

DESIGN, SYNTHESIS, CHARACTERISATION, *IN VITRO* AND *IN SILICO* STUDY OF *S*-
BENZYL DITHIOCARBAZATE DERIVATIVES AND
THEIR METAL COMPLEXES AS *ESCHERICHIA COLI*
DIHYDROFOLATE REDUCTASE INHIBITORS

BY

SITI KHADIJAH ROSLAN

A thesis submitted in fulfillment of the requirement for the
degree of Doctor of Philosophy in Science

Kulliyyah of Science
International Islamic University Malaysia

AUGUST 2025

ABSTRACT

Dihydrofolate reductase (DHFR) is a key enzyme required for bacterial growth because it reduces 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), an essential compound for DNA synthesis and cell survival. Since DHFR plays a critical role in bacterial survival, it has become an important target for developing drugs to treat infections. With *Escherichia coli* (*E. coli*) infections on the rise, there is an urgent need to develop novel antibacterial agents specifically targeting *E. coli* DHFR to combat this growing threat. In response to this challenge, four new SBDTC derivatives and their sixteen corresponding metal complexes were designed, synthesised, and thoroughly characterised, followed by *in vitro* and *in silico* studies. Structural analysis revealed that SB2OME, SB3OME, and SB3NO were formed through substitution at the β -nitrogen of SBDTC, while SB4OME resulted from substitution at the α -nitrogen. Upon complexation, SB2OME, SB3OME, and SB4OME functioned as tridentate ligands via NOS donor atoms, while SB3NO acted as a bidentate ligand through OS donor atoms. ADMET predictions indicated that the SBDTC derivatives adhered to drug-likeness criteria while the metal complexes exhibited some violations. Nonetheless, all compounds were predicted to have low toxicity profiles. Subsequently, antibacterial activity evaluated using minimum inhibitory concentration (MIC) assays, revealed notable efficacy against both gram-positive and gram-negative bacteria. Most metal complexes significantly enhanced the antibacterial activity of their SBDTC derivatives alone. Cu(II), Zn(II), Co(II), and Ni(II) complexes derived from SB4OME reduced the MIC of SB4OME against *E. coli* (ATCC 25922) by half (MIC = 0.437 mg/mol) outperforming trimethoprim (MIC = 1.750 mg/mol). Molecular docking studies on SB4OME and its metal complexes demonstrated interactions with active site residues of *E. coli* DHFR (PDB ID: 1RX7), similar to the natural substrate (folic acid) and the commercial antibiotic (trimethoprim), justifying their superior *in-vitro* activity. To further validate their efficacy, Molecular dynamics (MD) simulations were performed on SB4OME and its Cu(II), Co(II), and Ni(II) complexes over a 100 ns timeframe. All systems exhibited stability throughout the simulation, with optimal stability observed from 30 ns onward. Among all these compounds, the DHFR-CUSB4 complex demonstrated the most favourable binding free energy (-36.67 kcal/mol) in MMPBSA calculations, surpassing both folic acid and trimethoprim. Furthermore, several of the investigated compounds, including Cu(SB4OME)₂, demonstrated stable binding at the mutated residues L28R and I94L, highlighting their potential to overcome trimethoprim resistance associated with these clinically relevant DHFR mutations. In conclusion, the exceptional performance of Cu(SB4OME)₂ in both *in vitro* and *in silico* studies underscores its potential as a promising antibacterial agent and a targeted inhibitor of *E. coli* DHFR.

ملخص البحث

ديهايدروفولات ريدوكتاز (DHFR) هو إنزيم رئيسي مطلوب لنمو البكتيريا لأنه يقوم باختزال -7,8 ديهيدروفولات (DHF) إلى -5,6,7,8 تيتراهيدروفولات (THF)، وهو مركب أساسي لتخليق الحمض النووي وبقاء الخلية. نظرًا لأن DHFR يلعب دورًا حاسمًا في بقاء البكتيريا، فقد أصبح هدفًا مهمًا لتطوير الأدوية لعلاج الالتهابات. مع تزايد حالات عدوى الإشريكية القولونية (*E. coli*)، هناك حاجة ملحة لتطوير مثبطات جديدة تستهدف بشكل خاص DHFR في *E. coli* لمكافحة هذا التهديد المتزايد. استجابةً لهذا التحدي، تم تصميم أربعة مشتقات جديدة من SBDTC وستة عشر من معقداتها المعدنية المقابلة، وتصنيعها، وتوصيفها بدقة، تليها دراسات في المختبر (*in vitro*) وحاسوبية (*in silico*). كشفت التحليلات الهيكلية أن SB2OME و SB3OME و SB3NO تم تكوينها من خلال الاستبدال عند النيتروجين β لـ SBDTC، بينما نتج SB4OME من الاستبدال عند النيتروجين α . عند التكوين المعقد، عملت SB2OME و SB3OME و SB4OME كروابط ثلاثية الأسنان عبر ذرات مانحة من النوع NOS، بينما عملت SB3NO كروابط ثنائي الأسنان عبر ذرات مانحة من النوع OS. أشارت توقعات ADMET إلى أن مشتقات SBDTC التزمت بمعايير تشبه الأدوية، بينما أظهرت المعقدات المعدنية بعض الانحرافات. ومع ذلك، تم توقع أن جميع المركبات لديها مستويات منخفضة من السمية. بعد ذلك، أظهرت النشاطات المضادة للبكتيريا التي تم تقييمها باستخدام اختبارات التركيز المثبطة الدنيا (MIC) فعالية ملحوظة ضد كل من البكتيريا إيجابية الجرام وسالبة الجرام. عززت معظم المعقدات المعدنية بشكل كبير النشاط المضاد للبكتيريا لمشتقات SBDTC بمفردها. قللت معقدات Cu(II) و Zn(II) و Co(II) و Ni(II) المشتقة من SB4OME من قيمة MIC لـ

SB4OME ضد *E. coli* (ATCC 25922) إلى النصف (MIC=0.437 mg/mol)

، متفوقة على تريميمثوبريم (MIC=1.750 mg/mol). أظهرت دراسات الالتحام الجزيئي على SB4OME ومعقداتها المعدنية تفاعلات مع بقايا الموقع النش لـ *E. coli* DHFR (PDB ID: 1RX7) مشابهة للركيزة الطبيعية (حمض الفوليك) والمضاد الحيوي التجاري (تريميمثوبريم)، مما يبرر تفوقها في النشاط المخبري. ولتأكيد فعاليتها، تم إجراء محاكاة الديناميكا الجزيئية (MD) على SB4OME ومعقداتها من Cu(II) و Co(II) و Ni(II) على مدى 100 نانوثانية. أظهرت جميع الأنظمة استقرارًا طوال فترة المحاكاة، مع ملاحظة الاستقرار الأمثل من 30 نانوثانية فصاعدًا. من بين جميع هذه المركبات، أظهر معقد DHFR-CUSB4 أفضل طاقة ارتباط حرة (-36.67 كيلو كالوري/مول) في حسابات MMPBSA، متفوقًا على كل من حمض الفوليك وتريميمثوبريم. علاوة على ذلك، أظهرت عدة مركبات من بين التي تم التحقيق فيها، بما في ذلك $\text{Cu}(\text{SB4OME})_2$ ، ارتباطًا مستقرًا عند بقايا الطفرات L28R و I94L، مما يسلط الضوء على قدرتها المحتملة في التغلب على مقاومة التريميمثوبريم المرتبطة بهذه الطفرات في إنزيم DHFR ذات الأهمية السريرية. في الختام، يسلط الأداء الاستثنائي لمركب $\text{Cu}(\text{SB4OME})_2$ في كل من الدراسات المخبرية والمحاكاة الحاسوبية الضوء على إمكانية استخدامه كمضاد للبكتيريا واعد ومثبط مستهدف لإنزيم DHFR في *E. coli*.

APPROVAL PAGE

The thesis of Siti Khadijah Roslan has been approved by the following:



Fiona How Ni Foong
Supervisor

Zalikha Ibrahim
Co-Supervisor

Nurasyikin Hamzah
Co-Supervisor

Azzmer Azzar Abdul Hamid
Internal Examiner

Vannajan Sanghiran Lee
External Examiner

Mohd Said Nurumal
Chairman

DECLARATION

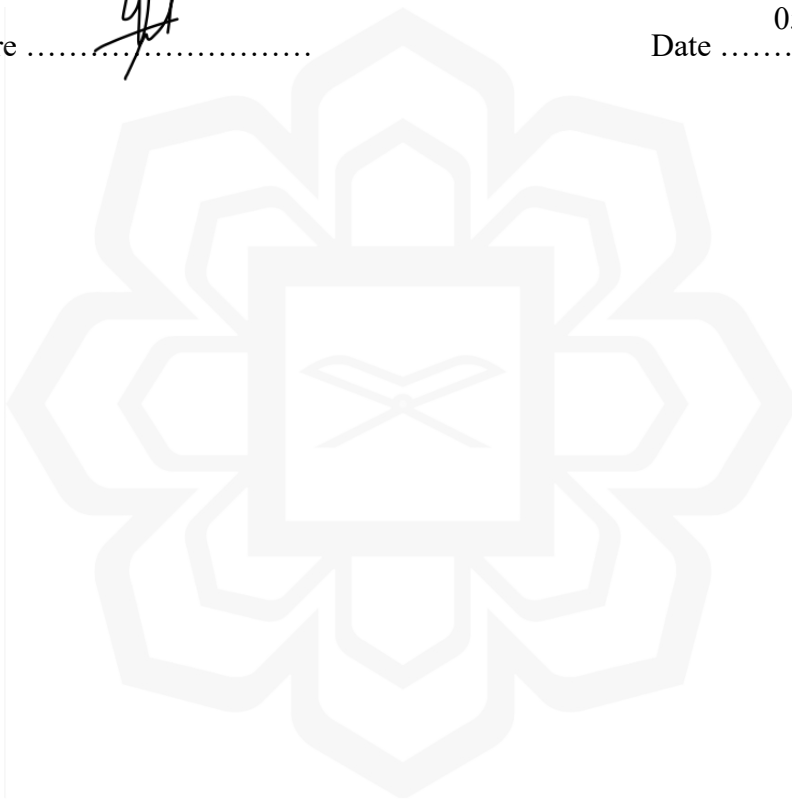
I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Siti Khadijah Roslan

Signature



Date 05/08/2025



INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

**DECLARATION OF COPYRIGHT AND AFFIRMATION OF
FAIR USE OF UNPUBLISHED RESEARCH**

**DESIGN, SYNTHESIS, CHARACTERISATION, *IN VITRO* AND
IN SILICO STUDY OF S-BENZYLDITHIOCARBAZATE
DERIVATIVES AND THEIR METAL COMPLEXES AS
ESCHERICHIA COLI DIHYDROFOLATE REDUCTASE
INHIBITORS**

I declare that the copyright holder of this thesis are jointly owned by the student and IIUM.

Copyright © 2024 Siti Khadijah Roslan and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may only be used by others in their writing with due acknowledgement.
2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purpose.
3. The IIUM library will have the right to make, store in a retrieval system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Siti Khadijah Roslan



Signature

05/08/2025

Date



This thesis is dedicated to my beloved family.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful.

All praise and thanks are due to Allah, the Most Gracious and the Most Merciful, for His boundless blessings that have guided me through the successful completion of this research endeavour. Without His grace and guidance, this journey would not have been possible. This work would not have been possible without the advice and support of many people.

I would like to express my deep and sincere gratitude to my research supervisor, Assoc. Prof. Dr Fiona How Ni Foong for her guidance, wisdom, and unwavering support throughout this research endeavour. She continually and convincingly conveyed a spirit of adventure regarding research. A big appreciation also goes to my co-supervisors, Asst. Prof. Dr. Zalikha binti Ibrahim and Asst. Prof. Dr. Nurasyikin binti Hamzah, for their insightful advice and constructive feedback that significantly enhanced the quality of this work. Their brilliant ideas help me a lot in preparing this research works. It was a great privilege and honour to work and study under all my supervisors' guidance. I could not have imagined having better advisors for my PhD study.

I am indebted to the dedicated science officers and laboratory assistants whose tireless efforts have greatly contributed to the success of this research. My sincere thanks to Br. Mizan for being just a call away and providing assistance whenever needed, Kak Hafiah and Br. Ikram for their invaluable assistance with CHNS analysis, Br. Wadi for his expertise in FTIR analysis, Kak Ain and Br. Arif for their support with GCMS analysis, Kak Fara, Kak Afzan, Sr. Zura and Sr. Yanti for their assistance with TGA, Pok Ya for his help with AAS, Br. Afiq for his assistance with molar conductivity, Puan Hafizah (UMP) for her help with NMR, Dr. Nadiah (UM) for her guidance on magnetic susceptibility and lastly, Sr. Syafiqah for her support with microbe assay.

I would also like to say thanks to my friends and fellow laboratory colleagues. To my friends in KOS, Sadina, Amanina, Kamilah, Aisyah and Rashidah, thank you for your camaraderie and support as labmates and housemates. Not to forget my friends in KOP, Min Mardhiyah, Najieha, Kak Mariam, Kak Nadiah, Kak Aqilah and Farisya, for being wonderful lunchmates and providing a supportive environment. A big thanks also to Br. Helmi, for his senior guidance in Molecular dynamics simulation and patience in answering my queries.

Additionally, my deepest gratitude goes to my beloved parents, Encik Roslan and Puan Fatimah for their unconditional love, unwavering support, and sacrifices that made this journey possible. I am also thankful to my beloved yet annoying younger siblings, extended family members and my friends, Nabihah, Syafiqah and Nurbahya for their understanding and encouragement. Even if their contributions may not be apparent, their presence was felt and appreciated. I am also grateful to JPA for their generous financial sponsorship, which enabled me to pursue my studies and provided necessary allowances.

To everyone mentioned, and to those who may not be listed but have contributed in various ways, I extend my sincerest appreciation. Your contributions have made a profound impact on my academic and personal growth, and I am truly grateful for your support.

Thank You.



TABLE OF CONTENTS

ABSTRACT.....	ii
ABSTRACT IN ARABIC.....	iii
APPROVAL PAGE	v
DECLARATION	vi
COPYRIGHT.....	vii
ACKNOWLEDGEMENTS.....	ix
TABLE OF CONTENTS.....	xi
LIST OF TABLES.....	xvii
LIST OF FIGURES	xx
LIST OF SCHEMES.....	xxviii
LIST OF SYMBOLS	xxix
LIST OF ABBREVIATIONS.....	xxx
LIST OF APPENDICES.....	xxxvi
CHAPTER ONE: INTRODUCTION.....	1
1.1 Research Background.....	1
1.2 Problem Statement	7
1.3 Research Significance	8
1.4 Research Questions	8
1.5 Research Hypothesis	9
1.6 Research Objectives.....	9
CHAPTER TWO: LITERATURE REVIEW.....	11
2.1 Dihydrofolate Reductase (DHFR).....	11
2.1.1 FDA-approved DHFR Inhibitors	16
2.1.2 Bacteria DHFR inhibitors	20
2.2 Dithiocarbamate Derivatives	25
2.2.1 Biological Activities of SBDTC Derivatives and Their Metal Complexes.....	29
2.3 Characterisation.....	36
2.3.1 Melting Point	36
2.3.2 Elemental Analysis	37
2.3.3 Fourier Transform Infrared (FTIR) spectroscopy.....	38
2.3.4 Nuclear magnetic resonance (NMR) spectroscopy.....	41

2.3.4.1	¹ H-NMR	41
2.3.4.2	¹³ C-NMR	42
2.3.5	Mass Spectrometry (MS)	43
2.3.6	Ultraviolet-Visible (UV-Vis) spectroscopy	45
2.3.7	Molar Conductivity	47
2.3.8	Magnetic Susceptibility	47
2.3.9	Thermogravimetric Analysis (TGA).....	49
2.4	<i>In Silico</i> Techniques	50
2.4.1	SwissADME	50
2.4.2	ProTox-II.....	52
2.4.3	Molecular Docking	53
2.4.3.1	AutoDock4 Software.....	54
2.4.4	Molecular Dynamics (MD) Simulation	55
2.4.4.1	Procedure in Molecular Dynamics (MD) Simulation	55
2.4.4.2	Analysis of Molecular Dynamics (MD) Simulation	56
2.4.4.3	In Silico-In Vitro Integration.....	57
CHAPTER THREE: METHODOLOGY.....		60
3.1	List Of Chemicals, Apparatus, Glassware, Consumable Items, Equipment And Instruments Used	60
3.2	List Of Hardware And Software Used	63
3.3	Synthesis Method	64
3.3.1	Synthesis of <i>S</i> -benzylidithiocarbazate (SBDTC).....	67
3.3.2	General synthesis of <i>S</i> -benzyl-β- <i>N</i> -2-methoxybenzoyl dithiocarbazate (SB2OME), <i>S</i> -benzyl-β- <i>N</i> -3- methoxybenzoyl dithiocarbazate (SB3OME) and <i>S</i> -benzyl-α- <i>N</i> -4-methoxybenzoyl dithiocarbazate (SB4OME)	67
3.3.3	Synthesis of <i>S</i> -benzyl-β- <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate (SB3NO).....	68
3.3.4	General synthesis of the metal complexes from SB2OME, SB3OME and SB4OME	68
3.3.5	Synthesis of the metal complexes from SB3NO.....	68
3.4	Characterisation.....	69
3.4.1	Melting Point Determination	69
3.4.2	CHNS Elemental Analysis.....	69
3.4.3	Atomic Absorption Spectroscopy (AAS)	69
3.4.4	Fourier Transform Infrared (FTIR) Spectroscopy	70

3.4.5	¹ H and ¹³ C Nuclear Magnetic Resonance (NMR) Spectroscopy	70
3.4.6	Mass Spectrometry (MS)	70
3.4.7	Ultraviolet-Visible (UV-Vis) Spectroscopy	71
3.4.8	Molar Conductivity	71
3.4.9	Magnetic Susceptibility	72
3.4.10	Thermogravimetric Analysis (TGA).....	72
3.5	Absorption, Distribution, Metabolism, Excretion, Toxicity (Admet).....	72
3.6	Antimicrobial Assay.....	74
3.6.1	Bioactivity and Target Microorganisms	74
3.6.2	Preparation of Nutrient Agar and Nutrient Broth	75
3.6.3	Bacteria Culture	75
3.6.4	Minimum Inhibitory Concentration (MIC).....	75
3.6.5	Analysis of Minimum Inhibitory Concentration (MIC) Result	76
3.7	Molecular Docking.....	77
3.7.1	Preparation of Protein (Wild-type)	77
3.7.2	Preparation of Ligand	78
3.7.3	Preparation of Input Files.....	78
3.7.3.1	Grid Parameter File (GPF) Preparation.....	78
3.7.3.2	Docking Parameter File (DPF) Preparation	79
3.7.4	Docking Running	80
3.7.5	Validation of Docking Parameters.....	80
3.7.6	Blind Docking.....	80
3.7.7	Analysis of Docking	81
3.8	Molecular Dynamics Simulation	81
3.8.1	Preparation of Protein Topology Files	82
3.8.2	Preparation of Ligand Topology Files	82
3.8.3	Solvation	83
3.8.4	Additions of Ions.....	84
3.8.5	Energy Minimisation	84
3.8.6	Equilibration (NVT & NPT).....	84
3.8.7	Production Molecular Dynamics Simulation.....	85
3.8.8	Molecular Dynamics Simulation Analysis	85
3.9	Molecular Docking For Mutant Type <i>E. Coli</i> DHFR	86
3.9.1	Preparation of Protein Topology Files	87

CHAPTER FOUR: RESULT & DISCUSSION.....	88
4.1 Design Of Compounds.....	88
4.1.1 Rationale Design of SBDTC Derivatives and Their Metal Complexes.....	88
4.1.2 SBDTC Derivatives And Their Metal Complexes As <i>E. coli</i> DHFR Inhibitors	98
4.2 Synthesis And Physical Properties.....	102
4.3 Elemental Analysis.....	104
4.4 Fourier Transform Infrared (FTIR) Spectroscopy	106
4.4.1 SBDTC, SB2OME, SB3OME and SB4OME.....	106
4.4.2 Metal Complexes from SB2OME.....	111
4.4.3 Metal Complexes from SB3OME.....	115
4.4.4 Metal Complexes from SB4OME.....	119
4.4.5 SBDTC, SB3NO and Its Metal Complexes.....	123
4.4.6 Tautomerism In Solid Form.....	129
4.5 Nuclear Magnetic Resonance (NMR) Spectroscopy	131
4.5.1 SB2OME.....	131
4.5.2 SB3OME.....	135
4.5.3 SB4OME.....	138
4.5.4 SB3NO	141
4.5.5 Tautomerism In Liquid Form.....	144
4.6 Mass Spectrometry (MS)	145
4.6.1 SBDTC.....	145
4.6.2 SB2OME.....	147
4.6.3 SB3OME.....	148
4.6.4 SB4OME.....	150
4.6.5 SB3NO	151
4.7 Ultraviolet-Visible (UV-VIS) Spectroscopy	153
4.7.1 SB2OME and Its Metal Complexes.....	153
4.7.2 SB3OME and Its Metal Complexes.....	155
4.7.3 SB4OME and Its Metal Complexes.....	157
4.7.4 SB3NO and Its Metal Complexes.....	159
4.8 Molar Conductivity And Magnetic Moment	161
4.9 Plausible Mechanism	164
4.9.1 SBDTC.....	164
4.9.2 SB2OME, SB3OME and their metal complexes.....	164

4.9.1	SB4OME and its metal complexes	166
4.9.2	SB3NO and its metal complexes	168
4.10	Thermogravimetric Analysis (TGA).....	169
4.10.1	SBDTC, SB2OME, SB3OME and SB4OME.....	169
4.10.2	Metal Complexes from SB2OME.....	171
4.10.3	Metal Complexes from SB3OME.....	172
4.10.4	Metal Complexes from SB4OME.....	174
4.10.5	SB3NO And Its Metal Complexes.....	176
4.10.6	Summarisation of Thermogravimetric Analysis (TGA).....	178
4.11	Absorption, Distribution, Metabolism, Excretion, And Toxicity (ADMET).....	179
4.11.1	ADME Prediction	179
4.11.2	Toxicity Prediction.....	185
4.12	Biological Activity	187
4.12.1	SBDTC, SB2OME, SB3OME and SB4OME.....	189
4.12.2	Metal Complexes from SB2OME.....	190
4.12.3	Metal Complexes from SB3OME.....	192
4.12.4	Metal Complexes from SB4OME.....	193
4.12.5	SB3NO And its Metal Complexes	195
4.12.6	Comparison Antibacterial Effectiveness of Synthesised Compounds with Trimethoprim.....	196
4.12.7	Structure-Activity Relationship (SAR).....	197
4.12.7.1	Structure-Activity Relationship (SAR) of SBDTC Derivatives.....	197
4.12.7.2	Structure-Activity Relationship (SAR) of Metal Complexes	199
4.12.8	Gram-Positive vs Gram-Negative Bacteria	203
4.12.9	Selection Compounds for the Computational Study.....	204
4.13	Molecular Docking.....	205
4.13.1	Docking and Binding Site Validation	205
4.13.2	Binding Energies and Binding Interactions	207
4.14	Molecular Dynamics Simulation	215
4.14.1	Root Mean Square Deviation (RMSD).....	216
4.14.2	Radius Gyration (Rg).....	219
4.14.3	Root Mean Square Fluctuation (RMSF).....	221
4.14.4	Pocket Volume.....	226

4.14.5	MET20 Loop Stability	228
4.14.6	Binding Free Energy Calculation.....	231
4.14.7	Binding Interaction Analysis	233
4.14.7.1	Interaction Analysis of FOL.....	233
4.14.7.2	Interaction Analysis of TMP	235
4.14.7.3	Interaction Analysis of SB4	237
4.14.7.4	Interaction Analysis of CUSB4.....	239
4.14.7.5	Interaction Analysis of COSB4.....	241
4.14.7.6	Interaction Analysis of NISB4	244
4.14.8	Molecular Distance analysis	246
4.14.9	Comparative Binding Analysis of SB4 and Its Metal Complexes with <i>E. coli</i> DHFR	249
4.15	Potential To Counteract <i>E. Coli</i> DHFR Mutations Via Molecular Docking Study.....	252
4.15.1	Validation of Protein Structure	252
4.15.2	Molecular Docking of the Compounds Against L28R mutant <i>E. coli</i> DHFR	253
4.15.3	Molecular Docking of the Compounds Against I94L mutant <i>E. coli</i> DHFR	259
4.16	Determination Of New Antibacterial Agent	264
CHAPTER FIVE: CONCLUSION		267
5.1	Summary	267
5.2	Future Works.....	270
REFERENCES		271
APPENDICES		310

LIST OF TABLES

Table 3.1	List of chemicals used	60
Table 3.2	List of apparatus and glassware used	61
Table 3.3	List of consumable items used	62
Table 3.4	List of equipment used	62
Table 3.5	List of instruments used	62
Table 3.6	List of hardware used	63
Table 3.7	List of software used	63
Table 3.8	SMILES notation of compounds for ADMET study	73
Table 3.9	The grid box parameters for protein	79
Table 3.10	The grid centres and the number of points for protein in blind docking	80
Table 3.11	Ligand-protein complex investigated with MD simulation in this study	81
Table 3.12	The grid box parameters for mutated protein	87
Table 4.1	Physical properties of all synthesised compounds	102
Table 4.2	Elemental data of SBDTC derivatives and their metal complexes	104
Table 4.3	Important IR bands for SBDTC, SB2OME, SB3OME and SB4OME	109
Table 4.4	Important IR bands for metal complexes from SB2OME	113
Table 4.5	Important IR bands for metal complexes from SB3OME	117

Table 4.6	Important IR bands for metal complexes from SB4OME	121
Table 4.7	Important IR bands for SB3NO and its metal complexes	126
Table 4.8	Electronic spectra of SB2OME and its metal complexes	154
Table 4.9	Electronic spectra of SB3OME and its metal complexes	156
Table 4.10	Electronic spectra of SB4OME and its metal complexes	158
Table 4.11	Electronic spectra of SB3NO and its metal complexes	160
Table 4.12	Molar conductivity and magnetic moments of metal complexes	161
Table 4.13	ADME analysis of all compounds	183
Table 4.14	Toxicity analysis of all compounds	186
Table 4.15	Minimum inhibition concentration of all synthesised compounds and positive control	188
Table 4.16	AutoDock results show binding energy, interaction type, and interacting amino acid residue at <i>E. coli</i> DHFR	208
Table 4.17	RMSD data for the investigated compounds at <i>E. coli</i> DHFR	216
Table 4.18	Radius gyration of the investigated compounds at <i>E. coli</i> DHFR	219
Table 4.19	Pocket volume of the investigated compounds at the binding site of <i>E. coli</i> DHFR	226
Table 4.20	MMPBSA of the investigated compounds at <i>E. coli</i> DHFR	231
Table 4.21	Interacting residues of DHFR-FOL	234
Table 4.22	Interacting residues of DHFR-TMP	236
Table 4.23	Interacting residues of DHFR-SB4	238

Table 4.24	Interacting residues of DHFR-CUSB4	240
Table 4.25	Interacting residues of DHFR-COSB4	243
Table 4.26	Interacting residues of DHFR-NISB4	245
Table 4.27	Molecular distance of the investigated compounds at <i>E. coli</i> DHFR	246
Table 4.28	Comparison of binding energy, interaction type, and interacting amino acid residue in wild-type and L28R mutant <i>E. coli</i> DHFR. (Mutated residue in bold)	257
Table 4.29	Comparison of binding energy, interaction type, and interacting amino acid residue in wild-type and I94L mutant <i>E. coli</i> DHFR. (Mutated residue in bold)	262

LIST OF FIGURES

Figure 1.1	The number of pathogens attributed to AMR in 2019. (Adopted from Nature, (2022))	2
Figure 1.2	Chemical structure of trimethoprim (TMP)	3
Figure 1.3	Chemical structure of <i>S</i> -benzylthiocarbamate (SBDTC)	5
Figure 2.1	Structures representing the occluded loop conformation of <i>E. coli</i> DHFR (PDB ID: 1RX7)	13
Figure 2.2	Cartoon representation of <i>E. coli</i> DHFR with the MET20 highlighted in different structural conformations. (Green = Open/disordered, Blue = Closed, Red = Occluded)	14
Figure 2.3	Cartoon representative of hydrogen bond interaction between ASN18 with SER148	15
Figure 2.4	Residues interacting with folic acid at <i>E. coli</i> DHFR (PDB ID: 1RX7)	16
Figure 2.5	Chemical structure of methotrexate (MTX)	17
Figure 2.6	Structures of selected non-classical DHFR inhibitors	17
Figure 2.7	Residues interacting with TMP at <i>E. coli</i> DHFR (PDB ID: 6XG5)	19
Figure 2.8	Structure of formazan derivative	20
Figure 2.9	Structures of thiophenyl-pyrazolylthiazole hybrids derivatives	21
Figure 2.10	Structures of quinazolinone derivatives	22
Figure 2.11	Structure of Schiff base hybrids of 1,2,4-triazole-pyridine	22
Figure 2.12	Structures of triazole derivatives	23

Figure 2.13	Structure of caffeic acid analogue	24
Figure 2.14	Structure of TMP analogue	24
Figure 2.15	Structure of novel hydroxyl TMP analogue	25
Figure 2.16	Chemical structure of dithiocarbazate backbone	26
Figure 2.17	Tautomeric forms of SBDTC	26
Figure 2.18	Tautomeric form of <i>S</i> -benzyl- β -N-(benzoyl) dithiocarbazate (How et al., 2008)	27
Figure 2.19	NS chelation mode of metal complex derived from Schiff base of SBDTC with 2, 3- dimethoxybenzaldehyde	28
Figure 2.20	Complexation modes of SBDTC derivative	29
Figure 2.21	Nickel(II) acetate of SBDTC complex	31
Figure 2.22	Structure of Schiff base from SBDTC with 2, 3- dimethoxybenzaldehyde	31
Figure 2.23	Structure of Schiff base from SBDTC with 4- acetylbenzenesulfonamide	32
Figure 2.24	Structure of Schiff base from SBDTC with; (a) 2-hydroxy-3- methoxybenzaldehyde and (b) 2,3-dihydroxybenzaldehyde	33
Figure 2.25	Structure of Schiff base from SBDTC with 2- methoxybenzaldehyde	34
Figure 2.26	Structure of Schiff base from SBDTC with dibenzalacetone	34
Figure 2.27	Structure of Schiff base from SBDTC with; (a) 4- carboxybenzaldehyde (b) 4-formyl-3-hydroxybenzoic acid	35
Figure 2.28	Structure of Schiff base from SBDTC with cinnamaldehyde	35

Figure 2.29	Enolisation followed by deprotonation of Schiff base from SBDTC and isatin prior to coordination to Ni(II)	39
Figure 2.30	NOS mode of benzyl- <i>N</i> -(2-methoxybenzoyl) hydrazinecarbodithioate	40
Figure 3.1	General workflow of molecular docking	77
Figure 3.2	General workflow of molecular dynamics simulation	81
Figure 3.3	Dodecahedron solvation box	83
Figure 3.4	Energy minimisation graph of a system in molecular dynamics	84
Figure 4.1	Structure integral of TMP highlighting two important moiety	89
Figure 4.2	Structure integral of TMP and SBDTC highlighting amino group	89
Figure 4.3	Structure integral of TMP and SBDTC highlighting aromatic group	90
Figure 4.4	Structure integral of TMP highlighting 3',4',5'-trimethoxybenzyl	91
Figure 4.5	The proposed pharmacophore structure of isomeric methoxy derivatives	92
Figure 4.6	Structure integral of SBDTC highlighting thiol group	93
Figure 4.7	The proposed pharmacophore structure of the amide group	93
Figure 4.8	The proposed pharmacophore structure of <i>p</i> -chloro and <i>m</i> -nitro	95
Figure 4.9	The proposed tautomeric form of the designed compound	95
Figure 4.10	The proposed structure of the designed SBDTC derivatives	100

Figure 4.11	The proposed structure of the designed metal complexes from SBDTC derivatives	101
Figure 4.12	Overlay IR spectra of SBDTC with SB2OME, SB3OME and SB4OME	110
Figure 4.13	Coordination mode of metal complexes from SB2OME	112
Figure 4.14	Overlay IR spectra of metal complexes from SB2OME	114
Figure 4.15	Coordination mode of metal complexes from SB3OME	116
Figure 4.16	Overlay IR spectra of metal complexes from SB3OME	118
Figure 4.17	Coordination mode of metal complexes from SB4OME	120
Figure 4.18	Overlay IR spectra of metal complexes from SB4OME	122
Figure 4.19	Coordination mode of metal complexes from SB3NO	125
Figure 4.20	Overlay IR spectra of SB3NO and its starting material	127
Figure 4.21	Overlay IR spectra of metal complexes from SB3NO	128
Figure 4.22	Possible tautomeric forms of SB2OME, SB3OME and SB3NO	129
Figure 4.23	Thione-thiol tautomer of SB3NO during complexation	130
Figure 4.24	¹ H NMR spectra of SB2OME	133
Figure 4.25	¹³ C NMR spectra of SB2OME	134
Figure 4.26	¹ H NMR spectra of SB3OME	136
Figure 4.27	¹³ C NMR spectra of SB3OME	137
Figure 4.28	¹ H NMR spectra of SB4OME	139
Figure 4.29	¹³ C NMR spectra of SB4OME.	140
Figure 4.30	¹ H NMR spectra of SB3NO	142

Figure 4.31	^{13}C NMR spectra of SB3NO	143
Figure 4.32	Mass spectrum of SBDTC	146
Figure 4.33	Fragmentation pathway of SBDTC	146
Figure 4.34	Mass spectrum of SB2OME	147
Figure 4.35	Fragmentation pathway of SB2OME	148
Figure 4.36	Mass spectrum of SB3OME	149
Figure 4.37	Fragmentation pathway of SB3OME	150
Figure 4.38	Mass spectrum of SB4OME	150
Figure 4.39	Fragmentation pathway of SB4OME	151
Figure 4.40	Mass spectrum of SB3NO	152
Figure 4.41	Fragmentation pathway of SB3NO	152
Figure 4.42	Electronic spectra of SB2OME and its metal complexes	154
Figure 4.43	Electronic spectra of SB3OME and its metal complexes	156
Figure 4.44	Electronic spectra of SB4OME and its metal complexes	158
Figure 4.45	Electronic spectra of SB3NO and its metal complexes	160
Figure 4.46	Decomposing stages of SBDTC, SB2OME, SB3OME, and SB4OME	170
Figure 4.47	Decomposing stages of metal complexes from SB2OME	171
Figure 4.48	Decomposing stages of metal complexes from SB3OME	173
Figure 4.49	Decomposing stages of metal complexes from SB4OME	175
Figure 4.50	Decomposing stages of metal complexes from SB3NO	176
Figure 4.51	The Bioavailability Radar of the representative compounds	180

Figure 4.52	LD ₅₀ values of all compounds	187
Figure 4.53	MIC values of SBDTC, SB2OME, SB3OME and SB4OME	190
Figure 4.54	MIC values of metal complexes from SB2OME	191
Figure 4.55	MIC values of metal complexes from SB3OME	192
Figure 4.56	MIC values of metal complexes from SB4OME	194
Figure 4.57	MIC values of SB3NO and its metal complexes	195
Figure 4.58	SAR studies of SBDTC derivatives	198
Figure 4.59	SAR studies of metal complexes from SBDTC derivatives	201
Figure 4.60	Superimposition of the predicted (yellow) and experimental (green) positions of FOL	206
Figure 4.61	Localisation of FOL from blind docking (green) with the local docking result (red)	206
Figure 4.62	Localisation of all compounds at <i>E. coli</i> DHFR from molecular docking	207
Figure 4.63	Binding interactions of FOL and TMP at <i>E. coli</i> DHFR	210
Figure 4.64	Binding interactions of SB4 at <i>E. coli</i> DHFR	211
Figure 4.65	Binding interactions of CUSB4 at <i>E. coli</i> DHFR	212
Figure 4.66	Binding interactions of ZNSB4 at <i>E. coli</i> DHFR	213
Figure 4.67	Binding interactions of COSB4 at <i>E. coli</i> DHFR	214
Figure 4.68	Binding interactions of NISB4 at <i>E. coli</i> DHFR	215
Figure 4.69	RMSD plots of the investigated compounds extracted by protein RMSD at <i>E. coli</i> DHFR	217

Figure 4.70	RMSD plots of the investigated compounds extracted by ligand RMSD at <i>E. coli</i> DHFR	218
Figure 4.71	Compound binding poses of NISB4 at 0 ns (red) and 20 ns (orange) at <i>E. coli</i> DHFR	219
Figure 4.72	Radius gyration of the investigated compounds at <i>E. coli</i> DHFR	220
Figure 4.73	RMSF plot of the investigated compounds extracted by protein RMSF at <i>E. coli</i> DHFR	222
Figure 4.74	B-factor putty representative of the investigated compounds at <i>E. coli</i> DHFR	224
Figure 4.75	RMSF plot of the investigated compound extracted by ligands RMSF at <i>E. coli</i> DHFR	225
Figure 4.76	The pocket volume of the investigated compounds at <i>E. coli</i> DHFR	227
Figure 4.77	Hydrogen bond interaction between SER148 with ASN23 of the investigated compounds at <i>E. coli</i> DHFR. The hydrogen bonds were shown in yellow dashed lines, and the distances are written in Angstrom units	230
Figure 4.78	MMPBSA of the investigated compounds at <i>E. coli</i> DHFR	232
Figure 4.79	Binding interactions of FOL at <i>E. coli</i> DHFR	234
Figure 4.80	Binding interactions of TMP at <i>E. coli</i> DHFR	236
Figure 4.81	Binding interactions of SB4 at <i>E. coli</i> DHFR	238
Figure 4.82	Binding interactions of CUSB4 at <i>E. coli</i> DHFR	240
Figure 4.83	Binding interactions of COSB4 at <i>E. coli</i> DHFR	242
Figure 4.84	Binding interactions of NISB4 at <i>E. coli</i> DHFR	245

Figure 4.85	Molecular distance of the investigated compounds at specific residue of <i>E. coli</i> DHFR	247
Figure 4.86	Localisation of SB4 and its metal complexes with FOL (red) from molecular docking study at <i>E. coli</i> DHFR	250
Figure 4.87	Localisation of SB4 (yellow) and its metal complexes (CUSB4: green, COSB4: magenta, NISB4: orange) from MD simulation study at <i>E. coli</i> DHFR	251
Figure 4.88	Localisation of TMP (blue) at the wild-type and L28R mutant <i>E. coli</i> DHFR	254
Figure 4.89	Localisation of CUSB4 (green) at the wild-type and L28R mutant <i>E. coli</i> DHFR	255
Figure 4.90	Localisation of TMP (blue) at the wild-type and I94L mutant <i>E. coli</i> DHFR	260
Figure 4.91	Localisation of CUSB4 (green) at the wild-type and I94L mutant <i>E. coli</i> DHFR	261
Figure 4.92	Pathway strategy for discovering new antibacterial agent from SBDTC derivatives and their metal complexes targeting <i>E. coli</i> DHFR.	266

LIST OF SCHEMES

Scheme 1.1	Reduction of dihydrofolate (DHF) to tetrahydrofolate (THF)	3
Scheme 2.1	Folate biosynthesis pathway	11
Scheme 3.1	Synthesis pathway of the designed SBDTC derivatives and their respective metal complexes	66
Scheme 4.1	Plausible reaction mechanism of SBDTC	164
Scheme 4.2	Plausible reaction mechanism of SB2OME, SB3OME and their metal complexes	166
Scheme 4.3	Plausible reaction mechanism of SB4OME and its metal complex	167
Scheme 4.4	Plausible reaction mechanism of SB3NO and its metal complexes	168

LIST OF SYMBOLS

°C	Celsius
μ_{eff}	Effective magnetic moment
$\mu\text{g}/\text{cm}^3$	Micrograms per cubic centimeter
μL	Microliter
μM	Micromolar
Å	Angstrom
B.M	Bohr magneton
CFU/mL	Colony-Forming Units per milliliter
cm	Centimeter
$\chi\mu$	Molar susceptibility
e	Euler's number
fs	Femtosecond
g	Gram
g/mol	Gram per mole
Hz	Hertz
J	Joule
K	Kelvin
kcal/ mol	kilocalories per mole
kg	kilogram
L	Liter
m	Meta-substitution
m	meter
M	Molarity
m/z	Mass-to-charge ratio
M^+	Molecular ion peak
mg	Milligram
MHz	Megahertz
min	minutes
mL	Milliliter
mm	Millimeter

mmol	Millimole
mol	Mole
n	nano
<i>o</i>	Ortho-substitution
p	Pico
<i>p</i>	Para-substitution
ppm	Parts per million
S	Siemen
s	second
α	Alpha
β	Beta
δ	Delta
ΔG	Gibbs Free Energy
λ_{max}	Wavelength of maximum absorbance
μ	Micro
π	Pi
ν	Frequency
χ	Magnetic susceptibility
Ω	Ohm

LIST OF ABBREVIATIONS

^{13}C -NMR	Carbon-13 Nuclear Magnetic Resonance
^1H -NMR	Proton Nuclear Magnetic Resonance
2OME	2-methoxybenzoyl chloride
3NO	4-chloro-3-nitrobenzoyl chloride
3OME	3-methoxybenzoyl chloride
4OME	4-methoxybenzoyl chloride
AAS	Atomic Absorption Spectroscopy
ADMET	Absorption, distribution, metabolism, excretion and toxicity
AM1-BCC	Austin Model 1 Bond Charge Correction
AMBER	Assisted Model Building with Energy Refinement
BACE-1	Beta-site Amyloid Precursor Protein Cleaving Enzyme 1
BSA	Bovine Serum Albumin
CD ₅₀	Median curative dose
CEM-SS	Human T-lymphoblast-like cell line
CHARMM	Chemistry at HARvard Macromolecular Mechanics
CHNS	Carbon, Hydrogen, Nitrogen, Sulfur (elements)
CLSI	Clinical and Laboratory Standards Institute
Co(SB2OME) ₂	Cobalt complex of <i>S</i> -benzyl- β - <i>N</i> -2-methoxybenzoyl dithiocarbazate
Co(SB3NO) ₂	Cobalt complex of <i>S</i> -benzyl- β - <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate
Co(SB3OME) ₂	Cobalt complex of <i>S</i> -benzyl- β - <i>N</i> -3-methoxybenzoyl dithiocarbazate
Co(SB4OME) ₂ / COSB4	Cobalt complex of <i>S</i> -benzyl- α - <i>N</i> -4-methoxybenzoyl dithiocarbazate
CS ₂	Carbon Disulfide
Csp ³	Aliphatic Carbon

Cu(SB2OME) ₂	Copper complex of <i>S</i> -benzyl-β- <i>N</i> -2-methoxybenzoyl dithiocarbazate
Cu(SB3NO) ₂	Copper complex of <i>S</i> -benzyl-β- <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate
Cu(SB3OME) ₂	Copper complex of <i>S</i> -benzyl-β- <i>N</i> -3-methoxybenzoyl dithiocarbazate
Cu(SB4OME) ₂ / CUSB4	Copper complex of <i>S</i> -benzyl-α- <i>N</i> -4-methoxybenzoyl dithiocarbazate
DFT	Density Functional Theory
DHF	Dihydrofolate
DHFR	Dihydrofolate Reductase
DLG	Docking log file
DMSO	Dimethyl Sulfoxide
DMSO-d ₆	Deuterated Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPF	Docking Parameter File
DTG	Differential thermogravimetry
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
FDA	U.S. Food and Drug Administration
FLEX	Flexible Ligand Docking
FOL	Folic acid
FTIR	Fourier Transform Infrared Spectroscopy
GAFF	General Amber Force Field
GC-MS	Gas Chromatography-Mass Spectrometry
GI ₅₀	Growth Inhibition 50%
GPF	Grid Parameter File
GROMACS	GRoningen MACHine for Chemical Simulations
GSK-3β	Glycogen Synthase Kinase-3 Beta
GyrB	DNA gyrase subunit B
H-bond	Hydrogen bond
HeLa	Human cervical cancer cells
HT29	Human colon cancer cell line

IC ₅₀	Half maximal inhibitory concentration
INSATU	Degree of unsaturation in organic compounds
INSOLU	Insoluble in aqueous solvents
KBr	Potassium bromide
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
LD ₅₀	Median lethal dose
LIPO	Lipophilicity
LMCT	Ligand-to-metal charge transfer
log P	Octanol-water partition coefficient
MCF-7	Michigan Cancer Foundation-7 (breast cancer cell line)
MD	Molecular dynamics
MDA-MB-231	Human breast cancer cell line
MIC	Minimum inhibitory concentration
MM	Molecular mechanics
MMPBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NADPH	Nicotinamide adenine dinucleotide phosphate
Ni(SB2OME) ₂	Nickel complex of <i>S</i> -benzyl-β- <i>N</i> -2-methoxybenzoyl dithiocarbazate
Ni(SB3NO) ₂	Nickel complex of <i>S</i> -benzyl-β- <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate
Ni(SB3OME) ₂	Nickel complex of <i>S</i> -benzyl-β- <i>N</i> -3-methoxybenzoyl dithiocarbazate
Ni(SB4OME) ₂ / NISB4	Nickel complex of <i>S</i> -benzyl-α- <i>N</i> -4-methoxybenzoyl dithiocarbazate
NMR	Nuclear Magnetic Resonance

NorA	Bacterial multidrug efflux pump
NPT	Constant Pressure and Temperature ensemble in Molecular dynamicss
NVT	Constant Volume and Temperature ensemble in Molecular dynamicss
OD	Optical density
PBC	Periodic Boundary Conditions
PDB	Protein Data Bank
PDBQT	PDB with charges and atom types
POLAR	Partial Order Optimal Likelihood Algorithm
Rg	Radius of gyration
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RNA	Ribonucleic Acid
Ro5	Rules of Five
SAR	Structure-Activity Relationship
SASA	Solvent Accessible Surface Area
SB2OME	<i>S</i> -benzyl- β - <i>N</i> -2-methoxybenzoyl dithiocarbazate
SB3NO	<i>S</i> -benzyl- β - <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate
SB3OME	<i>S</i> -benzyl- β - <i>N</i> -3-methoxybenzoyl dithiocarbazate
SB4OME / SB4	<i>S</i> -benzyl- α - <i>N</i> -4-methoxybenzoyl dithiocarbazate
SBDTC	<i>S</i> -benzyldithiocarbazate
SIZE	Molecular weight
SMDTC	<i>S</i> -methyldithiocarbazate
SMILES	Simplified Molecular Input Line Entry System
SN ₂	Substitution Nucleophilic Bimolecular
SPC	Single Point Charge
TGA	Thermogravimetric Analysis
THF	Tetrahydrofolate
TIP3P	Transferable Intermolecular Potential with 3 Points
TMP	Trimethoprim

TMS	Trimethylsilyl
TPSA	Topological Polar Surface Area
UTI	Urinary Tract Infection
UV-Vis	Ultraviolet-Visible Spectroscopy
VMD	Visual Molecular dynamics
WHO	World Health Organization
XLOGP3	Calculated Log P (predicted octanol-water partition coefficient)
Zn(SB2OME) ₂	Zinc complex of <i>S</i> -benzyl-β- <i>N</i> -2-methoxybenzoyl dithiocarbazate
Zn(SB3NO) ₂	Zinc complex of <i>S</i> -benzyl-β- <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate
Zn(SB3OME) ₂	Zinc complex of <i>S</i> -benzyl-β- <i>N</i> -3-methoxybenzoyl dithiocarbazate
Zn(SB4OME) ₂ / ZNSB4	Zinc complex of <i>S</i> -benzyl-α- <i>N</i> -4-methoxybenzoyl dithiocarbazate

LIST OF APPENDICES

Appendix 1	Picture of all synthesised compounds	310
Appendix 2	All instruments used	311
Appendix 3	Raw data from CHNS	313
Appendix 4	Raw data from AAS	314
Appendix 5	Wavenumber of FTIR	315
Appendix 6	Overlay IR spectra of SBDTC	318
Appendix 7	Overlay IR spectra of SB2OME	319
Appendix 8	Overlay IR spectra of SB3OME	320
Appendix 9	Overlay IR spectra of SB4OME	321
Appendix 10	Overlay IR spectra of metal acetate	322
Appendix 11	Important NMR bands for all synthesised compounds	323
Appendix 12	Raw data from molar conductivity	325
Appendix 13	Raw data from magnetic susceptibility	326
Appendix 14	Raw data from TGA analysis	327
Appendix 15	TGA and DTG graph of all synthesised compounds	331
Appendix 16	Bioavaibility Radar from SwissADME	342
Appendix 17	MIC assay against gram-positive bacteria	346
Appendix 18	MIC assay against gram-negative bacteria	347
Appendix 19	Grid parameter file (GPF) from molecular docking	348
Appendix 20	Docking parameter file (DPF) from molecular docking	348

Appendix 21	Parameter of metal ions file	349
Appendix 22	Docking log file (DLG) from molecular docking	349
Appendix 23	Localisation of all compounds at <i>E. coli</i> DHFR from molecular docking	350
Appendix 24	Binding energy of all investigated compounds from molecular docking in triplicate	350
Appendix 25	Protein coordination file (.gro) from MD simulation	351
Appendix 26	Ligand coordination file (.gro) from MD simulation	352
Appendix 27	Equilibration parameter file (NVT) from MD simulation	353
Appendix 28	Equilibration parameter file (NPT) from MD simulation	354
Appendix 29	RMSD plot of DHFR-APO in triplicate from MD simulation	355
Appendix 30	RMSD plot of DHFR-FOL in triplicate from MD simulation	355
Appendix 31	RMSD plot of DHFR-TMP in triplicate from MD simulation	356
Appendix 32	RMSD plot of DHFR-SB4 in triplicate from MD simulation	356
Appendix 33	RMSD plot of DHFR-CUSB4 in triplicate from MD simulation	357
Appendix 34	RMSD plot of DHFR-COSB4 in triplicate from MD simulation	357
Appendix 35	RMSD plot of DHFR-NISB4 in triplicate from MD simulation	358
Appendix 36	RMSD plots of protein ligand complexes from MD simulation	359
Appendix 37	RMSD plot of ligand from MD simulation	360
Appendix 38	Radius of gyration plot of protein ligand complexes from MD simulation	361

Appendix 39	RMSF plot of protein ligand complexes from MD simulation	362
Appendix 40	Pocket volume plot of protein ligand complexes from MD simulation	363
Appendix 41	List of publications extracted from this study	364
Appendix 42	List of conferences attended and presentations extracted from this study	365



CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Bacterial infections have long posed a major threat to human health, leading to high morbidity and mortality (Murphy et al., 2024; Russu et al., 2024; Soni et al., 2024). The rise of antibiotic resistance has worsened the situation, making infections harder to treat. Among them, *Escherichia coli* (*E. coli*) has become a growing concern due to its rapid global spread (Ashinze et al., 2024; Asif et al., 2024; Mandujano-Hernández et al., 2024; Mujahid et al., 2024.) (Figure 1.1). *E. coli* infections range in various clinical conditions from urinary tract infections (UTIs) and food poisoning to severe cases like hemolytic uremic syndrome (HUS), which can be life-threatening (Ahmed & Ahmed, 2024; Eissa, 2024). According to the World Health Organization (WHO), drug-resistant *E. coli* cases have increased significantly over the past decade, with resistance rates to common antibiotics exceeding 50% in some regions (WHO, 2022). Of particular concern are strains producing extended-spectrum beta-lactamases (ESBLs) and carbapenemases, rendering many first-line antibiotics ineffective (Husna et al., 2023; Mahamat et al., 2024). In Malaysia, bacterial infections remain a significant health concern. The Ministry of Health's 2024 Health Facts report indicates that certain infectious and parasitic diseases accounted for 7.70% of hospitalisations in public hospitals (Health Facts, 2024). Notably, diseases of the respiratory system, which can include bacterial infections like pneumonia, constituted 15.30% of hospitalisations. These challenges have greatly limited treatment options and placed immense pressure on healthcare systems.

DEADLY INFECTIONS

These 6 pathogens were responsible for almost 80% of the 1.27 million deaths attributed directly to antimicrobial resistance in 2019.

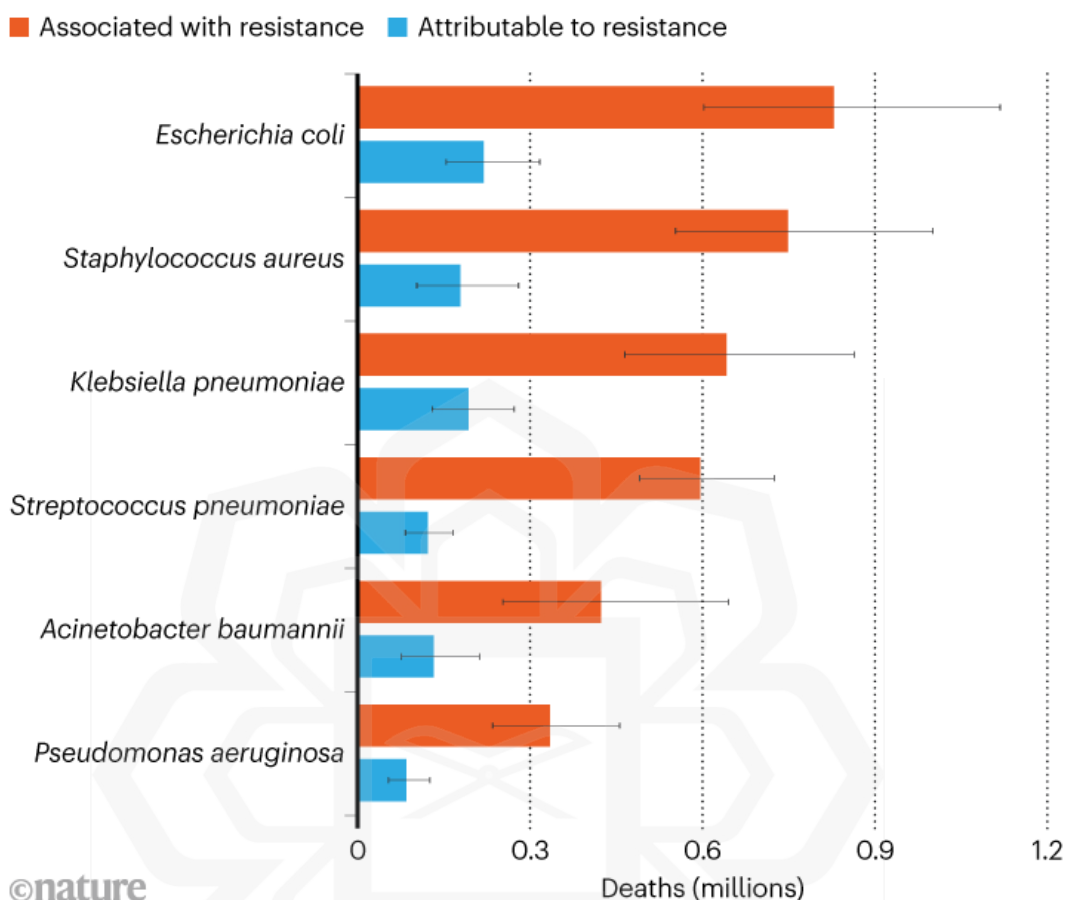
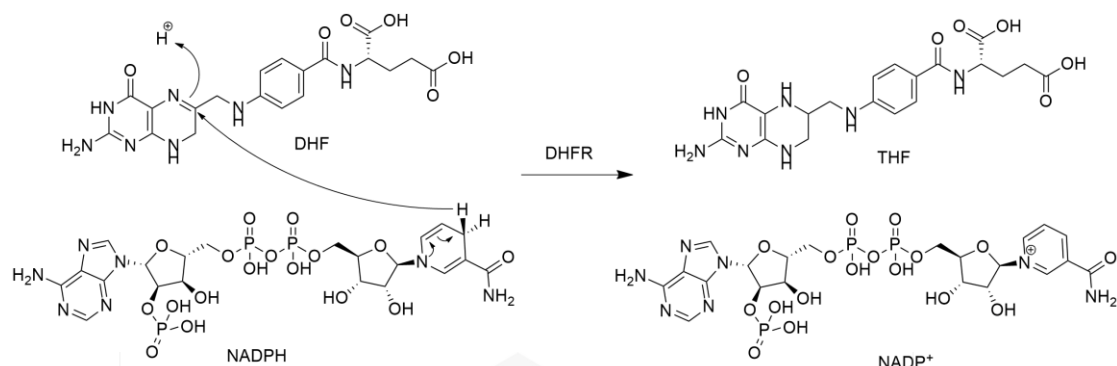


Figure 1.1 The number of pathogens attributed to AMR in 2019. (Adopted from Nature, (2022))

As bacterial infections continue to pose a serious global challenge, the need for new antibacterial agents targeting key bacterial pathways has become increasingly urgent. One such pathway is the folate biosynthesis pathway, which is essential for bacterial growth and reproduction. This pathway is involved in producing nucleotides, purines, and certain amino acids, all necessary for DNA replication and cell division (Bertacine Dias et al., 2018). A crucial enzyme in this pathway is dihydrofolate reductase (DHFR), which converts dihydrofolate (DHF) into tetrahydrofolate (THF), a compound needed for nucleotide production (Loveridge et al., 2017). DHFR performs this conversion with the help of NADPH as a coenzyme (Scheme 1.1). Inhibiting DHFR effectively disrupts DNA synthesis and cell division, leading to bacterial growth arrest. Notably, bacteria rely heavily on DHFR for survival and lack backup systems to

overcome its inhibition (T. Li et al., 2023). Thus, targeting DHFR is a promising approach for treating bacterial infections and could lead to the development of effective new treatments.



Scheme 1.1 Reduction of dihydrofolate (DHF) to tetrahydrofolate (THF)

The inhibition of bacterial DHFR has been extensively studied, and several compounds have demonstrated significant potential. Among these, trimethoprim (TMP) is one of the most widely used antibacterial agents in clinical practice. TMP was discovered in the 1940s when George Hitchings and Gertrude Elion found that 2,4-diaminopyrimidines could disrupt folate metabolism (Rubin, 2007). Their work led to the development of TMP, the first effective and selective inhibitor of bacterial DHFR (Figure 1.2). TMP is highly selective for bacterial DHFR, such as those in *Staphylococcus aureus* and *Escherichia coli*, with minimal effect on the human enzyme (Estrada et al., 2016). This selectivity, combined with its strong antibacterial activity and the ability to be taken orally, has made TMP a crucial drug for treating bacterial infections.

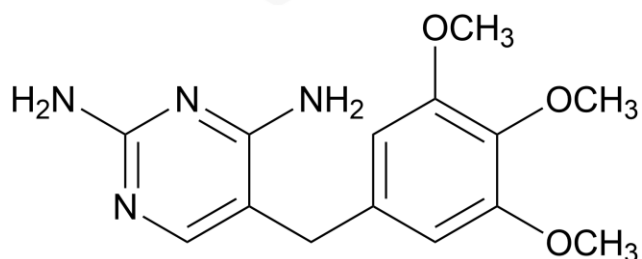


Figure 1.2 Chemical structure of trimethoprim (TMP)

Trimethoprim's chemical structure consists of pyrimidine-2,4-diamine and 2,3,4-trimethoxybenzene connected by a methylene bridge. This arrangement allows

for its selective targeting of bacterial DHFR (Waghchaure et al., 2023). X-ray crystallographic studies have confirmed that TMP poorly binds to the nucleotide-binding site of mammalian DHFR but fits well within the homologous site of bacterial DHFR (Lange et al., 2023). This structural specificity makes bacterial DHFR enzymes 20-60 times more susceptible to TMP than their mammalian counterparts, providing the drug with a favourable safety profile and minimising side effects (Corral et al., 2018). However, despite its effectiveness, the widespread TMP has led to bacterial resistance (Manna et al., 2021). TMP resistance in *E. coli* DHFR often arises from point mutations that reduce TMP binding affinity while preserving enzymatic activity. One important mutation is at LEU28, where a change to arginine (L28R) disrupts the hydrophobic pocket near the TMP binding site (Manna et al., 2021; Zhang et al., 2021). These mutation significantly lowered TMP binding affinity and are frequently found in clinical and laboratory-evolved resistant strains of *E. coli*. Hence, these challenges highlight the pressing need for the development of new DHFR inhibitors.

Trimethoprim's success as a first-generation DHFR inhibitor has established a strong foundation for the advancement of antibacterial therapies. With progress in drug design and structural biology, next-generation DHFR inhibitors have been developed. In the search for alternative scaffolds, dithiocarbazate derivatives have emerged as a promising class of compounds. Among these, derivatives of *S*-benzylidithiocarbazate (SBDTC) (Figure 1.3) have garnered particular attention due to their versatility and significant biological activity. First synthesised by Ali and Bose in 1977, SBDTC features a dithiocarbazate moiety (-NH-NH-C(=S)-SH), which allows for extensive chemical modifications to enhance its pharmacological properties (M. A. Ali & Bose, 1977; Azam et al., 2021). Research has demonstrated that SBDTC exhibited potent antibacterial activity against both gram-positive and gram-negative bacteria, including strains resistant to antibiotics (Bhattacharjee et al., 2021; Sahu et al., 2023; Sohtun et al., 2022). These findings demonstrate the potential of SBDTC as a novel scaffold for the development of antibacterial drugs for effective treatments in the face of increasing bacterial infections.

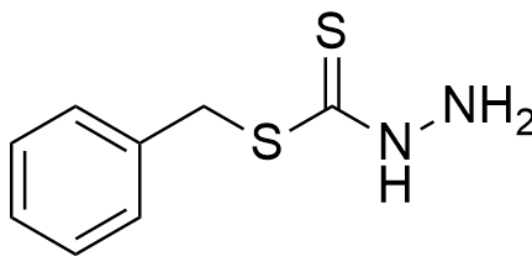


Figure 1.3 Chemical structure of *S*-benzylthiocarbamate (SBDTC)

Due to its flexibility, SBDTC can be easily modified to form various SBDTC derivatives. SBDTC derivatives are well-suited for metal coordination due to the presence of sulfur and nitrogen atoms, which serve as donor sites to form stable metal complexes. Integrating metal ions into SBDTC derivatives offers a promising strategy to enhance their therapeutic potential. Studies have shown that these metal complexes exhibit improved antibacterial, anticancer, and antitumor activities compared to their parent ligands (Elzaref et al., 2023; Hossain et al., 2021; Kudrat-E-Zahan & Islam, 2015; Latif et al., 2023). Transition metals such as copper(II), zinc(II), cobalt(II), and nickel(II) exhibit unique physicochemical properties, including varied geometries, thermal stability, and electronic configurations, which contribute to their biological activity (Dutta et al., 2002; Pavan et al., 2010; Sahu et al., 2023). These properties allow metal complexes to engage in novel mechanisms of action that are difficult to achieve with organic compounds alone (B. Sharma et al., 2022). As a result, metal complexes are widely studied for their potential in treating human diseases.

Since the 1970s, when SBDTC derivatives were first synthesised, significant advancements have been made in their development. Early research primarily focused on synthesising and characterising these compounds, while studies on their biological activities began gaining attention in the early 2000s (Dutta et al., 2002; Khoo Teng Jin, 2008). In recent years, computational tools like molecular docking have been employed to predict their interactions with protein targets (Moustafa et al., 2021). However, despite their promising *in vitro* activity, many studies have remained limited to biological assays or preliminary docking analyses, leaving the molecular mechanisms of action, largely unexplored. Additionally, while metal complexes derived from SBDTC derivatives have shown enhanced antibacterial efficacy, their potential as *E. coli* DHFR inhibitors remain underexplored, highlighting a valuable direction for future research.

Computational methods have greatly transformed drug discovery, providing cost-effective and efficient alternatives to traditional experimental screening. These methods make the drug discovery process more accessible to researchers with limited resources (Tiwari et al., 2022). Among these, absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies are essential for evaluating the pharmacokinetic and safety profiles of compounds (Butina et al., 2002; Y. Wang et al., 2015). They provide critical insights into properties such as solubility, permeability, metabolic stability, and toxicity (Norinder & Bergström, 2006; Y. Wang et al., 2015). Additionally, molecular docking has emerged as a widely used computational technique for predicting the binding affinity and interaction between ligands and target proteins (Seeliger & De Groot, 2010). While docking simulations offer valuable insights into binding patterns and ligand affinities, it is limited by their static nature (Sousa et al., 2006). Consequently, essential aspects such as structural flexibility and the role of metal ions remain underexplored. To complement docking studies, molecular dynamics (MD) simulations offer a dynamic perspective on the stability and behaviour of protein-ligand complexes over time (Bekker et al., 1993; E. Wang et al., 2019). MD simulations account for conformational flexibility, solvent effects, and thermal fluctuations, offering a more accurate depiction of molecular interactions (E. Wang et al., 2019).

Given the significant antibacterial potential of SBDTC derivatives and the additional benefits provided by metal complexes, this study aimed to identify a new antibacterial agent as an alternative to TMP. The research began with the design and synthesis of four new SBDTC derivatives and their corresponding copper(II), zinc(II), cobalt(II), and nickel(II) complexes as potential *E. coli* DHFR inhibitors. The synthesised compounds were characterised using various analytical techniques, including melting point determination, elemental analysis (CHNS), atomic absorption spectroscopy (AAS), Fourier-transform infrared (FTIR) spectroscopy, ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), ultraviolet-visible (UV-Vis) spectroscopy, molar conductivity, magnetic susceptibility and thermogravimetric analysis (TGA). ADMET predictions were conducted using SwissADME and Protox-II to evaluate the drug-likeness and toxicity of the compounds.

To assess the antibacterial efficacy of these compounds, both *in vitro* and *in silico* approaches were employed. The antibacterial activity was evaluated using the

minimum inhibitory concentration (MIC) assay against gram-positive and gram-negative bacteria, including *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922). Molecular docking was conducted to evaluate the binding interactions of selected compounds with *E. coli* DHFR (PDB ID: 1RX7) using AutoDock 4.2. Furthermore, molecular dynamics simulations were performed using GROMACS to validate the stability of the selected compounds within the *E. coli* DHFR binding site. This combined experimental and computational approach provides a comprehensive evaluation of the potential of SBDTC derivatives and their metal complexes as *E. coli* DHFR inhibitors.

1.2 PROBLEM STATEMENT

Bacterial infections, especially those caused by *E. coli*, remain a major global health concern, leading to high rates of illness and death (Ashinze et al., 2024; Husna et al., 2023). *E. coli* is a versatile pathogen, causing conditions ranging from urinary tract infections to severe sepsis (Mandujano-Hernández et al., 2024). One critical enzyme that makes an excellent target for antibacterial drugs is dihydrofolate reductase (DHFR). Trimethoprim (TMP), a widely used DHFR inhibitor, has been a key player in treating bacterial infections. However, its effectiveness has been greatly reduced due to the rise of drug-resistant *E. coli* strains driven by bacterial mutations (Manna et al., 2021). Point mutations at key active site residues, such as L28R and I94L, significantly reduce TMP binding affinity, undermining its antibacterial efficacy (Zhang et al., 2021). This growing resistance crisis highlights the urgent need for innovative antibacterial agents that can effectively target bacterial DHFR and capable of overcoming these resistance-conferring mutations.

Among the promising candidates for antibacterial drug development are dithiocarbazate derivatives, particularly those from *S*-benzylidithiocarbazate (SBDTC). These compounds have demonstrated strong antimicrobial properties, which can be notably enhanced when coordinated with metal ions such as copper(II), zinc(II), cobalt(II), and nickel(II). Metal coordination not only improves the physical and chemical properties of these derivatives but also introduces novel mechanisms of action, positioning them as potential candidates for the development of new antibacterial agents (B. Sharma et al., 2022). However, despite these advancements, the precise mechanism

by which SBDTC derivatives and their metal complexes exert their antibacterial effects, especially in targeting *E. coli* DHFR, remains inadequately understood.

To address these gaps, this research focused on the design, synthesis, and evaluation of SBDTC derivatives and their metal complexes as potential inhibitors of *E. coli* DHFR. Advanced computational methods, particularly molecular dynamics (MD) simulations, were employed to complement and extend the insights provided by molecular docking studies. Overall, the ultimate goal of this work is to contribute to the development of next-generation antibacterial agents that could serve as effective alternatives to TMP.

1.3 RESEARCH SIGNIFICANCE

The significance of this study lies in its potential to contribute to the development of new antibacterial agents that target *E. coli* DHFR. By designing and synthesising SBDTC derivatives and their metal complexes, this research opens the door for creating compounds with improved antibacterial activity of TMP. These derivatives, with their ability to bind metal ions, may offer new ways to tackle bacterial infections that are difficult to treat with current antibiotics.

Additionally, this study advances the understanding of how these compounds interact with bacterial DHFR and their potential as effective alternatives to TMP. The use of computational tools, including molecular dynamics simulations, allows for a deeper insight into the binding mechanisms and stability of these compounds, which could lead to more targeted and efficient drug development.

1.4 RESEARCH QUESTIONS

1. How SBDTC derivatives and their metal complexes be rationally designed as *E. coli* DHFR inhibitors using TMP as a reference?
2. Can the designed SBDTC derivatives and their metal complexes be synthesised and characterised to confirm their structural, physical and chemical properties?
3. Can SBDTC derivatives and their metal complexes be considered potential drug candidates based on ADMET prediction evaluation?
4. How effective are SBDTC derivatives and their metal complexes against gram-positive and gram-negative bacteria compared to TMP?

5. What are the binding affinities, binding interactions and stabilities exhibited by SBDTC derivatives and their metal complexes with *E. coli* DHFR compared to TMP, as determined by molecular docking studies and molecular dynamics simulation?

1.5 RESEARCH HYPOTHESIS

Four series of SBDTC derivatives and their metal complexes will be rationally designed, guided by TMP. Following the design phase, these compounds will be successfully synthesised as potential *E. coli* DHFR inhibitors. Comprehensive characterisation of these compounds will confirm their structures, physical and chemical properties. The ADMET evaluation of these compounds will satisfy essential drug-like criteria, indicating favourable pharmacokinetic profiles and minimal toxicity, thereby establishing their potential as promising drug candidates.

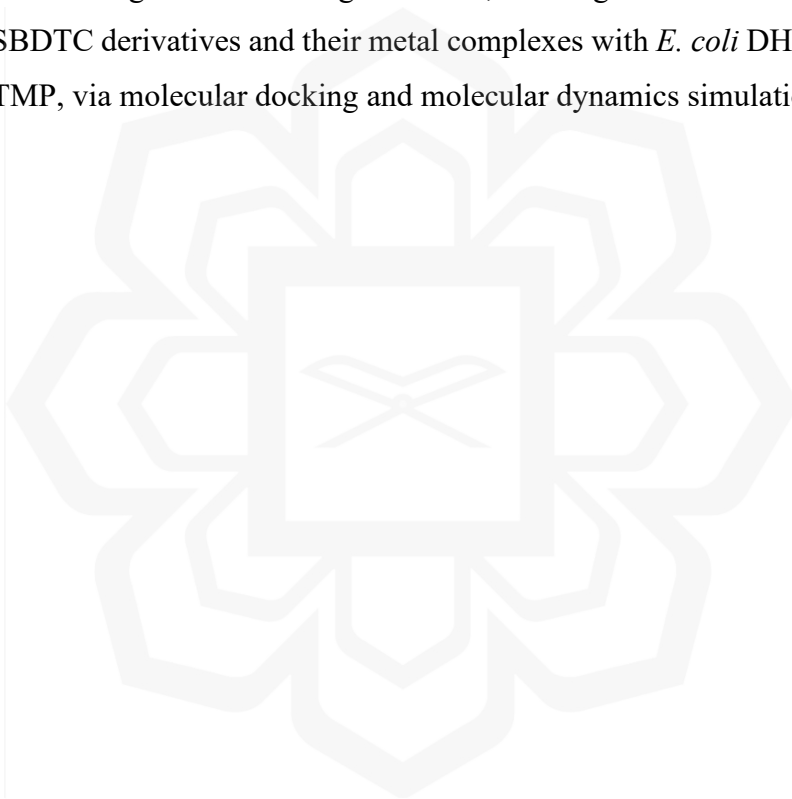
In vitro antibacterial assays will reveal that these compounds exhibit significant inhibitory activity against both gram-positive and gram-negative bacteria, surpassing the efficacy of TMP. Molecular docking studies will provide valuable insights into the binding interactions of the selected compounds with *E. coli* DHFR. These analyses will show that these compounds interact with key active site residues through specific hydrogen bonds and hydrophobic interactions, similar to TMP. These interactions will contribute to strong binding affinities, further supporting their role as effective DHFR inhibitors.

Furthermore, MD simulations will validate the stability of these compounds within the *E. coli* DHFR binding site over time. The simulations will highlight consistent binding patterns, conformational stability, and favorable protein-ligand dynamics, demonstrating improved stability and interactions compared to TMP. Collectively, all these findings will highlight the therapeutic potential of SBDTC derivatives and their metal complexes as novel antibacterial agents, especially as *E. coli* DHFR inhibitors, with superior efficacy compared to TMP.

1.6 RESEARCH OBJECTIVES

The aims of this research are:

1. To rationally design four series of SBDTC derivatives and their metal complexes as *E. coli* DHFR inhibitors, using TMP as reference.
2. To synthesise and characterise the designed SBDTC derivatives and their metal complexes.
3. To predict the ADMET properties of SBDTC derivatives and their metal complexes using computational tools.
4. To evaluate the antibacterial activity of SBDTC derivatives and their metal complexes against gram-positive and gram-negative bacteria in comparison to TMP.
5. To investigate the binding affinities, binding interactions and stability of SBDTC derivatives and their metal complexes with *E. coli* DHFR, compared to TMP, via molecular docking and molecular dynamics simulation.

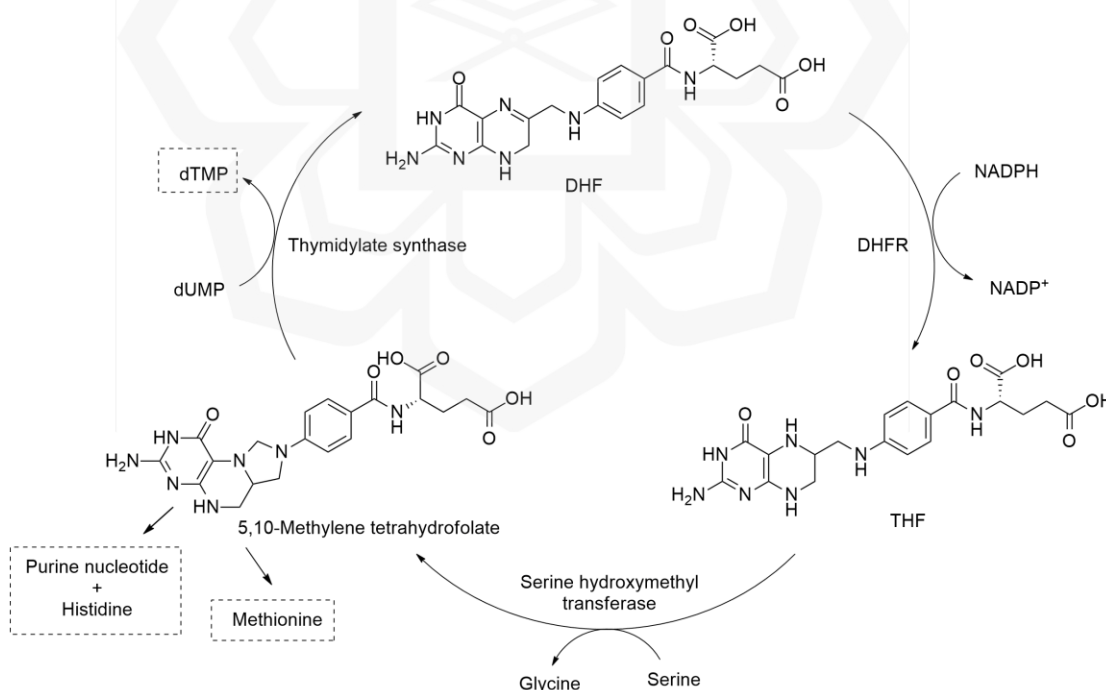


CHAPTER TWO

LITERATURE REVIEW

2.1 DIHYDROFOLATE REDUCTASE (DHFR)

Dihydrofolate reductase (DHFR) is a key enzyme in the folate biosynthesis pathway. It plays an essential role in DNA synthesis, repair, and cellular growth in both prokaryotic and eukaryotic organisms (Farnum et al., 1991). Due to its critical function in cellular metabolism, DHFR has become a major target for antimicrobial and anticancer therapies. DHFR catalyses the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF) using NADPH as a coenzyme as illustrated in Scheme 2.1 (Bertacine Dias et al., 2018). THF is the active form of folic acid and serves as a key carrier of one-carbon units. It participates in more than 20 metabolic reactions, including the synthesis of deoxythymidine monophosphate (dTMP) and deoxyuridine monophosphate (dUMP), which are crucial for DNA synthesis and cell division.



Scheme 2.1 Folate biosynthesis pathway

DHFR is an enzyme found in bacteria, fungi, parasites, and humans (Garcia-Viloca et al., 2003). While human and bacterial DHFR perform similar functions, their

structures and sequences differ significantly (Schnell et al., 2004). Human DHFR is a monomeric protein with a sequence similarity of 75–90% across vertebrates but only 30% similarity to its bacterial counterpart (Schnell et al., 2004). Structurally, bacterial DHFR possesses a smaller and more hydrophobic active site, containing key residues such as ILE5, ALA6, ASP27, and TYR100, whereas human DHFR features ASP30 and ARG70 (Bystroff & Kraut, 1991; Matthews et al., 1977). The hydrophobic nature of bacterial DHFR enhances its affinity for inhibitors like trimethoprim (Bystroff & Kraut, 1991). In contrast, the human DHFR active site is larger and less hydrophobic, making it less efficient at binding bacterial-specific inhibitors (Beard et al., 1991). These structural differences make bacterial DHFR an attractive target for the development of selective antimicrobial agents.

In this study, the bacterial DHFR protein was obtained from the crystal structure with PDB ID: 1RX7, representing *E. coli* DHFR in its occluded conformational state (Sawaya & Kraut, 1997). The protein has a molecular weight of approximately 18 kDa, consisting of eight β -strands and four α -helices (Figure 2.1). The active site is situated within the major subdomain, comprising approximately 100 residues from both the N- and C-terminal regions. This region is defined by three key loops collectively forming nearly half of the major subdomain and referred to as the "loop subdomain" (Osborne et al., 2001). As illustrated in Figure 2.1, these loops are color-coded, green for the MET20 loop (residues 9-24), blue for the F-G loop (residues 116-132), and red for the G-H loop (residues 142-150) (Boehr et al., 2006). The occluded form of DHFR is stabilised by hydrogen bonding interactions, particularly between ASN23 and SER148, which play a crucial role in maintaining loop conformation (Mhashal et al., 2017).

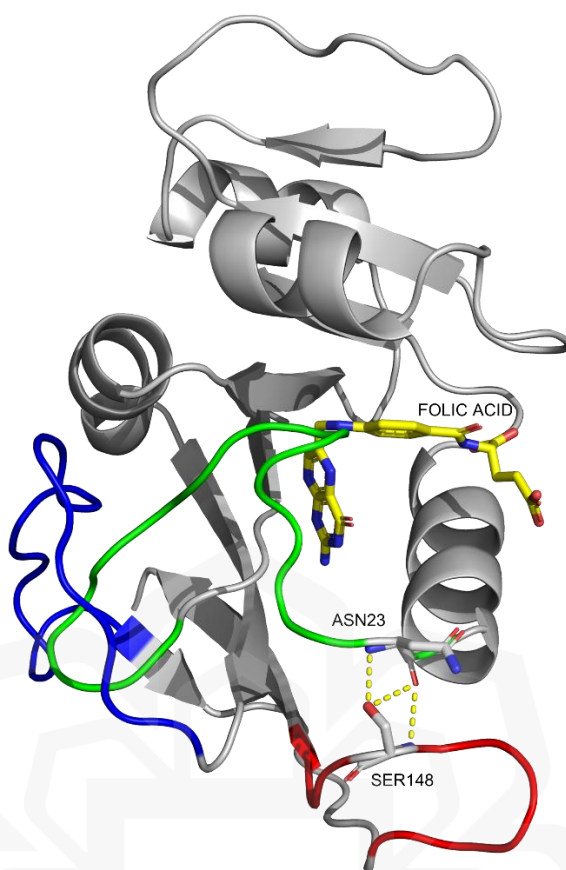


Figure 2.1 Structures representing the occluded loop conformation of *E. coli* DHFR (PDB ID: 1RX7)

The movement of the MET20 loop in *E. coli* DHFR plays a crucial role in the enzyme's catalytic activity. As described by Sawaya and Kraut (1997), the MET20 loop shifts between closed and occluded conformations, depending on the ligand bound to the enzyme (Figure 2.2). In the closed conformation, observed in the holoenzyme ($E \cdot \text{NADPH}$), the Michaelis complex ($E \cdot \text{NADPH} \cdot \text{DHF}$), and the transition state ($E \cdot \text{NADPH} \cdot \text{THF}$), the loop tightly interacts with NADPH, shielding the active site from solvent exposure (Sawaya & Kraut, 1997). On the other hand, in product complexes such as $E \cdot \text{THF} \cdot \text{NADP}^+$, $E \cdot \text{THF}$, and $E \cdot \text{THF} \cdot \text{NADPH}$, the MET20 loop adopts an occluded conformation. In this state, the loop blocks the nicotinamide-binding pocket, forcing the nicotinamide-ribose moiety of NADPH to be exposed to the solvent instead of binding to its site. While the free form of *E. coli* DHFR during catalysis has not been directly observed, crystallographic studies suggest that the apo form may exist in an open or disordered state (Venkitakrishnan et al., 2004). Computational simulations indicate that the energy barriers between these conformations are minimal, with ligand

binding affecting their relative stabilities. Therefore, the dynamic shifts in the MET20 loop's conformation are closely linked to the enzyme's catalytic cycle, and understanding these structural changes is essential for exploring the enzyme's mechanism and its potential as a drug target.

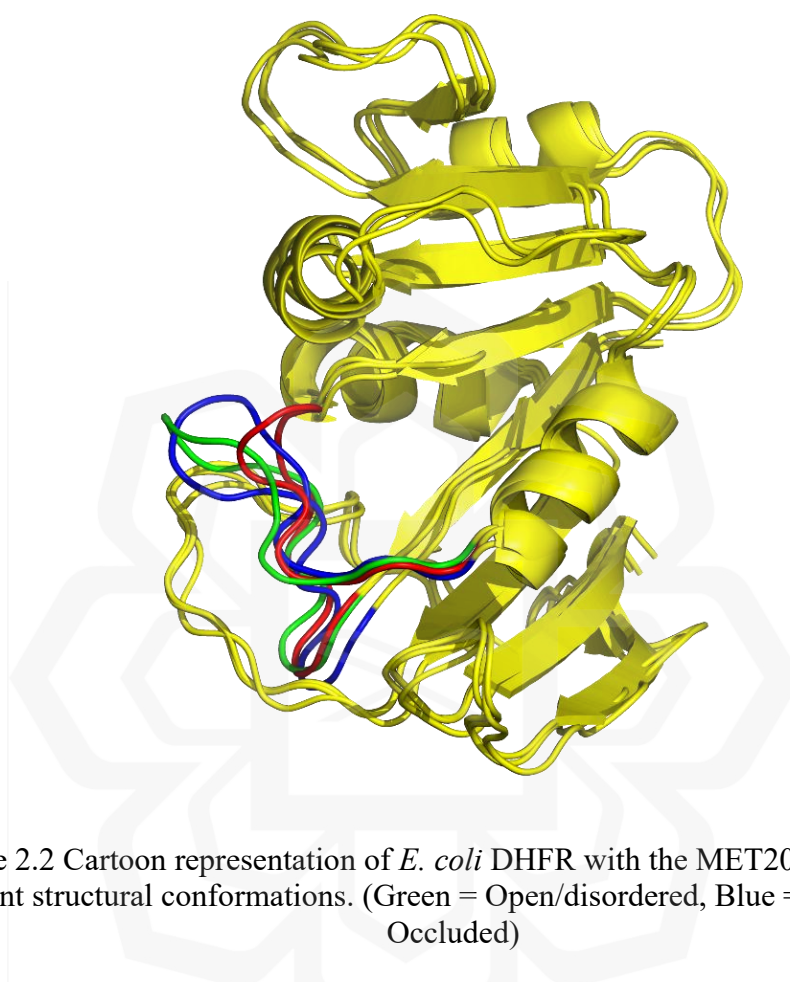


Figure 2.2 Cartoon representation of *E. coli* DHFR with the MET20 highlighted in different structural conformations. (Green = Open/disordered, Blue = Closed, Red = Occluded)

Behiry (2013) identified the occluded conformation as a promising target for antibacterial drug development due to its specificity to bacterial DHFR. In *E. coli* DHFR, the occluded conformation is stabilised by hydrogen bonds between SER148 in the GH loop and ASN23 in the MET20 loop, as illustrated in Figure 2.3. Many other DHFR enzymes lack the critical interaction with SER148, which is essential for stabilising the occluded conformation (Behiry, 2013). This suggests that the occluded state is not universally conserved across DHFR enzymes and varies between organisms. Supporting this, Hughes et al. (2017) demonstrated that the occluded conformation in *E. coli* DHFR is crucial for its function. In *E. coli*, the products of the enzyme's reaction, NADP⁺ and THF, can accumulate and slow down enzyme activity (Hughes et al., 2017). To prevent this, the enzyme adopts the occluded conformation, blocking the cofactor

binding site and maintaining balanced activity. In contrast, human DHFR works differently and does not rely on the occluded conformation. Human cells have lower levels of NADP^+ and THF, so human DHFR binds the cofactor tightly and releases it slowly to stay efficient. These differences confirm that the occluded conformation in *E. coli* DHFR could be a unique and selective target for antibacterial drugs.

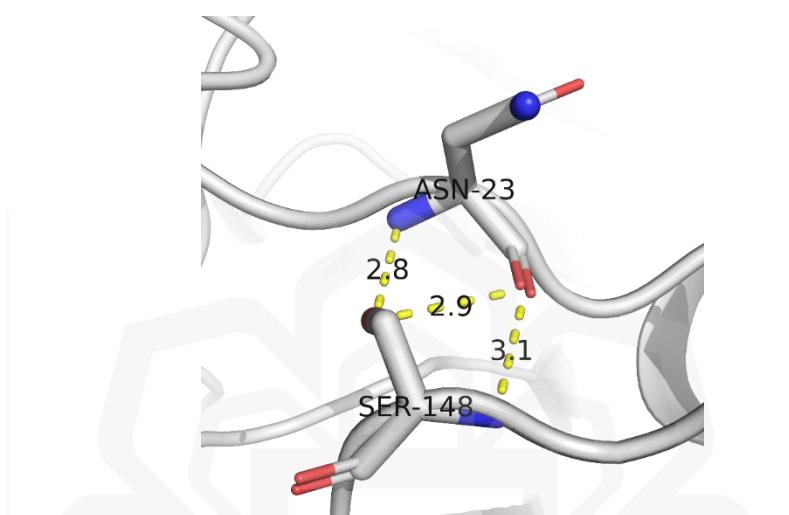


Figure 2.3 Cartoon representative of hydrogen bond interaction between ASN18 with SER148

In occluded conformation, as observed in the DHFR crystal structure (PDB ID: 1RX7), the enzyme is bound to folic acid, a precursor to dihydrofolate (DHF). Srinivasan et al. (2015) demonstrated that the folate molecule can be divided into three fragments, each interacting uniquely with the active-site pocket and surrounding residues, as illustrated in Figure 2.4 (Srinivasan et al., 2015). First, the 2-amino-4-oxo-1,4-dihydropteridine moiety (in red dashed circle) interacts with key residues within the active-site pocket, including ILE5, ALA6, ALA7, ASP27, ILE94, and THR113. Second, the central methylaminobenzoyl group of folates (in a blue dashed circle) interacts with a hydrophobic region formed by LEU28, PHE31, LYS32, THR46, ILE50, and LEU54, which accommodates compounds with similar hydrophobic properties. Finally, the polar and negatively charged glutamic acid tail of folate (in green dashed circle) interacts with a positively charged triad, ARG52, and ARG5 located at the entrance of the binding site. These residues facilitate binding by readily interacting with charged amino acids.

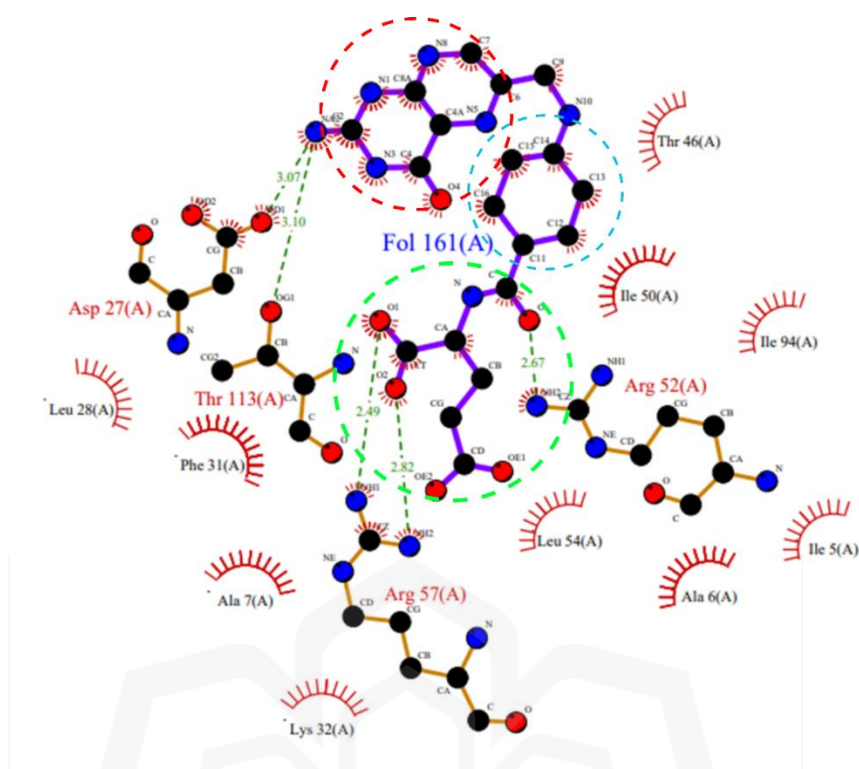


Figure 2.4 Residues interacting with folic acid at *E. coli* DHFR (PDB ID: 1RX7)

2.1.1 FDA-approved DHFR Inhibitors

DHFR inhibitors are classified into classical and non-classical antifolates based on their chemical scaffold (Sehrawat et al., 2023). Classical inhibitors mimic the substrate, dihydrofolate, and compete for the enzyme's binding site (Beard et al., 1991). These inhibitors resemble folic acid and include a para-aminobenzoyl glutamic acid side chain. They are competitive in nature and contain a 2,4-diamino-1,3-diaza pharmacophore group. Classical DHFR inhibitors include diaminoquinazoline, diaminopyrimidine, diaminopteridine, and diaminotriazine derivatives (Bolstad et al., 2008; Riyadh et al., 2018; Srinivasan et al., 2015).

Methotrexate (MTX) is one of the most well-known classical DHFR inhibitors (Figure 2.5). It is used to treat cancers such as leukaemia, breast cancer, and non-Hodgkin lymphoma, as well as psoriasis and rheumatoid arthritis (McGuire, 2005). Cronstein & Bertino (2000) showed its effectiveness in inducing remission in leukaemia. Later studies explored its use in managing autoimmune diseases and combination therapies (Cronstein & Bertino, 2000).

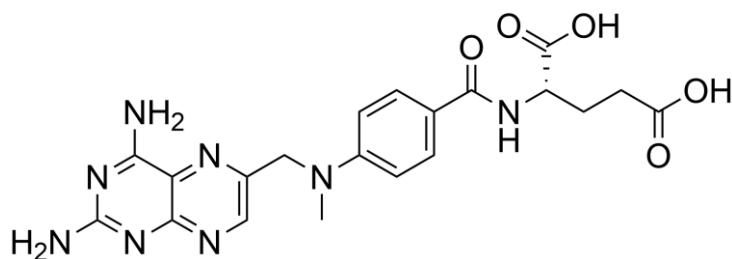


Figure 2.5 Chemical structure of methotrexate (MTX)

Non-classical DHFR inhibitors, such as trimethoprim, pyrimethamine, and iclaprim (Figure 2.6), differ structurally from classical inhibitors (Then, 2004; Wright & Anderson, 2011). Unlike classical inhibitors, which have a *p*-aminobenzoyl glutamic acid side chain, non-classical inhibitors contain a lipophilic side chain. This modification helps overcome resistance mechanisms like reduced active transport, diminished polyglutamation, and mutations in DHFR that affect classical inhibitors' efficacy (M. Sharma & Chauhan, 2012). Rao et al. (2013) highlighted several advantages of non-classical inhibitors, including higher potency, improved lipid solubility, and better cellular uptake (Rao et al., 2013). These properties allow selective targeting of microbial DHFR while minimising effects on human DHFR, reducing potential side effects. Therefore, non-classical antifolates are promising candidates for next-generation bacteria DHFR inhibitors.

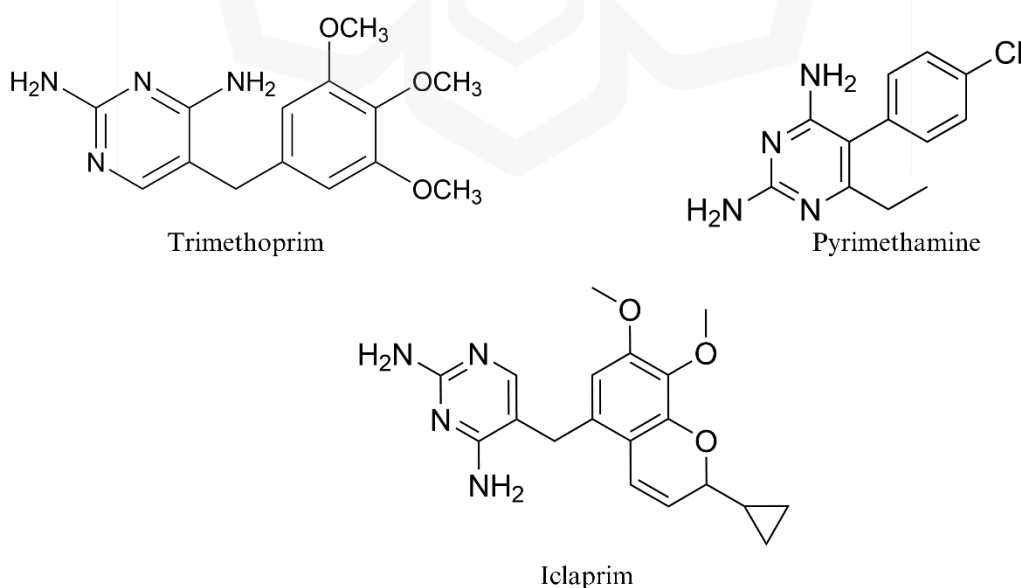


Figure 2.6 Structures of selected non-classical DHFR inhibitors

Trimethoprim (TMP) is a well-known non-classical DHFR inhibitor that targets bacterial DHFR. It was first synthesised in 1962 and introduced clinically in 1968 as part of a combination therapy with sulfamethoxazole (Bushby, 1973; Forgacs et al., 2009). TMP acts as a structural analogue of dihydrofolate (DHF), binding strongly to bacterial DHFR and blocking its ability to convert DHF to THF (Shivashankar & Kannan, 2021). TMP quickly became a widely used treatment for urinary tract infections (UTIs), especially in developing countries, due to its affordability (Jorba et al., 2021). In Malaysia, TMP combined with sulfamethoxazole accounts for over one-third of antibiotics prescribed for UTIs. However, its widespread use has contributed to the emergence of resistant bacterial strains, reducing its efficacy and leading to frequent treatment failures (L. Yu, 2024). As a result, TMP is now prescribed more cautiously for infections where resistance is less likely to preserve its clinical utility.

A key feature of TMP's structure (Figure 2.7) is its 2,4-diaminopyrimidine ring (in red dashed circle) which is a crucial pharmacophore for DHFR inhibition (Pavithra et al., 2024). Upon binding to bacterial DHFR, the protonated aminopyrimidine group forms hydrogen bonds with key residues like ASP27, ILE94, and ILE5 (Wróbel, Arciszewska, et al., 2019). Its trimethoxybenzyl group (in a blue dashed circle) fits into the hydrophobic pocket, interacting with MET20, LEU28, ILE50, PHE31, and ILE94. In *E. coli* DHFR, TMP's benzyl side chain is positioned "low" within the active site pocket, enhancing its binding affinity and antibacterial effectiveness (Wróbel, Maliszewski, et al., 2019). These structural insights into TMP's interactions with bacterial DHFR provide valuable guidance for designing more effective therapeutics by modifying TMP's structure.

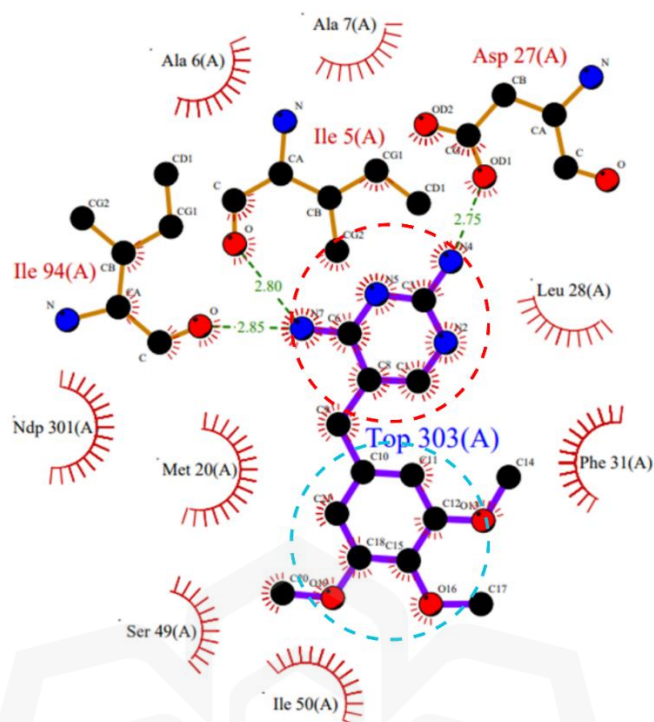


Figure 2.7 Residues interacting with TMP at *E. coli* DHFR (PDB ID: 6XG5)

TMP resistance in *E. coli* DHFR is primarily driven by point mutations that alter the drug-binding pocket while preserving the enzyme's ability to catalyse folate reduction. One of the most critical residues is LEU28, which lies within a hydrophobic pocket near the TMP binding site (Manna et al., 2021). In resistant strains, this residue is often mutated to arginine (L28R) or glutamine (L28Q). These substitutions introduce polar or charged side chains that disrupt the hydrophobic interactions essential for TMP binding, thereby reducing drug affinity without significantly affecting substrate binding (Zhang et al., 2021). Another important residue is ILE94, located near the active site. It is commonly mutated to leucine (I94L) (Manna et al., 2021). Although this is a conservative hydrophobic change, it subtly alters side-chain geometry and can shift the conformation of the binding pocket, reducing TMP compatibility. These mutations, either individually or in combination, significantly lower TMP binding affinity while retaining enzymatic activity, thus enabling bacterial survival under TMP treatment.

In response to TMP resistance, several strategies have been explored, including the development of novel bacterial DHFR inhibitors. Many of these inhibitors are derived from natural products or synthetic compounds that resemble TMP structurally. Examples include quinolizines and diaminopyrimidines, which maintain the same mechanism of action but offer enhanced potency and broader spectrum activity (X. Li

et al., 2011; Y. Li et al., 2022). Some of these inhibitors also show synergistic effects when combined with other antibiotics, boosting their antimicrobial efficacy. However, despite their effectiveness, these inhibitors often exhibit toxicity due to a lack of selectivity, presenting challenges for clinical use.

2.1.2 Bacteria DHFR inhibitors

The search for new DHFR inhibitors has gained significant attention in recent years due to the enzyme's crucial role in bacterial survival and replication. Researchers have explored various chemical scaffolds and structural modifications to develop compounds with increased potency and specificity against bacterial DHFR. Additionally, next-generation DHFR inhibitors have been designed to overcome the limitations of older drugs like trimethoprim (TMP) and methotrexate (MTX). This review highlights key findings from recent studies, revealing promising bacterial DHFR inhibitors and their potential for advancing therapeutic strategies.

Bayazeed et al. (2022) synthesised a series of new formazan derivatives and tested their antimicrobial properties (Bayazeed et al., 2022). Among these, compound (1) in Figure 2.8 stood out with significant activity against methicillin-resistant *Staphylococcus aureus* (MRSA), achieving a minimum inhibitory concentration (MIC) of 3.91 µg/mL. Additionally, compound (1) showed exceptional DHFR inhibition, with an IC₅₀ value of 0.142 µM, outperforming the standard drug TMP, which had an IC₅₀ of 0.188 µM. Notably, this study demonstrated that the presence of a nitro group enhanced antibacterial activity, while compounds lacking this group showed lower efficacy. These findings highlighted the potential of compound (1) as a promising dual-action agent, both as an antimicrobial and a potent DHFR inhibitor.

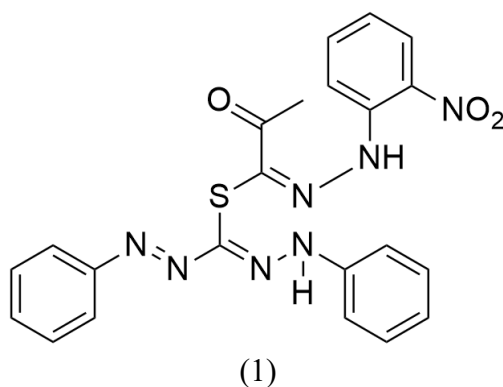


Figure 2.8 Structure of formazan derivative

Additionally, Dawood et al. (2023) synthesised a series of thiophenyl-pyrazolylthiazole hybrid derivatives (Dawood et al., 2023). Among these, compounds (2) and (3) in Figure 2.9 exhibited strong DHFR inhibition, with IC_{50} values of 4.21 μ M and 5.70 μ M, respectively. Remarkably, both compounds outperformed TMP, which has an IC_{50} value of 6.23 μ M. These compounds exhibited significant binding scores of -11.26 and -11.08 kcal/mol, respectively against *M. tuberculosis* DHFR. Notably, the study highlighted the role of sulfur in the thiazole moiety, which formed hydrogen bonds with the backbone of the key amino acid ILE94 at distances of 4.09 and 3.60 Å, respectively. This emphasises the importance of sulfur in enhancing interactions and contributing to antibacterial potency.

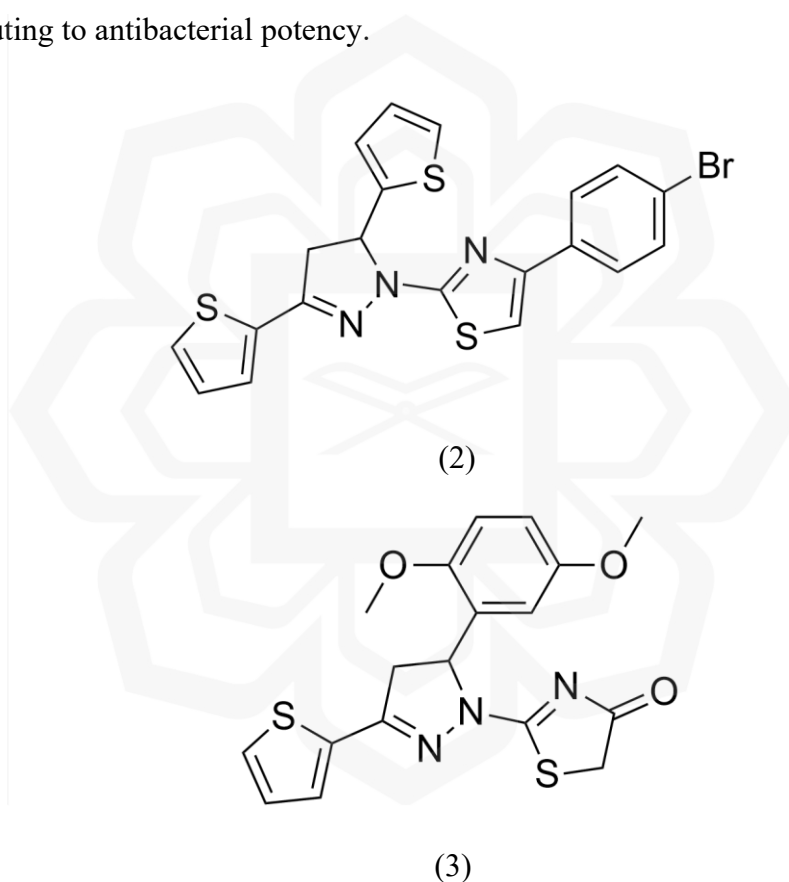
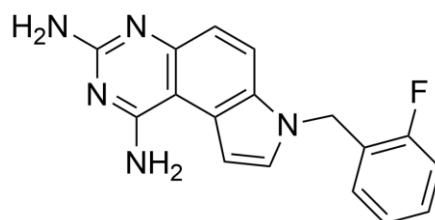


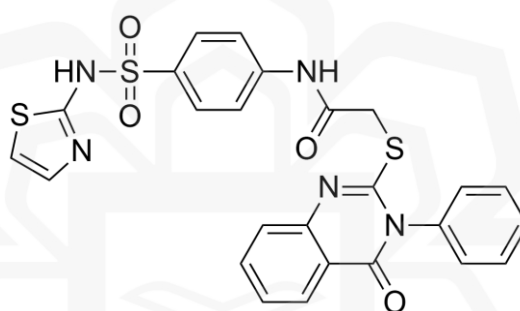
Figure 2.9 Structures of thiophenyl-pyrazolylthiazole hybrids derivatives

Another promising scaffold for DHFR inhibition is quinazoline. Li et al. (2022) synthesised a series of 1,3-diamino-7H-pyrrol [3,2-f]quinazoline derivatives, identifying compound (4) (Figure 2.10) as a standout compound (Y. Li et al., 2022). It demonstrated remarkable potency against *E. coli* DHFR, with an exceptionally low IC_{50} value of 2.36 ± 1.18 nM, making it over 20 times more effective than TMP ($IC_{50} = 61.60 \pm 11.78$ nM). Similarly, Osman et al. (2023) investigated quinazolinone derivatives,

with compound (5) showing strong inhibition of *E. coli* DHFR ($IC_{50} = 0.158 \pm 0.01 \mu M$), outperforming TMP ($IC_{50} = 0.226 \pm 0.014 \mu M$) (Osman et al., 2023). These studies emphasised the potential of quinazolinone-based scaffolds as a platform for developing the next generation of highly potent DHFR inhibitors.



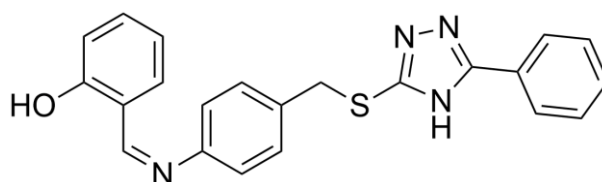
(4)



(5)

Figure 2.10 Structures of quinazolinone derivatives

In addition to the quinazoline scaffold, the triazole scaffold has emerged as another significant class of DHFR inhibitors. Dewangan et al. (2021) synthesised and evaluated Schiff base hybrids of 1,2,4-triazole-pyridine, with compound (6) (Figure 2.11) displaying notable antibacterial activity (Dewangan et al., 2021). The pyridine and nitrogen atoms in the triazole ring were found to strongly interact with amino acid residues in the DHFR active site. This study highlighted the potential of triazole-pyridine hybrids as promising DHFR inhibitors.



(6)

Figure 2.11 Structure of Schiff base hybrids of 1,2,4-triazole-pyridine

Similarly, Wahaibi et al. (2024) studied 1,2,4-triazolo[1,5-a]pyrimidine-based derivatives, identifying compounds (7) and (8), illustrated in Figure 2.12 as potent DHFR inhibitors with IC_{50} values of 4.60 μ M and 3.90 μ M, respectively, outperforming TMP (IC_{50} = 5.20 μ M) (Al-Wahaibi et al., 2024). The study highlighted the importance of the triazolo[1,5-a]pyrimidine scaffold and emphasised the structure-activity relationship (SAR) analysis of substituents on this scaffold. The methoxy group (-OMe) at the para-position significantly enhanced antibacterial activity, following the reactivity trend OMe > Me > Cl > H. Its oxygen atom forms a hydrogen bond with TYR59 in the DHFR binding site, improving binding affinity. Meanwhile, the chloro group (-Cl) at the para position also influences activity, with the reactivity order Cl > OMe. This effect is likely due to enhanced hydrophobic interactions with key amino acids in the enzyme pocket, contributing to the compound's antibacterial potential. Overall, this study highlighted the crucial role of methoxy and chloro groups in optimising antibacterial efficacy by strengthening molecular interactions at target sites.

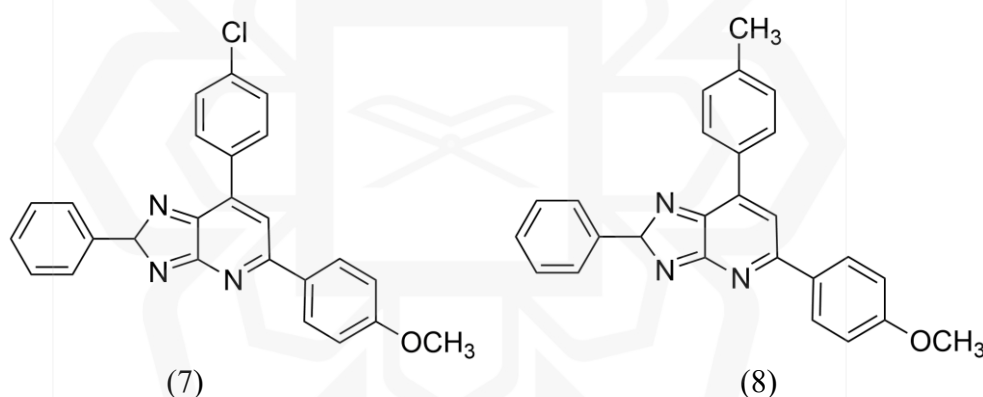
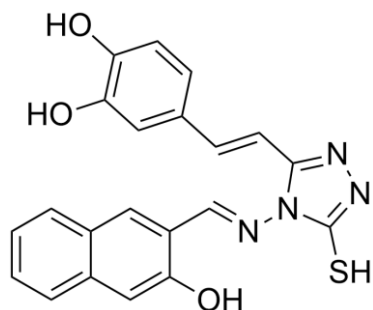


Figure 2.12 Structures of triazole derivatives

Sehrawat et al. (2024) developed a novel series of 1,2,4-triazole analogues based on caffeic acid, identifying key structural features that enhance DHFR inhibition and antibacterial activity. Among these, compound (9) (Figure 2.13) emerged as the most potent DHFR inhibitor, with an IC_{50} of 0.15 μ M, making it over 100 times more effective than TMP (IC_{50} = 15.33 μ M). This study highlights the role of bulky aromatic groups and electron-withdrawing groups (NO_2 , Cl, OH) on the aromatic ring in improving DHFR inhibition. The chlorine atom strengthened binding through halogen bonding and hydrophobic interactions with nonpolar regions of the DHFR active site. Additionally, nitro groups at the ortho and meta positions enhance both DHFR inhibition and antibacterial potency by forming hydrogen bonds, π -cation interactions,

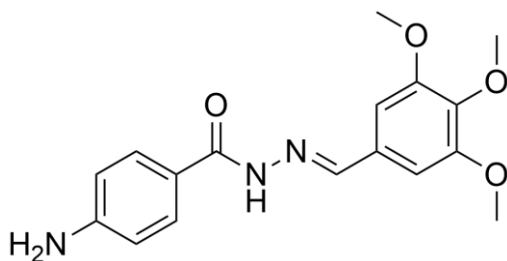
and salt bridges with key DHFR residues. Overall, this study emphasised that incorporating bulky aromatics, nitro, and chloro groups significantly enhances the antibacterial efficacy of DHFR inhibitors.



(9)

Figure 2.13 Structure of caffeic acid analogue

Ghany et al., (2024) synthesised a series of novel TMP analogues (Ghany et al., 2024), with compound (10) (Figure 2.14) demonstrating outstanding antimicrobial activity against a wide range of bacterial and fungal strains. Enzyme inhibition assays showed that compound (10) had an IC_{50} value of 3.731 $\mu\text{g/mL}$, making it three times more potent than TMP. SAR analysis revealed that adding hydrophobic or bulky substituents to the DHFR pharmacophoric scaffold significantly improved interactions with the bacterial DHFR active site. This study also highlights the importance of structural modifications, such as the incorporation of amide and amino groups, in enhancing the antimicrobial potency of existing drugs like TMP for more effective treatments. The amide and amino groups formed strong hydrogen bonds with DHFR's key residue, ILE50 and LEU5, respectively. Overall, these modifications contribute to stronger molecular interactions with the DHFR binding site, ultimately leading to enhanced antibacterial efficacy against resistant pathogens.



(10)

Figure 2.14 Structure of TMP analogue

A study by Rashid et al. (2016) explored novel TMP analogues and their antibacterial properties (Rashid et al., 2016). They synthesised hydroxyl trimethoprim by adding benzyloxy and phenyl ethanone groups at the 4-position of the dimethoxy benzyl ring to evaluate the effect of modification at this position. Among the derivatives, the benzyloxy TMP derivative (11) (Figure 2.15) exhibited the most promising activity, with MIC values of 5.0 μM against *E. coli* and 4.0 μM against *S. aureus*. In comparison, the standard TMP exhibited much higher MIC values of 55.1 μM against *E. coli* and 22.7 μM against *S. aureus*. These results demonstrated that modification at the 4-methoxy benzyl position significantly enhanced the antibacterial activity of TMP (Figure 2.15).

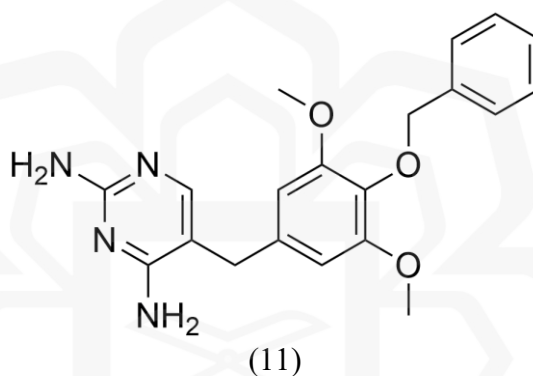


Figure 2.15 Structure of novel hydroxyl TMP analogue

In summary, these studies show how different chemical scaffolds and structural modifications are used to improve DHFR inhibition. In addition to scaffold design, key pharmacophores such as methoxy, nitro, chloro, and amide groups are important for antibacterial activity. These findings also highlight the need to combine the right scaffold with pharmacophore modifications to develop more potent DHFR inhibitors for treating bacterial infections. Furthermore, they provide a foundation for the rational design of novel bacterial DHFR inhibitors.

2.2 DITHIOCARBAZATE DERIVATIVES

Dithiocarbazate derivatives are organic compounds characterised by a thiosemicarbazide moiety ($-\text{NH}-\text{NH}-\text{C}(=\text{S})-$) with a thiol group ($-\text{SH}$) attached to a central carbon atom (Figure 2.16). Over the decades, dithiocarbazate and its substituted derivatives have gained significant attention. The first synthesis of a ligand from dithiocarbazate derivatives was accomplished by Ali and Livingstone in 1974 (Akbar

Ali & Livingstone, 1974). Since then, numerous studies have been published on dithiocarbazate derivatives substituted at the sulfur atom, with a predominant focus on *S*-benzyl dithiocarbazate (SBDTC).

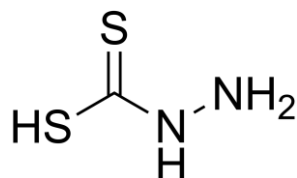


Figure 2.16 Chemical structure of dithiocarbazate backbone

SBDTC possesses four potential donor atoms, allowing it to coordinate with metal ions via either the NS or SS mode, resulting in SBDTC complexes (Akbar Ali & Livingstone, 1974). However, the IR spectra of most SBDTC complexes suggest a preference for NS chelated structures, as the changes in the C=S bond order are negligible compared to those in the C-N bond order (Akbar Ali & Livingstone, 1974). Furthermore, structural investigations indicate that SBDTC can exist in thione, thiol, or mixed tautomeric forms (Figure 2.17) (Akbar Ali & Tarafdar, 1977). SBDTC features a proton adjacent to the thione group, which is relatively unstable in its monomeric form and tends to convert to the more stable C-S bond through enethiolisation (Akbar Ali & Tarafdar, 1977).

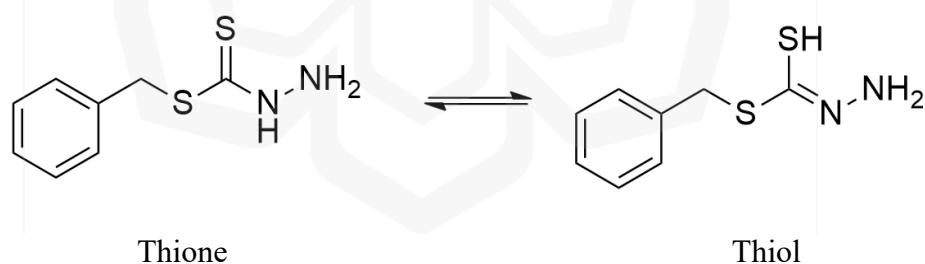


Figure 2.17 Tautomeric forms of SBDTC

Indeed, SBDTC continues to receive considerable attention due to its properties, which can be significantly modified by introducing different substituents to form new SBDTC derivatives or Schiff bases. Similar to SBDTC, SBDTC derivatives can undergo thione-thiol tautomerism to achieve stability (Tarafder et al., 2002). Hossain et al. (2021) reported on the thione-thiol tautomerism of their Schiff base derived from SBDTC with 2,3-dimethoxybenzaldehyde (Hossain et al., 2021). Interestingly, How et al. (2008) demonstrated another type of tautomerism in their SBDTC derivatives, which

possesses a carbonyl group, allowing for the formation of four tautomeric forms, thione-thiol-thionol-thiolol tautomerism (Figure 2.18) (How et al., 2008). The thione-thiol tautomerism, characterised by the transition of a C=S double bond to a single C-S bond, occurs due to the conversion of the dithiocarbazate ligand to its imino thiolate (Ravoof et al., 2010). Meanwhile, the conversion of a C=O double bond to a single C-O bond is attributed to the tautomerism of the dithiocarbazate ligand to its enol form, resulting in thionol-thiolol tautomerism.

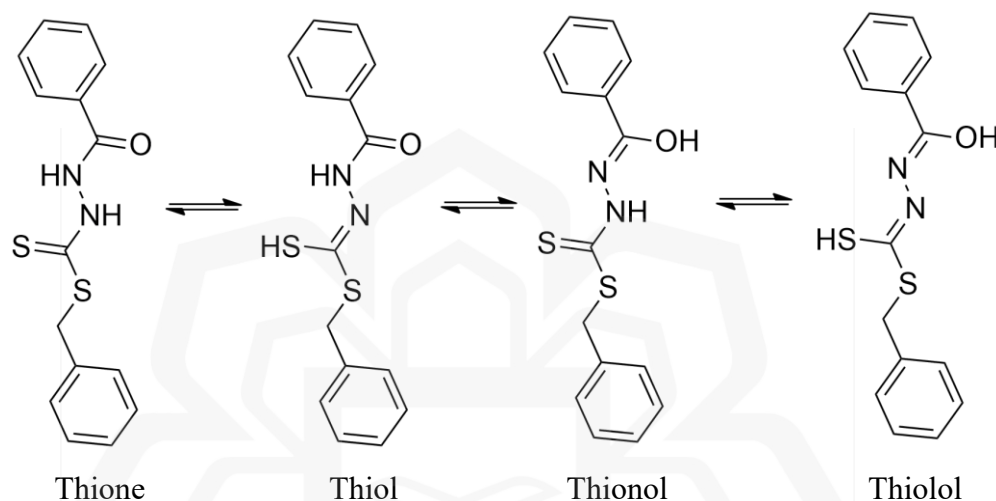


Figure 2.18 Tautomeric form of *S*-benzyl- β -N-(benzoyl) dithiocarbazate (How et al., 2008)

Besides SBDTC complexes, SBDTC derivatives also have been extensively studied due to their ability to form stable metal complexes with transition metal ions such as copper, cobalt, nickel, and zinc. These ligands exhibit a strong ability to coordinate with metal ions because they contain nitrogen and sulfur donor atoms, classifying them as hard-soft ligands resulting in metal complexes with unique structural and electronic properties. These ligands can coordinate with metal ions in bidentate, tridentate, or tetradentate modes, leading to various geometries, including tetrahedral, square planar, square pyramidal, and octahedral. Bidentate ligands coordinate with metal ions through the thiolate-sulfur atom and the β -nitrogen atom, typically forming four coordinated metal complexes. For instance, the Schiff base of SBDTC with 2,3-dimethoxybenzaldehyde has been found to form metal complexes with transition metal ions, primarily acting as bidentate NS chelating agents (Figure 2.19) (Hossain et al., 2021). However, different metal ions result in various geometries,

such as Ni(II) and Cu(II) complexes exhibiting square planar geometry, while Zn(II) and Cd(II) complexes are tetrahedral.

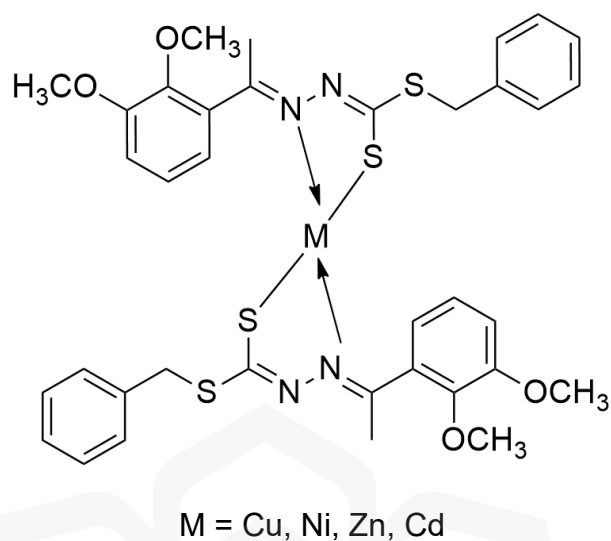
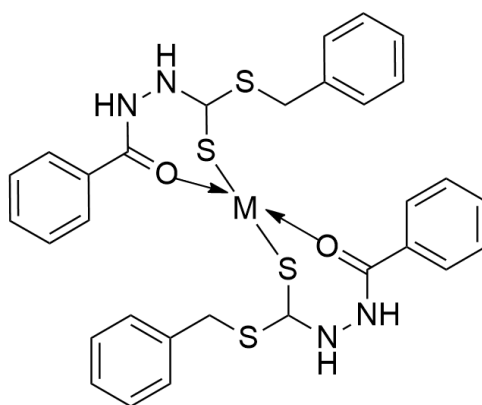


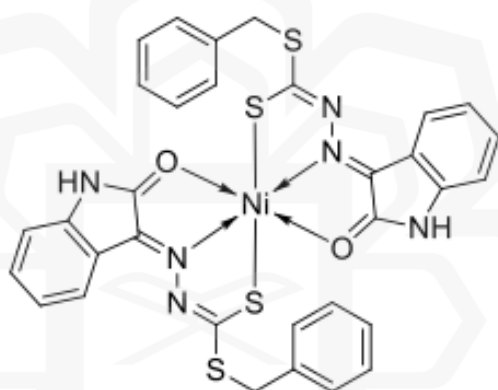
Figure 2.19 NS chelation mode of metal complex derived from Schiff base of SBDTC with 2, 3- dimethoxybenzaldehyde

Additionally, different chelating agents have been reported to form metal complexes through varying coordination modes. One study revealed that certain SBDTC derivatives behave as bidentate OS donor ligands. How et al. (2008) disclosed that *S*-benzyl- β -N-(benzoyl)dithiocarbazate derived from the substitution of SBDTC with benzoyl chloride forms bidentate complexes through OS coordination (Figure 2.20) with metal ions (How et al., 2008). Plus, SBDTC derivatives containing extra donor atoms, such as nitrogen or oxygen, in suitable positions can result in tridentate ligands with different structural properties during complexation. A tridentate ligand may coordinate with metal ions through thiolate sulfur, β -nitrogen, and an additional donor atom (Manan & Mohammat, 2022). According to Bhat (2019), the presence of an additional donor atom can enhance the coordination ability of the ligand, leading to various coordination geometries (Bhat, 2019). For instance, Manan and Mohammat (2022) reported that the spectral data of their Ni(II) complex derived from SBDTC with isatin indicates an ONS coordination mode, resulting in a distorted octahedral geometry (Figure 2.20) (Manan & Mohammat, 2022).



M = Cu, Ni, Cd, Co, Pb

OS mode (How et al., 2008)



ONS mode (Manan & Mohammat, 2022)

Figure 2.20 Complexation modes of SBDTC derivative

Overall, dithiocarbazate derivatives have been extensively studied for their various properties, including biological and catalytic activities. The addition of metal ions to form metal complexes was said to assist the compounds to be more potent than their ligands alone. The biological activities included antibacterial properties (Elzaref et al., 2023; Hossain et al., 2021; Kudrat-E-Zahan et al., 2020) anti-dengue activity (Maryam et al., 2020), as well as antitumor (Ramilo-Gomes et al., 2021) and anticancer activities (Khirudin et al., 2023; Yusof et al., 2022).

2.2.1 Biological Activities of SBDTC Derivatives and Their Metal Complexes

Numerous studies have demonstrated the antibacterial and cytotoxic efficacy of dithiocarbazate derivatives, particularly SBDTC, highlighting its potential as an

antimicrobial and anticancer agent. For instance, Tarafder et al. (2001) showed that SBDTC exhibited strong antimicrobial activity, achieving the highest inhibition zone of 36 mm against *Candida lypolytica* (2075). This finding was further supported by Crouse et al. (2004), who reported that SBDTC was highly effective in antibacterial assays, producing an inhibition zone of 30 mm against *Pseudomonas aeruginosa* (60690).

In addition to its antimicrobial properties, SBDTC has demonstrated significant cytotoxic activity. The study by Crouse et al. (2004) revealed that SBDTC exhibited a CD_{50} value of $2.6 \mu\text{g}/\text{cm}^3$ against CEM-SS (T-lymphoblastic leukemic cells), underscoring its potential as a cytotoxic agent. These findings collectively highlight the versatility of SBDTC as a bioactive compound, with strong efficacy against specific microbial strains and notable toxicity to cancer cells. Consequently, SBDTC represents a promising candidate for further development as both an antimicrobial and anticancer agent.

While there are limited studies on the bioactivity of SBDTC complexes, one notable study explored the bioactivity of a nickel complex of SBDTC. Zahan et al. (2020) synthesised this complex by reacting SBDTC with nickel(II) acetate tetrahydrate and tested it in vitro using the MTT colourimetric assay (Figure 2.21) (Zahan et al., 2021). The complex was found to reduce the growth of EAC (Ehrlich ascites carcinoma) cells in a dose-dependent manner, with a 26.62% inhibition at a concentration of $31.25 \mu\text{g}/\text{mL}$. In comparison, the standard anticancer drug bleomycin exhibited a significantly higher inhibition of 79.64% at the same concentration. This observed effect is attributed to the positive charge of the nickel ion, which enhances the acidity of the ligand, promoting the formation of strong hydrogen bonds and enhancing the complex's bioactivity.

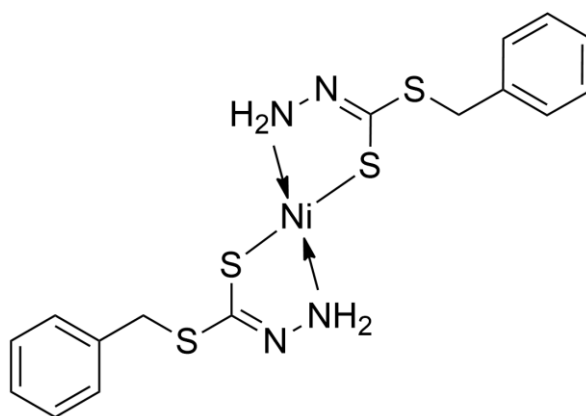


Figure 2.21 Nickel(II) acetate of SBDTC complex

Substituting SBDTC to form new SBDTC derivative ligands has been shown to improve the bioactivity of the scaffold. Hossain et al. (2021) reported that the Schiff base of SBDTC with 2,3-dimethoxybenzaldehyde, along with its metal complexes, exhibited promising antimicrobial activity (Hossain et al., 2021). The Schiff base (Figure 2.22), which features two methoxy groups attached to the benzyl group, showed moderate antibacterial activity against *E. coli*, *S. sonnei*, and *B. subtilis*, with inhibition zones of 12 mm, 14 mm, and 13 mm, respectively. When the Schiff base was chelated with Ni(II), Cu(II), Zn(II), and Cd(II), the resulting metal complexes exhibited enhanced antimicrobial activity. These metal complexes demonstrated clear inhibition zones around the discs and showed moderate to strong antibacterial effects against both gram-positive and gram-negative bacteria. Notably, their antibacterial activity surpassed that of the standard antibiotic, kanamycin, highlighting the enhanced effectiveness of the metal complexes.

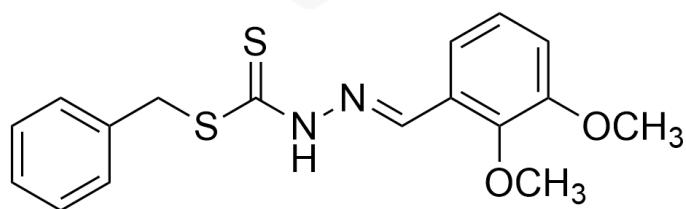


Figure 2.22 Structure of Schiff base from SBDTC with 2, 3- dimethoxybenzaldehyde

Additionally, another benzyl substitution at SBDTC by Elzaref et al. (2023) reported significant bioactivity. The study synthesised the Schiff base of SBDTC with 4-acetylbenzenesulfonamide, along with its Cu(II) and Zn(II) complexes (Elzaref et al.,

2023). The antibacterial activity of the synthesised derivatives was evaluated *in vitro* using a well diffusion method against *S. aureus* (ATCC 33592) as representative of gram-positive bacteria as well as *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), and *K. pneumoniae* (ATCC27736) as representatives of gram-negative bacteria. Ofloxacin (Oxoid) was used as the standard antibiotic, and DMSO was used as a solvent control. Schiff base, Cu(II) and Zn(II) complexes scored inhibition zones of 1.4 cm, 1.2 cm, and 1.9 cm, respectively, against *S. aureus* (ATCC 33592). Meanwhile, only the Schiff base (Figure 2.23) showed inhibition zones of 0.8 cm and 0.7 cm against *P. aeruginosa* (ATCC 27853) and *K. pneumoniae* (ATCC 27736), respectively.

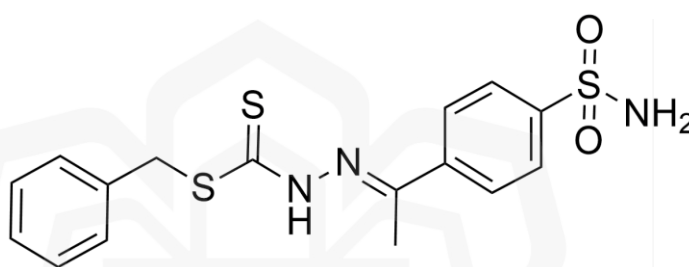


Figure 2.23 Structure of Schiff base from SBDTC with 4-acetylbenzenesulfonamide

To further assess the antibacterial efficiency, a MIC assay was conducted against *S. aureus* (ATCC 33592). The results revealed that the Cu(II) derivative was the most effective, with a MIC of 64 mg/mL, followed by the Zn(II) derivative with a MIC of 128 mg/mL. The Schiff base, on the other hand, exhibited a MIC of 256 mg/mL. In addition, the Schiff base showed a MIC of 256 mg/mL against both *P. aeruginosa* (ATCC 27853) and *K. pneumoniae* (ATCC 27736). Based on the findings from both the disc diffusion and MIC assays, the study concluded that the metal complexes, especially the Cu(II) complex, were particularly effective against *S. aureus*. In contrast, the Schiff base demonstrated a broader range of activity, though its effectiveness varied depending on the bacterial strain.

Furthermore, a study by Yusof et al. (2020) disclosed the superior bioactivity of SBDTC derivatives over their metal complexes. The research focused on the cytotoxicity testing of Schiff bases and tin complexes derived from SBDTC with 2-hydroxy-3-methoxybenzaldehyde (a) and 2,3-dihydroxybenzaldehyde (b) using the MTT assay (Yusof et al., 2020). Both Schiff bases (Figure 2.24) demonstrated activity in the cytotoxicity assay compared to their tin complexes, with a GI₅₀ value of 2.2 µM

and 2.1 μM against HT29 (colon), respectively. The study concluded that the presence of tin did not significantly enhance cytotoxicity, suggesting that the compounds' bulkiness and the Schiff bases' ability to bind to macromolecules were likely responsible for their observed activity.

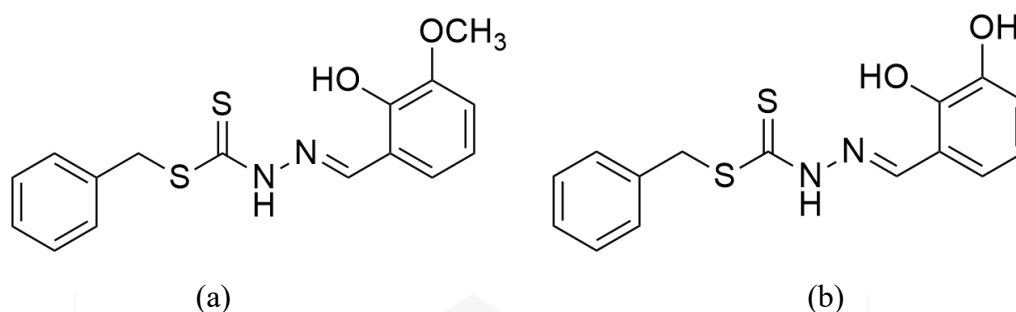


Figure 2.24 Structure of Schiff base from SBDTC with; (a) 2-hydroxy-3-methoxybenzaldehyde and (b) 2,3-dihydroxybenzaldehyde

Indeed, while some studies suggest that SBDTC derivatives are more active than their metal complexes, the coordination of metal ions to these ligands can significantly enhance bioactivity under certain conditions. For instance, Khirudin et al. (2023) investigated the cytotoxicity of a Schiff base and its Cu(II) complex derived from SBDTC with 2-methoxybenzaldehyde against two human breast cancer cell lines: estrogen-positive (MCF-7) and triple-negative estrogen (MDA-MB-231) receptors. Interestingly, the Schiff base (Figure 2.25) exhibited no significant growth inhibition activity against either cell line (Khirudin et al., 2023). Similarly, the Cu(II) complex was inactive against MCF-7 cells. However, the Cu(II) complex demonstrated notable cytotoxic activity against MDA-MB-231 cells, with a GI_{50} value of 2.71 μM . This result is particularly significant as it surpasses the activity of cisplatin ($\text{GI}_{50} = 12.0 \mu\text{M}$) against the same cell line. These findings suggest that the effectiveness of the Cu(II) complex is cell-specific, with enhanced activity observed in the more aggressive MDA-MB-231 cells while being inactive against the less aggressive MCF-7 cells.

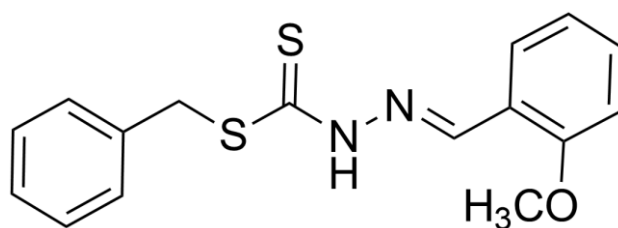


Figure 2.25 Structure of Schiff base from SBDTC with 2- methoxybenzaldehyde

Sultana et al. (2020) demonstrated the enhanced antibacterial activity of metal complexes over their corresponding Schiff base. Their study focused on the Schiff base of SBDTC with dibenzalacetone (Figure 2.26), which showed weak inhibition against *B. cereus* (5 mm) and *B. subtilis* (6 mm). However, upon coordination with Ni(II), Cu(II), and Co(II), antibacterial activity improved significantly. At the dose of 256 mg/mL, the Ni(II) complex showed the strongest effect, with inhibition zones of 16 mm and 11 mm against *B. cereus* and *B. subtilis*, respectively. The Cu(II) complex inhibited *B. cereus* (12 mm) but had little effect on *B. subtilis*. Against *E. coli*, only the Ni(II) (9 mm) and Cu(II) (6 mm) complexes showed activity, while the Schiff base and Co(II) complexes were inactive. These findings highlight how metal coordination enhances the bioactivity of Schiff bases derived from SBDTC, particularly against gram-positive bacteria and to a lesser extent against *E. coli*.

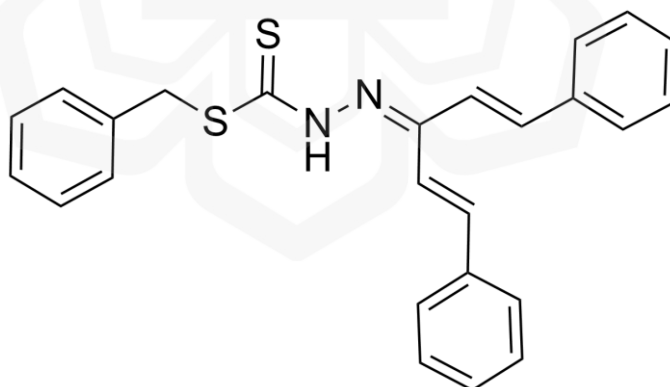


Figure 2.26 Structure of Schiff base from SBDTC with dibenzalacetone

Gan et al. (2022) investigated the antibacterial efficiency of Cu(II) complexes derived from SBDTC against two types of *Staphylococcus aureus* strains, antibiotic-susceptible strains (ATCC 35923) and multidrug-resistant strains (ATCC 43300). Using the MIC assay, the study evaluated two Schiff base ligands (Figure 2.27) synthesised

via the condensation of SBDTC with (a) 4-carboxybenzaldehyde and (b) 4-formyl-3-hydroxybenzoic acid, along with their corresponding Cu(II) complexes. The findings demonstrated that the Cu(II) complexes exhibited significantly enhanced antibacterial activity compared to the Schiff bases alone. The Cu(II) complex of the Schiff base (b) (designated as CuB) displayed the lowest MIC value of 4 μ M against methicillin-susceptible *S. aureus*, while the Cu(II) complex of Schiff base (a) (CuA) exhibited a MIC value of 8 μ M. Remarkably, against methicillin-resistant *S. aureus* (MRSA), the MIC values of CuB and CuA were 8 μ M and 4 μ M, respectively. This study underscores the significant role of metal coordination in enhancing the antibacterial properties of SBDTC derivatives, especially against challenging multidrug-resistant pathogens.

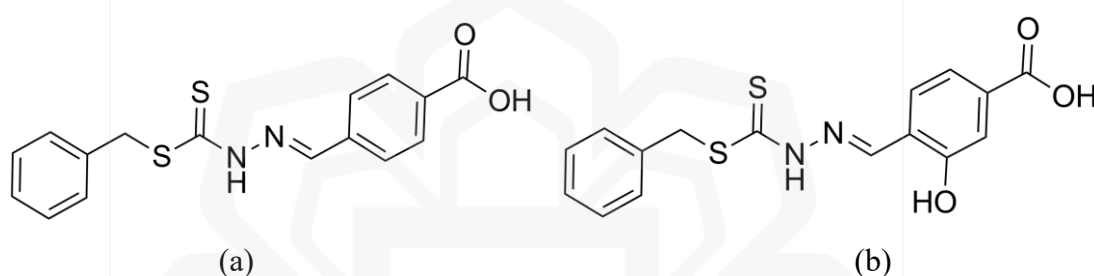


Figure 2.27 Structure of Schiff base from SBDTC with; (a) 4-carboxybenzaldehyde (b) 4-formyl-3-hydroxybenzoic acid

Hossen et al. (2020) compared the superiority of different metals in their study on the antimicrobial activity of a Schiff base and metal complexes from SBDTC with cinnamaldehyde (Figure 2.28) (Hossen et al., 2020). They found that Ni(II) and Cu(II) complexes were active against *B. subtilis* with the highest inhibition zones of 22 mm and 24 mm, respectively. Overall, the study highlights the potential of nickel and copper as a preferred metal ion for enhancing the antimicrobial efficacy of SBDTC-based Schiff bases, making it a promising candidate for further development in antimicrobial research.

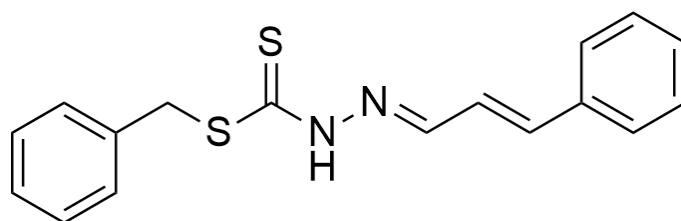


Figure 2.28 Structure of Schiff base from SBDTC with cinnamaldehyde

While earlier studies have suggested that SBDTC derivatives often exhibit higher bioactivity compared to their complexes, the majority of recent research indicates that metal complexes of SBDTC derivatives consistently demonstrate significantly enhanced activity compared to the ligands alone, as discussed above. This enhanced bioactivity can be explained by the chelation theory, as highlighted by Hossen et al. (2020). Chelation reduces the polarity of metal ions by delocalizing π -electrons over the ligand's aromatic system, thereby increasing the lipophilic character of the complexes (Hossen et al., 2020). This increased lipophilicity facilitates better penetration of the complexes into the lipid membranes of microorganisms, enhancing their diffusion across bacterial cell walls and improving antibacterial efficacy.

Additionally, the enhanced activity of metal complexes can be attributed to several factors, such as the choice of metal ions. Metal ions play a pivotal role in modulating the redox properties, coordination geometry, and overall reactivity of the metal complexes, which are crucial for antibacterial activity (Claudel et al., 2020). Among the various metal complexes discussed in previous studies, copper, zinc, cobalt, and nickel have shown exceptional performance, consistently outperforming their respective Schiff base ligands.

Despite the potential shown by SBDTC derivatives and their metal complexes, a clear structure-activity relationship (SAR) has not been established. Changes in their chemical structure have led to varying biological activities, but a consistent link between specific structural modifications and bioactivity remains unclear. To fill this gap, insights from previous studies on structural differences and biological activity were analysed. This information provided a basis for designing new SBDTC derivatives and their metal complexes, using SBDTC as the scaffold.

2.3 CHARACTERISATION

2.3.1 Melting Point

The melting point of a compound serves as a vital indicator of its purity. Typically, pure compounds exhibit a sharp, well-defined melting point, while impure compounds tend to show a range of melting points (Brittain, 2009). Researchers often compare the observed melting point with the known or literature value to evaluate the purity of a synthesised compound. A significant deviation from the expected melting point can

detect the presence of impurities or a different compound altogether. In this context, SBDTC is a well-documented compound, and according to Ali and Livingstone (1974), its reported melting point is approximately 125 °C (Akbar Ali & Livingstone, 1974). Similarly, other studies also mention the melting point of SBDTC observed around 123 to 125 °C (Z. Chen et al., 2007; Manan et al., 2011). Moreover, melting point analysis plays a crucial role in compound identification because each compound possesses a unique melting point (Brittain, 2009).

2.3.2 Elemental Analysis

Carbon, Hydrogen, Nitrogen, Sulfur (CHNS) analysis and Atomic Absorption Spectroscopy (AAS) are essential techniques for determining the molecular composition of compounds. CHNS analysis measures the precise amounts of carbon, hydrogen, nitrogen, and sulfur in a substance, helping to confirm its structure. For instance, in the synthesis of a Schiff base of SBDTC with acetophenone, the CHNS analysis yielded results closely aligning with the calculated percentages. Thus, these results validated the proposed molecular structure (Ameram & Othman, 2013). Similarly, the formation of Schiff bases from SBDTC and various aldehydes has been corroborated by CHNS analysis, with the obtained elemental compositions matching the theoretical values. This confirms the successful synthesis of the ligands (P. Ghosh et al., 2019).

On the other hand, AAS is a powerful analytical technique specifically used for quantitatively determining metal ions in a sample. This technique is particularly useful for characterising metal-containing compounds. In studies involving SBDTC-derived Schiff bases complexed with metals such as Co(II), Cu(II), Cd(II), Zn(II), and Ni(II), AAS has been employed to determine the metal-to-ligand ratios (Ameram & Othman, 2013). The results consistently indicate a 1:2 metal-to-ligand stoichiometry, supporting the proposed coordination structures. In another study on cis-dioxomolybdenum(VI) complexes with Schiff base ligands derived from SBDTC, AAS was used to determine the molybdenum content. The results matched the theoretical values, confirming the successful formation of the metal complexes (Kathale et al., 2001).

Hence, these analytical techniques not only confirm the elemental composition and metal content but also confirm the proposed molecular structures.

2.3.3 Fourier Transform Infrared (FTIR) spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is a powerful analytical tool for identifying functional groups within a compound by detecting the characteristic vibrations of chemical bonds (Berthomieu & Hienerwadel, 2009). FTIR operates within a wavelength range of 4000 to 400 cm^{-1} , where infrared radiation interacts with specific functional groups, producing characteristic peaks in the spectrum. These peaks allow researchers to identify bond types and functional groups and comparing experimental spectra with reference data or databases aids in deducing a compound's structural features (Lavine et al., 2020). Furthermore, FTIR spectroscopy is particularly useful in identifying ligand donor sites in metal complexes (Pharmaceutica, 2019). The coordination of ligands with metals alters bond lengths, angles, and interatomic forces, which is reflected in changes in the IR spectrum (Cotton et al., 2005). These changes include shifts in band positions, the appearance of new bands, the broadening or splitting of existing bands, and the disappearance of certain bands upon complexation (Bhat, 2014).

In the FTIR spectra of the SBDTC derivative, notable peaks include a broad band in the 2900-3300 cm^{-1} region, attributed to N-H stretching frequencies. Ameram & Othman (2013) observed peaks at 3303 cm^{-1} and 3180 cm^{-1} in SBDTC, corresponding to $\nu(\text{NH}_2)$ and $\nu(\text{NH})$ groups, respectively. They also reported that the $\nu(\text{NH}_2)$ peak disappears during Schiff base formation due to deprotonation in the condensation reaction (Ameram & Othman, 2013). Similarly, Singh et al. (2009) reported two N-H stretching bands in a Schiff base derived from SBDTC at 3244 cm^{-1} and 3120 cm^{-1} , corresponding to N-H groups attached to C=O and C=S, respectively (M. Singh et al., 2009). In metal complexes, these bands typically shift to lower frequencies or disappear altogether, indicating coordination involving the N-H group (Hossain et al., 2021). Additionally, Battistoni et al. (1971) noted that N-H bending vibrations shift to lower frequencies (approximately 1580 cm^{-1}) upon complexation (Battistoni et al., 1971).

Another critical feature is the N-N stretching band, which appears at 1346 cm^{-1} in SBDTC and shifts to around 1272 cm^{-1} in Schiff bases (How et al., 2008). Upon complexation, this band may shift further due to deprotonation. For example, Khirudin

et al. (2023) observed a shift in the hydrazinic N-N band from 1158 cm^{-1} to 1099 cm^{-1} in metal complexes, signifying coordination through the azomethine nitrogen.

The $\nu(\text{C}=\text{S})$ stretching band is another prominent feature, typically observed around 1048 cm^{-1} in SBDTC and within the $1000\text{--}1060\text{ cm}^{-1}$ range in Schiff bases. The presence of this band indicates the thione form of the compound. Khoo et al. (2014) confirmed the thione form by noting the presence of $\nu(\text{C}=\text{S})$ and $\nu(\text{N-H})$ peaks at 1148 cm^{-1} and 2920 cm^{-1} , respectively (Khoo et al., 2014). The absence of a $\nu(\text{S-H})$ band ($2600\text{--}2700\text{ cm}^{-1}$) in Schiff base spectra further supports the conclusion that these compounds exist predominantly in the thione form. However, upon complexation, the $\nu(\text{C}=\text{S})$ band typically disappears due to the conversion of the thione form to the thiol form via enolisation, involving deprotonation of the thiolate sulfur before coordination with the metal ion (Figure 2.29) (Manan & Mohammad, 2022). This transformation is evidenced by the emergence of a new strong band, $\text{C}=\text{N}$ at lower frequencies ($1590\text{--}1620\text{ cm}^{-1}$) in the metal complexes (Bitu et al., 2020).

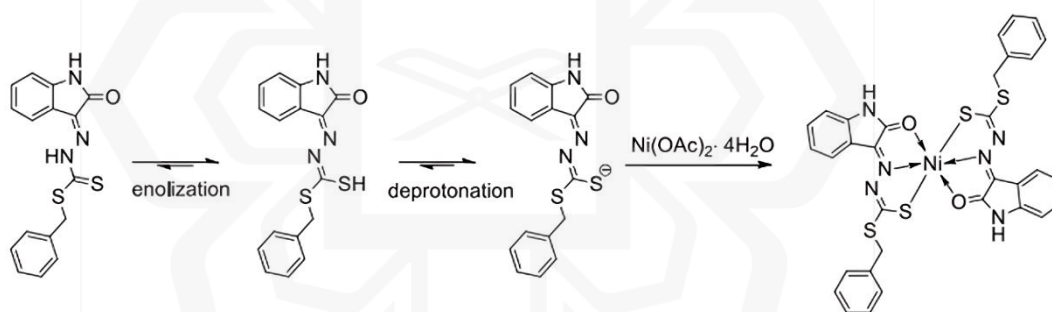
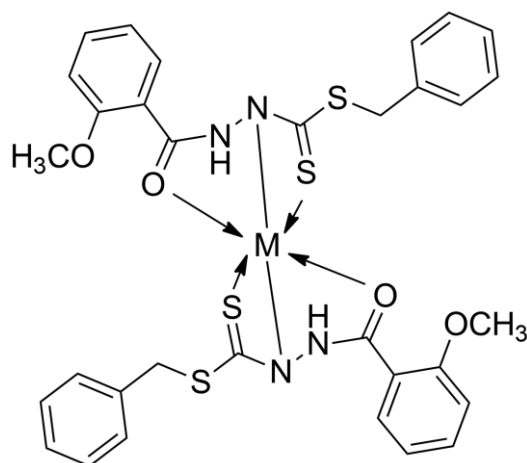


Figure 2.29 Enolisation followed by deprotonation of Schiff base from SBDTC and isatin prior to coordination to Ni(II)

In some cases, the $\text{C}=\text{S}$ band remains present in the spectra of metal complexes, though shifted from its position in the spectrum of the SBDTC derivative. This shift indicates that complexation occurs directly through the thione sulfur. For example, Singh et al. (2009) reported that benzyl-*N*-(2-methoxybenzoyl)hydrazinecarbodithioate formed complexes with $\text{Mn}(\text{II})$, $\text{Co}(\text{II})$, and $\text{Ni}(\text{II})$ via an NOS donor sequence, involving the deprotonation of the amino group, thione sulfur, and carbonyl oxygen (M. Singh et al., 2009). The negative shift of $58\text{--}63\text{ cm}^{-1}$ observed in the $\text{C}=\text{S}$ band of the complexes' spectra confirms the involvement of thione sulfur in bonding (Figure 2.30).



M = Mn, Co, Ni

Figure 2.30 NOS mode of benzyl-*N*-(2-methoxybenzoyl) hydrazinecarbodithioate

The participation of sulfur in complexation can also be confirmed through changes in the S-C-S band, typically found in the 1000–950 cm^{-1} range for dithiocarbazate derivatives. This band may either split or shift upon complexation. For instance, How et al. (2008) demonstrated that *S*-benzyl- β -*N*-(benzoyl)dithiocarbazate coordinated through thio sulfur, as evidenced by the splitting of $\nu(\text{CSS})$ in the spectra of metal complexes, which appeared between 966 and 900 cm^{-1} (How et al., 2008). Similarly, Ameram & Othman (2013) observed that the $\nu(\text{S-C-S})$ band shifted toward lower frequencies by 8-17 cm^{-1} in metal complexes compared to the Schiff bases, further confirming sulfur's role in the coordination process. These observations highlight the ability of sulfur atoms in dithiocarbazate derivatives to participate actively in bonding during metal complexation.

The C=O band in SBDTC derivative results from the substitution of SBDTC with organic compounds containing a carbonyl group. Typically, C=O bands are observed in the range of 1680–1600 cm^{-1} (How et al., 2008). Upon complexation, this band undergoes a significant negative shift, indicating coordination via the carbonyl oxygen. For instance, Singh et al. (2009) reported a major shift of approximately $\sim 50 \text{ cm}^{-1}$ in the $\nu(\text{C=O})$ band in all metal complexes, confirming the involvement of the carbonyl oxygen in metal coordination (M. Singh et al., 2009).

Overall, these characteristic peaks offer key information about the functional groups and bonding of the SBDTC derivatives and their metal complexes, helping to understand their coordination and structure.

2.3.4 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is indeed a crucial tool in structural chemistry, offering detailed insights into the molecular structure of compounds. Proton NMR (^1H -NMR) is particularly valuable for identifying the hydrogen atoms in a molecule, providing information about their chemical environment. By examining the chemical shifts, splitting patterns and integration of the proton signals, one can deduce molecular structure, functional groups, and proton environments within the compound (Günther, 2013). Carbon-13 NMR complements ^1H -NMR by revealing details about the carbon atoms. Carbon atoms exhibit characteristic chemical shifts depending on their bonding environment, such as whether they are part of an alkyl chain, an aromatic ring, or a functional group. This helps in pinpointing the exact positions of carbon atoms in the molecular structure.

The ability of NMR spectroscopy to differentiate structural isomers is one of its most powerful features. Structural isomers share the same molecular formula but have different arrangements of atoms. NMR's sensitivity to the unique environments of protons and carbon atoms allows it to distinguish between isomers that might appear identical using other techniques like mass spectrometry or infrared spectroscopy (Bifulco et al., 2007).

2.3.4.1 ^1H -NMR

The ^1H NMR spectrum of the SBDTC derivative provides significant insights into the structural features, functional groups, and tautomeric behaviour of these compounds. One of the primary indicators is the presence of secondary N-H protons, which typically appear in the chemical shift range of 7.73-10.14 ppm. This deshielding is due to the electronegative nature of the nitrogen atom, as noted by Shuhada et al. (2017). Additionally, M.A. Ali et al. (2004) reported that a secondary N-H proton signal can also appear at a much higher chemical shift, around 13.4 ppm (M. A. Ali et al., 2004). For aromatic groups, the proton signals are generally observed between 6.2 and 7.1

ppm, reflecting the protons attached to the aromatic rings, as evidenced by studies from Gwaram et al. (2012) and Tarafder et al. (2002). These aromatic signals provide crucial information regarding the presence and arrangement of aromatic moieties within the molecule.

Methoxy groups (-OCH₃), commonly present in SBDTC derivatives, exhibited distinct proton signals in the range of 3.3-3.9 ppm. Singh et al. (2009) specifically identified methoxyl protons resonating at 3.34 ppm, confirming their incorporation into the compound. Additionally, the methylene protons (-CH₂) of the dithiocarbazate moiety typically appear as singlet peaks around 4.5 ppm, as reported by Break et al. (2013). In SBDTC, these peaks are deshielded due to the neighbouring sulfur atom. Supporting this, Khirudin et al. (2023) observed methylene and methoxy proton signals at 4.43 ppm and 3.80 ppm, respectively, in the spectrum of Schiff base of SBDTC with 2-methoxybenzaldehyde.

Beyond structural confirmation, the ¹H NMR spectrum is instrumental in identifying tautomeric forms of SBDTC derivatives, particularly the thione and thiol tautomer. How et al. (2008) demonstrated that *S*-benzyl-β-*N*-(benzoyl) dithiocarbazate exists predominantly in the thione form in the solid state. However, when dissolved in DMSO-d₆, the compound shifted towards the thiol form, evident from an S-H proton peak at 3.38 ppm and the absence of an O-H signal in the spectrum. This transition highlights the dynamic nature of SBDTC dithiocarbazates in solution.

Bera et al. (2008) further corroborated the thione-thiol tautomerism in SBDTC derivatives. In their study, a sharp singlet at approximately 10.80 ppm in the ¹H NMR spectrum indicated the α-NH group of the thione form (Bera et al., 2008). For *S*-methyldithiocarbazate (SMDTC), a singlet at 5.06 ppm was attributed to NH₂ protons, but this signal was absent in the spectrum of SBDTC. Instead, overlapping CH₂ signals at 4.37 and 4.38 ppm in the SBDTC spectrum suggested a shift to the thiol form in solution.

2.3.4.2 ¹³C-NMR

While much research has predominantly focused on ¹H NMR spectroscopy for analysing SBDTC derivatives, the utility of ¹³C NMR spectroscopy cannot be ignored, particularly in elucidating the structures of new substituted ligands. In ¹³C NMR, the

SBDTC derivative exhibited a distinct deshielded signal at 196.7 ppm, attributed to the carbon atom in the -HN-C(=S)S moiety, as reported by Khirudin et al. (2023). This downfield peak confirms the presence of the thione tautomer in solution, supported by its characteristic chemical environment. Similarly, Elzaref et al. (2023) assigned a resonance at 199.20 ppm to the C=S group based on its low intensity and highly deshielded position. The pronounced deshielding of this carbon arises from the influence of adjacent electronegative sulfur and nitrogen atoms, as highlighted by Shuhada et al. (2017).

The carbonyl carbon (C=O) also provides significant insight, with signals typically appearing around 164.52 ppm (M. Singh et al., 2009). This range aligns with Bhat's (2014) observations, which noted the signals of aldehyde and ketone carbonyl carbons between 160-210 ppm. Such resonances confirm the presence of the carbonyl group in SBDTC derivatives. Aromatic carbons, on the other hand, resonate within the 150-130 ppm range, as noted in multiple studies. For example, Khirudin et al. (2023) reported carbon aromatic resonances within 112.5-142.8 ppm, further refining the assignments of aromatic ring carbons in SBDTC derivatives.

In addition to aromatic and carbonyl carbons, aliphatic carbons also feature prominently in the ^{13}C NMR spectrum of SBDTC derivatives. The -CH_2 carbons typically resonate around 38.1 ppm, while -OCH_3 carbons are observed near 56.3 ppm (Khirudin et al., 2023). This observation is consistent with findings by Singh et al. (2009), who reported methoxy carbons at 56.27 ppm. These peaks provide direct evidence of aliphatic substituents, confirming the presence of methylene and methoxy groups. Overall, the ^{13}C NMR spectrum complements the insights gained from ^1H NMR by providing critical information about the structural arrangement of carbons in SBDTC derivatives.

2.3.5 Mass Spectrometry (MS)

Mass spectrometry (MS) is a technique that provides important information about a compound's molecular weight and structure. It works by ionising the molecule, which produces various fragment ions (Rotzsche, 1991). The ions are identified based on their mass-to-charge (m/z) ratio, with the highest m/z value representing the parent molecular ion, which shows the compound's molecular weight (Chemistry, 2005). The parent

molecular ion, often called M^+ , is the ionised form of the whole molecule. The molecular ion peak usually appears one mass unit lower than the actual molecular weight due to the radical cation.

In the synthesis of Schiff bases derived from SBDTC, MS has been instrumental in verifying molecular structures. For instance, the reaction of SBDTC with 2,4,5-trimethoxybenzaldehyde yielded a bidentate NS Schiff base (Islam et al., 2016). The mass spectrum of this compound displayed a molecular ion peak at m/z 376 corresponding to its calculated molecular weight (377 g/mol), thereby confirming the successful formation of the desired Schiff base. Another study also confirms the successful formation of ligands from mass spectrometry. The synthesis of a Schiff base from SBDTC and 4-benzyloxybenzaldehyde revealed a molecular ion peak consistent with the expected molecular weight (Hasibul Islam et al., 2020).

The mass spectrum also reveals the fragmentation patterns of the molecule, which are specific to its chemical structure (Hübschmann, 2015). By studying these patterns, researchers can identify the molecule, its functional groups, and other structural features. The most intense peak in the spectrum is the base peak, assigned a relative intensity of 100% (Shrader, 2013). This peak represents the most stable and abundant fragment ion produced during ionisation. A common and stable fragment in compounds with aromatic rings is the tropylium ion, which appears at m/z 91 (Smith, 2004). This ion is typically formed from aromatic compounds, especially those containing a benzyl group ($C_6H_5CH_2$). The tropylium ion forms when the benzyl cation rearranges to create a seven-membered aromatic ring with a positive charge. For example, the mass spectrum of a Schiff base from SBDTC and 4-hydroxy-2-methoxybenzaldehyde exhibited a molecular ion peak at m/z 331.1, corresponding to the expected molecular weight (Pal et al., 2021). Additionally, a significant fragment peak at m/z 91.0 was observed, indicative of a benzyl moiety, which aligns with the proposed structure in the Schiff base.

Ionised molecules can break apart in predictable ways, which are often determined by the chemical bonds and functional groups present. One common fragmentation pattern is alpha cleavage (α -cleavage) (Steckel et al., 2019). This occurs when a bond next to a functional group breaks, usually between a carbon atom and a heteroatom like oxygen, nitrogen, or sulfur. This is common in compounds with

carbonyl groups, alcohols, amines, and ethers. The resulting fragment ions provide valuable information about the molecule's functional groups.

2.3.6 Ultraviolet-Visible (UV-Vis) spectroscopy

UV-Vis spectroscopy is an analytical technique used to measure the absorbance or transmittance of a substance across the ultraviolet (200-400 nm) and visible (400-700 nm) light ranges (Weckhuysen, 2004). It is commonly used to study electronic transitions within compounds, particularly those between molecular orbitals, such as from the ground state to excited states (Catalysis, 2009). When a compound absorbs light, electrons are excited from lower-energy bonding or non-bonding orbitals in the Highest Occupied Molecular Orbital (HOMO) to higher-energy anti-bonding orbitals in the Lowest Unoccupied Molecular Orbital (LUMO) (Vogt et al., 2023). The resulting absorbance spectrum provides valuable information about a compound's electronic structure and can reveal details about its functional groups. These transitions include $\pi \rightarrow \pi^*$ transitions, $n \rightarrow \pi^*$ transitions, ligand-to-metal charge transfer (LMCT), and $d \rightarrow d$ transitions (Vogt et al., 2023).

In the SBDTC derivative, bands corresponding to the $\pi \rightarrow \pi^*$ transition of the imine group and the $n \rightarrow \pi^*$ transition of the thione group are observed at 297-307 nm and 335-354 nm, respectively. Upon metal chelation, the spectra experience a slight shift. Liu et al. (2013) observed that the Schiff base of SBDTC with ferrocenyl-3-4-methylbenzeneacrylketone exhibited three absorption bands in the ultraviolet region at 246, 275, and 369 nm (Y. T. Liu et al., 2013). The 246 and 275 nm bands are attributed to the $\pi \rightarrow \pi^*$ transitions of the benzene rings and azomethine group (C=N), while the 369 nm band corresponds to the $n \rightarrow \pi^*$ transitions associated with the azomethine group.

In metal complexes, metalation leads to shifts in absorption bands and changes in intensity, which affect the energy of both $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions in the conjugated chromophore (Weckhuysen, 2004). The type of metal ion, such as Ni(II), Cu(II), Co(II), Hg(II), Cd(II), Zn(II), and Pb(II), plays a significant role in determining the position of absorption bands (T. Liu, 2003). According to molecular orbital theory, any factor that alters the electronic density of a conjugated system can result in bathochromic or hypochromic shifts in absorption bands (Liauw et al., 2010).

Therefore, when a ligand forms complexes with different metal ions, bathochromic shifts are typically associated with the electronegativity of the metal ions. Low et al. (2015) reported a slight hypsochromic shift from 344 nm to 325 nm upon complexation in the Re(I) complex of SBDTC with 3- acetylcoumarin. Moreover, a bathochromic shift from 286 nm to 292 nm was observed for the Zn(II) complex (Low et al., 2015). This demonstrates that the position of absorption bands can shift depending on the metal ion involved.

Additionally, the spectral region from 407 nm to approximately 500 nm is characteristic of LMCT. These bands form particularly from the charge transfer nitrogen atom of the ligand to the metal centre (Gwaram et al., 2012). Another LMCT observed from the lone-pair p-orbital of the sulfur atom in the ligand to the metal's d-orbital appears as a band around 397-418 nm (Bhat, 2014). Another study observed sulfur to Cu(II) charge transfer bands at 407-410 nm, confirming that the Schiff base from SBDTC with saccharinate anion coordinates with Cu(II) via the thiolate-sulfur atom (Shuhada et al., 2017). Both of these bands support the involvement of nitrogen and sulfur donor atoms in the coordination of the SBDTC derivative.

The final spectral band, found between 600 and 750 nm, corresponds to $d \rightarrow d$ transitions of the metal ions. In a study involving Cu(II) complexes with Schiff bases derived from SBDTC, UV-Vis spectra exhibited d-d transition bands around 601 nm, corresponding to the ${}^2E_g \rightarrow {}^2T_{2g}$ transition (Md Yusof et al., 2015). This band is indicative of a distorted octahedral geometry around the Cu(II) ion. The study also stated that nickel(II) complexes with SBDTC-derived Schiff bases have shown characteristic d-d transitions in their UV-Vis spectra. For instance, bands observed at approximately 609 nm are attributed to the ${}^3A_{2g}(F) \rightarrow {}^3T_{2g}(F)$ transition, suggesting an octahedral geometry. Cobalt(II) complexes derived from SBDTC Schiff bases display d-d transition bands in the UV-Vis region, typically around 609 nm, corresponding to the ${}^4T_{1g}(F) \rightarrow {}^4T_{2g}(F)$ transition. This observation is indicative of an octahedral geometry around the Co(II) ion. However, zinc(II) complexes with SBDTC-derived Schiff bases generally do not exhibit d-d transitions in the UV-Vis spectra due to the d^{10} electronic configuration of Zn(II).

2.3.7 Molar Conductivity

Molar conductivity analysis, also referred to as specific conductivity, is a technique for determining a compound's ability to conduct electricity in a solution, directly linked to its electrolyte strength (Shcherbakov et al., 2021). Strong electrolytes like salts and acids readily dissociate into ions in solution, resulting in elevated molar conductivity values. Conversely, weak electrolytes, encompassing many organic compounds and metal complexes, have lower molar conductivity due to partial ionization or complex formation (El-Shenawy et al., 2014).

Researchers often compare conductance values with standard literature values to ascertain whether complexes act as electrolytes or nonelectrolytes (Geary, 1971). For instance, the conductivity of non-complex electrolytes in DMSO solvent at 10^{-3} M for given solutes is typically $35 \Omega^{-1} \text{ cm}^2 \text{ mol}^{-1}$ (Sears et al., 1955). Greenwood et al. (1967) suggested a range of $50\text{-}70 \Omega^{-1} \text{ cm}^2 \text{ mol}^{-1}$ for electrolytes in DMSO (Greenwood & Travers, 1967), corroborated by Ali et al. (2013), where conductivity values for non-electrolytes usually fall below $50 \Omega^{-1} \text{ cm}^2 \text{ mol}^{-1}$ in DMSO solution (I. Ali et al., 2013).

2.3.8 Magnetic Susceptibility

Magnetic susceptibility, χ is a measurement of a material's degree of magnetisation in response to an applied magnetic field (Kriessman & Callen, 1954). According to Nemade and Kolhe (2016), the magnetic properties give an idea about the number of unpaired electrons, oxidation state, the spin state of the metal ion as well as information about the geometry of the metal complex (Nemade & Kolhe, 2016). Materials can exhibit either paramagnetism or diamagnetism. Paramagnetism is where they are attracted to a magnetic field due to the presence of unpaired electrons, leading to a positive magnetic susceptibility. Meanwhile, diamagnetism is where they are repelled by the field because of induced magnetic moments of paired electrons, resulting in a negative magnetic susceptibility (Kriessman & Callen, 1954). Moreover, the effective magnetic moment (μ_{eff}), representing the number of unpaired electrons, can be derived from the magnetic susceptibility value, which is calculated using the equation below (2.1). Comparing μ_{eff} values with literature allows for predicting the geometry of metal complexes and each metal type has specific literature values for its structural configurations, such as square planar, tetrahedral, and octahedral.

Equation 2.1:

$$\mu_{\text{eff}} = 2.83 (\chi_{\text{m}} T)^{1/2} \text{ BM}$$

where, χ_{m} = molar susceptibility, T = temperature in Kelvin

For copper complexes in the Cu(II) oxidation state with a d^9 electron configuration, the common geometries observed are square planar or distorted octahedral (Enamullah et al., 2014). Bhat (2014) reported that the magnetic moments of synthesised Cu(II) complexes ranged from 1.43 to 1.56 B.M, indicating a square planar geometry (Bhat, 2014). However, the ideal magnetic moment for square planar complexes is typically around 1.6-1.7 B.M due to the covalent nature of the metal-sulfur bond (Adsule et al., 2006). Octahedral Cu(II) complexes, by contrast, show magnetic moments between 1.7 and 2.2 B.M (Turk et al., 2022).

In the case of d^{10} configuration metal complexes, such as Zinc(II), the zinc ion is diamagnetic due to its fully occupied d^0 electron configuration (Ravoof et al., 2011). Bhat (2019) noted that the d^{10} configuration is symmetrically stable (singlet-singlet) and does not split in a magnetic field. As a result, a non-degenerate singlet state makes it difficult to assign a specific structure (Bhat, 2019). Typically, four-coordinate Zn(II) complexes prefer tetrahedral stereochemistry via sp^3 hybridization, while six-coordinate Zn(II) complexes adopt an octahedral geometry.

Cobalt(II) complexes can adopt various coordination geometries, including tetrahedral, octahedral, and square planar, depending on the magnetic moment. Bhat (2014) reported that high-spin octahedral Co(II) complexes have magnetic moments ranging from 4.7 to 5.2 B.M. This is consistent with findings from Hosny (2007), where a magnetic moment of 5.1 B.M indicated an octahedral geometry around Co(II) (Hosny, 2007). Square planar Co(II) complexes generally have magnetic moments ranging from 1.92 to 2.47 B.M. In the case of the Co(II) complex of SBDTC with benzoyl chloride, it was expected to adopt a tetrahedral geometry with a magnetic moment of 4.5 B.M (How et al., 2008). Bhat (2014) also highlighted that a highly distorted tetrahedral environment around the Co(II) ion, potentially due to bulky groups such as $-C_6H_5$ and OCH_3 , which lead to magnetic moments between 4.2 and 4.8 B.M (Bhat, 2014).

Nickel(II) complexes, being a d^8 system, can exhibit both paramagnetic and diamagnetic properties. In paramagnetic conditions, the complex may adopt either

tetrahedral or octahedral geometries, depending on the magnetic moment value. In diamagnetic conditions, the metal complex typically assumes a square planar geometry. Literature suggests that a magnetic moment around 3 to 4.1 B.M is indicative of a tetrahedral geometry (S. Ghosh et al., 2020; Kriessman & Callen, 1954; Nemade & Kolhe, 2016). Hosny and El-Dossoki (2008) reported that a magnetic moment of 3.5 B.M aligns with the range for tetrahedral Ni(II) complexes (Hosny & El-Dossoki, 2008). How et al. (2008) also described a Ni(II) complex with a tetrahedral geometry and a magnetic moment of 3.2 B.M (How et al., 2008). On the other hand, magnetic moments between 2.9 and 3.4 B.M are associated with an octahedral geometry. Lobana et al. (2009) synthesised a tridentate Ni(II) coordination complex and reported an octahedral geometry based on an effective magnetic moment ranging from 3.03 to 3.06 B.M (Lobana et al., 2009).

2.3.9 Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis (TGA) is a thermal analysis technique used to assess the thermal stability of substances by monitoring the change in mass as the sample is heated (Menczel & Prime, 2008). TGA measures the mass loss as a function of temperature or time, helping to identify the proportions of components within the sample. TGA analysis of the SBDTC derivative revealed two main mass loss stages. The first loss, around 46.07%, occurs between 110-191 °C, attributed to the loss of the benzyl unit. The second significant mass loss, about 52.16%, occurs between 191-700 °C, corresponding to the loss of the residual part of the ligand (Bhat, 2014). Similarly, Sultana et al. (2020) reported a large weight loss of 63% between 175-400 °C, which they attributed to the removal of the ligand and the =N-NH-CS-CH₂-Ph unit (Sultana et al., 2020).

While ligands generally decompose in two or three stages, metal complexes typically decompose in four stages (Bhat, 2014). For example, the first stage of mass loss, about 30.36%, occurs between 110-222 °C, attributed to the loss of specific ligand units like =N-NH-CS-CH₂-Ph. This first stage is similar to the composition of the ligand itself (Sultana et al., 2020). The second mass loss, around 18.73%, occurs between 222-590 °C and corresponds to the loss of another part of the ligand. The remaining two stages, observed at 590-678 °C and 678-800 °C, are attributed to the loss of coordinated NH₂ and oxygen, respectively. After complete decomposition, residues

such as metal ions, metal oxides, and metal sulfides remain. For example, Elzaref et al. (2023) reported that $\text{ZnO} + 3\text{C}$ remains as a final residue (Elzaref et al., 2023), with Ghasemi et al. (2020) observing that ZnO remained as the final product with a mass of 19.20% (Calc. 21.80%) (Yekke-Ghasemi et al., 2020). Ghasemi also reported CoO_2 with a mass of 14.49% (Calc. 15.47%) and NiO with a mass of 15.33% (Calc. 15.47%) as final products.

Further supporting this, Singh et al. (2014) examined the thermal properties of manganese(II) and zinc(II) complexes of 4-phenyl(2-methoxybenzoyl)-3-thiosemicarbazide. For the Mn(II) complex, four decomposition steps occurred at 200, 360, 550, and 700 °C, while the Zn(II) complex decomposed at 200, 320, 410, and 720 °C. The first decomposition for both complexes (~220°C) was due to the loss of the $-\text{OCH}_3$ group. The second decomposition (320-360°C) corresponded to the loss of *o*-phen, resulting in a 24% weight loss. In the Mn(II) complex, the third decomposition (500-550°C) involved the loss of two PhNHCSN groups (52% weight loss), leaving $\text{Mn}(\text{NCO})_2$ as the residue. In the Zn(II) complex, the third decomposition (410-450°C) involved the loss of two PhCONH groups (42% weight loss), leaving $\text{Zn}(\text{NCSNH})_2$ as the final product. These findings demonstrated that metal complexes tend to exhibit higher thermal stability than the ligands, as evidenced by their higher decomposition stages (Elzaref et al., 2023).

2.4 *IN SILICO* TECHNIQUES

2.4.1 SwissADME

SwissADME, developed by the Swiss Institute of Bioinformatics (SIB), is a web tool used to predict and evaluate the pharmacokinetic and drug-likeness properties of small molecules (Daina et al., 2017). One of its key features is the Bioavailability Radar, which assesses six crucial physicochemical parameters influencing oral bioavailability, including lipophilicity, size, polarity, solubility, flexibility, and saturation. Lipophilicity (LIPO), typically measured by the partition coefficient ($\log P$), plays a major role in a compound's absorption and membrane permeability. Daina et al. (2017) highlighted SwissADME's ability to estimate $\log P$ using multiple predictive models, making it a valuable tool for evaluating a molecule's lipophilicity.

According to Daina et al. (2017), molecular size (SIZE), determined by molecular weight (MW), affects a compound's ability to traverse biological membranes. For optimal drug-likeness, the Bioavailability Radar recommends a molecular weight between 150 and 500 g/mol. Next was polarity (POLAR) which is measured by topological polar surface area (TPSA). It significantly impacts both solubility and membrane permeability. A TPSA within 20 to 130 Å² is considered ideal for oral absorption. Additionally, solubility (INSOLU) is a key factor in drug absorption and distribution. SwissADME employs various predictive models to estimate a compound's aqueous solubility, ensuring it meets the required threshold for effective bioavailability.

Saturation (INSATU), represented by the fraction of sp³-hybridized carbons (Csp³), influences a molecule's three-dimensionality and interactions with biological targets. A Csp³ value above 0.25 is generally preferred, as it enhances molecular stability and receptor binding. Lastly, molecular flexibility (FLEX), indicated by the number of rotatable bonds, determines how easily a molecule can adjust its conformation. The Bioavailability Radar suggests that maintaining nine or fewer rotatable bonds is optimal for drug absorption (Daina et al., 2017).

On the other hand, drug-likeness rules are used in drug discovery to assess a compound's potential as a successful drug candidate (Y. Wang et al., 2015). These rules are based on a statistical analysis of properties found in known drugs and their derivatives. SwissADME evaluates drug-likeness by applying several rules, including Lipinski's Rule, the Ghose rule, the Veber rule, the Egan rule, and the Muegge rule. One of the most well-known guidelines is Lipinski's Rule of Five, proposed by Christopher Lipinski in 1997 (Lipinski, 2004; Lipinski et al., 1997).

According to Lipinski's Rule of Five, a compound should meet the following criteria:

1. Molecular weight under 500 daltons.
2. Not more than 5 hydrogen bond donors.
3. Not more than 10 hydrogen bond acceptors.
4. Log P value should not exceed 5.

Compounds within these limits are more likely to have good oral bioavailability and permeability, increasing their chances of being successful drug candidates. However, it is important to note that these rules are not strict, and some successful drugs may not fully meet these criteria. To date, SwissADME analyses of SBDTC derivatives and their metal complexes were limited in the literature. Thus, comprehensive SwissADME parameter evaluations remain an area for future research.

2.4.2 ProTox-II

Protox-II is an advanced web-based tool designed to predict the acute oral toxicity of chemical compounds based on their structure and bioactivity. One of its key features is the classification of substances into six LD₅₀ toxicity classes, which range from Class 1 (extremely toxic) to Class 6 (non-toxic) (Banerjee et al., 2018). LD₅₀ refers to the dose required to kill 50% of the test population when administered orally (Abal et al., 2017). According to Drwal et al. (2014), toxicity is divided into six categories based on LD₅₀ values as outlined below (Drwal et al., 2014):

1. Class I: Compounds with an LD₅₀ of 5 mg/kg or less are extremely toxic. They pose a high fatality risk even in small amounts, such as cyanide and strychnine (Diener et al., 1994; Schlede et al., 1995).
2. Class II: Compounds with an LD₅₀ between 5 mg/kg and 50 mg/kg are highly toxic. These compounds, including some pesticides and toxic pharmaceuticals, require strict safety measures (Stallard et al., 2004).
3. Class III: Compounds with an LD₅₀ ranging from 50 mg/kg to 300 mg/kg are moderately toxic. They may lead to serious health effects and require medical intervention. Examples include certain industrial chemicals and over-the-counter medications (Schlede et al., 1995).
4. Class IV: Compounds with LD₅₀ values between 300 mg/kg and 2000 mg/kg are slightly toxic. They can still cause adverse health effects, such as some household cleaning products and food additives (Schlede et al., 1995).

5. Class V: Compounds with LD₅₀ values from 2000 mg/kg to 5000 mg/kg are practically non-toxic. These substances, like cosmetics and food colourings, have lower acute toxicity profiles (Schlede et al., 1995).
6. Class VI: Compounds with an LD₅₀ greater than 5000 mg/kg are considered non-toxic. They pose minimal risk and are generally regarded as safe for human exposure, such as water or some dietary supplements (Diener et al., 1994).

In summary, ProTox-II's LD₅₀ classification is crucial for assessing acute oral toxicity. It helps guide safety protocols and decision-making throughout drug development, allowing researchers to prioritise safer compounds and minimise the risk of toxicity in potential drug candidates. However, a review of the existing literature reveals that studies applying ProTox-II to predict the acute oral toxicity of SBDTC derivatives and their metal complexes are also limited. Therefore, a more comprehensive evaluation of these predictions remains an important direction for future research.

2.4.3 Molecular Docking

Molecular docking is a vital tool in drug discovery that predicts how a potential drug (ligand) interacts with its target protein (Meng et al., 2011). It helps researchers understand how a ligand binds to proteins, such as enzymes or receptors, which are involved in important biological functions. Through simulations, docking provides information on the binding orientation, affinity, and the nature of interactions between the ligand and the protein's active site (Saikia & Bordoloi, 2018). Two primary types of interactions observed in molecular docking are hydrogen bonds and hydrophobic interactions (Meng et al., 2011). Hydrogen bonds form when a hydrogen atom attached to an electronegative atom, like oxygen or nitrogen, interacts with another electronegative atom. Strong hydrogen bonds are characterised by short distances and favourable angles, significantly enhancing binding efficiency. These bonds are essential for the stability of ligand-protein complexes (Vaidyanathan et al., 2023).

Hydrophobic interactions, on the other hand, occur between non-polar molecules or non-polar parts of molecules in an aqueous environment. These interactions are important for stabilising the complex by hiding hydrophobic regions of the ligand within the hydrophobic pockets of the protein. Several types of hydrophobic

interactions include π - π interactions, π -alkyl interactions, and van der Waals forces (Brylinski, 2018). While weaker than hydrogen bonds, hydrophobic interactions complement hydrogen bonding by improving ligand-protein affinity. Rashid et al. (2016) demonstrated that although TMP forms critical hydrophilic interactions with DHFR, compounds exhibiting greater hydrophobicity tend to have higher potential as inhibitors of *E. coli* DHFR.

Additionally, molecular docking also plays a key role in Structure-Activity Relationship (SAR) analysis by connecting the structural features of drugs with their binding affinity to target proteins. By understanding how changes in functional groups affect binding, researchers can refine the design of more effective compounds (Fan et al., 2019). For example, trimethoprim (TMP) has an amino nitrogen that acts as a hydrogen bond acceptor, interacting with ASP27, which is crucial for its activity as a DHFR inhibitor (Wróbel, Arciszewska, et al., 2019). TMP's trimethoxybenzyl group also forms hydrophobic interactions with residues like LEU28, ILE50, PHE31, and ILE94, enhancing its lipophilicity (Pavithra et al., 2024). These docking insights are essential for optimising and developing new drug candidates.

2.4.3.1 AutoDock4 Software

AutoDock4 is a widely recognised molecular modelling software that is extensively used in docking studies. Developed by the Olson Laboratory at The Scripps Research Institute (Trott & Olson, 2010), it employs an advanced scoring function to predict binding affinities accurately by incorporating various factors that influence molecular interactions (Huey et al., 1998; Pagadala et al., 2017). AutoDock4 is freely available to academic and non-profit users, making it an accessible tool for researchers worldwide. The software's user-friendly graphical interface simplifies the setup of docking experiments and the analysis of results, making it suitable for both novice and experienced users (Santos-Martins et al., 2021).

A key strength of AutoDock4 is its ability to accurately parameterise ligands containing metal ions. This capability allows AutoDock4 to model metal-containing compounds effectively, facilitating robust studies of metal-ion complexes in molecular docking. For instance, Anupama et al. (2020) demonstrated the docking of a Cu(II) complex to a double-stranded DNA structure using AutoDock 4.2 (Anupama, 2020).

Similarly, Malella et al. (2018) employed AutoDock 4.2 to dock Zn(II), Cd(II), and Hg(II) metal complexes of 2-aminonicotinaldehyde against the EGFR protein receptor (Malella et al., 2018). Additional studies, such as those of Islam & Pillay (2020), and Bursal (2021), further showcase AutoDock4's versatility and effectiveness in docking metal complexes (Bursal et al., 2021; M. A. Islam & Pillay, 2020).

2.4.4 Molecular Dynamics (MD) Simulation

Molecular dynamics (MD) simulation is a powerful computational method that provides insights into the behaviour of molecules and atoms over time. Unlike molecular docking, which offers a static view of ligand binding to a protein, MD simulations track the dynamic nature of the interaction. This includes the flexibility and conformational changes of both the ligand and the protein (Alonso et al., 2006). By simulating the movements of molecules in real time, MD simulations contribute to designing more potent and specific drug candidates.

2.4.4.1 Procedure in Molecular Dynamics (MD) Simulation

In molecular dynamics (MD) simulations, several crucial steps must be taken to ensure accurate and reliable results. These steps involve preparing the ligand and protein structures, adding solvent and ions, performing energy minimization, and conducting equilibration. The initial step is the preparation of topology files for both the ligand (drug molecule) and protein. These files describe the interactions between atoms within each molecule, specifying key parameters such as charges, bond lengths, and angles (Ramos et al., 2021). The force field, which defines how atomic interactions are computed, must be selected carefully to ensure that the simulation provides accurate results. A commonly used force field is AMBER, known for its reliability in computational chemistry (J. Wang et al., 2004). AMBER calculates the forces between atoms in molecules, including those in complex biomolecules such as proteins (González, 2011). It is particularly advantageous for modelling metal-containing ligands, such as copper, cobalt, and nickel (Haghshenas et al., 2020; Macchiagodena et al., 2020). However, a limitation of AMBER is its lack of parameters for zinc, which can hinder simulations involving zinc-based complexes.

Once the topology files are prepared, the next step involves solvation. During this step, the system is surrounded by a solvent, usually water, to simulate a biological environment (Heenen et al., 2020). Ions are then added to neutralize the system and simulate the ionic strength typical of living organisms (Idris et al., 2021). Following solvation and ion addition, energy minimization is performed to eliminate any initial stress or unrealistic structures in the system. This step ensures that the system is prepared for stable simulation (Panwar & Kumar, 2021). Equilibration then follows, where the system is adjusted to a stable state by controlling temperature and pressure. During NVT (constant volume and temperature) equilibration, the temperature is controlled, and during NPT (constant pressure and temperature) equilibration, pressure is regulated. Specialized algorithms, such as thermostats and barostats, are used to manage these parameters (Vieira et al., 2023).

Once equilibration is complete and the system is stable, the final MD simulation can proceed. Simulations often run for extended periods, typically up to 100 ns, as seen in studies such as Zarenezhad (2023). Zarenezhad (2023) ran a 100 ns simulation to ensure the stability of methotrexate when bound to its target (Zarenezhad et al., 2023).

2.4.4.2 Analysis of Molecular Dynamics (MD) Simulation

Several key analyses are extracted from molecular dynamics (MD) simulations to provide a thorough understanding of protein-ligand interactions and overall system stability. These analyses, including Root Mean Square Deviation (RMSD), Radius of Gyration (Rg), Root Mean Square Fluctuation (RMSF), pocket volume, hydrogen bonding (H-bonds), and Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) calculations.

RMSD measures the average distance between atoms in a simulated structure and a reference structure (Bagewadi et al., 2023). It tracks how the protein-ligand complex changes over time, revealing structural stability or fluctuations. A significant increase in RMSD suggests instability, while a stable RMSD profile indicates the complex is in equilibrium (Aier et al., 2016). Rg measures the compactness of the protein structure, calculated as the root-mean-square distance of atoms from the centre of mass (Alarcón et al., 2013). A consistent and low Rg value indicates a stable protein, while large fluctuations suggest instability or unfolding (Bekker et al., 1993). RMSF

tracks the fluctuation of individual atoms within the protein over time (Kumar et al., 2014). Higher RMSF values indicate more flexibility, while lower values suggest rigidity. RMSF also includes B-factor values, which reflect atomic mobility in crystal structures (Sun et al., 2019). High B-factor values correspond to increased mobility, while low values indicate less movement. The B-factor visualization, also known as B-factor putty representative aids in understanding protein flexibility during simulations.

Pocket volume, on the other hand, refers to the dynamic cavity in the protein where ligands bind. Monitoring pocket volume during MD simulations reveals how ligand binding affects the active site (Rampogu et al., 2022). Besides, hydrogen bonds play a crucial role in stabilising biomolecular structures and ensuring the function of proteins (Hubbard & Kamran Haider, 2010). A higher number of stable hydrogen bonds (high occupancy) typically indicates a stronger binding affinity and greater complex stability. Similar to docking, binding energy also can be measured from MD simulations using MMPBSA. MMPBSA calculations estimate the binding free energy between the ligand and protein, incorporating both molecular mechanics energies and solvation effects (Miller et al., 2012). However, MMPBSA is more reliable than molecular docking because it accounts for solvent effects and fully captures protein-ligand flexibility, which docking simulations cannot (Tuccinardi, 2021; E. Wang et al., 2019). As a result, MMPBSA calculations often align well with experimental results (B. Sharma et al., 2022).

By combining these analyses, MD simulations offer a comprehensive understanding of the stability, flexibility, and binding dynamics of protein-ligand complexes. This helps researchers predict the molecular mechanisms behind biological processes and design more effective drugs or inhibitors. Notably, prior to this research, studies focusing on MD simulations of metal complexes derived from SBDTC derivatives interacting with bacterial proteins, such as *E. coli* DHFR, were limited. Thus, this work not only deepens the understanding of ligand-protein interactions but also paves the way for designing and optimising more effective metal-based antibacterial compounds.

2.4.4.3 In Silico-In Vitro Integration

The integration of *in silico* techniques, particularly molecular docking and MD simulations, with *in vitro* assays has provided strong support for the identification of new inhibitors especially targeting DHFR. Several studies have demonstrated how computational methods validate and explain biological activity observed in experimental assays.

In the study conducted by Mishra and Muthukaliannan (2024), the peptide AM1 derived from *Aegle marmelos* was evaluated for its antimicrobial activity through both *in vitro* and *in silico* approaches, specifically targeting the DHFR enzyme (Awdhesh & Kodiveri, 2024). The peptide demonstrated potent inhibitory effects against Methicillin-Susceptible *Staphylococcus aureus* (MSSA), Methicillin-Resistant *Staphylococcus aureus* (MRSA), and Multi-Drug-Resistant *Staphylococcus aureus* (MDR-SA), with MICs of 2, 4, and 8.5 µg/mL, respectively. These values were notably lower than those of TMP, which exhibited MICs of 2.5, 5, and 9.5 µg/mL for the same strains.

Molecular docking results showed strong interactions between AM1 and the DHFR enzyme, with a maximum binding affinity of -10.2 kcal/mol, surpassing that of TMP (-7.4 kcal/mol). Furthermore, 300 ns MD simulations confirmed the stability of the AM1–DHFR complex, as assessed by RMSD, RMSF, and additional statistical analyses. The MMPBSA binding free energy analysis further supported these findings, revealing a significantly more favorable binding energy for AM1 (-47.69 kcal/mol) compared to TMP (-13.85 kcal/mol). Collectively, these computational results corroborated the *in vitro* antibacterial activity, indicating that peptide AM1 interacts more effectively with bacterial DHFR than TMP.

Sehrawat et al. (2024) synthesised a novel series of 1,2,4-triazole derivatives of caffeic acid and evaluated their potential as antimicrobial agents (Sehrawat et al., 2024). Among the synthesised compounds, 4-(2-(4-(3-nitrobenzylideneamino)-5-mercapto-4H-1,2,4-triazol-3-yl)vinyl)benzene-1,2-diol (CTh3) showed notable antibacterial activity against *S. aureus*, with a MIC of 5 µM. Similarly, 4-(2-(4-(4-hydroxybenzylidene amino)-5-mercapto-4H-1,2,4-triazol-3-yl)vinyl)benzene-1,2-diol (CTh4) displayed significant antibacterial effects against both *E. coli* and *P. aeruginosa*, with MIC values of 5 µM for each strain. Molecular docking analysis of CTh3, the most active antimicrobial compound revealed key interactions with the DHFR active site, including hydrogen bonds with THR121 and ASN18, a π -cation interaction with

PHE92, and a salt bridge with the polar residue ASP27. Thus, these *in silico* findings support the hypothesis that the observed antibacterial activity arises from effective DHFR inhibition.

Nakashima et al. (2022) employed a combined *in vitro* and *in silico* approach to identify novel antimicrobial compounds targeting *S. aureus* DHFR (Nakashima et al., 2022). The antibacterial activity of the candidate compounds was initially evaluated against *Staphylococcus epidermidis*, a model organism closely related to *S. aureus*. Among the screened candidates, two compounds demonstrated strong growth inhibitory effects, with IC₅₀ values of 6.34 μ M and 56.94 μ M, respectively. MD simulations further predicted stable and direct interactions between these active compounds and the TMP-resistant DHFR from *S. aureus* (saDHFR). Overall, the correlation between high stability and observed biological activity highlights the effectiveness of *in silico* tool in predicting therapeutic efficacy and guiding drug discovery efforts.

Adhikari et al. (2022) investigated the antimalarial potential of novel *N*-(4-aminobenzoyl)-*L*-glutamic acid-conjugated 1,3,5-triazine derivatives as inhibitors of *Plasmodium falciparum* dihydrofolate reductase (PfDHFR) (Adhikari et al., 2022). *In vitro* assays revealed that among the ten compounds tested, 2-[(4-{[4-(Methylamino)-6-(piperidin-1-yl)-1,3,5-triazin-2-yl]amino}phenyl)formamido]pentanedioic acid (Df3) exhibited the highest antimalarial activity against the chloroquine-resistant Dd2 strain, with an IC₅₀ value of 9.54 μ g/mL. Molecular docking analysis showed that Df3 established strong interactions with key residues such as SER108, SER167, THR107 (PDB ID: 1J3I), and ARG122 (PDB ID: 1J3K). Furthermore, MD simulations indicated that Df3 bound to PfDHFR with greater stability and affinity compared to the co-crystallized reference ligand WRA609. These findings support the potential of Df3 as a promising PfDHFR inhibitor and highlight the compound's suitability for further development as a new class of antimalarial agents.

In summary, these studies demonstrated that *in silico* methods not only aid in predicting potential protein inhibitors but also support and validate the findings of *in vitro* antibacterial assays. The combined use of molecular docking and MD simulations enables the identification of key molecular interactions, evaluation of binding stability, and prediction of binding free energies, making them essential tools in contemporary antibacterial drug discovery efforts.

METHODOLOGY

3.1 LIST OF CHEMICALS, APPARATUS, GLASSWARE, CONSUMABLE ITEMS, EQUIPMENT AND INSTRUMENTS USED

All chemicals, apparatus, glassware, consumable items, equipment and instruments used in this study were listed in Table 3.1 to Table 3.5. The chemicals and commercial reagents in Table 3.1 were used as received in the synthesis process. The synthesis was conducted at Research Laboratory 4.2, Physical Science Building, Kulliyah of Science, IIUM Kuantan.

Table 3.1 List of chemicals used

Chemicals	Supplier	Chemical grade (%)
Absolute ethanol	HmBG® Chemicals	99
Methanol	HmBG® Chemicals	99
Benzyl chloride	Acros Organics	99.5
Hydrazine hydrate	Merck	-
Carbon disulfide	Merck	-
Potassium hydroxide	Merck	-
Nitric acid	Merck	65
2-methoxybenzoyl chloride	Sigma Aldrich	99
3-methoxybenzoyl chloride	Sigma Aldrich	99
4-methoxybenzoyl chloride	Sigma Aldrich	99
4-chloro-3-nitrobenzoyl chloride	Sigma Aldrich	99
Copper(II) acetate monohydrate	R&M Chemicals	99.5
Nickel(II) acetate tetrahydrate	Acros Organics	99.5
Zinc(II) acetate dihydrate	Merck	99.5
Cobalt(II) acetate tetrahydrate	Acros Organics	99.5
Silica gel	Bendosen	-
Dimethyl sulfoxide	Fisher Scientific	99
Cystine	Leco	-
Potassium bromide	Uvasol	-

12.88 mS/cm conductivity standard solution	Eutech Instruments	-
Nutrient agar	Himedia	-
Nutrient broth	Himedia	-
Gentamycin	VWR Life Science	99
Ampicillin	VWR Life Science	99
Novobiocin	Fisher Scientific	99
Trimethoprim	MedChem Express	99

Table 3.2 List of apparatus and glassware used

Apparatus and glassware	Supplier
Beakers - 50, 100, 250, 500 & 600 mL	HmBG® Chemicals
Glass bottle - 50, 250, 500 & 1000 mL	SCHOTT DURAN®
Glass dropper	HmBG® Chemicals
Measuring cylinder - 10 & 100 mL	DURAN®
Volumetric flask 50, 100 & 500 mL	NICE
Filter funnel	HmBG® Chemicals
Burette 50 mL	NICE
Separatory funnel 500 mL	HmBG® Chemicals
Buchner funnel	-
Spatula	-
Thermometer	-
Glass rod	-
Melting point tube	-
Watch glass	DURAN®
Agate mortar and pestle	-
UV Quartz Glass	Shimadzu
Micropipette	Thermo Scientific
Hockey stick L-shape	-
Inoculation loop	-

Table 3.3 List of consumable items used

Disposable / Consumable Item	Supplier
20 mL disposable scintillation vials	Favorit
Gloves	Cleanguard
Parafilm	Bemis®
Filter paper (Grade No.1)	Whatman
Aluminium foil	Diamond
Plastic dropper	-
Plastic cuvette	-
Magnetic bar	-
Micropipette tips	Thermo Scientific
Agar disc	Favorit
96 well microplate	NICE

Table 3.4 List of equipment used

Equipment	Model
Analytical balance	Shimadzu
Hot plate	Favorit
Magnetic Stirrer	-
Autoclave machine	Hirayama
Ultrasonic Cleaner Set	DAIHAN Scientific WUC-D10H
Drying oven	Memmert UFB 400
-80 C Freezer	Medfrez
Incubator	Binder
Chiller	-
Laminar flow cabinet	Esco

Table 3.5 List of instruments used

Instrument	Model
Melting point apparatus	Electrothermal™ IA9300
CHNS elemental analyzer	Perkin Elmer EA 2400
Atomic Absorption Spectrometer	Perkin Elmer The AAnalyst™ 700

FTIR Spectrophotometer	Perkin Elmer 96255 FTIR Frontier
¹ H and ¹³ C NMR Spectrometer	Bruker Ultra Shield Plus 500MHz
GC-MS Spectrometry	Perkin Elmer CLARUS 680 GC / CLARUS 5S8T MS
Double beam UV-VIS Spectrometer	Shimadzu UV-1900 Series
Single-beam UV-Vis Spectrophotometer	Shimadzu UV mini-1240 Series
Conductivity meter	Eutech CON 700
Magnetic Susceptibility Balance	Sherwood Scientific MSB AUTO
Thermogravimetric Analyzer	Shimadzu TGA-50 Series

3.2 LIST OF HARDWARE AND SOFTWARE USED

For the computational study, all the hardware and software used were listed in Table 3.6 and Table 3.7 respectively. All software used was open-source software. Furthermore, the computational study was conducted at the Computer Aided Drug Discovery Development Laboratory, Kulliyyah of Pharmacy, IIUM Kuantan.

Table 3.6 List of hardware used

Computers (Processor)	Operating system
Custom PC (Intel Core i9-12900F processor with 2.4 GHz)	Ubuntu 2024.4 LTS
Gaming Computer (Intel i7-10700F processor with 2.9 GHz)	Windows 11
Acer Notebook (12 th Gen Intel® Core™ processors)	Windows 11

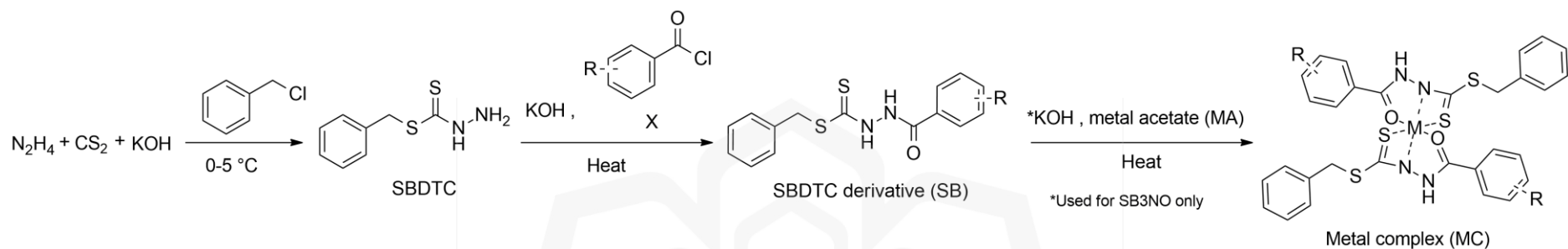
Table 3.7 List of software used

Software	Company / Organizations
ChemDraw Ultra 12.0	PerkinElmer Informatics
Avogadro	Open-Source Molecular Graphics
UCSF Chimera	University of California, San Francisco (UCSF)

AutoDockTools (ADT)	The Scripps Research Institute
AutoDock 4.2	The Scripps Research Institute
GROMACS 2023.3	KTH Royal Institute of Technology and Uppsala University
AmberTools23 (part of the Amber Molecular dynamics Package)	University of California, San Francisco (UCSF)
BIOVIA Discovery Studio 2021 visualizer	Dassault Systèmes
PyMOL Edu version	Schrödinger, LLC
QtGrace	Ion Vasilief
Visual Molecular dynamics (VMD)	Theoretical and Computational Biophysics Group of the University of Illinois, Urbana-Champaign

3.3 SYNTHESIS METHOD

This study utilised two primary synthesis methodologies based on the precursor *S*-benzyl dithiocarbazate (SBDTC) which is the synthesis of SBDTC derivatives and the subsequent synthesis of their metal complexes. Four SBDTC derivatives were synthesised, namely *S*-benzyl- β -*N*-2-methoxybenzoyl dithiocarbazate (SB2OME), *S*-benzyl- β -*N*-3-methoxybenzoyl dithiocarbazate (SB3OME), *S*-benzyl- α -*N*-4-methoxybenzoyl dithiocarbazate (SB4OME), and *S*-benzyl- β -*N*-4-chloro-3-nitrobenzoyl dithiocarbazate (SB3NO). Each derivative was subsequently reacted with four different transition metal ions, yielding a total of sixteen metal complexes. The synthesis pathway for all designed compounds was illustrated in Scheme 3.1.



R substitution	Compound X	SBDTC derivative (SB)	Metal acetate (MA)	Element M	Metal complex (MC)
<i>ortho</i> -OCH ₃	2-methoxybenzoyl chloride (2OME)	<i>S</i> -benzyl-β- <i>N</i> -2-methoxybenzoyl dithiocarbazate (SB2OME)	Copper(II) acetate monohydrate	Cu	Cu(SB2OME) ₂
			Zinc(II) acetate dihydrate	Zn	Zn(SB2OME) ₂
			Cobalt(II) acetate tetrahydrate	Co	Co(SB2OME) ₂
			Nickel(II) acetate tetrahydrate	Ni	Ni(SB2OME) ₂
<i>meta</i> -OCH ₃	3-methoxybenzoyl chloride (3OME)	<i>S</i> -benzyl-β- <i>N</i> -3-methoxybenzoyl dithiocarbazate (SB3OME)	Copper(II) acetate monohydrate	Cu	Cu(SB3OME) ₂
			Zinc(II) acetate dihydrate	Zn	Zn(SB3OME) ₂
			Cobalt(II) acetate tetrahydrate	Co	Co(SB3OME) ₂
			Nickel(II) acetate tetrahydrate	Ni	Ni(SB3OME) ₂
<i>para</i> -OCH ₃	4-methoxybenzoyl chloride (4OME)	<i>S</i> -benzyl-α- <i>N</i> -4-methoxybenzoyl	Copper(II) acetate monohydrate	Cu	Cu(SB4OME) ₂
			Zinc(II) acetate dihydrate	Zn	Zn(SB4OME) ₂

<i>para</i> -Cl- <i>meta</i> -NO ₂	4-chloro-3-nitrobenzoyl chloride (3NO)	<i>S</i> -benzyl-β- <i>N</i> -4-chloro-3-nitrobenzoyl dithiocarbazate (SB3NO)	dithiocarbazate (SB4OME)	Cobalt(II) acetate tetrahydrate	Co	Co(SB4OME) ₂
				Nickel(II) acetate tetrahydrate	Ni	Ni(SB4OME) ₂
				Copper(II) acetate monohydrate	Cu	Cu(SB3NO) ₂
				Zinc(II) acetate dihydrate	Zn	Zn(SB3NO) ₂
				Cobalt(II) acetate tetrahydrate	Co	Co(SB3NO) ₂
				Nickel(II) acetate tetrahydrate	Ni	Ni(SB3NO) ₂

Scheme 3.1 Synthesis pathway of the designed SBDTC derivatives and their respective metal complexes

3.3.1 Synthesis of *S*-benzylthiocarbamate (SBDTC)

SBDTC was prepared with some modifications (Scheme 3.1) as previously reported by (M. A. Ali & Bose, 1977). Potassium hydroxide (0.1 mol, 5.7 g) was dissolved in 35 mL of cold 90% ethanol and was added with hydrazine hydrate (0.1 mol, 5 mL). The mixture was cooled in an ice salt bath until the temperature reached 0 °C. To the resulting cooled solution, carbon disulphide (0.1 mol, 6 mL) was added dropwise over an hour with constant stirring using a mechanical stirrer. Two layers were formed with the brown oil (lower layer) was separated and dissolved in 30 mL of cold 40% ethanol. The solution was kept in an ice salt bath (0-5 °C) and benzyl chloride (0.1 mol, 11.37 mL) was added dropwise with vigorous stirring. After the addition of benzyl chloride was completed, a white precipitate was obtained, which was filtered and dried overnight over silica gel. Finally, it was recrystallised from 40 ml of absolute ethanol to obtain the pure product.

3.3.2 General synthesis of *S*-benzyl- β -*N*-2-methoxybenzoyl dithiocarbamate (SB2OME), *S*-benzyl- β -*N*-3-methoxybenzoyl dithiocarbamate (SB3OME) and *S*-benzyl- α -*N*-4-methoxybenzoyl dithiocarbamate (SB4OME)

The experimental procedure was prepared according to previous literature (How et al., 2008). SBDTC (0.01 mol, 1.983 g) dissolved in 30 mL absolute ethanol was mixed with potassium hydroxide (0.01 mol, 0.56 g) in 10 mL ethanol. An equimolar amount of compound X was added to the solution with constant stirring and heating. After the complete addition of compound X, the resulting solution was heated for approximately one hour to reduce the volume to half and give precipitation. The precipitate was filtered while the solution was hot and dried overnight over silica gel. The precipitate was later recrystallised from 30 ml of absolute ethanol.

SBDTC derivatives	Compound X	Volume (ml)
<i>S</i> -benzyl- β - <i>N</i> -2-methoxybenzoyl dithiocarbamate (SB2OME)	2-methoxylbenzoyl chloride (2OME)	1.4887
<i>S</i> -benzyl- β - <i>N</i> -3-methoxybenzoyl dithiocarbamate (SB3OME)	3-methoxylbenzoyl chloride (3OME)	1.4053
<i>S</i> -benzyl- α - <i>N</i> -4-methoxybenzoyl dithiocarbamate (SB4OME)	4-methoxylbenzoyl chloride (4OME)	1.3540

3.3.3 Synthesis of *S*-benzyl- β -*N*-4-chloro-3-nitrobenzoyl dithiocarbazate (SB3NO)

The experimental procedure was prepared according to previous literature (How et al., 2008). SBDTC (0.01 mol, 1.983 g) dissolved in 30 ml absolute ethanol was mixed with potassium hydroxide (0.01 mol, 0.56 g) in 10 ml ethanol. 4-chloro-3-nitrobenzoyl chloride (0.01 mol, 2.20 g) in 30 ml absolute ethanol was added dropwise to the SBDTC solution with constant stirring and heating. After the completion addition of 4-chloro-3-nitrobenzoyl chloride, the resulting solution was reduced to half the volume. After heating the reaction for approximately 20 minutes, the final product still did not form so filtration could not be done during hot. However, the product started to form after a few hours left on the laboratory's bench for one day. Thus, the solution was allowed to stand overnight before the precipitate was filtered and dried over silica gel. The product was recrystallised from 30 ml of absolute ethanol.

3.3.4 General synthesis of the metal complexes from SB2OME, SB3OME and SB4OME

The metal complexes were prepared following the method from previous literature (Khoo et al., 2014). SBDTC derivatives (0.9 mmol, 0.3 g) dissolved in absolute ethanol (30 mL) were added to a hot solution of the metal salt (0.9 mmol) in the same solvent (30 mL). The mixture was heated and stirred until the volume was reduced by half at approximately two hours. The precipitate formed was filtered while the solution was hot and dried overnight over silica gel. The metal salts used are copper(II) acetate monohydrate (0.09 g), zinc(II) acetate dihydrate (0.098 g), cobalt(II) acetate tetrahydrate (0.11 g) and nickel(II) acetate tetrahydrate (0.11 g).

3.3.5 Synthesis of the metal complexes from SB3NO

The metal complexes were prepared based on the method from previous literature (Break et al., 2013). Potassium hydroxide (0.9 mmol, 0.08 g) dissolved in absolute ethanol (30 ml) was added to SB3NO (0.9 mmol, 0.3 g) dissolved in boiling ethanol (30 ml). The metal salt (0.9 mmol) dissolved in the same solvent (30 ml) was later added to the mixture. The mixture was heated and stirred until the volume was reduced at approximately two hours. The precipitate formed was filtered while the solution was hot and dried overnight over silica gel. The metal salts used are copper(II) acetate

monohydrate (0.09 g), zinc(II) acetate dihydrate (0.098 g), cobalt(II) acetate tetrahydrate (0.11 g) and nickel(II) acetate tetrahydrate (0.11 g).

3.4 CHARACTERISATION

3.4.1 Melting Point Determination

A small amount of the sample was filled into a capillary tube about 3 to 4 mm long. The filled capillary tube was then inserted into the observation chamber. The initial heating temperature was set at 50 °C with a heating rate of 5 °C/min. Then, the heating process was started and the temperature at which the first sign of melting was reported. The temperature was also reported when the entire sample was melted after the heating process continued. In some cases, the entire sample was not fully melted up to 400 °C thus the decomposition temperature was recorded instead. The decomposition temperature was recorded when the colour or quantity of the sample changed significantly.

3.4.2 CHNS Elemental Analysis

Approximately 1.9 to 2.1 mg of the sample was weighed into a tared tin capsule. The top of the capsule was then pinched with forceps to seal it and folded twice over the edge to ensure that the sample was securely enclosed. The capsule was flattened and gently squeezed to form a ball or cube before being placed in 96-well microtitre plates. Once all samples had been prepared and weighed, they were placed in the autosampler of the elemental analyser. The analyser was first calibrated with the standard cystine and the blank sample (empty tin capsule) before the samples were analysed. The samples were combusted in a reactor at 1000°C. In the presence of excess oxygen and combustion reagents, the samples were combusted completely and reduced to the elemental gases CO₂, H₂O, SO₂ and N₂. The combustion products were carried by a constant carrier gas stream (helium) to be separated and finally detected with the detector. The CHNS element contents were expressed in weight per cent.

3.4.3 Atomic Absorption Spectroscopy (AAS)

Approximately 1 mg of the sample was accurately weighed and completely digested in 60% concentrated nitric acid. The sample solution was then slowly heated to remove

nitrogen oxides before being diluted to 10 mL volumetric flasks and maintained in 5% nitric acid. A blank solution was prepared by diluting the 60% nitric acid to 5% nitric acid with distilled water. Prior to analysis, metal standard solutions were prepared to obtain a linear calibration curve with an R-value greater than 0.995. They were prepared from a stock standard solution of the metal of interest with a concentration of 1000 mg/L. Then, a series of working standard solutions were obtained by diluting the stock standard solution to cover the expected concentration range of the samples. Finally, after successful calibration, the sample was run in triplicate.

3.4.4 Fourier Transform Infrared (FTIR) Spectroscopy

The apparatuses were cleaned with acetone before sample preparation. Approximately 1-2 mg of the sample was ground to a fine powder with potassium bromide (KBr) salt in a ratio of 1:10 using an agate mortar and pestle. The fine sample was placed in a pellet press with a spatula and scattered evenly so that the surface of the mould was covered. The sample was then compressed in a manual hydraulic press with 10 tonnes at about 1 minute. Then, the pellet was removed from the hydraulic press before it was placed in the sample holder and inserted in the spectrometer. The samples were analysed in the range from 4000 cm^{-1} to 400 cm^{-1} .

3.4.5 ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy

The sample was sent to the Central Laboratory of University Malaysia Pahang for NMR analyses. Approximately 0.01 to 0.03 g of sample was dissolved in 600 μL deuterated dimethyl sulphoxide (DMSO-d_6) solvent and transferred into a 5 mm NMR tube. Tetramethylsilane (TMS) was used as an internal standard. The sample is then inserted into the NMR spectrometer for analysis. The chemical shift of the ^1H NMR spectra was in the range of 0 to 12 ppm while ^{13}C NMR spectra were in the range of 0 to 200 ppm.

3.4.6 Mass Spectrometry (MS)

Prior to sample preparation, all glass vials were ultrasonically cleaned using soap in an ultrasonic cleaner for approximately 10 minutes. This cleaning step was repeated, first with distilled water and subsequently with methanol to ensure the removal of contaminants. After cleaning, approximately 0.1 g of the sample was weighed and

dissolved in 10 mL of methanol. The solution was then filtered using a 0.22 μm filter membrane attached to a syringe to remove any particulate matter. The filtered solution was carefully transferred into a clean vial, which was sealed and placed into the sample holder for analysis.

The GC was equipped with an Elite-5MS column (30 m $\times$ 0.25 mm internal diameter $\times$ 250 μm film thickness), consisting of a stationary phase made of 5% biphenyl and 95% dimethylpolysiloxane. Helium, an inert gas, was used as the carrier gas at a constant flow rate. The oven temperature was initially set at 35 $^{\circ}\text{C}$ and then programmed to increase at a rate of 5 $^{\circ}\text{C}/\text{min}$ until reaching a final temperature of 250 $^{\circ}\text{C}$. This temperature ramp allowed for the gradual separation of the components present in the sample. The mass spectrometer was operated using electron ionization (EI) mode, and the resulting spectra were processed using TurboMass software version 5.4.2.

3.4.7 Ultraviolet-Visible (UV-Vis) Spectroscopy

Samples were prepared in two dilutions of 10^{-3} M and 10^{-4} M in DMSO. First, the baseline was set with the range of wavelength from 200 nm to 800 nm. Then 4 mL of DMSO was added to the clean quartz cuvette and this was placed in the instrument. The sample was then poured into the second quartz cuvette before it was inserted into the instrument. The analysis was then started by simply pressing the start button. After each run, the cuvette was rinsed with distilled water and dried before the next sample was inserted. This process was repeated until all samples had been analysed.

3.4.8 Molar Conductivity

The molar conductivity analysis was started with calibration. A dip-type conductivity cell with a platinised electrode was calibrated using a 12.88 mS/cm conductivity standard calibration solution. After successful calibration, sample dilution of 10^{-3} M was prepared in DMSO. Once the solution was prepared, the next step was to run the conductivity measurement. The analysis was performed by immersing the electrode in the solution and stirring to ensure that the ions in the solution were evenly distributed. The electrode was left in the solution for about 5 minutes before the measurement was performed. The conductivity of each solution was measured at a fixed temperature of 25 $^{\circ}\text{C}$.

3.4.9 Magnetic Susceptibility

The magnetic susceptibility of the metal complexes was determined at the University of Malaya, Malaysia, and mercury (II) tetrathiocyanatocobaltate (II) was used as a standard. The empty capillary tube was weighed and placed in the magnetic susceptibility balance for the 'TARE' step. Then 30-80 mg of the metal complex was ground with an agate mortar and pestle and added to the empty capillary tube. The tube filled with the sample was weighed again to determine the actual weight of the sample. The length of the sample in the tube was measured and must be at least 30 mm before it was inserted into the magnetic susceptibility balance. The values of length and weight of the sample were entered into the RS232 computer interface and the readings were then recorded.

3.4.10 Thermogravimetric Analysis (TGA)

The sample was prepared by weighing approximately 4-11 mg of the sample into a tared crucible before loading it into the instrument. All parameters were set in the programme, including the mass of the sample, the maximum temperature, the heating rate and the purge gas. Nitrogen gas was used at a flow rate of 10 mL/min. The initial temperature was set to 30 °C and the maximum temperature was set up to 500 °C for SBDTC derivatives and 800 °C for the metal complexes at a heating rate of 10 °C/min. Finally, the analysis was started by simply pressing the start button.

3.5 ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION, TOXICITY (ADMET)

ADMET properties of the synthesised compounds and control samples were evaluated using two online tools which are SwissADME (Daina et al., 2017) and Pro-Tox II (Banerjee et al., 2018). Initially, the 2D chemical structures of the synthesised compounds were drawn using ChemDraw software. Once finalised, the 2D structures were converted into SMILES (Simplified Molecular Input Line Entry System) notation using ChemDraw. This text-based format, which encodes molecular structures, was then used as input for analysis in SwissADME and Pro-Tox II, as summarized in Table 3.8.

Table 3.8 SMILES notation of compounds for ADMET study

Compounds	SMILES
SBDTC	<chem>NNC(=S)SCc1ccccc1</chem>
SB2OME	<chem>COc1ccccc1C(=O)NNC(=S)SCc1ccccc1</chem>
Cu(SB2OME) ₂	<chem>COc1ccccc1C1=[O][Cu]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccccc1OC)SCc1ccccc1</chem>
Zn(SB2OME) ₂	<chem>COc1ccccc1C1=[O][Zn]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccccc1OC)SCc1ccccc1</chem>
Co(SB2OME) ₂	<chem>COc1ccccc1C1=[O][Co]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccccc1OC)SCc1ccccc1</chem>
Ni(SB2OME) ₂	<chem>COc1ccccc1C1=[O][Ni]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccccc1OC)SCc1ccccc1</chem>
SB3OME	<chem>COc1cccc(c1)C(=O)NNC(=S)SCc1ccccc1</chem>
Cu(SB3OME) ₂	<chem>COc1cccc(c1)C1=[O][Cu]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1cccc(c1)OC)SCc1ccccc1</chem>
Zn(SB3OME) ₂	<chem>COc1cccc(c1)C1=[O][Zn]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1cccc(c1)OC)SCc1ccccc1</chem>
Co(SB3OME) ₂	<chem>COc1cccc(c1)C1=[O][Co]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1cccc(c1)OC)SCc1ccccc1</chem>
Ni(SB3OME) ₂	<chem>COc1cccc(c1)C1=[O][Ni]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1cccc(c1)OC)SCc1ccccc1</chem>
SB4OME	<chem>COc1ccc(cc1)C(=O)NNC(=S)SCc1ccccc1</chem>
Cu(SB4OME) ₂	<chem>COc1ccc(cc1)C1=[O][Cu]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccc(cc1)OC)SCc1ccccc1</chem>
Zn(SB4OME) ₂	<chem>COc1ccc(cc1)C1=[O][Zn]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccc(cc1)OC)SCc1ccccc1</chem>
Co(SB4OME) ₂	<chem>COc1ccc(cc1)C1=[O][Co]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccc(cc1)OC)SCc1ccccc1</chem>
Ni(SB4OME) ₂	<chem>COc1ccc(cc1)C1=[O][Ni]234(N1N=C(S2)SCc1ccccc1)SC(=NN4C(=[O]3)c1ccc(cc1)OC)SCc1ccccc1</chem>
SB3NO	<chem>S=C(SCc1ccccc1)NNC(=O)c1ccc(c(c1)[N+](=O)[O-])Cl</chem>

	[O-
Cu(SB3NO) ₂	][N+](=O)c1cc(ccc1Cl)C1=[O][Cu]2(SC(=NN1)SCc1cccc1)SC(=NNC(=[O]2)c1ccc(c(c1)[N+](=O)[O-])Cl)SCc1cccc1
	[O-
Zn(SB3NO) ₂	][N+](=O)c1cc(ccc1Cl)C1=[O][Zn]2(SC(=NN1)SCc1cccc1)SC(=NNC(=[O]2)c1ccc(c(c1)[N+](=O)[O-])Cl)SCc1cccc1
	[O-
Co(SB3NO) ₂	][N+](=O)c1cc(ccc1Cl)C1=[O][Co]2(SC(=NN1)SCc1cccc1)SC(=NNC(=[O]2)c1ccc(c(c1)[N+](=O)[O-])Cl)SCc1cccc1
	[O-
Ni(SB3NO) ₂	][N+](=O)c1cc(ccc1Cl)C1=[O][Ni]2(SC(=NN1)SCc1cccc1)SC(=NNC(=[O]2)c1ccc(c(c1)[N+](=O)[O-])Cl)SCc1cccc1
Trimethoprim	COc1cc(Cc2cnc(nc2N)N)cc(c1OC)OC
Rifampin	CO[C@@H]1/C=C/O[C@@@]2(C)Oc3c(C2=O)c2C(=O)C(=C(C(=O)c2c(c3C)O)NC(=O)/C(=C\C=C\[C@@H]([C@@H]([C@H]([C@H]([C@H]([C@@H]([C@@H]([C@@H]1C)OC(=O)C)C)O)C)O)C)/C)/C=N/N1CCN(CC1)C

For ADME prediction, the SMILES notation for each compound was submitted to the SwissADME web portal. The output from SwissADME comprised graphical representations and tabulated data for each pharmacokinetic parameter. These results were subsequently downloaded and analysed to assess the overall pharmacokinetic profiles of the compounds. Meanwhile, for toxicity analysis, the same SMILES notations utilised for the SwissADME evaluation were submitted to the Pro-Tox II web portal. The classification of LD₅₀ values and their respective toxicity classes was examined in detail.

3.6 ANTIMICROBIAL ASSAY

3.6.1 Bioactivity and Target Microorganisms

The antimicrobial activity was investigated in the Microbiological Laboratory of Physical Science Building, Kulliyah of Science, International Islamic University Malaysia. The four pathogenic bacteria used were *Staphylococcus aureus* (ATCC

25923), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922).

3.6.2 Preparation of Nutrient Agar and Nutrient Broth

Nutrient agar and nutrient broth were selected as medium due to their compatibility with the microorganisms (Balouiri et al., 2016). The agar was prepared by dissolving 28 g of agar powder in 1 L of distilled water and transferring it into a Schott bottle. Meanwhile, 13 g of broth powder was also dissolved in 1 L of distilled water. Both media together with all apparatus used for the microbial test were autoclaved at 121 °C for about 30 minutes. After autoclaving, the agar medium was poured into a sterile Petri dish, filling about three-quarters of the dish. The dish was then set aside to cool at room temperature. Finally, both media were kept in a chiller, in which the medium agar was kept upside down to avoid contamination with the water vapour. The preparation process was carried out in a laminar flow with the presence of UV light.

3.6.3 Bacteria Culture

Four pathogenic bacteria were cultured according to the reported literature (Malmberg et al., 2016). All strains, *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were stored at -80°C and streaked on nutrient agar plates. Strains were grown at 37°C on a nutrient medium 24 hours before the MIC assay. Bacterial inocula were prepared by picking 3-4 colonies from the agar plate and adjusting to the 0.5 Mc Farland standard solutions in nutrient broth. The turbidity of the bacteria was determined by the optical density (OD) using a single-beam UV-Vis Spectrophotometer at 600 nm. The ideal OD for the bacteria is 0.1, which is also equivalent to 1.5×10^8 CFU/mL.

3.6.4 Minimum Inhibitory Concentration (MIC)

Antimicrobial activity was examined by the minimal inhibitory concentration (MIC) method under standard procedures provided by the Clinical & Laboratory Standards Institute (CLSI) with some modifications. MIC was determined by the two-fold serial broth microdilution method in 96-well microtitration plates using a nutrient broth

medium. The stock solution of investigated compounds (7 mg/mL) was prepared in 5% dimethyl sulfoxide (DMSO) and diluted using distilled water.

The analysis was started by adding 100 μ L nutrient broth into all sterilized 96-well plates except for the first row of the well plate. Following that, 200 μ L of the sample or positive control was added to the first row of the well plate, and serial dilutions were carried out by transferring 100 μ L into the second row of the well using a micropipette. The mixture was mixed on the second row of the well before 100 μ L was transferred to the third row well. The step was repeated to the next row well till the last row of the well. The concentration of the first row to the last row of the well was reported at 7 mg/mL to 0.109 mg/mL. The fresh bacteria were then introduced to each well at a volume of 100 μ L. The plate was sealed and stored in the incubator overnight.

Three types of controls were included in the study alongside the tested compounds. The first was the positive control, which consisted of bacteria treated with antibiotics. The antibiotics used as positive controls were trimethoprim, ampicillin, novobiocin, and gentamicin. The second was the negative control, where only the bacterial culture and broth were combined to confirm consistent bacterial growth during incubation. Finally, a solvent control was prepared by adding 5% DMSO to the bacterial culture to verify that the solvent did not affect bacterial inhibition. All experiments were performed in triplicate to ensure the accuracy, reliability, and reproducibility of the results.

3.6.5 Analysis of Minimum Inhibitory Concentration (MIC) Result

After 24 hours of incubation at 37°C, the MIC value was determined as the lowest concentration of the compounds that did not display any observable growth of bacteria (expressed in mg/mL). Among the triplicate MIC values obtained, the modal values are selected to be in the data. Modal values are the ones that appear most frequently. Selecting the modal values ensures that the reported MIC are the most representative and consistent values observed in the experiments.

3.7 MOLECULAR DOCKING

Molecular docking was performed on an HP Pavilion gaming computer equipped with an Intel i7-10700F processor (2.9 GHz) running Windows 11. The methodology for molecular docking is outlined in the workflow below (Figure 3.1).

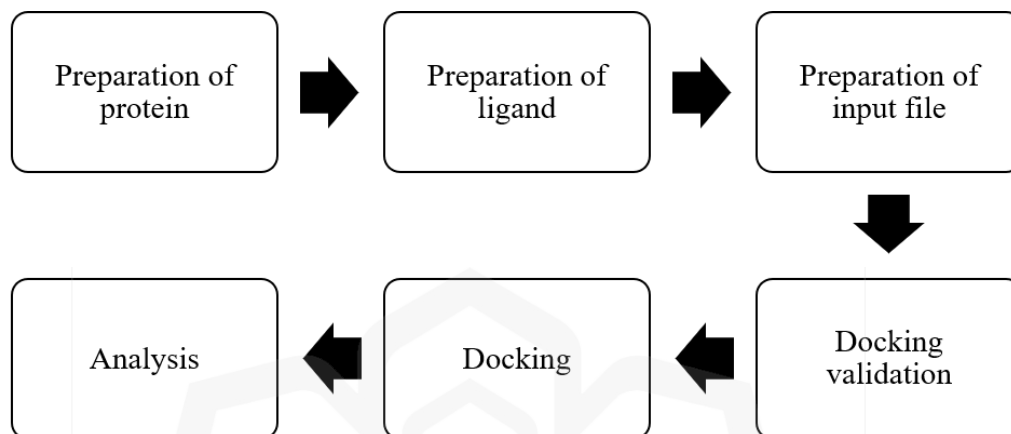


Figure 3.1 General workflow of molecular docking

3.7.1 Preparation of Protein (Wild-type)

In this study, *E. coli* DHFR was chosen as the target for the *in silico* analysis. This decision was based on several scientific reasons. First, *E. coli* DHFR is one of the most well-studied bacterial DHFR enzymes. There is a large amount of structural and kinetic data available, which makes it easier to perform accurate docking and simulation studies (Berman et al., 2000; Blakley, 1969). Second, its crystal structure has been solved at good resolution and is available in the Protein Data Bank, making it a reliable model for drug design (Sawaya & Kraut, 1997). Third, *E. coli* DHFR shares similar structural and functional features with DHFR enzymes from other gram negative bacteria. This makes it a useful model for predicting how ligands might interact with related enzymes (Srinivasan et al., 2018). Lastly, *E. coli* is an important clinical pathogen, especially with the rise of multidrug-resistant strains, which makes it a relevant target for new antibacterial agents (Karam et al., 2016). For these reasons, *E. coli* DHFR was selected as the model for computational study, allowing focused but meaningful insights that also support the broader findings from the *in vitro* antibacterial tests.

The structure of *E. coli* DHFR used in this study was retrieved from the Protein Data Bank (PDB ID: 1RX7). It was solved by X-ray diffraction at a resolution of 2.30

Å, which provides sufficient detail for studying the active site and ligand interactions. This crystal structure includes folate as a co-crystallised ligand, bound at the active site. The protein is a wild-type enzyme with no mutations and was expressed in *E. coli*, making it relevant for bacterial studies. One important feature of this structure is the occluded conformation of the MET20 loop (Sawaya & Kraut, 1997). This conformation is unique to bacterial DHFR and is not commonly found in the human version of the enzyme. Overall, this structure was chosen because it is complete, includes a natural ligand, and represents a biologically meaningful form of the enzyme. Thus, it was suitable for use in molecular docking and MD simulations.

For the preparation of protein, firstly, the three-dimensional structure of *E. coli* DHFR (PDB ID: 1RX7) was obtained from the Protein Data Bank (PDB) (<http://www.pdb.org>) and prepared using AutoDockTools (ADT) (Huey et al., 1998). The protein was cleaned by removing water molecules, ligands, and other heteroatoms which not involved in docking. Polar hydrogens were then added to the protein structure, and Kollman charges were assigned to ensure proper interaction with the ligand. The prepared protein was saved in PDBQT format for docking.

3.7.2 Preparation of Ligand

For ligand preparation, the 2D structures of the compounds were sketched in ChemDraw to generate SMILES notation. This notation was then submitted to Avogadro 2.0 (Hanwell et al., 2012) to generate the 3D structures of the ligands. The ligand structures were subsequently optimised using the Auto Optimization tool within Avogadro 2.0. The final optimised structures were saved in PDB format. In ADT, gasteiger charges were assigned to the ligands, and rotatable bonds were defined to ensure flexibility during docking. The prepared ligands were saved in PDBQT format.

3.7.3 Preparation of Input Files

Two input files were prepared for the docking process including the Grid Parameter File (GPF) and the Docking Parameter File (DPF).

3.7.3.1 Grid Parameter File (GPF) Preparation

The GPF file was generated by defining a grid box around the protein's active site, with grid dimensions and spacing set to ensure the ligand had sufficient space to explore binding poses. To cover all the active site residues for ligand binding, the grid box centre was positioned at the coordinates of the active sites. The active site residues include ILE5, ALA6, ALA7, TRP22, ASN23, LEU24, PRO25, ASP27, LEU28, PHE31, LYS32, ILE50, ARG52, LEU54, ARG57, ILE94, VAL99, and TYR100. The grid space was 0.3750 Å, and docking was done using the default AutoDock parameter values. Table 3.9 displays the grid centres and the number of points for the protein. The grid parameter file (GPF) file format was used to save the output. For metal complexes, parameters charged for each metal (copper, zinc, cobalt and nickel) were added manually to the GPF's input file before running the docking.

Table 3.9 The grid box parameters for protein

Grid box parameters	Values
Grid center x	33.55
Grid center y	42.49
Grid center z	7.131
No. of points x (Å)	50
No. of points y (Å)	50
No. of points z (Å)	50

3.7.3.2 Docking Parameter File (DPF) Preparation

Following the successful execution of the GPF file, the DPF file was created. The DPF was generated using the default with the genetic algorithm parameters as follows:

- i) The number of GA runs: 50
- ii) Population size: 150
- iii) The number of energy evaluations: 2.5 million (2.0 Å clustered tolerance)
- iv) The number of generations: 27000.

The DPF file format was used to save the output of the Lamarckian genetic algorithm (LGA). Similar to the GPF file, parameters charged for each metal (copper, zinc, cobalt and nickel) were added manually to the DPF's input file before running the docking.

3.7.4 Docking Running

The molecular docking simulations were performed using AutoDock 4.2.6 (Morris, 1998). The process began with the generation of grid maps for protein-ligand interactions using AutoGrid based on the GPF file. Subsequently, AutoDock 4.2.6 was executed using the DPF file as input, and the docking log file (DLG) was generated. Each docking simulation was carried out in triplicate to ensure consistency, reproducibility, and to account for any variability in the docking poses and scores. The compiled triplicate results are presented in Appendix 23.

3.7.5 Validation of Docking Parameters

Docking validation involved redocking folic acid into *E. coli* DHFR's binding site to check its alignment with the original position. The preparation of the protein and ligand, as well as the docking parameters and procedures, followed the methodology outlined in Subsections 3.7.1–3.7.4. After the docking process, the Root Mean Square Deviation (RMSD) value was calculated using PyMOL (Schrodinger, L., 2010) to evaluate the accuracy of the docking simulation.

3.7.6 Blind Docking

Blind docking was done to validate the possible ligand binding site in the proteins. As the active site of *E. coli* DHFR was known from previous literature, confirmation of the active site was performed through blind docking. The blind docking was generated in which the grid box was set up to cover the whole protein. The procedure of preparing protein and ligand was also similar to the Subsections 3.7.1 and 3.7.2. The docking parameter files were prepared by setting up the grid parameters as shown in Table 3.10.

Table 3.10 The grid centres and the number of points for protein in blind docking

Grid box parameters	Values
Grid spacing (Å)	0.7
Grid center x	27.148
Grid center y	44.067
Grid center z	13.191
No. of points x (Å)	50

No. of points y (Å)	50
No. of points z (Å)	50

3.7.7 Analysis of Docking

Following docking, the docking log file (DLG) was executed as an output. The DLG file contains information such as the top 10 free binding energy energies for each run in the histogram table. The lowest docking score (indicating the most favorable binding affinity) within the most populated or relevant cluster was chosen as the representative binding structure. Finally, the representative structures extracted were viewed and analysed such as their interactions with protein using BIOVIA Discovery Studios 2021 and PyMOL.

3.8 MOLECULAR DYNAMICS SIMULATION

Molecular dynamics (MD) simulations were conducted using a Custom PC (Intel Core i9-12900F processor (2.4 GHz) with GeForce RTX (Ubuntu 2024.4 LTS). The simulations were performed using GROMACS version 2023.3 (<http://www.gromacs.org/>) with the general workflow as below (Figure 3.2). Furthermore, in this MD simulation study, there are seven systems were conducted as tabulated in Table 3.11.

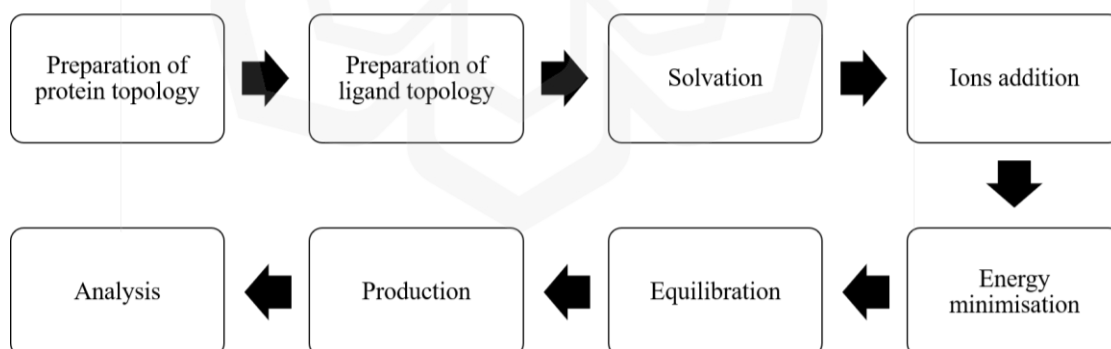


Figure 3.2 General workflow of molecular dynamics simulation

Table 3.11 Ligand-protein complex investigated with MD simulation in this study

Systems	Ligand	No. of atoms	No. of water molecules	No. of ions
DHFR-apo	-	2489	10478	-

DHFR-FOL	Folic acid	2537	7128	11 Na
DHFR-TMP	Trimethoprim	2514	7138	11 Na
DHFR-SB4	SB4OME	2529	7132	11 Na
DHFR-CUSB4	Cu(SB4OME) ₂	2568	7122	11 Na
DHFR-COSB4	Co(SB4OME) ₂	2568	7120	11 Na
DHFR-NISB4	Ni(SB4OME) ₂	2568	7124	11 Na

3.8.1 Preparation of Protein Topology Files

The three-dimensional structure of *E. coli* DHFR (PDB ID: 1RX7) was retrieved from the Protein Data Bank (PDB) and prepared for simulation using the 'pdb2gmx' tool in GROMACS. The protein was parameterised with the AMBER99SB-ILDN force field. Water molecules and other heteroatoms were removed from the structure, and missing hydrogen atoms were added where required. The output from the protein preparation included a set of files including the gro file (molecular structure), the top file (topology), and the itp file (include topology). These files served as inputs for the subsequent steps of solvation and energy minimisation.

3.8.2 Preparation of Ligand Topology Files

The preparation of the ligand structure for MD simulation began with the extraction of the best-docked conformation from the docking results. The selected ligand pose was isolated from the protein–ligand complex using PyMOL. Following this, molecular mechanics parameters were individually assigned to the ligand. The 'antechamber' module of AmberTools23 was utilised to generate ligand topologies. Atomic charges were assigned using the Austin Model 1 with Bond Charge Corrections (AM1-BCC) charge method (Jakalian et al., 2002), while force-field parameters were derived using the Generalised Amber Force Field (GAFF) (X. Wang et al., 2020). For metal complexes, an additional preparation step was performed using the 'pdb4amber' module in AmberTools23 to accurately account for metal coordination. The necessary parameters for these ligands were obtained from the AMBER parameter database. (<http://research.bmh.manchester.ac.uk/bryce/amber/>).

To convert ligand files into a format compatible with GROMACS, the 'Acpyype.py' Python script (Sousa Da Silva & Vranken, 2012) was employed. It

generated files such as the top file (topology), itp file (include topology), and gro file (molecular structure). Once the ligand topology files were successfully generated, they were merged with the protein topology using a text editor, resulting in a combined protein-ligand complex ready for subsequent simulation steps.

3.8.3 Solvation

In solvation, water molecules are added around the solute molecules to create a solvent shell, effectively surrounding and hydrating the solute (X. Wang et al., 2020). According to a previous study, the size of the solvation box, which determines the distance between the solute and the box's edge, should be large enough to prevent artificial interactions between periodic images of the solute but small enough to be computationally feasible (Y. Li et al., 2021). Typically, the solvation box extends several angstroms beyond the solute in all directions. Thus, in this work, the protein-ligand complex was solvated in a dodecahedron box with a minimum distance of 1.0 nm between the complex and the box edges. Using the 'editconf' tool, periodic boundary conditions (PBC) in all directions (x,y,z) were applied, and water molecules were added to fill the box using the SPC water model as illustrated in Figure 3.3. The solvated system was stored in a gro file for further steps.

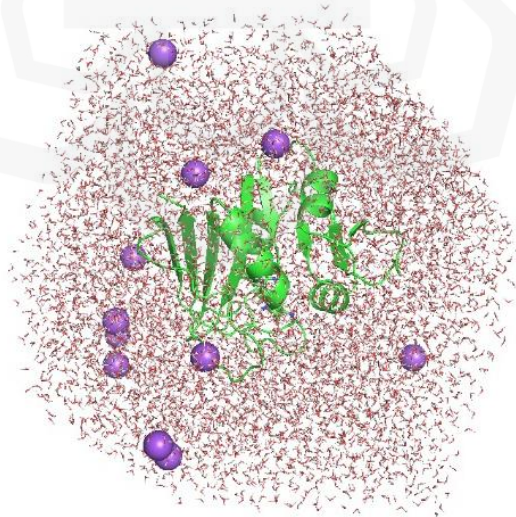


Figure 3.3 Dodecahedron solvation box

3.8.4 Additions of Ions

Ions were added to neutralise the system. The 'genion' tool was used to add ions to the solvated protein-ligand complex. For all ligands which had a net charge of zero, 6 Na⁺ ions were added to neutralise the system. However, in the case of FOL, which had a net charge of -2, 8 Na⁺ ions were added to achieve neutrality. The output was an updated gro file containing the water molecules and ions, as well as a revised top file reflecting the new system composition.

3.8.5 Energy Minimisation

Energy minimisation was carried out using the steepest descent algorithm to resolve steric clashes and unfavourable contacts within the solvated protein-ligand system (Vieira et al., 2023). This process was iterated until no further changes in the potential energy were observed, as depicted in Figure 3.4. The minimised structure was then saved in a gro file, which was subsequently used in the equilibration stage.

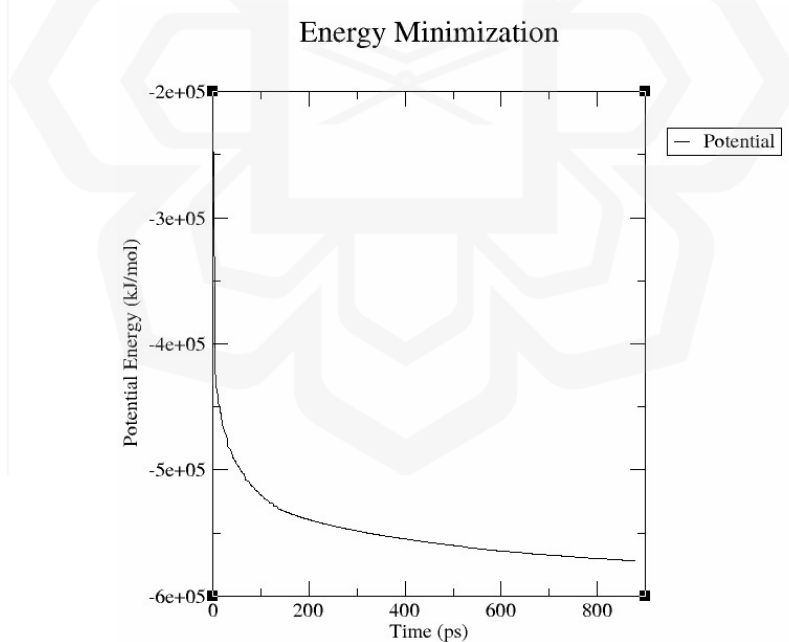


Figure 3.4 Energy minimisation graph of a system in molecular dynamics

3.8.6 Equilibration (NVT & NPT)

Equilibration of the system was conducted in two stages. First, NVT (constant Number of particles, Volume, and Temperature) equilibration was performed at 300 K for 100

ps using the V-rescale thermostat. Following this, an NPT (constant Number of particles, Pressure, and Temperature) equilibration was carried out at 1 bar for 100 ps using the Berendsen barostat. During both equilibration steps, position restraints were applied to the protein-ligand complex to maintain its structural integrity. The output from this stage was an equilibrated system prepared for the production MD run.

3.8.7 Production Molecular Dynamics Simulation

Once equilibration was completed and the system had reached a stable state, the production MD run commenced. The system underwent a final production run for 100 ns in triplicate, with a time-step of 2 fs. During this stage, all restraints were lifted, allowing free movement of the entire system. The resulting simulation trajectory was centred using the "gmx trajconv" tool to ensure that no atoms moved outside the simulation box. Subsequently, the trajectory was visualised with VMD to confirm that all atoms remained within the box. The resulting xtc, gro, tpr, and edr files from the production run were then used for further analysis.

3.8.8 Molecular Dynamics Simulation Analysis

Several analyses were performed to assess the stability and behaviour of the protein-ligand complex during the simulation. First, the root-mean-square deviation (RMSD) of the alpha carbon atoms was calculated using alpha carbon atoms over 100 ns, following least-squares fitting. The triplicate RMSD data for each system are compiled in Appendix 29-35. Similarly, the RMSD for each ligand was calculated after the least-squares fitting of the ligand atoms over the same period. Upon establishing stability through the RMSD analysis, specific time points from the trajectory were extracted. Consistent stability for all ligands was observed from 30 to 100 ns, and subsequent analyses were based on this time frame.

Next, the radius of gyration (Rg) was computed using alpha carbon atoms between 30 and 100 ns. Additionally, the root-mean-square fluctuation (RMSF) per residue was calculated which was extracted from the alpha carbon atoms of the protein-ligand complex over the same time frame. Similarly, the RMSF for each ligand was calculated after the least-squares fitting of the ligand atoms over the same period. B-factor calculations were also performed under similar conditions. The solvent-

accessible surface area (SASA) was determined using the 'gmx sasa' tool to measure the binding pocket volume. This pocket site is generated by selecting all amino acid residues within 4 Å of the ligand. Hydrogen bond analysis and its occupancy were conducted using VMD software, employing a cutoff distance of 3.5 Å and an angle of 60°. Additionally, to analyse these interactions in detail, the distances between the ligand and selected residues were also measured using 'g_dist'.

Binding free energy (ΔG_{bind}) was calculated using the Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) method with the 'g_mmpbsa' tool (Valdés-Tresanco et al., 2021). Snapshots were collected between 30 and 100 ns at 200 ps intervals, yielding 350 frames for analysis. Finally, interaction analysis was performed using a 2D representative structure derived from clustering. Clustering was extracted from the binding pocket using the Gromos method in 'g_cluster', with an RMSD cutoff of 0.15 nm. The structure from the largest cluster was selected for detailed binding mode analysis. All graphical plots were generated using the 'Qtgrace' tool, and visualisations of the molecular structures were carried out with PyMOL and Discovery Studio Visualizer (2021).

3.9 MOLECULAR DOCKING FOR MUTANT TYPE *E. COLI* DHFR

In this section, two mutated strains, L28R and I94L, were selected for further analysis. As reported in previous literatures, the L28R mutation was one of the most frequently observed alterations in *E. coli* DHFR associated with resistance to TMP (Manna et al., 2021). As a result, many resistant clinical isolates exhibited this mutation as a key mechanism of TMP evasion. In contrast, the I94L mutation, while not as widely reported or characterised as L28R, also occurs within the active site of DHFR. Thus, despite being less prominent in previous studies, mutations like I94L may still contribute significantly to TMP resistance and merit further investigation in the context of rational inhibitor design. Overall, the molecular docking for mutant strains' procedure followed the same steps as described in Subsection 3.7, with the exception of the protein preparation step. Therefore, the procedures specific to the introduction and preparation of the mutated residues are detailed below.

3.9.1 Preparation of Protein Topology Files

This study focused on two mutated strains of *E. coli* DHFR, namely L28R and I94L. The first structure, with PDB ID 6XG4, represented the L28R mutant, where leucine at position 28 is replaced with arginine. This structure was co-crystallized with the antibiotic TMP and was resolved at a 2.10 Å resolution. Meanwhile, the second structure, PDB ID 6XG5, represented the wild-type *E. coli* DHFR in complex with TMP. Since no crystal structure has been reported for the I94L mutant of *E. coli* DHFR, this wild-type structure was used as the template to introduce the mutation. The I94L mutation was generated using UCSF Chimera by substituting isoleucine at position 94 with leucine.

Following the mutation, the structure underwent energy minimisation to resolve any steric clashes and to ensure a stable, realistic conformation of the protein-ligand complex. To confirm the quality of the mutated structure, it was validated using the SAVES v6.0 web server (<https://www.saves.mbi.ucla.edu/>). This server includes tools like ERRAT, Verify3D, and PROCHECK, which check for stereochemical quality, residue environments, and overall structural reliability. These steps ensured the mutant model was suitable for further computational analysis.

Finally, for the grid box, it was set up around the active site of each mutated protein, following the same region used for the wild-type. The grid center coordinates and number of grid points for each mutant was listed in Table 3.12.

Table 3.12 The grid box parameters for mutated protein

Grid box parameters	L28R (PDB ID:6XG4)	I94L (PDB ID: 6XG5)
Grid center x	-7.325	5.464
Grid center y	28.019	-1.987
Grid center z	18.649	4.646
No. of points x (Å)	50	50
No. of points y (Å)	50	50
No. of points z (Å)	50	50

CHAPTER FOUR

RESULT & DISCUSSION

4.1 DESIGN OF COMPOUNDS

In this study, 20 new potential *E. coli* DHFR inhibitors were designed based on the dithiocarbazate compound, with *S*-benzylidithiocarbazate (SBDTC) acting as the scaffold. The design was inspired by the structure of the non-classical DHFR inhibitor trimethoprim (TMP), to match or improve TMP's antibacterial effectiveness.

4.1.1 Rationale Design of SBDTC Derivatives and Their Metal Complexes

The strategy to design DHFR inhibitors from SBDTC is based on three key approaches. The first involved designing SBDTC derivatives by incorporating substituents similar to TMP, such as methoxy and amino groups, into the biologically active SBDTC scaffold. The second approach focused on introducing promising pharmacophores identified in previous studies, such as carbonyl, nitro, and chloro groups. Lastly, a novel approach introduced metal ions into SBDTC derivatives, aiming to enhance bioactivity while improving their physicochemical and pharmacokinetic properties.

To apply the first strategy effectively, a detailed understanding of the structural features of TMP is crucial. TMP's core structure consists of two key components including the 2,4-diaminopyrimidine moiety and the 3',4',5'-trimethoxybenzyl group (Figure 4.1). The 2,4-diaminopyrimidine moiety consists of two amino groups attached to the pyrimidine aromatic ring. According to Wahaibi et al. (2024), these amino group plays a vital role by interacting with ASP27, thereby blocking the transfer of a hydride ion from dihydrofolate (DHF) to tetrahydrofolate (THF). Furthermore, these amino group also interacts with active site residues such as ILE5 and ILE94, enhancing their binding affinity to the DHFR protein (Al-Wahaibi et al., 2024). These interactions showed the significance of the amino group moiety in TMP's effectiveness as a DHFR inhibitor.

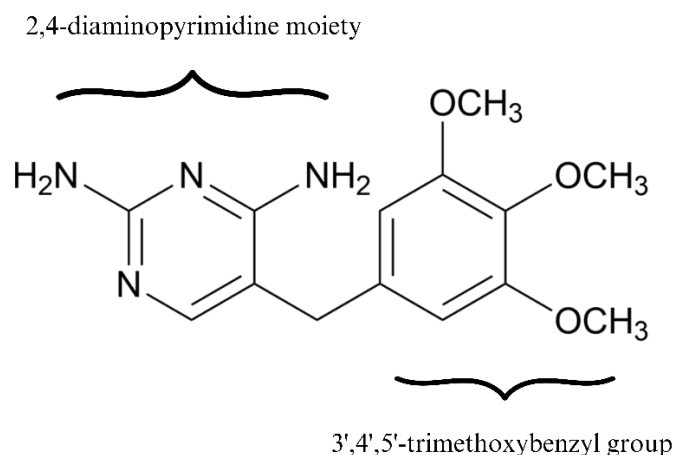


Figure 4.1 Structure integral of TMP highlighting two important moiety

To compare with the structural feature of the SBDTC scaffold, it does contain two amino groups as part of its dithiocarbazate backbone (Figure 4.2). Research has shown that amino groups play a crucial role in antibacterial activity by forming strong hydrogen bond interactions with DHFR's active site residues. For example, Rashid et al. (2016) reported that substituting a primary amine group with a secondary amine significantly increased the MIC against *E. coli* from 13.3 mg/mL to 256 mg/mL (Rashid et al., 2016). Further evidence supporting the importance of amino groups as pharmacophores in *E. coli* DHFR inhibition comes from Eldaly (2024). The study found that primary amino groups improved antibacterial activity against *E. coli* compared to the standard antibiotic TMP (Eldaly, 2024). This enhancement was attributed to the NH_2 group in the pyrazole ring, which forms strong hydrogen bond interactions with SER49, a key residue in *E. coli* DHFR.

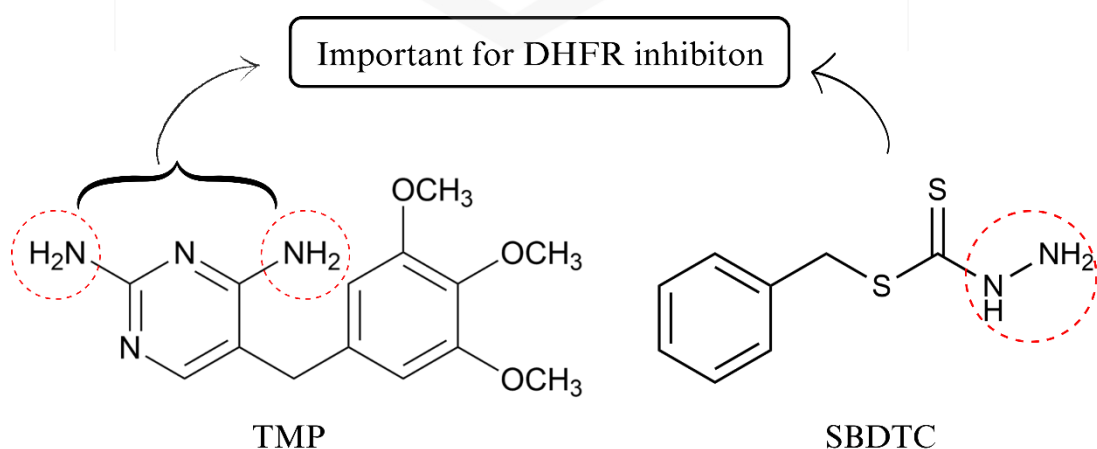


Figure 4.2 Structure integral of TMP and SBDTC highlighting amino group

Beyond the amino groups, the SBDTC scaffold lacks the pyrimidine ring found in TMP. However, this aromatic ring shares structural similarities with the benzyl substituent in the SBDTC scaffold (Figure 4.3). Benzyl derivative in SBDTC was found to enhance bacterial membrane penetration (Bhattacharjee et al., 2021). A comparative study by Sengottiyar et al. (2022) investigated the binding affinities of benzyl-substituent (SBDTC) with methyl-substituent (SMDTC) dithiocarbamate derivatives (Sengottiyar et al., 2022). The results showed that SBDTC derivatives displayed better binding affinity to the BSA protein, with a lower binding energy (-8.64 kcal/mol) compared to SMDTC derivatives (-7.44 kcal/mol). While SMDTC derivatives formed two hydrogen bonds, contributing to stronger polar interactions, the benzyl group in SBDTC derivatives offered enhanced hydrophobic interactions. These hydrophobic interactions proved more dominant, leading to the higher overall binding affinity observed for SBDTC derivatives.

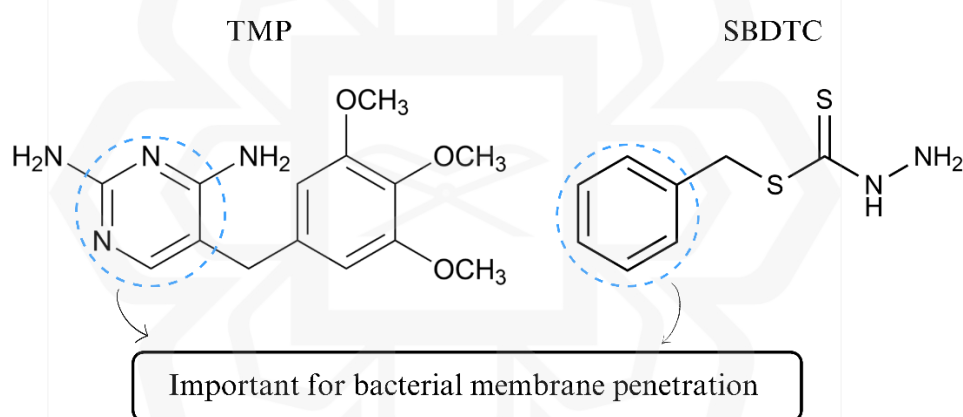


Figure 4.3 Structure integral of TMP and SBDTC highlighting aromatic group

Furthermore, Sehrawat et al. (2024) proposed that the effectiveness of DHFR inhibitors could be improved by optimising van der Waals interactions through the incorporation of bulky aromatic groups (Sehrawat et al., 2024). Accordingly, the two amino groups and the benzyl-substituent in the SBDTC scaffold were expected to facilitate similar interactions to those mediated by the 2,4-diaminopyrimidine moiety in TMP.

Another important feature of TMP is the presence of the 3',4',5'-trimethoxybenzyl groups (Figure 4.4). According to Jorba et al. (2021), this group enhances lipophilicity and supports hydrophobic interactions within the enzyme's active

site (Jorba et al., 2021). Wahaibi et al. (2024) specifically noted that these groups interact hydrophobically with key residues such as LEU28, ILE50, and PHE31. This not only increases TMP's lipophilicity but also improves its selectivity for bacterial DHFR over human DHFR, making it a highly effective antibacterial agent.

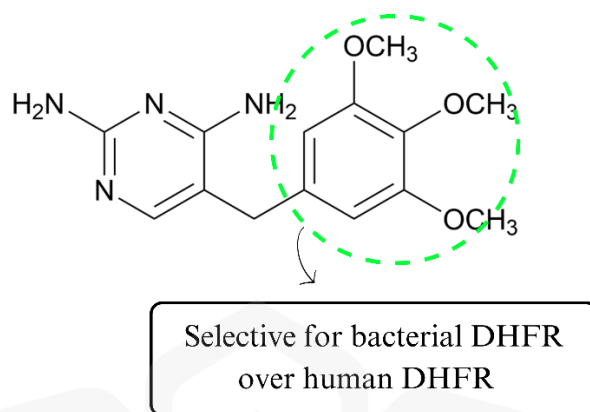


Figure 4.4 Structure integral of TMP highlighting 3',4',5'-trimethoxybenzyl

The SBDTC scaffold does not naturally contain methoxy groups, so these functional groups were introduced as substituents to enhance biological activity. Methoxy groups are well-known for their electron-donating properties, which can significantly impact lipophilicity, electronic effects, and steric factors (Bharty et al., 2012; Emam et al., 2009). These properties are the key elements for effective binding to the DHFR active site (Chang et al., 2024; Maia et al., 2021). Many researchers have designed DHFR inhibitors with methoxy substituents, often modifying one or more of these groups with alternative pharmacophores to assess their relative effectiveness. For example, Zavala et al. (2024) reported that compounds featuring methoxy groups demonstrated superior activity compared to those with hydroxyl groups (Alcántar-Zavala et al., 2024). The study proposed that methoxy groups could function as hydrogen bond donors or acceptors when interacting with the DHFR protein, further enhancing their binding capability.

Interestingly, Dawood et al. (2023) showed that replacing the methyl group with a 4-methoxybenzylidene group significantly improved antituberculosis activity (Dawood et al., 2023). This emphasised the critical role of methoxy groups in enhancing bioactivity. Building on these findings, this study designed three isomeric derivatives of SBDTC by introducing methoxy substituents at the ortho, meta, and para positions of the benzyl ring (Figure 4.5). This approach was undertaken to systematically examine

how the position of methoxy groups affects antibacterial activity, as no previous research has fully explored these positional effects. The goal was to not only maintain efficient bacterial membrane penetration but also identify the most effective methoxy substitution site for optimal inhibitory activity.

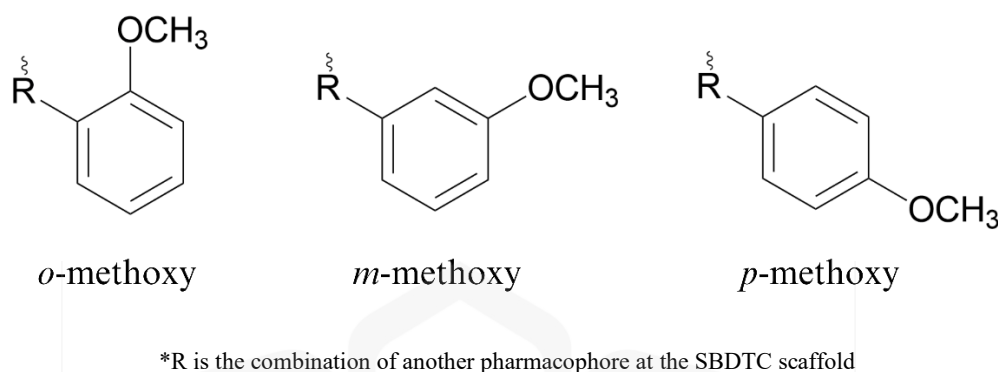


Figure 4.5 The proposed pharmacophore structure of isomeric methoxy derivatives

Expanding on the first strategy, the second strategy emphasised incorporating pharmacophores identified in previous studies as effective DHFR inhibitors. Within the SBDTC scaffold, the C=S and C–S groups (Figure 4.6) associated with the dithiocarbazate backbone are particularly noteworthy, as these groups have been extensively recognised for their contribution to antibacterial activity (Garcia-Vazquez et al., 1999; Yoshinari et al., 2023). Beyond dithiocarbazate compounds, sulfur-containing functional groups are well known for their critical role in enhancing biological efficacy. For example, Dawood et al. (2023) reported that thiophenyl-pyrazolylthiazole hybrids demonstrated strong antibacterial activity with an IC_{50} value of 4.21 μ M, surpassing TMP (IC_{50} = 6.23 μ M). In this case, the sulfur atom in the thiazole moiety formed hydrogen bond interactions with ILE94, underscoring the key role of sulfur in improving activity. Similarly, Summerfield et al. (2006) showed that isothiurea groups could optimise interactions with carbonyl groups in the MET20 loop, thereby enhancing the binding interactions with *E. coli* DHFR (Summerfield et al., 2006).

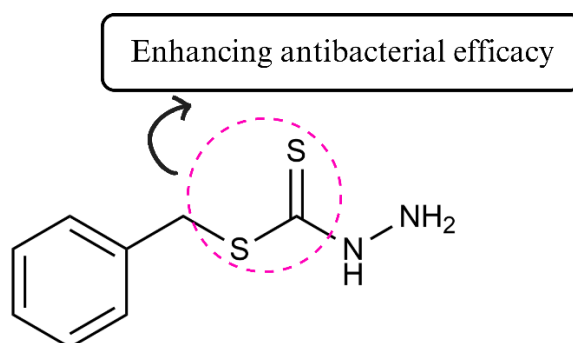


Figure 4.6 Structure integral of SBDTC highlighting thiol group

Another important pharmacophore is the amide group, which has demonstrated significant potential as a DHFR inhibitor in bacteria. For example, Wrobel et al. (2019) incorporated an amide bond into the TMP scaffold, resulting in a notable enhancement of DHFR inhibitory potency (Wróbel, et al., 2019). The amide bond facilitates additional hydrogen bonding with active site residues, improving both the binding affinity and specificity for DHFR. Supporting this, Yadav et al. (2024) investigated thiophenyl scaffolds with carbonyl group substituents and reported considerable antibacterial activity (Yadav et al., 2024). Further studies by Drozdowska et al. (2024) and Ghany et al. (2024) also confirmed the utility of amide bonds in medicinal chemistry as essential structural components for active lead compounds (Drozdowska et al., 2024; Ghany et al., 2024). Inspired by these findings, the amide group was used as a bridge between the SBDTC scaffold and pharmacophore substituents in all the SBDTC derivatives in this study (Figure 4.7).

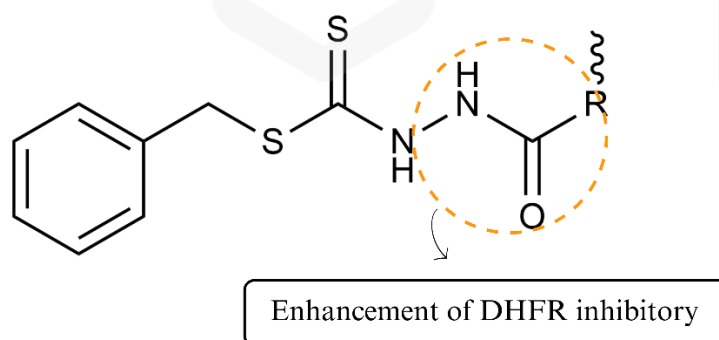


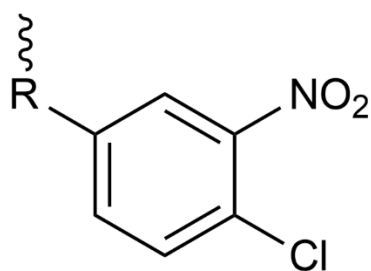
Figure 4.7 The proposed pharmacophore structure of the amide group

Previous studies on DHFR inhibitors have demonstrated that electron-withdrawing groups, such as nitro (NO_2) groups, significantly enhance antibacterial

activity. For instance, Pavithra et al. (2024) reported that substituting a nitro group at the meta position of the aromatic ring in the TMP scaffold resulted in substantial inhibition, with 70% effectiveness when compared to conventional Butylated Hydroxytoluene antioxidants (Pavithra et al., 2024). The study attributed this enhanced activity to the presence of the NO₂ group on the phenyl ring, which increases the resonance effect and thereby strengthens its antibacterial properties. This finding was further supported by Bayazeed et al. (2022), who highlighted the notable antibacterial potential of nitro groups against *S. faecalis*, *B. pumilis*, and multi-drug-resistant bacteria such as MRSA (Bayazeed et al., 2022).

In addition to nitro groups, chloro groups have also demonstrated a significant impact on DHFR activity. According to Eldaly (2024), the introduction of electron-withdrawing groups, such as the 4-chlorophenylazo moiety, notably enhanced antibacterial activity. The compound exhibited superior potency against *E. coli*, *K. pneumoniae*, and *A. baumannii* when compared to standard antibiotics, primarily due to the formation of strong hydrogen bond interactions between the chloro group and THR123 in the *E. coli* DHFR active site. Similarly, Jays et al. (2019) highlighted the effectiveness of chloro-substituted derivatives, with the 2,4-dichloro derivative demonstrating the highest enzyme inhibition, which correlated with potent antibacterial activity (Jays et al., 2019). Furthermore, Jadhav et al. (2023) provided additional evidence supporting the role of chloro groups in enhancing DHFR inhibition and overall antibacterial efficacy (Jadhav et al., 2023).

Building on these findings, this study incorporated both meta-nitro and para-chloro group substituents into the aromatic ring of the SBDTC scaffold (Figure 4.8). This approach aimed to explore their combined effect on bioactivity and investigate whether the synergistic influence of these electron-withdrawing groups could further optimise antibacterial potency.



p-chloro-*m*-nitro

Figure 4.8 The proposed pharmacophore structure of *p*-chloro and *m*-nitro

Moreover, SBDTC and its derivatives are expected to exhibit tautomerism (Figure 4.9), a phenomenon that can significantly influence their stability and biological activity. According to How et al. (2008), four possible tautomeric states can be proposed for these compounds, involving thione, thiol, thiolo, and thionol. In the development of DHFR inhibitors, it is crucial to carefully account for tautomeric states to maximise the functional groups' efficiency and refine pharmacophore design. Tautomerism can significantly influence how inhibitors interact with DHFR active site residues, affecting their antibacterial activity, binding affinity, and stability under physiological conditions.

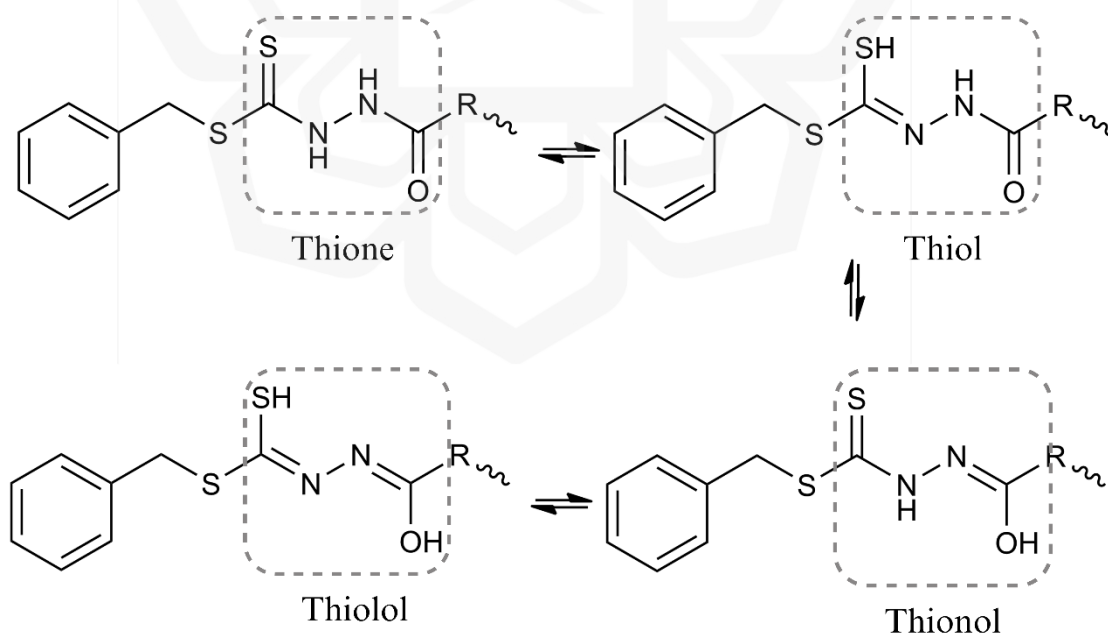


Figure 4.9 The proposed tautomeric form of the designed compound

For example, Dhaked & Nicklaus (2024) demonstrated that erythromycin, a widely used antibiotic, can exist in three different forms, one ketonic form and two

cyclic hemiketal forms (Dhaked & Nicklaus, 2024). The ketonic form is the biologically active version that binds to bacterial ribosomes and inhibits protein synthesis. However, under gastrointestinal conditions, up to 20% of the erythromycin may convert into inactive hemiketal forms, which are not effective against bacteria. As a result, higher doses of erythromycin may be required to ensure there is enough of the active ketonic form available to achieve therapeutic effects. Similarly, studies on curcumin-based molecules have shown that keto-enol tautomerism can influence activity. While keto tautomers exhibit little to no biological activity, keto-enol forms demonstrate potent inhibitory effects against enzymes such as BACE-1 and GSK-3 β (Matsui et al., 2023).

The third strategy in DHFR inhibitor development involved incorporating metal ions into SBDTC derivatives. This approach aims to enhance bioactivity and improve the physicochemical and pharmacokinetic properties of the compounds (Evans & Kavanagh, 2021). The inclusion of metal ions in biologically active compounds results in metal complex drugs. These drugs enhance antibacterial efficacy through novel mechanisms.

Firstly, the rationale behind integrating metal ions is their ability to stabilise ligands by forming rigid chelate structures. This reduces the degradation of the ligand under physiological conditions (Nemade & Kolhe, 2016). Studies have shown that metal complexes are more thermally stable than their parent ligands. Consequently, this stability improves both bioavailability and antibacterial potential (Z. Chen et al., 2007; Costa et al., 2019; El-Shenawy et al., 2014).

Additionally, metal ion coordination was expected to increase lipophilicity. This enables better penetration through bacterial cell membranes and improves access to intracellular bacterial targets (Claudel et al., 2020). For example, a Cu(II) complex of SBDTC derivative with 4-formyl-3-hydroxybenzoic acid demonstrated potent inhibition of the *S. aureus* efflux pump. This revealed that metal complexes can modulate bacterial efflux mechanisms, thereby influencing antibacterial efficacy (Gan et al., 2022)

From an electronic perspective, metal ions modulate the charge distribution and electronic properties of ligands. These properties were expected to enhance their reactivity and binding potential (Lobana et al., 2009). For instance, cisplatin, an

anticancer drug, that is essential for DNA cross-linking and replication inhibition (Dasari & Bernard Tchounwou, 2014). In contrast, its trans-isomer (transplatin) is inactive. This demonstrated the critical role of geometry in drug efficacy.

This study selected copper, zinc, cobalt, and nickel ions for their well-documented antimicrobial properties. Copper ions disrupt bacterial membranes by binding to phospholipids and proteins, causing leakage and cell death (Ingle et al., 2014). Additionally, Cu(II) catalyses Reactive Oxygen Species (ROS) formation, leading to oxidative damage to bacterial DNA, proteins, and lipids (Anupama, 2020). The superior antibacterial activity of Cu(II) complexes has been demonstrated in several studies. Break et al. (2023) synthesised a macroacyclic Schiff base from SBDTC and complexed it with Cu(II) ions. These complexes exhibited potent antibacterial activity against *Kocuria rhizophila* and *Staphylococcus aureus*, with MIC values below 24.4 µg/mL (Break et al., 2023). Similarly, Zangrando et al. (2015) investigated a Schiff base derived from SBDTC and 4-benzyloxybenzaldehyde, showing that its Cu(II) complex had enhanced antibacterial activity compared to the free ligand. This suggests that copper coordination plays a key role in boosting antibacterial properties (Zangrando et al., 2015).

Meanwhile, zinc ions bind to bacterial proteins and ribosomes, inhibiting critical enzymatic activities and disrupting protein synthesis (Hussain et al., 2022). Zn(II) also competes with essential metal ions in bacterial cells, leading to metabolic imbalances (Cavalcante et al., 2019). Research by Singh et al. (2006) assessed the antibacterial activity of Zn(II) complexes with Schiff bases derived from 1,2,4-triazoles against *S. aureus* and *P. aeruginosa*. These complexes exhibited superior antibacterial activity compared to the free ligands, indicating that zinc coordination enhances the antibacterial efficacy (Singh et al., 2006). The findings suggest that Zn(II) complexes exert their antibacterial effects by disrupting bacterial membranes and interfering with essential enzymatic processes.

On the other hand, cobalt ions interfere with bacterial DNA replication by replacing essential metal cofactors in DNA polymerases and enzymes (Girish et al., 2019). Co(II) complexes also generate oxidative stress and inhibit bacterial respiration (Fasnacht & Polacek, 2021). The superiority of Co(II) complexes in antibacterial activity also have been shown in several past studies. Singh et al. (2006) evaluated the

antibacterial properties of Co(II) complexes with Schiff bases derived from 1,2,4-triazoles. These complexes showed potent activity against both gram-positive and gram-negative bacteria, surpassing the efficacy of the free ligands. The enhanced activity is likely due to cobalt's ability to interfere with bacterial DNA synthesis and enzyme function (Singh et al., 2006).

Lastly, the choice of metal was nickel. Nickel ions inhibit bacterial metalloenzymes, including urease and hydrogenase, disrupting essential metabolic functions (Benoit & Maier, 2013). Ni(II) also affects bacterial cell wall synthesis, making bacteria more susceptible to external stressors (Lusi et al., 2017). A study by Lima et al. (2021) focused on the synthesis of Ni(II) complexes with symmetrical and asymmetrical 4,6-diacetylresorcinol-dithiocarbazate ligands (Lima et al., 2021). The antimicrobial evaluation indicated that these Ni(II) complexes possess significant antibacterial activity, which may be due to their interaction with bacterial DNA and inhibition of replication processes.

Comparative studies of SBDTC-derived metal complexes have demonstrated their potent antibacterial activities (Hossen et al., 2020). The enhancement of bioactivity by metal complexes has been confirmed by numerous studies especially with copper, zinc, cobalt and nickel. Overall, these findings point out the synergistic effects of metal ion coordination, which enhances both the physicochemical stability and biological activity of SBDTC derivatives.

4.1.2 SBDTC Derivatives And Their Metal Complexes As *E. coli* DHFR Inhibitors

The structure of SBDTC scaffold is highly flexible, making them easy to modify chemically and allowing for the creation of various derivatives. In this study, 20 compounds were designed, including 4 SBDTC derivatives, namely *S*-benzyl-2-methoxybenzoyl dithiocarbazate (SB2OME), *S*-benzyl-3-methoxybenzoyl dithiocarbazate (SB3OME), *S*-benzyl-4-methoxybenzoyl dithiocarbazate (SB4OME) and *S*-benzyl-3-nitro-4-chlorobenzoyl dithiocarbazate (SB3NO) with their 16 respective metal complexes. The proposed structure for the designed SBDTC derivatives is shown in Figure 4.10.

Each SBDTC derivative features a carbonyl group ($\text{C}=\text{O}$) connected to a nitrogen atom, forming an amide group. A benzyl ring was also attached to the carbonyl group, and the differences between these derivatives were substituent groups attached to the benzyl ring. In SB2OME, a methoxy group ($-\text{OCH}_3$) is attached to the benzyl ring at the ortho position. In SB3OME, the methoxy group is placed at the meta position, while in SB4OME, it is located at the para position. SB3NO differs because it contains both a nitro group ($-\text{NO}_2$) at the meta position and a chloro group ($-\text{Cl}$) at the para position.

The design of metal complexes involves introducing metal ions, including copper, zinc, cobalt, and nickel, to each of the SBDTC derivatives. However, predicting the exact structures of these metal complexes proved challenging. The metal-to-ligand ratios could vary and the coordination modes could differ depending on the ligand and metal used. For example, the SBDTC derivatives, which contain donor atoms like sulfur, nitrogen, and oxygen, could coordinate with metal ions in bidentate, tridentate, or even tetradentate modes. Moreover, the complexes could interact with other molecules, such as water or acetate ions from metal salts, making the predicted structures more complex.

Thus, predictions were made based on the majority of characterisation data from previous studies. Given that all SBDTC derivatives contain donor atoms such as sulfur, nitrogen, and oxygen, these compounds were predicted to act as tridentate ligands upon complexation. Additionally, most publications reported a metal-to-ligand ratio of 1:2, which was also considered for all metal complexes in this study, as illustrated in Figure 4.11.

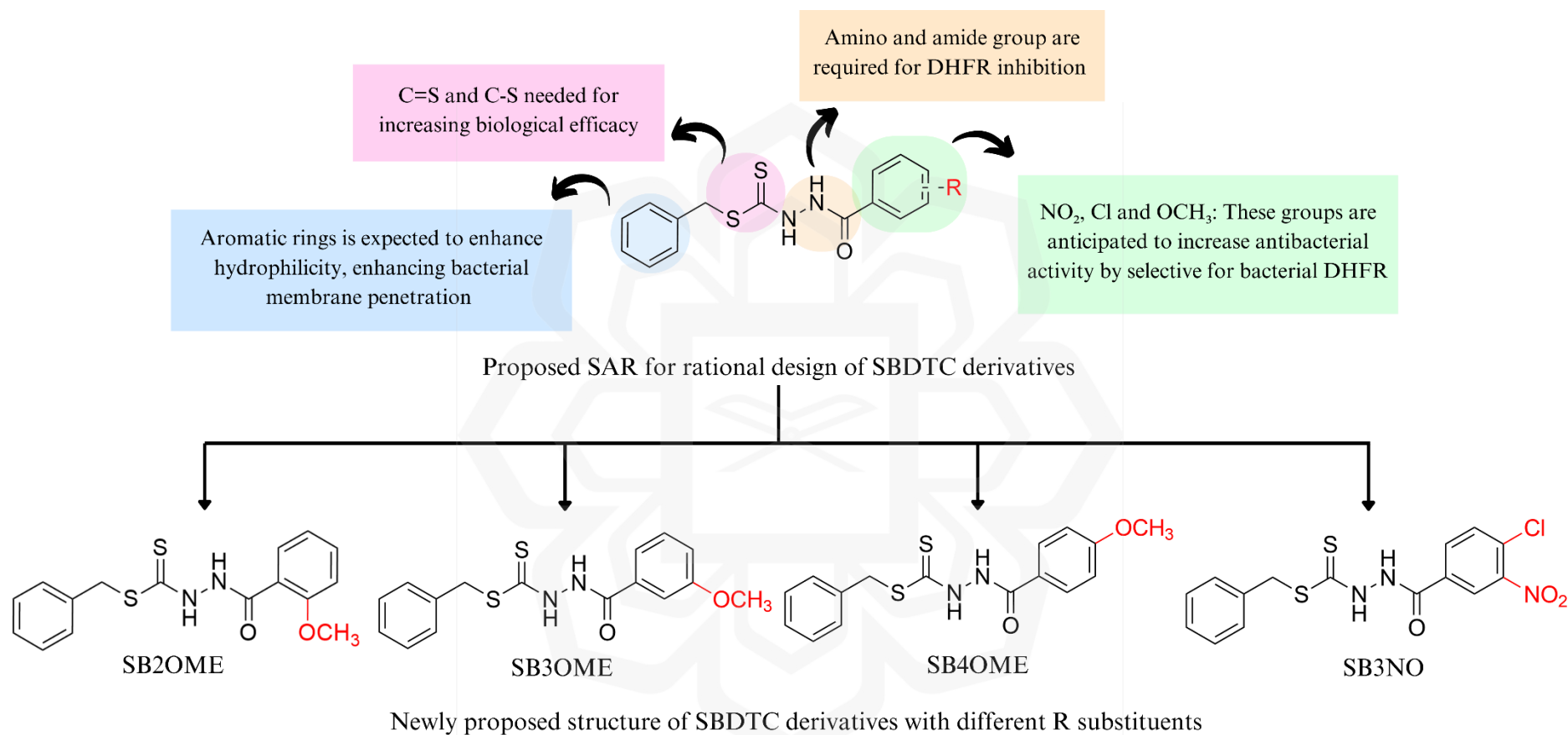
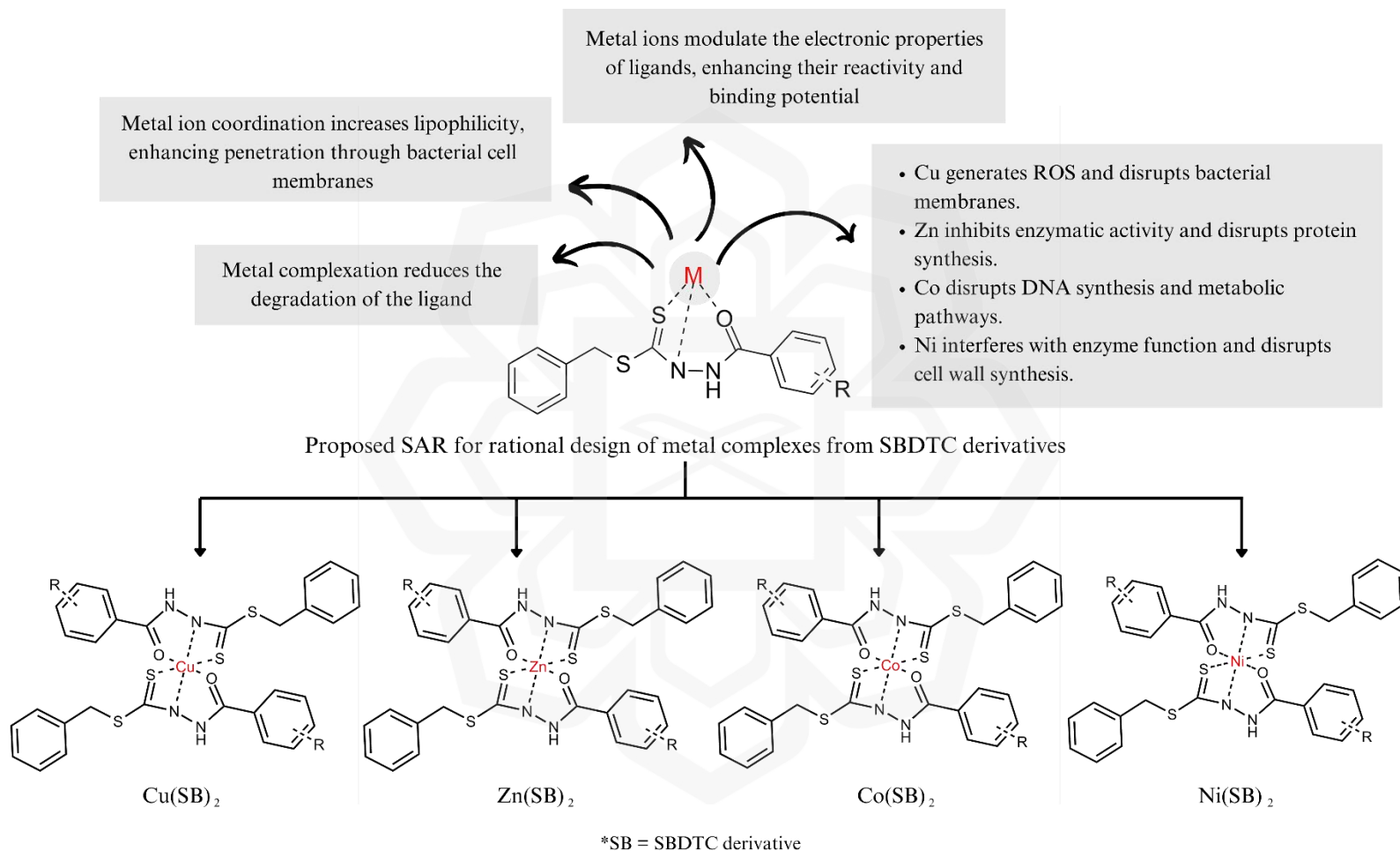


Figure 4.10 The proposed structure of the designed SBDTC derivatives



Newly proposed structure of metal complexes from SBDTC derivatives with different M substituents

Figure 4.11 The proposed structure of the designed metal complexes from SBDTC derivatives

4.2 SYNTHESIS AND PHYSICAL PROPERTIES

All designed compounds were successfully synthesised following the procedure in methodology and the physical properties of these compounds were tabulated in Table 4.1 and displayed in Appendix 1. SBDTC was obtained as a white precipitate, with a modest yield of 73%, attributable to the inconsistent temperature fluctuations during synthesis. The purity of SBDTC was confirmed by comparing its melting points with previously reported literature, demonstrating a sharp melting point of 122-123°C. This temperature falls in between the range of 2 °C, aligning closely with the literature value of 124-125°C (Akbar Ali & Tarafdar, 1977).

Table 4.1 Physical properties of all synthesised compounds

Compound	Molecular formula	State	Colour	Yield (%)	Melting point (°C)
SBDTC	C ₈ H ₁₀ N ₂ S ₂	Solid	White	73	122-123
SB2OME	C ₁₆ H ₁₆ N ₂ O ₂ S ₂	Solid	White	65	156-158
Cu(SB2OME) ₂	Cu(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Black	63	230-232
Zn(SB2OME) ₂	Zn(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	White	68	217-220
Co(SB2OME) ₂	Co(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Green	62	260-263
Ni(SB2OME) ₂	Ni(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Brown	75	230-232
SB3OME	C ₁₆ H ₁₆ N ₂ O ₂ S ₂	Solid	White	40	138-141
Cu(SB3OME) ₂	Cu(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Black	61	245-246
Zn(SB3OME) ₂	Zn(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	White	65	236-238
Co(SB3OME) ₂	Co(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Green	56	256-258
Ni(SB3OME) ₂	Ni(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Brown	70	226-228
SB4OME	C ₁₆ H ₁₆ N ₂ O ₂ S ₂	Solid	White	70	138-140
Cu(SB4OME) ₂	Cu(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Black	64	296-297
Zn(SB4OME) ₂	Zn(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	White	72	265-266
Co(SB4OME) ₂	Co(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Green	73	269-270
Ni(SB4OME) ₂	Ni(C ₁₆ H ₁₆ N ₂ O ₂ S ₂) ₂	Solid	Brown	54	260-261
SB3NO	C ₁₅ H ₁₂ ClN ₃ O ₃ S ₂	Solid	Cream	84	188-190
Cu(SB3NO) ₂	Cu(C ₁₅ H ₁₂ ClN ₃ O ₃ S ₂) ₂	Solid	Green	60	318 d
Zn(SB3NO) ₂	Zn(C ₁₅ H ₁₂ ClN ₃ O ₃ S ₂) ₂	Solid	Black	49	346 d

Co(SB3NO) ₂	Co(C ₁₅ H ₁₂ ClN ₃ O ₃ S ₂) ₂	Solid	Brown	66	310 d
Ni(SB3NO) ₂	Ni(C ₁₅ H ₁₂ ClN ₃ O ₃ S ₂) ₂	Solid	Yellow	75	256 d

d-decompose temperature

SB2OME, SB3OME and SB4OME were obtained in a white precipitate. SB2OME recorded a moderate yield of 65% with a melting point of 156-158 °C. SB4OME also exhibited a moderate yield, 70% with a melting point of 138-140 °C. Despite many attempts have been made to synthesise SB3OME in high yield, the highest yield was obtained only at 40% with a melting point of 138-141 °C. The low yield of SB3OME is due to its incompatibility with the strong base, potassium hydroxide (Sigma-Aldrich, 2022). Generally, all the melting points obtained were sharp (over 1-3 °C) indicating that these SBDTC derivatives were free of impurities. SB3NO was obtained as a cream precipitate with a high yield of 84% and melted in a range of 188-190 °C.

Initial observations of the metal complexes from the SB2OME, SB3OME and SB4OME series revealed varying colours but similar shades for each metal. According to Cotton (2005), the formation of complexes in many colours is attributed to their partially filled shells, and the colours of the products confirm the occurrence of the reaction between the ligand and metal (Cotton et al., 2005). Cu(II), Zn(II), Co(II), and Ni(II) complexes from SB2OME, SB3OME and SB4OME series exhibited black, white, green, and brown colours, respectively. All metal complexes are coloured except for zinc (II) complexes which followed the colour of the ligands due to the filled d orbital of Zn²⁺ which does not allow electronic transition between d orbital (Yusof, 2014).

The observed melting points of the metal complexes ranged from 200 to 300 °C, higher than those of their SBDTC derivatives, indicating increased stability and complexation (Dalha et al., 2021). Metal complexes possess closed-packed structures with a coordination number, along with strong metallic bonds and small atomic radii. This necessitates more energy to break these bonds, resulting in higher melting points for the metal complexes (Ivanov, 2012). Generally, the metal complexes of SB2OME, SB3OME and SB4OME exhibited varying yields from 43-73%.

Metal complexes of SB3NO exhibited various colours with percentage yields ranging from 47-66%. Cu(II), Zn(II), Co(II), and Ni(II) complexes displayed green, black, brown, and yellow colours, respectively. Unlike metal complexes from SB2OME, SB3OME and SB4OME, metal complexes from SB3NO did not melt until 400°C, hence, the decomposition temperature was observed when the colour and amounts of metal complexes significantly changed in the capillary tube. Consequently, the decomposition temperature ranged from 256-350°C.

4.3 ELEMENTAL ANALYSIS

The molecular formula of the compounds can be predicted via elemental analysis. Two elemental analyses were conducted which are carbon, hydrogen, nitrogen, sulfur (CHNS) analysis and metal analysis. All the results from the analysis are shown in Table 4.2 and the raw data from experiments were compiled in Appendix 3 and 4. The experimental results obtained from this study were compared to theoretical or calculated studies.

Table 4.2 Elemental data of SBDTC derivatives and their metal complexes

Compound	Percentage weight (Theoretical) (%)				
	C	H	N	S	M
SB2OME	58.08 (57.81)	4.24 (4.85)	8.22 (8.43)	19.36 (19.29)	-
Cu(SB2OME) ₂	52.68 (52.76)	4.35 (4.43)	7.95 (7.69)	17.75 (17.61)	8.92 (8.72)
Zn(SB2OME) ₂	52.19 (52.63)	4.30 (4.42)	7.62 (7.67)	17.79 (17.56)	8.636 (8.96)
Co(SB2OME) ₂	52.97 (53.10)	4.40 (4.46)	7.60 (7.74)	17.62 (17.72)	-
Ni(SB2OME) ₂	52.71 (53.12)	4.29 (4.46)	7.67 (7.74)	17.09 (17.72)	8.005 (8.11)
SB3OME	56.81 (57.81)	4.30 (4.85)	8.37 (8.43)	19.54 (19.29)	-
Cu(SB3OME) ₂	51.76 (52.76)	4.22 (4.43)	7.51 (7.69)	17.03 (17.61)	8.25 (8.72)

Zn(SB3OME) ₂	52.40 (52.63)	4.70 (4.42)	7.43 (7.67)	17.39 (17.56)	8.69 (8.96)
Co(SB3OME) ₂	54.90 (53.10)	4.35 (4.46)	7.47 (7.74)	18.87 (17.72)	-
Ni(SB3OME) ₂	51.30 (53.12)	4.67 (4.46)	7.68 (7.74)	17.49 (17.72)	8.30 (8.11)
SB4OME	58.28 (57.81)	4.39 (4.85)	8.01 (8.43)	19.62 (19.29)	-
Cu(SB4OME) ₂	52.35 (52.76)	4.11 (4.43)	7.44 (7.69)	16.94 (17.61)	8.21 (8.72)
Zn(SB4OME) ₂	53.80 (52.63)	4.81 (4.42)	7.83 (7.67)	17.42 (17.56)	8.23 (8.96)
Co(SB4OME) ₂	54.20 (53.10)	4.80 (4.46)	7.91 (7.74)	17.45 (17.72)	-
Ni(SB4OME) ₂	52.14 (53.12)	4.34 (4.46)	7.77 (7.74)	17.11 (17.72)	8.07 (8.11)
SB3NO	46.56 (47.18)	2.97 (3.17)	10.65 (11.00)	15.04 (16.79)	-
Cu(SB3NO) ₂	42.48 (43.56)	2.80 (2.92)	11.18 (10.16)	14.35 (15.50)	7.73 (7.68)
Zn(SB3NO) ₂	42.73 (43.46)	3.23 (2.92)	10.27 (10.14)	14.53 (15.47)	7.57 (7.89)
Co(SB3NO) ₂	44.76 (43.80)	2.82 (2.94)	10.53 (10.22)	14.55 (15.59)	-
Ni(SB3NO) ₂	44.05 (43.81)	2.94 (2.94)	9.87 (10.22)	14.36 (15.59)	7.12 (7.14)

Elemental analysis, especially the CHNS analysis, confirms their molecular structure. All the experimental results matched the expected values, confirming that all the compounds were successfully synthesised. Most of the experimental values obtained were quite close to the theoretical values (less than 5% differences). However, few compounds have more than a 5% difference with at most 12.58 % observed. The differences may be due to several factors, which include the loss of product during preparation and handling throughout the experiment, even before loading into the

instrument for analysis. Besides, impurities and incomplete drying can also create small differences between the experimental and theoretical values.

The metals analysis gave the compositions of metals (copper, zinc, and nickel) present in the metal complexes. Due to the absence of a cobalt lamp, the percentage of cobalt was unable to be determined. However, the elemental CHNS analysis still corresponded to the experimental elemental values for all the cobalt metal complexes. From the results reported in Table 4.3, the percentages of metals closely corresponded with the theoretical values, providing strong evidence of successful synthesis of the metal complexes.

Though the synthesised metal complexes were prepared with a 1:1 ratio of metal to ligand, AAS analysis consistently gave a 1:2 ratio of metal to ligand for the synthesised metal complexes. In fact, metal complexes containing a 1:2 ratio of metal to ligand are usually termed bis-chelate complexes (Hider & Ma, 2016). Nevertheless, there was a minor difference (less than 5% differences) between experimental and theoretical concentrations of metal. However, few metals have more than a 5% difference with at most 12.15%. These differences may be due to various factors related to the incomplete dissolution of the compounds during the dilution process carried out before analysis. Overall, both CHNS and AAS results agreed well with the proposed formula for all synthesised compounds.

4.4 FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

Dithiocarbazate derivatives are renowned for exhibiting characteristic infrared (IR) absorption bands, particularly associated with important functional groups such as (N-H), (N-N), (C=S), and (SCS). In this study, however, the newly synthesised SBDTC derivatives also include carbonyl groups, adding an additional functional group, (C=O), to the compounds. Detailed wavenumber data for these groups is provided in Appendix 5.

4.4.1 SBDTC, SB2OME, SB3OME and SB4OME

The IR analysis of SBDTC, shown in Table 4.3, confirms that the compound matches the structure described in prior literature (Akbar Ali & Tarafdar, 1977). Thus, these

results validated its suitability for use as a precursor in reactions with other organic ligands to form new SBDTC derivatives.

Upon the derivation of SBDTC to its derivatives, SB2OME, SB3OME, and SB4OME, notable changes were observed in the IR spectra, particularly in the region around 3300–3000 cm^{-1} , as shown in Figure 4.12. For SB2OME and SB3OME, two medium intensity bands were identified, appearing at 3209 cm^{-1} and 3121 cm^{-1} for SB2OME, and at 3258 cm^{-1} and 3135 cm^{-1} for SB3OME. These bands correspond to N-H stretching vibrations adjacent to the carbonyl (C=O) and thiocarbonyl (C=S) groups, respectively, as reported by Singh et al. (2009).

Interestingly, no band for primary amine (N-H₂) stretching was detected in SB2OME and SB3OME. This absence suggested that the methoxybenzoyl groups selectively interacted with the β -nitrogen of SBDTC, which originally existed as a primary amine (N-H₂) (Figure 4.12). This interaction converted the β -nitrogen into a secondary amine (N-H), thereby removing the N-H₂ stretching signature from the spectra and resulting in two bands indicative of N-H.

In contrast, the spectrum for SB4OME revealed two strong bands at 3258 cm^{-1} and 3329 cm^{-1} , corresponding to N-H and N-H₂ stretching vibrations, respectively. This pattern aligned with previous findings (Ameram & Othman, 2013). The presence of the N-H₂ band implied that the 4-methoxybenzoyl group targeted the α -nitrogen of SBDTC, which was originally observed as the N-H group. As shown in Figure 4.12, this interaction transformed the α -nitrogen into a tertiary amine while leaving the β -nitrogen as a primary amine (N-H₂). Consequently, the spectrum of SB4OME exhibited both primary and secondary amine vibrations, setting it apart from SB2OME and SB3OME.

Additionally, the spectra of all derivatives showed a strong C=O stretching band in the range of 1630–1650 cm^{-1} . These bands were shifted to lower wavenumbers compared to their starting material 2-methoxybenzoyl chloride (2OME), 3-methoxybenzoyl chloride (3OME) and 4-methoxybenzoyl chloride (4OME) (Table 4.3). This shift provided clear evidence of new bond formation involving the carbonyl carbon. This observation was consistent with previous literature reports by Singh et al. (2008) and How et al. (2008), where the band was assigned at 1641 cm^{-1} and 1638 cm^{-1} , respectively. Additionally, the successful formation of SB2OME, SB3OME and

SB4OME was further corroborated by the disappearance of the $\nu(\text{ClC}=\text{O})$ band, typically observed around $600\text{--}670\text{ cm}^{-1}$ (Table 4.3). The absence of this band indicates the cleavage of the bond between chloride and the carbonyl carbon during the reaction process.

Moreover, there were a few other characteristic bands that further supported the successful synthesis of these derivatives. A sharp band in the range of $1035\text{--}1047\text{ cm}^{-1}$ was assigned to $\text{C}=\text{S}$ stretching, while S-C-S stretching vibrations were observed between $980\text{--}1016\text{ cm}^{-1}$ (Table 4.3) (Khoo et al., 2014). Weak bands corresponding to N-N stretching appeared in the $1276\text{--}1308\text{ cm}^{-1}$ range, showing a slight negative shift compared to the scaffold SBDTC compound (Table 4.3) (Khirudin et al., 2023). This shift indicated the formation of new bonds at the nitrogen atoms. Altogether, these spectral features strongly confirmed the structural identities of SB2OME, SB3OME, and SB4OME (as shown in Figure 4.12) providing compelling evidence of their successful synthesis.

Table 4.3 Important IR bands for SBDTC, SB2OME, SB3OME and SB4OME

Compound	Wavenumber (cm ⁻¹)							
	$\nu(\text{N-H})$	$\nu(\text{N-H}_2)$	$\nu(\text{N-N})$	$\nu(\text{C=S})$	$\nu(\text{SCS})$	$\nu(\text{ClC=O})$	$\nu(\text{C=O})$	$\nu(\text{OCH}_3)$
SBDTC	3220, 3180 s	3304 s	1347 m	1048 s	952 m	-	-	-
2OME	-	-	-	-	-	654 s	1696 s	1465 s
3OME	-	-	-	-	-	667 s	1764 s	1486 s
4OME	-	-	-	-	-	613 s	1771 s	1506 s
SB2OME	3209, 3121 m	-	1308 m	1035 s	980 s	-	1638 s	1446 s
SB3OME	3258, 3135 m	-	1297 m	1047 s	988 s	-	1658 s	1437 s
SB4OME	3258 s	3329 s	1276 m	1043 m	1016 m	-	1658 s	1495 s

s-strong; m-medium; w-weak

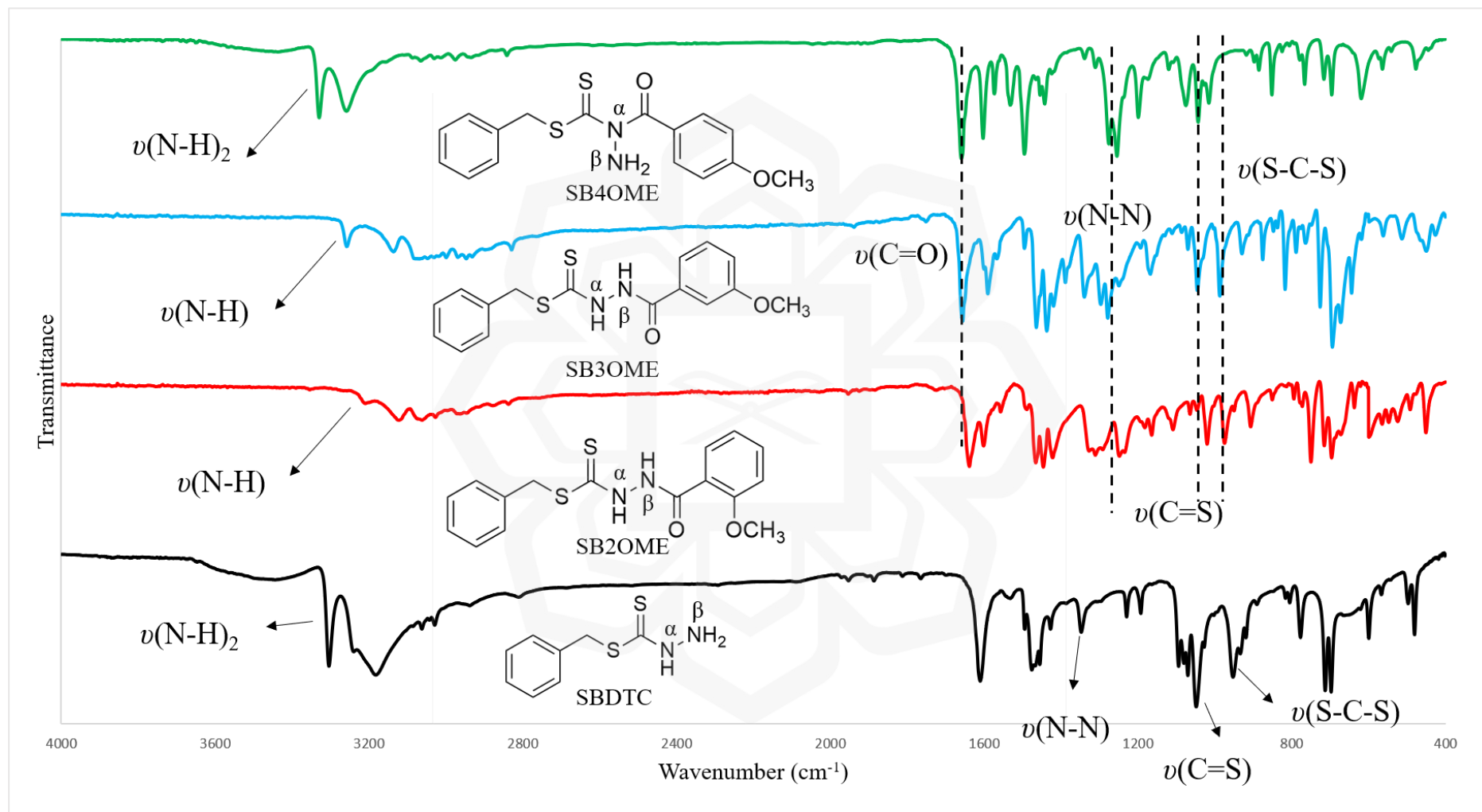


Figure 4.12 Overlay IR spectra of SBDTC with SB2OME, SB3OME and SB4OME

4.4.2 Metal Complexes from SB2OME

The coordination of SB2OME was analysed using IR data from Table 4.4 and Figure 4.14. From Figure 4.14, a key observation was the disappearance of one of the N-H stretching bands, which indicated the involvement of the amino group in complexation. SB2OME initially exhibited two N-H stretching bands at 3209 cm^{-1} and 3121 cm^{-1} , corresponding to the N-H groups adjacent to the carbonyl (C=O) and thiocarbonyl (C=S) groups, respectively. Upon complexation, the N-H band shifted and reduced to a single band in the metal complexes, appearing at 3210 cm^{-1} for $\text{Cu}(\text{SB2OME})_2$, 3206 cm^{-1} for $\text{Zn}(\text{SB2OME})_2$, and 3180 cm^{-1} for both $\text{Co}(\text{SB2OME})_2$ and $\text{Ni}(\text{SB2OME})_2$. This shift indicates that the N-H group adjacent to C=S was deprotonated and coordinated with the metal ions. This is consistent with findings from previous studies where the disappearance of one N-H band indicated complexation via the amino group (Ameram & Othman, 2013; M. Singh et al., 2009).

Additionally, the N-N stretching vibration in SB2OME initially observed at 1308 cm^{-1} , shifted significantly in the metal complexes. For all metal complexes, the N-N band shifted to approximately $1234\text{--}1243\text{ cm}^{-1}$, reflecting a negative shift of $60\text{--}70\text{ cm}^{-1}$. This shift confirms that the hydrazinic nitrogen participated in coordination, altering the electronic environment of the N-N bond (How et al., 2008).

The carbonyl stretching band (C=O), initially at 1638 cm^{-1} in SB2OME, also experienced marked shifts upon complexation. In the metal complexes, the C=O band appeared at 1602 cm^{-1} for $\text{Cu}(\text{SB2OME})_2$, $\text{Co}(\text{SB2OME})_2$, $\text{Ni}(\text{SB2OME})_2$ and 1604 cm^{-1} for $\text{Zn}(\text{SB2OME})_2$, with an average shift of $34\text{--}36\text{ cm}^{-1}$. These shifts are indicative of strong metal-oxygen bond formation, confirming coordination through the carbonyl oxygen (M. Singh et al., 2009).

Similarly, the thiocarbonyl (C=S) stretching band in SB2OME, observed at 1035 cm^{-1} , shifted to 1019 cm^{-1} for $\text{Cu}(\text{SB2OME})_2$, 1023 cm^{-1} for $\text{Zn}(\text{SB2OME})_2$, 1013 cm^{-1} for $\text{Co}(\text{SB2OME})_2$, and 1020 cm^{-1} for $\text{Ni}(\text{SB2OME})_2$. The average negative shift of $12\text{--}22\text{ cm}^{-1}$ confirmed that coordination occurred via the thione sulfur (M. Singh et al., 2009). Furthermore, the S-C-S stretching vibration at 980 cm^{-1} in SB2OME also shifted upon complexation, appearing at $961\text{--}965\text{ cm}^{-1}$ across all metal complexes, with

a shift of 12–19 cm^{-1} , further confirming the role of sulfur in metal coordination (Ameram & Othman, 2013).

Interestingly, the methoxy group (OCH_3) did not show significant changes in its stretching frequency, indicating that it did not participate in coordination. The $\nu(\text{OCH}_3)$ band (Table 4.4), observed at 1446 cm^{-1} in SB2OME, shifted minimally to 1444 cm^{-1} in $\text{Cu}(\text{SB2OME})_2$, and to 1451 cm^{-1} in the $\text{Zn}(\text{SB2OME})_2$, $\text{Co}(\text{SB2OME})_2$, and $\text{Ni}(\text{SB2OME})_2$. These negligible shifts suggest that the methoxy group remained uncoordinated due to steric hindrance caused by the bulky aromatic ring. Therefore, the steric hindrance prevents it from interacting with the metal ions, as also reported in the previous study (Sibaud et al., 2016).

Lastly, the acetate (OAc) stretching bands, initially observed at $\sim 1600\text{ cm}^{-1}$ and $\sim 1400\text{ cm}^{-1}$ in the metal salts disappeared in all metal complex spectra (Table 4.4) (Hosny, 2007). This reveals that the acetate ions did not participate in coordination and remained in solution, with SB2OME coordinating directly with the metal ions. In conclusion, SB2OME formed tridentate metal complexes with $\text{Cu}(\text{II})$, $\text{Zn}(\text{II})$, $\text{Co}(\text{II})$ and $\text{Ni}(\text{II})$ ions. The coordination involved the deprotonation of the amino group, the carbonyl oxygen, and the thione sulfur, forming six coordinated metal complexes as illustrated in Figure 4.13.

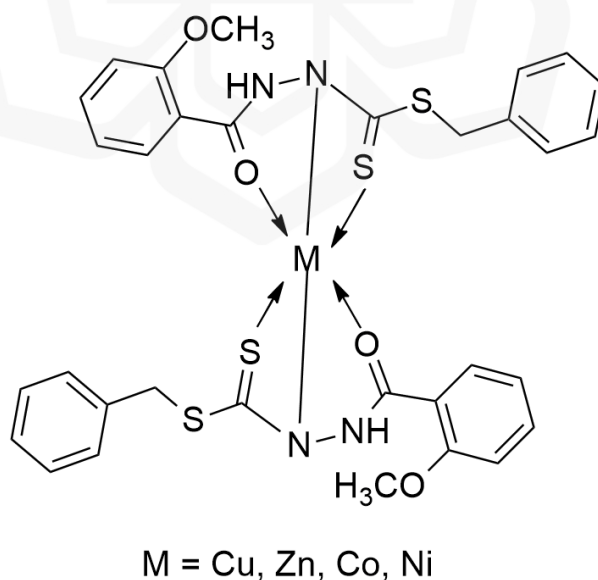


Figure 4.13 Coordination mode of metal complexes from SB2OME

Table 4.4 Important IR bands for metal complexes from SB2OME

Compound	Wavenumber (cm ⁻¹)						
	$\nu(\text{OAc})$	$\nu(\text{N-H})$	$\nu(\text{N-N})$	$\nu(\text{C=S})$	$\nu(\text{SCS})$	$\nu(\text{C=O})$	$\nu(\text{OCH}_3)$
Copper(II) acetate monohydrate	1603 s, 1445 s	-	-	-	-	-	-
Zinc (II) acetate dihydrate	1558 s, 1456 s	-	-	-	-	-	-
Cobalt (II) acetate tetrahydrate	1569 s, 1453 m	-	-	-	-	-	-
Nickel (II) acetate tetrahydrate	1606 s, 1456 s	-	-	-	-	-	-
SB2OME	-	3209, 3121 m	1308 m	1035 s	980 s	1638 s	1446 s
Cu(SB2OME) ₂	-	3210 m	1234 m	1019 s	971 s	1602 s	1444 s
Zn(SB2OME) ₂	-	3206 m	1243 m	1023 s	965 s	1604 s	1451 s
Co(SB2OME) ₂	-	3180 m	1242 m	1013 s	963 s	1602 s	1451 s
Ni(SB2OME) ₂	-	3180 m	1239 m	1020 s	963 s	1602 s	1451 s

s-strong; m-medium; w-weak

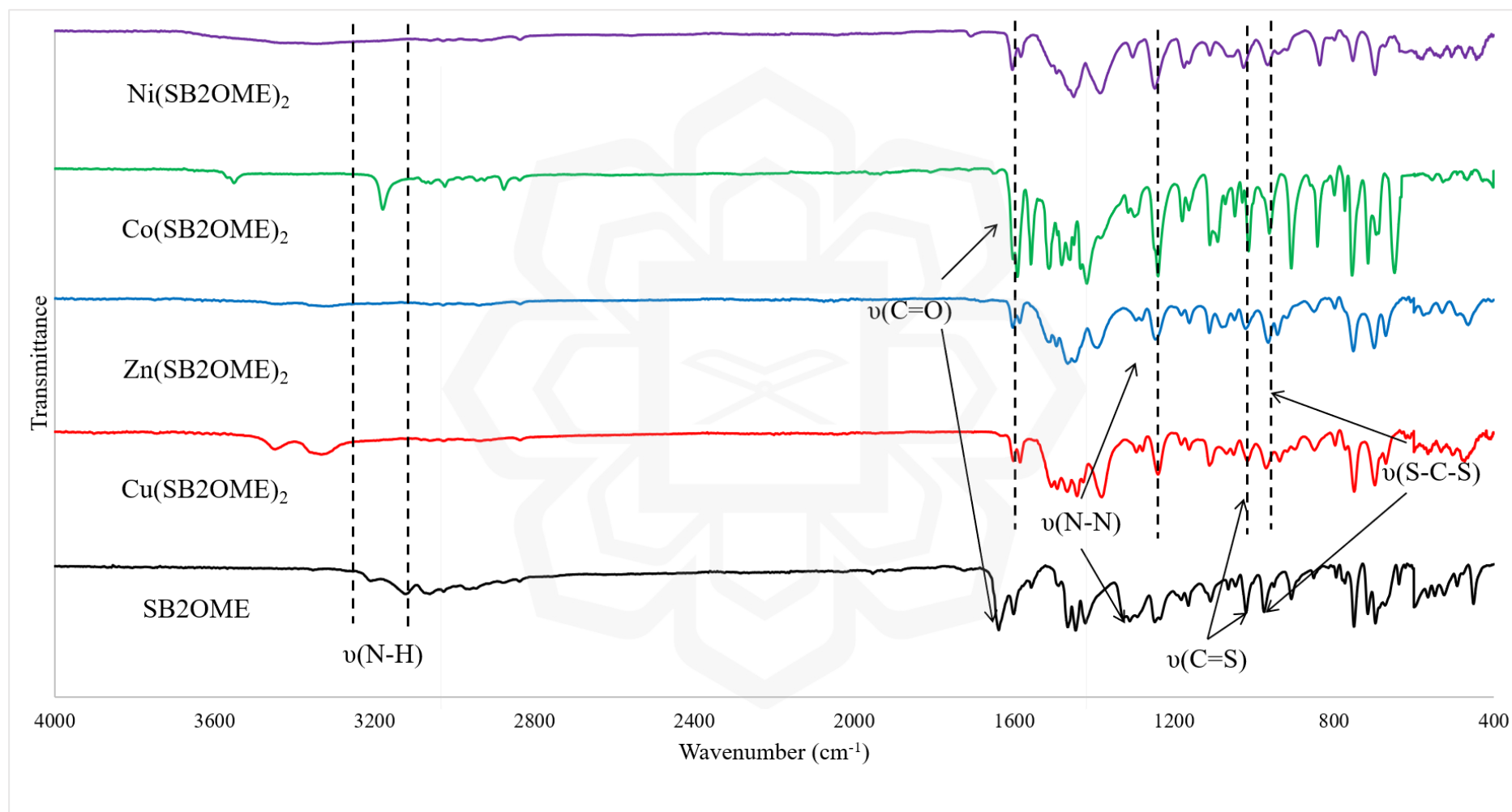


Figure 4.14 Overlay IR spectra of metal complexes from SB2OME

4.4.3 Metal Complexes from SB3OME

The coordination of SB3OME was analysed using IR data from Table 4.5. From Figure 4.16, one of the most prominent observations was the broadening of the $\nu(\text{O-H})$ stretching band around 3400 cm^{-1} , which corresponds to the water molecules. This broad band, attributed to the presence of water, was observed in all metal complexes. However, the absence of bands around $931\text{--}937\text{ cm}^{-1}$ and $625\text{--}627\text{ cm}^{-1}$, which typically correspond to the rocking and wagging modes of water, suggested that the water molecules were not coordinated with the metal ions but were likely trapped in the lattice. This broad $\nu(\text{O-H})$ band could be a result of incomplete drying during sample preparation, as noted in a previous study (Emam et al., 2009).

The coordination of the amino group in SB3OME was observed from the shifting of the N-H stretching bands. Initially, SB3OME displayed two bands at 3258 cm^{-1} and 3135 cm^{-1} , corresponding to the N-H protons adjacent to C=O and C=S, respectively (M. Singh et al., 2009). Upon complexation with metal ions, one of these bands disappeared, while the remaining band shifted to 3196 cm^{-1} for $\text{Cu}(\text{SB3OME})_2$, 3194 cm^{-1} for $\text{Zn}(\text{SB3OME})_2$, 3192 cm^{-1} for $\text{Co}(\text{SB3OME})_2$, and 3195 cm^{-1} for $\text{Ni}(\text{SB3OME})_2$. This disappearance of one of the N-H bands confirmed the involvement of the amino group proton adjacent to C=S in the coordination with the metal ions.

The N-N stretching vibration initially observed at 1297 cm^{-1} in SB3OME, exhibited a significant negative shift of $102\text{--}106\text{ cm}^{-1}$ in all the metal complexes, shifting to the region of $1242\text{--}1246\text{ cm}^{-1}$. This large shift is indicative of the participation of the hydrazinic nitrogen in the coordination process, further supporting the formation of metal-ligand bonds through this nitrogen (How et al., 2008). The carbonyl (C=O) stretching vibration, initially at 1658 cm^{-1} in SB3OME, also underwent a substantial negative shift upon complexation. In the metal complexes, the C=O band shifted to 1600 cm^{-1} for $\text{Cu}(\text{SB3OME})_2$, $\text{Co}(\text{SB3OME})_2$, and $\text{Ni}(\text{SB3OME})_2$, and to 1601 cm^{-1} for $\text{Zn}(\text{SB3OME})_2$. These shifts of $57\text{--}58\text{ cm}^{-1}$ are indicative of the participation of the carbonyl oxygen in coordination with all the metal ions, reinforcing the coordination through the oxygen atom (Gatto et al., 2024).

In addition, the thione (C=S) stretching band, initially at 1047 cm^{-1} in SB3OME, shifted to 1031 cm^{-1} for $\text{Cu}(\text{SB3OME})_2$, 1035 cm^{-1} for $\text{Zn}(\text{SB3OME})_2$ and

Co(SB3OME)₂, and 1030 cm⁻¹ for Ni(SB3OME)₂. These shifts of 16–18 cm⁻¹ in all metal complexes confirm that thione sulfur coordinates with the metal ions. The S-C-S stretching band in SB3OME, initially at 988 cm⁻¹, underwent a shift to 954 cm⁻¹ for Cu(SB3OME)₂ and 958 cm⁻¹ for Zn(SB3OME)₂, Co(SB3OME)₂, and Ni(SB3OME)₂. These shifts further support the involvement of sulfur atoms in coordination, confirming that the sulfur atoms play a role in metal binding.

Interestingly, the methoxy group (OCH₃) did not show significant changes in its stretching frequencies (Table 4.5). The $\nu(\text{OCH}_3)$ band in SB3OME was observed at 1047 cm⁻¹ and shifted slightly in the metal complexes. These bands were observed at 1440 cm⁻¹ for Cu(SB3OME)₂, 1439 cm⁻¹ for Zn(SB3OME)₂, 1443 cm⁻¹ for Co(SB3OME)₂, and remained at 1047 cm⁻¹ for Ni(SB3OME)₂. These minimal shifts prove that the methoxy group does not participate in coordination, which is consistent with previous findings (Sibaud et al., 2016). Furthermore, the absence of acetate (OAc) stretching bands around 1603 cm⁻¹ and 1445 cm⁻¹ in all metal complexes suggests that the acetate ions did not participate in coordination (Table 4.5). Instead, the acetate ions remained in the solution, while SB3OME coordinated directly with the metal ions.

In conclusion, the IR spectral data confirmed that SB3OME functions as a tridentate ligand, coordinating with the metal ions via the deprotonated amino group, carbonyl oxygen, and thione sulfur atoms as illustrated in Figure 4.15. These findings are consistent with the coordination behaviour observed in the metal complexes of SB2OME, showing a similar mode of coordination for both ligands.

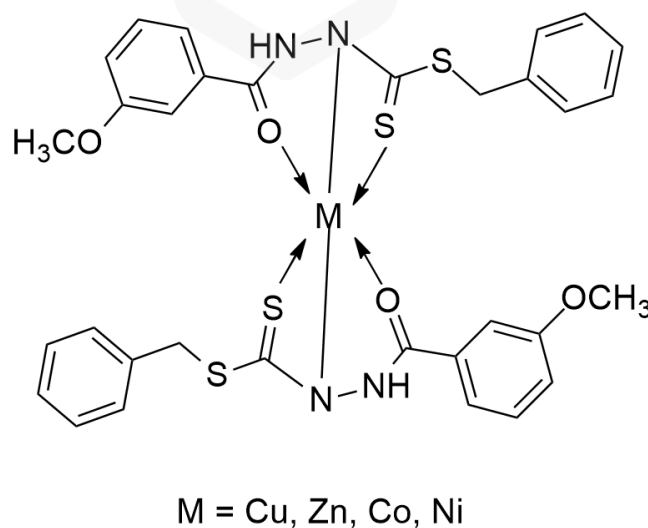


Figure 4.15 Coordination mode of metal complexes from SB3OME

Table 4.5 Important IR bands for metal complexes from SB3OME

Compound	Wavenumber (cm ⁻¹)						
	$\nu(\text{OAc})$	$\nu(\text{N-H})$	$\nu(\text{N-N})$	$\nu(\text{C=S})$	$\nu(\text{SCS})$	$\nu(\text{C=O})$	$\nu(\text{OCH}_3)$
Copper(II) acetate monohydrate	1603 s, 1445 s	-	-	-	-	-	-
Zinc (II) acetate dihydrate	1558 s, 1456 s	-	-	-	-	-	-
Cobalt (II) acetate tetrahydrate	1569 s, 1453 m	-	-	-	-	-	-
Nickel (II) acetate tetrahydrate	1606 s, 1456 s	-	-	-	-	-	-
SB3OME	-	3258, 3135 m	1297 m	1047 s	988 s	1658 s	10.437 s
Cu(SB3OME) ₂	-	3196 w	1242 m	1031 s	954 s	1600 s	1440 s
Zn(SB3OME) ₂	-	3194 w	1243 m	1035 s	958 s	1601 s	1439 s
Co(SB3OME) ₂	-	3192 w	1246 m	1035 s	954 s	1600 s	1443 s
Ni(SB3OME) ₂	-	3195 w	1243 m	1030 s	955 s	1600 s	1437 s

s-strong; m-medium; w-weak

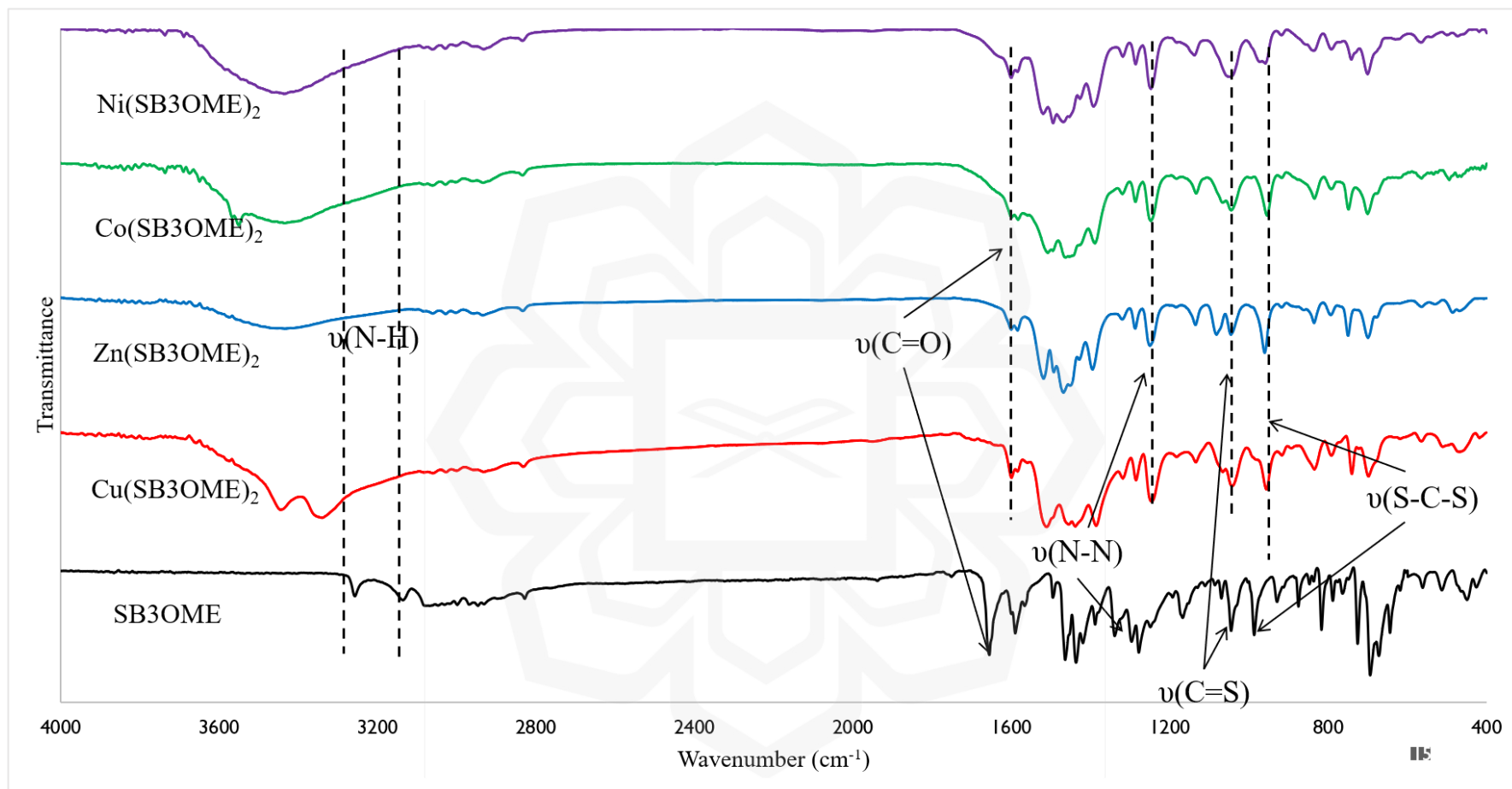


Figure 4.16 Overlay IR spectra of metal complexes from SB3OME

4.4.4 Metal Complexes from SB4OME

The coordination behaviour of SB4OME, presented in Table 4.6, is similar to that of SB2OME and SB3OME, even though SB4OME has a slightly different structure. The N-H stretching frequencies in SB4OME were observed at 3329 cm^{-1} and 3258 cm^{-1} , corresponding to the $\nu(\text{N-H}_2)$ and $\nu(\text{N-H})$ vibrations, respectively (Ameram & Othman, 2013). Upon complexation with metal ions, one of these N-H bands disappeared (Figure 4.18), demonstrating the involvement of the amino group in coordination. The remaining N-H stretching band shifted to a lower frequency, appearing around $3212\text{--}3209\text{ cm}^{-1}$ in all metal complexes, confirming that the proton of the amino group from $\nu(\text{N-H}_2)$ has taken part in bonding with the metal ion.

The N-N stretching vibration initially observed at 1276 cm^{-1} in SB4OME, experienced a negative shift of 23 cm^{-1} , moving to 1253 cm^{-1} in all metal complexes. This significant shift further confirmed that hydrazinic nitrogen was involved in the coordination process (How et al., 2008). The carbonyl stretching frequency in SB4OME, observed at 1658 cm^{-1} , shifted to 1606 cm^{-1} upon complexation with all metal ions, indicating the involvement of the carbonyl oxygen in coordination (Taraferder et al., 2000). The observed negative shift of 52 cm^{-1} is consistent with the coordination of the carbonyl oxygen with the metal ions, a pattern seen across all metal complexes.

The thione ($\text{C}=\text{S}$) stretching frequency in SB4OME, which was initially observed at 1043 cm^{-1} , shifted upon complexation, confirming the participation of sulfur in coordination. The $\text{C}=\text{S}$ band shifted to 1029 cm^{-1} in the $\text{Cu}(\text{SB4OME})_2$ and to 1028 cm^{-1} in the $\text{Zn}(\text{SB4OME})_2$, $\text{Co}(\text{SB4OME})_2$, and $\text{Ni}(\text{SB4OME})_2$ complexes. The S-C-S bending frequency, which occurred at 1016 cm^{-1} in SB4OME, also underwent a shift upon complexation. The band shifted to 963 cm^{-1} for $\text{Cu}(\text{SB4OME})_2$, 966 cm^{-1} for $\text{Zn}(\text{SB4OME})_2$, 959 cm^{-1} for $\text{Co}(\text{SB4OME})_2$, and 971 cm^{-1} for $\text{Ni}(\text{SB4OME})_2$. These shifts further support the involvement of sulfur in coordination.

The absence of characteristic acetate (OAc) stretching bands in all the metal complexes, though these bands were present in the original salts, indicates that the acetate groups were displaced by SB4OME upon complexation. This observation suggests that acetate ions did not participate in the coordination process, and SB4OME was successfully bound to the metal ions in a tridentate mode. In addition, the stretching

frequency of the methoxy group (OCH_3) in SB4OME, observed at 1495 cm^{-1} , showed only minor shifts ($1\text{-}2\text{ cm}^{-1}$) upon complexation with the metal ions, supporting that the methoxy group also does not participate in coordination (Table 4.6). This lack of significant shift supports the conclusion that the methoxy group remains non-involved in bonding with the metal ions.

In conclusion, the IR spectral data confirm that SB4OME coordinates with metal ions in a tridentate NOS pattern, involving the deprotonation of the amino group, carbonyl oxygen, and thione sulfur atoms as illustrated in Figure 4.17. These results demonstrate that SB4OME exhibits consistent coordination properties with other isomeric SBDTC derivatives, forming six coordinated metal complexes.

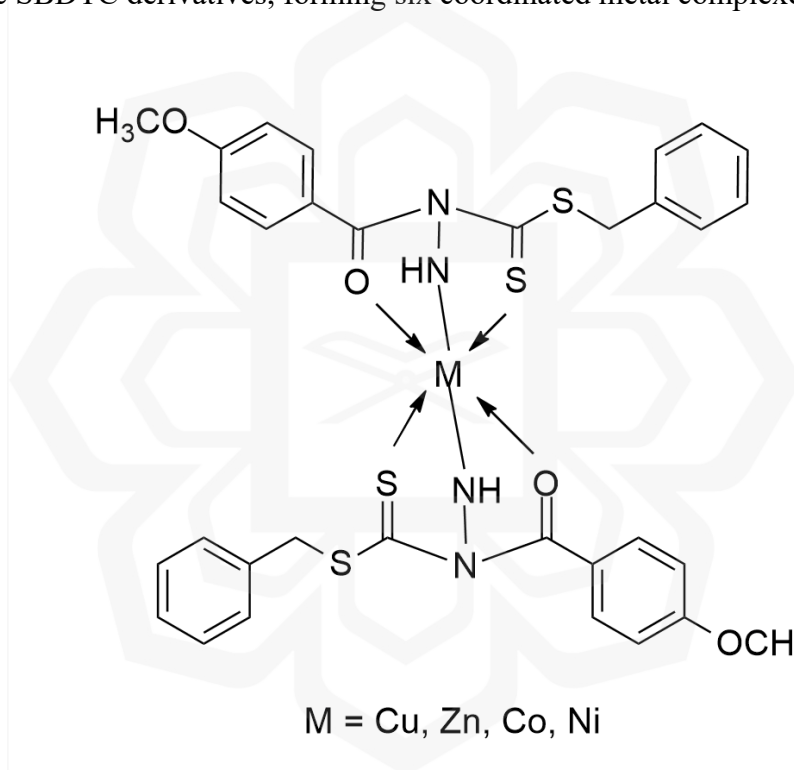


Figure 4.17 Coordination mode of metal complexes from SB4OME

Table 4.6 Important IR bands for metal complexes from SB4OME

Compound	Wavenumber (cm ⁻¹)							
	$\nu(\text{OAc})$	$\nu(\text{N-H}_2)$	$\nu(\text{N-H})$	$\nu(\text{N-N})$	$\nu(\text{C=S})$	$\nu(\text{SCS})$	$\nu(\text{C=O})$	$\nu(\text{OCH}_3)$
Copper(II) acetate monohydrate	1603 s, 1445 s	-	-	-	-	-	-	-
Zinc (II) acetate dihydrate	1558 s, 1456 s	-	-	-	-	-	-	-
Cobalt (II) acetate tetrahydrate	1569 s, 1453 m	-	-	-	-	-	-	-
Nickel (II) acetate tetrahydrate	1606 s, 1456 s	-	-	-	-	-	-	-
SB4OME	-	3329 s	3258 s	1276 m	1043 m	1016 m	1658 s	1495 s
Cu(SB4OME) ₂	-	-	3213 w	1253 m	1029 s	963 s	1606 s	1494 s
Zn(SB4OME) ₂	-	-	3212 w	1253 m	1028 s	966 s	1606 s	1491 s
Co(SB4OME) ₂	-	-	3212 w	1253 m	1028 s	959 s	1606 s	1491 s
Ni(SB4OME) ₂	-	-	3209 w	1253 m	1028 s	971 s	1606 s	1493 s

s-strong; m-medium; w-weak

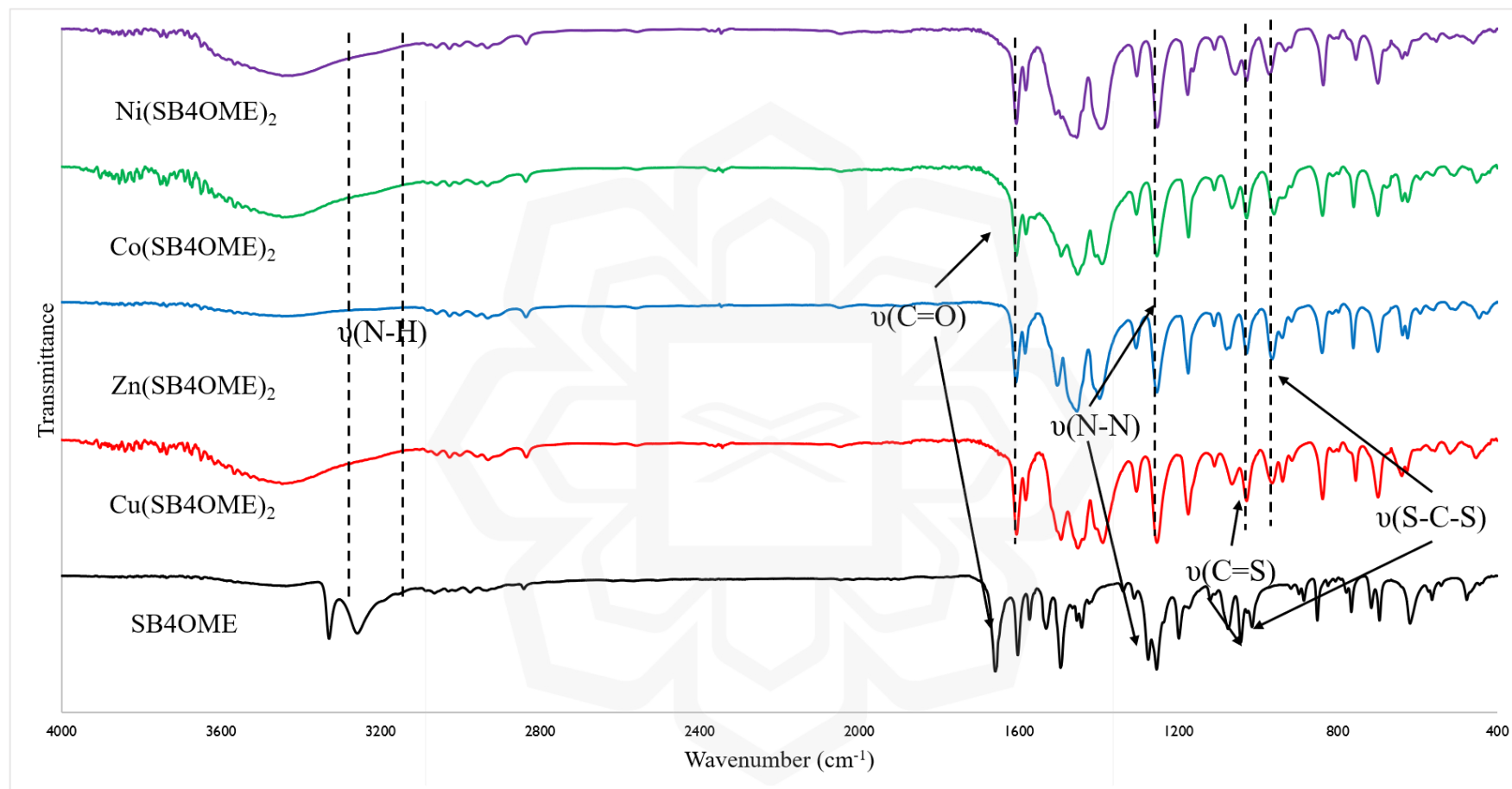


Figure 4.18 Overlay IR spectra of metal complexes from SB4OME

4.4.5 SBDTC, SB3NO and Its Metal Complexes

The overlay of IR spectra for SB3NO was presented in Figure 4.20. Unlike isomeric SB2OME, SB3OME and SB4OME, the spectrum of SB3NO showed a lone weak band at 3094 cm^{-1} , which was attributed to secondary $\nu(\text{N-H})$. Notably, none $\nu(\text{NH}_2)$ was observed, showing the behaviour of the 4-chloro-3-nitrobenzoyl chloride (3NO), which favours attachment to the β -nitrogen of SBDTC, leading to the formation of SB3NO. The SB3NO spectrum also displayed a sharp, intense C=O stretch at 1685 cm^{-1} which is one of the features from 3NO (Table 4.7). Furthermore, the C=S stretching frequency was observed at 1107 cm^{-1} , and the N-N stretch appeared at 1353 cm^{-1} , with the nitro (NO_2) group showing a peak at 1382 cm^{-1} (Kudrat-E-Zahan & Islam, 2015). Additionally, the starting material, 3NO showed a ClC=O stretch at 663 cm^{-1} (Krishnakumar et al., 2013), which disappeared in the SB3NO spectrum (Table 4.7). This suggested that the ClC=O bond breaks during the formation of SB3NO, leading to the formation of SB3NO that readily coordinate with metal ions.

When examining the metal complexes of SB3NO (Figure 4.21), a broad band near 3400 cm^{-1} was observed, corresponding to the O-H stretching of water molecules. The lack of bands in the $931\text{-}937\text{ cm}^{-1}$ and $625\text{-}627\text{ cm}^{-1}$ regions, which correspond to water's rocking and wagging vibrations (Emam et al., 2009), indicates that the water molecules are not coordinating with the metal ions but are merely present in the lattice. This broad band could be attributed to incomplete drying during sample preparation.

Notably, weak N-H stretching band in all metal complexes' spectra at 3094 cm^{-1} remained relatively unchanged from SB3NO's spectrum, with only slight shifts between 3079 and 3084 cm^{-1} (Table 4.7). This demonstrates that the amino group does not participate in the coordination. For instance, in $\text{Cu}(\text{SB3NO})_2$, the N-H band shifted to 3080 cm^{-1} , and in $\text{Ni}(\text{SB3NO})_2$, it shifted to 3084 cm^{-1} . The N-N stretch in SB3NO observed at 1353 cm^{-1} , showed minimal shifting in the metal complexes, further supporting that the amino group does not participate in coordination (Table 4.7).

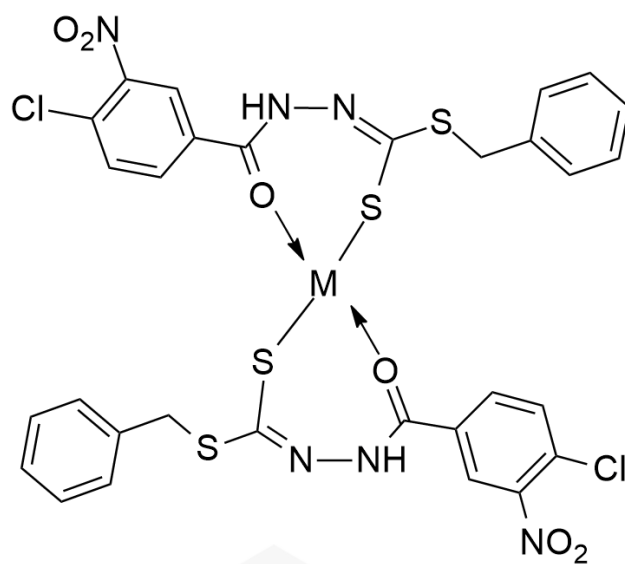
For the carbonyl group, the stretching frequency in SB3NO was initially observed at 1685 cm^{-1} . However, upon metal complexation, this band shifted to lower frequencies, confirming that the carbonyl oxygen participates in coordination (How et al., 2008). In $\text{Cu}(\text{SB3NO})_2$, the C=O band shifted to 1623 cm^{-1} , and in $\text{Zn}(\text{SB3NO})_2$, it

shifted to 1618 cm^{-1} . Similarly, in $\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$, the shift occurred at 1630 cm^{-1} , confirming the involvement of carbonyl oxygen in metal binding across all metal complexes.

The C=S stretch in SB3NO, initially observed at 1107 cm^{-1} , disappeared in all the metal complexes, indicating that the C=S bond was no longer involved in coordination. Instead, coordination occurred through the thiolate (C-S) group, as evidenced by the splitting of the S-C-S bending mode, with bands appearing between 1123 cm^{-1} to 1049 cm^{-1} . This confirms that sulfur participates in coordination through the thiol group (Manan & Mohammat, 2022). The splitting of the S-C-S band is common in SBDTC derivatives, according to previous studies (Moustafa et al., 2021; Ramilo-Gomes et al., 2021; Sahu et al., 2023).

Additionally, a new C=N stretch was observed in the metal complexes, supporting the thiol form of SB3NO. In the free SB3NO ligand, no C=N band was present, but its appearance in the metal complexes suggests the tautomerisation of the C=S group to form the thiol C-S group. For example, in $\text{Cu}(\text{SB3NO})_2$, the C=N stretch occurred at 1602 cm^{-1} , in $\text{Zn}(\text{SB3NO})_2$ at 1597 cm^{-1} , and in $\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$ at 1600 cm^{-1} (Break et al., 2013). Further details on this tautomerisation reaction are discussed in Subsection 4.4.6.

The nitro group stretching, initially at 1382 cm^{-1} in SB3NO (Table 4.7), showed minimal variation in all metal complexes, with shifts to 1385 cm^{-1} in $\text{Cu}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$, 1387 cm^{-1} in $\text{Zn}(\text{SB3NO})_2$, and 1386 cm^{-1} in $\text{Co}(\text{SB3NO})_2$. This indicates that the nitro group does not participate in coordination and remains unchanged during the complexation process. Moreover, the absence of acetate bands in all the metal complexes suggests that the acetate group does not coordinate with the metal ions (Table 4.7). In conclusion, the IR spectral data suggest that SB3NO acts as a bidentate ligand through the thiolate sulfur and carbonyl oxygen atom, forming four coordinated metal complexes as illustrated in Figure 4.19.



M = Cu, Zn, Co, Ni

Figure 4.19 Coordination mode of metal complexes from SB3NO

Table 4.7 Important IR bands for SB3NO and its metal complexes

Compound	Wavenumber (cm ⁻¹)							
	$\nu(\text{N-H})$	$\nu(\text{N-N})$	$\nu(\text{C=S})$	$\nu(\text{SCS})$	$\nu(\text{ClC=O})$	$\nu(\text{C=O})$	$\nu(\text{C=N})$	$\nu(\text{NO}_2)$
SBDTC	3304, 3220, 3180 s	1347 m	1048 s	952 m	-	-	-	-
3NO	-	-	-	-	663 w	1694 s	-	1386 s
SB3NO	3094 w	1353 m	1107 s	1037 s	-	1685 s	-	1382 s
Cu(SB3NO) ₂	3080 w	1342 m	-	1123, 1049 m	-	1623 s	1602 m	1385 s
Zn(SB3NO) ₂	3083 w	1350 m	-	1123, 1049 m	-	1618 s	1597 s	1387 s
Co(SB3NO) ₂	3079 w	1345 m	-	1123, 1049 m	-	1630 s	1600 s	1386 s
Ni(SB3NO) ₂	3084 w	1349 m	-	1123, 1043 m	-	1630 s	1600 s	1385 s

s-strong; m-medium; w-weak

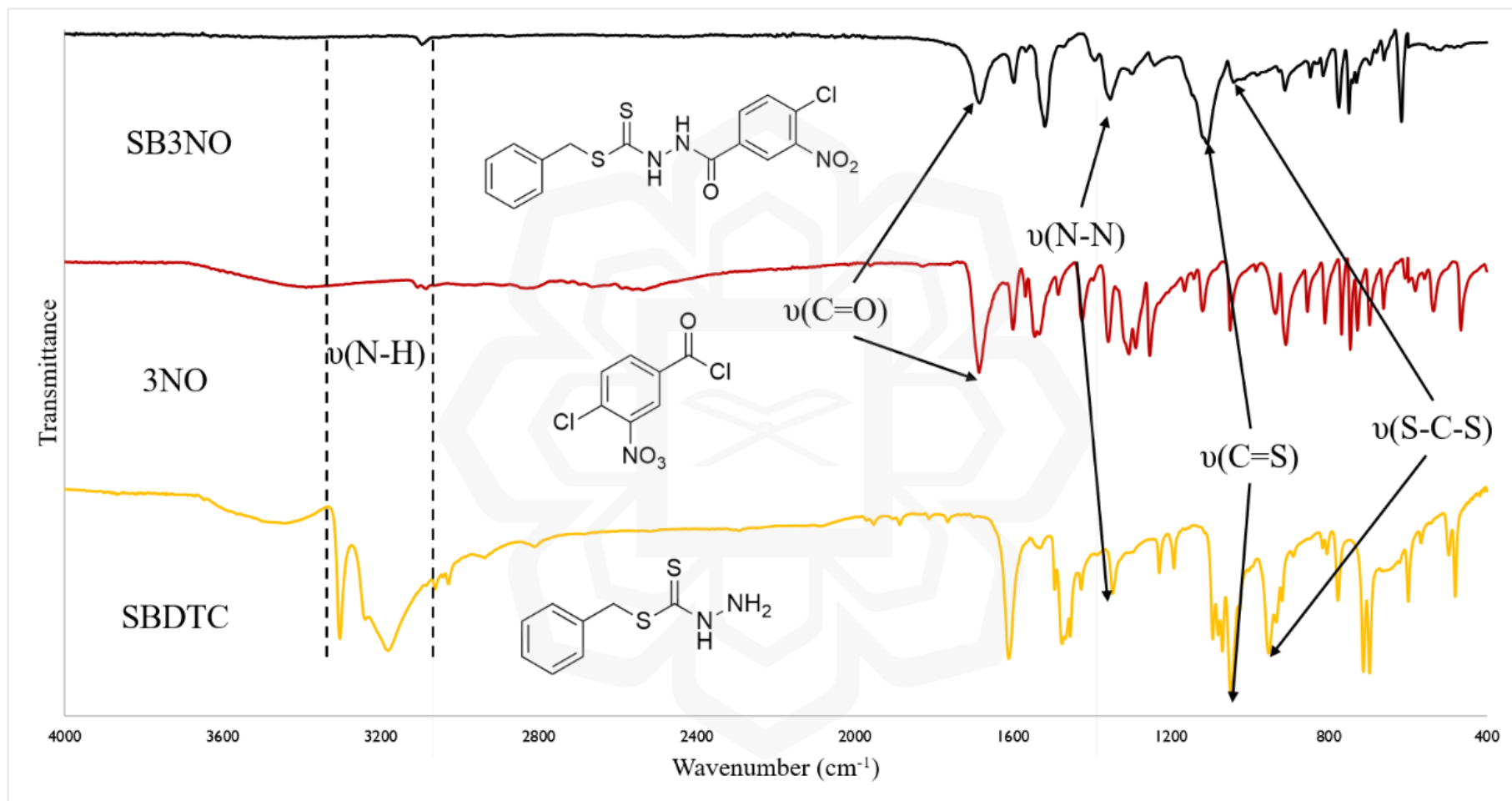


Figure 4.20 Overlay IR spectra of SB3NO and its starting material

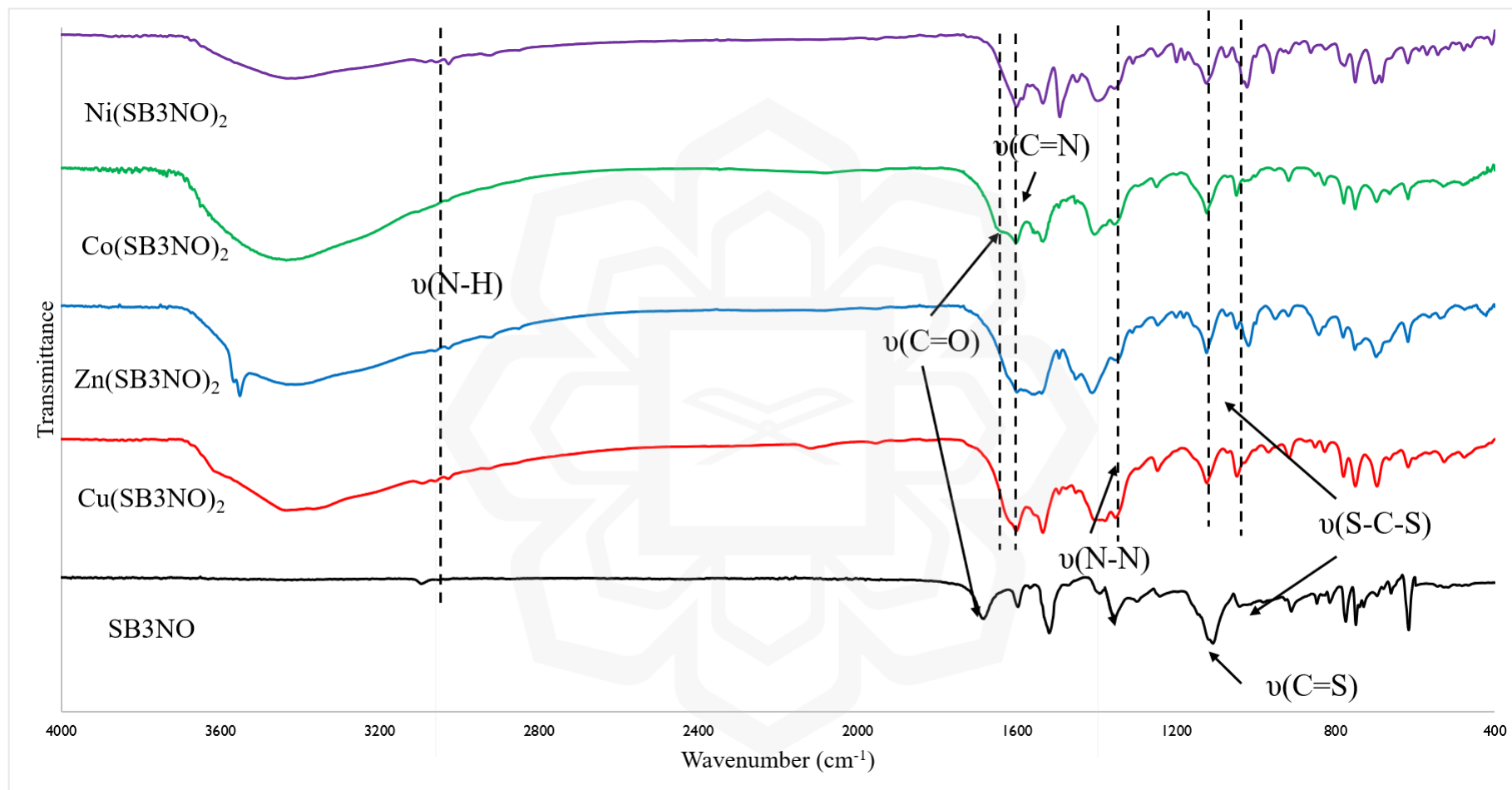


Figure 4.21 Overlay IR spectra of metal complexes from SB3NO

4.4.6 Tautomerism In Solid Form

The ability of SBDTC derivatives to undergo thione-thiol tautomerism is well-documented, with SB2OME, SB3OME, and SB3NO capable of forming four tautomeric forms (thione, thiol, thionol, and thiolol), as illustrated in Figure 4.22. However, SB4OME lacks the α -hydrogen necessary for tautomerism, which supports the fact that tautomeric shifts are not possible for this derivative. According to Bhat (2014), at least one proton adjacent to the α -amine group is required for tautomerism in dithiocarbazate derivatives, and this is absent in SB4OME.

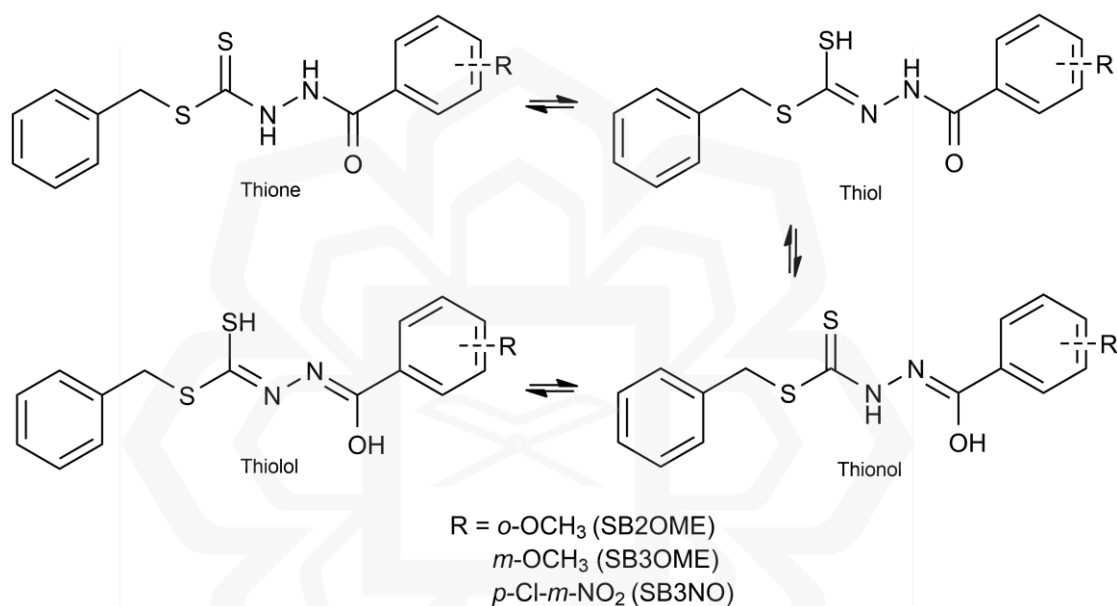


Figure 4.22 Possible tautomeric forms of SB2OME, SB3OME and SB3NO

In the solid state, the IR spectra of SB2OME, SB3OME, and SB3NO indicated that these ligands predominantly existed in the thione form, as evidenced by the strong absorption of the C=S and C=O bands. This was further supported by the absence of the key functional group absorptions associated with the thiol ($\nu(\text{S-H})$ at 2603-2707 cm^{-1}), thionol ($\nu(\text{O-H})$ at 3400-3500 cm^{-1}), and thiolol ($\nu(\text{C=N})$ at 1640-1690 cm^{-1}) forms. This solid-state behaviour is consistent with previous studies showing that SBDTC derivatives typically exist in the thione form in the solid state (Bitu et al., 2020; Hossain et al., 2021).

In solution, however, SB2OME, SB3OME and SB3NO may be present in equilibrium with the four tautomeric structures (How et al., 2008). This claim was supported by attempts to synthesise the metal complexes with the addition of KOH in

all metal complexes from SB3NO. As depicted in metal complexes' spectra of SB3NO, the absence of $\nu(\text{C}=\text{S})$ indicated that SB3NO was no longer in thione form in complexation. As tautomerism converts $\text{C}=\text{S}$ to $\text{C}-\text{S}$, the thiol SB3NO underwent deprotonation, to form negatively charged sulfur before forming a new bond with the positive charge of metal ion (Figure 4.23). As a result, the $\text{C}=\text{S}$ and $\text{S}-\text{H}$ frequency was absent in metal complexes while $\nu(\text{C}=\text{N})$ bands appeared in all metal complexes spectra of SB3NO around $1588\text{--}1550\text{ cm}^{-1}$.

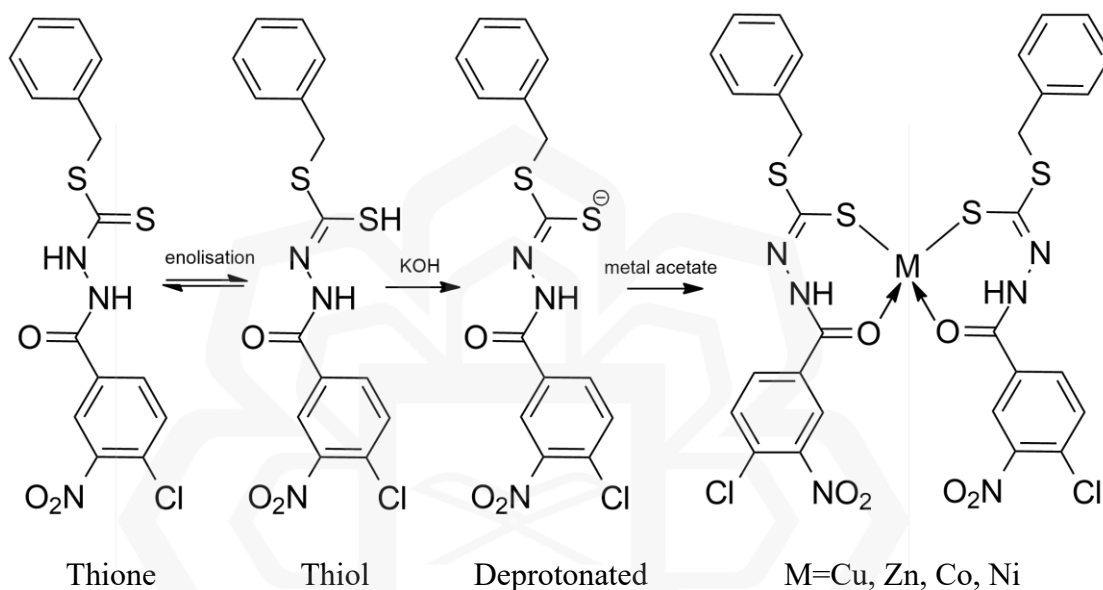


Figure 4.23 Thione-thiol tautomer of SB3NO during complexation

Thus, this inability to synthesise metal complexes of SB3NO without the addition of KOH presented two important pieces of information. Firstly, this failure confirms that SB3NO was not present in the thione form in the solution. Without KOH, SB3NO could not carry out deprotonation essential for metal coordination. Secondly, the success of the reaction in the presence of KOH itself gave support to the fact that SB3NO undergoes enolisation followed by deprotonation, thereby allowing coordination of sulfur with the metal ion.

In contrast, SB2OME and SB3OME remained predominantly in the thione form during complexation, as evidenced by the presence of the $\text{C}=\text{S}$ band in their metal complex IR spectra. This suggests that these ligands do not require the tautomeric transition to the thiol form for metal coordination, differentiating them from SB3NO.

In conclusion, while the SBDTC derivatives exist in the thione form in the solid state, their tautomeric behaviour in solution allows for the transition between different forms, particularly when coordinating with metal ions. These results indicate that the tautomeric shifts of SB2OME, SB3OME, and SB3NO are crucial for their ability to interact with metal ions, whereas SB4OME, lacking the α -hydrogen, does not exhibit such behaviour. However, further structural details of each SBDTC derivative in the liquid form could be confirmed through the next analytical technique, which is Nuclear Magnetic Resonance (NMR) spectroscopy.

4.5 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

In all ^1H NMR spectra, singlet bands around 2.5 and 3.3 ppm were observed, corresponding to the deuterated dimethyl sulfoxide (DMSO) solvent and water, respectively. Meanwhile, in the ^{13}C NMR spectrum, the solvent signal was observed at 40.0 ppm. These solvent and water peaks were excluded from the structural analysis.

4.5.1 SB2OME

The NMR data reveals that SB2OME exists in a 5:3 ratio of thione to thiol tautomers, which is evident from the significance signals for each form. In the ^1H NMR spectrum (Figure 4.24), the thione tautomer was indicated by singlets at $\delta = 11.50$ ppm and $\delta = 10.50$ ppm, corresponding to the two secondary NH protons of the thione form (Shuhada et al., 2017). The integration value of one proton for each NH group supported the formation of SB2OME through the β -nitrogen of SBDTC, as also indicated by IR data. The thione CH_2 protons resonated as a singlet at 4.45 ppm (Khoo et al., 2014), while the thione CH_3 group on the methoxy ring appeared as a singlet at $\delta = 3.83$ ppm (Emam et al., 2009). Additionally, a multiplet between $\delta = 7.04$ ppm and $\delta = 7.87$, with coupling constants of $J = 7.5$ and 9 Hz, indicated the presence of aromatic protons (Gwaram et al., 2012).

Meanwhile, the thiol tautomer was represented in the ^1H NMR spectrum by a singlet at $\delta = 11.19$ ppm, corresponding to the one NH proton. The CH_2 group in the thiol form resonated slightly upfield at $\delta = 4.55$ ppm compared to the thione form, and the thiol methoxy CH_3 protons resonated at $\delta = 3.95$ ppm. The SH proton of the thiol form was observed at $\delta = 4.50$ ppm (Bera et al., 2008), confirming the coexistence of

both tautomers. The absence of peaks between $\delta = 10\text{--}13$ ppm, corresponding to OH peaks, eliminated the possibility of SB2OME existing in either the thionol or thiolol form. To determine the ratio of thione to thiol tautomers, a comparison of the integration values of the NH and CH₂ groups was performed. The thione NH proton had an integration value of 1.65, while the thiol NH proton had an integration value of 1.00, yielding a 5:3 ratio. Similarly, the integration values for the CH₂ protons of the thione and thiol forms were 3.41 and 2.08, respectively, further supporting this ratio.

In the ¹³C NMR spectrum (Figure 4.25), the thione form was confirmed by the characteristic C=S resonance at $\delta = 189.64$ ppm and the C=O signal at $\delta = 169.21$ ppm (Elzaref et al., 2023). Signals at $\delta = 56.23$ ppm and $\delta = 37.83$ ppm corresponded to the CH₃ and CH₂ groups, respectively (Khirudin et al., 2023). Aromatic carbon signals for SB2OME were observed between $\delta = 112.58$ and $\delta = 157.62$ ppm. Meanwhile, the thiol form was confirmed by the presence of a C=N signal at $\delta = 165.16$ ppm, indicating its coexistence with the thiol form. Overall, SB2OME existed in thione-thiol tautomeric equilibrium, and predominantly exists in the thione form, as evidenced by the higher intensity of the NH and C-H₂ proton signals, with a 5:3 ratio of thione to thiol tautomers.

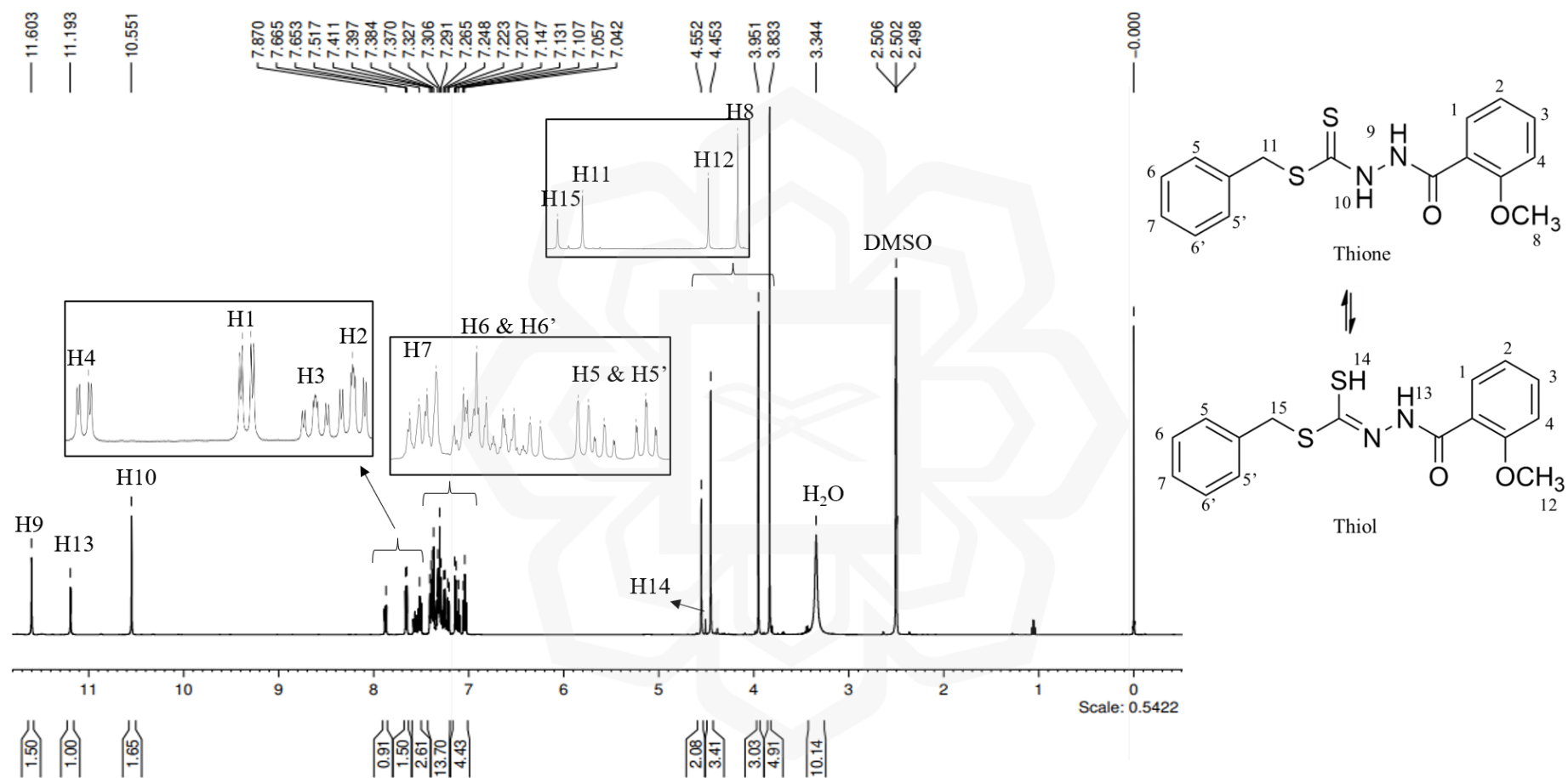


Figure 4.24 ^1H NMR spectra of SB2OME

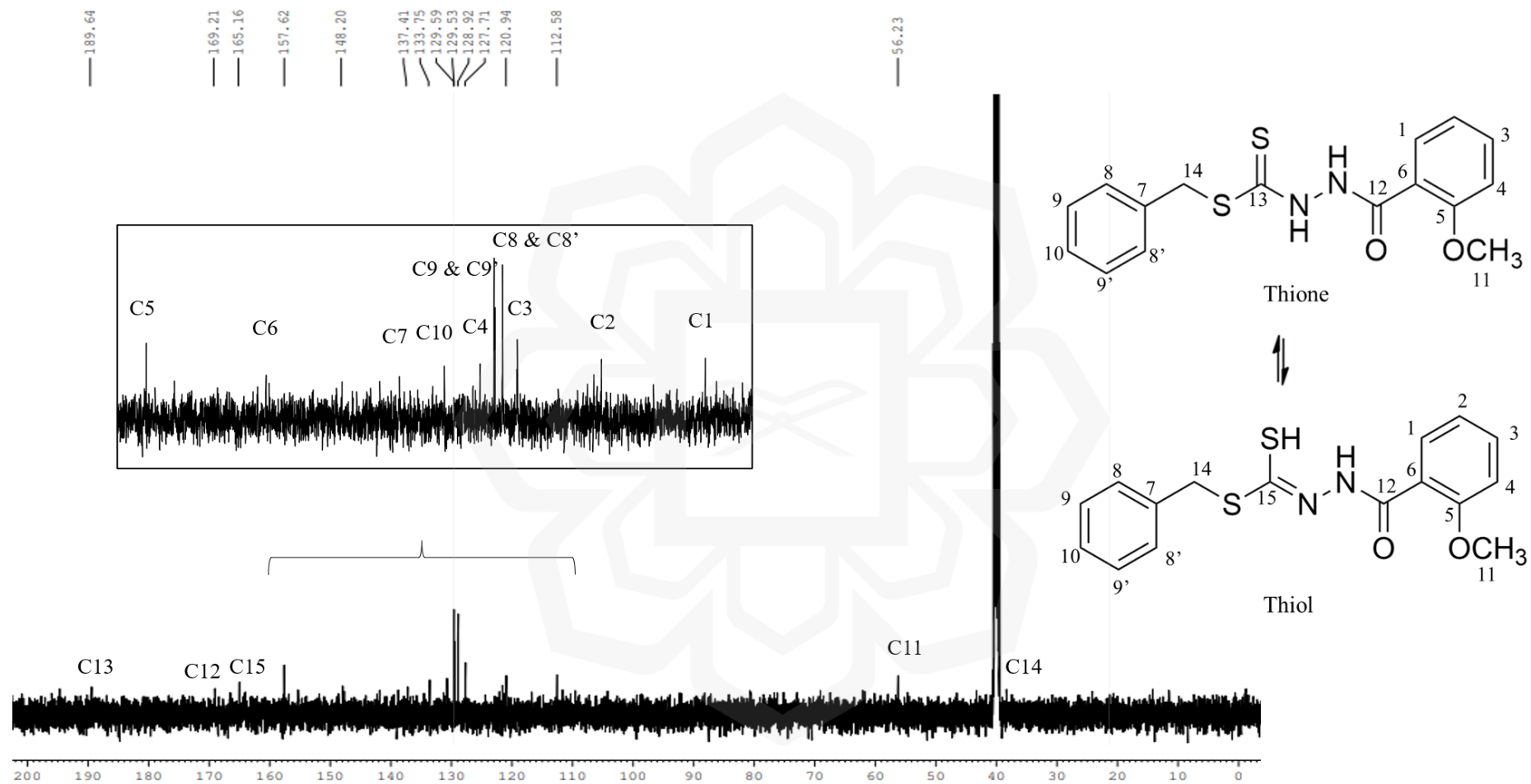


Figure 4.25 ^{13}C NMR spectra of SB2OME

4.5.2 SB3OME

Unlike SB2OME, which exists as a mixture of tautomers, SB3OME was found to be entirely in the thiol form, as confirmed by the NMR data. In the ^1H NMR spectrum (Figure 4.26), only one NH proton was observed, resonating as a singlet at $\delta = 10.53$ ppm, which indicates the absence of the thione form. The integration value for this proton was low (0.41), likely due to proton exchange with water causing peak broadening and reduced integration (Guthrie, 1979). Additionally, the lack of peaks in the $\delta = 10\text{--}13$ ppm range, which would correspond to O-H protons, rules out the presence of either thionol or thiolol forms. The CH_2 group attached to sulfur appeared as a singlet at $\delta = 4.51$ ppm, and the CH_3 group on the methoxy ring was observed at $\delta = 3.81$ ppm. Aromatic protons showed a multiplet between $\delta = 7.16$ ppm and $\delta = 7.56$ with a coupling constant of $J = 7.5$ and 9 Hz, confirming the substitution pattern on the benzene ring (Tarafter et al., 2001).

In the ^{13}C NMR spectrum (Figure 4.27), the absence of a $\text{C}=\text{S}$ signal further supports the non-existence of the thione form. Instead, the $\text{C}=\text{N}$ peak for the thiol form resonated at $\delta = 159.76$ ppm (Kudrat-E-Zahan & Islam, 2015), while the $\text{C}=\text{O}$ signal appeared at $\delta = 166.03$ ppm. Aromatic carbon peaks for SB3OME ranged from $\delta = 114.27$ ppm to 136.77 ppm (Khirudin et al., 2023). These findings confirm that SB3OME exists exclusively in the thiol form in the liquid state.

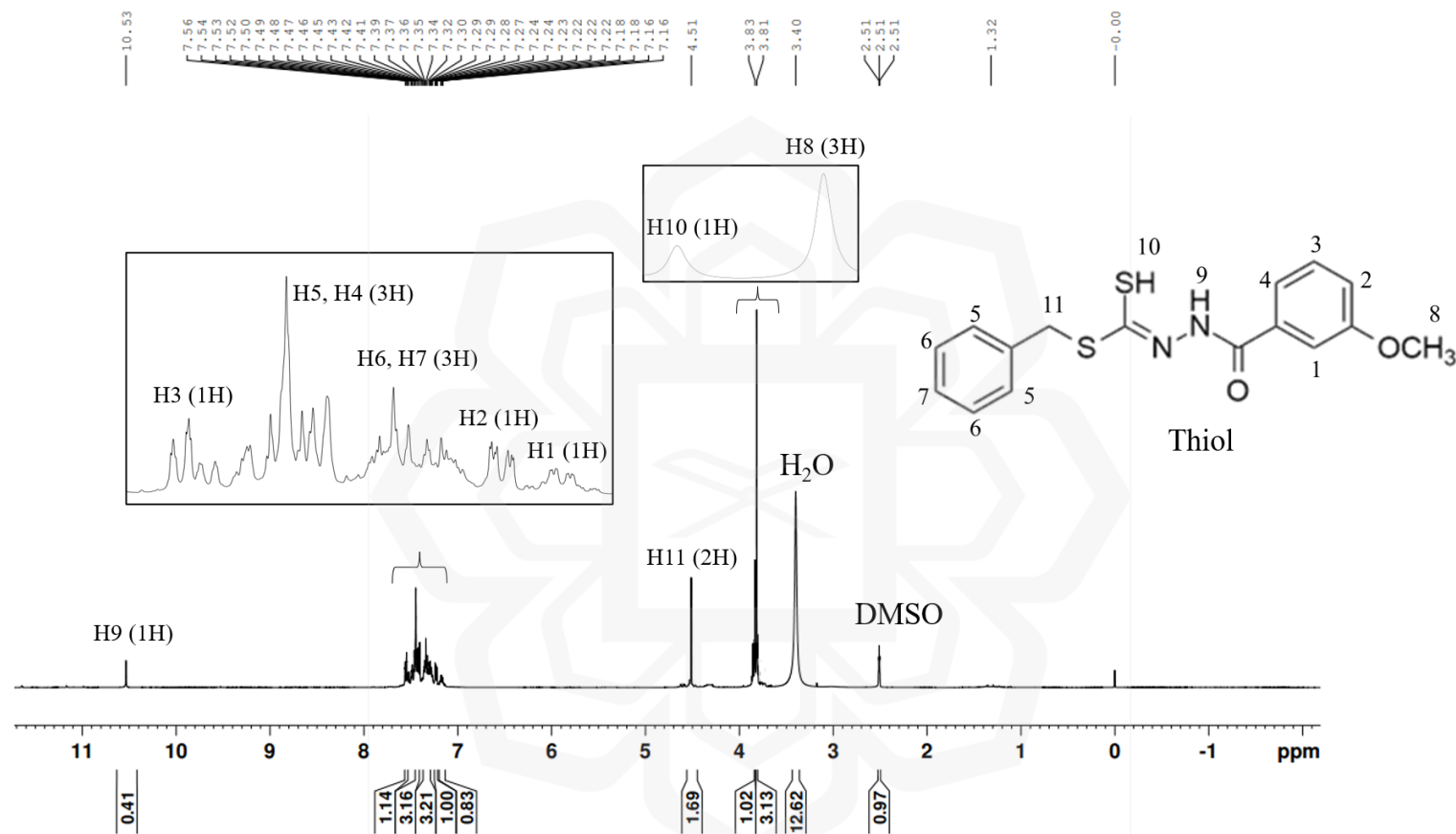


Figure 4.26 ^1H NMR spectra of SB3OME

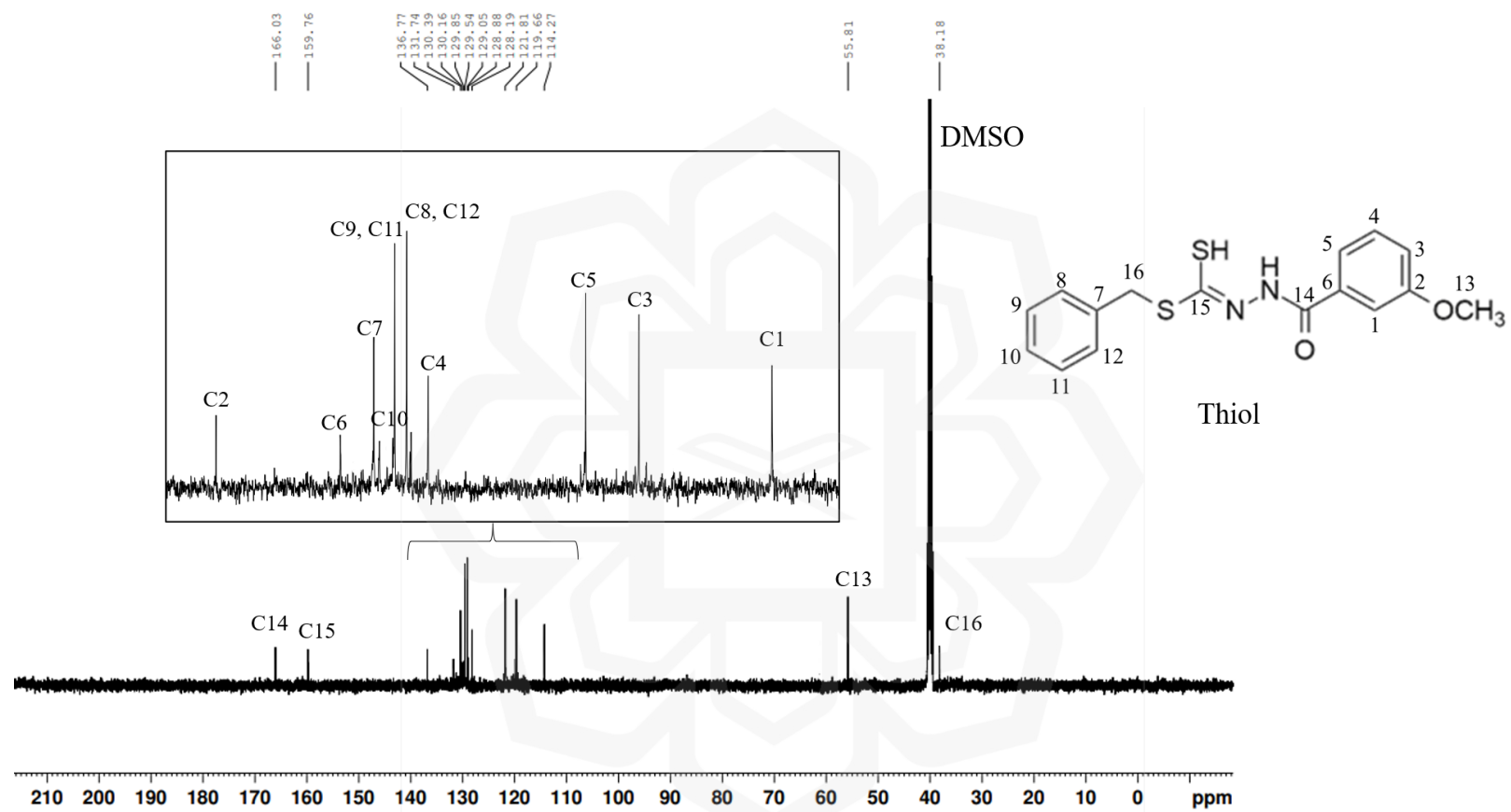


Figure 4.27 ^{13}C NMR spectra of SB3OME

4.5.3 SB4OME

In contrast to SB3OME, which exists in the thiol form, SB4OME was present solely in the thione form in the liquid state, as evidenced by the NMR data. In the ^1H NMR spectrum (Figure 4.28), NH protons resonated as a singlet with an integration of two at $\delta = 9.71$ ppm, indicating the presence of two hydrogens. This observation confirmed that SB4OME was directed toward the α -nitrogen of SBDTC, with the primary amine group still present in the compound. The CH_2 group attached to sulfur appeared as a singlet at $\delta = 4.50$ ppm, and the CH_3 group of the methoxy ring resonates at $\delta = 3.79$ ppm (Günther, 2013). Aromatic protons showed a multiplet between $\delta = 6.82$ ppm and $\delta = 8.13$ with a coupling constant of $J = 7.5$ and 9 Hz, consistent with the substitution pattern on the benzene ring. Notably, no SH or OH signals were detected, which confirms the absence of the thiol, thiolol, and thionol forms.

In the ^{13}C NMR spectrum (Figure 4.29), the $\text{C}=\text{S}$ group was observed at $\delta = 195.80$ ppm, while the $\text{C}=\text{O}$ group appeared at $\delta = 161.69$ ppm (Ali et al., 2004), supporting the presence of the thione structure. The CH_3 and CH_2 groups resonated at $\delta = 56.05$ ppm and $\delta = 37.86$ ppm, respectively. The absence of a $\text{C}=\text{N}$ signal further supported the non-existence of the thiol and thiolol forms. Aromatic carbon signals ranged from 110.65 ppm to $\delta = 150.26$, indicating the electronic environment of the aromatic ring. These findings confirmed that SB4OME exists exclusively in the thione form in the liquid state.

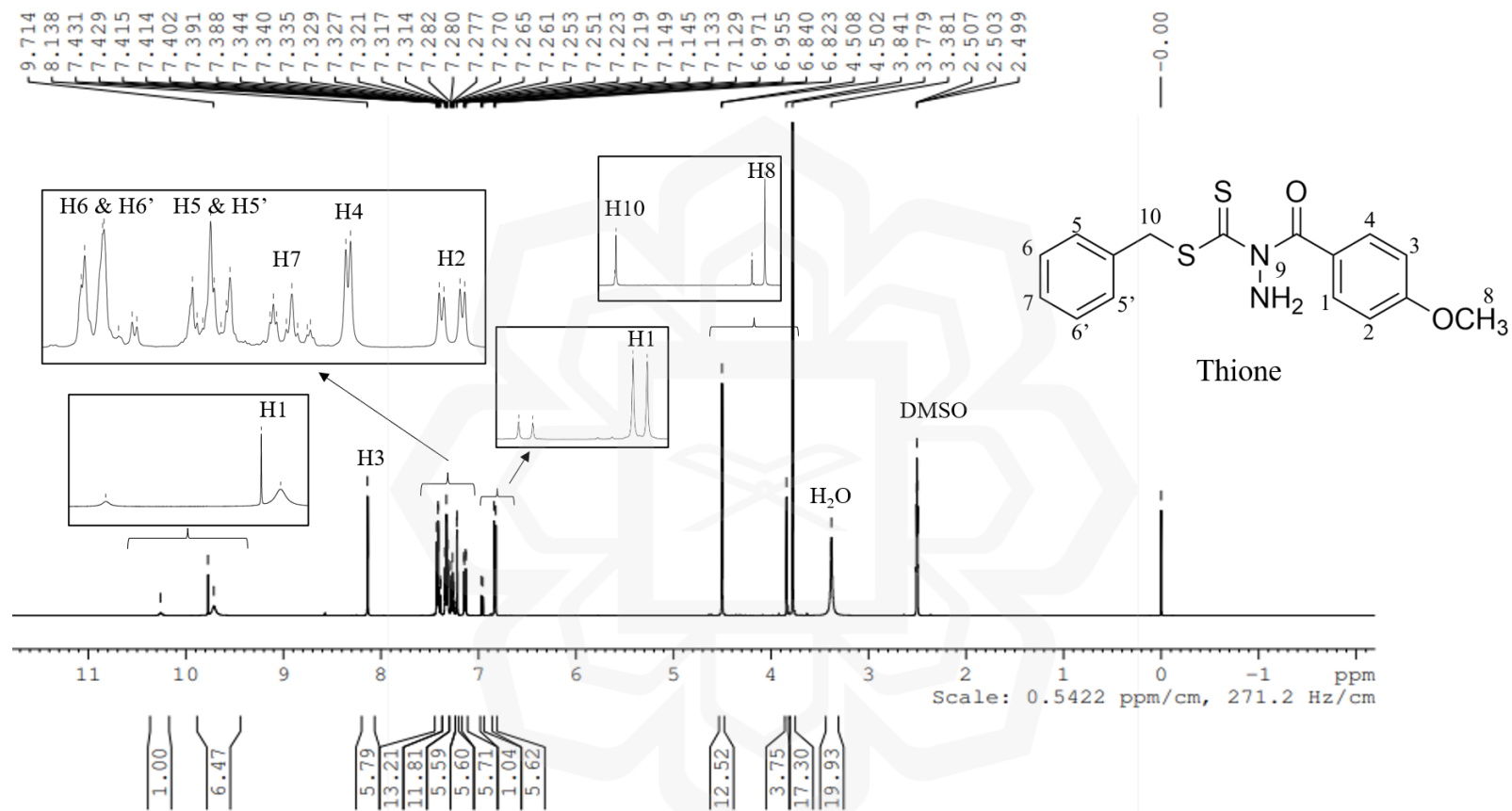


Figure 4.28 ^1H NMR spectra of SB4OME

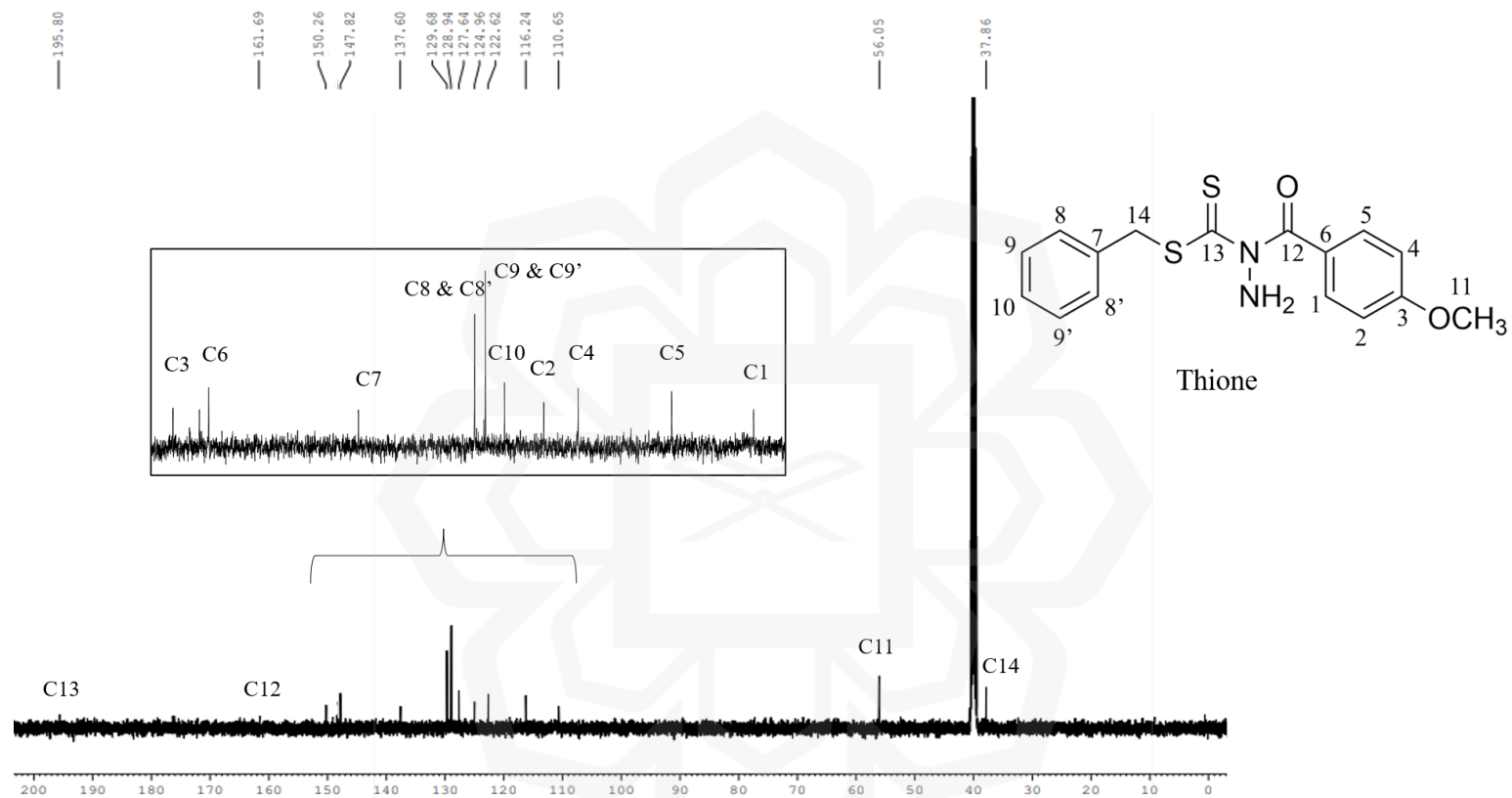


Figure 4.29 ^{13}C NMR spectra of SB4OME.

4.5.4 SB3NO

Similar to SB2OME, SB3NO exists as a mixture of thione and thiol tautomers, but in a different ratio of 2:1, with the thione form being predominant. The ^1H NMR spectrum (Figure 4.30) reveals that the NH proton of the thione form resonated as a doublet at $\delta = 4.37$ and $\delta = 4.40$ ppm (M. A. Ali et al., 2004). The CH_2 protons in the thione tautomer were observed as a singlet at $\delta = 4.59$ ppm. In the aromatic region, a multiplet between $\delta = 7.22$ ppm and $\delta = 9.53$ with a coupling constant of $J = 7.0$ and 8 Hz corresponds to the aromatic protons (Gwaram et al., 2012).

In contrast, the thiol tautomer was indicated by an NH proton resonance as a singlet at $\delta = 4.50$ ppm and an SH peak at $\delta = 3.74$ ppm (How et al., 2008). The absence of any peaks around $\delta = 10\text{--}13$ ppm, which would correspond to O-H, suggests that SB3NO was not in either thionol or thiolo forms. The ratio of the tautomers was determined by comparing the integration values of the N-H peaks. The thione N-H showed an integration value of 2.02 while the thiol N-H showed 1.11. This indicates that SB3NO existed as a mixture of thione and thiol forms in a 2:1 ratio, with the thione form being more prevalent.

In the ^{13}C NMR spectrum (Figure 4.31), the $\text{C}=\text{S}$ signal appeared at $\delta = 200.17$ ppm and the $\text{C}=\text{O}$ group was observed at $\delta = 165.81$ ppm (Khirudin et al., 2023), which identifies the presence of the thione tautomer. The thiol tautomer was supported by the presence of a $\text{C}=\text{N}$ signal at $\delta = 154.66$ ppm. The CH_2 group showed signals at $\delta = 36.99$ and $\delta = 37.53$ ppm, corresponding to the thione and thiol tautomers, respectively. Additionally, the chemical shifts in the range of $\delta = 126.37\text{--}147.67$ ppm were attributed to the carbons of the aromatic rings. Thus, SB3NO existed as a mixture of thione and thiol tautomers in a 2:1 ratio, with the thione form being more dominant in the liquid state.

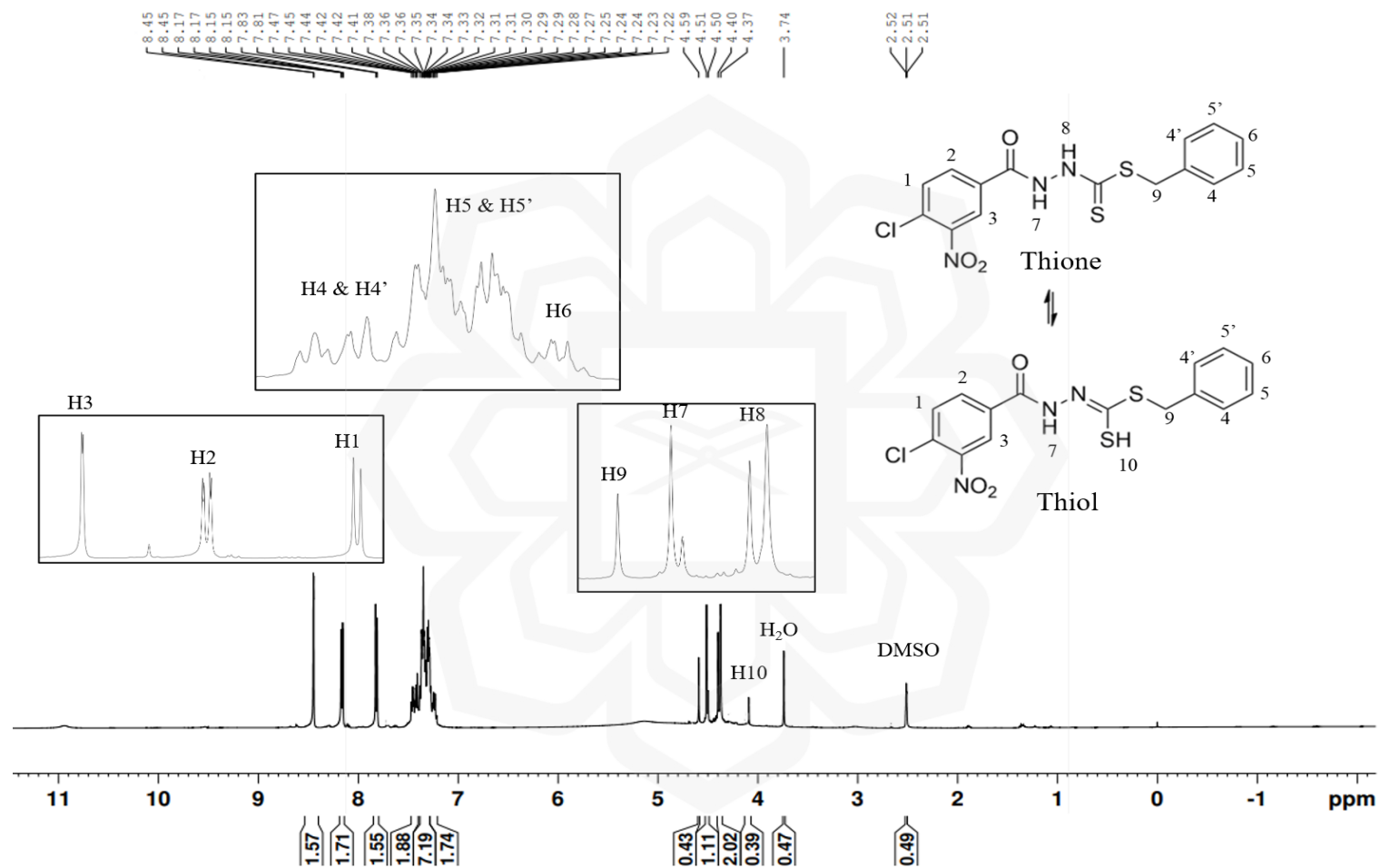


Figure 4.30 ^1H NMR spectra of SB3NO

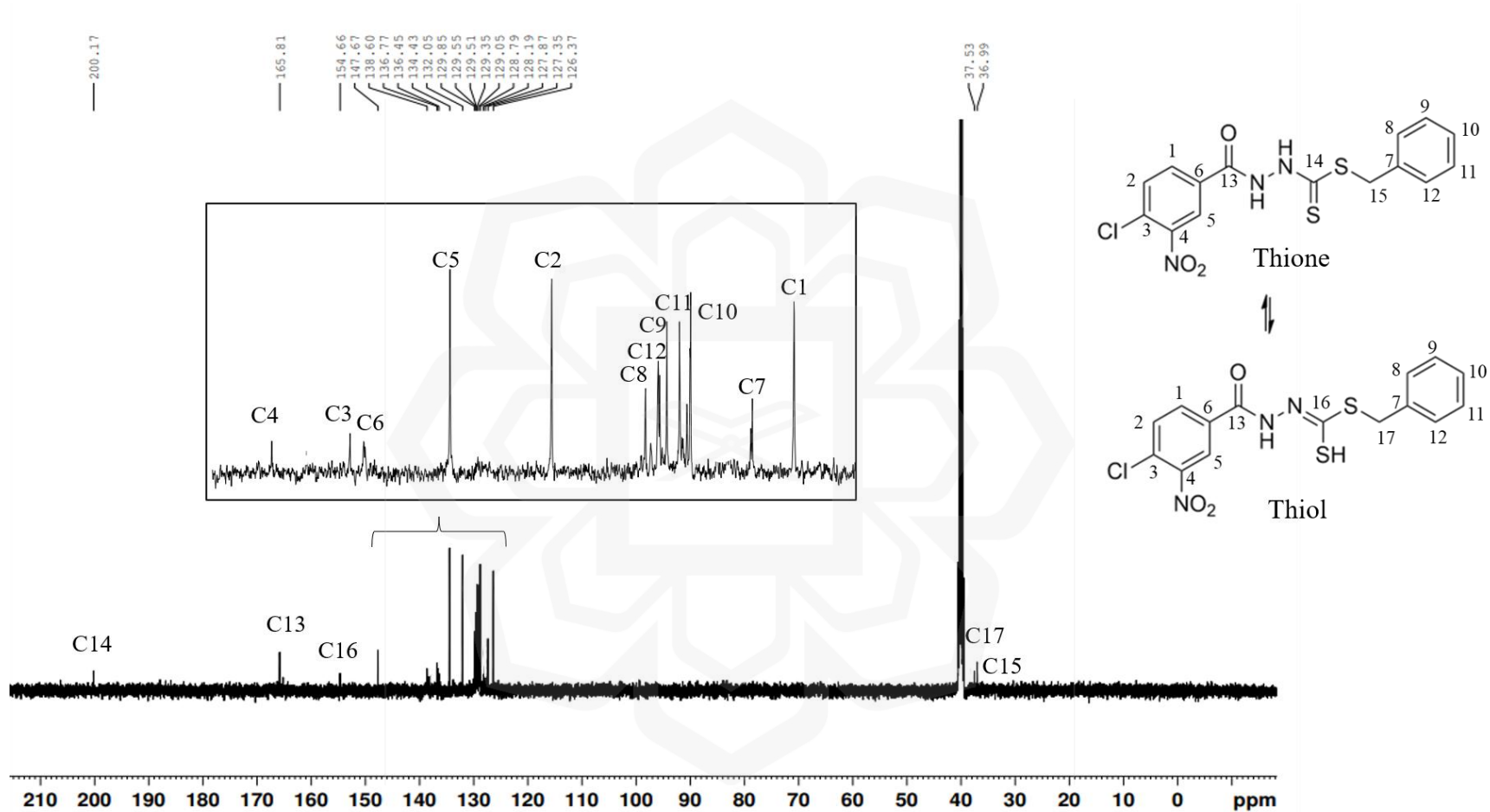


Figure 4.31 ¹³C NMR spectra of SB3NO

4.5.5 Tautomerism In Liquid Form

The tautomerism of SB2OME, SB3OME, SB4OME, and SB3NO revealed different behaviours, characterised by their NH, C=S, and C=N signals in the NMR spectra (Günther, 2013). For SB2OME, NMR data indicated a mixture of thione and thiol tautomers in a 5:3 ratio. This dual existence suggested that SB2OME could stabilise both tautomeric forms in a liquid state. This finding differed from the IR analysis, which showed that in the solid and liquid state (during complexation), SB2OME existed only in the thione form. This suggested that the shift to a thione-thiol tautomeric mixture in solution during NMR analysis might be influenced by the solvent. As reported by Evangelio et al. (2009) and Zhao et al. (2022), solvent effects are one of the key factors driving tautomerism in NMR analysis (Evangelio et al., 2009; Zhao et al., 2022).

As described by Forlani et al. (2001), in DMSO- d_6 , the solvent acted as a weak base and interacted with acidic protons (Forlani et al., 2001), such as NH in the thione form. This weak interaction reduced the NH proton's ability to donate, shifting the equilibrium toward the thiol form, where the proton was localised on sulfur. Thus, this phenomenon explained the observation of SB2OME as a thione-thiol mixture in DMSO- d_6 . On the other hand, SB3OME was exclusively in the thiol form, as demonstrated by its NMR spectrum. This result also contradicted the IR findings, validating that SB3OME likely experienced a similar solvent-induced tautomeric shift as SB2OME. In deuterated DMSO, SB3OME is completely converted to the thiol form, likely to achieve greater stability.

Notably, SB4OME was confirmed to be in the thione form based on NMR results. This finding aligned perfectly with the IR data, where it remained in the thione form in both solid and liquid states. This consistency supported the conclusion that SB4OME could not undergo tautomerism due to the absence of an α -hydrogen adjacent to the thione and carbonyl groups. Conversely, in NMR analysis, SB3NO exhibited both thione and thiol forms in a 2:1 ratio, favouring the thione form. This showed that SB3NO could stabilise both forms in solution, with the thione form being more prevalent. Interestingly, this finding supported the IR data, which indicated that SB3NO shifted to the thiol form during complexation. However, in the IR analysis, enolisation had to be induced by potassium hydroxide (KOH), whereas in NMR, the solvent most likely facilitated the transformation.

Overall, aside from solvent effects, the tautomeric states among these SBDTC derivatives were primarily influenced by the SBDTC scaffold. As observed by a past study, SBDTC existed as a mixture of thione-thiol forms in the liquid state, as indicated by NMR interpretation (Bera et al., 2008). This study was further supported by How et al. (2008), which stated that SBDTC readily underwent tautomerism in liquid form, with their compounds existing predominantly in the thiol form, as revealed by NMR. Thus, NMR spectroscopy proved to be a powerful tool not only for confirming the structures of compounds but also for determining their tautomeric forms. This capability was essential for understanding the chemical behaviour and reactivity of these compounds, as discussed in Subsection 4.12.

4.6 MASS SPECTROMETRY (MS)

4.6.1 SBDTC

As illustrated in Figure 4.32, the molecular ion peak in the SBDTC spectrum was observed at m/z 199, close to its molecular weight of 198 g/mol. However, it was detected at a very low intensity of 2%. This indicates that the parent molecule was not particularly stable and readily fragmented into smaller ions (Hübschmann, 2015). This instability may be attributed to weak bonds within the molecule, making it prone to fragmentation after ionization.

The fragmentation process of SBDTC led to several notable peaks, including m/z 124, m/z 91, m/z 77, m/z 65, and m/z 51 (Figure 4.32), all following a single fragmentation pathway as illustrated in Figure 4.33. The peak at m/z 124 corresponded to the loss of a $\text{CH}_3\text{N}_2\text{S}$ ion (75 g/mol) from the parent molecule, leading to the formation of the fragment $\text{C}_6\text{H}_5\text{-S}$ at m/z 124 (47%). This fragmentation likely occurred through α -cleavage of the S-C bond, a common fragmentation pattern involving the cleavage of a bond adjacent to heteroatoms like sulfur and nitrogen (Shrader, 2013). The base peak, observed at m/z 91 (100%), represents the tropylium ion (C_7H_7^+), a highly stable aromatic ion formed by the rearrangement of the benzyl carbocation. This occurred through the loss of sulfur (32 g/mol), allowing the aromatic ring to stabilise.

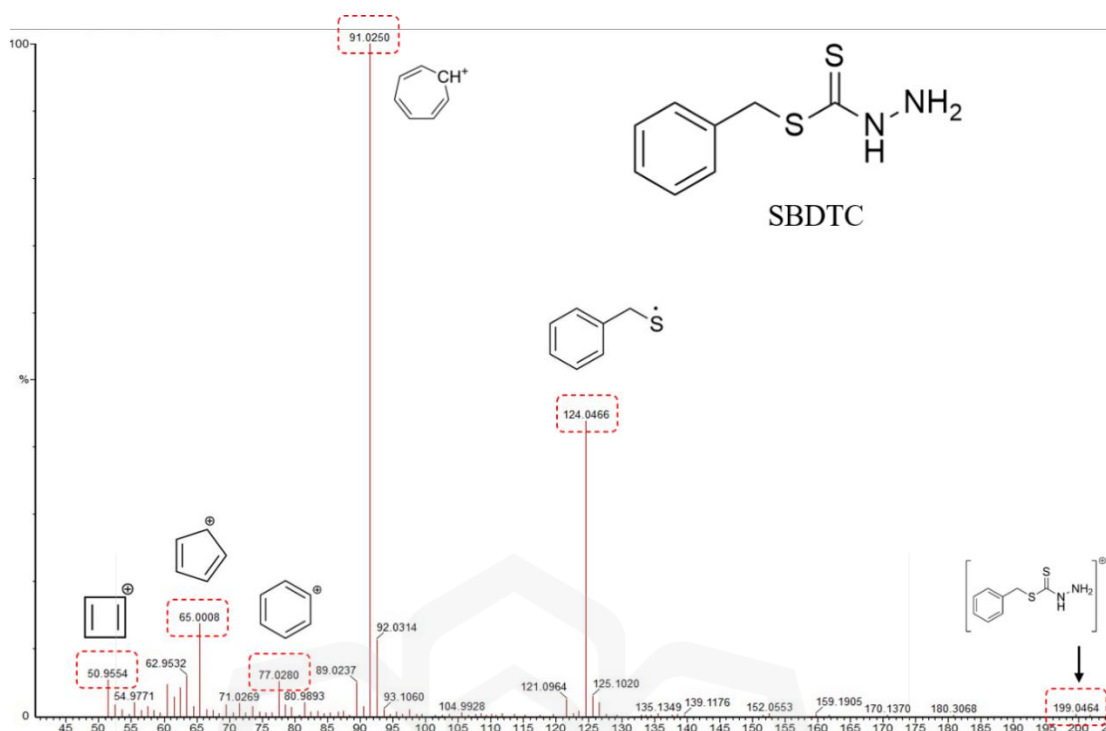


Figure 4.32 Mass spectrum of SBDTC

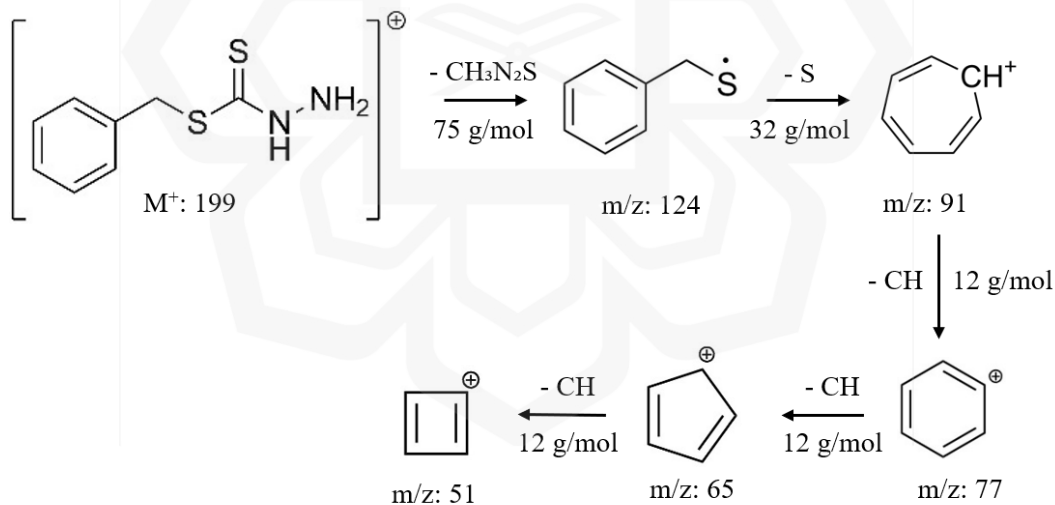


Figure 4.33 Fragmentation pathway of SBDTC

The peak at m/z 77 (5%) corresponded to the phenyl cation ($C_6H_5^+$), formed by the loss of a carbon atom, leaving behind the phenyl ring. The peak at m/z 65 (13%) likely resulted from the loss of a CH group from the phenyl ring, producing the fragment $C_5H_5^+$. Finally, the peak at m/z 51 (8%) represented further fragmentation of the aromatic ring, resulting in $C_4H_3^+$ (Steckel et al., 2019). Overall, these fragmentation

(56 g/mol) from m/z 148. The tropylium ion is highly stable and forms via rearrangement of the benzyl cation, where a hydrogen atom shifts to stabilise the structure into a cyclic form (Smith, 2004). This ion reflects the aromatic nature of the SB2OME structure.

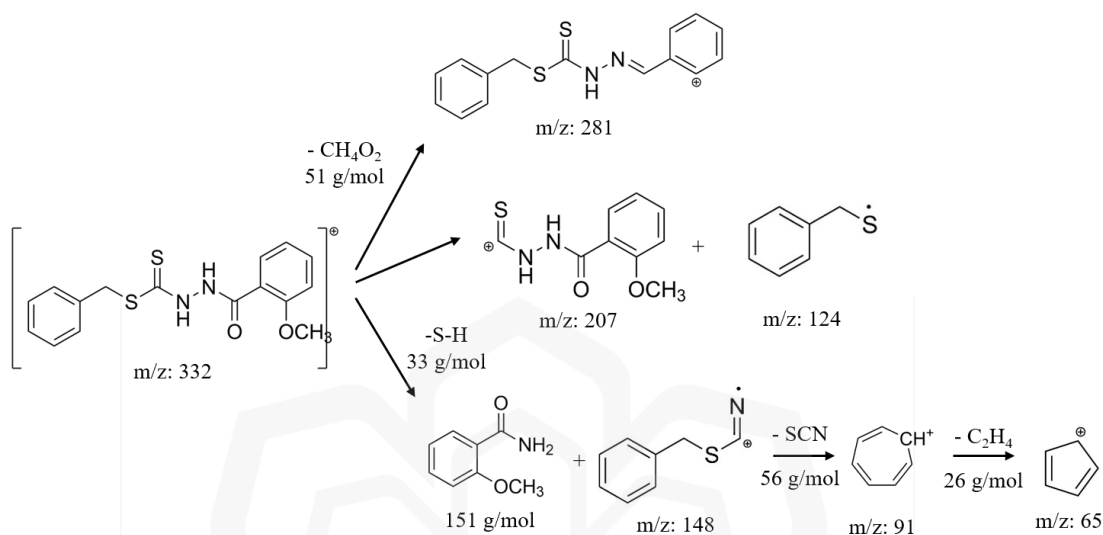


Figure 4.35 Fragmentation pathway of SB2OME

Finally, the peak at m/z 65 (30%) resulted from the loss of C_2H_4 from the tropylium ion, yielding the C_5H_5^+ fragment. Overall, the mass spectrum of SB2OME, with a parent ion at m/z 330 confirmed the formation of SB2OME, which was further supported by other spectroscopic data.

4.6.3 SB3OME

Similar to SB2OME, the parent peak of SB3OME appeared at m/z 330 (30%) (Figure 4.36), slightly lower than the molecular weight of 332 g/mol. These two g/mol differences were attributed to the loss of two hydrogen atoms during the ionisation process (Cohen et al., 2007).

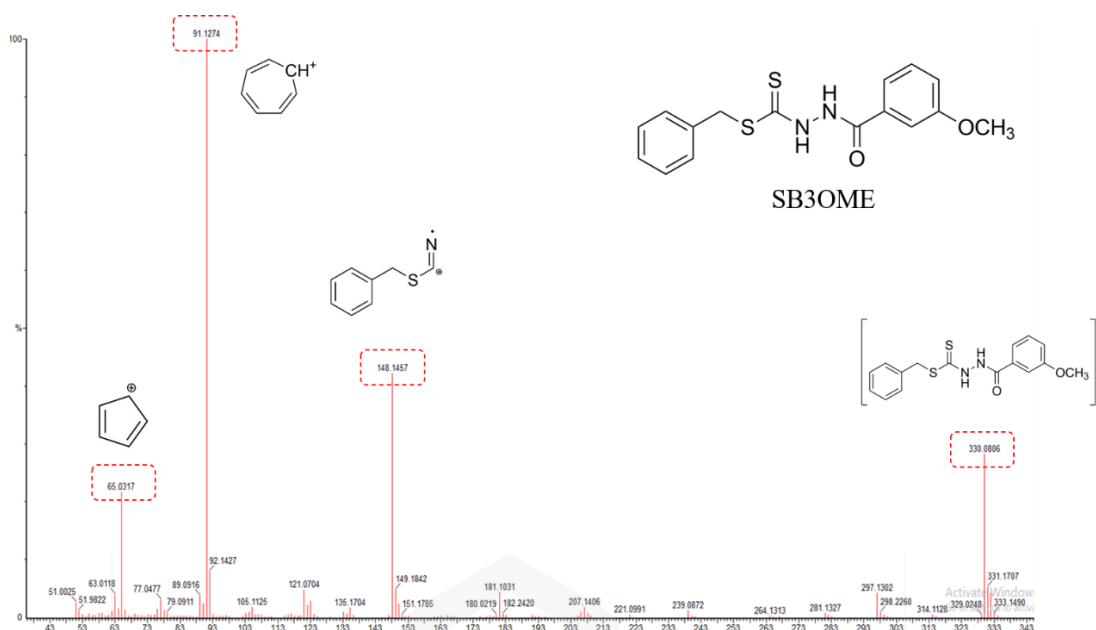


Figure 4.36 Mass spectrum of SB3OME

Several significant fragmentation peaks were observed, notably at m/z 148, m/z 91, and m/z 65, with one primary fragmentation pathway as illustrated in Figure 4.37. The peak at m/z 148 (47%) corresponded to the loss of $C_8H_8NO_2$ (151 g/mol) and S-H (33 g/mol), resulting in the fragment ion C_8H_7NS . This fragmentation likely involved α -cleavage at the S–C bond, followed by the detachment of the NH_2 group, which stabilised the fragment at m/z 148 (Smith, 2004). From this fragment, the peak at m/z 91 (100%) emerged, representing the tropylium ion ($C_7H_7^+$), a highly stable ion commonly seen in aromatic compounds. This ion formed through the rearrangement of a benzyl cation after the loss of a CNS ion (56 g/mol), confirming the presence of a benzyl group in SB3OME.

Finally, the peak at m/z 65 (23%) resulted from the further fragmentation of the aromatic ring. The tropylium ion ($C_7H_7^+$) lost a C_2H_2 group (26 g/mol), leading to the formation of the $C_5H_5^+$ fragment. In conclusion, the mass spectrum of SB3OME, with a parent ion at m/z 330, confirmed the formation of SB3OME, which was further supported by other spectroscopic data.

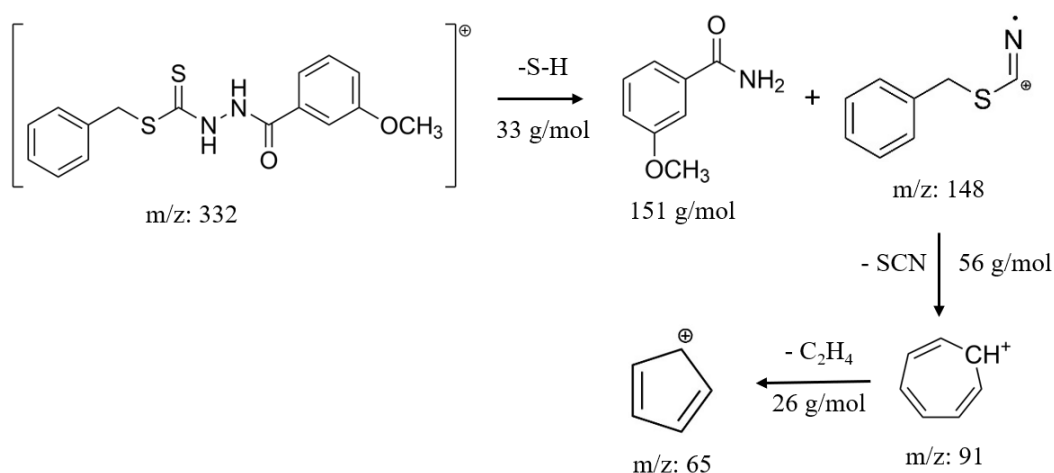


Figure 4.37 Fragmentation pathway of SB3OME

4.6.4 SB4OME

In the mass spectrum of SB4OME, the parent ion was also observed at m/z 330 (Figure 4.38), despite the compound having a molecular weight of 332 g/mol. This two atomic masses units' discrepancy was likely due to the loss of two hydrogen atoms, which commonly occurs during ionisation in mass spectrometry (Cohen et al., 2007).

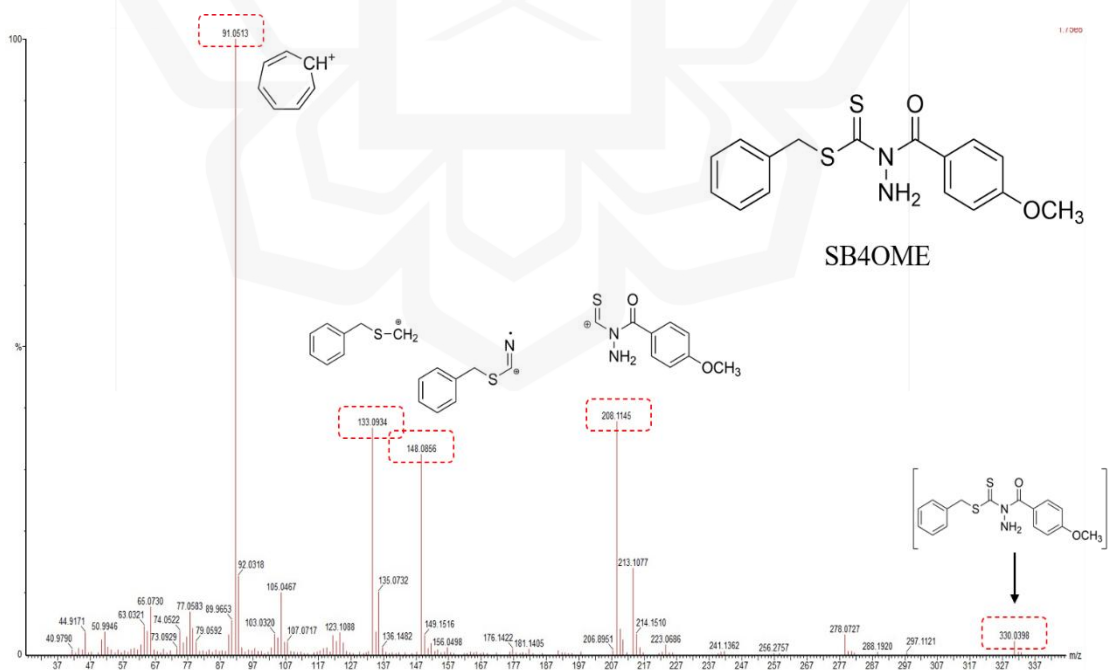


Figure 4.38 Mass spectrum of SB4OME

Several key peaks were observed in the spectrum, notably at m/z 208, 148, 133, and 91, indicating two primary fragmentation routes as shown in Figure 4.39. As

depicted in Figure 4.39, the first fragmentation pathway led to a peak at m/z 208, likely resulting from the loss of a benzylthiol group (C_7H_7S , 124 g/mol) from the parent ion. A second fragmentation route resulted in the formation of an ion at m/z 148 (33%), corresponding to the loss of $C_8H_6O_2$ (182 g/mol), $S-H$ (33 g/mol), and $N-H_2$ (16 g/mol), yielding the C_8H_7NS (148 g/mol) fragment. Following this, the peak at m/z 133 (38%) formed through α -cleavage between the $C-N$ bond, resulting in the loss of a nitrogen atom (14 g/mol) and producing a stable ion at m/z 133.

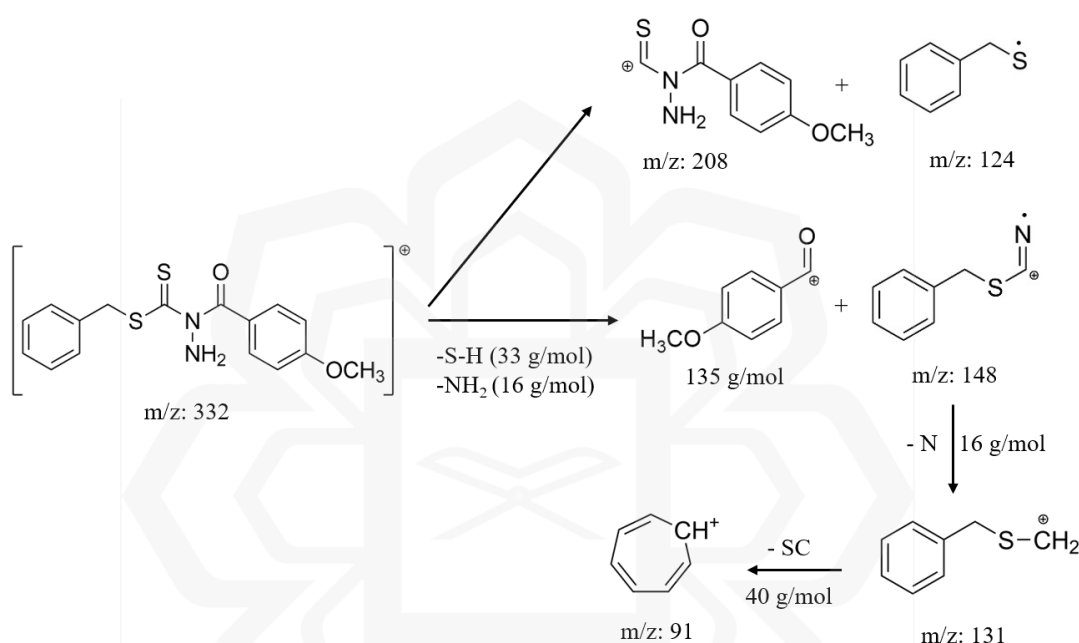


Figure 4.39 Fragmentation pathway of SB4OME

Finally, the ion at m/z 91 (100%) represented the formation of a tropylium ion ($C_7H_7^+$), a highly stable aromatic ion. This peak resulted from the rearrangement of a benzyl group after the loss of a sulfur atom (32 g/mol), confirming the presence of an aromatic benzyl group in the SB4OME structure. Thus, the mass spectrum of SB4OME, with a parent ion at m/z 330, confirmed the formation of SB4OME, which was further supported by other spectroscopic data.

4.6.5 SB3NO

In the mass spectrum of SB3NO (Figure 4.40), the parent ion was observed at m/z 382, which closely corresponds to the molecular weight of the compound (381 g/mol). Several significant peaks were detected in the spectrum, notably at m/z 207, 171, 121,

91, and 54, suggesting one primary fragmentation routes, as illustrated in Figure 4.41. The fragmentation route began with the peak at m/z 207 (18%), which resulted from the loss of the dithiocarbazate moiety ($C_8H_8S_2$, 171 g/mol), observed at m/z 171 (13%). This cleavage formed the $C_7H_5ClN_2O_3$ ion via homolytic cleavage at the N-N bond (Chemistry, 2005). From the m/z 171 peak, the loss of the SCN group (56 g/mol) resulted in a benzylthio group (m/z 121), observed at m/z 121 (50%).

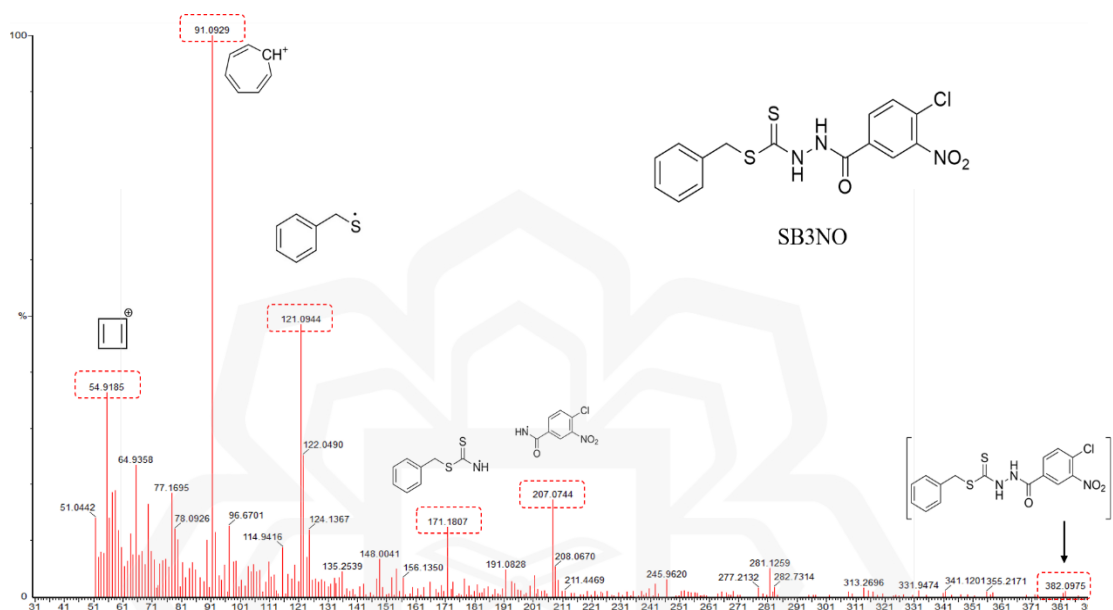


Figure 4.40 Mass spectrum of SB3NO

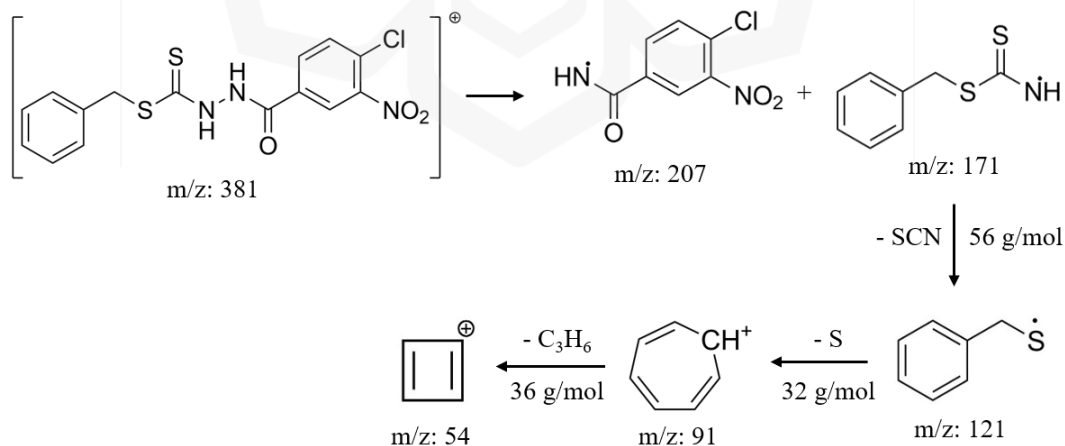


Figure 4.41 Fragmentation pathway of SB3NO

Following this pathway, the peak at m/z 91 (100%) formed after the loss of sulphur (32 g/mol). The peak at m/z 91 represented the tropylium ion ($C_7H_7^+$), observed after the m/z 121 fragment underwent cleavage of the C–S bond. Lastly, the final fragment at m/z 54 represented a further breakdown of the tropylium ion ($C_7H_7^+$), resulting in the $C_4H_3^+$ ion through sequential cleavage of carbon atoms (Cohen et al., 2007). In conclusion, the mass spectrum of SB3NO, with a parent ion at m/z 382, confirmed the formation of SB3NO, which was further supported by other spectroscopic data.

4.7 ULTRAVIOLET-VISIBLE (UV-VIS) SPECTROSCOPY

UV-Vis spectroscopy was employed by analysing their electronic transitions within the 200–800 nm range. This technique helps determine key properties such as $\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$, ligand-to-metal charge transfer (LMCT) and $d \rightarrow d$ transitions, providing critical insights into electronic and structural properties of compounds, especially metal complexes.

4.7.1 SB2OME and Its Metal Complexes

In the electronic spectra of SB2OME (Figure 4.42), a strong absorbance band appeared at 259 nm, corresponding to the $\pi \rightarrow \pi^*$ transitions of the benzene rings. This transition occurred due to electron movement within the aromatic system. Upon complexation with metal ions, only minimal shifts were observed, with absorbance bands recorded between 259 and 268 nm (Table 4.8). Specifically, the $\pi \rightarrow \pi^*$ transition was detected at 266 nm for $Cu(SB2OME)_2$, 259 nm for $Zn(SB2OME)_2$, 268 nm for $Co(SB2OME)_2$, and 265 nm for $Ni(SB2OME)_2$. The minimal shift indicated that the benzene ring remained in the conjugated system even after complexation (Liu et al., 2013).

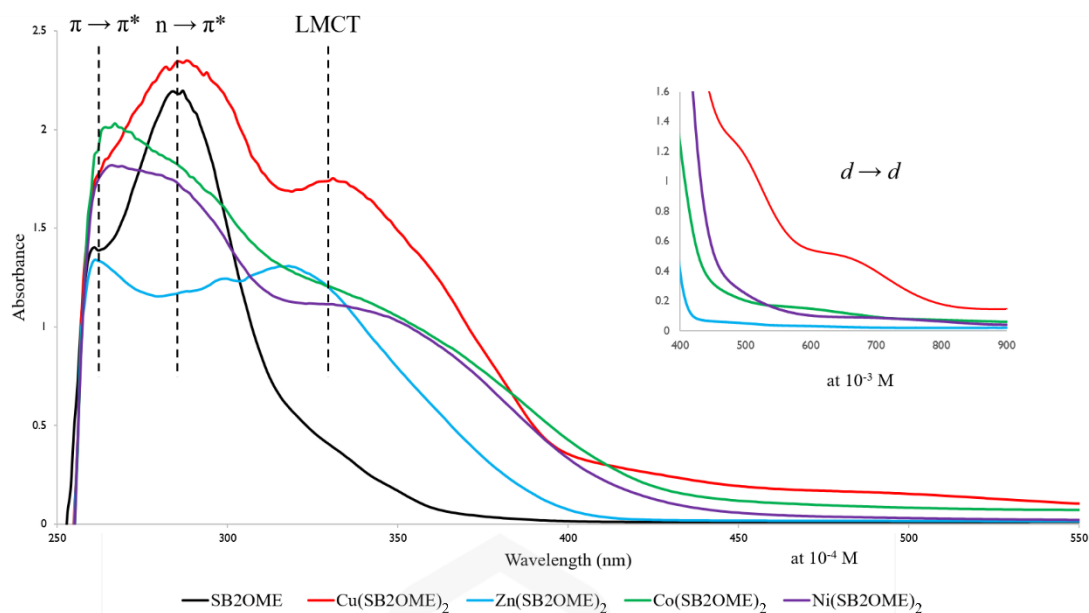


Figure 4.42 Electronic spectra of SB2OME and its metal complexes

Table 4.8 Electronic spectra of SB2OME and its metal complexes

Compounds	The wavelength for each electronic transition λ_{max} , nm (log ϵ , $cm^{-1} mol^{-1} L$)			
	$\pi \rightarrow \pi^*$	$n \rightarrow \pi^*$	LMCT	$d \rightarrow d$
SB2OME	259 (4.145)	286 (4.338)	-	-
Cu(SB2OME) ₂	266 (4.260)	291 (4.366)	334 (4.24)	496 (3.072), 672 (2.662)
Zn(SB2OME) ₂	259 (4.125)	300 (4.094)	322 (4.113)	-
Co(SB2OME) ₂	268 (4.303)	292 (4.235)	354 (4.04)	604 (2.17)
Ni(SB2OME) ₂	265 (4.254)	288 (4.239)	351 (4.012)	718 (1.934)

Additionally, the $n \rightarrow \pi^*$ transition in SB2OME, occurring at 286 nm, was attributed to the excitation of non-bonding electrons, mainly from sulfur or oxygen to anti-bonding π orbitals (Newberry & Raines, 2017). In the metal complexes, this transition shifted between 288 and 300 nm, indicating a bathochromic shift (Tarafter et al., 2002). Specifically, the $n \rightarrow \pi^*$ transition was recorded at 291 nm for Cu(SB2OME)₂, 300 nm for Zn(SB2OME)₂, 292 nm for Co(SB2OME)₂, and 288 nm for Ni(SB2OME)₂. These shifts suggested that the lone pairs on the nitrogen atoms in SB2OME interacted differently with each metal ion, thereby altering the transition

energy. Moreover, the metal ion choice significantly influenced the position of the absorption band, as explained by molecular orbital theory (Z. Chen et al., 2007; Kasumov & Köksal, 2003).

Another absorbance band, appearing between 322 and 354 nm, was attributed to LMCT transitions. These transitions were present in the metal complexes but absent in SB2OME (Gwaram et al., 2012). The presence of LMCT bands confirmed strong interactions between SB2OME and the metal ions, with Cu(SB2OME)₂ exhibiting the lowest energy transition at 334 nm and Co(SB2OME)₂ showing the highest at 354 nm. Additionally, only the Zn(II) complex lacked low-intensity bands in the 500–800 nm range (Figure 4.42), which corresponded to forbidden $d \rightarrow d$ transitions. These transitions, occurring at higher wavelengths, indicated lower energy transitions within the d -orbitals of the metal centers (Gwaram et al., 2012). The number and position of these bands provided important information about the geometry and ligand field strength around the metal ions.

For instance, Cu(SB2OME)₂ exhibited two low-energy $d \rightarrow d$ transitions at 496 and 672 nm (Table 4.8), corresponding to the ${}^2E_g \rightarrow {}^2T_{2g}$ transition, suggesting a distorted octahedral geometry. This observation was further supported by the Jahn-Teller effect which is a geometric distortion in octahedral systems that reduces symmetry and energy (Wilkinson, 1956). The Jahn-Teller effect is commonly observed in octahedral Cu(II) complexes, resulting in elongation or compression of axial bonds relative to equatorial bonds. Meanwhile, Co(SB2OME)₂ and Ni(SB2OME)₂ exhibited broad bands at 604 and 718 nm, corresponding to the ${}^4T_{1g}(F) \rightarrow {}^4T_{2g}(F)$ and ${}^3A_{2g}(F) \rightarrow {}^3T_{2g}(F)$ transitions, respectively. These transitions further confirm an octahedral geometry for these metal complexes.

Overall, these transitions validate the electronic behaviour of SB2OME and its metal complexes. Moreover, these transitions confirm the successful formation of metal complexes from SB2OME, further supported by other spectroscopic data.

4.7.2 SB3OME and Its Metal Complexes

The electronic spectrum data of SB3OME and its metal complexes are presented in Table 4.9. According to Guo et al. (2006), the spectrum of SB3OME showed high-intensity bands at 260 nm (Figure 4.43), which correspond to the $\pi \rightarrow \pi^*$ transitions

associated with the benzene rings. These transitions were intense due to electron movement within the aromatic system. Upon complexation with metal ions, minimal shifts in this transition were observed. These absorbance bands appear between 258 to 269 nm. Specifically, the $\pi \rightarrow \pi^*$ transition was recorded at 269 nm for Cu(SB3OME)₂, 261 nm for Zn(SB3OME)₂, 258 nm for Co(SB3OME)₂, and 264 nm for Ni(SB3OME)₂. Additionally, the $n \rightarrow \pi^*$ transition in SB3OME was observed at 293 nm. This band was attributed to the excitation of non-bonding electrons from sulfur or oxygen into anti-bonding π orbitals. According to the data in Table 4.9, only Ni(SB3OME)₂ exhibited a hypsochromic shift while the others underwent a bathochromic shift. Ni(SB3OME)₂ showed $n \rightarrow \pi^*$ transition to shorter wavelength 289 nm while Cu(SB3OME)₂, Zn(SB3OME)₂ and Co(SB3OME)₂ to 297, 330 and 318 nm respectively.

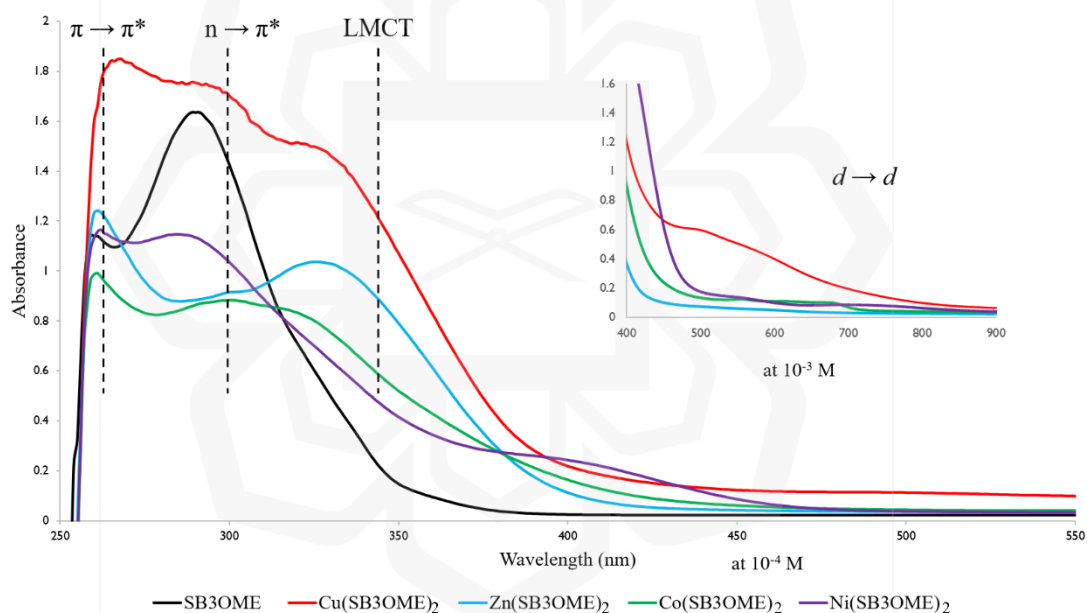


Figure 4.43 Electronic spectra of SB3OME and its metal complexes

Table 4.9 Electronic spectra of SB3OME and its metal complexes

Compounds	The wavelength for each electronic transition λ_{max} , nm (log ϵ , $cm^{-1} mol^{-1} L$)			
	$\pi \rightarrow \pi^*$	$n \rightarrow \pi^*$	LMCT	$d \rightarrow d$
SB3OME	260 (4.056)	293 (4.214)	-	-
Cu(SB3OME) ₂	269 (4.266)	297 (4.238)	329 (4.17)	506 (2.767)

Zn(SB3OME) ₂	261 (4.091)	330 (3.946)	-	-
Co(SB3OME) ₂	258 (4.014)	318 (3.945)	-	674 (1.944)
Ni(SB3OME) ₂	264 (4.061)	289 (4.059)	407 (3.359)	563 (2.107), 750 (1.903)

Moreover, bands between 329 to 407 nm were identified as LMCT transitions (Figure 4.43) (Gwaram et al., 2012). Interestingly, the LMCT transition was only present in the copper and nickel complexes, with Cu(SB3OME)₂ showing LMCT at 329 nm and Ni(SB3OME)₂ at 407 nm. Additionally, $d \rightarrow d$ transitions were observed in the Cu(II), Co(II) and Ni(II) complexes but were absent in the Zn(II) complex (Figure 4.43), due to its filled d-electron configuration (Bhat, 2014). Cu(SB3OME)₂ showed low-energy $d \rightarrow d$ transitions at 506 nm, assigned to the $^2E_g \rightarrow ^2T_{2g}$ transition, indicating an octahedral geometry (Zahradník, 1965). Co(SB3OME)₂ exhibited a broad band at 674 nm, corresponding to the $^4T_{1g}(F) \rightarrow ^4T_{2g}(F)$ transition, also suggesting octahedral geometry (Mumit et al., 2020). Similarly, Ni(SB3OME)₂ complex showed low-intensity bands at 563 and 750 nm, attributed to the $^3A_{2g} \rightarrow ^3T_{1g}(F)$ and $^3A_{2g} \rightarrow ^3T_{2g}(F)$ transitions, respectively, further supporting an octahedral geometry.

Overall, these transitions provide evidence of the electronic properties of SB3OME and its metal complexes. Furthermore, they confirm the successful formation of metal complexes from SB3OME, as corroborated by other spectroscopic data.

4.7.3 SB4OME and Its Metal Complexes

The electronic spectrum data of SB4OME and its metal complexes were presented in Table 4.10. According to the spectrum in Figure 4.44, the $\pi \rightarrow \pi^*$ transitions were observed in both the SB4OME and its metal complexes, indicating the excitation of an electron from a bonding π orbital to an antibonding π orbital. In SB4OME, this transition occurred at 259 nm, with high molar absorptivity (Table 4.10) (Liu et al., 2013). Upon complexation, the $\pi \rightarrow \pi^*$ transition underwent slight shifts (as illustrated in Table 4.10). In the Cu(SB4OME)₂, the transition shifted to 256 nm, while Zn(SB4OME)₂ showed it at 258 nm, Co(SB4OME)₂ at 260 nm, and Ni(SB4OME)₂ exhibited a transition at 263 nm.

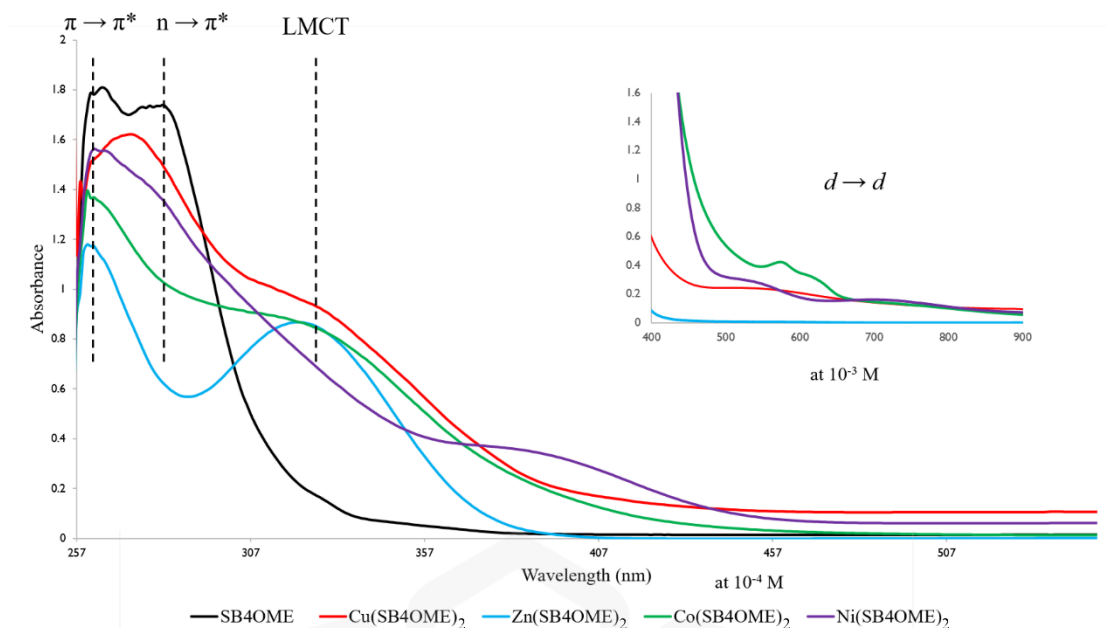


Figure 4.44 Electronic spectra of SB4OME and its metal complexes

Table 4.10 Electronic spectra of SB4OME and its metal complexes

Compounds	The wavelength for each electronic transition λ_{max} , nm (log ϵ , $\text{cm}^{-1} \text{mol}^{-1} \text{L}$)			
	$\pi \rightarrow \pi^*$	$n \rightarrow \pi^*$	LMCT	$d \rightarrow d$
SB4OME	259 (4.250)	281 (4.239)	-	-
Cu(SB4OME) ₂	256 (4.206)	327 (3.965)	-	554 (2.371)
Zn(SB4OME) ₂	258 (4.070)	326 (3.928)	-	-
Co(SB4OME) ₂	260 (4.132)	320 (3.931)	-	624 (2.460)
Ni(SB4OME) ₂	263 (4.192)	273 (4.192)	391 (3.531)	555 (2.426), 720 (2.195)

Next, the $n \rightarrow \pi^*$ transitions involved the excitation of non-bonding electrons from heteroatoms, such as sulfur or oxygen, to an antibonding π orbital (Zahradník, 1965). These transitions were generally less intense than $\pi \rightarrow \pi^*$ transitions. In SB4OME, the $n \rightarrow \pi^*$ transition was observed at 281 nm. Upon complexation, bathochromic and hypochromic shifts were observed (Figure 4.44) (Zahradník, 1965). For Cu(SB4OME)₂, the bathochromic transition shifted significantly to 327 nm. Zn(SB4OME)₂ showed a similar bathochromic shift to 326 nm and Co(SB4OME)₂ had

the transition at 320 nm. However, Ni(SB4OME)₂ displayed a hypochromic shift to 273 nm.

A distinguishing feature of metal complexes was the presence of LMCT transitions (Lever, 1984). In Ni(SB4OME)₂, an LMCT transition was observed at 391 nm. However, no LMCT bands were observed in Cu(SB4OME)₂, Zn(SB4OME)₂, or Co(SB4OME)₂, likely due to overlap with the broad $n \rightarrow \pi^*$ transition. Lastly, $d \rightarrow d$ transitions were observed in all metal complexes' spectra except for the Zn(II) complex. For Cu(SB4OME)₂, $d \rightarrow d$ transitions were observed at 554 nm (Table 4.10), corresponding to the ${}^2E_g \rightarrow {}^2T_{2g}$, suggesting an octahedral geometry. Similarly, Co(SB4OME)₂ exhibited a $d \rightarrow d$ transition at 624 nm, corresponding to ${}^4T_{1g}(F) \rightarrow {}^4T_{2g}(F)$ transitions, characteristic of Co(II) in an octahedral geometry (Vogt et al., 2023). In Ni(SB4OME)₂, two $d \rightarrow d$ transitions were observed at 555 nm and 720 nm, corresponding to ${}^3A_{2g} \rightarrow {}^3T_{2g}(F)$ and ${}^3A_{2g} \rightarrow {}^3T_{1g}(F)$ transitions respectively, which were consistent with the electronic configuration of Ni(II) in an octahedral environment. Zn(SB4OME)₂, with its filled d^{10} configuration, did not exhibit $d \rightarrow d$ transitions, as such transitions were not possible.

Overall, these transitions confirm the electronic properties of SB4OME and its metal complexes. Additionally, they provide evidence for the successful formation of metal complexes from SB4OME, as further supported by other spectroscopic data.

4.7.4 SB3NO and Its Metal Complexes

The electronic spectrum data of SB3NO and its metal complexes were presented in Table 4.11. The $\pi \rightarrow \pi^*$ transition, characteristic of the benzene rings in the SB3NO, was observed at 258 nm. This transition is highly intense due to electron movement within the aromatic system (Vogt et al., 2023) as illustrated in Figure 4.45. Upon complexation with metal ions, minimal shifts were observed, with absorbance bands appearing between 261 and 274 nm. Specifically, the $\pi \rightarrow \pi^*$ transition was recorded at 263 nm for Cu(SB3NO)₂ and Zn(SB3NO)₂, at 261 nm for Co(SB3NO)₂, and at 274 nm for Ni(SB3NO)₂.

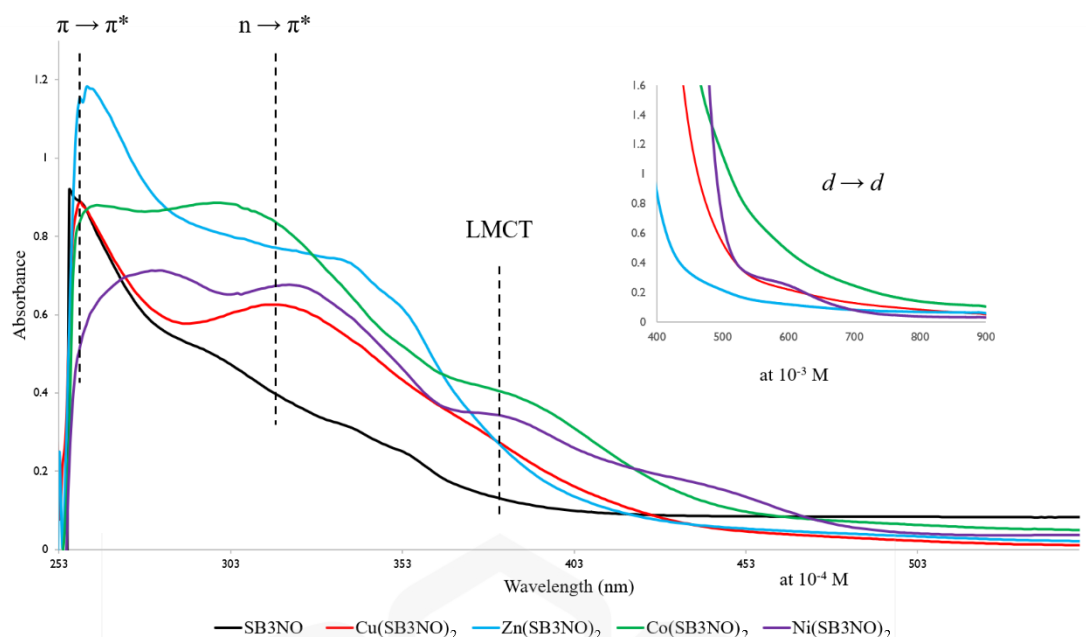


Figure 4.45 Electronic spectra of SB3NO and its metal complexes

Table 4.11 Electronic spectra of SB3NO and its metal complexes

Compounds	The wavelength for each electronic transition λ_{max} , nm (log ϵ , $cm^{-1} mol^{-1} L$)			
	$\pi \rightarrow \pi^*$	$n \rightarrow \pi^*$	LMCT	$d \rightarrow d$
SB3NO	258 (3.963)	302 (3.684)	-	-
Cu(SB3NO) ₂	263 (3.93)	319 (3.795)	-	-
Zn(SB3NO) ₂	263 (4.070)	338 (3.865)	354 (3.800)	-
Co(SB3NO) ₂	261 (3.943)	301 (3.945)	389 (3.588)	-
Ni(SB3NO) ₂	274 (3.851)	326 (3.828)	383 (3.527)	456 (3.136), 606 (2.397)

Meanwhile, the $n \rightarrow \pi^*$ transition in SB3NO was observed at 302 nm. This band was attributed to the excitation of non-bonding electrons, primarily from sulfur or oxygen, to an antibonding π orbital (Zahradník, 1965). After complexation, all metal complexes except Co(SB3NO)₂ exhibited a bathochromic shift in the $n \rightarrow \pi^*$ transition (Table 4.11). Cu(SB3NO)₂ showed a band at 319 nm, Zn(SB3NO)₂ at 338 nm, and Ni(SB3NO)₂ at 326 nm, while Co(SB3NO)₂ remained close to the SB3NO at 301 nm.

The variation in these shifts was largely influenced by the electronic properties of metal ions.

A significant band around 354–389 nm (Figure 4.45) was observed in all the metal complexes except for Cu(SB3NO)₂, which is attributed to LMCT transitions. Zn(SB3NO)₂ showed an LMCT band at 354 nm, Co(SB3NO)₂ at 389 nm, and Ni(SB3NO)₂ at 383 nm. Furthermore, only Ni(SB3NO)₂ uniquely displayed $d \rightarrow d$ transitions in the visible region, with bands at 456 nm and 606 nm (Table 4.11). These bands correspond to the $^3A_{2g} \rightarrow ^3T_{2g}(F)$ transitions, indicative of a tetrahedral geometry for Ni(SB3NO)₂.

In summary, these transitions demonstrate the electronic characteristics of SB3NO and its metal complexes. Furthermore, they serve as evidence of the successful formation of metal complexes from SB3NO, reinforced by other spectroscopic data.

4.8 MOLAR CONDUCTIVITY AND MAGNETIC MOMENT

The molar conductivity data obtained in this study are comprehensively presented in Table 4.12 and Appendix 12. Analyses were performed in DMSO due to its excellent solubility for the metal complexes. The molar conductivities of the metal complexes ranged from 2.45 to 37.8 $\Omega^{-1} \text{ mol}^{-1} \text{ cm}^2$, consistent with their non-electrolytic nature. These values align with previously reported data, which indicate that molar conductivity values for non-electrolytes in DMSO solutions typically remain below 50 $\Omega^{-1} \text{ mol}^{-1} \text{ cm}^2$ (I. Ali et al., 2013). This suggests that SB2OME, SB3OME, SB4OME, and SB3NO ligands are directly coordinated with the metal ions, as the metal complexes do not dissociate in solution (Emam et al., 2009; Wahba et al., 1860).

Table 4.12 Molar conductivity and magnetic moments of metal complexes

Compounds	Molar conductivity (Ω^{-1}	Magnetic moment, μ_{eff}
	$\text{mol}^{-1} \text{ cm}^2$)	(B.M.)
Cu(SB2OME) ₂	4.82	1.960
Zn(SB2OME) ₂	8.71	Diamagnetic
Co(SB2OME) ₂	7.89	4.267

Ni(SB2OME) ₂	5.94	3.288
Cu(SB3OME) ₂	2.81	1.897
Zn(SB3OME) ₂	2.45	Diamagnetic
Co(SB3OME) ₂	8.37	4.944
Ni(SB3OME) ₂	21.5	2.270
Cu(SB4OME) ₂	31.4	1.648
Zn(SB4OME) ₂	33.6	Diamagnetic
Co(SB4OME) ₂	32.4	4.740
Ni(SB4OME) ₂	37.8	2.385
Cu(SB3NO) ₂	15.51	1.536
Zn(SB3NO) ₂	19.83	Diamagnetic
Co(SB3NO) ₂	18.5	4.564
Ni(SB3NO) ₂	12.29	3.874

Furthermore, to obtain further structural information and the magnetic behaviour of the metal complexes, the magnetic moments were measured and the results were tabulated in Table 4.12 and Appendix 13. Paramagnetic compounds typically exhibit positive magnetic moment values, while diamagnetic compounds display zero values. Among all the metal complexes synthesised, only the Zn(II) complex showed zero value for magnetic moment indicating the diamagnetic properties. In fact, the Zn(II) complex has a d¹⁰ electron configuration making it have a diamagnetic system (Ravoof et al., 2010). According to Bhat (2019), the highly symmetric d¹⁰ configuration, in a singlet state, remains unsplit under an applied magnetic field, resulting in a non-degenerate state. Consequently, definitive structural assignments become challenging. Nevertheless, it is anticipated that the six-coordinated Zn(II) complexes of SB2OME, SB3OME, and SB4OME adopt an octahedral structure. Meanwhile, the diamagnetic of Zn(SB3NO)₂, being four-coordinated, typically favours a tetrahedral stereochemistry through sp³ hybridization.

Next, the Cu(II) complex, which has an electronic configuration of d⁹ electrons showed magnetic moment values in a range of 1.6 to 1.960 B.M. From the analysis, the results suggested that they are paramagnetic with the presence of unpaired electrons.

Therefore, it was expected that the Cu(II) complex of SB2OME, SB3OME and SB4OME had an octahedral shape surrounding the Cu(II) ion (Xuan Hung et al., 2020). Besides, Cu(SB3NO)₂ showed magnetic moments of 1.536 B.M, confirming that Cu(SB3NO)₂ had a square planar geometry as supported by previous literature (Break et al., 2013). According to Adsule et al. (2006), the ideal range of magnetic moment in square planar should be low in the range of 1.5-1.7 B.M due to the covalent nature of the metal-sulfur bond (Adsule et al., 2006).

On the other hand, the Co(II) complex has an electronic configuration of d⁷ electrons that can be readily oxidised to cobalt(III) (Akbar Ali & Tarafdar, 1977). The cobalt(II) ion in Co(SB2OME)₂, Co(SB3OME)₂, and Co(SB4OME)₂ were predicted to adopt an octahedral geometry based on the magnetic moment, 4.267, 4.944 and 4.740 B.M respectively (Emam et al., 2009b). It was stated by Bhat (2019), that high-spin octahedral Co(II) complexes have magnetic moments ranging from 4.7 to 5.2 B.M. Besides, Co(SB3NO)₂ exhibited a magnetic moment of 4.564 B.M suggesting that it will be in tetrahedral form. This is because according to Bhat (2019), in high-spin tetrahedral complexes, the range was between 4.3-4.7 B.M. Plus, the Co(II) ion is usually in a highly distorted tetrahedral environment due to the presence of bulky groups such as -C₆H₅ and OCH₃, which is aligned with the previous study (How et al., 2008).

Last but not least, the Ni(II) complexes constituting a d⁸ system can exhibit both paramagnetic and diamagnetic properties. In paramagnetic conditions, the complex may adopt a tetrahedral or octahedral geometry depending on its magnetic moment value (Kriessman & Callen, 1954). However, in diamagnetic conditions, the complex tends to favour square planar or octahedral based on the coordination (Bhattacharjee et al., 2021). Paramagnetic of Ni(SB2OME)₂, Ni(SB3OME)₂ and Ni(SB4OME)₂ showed magnetic moment values of 3.288, 2.270 and 2.385 B.M respectively indicating the presence of two unpaired electron configurations. This suggests an octahedral geometry (Lever, 1984). Meanwhile, Ni(SB3NO)₂ displayed 3.874 B.M as the magnetic moment that is within the range of tetrahedral properties of the complex.

Despite numerous attempts, the confirmation of the metal complexes' structure remains inconclusive due to the inability to obtain crystals for a single X-ray diffraction analysis. Therefore, it is inferred that all the metal complexes of SB2OME, SB3OME and SB4OME possess an octahedral geometry structure, while the metal complexes of

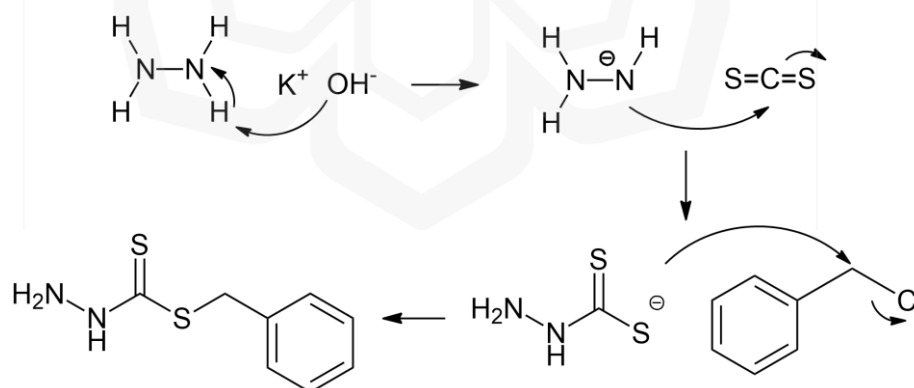
the SB3NO complexes exhibit coordination geometry either square planar or tetrahedral as supported by other spectroscopic studies.

4.9 PLAUSIBLE MECHANISM

Based on the spectroscopic techniques discussed above, the plausible mechanisms for each compound are proposed below.

4.9.1 SBDTC

As illustrated in Scheme 4.1, the formation of SBDTC begins with the deprotonation of hydrazine (N_2H_4) by potassium hydroxide (KOH). In this step, the strong base KOH abstracts a proton from one of the amine groups of hydrazine, generating a hydrazine anion (N_2H_3^-). This anion acts as a nucleophile and undergoes a nucleophilic addition reaction with carbon disulfide (CS_2). As a result, dithiocarbazate moiety was formed (Solomons et al., 2016). In the final step, the sulfur atom within the dithiocarbazate moiety, acting as a nucleophile, participates in a nucleophilic substitution ($\text{S}_\text{N}2$) reaction with benzyl chloride ($\text{C}_6\text{H}_5\text{CH}_2\text{Cl}$). During this reaction, the sulfur atom attacks the electrophilic carbon of benzyl chloride, displacing the chloride ion (Cl^-) and producing SBDTC as the main product. Potassium chloride (KCl) and water are generated as byproducts of the reaction.



Scheme 4.1 Plausible reaction mechanism of SBDTC

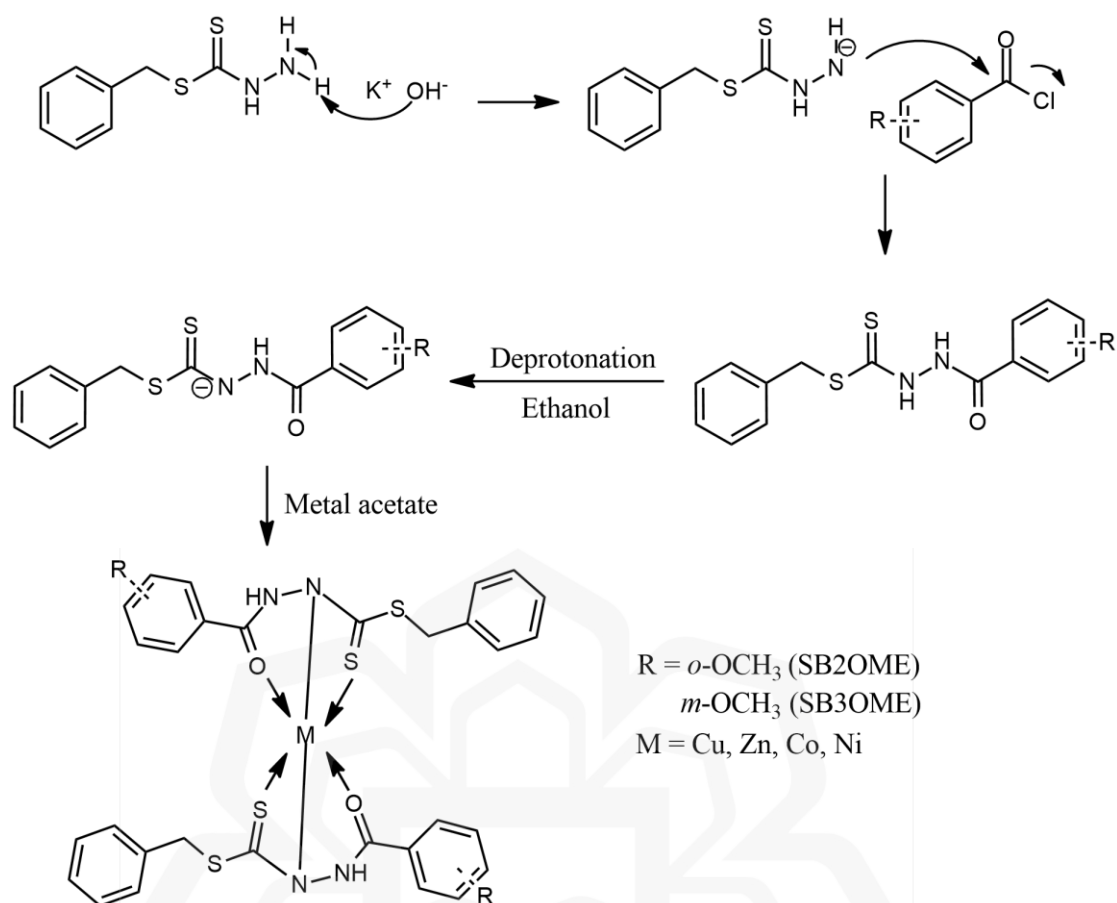
4.9.2 SB2OME, SB3OME and their metal complexes

Since SB2OME and SB3OME share a similar formation pathway and coordination mode in their metal complexes, their mechanisms are predicted to be identical. Their reaction mechanisms are illustrated in Scheme 4.2. The process begins with the

deprotonation of SBDTC by potassium hydroxide (KOH). The hydroxide ion (OH^-) abstracts a proton from the nitrogen atom of the hydrazine ($-\text{NH}$) group in SBDTC. This step generates a nucleophilic nitrogen species, significantly increasing the nucleophilicity of the nitrogen atom within the SBDTC moiety (Solomons et al., 2016).

The deprotonated SBDTC then undergoes a nucleophilic acyl substitution reaction with *o*- or *m*-methoxybenzoyl chloride to form SB2OME and SB3OME, respectively. During this reaction, the nucleophilic nitrogen of the SBDTC moiety attacks the electrophilic carbonyl carbon ($\text{C}=\text{O}$) of the acyl chloride, forming a bond directly with the β -nitrogen of SBDTC. The electrophilicity of the carbonyl carbon is further enhanced by the electron-withdrawing effects of both the carbonyl oxygen and the chlorine atom. The reaction concludes with the expulsion of a chloride ion (Cl^-) from the carbonyl carbon, resulting in the formation of SB2OME or SB3OME along with potassium chloride (KCl) and water (H_2O) as byproducts.

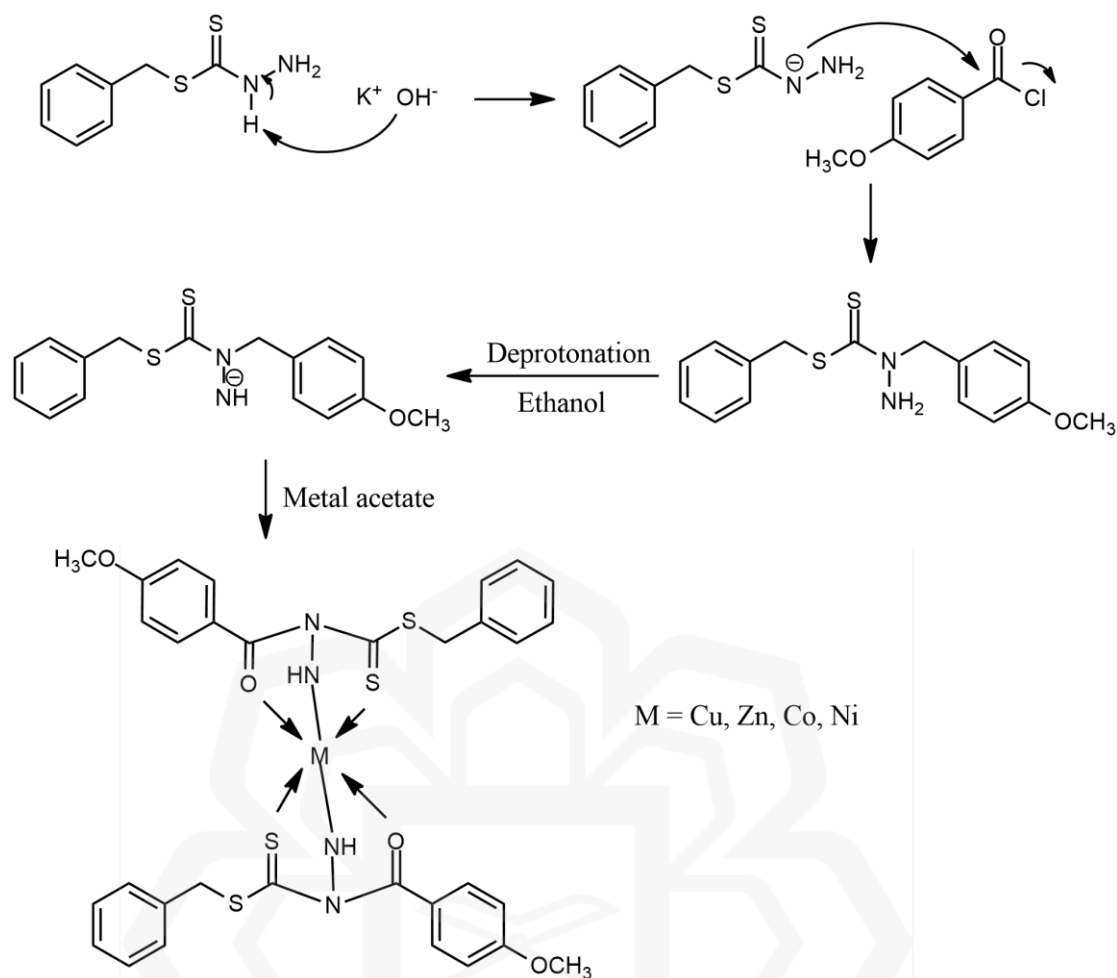
For the formation of metal complexes from SB2OME and SB3OME (Scheme 4.2), the process begins with the deprotonation of the α -nitrogen atom by an acetate ion. This deprotonation enhances the nucleophilicity of the nitrogen, enabling it to serve as a key donor site in the metal-binding process. The positively charged metal ion is subsequently coordinated with the nucleophilic nitrogen. Meanwhile, the carbonyl oxygen and thione sulfur also coordinate with metal ions through electron donation. Following this, SB2OME and SB3OME act as tridentate ligands, coordinating with the metal ion through the nitrogen atom, the sulfur atom from the $\text{C}=\text{S}$ group, and the oxygen atom from the $\text{C}=\text{O}$ group. This coordination results in the formation of six-coordinated metal complexes.



Scheme 4.2 Plausible reaction mechanism of SB2OME, SB3OME and their metal complexes

4.9.1 SB4OME and its metal complexes

Although SB4OME is an isomer of SB2OME and SB3OME, its formation involves a different nitrogen atom, leading to slight differences in its mechanism compared to its isomers. Similar to the formation of SB2OME and SB3OME, the synthesis of SB4OME begins with the deprotonation of SBDTC by potassium hydroxide (KOH), producing a nucleophilic nitrogen species (Scheme 4.3). This deprotonated SBDTC then undergoes a nucleophilic acyl substitution reaction with *p*-methoxybenzoyl chloride to form SB4OME. In this reaction, the nucleophilic nitrogen in the SBDTC moiety attacks the electrophilic carbonyl carbon (C=O) of the acyl chloride, resulting in the formation of a bond directly with the α -nitrogen of SBDTC. Unlike SB2OME and SB3OME, which are directed toward the β -nitrogen of SBDTC, SB4OME is directed toward the α -nitrogen, leading to the different structure of SB4OME.

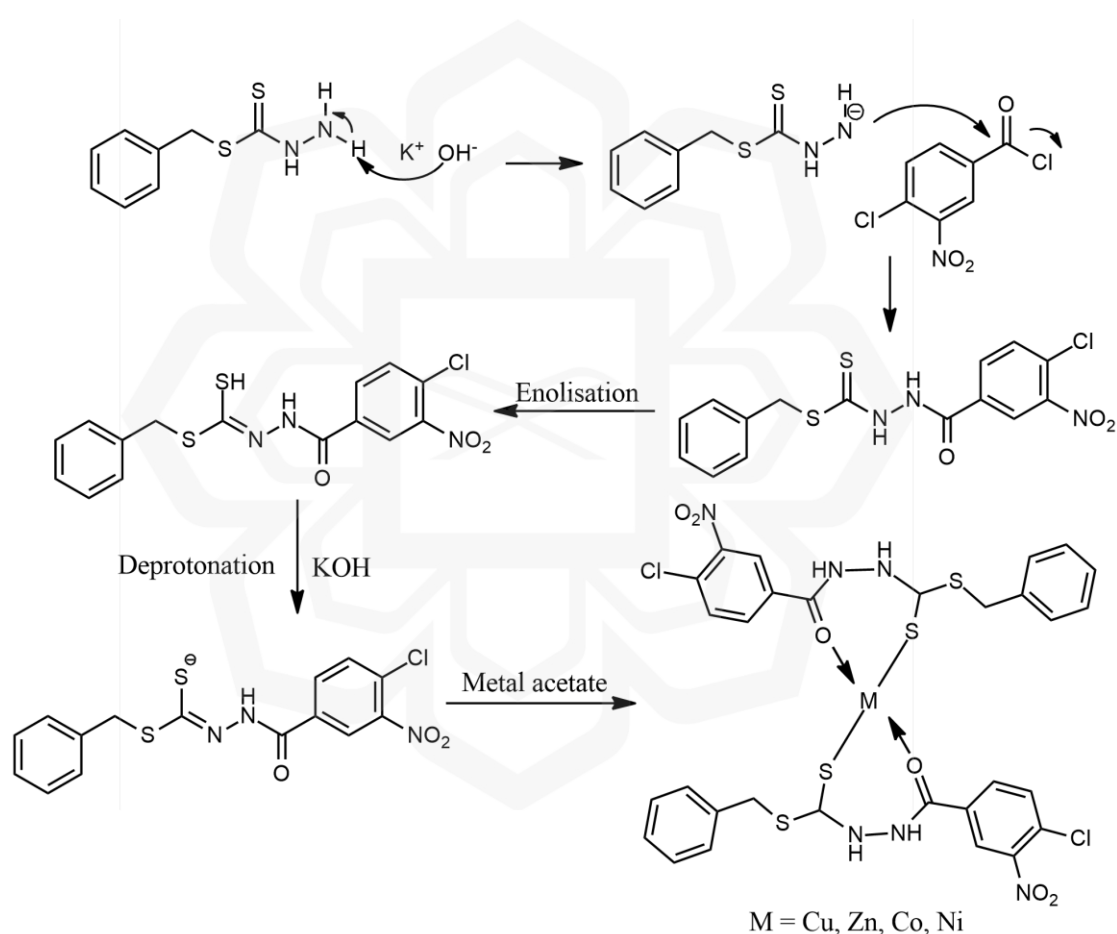


Scheme 4.3 Plausible reaction mechanism of SB4OME and its metal complex

Nevertheless, the mechanism for the metal complexes from SB4OME (Scheme 4.3) is expected to be similar to those observed for SB2OME and SB3OME, as it exhibits the same coordination mode via the NOS donor atoms. The process begins with the deprotonation of the nitrogen atom by the acetate ion. The positively charged metal ion is then coordinated with the nucleophilic nitrogen. Meanwhile, the carbonyl oxygen and thione sulfur also coordinate with metal ions through electron donation. Subsequently, SB4OME acts as a tridentate ligand, coordinating with the metal ion through the nitrogen atom, the sulfur atom from the $\text{C}=\text{S}$ group, and the oxygen atom from the $\text{C}=\text{O}$ group. This coordination results in the formation of six-coordinated metal complexes.

4.9.2 SB3NO and its metal complexes

The reaction mechanism of SB3NO (Scheme 4.4) was expected to be similar to that of SB2OME and SB3OME, as it involves the same β -nitrogen of SBDTC. The process begins with the deprotonation of SBDTC by potassium hydroxide (KOH), producing a nucleophilic nitrogen species. This deprotonated SBDTC then undergoes a nucleophilic acyl substitution reaction with *p*-chloro-*m*-nitrobenzoyl chloride, resulting in the formation of SB3NO. In this reaction, the nucleophilic nitrogen of the SBDTC moiety attacks the electrophilic carbonyl carbon (C=O) of the acyl chloride, forming a bond directly with the β -nitrogen of SBDTC.



Scheme 4.4 Plausible reaction mechanism of SB3NO and its metal complexes

However, the mechanism of metal complexes from SB3NO (Scheme 4.4) was expected to differ from metal complexes from SB2OME, SB3OME and SB4OME due to its different coordination modes. The formation of metal complexes from SB3NO begins with the enolisation of SB3NO, which undergoes tautomerisation from the thione (C=S) form to the thiol (C-SH) form (Scheme 4.4). In the thiol form, SB3NO is

deprotonated by KOH, generating a highly nucleophilic thiolate sulfur. This transformation enhances SB3NO's ability to act as a bidentate ligand by coordinating with the metal ion. The thiolate sulfur forms a strong covalent bond with the metal ion, while the carbonyl oxygen contributes to the overall stability of the metal complex through electron donation, further stabilising the structure.

4.10 THERMOGRAVIMETRIC ANALYSIS (TGA)

Thermogravimetric analysis was performed on SBDTC derivatives and their metal complexes to assess their thermal stability. The data gathered from the thermal studies of all compounds are shown in Appendix I4. During analysis, some weight loss stages were very close together, making it hard to interpret, so these cases were ignored. Notably, compounds exhibited stability below 100°C, indicative of the loss of water or water impurities (Gabbott, 2008). However, there are some compounds decomposing at below than 100 °C signalling the presence of impurities. Nevertheless, the decomposed temperature below 100 °C was not recorded due to insignificant weight loss.

4.10.1 SBDTC, SB2OME, SB3OME and SB4OME

From the overlays in Figure 4.46, SBDTC began to decompose at 122°C, which was close to its reported melting point. The first decomposition phase, occurring between 122 and 182°C, resulted in a mass loss of 14.40% (calculated: 15.65%), which was attributed to the detachment of the hydrazine group (Sultana et al., 2020). Subsequently, the benzyl group underwent decomposition in the next stage, leading to a mass loss of 66.95% (calculated: 68.18%) between 182 and 314°C. Thereafter, a gradual mass reduction was observed up to 500°C, corresponding to an anticipated carbon loss of 5.73% (calculated: 6.06%). The residual content likely contained trace amounts of the S-C=S group, which persisted up to 500°C and accounted for 14.51% (calculated: 13.26%) of the total mass.

In the case of SB2OME, decomposition was initiated at 151°C (Figure 4.46). The first mass loss phase, occurring up to 222°C, accounted for approximately 19.74% (calculated: 23.19%), corresponding to the loss of the benzyl group from SBDTC (Bhat, 2014). This was followed by the expulsion of the anisole group between 222 and 327°C, resulting in a loss of 36.53% (calculated: 35.84%). Beyond 327°C, a minor weight

reduction was attributed to oxide formation, contributing to a loss of 4.73% (calculated: 4.81%). A substantial residue of 41.00% (calculated: 39.00%) was observed for SB2OME, indicating the stability of the dithiocarbazate moiety even at temperatures up to 500°C.

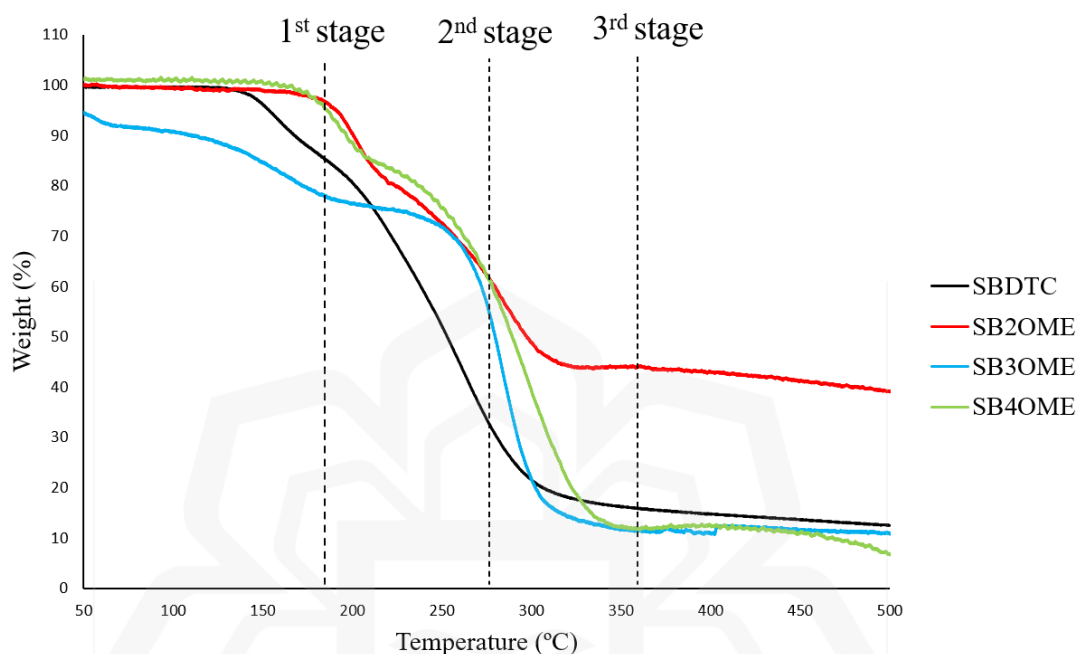


Figure 4.46 Decomposing stages of SBDTC, SB2OME, SB3OME, and SB4OME

The decomposition of SB3OME began at 125°C (Figure 4.46), with an initial mass loss of 26.56% (calculated: 27.40%) observed up to 239°C, which was attributed to the loss of the benzyl group from SBDTC. A significant mass loss of 58.83% (calculated: 58.43%) occurred between 239°C and 317°C, corresponding to the combination of the hydrazine and anisole groups. Following this, a minor mass loss was noted after 317°C, attributed to oxide formation, with a weight loss of 4.064% (calculated: 4.81%). The remaining 9.33% (calculated: 10.546%) residue was assigned to the S-C=S group.

SB4OME exhibited the highest degree of decomposition, with only 6.673% (calculated: 9.33%) residue attributed to the C=S group. Decomposition commenced at 155°C and extended to 288°C, resulting in a weight loss of 14.56% (calculated: 15.65%) due to the loss of the hydrazine group. Subsequent decomposition of other components from SBDTC and the *p*-methoxybenzoyl group occurred between 208°C and 347°C (A.

Singh et al., 2014), leading to a substantial loss of 73.19% (calculated: 73.19%). SB4OME exhibited a minor loss of 5.577% (calculated: 4.81%) attributed to oxide formation up to 347°C.

4.10.2 Metal Complexes from SB2OME

In contrast to the ligands, which were heated up to 500°C, the metal complexes were heated up to 800°C to assess the stability of the coordination bond. All metal complexes from SB2OME decomposed in four significant stages (Figure 4.47). For $\text{Cu}(\text{SB2OME})_2$, the benzyl group decomposed between 100°C and 255°C, resulting in a mass loss of 32.61% (calculated: 33.88%). This was followed by the decomposition of the methoxy group, which caused a mass loss of 11.28% (calculated: 11.29%) within the temperature range of 255°C to 356°C (M. Singh et al., 2009). Subsequently, the phenyl group underwent degradation between 356°C and 691°C, leading to a mass loss of 20.84% (calculated: 20.90%). Finally, hydrazine removal occurred at higher temperatures, from 691°C to 800°C, accounting for 5.80% (calculated: 7.41%) of the mass. The remaining residue, approximately 26.03% (calculated: 24.45%), was identified as copper oxide and the S-C=S group (Elzaref et al., 2023).

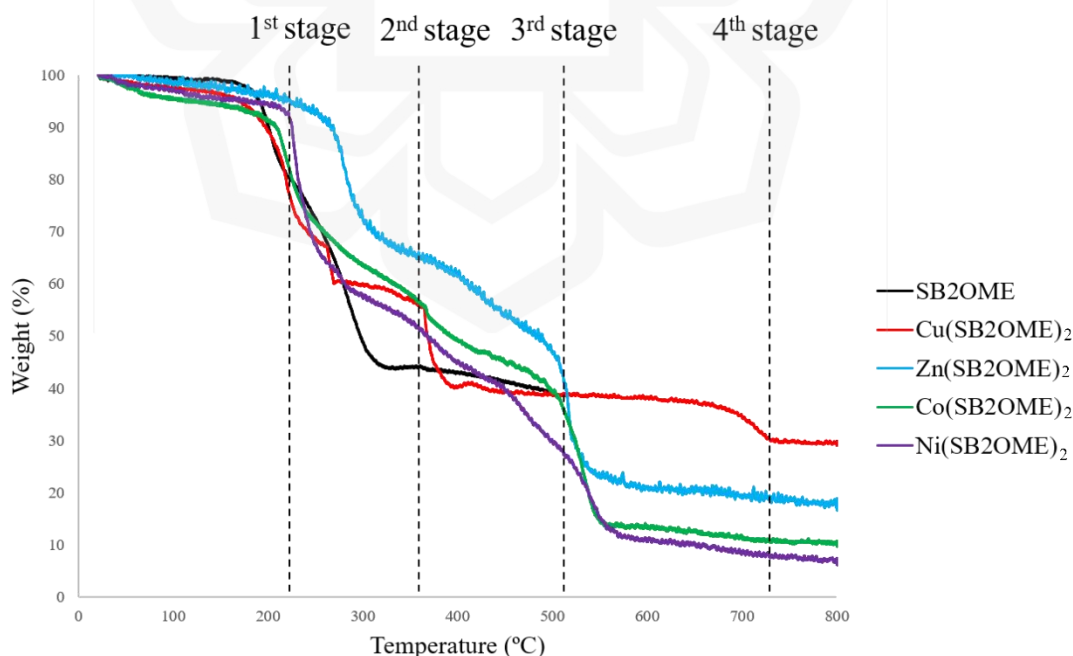


Figure 4.47 Decomposing stages of metal complexes from SB2OME

In the case of $\text{Zn}(\text{SB2OME})_2$, the decomposition began around 146°C to 334°C (Figure 4.47), predominantly involving the benzyl group, resulting in a mass loss of 33.26% (calculated: 33.88%). The phenyl group then underwent decomposition between 334°C and 495°C, with a mass loss of 19.33% (calculated: 20.90%). The subsequent stages, occurring between 495°C and 550°C, led to a loss of 24.72% (calculated: 24.24%) and were attributed to the dithiocarbazate moiety and methoxy group. Similar to $\text{Cu}(\text{SB2OME})_2$, the final stage involved the degradation of the hydrazine group, with a marginal loss of 4.51% (calculated: 7.39%) at temperatures exceeding 550°C. The resultant products of $\text{Zn}(\text{SB2OME})_2$ constituted about 20.80% (calculated: 17.35%), primarily composed of zinc oxide, aligning with previous studies (Elzaref et al., 2023).

Meanwhile, $\text{Co}(\text{SB2OME})_2$ and $\text{Ni}(\text{SB2OME})_2$ exhibited similar decomposition patterns (Figure 4.45). In both cases, all aromatic groups decomposed between 146°C and 366°C, with mass losses of 44.92% (calculated: 46.00%) and 44.50% (calculated: 46.38%), respectively. The methoxy group was then expelled between 320°C and 493°C, resulting in weight losses of 14.14% (calculated: 15.81%) and 13.42% (calculated: 14.72%), respectively. Furthermore, the S-C=S group underwent decomposition between 424°C and 569°C, with mass losses of 27.55% (calculated: 25.24%) and 30.62% (calculated: 25.30%), respectively. The final decomposition stage, as usual, involved the expulsion of the hydrazine group, leading to losses of 3.34% (calculated: 4.75%) and 5.035% (calculated: 4.76%), respectively. The remaining residues of these metal complexes are anticipated to comprise metal oxide (Yekke-Ghasemi et al., 2020).

4.10.3 Metal Complexes from SB3OME

In contrast, among the metal complexes derived from SB3OME, only $\text{Zn}(\text{SB3OME})_2$ and $\text{Co}(\text{SB3OME})_2$ exhibited three and two stages of decomposition, respectively, while the remaining complexes followed a four-stage pattern, as shown in Figure 4.48. Starting with $\text{Cu}(\text{SB3OME})_2$, its decomposition began at approximately 142-247°C, leading to a mass loss of 26.78% (calculated: 25.06%), attributed to the dissociation of the benzyl group from SBDTC (M. Singh et al., 2009). The next stage involved the decomposition of the anisole group between 247°C and 347°C, resulting in a mass loss of 24.70% (calculated: 24.24%). The third stage, between 327°C and 566°C, saw the

degradation of the hydrazine group, leading to a mass loss of 10.51% (calculated: 11.29%). Finally, the S-C=S group decomposed at temperatures reaching up to 566°C, with a significant mass loss of 12.46% (calculated: 13.22%). The remaining residue of $\text{Cu}(\text{SB3OME})_2$, comprising copper oxide and a few carbon residues, accounted for 26.03% (calculated: 25.547%).

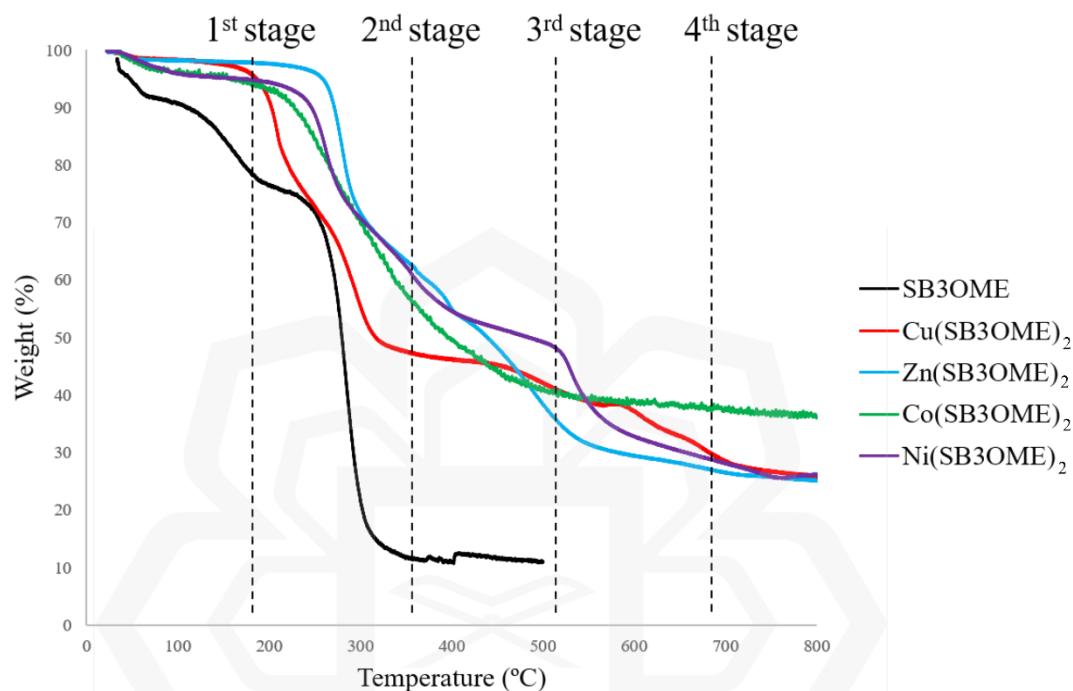


Figure 4.48 Decomposing stages of metal complexes from SB3OME

Similarly, $\text{Zn}(\text{SB3OME})_2$ underwent initial decomposition with the removal of the anisole group, resulting in a mass loss of 30.68% (calculated: 33.88%) between 151°C and 309°C. The next stage, between 309°C and 549°C, involved the decomposition of the benzyl and S-C=S groups, with a combined mass loss of 37.96% (calculated: 39.94%). A minor mass loss of 5.82% (calculated: 4.46%) was observed between 549°C and 800°C, which is attributed to the removal of the hydrazine group. The remaining constituents, primarily zinc oxide and a few carbon residues, accounted for 26.03% (calculated: 25.547%).

$\text{Co}(\text{SB3OME})_2$ exhibited the lowest number of decomposition stages, initiating at approximately 141°C and continuing until 450°C (Figure 4.48), resulting in a significant mass loss of 56.10% (calculated: 58.52%). This loss likely originated from the dissociation of the benzyl and anisole groups. In the subsequent stage, between

450°C and 800°C, a minor mass loss of 7.94% (calculated: 8.04%) was attributed to the removal of the hydrazine group. The remaining 33.19% (calculated: 35.96%) of $\text{Co}(\text{SB3OME})_2$ was ascribed to cobalt oxide and the S-C=S group, consistent with the findings of Yekke-Ghasemi et al. (2020).

Lastly, $\text{Ni}(\text{SB3OME})_2$ followed a decomposition pattern similar to that of $\text{Cu}(\text{SB3OME})_2$ (Figure 4.48). The compound began decomposing between 130°C and 302°C, resulting in a mass loss of 29.72% (calculated: 34.16%), corresponding to the removal of the anisole group. The next phase, from 302°C to 510°C, involved the removal of the benzyl group, leading to a mass loss of 21.88% (calculated: 21.11%). Following this, between 510°C and 628°C, the S-C=S group decomposed, resulting in a mass loss of 16.94% (calculated: 17.68%). Lastly, from 628°C to 800°C, a minor mass loss of 5.14% (calculated: 4.44%) was observed, which is associated with the removal of the hydrazine group. The residual mass of 22.61% (calculated: 26.32%) was composed of nickel oxide and some carbon residues.

4.10.4 Metal Complexes from SB4OME

In parallel with the metal complexes from SB2OME, the metal complexes from SB4OME also exhibited four decomposition stages for each metal complex (Figure 4.49). Starting with $\text{Cu}(\text{SB4OME})_2$, the decomposition occurred within the temperature range of 145°C to 303°C, resulting in a weight loss of 41.78% (calculated: 41.87%), indicative of the removal of all aromatic groups (Sultana et al., 2020). The subsequent stage, occurring between 303°C and 349°C, involved the expulsion of the dithiocarbazate moiety and methoxy group, leading to a weight loss of 31.05% (calculated: 31.68%). An insignificant weight loss of 0.61% was observed between 349°C and 661°C, which was deemed negligible and disregarded. The final decomposition stage, from 661°C to 800°C, corresponded to the degradation of the hydrazine group, resulting in a weight loss of 4.78% (calculated: 4.4%). The residual components of $\text{Cu}(\text{SB4OME})_2$, which accounted for a weight loss of 26.03% (calculated: 21.79%), primarily consisted of copper oxide and a small amount of carbon residues.

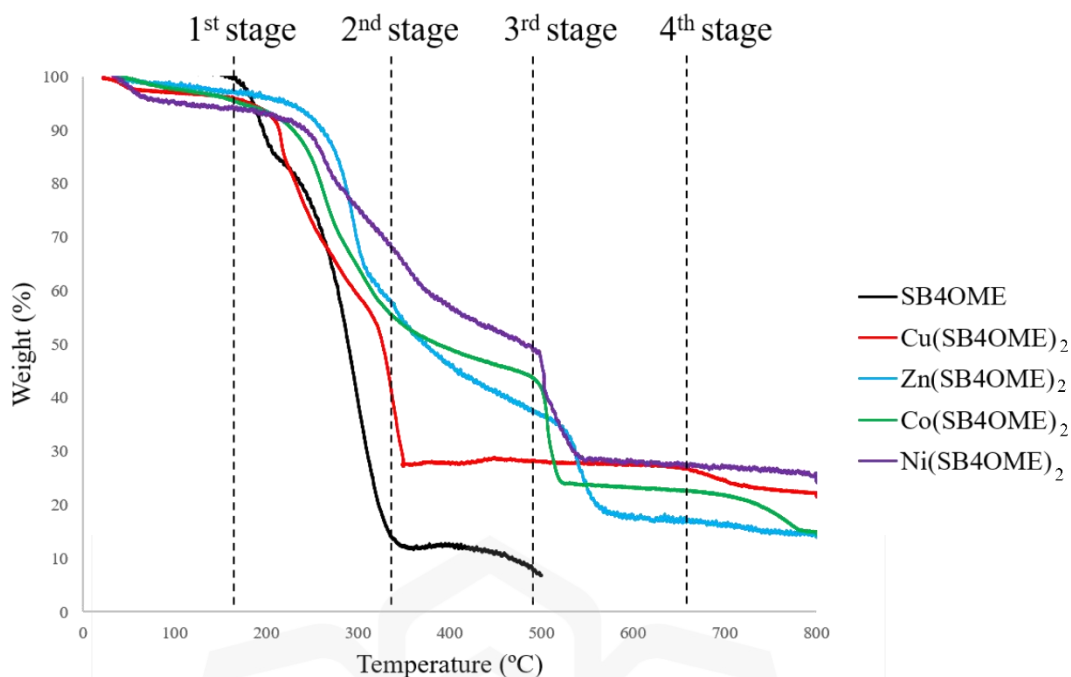


Figure 4.49 Decomposing stages of metal complexes from SB4OME

Zn(SB4OME)₂ initiated decomposition at 139-314 °C (Figure 4.49) with the loss of the benzyl group resulting in a mass loss of 37.98% (calculated: 36.80%). Subsequently, the anisole group underwent decomposition at 314-519 °C, leading to a weight loss of 27.14% (calculated: 29.47%). The subsequent removal of the S-C=S group occurred at 519-584 °C, resulting in a weight loss of 17.02% (calculated: 17.63%). A minor loss observed at 584-800 °C, amounting to 3.345% (calculated: 4.35%), corresponded to hydrazine group removal. The remaining compounds in the Zn(SB4OME)₂ complex consisted of zinc oxide as reported by A. Singh et al., (2014).

Besides, Co(SB4OME)₂ decomposed within the temperature range of 140-477 °C (Figure 4.49), resulting in a weight loss of 55.37% (calculated: 57.81%) due to the loss of all aromatic groups and the methoxy group. S-C=S group was then decomposed in the subsequent stage around 477-522 °C, resulting in a weight loss of 20.79% (calculated: 17.70%). Subsequently, the hydrazine group was removed at 522-778 °C, leading to a weight loss of 8.67% (calculated: 8.85%). The insignificant loss observed at 778-800 °C, amounting to 0.638%, was disregarded. Therefore, the remaining weight loss of 12.58% (calculated: 14.535%) is attributed to cobalt oxide.

In the case of $\text{Ni}(\text{SB4OME})_2$, the benzyl group and the S-C=S group underwent removal in the initial decomposing stage around 148-379 °C (Figure 4.49), resulting in a weight loss of 41.13% (calculated: 40.11%). Next, at 379-495 °C, the methoxy group was decomposed, leading to a weight loss of 10.60% (calculated: 8.57%). It was followed by the decomposition of the phenyl group at 495-546 °C, resulting in a weight loss of 20.15% (calculated: 21.02%). The final stage involved decomposition by the hydrazine group at 546-800 °C, resulting in a weight loss of 5.057% (calculated: 4.42%). The residual components remaining from $\text{Ni}(\text{SB4OME})_2$ consisted of nickel oxide, accounting for a mass of 25.72% (calculated: 23.063%).

4.10.5 SB3NO And Its Metal Complexes

SB3NO also depicted three decomposing stages as illustrated in Figure 4.50. SB3NO started to decompose at 130°C, continuing until 226°C with a mass loss of 26.04% (calculated: 23.88%) due to the loss of the benzyl group from SBDTC (Bhat, 2014). Then, a dual weight loss of 47.70% (calculated: 46.45%) was observed between 226°C and 295°C, attributable to the loss of the 4-chloro-3-nitrobenzoyl group. The decomposition of the hydrazine group occurred above 295°C, resulting in a mass loss of 8.034% (calculated: 7.87%). Lastly, the remaining residue comprised the S-C=S group accounting for a loss of 20.47% (calculated: 19.94%).

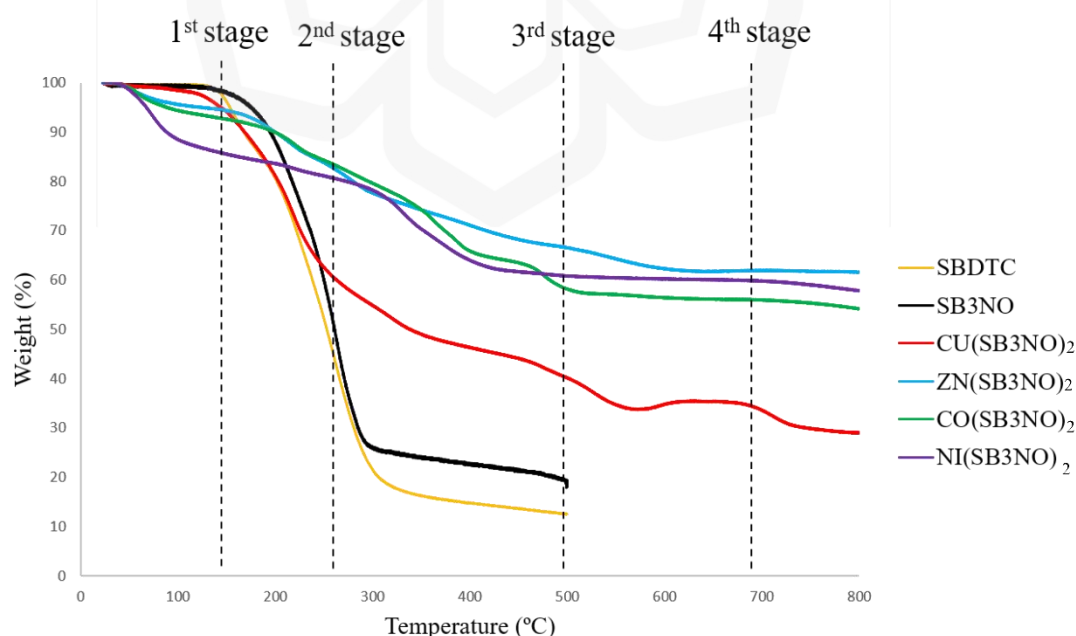


Figure 4.50 Decomposing stages of metal complexes from SB3NO

The metal complexes from SB3NO, on the other hand, demonstrated a complicated decomposition process (Figure 4.50). Notably, only $\text{Cu}(\text{SB3NO})_2$ exhibited significant weight loss exceeding half of its mass (as illustrated in Figure 4.50), implying the necessity for higher heating temperatures for other metal complexes (Menczel & Prime, 2008). The decomposition of $\text{Cu}(\text{SB3NO})_2$ occurred within the range of 127-372 °C, resulting in a mass loss of 52.50% (calculated: 59.95%). This indicates the dissociation of all aromatic rings from SB3NO along with the dissociation of chloro and nitro group (M. Singh et al., 2009b). Next, the hydrazine group was removed in the subsequent stage around 372-564 °C, leading to a mass loss of 13.72% (calculated: 10.19%). A minor weight loss observed at 564-735 °C, accounting for 3.55% (calculated: 3.88%), corresponds to carbon removal. Negligible weight loss of 1.15% observed at 735-800 °C was disregarded. Consequently, the residual mass of 26.61% (calculated: 29.0%) comprises copper oxide and S-C=S group (Yekke-Ghasemi et al., 2020).

$\text{Zn}(\text{SB3NO})_2$ only decomposed 38.442 % (Figure 4.50) of its mass with the majority loss from the first stage at 132-298 with a mass loss of 22.39% (calculated: 22.08%) which was assigned to the benzyl group from SBDTC. Then, the nitro group was removed in the second stage around 298-467 °C, resulting in a mass loss of 10.17% (calculated: 11.15%). The third stage involved chloride group decomposition at 467-615 °C, resulting in a mass loss of 5.60% (calculated: 8.23%). Negligible mass loss observed in the final stage was disregarded, leaving the remaining products, including zinc oxide, dithiocarbazate moiety and aromatic ring from the 4-chloro-3-nitrobenzoyl group with a mass of 61.16% (Calculated 61.56%).

$\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$ exhibited similar total mass and decomposition patterns (Figure 4.50). In the initial stage, both compounds underwent decomposition of the benzyl group from SBDTC around 136-323 °C and 110-273 °C, resulting in mass losses of 22.71% (calculated: 22.22%) and 20.09% (calculated: 22.24%), respectively. Subsequently, nitro and chloro groups were separately removed from $\text{Co}(\text{SB3NO})_2$ at 323-420 °C and 420-518 °C, resulting in mass losses of 10.17% (calculated: 11.15%) and 5.60% (calculated: 8.17%), respectively. However, both fragments decomposed at the same stage in $\text{Ni}(\text{SB3NO})_2$ around 273-426 °C resulting in a mass loss of 17.77% (calculated: 16.85%). For $\text{Co}(\text{SB3NO})_2$, a minor loss observed at 518-800 °C,

accounting for 7.45% (calculated: 7.27%), corresponds to the removal of the hydrazine group. Meanwhile, $\text{Ni}(\text{SB3NO})_2$ exhibited negligible losses in both final stages at 426-733 °C and 733-800 °C, with mass losses of 2.95% and 1.547%, respectively. As a result, the remaining compounds from both $\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$ consisted of metal oxide, dithiocarbazate moiety and aromatic ring from the 4-chloro-3-nitrobenzoyl group with masses of 52.38% (calculated: 54.07%) and 60.88% (calculated: 57.643%), respectively.

4.10.6 Summarisation of Thermogravimetric Analysis (TGA)

The decomposition pattern of SBDTC derivatives begins with the loss of the benzyl group, consistent with previous studies. This stage occurs between 150°C and 240°C and is accompanied by significant weight loss, as observed in SBDTC dithiocarbazate ligands (Bhat, 2014). The second stage involves the decomposition of the dithiocarbazate backbone, while the final stage typically involves oxide decomposition with minimal weight loss. The residual content at the end of the decomposition process often includes elemental sulfur or nitrogen (Costa et al., 2019).

In contrast, the decomposition of metal complexes derived from SBDTC derivatives occurs at higher temperatures and involves more stages. Unlike free ligands, which typically exhibit two decomposition stages, metal complexes undergo three to four distinct stages (Elzaref et al., 2023). In the first stage, occurring between 120°C and 300°C, the benzyl group is lost, resulting in a 40% to 60% weight reduction. This temperature range aligns with the degradation profile of the ligand itself. The second stage, occurring between 400°C and 550°C, involves the decomposition of substituents such as phenyl, methoxy, or nitro groups (Sultana et al., 2020). The third stage, at temperatures ranging from 700°C to 800°C, corresponds to the breakdown of groups such as thiol, hydrazine, or chloro groups, leaving 10% to 30% of the complex's original weight (A. Singh et al., 2014).

The undecomposed residue was stabilised by strong coordination bonds between donor atoms such as nitrogen, oxygen, and sulfur with the metal ions (Saad et al., 2018). Due to their high bond dissociation energies, breaking these bonds required temperatures exceeding 1000°C (Kudrat-E-Zahan et al., 2020). Furthermore, during decomposition, metal complexes often formed thermally stable products such as metal

oxides or sulfides, which had melting points above 1000°C (Costa et al., 2019). However, the residue obtained from metal complexes of SB3NO was notably large, particularly for $\text{Zn}(\text{SB3NO})_2$, $\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$ which can be attributed to the coordination mode of SB3NO. As reported in the IR analysis, SB3NO coordinated with metal ions through thiolate sulfur and carbonyl oxygen. Previous studies indicated that thiolate sulfur coordination formed much stronger interactions compared to other coordination modes (Kreider-Mueller et al., 2017; Turnley et al., 2023).

In contrast, the ONS coordination mode observed in SB2OME, SB3OME, and SB4OME, although tridentate, primarily involved deprotonation of nitrogen. According to Chen et al. (2006), coordination through the amino group resulted in significantly weaker binding energy than thiol coordination (F. Chen et al., 2006). As a result, metal complexes from SB3NO formed stronger interactions, leading to a higher percentage of residual mass after decomposition. Nevertheless, all metal complexes exhibited significantly higher thermal stability than SBDTC derivatives, as evidenced by their higher decomposition temperatures and additional decomposition stages.

4.11 ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION, AND TOXICITY (ADMET)

In this study, all synthesised compounds were evaluated using the ADMET parameter to gain preliminary knowledge about their potential for future use as marketable drugs.

4.11.1 ADME Prediction

First, ADME predictions were performed for all compounds, with the results summarised in Table 4.13. The bioavailability radar for SB4OME and $\text{Cu}(\text{SB4OME})_2$ was selected as a representative example for the SBDTC derivatives and metal complexes, respectively (Figure 4.51), while the bioavailability radars for the other compounds can be found in Appendix 16. This bioavailability radar from SwissADME provided a graphical representation of each compound's drug-likeness based on six key physicochemical parameters, which were further discussed in detail.

Firstly, lipophilicity (LIPO) was assessed using XLOGP3, with the ideal range being between -0.7 and 5 (Dong et al., 2018). The SBDTC derivatives and their metal complexes showed XLOGP3 values of approximately 3.72 and 7.84, respectively

(Table 4.13). These results indicate that the lipophilicity of all metal complexes exceeded the optimal range for LIPO, as depicted in Figure 4.51. Next, membrane permeability was evaluated through molecular size (SIZE) to ensure appropriate permeability and binding (J. Ghosh et al., 2016). According to Daina et al. (2017), the ideal molecular weight range is between 150 and 500 g/mol. The metal complexes exhibited molecular weights ranging from 700 to 800 g/mol (Table 4.13), which places their SIZE point outside the optimal range (Figure 4.51). In contrast, the SBDTC derivatives remained within the ideal molecular weight range.

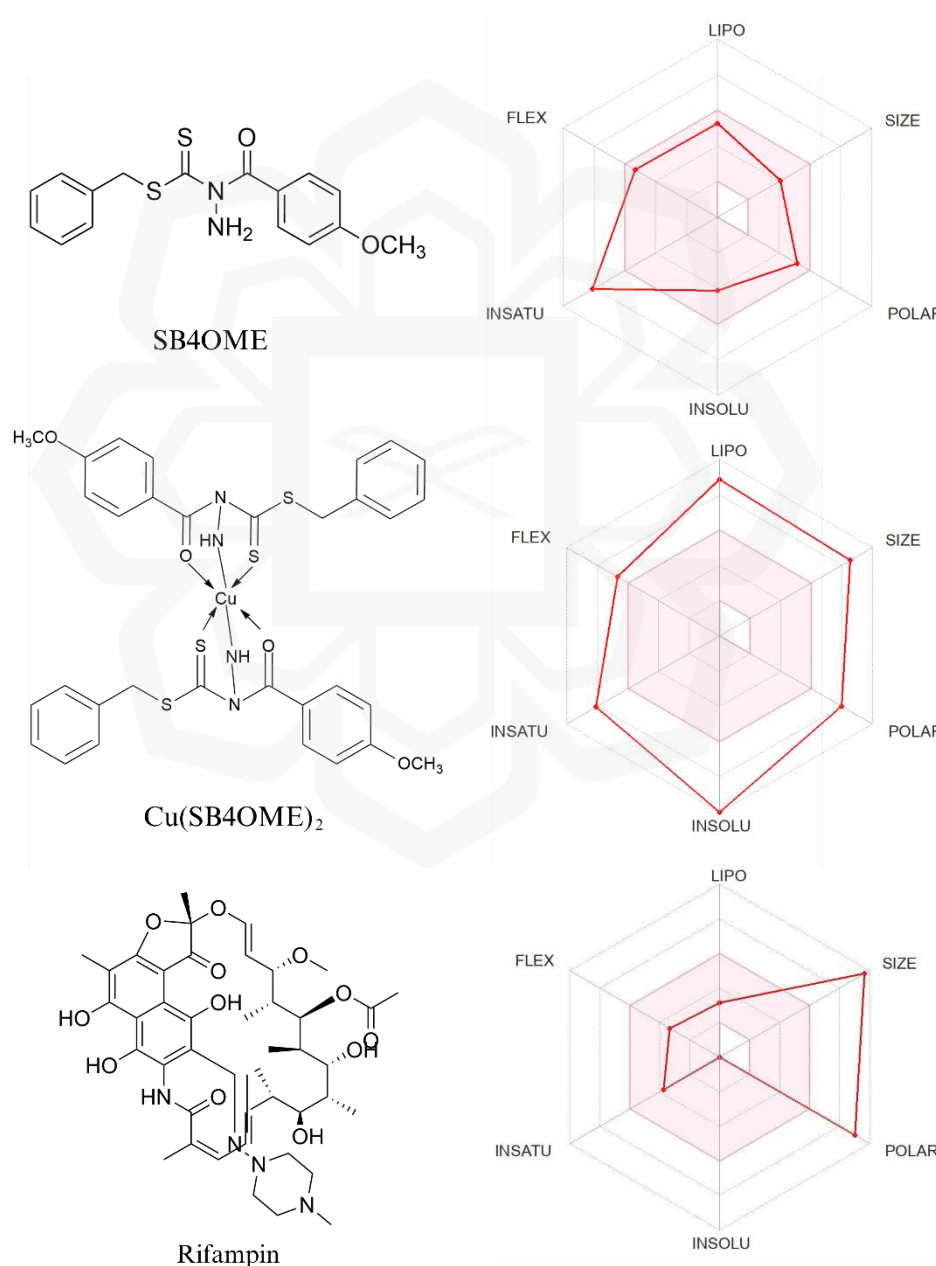


Figure 4.51 The Bioavailability Radar of the representative compounds

Additionally, the Topological Polar Surface Area (TPSA) was considered a crucial parameter for predicting a molecule's drug likeness (van de Waterbeemd & Gifford, 2003). According to Daina et al. (2017), the ideal TPSA range lies between 20 and 130 Å², as represented by polarity (POLAR) in the bioavailability radar, which helps balance solubility and permeability. In this study, only SB2OME, SB3OME, and SB4OME met this criterion, with TPSA values around 107.75 Å² (Table 4.13). In contrast, other compounds, including SB3NO and all metal complexes, showed TPSA values exceeding 130 Å², which places them outside the optimal POLAR range (Figure 4.51).

To assess aqueous solubility, the solubility (INSOLU) parameter was evaluated. Only the SBDTC derivatives fell within the optimal range, with LogS (ESOL) values around -4 (Daina et al., 2017). Meanwhile, the metal complexes exhibited LogS (ESOL) values lower than -6, placing them outside the ideal INSOLU range (Figure 4.51). Next, the fraction of sp³ carbon atoms was used to characterise the aliphatic degree of the molecules and predict their solubility. It has been suggested that increasing saturation enhances the clinical success rate of a compound. According to Daina et al. (2017), the ideal fraction of sp³ carbon atoms should be at least 0.25. All synthesised compounds displayed lower values for the fraction of sp³ carbons, ranging from 0.07 to 0.12 (Table 4.13). As a result, it deviated from the optimal INSATU range (Figure 4.51), which predicts a lower clinical success rate.

Finally, Daina et al. (2017) also recommended that a potential drug should have no more than nine rotatable bonds, as depicted by flexibility (FLEX) in the bioavailability radar (Figure 4.51). All metal complexes, however, had ten rotatable bonds, which exceeded this optimal range (Table 4.13). Overall, while the SBDTC derivatives did not deviate from Lipinski's Rule of Five (Ro5), the metal complexes were predicted to violate at least one rule (Table 4.13). These violations and several out-of-range values identified by SwissADME suggest that the metal complexes may not be ideal drug candidates.

However, it is important to note that SwissADME, while helpful, is not absolute (Dong et al., 2018). The software is known to be less accurate when evaluating large compounds, such as metal complexes, even if they possess favourable drug-like properties. This is exemplified by rifampin, a large commercial drug that shares several

similarities with the metal complexes, particularly in terms of molecular weight (around 700–800 g/mol) (Table 4.13). As expected, this molecular weight results in deviations from the optimal SIZE range in the bioavailability radar, as shown in Figure 4.51.

Past studies also have highlighted the limitations of SwissADME when evaluating large compounds. Benet et al. (2016) emphasised that large compounds often do not meet the typical requirements of the Ro5 and may violate one or more of its criteria. For example, certain large cyclic peptide immunosuppressants, such as cyclosporine, sirolimus, tacrolimus, and everolimus, are known exceptions (Benet et al., 2016). Similarly, macrolide antibiotics like clarithromycin, erythromycin, and roxithromycin often break Ro5 rules.

Young and Leeson (2018) further emphasised this trend, noting that many HIV protease inhibitors and hepatitis C virus (HCV) drugs break Ro5 rules multiple times (Young & Leeson, 2018). For instance, among 12 HIV protease inhibitors, seven had two violations, one (atazanavir) had three, and the others had one violation. These examples demonstrate that despite violating the Ro5, metal complexes and other large drugs can still be effective and successful.

Table 4.13 ADME analysis of all compounds

Compounds	Molecular weight (g/mol)	Fraction Csp ³	Rotatable bonds	H-bond acceptors	H-bond donors	TPSA	XLOGP3	ESOL Log S	Lipinski violations
SBDTC	198.31	0.12	4	1	2	95.44	1.83	-2.33	0
SB2OME	332.44	0.12	8	2	2	107.75	3.72	-4.12	0
Cu(SB2OME) ₂	724.4	0.12	10	6	0	185	10.01	-10.37	2
Zn(SB2OME) ₂	726.23	0.12	10	6	0	185	10.01	-10.38	2
Co(SB2OME) ₂	719.78	0.12	10	6	0	185	10.01	-10.34	2
Ni(SB2OME) ₂	719.54	0.12	10	6	0	185	10.01	-10.34	2
SB3OME	332.44	0.12	8	2	2	107.75	3.72	-4.12	0
Cu(SB3OME) ₂	724.4	0.12	10	6	0	185	10.01	-10.37	2
Zn(SB3OME) ₂	726.23	0.12	10	6	0	185	10.01	-10.38	2
Co(SB3OME) ₂	719.78	0.12	10	6	0	185	10.01	-10.34	2
Ni(SB3OME) ₂	719.54	0.12	10	6	0	185	10.01	-10.34	2
SB4OME	332.44	0.12	7	3	1	112.95	3.43	-4	0
Cu(SB4OME) ₂	726.41	0.12	10	6	2	197.92	7.84	-8.49	1
Zn(SB4OME) ₂	728.24	0.12	10	6	2	197.92	7.84	-8.5	1
Co(SB4OME) ₂	721.8	0.12	10	6	2	197.92	7.84	-8.46	1
Ni(SB4OME) ₂	721.56	0.12	10	6	2	197.92	7.84	-8.46	1

SB3NO	381.86	0.07	8	3	2	144.34	4.2	-4.7	0
Cu(SB3NO) ₂	825.24	0.07	10	8	2	275.76	10.73	-11.42	3
Zn(SB3NO) ₂	827.08	0.07	10	8	2	275.76	10.73	-11.43	3
Co(SB3NO) ₂	820.63	0.07	10	8	2	275.76	10.73	-11.39	3
Ni(SB3NO) ₂	820.39	0.07	10	8	2	275.76	10.73	-11.39	3
Trimethoprim	290.32	0.29	5	5	2	105.51	0.91	-2.31	0
Rifampin	820.92	0.53	5	14	4	213.83	3.44	0	2

4.11.2 Toxicity Prediction

To predict the toxicity profile of the synthesised compounds, toxicity analysis was performed using Protox-II webserver and the results are summarised in Table 4.14. SBDTC exhibited among the lowest toxicity profiles with an LD₅₀ of 300 mg/kg, placing it in toxicity class 3 (toxic if swallowed). As stated by a previous study, class 3 suggests moderate toxicity and this highlights the need for chemical modifications to improve its safety profile (J. Ghosh et al., 2016). SB2OME and SB3OME, both have LD₅₀ values of 1500 mg/kg, falling into toxicity class 4 (harmful if swallowed). These modifications appear to substantially reduce the toxicity risk of the scaffold. SB4OME, had the highest LD₅₀ value of 2050 mg/kg, placing it in class 5 (may be harmful if swallowed). This class also points out a better toxicity profile compared to its scaffold and other isomers. Similarly, SB3NO exhibits an LD₅₀ of 500 mg/kg, making it slightly more toxic than SB2OME and SB3OME but still within class 4.

On the other hand, the metal complexes generally demonstrated varied toxicity profiles as depicted in Figure 4.52. For the metal complexes from SB2OME, LD₅₀ values range from 73 mg/kg for Ni(SB2OME)₂ (class 3) to 1500 mg/kg for Zn(SB2OME)₂ (class 4). Meanwhile, metal complexes from SB3OME exhibited LD₅₀ values between 1000 and 2000 mg/kg, all categorised under class 4, with Zn(SB3OME)₂ showing the highest LD₅₀ (2000 mg/kg). Interestingly, metal complexes from SB4OME, particularly those of Cu(SB4OME)₂ and Ni(SB4OME)₂, demonstrated outstanding safety profiles with LD₅₀ values of 2050 mg/kg, placing them in class 5. In the case of metal complexes from SB3NO, most metal complexes, including those Zn(SB3NO)₂, Co(SB3NO)₂ and Ni(SB3NO)₂ have LD₅₀ values of 600 mg/kg, placing them in class 4. This consistent toxicity profile suggests that the metal ion does not significantly alter the safety of SB3NO.

Trimethoprim, a widely used commercial antibiotic, has an LD₅₀ of 3500 mg/kg, categorising it as class 5. This higher LD₅₀ value reflects its well-established safety profile in clinical use. While none of the SBDTC derivatives or metal complexes reached this level of safety, SB4OME and its Cu(II) and Ni(II) complexes shared similar toxicity class with LD₅₀ values of 2050 mg/kg (Figure 4.52), validating their potential for therapeutic use. Overall, the toxicity predictions indicated that most of the SBDTC

derivatives and their metal complexes exhibit good toxicity profiles, which are comparable to those of known therapeutic agents.

Table 4.14 Toxicity analysis of all compounds

Compounds	LD ₅₀ (mg/kg)	Toxicity class
SBDTC	300	3
SB2OME	1500	4
Cu(SB2OME) ₂	1000	4
Zn(SB2OME) ₂	1500	4
Co(SB2OME) ₂	900	4
Ni(SB2OME) ₂	73	3
SB3OME	1500	4
Cu(SB3OME) ₂	1000	4
Zn(SB3OME) ₂	2000	4
Co(SB3OME) ₂	1000	4
Ni(SB3OME) ₂	1500	4
SB4OME	2050	5
Cu(SB4OME) ₂	2050	5
Zn(SB4OME) ₂	1500	4
Co(SB4OME) ₂	1500	4
Ni(SB4OME) ₂	2050	5
SB3NO	500	4
Cu(SB3NO) ₂	1994	4
Zn(SB3NO) ₂	600	4
Co(SB3NO) ₂	600	4
Ni(SB3NO) ₂	600	4
Trimethoprim	3500	5

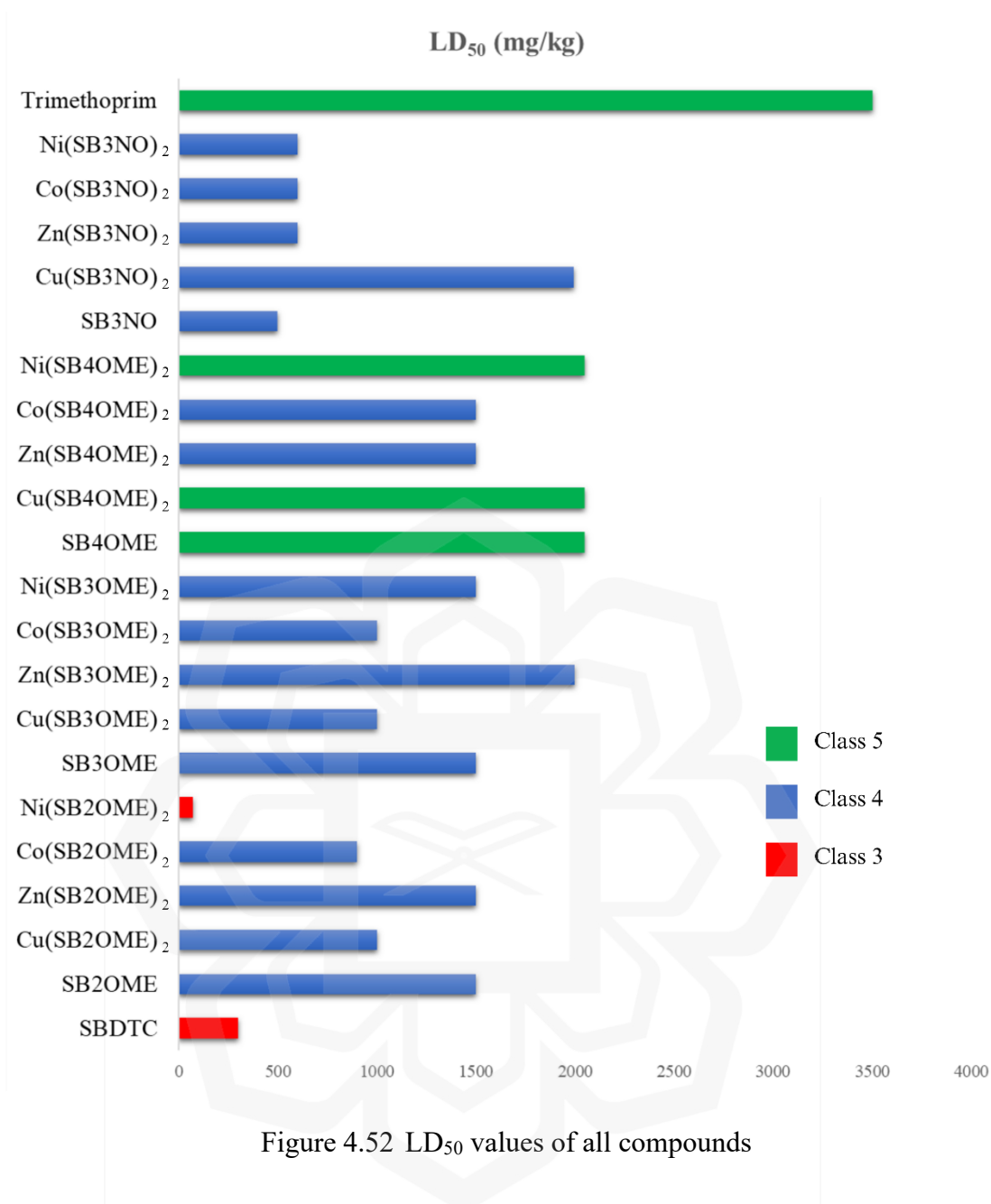


Figure 4.52 LD₅₀ values of all compounds

4.12 BIOLOGICAL ACTIVITY

Antibacterial activity of SBDTC derivatives and their metal complexes was determined using Minimum Inhibition Concentration (MIC) (in mg/mL) against four bacterial strains, two gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778) and two gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The data are presented in Table 4.15. In this study, MIC values below 0.437 mg/mL indicated strong antibacterial activity, while values above 1.750 mg/mL reflected weak activity. MIC values between these thresholds were considered to indicate moderate activity. The values were selected due

to the distribution of MIC results among the investigated compounds, allowing for a clear distinction between strong, moderate, and weak antibacterial activity. Additionally, these thresholds were set only for this study ensuring a meaningful evaluation of the antibacterial potency of the SBDTC derivatives and their metal complexes. The triplicate data of MIC values were compiled in Appendix 17-18.

Table 4.15 Minimum inhibition concentration of all synthesised compounds and positive control

Compounds	Minimum inhibition concentration (mg/mL)			
	Gram-positive bacteria		Gram-negative bacteria	
	<i>Staphylococcus aureus</i> (ATCC 25923)	<i>Bacillus cereus</i> (ATCC 11778)	<i>Escherichia coli</i> (ATCC 25922)	<i>Pseudomonas aeruginosa</i> (ATCC 27853)
SBDTC	0.875	0.437	0.437	0.875
SB2OME	1.750	0.875	1.750	0.875
Cu(SB2OME) ₂	0.875	0.875	0.875	0.437
Zn(SB2OME) ₂	1.750	1.750	1.750	0.875
Co(SB2OME) ₂	1.750	0.875	0.875	0.875
Ni(SB2OME) ₂	1.750	0.875	1.750	0.875
SB3OME	0.875	0.875	0.875	0.437
Cu(SB3OME) ₂	0.437	0.875	0.875	0.437
Zn(SB3OME) ₂	0.437	1.750	0.875	0.437
Co(SB3OME) ₂	0.437	1.750	0.875	0.875
Ni(SB3OME) ₂	0.437	1.750	0.875	0.875
SB4OME	0.875	0.875	0.875	1.750
Cu(SB4OME) ₂	0.437	0.875	0.437	0.875
Zn(SB4OME) ₂	0.437	0.875	0.437	1.750
Co(SB4OME) ₂	0.437	0.875	0.437	0.875
Ni(SB4OME) ₂	0.437	0.875	0.437	0.875
SB3NO	3.500	1.750	1.750	1.750
Cu(SB3NO) ₂	1.750	1.750	1.750	1.750
Zn(SB3NO) ₂	0.875	0.875	0.875	0.437

Co(SB3NO) ₂	0.875	0.875	1.750	1.750
Ni(SB3NO) ₂	0.875	1.750	1.750	1.750
Trimethoprim	0.437	0.437	1.750	1.750
Novobiocin	0.218	0.437	0.437	0.109
Ampicillin	0.109	0.109	0.218	0.109
Gentamycin	0.054	0.027	0.027	0.013

4.12.1 SBDTC, SB2OME, SB3OME and SB4OME

As illustrated in Figure 4.53, against *S. aureus*, SBDTC and its derivatives, SB3OME and SB4OME, exhibited the same MIC of 0.875 mg/mL, while SB2OME showed reduced activity with a MIC of 1.750 mg/mL. This suggests that SB2OME decreases its bioactivity compared to the precursor, SBDTC which could be attributed to the larger steric hindrance caused by the 2-position methoxy group and may affect its interaction with bacterial targets (Kasumov & Köksal, 2003). However, the trend in bioactivity shifts slightly for *B. cereus*. Here, SBDTC showed strong activity with a MIC of 0.437 mg/mL, but its derivatives, SB2OME, SB3OME, and SB4OME, all exhibited a MIC of 0.875 mg/mL. This indicates that the substitution of methoxybenzoyl groups did not enhance bioactivity relative to the parent compound. Instead, the modifications reduced the antimicrobial effects observed in the precursor.

For *E. coli*, all isomeric derivatives increased the MIC value of the precursor with SB3OME and SB4OME showed consistent MIC values of 0.875 mg/mL. This suggests that the modifications of the methoxybenzoyl groups reduced bioactivity in this species. Against *P. aeruginosa*, SB3OME demonstrated the strongest bioactivity, with a MIC of 0.437 mg/mL, significantly better than the precursor SBDTC (0.875 mg/mL). SB2OME and SB4OME, however, showed lower activity, with MICs of 0.875 mg/mL and 1.750 mg/mL, respectively. The enhanced activity of SB3OME suggested that the 3-position methoxy group provides an advantage in terms of molecular interactions with the *P. aeruginosa*'s binding pocket (Muddala et al., 2020).

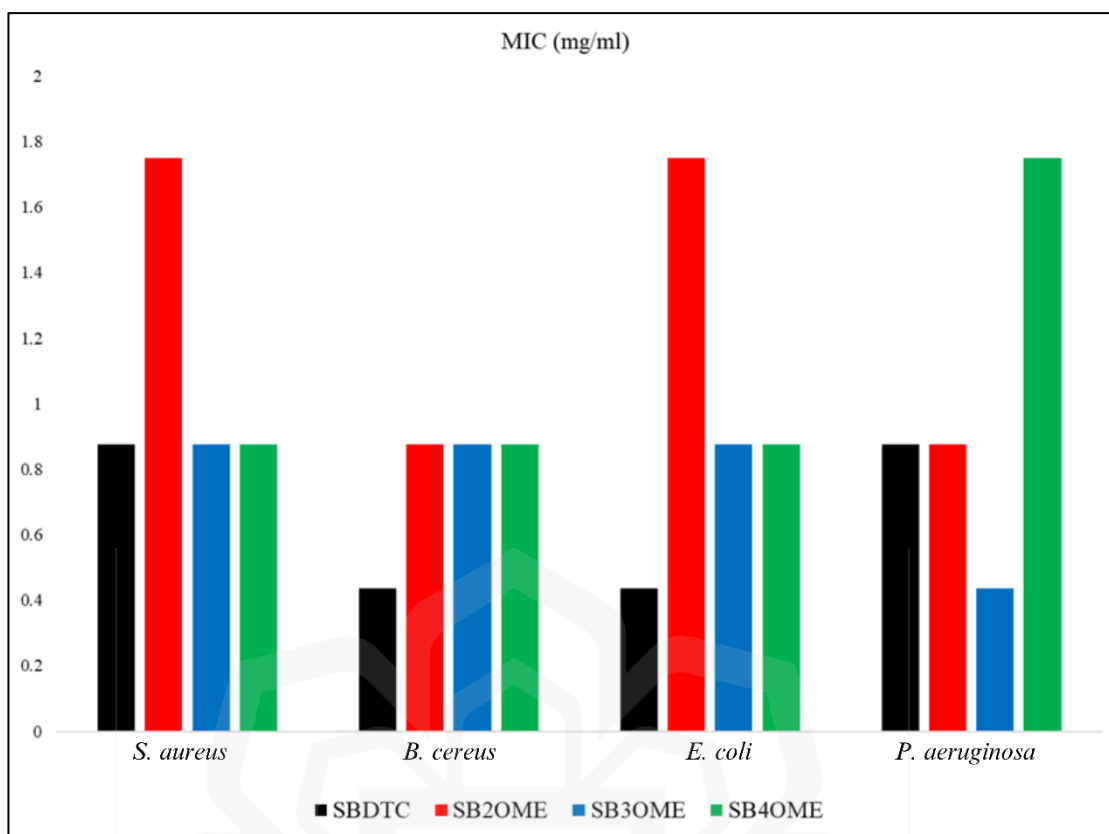


Figure 4.53 MIC values of SBDTC, SB2OME, SB3OME and SB4OME

Overall, among the isomeric derivatives, SB3OME stood out as the most promising, particularly for its activity against *P. aeruginosa*. Its enhanced bioactivity is discussed in further detail in Subsection 4.12.6.

4.12.2 Metal Complexes from SB2OME

As illustrated in Figure 4.54, in *S. aureus*, SB2OME demonstrated weak bioactivity with a MIC of 1.750 mg/mL. Upon coordination with metals, Cu(SB2OME)₂ significantly improved the bioactivity, reducing the MIC to 0.875 mg/mL. In contrast, Zn(SB2OME)₂, Co(SB2OME)₂, and Ni(SB2OME)₂ did not show any improvement, maintaining the same MIC of SB2OME. This suggests that these metal complexes did not enhance interaction with bacterial targets in this species.

For *B. cereus*, the ligand SB2OME exhibited a MIC of 0.875 mg/mL, indicating better inhibition compared to *S. aureus*. However, Cu(SB2OME)₂, Co(SB2OME)₂, and Ni(SB2OME)₂ all retain this MIC value, showing no significant enhancement upon coordination. In this case, the absence of a further reduction in MIC by Zn(SB2OME)₂ might be due to bacterial resistance mechanisms (Giedraitiene et al., 2011).

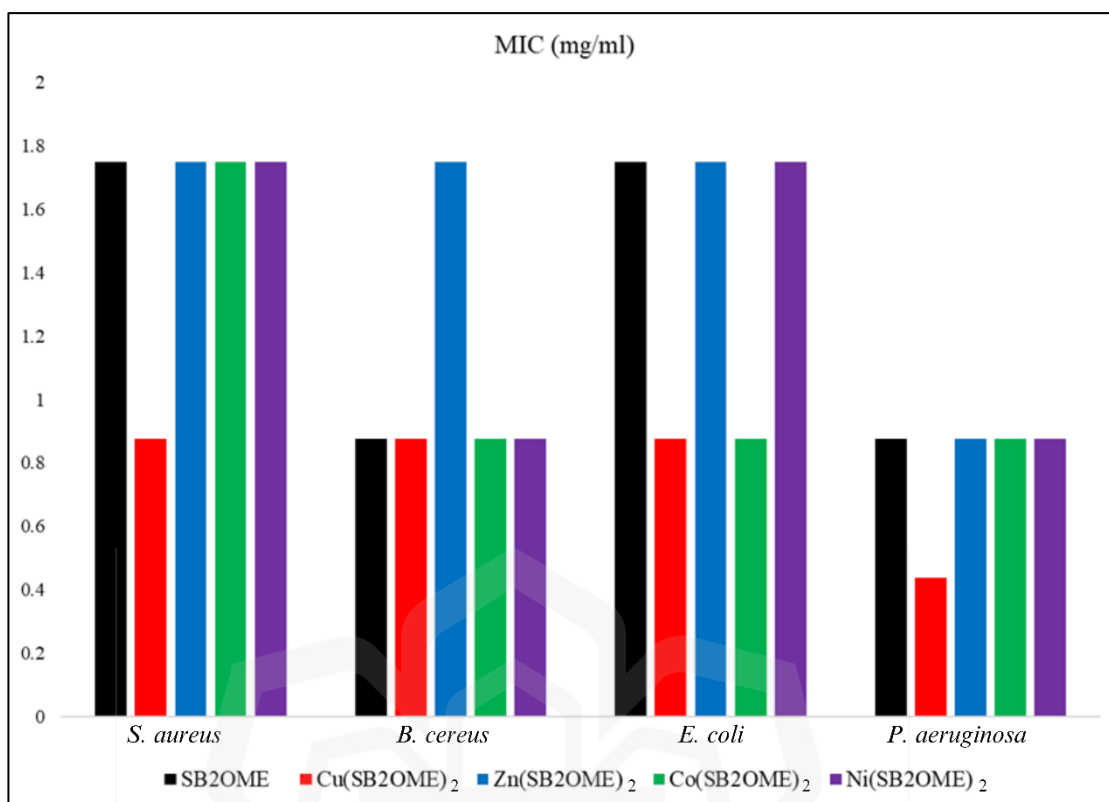


Figure 4.54 MIC values of metal complexes from SB2OME

For *E. coli*, SB2OME, as well as Zn(SB2OME)₂ and Ni(SB2OME)₂ complexes, exhibited a MIC of 1.750 mg/mL. This suggests that metal coordination of Zn(II) and Ni(II) did not enhance the ligand's bioactivity against this gram-negative bacterium. This could be due to the complex structure of the gram-negative membrane, which was further explained in Subsection 4.11.7. However, Cu(SB2OME)₂ and Co(SB2OME)₂ showed better antibacterial activity than their free ligand with MICs of 0.875 mg/mL.

In *P. aeruginosa*, SB2OME alone demonstrated a MIC of 0.875 mg/mL. Upon coordination with copper, the bioactivity is significantly enhanced, with Cu(SB2OME)₂ showing a MIC of 0.437 mg/mL. In contrast, Zn(SB2OME)₂, Co(SB2OME)₂, and Ni(SB2OME)₂ retained the MIC of 0.875 mg/mL, indicating that these metals did not improve the bioactivity of SB2OME in this species.

Overall, among the metal complexes, Cu(SB2OME)₂ showed the most significant bioactivity enhancement, particularly against *P. aeruginosa*. In contrast, Zn(SB2OME)₂, Co(SB2OME)₂ and Ni(SB2OME)₂ complexes did not significantly enhance bioactivity, with MIC values remaining similar to SB2OME.

4.12.3 Metal Complexes from SB3OME

As illustrated in Figure 4.55, against *S. aureus*, SB3OME demonstrated moderate bioactivity, with a MIC of 0.875 mg/mL. However, upon coordination with metals, all metal complexes exhibited improved bioactivity, reducing the MIC to 0.437 mg/mL. This indicates that metal coordination enhances the SB3OME's ability to interact with bacterial targets, making the metal complexes more effective inhibitors against *S. aureus*. In *B. cereus*, SB3OME also displayed a MIC of 0.875 mg/mL, similar to its effectiveness in *S. aureus*. Metal complex, however, revealed mixed effects. Cu(SB3OME)₂ retained the same MIC of 0.875 mg/mL, suggesting no further enhancement. In contrast, Zn(SB3OME)₂, Co(SB3OME)₂, and Ni(SB3OME)₂ showed reduced bioactivity, with higher MIC values of 1.750 mg/mL. Against *E. coli*, SB3OME showed a MIC of 0.875 mg/mL, and all metal complexes also maintained the same MIC of 0.875 mg/mL. Against *P. aeruginosa*, SB3OME showed a MIC of 0.437 mg/mL, while Cu(SB3OME)₂ and Zn(SB3OME)₂ also showed a MIC of 0.437 mg/mL. Co(SB3OME)₂ and Ni(SB3OME)₂ showed a MIC of 0.875 mg/mL.

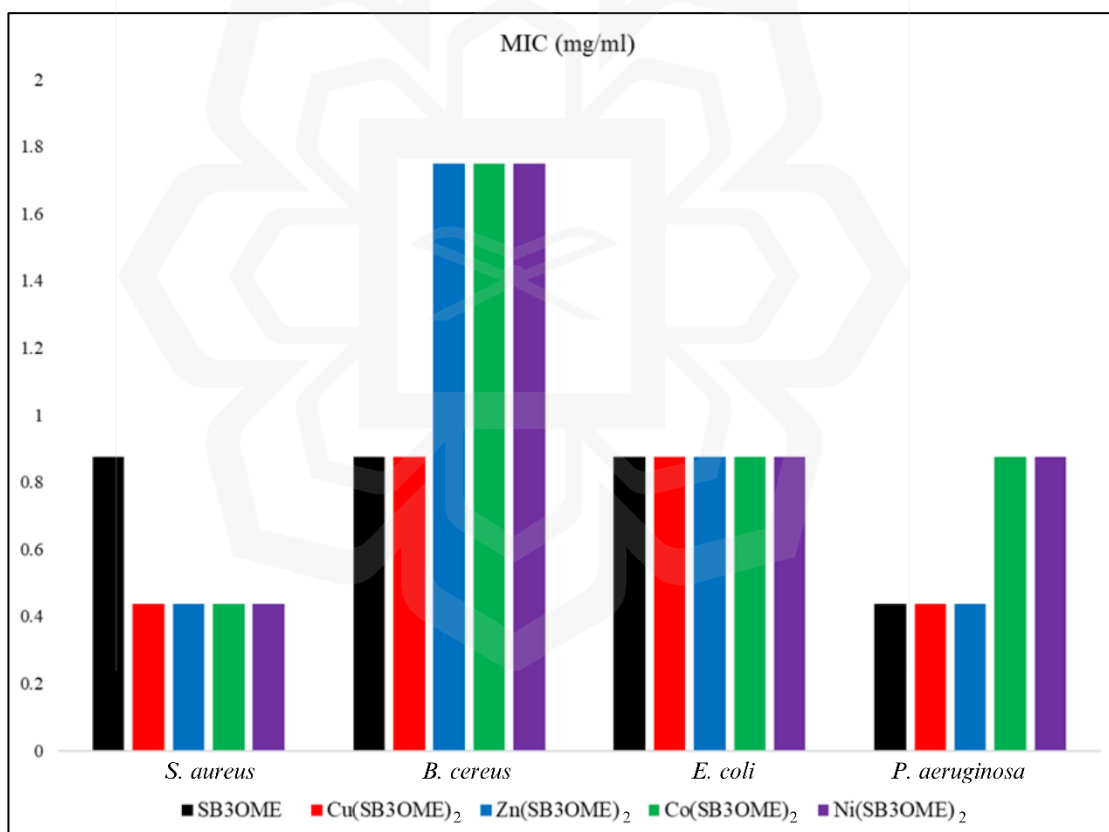


Figure 4.55 MIC values of metal complexes from SB3OME

For *E. coli*, SB3OME showed a MIC of 0.875 mg/mL, and coordination with metals did not improve bioactivity. All metal complexes from SB3OME maintain the same MIC of 0.875 mg/mL, indicating that metal coordination did not enhance SB3OME's activity against *E. coli*. Against *P. aeruginosa*, SB3OME showed good

activity with a MIC of 0.437 mg/mL. Coordination with copper and zinc did not lead to further enhancement, as $\text{Cu}(\text{SB3OME})_2$ and $\text{Zn}(\text{SB3OME})_2$ retained the same MIC value of 0.437 mg/mL. Conversely, $\text{Co}(\text{SB3OME})_2$ and $\text{Ni}(\text{SB3OME})_2$ showed reduced activity, with MIC values of 0.875 mg/mL. This suggests that these metals hinder the SB3OME's ability to inhibit *P. aeruginosa*, possibly due to resistance mechanisms or limited permeability in gram-negative bacteria (Giedraitiene et al., 2011).

Overall, all metal complexes from SB3OME stood out as the most effective inhibitor, particularly against *S. aureus*. However, the impact of metal complexes is less pronounced in gram-negative bacteria like *E. coli*, suggesting that they are more suited for inhibiting gram-positive bacteria.

4.12.4 Metal Complexes from SB4OME

The bioactivity of SB4OME and its metal complexes against gram-positive and gram-negative bacteria exhibits varying degrees of inhibition (Figure 4.56). Against *S. aureus*, SB4OME demonstrated moderate activity with a MIC of 0.875 mg/mL. Coordination with metals significantly enhanced its antibacterial effect, as metal complexes reduced the MIC to 0.437 mg/mL, indicating a two-fold improvement in inhibition. This suggests that the metal complex enhanced SB4OME's interaction with *S. aureus*, resulting in stronger bioactivity. In contrast, against *B. cereus*, SB4OME also showed moderate activity with a MIC of 0.875 mg/mL. However, unlike in *S. aureus*, the metal complex did not improve the bioactivity, as all metal complexes retained the MIC value of 0.875 mg/mL.

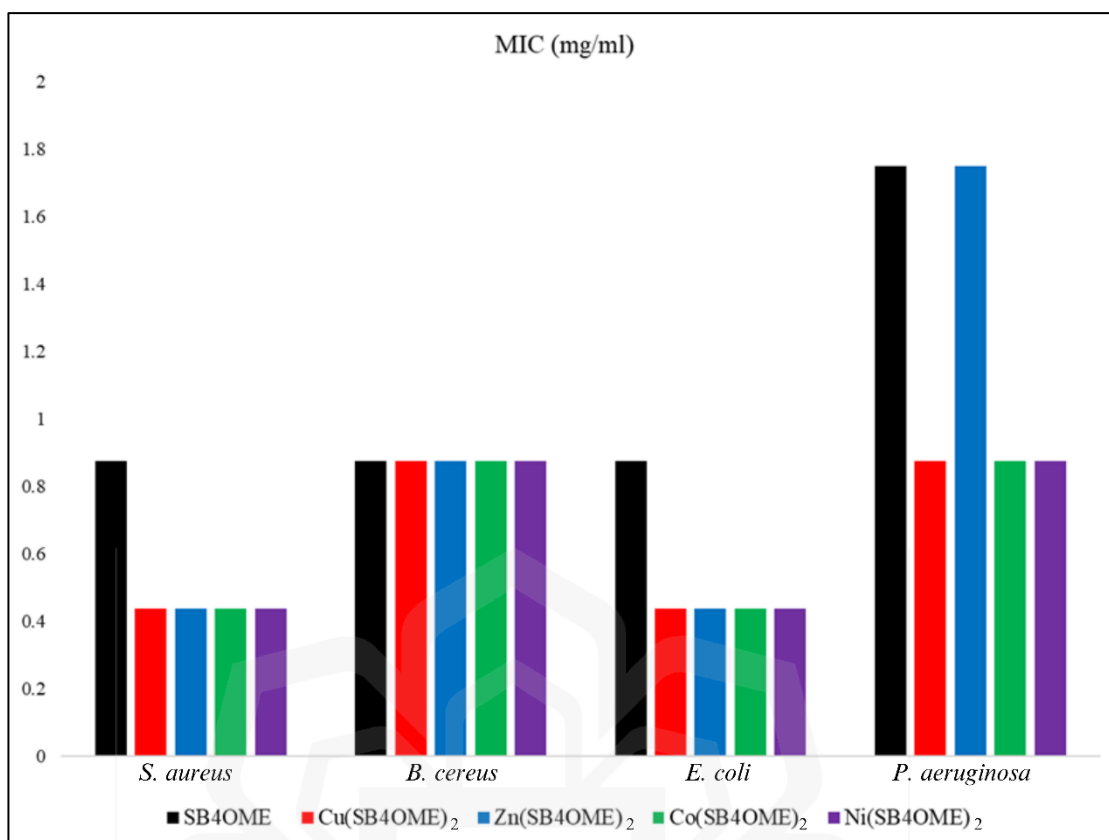


Figure 4.56 MIC values of metal complexes from SB4OME

The activity of SB4OME and its metal complexes against gram-negative bacteria also varies. Against *E. coli*, SB4OME exhibited moderate activity with a MIC of 0.875 mg/mL. Fortunately, complexation with metals resulted in a two-fold enhancement, reducing the MIC to 0.437 mg/mL for all metal complexes. This suggested that the metal complexes enhanced SB4OME's ability to penetrate the outer membrane of *E. coli* and effectively inhibit bacterial growth. For *P. aeruginosa*, SB4OME showed weak inhibition with a MIC of 1.750 mg/mL. However, metal coordination with copper, cobalt, and nickel enhanced the activity, reducing the MIC to 0.875 mg/mL. Zn(SB4OME)₂ however, failed to improve the bioactivity, with the MIC remaining at 1.750 mg/mL.

In summary, the Cu(SB4OME)₂, Co(SB4OME)₂ and Ni(SB4OME)₂ exhibited the most consistent and enhanced bioactivity across all bacterial species, especially against *S. aureus* and *E. coli*. Thus, metal complexes from SB4OME showed potential as promising candidates for further optimisation, particularly due to their consistent performance across different bacterial strains.

4.12.5 SB3NO And its Metal Complexes

The activity of SB3NO and its metal complexes against both gram-positive and gram-negative bacteria showed a range of improvements and effectiveness (Figure 4.57). For *S. aureus*, SB3NO exhibited the weakest antimicrobial activity with a MIC of 3.500 mg/mL. However, metal complexes from SB3NO significantly improved this bioactivity. The complexation with zinc, cobalt, and nickel reduced the MIC to 0.875 mg/mL, representing the best result among the metal complexes tested. Cu(SB3NO)₂, reduced the MIC to 1.750 mg/mL, reflecting a notable but less pronounced improvement compared to the other metal complexes. In the case of *B. cereus*, SB3NO had a weak MIC of 1.750 mg/mL. For this bacterium, Zn(SB3NO)₂ and Co(SB3NO)₂ were particularly effective, reducing the MIC to 0.875 mg/mL. However, Cu(SB3NO)₂ and Ni(SB3NO)₂ maintained the MIC of 1.750 mg/mL, showing no enhancement over SB3NO.

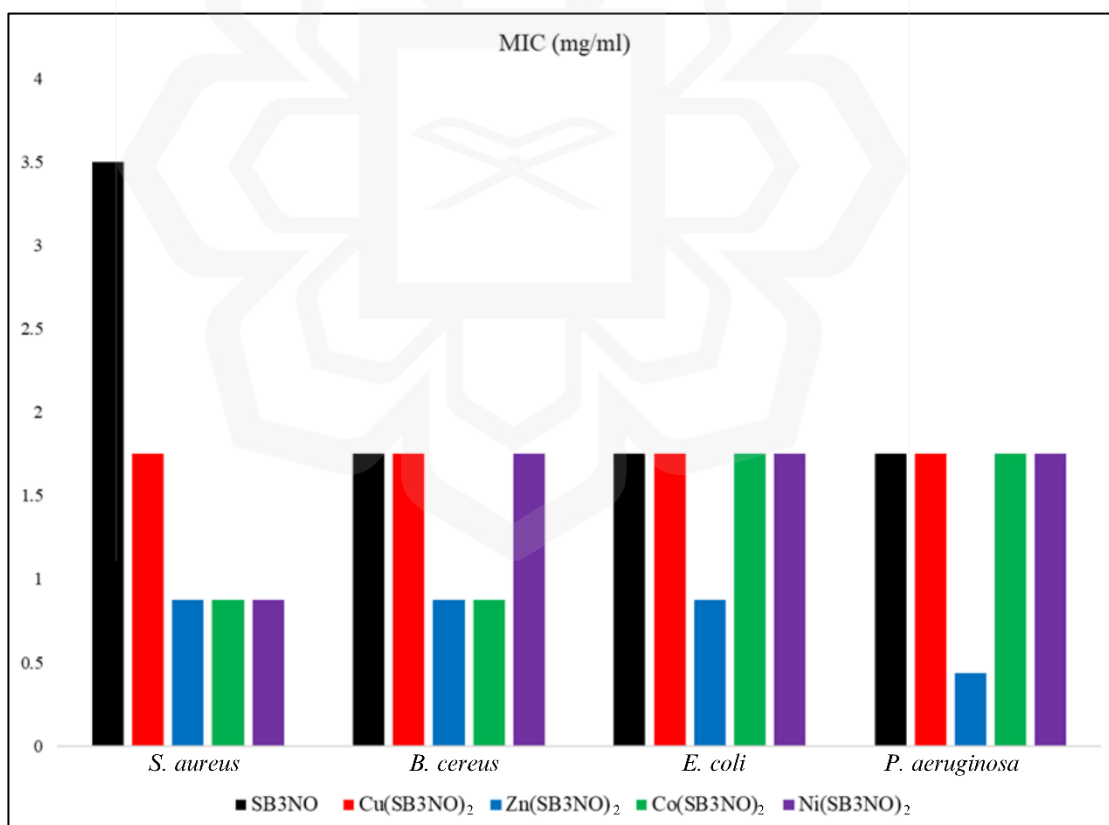


Figure 4.57 MIC values of SB3NO and its metal complexes

For gram-negative bacteria, SB3NO showed weak activity against *E. coli* with an MIC of 1.750 mg/mL. Among the metal complexes, only Zn(SB3NO)₂ significantly improved activity, reducing the MIC to 0.875 mg/mL. The Cu(SB3NO)₂, Co(SB3NO)₂

and $\text{Ni}(\text{SB3NO})_2$ did not enhance the activity, retaining the MIC of 1.750 mg/mL. Against *P. aeruginosa*, SB3NO had a MIC of 1.750 mg/mL, indicating weak inhibition. $\text{Zn}(\text{SB3NO})_2$ achieved the most significant improvement with a MIC of 0.437 mg/mL, a four-fold reduction compared to SB3NO. The $\text{Cu}(\text{SB3NO})_2$, $\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$ did not show any enhancement, maintaining the MIC of 1.750 mg/mL.

In summary, among the metal complexes of SB3NO, $\text{Zn}(\text{SB3NO})_2$ proved to be the most effective inhibitor, consistently reducing MIC values across all tested bacteria. The observed trend in antibacterial activity followed the order; $\text{Zn}(\text{SB3NO})_2 > \text{Co}(\text{SB3NO})_2 > \text{Ni}(\text{SB3NO})_2 > \text{Cu}(\text{SB3NO})_2$.

4.12.6 Comparison Antibacterial Effectiveness of Synthesised Compounds with Trimethoprim

Given that this study aims to design potential drug candidates as alternatives to TMP, therefore the antibacterial activity of the synthesised compounds was compared to TMP. Although antibacterial activity was assessed against four positive controls (antibiotic) including TMP, novobiocin, ampicillin and gentamicin but only the comparison with TMP is discussed in detail.

For *S. aureus*, TMP exhibited good activity with a MIC of 0.437 mg/mL. Among the synthesised compounds, the metal complexes of SB3OME and SB4OME showed the strongest activity, achieving MIC values of 0.437 mg/mL, matching the efficacy of TMP. However, other derivatives displayed moderate to weak activity, with MIC values ranging from 0.875 to 1.750 mg/mL, indicating that they were less potent than TMP. Against *B. cereus*, TMP recorded MIC values of 0.437 mg/mL. Among the synthesised compounds, SB4OME and its metal complexes demonstrated the strongest activity, with MIC values of 0.875 mg/mL. While this represents significant antibacterial potential, it remains weaker than the performance of TMP.

In the case of *E. coli*, TMP showed weak efficacy with a MIC of 1.750 mg/mL. Interestingly, most of the synthesised compounds surpassed the activity of TMP. Particularly, SB4OME and its metal complexes had MIC values between 0.437 and 0.875 mg/mL, thus demonstrating superior effectiveness against this strain. For *P. aeruginosa*, again, TMP showed weak activity with an MIC of 1.750 mg/mL. Among the synthesised compounds, the metal complexes of SB2OME and SB3OME

demonstrated significant antibacterial activity, with MIC values ranging from 0.437 mg/mL to 0.875 mg/mL, outperforming TMP.

Overall, several synthesised compounds in this study demonstrated superior antibacterial effectiveness compared to TMP, highlighting their potential as stronger antibacterial agents, particularly as DHFR inhibitors. Notably, TMP showed lower antibacterial activity than several synthesised compounds, especially against gram-negative bacteria. Its lower efficacy had been documented in previous studies (Kemnic & Coleman, 2022; Manna et al., 2021; L. Yu, 2024). TMP was often used in combination with other antibiotics, notably sulfamethoxazole, to enhance its therapeutic effect.

For example, the introduction of Bactrim, a synergistic combination of TMP and sulfamethoxazole, marked a significant milestone in antibacterial treatment (Vilchèze & Jacobs, 2012). This combination targets two sequential steps in the folate biosynthesis pathway, resulting in enhanced efficacy. Bactrim has been widely used to treat a range of infections caused by both gram-positive bacteria (e.g., skin and soft-tissue infections, pneumonia) and gram-negative bacteria (e.g., urinary tract infections) (Bushby, 1973; Forgacs et al., 2009). To this day, TMP and sulfamethoxazole remain one of the few truly synergistic antibacterial combinations and the only clinically approved antifolates for treating bacterial infections (Kemnic & Coleman, 2022).

4.12.7 Structure-Activity Relationship (SAR)

To investigate the correlation between biological activity and the structures of the newly synthesised SBDTC derivatives and their metal complexes, a Structure-Activity Relationship (SAR) analysis was conducted. SAR examines how a molecule's chemical structure influences its biological activity (Tong et al., 2003). This approach serves as a strategic method for designing more effective drug candidates.

4.12.7.1 Structure-Activity Relationship (SAR) of SBDTC Derivatives

SAR of the SBDTC derivatives was assessed by comparing the antibacterial activity of SB2OME, SB3OME, SB4OME, and SB3NO to their respective pharmacophores, as summarised in Figure 4.58. The SAR analysis revealed that the methoxy substituent group generally demonstrated better antibacterial performance compared to the chloro

and nitro substituent groups. This is evidenced by the MIC values, where SB2OME, SB3OME, and SB4OME (with MIC of 0.875 mg/mL) outperformed SB3NO (with MIC of 3.500 mg/mL) against *S. aureus*. Similar observations have been reported by Palmer et al (2010) and Shen et al. (2007), who found that methoxy groups showed superior activity compared to nitro and chloro substitutions (Palmer et al., 2010; Shen et al., 2007).

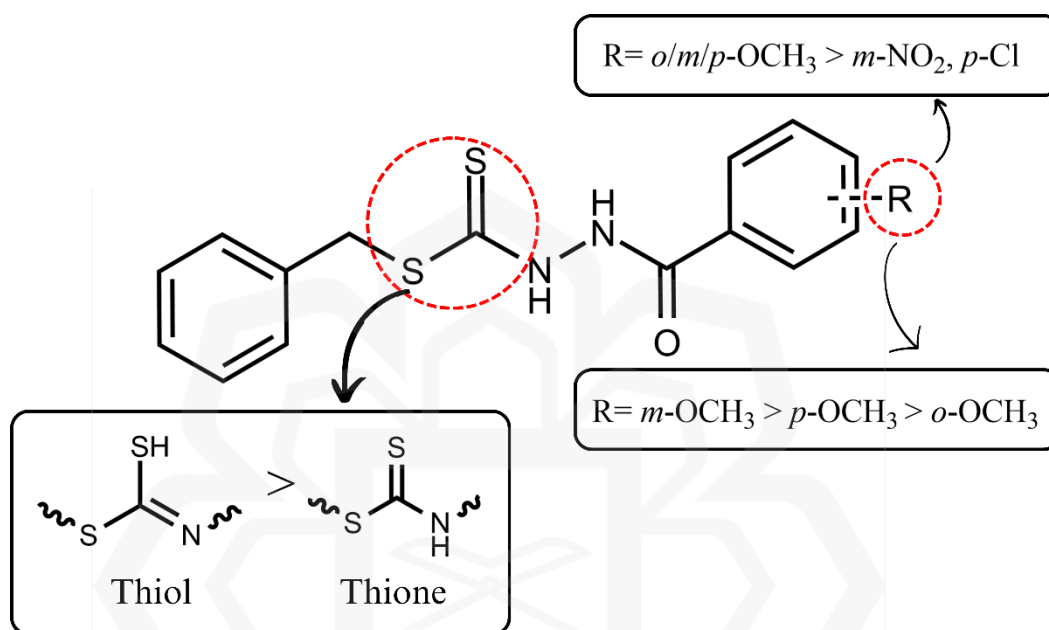


Figure 4.58 SAR studies of SBDTC derivatives

Among the isomeric methoxy substituent group, the meta position group demonstrated better antibacterial activity than the ortho and para positions. For example, SB3OME exhibited an MIC of 0.437 mg/mL against *P. aeruginosa*, while SB2OME and SB4OME showed MIC values of 0.875 mg/mL and 1.750 mg/mL, respectively. This finding contrasts with other past studies, which typically report that para-substitution provided better activity than ortho and meta substitutions. Yin et al. (2012) found higher activity for para-substituted Schiff bases due to optimal electronic and spatial properties for bacterial interaction (Yin et al., 2012). Similarly, Break et al. (2013) noted that para-substituted compounds exhibit enhanced antibacterial activity due to improved electronic distribution, which enhances interactions with bacterial targets.

However, it is important to note that SBDTC derivatives may exhibit tautomerism in their liquid form. Given that the analysis was performed in liquid, the

compounds likely exist in their tautomeric forms. NMR analysis confirmed that SB3OME remained predominantly in the thiol form, SB4OME in the thione form, and SB2OME and SB3NO existed as a mixture of thione and thiol forms. This study demonstrated that superior performance by SB3OME might be due to its thiol form. Previous studies depicted that the dynamic tautomeric equilibrium between thione and thiol forms allows compounds to adapt to different binding environments, thus affecting antibacterial efficacy (Bitu et al., 2020). As reflected in research by Rani et al. (2023), the thiol form provides better interactions compared to the thione form (Rani et al., 2023). Consistent with other studies, they observed improved antibacterial properties in compounds with the thiol tautomer due to increased adaptability in biological environments (Kemnic & Coleman, 2022; Manna et al., 2021; L. W. Yu, 2024). Therefore, the effectiveness of SB3OME can be attributed to its structural difference from other derivatives, particularly its thiol form.

Overall, these findings suggest that specific substitutions and modifications significantly impact the biological activities of the compounds. Introducing methoxy substitutions is preferable to nitro and chloro groups. Additionally, meta substitution of the methoxy group is more effective than other positions, likely due to the thiol tautomeric form.

4.12.7.2 Structure-Activity Relationship (SAR) of Metal Complexes

The MIC results above clearly demonstrated that metal complexes consistently achieved lower MIC values than their free ligand against all tested bacterial strains, highlighting their superior antibacterial potency. For instance, while SB2OME exhibited a MIC of 1.750 mg/mL against *S. aureus*, Cu(SB2OME)₂ showed a significantly reduced MIC of 0.875 mg/mL. A similar pattern was observed for *P. aeruginosa*, where SB2OME's MIC of 0.875 mg/mL was halved to 0.437 mg/mL in its copper complex. This trend extended to other ligands and bacterial species. For example, SB3OME had a MIC of 0.875 mg/mL against *B. cereus*, while Co(SB3OME)₂ halved it to 0.437 mg/mL. These findings indicate that metal complexes consistently outperformed their free ligands and were supported by previous studies from (Khirudin et al., 2023; Md Yusof et al., 2020; Sultana et al., 2020).

The enhanced antibacterial activity of the metal complexes can be attributed to several factors. A primary contributor is the chelation effect, where the metal ion coordinates with the ligand to form a stable complex. As a result, it improved the compound's ability to penetrate bacterial cell walls (Claudel et al., 2020; Haas & Franz, 2009). Furthermore, according to Haas & Franz, (2009), this coordination reduces the complex's polarity and increases its lipophilicity, enabling it to interact more effectively with bacterial membranes, disrupt essential processes, and ultimately induce cell death (Haas & Franz, 2009). Thus, the synergistic interaction between the metal ion and the organic ligand amplifies these bactericidal effects and might target multiple pathways in bacterial metabolism which reduces the likelihood of resistance development.

Expanding on this data, the SAR of the metal complex was assessed by comparing the antibacterial activity of metal complexes from SB2OME, SB3OME, SB4OME, and SB3NO as summarised in Figure 4.59. The SAR analysis revealed that metal complexes with methoxy substituents demonstrated superior antibacterial activity compared to those with nitro and chloro substituents. For example, against *E. coli*, Co(SB2OME)₂, Co(SB3OME)₂, and Co(SB4OME)₂ exhibited MIC values of 0.875 mg/mL, 0.875 mg/mL and 0.437 mg/mL, respectively. In contrast, Co(SB3NO)₂ had a higher MIC of 1.750 mg/mL, indicating reduced potency. This trend is consistent with the SAR activity observed in SBDTC derivatives (Figure 4.58), where the isomeric methoxy compounds SB2OME, SB3OME, and SB4OME showed better activity than SB3NO.

Furthermore, among the methoxy-substituted isomers, the metal complexes derived from SB4OME exhibited the best antibacterial activity, followed by those from SB3OME and SB2OME. Specifically, against *E. coli*, the metal complexes from SB4OME and SB3OME had MIC values of 0.437 mg/mL and 0.875 mg/mL, respectively. Meanwhile, metal complexes from SB2OME ranged from 0.875 mg/mL to 1.750 mg/mL, demonstrating lower potency. Although this trend differs from the SAR study of SBDTC derivatives (Figure 4.58), it was supported by most of the previous studies (Break et al., 2013; Eldaly, 2024; Yin et al., 2012).

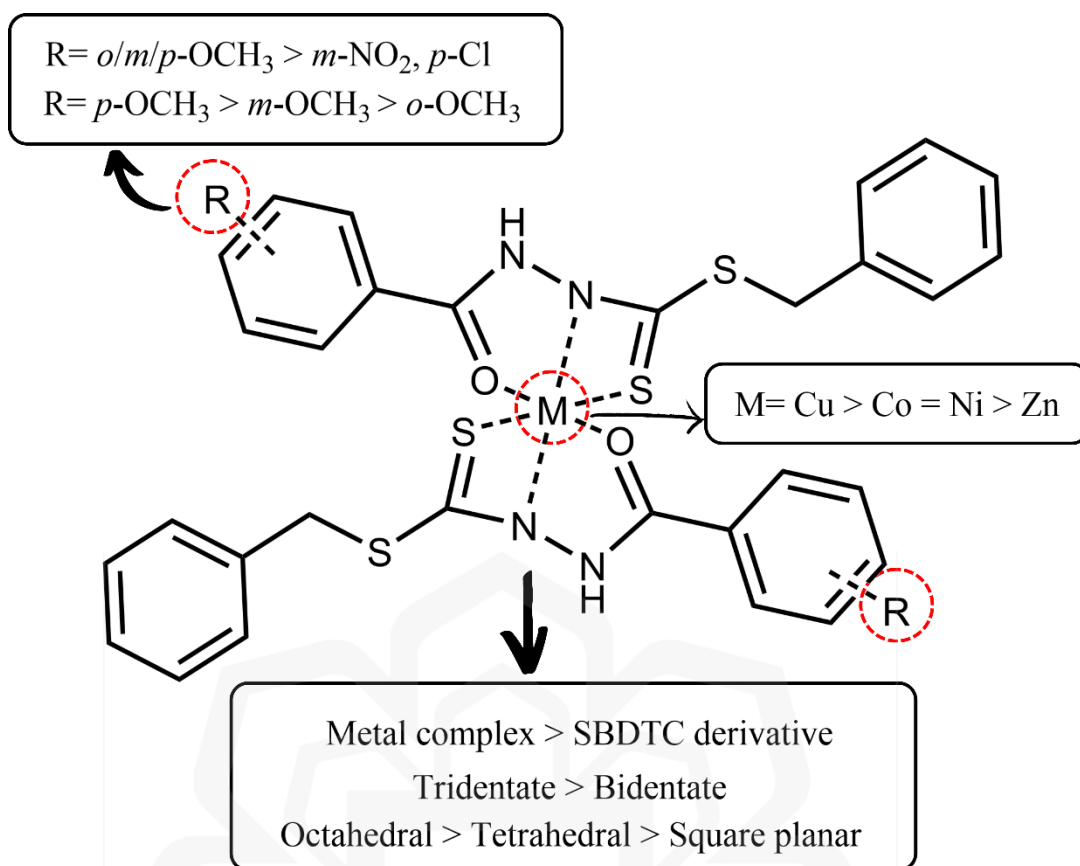


Figure 4.59 SAR studies of metal complexes from SBDTC derivatives

Among the metal complexes, the Cu(II) complex stood out as the most effective. For instance, Cu(SB4OME)₂ exhibited a MIC of 0.437 mg/mL against all tested bacterial strains, including both gram-positive and gram-negative species. Another example, Zn(SB2OME)₂ achieved a MIC of 1.750 mg/mL against *S. aureus* and *E. coli*, which, while effective, was less potent than Cu(SB2OME)₂. This was consistently the lowest MIC value across all compounds, emphasising copper's superior antibacterial properties compared to zinc, cobalt, and nickel. Gan et al. (2022) agreed that the antibacterial efficiency of copper complexes is superior to other metal ions. Although zinc and nickel complexes also showed enhanced activity relative to the SBDTC derivatives, their MIC values were generally higher.

The relationship between coordination mode and antibacterial activity was also explored. Metal complexes of SB3NO exhibited a bidentate coordination mode, whereas other metal complexes adopted a tridentate coordination mode. Tridentate coordination consistently correlated with enhanced antibacterial activity. For example, Cu(SB2OME)₂, Cu(SB3OME)₂, and Cu(SB4OME)₂, all of which feature tridentate

coordination, exhibited MIC values of 0.875, 0.875, and 0.437 mg/mL against *E. coli*, respectively. In contrast, the bidentate $\text{Cu}(\text{SB3NO})_2$ displayed a higher MIC of 1.750 mg/mL. These findings align with prior studies, such as from El-Sonbati et al. (2016), which demonstrated that tridentate Schiff base complexes exhibit stronger bacterial interactions due to their enhanced stability (El-Sonbati et al., 2016). Gatto et al., (2024) further noted that NOS donor ligands are particularly potent due to their strong electron-donating abilities and increased stability.

The electronic properties of metal complexes also appeared to influence their antibacterial activity. Majumdar et al., (2017) reported that octahedral geometries offer superior antibacterial performance compared to tetrahedral or square planar geometries, as they provide greater binding versatility. This was consistent with this study's findings, where octahedral complexes of SB2OME, SB3OME and SB4OME demonstrated higher activity than the tetrahedral or square planar geometries of SB3NO. Moreover, in this study, among metal complexes from SB3NO, only $\text{Cu}(\text{SB3NO})_2$ had square planar geometry while the others were tetrahedral. Interestingly, $\text{Cu}(\text{SB3NO})_2$ had the least performance in antibacterial activity across all bacteria. Hence, it was suggested that $\text{Zn}(\text{SB3NO})_2$, $\text{Co}(\text{SB3NO})_2$ and $\text{Ni}(\text{SB3NO})_2$ antibacterial activity might be enhanced due to electronic properties by tetrahedral, as reflected by a previous study (A. Singh et al., 2022). Therefore, this study concludes that the electronic properties, in terms of activity, follow the trend: octahedral > tetrahedral > square planar, from highest to lowest.

Other properties, including thermal stability and electrolytic behaviour, also played a role in the biological activity of the complexes. Metal complexes generally exhibit higher thermal stability than free ligands, which enhances their persistence in biological environments. According to Menczel & Prime (2008), increased thermal stability prevents degradation under physiological conditions, ensuring prolonged activity. Khalaf et al. (2024) similarly emphasised the importance of thermal stability in maintaining the structural integrity of metal complexes during antibacterial applications. Since all metal complexes in this study exhibited greater thermal stability than their corresponding SBDTC derivatives, this factor likely contributed to their superior performance. Regarding electrolytic properties, all metal complexes were neutral and non-conductive. Although this study showed limited comparisons between

different charge states, previous studies have indicated that neutral drugs generally pose lower toxicity risks. This is because they are less likely to interact with charged biomolecules, thereby reducing potential side effects (S. Sharma et al., 2021).

In conclusion, the metal complexes of copper, zinc, cobalt, and nickel demonstrated markedly enhanced antibacterial activity compared to their SBDTC derivatives, as evidenced by significantly lower MIC values. Among the metals, copper complexes consistently exhibited the greatest potency. Furthermore, factors such as coordination mode, electronic properties, thermal stability, and electrolytic properties were found to influence antibacterial efficacy. Overall, these findings highlight the potential of metal complexes as powerful tools in combating bacterial infections.

4.12.8 Gram-Positive vs Gram-Negative Bacteria

The data revealed a notable trend in the antimicrobial activity of the synthesised compounds, with a stronger inhibitory effect observed against gram-positive bacteria, such as *S. aureus* and *B. cereus*, compared to gram-negative bacteria, like *E. coli* and *P. aeruginosa*. For instance, SB2OME exhibited a MIC of 1.750 mg/mL against *E. coli* (a gram-negative bacterium) but showed a more effective MIC of 0.875 mg/mL against *B. cereus* (a gram-positive bacterium), indicating it was twice as effective against the gram-positive strain.

Similarly, Co(SB3NO)₂ showed an equal MIC value of 0.875 mg/mL against both gram-positive bacteria, *S. aureus* and *B. cereus*, but a significantly higher MIC of 1.750 mg/mL against *E. coli* and *P. aeruginosa* (gram-negative bacteria), suggesting reduced potency toward gram-negative bacteria. A more pronounced example was the Ni(SB3OME)₂ complex, which had a MIC of 0.875 mg/mL against both *E. coli* and *P. aeruginosa*, but showed stronger inhibition against *S. aureus* with a MIC of 0.437 mg/mL, indicating superior activity against gram-positive bacteria.

This pattern of increased effectiveness against gram-positive bacteria could be attributed to several key factors. Gram-positive bacteria had a simpler cell wall structure, primarily composed of a thick layer of peptidoglycan, which is more accessible to antimicrobial agents (Malanovic & Lohner, 2016). In contrast, gram-negative bacteria possess an additional outer membrane that acts as a barrier, reducing the permeability of compounds and hindering the penetration of many antimicrobial

agents, especially those that are large or hydrophobic (Saxena et al., 2023). Additionally, gram-negative bacteria often have efflux pumps and other resistance mechanisms that can expel or neutralise antibiotics before they reach their target, further contributing to their higher resistance (Silhavy et al., 2010).

Overall, the tested compounds exhibited stronger inhibition against gram-positive bacteria, which can be attributed to the structural differences in bacterial cell walls. This draws attention to the importance of understanding bacterial structural differences in developing new antimicrobial agents that can effectively target both gram-positive and gram-negative bacteria.

4.12.9 Selection Compounds for the Computational Study

To identify the most promising series compounds for further study, additional analysis was conducted with a focus on *E. coli* as the bacterial model. This choice was in line with the next phase of computational studies, where *E. coli* dihydrofolate reductase (DHFR) would be the target protein. By using *E. coli* for both the experimental and computational analyses, consistency between the two approaches was ensured. The results (Table 4.15) clearly showed that SB4OME and its metal complexes had better antibacterial activity compared to other series compounds. For example, SB4OME had a MIC of 0.875 mg/mL against *E. coli*, which was better than SB2OME and SB3NO, and similar to SB3OME.

Interestingly, the metal complexes from SB4OME also showed even better antibacterial activity than metal complexes from other series. Cu(SB4OME)₂, for example, had a MIC of 0.437 mg/mL against *E. coli*, which was much lower than the MICs for Cu(SB2OME)₂ and Cu(SB3OME)₂, both of which had MICs of 0.875 mg/mL. This significant improvement in activity suggested that the copper complex with SB4OME was much more effective at fighting bacteria compared to SB2OME and SB3OME. Similarly, Zn(SB4OME)₂ showed an MIC of 0.437 mg/mL, whereas Zn(SB2OME)₂ had an MIC of 1.750 mg/mL, making the zinc complex with SB4OME about four times more effective. Co(SB4OME)₂ also had a better MIC of 0.437 mg/mL compared to Co(SB3NO)₂, which had a MIC of 1.750 mg/mL. Lastly, Ni(SB4OME)₂ showed the lowest MIC observed among all nickel complexes, at 0.437 mg/mL.

Given their outstanding bioactivity, SB4OME and its Cu(II), Zn(II), Co(II) and Ni(II) metal complexes were selected for further computational studies to explore their potential mechanisms of action at the molecular level. Although these compounds showed promising antimicrobial activity, the precise mechanisms by which they inhibit bacterial growth remain still not completely clear. To elucidate these mechanisms, *in-silico* approaches, including molecular docking and Molecular dynamics (MD) simulations, have been employed. By combining experimental results with detailed molecular insights, these studies offered crucial information that would be hard to get through experiments alone.

4.13 MOLECULAR DOCKING

4.13.1 Docking and Binding Site Validation

Before conducting docking studies, several validations must be performed to ensure the reliability and accuracy of the docking algorithm in predicting ligand binding poses. Two primary validation techniques were employed which are redocking and blind docking, each serving different purposes in assessing the performance of the docking algorithm (Reynisson & Söhnle, 2022).

The first step involved redocking the co-crystallised ligand, folic acid (FOL), to validate the docking methodology and parameters. The redock DHFR-FOL complex produced a binding energy of -12.72 kcal/mol with a Root Mean Square Deviation (RMSD) of 1.303 Å. As shown in Figure 4.60, the superimposition of the predicted (yellow) and experimental (green) positions demonstrates the docking accuracy. According to Bursal et al. (2021), a satisfactory redocking result is obtained when the atomic deviation between the redocked and crystal ligand coordinates is below 2 Å. Thus, the redocking results confirm the ability of the docking parameters to accurately reproduce the conformations of the crystallised ligand.

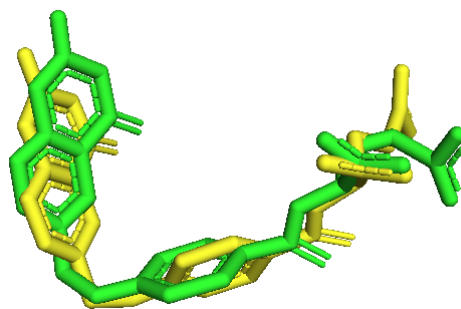


Figure 4.60 Superimposition of the predicted (yellow) and experimental (green) positions of FOL

Additionally, validation of the binding sites was carried out through blind docking. During blind docking, the ligand is docked into various regions of the protein surface, and the resulting poses are evaluated based on their proximity to known binding sites. As illustrated in Figure 4.61, localisation of FOL (green) from blind docking was consistent with the local docking result (red). This further supports the conclusion that the local docking site was well-selected and likely represents a robust binding region on the protein.

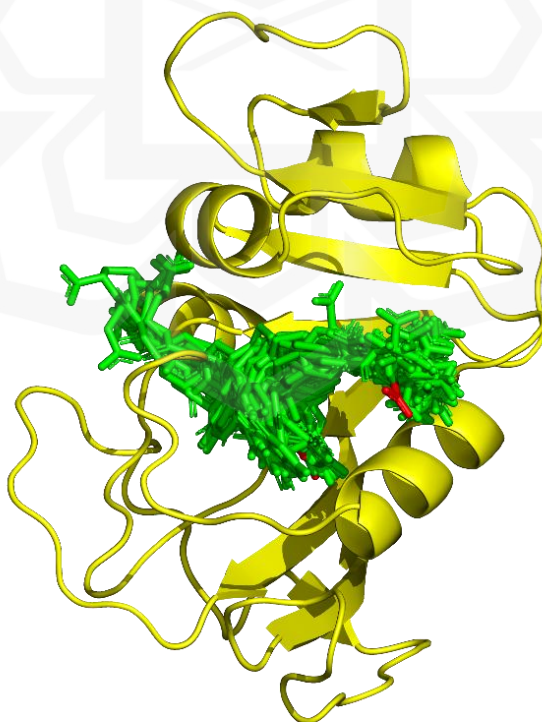


Figure 4.61 Localisation of FOL from blind docking (green) with the local docking result (red)

4.13.2 Binding Energies and Binding Interactions

During molecular docking, several potential binding orientations and conformations of the ligand within the binding site of the target protein were assessed (Mohanty & Mohanty, 2023). The primary aim was to discover the interactions that contributed to the remarkable inhibitory activity of these compounds against bacterial targets. These interactions (Figure 4.63-4.68) predominantly were hydrogen bonds (green) and hydrophobic interactions (pink). Table 4.16 summarises the results of the binding energies and interactions for the selected compounds, including SB4OME (SB4) and its metal complexes including Cu(SB4OME)₂ (CUSB4), Zn(SB4OME)₂ (ZNSB4), Co(SB4OME)₂ (COSB4) and Ni(SB4OME)₂ (NISB4) as well as the control ligand (FOL) and the commercial antibiotic, trimethoprim (TMP). The localisation of all compounds at *E. coli* DHFR were illustrated in Figure 4.62. Meanwhile, their binding energy data, presented in triplicate, can be found in Appendix 23 and 24.

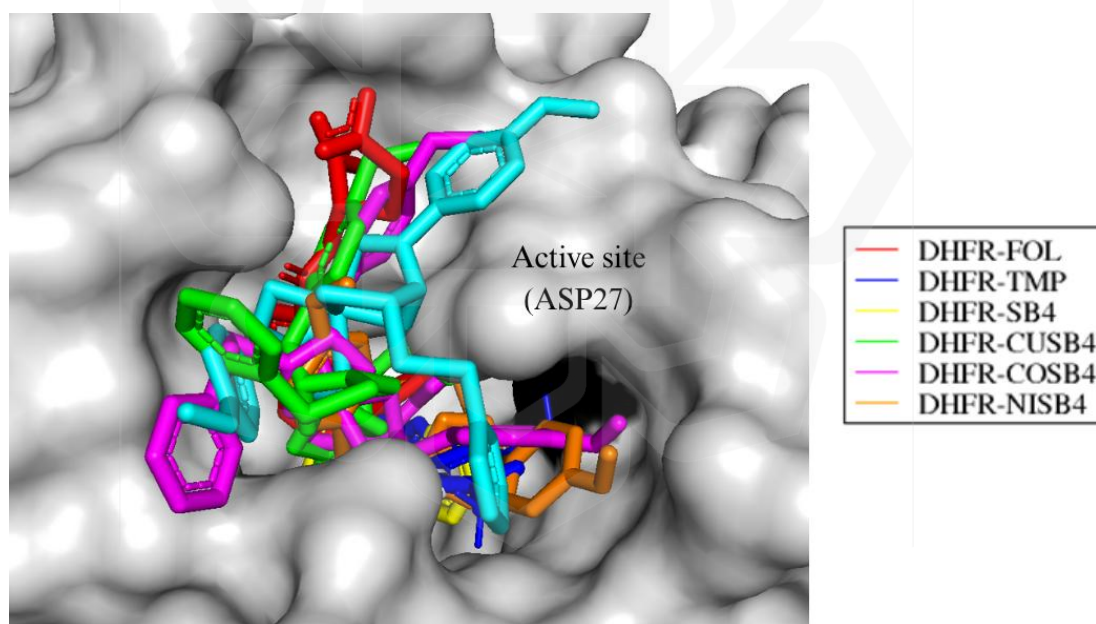


Figure 4.62 Localisation of all compounds at *E. coli* DHFR from molecular docking

Table 4.16 AutoDock results show binding energy, interaction type, and interacting amino acid residue at *E. coli* DHFR

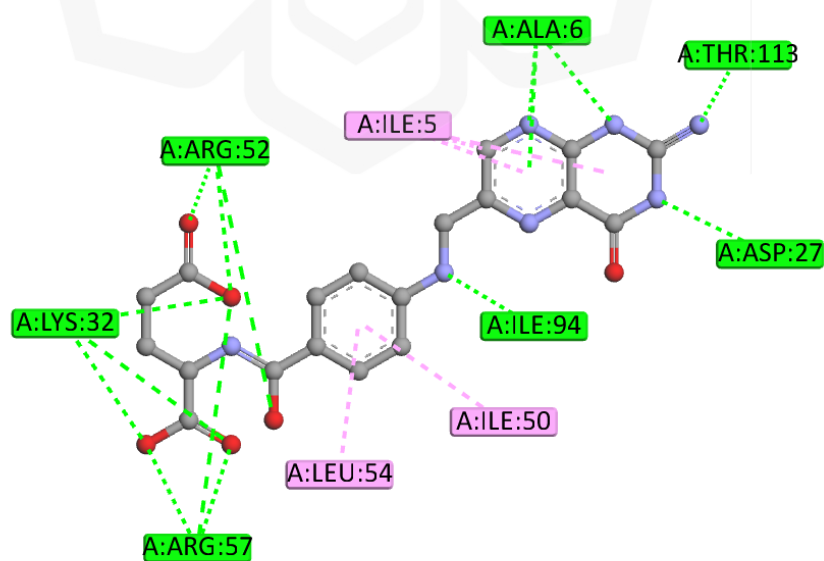
Complex	Binding energy (kcal/mol)	Hydrogen bond interaction	Hydrophobic bond interaction
DHFR-FOL (co-crystal)	-	ILE5, ALA6, ASP27, LYS32, ARG52, ARG57, THR113	ALA7, PHE31, ILE50
DHFR-FOL (redocked)	-12.72	ALA6, ASP27, LYS32, ARG52, ARG57, ILE94, THR113	ILE5, ILE50, LEU54
DHFR-TMP	-6.99	GLU17, TRP22, ASP27, TYR100	ILE5, ALA6, ALA7, ILE14, MET20, LEU28, PHE31, ILE94
DHFR-SB4	-6.92	ILE5, ALA7, TRP22, ILE94, TYR100	ILE14, LEU28
DHFR-CUSB4	-8.72	GLU17	ILE5, ALA7, ILE14, MET16, LEU28, LYS32, ARG52, LEU54
DHFR-ZNSB4	-7.24	ARG52	LEU28, ALA29, PHE31, LYS32, ILE50, ILE94
DHFR-COSB4	-9.30	LEU24, ARG52	ALA7, LEU28, PRO25, PHE31, LYS32, ILE50
DHFR-NISB4	-10.92	GLU17, LEU24, ARG52	ALA7, ILE14, MET16, LEU28, PHE31, ILE50, ILE94

Among all the compounds, redocked FOL demonstrated the strongest binding energy (-12.72 kcal/mol), followed by NISB4 (-10.92 kcal/mol), COSB4 (-9.30 kcal/mol), CUSB4 (-8.72 kcal/mol), and ZNSB4 (-7.24 kcal/mol) (Table 4.16). The compounds with the lowest binding affinities were TMP and SB4, with binding energies of -6.99 kcal/mol and -6.92 kcal/mol, respectively. This trend suggested that the

inclusion of metal ions enhanced the binding affinity of SB4 derivatives to DHFR, with NISB4 showing the most promising interaction profile.

This observation was consistent with the experimental study, where metal complexes exhibited superior performance compared to TMP and SB4, supporting the DHFR inhibition as a potential mechanism for the SBDTC derivative and its metal complexes. However, despite TMP demonstrating a slightly higher affinity for DHFR than SB4, the latter compound showed better *in vitro* activity than TMP. The difference in binding energy was minimal, with only a 0.07 kcal/mol gap, indicating that factors beyond binding affinity may have contributed to SB4's superior biological activity. To gain further insight, a detailed analysis of the interactions was conducted to elucidate the key interactions responsible for their promising *in vitro* performance.

FOL (Figure 4.63), the control ligand in this study, formed seven hydrogen bond interactions with ALA6, ASP27, LYS32, ARG52, ARG57, ILE94, and THR113, in addition to three hydrophobic interactions with ILE5, ILE50, and LEU54. Meanwhile, TMP (Figure 4.63), the reference compound, exhibited crucial hydrogen bond interactions with GLU17, TRP22, and ASP27, which played a significant role in its DHFR inhibitory activity (Sehrawat et al., 2024). TMP also formed hydrophobic interactions with residues ILE5, ALA6, ALA7, ILE14, MET20, LEU28, PHE31, and ILE94.



FOL

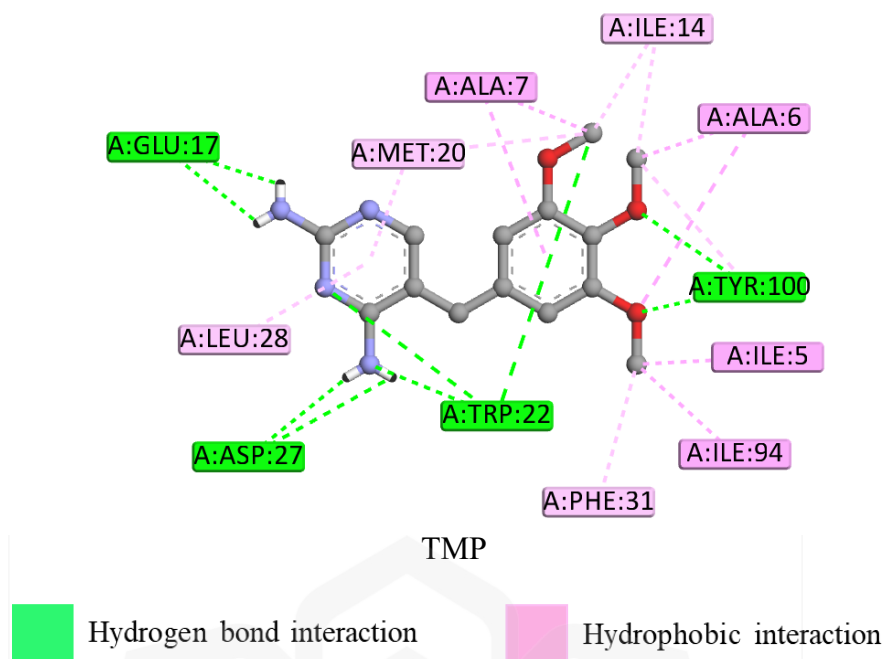


Figure 4.63 Binding interactions of FOL and TMP at *E. coli* DHFR

While FOL and TMP act as substrate and inhibitor respectively, their interactions serve as benchmarks for effective DHFR inhibitors. Both compounds formed hydrogen bonds with ASP27. In TMP, the amino group functions as a hydrogen bond donor to ASP27, as depicted in Figure 4.63. The interaction with ASP27 is significant, as it plays a crucial role in inhibiting DHFR and is pivotal for TMP's antibacterial efficacy (Al-Wahaibi et al., 2024). However, none of the investigated compounds, including SB4 and its metal complexes, formed interactions with ASP27, even though they demonstrated superior antibacterial activity than TMP. This suggests that other residues are also significant in inhibiting DHFR, aside from ASP27. Therefore, it is essential to explore the interactions that contribute to their effectiveness.

SB4 formed five hydrogen bond interactions with ILE5, ALA7, TRP22, ILE94, and TYR100, along with two hydrophobic interactions with ILE14 and LEU28 (Figure 4.64). Notably, the promising *in vitro* antibacterial activity of SB4 may be attributed to the strong hydrogen bonds formed between the para-methoxy group with TRP22, which are also involved in the binding interaction of TMP. Thus, SB4 is said to successfully mimic TMP as a DHFR inhibitor. Furthermore, the presence of the para-methoxy substitution group was significant in the design of SB4.

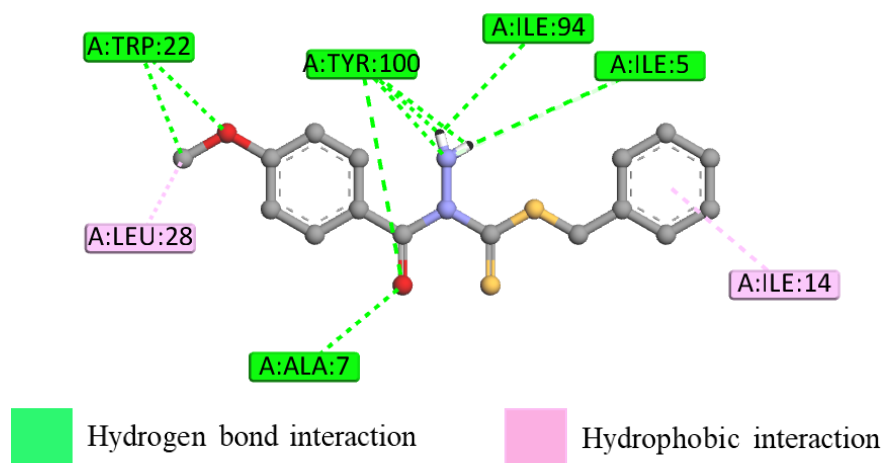


Figure 4.64 Binding interactions of SB4 at *E. coli* DHFR

Numerous studies have shown that a higher number of hydrogen bonds generally correlates with higher binding efficiency due to their strength. Nevertheless, for DHFR inhibition, an increased number of hydrophobic interactions enhances its effectiveness (Ghany et al., 2024). However, SB4 exhibited fewer hydrophobic interactions compared to TMP. Therefore, the relatively low number of hydrophobic interactions in SB4 was expected to contribute to its comparatively lower binding affinity than TMP.

On the other hand, CUSB4 formed a hydrogen bond only with GLU17 (Figure 4.65), which is also observed in the interaction of TMP. This hydrogen bond interaction arises due to the presence of the amino group in the SBDTC scaffold, highlighting the significance of the amino group in mimicking the 2,4-aminopyridine moiety pharmacophore of TMP. CUSB4 also formed numerous hydrophobic interactions with residues such as ILE5, ALA7, ILE14, MET16, LEU28, LYS32, ARG52, and LEU54. Notably, four of these residues were also involved in interactions with TMP. Therefore, aside from the hydrogen bond with GLU17, CUSB4 was further stabilised by hydrophobic interactions, particularly with LEU28.

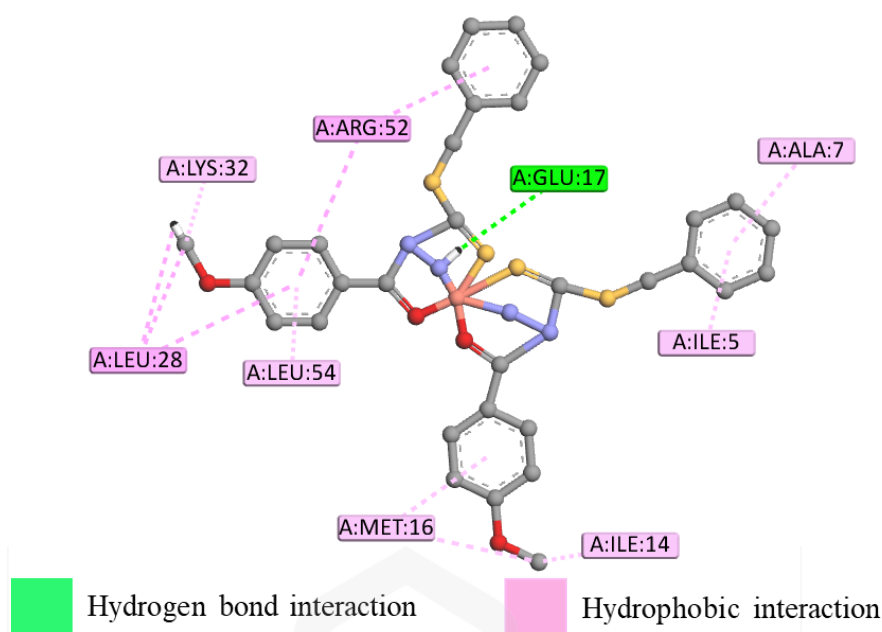


Figure 4.65 Binding interactions of CUSB4 at *E. coli* DHFR

According to past studies, TMP was stabilised by hydrophobic interactions between the aromatic ring of the 3,4,5-trimethoxybenzyl moiety with LEU28 and PHE31 (Jorba et al., 2021; Pavithra et al., 2024; Wróbel, Maliszewski, et al., 2019). These interactions contribute to the lipophilicity of TMP, enhancing its permeability through bacterial membranes. As a result, CUSB4, aided by these hydrophobic interactions, is more readily able to diffuse through the bacterial membrane, which explains its superior performance in the experimental assays.

Similar to CUSB4, ZNSB4 formed only one hydrogen bond, which is with ARG52 (Figure 4.66). This hydrogen bond is formed between the carbonyl group and the sulfur atom, demonstrating that both contribute to the antibacterial activity of the metal complex. Although ARG52 was not observed in DHFR-TMP, it was present in DHFR-FOL, suggesting its significance in ligand binding towards DHFR. According to Jays et al. (2019), ARG52 is a key active site residue for DHFR inhibitors. ZNSB4 also exhibited six hydrophobic interactions, primarily with residues LEU28, ALA29, PHE31, LYS32, ILE50, and ILE94, three of which are also found in TMP. These interactions suggest that the ZNSB4 mimics TMP as a DHFR inhibitor by forming crucial hydrophobic interactions with LEU28 and PHE31, further supporting its potential to inhibit DHFR.

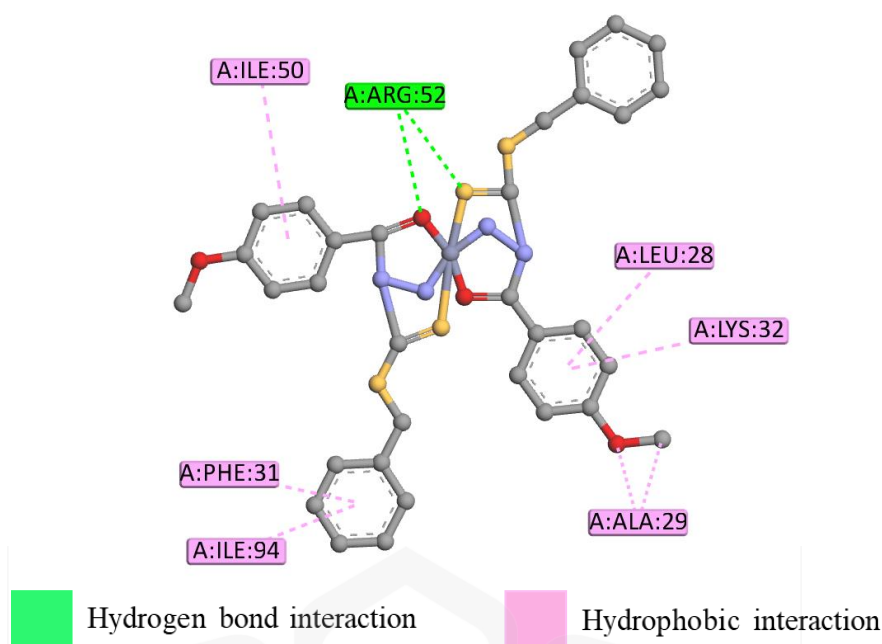


Figure 4.66 Binding interactions of ZNSB4 at *E. coli* DHFR

COSB4 (Figure 4.67) exhibited two hydrogen bonds, one with LEU24 and the other with ARG52. Notably, only the ARG52 residue was present in the interactions of DHFR-FOL, with the sulfur atom contributing to the bond formation. This indicates that COSB4 effectively mimics FOL and functions as a DHFR inhibitor, largely due to the involvement of the sulfur atom. Similar to ZNSB4, COSB4 also formed six hydrophobic interactions with ALA7, LEU28, PRO25, PHE31, LYS32, and ILE50. Previous studies have indicated that ALA7, LEU28, PHE31, and ILE50 are critical residues involved in the effective inhibition of DHFR by TMP (Wróbel, Arciszewska, et al., 2019). Therefore, COSB4 likely enhances its DHFR inhibitory activity not only through hydrogen bond interactions but also through stable hydrophobic interactions, similar to those observed in the DHFR-CUSB4 and DHFR-ZNSB4.

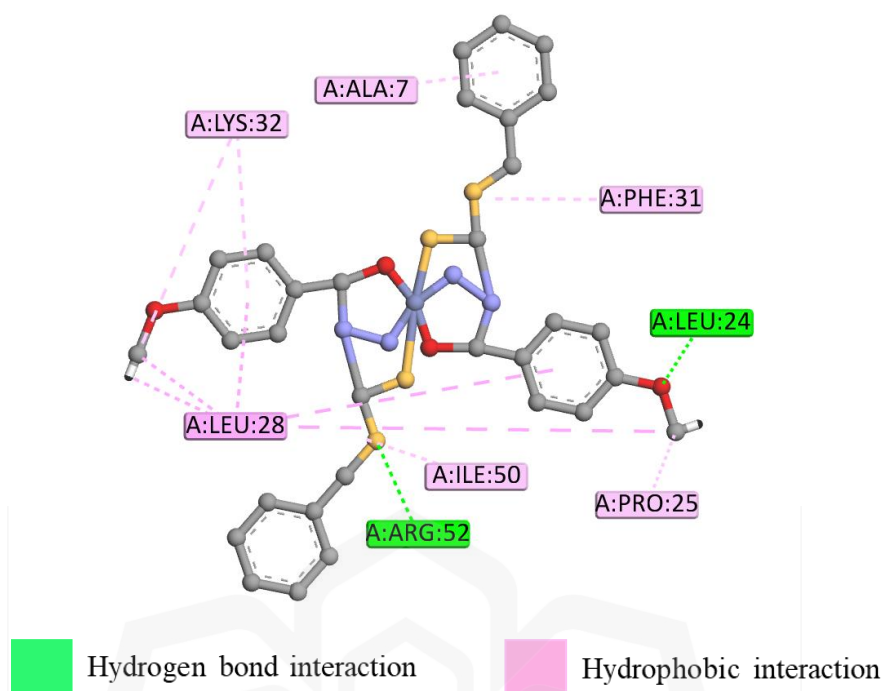


Figure 4.67 Binding interactions of COSB4 at *E. coli* DHFR

Among the compounds investigated, NISB4 exhibited the best binding affinity for DHFR, despite its experimental assay results being similar to those of the other metal complexes. This superior binding affinity is likely attributed to its highest number of hydrogen bond interactions, combined with a significant number of hydrophobic interactions (Figure 4.68). NISB4 formed three hydrogen bond interactions with GLU17, LEU24, and ARG52. Among these, GLU17 was also present in TMP's binding, while ARG52 was found in FOL's binding. Similar to CUSB4, GLU17 formed a hydrogen bond with the amino group of NISB4, pointing out the importance of the amino group in the design of DHFR inhibitors. The other two hydrogen bond interactions were formed via the para-methoxy substitution group, which enhances the affinity of NISB4 at the active site of DHFR.

Additionally, NISB4 displayed seven hydrophobic interactions with ALA7, ILE14, MET16, LEU28, PHE31, ILE50, and ILE94, five of which are also found in TMP's interactions. Therefore, both hydrogen and hydrophobic interactions in NISB4 are comparable to those observed in TMP.

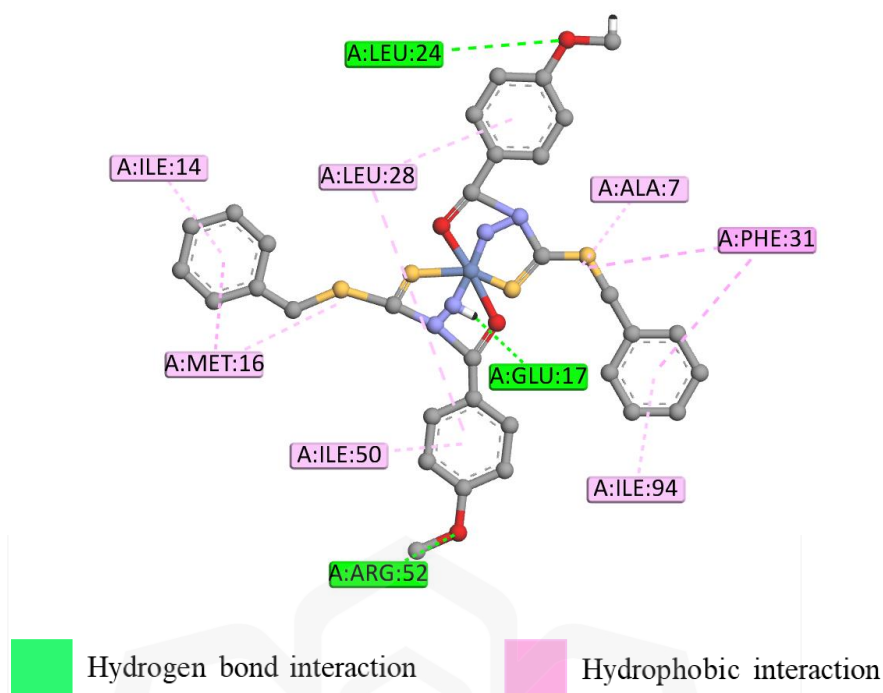


Figure 4.68 Binding interactions of NISB4 at *E. coli* DHFR

Overall, the superior MIC activity of TMP is primarily attributed to its critical hydrogen bond interaction with ASP27, which is a key feature absent in all SB4 and metal complexes. However, the interactions of CUSB4 and NISB4 with GLU17, as well as ZNSB4 and COSB4 with ARG52, coupled with their hydrophobic interactions with LEU28, PHE31, and ILE50, highlighted the potential of these compounds as promising DHFR inhibitors.

4.14 MOLECULAR DYNAMICS SIMULATION

Molecular dynamics (MD) simulations were performed to evaluate the stability and behaviour of the protein-ligand complexes over a 100 ns simulation period. All the systems studied were listed in Table 3.11. In this study, several analyses were conducted to understand the complexes' behaviour in detail, including root mean square deviation (RMSD), radius of gyration (Rg), root mean square fluctuation (RMSF), pocket volume analysis, hydrogen bond (H-bond) analysis, molecular mechanics Poisson-Boltzmann surface area (MMPBSA) calculations, and binding interaction studies. Each of these analyses is presented and discussed in detail to provide a comprehensive evaluation of the protein-ligand systems.

4.14.1 Root Mean Square Deviation (RMSD)

Root mean square deviation (RMSD) analysis was conducted to examine the stability and conformational changes of the protein-ligand complexes throughout the simulation over time. The results are summarised in Table 4.17, with the corresponding plots illustrated in Figure 4.69.

Table 4.17 RMSD data for the investigated compounds at *E. coli* DHFR

System	RMSD (nm)	RMSD of the ligand (nm)
DHFR-apo	0.1018 ± 0.0138	-
DHFR-FOL	0.1524 ± 0.0150	0.1790 ± 0.0692
DHFR-TMP	0.1479 ± 0.0130	0.1129 ± 0.0141
DHFR-SB4	0.1425 ± 0.0132	0.1256 ± 0.0159
DHFR-CUSB4	0.1661 ± 0.0195	0.2495 ± 0.0200
DHFR-COSB4	0.1658 ± 0.0213	0.3192 ± 0.0271
DHFR-NISB4	0.1505 ± 0.0128	0.3215 ± 0.0426

The apoprotein exhibited the lowest deviation, with an average RMSD of 0.1018 nm. Among the investigated compounds, SB4 demonstrated the greatest stability in its interaction with *E. coli* DHFR, with an RMSD of 0.1425 nm. The DHFR-TMP complex followed closely, showing an average RMSD of 0.1479 nm. Meanwhile, the DHFR-NISB4 and DHFR-FOL complexes displayed slightly higher RMSD values of 0.1505 nm and 0.1524 nm, respectively. In contrast, the DHFR-COSB4 and DHFR-CUSB4 complexes exhibited the highest deviations, with RMSD averages of 0.1658 nm and 0.1661 nm, respectively. These findings suggest that the protein adopts a more stable conformation in the presence of SB4, TMP, and NISB4 compared to the co-crystallized ligand, FOL.

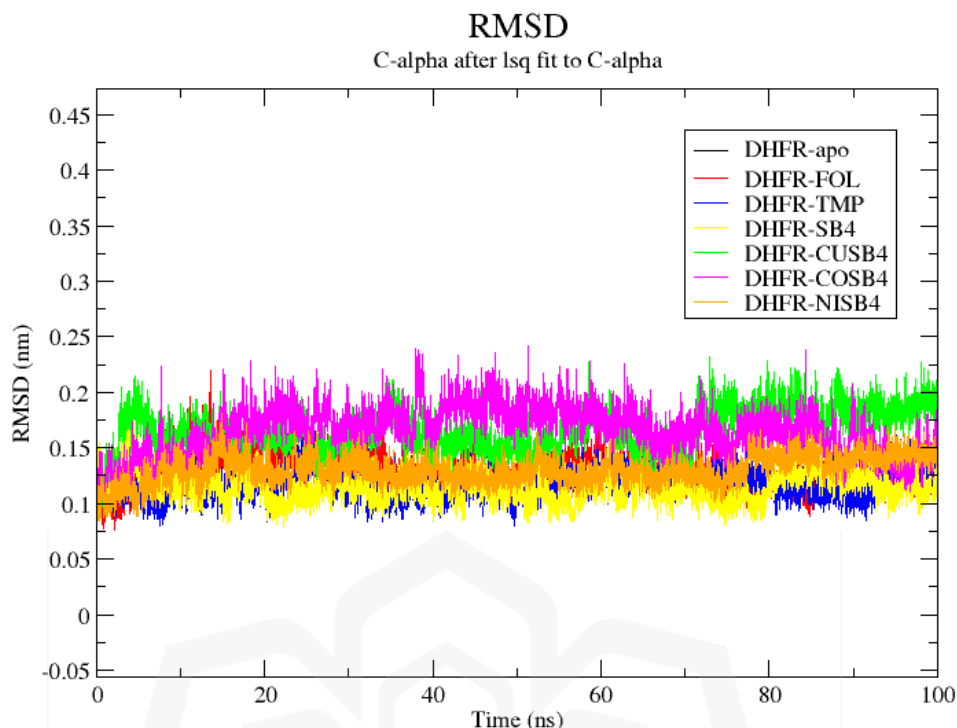


Figure 4.69 RMSD plots of the investigated compounds extracted by protein RMSD at *E. coli* DHFR

According to Aier et al. (2016), an increase in the RMSD indicates structural fluctuations or unfolding of the protein, whereas a stable RMSD trend reflects structural equilibrium or stability. Thus, detail stability was analysed for each system. The apoprotein began to stabilise around 18 ns, while the DHFR-FOL reached early stability at approximately 14 ns. The DHFR-TMP complex exhibited the longest period of stabilisation, remaining stable from approximately 2 ns till the end of the simulation. However, DHFR-SB4, DHFR-CUSB4, DHFR-COSB4 and DHFR-NISB4, all reached stability around 10 ns, with RMSD values remaining steady until the end of the simulation.

To further confirm the stability, the RMSD of the ligands at the DHFR's active site was assessed (Figure 4.70). NISB4 exhibited the highest RMSD value of 0.3215 nm, followed by COSB4 (0.3193 nm) and CUSB4 (0.2495 nm). As the metal complexes consistently showed higher RMSD ligand values compared to other compounds, this suggests that the larger size of the metal complexes might contribute to the greater RMSD values observed. To support this, FOL had a higher RMSD (0.179 nm) than the SB4 and TMP but lower than metal complexes. This was likely due to its smaller size

compared to the metal complexes. TMP and SB4, the smallest compounds, showed the lowest RMSD values at 0.1129 nm and 0.1256 nm, respectively.

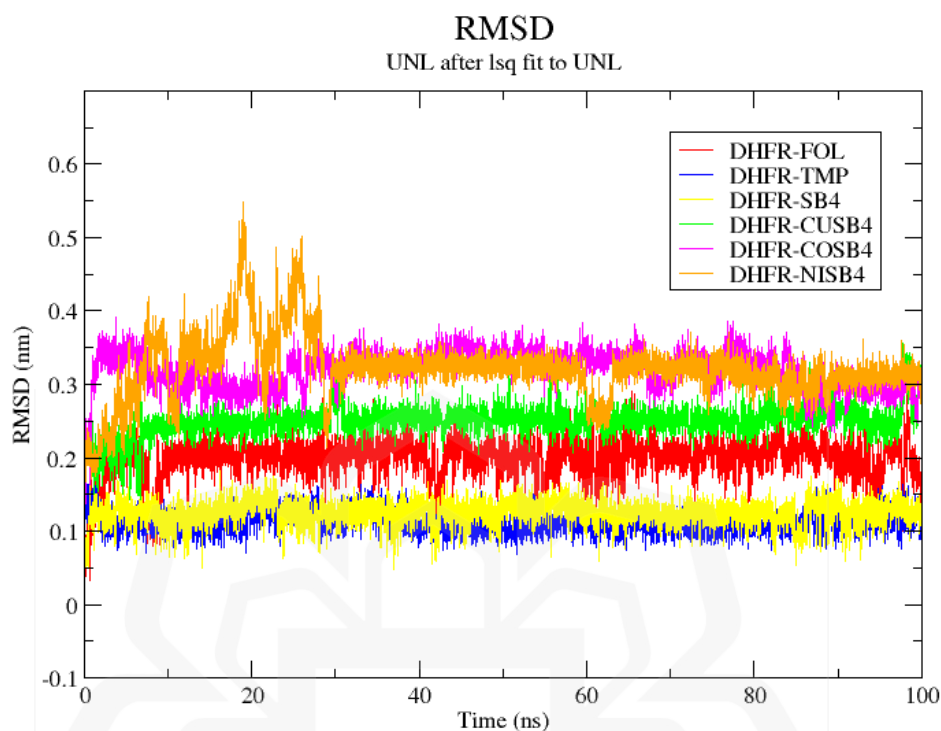


Figure 4.70 RMSD plots of the investigated compounds extracted by ligand RMSD at *E. coli* DHFR

Although DHFR-NISB4 had the lowest overall RMSD, NISB4 alone exhibited the highest RMSD, as shown in Table 4.17. Further analysis revealed that only NISB4 showed instability before 30 ns, with a sharp increase at 20 ns (Figure 4.70). Snapshots at 20 ns (Figure 4.71) indicated that NISB4 is primarily bound to regions outside the protein pocket. This demonstrates that NISB4 was exploring alternative binding modes or interacting with adjacent protein regions. Despite this, RMSD analysis confirmed that all compounds remained stable after 30 ns, so further analysis focused on the 30 to 100 ns simulation period.

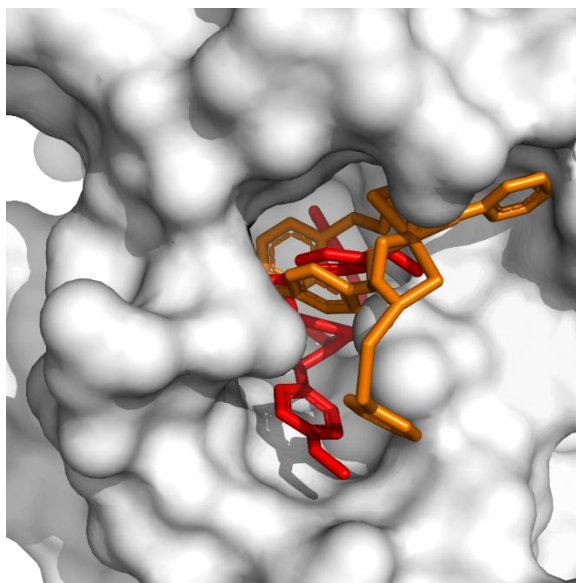


Figure 4.71 Compound binding poses of NISB4 at 0 ns (red) and 20 ns (orange) at *E. coli* DHFR

4.14.2 Radius Gyration (Rg)

The radius of gyration (Rg) was calculated to evaluate the compactness of the protein structure (Alarcón et al., 2013). By tracking Rg values throughout the simulation, changes in the size and shape of the protein-ligand complexes were monitored. Table 4.18 summarises the Rg values for all five systems, with their trends shown in Figure 4.72.

Table 4.18 Radius gyration of the investigated compounds at *E. coli* DHFR

Complex	Average radius gyration (nm)
DHFR-apo	1.5470 ± 0.0113
DHFR-FOL	1.5682 ± 0.0073
DHFR-TMP	1.5685 ± 0.0069
DHFR-SB4	1.5696 ± 0.0076
DHFR-CUSB4	1.5883 ± 0.0106
DHFR-COSB4	1.5806 ± 0.0081
DHFR-NISB4	1.5667 ± 0.0062

Radius of gyration (total and around axes)

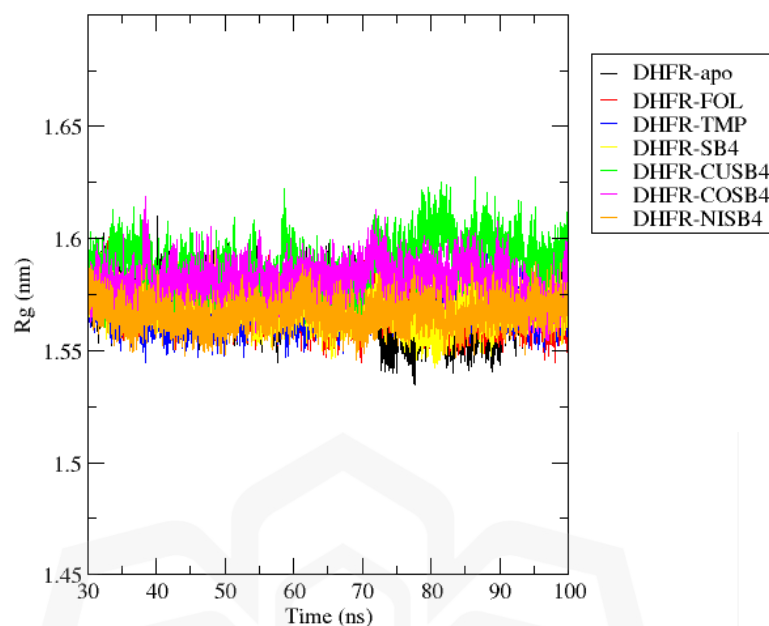


Figure 4.72 Radius gyration of the investigated compounds at *E. coli* DHFR

In its apo form, *E. coli* DHFR exhibited the smallest Rg value of 1.5470 nm, indicating that the protein maintained a compact conformation without any compound bound. The binding of FOL slightly increased the Rg to 1.5682 nm, suggesting a modest structural expansion. Similarly, the DHFR-TMP complex showed an Rg of 1.5685 nm, which was comparable to FOL. This indicates that both FOL and TMP induced similar effects on the protein's compactness.

The DHFR-SB4 complex had an Rg of 1.5696 nm, slightly higher than DHFR-FOL and DHFR-TMP, suggesting a marginally greater structural expansion upon SB4 binding. Among the investigated compounds, the DHFR-CUSB4 complex displayed the largest Rg value of 1.5883 nm, indicating that this compound caused the greatest and introduced structural rearrangements within the protein. DHFR-COSB4 complex also showed an increased Rg of 1.5806 nm, slightly less than DHFR-CUSB4 but still reflecting a notable expansion of the DHFR structure. Interestingly, the DHFR-NISB4 complex had an Rg of 1.5667 nm, which was smaller than FOL, TMP, and SB4. This validates that NISB4 binding induced a relatively compact conformation compared to the other compounds, although it contains a larger size than FOL, TMP and SB4.

Overall, all compounds consistently increased the Rg compared to the apoprotein, reflecting that they induced structural expansion. Among the investigated compounds, CUSB4 caused the most significant increase in protein size, while NISB4 resulted in the smallest expansion. Importantly, the average Rg values showed no abrupt changes during the simulations, indicating that the protein-ligand systems remained dynamically stable despite these structural adjustments.

4.14.3 Root Mean Square Fluctuation (RMSF)

RMSF was used to measure the flexibility of each amino acid during the MD simulation. Higher RMSF values indicated greater movement, while lower values showed that the protein region was more stable or rigid (Kumar et al., 2014). Similarly, B-factor values, derived from RMSF, reflected the thermal motion of atoms in a protein crystal, where higher values suggested more mobility and lower values indicated rigidity (Sun et al., 2019).

The RMSF graph (Figure 4.73) revealed two key regions that exhibited notable changes upon compound binding, specifically residues around 15-21 and 64-70. These regions were also highlighted in the B-factor putty model (Figure 4.74). The first region, residues 15-21 (marked in magenta dashed box), is close to the MET20 loop at the active site. In the apoprotein, this loop was highly flexible. This flexibility is critical for the DHFR's function, as it helps accommodate substrates during catalysis (Nishimura et al., 2013). When FOL and TMP were bound, the flexibility of the MET20 loop decreased. This reduction in flexibility suggested that the compounds partially stabilised the loop while still allowing some movement for enzymatic activity.

RMS fluctuation

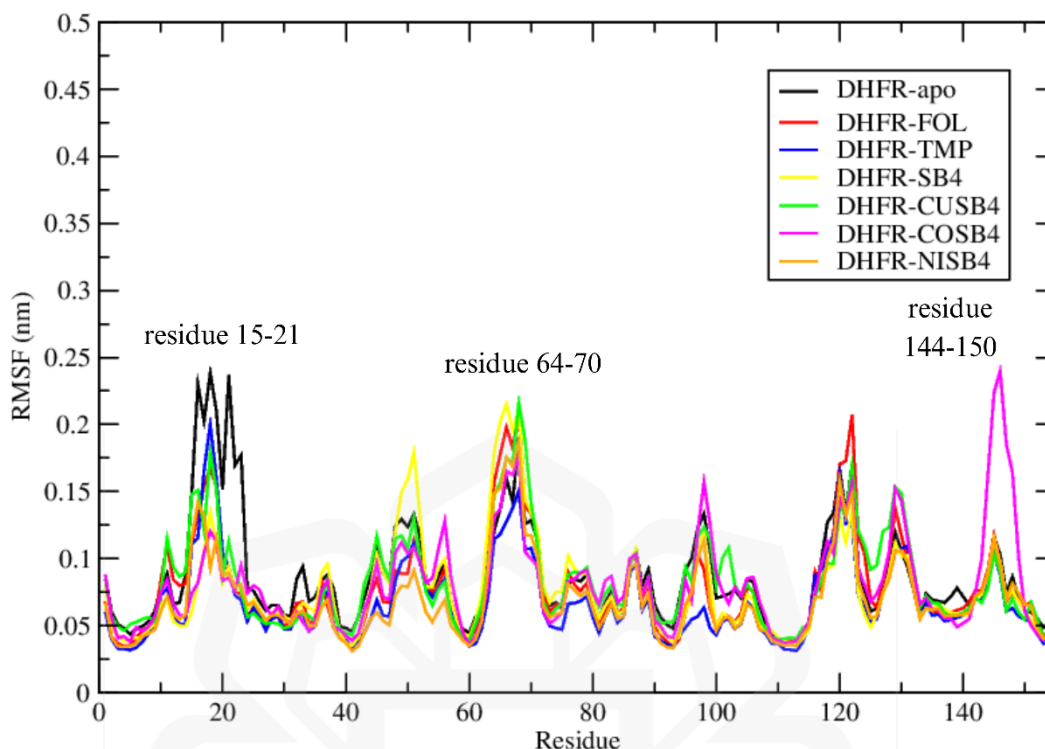


Figure 4.73 RMSF plot of the investigated compounds extracted by protein RMSF at *E. coli* DHFR

In contrast, the binding of SB4, CUSB4, COSB4, and NISB4 resulted in a significant increase in rigidity of the MET20 loop as illustrated in Figure 4.74. This rigidity suggested that these compounds stabilised the DHFR backbone, making it less flexible. The MET20 loop is crucial for enzyme activity, as its movement enables THF release and hydride transfer from NADPH to DHF (Nishimura et al., 2013; Sawaya & Kraut, 1997). Thus, to effectively inhibit DHFR, targeting the flexibility of the MET20 loop presents a promising strategy. Restricting its movement impairs the DHFR's ability to accommodate and process natural substrates, ultimately disrupting enzymatic activity. Hence, this study's findings discovered that the rigidity induced by these compounds significantly hindered enzyme function, highlighting their potential as effective DHFR inhibitors.

Another affected region, located between residues 64-70 (marked in a grey dashed box), showed an opposite trend in flexibility. In the apoprotein, this region exhibited moderate flexibility. When FOL and TMP were bound, this region became

more flexible. However, the binding of SB4, CUSB4, COSB4, and NISB4 caused a further increase in flexibility across this region. According to past studies, this area is linked to NADPH binding, which is absent in the occluded form of *E. coli* DHFR (Lange et al., 2023; Mamta & Chaudhary, 2024). Therefore, the changes in flexibility within this region did not significantly affect the enzyme's activity.

Interestingly, another region attracted attention due to its significant flexibility. This region, located at residues 144-150, was observed in DHFR-COSB4. Notably, only this compound induced flexibility in this region, which was expected to remain rigid based on the apoprotein. According to previous literature, this region is known as the GH loop subdomain (Sawaya & Kraut, 1997). Important hydrogen bond interactions were observed within this region, particularly between ASN23 and SER148, which function to stabilise the MET20 loop. However, the induced flexibility in DHFR-COSB4 suggests that these interactions might have diminished. A detailed analysis of this region was further discussed in Subsection 4.14.5.

Overall, protein flexibility analysis revealed that several residues located near the active site were affected, indicating that both minor and significant fluctuations could influence DHFR's enzymatic activity. These results reinforce that these compounds caused noticeable rigidification in the MET20 loop which became a promising approach for inhibiting DHFR activity.

Subsequently, RMSF of the ligands was analysed to evaluate their atomic flexibility when bound to *E. coli* DHFR during MD simulations (Figure 4.75). The results revealed that TMP exhibited the lowest RMSF values across most of its atoms, indicating a highly stable binding conformation. This observation is consistent with TMP's well-established role as a potent DHFR inhibitor. Its low atomic fluctuations throughout the simulation suggest a strong and stable interaction with the active site, contributing to its inhibitory effectiveness. Similarly, FOL also demonstrated relatively low RMSF values, reflecting stable binding. However, moderate fluctuations were observed between atoms 10-40, likely corresponding to flexible side-chain regions that do not significantly disrupt the overall binding stability.

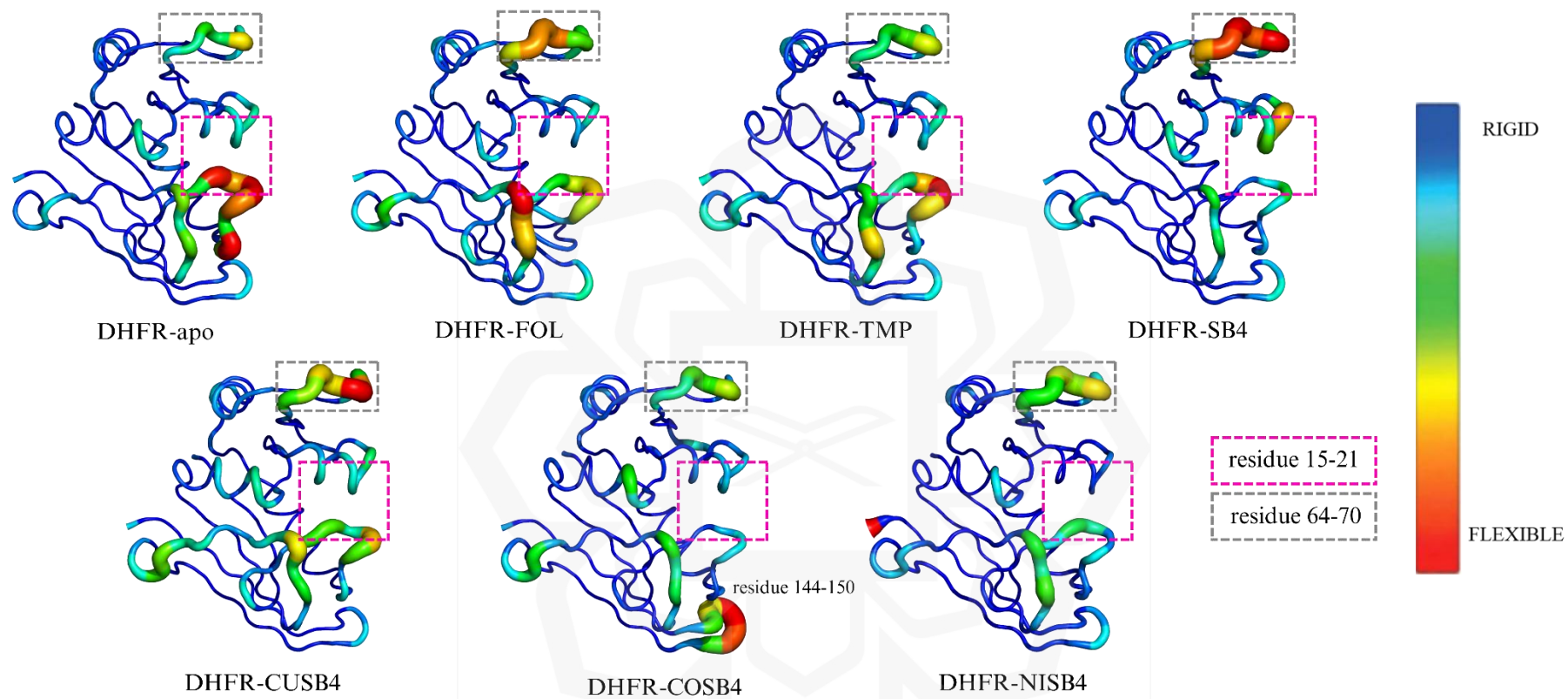


Figure 4.74 B-factor putty representative of the investigated compounds at *E. coli* DHFR

RMS fluctuation of ligands

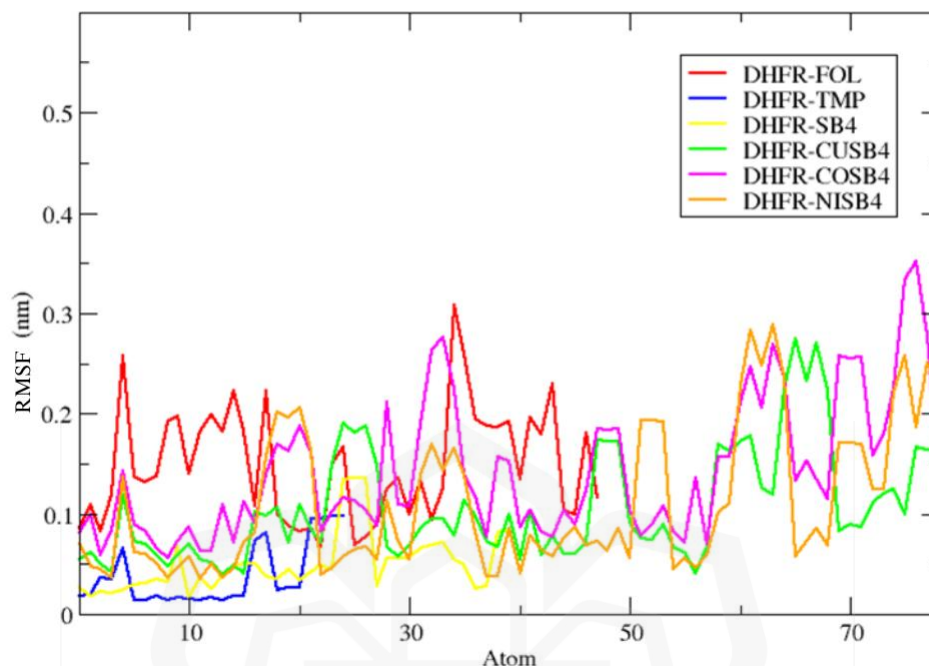


Figure 4.75 RMSF plot of the investigated compound extracted by ligands RMSF at *E. coli* DHFR

In contrast to TMP and FOL, COSB4 exhibited the highest atomic fluctuations among all ligands, particularly between atoms 20-40 and again after atom 60. This observation corresponds well with the RMSF analysis of the protein, where disruption of key interactions with the MET20 loop was noted. The loss of stabilising contacts likely contributed to increased mobility of COSB4 within the binding pocket, indicating reduced conformational stability. CUSB4 also showed slightly elevated RMSF values, but overall demonstrated more consistent behavior than COSB4. This suggests that the CUSB4 may form a more stable interaction with DHFR compared to the COSB4.

SB4 exhibited moderate atomic fluctuations with occasional peaks, indicating a reasonable degree of stability, although not as consistent as TMP or FOL. In contrast, NISB4 showed increased atomic movements toward the terminal region (atoms 60-75), likely due to the presence of flexible methoxy groups that are not fully engaged in binding interactions. Despite this localised flexibility, NISB4 demonstrated greater overall stability compared to COSB4, suggesting a better structural fit and stronger binding potential within the DHFR active site.

In conclusion, the RMSF analysis of the ligands highlighted TMP and FOL as the most stable binders, in line with their established biological functions. Among the synthesised compounds, NISB4 and CUSB4 demonstrated relatively stable binding conformations, supporting their potential as effective DHFR inhibitors. In contrast, the pronounced flexibility of COSB4 may compromise its binding efficiency, underscoring the importance of ligand rigidity within the binding pocket for the development of potent DHFR inhibitors.

4.14.4 Pocket Volume

Pocket volume analysis was conducted to evaluate changes in the binding pocket size upon ligand binding. The conformational shifts induced by compound binding often altered the pocket's shape and volume, influencing the overall stability and accessibility of the active site. Table 4.19 summarises the impact of different compounds on the pocket volume of the DHFR protein during the simulation which is also illustrated in Figure 4.76.

Table 4.19 Pocket volume of the investigated compounds at the binding site of *E. coli* DHFR

Complex	Pocket volume (nm ³)
DHFR-apo	6.3141 ± 0.1266
DHFR-FOL	6.6154 ± 0.1200
DHFR-TMP	6.4137 ± 0.1325
DHFR-SB4	6.2951 ± 0.1182
DHFR-CUSB4	6.6164 ± 0.1246
DHFR-COSB4	6.4564 ± 0.1234
DHFR-NISB4	6.3098 ± 0.1169

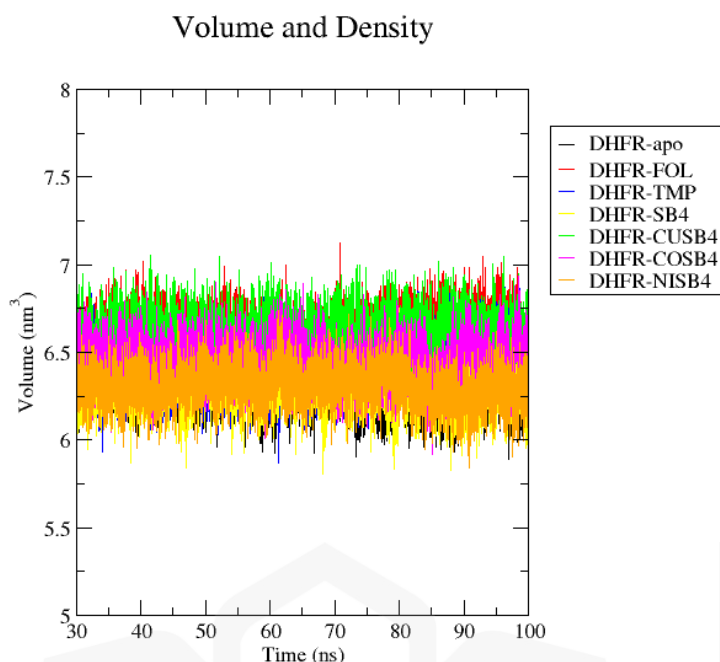


Figure 4.76 The pocket volume of the investigated compounds at *E. coli* DHFR

The pocket volume of the apo form was recorded at 6.3141 nm³, serving as the baseline and representing the binding pocket's native conformation in the absence of compounds. When FOL was bound, the pocket volume increased to 6.6154 nm³, suggesting structural adjustments to accommodate FOL, leading to an expanded pocket. In the case of the TMP complex, the pocket volume measured 6.4137 nm³, indicating a moderate expansion compared to the apo form. This increase, while less pronounced than with FOL, could be attributed to TMP's smaller size, resulting in a lower overall pocket volume.

In contrast, the SB4 complex showed a reduced pocket volume of 6.2951 nm³, suggesting a slight contraction of the binding site upon SB4 binding. This could be linked to SB4's tighter interaction with the protein. As noted in the RMSF analysis, the flexible MET20 loop in the apo form appeared to become more rigid upon interaction with SB4. As a result, it might contribute to the smaller pocket volume. The CUSB4 complex displayed the largest pocket volume at 6.6164 nm³, reflecting significant expansion to accommodate the compound. This observation mirrored the radius of gyration results, where CUSB4 binding resulted in reduced compactness of protein, leading to a larger pocket volume.

COSB4 exhibited a pocket volume of 6.4564 nm³, demonstrating moderate expansion relative to the apo form. This indicated structural adjustments to accommodate COSB4, although the increase was less substantial compared to CUSB4 or FOL. Interestingly, NISB4, despite its larger size, had a pocket volume of 6.3098 nm³, lower than that of the apoprotein. This suggested minimal structural changes in the binding site, implying a snug fit with minimal expansion. The reduced pocket volume of NISB4 aligned with findings from the radius of gyration analysis, which showed NISB4 induced minimal compactness in the protein.

Overall, the binding of FOL, TMP, CUSB4, and COSB4 resulted in an expansion of the pocket volume compared to the apo form, with CUSB4 inducing the most significant expansion. Conversely, SB4 and NISB4 binding led to smaller pocket volumes, indicating tighter binding or reduced flexibility of the binding site. Despite these variations, the consistent trends observed in Figure 4.76 confirming that there were no abrupt changes in pocket volume following the binding of all investigated compounds throughout the simulation.

4.14.5 MET20 Loop Stability

The inhibition of *E. coli* DHFR involves not just active site interactions but also movements of loops and subdomains (Nishimura et al., 2013). The MET20 loop plays a crucial role in stabilising the transition state during catalysis (Mhashal et al., 2017). In its occluded conformation, this loop helps accommodate substrates, especially without nicotinamide. SER148 is key to stabilising this loop by forming hydrogen bonds with ASN23, maintaining active site integrity and enhancing substrate binding (Arora & Brooks, 2009; Goldstein & Goodey, 2021). These hydrogen bonds act as a "lock" for the MET20 loop, ensuring proper configuration of the active site for catalytic activity. In this study, the stability of the MET20 loop was assessed by examining hydrogen bond interactions between SER148 and ASN23 in the presence of all investigated compounds.

As illustrated in Figure 4.77, in the DHFR-FOL complex, there were three primary interactions identified. The first interaction involved a hydrogen bond between the amine group of SER148 and the carbonyl oxygen of ASN23, with a distance of 3.1 Å. The second was a hydrogen bond between the hydroxyl group of SER148 and the

carbonyl oxygen of ASN23, occurring at a distance of 2.9 Å. Lastly, a hydrogen bond formed between the hydroxyl group of SER148 and the amine group of ASN23 at a distance of 2.8 Å.

When comparing these interactions in DHFR-FOL to other complexes, a reduction in the number of hydrogen bonds was observed, except NISB4. For DHFR-TMP, DHFR-SB4, and DHFR-CUSB4, two consistent interactions were noted. In the DHFR-TMP complex, the amine group of SER148 formed a hydrogen bond with the carbonyl oxygen of ASN23 at a distance of 2.1 Å, while the hydroxyl group of SER148 formed a hydrogen bond with the amine group of ASN23 at 1.1 Å. Similarly, in the DHFR-SB4 complex, both interactions were observed at a distance of 2.1 Å. For the DHFR-CUSB4 complex, the interaction distances were 2.1 Å and 2.6 Å, respectively.

Notably, no interactions were observed between ASN23 and SER148 in the DHFR-COSB4 complex. This showed that COSB4 could not stabilise the active site loop. As a result, these residues were likely more flexible, as indicated by the RMSF analysis. Previous studies have shown that when the interaction between ASN23 and SER148 is lost during compound binding, *E. coli* DHFR may no longer be in the occluded form (Arora & Brooks, 2009). *E. coli* DHFR typically shifts between closed and occluded forms. This suggests that COSB4 might have triggered a shift from the occluded to the closed form of DHFR. As a result, COSB4 might have a reduced selectivity towards bacterial DHFR since the important interactions of ASN23-SER148 are missing.

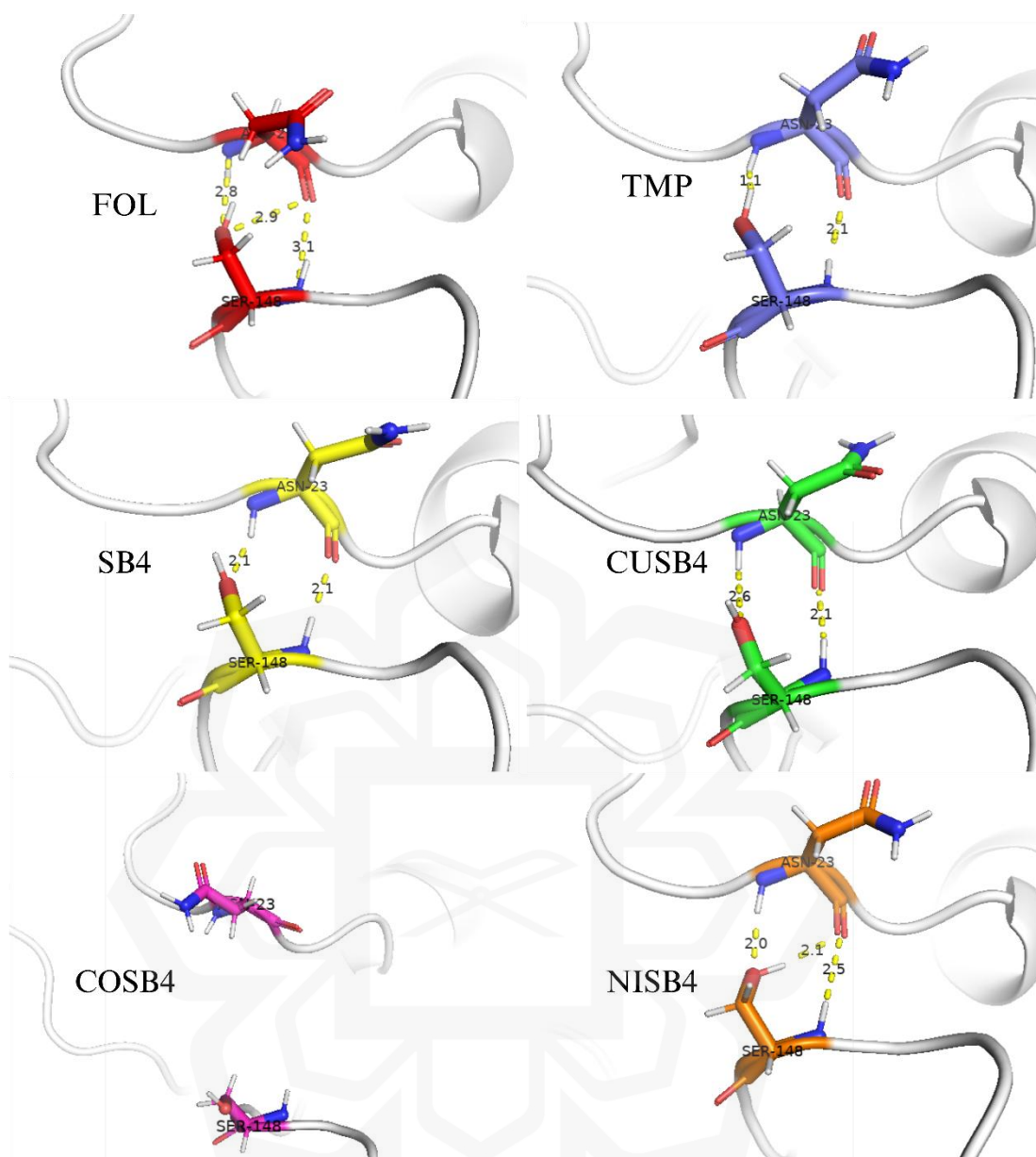


Figure 4.77 Hydrogen bond interaction between SER148 with ASN23 of the investigated compounds at *E. coli* DHFR. The hydrogen bonds were shown in yellow dashed lines, and the distances are written in Angstrom units

Interestingly, the DHFR-NISB4 complex exhibited three interactions, closely resembling those in the DHFR-FOL complex. The hydrogen bond between the amine group of SER148 and the carbonyl oxygen of ASN23 was recorded at a distance of 2.5 Å. Additionally, the hydroxyl group of SER148 formed a hydrogen bond with the carbonyl oxygen of ASN23 at 2.1 Å, and another hydrogen bond was observed between the hydroxyl group of SER148 and the amine group of ASN23 at a distance of 2.0 Å.

Overall, the results of this study demonstrated that all investigated compounds, except for the COSB4, formed significant hydrogen bond interactions between ASN23 and SER148. These interactions closely mirrored those observed in the DHFR-FOL complex, suggesting that, apart from the COSB4, these compounds effectively maintain the critical interactions necessary for MET20 loop stabilisation, thereby mimicking the binding mode of the native substrate.

4.14.6 Binding Free Energy Calculation

MMPBSA was employed to estimate the binding free energy from the MD trajectories using the *g_mmpbsa* tool. The binding free energies were tabulated in Table 4.20. In the DHFR-FOL complex, the binding free energy was -29.97 kcal/mol, indicating a strong interaction between DHFR and FOL. This was consistent with FOL's role as a natural substrate of DHFR and its high affinity for the enzyme's active site (Sawaya & Kraut, 1997).

Table 4.20 MMPBSA of the investigated compounds at *E. coli* DHFR

Complex	Binding energy (kcal/mol)
DHFR-FOL	-29.97
DHFR-TMP	-11.43
DHFR-SB4	-18.99
DHFR-CUSB4	-36.67
DHFR-COSB4	-34.95
DHFR-NISB4	-34.11

The DHFR-TMP complex showed the least negative binding free energy (-11.43 kcal/mol). This suggested that TMP had a weaker binding affinity compared to the other compounds. In contrast, DHFR-SB4 exhibited a binding free energy of -18.99 kcal/mol, which was more negative than TMP. This indicates that SB4 showed stronger binding than TMP, which was supported by its *in vitro* studies (Subsection 4.12.6). Nevertheless, their binding affinity was weaker compared to the metal complexes. Among the metal complexes, DHFR-CUSB4 had the most negative binding free energy of -36.67 kcal/mol, indicating the strongest binding affinity. This is followed by DHFR-COSB4 (-34.95 kcal/mol) and DHFR-NISB4 (-34.11 kcal/mol).

Overall, the metal complexes, including CUSB4, COSB4, and NISB4, exhibited the most negative binding free energy values (Figure 4.78). This finding is in line with their superior performance in their biological activity. However, the binding energy differences obtained between the metal complexes were small, which explained their similar MIC values. In contrast, TMP displayed the weakest binding, mirrored perfectly with *in vitro* results. Although SB4 binds more strongly than TMP, it still lagged behind the metal complexes.

When comparing both *in silico* methods, it was observed that the binding free energy values obtained from MMPBSA analysis during MD simulations correlated better with experimental MIC values than the binding energies derived from molecular docking. For example, MMPBSA calculations showed that TMP had weaker binding affinity than SB4 with a free energy difference of 7.56 kcal/mol. However, molecular docking showed that SB4 had weaker affinity than TMP with only a 0.07 kcal/mol difference. This observation on MMPBSA prediction is supported by MIC assay, which confirmed SB4's superior antibacterial activity than TMP against *E. coli*, confirming that MMPBSA predictions were more accurate.

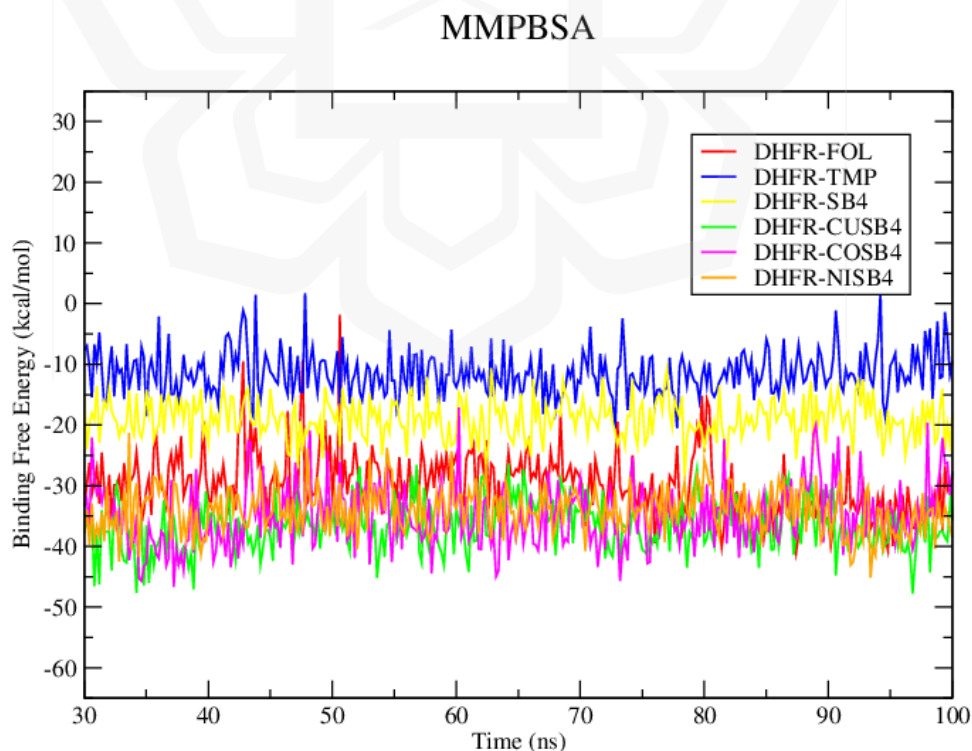


Figure 4.78 MMPBSA of the investigated compounds at *E. coli* DHFR

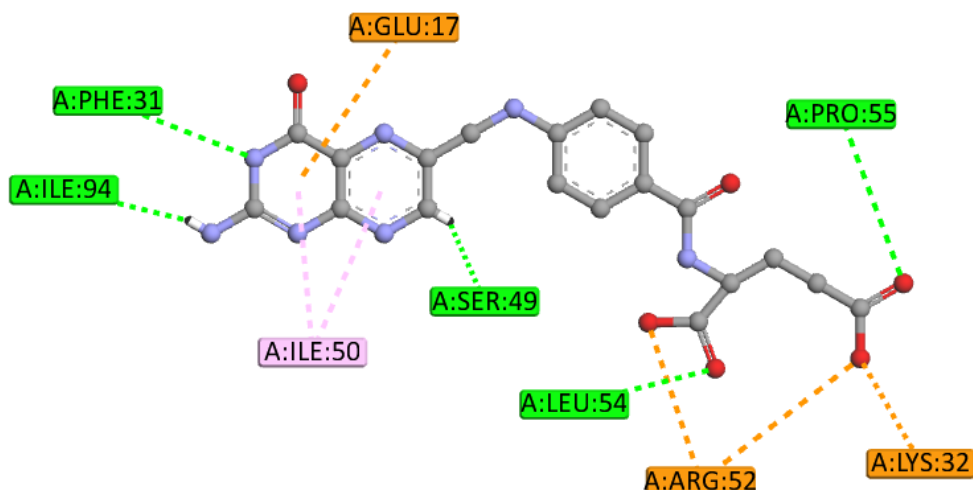
The higher accuracy of MMPBSA came from the dynamic nature of MD simulations, which account for conformational changes and solvent effects (Alonso et al., 2006). In contrast, molecular docking offers only a static snapshot and does not consider time-dependent structural changes, reducing its predictive reliability (Salo-Ahen et al., 2020; Vivo et al., 2016). Therefore, MD simulations provided better agreement with experimental findings.

4.14.7 Binding Interaction Analysis

To further illustrate the interaction between the compounds and the *E. coli* DHFR protein, representative structures obtained from clustering analysis were examined. The interacting residues identified from clustering were supported by hydrogen bond occupancy and MMPBSA decomposition data. These interactions (Figure 4.79-4.84) showed in hydrogen bonding (green), hydrophobic interactions (pink), and electrostatic interactions (orange).

4.14.7.1 Interaction Analysis of FOL

FOL demonstrated a variety of interactions with key residues in the active site of DHFR, including hydrogen bonding, hydrophobic interactions, and electrostatic interactions as illustrated in Figure 4.79. The most notable hydrogen bond was observed with ILE94, exhibiting the highest occupancy of 33.92% (Table 4.21) This demonstrated a stable and significant contribution of ILE94 to the binding of FOL. However, the decomposed residue energy showed a positive contribution of 0.64 kcal/mol. This positive value was attributed to the desolvation effect observed during the MD simulation. According to Rudling et al. (2018), residues forming hydrogen bonds with the compound during the simulation tended to experience desolvation due to their polar interactions with water. As the compound bound to the protein, the protein-solvent interaction diminished, leading to new interactions with the compound. This phenomenon caused the desolvation effect, resulting in a positive decomposed residue value (Eberhardt & Forli, 2023).



Hydrogen bond interaction
 Hydrophobic interaction
 Electrostatic interaction

Figure 4.79 Binding interactions of FOL at *E. coli* DHFR

Table 4.21 Interacting residues of DHFR-FOL

Residues	Type of interactions	H-bond occupancy (%)	Binding free energy (kcal/mol)
GLU17	Electrostatic interaction/ hydrogen bond	-	2.44
PHE31	Hydrogen bond/hydrophobic interaction	-	-1.28
LYS32	Electrostatic interaction	-	-
SER49	Hydrogen bond	17.44	-
ILE50	Hydrophobic interaction	-	-2.21
ARG52	Electrostatic interaction	-	-
LEU54	Hydrogen bond/ hydrophobic interaction	-	-1.3
PRO55	Hydrogen bond	22.21	-
ILE94	Hydrogen bond	33.92	0.64

FOL also formed a prominent hydrogen bond interaction with PRO55, which had an occupancy of 22.21%. Similarly, a hydrogen bond with SER49 was observed with an occupancy of 17.44%. LEU54 and PHE31 also engaged in hydrogen bonding with FOL, though their occupancies were insignificant. Nonetheless, they contributed to binding affinity with decomposed energy values of -1.3 and -1.28 kcal/mol, respectively.

As illustrated in Figure 4.79, FOL only formed one hydrophobic interaction at DHFR. FOL exhibited a hydrophobic interaction with ILE50, with a significant decomposed binding free energy contribution of -2.21 kcal/mol, demonstrating its role in stabilising the FOL-DHFR complex. Additionally, electrostatic interactions were observed between FOL and residues such as GLU17, LYS32, and ARG52. Among these residues, only GLU17 contributed a decomposed binding free energy of 2.44 kcal/mol through hydrogen bond interactions in MMPBSA due to desolvation effects. Although LYS32 and ARG52 do not exhibit explicit occupancy or energy values, they likely play a role in electrostatic balance within the binding site.

In summary, while FOL acted as a substrate for DHFR, these interactions were also significant for potential DHFR inhibition. The hydrogen bond interactions with ILE94 and PRO55, along with the hydrophobic interaction with ILE50, were the most significant in the DHFR-FOL complex.

4.14.7.2 Interaction Analysis of TMP

TMP formed a strong hydrogen bond with ASP27 (Figure 4.80), which exhibited a 100% occupancy rate. Plus, it significantly contributed to the positive binding free energy (6.99 kcal/mol) due to the desolvation effect (Table 4.22). According to the literature, hydrogen bond interactions with ASP27 played a pivotal role in stabilising the DHFR-TMP complex during the MD simulation (Wróbel, Arciszewska, et al., 2019). ASP27 is crucial for the transfer of hydrogen from NADPH to DHF (Krucinska et al., 2022). By blocking this interaction, TMP directly interfered with the reduction of DHF to THF, ultimately leading to cell death.

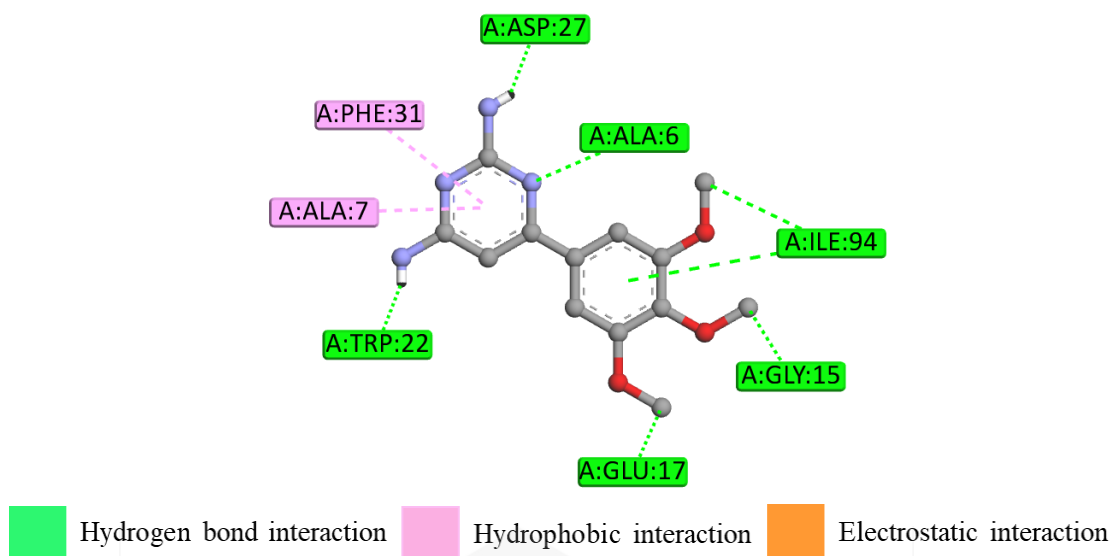


Figure 4.80 Binding interactions of TMP at *E. coli* DHFR

Table 4.22 Interacting residues of DHFR-TMP

Residues	Type of interactions	H-bond occupancy (%)	Binding free energy (kcal/mol)
ALA6	Hydrogen bond	11.70	-1.06
ALA7	Hydrophobic interaction	-	-0.68
GLY15	Hydrogen bond	-	0.98
GLU17	Hydrogen bond	11.37	-
TRP22	Hydrogen bond	-	-
ASP27	Hydrogen bond	100	6.99
PHE31	Hydrophobic interaction	-	-1.14
ILE94	Hydrogen bond	10.87	-

TMP also interacted with ALA6 through a hydrogen bond, which showed a moderate hydrogen bond occupancy of 11.70% and contributed -1.06 kcal/mol to the binding free energy. Similarly, TMP showed moderate hydrogen bond occupancy with

GLU17 and ILE94, with occupancy rates of 11.37% and 10.87%, respectively. However, no energy values were recorded. Other hydrogen bond interactions with TMP included those formed with GLY15 and TRP22. Among them, only GLY15 contributed to binding affinity with a binding free energy of -0.98 kcal/mol.

Hydrophobic interactions further contributed to the dynamic stability of TMP within the DHFR binding site. TMP formed a notable hydrophobic interaction with PHE31, which significantly contributed to the binding free energy (-1.14 kcal/mol). The interaction with PHE31 enhanced TMP's antibacterial activity by facilitating its permeability across the bacterial membrane (Jays et al., 2019). TMP also engaged in hydrophobic interactions with ALA7, contributing -0.68 kcal/mol to the binding free energy.

In conclusion, the stability of the DHFR-TMP complex was primarily driven by hydrogen bond interactions, particularly the strong and consistent interaction with ASP27. Hydrophobic interactions, especially with PHE31, complemented these hydrogen bonds by positioning TMP favourably within the hydrophobic regions of the binding pocket. When comparing the binding of TMP to FOL, three similar residues were observed, including GLU17, PHE31, and ILE94.

4.14.7.3 Interaction Analysis of SB4

Similar to TMP, SB4 exhibited only two types of interactions including hydrogen bonding and hydrophobic interactions (Figure 4.81). SB4 formed a hydrogen bond with ILE94, which showed a perfect hydrogen bond occupancy of 100% (Table 4.23). ILE94 was a crucial residue involved in both FOL and TMP, suggesting that SB4 mimicked both, as DHFR inhibitor and substrate. Additionally, SB4 formed strong hydrogen bond interactions with ALA7 and TYR100, with hydrogen bond occupancies of 97.84% and 53.07%, respectively. The hydrogen bond with TYR100 was also present in TMP's binding, but its occupancy was much weaker (<10%), making SB4's interaction with TYR100 significantly stronger. This difference in interaction strength explains why SB4 binds more strongly than TMP, a finding confirmed by the experimental assays.

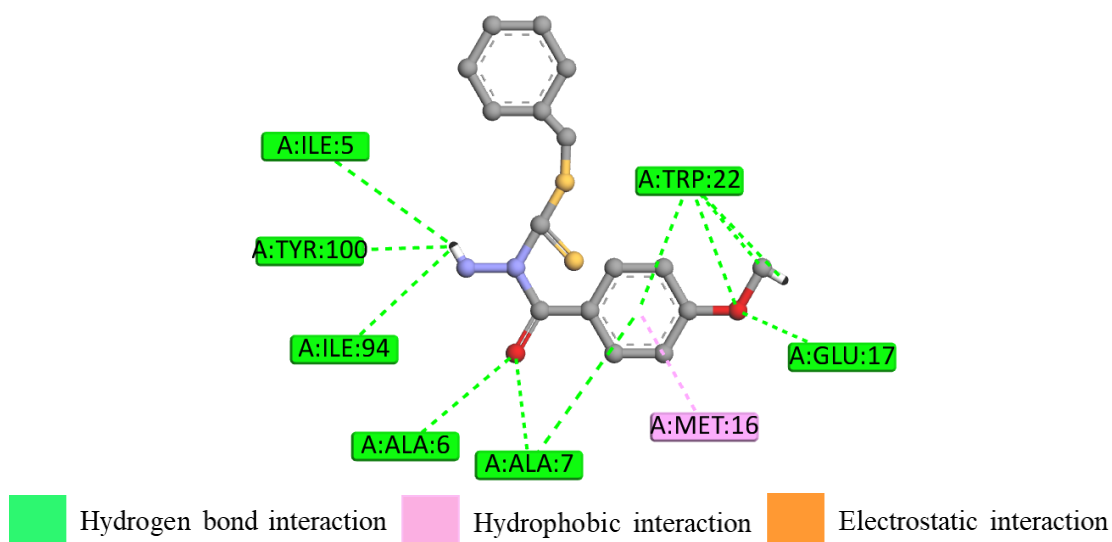


Figure 4.81 Binding interactions of SB4 at *E. coli* DHFR

Table 4.23 Interacting residues of DHFR-SB4

Residues	Type of interactions	H-bond occupancy (%)	Binding free energy (kcal/mol)
ILE5	Hydrogen bond	-	-
ALA6	Hydrogen bond / Electrostatic interaction	-	-1.22
ALA7	Hydrogen bond	97.84	-
MET16	Hydrophobic interaction	-	-0.93
GLU17	Hydrogen bond	22.42	-
TRP22	Hydrogen bond	25.72	-
ILE94	Hydrogen bond	100	-
TYR100	Hydrogen bond	53.07	-

SB4 also formed hydrogen bond interactions with GLU17 and TRP22, with occupancies of 22.42% and 25.72%, respectively. Both residues were found in TMP's binding, but SB4 showed better occupancy, further justifying its stronger binding affinity at DHFR. Moreover, the interaction with GLU17 observed in the docking analysis was maintained, indicating that this interaction is not only significant but also

stable. Weak hydrogen bond interactions with ILE5 and ALA6 were also observed. Notably, MMPBSA decomposition analysis revealed that ALA6 formed an electrostatic interaction with SB4, contributing -1.22 kcal/mol to the binding free energy. This contribution was significantly higher than that of TMP, which contributed -1.06 kcal/mol, suggesting a stronger interaction between SB4 and ALA6.

On the hydrophobic side, SB4 only formed a hydrophobic interaction with MET16, contributing -0.93 kcal/mol. Overall, SB4 demonstrated stronger binding interactions than TMP, which justified its higher binding affinity observed in the MMPBSA analysis. This result was consistent with the MIC assay findings, where SB4 exhibited superior antibacterial activity compared to TMP. Hence, the improved performance of SB4 could be attributed to its consistent and significant hydrogen bond interactions, particularly with ILE94, ALA7, TYR100, GLU17, and TRP22.

4.14.7.4 Interaction Analysis of CUSB4

Unlike SB4, CUSB4 favours hydrophobic interactions over hydrogen bonding interactions. CUSB4 formed three hydrogen bond interactions with SER49, ARG52, and ARG57 (Figure 4.82). Interestingly, all these interactions are mediated through oxygen atoms, either from the carbonyl or methoxy group. However, none of these interactions were detected in the hydrogen bond occupancy or decomposed residue analysis (Table 4.24).

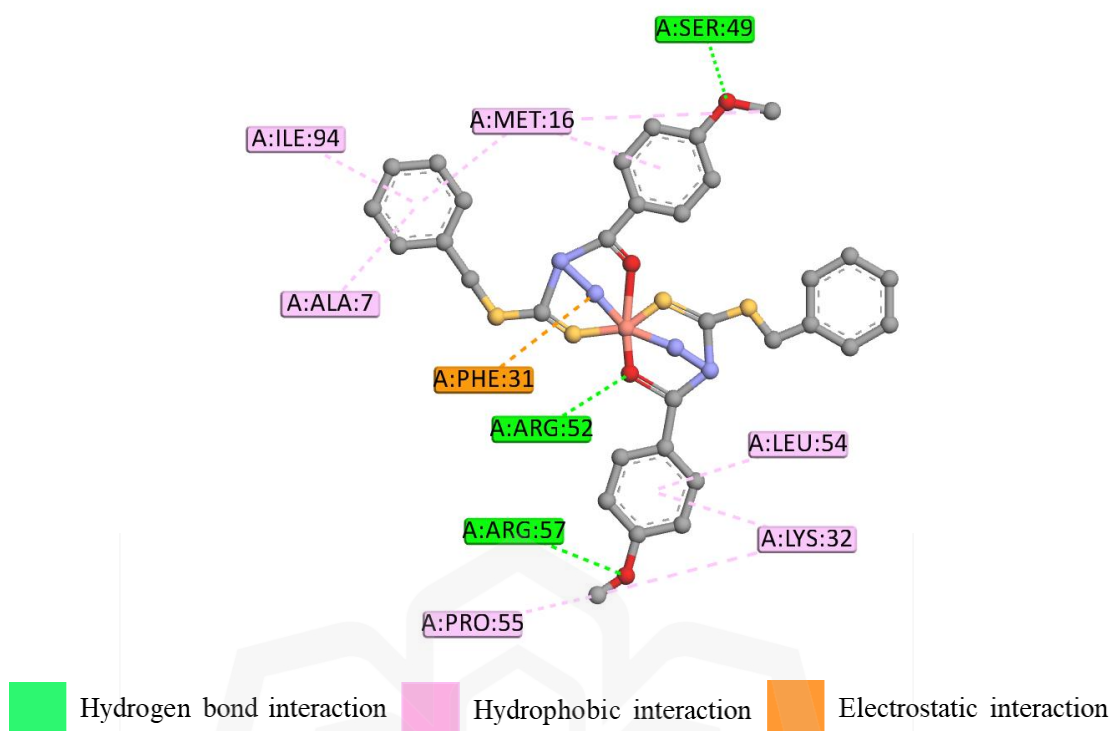


Figure 4.82 Binding interactions of CUSB4 at *E. coli* DHFR

Table 4.24 Interacting residues of DHFR-CUSB4

Residues	Type of interactions	H-bond occupancy (%)	Binding free energy (kcal/mol)
ALA7	Hydrophobic interaction	-	-0.51
MET16	Hydrophobic interaction	-	-1.48
PHE31	Electrostatic interaction/ hydrophobic interaction	14.52	-1.21
LYS32	Hydrophobic interaction	-	-
SER49	Hydrogen bond	-	-
ARG52	Hydrogen bond	-	-
LEU54	Hydrophobic interaction	-	-0.92
PRO55	Hydrophobic interaction	-	-0.87
ARG57	Hydrogen bond	-	-
ILE94	Hydrophobic interaction	-	-0.85

In contrast, CUSB4 formed six hydrophobic interactions with DHFR. Notably, the complex interacted with ALA7 and MET16, with significant binding free energy contributions of -0.51 and -1.48 kcal/mol, respectively. The interaction with ALA7 was also observed in TMP's binding, highlighting its importance in targeting DHFR. CUSB4 further formed hydrophobic interactions with LYS32, LEU54, PRO55, and ILE94. The interaction with LYS32 does not contribute to the binding affinity of CUSB4, while the interactions with LEU54, PRO55, and ILE94 contributed binding free energies of -0.92, -0.87, and -0.85 kcal/mol, respectively. These four residues were also involved in FOL's binding, suggesting that CUSB4 inhibits DHFR by mimicking the substrate rather than TMP.

CUSB4 also formed an electrostatic interaction with PHE31, a residue found in both FOL and TMP. In the MMPBSA analysis, PHE31 contributed through hydrophobic interaction, with a binding free energy of -1.21 kcal/mol. While this value is lower than FOL's, it is significantly higher than TMP's, validating the superior antibacterial activity of CUSB4 compared to TMP. Additionally, only the significant interaction with PHE31 was retained from the docking results, while the main interactions with GLU17, LEU28, and ILE50 were lost during the MD simulation, indicating that the interaction with PHE31 was the most stable.

Overall, CUSB4 was stabilised by three hydrogen bond interactions, six hydrophobic interactions, and one electrostatic interaction. Among all the compounds investigated, CUSB4 exhibited the most hydrophobic interactions, which likely contributed to its highest affinity for DHFR, from MMPBSA calculation. According to the literature, the formation of more hydrophobic interactions strengthens the affinity for DHFR (Ghany et al., 2024). Thus, these findings support the conclusion that CUSB4 has the most favourable interactions among the compounds studied.

4.14.7.5 Interaction Analysis of COSB4

COSB4 formed four hydrogen bond interactions with GLU17, ASP27, LYS32, and THR35 (Figure 4.83). Among these, the interaction with LYS32 was the most persistent, with a hydrogen bond occupancy of 50.50% (Table 4.25). This interaction contributed significantly to the overall binding, with a binding free energy of -0.73

kcal/mol. LYS32 is also involved in FOL's binding, but with a much weaker contribution, indicating that the interaction with LYS32 enhances COSB4's binding affinity at DHFR. It also justified its higher affinity in the MMPBSA calculations compared to FOL. COSB4 also formed an interaction with ASP27, a crucial residue involved in TMP binding, with an occupancy of 32.05%. Although this occupancy is lower than in TMP, it still contributed to the overall binding with a positive free energy of 4.95 kcal/mol due to desolvation effect.

Additionally, COSB4 formed a significant hydrogen bond interaction with GLU17, with an occupancy of 17.78%. This residue, which is also involved in TMP and FOL binding, showed a stronger and more persistent interaction with COSB4, leading to a higher affinity for DHFR. This is consistent with experimental assays, where COSB4 demonstrated better antibacterial activity against *E. coli* than TMP. The weakest hydrogen bond interaction occurred between COSB4 with THR35, which showed no occupancy or decomposed residue in the analysis. This residue was not found in FOL or TMP binding, clarifying that it is unique to COSB4 at DHFR.

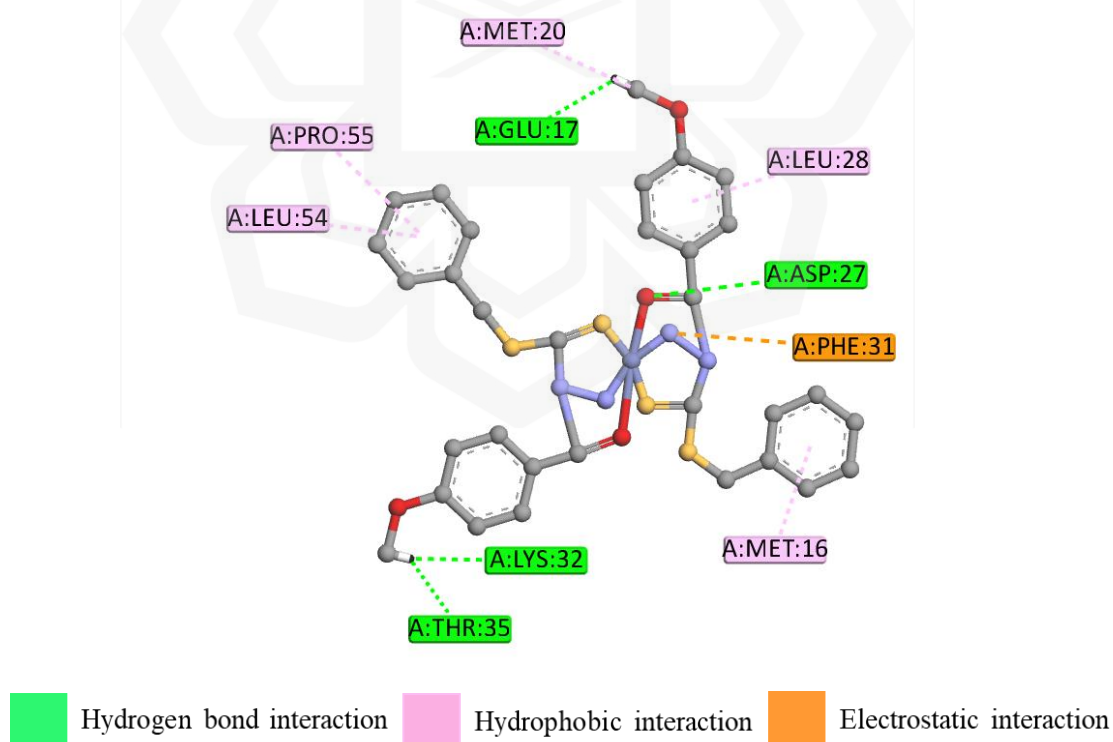


Figure 4.83 Binding interactions of COSB4 at *E. coli* DHFR

Table 4.25 Interacting residues of DHFR-COSB4

Residues	Type of interactions	H-bond occupancy (%)	Binding free energy (kcal/mol)
MET16	Hydrophobic Interaction	-	-
GLU17	Hydrogen bond	17.78	-
MET20	Hydrophobic Interaction	-	-1.07
ASP27	Electrostatic Interaction/Hydrogen Bond	32.05	4.95
LEU28	Hydrophobic Interaction	-	-2.99
PHE31	Electrostatic Interaction/ Hydrophobic Interaction	22.09	-1.61
LYS32	Hydrogen Bond	50.50	-0.73
THR35	Hydrogen Bond	-	-
LEU54	Hydrophobic Interaction	-	-1.84
PRO55	Hydrophobic Interaction	-	-1.58

In addition to hydrogen bonds, COSB4 also formed several hydrophobic interactions with MET16, MET20, LEU28, LEU54, and PRO55. The most significant hydrophobic interaction was with LEU28, with a binding free energy of -2.99 kcal/mol. Although LEU28 was not involved in TMP or FOL binding during MD simulations, it plays an important role at DHFR. Previous studies have shown that LEU28 interacts with TMP through hydrophobic interactions, and its mutation reduces TMP's effectiveness by 400-fold (Reeve et al., 2016). Therefore, this highlights the crucial role of LEU28 in DHFR's enzymatic activity. Additionally, the interaction with LEU28 was retained from the docking results, indicating its strong stability within the DHFR binding site.

Other hydrophobic interactions in COSB4 involved LEU54 and PRO55, with binding free energies of -1.84 and -1.58 kcal/mol, respectively. These residues were

part of FOL's binding, but COSB4 formed stronger interactions with both residues. Hence, this further justifies COSB4's higher affinity in the MMPBSA calculations than FOL. COSB4 also interacted hydrophobically with MET20 and MET16, with MET20 contributing a binding free energy of -1.07 kcal/mol.

Lastly, COSB4 formed an electrostatic interaction with PHE31, a residue present in both TMP and FOL. The binding contribution from PHE31 in COSB4 was significantly higher than in TMP, with a binding free energy of -1.61 kcal/mol through hydrophobic interaction. This indicates that PHE31 binds more strongly with COSB4 than with TMP, further validating the higher antibacterial activity observed with COSB4. Moreover, similar to CUSB4, the interaction with PHE31 was also observed in the docking study, indicating the stability of this interaction throughout the simulation.

In conclusion, COSB4, which showed the second-highest binding affinity to DHFR, demonstrated significant hydrogen bonds and hydrophobic interactions with the protein. Although it had fewer hydrophobic interactions compared to CUSB4, which may explain its slightly lower binding affinity, its affinity remains significantly higher than TMP.

4.14.7.6 Interaction Analysis of NISB4

NISB4 formed two hydrogen bond interactions, one with LYS32 and the other with ARG52, showing occupancies of 23.81% and 34.70%, respectively (Figure 4.84). However, only the interaction with ARG52 contributed significantly to the overall binding, with a binding free energy of 0.7 kcal/mol (Table 4.26). Both of these residues are involved in the binding of FOL through electrostatic interactions, suggesting that NISB4 mimics the natural substrate rather than TMP.

In addition, NISB4 formed four more hydrophobic interactions with LEU28, LEU54, PRO55, and ILE94. Only LEU28, LEU54, and PRO55 contributed to binding free energies of -1.83, -1.42, and -1.06 kcal/mol, respectively. As previously noted, LEU28, although not involved in TMP or FOL binding during MD simulations, plays an important role in DHFR by interacting with TMP through hydrophobic interactions. Furthermore, this interaction was maintained from the docking analysis, indicating its strong stability within the DHFR binding site. On the other hand, LEU54, PRO55, and

ILE94 are part of FOL's binding interactions, further supporting the idea that the NISB4 mimics the substrate binding at DHFR.

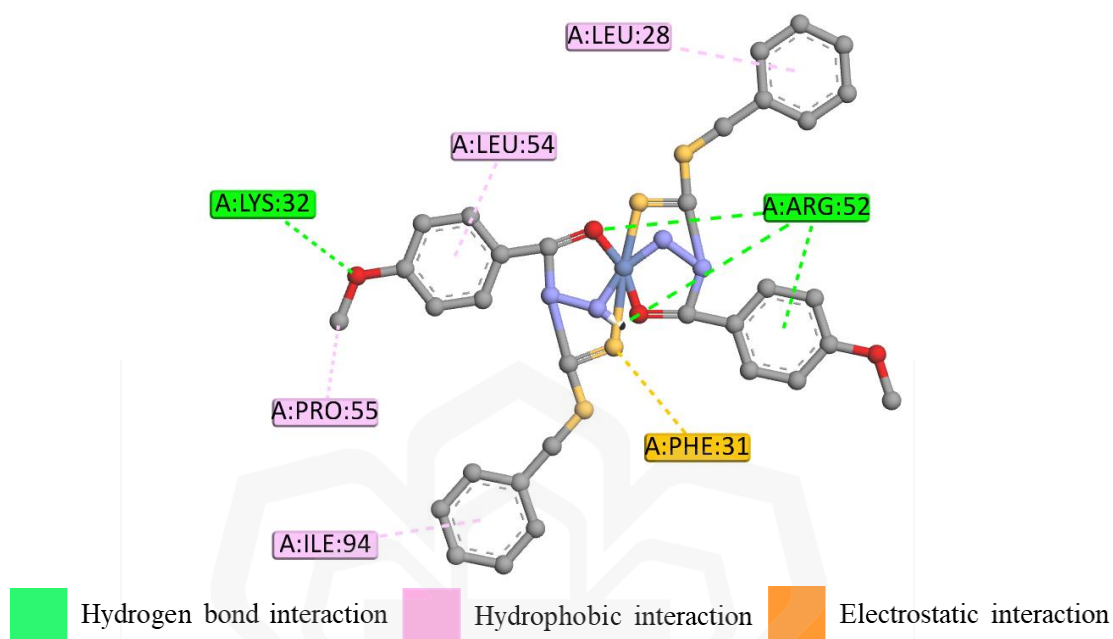


Figure 4.84 Binding interactions of NISB4 at *E. coli* DHFR

Table 4.26 Interacting residues of DHFR-NISB4

Residues	Type of interactions	H-bond occupancy (%)	Binding free energy (kcal/mol)
LEU28	Hydrophobic Interaction	-	-1.83
PHE31	Electrostatic Interaction/ Hydrophobic Interaction	17.69	-1.03
LYS32	Hydrogen Bond	23.81	-
ARG52	Hydrogen Bond	34.70	0.7
LEU54	Hydrophobic Interaction	-	-1.42
PRO55	Hydrophobic Interaction	-	-1.06
ILE94	Hydrophobic Interaction	-	-

NISB4 also formed an electrostatic interaction with PHE31, a residue that is also involved in TMP and FOL binding. However, in the MMPBSA analysis, this interaction contributed -1.03 kcal/mol to the overall binding, primarily through hydrophobic interactions. This interaction was also retained from the docking results, highlighting the significance of PHE31 in the stability of NISB4. Overall, among the metal complexes, it is clear that NISB4 interacts the least with DHFR, which explains its lowest binding affinity in the MMPBSA calculations. Rather than mimicking TMP, NISB4 more closely resembles FOL's binding at the DHFR active site.

4.14.8 Molecular Distance analysis

The lack of hydrogen bond occupancy exhibited by investigated compounds especially metal complexes in Table 4.24-4.26 suggest that these interactions may not be consistently maintained throughout the simulation. According to standard conventions, a hydrogen bond is typically favorable and stable when the donor–acceptor distance is less than 3.5 Å (0.35 nm), with optimal hydrogen bonding often observed in the range of 2.7–3.2 Å (0.27-0.35 nm) (Jeffrey, 1997; McDonald & Thornton, 1994). Therefore, further analysis of the hydrogen bond distances between selected residues and each compound was conducted to determine whether these interactions remained within a favorable range. Table 4.27 presents the average molecular distances for each complex, which are further illustrated in Figure 4.85.

Table 4.27 Molecular distance of the investigated compounds at *E. coli* DHFR

Complex	Molecular distance (nm)
FOL-LEU54	0.6256 ± 0.1496
TMP-ASP27	0.2957 ± 0.0905
SB4-ILE94	0.2858 ± 0.0527
CUSB4-ARG57	1.0377 ± 0.0739
COSB4-LYS32	0.4507 ± 0.1342
NISB4-ARG52	0.3154 ± 0.0386

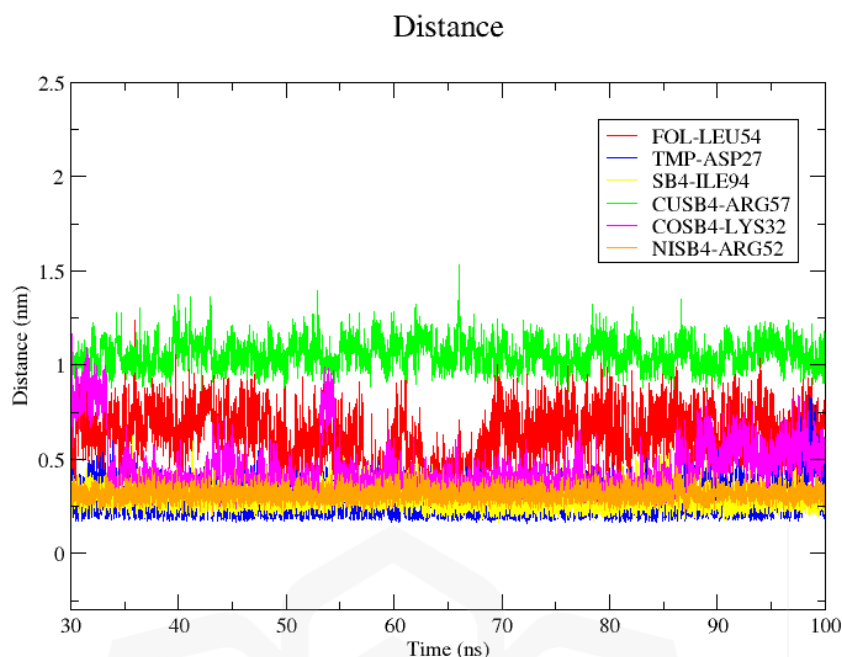


Figure 4.85 Molecular distance of the investigated compounds at specific residue of *E. coli* DHFR

Two control compounds, TMP and FOL, were included in the analysis. For TMP, the interaction with ASP27 was selected, as it exhibited 100% hydrogen bond occupancy. Plus, this residue was also known to play a crucial role in DHFR activity (Wahaibi et al., 2024). Accordingly, the molecular distance between TMP and ASP27 was analysed to evaluate the consistency and stability of this key interaction. In contrast, the weakest hydrogen bond formed by FOL, which was with LEU54, was chosen to illustrate how an unfavorable interaction would be reflected in the molecular distance analysis.

As expected, the TMP-ASP27 complex showed the shortest average distance (0.2957 nm) with minimal fluctuations throughout the simulation. This indicates a highly stable interaction between TMP with ASP27. Conversely, the FOL-LEU54 pair exhibited a higher average distance (0.6256 nm) with significant fluctuations. This observation suggests an unstable and less favorable interaction between FOL and LEU54. Therefore, both control plots revealed that stable hydrogen bonds with high occupancy are associated with shorter and more consistent molecular distances, while weaker interactions showed as longer and more variable distances over time.

Among the synthesised compounds, the hydrogen bond between SB4 and ILE94 was evaluated. The occupancy analysis revealed that ILE94 maintained 100% hydrogen bond occupancy, similar to ASP27 in the TMP complex. As illustrated in Figure 4.85, the SB4-ILE94 pair showed the shortest average molecular distance (0.2858 nm), even shorter than the TMP-ASP27. In addition, the distance profile remained highly consistent throughout the simulation, indicating a favourable and stable interaction that is likely crucial for complex stability.

In the case of metal complexes, the pattern differed, as these compounds tended to favor hydrophobic interactions over hydrogen bonding, as discussed in Subsection 4.14.7. For example, CUSB4 formed three hydrogen bonds with DHFR residues. However, none of these residues exhibited significant occupancy. Therefore, the molecular distance between CUSB4 and ARG57 was measured as a representative hydrogen bond. The results showed that this interaction had the highest average distance (1.0377 nm), suggesting it was an unfavourable hydrogen bond. Nonetheless, the distance remained consistent, implying that despite its weakness, the interaction persisted throughout the simulation.

Further analysis of COSB4 revealed four hydrogen bonds, with LYS32 showing the highest occupancy at 50.50% (Table 4.25). The average distance of this interaction was 0.4507 nm, reflecting a moderately stable hydrogen bond. However, distance fluctuations were observed, particularly between 85-100 ns, suggesting a possible rearrangement during the latter part of the simulation. Finally, for NISB4, which formed three hydrogen bonds, the interaction with ARG52 was selected based on its highest hydrogen bond occupancy (34.70%, Table 4.26). Interestingly, the NISB4-ARG52 interaction exhibited a short average distance (0.3154 nm) and remained highly consistent throughout the simulation. This suggests a robust and stable interaction that may be critical for effective binding. Moreover, this consistent proximity between NISB4 and ARG52 indicates a strong likelihood of effective binding at this site.

Overall, the analysis highlights that SB4 and NISB4 maintain the most consistent and closest interactions with their respective residues, suggesting stronger and more stable binding profiles. In contrast, the CUSB4-ARG57 interaction was the weakest, which may indicate a preference for hydrophobic interactions over hydrogen bonding in this complex.

4.14.9 Comparative Binding Analysis of SB4 and Its Metal Complexes with *E. coli* DHFR

In silico revealed differences in the behaviour of SB4 and its metal complexes when binding to DHFR. This study has shown that metal complexes exhibited stronger binding than SB4 due to a few significant reasons. An exploration of the factors contributing to the enhanced binding affinity of the metal complexes for DHFR, as compared to the free ligand, is outlined below.

Firstly, molecular docking showed that metal ions themselves did not directly interact with the protein rather they influence the positioning of the ligand within the active site. To investigate how metal ions affect the ligand's positioning, Figure 4.86 compared the localisation of SB4 and its metal complexes from a molecular docking study with the control ligand (red). NISB4, showing the highest affinity, is deeply embedded within the protein's active pocket. ZNSB4 showed the least affinity, positioned at the outer edge of the pocket. COSB4 and CUSB4 also bound within the pocket, but their flexibility caused parts of the ligands to be positioned away from active residues, resulting in moderate affinity. In contrast, SB4 deviates from FOL's original position, occupying less than half of its location, leading to the lowest binding affinity. These observations suggest that the metal ions act as “anchors”, locking the ligand into a conformation that is more favourable within the binding site.

This anchoring effect aligns with Jevtovic et al. (2023). The study showed that different metal ions induce specific structural rearrangements within the ligand, altering its overall shape and positioning in the protein's active site (Jevtovic et al., 2023). While docking provides a static snapshot of these interactions, MD simulations offer a dynamic perspective by capturing the conformational flexibility of both the compounds and the protein over time. MD results showed that metal ions restrict the movement of the ligand and stabilise its conformation. This is evident in stable and less flexibility from RMSD (Subsection 4.14.1) and RMSF analysis (Subsection 4.14.3), respectively discussed during MD simulations (J. Chen et al., 2007; Diab et al., 2019)

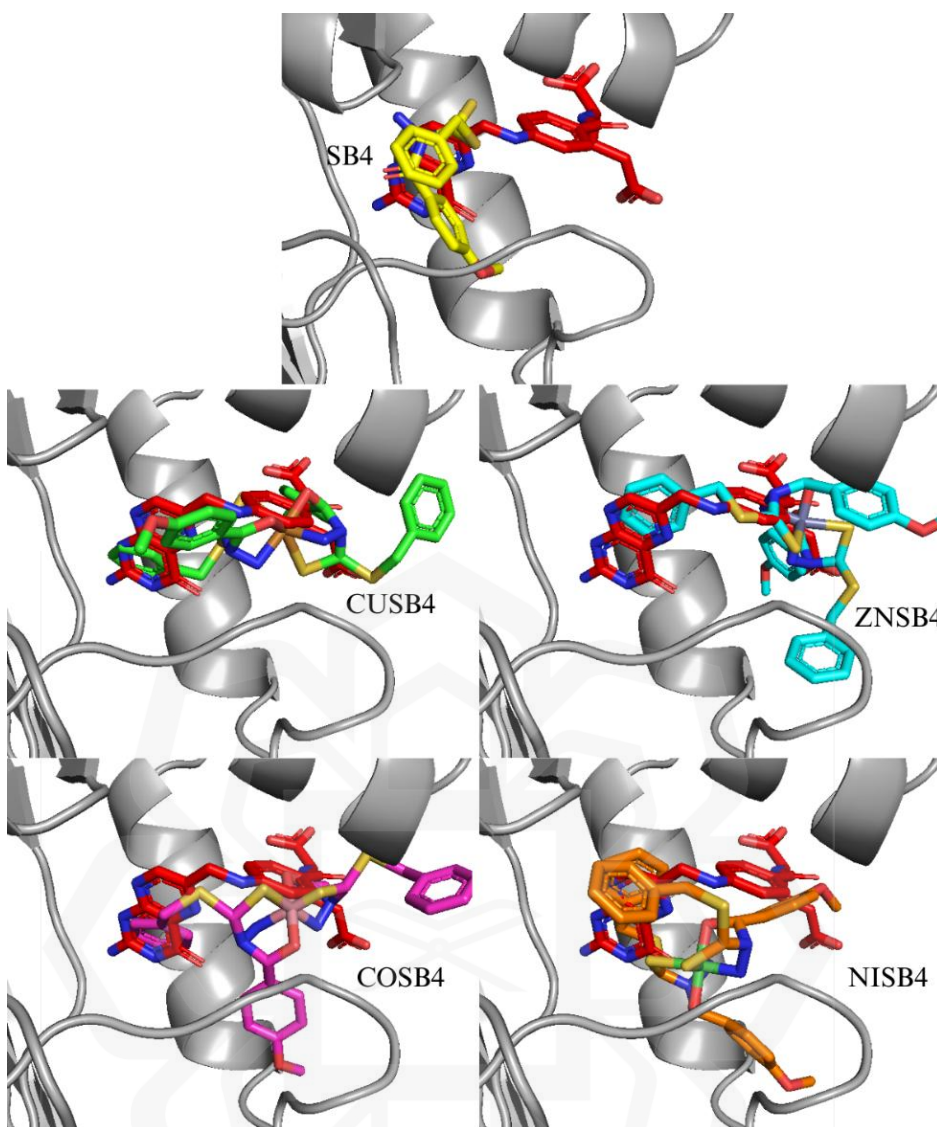


Figure 4.86 Localisation of SB4 and its metal complexes with FOL (red) from molecular docking study at *E. coli* DHFR

Moreover, the enhanced binding affinity of metal complexes compared to SB4 might not solely be due to the metal ion itself. It could also be attributed to the larger molecular size of the metal complexes. The larger size likely contributes to the rigidity of these complexes, improving their fit within the protein's active site. This is evident in the MD simulation shown in Figure 4.87 and B-factor putty representative in Figure 4.74. The larger structures of CUSB4, COSB4, and NISB4 make them more rigid and concentrated at the binding site. In contrast, the smaller structure of SB4 makes it less rigid in the binding site. This observation supports findings from Johnson et al. (2018) and C. Li et al. (2020), noted that the rigidity of large metal complexes enhances their binding efficiency.

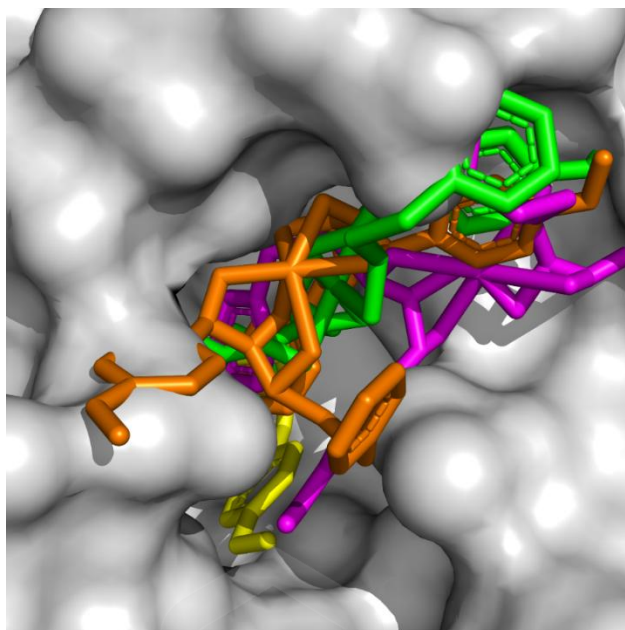


Figure 4.87 Localisation of SB4 (yellow) and its metal complexes (CUSB4: green, COSB4: magenta, NISB4: orange) from MD simulation study at *E. coli* DHFR

In addition, bulky metal complexes also enhance crucial interactions with binding sites. Previous studies showed that bacterial DHFR has a more hydrophobic active site, which enables stronger binding of inhibitors like TMP (Bystroff & Kraut, 1991). The inclusion of hydrophobic groups, such as aromatic rings in these compounds, allows them to form numerous hydrophobic interactions at DHFR's hydrophobic pockets, as illustrated in Figure 4.81-4.83. These crucial interactions strengthen the binding interactions, as reflected by the binding free energy values from MMPBSA analysis. To compare with SB4, it lacks a hydrophobic group, resulting in less affinity for DHFR's binding site as proved by higher binding free energy observed in MMPBSA calculation (Subsection 4.14.6).

Moreover, bulky metal complexes also reduce the exposure of water molecules at the binding site. Water molecules can disrupt the bond between the ligand and the protein, destabilising the interaction. The larger size of the metal complexes creates a shield, preventing water from interfering with critical binding regions. As a result, this situation helps maintain a stable ligand-protein interaction. Similarly, the MET20 loop in DHFR helps shield the active site from water, creating an environment that supports stronger binding (Goldstein & Goodey, 2021). Goldstein & Goodey (2021) explained that this solvent shielding is crucial for DHFR's activity, as it prevents water from interfering with the protein's function.

In conclusion, both docking and MD simulations highlight the stabilising effects of metal complexes on DHFR binding. Docking provides an initial insight into binding localisation, while MD simulation demonstrates how these binding poses are maintained dynamically. Traditionally, metal ions in metal-based drugs are considered contributors to direct coordination bonding. However, these findings suggest that the metal ions serve as anchors that optimise ligand positioning. Additionally, the bulkiness of the metal complexes contributes to rigidity, hydrophobic interactions, and reduced water exposure at the binding site, leading to a stronger affinity for DHFR.

4.15 POTENTIAL TO COUNTERACT *E. COLI* DHFR MUTATIONS VIA MOLECULAR DOCKING STUDY

To assess the potential of SB4OME and its metal complexes in combating drug-resistant *E. coli* strains, this subsection investigates their binding affinities and interactions with mutant variants of the DHFR enzyme. This analysis focuses on two well-characterised resistance-associated point mutations, occurring at positions L28R and I94L, both of which have been shown to significantly reduce the binding efficiency of TMP (Manna et al., 2021). Thus, molecular docking studies were conducted to systematically evaluate whether these compounds can retain or enhance their binding affinity despite the structural changes introduced by these mutations. The findings aim to provide insight into the potential of these compounds as alternative DHFR inhibitors and contribute to the rational design of novel therapeutics capable of overcoming TMP resistance in *E. coli*.

4.15.1 Validation of Protein Structure

Since the wild-type *E. coli* DHFR structure (PDB ID: 6XG5) was manually mutated to I94L using UCSF Chimera, structural validation of the resulting model was essential to ensure its suitability for subsequent *in silico* analyses. To assess the impact of the mutation, the I94L model was aligned with the wild-type DHFR structure, and the RMSD was calculated. The resulting low RMSD value of 0.071 Å indicated that the mutation was localized and did not induce significant conformational changes in the overall protein structure (Binbay et al., 2023).

Further validation was conducted using the ERRAT tool, which evaluates the overall quality of protein models. The I94L mutant achieved an ERRAT quality factor

of 96%, which is consistent with the benchmark for high-resolution crystallographic structures typically scoring above 95% (Tahir et al., 2014). Complementing this, the Verify3D analysis demonstrated that 84.85% of the amino acid residues had compatibility scores above the 0.1 threshold in the 3D-1D profile, further affirming the structural integrity of the model.

Stereochemical quality was also examined using PROCHECK through Ramachandran plot analysis. The results showed that 90.9% of residues were located in the most favoured regions, 8.4% in additionally allowed regions, and only 0.7% in disallowed regions. As more than 80% of the residues occupied the favoured regions, the structure falls within acceptable parameters for molecular modelling (Sobolev et al., 2020).

Collectively, the validation outcomes from RMSD, ERRAT, Verify3D, and Ramachandran plot analyses confirm the overall structural quality and reliability of the I94L DHFR mutant model. These results establish the model's suitability for further computational study including molecular docking.

4.15.2 Molecular Docking of the Compounds Against L28R mutant *E. coli* DHFR

The binding energy values and molecular interactions of all investigated compounds were compared between wild-type *E. coli* DHFR (as detailed in Subsection 4.13.2) and the L28R mutant variant (Table 4.28). This comparison aimed to evaluate the impact of the L28R mutation on ligand binding affinity and interaction profiles.

Overall, most compounds exhibited stronger binding affinities toward the L28R mutant than the wild-type enzyme. Notably, SB4, CUSB4, and ZNSB4 demonstrated enhanced binding to the mutant, suggesting their potential to overcome resistance conferred by the L28R mutation. Among these, CUSB4 exhibited the strongest interaction, with a binding free energy of -9.33 kcal/mol, highlighting its promise as a potential inhibitor targeting the resistant mutant. In contrast, COSB4 and NISB4 showed comparatively better binding to the wild-type DHFR, yet still maintained high affinity for the L28R variant, with binding energies of -9.14 and -9.47 kcal/mol, respectively. This dual binding capability suggests that the metal complexes may remain effective across both wild-type and mutant forms of the enzyme.

To better understand the basis for these differences, detailed interaction analyses were conducted for both wild-type and L28R mutant DHFR, with particular emphasis on mutation at position 28. In the wild-type enzyme, all compounds including TMP, SB4, and its metal complexes consistently formed hydrophobic interactions with LEU28. This emphasises LEU28's role in stabilising ligand binding and showing its contribution to enzymatic function. However, in the L28R mutant, this interaction was completely lost for TMP and SB4, indicating a reduced ability to engage the mutated residue. As illustrated in Figure 4.88, the substitution of LEU28 with the positively charged ARG28 introduces a significant change to the physicochemical environment of the binding pocket, disrupting previously established hydrophobic interaction. This loss likely reduces TMP's binding ability in strains with the L28R mutation. These structural insights are consistent with clinical observations of TMP-resistant *E. coli* isolates (Manna et al., 2021).

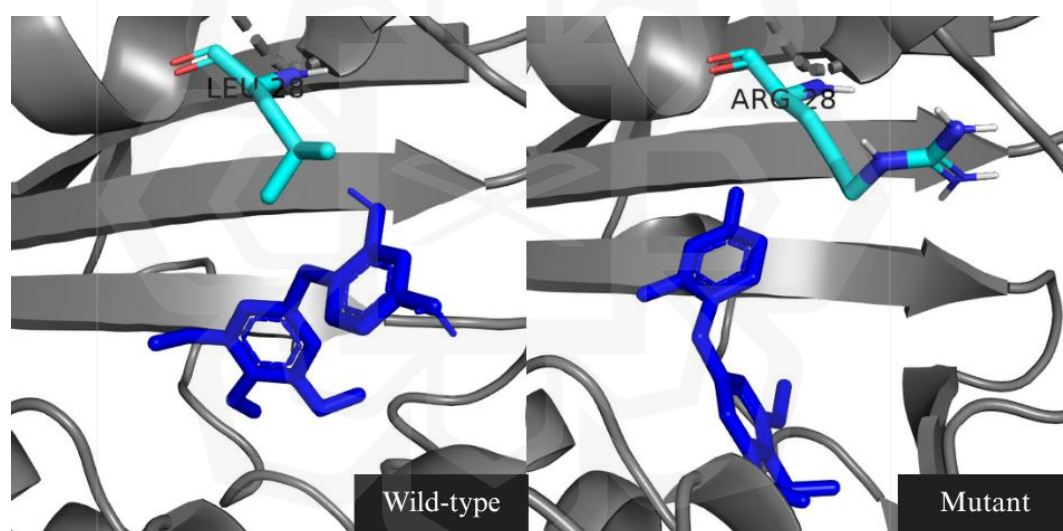


Figure 4.88 Localisation of TMP (blue) at the wild-type and L28R mutant *E. coli* DHFR

Remarkably, all metal complexes, including CUSB4, ZNSB4, COSB4, and NISB4 retained hydrophobic interactions with ARG28. This suggests that the metal complexes exhibited an adaptive binding capacity, enabling them to accommodate both the longer side chain and electrostatic properties of arginine. For instance, as illustrated in Figure 4.89, CUSB4 was able to form interactions with ARG28, likely due to its structural flexibility and relatively large molecular size. The ability to maintain binding interactions may reflect the general tendency of these metal complexes to engage hydrophobic regions within the DHFR active site. Notably, a similar interaction pattern

was observed in the wild-type enzyme (refer to Subsection 4.13.2). Such adaptability likely contributes to their superior binding affinity relative to TMP and SB4.

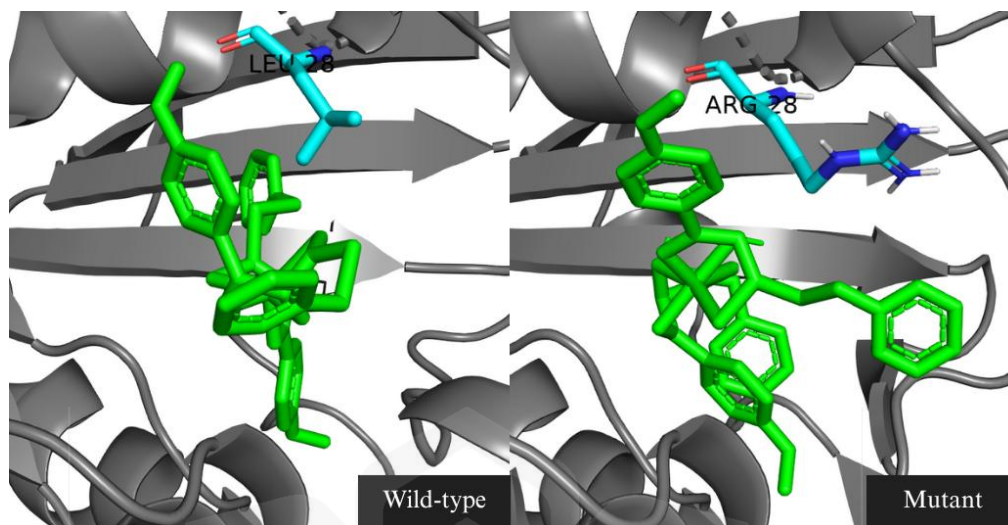


Figure 4.89 Localisation of CUSB4 (green) at the wild-type and L28R mutant *E. coli* DHFR

Notably, binding interactions with GLU17 and ILE94 were disrupted, indicating broader structural effects introduced by the mutation (Table 4.28). Conversely, interactions with ALA7 and ARG52 were preserved across both wild-type and mutant variant, continuing to provide hydrophobic and hydrogen bonding stabilisation, respectively. Interestingly, new interactions emerged in the L28R mutant, particularly involving LYS32 and THR113. For example, LYS32 formed hydrogen bonds with several compounds including CUSB4, ZNSB4, and COSB4 which may provide additional stabilisation in the altered binding site (Table 4.28).

These findings indicated that although the L28R mutation disrupts a key hydrophobic interaction at position 28, it does not entirely restrict ligand binding. Compounds such as CUSB4, ZNSB4, COSB4, and NISB4 exhibited adaptive binding behavior by forming new interactions with ARG28 while maintaining strong binding affinity. These observations align with prior studies aimed at overcoming TMP resistance by targeting the L28R mutation. For instance, Manna et al. (2021) developed a TMP derivative, 4'-desmethyltrimethoprim (4'-DTMP) which incorporates polar substituent on the aryl ring to enhance electrostatic and hydrogen bonding interactions with ARG28. Biochemical assays revealed that 4'-DTMP binds the L28R variant with nearly twice the affinity of TMP and demonstrates a 30- to 90-fold increase in

antimicrobial activity against *E. coli* strains expressing L28R, while retaining potency against wild-type strains. Moreover, laboratory evolution experiments showed that 4'-DTMP delays the emergence resistance by diverting adaptive pressure toward less favorable evolutionary pathways.

Similarly, Zhang et al. (2020) identified the novel inhibitor, CD15-3 through a combined computational and experimental approach. CD15-3 effectively inhibited the L28R variant, with IC_{50} values ranging from 50 to 75 μ M, and significantly slowed the evolution of resistance. Resistance to CD15-3 emerged via gene duplication rather than point mutations in *folA*, indicating a less favorable and more complex resistance mechanism.

In conclusion, these results support the rational design of metal complexes as inhibitors for overcoming resistance associated with DHFR mutations such as L28R. The ability of metal complexes derived from SB4OME to retain key interactions with ARG28, despite structural alterations at the active site, is noteworthy. Thus, this finding highlights their potential as promising leads for the development of next-generation DHFR inhibitors targeting antimicrobial-resistant *E. coli*.

Table 4.28 Comparison of binding energy, interaction type, and interacting amino acid residue in wild-type and L28R mutant *E. coli* DHFR.
(Mutated residue in bold)

Complex	Wild-type			Mutant L28R		
	Binding energy (kcal/mol)	Hydrogen bond interaction	Hydrophobic bond interaction	Binding energy (kcal/mol)	Hydrogen bond interaction	Hydrophobic bond interaction
DHFR-TMP	-6.99	GLU17, TRP22, ASP27, TYR100	ILE5, ALA6, ALA7, ILE14, MET20, LEU28 , PHE31, ILE94	-7.19	ILE5, ALA6, ASP27, ILE94, TYR100, THR113	ILE14, MET20
DHFR-SB4	-6.92	ILE5, ALA7, TRP22, ILE94, TYR100	ILE14, LEU28	-7.90	ALA7, ILE94, TYR100, THR113	ILE5, ILE14, PHE31
DHFR-CUSB4	-8.72	GLU17	ILE5, ALA7, ILE14, MET16, LEU28 , LYS32, ARG52, LEU54	-9.33	MET20, LYS32, ARG52	ALA7, ILE94, ALA19, ARG28
DHFR-ZNSB4	-7.24	ARG52	LEU28 , ALA29, PHE31, LYS32, ILE50, ILE94	-9.20	LYS32	ALA6, ALA7, ILE14, ALA19, ARG28 , ARG52

DHFR- COSB4	-9.30	LEU24, ARG52	ALA7, LEU28 , PRO25, PHE31, LYS32, ILE50	-9.14	LYS32, THR113	ILE5, ALA7, ALA19, MET20, ARG28 , LEU54
DHFR- NISB4	-10.92	GLU17, LEU24, ARG52	ALA7, ILE14, MET16, LEU28 , PHE31, ILE50, ILE94	-9.47	MET20, ARG52	ALA7, ARG28 , PHE31, LYS32, ILE50

4.15.3 Molecular Docking of the Compounds Against I94L mutant *E. coli* DHFR

Following the analysis of the L28R mutation, this section evaluates the binding behaviour of investigated compounds in the I94L mutant compared to the wild-type *E. coli* DHFR (Table 4.29). This comparison provides insight into how the substitution from isoleucine to leucine at position 94 influences ligand binding affinity and interaction. Although the I94L mutation does not alter the charge or polarity of the residue, it may introduce subtle spatial rearrangements within the binding pocket that impact ligand positioning and stabilisation.

As anticipated, TMP displayed a reduced binding affinity in the I94L mutant relative to the wild-type enzyme with the minimal differences of 0.19 kcal/mol (Table 4.29). This observation aligns with previous studies reporting that substitutions at position 94 can significantly diminish TMP's inhibitory effect, thereby contributing to antimicrobial resistance (L. Yu, 2024). In contrast, most of the synthesised compounds demonstrated improved binding affinity toward the I94L mutant. For example, SB4 exhibited an enhanced binding free energy, improving from -6.92 kcal/mol in the wild-type to -7.82 kcal/mol in the mutant. Similarly, CUSB4 improved from -8.72 to -9.06 kcal/mol, while ZNSB4 showed a modest increase from -7.24 to -7.61 kcal/mol. These trends were consistent with those observed in the L28R mutant, where SB4, CUSB4, and ZNSB4 also demonstrated enhanced binding, collectively reinforcing their potential to overcome DHFR mutation-associated resistance mechanisms.

Conversely, COSB4 and NISB4 exhibited a decrease in binding affinity from wild-type to I94L mutant, dropping from -9.30 to -8.56 kcal/mol and -10.92 to -8.06 kcal/mol, respectively. A similar pattern was noted in the L28R mutant, suggesting that these compounds may be more sensitive to minor conformational changes induced by the I94L substitution. Nevertheless, their binding affinities remained significantly stronger than TMP, highlighting their continued potential as alternative DHFR inhibitors.

To further investigate these observations, a detailed interaction analysis was performed with a specific focus on residue 94. In the wild-type enzyme, TMP formed a hydrophobic interaction with ILE94. However, in the I94L mutant, this interaction shifted to a hydrogen bond with LEU94. As illustrated in Figure 4.90, this shift was

attributed to a notable change in TMP's binding orientation. In the wild-type, the trimethoxybenzyl group of TMP aligned with ILE94, enabling hydrophobic interaction. Upon mutation, the binding orientation of TMP rotated by approximately 180°, positioning the diaminopyrimidine moiety near LEU94 and facilitating hydrogen bond formation. Although this reorientation preserved an interaction with the mutated residue, the nature and strength of the interaction were altered, likely contributing to the observed reduction in binding affinity.

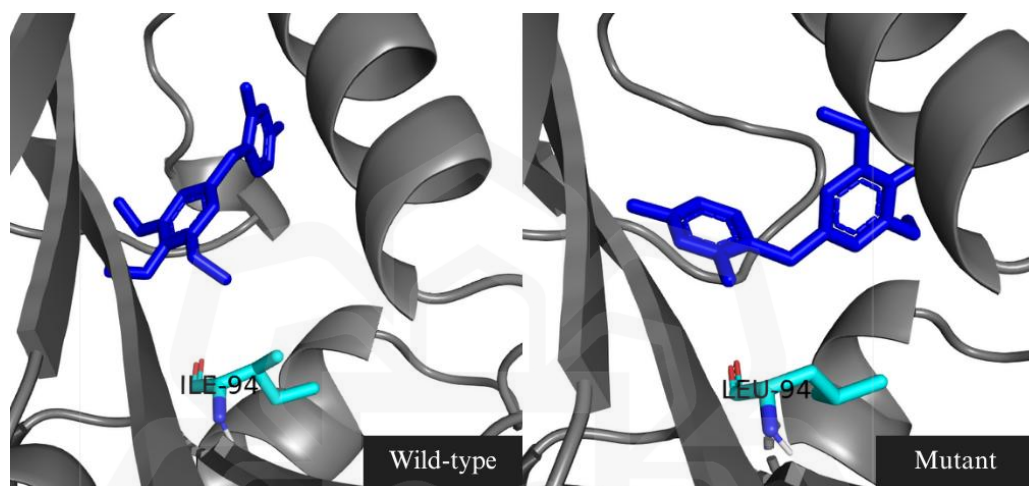


Figure 4.90 Localisation of TMP (blue) at the wild-type and I94L mutant *E. coli* DHFR

Moreover, among the investigated compounds, SB4, CUSB4, and COSB4 retained interactions with residue 94 in the I94L mutant protein (Table 4.29). Notably, SB4 preserved this interaction across both the wild-type and mutant forms, suggesting good adaptability to structural changes induced by the mutation. In contrast, CUSB4 and COSB4 established interactions with LEU94 only in the mutant form, with no corresponding interaction observed in the wild-type. Structural analysis in Figure 4.91 revealed that, in the mutant, CUSB4 adopted a new orientation that positioned the sulfur atom of the SBDTC scaffold close to LEU94, enabling a new stabilising interaction. This newly formed interaction likely contributes to the improved binding energy of CUSB4 in the mutant. Meanwhile, ZNSB4 and NISB4 lost interactions with residue 94 upon mutation yet both compounds still exhibited substantially higher binding affinities than TMP. Hence, this suggests their potential to maintain inhibitory activity despite the structural changes induced by I94L.

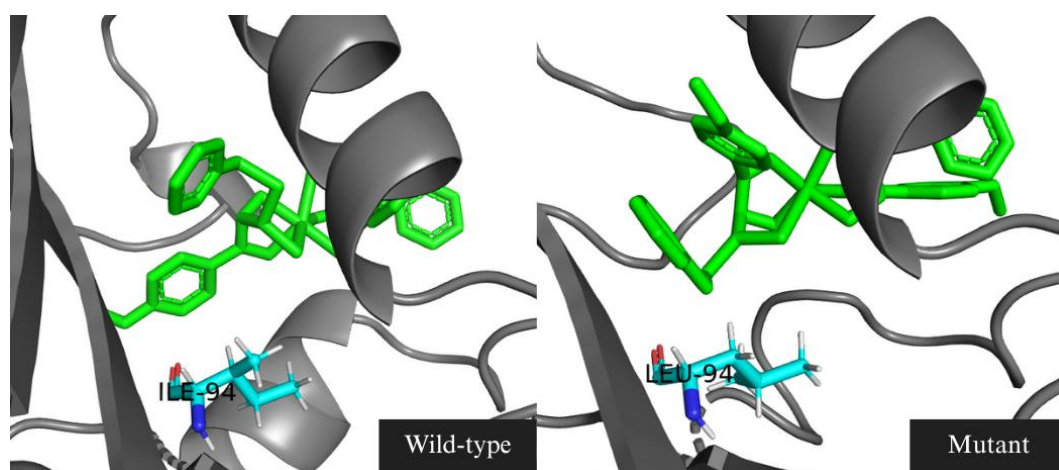


Figure 4.91 Localisation of CUSB4 (green) at the wild-type and I94L mutant *E. coli* DHFR

In addition to residue 94, LEU28 remained a consistent site of hydrophobic interaction across nearly all compounds in both wild-type and mutant forms, highlighting its vital role in ligand stabilisation (Table 4.29). Furthermore, new interactions emerged in the mutant protein, likely reflecting local conformational shifts. Notably, ASN18 formed new hydrogen bonds with SB4, CUSB4, ZNSB4, and COSB4, in which these interactions not present in the wild-type structure. Similarly, ZNSB4 and NISB4 interacted with ARG57 in the mutant, suggesting slight changes in their position to fit the altered binding environment. Hydrophobic residues such as PRO55 and LEU54 also contributed more frequently to stabilisation in the mutant, potentially enhancing the overall binding of several ligands.

In summary, the I94L mutation did not significantly impair the binding ability of most investigated compounds. Particularly, CUSB4 and COSB4 maintained strong affinity toward the I94L mutant DHFR, highlighting their potential as promising leads in the development of inhibitors targeting TMP-resistant strains. Notably, to date, no clinically validated inhibitors have been specifically designed to target the I94L mutation in *E. coli* DHFR. Nevertheless, the present computational findings offer a compelling rationale for exploring this direction. The binding affinity and interaction analyses support the design of next-generation DHFR inhibitors that overcome I94L-mediated TMP resistance, helping to develop more effective antibacterial agents.

Table 4.29 Comparison of binding energy, interaction type, and interacting amino acid residue in wild-type and I94L mutant *E. coli* DHFR.
(Mutated residue in bold)

Complex	Wild-type			Mutant I94L		
	Binding energy (kcal/mol)	Hydrogen bond interaction	Hydrophobic bond interaction	Binding energy (kcal/mol)	Hydrogen bond interaction	Hydrophobic bond interaction
DHFR-TMP	-6.99	GLU17, TRP22, ASP27, TYR100	ILE5, ALA6, ALA7, ILE14, MET20, LEU28, PHE31, ILE94	-6.80	ILE5, ALA7, LEU94 , TYR100	LEU28, PHE31, ILE50
DHFR-SB4	-6.92	ILE5, ALA7, TRP22, ILE94 , TYR100	ILE14, LEU28	-7.82	MET16, ASN18, LEU94	ALA7, MET20, LEU28, ILE50
DHFR-CUSB4	-8.72	GLU17	ILE5, ALA7, ILE14, MET16, LEU28, LYS32, ARG52, LEU54	-9.06	ASN18	ALA7, LEU28, PHE31, ILE50, ARG52, LEU54, PRO55, LEU94
DHFR-ZNSB4	-7.24	ARG52	LEU28, ALA29, PHE31, LYS32, ILE50, ILE94	-7.61	ASN18, ARG57	ILE5, ALA7, LEU28, ARG52, LEU54, PRO55

DHFR- COSB4	-9.30	LEU24, ARG52	ALA7, LEU28, PRO25, PHE31, LYS32, ILE50	-8.56	ASN18, LYS32	PRO25, PHE31, ILE50, ARG52, PRO55, LEU94
DHFR- NISB4	-10.92	GLU17, LEU24, ARG52	ALA7, ILE14, MET16, LEU28, PHE31, ILE50, ILE94	-8.06	ARG57	ALA29, ILE50, ARG52, PRO55, LEU54

4.16 DETERMINATION OF NEW ANTIBACTERIAL AGENT

To identify the most promising new antibacterial agent among the 20 synthesised compounds of SBDTC derivatives and their metal complexes, a two-step filtration process was employed, involving both *in vitro* and *in silico* studies. The *in vitro* assays revealed that SB4OME and its metal complexes demonstrated the most effective antibacterial activity, as evidenced by their lowest MIC values compared to other SBDTC derivatives and their respective metal complexes. Consequently, SB4OME and its Cu(II), Zn(II), Co(II), and Ni(II) complexes were selected for further analysis through *in silico* studies to validate their potential.

Molecular docking studies supported the *in vitro* findings by highlighting the specific interactions between these compounds and the *E. coli* DHFR protein. However, since docking inherently assumes rigid ligand-protein interactions and does not account for flexibility, MD simulations were conducted to assess the stability of these interactions more precisely. Results from the MMPBSA calculations revealed that the metal complexes displayed stronger binding to DHFR compared to the free ligand alone. Among these, Cu(SB4OME)₂ exhibited the most negative binding free energy, marking it as the top candidate for further investigation. Furthermore, Cu(SB4OME)₂ demonstrated strong potential in overcoming TMP resistance, as evidenced by its high binding affinity and sustained interactions with both L28R and I94L mutant forms of *E. coli* DHFR.

Notably, to support the Cu(SB4OME)₂ as a new potential antibacterial agent, information from characterisation and toxicity studies was reviewed. Characterisation results showed that Cu(SB4OME)₂ possessed higher thermal stability than the uncoordinated ligand, reducing the risk of potential decomposition during storage, handling, and biological applications. Furthermore, toxicity predictions indicated that Cu(SB4OME)₂ belongs to the same toxicity class as TMP, suggesting favourable safety for therapeutic use.

Overall, based on the comprehensive results discussed above and illustrated in Figure 4.92, this study concludes that Cu(SB4OME)₂ stands out as the most promising new antibacterial agent. Furthermore, the *in silico* findings align perfectly with the *in vitro* data, indicating that Cu(SB4OME)₂ is likely to inhibit the *E. coli* DHFR enzyme

during folate synthesis, following a mechanism similar to that of TMP. Therefore, given the ultimate goal of this research to contribute to the development of next-generation antibacterial agents as effective alternatives to TMP, this study strongly recommends Cu(SB4OME)₂ for further exploration as an *E. coli* DHFR-targeting drug in drug development.



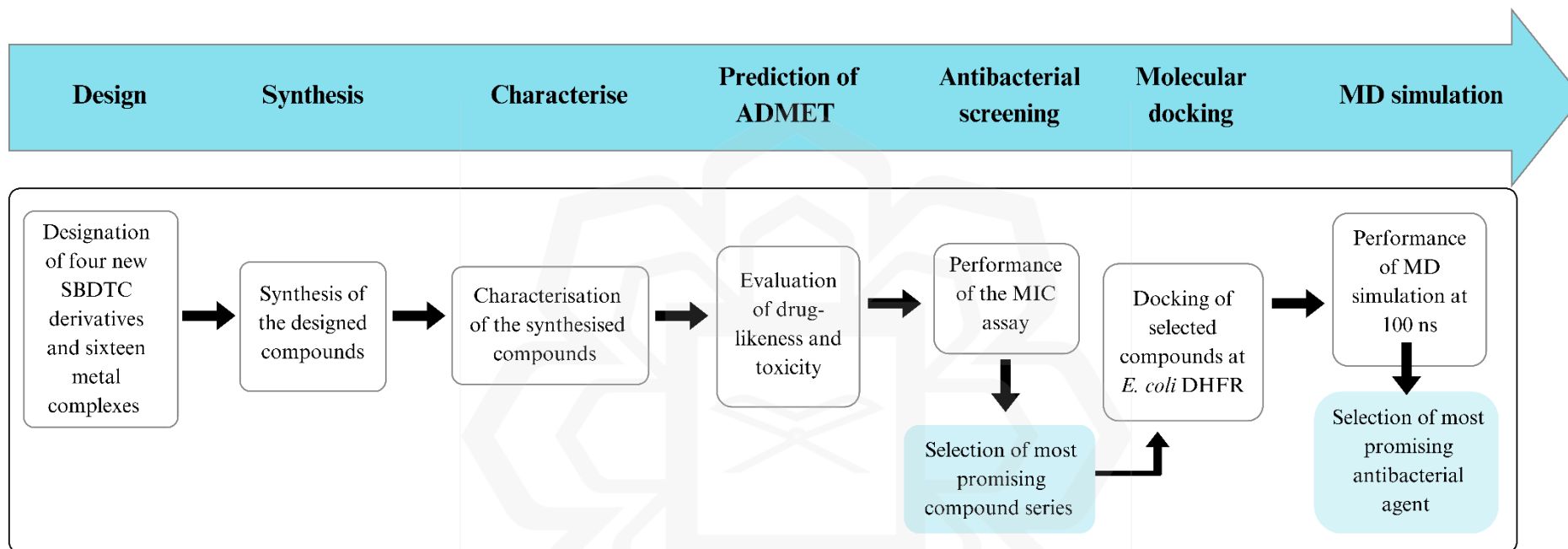


Figure 4.92 Pathway strategy for discovering new antibacterial agent from SBDTC derivatives and their metal complexes targeting *E. coli* DHFR.

CONCLUSION

5.1 SUMMARY

In summary, all objectives of this study were successfully achieved. A total of 20 compounds from SBDTC derivatives and their metal complexes, were rationally designed as inhibitors of *E. coli* DHFR, using SBDTC as the scaffold. The SBDTC derivatives were designed to resemble TMP, incorporating pharmacophores such as methoxy, amide, chloro and nitro groups. Plus, the design of metal complexes from SBDTC derivatives incorporating metal ions including copper, zinc, cobalt and nickel. Following the design phase, these compounds were successfully synthesised, including four new SBDTC derivatives and sixteen metal complexes. Comprehensive characterisation was performed using various analytical and spectroscopic techniques, such as melting point determination, elemental analysis, FTIR spectroscopy, NMR spectroscopy, GC-MS analysis, molar conductivity measurement, UV-Vis spectroscopy, magnetic susceptibility measurement, and TGA analysis. These methods confirmed the structural, physical and chemical of the synthesised compounds.

Elemental and metal analyses supported the proposed chemical formulas for all compounds, confirming the 2:1 ligand-to-metal ratio in metal complexes. Melting point and TGA analyses revealed that the metal complexes were more thermally stable than the SBDTC derivatives alone. FTIR analysis indicated that SB2OME, SB3OME, and SB3NO were substituted at the β -nitrogen of SBDTC, while SB4OME was substituted at the α -nitrogen. Further analysis revealed that SB2OME, SB3OME, and SB4OME acted as tridentate ligands, coordinating through the deprotonation of the amino group, carbonyl oxygen, and thione sulfur. In contrast, SB3NO acted as a bidentate ligand, coordinating through thiolate sulfur and carbonyl oxygen.

Additionally, FTIR and NMR spectra confirmed that SBDTC derivatives exhibited thione behaviour in the solid state. However, SB2OME and SB3NO displayed tautomeric mixtures of thione and thiol forms in solution, while SB3OME and SB4OME were present in thiol and thione forms, respectively. Mass spectrometry results further validated the structures of SB2OME, SB3OME, SB4OME, and SB3NO through molecular ion and fragmentation peaks. Magnetic susceptibility and UV-Vis

analyses indicated that metal complexes from SB2OME, SB3OME, and SB4OME exhibited octahedral geometry, while the metal complexes from SB3NO adopted tetrahedral or square planar geometries. All metal complexes demonstrated low conductivity in DMSO, suggesting that they remained undissociated in solution.

To address the third objective, ADMET properties were evaluated to assess the drug-likeness and toxicity of these compounds. The SBDTC derivatives met all drug-likeness criteria, while the metal complexes presented minor violations. However, the limitations of SwissADME in assessing complex compounds were noted, as it is primarily designed for small organic molecules. Nevertheless, all compounds exhibited low toxicity levels, demonstrating their potential for further drug development.

Following that, the *in vitro* study was conducted by determining their MIC against gram-positive and gram-negative bacteria. Most metal complexes significantly enhanced the antibacterial activity of their SBDTC derivatives alone. Remarkably, Cu(II), Zn(II), Co(II), and Ni(II) complexes derived from SB4OME reduced the MIC of SB4OME against *E. coli* (ATCC 25922) by half (MIC = 0.437 mg/mol). Furthermore, they also demonstrated superior antibacterial activity compared to TMP (MIC = 1.750 mg/mol). From structure-activity relationship analysis, among the SBDTC derivatives, SB3OME exhibited the highest antibacterial activity, likely due to its thiol tautomer state. Moreover, among all the metal complexes, those derived from SB4OME exhibited the most potent antibacterial activity compared to the metal complexes of other ligands.

Molecular docking studies were performed on SB4OME and its Cu(II), Zn(II), Co(II), and Ni(II) complexes to investigate their interactions with *E. coli* DHFR (PDB ID: 1RX7). These compounds exhibited hydrogen bond interactions with active site residues such as GLU17, TRP22 and ARG52. These residues are key in the binding of the natural substrate (folic acid) and TMP, justifying their superiority in *in-vitro* activity. Furthermore, their hydrophobic interactions with LEU28, PHE31, and ILE50 further underscored their potential as promising DHFR inhibitors.

Following the molecular docking results, molecular dynamics (MD) simulations were conducted to validate these compounds' stability at the *E. coli* DHFR (PDB ID: 1RX7), achieving the final objective. Seven systems were simulated using GROMACS

for 100 ns including DHFR-apo, DHFR-FOL, DHFR-TMP, DHFR-SB4, DHFR-CUSB4, DHFR-COSB4 and DHFR-NISB4. RMSD and Rg analyses confirmed the stability of all systems, with optimal stability observed from 30 ns onwards. RMSF and pocket volume analyses revealed no significant changes in active site residues, suggesting secure binding. However, the flexibility of the MET20 loop was decreased upon SB4, CUSB4, COSB4 and NISB4's binding, thereby inhibiting the natural substrate's affinity for the active site. Further analysis showed that all compounds maintained hydrogen bond interactions between ASN23 and SER148, except for COSB4.

MMPBSA calculations revealed that the DHFR-CUSB4 complex exhibited the most favourable binding free energy (-36.67 kcal/mol), surpassing both DHFR-FOL and DHFR-TMP. These results were perfectly aligned with the MIC assay, showing that the *in silico* study fully supports their *in vitro* activity in this study. In the *E. coli* DHFR's binding interaction analysis, SB4, CUSB4, COSB4 and NISB4 successfully mimicked several interactions of TMP and FOL. Plus, these compounds exhibited better interactions than TMP and FOL, based on the hydrogen bond occupancy, decomposed residue and molecular distance analysis. Additionally, binding analysis between SBDTC derivatives and metal complexes concluded that metal ions act as "anchors" in metal complexes and the bulkiness of metal complexes enhances rigidity, and hydrophobic interactions, and reduces water exposure, leading to higher DHFR affinity and antibacterial potency.

Further molecular docking studies were performed on the L28R and I94L mutant forms of *E. coli* DHFR to evaluate the ability of the synthesised compounds to overcome TMP resistance. Based on binding affinity and interaction analyses, the SB4OME-derived metal complexes showed strong potential in addressing antimicrobial resistance associated with these mutations. Most compounds either retained or improved their binding affinity in the mutant proteins, with notable interactions formed at critical residues. Among all, CUSB4 consistently demonstrated the strongest binding affinity and maintained key interactions with ARG28 and LEU94.

Overall, the combined results from the *in vitro* assay and *in silico* studies identified Cu(SB4OME)₂ as the most promising antibacterial agent. It exhibited the highest antibacterial activity in MIC determination. Moreover, the *in silico* study of

Cu(SB4OME)₂ showed that its affinity with the *E. coli* DHFR was better than that of the natural substrate and TMP. Plus, Cu(SB4OME)₂ also identified as the most promising lead compound for targeting resistant *E. coli* DHFR variants. Therefore, this study strongly recommends Cu(SB4OME)₂ for further development as a novel *E. coli* DHFR-targeting drug.

5.2 FUTURE WORKS

In future studies, several key areas require further investigation to strengthen the development of these compounds as antibacterial agents. First, enzyme inhibition assays targeting *E. coli* DHFR should be conducted to experimentally confirm the inhibitory potential observed in this study. *In vivo* toxicity studies are also crucial to evaluate the safety of these compounds before considering clinical or industrial applications. Additionally, exploring the use of alternative metal ions may enhance antibacterial activity by improving coordination properties. Further work should also include both *in vitro* and *in silico* approaches, such as molecular dynamics simulations on mutant *E. coli* DHFR strains, to assess the compounds' ability to overcome antibiotic resistance. Finally, Density Functional Theory (DFT) calculations should be employed to optimize molecular geometry and gain deeper insights into the electronic properties of the ligands, guiding the future design of more effective agents.

REFERENCES

- Abal, P., Louzao, M. C., Antelo, A., Alvarez, M., Cagide, E., Vilariño, N., ... & Botana, L. M. (2017). Acute oral toxicity of tetrodotoxin in mice: Determination of lethal dose 50 (LD50) and no observed adverse effect level (NOAEL). *Toxins*, 9(3), 75.
- Adhikari, N., Choudhury, A. A. K., Shaky, A., Ghosh, S. K., Patgiri, S. J., Singh, U. P., & Bhat, H. R. (2022). Molecular docking and antimalarial evaluation of novel N-(4-aminobenzoyl)-l-glutamic acid conjugated 1, 3, 5-triazine derivatives as Pf-DHFR inhibitors. *3 Biotech*, 12(12), 347.
- Adsule, S., Barve, V., Chen, D., Ahmed, F., Dou, Q. P., Padhye, S., & Sarkar, F. H. (2006). Novel Schiff base copper complexes of quinoline-2 carboxaldehyde as proteasome inhibitors in human prostate cancer cells. *Journal of Medicinal Chemistry*, 49(24), 7242–7246.
- Ahmed, H. K., & Ahmed, W. M. (2024). New Discoveries Toxins from Gram Negative Bacteria Escherichia coli (E. coli). *Microbial Toxins in Food Systems: Causes, Mechanisms, Complications, and Metabolism*, 263–276.
- Aier, I., Varadwaj, K., & Raj, U. (2016). Structural insights into conformational stability of both wild-type and mutant EZH2 receptor OPEN. *Nature Publishing Group*.
- Akbar Ali, M., & Livingstone, S. E. (1974). Metal complexes of sulphur-nitrogen chelating agents. *Coordination Chemistry Reviews*, 13(2–3), 101–132.
- Akbar Ali, M., & Tarafdar, M. T. H. (1977). Metal complexes of sulphur and nitrogen-containing ligands: Complexes of S-benzylidithiocarbamate and a schiff base formed by its condensation with pyridine-2-carboxaldehyde. *Journal of Inorganic and Nuclear Chemistry*, 39(10), 1785–1791.
- Alarcón, F., Pérez-Hernández, G., Pérez, E., & Gama Goicochea, A. (2013). Coarse-grained simulations of the salt dependence of the radius of gyration of

polyelectrolytes as models for biomolecules in aqueous solution. *European Biophysics Journal*, 42(9), 661–672.

- Alcántar-Zavala, E., Delgado-Vargas, F., Marín-González, F., López Angulo, G., Aguirre-Madrigal, H. E., Ochoa-Terán, A., Rodríguez-Vega, G., Aguirre-Hernández, G., & Montes-Avila, J. (2024). Design, synthesis, and exploration of antibacterial activity of 6H-1,2-oxazin-6-ones. *RSC Advances*, 14(33), 23828–23839.
- Ali, I., Wani, W. A., & Saleem, K. (2013). Empirical formulae to molecular structures of metal complexes by molar conductance. *Synthesis and Reactivity in Inorganic, Metal-Organic and Nano-Metal Chemistry*, 43(9), 1162–1170.
- Ali, M. A., & Bose, R. (1977). Metal complexes of schiff bases formed by condensation of 2-methoxybenzaldehyde and 2-hydroxybenzaldehyde with s-benzylidithiocarbazate. In *J. inorg nucl. Chem* (Vol. 39). Pergamon Press.
- Ali, M. A., Mirza, A. H., Hamid, M. H. S. A., Bujang, F. H., & Bernhardt, P. V. (2004). The preparation and characterization of tin(IV) complexes of 2-quinolinecarboxaldehyde Schiff bases of S-methyl- and S-benzylidithiocarbazates and the X-ray crystal and molecular structures of the 2-quinolinecarboxaldehyde Schiff base of S-benzylidithiocarbazate (Hqaldsbz) and its tin(IV) complex [Sn(qaldsbz)I 3]. *Polyhedron*, 23(15), 2405–2412.
- Alonso, H., Bliznyuk, A. A., & Gready, J. E. (2006). Combining docking and molecular dynamic simulations in drug design. *Medicinal research reviews*, 26(5), 531–568.
- Al-Wahaibi, L. H., Rabea, S. M., Mahmoud, M. A., Youssif, B. G. M., Bräse, S., & Abdel-Aziz, S. A. (2024). Synthesis and Antimicrobial Evaluation of New 1,2,4-Triazolo[1,5- a]pyrimidine-Based Derivatives as Dual Inhibitors of Bacterial DNA Gyrase and DHFR. *ACS Omega*, 9(47).
- Ameram, N., & Othman, U. (2013). Synthesis And Characterization Of Co(II), Cu(II), Cd(II), Zn(II) And Ni(II) Complexes of Schiff Base Ligand Derived From S-Benzylidithiocarbazate (SBDTC) And Acetophenone With Their Biological Activity Studies. In *IOSR Journal of Engineering*, 3(8).

- Anupama, B. (2020). DNA binding interactions, docking and antioxidative studies of ternary copper (II) complexes. *Journal of Molecular Structure*, 1210, 127988.
- Arora, K., & Brooks, C. L. (2009). Functionally important conformations of the met20 loop in dihydrofolate reductase are populated by rapid thermal fluctuations. *Journal of the American Chemical Society*, 131(15), 5642–5647.
- Ashinze, P., Banerjee, S., Ademola, A. S., Aji, N., Abdul-Rahman, T., & Wireko, A. A. (2024). Outbreak investigation and implications: assessing the severity of E. coli outbreak in Alberta, pediatric hospitalizations, and the rise of hemolytic uremic syndrome. *IJS Global Health*, 7(1), e0399.
- Asif, A., Chen, J., Hussain, B., Hsu, G. (2024). The escalating threat of human-associated infectious bacteria in surface aquatic resources: Insights into prevalence, antibiotic resistance, survival mechanisms. *Elsevier*.
- Awdhesh Kumar Mishra, R., & Kodiveri Muthukaliannan, G. (2024). In-silico and in-vitro study of novel antimicrobial peptide AM1 from Aegle marmelos against drug-resistant Staphylococcus aureus. *Scientific Reports*, 14(1), 25822.
- Bagewadi, Z. K., Yunus Khan, T. M., Gangadharappa, B., Kamalapurkar, A., Mohamed Shamsudeen, S., & Yaraguppi, D. A. (2023). Molecular dynamics and simulation analysis against superoxide dismutase (SOD) target of Micrococcus luteus with secondary metabolites from Bacillus licheniformis recognized by genome mining approach. *Saudi Journal of Biological Sciences*, 30(9).
- Balouiri, M., Sadiki, M., & Ibensouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6(2), 71–79.
- Banerjee, P., Eckert, A. O., Schrey, A. K., & Preissner, R. (2018). ProTox-II: a webserver for the prediction of toxicity of chemicals. *Nucleic Acids Research*, 46(Web Server issue), W257.
- Battistoni, C., Mattogno, G., Monaci, A., & Tarli, F. (1971). Metal complexes of dithiocarbazic acid and its derivatives—I: Ni(II) and Zn(II) complexes of N-

- substituted dithiocarbazic acids. *Journal of Inorganic and Nuclear Chemistry*, 33(11), 3815–3832.
- Bayazeed, A., Alenazi, N. A., Alsaedi, A. M. R., Ibrahim, M. H., Al-Qurashi, N. T., & Farghaly, T. A. (2022). Formazan analogous: Synthesis, antimicrobial activity, dihydrofolate reductase inhibitors and docking study. *Journal of Molecular Structure*, 1258.
- Beard, W. A., Appleman, J. R., Blakley, R. L., Huang, S., Delcamp, T. J., Freisheim, J. H., & Blakley, R. L. (1991). Role of the Conserved Active Site Residue Tryptophan-24 of Human Dihydrofolate Reductase As Revealed by Mutagenesis. *Biochemistry*, 30(5), 1432–1440.
- Behiry, E. (2013). *Dihydrofolate reductase and the physical basis of enzyme catalysis*.
- Bekker, H., Berendsen, H., Dijkstra, E., Achterop, S., Vondrumen, R., Vanderspoel, D., Sijbers, A., Keegstra, H., & Renardus, M. (1993). Gromacs - A Parallel Computer For Molecular-Dynamics Simulations. 252–256.
- Belay, W. Y., Getachew, M., Tegegne, B. A., Teffera, Z. H., Dagne, A., Zeleke, T. K., Abebe, R. B., Gedif, A. A., Fenta, A., Yirdaw, G., Tilahun, A., & Aschale, Y. (2024). Mechanism of antibacterial resistance, strategies and next-generation antimicrobials to contain antimicrobial resistance: a review. *Frontiers in Pharmacology*, 15.
- Benet, L. Z., Hosey, C. M., Ursu, O., & Oprea, T. I. (2016). BDDCS, the Rule of 5 and drugability. *Advanced Drug Delivery Reviews*, 101, 89–98.
- Benoit, S. L., & Maier, R. J. (2013). Nickel Ions in Biological Systems. *Encyclopedia of Metalloproteins*, 1501–1505.
- Bera, P., Kim, C. H., & Seok, S. Il. (2008). Synthesis, spectroscopic characterization and thermal behavior of cadmium(II) complexes of S-methyldithiocarbazate (SMDTC) and S-benzylidithiocarbazate (SBDTC): X-ray crystal structure of [Cd(SMDTC)₃] · 2NO₃. *Polyhedron*, 27(17), 3433–3438.

- Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., & Bourne, P. E. (2000). The Protein Data Bank. *Nucleic Acids Research*, 28(1), 235–242.
- Bertacine Dias, M. V., Santos, J. C., Libreros-Zúñiga, G. A., Ribeiro, J. A., & Chavez-Pacheco, S. M. (2018). Folate biosynthesis pathway: Mechanisms and insights into drug design for infectious diseases. *Future Medicinal Chemistry*, 10(8), 935–959.
- Berthomieu, C., & Hienerwadel, R. (2009). Fourier transform infrared (FTIR) spectroscopy. *Photosynthesis Research*, 101(2–3), 157–170.
- Bharty, M. K., Bharti, A., Dani, R. K., Dulare, R., Bharati, P., & Singh, N. K. (2012). Synthesis and X-ray structural studies of Cd(II) and Ni(II) complexes of 5-(4-methoxy phenyl), 5-(2-pyridyl) and 5-(2-methoxy phenyl)-1,3,4-oxadiazole-2-thione. *Journal of Molecular Structure*, 1011, 34–41.
- Bhat, R. A. (2014). Synthesis Characterisation and theoretical studies of some Transition metal complexes of Dithiocarbazate schiff bases and their potential biological activity investigations. *University*.
- Bhattacharjee, A., Das, S., Das, B., & Roy, P. (2021). Intercalative DNA binding, protein binding, antibacterial activities and cytotoxicity studies of a mononuclear copper(II) complex. *Inorganica Chimica Acta*, 514, 119961.
- Bifulco, G., Dambruoso, P., Gomez-Paloma, L., & Riccio, R. (2007). Determination of relative configuration in organic compounds by NMR spectroscopy and computational methods. *Chemical Reviews*, 107(9), 3744–3779.
- Binbay, F. A., Rathod, D. C., George, A. A. P., & Imhof, D. (2023). Quality Assessment of Selected Protein Structures Derived from Homology Modeling and AlphaFold. *Pharmaceuticals*, 16(12), 1662.
- Bitu, N. A., Hossain, S., Kader, A., Islam, M. S., Haque, M. M., Hossen, F., Asraf, A., & Kudrat-E-Zahan. (2020). SBDTC Derived Schiff Base Metal Complexation: Synthesis, Physicochemical, and Spectral Studies with Antibacterial Activity. *Russian Journal of General Chemistry*, 90(8), 1529–1532.

- Blakley, R. L. (1969). The biochemistry of folic acid and related pteridines. *Front Biol*, 13, 189.
- Boehr, D. D., McElheny, D., Dyson, H. J., & Wrightt, P. E. (2006). The dynamic energy landscape of dihydrofolate reductase catalysis. *Science*, 313(5793), 1638–1642.
- Bolstad, D. B., Bolstad, E. S. D., Wright, D. L., & Anderson, A. C. (2008). Dihydrofolate reductase inhibitors: Developments in antiparasitic chemotherapy. *Expert Opinion on Therapeutic Patents*, 18(2), 143–157.
- Break, M. K. Bin, Fung, T. Y., Koh, M. Z., Ho, W. Y., Tahir, M. I. M., Elfar, O. A., Syed, R. U., Khojali, W. M. A., Alluhaibi, T. M., Huwaimel, B., Wiart, C., & Khoo, T. J. (2023). Synthesis, Crystal Structure, Antibacterial and *In Vitro* Anticancer Activity of Novel Macroacyclic Schiff Bases and Their Cu (II) Complexes Derived from S-Methyl and S-Benzyl Dithiocarbazate. *Molecules*, 28(13), 5009.
- Break, M. K. Bin, Tahir, M. I. M., Crouse, K. A., & Khoo, T. J. (2013). Synthesis, characterization, and bioactivity of schiff bases and their Cd^{2+} , Zn^{2+} , Cu^{2+} , and Ni^{2+} complexes derived from chloroacetophenone isomers with S-benzyl dithiocarbazate and the X-ray crystal structure of S-benzyl- β -N-(4-chlorophenyl) methylenedithiocarbazate. *Bioinorganic Chemistry and Applications*, 3(8).
- Brittain, C. G. (2009). *Using Melting Point to Determine Purity of Crystalline Solids*.
- Brylinski, M. (2018). Aromatic interactions at the ligand-protein interface: Implications for the development of docking scoring functions. *Chemical Biology & Drug Design*, 91(2), 380.
- Bursal, E., Turkan, F., Buldurun, K., Turan, N., Aras, A., Çolak, N., Murahari, M., & Yegeri, M. C. (2021). Transition metal complexes of a multidentate Schiff base ligand containing pyridine: synthesis, characterization, enzyme inhibitions, antioxidant properties, and molecular docking studies. *BioMetals*, 34(2), 393–406.

- Bushby, S. R. M. (1973). Trimethoprim-sulfamethoxazole: In vitro microbiological aspects. *Journal of Infectious Diseases*, 128, S442–S462.
- Butina, D., Segall, M. D., & Frankcombe, K. (2002). Predicting ADME properties in silico: methods and models. *Drug Discovery Today*, 7(11), S83–S88.
- Bystroff, C., & Kraut, J. (1991). Crystal Structure of Unliganded Escherichia coli Dihydrofolate Reductase. Ligand-Induced Conformational Changes and Cooperativity in Binding. *Biochemistry*, 30(8), 2227–2239.
- Catalysis, F. J.-A. (2009). Ultraviolet–visible–near infrared spectroscopy in catalysis: theory, experiment, analysis, and application under reaction conditions. *Elsevier*.
- Cavalcante, C. D. Q. O., Arcanjo, D. D. S., Silva, G. G. D., Oliveira, D. M. D., & Gatto, C. C. (2019). Solution and solid behavior of mono and binuclear Zn(II) and Ni(II) complexes with dithiocarbazates: X-ray analysis, mass spectrometry and cytotoxicity against cancer cell lines. *New Journal of Chemistry*, 43(28), 11209–11221.
- Chang, H., Ji, R., Zhu, Z., Wang, Y., Yan, S., He, D., Jia, Q., Huang, P., Cheng, T., Wang, R., & Zhou, Y. (2024). Target identification, and optimization of dioxygenated amide derivatives as potent antibacterial agents with FabH inhibitory activity. *European Journal of Medicinal Chemistry*, 265, 116064.
- Chemistry, H. S.-C. A. (2005). GC-MS. I: Basic principles and technical aspects of GC-MS for pesticide residue analysis. *Elsevier*.
- Chen, F., Li, X., Hihath, J., Huang, Z., & Tao, N. (2006). Effect of anchoring groups on single-molecule conductance: Comparative study of thiol-, amine-, and carboxylic-acid-terminated molecules. *Journal of the American Chemical Society*, 128(49), 15874–15881.
- Chen, J., Dima, R. I., & Thirumalai, D. (2007). Allosteric Communication in Dihydrofolate Reductase: Signaling Network and Pathways for Closed to Occluded Transition and Back. *Journal of Molecular Biology*, 374(1), 250–266.

- Chen, Z., Wu, Y., Gu, D., & Gan, F. (2007). Spectroscopic, and thermal studies of some new binuclear transition metal(II) complexes with hydrazone ligands containing acetoacetanilide and isoxazole. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 68(3), 918–926.
- Claudel, M., Schwarte, J. V., & Fromm, K. M. (2020). New Antimicrobial Strategies Based on Metal Complexes. *Chemistry 2020, Vol. 2, Pages 849-899*, 2(4), 849–899.
- Cohen, L., Go, E., MS, G. S.-M. (2007). Small-molecule desorption/ionization mass analysis. *Wiley Online Library*.
- Corral, M. G., Haywood, J., Stehl, L. H., Stubbs, K. A., Murcha, M. W., & Mylne, J. S. (2018). Targeting plant Dihydrofolate Reductase with antifolates and mechanisms for genetic resistance. *Plant Journal*, 95(4), 727–742.
- Costa, A. R., de Menezes, T. I., Nascimento, R. R., dos Anjos, P. N. M., Viana, R. B., de Araujo Fernandes, A. G., & Santos, R. L. S. R. (2019). Ruthenium(II) dimethylsulfoxide complex with pyrazole/dithiocarbazate ligand: Synthesis, spectroscopy studies, DFT calculations and thermal behavior. *Journal of Thermal Analysis and Calorimetry*, 138(2), 1683–1696.
- Cotton, F., Murillo, C., & Walton, R. (2005). *Multiple bonds between metal atoms*.
- Cronstein, B., & Bertino, J. (2000). *Methotrexate*.
- Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, 7.
- Dalha, U., Ahmad, A., Yahaya, S., & Kutama, I. (2021). Characterization of Ni(II) and Cd(II) metal complexes using Schiff base ligand derived from 2-Thiophene carboxyldehyde and 2-Aminothiophenol. *International Journal of Chemical Studies*, 9(1), 45–52.
- Dasari, S., & Bernard Tchounwou, P. (2014). Cisplatin in cancer therapy: Molecular mechanisms of action. *European Journal of Pharmacology*, 740, 364–378.

- Dawood, D. H., Sayed, M. M., Tohamy, S. T. K., & Nossier, E. S. (2023). New Thiophenyl-pyrazolyl-thiazole Hybrids as DHFR Inhibitors: Design, Synthesis, Antimicrobial Evaluation, Molecular Modeling, and Biodistribution Studies. *ACS Omega*, 8(42), 39250–39268.
- Dewangan, D., Vaishnav, Y., Mishra, A., Jha, A. K., Verma, S., & Badwaik, H. (2021). Synthesis, molecular docking, and biological evaluation of Schiff base hybrids of 1,2,4-triazole-pyridine as dihydrofolate reductase inhibitors. *Current Research in Pharmacology and Drug Discovery*, 2, 100024.
- Dhaked, D. K., & Nicklaus, M. C. (2024). What impact does tautomerism have on drug discovery and development? *Expert Opinion on Drug Discovery*, 19(9), 1011–1016.
- Diab, M. A., Mohamed, G. G., Mahmoud, W. H., El-Sonbati, A. Z., Morgan, S. M., & Abbas, S. Y. (2019). Inner metal complexes of tetradentate Schiff base: Synthesis, characterization, biological activity and molecular docking studies. *Applied Organometallic Chemistry*, 33(7).
- Dias, R., & de Azevedo Jr., W. F. (2008). Molecular Docking Algorithms. *Current Drug Targets*, 9(12), 1040–1047.
- Diener, W., Siccha, L., Mischke, U., Kayser, D., & Schiede, E. (1994). The biometric evaluation of the acute-toxic-class method (oral). *Archives of Toxicology*, 68(10), 599–610.
- Dong, J., Wang, N. N., Yao, Z. J., Zhang, L., Cheng, Y., Ouyang, D., Lu, A. P., & Cao, D. S. (2018). Admetlab: A platform for systematic ADMET evaluation based on a comprehensively collected ADMET database. *Journal of Cheminformatics*, 10(1), 1–11.
- Drozdowska, D., Wróbel-Tałałaj, A., Parzych, C., & Ratkiewicz, A. (2024). Benzamide Trimethoprim Derivatives as Human Dihydrofolate Reductase Inhibitors- Molecular Modeling and In Vitro Activity Study. *Biomedicines*, 12(5).

- Drwal, M. N., Banerjee, P., Dunkel, M., Wettig, M. R., & Preissner, R. (2014). ProTox: a web server for the in silico prediction of rodent oral toxicity. *Nucleic Acids Research*, 42(Web Server issue), W53.
- Dutta, S. K., Samanta, S., Ghosh, D., Butcher, R. J., & Chaudhury, M. (2002). Oxovanadium(V) and cobalt(III) complexes of dithiocarbazate-based schiblanckff base ligands: Formation of a thiadiazole ring by vanadium-induced cyclization of the coordinated ligand. *Inorganic Chemistry*, 41(21), 5555–5560.
- Eberhardt, J., & Forli, S. (2023). WaterKit: thermodynamic profiling of protein hydration sites. *Journal of Chemical Theory and Computation*, 19(9), 2535.
- Eissa, M. (2024). Escherichia coli: Epidemiology, Impact, Antimicrobial Resistance and Prevention: A review. *Journal of Public Health and Community Medicine*, 1(1), 39.
- Eldaly, S. M. (2024). Green synthesis of some new azolopyrimidines as antibacterial agents based on thiophene-chalcone. *Synthetic Communications*, 54(5), 348–370.
- El-Shenawy, A., Atta, A. (2014). Complexation of Gadolinium (III) and terbium (III) with nalidixic acid (NDX): Molar conductivity, thermal and spectral investigation. *Elsevier*.
- El-Sonbati, A. Z., Diab, M. A., El-Bindary, A. A., Mohamed, G. G., Morgan, S. M., Abou-Dobara, M. I., & Nozha, S. G. (2016). Geometrical structures, thermal stability and antimicrobial activity of Schiff base supramolecular and its metal complexes. *Journal of Molecular Liquids*, 215, 423–442.
- Elzaref, A. S., Elgammal, W. E., Mosaad Abdallah, A., Al-Mhyawi, S. R., Abdellatif, H. A., Abdou, A. M., Eliwa, E. M., Ibrahim, I., & Elfeky, A. S. (2023). Thione tautomer of hydrazone-carbodithioate chelator-derived Cu(II) and Zn(II) complexes as potential antibacterial agents: DFT computations and UV-assisted photocatalytic degradation of Rose Bengal dye. *Journal of Molecular Liquids*, 385.

- Emam, S. M., El-Saied, F. A., Abou El-Enein, S. A., & El-Shater, H. A. (2009). Cobalt(II), nickel(II), copper(II), zinc(II) and hafnium(IV) complexes of N'-(furan-3-ylmethylene)-2-(4-methoxyphenylamino) acetohydrazide. *Molecular and Biomolecular Spectroscopy*, 72(2), 291–297.
- Enamullah, M., Uddin, A. K. M. R., Pescitelli, G., Berardozi, R., Makhloufi, G., Vasylyeva, V., Chamayou, A. C., & Janiak, C. (2014). Induced chirality-at-metal and diastereoselectivity at Δ/Λ -configured distorted square-planar copper complexes by enantiopure Schiff base ligands: combined circular dichroism, DFT and X-ray structural studies. *Dalton Transactions*, 43(8), 3313–3329.
- Enis Nadia Binti Yusof, B. (2014). *Synthesis, Structural Characterisation And Cytotoxic Study Of Multidentate Dithiocarbazate Schiff Bases And Their Divalent Cu, Ni, And Zn Complexes*.
- Estrada, A., Wright, D. L., & Anderson, A. C. (2016). Antibacterial Antifolates: From Development through Resistance to the Next Generation. *Cold Spring Harbor Perspectives in Medicine*, 6(8), a028324.
- Evangelio, E., Rodriguez-Blanco. (2009). Solvent effects on valence tautomerism: A comparison between the interconversion in solution and solid state. *Elsevier*.
- Evans, A., & Kavanagh, K. A. (2021). Evaluation of metal-based antimicrobial compounds for the treatment of bacterial pathogens. *Journal of Medical Microbiology*, 70(5).
- Fan, J., Fu, A., & Zhang, L. (2019). Progress in molecular docking. *Quantitative Biology*, 7(2), 83–89.
- Farnum, M. F., Magde, D., Hirai, J. T., Warren, M. S., Grimsley, J. K., Kraut, J., & Howell, E. E. (1991). Analysis of Hydride Transfer and Cofactor Fluorescence Decay in Mutants of Dihydrofolate Reductase: Possible Evidence for Participation of Enzyme Molecular Motions in Catalysis. *Biochemistry*, 30(49), 11567–11579.
- Fasnacht, M., & Polacek, N. (2021). Oxidative Stress in Bacteria and the Central Dogma of Molecular Biology. *Frontiers in Molecular Biosciences*, 8.

- Forgacs, P., Wengenack, N. L., Hall, L., Zimmerman, S. K., Silverman, M. L., & Roberts, G. D. (2009). Tuberculosis and trimethoprim-sulfamethoxazole. *Antimicrobial Agents and Chemotherapy*, 53(11), 4789–4793.
- Forlani, L., Mezzina, E. (2001). Tautomerism and Dimerization of Acetamidothiazole Derivatives– UV/Vis and NMR Spectroscopic Investigation. *Wiley Online Library*.
- Gabbott, P. (2008). Principles and Applications of Thermal Analysis. *Principles and Applications of Thermal Analysis*, 1–388.
- Gan, W. K., Liew, H. S., Pua, L. J. W., Ng, X. Y., Fong, K. W., Cheong, S. L., Liew, Y. K., & Low, M. L. (2022). Novel Cu(II) Schiff Base Complex Combination with Polymyxin B/Phenylalanine-Arginine β -Naphthylamide Against Various Bacterial Strains. *International Journal of Peptide Research and Therapeutics*, 28(2).
- Garcia-Vazquez, J. A., Romero, J., & Sousa, A. (1999). Electrochemical synthesis of metallic complexes of bidentate thiolates containing nitrogen as an additional donor atom. *Coordination Chemistry Reviews*, 193–195, 691–745.
- Garcia-Viloca, M., Truhlar, D. G., & Gao, J. (2003). Reaction-Path Energetics and Kinetics of the Hydride Transfer Reaction Catalyzed by Dihydrofolate Reductase. *Biochemistry*, 42(46), 13558–13575.
- Gatto, C. C., Cavalcante, C. de Q. O., Lima, F. C., Nascimento, É. C. M., Martins, J. B. L., Santana, B. L. O., Gualberto, A. C. M., & Pittella-Silva, F. (2024). Structural Design, Anticancer Evaluation, and Molecular Docking of Newly Synthesized Ni(II) Complexes with ONS-Donor Dithiocarbazate Ligands. *Molecules*, 29(12), 2759.
- Geary, W. J. (1971). The use of conductivity measurements in organic solvents for the characterisation of coordination compounds. *Coordination Chemistry Reviews*, 7(1), 81–122.
- Ghany, L. M. A. A., Ryad, N., Abdel-Aziz, M. S., El-Lateef, H. M. A., Zaki, I., & Beshay, B. Y. (2024). Design, synthesis, antimicrobial evaluation, and

- molecular modeling of new sulfamethoxazole and trimethoprim analogs as potential DHPS/DHFR inhibitors. *Journal of Molecular Structure*, 1309.
- Ghosh, J., Lawless, M. S., Waldman, M., Gombar, V., & Fraczekiewicz, R. (2016). Modeling ADMET. *Methods in Molecular Biology*, 1425, 63–83.
- Ghosh, P., Dey, S. K., Ara, M. H., Md. Rezaul Karim, K., & Nazmul Islam, A. B. M. (2019). A Review on Synthesis and Versatile Applications of Some Selected Schiff Bases with Their Transition Metal Complexes. *Egyptian Journal of Chemistry*, 62, 523–547.
- Ghosh, S., Kamilya, S., Das, M., Mehta, S., Boulon, M.-E., Nemec, I., Rouzières, M., Herchel, R., & Mondal, A. (2020). Effect of Coordination Geometry on Magnetic Properties in a Series of Cobalt(II) Complexes and Structural Transformation in Mother Liquor. *Inorganic Chemistry*, 59, 7067–7081.
- Giedraitiene, A., Vitkauskienė, A., Naginiene, R., & Pavilionis, A. (2011). Antibiotic Resistance Mechanisms of Clinically Important Bacteria. *Medicina 2011, Vol. 47, Page 19*, 47(3), 19.
- Girish, K., Channu, B. C., & Baba, A. R. (2019). Synthesis and antibacterial activity of cobalt(II) complex of curcumin. *Indian Journal of Pharmaceutical Sciences*, 81(1), 150–155.
- Goldstein, M., & Goodey, N. M. (2021). Distal Regions Regulate Dihydrofolate Reductase-Ligand Interactions. *Methods in Molecular Biology*, 2253, 185–219.
- González, M. A. (2011). Force fields and Molecular dynamics simulations. *Collection SFN*, 12, 169–200.
- Greenwood, N. N., & Travers, N. F. (1967). Cadmium derivatives of decaborane. *Journal of the Chemical Society A: Inorganic, Physical, Theoretical*, 0, 880–884.
- Günther, H. (Harald). (2013). *NMR spectroscopy: basic principles, concepts, and applications in chemistry*. 718.

- Guthrie, R. D. (1979). Introduction to Spectroscopy (Pavia, Donald; Lampman, Gary M.; Kriz, George S., Jr.). *Journal of Chemical Education*, 56(10), A323.
- Gwaram, N. S., Ali, H. M., Khaledi, H., Abdulla, M. A., Hadi, H. A., Lin, T. K., Ching, C. L., & Ooi, C. L. (2012). Antibacterial evaluation of some schiff bases derived from 2-acetylpyridine and their metal complexes. *Molecules*, 17(5), 5952–5971.
- Haas, K. L., & Franz, K. J. (2009). Application of metal coordination chemistry to explore and manipulate cell biology. *Chemical Reviews*, 109(10), 4921–4960.
- Haghshenas, H., Tavakol, H., Kaviani, B., & Mohammadnezhad, G. (2020). AMBER Force Field Parameters for Cobalt-Containing Biological Systems: A Systematic Derivation Study. *Journal of Physical Chemistry B*, 124(5), 777–787.
- Hanwell, M. D., Curtis, D. E., Lonie, D. C., Vandermeersch, T., Zurek, E., & Hutchison, G. R. (2012). Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics*, 4(8), 1–17.
- Hasibul Islam, M., Chanmiya Sheikh, M., Miyatake, R., Zahan, R., & Al-Amin-Al-Azadul Islam, M. (2020). Synthesis, characterization and bioactivity study of bidentate NS schiff base of S-Benzyl dithiocarbamate and its Zn(II) and Pd(II) complexes. *Asian Journal of Chemistry*, 32(8), 2091–2098.
- Heenen, H. H., Gauthier, J. A., Kristoffersen, H. H., Ludwig, T., & Chan, K. (2020). Solvation at metal/water interfaces: An ab initio Molecular dynamics benchmark of common computational approaches. *Journal of Chemical Physics*, 152(14).
- Hider, R. C., & Ma, Y. (2016). Basic Principles of Metal Chelation and Chelator Design. *RSC Metallobiology*, 2017-January(8), 24–55.
- Hosny, N. M. (2007). Synthesis and spectral characterization of Schiff-base complexes derived from alanine and 2-acetylpyridine with some divalent metal acetates. *Journal of Coordination Chemistry*, 60(24), 2755–2764.

- Hosny, N. M., & El-Dossoki, F. I. (2008). Schiff base complexes derived from 2-acetylpyridine, leucine, and some metal chlorides: Their preparation, characterization, and physical properties. *Journal of Chemical and Engineering Data*, 53(11), 2567–2572.
- Hossain, M., Bitu, N., Khan, M., Asraf, A., Hossen, F., & Mannan, M. (2021). 2,3-Dimethoxybenzaldehyde Schiff Base Of Sbdtc And Its Metal Complexes Of Ni(II), Cu(II), Zn(II) And Cd(II): Synthesis, Characterization And Biological Activity. *New Materials, Compounds and Applications*, 5(3), 182–191.
- Hossen, M. F., Zamir, R., Asraf, M. A., Kudrat-E-Zahan, M., Hossen, F., Ansary, R. H., & Asraf, A. (2018). Synthesis, Characterization And Biological Activity Evaluation Of Ni (II) And Cu (II) Complexes Of A Schiff Base Derived From S-Benzyldithiocarbazate (SBDTC) And Cinnamaldehyde. *Thematics Journal of Chemistry*, 2(2).
- How, F. N. F., Crouse, K. A., Tahir, M. I. M., Tarafder, M. T. H., & Cowley, A. R. (2008). Synthesis, characterization and biological studies of S-benzyl- β -N-(benzoyl) dithiocarbazate and its metal complexes. *Polyhedron*, 27(15), 3325–3329.
- Hubbard, R. E., & Kamran Haider, M. (2010). Hydrogen Bonds in Proteins: Role and Strength. *Encyclopedia of Life Sciences*.
- Hübschmann, H.-J. (2015). *Handbook of GC-MS: fundamentals and applications*.
- Huey, R., Morris, G. (2012). Using AutoDock 4 and AutoDock vina with AutoDockTools: a tutorial. *Researchgate.Net*.
- Huey, R., Morris, G. M., Olson, A. J., Goodsell, D. S., Lindstrom, W., Sanner, M. F., & Belew, R. K. (1998). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem*, 19(2), 2785–2791.
- Hughes, R. L., Johnson, L. A., Behiry, E. M., Loveridge, E. J., & Allemann, R. K. (2017). A rapid analysis of variations in conformational behavior during dihydrofolate reductase catalysis. *ACS Publications*, 56(15), 2126–2133.

- Husna, A., Rahman, M. M., Badruzzaman, A. T. M., Sikder, M. H., Islam, M. R., Rahman, M. T., Alam, J., & Ashour, H. M. (2023). Extended-Spectrum β -Lactamases (ESBL): Challenges and Opportunities. *Biomedicines*, 11(11), 2937.
- Hussain, S., Khan, M., Sheikh, T. M. M., Mumtaz, M. Z., Chohan, T. A., Shamim, S., & Liu, Y. (2022). Zinc Essentiality, Toxicity, and Its Bacterial Bioremediation: A Comprehensive Insight. *Frontiers in Microbiology*, 13, 900740.
- Idris, M. O., Yekeen, A. A., Alakanse, O. S., & Durojaye, O. A. (2021). Computer-aided screening for potential TMPRSS2 inhibitors: a combination of pharmacophore modeling, molecular docking and Molecular dynamics simulation approaches. *Journal of Biomolecular Structure and Dynamics*, 1–19.
- Ingle, A. P., Duran, N., & Rai, M. (2014). Bioactivity, mechanism of action, and cytotoxicity of copper-based nanoparticles: A review. *Applied Microbiology and Biotechnology*, 98(3), 1001–1009.
- Islam, M. A. A. A., Sheikh, M. C., Mumit, M. A., Miyatake, R., Alam, M. A., & Mondal, M. O. A. (2016). Synthesis, characterization and antimicrobial activity of a bidentate NS Schiff base of S-benzyl dithiocarbamate and its divalent complexes. *Journal of Coordination Chemistry*, 69(23), 3580–3592.
- Islam, M. A., & Pillay, T. S. (2020). Identification of promising anti-DNA gyrase antibacterial compounds using de novo design, molecular docking and Molecular dynamics studies. *Journal of Biomolecular Structure and Dynamics*, 38(6), 1798–1809.
- Ivanov, S. (2012). Multiferroic complex metal oxides: Main features of preparation, structure, and properties. *Science and Technology of Atomic, Molecular, Condensed Matter and Biological Systems*, 2(1), 163–238.
- Jadhav, P. A., Jagtap, J. R., Deodhar, M. N., Chavan, R. S., & Pawar, S. J. (2023). Design, Synthesis and Antimicrobial Activity of 1,3-Diazine Derivatives. *Current Indian Science*, 1(1), 1–15.

- Jakalian, A., Jack, D. B., & Bayly, C. I. (2002). Fast, efficient generation of high-quality atomic charges. AM1-BCC model: II. Parameterization and validation. *Journal of Computational Chemistry*, 23(16), 1623–1641.
- Jays, J., Mohan, S., & Saravanan, J. (2019). Molecular docking studies of novel furan-azetidinone hybrids as potential inhibitors of Escherichia coli. *Indian Journal of Pharmaceutical Education and Research*, 53(3), S325–S331.
- Jeffrey, G. A., & Jeffrey, G. A. (1997). *An introduction to hydrogen bonding* (Vol. 12, p. 228). New York: Oxford university press.
- Jevtovic, V., Alshamari, A. K., Milenković, D., Dimitrić Marković, J., Marković, Z., & Dimić, D. (2023). The Effect of Metal Ions (Fe, Co, Ni, and Cu) on the Molecular-Structural, Protein Binding, and Cytotoxic Properties of Metal Pyridoxal-Thiosemicarbazone Complexes. *International Journal of Molecular Sciences*, 24(15), 11910.
- Jorba, M., Pedrola, M., Ghashghaei, O., Herráez, R., Campos-Vicens, L., Luque, F. J., Lavilla, R., & Viñas, M. (2021). New Trimethoprim-Like Molecules: Bacteriological Evaluation and Insights into Their Action. *Antibiotics*, 10(6), 709.
- Karam, G., Chastre, J., Wilcox, M. H., & Vincent, J. L. (2016). Antibiotic strategies in the era of multidrug resistance. *Critical Care*, 20(1).
- Kasumov, V. T., & Köksal, F. (2003). Synthesis, spectroscopic characterization and redox reactivity of the copper(II) salicylaldiminates containing sterically hindered 2,6-diphenylphenol. *Transition Metal Chemistry* 2003 28:8, 28(8), 888–892.
- Kathale, N., Rao, N. S., Rao, N. N., & Munshi, K. N. (2001). Synthesis and characterization of some dioxomolybdenum(VI) complexes with O, N, S donor Schiff bases derived from S-methyldithiocarbazate, S-benzoyldithiocarbazate, 2-aminoethanethiol, and substituted salicylaldehydes. *Synthesis and Reactivity in Inorganic and Metal-Organic Chemistry*, 31(5), 859–871.

- Kemnic, T. R., & Coleman, M. (2022). Trimethoprim Sulfamethoxazole. *Mayo Clinic Proceedings*, 74(7), 730–734.
- Khalaf, M. M., El-Lateef, H. M. A., & Abdou, A. (2024). Structural, DFT, Molecular Docking and Biological Activity of New Albendazole-Norfloxacin Mixed-Ligand Complexes: Promising Metal Complexes for Combating Microbial Resistance and Inflammation. *Chemistry and Biodiversity*.
- Khirudin, N. N. M., Yusof, E. N. M., Arshad, S., & Sirat, S. S. (2023). Cu(II) complex of S-benzyl- β -N-(2-methoxybenzylmethylene)dithiocarbazate: Synthesis, DFT calculations, cell cytotoxicity assay and DNA binding studies. *Journal of Molecular Structure*, 1283.
- Khoo, T. J., Break, M. K. Bin, Crouse, K. A., Tahir, M. I. M., Ali, A. M., Cowley, A. R., Watkin, D. J., & Tarafder, M. T. H. (2014). Synthesis, characterization and biological activity of two Schiff base ligands and their nickel(II), copper(II), zinc(II) and cadmium(II) complexes derived from S-4-picolyldithiocarbazate and X-ray crystal structure of cadmium(II) complex derived from pyridine-2-carboxaldehyde. *Inorganica Chimica Acta*, 413, 68–76.
- Khoo Teng Jin, B. (2008). *Structure Elucidation and Biological Activity of Dithiocarbazate Derivatives, Their Schiff Base Ligands and Metal Complexes*.
- Kreider-Mueller, A., Quinlivan, P. J., Owen, J. S., & Parkin, G. (2017). Tris(2-mercaptoimidazolyl)hydroborato Cadmium Thiolate Complexes, [TmBut]CdSAr: Thiolate Exchange at Cadmium in a Sulfur-Rich Coordination Environment. *Inorganic Chemistry*, 56(8), 4643–4653.
- Kriessman, C. J., & Callen, H. B. (1954). The Magnetic Susceptibility of the Transition Elements. *Physical Review*, 94(4), 837.
- Krishnakumar, V., Mangaiarkkarasi, K., Mathammal, R., Prabavathi, N., & Surumbarkuzhali, N. (2013). FTIR, FT-Raman and NMR studies on 2,6-dichlorotoluene and 2-chloro-6-fluorotoluene based on density functional theory. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, 112, 429–439.

- Krucinska, J., Lombardo, M. N., Erlandsen, H., Estrada, A., Si, D., Viswanathan, K., & Wright, D. L. (2022). Structure-guided functional studies of plasmid-encoded dihydrofolate reductases reveal a common mechanism of trimethoprim resistance in Gram-negative pathogens. *Communications Biology* 2022 5:1, 5(1), 1–14.
- Kudrat-E-Zahan, M., Hossen, M. F., Zamir, R., Asraf, M. A., & Faruk Hossen, M. (2020). Antimicrobial Activities of Co(II) and Sb(III) Complexes of a Schiff Base Derived from S-benzylidithiocarbazate (SBDTC) and Cinnamaldehyde. *JMSRR*(1), 10–20.
- Kudrat-E-Zahan, M., & Islam, M. S. (2015). Synthesis, characterization, and antimicrobial activity of complexes of Cu(II), Ni(II), Zn(II), Pb(II), Co(II), Mn(II), and U(VI) containing bidentate Schiff base of [S-methyl-3-(4-methoxybenzylidene)dithiocarbazate]. *Russian Journal of General Chemistry*, 85(4), 979–983.
- Kumar, K. M., Anbarasu, A., & Ramaiah, S. (2014). Molecular docking and Molecular dynamicss studies on β -lactamases and penicillin binding proteins. *Molecular BioSystems*, 10(4), 891–900.
- Lange, K., Frey, K. M., Eck, T., Janson, C. A., Gubler, U., & Goodey, N. M. (2023). Crystal structure of dihydrofolate reductase from the filarial nematode *W. bancrofti* in complex with NADPH and folate. *PLoS Neglected Tropical Diseases*, 17(4).
- Latif, M. A., Ahmed, T., Hossain, M. S., Chaki, B. M., Abdou, A., & Kudrat-E-Zahan, M. (2023). Synthesis, Spectroscopic Characterization, DFT Calculations, Antibacterial Activity, and Molecular Docking Analysis of Ni(II), Zn(II), Sb(III), and U(VI) Metal Complexes Derived from a Nitrogen-Sulfur Schiff Base. *Russian Journal of General Chemistry*, 93(2), 389–397.
- Lavine, B., Almirall, J., Muehlethaler, C., Neumann, C., & Workman, J. (2020). Criteria for comparing infrared spectra – A review of the forensic and analytical chemistry literature. *Forensic Chemistry*, 18.

- Lever, A. B. P. (1984). Inorganic electronic spectroscopy. *Studies in Physical and Theoretical Chemistry*, 33, XVI-863 p.
- Li, T., Wang, Z., Guo, J., de la Fuente-Nunez, C., Wang, J., Han, B., Tao, H., Liu, J., & Wang, X. (2023). Bacterial resistance to antibacterial agents: Mechanisms, control strategies, and implications for global health. *Science of the Total Environment*, 860.
- Li, X., Hilgers, M., Cunningham, M., Chen, Z., Trzoss, M., Zhang, J., Kohnen, L., Lam, T., Creighton, C., Ge, K., Nelson, K., Kwan, B., Stidham, M., Brown-Driver, V., Shaw, K. J., & Finn, J. (2011). Structure-based design of new DHFR-based antibacterial agents: 7-aryl-2,4-diaminoquinazolines. *Bioorganic and Medicinal Chemistry Letters*, 21(18), 5171–5176.
- Li, Y., Ouyang, Y., Wu, H., Wang, P., Huang, Y., Li, X., Chen, H., Sun, Y., Hu, X., Wang, X., Li, G., Lu, Y., Li, C., Lu, X., Pang, J., Nie, T., Sang, X., Dong, L., Dong, W., ... You, X. (2022). The discovery of 1, 3-diamino-7H-pyrrol[3, 2-f]quinazoline compounds as potent antimicrobial antifolates. *European Journal of Medicinal Chemistry*, 228.
- Li, Y., Zhao, T., Qing, L., Yu, H., Xu, X., Li, P., & Zhao, S. (2021). Solvation dynamics in simple fluids: Effect of solute size and potential. *Chemical Engineering Science*, 232, 116371.
- Liau, M. (2010). UV-Visible Spectroscopy for On-Line Analysis. *Wiley Online Library*.
- Lipinski, C. A. (2004). Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discovery Today: Technologies*, 1(4), 337–341.
- Lipinski, C. A., Lombardo, F., Lombardo, F., Lipinski, C. A., Dominy, B. W., & Feeney, P. J. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Elsevier*, 23, 3–25.
- Liu, Y. T., Lian, G. D., Yin, D. W., & Su, B. J. (2013). Synthesis, characterization and biological activity of ferrocene-based Schiff base ligands and their metal (II)

complexes. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, 100, 131–137.

- Lobana, T. S., Sharma, R., Bawa, G., & Khanna, S. (2009). Bonding and structure trends of thiosemicarbazone derivatives of metals-An overview. *Coordination Chemistry Reviews*, 253(7–8), 977–1055.
- Loveridge, E. J., Hroch, L., Hughes, R. L., Williams, T., Davies, R. L., Angelastro, A., Luk, L. Y. P., Maglia, G., & Allemann, R. K. (2017). Reduction of Folate by Dihydrofolate Reductase from *Thermotoga maritima*. *Biochemistry*, 56(13), 1879–1886.
- Low, M. L., Paulus, G., Dorlet, P., Guillot, R., Rosli, R., Delsuc, N., Crouse, K. A., & Policar, C. (2015). Synthesis, characterization and biological activity of Cu(II), Zn(II) and Re(I) complexes derived from S-benzylthiocarbazate and 3-acetylcoumarin. *BioMetals*, 28(3), 553–566.
- Lusi, E. A., Patrissi, T., & Guarascio, P. (2017). Nickel-resistant bacteria isolated in human microbiome. *New Microbes and New Infections*, 19, 67–70.
- Macchiagodena, M., Pagliai, M., Andreini, C., Rosato, A., & Procacci, P. (2020). Upgraded AMBER Force Field for Zinc-Binding Residues and Ligands for Predicting Structural Properties and Binding Affinities in Zinc-Proteins. *ACS omega*, 5(25), 15301-15310.
- Mahamat, S., Founou, R. C., Founou, L. L., Tchouangueu, T. F., Dimani, B. D., Nkengkana, O. A., Mafo, L. D., Chelo, D., Fonkoua, M. C., Boum-Ii, Y., Gonsu, H., Noubom, M., Olivier Koki Ndombo, P., & Gatsing, D. (2024). Occurrence of Extended-spectrum β -lactamase (ESBL) and Carbapenemase-producing *Escherichia coli* isolated from Childhood Diarrhoea in Yaoundé, Cameroon. *BMC Microbiology*, 24(1), 401.
- Maia, M., Resende, D. I. S. P., Durães, F., Pinto, M. M. M., & Sousa, E. (2021). Xanthenes in Medicinal Chemistry – Synthetic strategies and biological activities. *European Journal of Medicinal Chemistry*, 210, 113085.

- Majumdar, D., Das, S., Biswas, J. K., & Mondal, M. (2017). Synthesis, structure, fluorescent property, and antibacterial activity of new Cd(II) metal complex based on multidentate Schiff base ligand N,N'-Bis(3-methoxysalicylideneimino)-1,3-diaminopropane. *Journal of Molecular Structure*, 1134, 617–624.
- Malanovic, N., & Lohner, K. (2016). Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1858(5), 936–946.
- Mallela, R., Konakanchi, R (2018). Zn (II), Cd (II) and Hg (II) metal complexes of 2-aminonicotinaldehyde: Synthesis, crystal structure, biological evaluation and molecular docking study.
- Malmberg, C., Yuen, P., Spaak, J., Cars, O., Tängdén, T., & Lagerbäck, P. (2016). A Novel Microfluidic Assay for Rapid Phenotypic Antibiotic Susceptibility Testing of Bacteria Detected in Clinical Blood Cultures. *PLOS ONE*, 11(12), e0167356.
- Mamta, & Chaudhary, A. (2024). Synthesis, spectroscopic characterization, in vitro antimicrobial activity, antioxidant study and theoretical approaches towards DNA gyrase, DHFR enzyme, NADPH enzyme of N8-tetraoxomacrocyclic complexes of Zn(II). *Journal of Molecular Structure*, 1295, 136743.
- Manan, M. A. F. A., Crouse, K. A., Tahir, M. I. M., Rosli, R., How, F. N. F., Watkin, D. J., & Slawin, A. M. Z. (2011). Synthesis, Characterization and Cytotoxic Activity of S-Benzyldithiocarbazate Schiff Bases Derived from 5-Fluoroisatin, 5-Chloroisatin, 5-Bromoisatin and Their Crystal Structures. *Journal of Chemical Crystallography*, 41(11), 1630–1641.
- Manan, M. A. F. A., & Mohammat, M. F. (2022). Synthesis, Structural Studies and Antimicrobial Evaluation of Nickel(II) Bis-Complex of Schiff Base of S-Benzyldithiocarbazate. *Trends in Sciences*, 19(23), 1500.
- Mandujano-Hernández, A., Animals, A. M.-V.-, & 2024, undefined. (n.d.). The Global Rise of ESBL-Producing Escherichia coli in the Livestock Sector: A Five-Year Overview. *Mdpi.Com*.

- Manna, M. S., Tamer, Y. T., Gaszek, I., Poulides, N., Ahmed, A., Wang, X., Toprak, F. C. R., Woodard, D. N. R., Koh, A. Y., Williams, N. S., Borek, D., Atilgan, A. R., Hulleman, J. D., Atilgan, C., Tambar, U., & Toprak, E. (2021a). A trimethoprim derivative impedes antibiotic resistance evolution. *Nature Communications* 2021 12:1, 12(1), 1–10.
- Maryam, M., Tan, S. L., Crouse, K. A., Mohamed Tahir, M. I., & Chee, H. Y. (2020). Synthesis, characterization and evaluation of antidengue activity of enantiomeric Schiff bases derived from S-substituted dithiocarbazate. *Turkish Journal of Chemistry*, 44(5), 1395–1409.
- Matsui, A., Bellier, J. P., Hayashi, D., Ishibe, T., Nakamura, Y., Taguchi, H., Naruse, N., & Mera, Y. (2023). Curcumin tautomerization in the mechanism of pentameric amyloid- β 42 oligomers disassembly. *Biochemical and Biophysical Research Communications*, 666, 68–75.
- Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M., & Hoogsteen, K. (1977). Dihydrofolate reductase: X-ray structure of the binary complex with methotrexate. *Science*, 197(4302), 452–455.
- McDonald, I. K., & Thornton, J. M. (1994). Satisfying hydrogen bonding potential in proteins. *Journal of molecular biology*, 238(5), 777-793.
- McGuire, J. J. (2005). Anticancer Antifolates: Current Status and Future Directions. *Current Pharmaceutical Design*, 9(31), 2593–2613.
- Md Yusof, E. N., Latif, M. A. M., Tahir, M. I. M., Sakoff, J. A., Veerakumarasivam, A., Page, A. J., Tiekink, E. R. T., & Ravoof, T. B. S. A. (2020). Homoleptic tin(IV) compounds containing tridentate ONS dithiocarbazate Schiff bases: Synthesis, X-ray crystallography, DFT and cytotoxicity studies. *Journal of Molecular Structure*, 1205, 127635.
- Md Yusof, E. N., Ravoof, T. B. S. A., Tiekink, E. R. T., Veerakumarasivam, A., Crouse, K. A., Tahir, M. I. M., & Ahmad, H. (2015). Synthesis, characterization and biological evaluation of transition metal complexes derived from N, S bidentate ligands. *International Journal of Molecular Sciences*, 16(5), 11034–11054.

- Menczel, J. D., & Prime, R. B. (2008). Thermal Analysis of Polymers: Fundamentals and Applications. *Thermal Analysis of Polymers: Fundamentals and Applications*, 1-688.
- Méndez, R., Leplae, R., De Maria, L., & Wodak, S. J. (2003). Assessment of blind predictions of protein–protein interactions: Current status of docking methods. *Proteins: Structure, Function, and Bioinformatics*, 52(1), 51-67.
- Meng, X. Y., Zhang, H. X., Mezei, M., & Cui, M. (2011). Molecular docking: a powerful approach for structure-based drug discovery. *Current computer-aided drug design*, 7(2), 146-157.
- Mhashal, A. R., Vardi-Kilshtain, A., Kohen, A., & Major, D. T. (2017). The role of the Met20 loop in the hydride transfer in Escherichia coli dihydrofolate reductase. *Journal of Biological Chemistry*, 292(34), 14229–14239.
- Miller, B. R., McGee, T. D., Swails, J. M., Homeyer, N., Gohlke, H., & Roitberg, A. E. (2012). MMPBSA.py: An efficient program for end-state free energy calculations. *Journal of Chemical Theory and Computation*, 8(9), 3314–3321.
- Mohanty, M., & Mohanty, P. S. (2023). Molecular docking in organic, inorganic, and hybrid systems: a tutorial review. *Monatshefte Fur Chemie*, 154(7), 683–707.
- Moustafa, A. H., Ahmed, D. H., El-Wassimy, M. T. M., & Mohamed, M. F. A. (2021). Synthesis, antimicrobial studies, and molecular docking of some new dihydro-1,3,4-thiadiazole and pyrazole derivatives derived from dithiocarbazates. *Synthetic Communications*, 51(4), 570–584.
- Muddala, N. P., White, J. C., Nammalwar, B., Pratt, I., Thomas, L. M., Bunce, R. A., Berlin, K. D., & Bourne, C. R. (2020). Inhibitor design to target a unique feature in the folate pocket of Staphylococcus aureus dihydrofolate reductase. *European Journal of Medicinal Chemistry*, 200, 112412.
- Mujahid, F., Rasool, M. H., Shafiq, M., Aslam, B., & Khurshid, M. (2024). Emergence of Carbapenem-Resistant Uropathogenic Escherichia coli (ST405 and ST167) Strains Carrying bla CTX-M-15, bla NDM-5 and Diverse Virulence Factors in Hospitalized Patients. *Pathogens*, 13(11), 964.

- Mumit, M. A., Pal, T. K., Alam, M. A., Islam, M. A. A. A., Paul, S., & Sheikh, M. C. (2020). DFT studies on vibrational and electronic spectra, HOMO–LUMO, MEP, HOMA, NBO and molecular docking analysis of benzyl-3-N-(2,4,5-trimethoxyphenylmethylene) hydrazinecarbodithioate. *Journal of Molecular Structure*, 1220.
- Murphy, M. P., & O'Neill, L. A. (2024). A break in mitochondrial endosymbiosis as a basis for inflammatory diseases. *Nature*, 626(7998), 271–279.
- Nakashima, J., Takeuchi, M., Kawamoto, S., Monobe, K., Taira, J., & Aoki, S. (2022). Establishing a parallel compound screening method and identifying novel antimicrobial compounds targeting *Staphylococcus aureus* dihydrofolate reductase. *Journal of Applied Pharmaceutical Science*, 12(8), 036–047.
- Nemade, A. M., & Kolhe, V. C. (2016). The Study of Thermal and Magnetic properties of Fe (II), Co (II), Ni (II) and Cu (II) Metal Complexes with 3-Aminolawsone oxime. *Journal of Chemical and Pharmaceutical Research*, 8(4), 878–888.
- Newberry, R. W., & Raines, R. T. (2017). The $n \rightarrow \pi^*$ Interaction. *Accounts of Chemical Research*, 50(8), 1838.
- Nishimura, M., Saito, H., Kawaguchi, K., & Nagao, H. (2013). Conformational stability of Met20 loop of DHFR: A Molecular dynamicss study. *AIP Conference Proceedings*, 1518(1), 654–657.
- Norinder, U., & Bergström, C. A. S. (2006). Prediction of ADMET Properties. *ChemMedChem*, 1(9), 920–937.
- Osborne, M. J., Schnell, J., Benkovic, S. J., Dyson, H. J., & Wright, P. E. (2001). Backbone Dynamics in Dihydrofolate Reductase Complexes: Role of Loop Flexibility in the Catalytic Mechanism†. *Biochemistry*, 40(33), 9846–9859.
- Osman, E. O., Emam, S. H., Sonousi, A., Kandil, M. M., Abdou, A. M., & Hassan, R. A. (2023). Design, synthesis, anticancer, and antibacterial evaluation of some quinazolinone-based derivatives as DHFR inhibitors. *Drug Development Research*, 84(5), 888–906.

- Pagadala, N. S., Syed, K., & Tuszynski, J. (2017). Software for molecular docking: a review. *Biophysical Reviews* 2017 9:2, 9(2), 91–102.
- Pal, T. K., Mumit, M. A., Hossen, J., Paul, S., Alam, M. A., Islam, M. A. A. A. A., & Sheikh, M. C. (2021). Computational and experimental insight into antituberculosis agent, (E)-benzyl-2-(4-hydroxy-2-methoxybenzylidene)hydrazinecarbodithioate: ADME analysis. *Heliyon*, 7(10), e08209.
- Palmer, B. D., Thompson, A. M., Sutherland, H. S., Blaser, A., Kmentova, I., Franzblau, S. G., Wan, B., Wang, Y., Ma, Z., & Denny, W. A. (2010). Synthesis and structure-activity studies of biphenyl analogues of the tuberculosis drug (6S)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5H-imidazo[2,1-b][1,3]oxazine (PA-824). *Journal of Medicinal Chemistry*, 53(1), 282–294.
- Panwar, A., & Kumar, A. (2021). In-silico Analysis and Molecular dynamics Simulations of Lysozyme by GROMACS 2020.2. *Annals of the Romanian Society for Cell Biology*, 25(6), 9679–9685.
- Pavan, F. R., Maia, P. I. da S., Leite, S. R. A., Deflon, V. M., Batista, A. A., Sato, D. N., Franzblau, S. G., & Leite, C. Q. F. (2010). Thiosemicarbazones, semicarbazones, dithiocarbazates and hydrazide/hydrazones: Anti – Mycobacterium tuberculosis activity and cytotoxicity. *European Journal of Medicinal Chemistry*, 45(5), 1898–1905.
- Pavithra, Pushpavathi, I., Pasha, K. M. M., & Nayaka, T. H. M. (2024). Synthesis, Biological Evaluation, Molecular Docking, and Molecular dynamics Simulation Studies of Some New 5-(3,4,5-Trimethoxybenzyl)pyrimidine-2,4-diamine (Trimethoprim) Derivatives via Modified Mannich-Type Reaction. *Russian Journal of Bioorganic Chemistry*, 50(2), 485–499.
- Pharmaceutica, H. A.-S. (2019). FT-IR spectroscopy for the identification of binding sites and measurements of the binding interactions of important metal ions with bovine serum albumin. *Mdpi.Com*.
- Ramilo-Gomes, F., Addis, Y., Tekamo, I., Cavaco, I., Campos, D. L., Pavan, F. R., Gomes, C. S. B., Brito, V., Santos, A. O., Domingues, F., Luís, Â., Marques, M.

- M., Pessoa, J. C., Ferreira, S., Silvestre, S., & Correia, I. (2021). Antimicrobial and antitumor activity of S-methyl dithiocarbazate Schiff base zinc(II) complexes. *Journal of Inorganic Biochemistry*, 216.
- Ramos, M. C., Quoika, P. K., Horta, V. A. C., Dias, D. M., Costa, E. G., Do Amaral, J. L. M., Ribeiro, L. M., Liedl, K. R., & Horta, B. A. C. (2021). PyPolyBuilder: Automated Preparation of Molecular Topologies and Initial Configurations for Molecular dynamics Simulations of Arbitrary Supramolecules. *Journal of Chemical Information and Modeling*, 61(4), 1539–1544.
- Rampogu, S., Lee, G., Park, J. S., Lee, K. W., & Kim, M. O. (2022). Molecular Docking and Molecular dynamics Simulations Discover Curcumin Analogue as a Plausible Dual Inhibitor for SARS-CoV-2. *International Journal of Molecular Sciences*, 23(3).
- Rani, M., Devi, J., & Kumar, B. (2023). Thiosemicarbazones-based Co(II), Ni(II), Cu(II) and Zn(II) complexes: synthesis, structural elucidation, biological activities and molecular docking. *Chemical Papers*, 77(10), 6007–6027.
- Rao, A. (2013). A study on dihydrofolate reductase and its inhibitors: A review. *Citeseer*.
- Rashid, U., Ahmad, W., Hassan, S. F., Qureshi, N. A., Niaz, B., Muhammad, B., Imdad, S., & Sajid, M. (2016). Design, synthesis, antibacterial activity and docking study of some new trimethoprim derivatives. *Bioorganic & Medicinal Chemistry Letters*, 26(23), 5749–5753.
- Ravoof, T. B. S. A., Crouse, K. A., Tahir, M. I. M., Rosli, R., Watkin, D. J., & How, F. N. F. (2010). Synthesis, Characterisation and Biological Activities of 2-Methylbenzyl 2-(dipyridin-2-yl methylene)hydrazinecarbodithioate. *Journal of Chemical Crystallography* 2010 41:4, 41(4), 491–495.
- Ravoof, T. B. S. A., Crouse, K. A., Tiekink, E. R. T., Tahir, M. I. M., Yusof, E. N. M., & Rosli, R. (2017). Synthesis, characterization and biological activities of S-2- or S-4-methylbenzyl- β -N-(di-2-pyridyl)methylenedithiocarbazate and Cu(II), Ni(II), Zn(II) and Cd(II) complexes. *Polyhedron*, 133, 383–392.

- Reeve, S. M., Scocchera, E. W., G-Dayananadan, N., Keshipeddy, S., Krucinska, J., Hajian, B., Ferreira, J., Nailor, M., Aeschlimann, J., Wright, D. L., & Anderson, A. C. (2016). MRSA Isolates from United States Hospitals Carry *dfrG* and *dfrK* Resistance Genes and Succumb to Propargyl-Linked Antifolates. *Cell Chemical Biology*, 23(12), 1458–1467.
- Reynisson, J., & Söhnle, P. T. (2022). *Computational chemistry approaches in modern drug discovery*.
- Riyadh, S. M., Ahmed, H., Profile, S., Khalil, K. D., Habib, E.-S. E., El-Motairi, S. A., & Ahmed, H. E. A. (2018). Synthesis, biological evaluation, and molecular docking of novel thiazoles and thiadiazoles incorporating sulfonamide group as DHFR Inhibitors. *Wiley Online Library*, 15(9).
- Rotzsche, H. (1991). *Stationary phases in gas chromatography*.
- Rubin, R. P. (2007). A brief history of great discoveries in pharmacology: In celebration of the centennial anniversary of the founding of the American Society of Pharmacology and Experimental Therapeutics. *Pharmacological Reviews*, 59(4), 289–359.
- Rudling, A., Orro, A., & Carlsson, J. (2018). Prediction of Ordered Water Molecules in Protein Binding Sites from Molecular dynamics Simulations: The Impact of Ligand Binding on Hydration Networks. *Journal of Chemical Information and Modeling*, 58(2), 350–361.
- Russu, E., Arbănași, E. M., & Șchiopu, A. (2024). Special Issue “COVID-19 Coagulopathy: Advances on Pathophysiology and Therapies”. *International Journal of Molecular Sciences*, 25(6), 3548.
- Sahu, G., Patra, S. A., Lima, S., Das, S., Görls, H., Plass, W., & Dinda, R. (2023). Ruthenium(II)-Dithiocarbazates as Anticancer Agents: Synthesis, Solution Behavior, and Mitochondria-Targeted Apoptotic Cell Death. *Chemistry - A European Journal*, 29(18).
- Saikia, S., & Bordoloi, M. (2018). Molecular Docking: Challenges, Advances and its Use in Drug Discovery Perspective. *Current Drug Targets*, 20(5), 501–521.

- Salo-Ahen, O. M. H., Alanko, I., Bhadane, R., Alexandre, A. M., Honorato, R. V., Hossain, S., Juffer, A. H., Kabedev, A., Lahtela-Kakkonen, M., Larsen, A. S., Lescrinier, E., Marimuthu, P., Mirza, M. U., Mustafa, G., Nunes-Alves, A., Pantsar, T., Saadabadi, A., Singaravelu, K., & Vanmeert, M. (2020). Molecular dynamicss Simulations in Drug Discovery and Pharmaceutical Development. *Processes* 2021, Vol. 9, Page 71, 9(1), 71.
- Santos-Martins, D., Solis-Vasquez, L., Tillack, A. F., Sanner, M. F., Koch, A., & Forli, S. (2021). Accelerating A uto D ock 4 with GPUs and Gradient-Based Local Search. *Journal of Chemical Theory and Computation*, 17(2), 1060–1073.
- Sawaya, M. R., & Kraut, J. (1997). Loop and subdomain movements in the mechanism of Escherichia coli dihydrofolate reductase: Crystallographic evidence. *Biochemistry*, 36(3), 586–603.
- Saxena, D., Maitra, R., Bormon, R., Czekanska, M., Meiers, J., Titz, A., Verma, S., & Chopra, S. (2023). Tackling the outer membrane: facilitating compound entry into Gram-negative bacterial pathogens. *Npj Antimicrobials and Resistance* 2023 1:1, 1(1), 1–22.
- Schlede, E., Mischke, U., Diener, W., & Kayser, D. (1995). The international validation study of the acute toxic class method (oral). *Archives of Toxicology*, 69(10), 659–670.
- Schnell, J. R., Dyson, H. J., & Wright, P. E. (2004). Structure, dynamics, and catalytic function of dihydrofolate reductase. *Annual Review of Biophysics and Biomolecular Structure*, 33, 119–140.
- Schrodinger, L. L. C. (2015). The PyMOL molecular graphics system. 1(8).
- Sears, P. G., Wilhoit, E. D., & Dawson, L. R. (1955). Conductances of Trimethylphenylammonium Chloride and Iodide in Water and in Dimethylformamide at 25°C. *The Journal of Chemical Physics*, 23(7), 1274–1276.

- Seeliger, D., & De Groot, B. L. (2010). Ligand docking and binding site analysis with PyMOL and Autodock/Vina. *Journal of Computer-Aided Molecular Design*, 24(5), 417–422.
- Sehrawat, R., Pasrija, R., Rathee, P., Kumari, D., & Khatkar, A. (2024). Molecular modeling, synthesis and biological evaluation of caffeic acid based Dihydrofolate reductase inhibitors. *BMC Chemistry*, 18(1).
- Sehrawat, R., Pasrija, R., Rathee, P., Kumari, D., Khatkar, A., Küpeli Akkol, E., & Sobarzo-Sánchez, E. (2024). Hybrid Caffeic Acid-Based DHFR Inhibitors as Novel Antimicrobial and Anticancer Agents. *Antibiotics*, 13(6).
- Sehrawat, R., Rathee, P., Khatkar, S., Akkol, E., Khayatkashani, M., Nabavi, S. M., & Khatkar, A. (2023). Dihydrofolate Reductase (DHFR) Inhibitors: A Comprehensive Review. *Current Medicinal Chemistry*, 31(7), 799–824.
- Sengottiyen, S., Malakar, K., Kathiravan, A., Velusamy, M., Mikolajczyk, A., & Puzyn, T. (2022). Integrated Approach to Interaction Studies of Pyrene Derivatives with Bovine Serum Albumin: Insights from Theory and Experiment. *Journal of Physical Chemistry B*, 126(21), 3831–3843.
- Sharma, B., Shukla, S., Rattan, R., Fatima, M., Goel, M., Bhat, M., Dutta, S., Ranjan, R. K., & Sharma, M. (2022). Antimicrobial Agents Based on Metal Complexes: Present Situation and Future Prospects. *International Journal of Biomaterials*, 2022.
- Sharma, M., & Chauhan, P. M. S. (2012). Dihydrofolate reductase as a therapeutic target for infectious diseases: Opportunities and challenges. *Future Medicinal Chemistry*, 4(10), 1335–1365.
- Shcherbakov, V. V., Artemkina, Y. M., Akimova, I. A., & Artemkina, I. M. (2021). Dielectric Characteristics, Electrical Conductivity and Solvation of Ions in Electrolyte Solutions. *Materials* 2021, Vol. 14, Page 5617, 14(19), 5617.
- Shen, B., Löffler, D., Zeller, K. P., Übele, M., Reischl, G., & Machulla, H. J. (2007). Effect of aldehyde and methoxy substituents on nucleophilic aromatic

- substitution by [18F]fluoride. *Journal of Fluorine Chemistry*, 128(12), 1461–1468.
- Shivashankar, S., & Kannan, S. M. (2021). Identification of biosimilar for trimethoprim-andrographis paniculata phytochemicals inhibits dihydrofolate reductase (DHFR). *Biointerface Research in Applied Chemistry*, 11(6), 14142–14154.
- Shrader, S. (2013). *Introductory mass spectrometry*.
- Shuhada, N., Mokhtaruddin, M., Yusof, N. M., Ravooof, T. B. S. A., Tiekink, E. R. T., Veerakumarasivam, A., Ibrahim, M., & Tahir, M. (2017). Unusual saccharin-N,O (carbonyl) coordination in mixed-ligand copper(II) complexes: Synthesis, X-ray crystallography and biological activity. *Journal of Molecular Structure*, 1139, 1-9.
- Sibeaud, M., Croutxé-Barghorn, C., Rigolet, S., Michelin, L., Josien, L., Vidal, L., Lebeau, B., Wörner, M., & Chemtob, A. (2016). UV aerosol synthesis: a one-step route to silica, organic-silica and surfactant/silica nanostructured materials. *RSC Advances*, 6(69), 65047–65054.
- Sigma-Aldrich. (2022). *Safety data sheet: 3-Methoxybenzoyl chloride*.
- Silhavy, T. J., Kahne, D., & Walker, S. (2010). The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives in Biology*, 2(5), a000414.
- Singh, A., Bharty, M. K., Dani, R. K., Singh, S., Kushawaha, S. K., & Singh, N. K. (2014). Manganese(II) and zinc(II) complexes of 4-phenyl(2-methoxybenzoyl)-3- thiosemicarbazide: Synthesis, spectral, structural characterization, thermal behavior and DFT study. *Polyhedron*, 73, 98–109.
- Singh, A., Gogoi, H. P., Barman, P., Das, A., & Pandey, P. (2022). Tetracoordinated ONNO donor purine-based Schiff base and its metal complexes: Synthesis, characterization, DNA binding, theoretical studies, and bioactivities. *Applied Organometallic Chemistry*, 36(10), e6852.

- Singh, K. (2006). Antibacterial Co (II), Ni (II), Cu (II) and Zn (II) complexes of Schiff bases derived from fluorobenzaldehyde and triazoles. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 21(5), 557-562.
- Singh, M., Aggarwal, V., Singh, U. P., & Singh, N. K. (2009). Synthesis, characterization and spectroscopic studies of a new ligand [N'-(2-methoxybenzoyl)hydrazinecarbodithioate] ethyl ester and its Mn(II) and Cd(II) complexes: X-ray structural study of Mn(II) complex. *Polyhedron*, 28(1), 107–112.
- Smith, R. (2004). *Understanding mass spectra: a basic approach*.
- Sohtun, W. P., Kathiravan, A., Asha Jhonsi, M., Aashique, M., Bera, S., & Velusamy, M. (2022). Synthesis, crystal structure, BSA binding and antibacterial studies of Ni(II) complexes derived from dithiocarbamate based ligands. *Inorganica Chimica Acta*, 536, 120888.
- Soni, J., Sinha, S., & Pandey, R. (2024). Understanding bacterial pathogenicity: a closer look at the journey of harmful microbes. *Frontiers in Microbiology*, 15.
- Sousa Da Silva, A. W., & Vranken, W. F. (2012). ACPYPE - AnteChamber PYthon Parser interface. *BMC Research Notes*, 5(1), 1–8.
- Sousa, S. F., Fernandes, P. A., & Ramos, M. J. (2006). Protein-ligand docking: Current status and future challenges. *Proteins: Structure, Function and Genetics*, 65(1), 15–26.
- Srinivasan, B., Tonddast-Navaei, S., & Skolnick, J. (2015). Ligand binding studies, preliminary structure-activity relationship and detailed mechanistic characterization of 1-phenyl-6,6-dimethyl-1,3,5-triazine-2,4-diamine derivatives as inhibitors of Escherichia coli dihydrofolate reductase. *European Journal of Medicinal Chemistry*, 103, 600.
- Stallard, N., Whitehead, A., & Indans, I. (2004). Statistical evaluation of an acute dermal toxicity test using the dermal fixed dose procedure.

- Steckel, A., & Schlosser, G. (2019). An organic chemist's guide to electrospray mass spectrometric structure elucidation. *Molecules*, 24(3), 611.
- Sultana, U. S., Ahsan, H. M., Amin, M. K., Mahiuddin, M., Kudrat-E-Zahan, M., & Nazmul Islam, A. B. M. (2020). Synthesis, Structural Properties of Hydrazine Carbodithioate Schiff Base and Their Metal Complexes and Evaluation of Their Biological Activity. *Egyptian Journal of Chemistry*, 63(10), 3811–3816.
- Summerfield, R. L., Daigle, D. M., Mayer, S., Mallik, D., Hughes, D. W., Jackson, S. G., Sulek, M., Organ, M. G., Brown, E. D., & Junop, M. S. (2006). A 2.13 Å structure of E. coli dihydrofolate reductase bound to a novel competitive inhibitor reveals a new binding surface involving the M20 loop region. *Journal of Medicinal Chemistry*, 49(24), 6977–6986.
- Sun, Z., Liu, Q., Qu, G., Feng, Y., & Reetz, M. T. (2019). Utility of B-Factors in Protein Science: Interpreting Rigidity, Flexibility, and Internal Motion and Engineering Thermostability. *Chemical Reviews*.
- T. W. Graham Solomons Craig B. Fryhle, & Snyder, S. A. (2016). *Organic Chemistry*. 1124.
- Tahir, A., Sehgal, A., Tahir, R. A., Shafique, S., Hassan, M., & Rashid, S. (2014). Molecular Modeling and Docking Analysis of CYP1A1 Associated with Head and Neck Cancer to Explore its Binding Regions. *J Theor Comput Sci*, 1(3), 112.
- Tarafder, M. T. H., Ali, M. A., Wee, D. J., Azahari, K., Silong, S., & Crouse, K. A. (2000). Complexes of a tridentate ONS Schiff base. Synthesis and biological properties. *Transition Metal Chemistry*, 25(4), 456–460.
- Tarafder, M. T. H., Kasbollah, A., Crouse, K. A., Ali, A. M., Yamin, B. M., & Fun, H. K. (2001). Synthesis and characterization of Zn(II) and Cd(II) complexes of S-benzyl-β-N-(2-pyridyl)methylenedithiocarbazate (HNNS): bioactivity of the HNNS Schiff base and its Zn(II), Cu(II) and Cd(II) complexes and the X-ray structure of the [Zn(NNS)₂] complex. *Polyhedron*, 20(18), 2363–2370.
- Tarafder, M. T. H., Khoo, T. J., Crouse, K. A., Ali, A. M., Yamin, B. M., & Fun, H. K. (2002). Coordination chemistry and bioactivity of some metal complexes

containing two isomeric bidentate NS Schiff bases derived from S-benzylthiocarbamate and the X-ray crystal structures of S-benzyl- β -N-(5-methyl-2-furylmethylene) dithiocarbamate and bis[S-benzyl- β -N-(2-furylmethylketone) dithiocarbamate]cadmium(II). *Polyhedron*, 21(27-28), 2691-2698.

Ten billion COVID vaccines, deadly bacteria and high-risk research. (2022). *Nature*, 602(7896), 189.

Then, R. L. (2004). Antimicrobial dihydrofolate reductase inhibitors - Achievements and future options: Review. *Journal of Chemotherapy*, 16(1), 3–12.

Tiwari, M., Gupta, S., & Bhargava, P. (2022). Virtual screening and Molecular dynamics simulation studies to predict the binding of *Sisymbrium irio* L. derived phytochemicals against *Staphylococcus aureus* dihydrofolate reductase (DHFR). *Journal of Applied and Natural Science*, 14(4), 1297-1307.

Tong, W., Welsh, W. J., Shi, L., Fang, H., & Perkins, R. (2003). Structure-activity relationship approaches and applications. *Environmental Toxicology and Chemistry*, 22(8), 1680–1695.

Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, 31(2).

Tuccinardi, T. (2021). What is the current value of MM/PBSA and MM/GBSA methods in drug discovery? *Expert Opinion on Drug Discovery*, 16(11), 1233–1237.

Turk, P., Singh, K., & Dhanda, A. (2022). Synthesis, spectroscopic, electrochemical, thermal and antimicrobial studies of Ni(II), Zn(II), Cu(II) and Co(II) metal complexes of novel bidentate schiff base ligand. *Journal of the Iranian Chemical Society*, 19(9), 3797–3813.

Turnley, J. W., Deshmukh, S. D., Boulos, V. M., Ellis, R. G., LiBretto, N. J., Liu, J. K. Y., Miller, J. T., Kenttämä, H. I., & Agrawal, R. (2023). Molecular Precursor

Approach to Sulfur-Free CuInSe₂: Replacing Thiol Coordination in Soluble Metal Complexes. *ACS Omega*, 8(49), 47262–47270.

- Vaidyanathan, R., Murugan Sreedevi, S., Ravichandran, K., Vinod, S. M., Hari Krishnan, Y., Babu, L. K., Parthiban, P. S., Basker, L., Perumal, T., Rajaraman, V., Arumugam, G., Rajendran, K., & Mahalingam, V. (2023). Molecular docking approach on the binding stability of derivatives of phenolic acids (DPAs) with Human Serum Albumin (HSA): Hydrogen-bonding versus hydrophobic interactions or combined influences? *JCIS Open*, 12, 100096.
- Valdés-Tresanco, M. S., Valdés-Tresanco, M. E., Valiente, P. A., & Moreno, E. (2021). Gmx_MMPBSA: A New Tool to Perform End-State Free Energy Calculations with GROMACS. *Journal of Chemical Theory and Computation*, 17(10), 6281–6291.
- Van de Waterbeemd, H., & Gifford, E. (2003). ADMET in silico modelling: Towards prediction paradise? *Nature Reviews Drug Discovery*, 2(3), 192–204.
- Venkitakrishnan, R. P., Zaborowski, E., McElheny, D., Benkovic, S. J., Dyson, H. J., & Wright, P. E. (2004). Conformational changes in the active site loops of dihydrofolate reductase during the catalytic cycle. *Biochemistry*, 43(51), 16046–16055.
- Vieira, I. H. P., Botelho, E. B., de Souza Gomes, T. J., Kist, R., Caceres, R. A., & Zanchi, F. B. (2023). Visual dynamics: a WEB application for Molecular dynamics simulation using GROMACS. *BMC Bioinformatics*, 24(1), 1–8.
- Vilchèze, C., & Jacobs, W. R. (2012). The Combination of Sulfamethoxazole, Trimethoprim, and Isoniazid or Rifampin Is Bactericidal and Prevents the Emergence of Drug Resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, 56(10), 5142.
- Vivo, M. D., Masetti, M., Bottegoni, G., & Cavalli, A. (2016). Role of Molecular dynamics and related methods in drug discovery. *Journal of medicinal chemistry*, 59(9), 4035–4061.

- Vogt, C., Wondergem, C. S., & Weckhuysen, B. M. (2023). Ultraviolet-Visible (UV-Vis) Spectroscopy. *Springer Handbooks*, 237–264.
- Waghchaure, P., Thange, T., & Vishal, G. (2023). Basic Chemistry, Mode Of Action And Clinical Uses Of Co-Trimoxazole. *International Journal in Pharmaceutical Sciences*, 1(12), 135-149.
- Wahba, M., El-Tabl, A. S., Abd-El Wahed, M. M., Wahba, M. A., EL-assaly, S. A., & Saad, L. M. (1860). Sugar Hydrazone Complexes; Synthesis, Spectroscopic Characterization and Antitumor Activity. *Journal: Journal of Advances in Chemistry*, 9(1).
- Wang, E., Sun, H., Wang, J., Wang, Z., Liu, H., Zhang, J. Z. H., & Hou, T. (2019). End-Point Binding Free Energy Calculation with MM/PBSA and MM/GBSA: Strategies and Applications in Drug Design. *Chemical Reviews*, 119(16), 9478-9508.
- Wang, J., Wolf, R. M., Caldwell, J. W., Kollman, P. A., & Case, D. A. (2004). Development and testing of a general amber force field. *Journal of Computational Chemistry*, 25(9), 1157–1174.
- Wang, X., Toroz, D., Kim, S., Clegg, S. L., Park, G. S., & Di Tommaso, D. (2020). Density functional theory based Molecular dynamicss study of solution composition effects on the solvation shell of metal ions. *Physical Chemistry Chemical Physics*, 22(28), 16301–16313.
- Wang, Y., Xing, J., Xu, Y., Zhou, N., Peng, J., Xiong, Z., Liu, X., Luo, X., Luo, C., Chen, K., Zheng, M., & Jiang, H. (2015). In silico ADME/T modelling for rational drug design. *Q Rev Biophys*, 48(4), 488–515.
- Weckhuysen, B. M. (2004). *Ultraviolet-visible spectroscopy*.
- Wilkinson, P. G. (1956). Absorption spectra of benzene and benzene- d 6 in the vacuum ultraviolet. *Canadian Journal of Physics*, 34(6), 596–615.
- Wright, D. L., & Anderson, A. C. (2011). Antifolate agents: A patent review (2006 2010). *Expert Opinion on Therapeutic Patents*, 21(9), 1293–1308.

- Wróbel, A., Arciszewska, K., Maliszewski, D., & Drozdowska, D. (2019). Trimethoprim and other nonclassical antifolates an excellent template for searching modifications of dihydrofolate reductase enzyme inhibitors. *The Journal of Antibiotics* 2019 73:1, 73(1), 5–27.
- Wróbel, A., Maliszewski, D., Baradyn, M., & Drozdowska, D. (2019). Trimethoprim: An Old Antibacterial Drug as a Template to Search for New Targets. Synthesis, Biological Activity and Molecular Modeling Study of Novel Trimethoprim Analogs. *Molecules* 2020, Vol. 25, Page 116, 25(1), 116.
- Xuan Hung, N., Anh Quang, D., Thanh Tam Toan, T., -, al, Guk, A., Krasnovskaya, O. O., Beloglazkina, E. K., Mahmoud, W. A., MHassan, Z., & Russel WAlI, and. (2020). Synthesis and spectral analysis of some metal complexes with mixed Schiff base ligands 1-[2-(2-hydroxybenzylideneamino) ethyl]pyrrolidine-2,5-dione (HL1) and (2-hydroxybenzalidine) glycine (HL2). *Journal of Physics: Conference Series*, 1660(1), 012027.
- Yadav, C. S., Azad, I., Nasibullah, M., Ahmad, N., Lohani, M. B., & Khan, A. R. (2024). Synthesis, characterization, quantum chemical modelling, molecular docking, in silico and in vitro assessment of 3-(2-bromo-5-fluorophenyl)-1-(thiophen-2-yl)prop-2-en-1-one. *Scientific Reports*, 14(1).
- Yekke-Ghasemi, Z., Ramezani, M., Mague, J. T., & Takjoo, R. (2020). Synthesis, characterization and bioactivity studies of new dithiocarbazate complexes. *New Journal of Chemistry*, 44(21), 8878–8889.
- Yin, X., Chen, J., Yuan, W., Lin, Q., Ji, L., & Liu, F. (2012). Preparation and antibacterial activity of Schiff bases from O-carboxymethyl chitosan and para-substituted benzaldehydes. *Polymer Bulletin*, 68(5), 1215–1226.
- Yoshinari, N., Kuwamura, N., Kojima, T., & Konno, T. (2023). Development of coordination chemistry with thiol-containing amino acids. *Coordination Chemistry Reviews*, 474.
- Young, R. J., & Leeson, P. D. (2018). Mapping the Efficiency and Physicochemical Trajectories of Successful Optimizations. *Journal of Medicinal Chemistry*, 61(15), 6421–6467.

- Yu, L. (2024). *The prevalence of trimethoprim-resistance-conferring dihydrofolate reductase genes in multidrug resistant bacteria from clinical isolates in Malaysia.*
- Yusof, E. N. M., Azam, M., Sirat, S. S., Ravooof, T. B. S. A., Page, A. J., Veerakumarasivam, A., Karunakaran, T., & Razali, M. R. (2022). Dithiocarbazate Ligand-Based Cu(II), Ni(II), and Zn(II) Complexes: Synthesis, Structural Investigations, Cytotoxicity, DNA Binding, and Molecular Docking Studies. *Bioinorganic Chemistry and Applications*, 2022(1), 2004052.
- Zahan, R., Ahmed, S., Sharmin, T., Halim, M. A., Rahi, M. S., Sheikh, M. C., Miyatake, R., Zangrando, E., Naz, T., Islam, M. A. A. A. A., & Reza, M. A. (2021). Synthesis of bis[benzyl-N'-hydrazinecarbodithioato- κ^2 N',S]nickel(II) complex as a novel lead molecule for cancer treatment. *Applied Organometallic Chemistry*, 35(1), 1–17.
- Zahradník, R. (1965). Electronic Structure of Heterocyclic Sulfur Compounds. *Advances in Heterocyclic Chemistry*, 5(C), 1–67.
- Zangrando, E., Islam, M. T., Islam, M. A. A. A. A., Sheikh, M. C., Tarafder, M. T. H., Miyatake, R., Zahan, R., & Hossain, M. A. (2015). Synthesis, characterization and bio-activity of nickel(II) and copper(II) complexes of a bidentate NS Schiff base of S-benzyl dithiocarbazate. *Inorganica Chimica Acta*, 427, 278–284.
- Zarenezhad, E., Behmard, E., Sadeghian, I., Sadeghian, S., Ghanbariasad, A., Ghasemian, A., Behrouz, S., Zarenezhad, A., & Rad, M. N. S. (2023). Synthesis, Cytotoxic Evaluation, Molecular Docking Studies and Molecular dynamics Simulation of some Metronidazole Analogues. *Journal of Molecular Structure*, 135378.
- Zhang, Y., Chowdhury, S., Rodrigues, J. V., & Shakhnovich, E. (2021). Development of antibacterial compounds that constrain evolutionary pathways to resistance. *Elife*, 10, e64518.
- Zhao, J., Liu, R., Zhang, Z., Xing, Q., Chang, H., Hou, X., & Wang, Y. (2022). Tautomer distributions of N-acetyl-D-glucosamine in the condition of

commonly utilized solvents and catalysts for biorefinery: NMR study. *Journal of Molecular Structure*, 1251, 131995.



APPENDICES

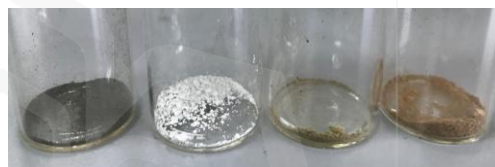
Appendix 1 Picture of all synthesised compounds



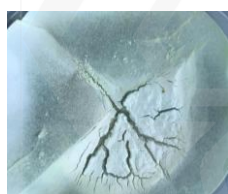
SBDTC



SB2OME



From left to right: $\text{Cu}(\text{SB2OME})_2$, $\text{Zn}(\text{SB2OME})_2$,
 $\text{Co}(\text{SB2OME})_2$, $\text{Ni}(\text{SB2OME})_2$



SB3OME



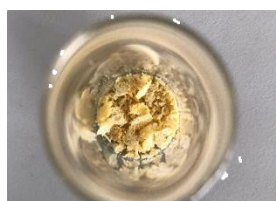
From left to right: $\text{Cu}(\text{SB3OME})_2$, $\text{Zn}(\text{SB3OME})_2$,
 $\text{Co}(\text{SB3OME})_2$, $\text{Ni}(\text{SB3OME})_2$



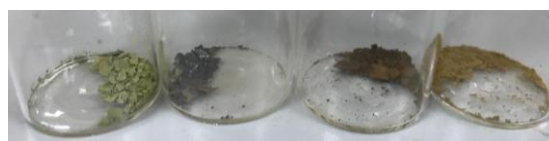
SB4OME



From left to right: $\text{Cu}(\text{SB4OME})_2$, $\text{Zn}(\text{SB4OME})_2$,
 $\text{Co}(\text{SB4OME})_2$, $\text{Ni}(\text{SB4OME})_2$



SB3NO



From left to right: $\text{Cu}(\text{SB3NO})_2$, $\text{Zn}(\text{SB3NO})_2$,
 $\text{Co}(\text{SB3NO})_2$, $\text{Ni}(\text{SB3NO})_2$

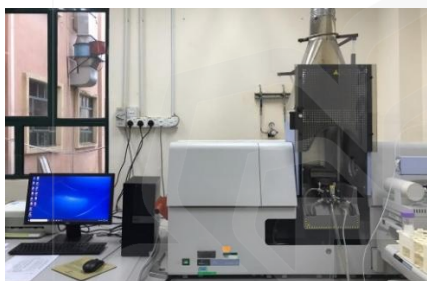
Appendix 2 All instruments used



Electrothermal™ IA9300 melting point apparatus



CHNS Perkin Elmer EA 2400



Perkin Elmer The AAnalyst™ 700



FTIR Perkin Elmer



Bruker Ultra Shield Plus 500MHz NMR



Perkin Elmer CLARUS GCMS



Double beam UV-Vis Spectrophotometer model Shimadzu UV-1900 Series



Conductivity meter model Eutech CON 700



Sherwood Scientific MSB AUTO
magnetic moment balance



SHIMADZU TGA-50 Series



Appendix 3 Raw data from CHNS

Compound	Weight used (mg)	Carbon	Hydrogen	Nitrogen	Sulfur
SB2OME	2.173	58.082	4.24	8.22	19.36
Cu(SB2OME) ₂	2.090	52.686	4.36	7.96	17.76
Zn(SB2OME) ₂	2.067	52.197	4.30	7.62	17.80
Co(SB2OME) ₂	2.055	52.97	4.41	7.60	17.63
Ni(SB2OME) ₂	2.027	52.712	4.30	7.68	17.09
SB3OME	2.090	56.81	4.31	8.38	19.55
Cu(SB3OME) ₂	1.997	51.767	4.23	7.52	17.04
Zn(SB3OME) ₂	1.981	52.404	4.70	7.43	17.39
Co(SB3OME) ₂	1.988	54.909	4.36	7.47	18.88
Ni(SB3OME) ₂	1.958	51.308	4.68	7.68	17.50
SB4OME	1.952	58.284	4.39	8.02	19.63
Cu(SB4OME) ₂	1.912	52.352	4.11	7.44	16.94
Zn(SB4OME) ₂	1.979	53.802	4.81	7.83	17.43
Co(SB4OME) ₂	1.986	54.206	4.80	7.91	17.45
Ni(SB4OME) ₂	2.083	52.14	4.35	7.77	17.12
SB3NO	2.068	46.561	2.97	10.65	15.04
Cu(SB3NO) ₂	2.263	42.487	2.81	11.18	14.35
Zn(SB3NO) ₂	2.156	42.73	3.23	10.27	14.53
Co(SB3NO) ₂	2.041	44.76	2.82	10.53	14.55
Ni(SB3NO) ₂	2.222	44.05	2.94	9.87	14.36

Appendix 4 Raw data from AAS

Compound	Weight (mg)	Metal (%)			
		1 st	2 nd	3 rd	Average
Cu(SB2OME) ₂	1.1	8.89	8.94	8.93	8.92
Zn(SB2OME) ₂	1	8.70	8.69	8.59	8.66
Ni(SB2OME) ₂	1	8.13	8.01	7.86	8.00
Cu(SB3OME) ₂	0.4	9.09	9.10	8.96	9.05
Zn(SB3OME) ₂	1	9.12	9.03	8.85	9.00
Ni(SB3OME) ₂	1	8.30	8.43	8.29	8.34
Cu(SB4OME) ₂	1	8.85	8.90	8.98	8.91
Zn(SB4OME) ₂	1	8.66	8.79	8.65	8.70
Ni(SB4OME) ₂	1	8.22	8.10	8.13	8.15
Cu(SB3NO) ₂	0.9	7.81	7.70	7.62	7.71
Zn(SB3NO) ₂	1	7.65	7.69	7.46	7.60
Ni(SB3NO) ₂	1	7.20	7.32	7.17	7.23

Appendix 5 Wavenumber of FTIR

Compound	Wavenumber (cm ⁻¹)								
	<i>v</i> (OAc)	<i>v</i> (N-H ₂)	<i>v</i> (N-H)	<i>v</i> (N-N)	<i>v</i> (C=S)	<i>v</i> (SCS)	<i>v</i> (ClC=O)	<i>v</i> (C=O)	<i>v</i> (OCH ₃)
SBDTC	-	3304 s	3220, 3180 s	1347 m	1048 s	952 m	-	-	-
2OME	-	-	-	-	-	-	654 s	1696 s	1465 s
3OME	-	-	-	-	-	-	667 s	1764 s	1486 s
4OME	-	-	-	-	-	-	613 s	1771 s	1506 s
Copper(II) acetate monohydrate	1603 s, 1445 s	-	-	-	-	-	-	-	-
Zinc (II) acetate dihydrate	1558 s, 1456 s	-	-	-	-	-	-	-	-
Cobalt (II) acetate tetrahydrate	1569 s, 1453 m	-	-	-	-	-	-	-	-
Nickel (II) acetate tetrahydrate	1606 s, 1456 s	-	-	-	-	-	-	-	-

Compound	Wavenumber (cm ⁻¹)								
	<i>v</i> (N-H ₂)	<i>v</i> (N-H)	<i>v</i> (N-N)	<i>v</i> (C=S)	<i>v</i> (SCS)	<i>v</i> (C=O)	<i>v</i> (OCH ₃)	(N-H) bending	<i>v</i> (M-N), <i>v</i> (M-O), <i>v</i> (M-S)
SB2OME	-	3209, 3121 m	1308 m	1035 s	980 s	1638 s	1446 s	1601, 1325 m	-
Cu(SB2OME) ₂	-	3210 m	1234 m	1019 s	971 s	1602 s	1444 s	1585 w	534, 497, 472 w
Zn(SB2OME) ₂	-	3206 m	1243 m	1023 s	965 s	1604 s	1451 s	1585 w	528, 495, 462 w

Co(SB2OME) ₂	-	3180 m	1242 m	1013 s	973 s	1602 s	1451 s	1592 w	530, 494, 462 w
Ni(SB2OME) ₂	-	3180 m	1239 m	1020 s	963 s	1602 s	1451 s	1582 w	531, 503, 469 w
SB3OME	-	3258, 3135 m	1297 m	1047 s	988 s	1658 s	10.437 s	1590, 1340 m	-
Cu(SB3OME) ₂	-	3196 w	1242 m	1031 s	954 s	1600 s	1440 s	1585 w	564, 516, 466 w
Zn(SB3OME) ₂	-	3194 w	1243 m	1035 s	958 s	1601 s	1449 s	1585 w	560, 490, 466 w
Co(SB3OME) ₂	-	3192 w	1246 m	1035 s	954 s	1600 s	1443 s	1582 w	562, 493, 463 w
Ni(SB3OME) ₂	-	3195 w	1243 m	1030 s	955 s	1600 s	10.437 s	1582 w	567, 495, 470 w
SB4OME	3329 s	3258 s	1276 s	1043 m	1016 m	1658 s	1495 s	1603 s	-
Cu(SB4OME) ₂	-	3213 w	1253 s	1029 s	963 s	1606 s	1494 s	1583 m	564, 516, 466 w
Zn(SB4OME) ₂	-	3212 w	1253 s	1028 s	966 s	1606 s	1491 s	1584 m	560, 490, 466 w
Co(SB4OME) ₂	-	3212 w	1253 s	1028 s	959 s	1606 s	1491 s	1582 m	562, 493, 463 w
Ni(SB4OME) ₂	-	3209 w	1253 s	1028 s	971 s	1606 s	1453 s	1581 m	567, 495, 470 w

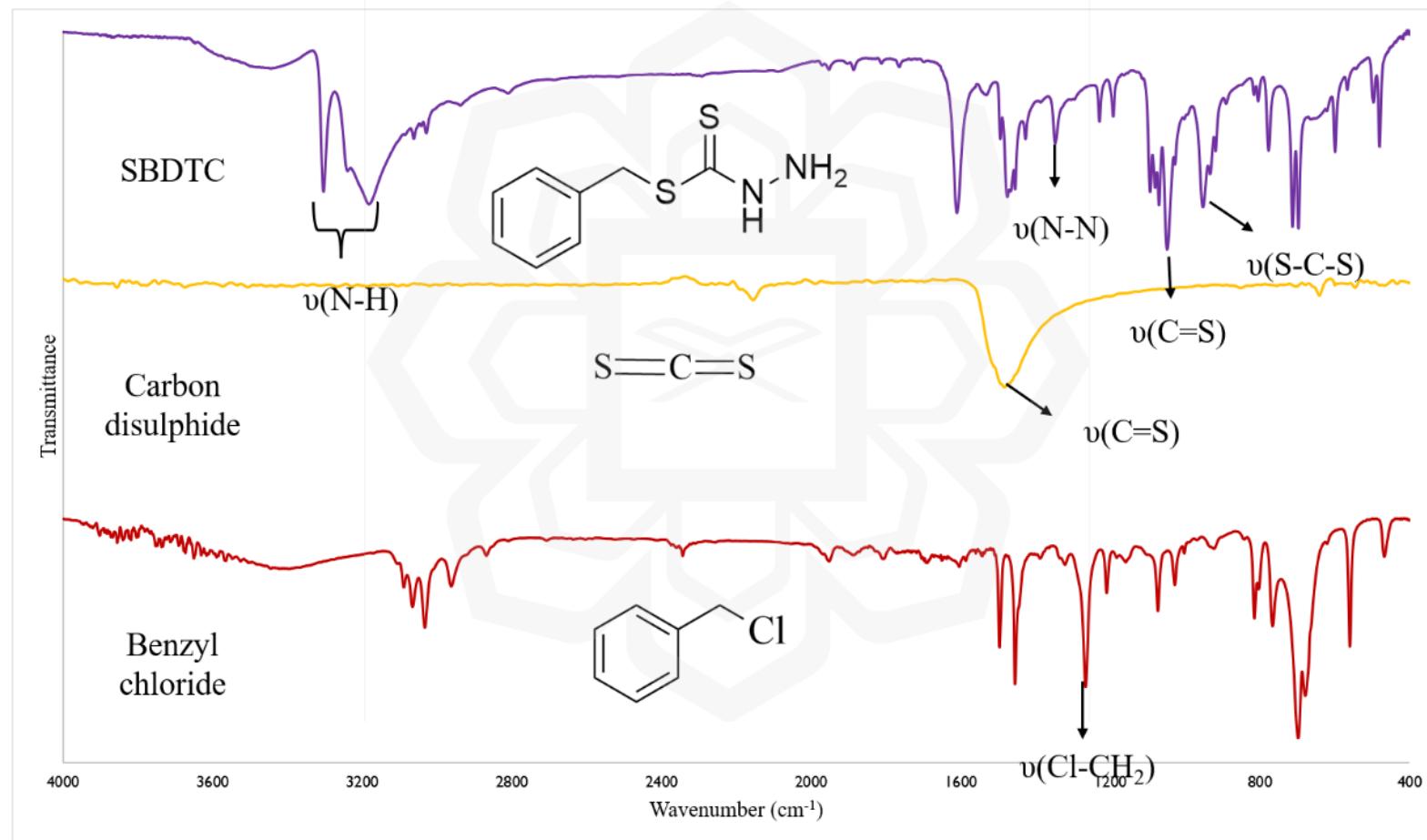
Compound	Wavenumber (cm ⁻¹)									
	<i>v</i> (N-H)	<i>v</i> (N-N)	<i>v</i> (C=S)	<i>v</i> (SCS)	<i>v</i> (C=O)	<i>v</i> (C=N)	<i>v</i> (NO ₂)	(N-H) bending	<i>v</i> (Ca-Cl)	<i>v</i> (M-S), <i>v</i> (M-O)
3NO	-	-	-	-	1694 s	-	1386 s	-	768 m	-
SB3NO	3094 w	1353 m	1107 s	1037 s	1685 s	-	1382 s	1598, 1393 m	775 m	-
Cu(SB3NO) ₂	3080 w	1342 m	-	1123, 1049 m	1623 s	1602 m	1385 s	1552 w	750 m	485, 536 w
Zn(SB3NO) ₂	3083 w	1350 m	-	1123, 1049 m	1618 s	1597 s	1387 s	1555 s	752 m	485, 537 w

Co(SB3NO) ₂	3079 w	1345 m	-	1123, 1049 m	1630 s	1600 s	1386 s	1551 s	752 m	485, 537 w
Ni(SB3NO) ₂	3084 w	1349 m	-	1123, 1043 m	1630 s	1600 s	1385 s	1557 w	751 m	481, 538 w

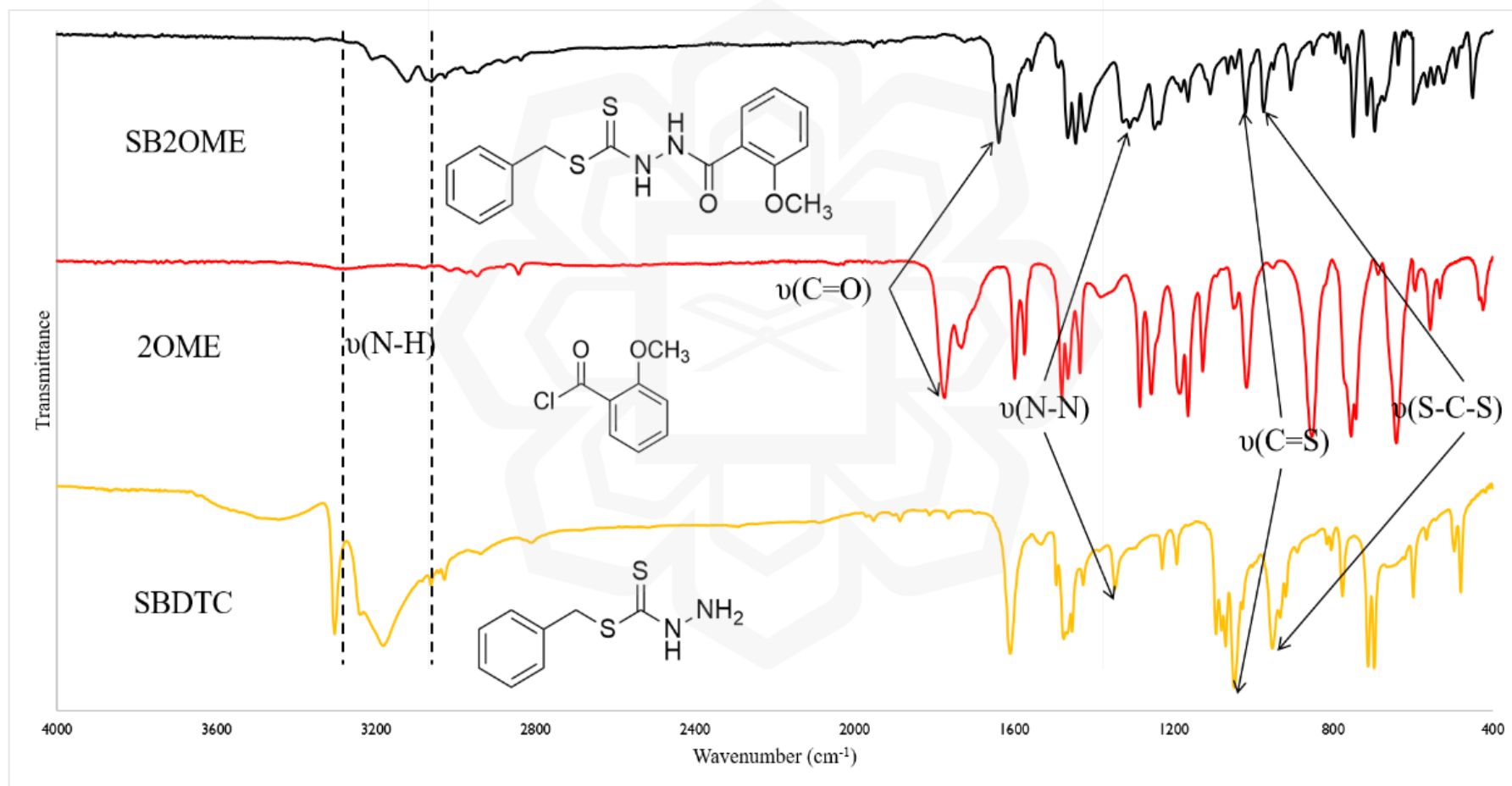
s-strong; m-medium; w-weak



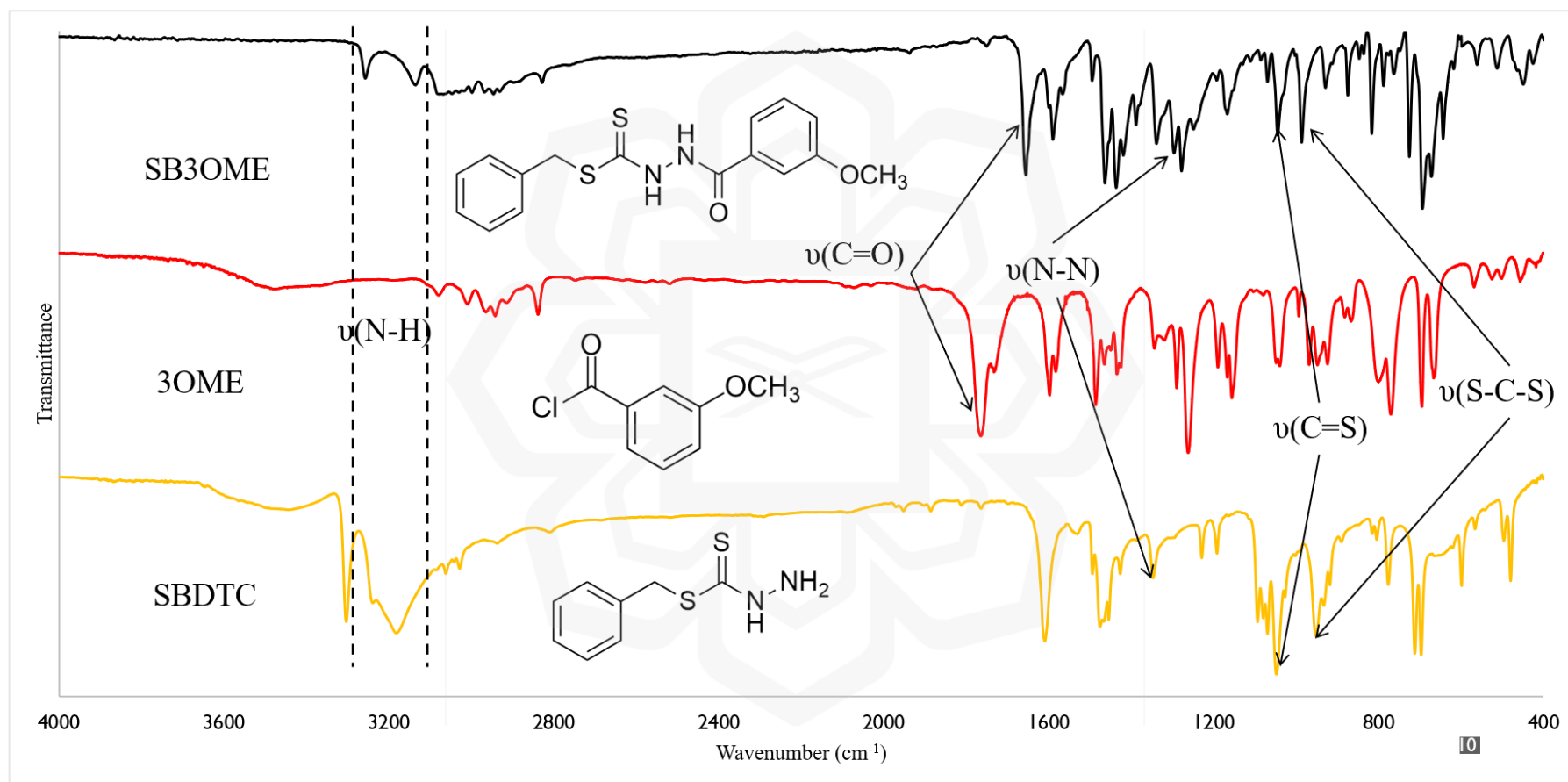
Appendix 6 Overlay IR spectra of SBDTC



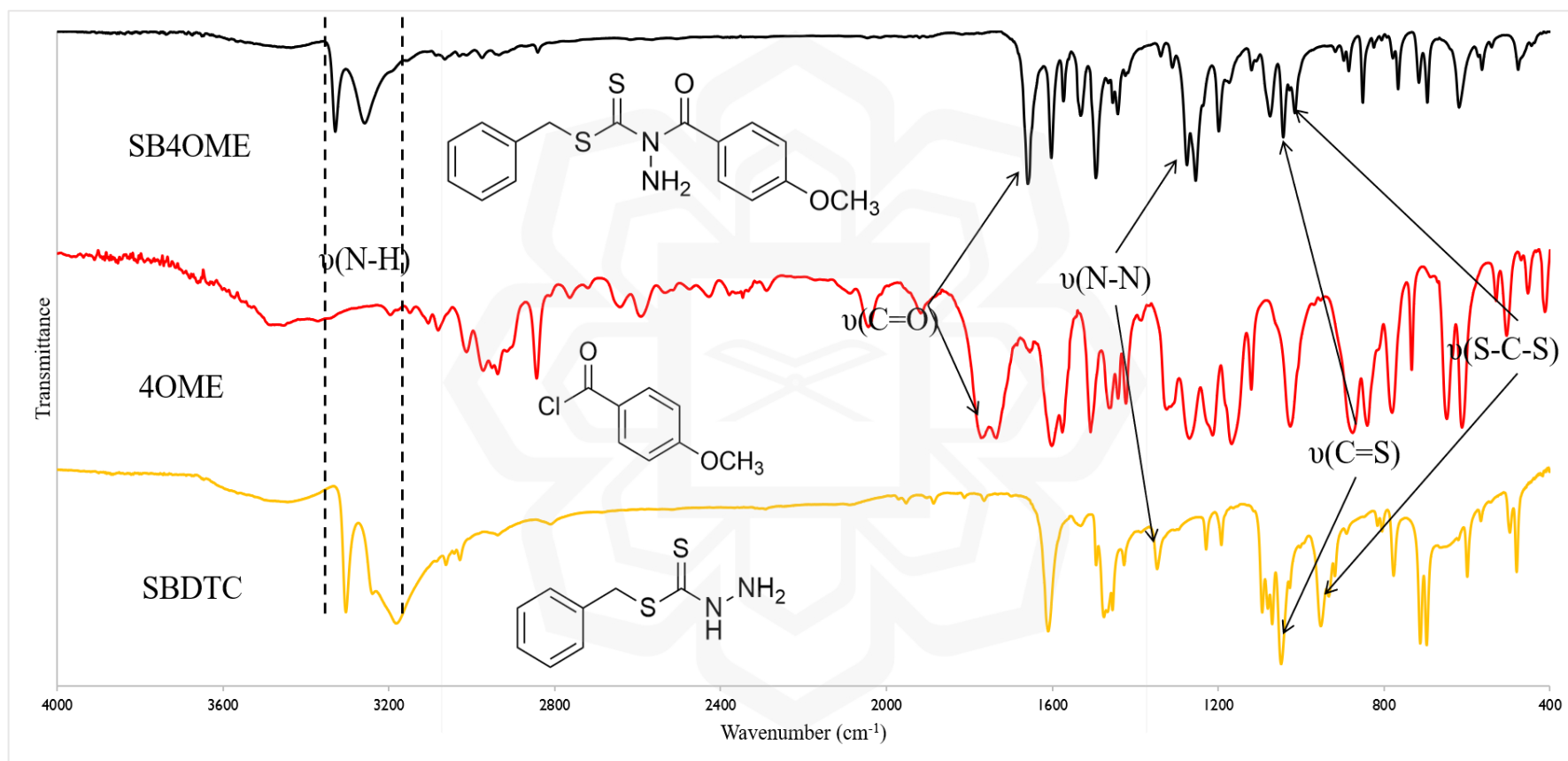
Appendix 7 Overlay IR spectra of SB2OME



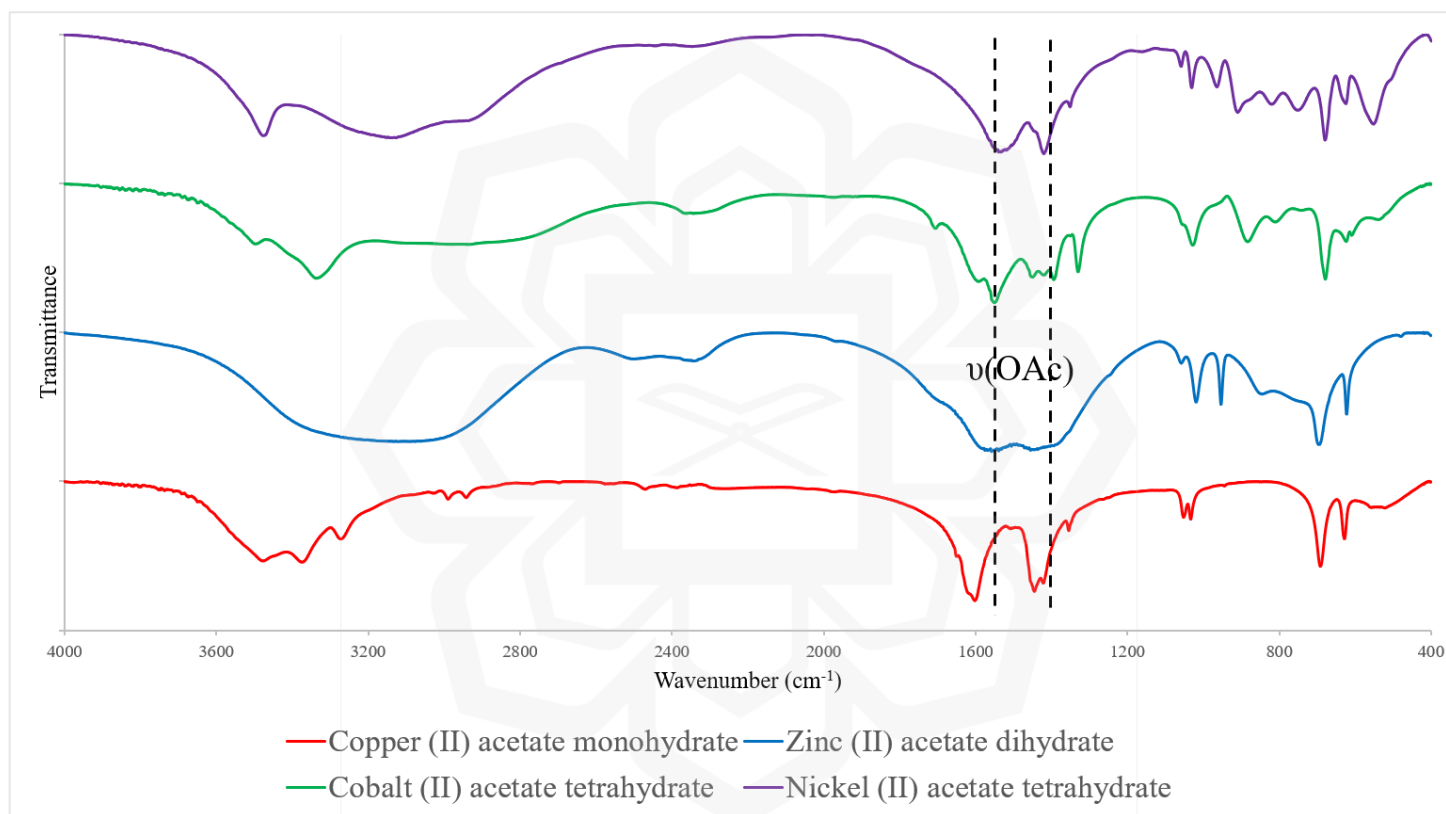
Appendix 8 Overlay IR spectra of SB3OME



Appendix 9 Overlay IR spectra of SB4OME



Appendix 10 Overlay IR spectra of metal acetate



Appendix 11 Important NMR bands for all synthesised compounds

Compounds	¹ H-NMR, δ (ppm)					
	N-H ₂	N-H	CH ₂	CH ₃	Aromatic	SH
SB2OME (thione-thiol, ratio of 5:3)	-	s, H (11.50), s, H (11.19) (thiol), s, H (10.50)	s, 2H (4.45) (thione), s, 2H (4.55) (thiol)	s, 3H (3.83) (thione), s, 3H (3.95) (thiol)	m, (7.87-7.04 J = 7.5 Hz, 9H)	s, H (4.50) (thiol)
SB3OME (thiol only)	-	s, H (10.53)	s, 2H (4.51)	s, 3H (3.81)	m, (7.56-7.16, J = 7.5 Hz, 9H)	s, H (3.83) (thiol)
SB4OME (thione only)	s, 2H (9.71)	-	s, 2H (4.50)	s, 3H (3.79)	m, (8.13-6.82, J = 7.5 Hz, 9H)	-
SB3NO (thione-thiol, ratio of 1:2)	-	d, H (4.37, 4.40), d, H (4.50 (thiol), 4.51)	s, 2H (4.59)	-	m, (9.53-7.22, J = 7.0 Hz, 8H)	s, H (3.74) (thiol)
Compounds	¹³ C-NMR, δ (ppm)					
	C=S	C=O	O-CH ₃	S-CH ₂	C=N	Aromatic

SB2OME (thione-thiol, ratio of 5:3)	189.64	169.21	56.23	37.83	165.16 (thiol)	157.62-112.58
SB3OME (thiol only)	-	166.03	55.81	38.18	159.76 (thiol)	136.77-114.27
SB4OME (thione only)	195.80	161.69	56.05	37.86	-	150.26-110.65
SB3NO (thione-thiol, ratio of 1:2)	200.17	165.81	-	37.53 (thiol), 36.99	154.66 (thiol)	147.66-126.37
s-singlet; d-doublet; m-multiplet						

Appendix 12 Raw data from molar conductivity

Complexes	Molar conductivity (10^{-5} M)			
	1 st	2 nd	3 rd	Average
Cu(SB2OME) ₂	7.40	5.02	2.04	4.82
Zn(SB2OME) ₂	9.67	8.49	7.97	8.71
Co(SB2OME) ₂	6.94	7.12	9.61	7.89
Ni(SB2OME) ₂	5.03	5.42	7.37	5.94
Cu(SB3OME) ₂	1.01	2.32	5.10	2.81
Zn(SB3OME) ₂	3.74	2.93	0.68	2.45
Co(SB3OME) ₂	8.51	7.43	9.17	8.37
Ni(SB3OME) ₂	20.91	18.56	25.03	21.50
Cu(SB4OME) ₂	29.73	32.05	32.42	31.40
Zn(SB4OME) ₂	29.81	32.41	38.58	33.60
Co(SB4OME) ₂	33.12	30.21	33.87	32.40
Ni(SB4OME) ₂	39.65	38.81	34.94	37.8
Cu(SB3NO) ₂	14.23	16.29	16.01	15.51
Zn(SB3NO) ₂	19.30	18.26	21.90	19.83
Co(SB3NO) ₂	19.72	19.02	16.76	18.50
Ni(SB3NO) ₂	11.20	12.82	12.85	12.29

Appendix 13 Raw data from magnetic susceptibility

Complexes	Mass magnetic susceptibility (X_m)				μ_{eff} (B.M)
	1 st	2 nd	3 rd	Average	
Cu(SB2OME) ₂	0.203	0.216	0.244	0.221	1.960
Zn(SB2OME) ₂	0	0	0	0	Diamagnetic
Co(SB2OME) ₂	1.162	1.002	1.001	1.055	4.267
Ni(SB2OME) ₂	0.549	0.674	0.658	0.627	3.288
Cu(SB3OME) ₂	0.275	0.210	0.136	0.207	1.897
Zn(SB3OME) ₂	0	0	0	0	Diamagnetic
Co(SB3OME) ₂	1.539	1.502	1.210	1.417	4.944
Ni(SB3OME) ₂	0.278	0.321	0.295	0.298	2.270
Cu(SB4OME) ₂	0.153	0.159	0.156	0.156	1.648
Zn(SB4OME) ₂	0	0	0	0	Diamagnetic
Co(SB4OME) ₂	1.439	1.328	1.139	1.302	4.740
Ni(SB4OME) ₂	0.302	0.299	0.386	0.329	2.385
Cu(SB3NO) ₂	0.109	0.121	0.127	0.119	1.536
Zn(SB3NO) ₂	0	0	0	0	Diamagnetic
Co(SB3NO) ₂	1.001	1.143	1.009	1.051	4.564
Ni(SB3NO) ₂	0.772	0.754	0.763	0.763	3.874

Appendix 14 Raw data from TGA analysis

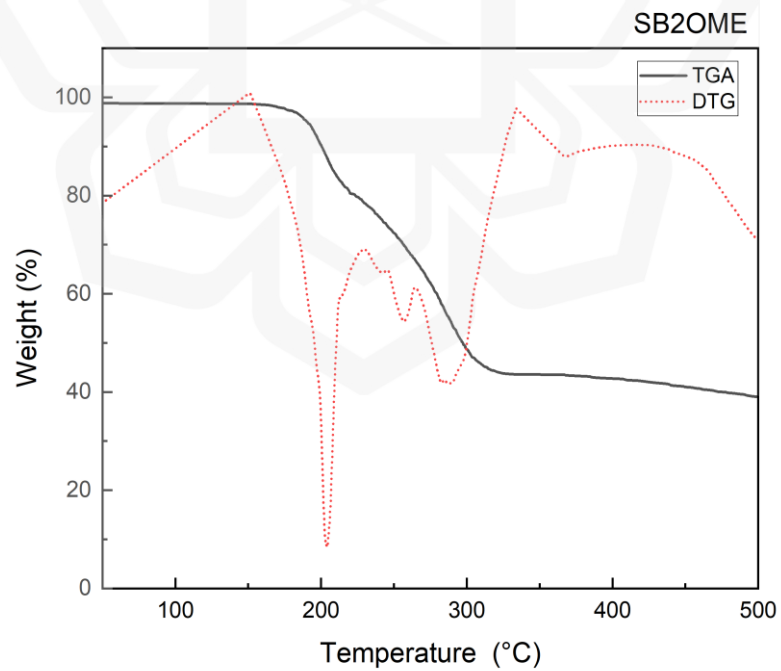
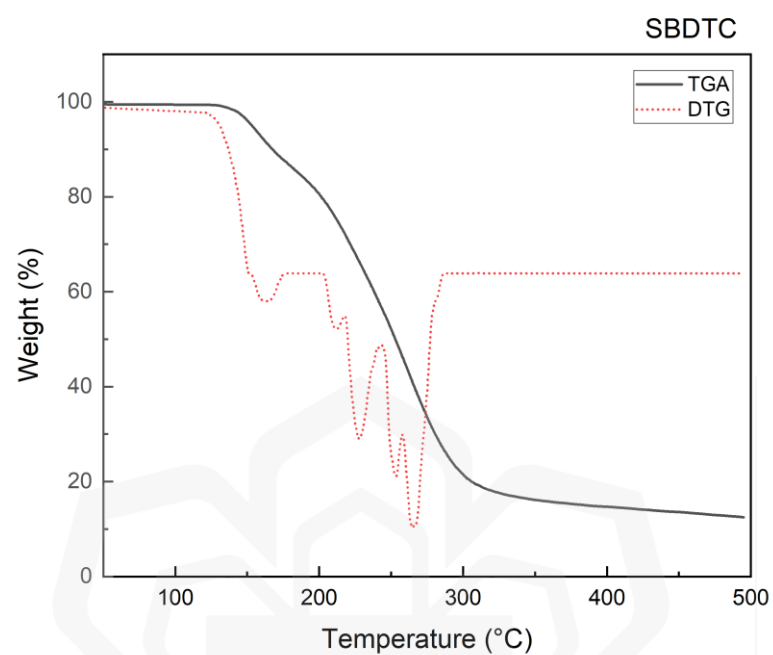
Compounds	Total Weight loss (%)	Temperature range (°C)	DTG (°C)	Mass loss Found (Calc.) (%)	Fragmentation loss
SBDTC	87.084	122-182	162.75	14.40 (15.65)	N ₂ H ₄
		182-314	265.77	66.95 (68.18)	C ₆ H ₅
		314-500	-	5.73 (6.06)	C
SB2OME	61.003	151-222	204.26	19.74 (23.19)	C ₆ H ₅
		222-327	287.10	36.53 (36.14)	C ₉ H ₇ N ₂
		327-500	367.95	4.733 (4.81)	O
SB3OME	89.454	125-239	171.58	26.56 (27.4)	C ₇ H ₇
		239-317	281.41	58.83 (58.43)	C ₈ H ₅ N ₂
		317-500	-	4.064 (4.81)	O
SB4OME	93.327	155-208	194.06	14.56 (15.65)	N ₂ H ₄
		208-347	292.05	73.19 (73.19)	C ₁₄ H ₁₁ S ₂
		347-500	426.75	5.577 (8.43)	O
Cu(SB2OME) ₂	70.526	100-255	217.98	32.61 (33.88)	(C ₇ H ₇) ₂
		255-356	265.25	11.28 (11.29)	(OCH ₃) ₂
		356-691	365.50	20.84 (20.90)	(C ₆ H ₄) ₂
		691-800	722.57	5.80 (4.75)	(N ₂ H ₄) ₂

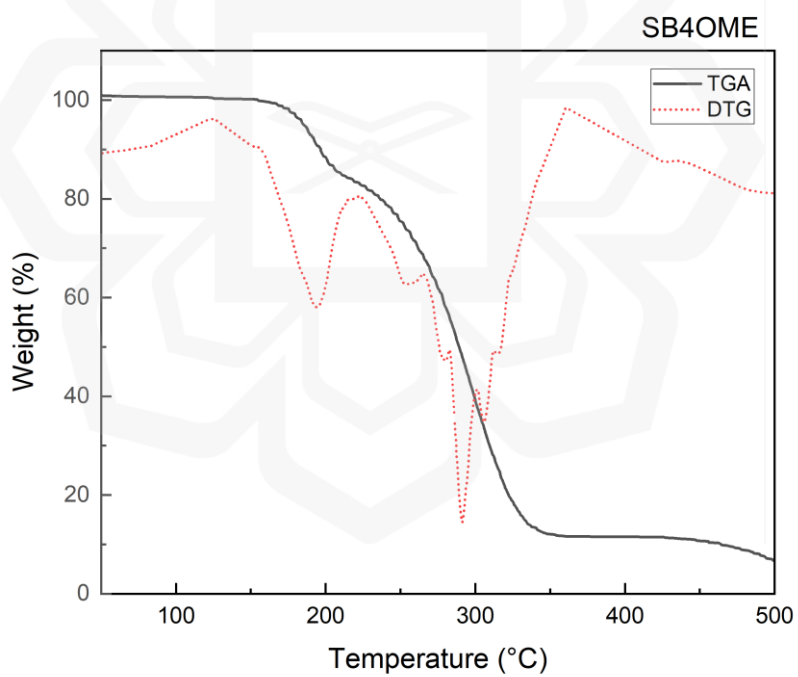
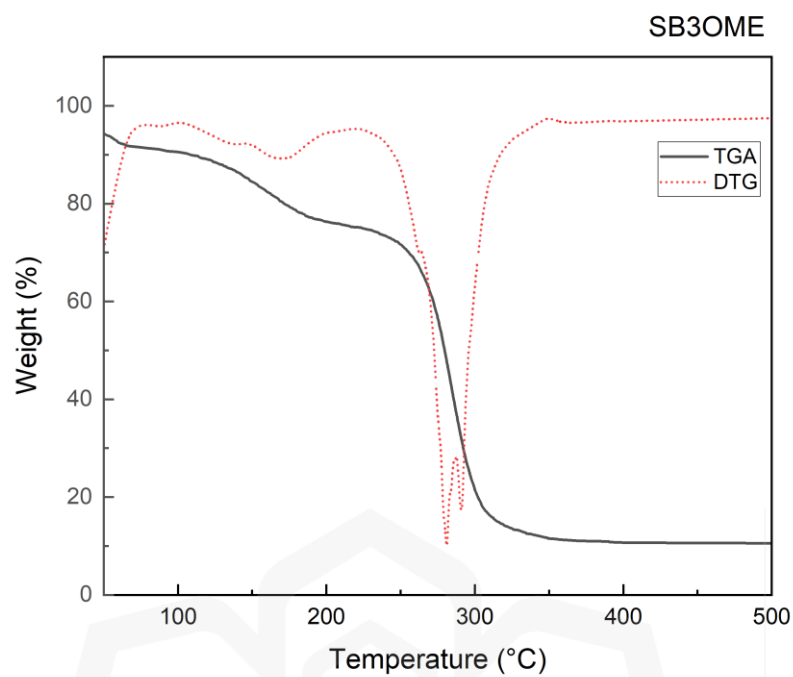
Zn(SB2OME) ₂	81.820	146-334	279.96	33.26 (33.88)	(C ₇ H ₇) ₂
		334-495	407.42	19.33 (20.9)	(C ₆ H ₄) ₂
		495-550	519.25	24.72 (24.24)	(C ₃ H ₅ N ₂ O) ₂
		550-800	-	4.51 (4.73)	(N ₂ H ₄) ₂
Co(SB2OME) ₂	89.950	146-366	215.48	44.92 (46.00)	(C ₁₃ H ₁₁) ₂
		366-493	373.66	14.14 (15.81)	(OCH ₃) ₂
		493-561	538.32	27.55 (25.24)	(CS ₂) ₂
		561-800	602.70	3.34 (4.75)	(N ₂ H ₄) ₂
Ni(SB2OME) ₂	93.575	151-320	228.55	44.50 46.38	(C ₁₃ H ₁₁) ₂
		320-424	370.34	13.42 (14.72)	(OCH ₃) ₂
		424-569	540.90	30.62 (25.30)	(CS ₂) ₂
		569-800	-	5.035 (4.76)	(N ₂ H ₄) ₂
Cu(SB3OME) ₂	74.453	142-247	207.6	26.78 (25.06)	(C ₇ H ₇) ₂
		247-327	299.03	24.70 (24.24)	(C ₆ H ₄) ₂
		327-566	500.92	10.51 (11.29)	(N ₂ H ₄) ₂
		566-800	610.08	12.46 (13.22)	(CS ₂) ₂
Zn(SB3OME) ₂	74.458	151-309	284.07	30.68 (33.88)	(C ₇ H ₇ O) ₂
		309-549	393.45	37.96 (39.94)	(C ₇ H ₆ N ₂) ₂
		549-800	-	5.82 (4.46)	(N ₂ H ₄) ₂
Co(SB3OME) ₂	64.044	141-450	292.04	56.10 (58.52)	(C ₁₄ H ₁₁ NO ₂) ₂

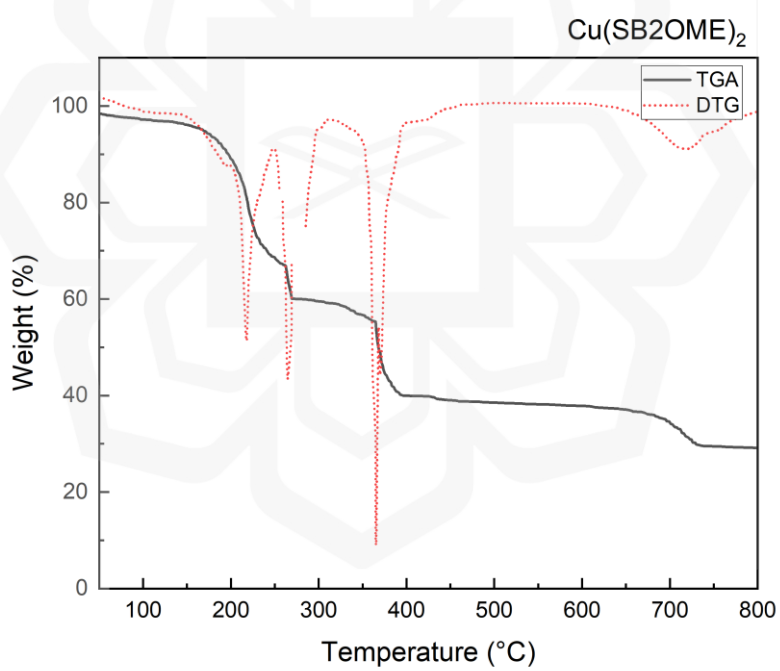
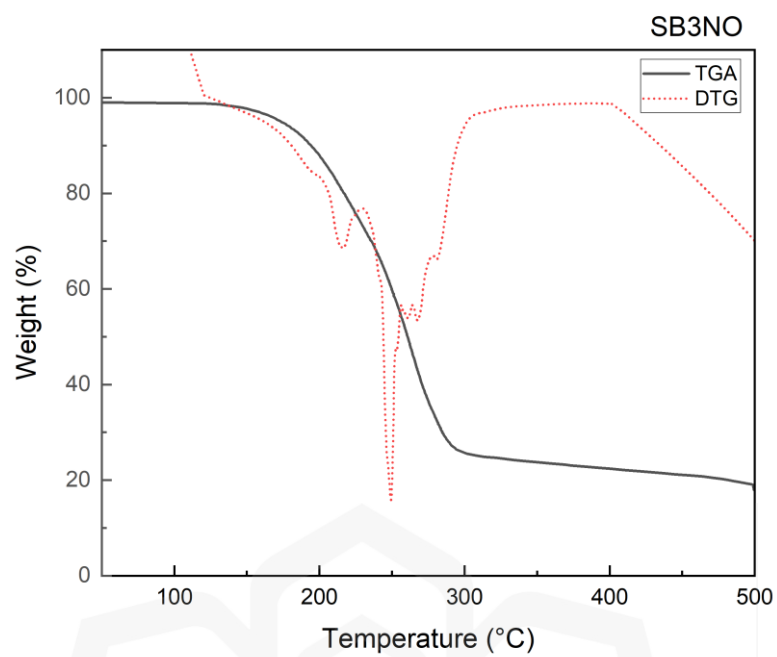
Ni(SB3OME) ₂	73.679	450-800	627.31	7.944 (8.04)	(N ₂ H ₄) ₂
		130-302	263.25	29.72 (34.16)	(C ₇ H ₇ O) ₂
		302-510	355.91	21.88 (21.11)	(C ₆ H ₄) ₂
		510-628	532.46	16.94 (17.68)	(CS ₂) ₂
		628-800	662.30	5.139 (4.44)	(N ₂ H ₄) ₂
Cu(SB4OME) ₂	78.215	145-303	217.65	41.78 (41.87)	(C ₁₂ H ₁₀) ₂
		303-349	341.03	31.05 (31.68)	(C ₄ H ₇ N ₂ S ₂ O) ₂
		349-661	-	0.61	-
		661-800	-	4.78 (4.4)	(N ₂ H ₄) ₂
		139-314	293.70	37.98 (36.8)	(C ₇ H ₇) ₂
Zn(SB4OME) ₂	85.485	314-519	341.81	27.14 (29.47)	(C ₇ H ₇ O) ₂
		519-584	541.58	17.02 (17.63)	(CS ₂) ₂
		584-800	-	3.345 (4.35)	(N ₂ H ₄) ₂
		140-477	262.94	55.37 (57.81)	(C ₁₄ H ₁₃ O) ₂
		477-522	506.88	20.79 (17.70)	(CS ₂) ₂
Co(SB4OME) ₂	85.468	522-778	556.07	8.67 (8.85)	(N ₂ H ₄) ₂
		778-800	-	0.638	-
		148-379	266.78	41.13 (40.11)	(C ₈ H ₉ S ₂) ₂
Ni(SB4OME) ₂	76.937	379-495	447.05	10.60 (8.57)	(OCH ₃) ₂
		495-546	501.67	20.15 21.02	(C ₆ H ₄) ₂

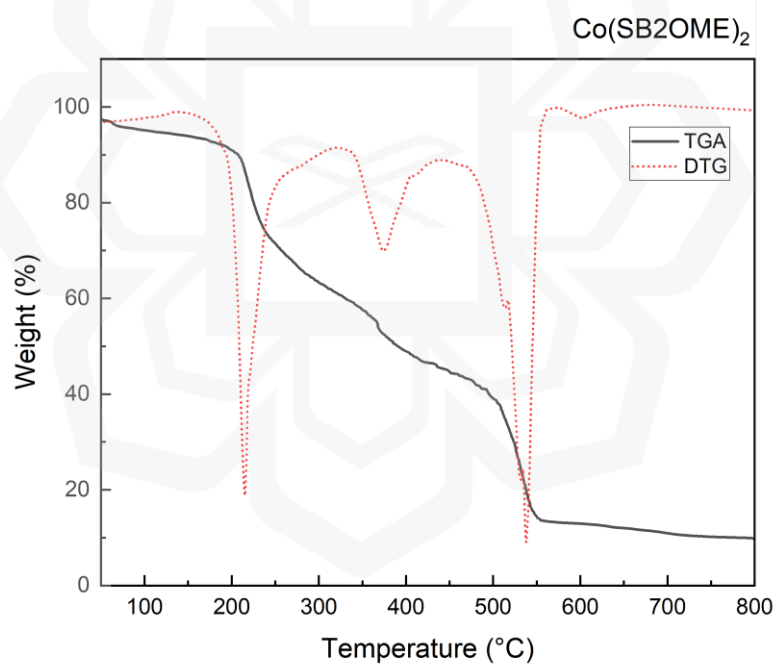
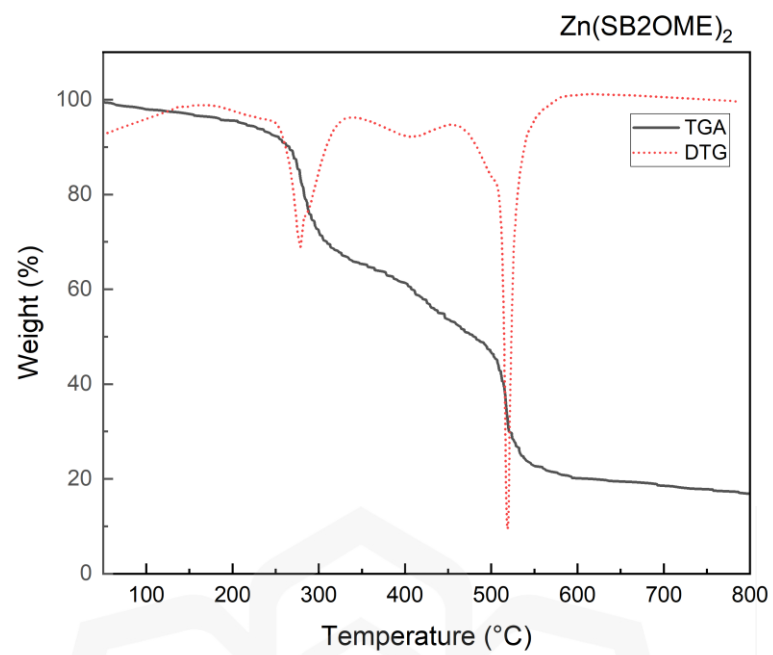
SB3NO	81.774	546-800	682.94	5.057 (4.42)	(N ₂ H ₄) ₂
		130-226	214.97	26.04 (23.88)	C ₇ H ₇
		226-295	249.37	47.70 (46.45)	C ₆ H ₄ NO ₂ Cl
		295-500	-	8.034 (9.18)	O
Cu(SB3NO) ₂	70.921	127-372	214.77	52.50 (53.22)	(C ₁₂ H ₁₀ Cl) ₂
		372-564	524.68	13.72 (16.28)	(NO ₂) ₂
		564-735	699.03	3.55 (3.88)	(N ₂ H ₄) ₂
		735-800	-	1.15	-
Zn(SB3NO) ₂	38.442	132-298	212.85	22.39 (22.08)	(C ₇ H ₇) ₂
		298-467	385.23	10.17 (9.95)	(NO ₂) ₂
		467-615	554.04	5.60 (6.79)	(Cl) ₂
		615-800	-	0.28	-
Co(SB3NO) ₂	45.93	136-323	218.20	22.71 (22.22)	(C ₇ H ₇) ₂
		323-420	364.82	10.17 (10.01)	(NO ₂) ₂
		420-518	477.32	5.60 (6.83)	(N ₂ H ₄) ₂
		518-800	-	7.45 (8.54)	(Cl) ₂
Ni(SB3NO) ₂	42.357	110-273	232.7	20.09 (22.24)	(C ₇ H ₇) ₂
		273-426	332.01	17.77 (16.85)	(NO ₂ Cl) ₂
		426-733	-	2.95	-
		733-800	-	1.547	-

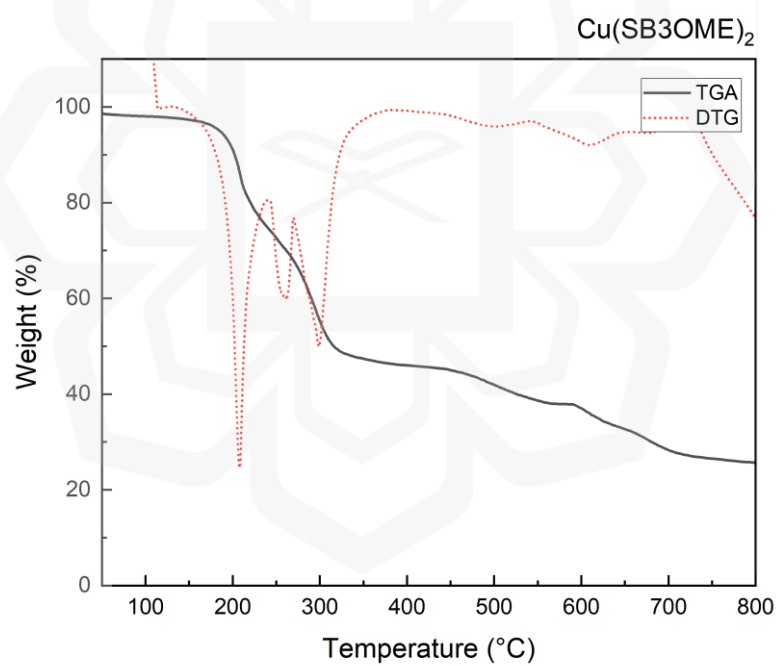
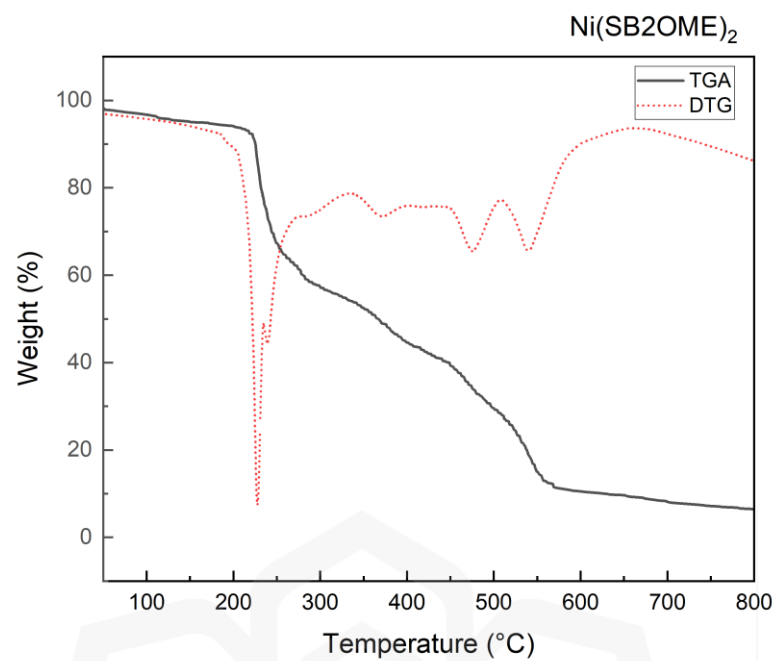
Appendix 15 TGA and DTG graph of all synthesised compounds

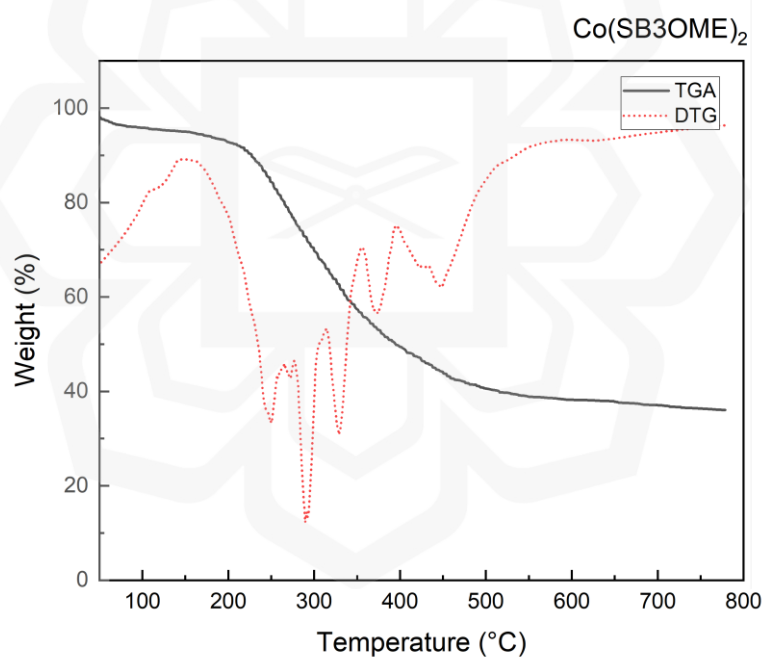
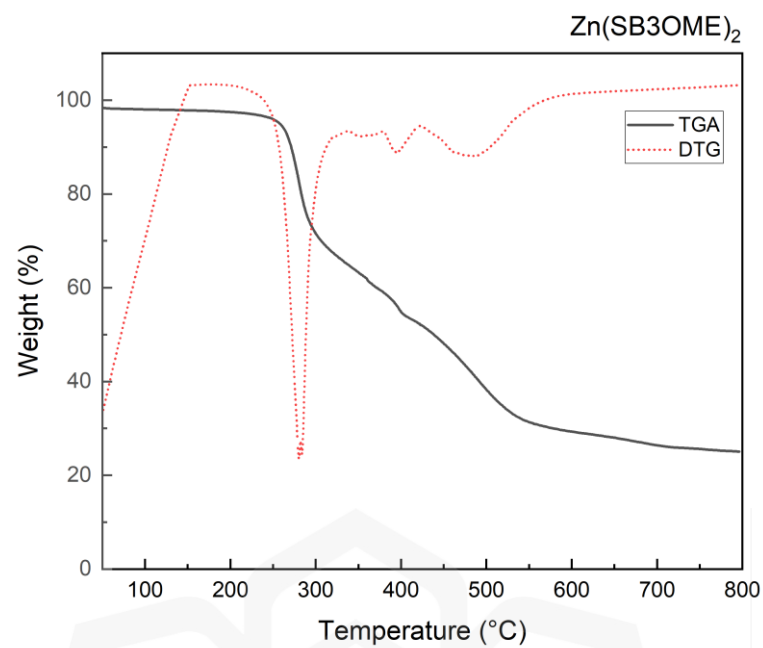


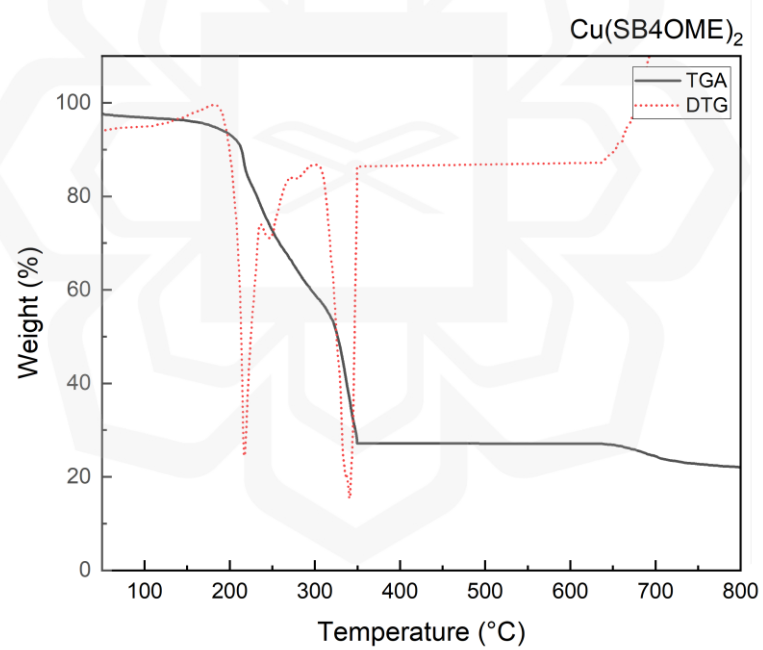
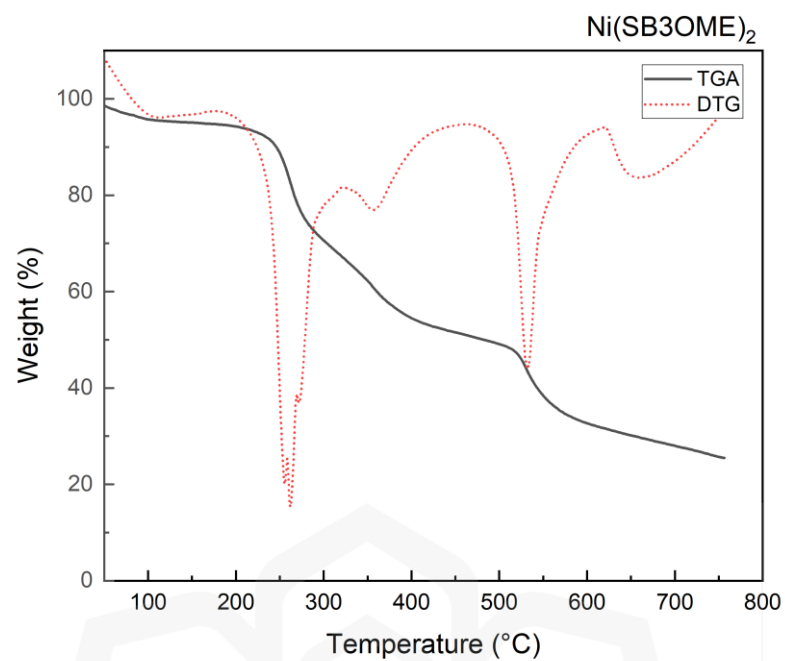


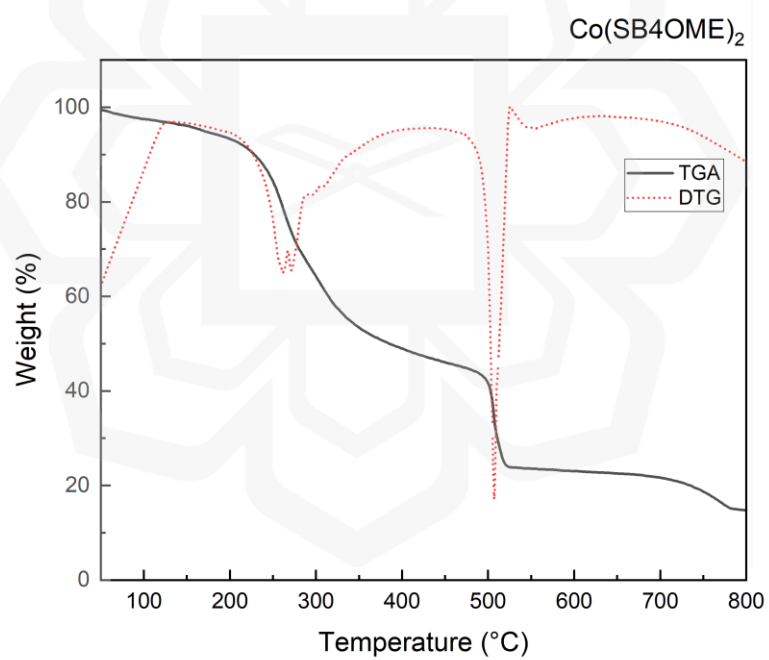
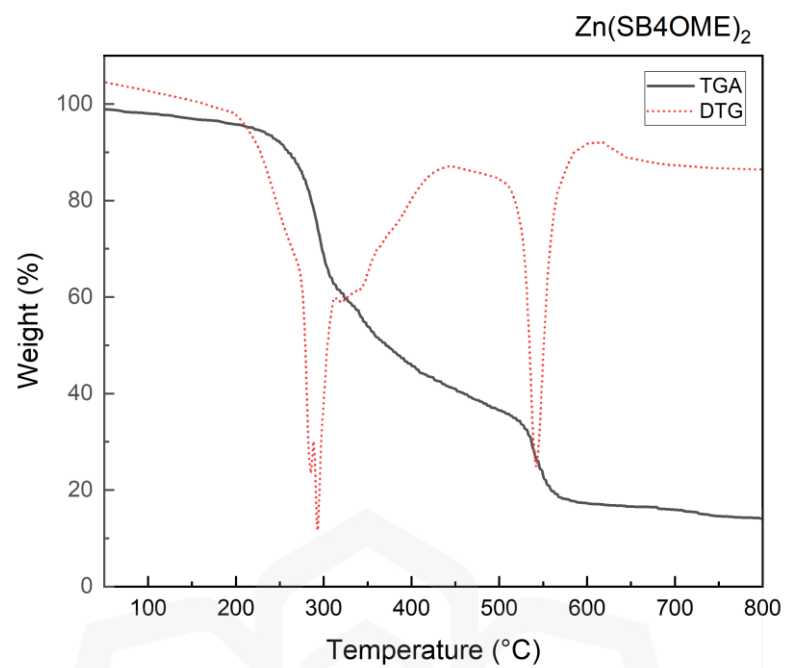


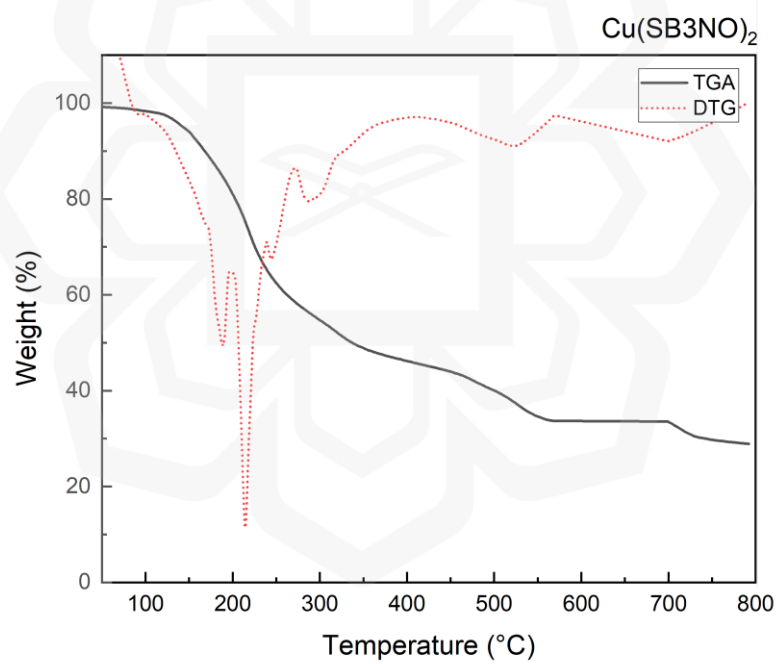
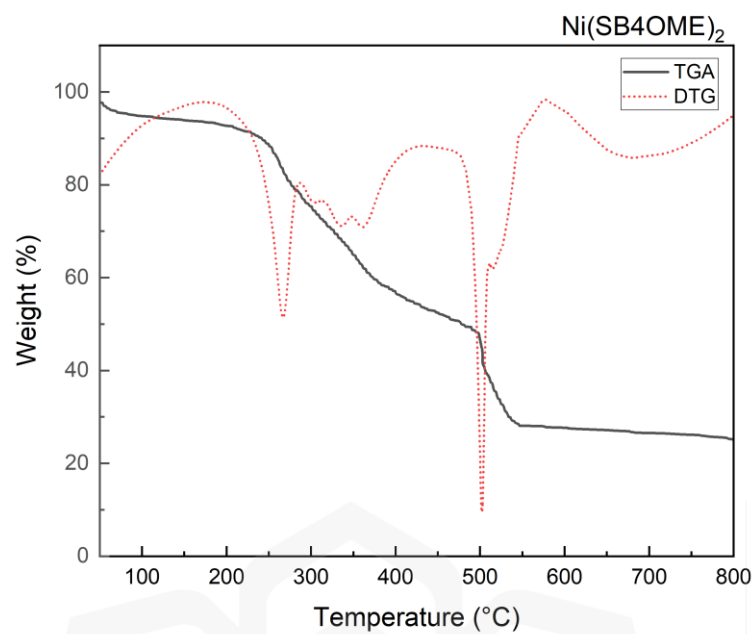


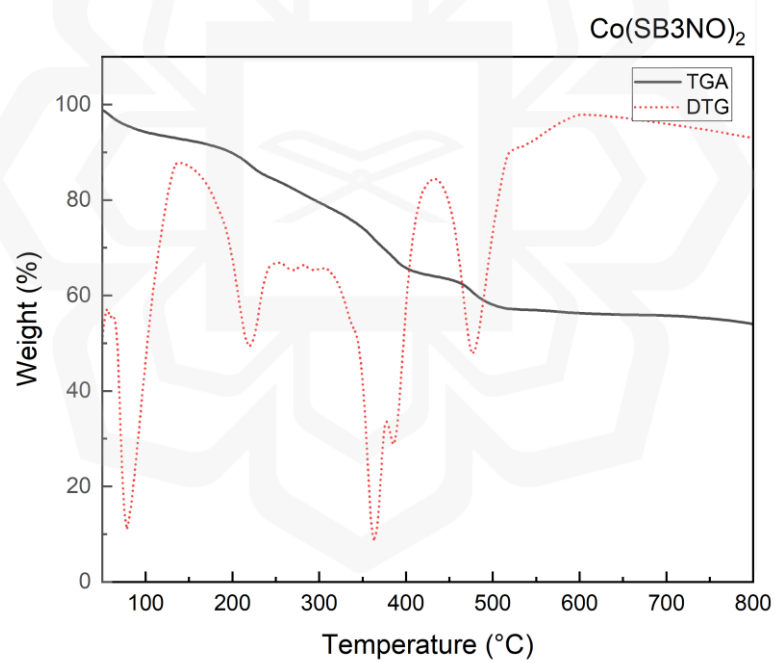
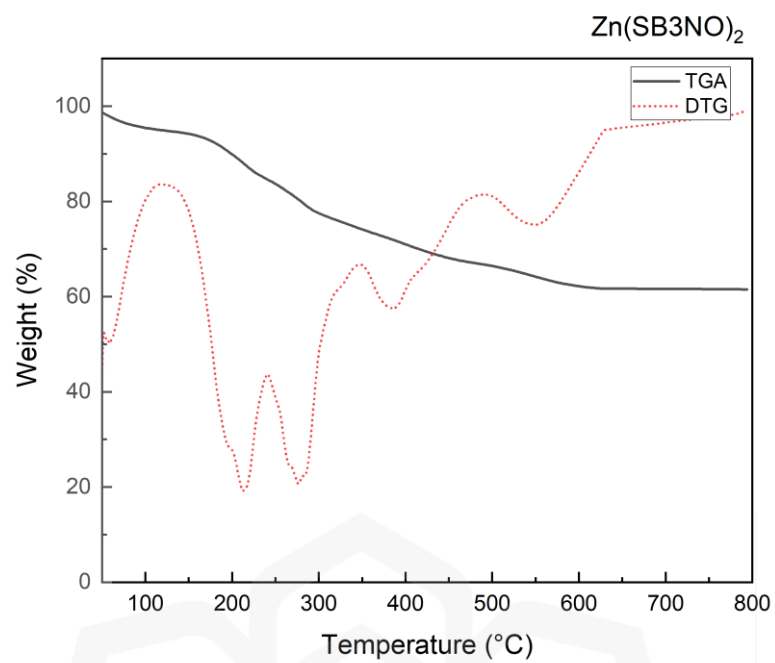


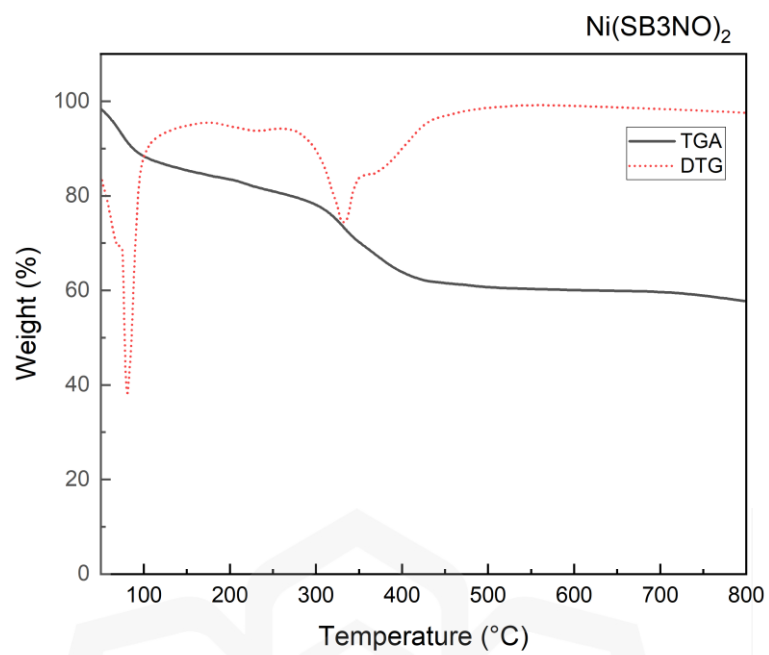




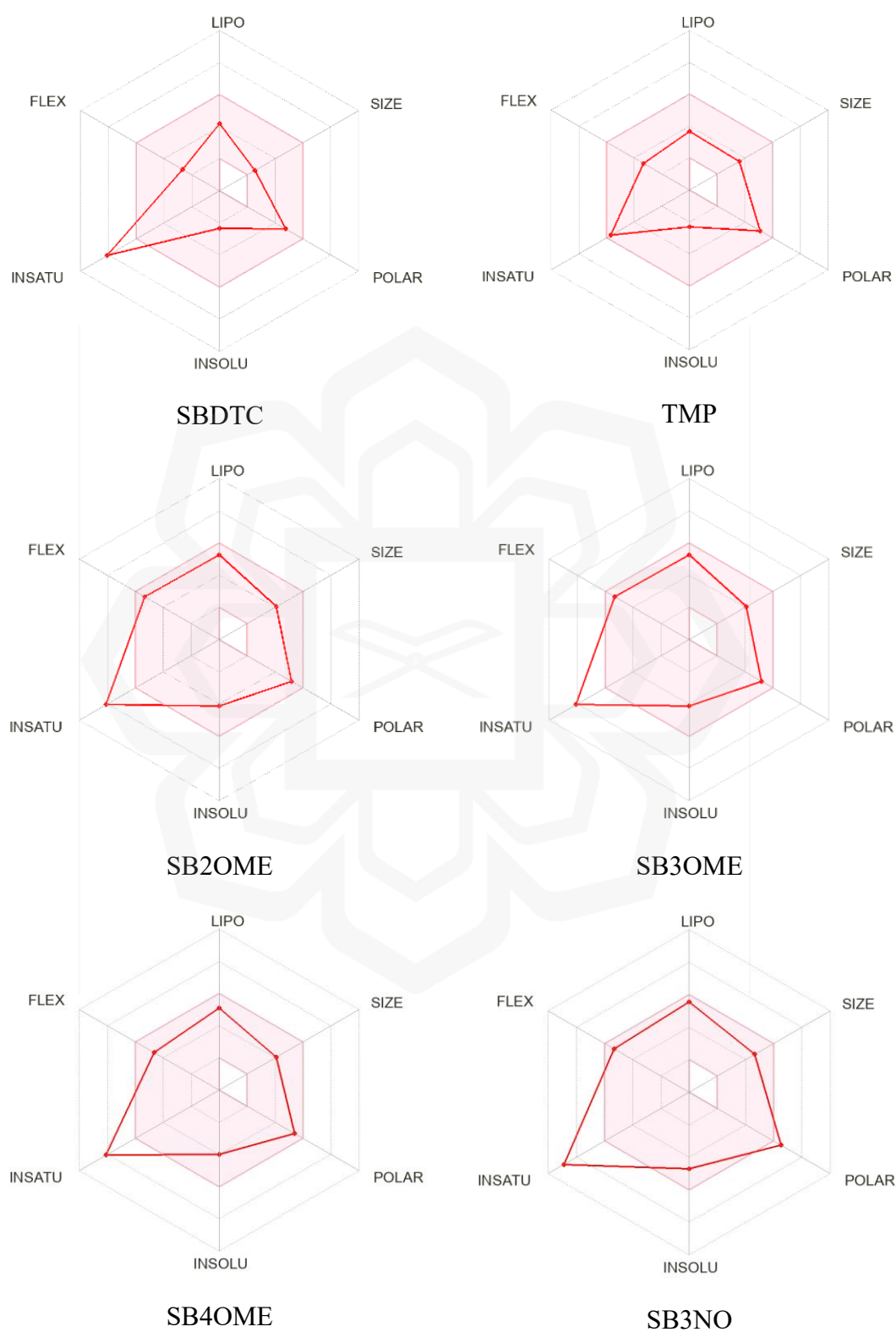


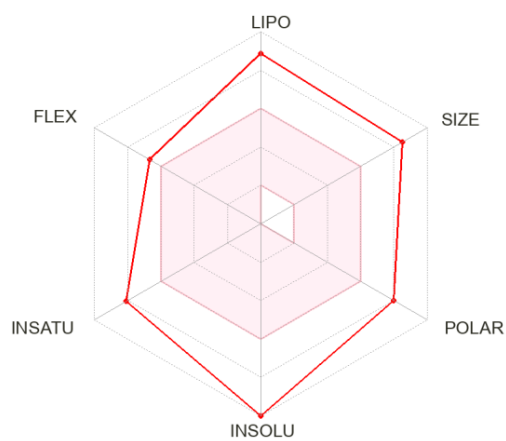




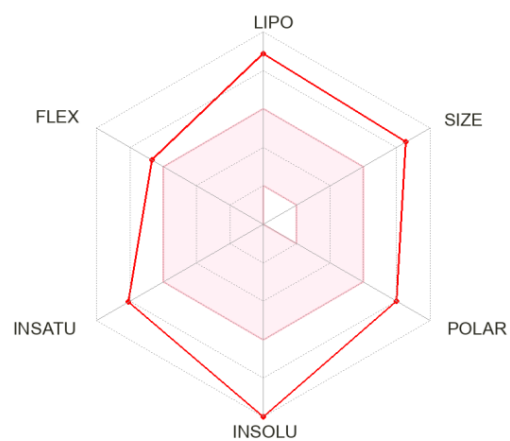


Appendix 16 Bioavaibility Radar from SwissADME

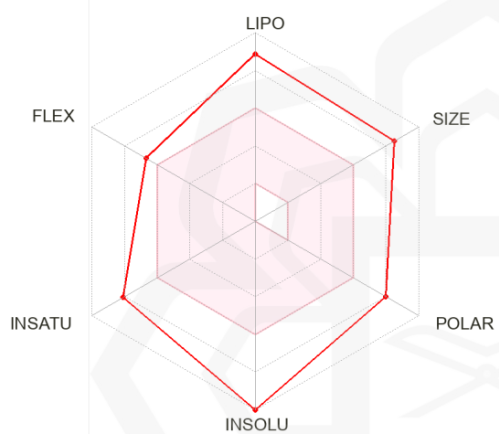




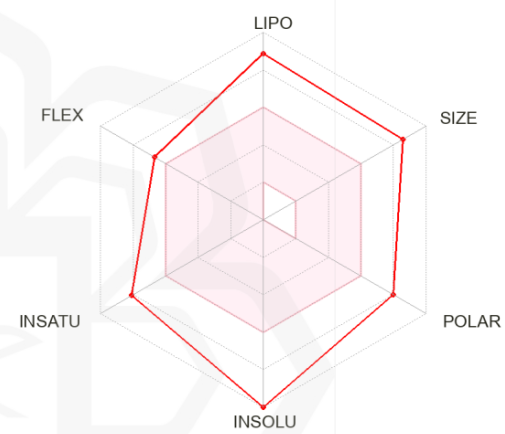
$\text{Cu}(\text{SB2OME})_2$



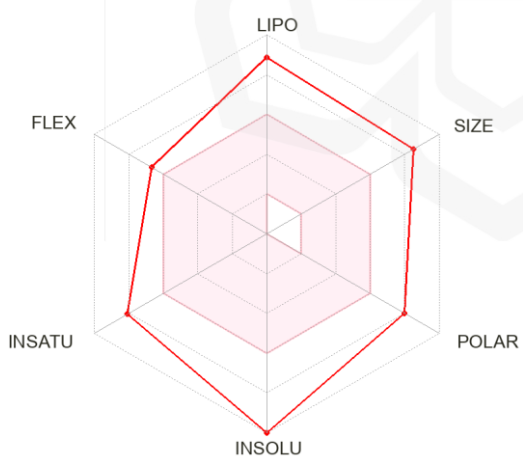
$\text{Zn}(\text{SB2OME})_2$



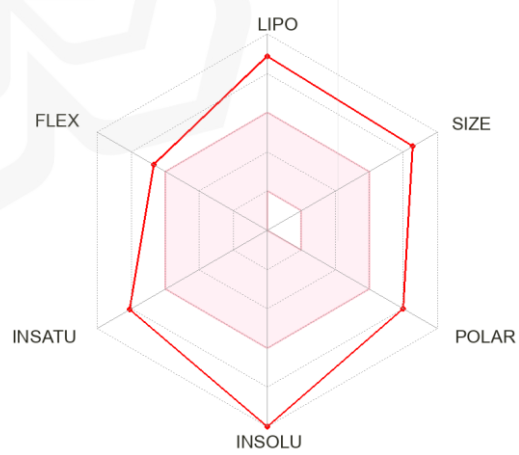
$\text{Co}(\text{SB2OME})_2$



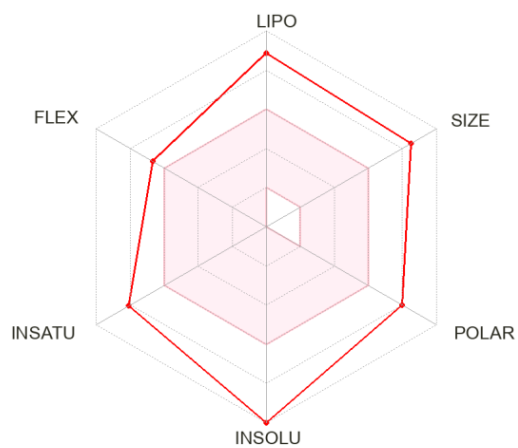
$\text{Ni}(\text{SB2OME})_2$



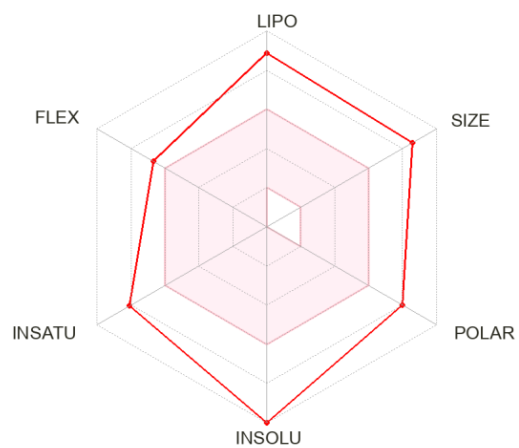
$\text{Cu}(\text{SB3OME})_2$



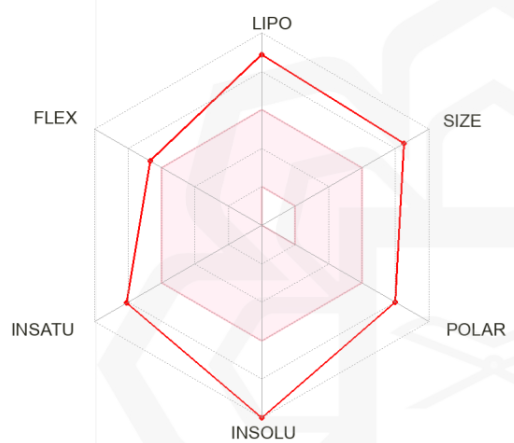
$\text{Zn}(\text{SB3OME})_2$



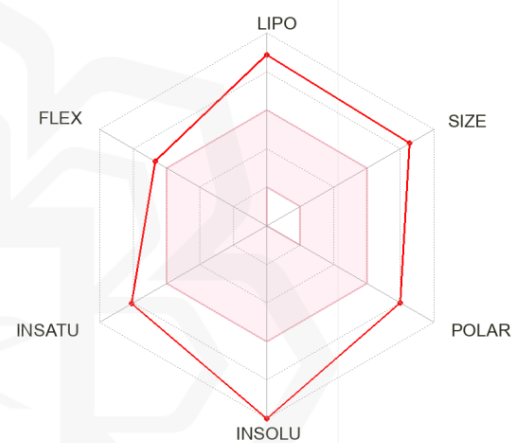
$\text{Co}(\text{SB3OME})_2$



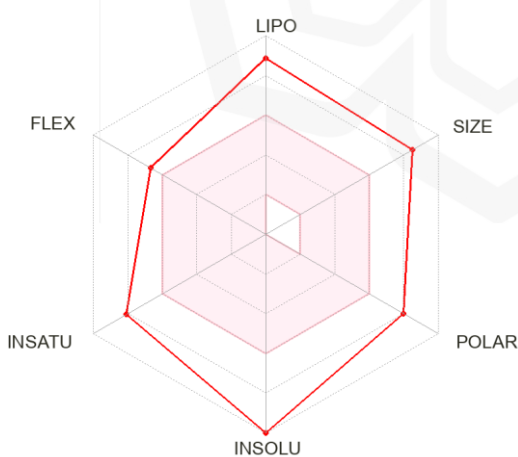
$\text{Ni}(\text{SB3OME})_2$



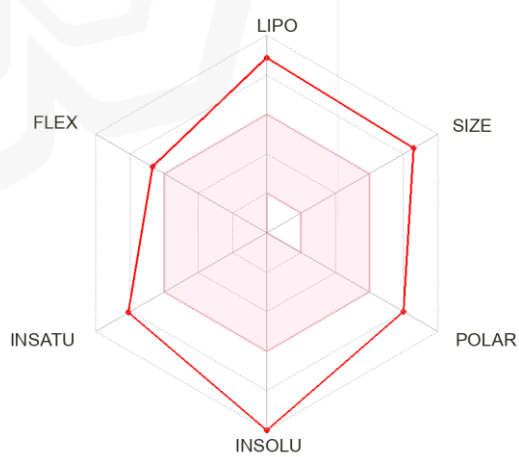
$\text{Cu}(\text{SB4OME})_2$



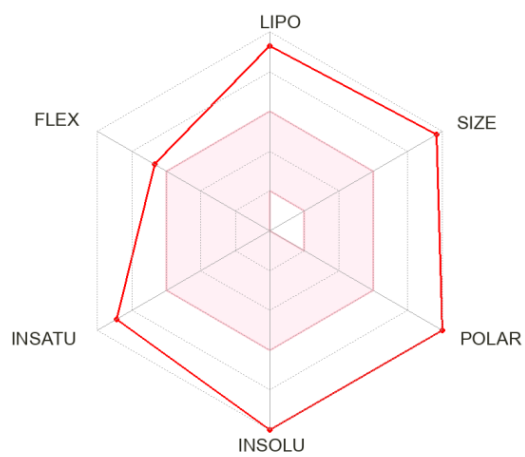
$\text{Zn}(\text{SB4OME})_2$



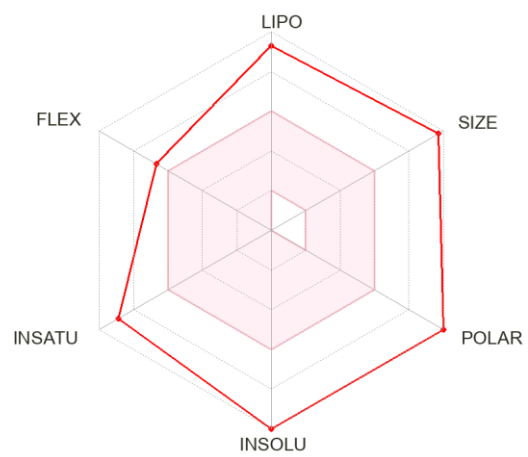
$\text{Co}(\text{SB4OME})_2$



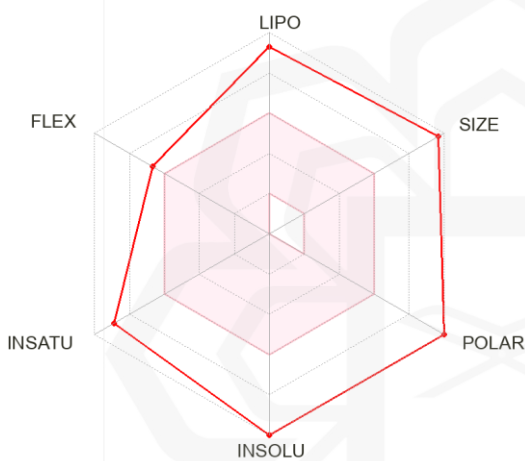
$\text{Ni}(\text{SB4OME})_2$



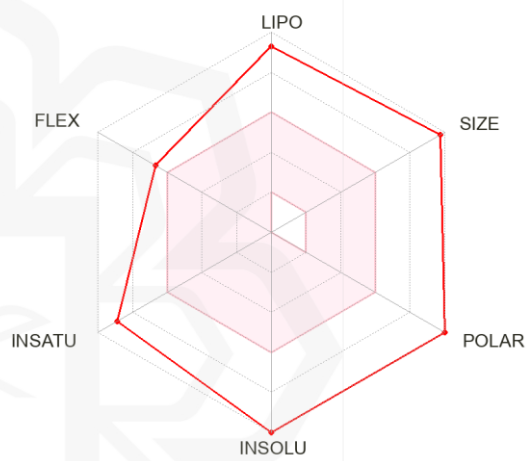
$\text{Cu}(\text{SB3NO})_2$



$\text{Zn}(\text{SB3NO})_2$



$\text{Co}(\text{SB3NO})_2$



$\text{Ni}(\text{SB3NO})_2$

Appendix 17 MIC assay against gram-positive bacteria

Compounds	Minimum inhibition concentration (mg/mL)							
	<i>Staphylococcus aureus</i> (ATCC				<i>Bacillus cereus</i> (ATCC			
	25923)				11778)			
	1 st	2 nd	3 rd	Modal	1 st	2 nd	3 rd	Modal
SBDTC	0.875	0.875	0.437	0.875	0.437	0.437	0.437	0.437
SB2OME	1.750	1.750	1.750	1.750	0.875	0.437	0.875	0.875
Cu(SB2OME) ₂	1.750	0.875	0.875	0.875	0.875	0.875	0.875	0.875
Zn(SB2OME) ₂	1.750	1.750	1.750	1.750	0.875	1.750	0.875	1.750
Co(SB2OME) ₂	0.875	1.750	1.750	1.750	0.875	0.875	0.875	0.875
Ni(SB2OME) ₂	0.875	1.750	1.750	1.750	0.875	0.875	0.875	0.875
SB3OME	0.875	0.875	1.750	0.875	0.875	0.875	0.875	0.875
Cu(SB3OME) ₂	0.437	0.437	0.437	0.437	0.875	0.875	0.875	0.875
Zn(SB3OME) ₂	0.437	1.750	0.437	0.437	0.875	1.750	1.750	1.750
Co(SB3OME) ₂	0.437	0.875	0.437	0.437	1.750	1.750	1.750	1.750
Ni(SB3OME) ₂	0.437	0.875	0.437	0.437	1.750	1.750	1.750	1.750
SB4OME	0.875	0.875	0.875	0.875	0.437	0.875	0.875	0.875
Cu(SB4OME) ₂	0.437	0.437	0.437	0.437	0.875	0.875	0.437	0.875
Zn(SB4OME) ₂	0.437	0.437	0.875	0.437	0.875	0.875	0.875	0.875
Co(SB4OME) ₂	0.437	0.875	0.437	0.437	0.875	0.875	0.875	0.875
Ni(SB4OME) ₂	0.437	0.437	0.437	0.437	0.437	0.875	0.875	0.875
SB3NO	-	3.500	3.500	3.500	1.750	3.500	1.750	1.750
Cu(SB3NO) ₂	3.500	1.750	1.750	1.750	1.750	1.750	1.750	1.750
Zn(SB3NO) ₂	1.750	0.875	0.875	0.875	0.875	1.750	0.875	0.875
Co(SB3NO) ₂	1.750	0.875	0.875	0.875	0.875	1.750	0.875	0.875
Ni(SB3NO) ₂	1.750	0.875	0.875	0.875	0.875	1.750	1.750	1.750
Trimethoprim	0.437	0.437	0.437	0.437	0.437	0.437	0.437	0.437
Novobiocin	0.218	0.437	0.218	0.218	0.218	0.437	0.437	0.437
Ampicillin	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109
Gentamycin	0.109	0.054	0.054	0.054	0.027	0.027	0.027	0.027

Appendix 18 MIC assay against gram-negative bacteria

Compounds	Minimum inhibition concentration (mg/mL)							
	<i>Escherichia coli</i> (ATCC 25922)				<i>Pseudomonas aeruginosa</i> (ATCC 27853)			
	1 st	2 nd	3 rd	Modal	1 st	2 nd	3 rd	Modal
SBDTC	0.437	0.437	0.875	0.437	0.875	0.875	0.875	0.875
SB2OME	1.750	1.750	1.750	1.750	0.875	0.437	0.875	0.875
Cu(SB2OME) ₂	0.875	0.875	0.875	0.875	0.875	0.437	0.437	0.437
Zn(SB2OME) ₂	0.875	1.750	1.750	1.750	0.875	0.875	0.875	0.875
Co(SB2OME) ₂	0.875	0.875	0.875	0.875	0.875	0.875	0.875	0.875
Ni(SB2OME) ₂	0.875	1.750	1.750	1.750	0.875	0.437	0.875	0.875
SB3OME	0.437	0.875	0.875	0.875	0.437	0.437	0.437	0.437
Cu(SB3OME) ₂	0.875	0.875	0.875	0.875	0.437	0.437	0.437	0.437
Zn(SB3OME) ₂	1.750	0.875	0.875	0.875	0.437	0.437	0.437	0.437
Co(SB3OME) ₂	1.750	0.875	0.875	0.875	0.875	0.875	0.875	0.875
Ni(SB3OME) ₂	0.875	0.437	0.875	0.875	0.875	0.875	0.875	0.875
SB4OME	0.875	0.875	0.875	0.875	1.750	1.750	1.750	1.750
Cu(SB4OME) ₂	0.437	1.750	0.437	0.437	0.875	0.875	0.875	0.875
Zn(SB4OME) ₂	0.437	0.875	0.437	0.437	1.750	1.750	1.750	1.750
Co(SB4OME) ₂	0.437	0.875	0.437	0.437	0.875	0.875	0.875	0.875
Ni(SB4OME) ₂	0.437	0.437	0.437	0.437	0.875	0.875	0.875	0.875
SB3NO	0.875	1.750	1.750	1.750	1.750	1.750	0.875	1.750
Cu(SB3NO) ₂	1.750	1.750	0.875	1.750	1.750	1.750	1.750	1.750
Zn(SB3NO) ₂	0.875	1.750	0.875	0.875	0.875	0.437	0.437	0.437
Co(SB3NO) ₂	1.750	1.750	1.750	1.750	1.750	1.750	1.750	1.750
Ni(SB3NO) ₂	0.875	1.750	1.750	1.750	0.875	1.750	1.750	1.750
Trimethoprim	1.750	0.875	1.750	1.750	1.750	1.750	3.500	1.750
Novobiocin	0.437	0.437	0.437	0.437	0.109	0.109	0.218	0.109
Ampicilin	0.218	0.218	0.437	0.218	0.109	0.109	0.109	0.109
Gentamycin	0.027	0.027	0.027	0.027	0.013	0.013	0.013	0.013

Appendix 19 Grid parameter file (GPF) from molecular docking

```
parameter_file AD4_parameters.dat
npts 50 50 50          # num.grid points in xyz
gridfld 1jijQT.maps.fld # grid_data_file
spacing 0.375          # spacing(A)
receptor_types A C HD N OA SA # receptor atom types
ligand_types A C NA OA HD SA Cu # ligand atom types
receptor 1jijQT.pdbqt # macromolecule
gridcenter -7.31 10.22 84.985 # xyz-coordinates or auto
smooth 0.5            # store minimum energy w/in rad(A)
map 1jijQT.A.map      # atom-specific affinity map
map 1jijQT.C.map      # atom-specific affinity map
map 1jijQT.NA.map     # atom-specific affinity map
map 1jijQT.OA.map     # atom-specific affinity map
map 1jijQT.HD.map     # atom-specific affinity map
map 1jijQT.SA.map     # atom-specific affinity map
map 1jijQT.Cu.map     # atom-specific affinity map
elecmap 1jijQT.e.map  # electrostatic potential map
dsolvmap 1jijQT.d.map # desolvation potential map
dielectric -0.1465    # <0, AD4 distance-dep.diel;>0, constant
```

Appendix 20 Docking parameter file (DPF) from molecular docking

```
parameter_file AD4_parameters.dat
autodock_parameter_version 4.2 # used by autodock to validate parameter set
outlev 1                       # diagnostic output level
intelec                       # calculate internal electrostatics
seed pid time                 # seeds for random generator
ligand_types A C NA OA HD SA Cu # atoms types in ligand
fld 1jijQT.maps.fld          # grid_data_file
map 1jijQT.A.map             # atom-specific affinity map
map 1jijQT.C.map             # atom-specific affinity map
map 1jijQT.NA.map            # atom-specific affinity map
map 1jijQT.OA.map            # atom-specific affinity map
map 1jijQT.HD.map            # atom-specific affinity map
map 1jijQT.SA.map            # atom-specific affinity map
map 1jijQT.Cu.map            # atom-specific affinity map
elecmap 1jijQT.e.map         # electrostatics map
desolvmap 1jijQT.d.map       # desolvation map
move cusb4a.pdbqt            # small molecule
about -0.990 2.250 5.164     # small molecule center
tran0 random                 # initial coordinates/A or random
quaternion0 random           # initial orientation
dihe0 random                 # initial dihedrals (relative) or random
torsdof 9                    # torsional degrees of freedom
rmstol 2.0                   # cluster_tolerance/A
extnrg 1000.0                # external grid energy
e0max 0.0 10000              # max initial energy; max number of retries
ga_pop_size 150              # number of individuals in population
ga_num_evals 2500000          # maximum number of energy evaluations
ga_num_generations 27000     # maximum number of generations
ga_elitism 1                  # number of top individuals to survive to next generation
ga_mutation_rate 0.02        # rate of gene mutation
ga_crossover_rate 0.8         # rate of crossover
ga_window_size 10            #
ga_cauchy_alpha 0.0          # Alpha parameter of Cauchy distribution
ga_cauchy_beta 1.0           # Beta parameter Cauchy distribution
set_ga                       # set the above parameters for GA or LGA
sw_max_its 300               # iterations of Solis & Wets local search
sw_max_succ 4                # consecutive successes before changing rho
sw_max_fail 4                # consecutive failures before changing rho
sw_rho 1.0                   # size of local search space to sample
sw_lb_rho 0.01               # lower bound on rho
ls_search_freq 0.06          # probability of performing local search on individual
set_pswl                     # set the above pseudo-Solis & Wets parameters
unbound_model bound          # state of unbound ligand
ga_run 50                    # do this many hybrid GA-LS runs
analysis                     # perform a ranked cluster analysis
```

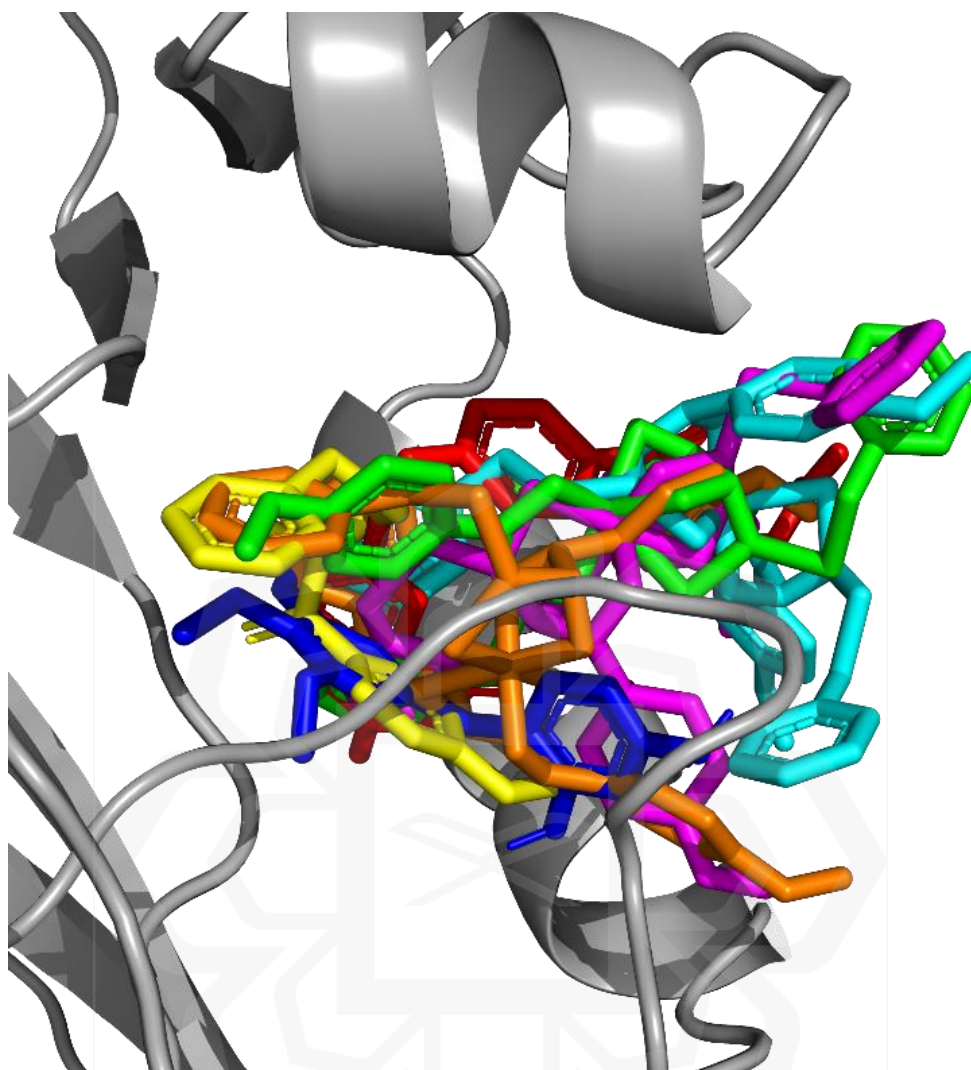

Appendix 21 Parameter of metal ions file

#	Atom	Rii	epsii	vol	solpar	Rij_hb	epsij_hb	rec_index	map_index	bond_index
#	Type						hbond			
atom_par H	H	2.00	0.020	0.0000	0.00051	0.0	0.0	0	-1	3 # Non H-bonding Hydrogen
atom_par HD	HD	2.00	0.020	0.0000	0.00051	0.0	0.0	2	-1	3 # Donor 1 H-bond Hydrogen
atom_par HS	HS	2.00	0.020	0.0000	0.00051	0.0	0.0	1	-1	3 # Donor S Spherical Hydrogen
atom_par C	C	4.00	0.150	33.5103	-0.00143	0.0	0.0	0	-1	0 # Non H-bonding Aliphatic Carbon
atom_par A	A	4.00	0.150	33.5103	-0.00052	0.0	0.0	0	-1	0 # Non H-bonding Aromatic Carbon
atom_par N	N	3.50	0.160	22.4493	-0.00162	0.0	0.0	0	-1	1 # Non H-bonding Nitrogen
atom_par NA	NA	3.50	0.160	22.4493	-0.00162	1.9	5.0	4	-1	1 # Acceptor 1 H-bond Nitrogen
atom_par NS	NS	3.50	0.160	22.4493	-0.00162	1.9	5.0	3	-1	1 # Acceptor S Spherical Nitrogen
atom_par OA	OA	3.20	0.200	17.1573	-0.00251	1.9	5.0	5	-1	2 # Acceptor 2 H-bonds Oxygen
atom_par OS	OS	3.20	0.200	17.1573	-0.00251	1.9	5.0	3	-1	2 # Acceptor S Spherical Oxygen
atom_par F	F	3.09	0.080	15.4480	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Fluorine
atom_par Mg	Mg	1.30	0.875	1.5600	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Magnesium
atom_par MG	MG	1.30	0.875	1.5600	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Magnesium
atom_par P	P	4.20	0.200	38.7924	-0.00110	0.0	0.0	0	-1	5 # Non H-bonding Phosphorus
atom_par SA	SA	4.00	0.200	33.5103	-0.00214	2.5	1.0	5	-1	6 # Acceptor 2 H-bonds Sulphur
atom_par S	S	4.00	0.200	33.5103	-0.00214	0.0	0.0	0	-1	6 # Non H-bonding Sulphur
atom_par Cl	Cl	4.09	0.276	35.8235	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Chlorine
atom_par CL	CL	4.09	0.276	35.8235	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Chlorine
atom_par Ca	Ca	1.98	0.550	2.7700	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Calcium
atom_par CA	CA	1.98	0.550	2.7700	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Calcium
atom_par Mn	Mn	1.30	0.875	2.1400	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Manganese
atom_par MN	MN	1.30	0.875	2.1400	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Manganese
atom_par Fe	Fe	1.30	0.010	1.8400	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Iron
atom_par FE	FE	1.30	0.010	1.8400	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Iron
atom_par Zn	Zn	1.48	0.550	1.7000	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Zinc
atom_par ZN	ZN	1.48	0.550	1.7000	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Zinc
atom_par Br	Br	4.33	0.389	42.5661	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Bromine
atom_par BR	BR	4.33	0.389	42.5661	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Bromine
atom_par I	I	4.72	0.550	55.0585	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding Iodine
atom_par Z	Z	4.00	0.150	33.5103	-0.00143	0.0	0.0	0	-1	0 # Non H-bonding covalent map
atom_par G	G	4.00	0.150	33.5103	-0.00143	0.0	0.0	0	-1	0 # Ring closure Glue Aliphatic Carbon # SF
atom_par GA	GA	4.00	0.150	33.5103	-0.00052	0.0	0.0	0	-1	0 # Ring closure Glue Aromatic Carbon # SF
atom_par J	J	4.00	0.150	33.5103	-0.00143	0.0	0.0	0	-1	0 # Ring closure Glue Aliphatic Carbon # SF
atom_par Q	Q	4.00	0.150	33.5103	-0.00143	0.0	0.0	0	-1	0 # Ring closure Glue Aliphatic Carbon # SF
atom_par Cu	Cu	3.50	0.005	12.0000	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding
atom_par Zn	Zn	1.48	0.550	1.7000	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding
atom_par Co	Co	2.87	0.014	12.0000	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding
atom_par Ni	Ni	2.83	0.015	12.0000	-0.00110	0.0	0.0	0	-1	4 # Non H-bonding
atom_par OA	OA	3.20	0.200	17.1573	-0.00251	1.9	5.0	3	-1	2 # Acceptor 2 H-bonds
atom_par OS	OS	3.20	0.200	17.1573	-0.00251	1.9	5.0	3	-1	2 # Acceptor S Spherical

Appendix 22 Docking log file (DLG) from molecular docking

CLUSTERING HISTOGRAM									
Clus	Lowest	Run	Mean	Num	Histogram				
-ter	Binding		Binding	in					
Rank	Energy		Energy	Clus	5	10	15	20	25 30
35									
1	-7.40	35	-6.88	11	#####				
2	-7.32	41	-6.83	9	#####				
3	-6.94	15	-6.79	2	##				
4	-6.83	3	-6.68	3	###				
5	-6.82	10	-6.54	5	#####				
6	-6.81	46	-6.76	3	###				
7	-6.75	23	-6.67	3	###				
8	-6.73	12	-6.72	2	##				
9	-6.72	38	-6.72	1	#				
10	-6.56	34	-6.39	2	##				
11	-6.51	22	-6.40	3	###				
12	-6.51	37	-6.22	3	###				
13	-6.37	36	-6.37	1	#				
14	-5.90	33	-5.90	1	#				
15	-5.47	8	-5.47	1	#				

Appendix 23 Localisation of all compounds at *E. coli* DHFR from molecular docking



Appendix 24 Binding energy of all investigated compounds from molecular docking in triplicate

PDB ID	Binding energy (kcal/mol) at PDB ID: 1RX7		
	1 st	2 nd	3 rd
FOL	-12.21	-12.72	-11.98
TMP	-6.99	-6.87	-6.34
SB4OME	-6.84	-6.92	-6.91
Cu(SB4OME) ₂	-8.72	-8.51	-7.81
Zn(SB4OME) ₂	-7.24	-7.04	-6.97
Co(SB4OME) ₂	-9.3	-9.17	-9.28
Ni(SB4OME) ₂	-10.87	-10.8	-10.92

Appendix 25 Protein coordination file (.gro) from MD simulation

```

OXIDOREDUCTASE
2489
1MET      N      1      1.163      4.234      1.064
1MET      H1      2      1.157      4.333      1.062
1MET      H2      3      1.206      4.201      0.980
1MET      H3      4      1.072      4.194      1.072
1MET      CA      5      1.244      4.193      1.179
1MET      HA      6      1.191      4.227      1.256
1MET      CB      7      1.254      4.039      1.182
1MET      HB1     8      1.315      4.008      1.109
1MET      HB2     9      1.163      3.999      1.169
1MET      CG     10     1.308      3.994      1.315
1MET      HG1     11     1.240      4.023      1.382
1MET      HG2     12     1.392      4.048      1.328
1MET      SD     13     1.339      3.817      1.321
1MET      CE     14     1.452      3.809      1.180
1MET      HE1     15     1.482      3.714      1.166
1MET      HE2     16     1.406      3.841      1.098
1MET      HE3     17     1.532      3.866      1.198
1MET      C      18     1.385      4.258      1.189
1MET      O      19     1.464      4.258      1.095
2ILE      N      20     1.419      4.316      1.303
2ILE      H      21     1.354      4.316      1.379
2ILE      CA      22     1.548      4.379      1.321
2ILE      HA      23     1.568      4.403      1.226
2ILE      CB      24     1.540      4.511      1.400
2ILE      HB      25     1.482      4.499      1.481
2ILE      CG2     26     1.682      4.544      1.437
2ILE      HG21    27     1.684      4.630      1.489
2ILE      HG22    28     1.721      4.471      1.492
2ILE      HG23    29     1.736      4.556      1.354
2ILE      CG1     30     1.479      4.627      1.320
2ILE      HG11    31     1.407      4.589      1.261
2ILE      HG12    32     1.552      4.665      1.262
2ILE      CD      33     1.418      4.742      1.402
2ILE      HD1     34     1.381      4.812      1.340
2ILE      HD2     35     1.344      4.707      1.459
2ILE      HD3     36     1.488      4.783      1.460
2ILE      C      37     1.663      4.285      1.370
2ILE      O      38     1.648      4.201      1.461
3SER      N      39     1.782      4.299      1.312
3SER      H      40     1.797      4.367      1.240
3SER      CA      41     1.889      4.212      1.355
3SER      HA      42     1.853      4.176      1.441

```

Appendix 26 Ligand coordination file (.gro) from MD simulation

```
fol_GMX.gro created by acpype (v: 2023.10.27) on Fri Dec 15 16:13:35 2023
48
1 FOL N 1 3.305 4.304 0.416
1 FOL CA 2 3.293 4.214 0.293
1 FOL C 3 3.383 4.385 0.429
1 FOL O 4 3.454 4.399 0.326
1 FOL NA2 5 3.079 3.923 1.285
1 FOL CB 6 3.369 4.089 0.323
1 FOL CG 7 3.505 4.098 0.240
1 FOL CD 8 3.609 3.982 0.223
1 FOL OE1 9 3.719 4.039 0.200
1 FOL OE2 10 3.589 3.859 0.224
1 FOL N1 11 3.026 4.134 1.245
1 FOL O1 12 3.048 4.178 0.355
1 FOL C2 13 3.108 4.027 1.244
1 FOL O2 14 3.109 4.193 0.141
1 FOL N3 15 3.242 4.040 1.189
1 FOL C4 16 3.290 4.160 1.137
1 FOL C4A 17 3.196 4.278 1.139
1 FOL O4 18 3.406 4.169 1.090
1 FOL N5 19 3.242 4.408 1.089
1 FOL C6 20 3.155 4.508 1.102
1 FOL C7 21 3.035 4.470 1.160
1 FOL C8A 22 3.074 4.258 1.193
1 FOL N8 23 2.991 4.348 1.205
1 FOL C9 24 3.190 4.639 1.056
1 FOL N10 25 3.310 4.634 0.965
1 FOL C11 26 3.374 4.452 0.563
1 FOL C12 27 3.476 4.535 0.631
1 FOL C13 28 3.447 4.594 0.771
1 FOL C14 29 3.317 4.573 0.845
1 FOL C15 30 3.218 4.498 0.789
1 FOL C16 31 3.240 4.433 0.645
1 FOL CT 32 3.139 4.187 0.262
1 FOL H01 33 3.238 4.290 0.491
1 FOL H02 34 3.336 4.259 0.204
1 FOL H03 35 3.145 3.847 1.282
1 FOL H04 36 2.987 3.909 1.325
1 FOL H05 37 3.311 4.000 0.297
1 FOL H06 38 3.390 4.083 0.430
1 FOL H07 39 3.562 4.166 0.302
1 FOL H08 40 3.475 4.124 0.138
1 FOL H09 41 2.963 4.551 1.170
1 FOL H10 42 3.214 4.701 1.142
1 FOL H11 43 3.106 4.681 1.001
1 FOL H12 44 3.394 4.680 0.998
1 FOL H13 45 3.572 4.554 0.583
1 FOL H14 46 3.524 4.654 0.818
1 FOL H15 47 3.124 4.483 0.842
1 FOL H16 48 3.161 4.373 0.601
15.12600 17.08200 23.73600
```

Appendix 27 Equilibration parameter file (NVT) from MD simulation

```

title                = Protein-ligand complex NVT equilibration
define               = -DPOSRES -DPOSRES_LIG ; position restrain the protein and ligand
; Run parameters
integrator           = md                ; leap-frog integrator
nsteps               = 50000             ; 2 * 50000 = 100 ps
dt                   = 0.002             ; 2 fs
; Output control
nstenergy            = 500               ; save energies every 1.0 ps
nstlog               = 500               ; update log file every 1.0 ps
nstxout-compressed    = 500               ; save coordinates every 1.0 ps
; Bond parameters
continuation         = no                ; first dynamics run
constraint_algorithm  = lincs             ; holonomic constraints
constraints          = h-bonds            ; bonds to H are constrained
lincs_iter           = 1                 ; accuracy of LINCS
lincs_order          = 4                 ; also related to accuracy
; Neighbor searching and vdW
cutoff-scheme        = Verlet
ns_type              = grid              ; search neighboring grid cells
nstlist              = 20                ; largely irrelevant with Verlet
rlist                = 1.2
vdwtype              = cutoff
vdw-modifier          = force-switch
rvdw-switch          = 1.0
rvdw                 = 1.2               ; short-range van der Waals cutoff (in nm)
; Electrostatics
coulombtype          = PME               ; Particle Mesh Ewald for long-range electrostatics
rcoulomb             = 1.2               ; short-range electrostatic cutoff (in nm)
pme_order            = 4                 ; cubic interpolation
fourierspacing       = 0.16             ; grid spacing for FFT
; Temperature coupling
tcoupl               = V-rescale          ; modified Berendsen thermostat
tc-grps              = Protein_FOL Water_and_ions ; two coupling groups - more accurate
tau_t                = 0.1 0.1           ; time constant, in ps
ref_t                = 300 300           ; reference temperature, one for each group, in K
; Pressure coupling
pcoupl               = no                ; no pressure coupling in NVT
; Periodic boundary conditions
pbc                  = xyz               ; 3-D PBC
; Dispersion correction is not used for proteins with the C36 additive FF
DispCorr             = no
; Velocity generation
gen_vel              = yes               ; assign velocities from Maxwell distribution
gen_temp             = 300               ; temperature for Maxwell distribution
gen_seed             = -1                ; generate a random seed

```

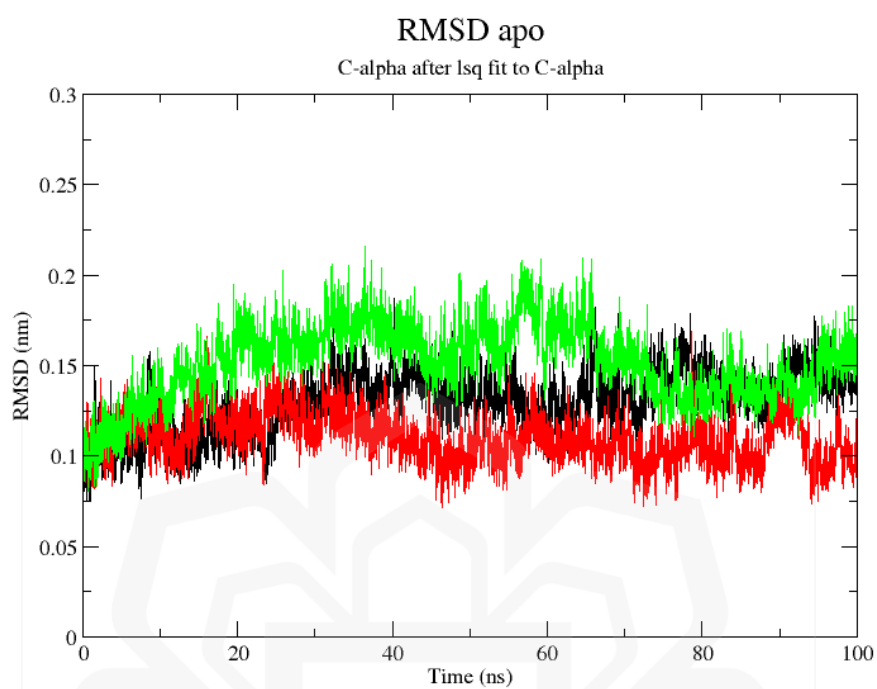
Appendix 28 Equilibration parameter file (NPT) from MD simulation

```

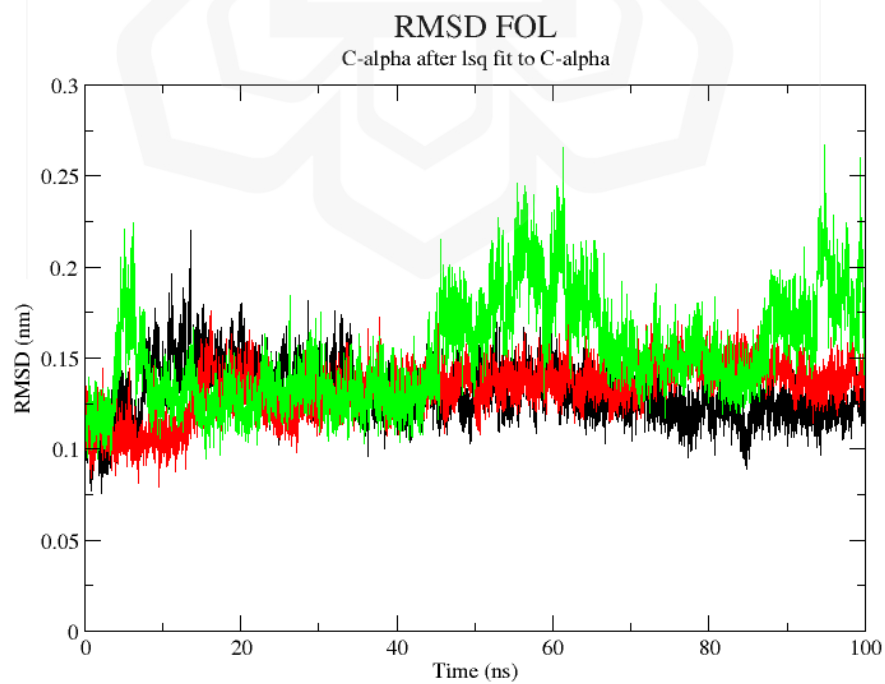
title                = Protein-ligand complex NPT equilibration
define               = -DPOSRES -DPOSRES_LIG ; position restrain the protein and ligand
; Run parameters
integrator           = md                ; leap-frog integrator
nsteps               = 50000             ; 2 * 50000 = 100 ps
dt                   = 0.002             ; 2 fs
; Output control
nstenergy            = 500               ; save energies every 1.0 ps
nstlog               = 500               ; update log file every 1.0 ps
nstxout-compressed    = 500               ; save coordinates every 1.0 ps
; Bond parameters
continuation         = yes               ; continuing from NVT
constraint_algorithm  = lincs             ; holonomic constraints
constraints           = h-bonds           ; bonds to H are constrained
lincs_iter           = 1                 ; accuracy of LINCS
lincs_order           = 4                 ; also related to accuracy
; Neighbor searching and vdW
cutoff-scheme        = Verlet
ns_type              = grid              ; search neighboring grid cells
nstlist              = 20                ; largely irrelevant with Verlet
rlist                = 1.2
vdwtype              = cutoff
vdw-modifier          = force-switch
rvdw-switch           = 1.0
rvdw                 = 1.2               ; short-range van der Waals cutoff (in nm)
; Electrostatics
coulombtype           = PME               ; Particle Mesh Ewald for long-range electrostatics
rcoulomb              = 1.2
pme_order             = 4                 ; cubic interpolation
fourierspacing        = 0.16             ; grid spacing for FFT
; Temperature coupling
tcoupl                = V-rescale          ; modified Berendsen thermostat
tc-grps               = Protein_FOL Water_and_ions ; two coupling groups - more accurate
tau_t                 = 0.1 0.1           ; time constant, in ps
ref_t                  = 300 300          ; reference temperature, one for each group, in K
; Pressure coupling
pcoupl                = Berendsen          ; pressure coupling is on for NPT
pcoupltype             = isotropic         ; uniform scaling of box vectors
tau_p                 = 2.0                ; time constant, in ps
ref_p                  = 1.0               ; reference pressure, in bar
compressibility         = 4.5e-5           ; isothermal compressibility of water, bar^-1
refcoord_scaling        = com
; Periodic boundary conditions
pbc                   = xyz                ; 3-D PBC
; Dispersion correction is not used for proteins with the C36 additive FF
DispCorr               = no
; Velocity generation
gen_vel                = no               ; velocity generation off after NVT

```

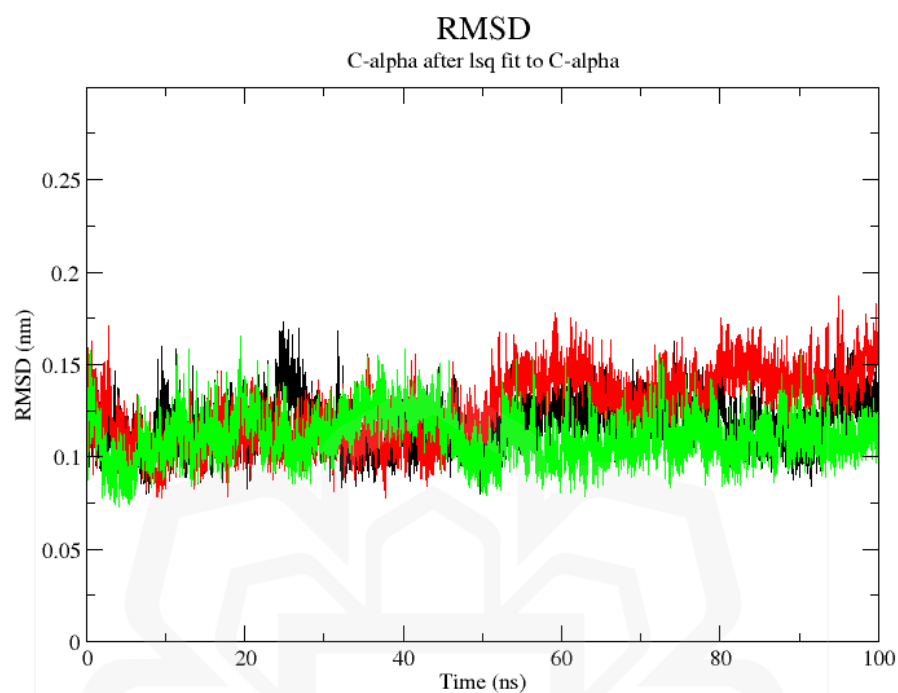
Appendix 29 RMSD plot of DHFR-APO in triplicate from MD simulation



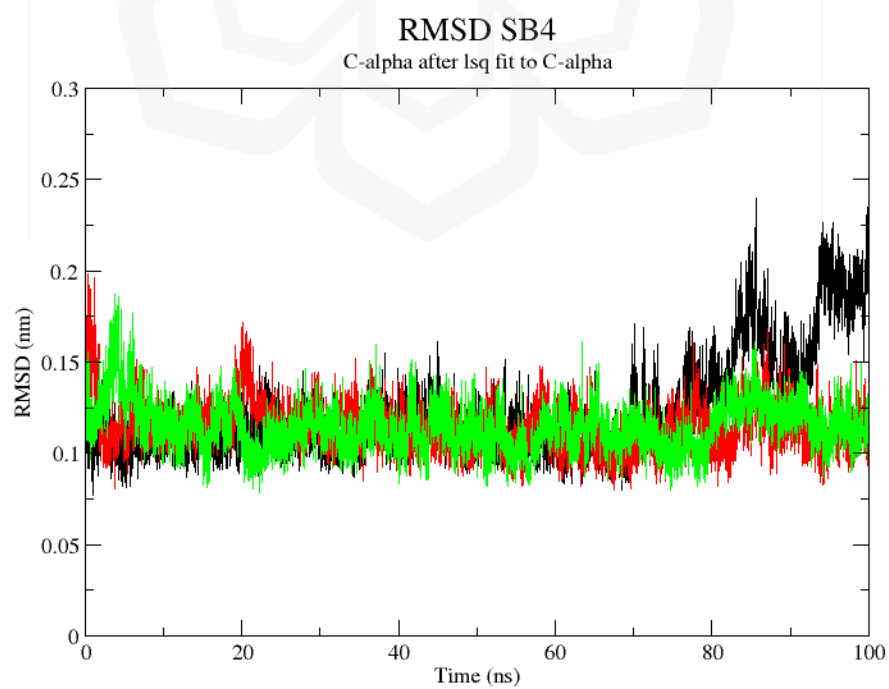
Appendix 30 RMSD plot of DHFR-FOL in triplicate from MD simulation



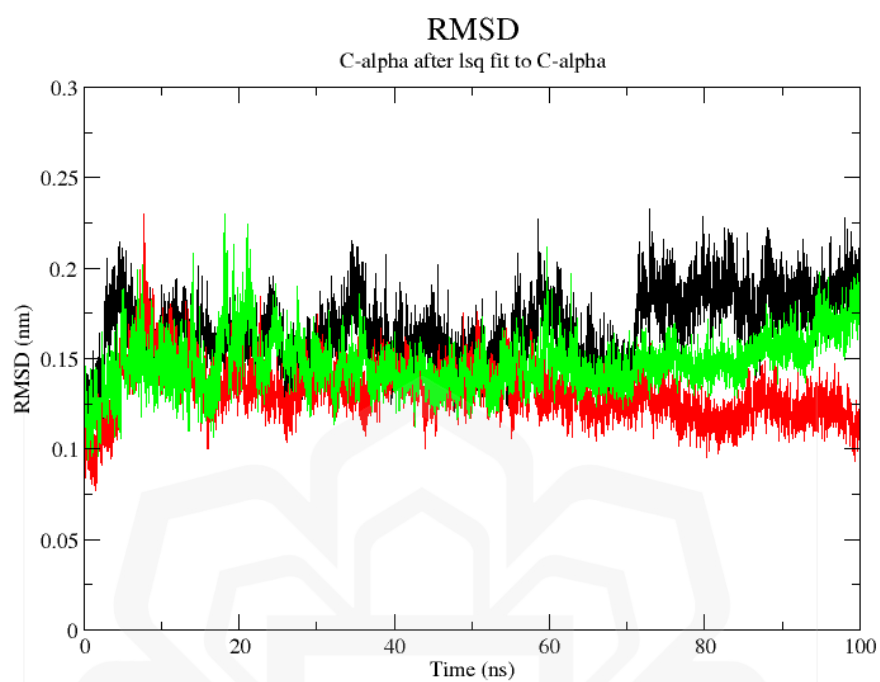
Appendix 31 RMSD plot of DHFR-TMP in triplicate from MD simulation



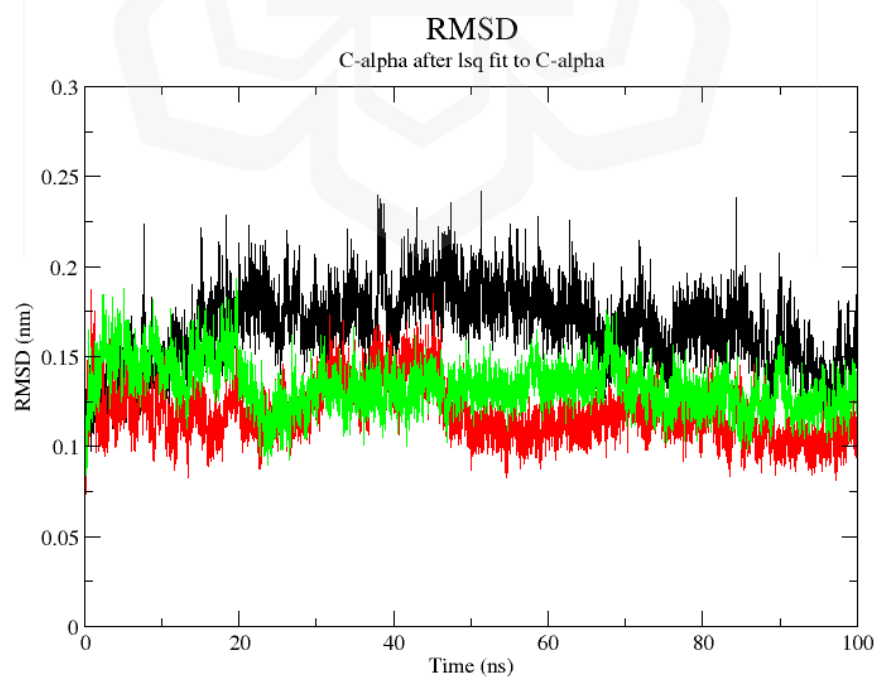
Appendix 32 RMSD plot of DHFR-SB4 in triplicate from MD simulation



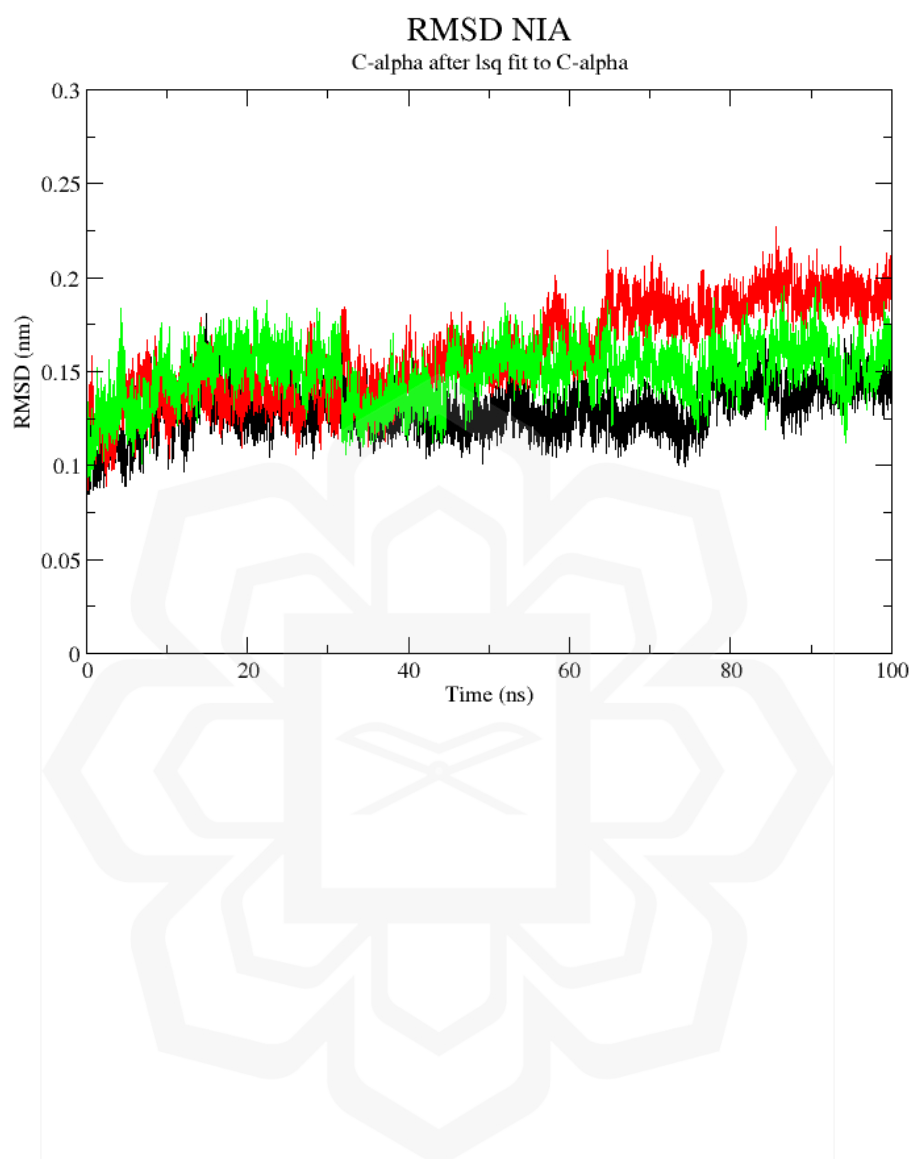
Appendix 33 RMSD plot of DHFR-CUSB4 in triplicate from MD simulation



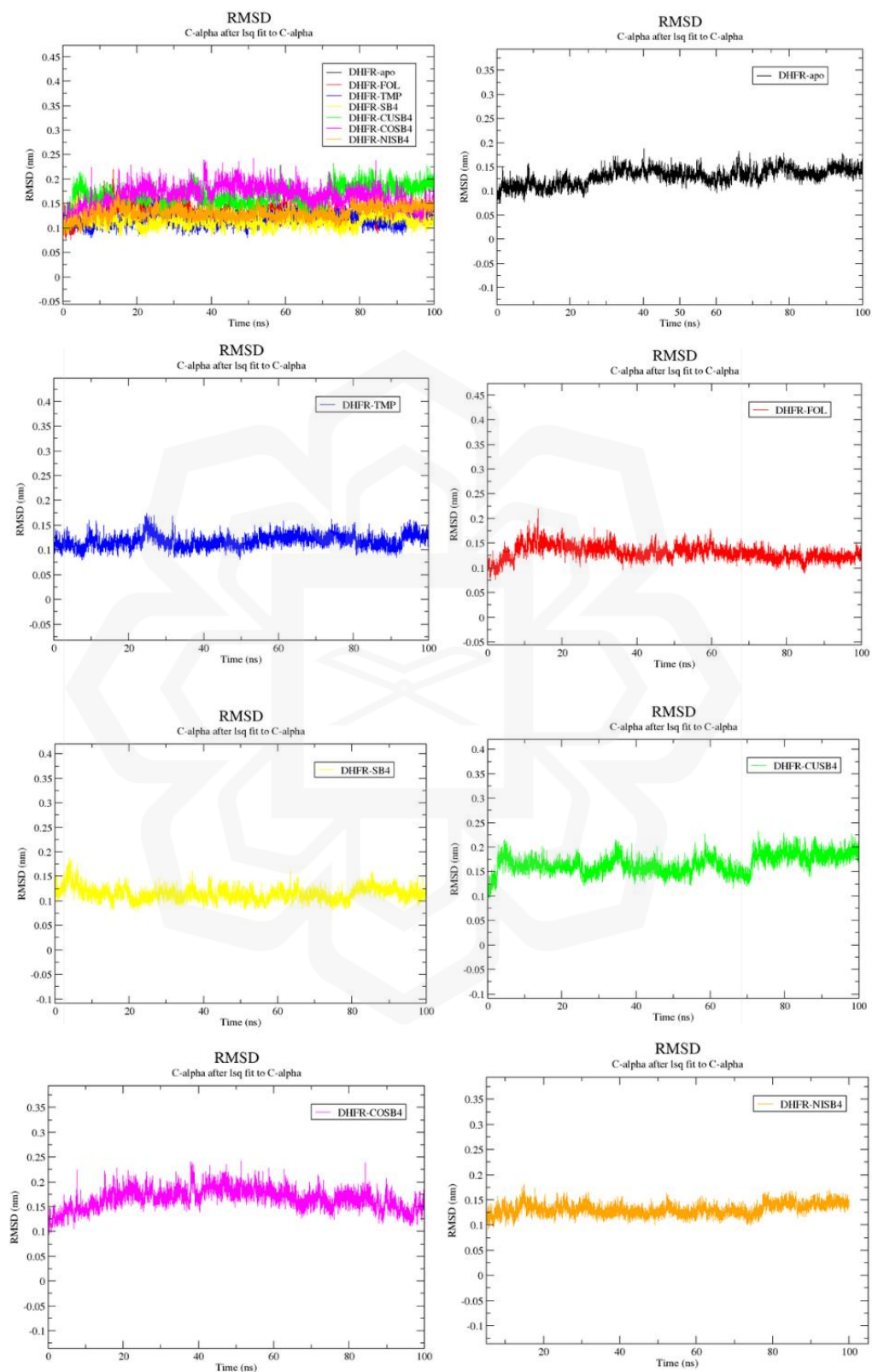
Appendix 34 RMSD plot of DHFR-COSB4 in triplicate from MD simulation



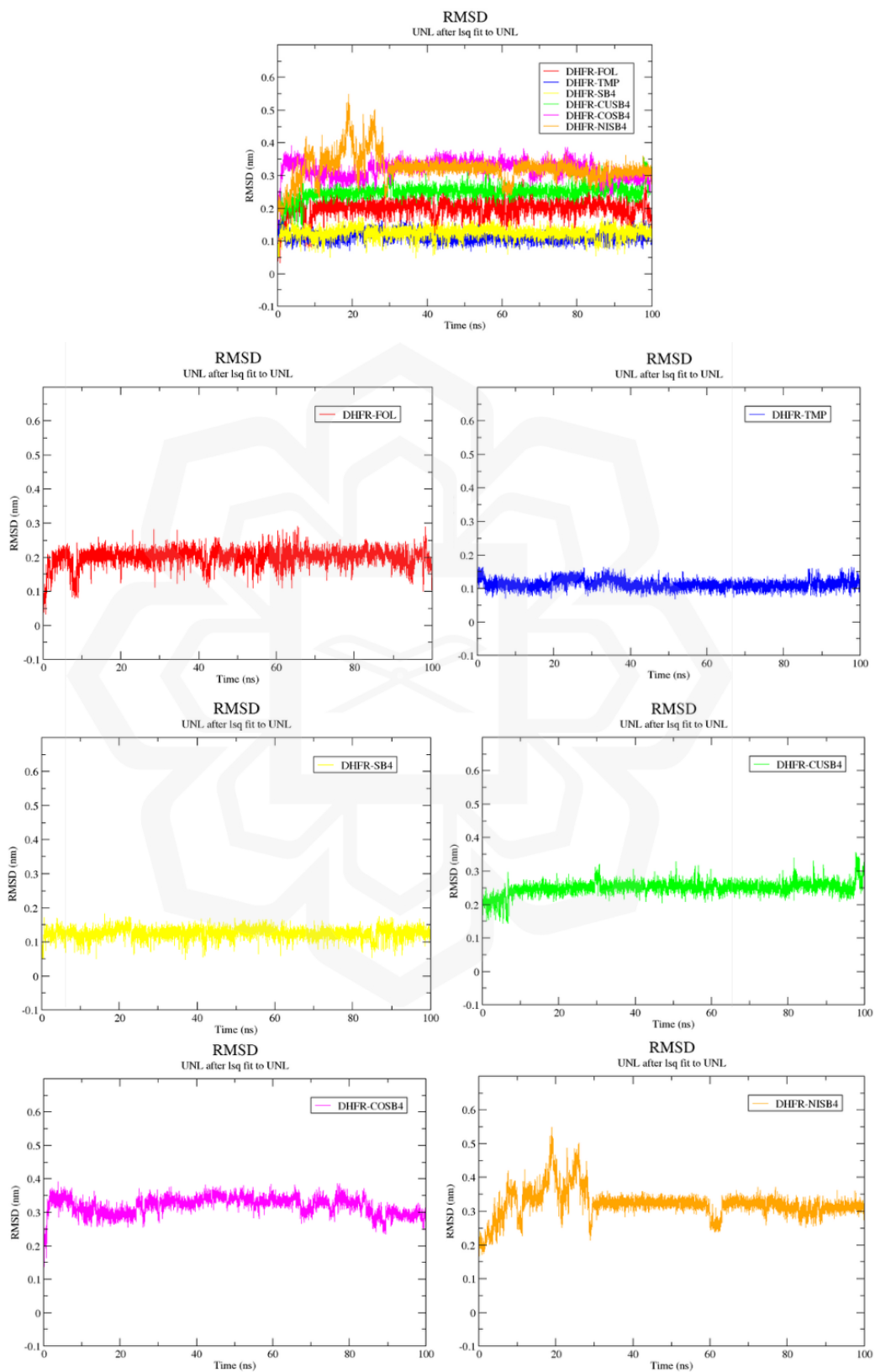
Appendix 35 RMSD plot of DHFR-NISB4 in triplicate from MD simulation



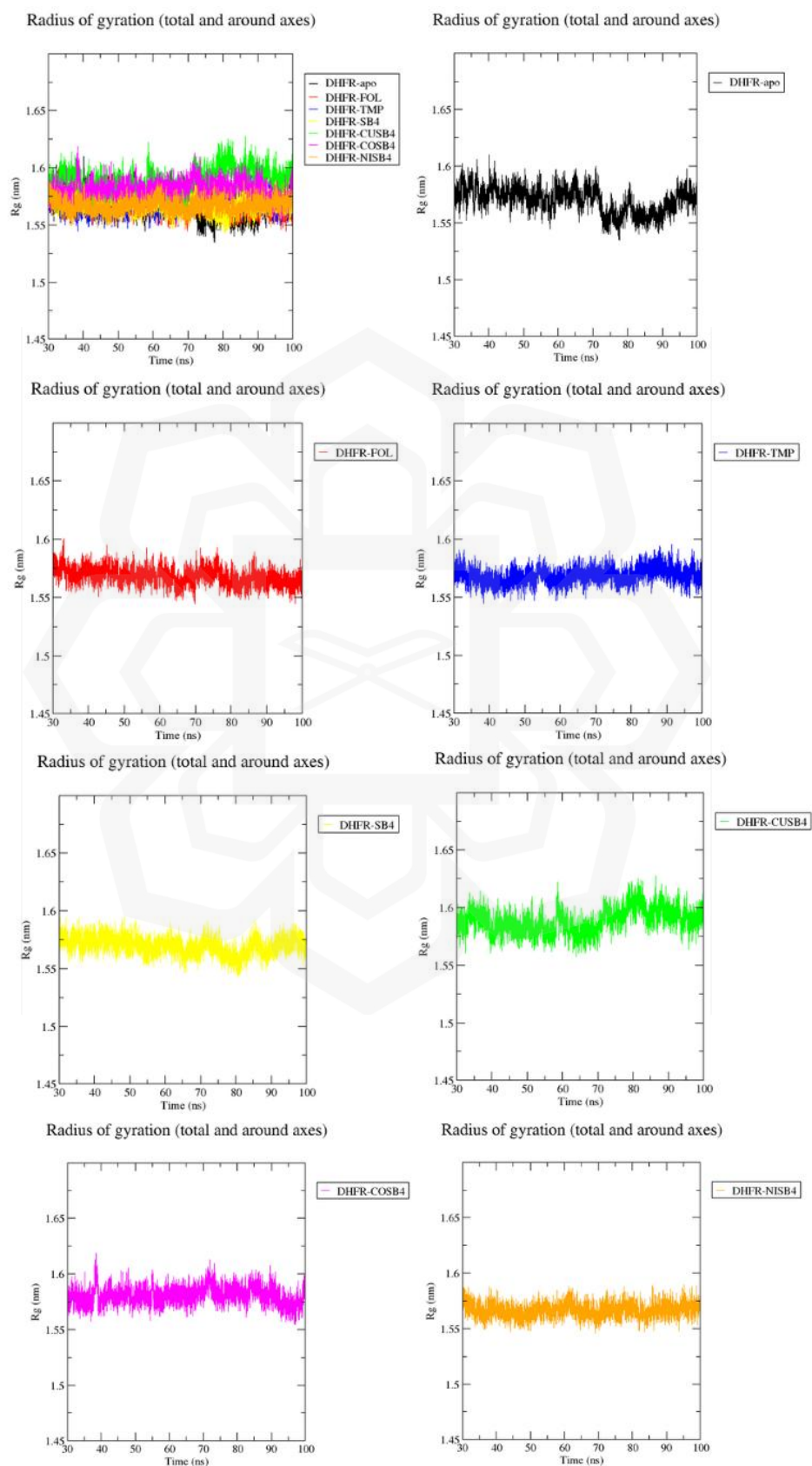
Appendix 36 RMSD plots of protein ligand complexes from MD simulation



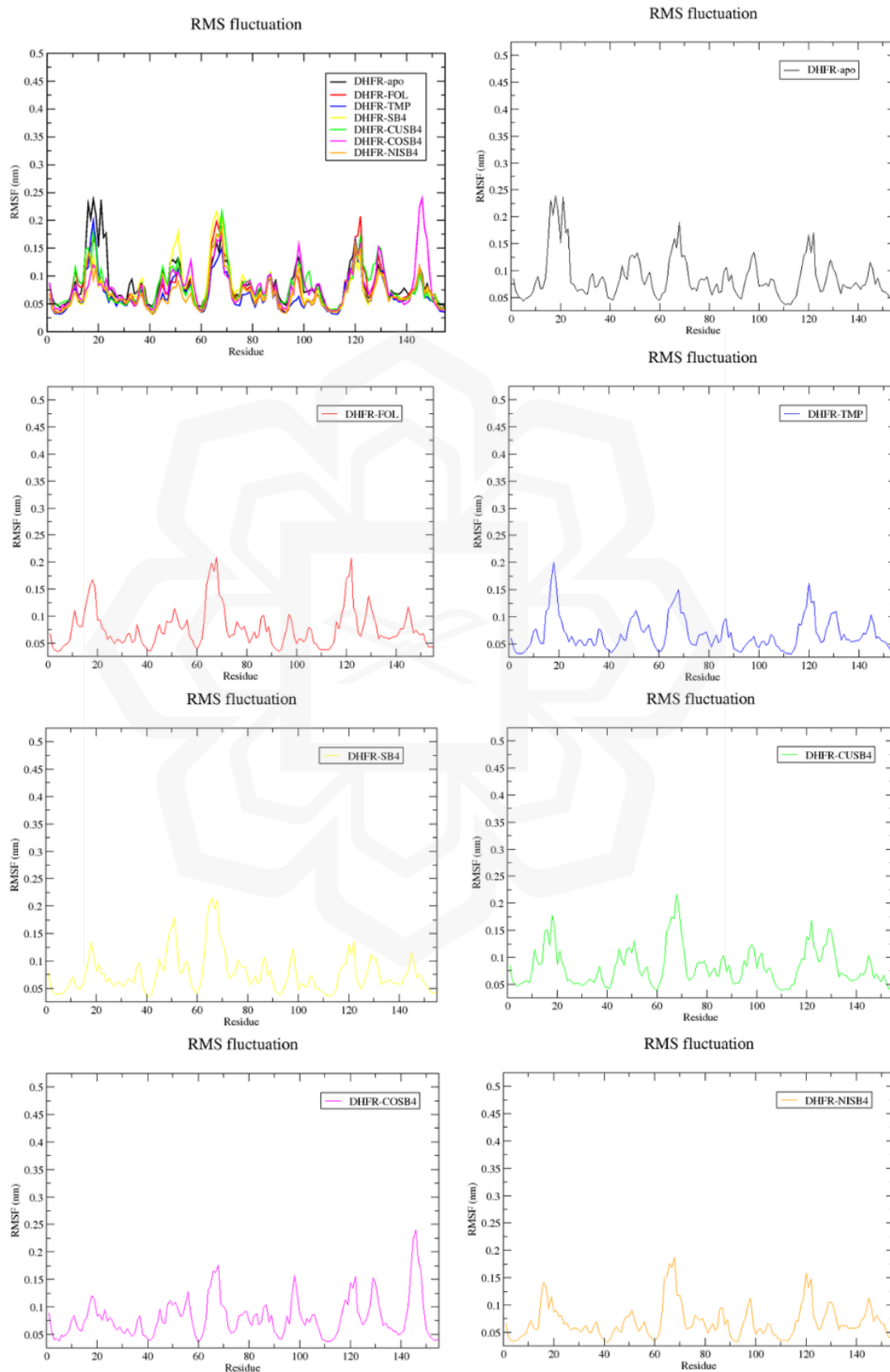
Appendix 37 RMSD plot of ligand from MD simulation



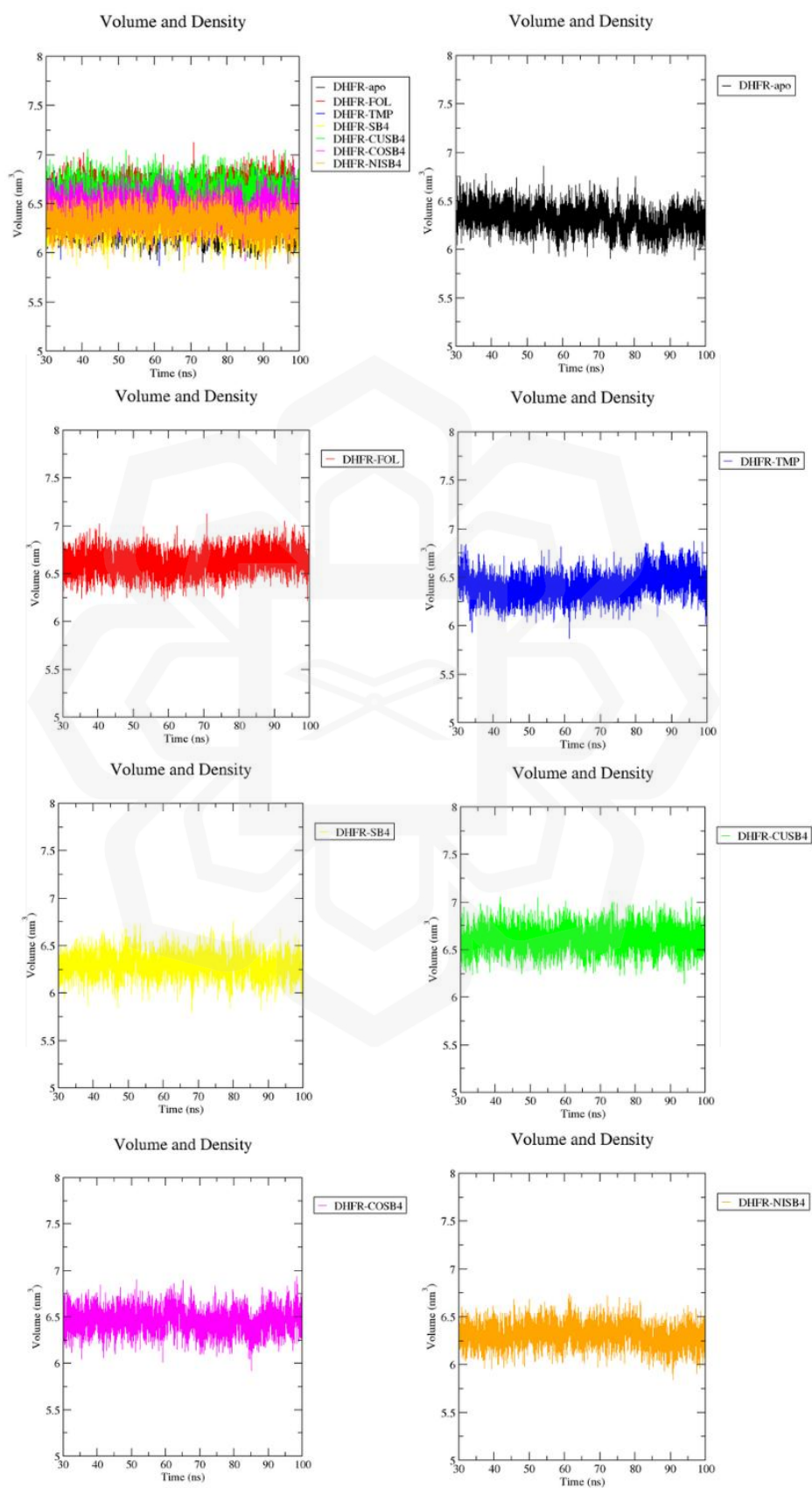
Appendix 38 Radius of gyration plot of protein ligand complexes from MD simulation



Appendix 39 RMSF plot of protein ligand complexes from MD simulation



Appendix 40 Pocket volume plot of protein ligand complexes from MD simulation



Appendix 41 List of publications extracted from this study

- Roslan, S. K., How, F. N. F., & Hamzah, N. (2023). Synthesis, characterization, antimicrobial activity and ADMET analysis of *S*-benzyl- α -*N*-(anisoyl)-dithiocarbazate (SB4OME) and its metal complexes. *Malaysian Journal of Chemistry*, 25(3), 481-491.
- Roslan, S. K., How, F. N. F., Ibrahim, Z., & Hamzah, N. (2024). Exploring SB2OME and Metal Complexes as Antibacterial Agents: Synthesis, Characterization, and Molecular Docking. *Malaysian Journal of Chemistry*, 26(5), 72-89.
- Roslan, S. K. & How, F. N. F. (2024). Synthesis, Characterisation, and Antimicrobial Activities of *S*-Benzyl- β -*N*-3-methoxybenzoyl Dithiocarbazate (SB3OME) and Its Metal Complexes. *Malaysian Journal of Chemistry*, 26(5), 465-476.
- Roslan, S. K. & How, F. N. F. (2024). A Novel OS Substituted Dithiocarbazate Derivatives: Synthesis, Characterisation, And Antibacterial Activity Of *S*-benzyl- β -*N*-4-chloro-3-nitrobenzoyl Dithiocarbazate (SB3NO) and its Cu(II), Zn(II), Co(II), and Ni(II) Complexes. *Malaysian Journal of Analytical Sciences*, 29(3), 1436.

Appendix 42 List of conferences attended and presentations extracted from this study

- “Synthesis, Characterization, Antimicrobial Activity and ADMET Analysis of SB4OME & its Metal Complexes”, 3rd IKMPB Online Symposium, Jan 2023, Zoom platform.
- “A new insight into substituted dithiocarbazate derivative, S-Benzyl- β -N-(2-methoxybenzoyl) dithiocarbazate (SB2OME) and its Cu(II), Zn(II), Co(II), and Ni(II) complexes as potential bioactive antibacterial agents: Synthesis, Characterization and ADMET Modelling”, 3rd Virtual International Symposium on Sciences (VISoS 2023), Sep 2023, Zoom platform.
- “Exploring the potential bioactive antibacterial agents of SB2OME Dithiocarbazate and Its Cu(II), Zn(II), Co(II), and Ni(II) Complexes: ADMET Modelling and Molecular Docking”, IIUM Postgraduate Colloquium, Oct 2023, Swiss Bell Hotel Kuantan.
- “Molecular Docking study of SB2OME & its metal complexes against mutant & wild-type DNA gyrase B *E. coli*”, American Chemical Society Global Innovation Imperatives The 7th International Conference on Computational for Science and Technology, Dec 2023, Connexion Bangsar Petaling Jaya.
- “Synthesis, Characterisation, and Antimicrobial Activities of SB3OME and Its Metal Complexes”, 4th IKMPB Online Symposium, Jan 2024, Zoom platform.
- “Unlocking the antibacterial potential of isomeric substituted dithiocarbazate derived from SBDTC: A computational study”, International Postgraduate Research Conference (IPRC), Aug 2024, Universiti Sultan Zainal Abidin (UniSZA) - Awarded with Best Poster Presenter.