CHEMOPREVENTION EFFECTS OF SELECTED MALAYSIAN HERBS VIA POLYAMINES PATHWAY IN CERVICAL CANCER

BY

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ABSTRACT

Polyamine biosynthesis, which is tightly regulated in normal cells to maintain homeostasis, is frequently dysregulated in carcinoma cells, resulting in uncontrolled proliferation, evasion of apoptosis, and enhanced cancer cell survival. As part of the World Health Organization's (WHO) initiative to eliminate cervical cancer as a public health concern by 2030, chemoprevention strategies offer a promising approach to target vulnerable populations and reduce cancer incidence. This study aims to investigate and elucidate the mechanistic and therapeutic potential of selected Malaysian herbs as chemopreventive agents targeting the polyamine pathway in human cervical cancer, SiHa cells. Aqueous extracts of 11 selected Malaysian herbs were prepared via maceration at 60 °C with a cosolvent and subsequently lyophilised at −80°C. An initial screening of the herbs –including *S. aromaticum, E. scaber, H. rosa sinensis, C. caudatus, C. ternatea, C. verum*, *C. zedoaria*, *F. deltoideia*, *S. crispus*, *C. tamala*, and *M. fragrans* –was conducted to assess cytotoxicity against SiHa cells. Phytochemical profiling was performed using Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS). The antiproliferative effects were evaluated using MTT and TBE assays, while protein levels were measured using Lowry's assay. Key genes involved in polyamine metabolism—*ODC, SSAT, OAZ1*, and *SMOX*—were analysed via quantitative PCR (qPCR). Cell death mechanisms were investigated through cell cycle analysis, annexin V assay, and caspase 3, 8, and 9 activity assays. Following cytotoxicity screening, *S. aromaticum* and *E. scaber* were selected for their highest cytotoxicity, with lowest IC_{50} values of 177.6 and 88.8 μg/ml at 48 hours, respectively. Both extracts significantly inhibited cell growth and reduced intracellular protein content ($p < 0.05$). Low endogenous polyamine levels were detected in *S. aromaticum* (0.585 nmol/mg) and *E. scaber* (0.192 nmol/mg) extracts. Polyamine biosynthesis was disrupted by the downregulation of *ODC* ($p < 0.05$) and upregulation of *OAZ1*, *SSAT*, and *SMOX* ($p < 0.05$), indicating a significant reduction in polyamine production in SiHa cells following treatment. Cell cycle analysis revealed G0/G1 arrest and G2/M accumulation, while annexin V assay indicated increased in late apoptotic cell populations. Caspase assays demonstrated that both extracts increased caspase 3 activity, with *E. scaber* predominantly activating caspase 8 and *S. aromaticum* significantly increasing caspase 9 activity. This study demonstrates the *in vitro* efficacy of *S. aromaticum* and *E. scaber* as chemopreventive agents against cervical cancer. The findings support their potential to inhibit cell proliferation, reduce polyamine content, induce apoptosis, and cause cell cycle arrest in SiHa cells, suggesting their promise as apoptosis-mediated therapeutic candidates for cervical cancer prevention and treatment.

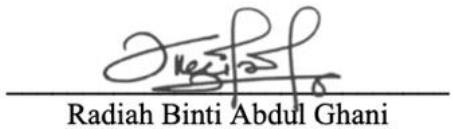
ABSTRACT IN ARABIC

يُعدّ تخليق البوليامينات عملية منظمة بدقة في الخلايا الطبيعية للحفاظ على التوازن الخلوي، إلا أنها غالبًا ما تكون مضطربة في الخلايا السرطانية، مما يؤدي إلى التكاثر غير المنضبط، وتجنّب الاستماتة، وزيادة بقاء للقضاء على سرطان عنق الرحم (WHO) الخلايا السرطانية. وفي إطار هدف منظمة الصحة العالمية كمشكلة صحية عامة بحلول عام 2030، تمثل استراتيجيات الوقاية الكيميائية وسيلة واعدة لاستهداف الفئات المعرضة للخطر وتقليل معدلات الإصابة بالسرطان. تهدف هذه الدراسة إلى التحقيق في الإمكانات العلاجية والآليات الخلوية للأعشاب الماليزية المختارة كعوامل وقاية كيميائية تستهدف مسار تم تحضير مستخلصات مائية لـ 11 .SiHa البوليامينات في خلايا سرطان عنق الرحم البشرية من نوع نوعًا من الأعشاب الماليزية عن طريق النقع عند 60 درجة مئوية باستخدام مساعد مذيب، ثم تجميدها *S.* بالتجفيف عند −80 درجة مئوية. أُجري اختبار أولي لتقييم السُمية الخلوية للأعشاب (بما في ذلك *C. ternatea*و *C. caudatus*و *H. rosa-sinensis*و *E. scaber*و *aromaticum* *M.* و *C. tamala*و *S. crispus*و *F. deltoidea*و *C. zedoaria*و *C. verum*و GC-MS كما تم إجراء تحليل كيميائي نباتي باستخدام تقنيتي ،SiHa على خلايا *fragrans* وتم قياس ،TBEو MTT جرى تقييم التأثيرات المضادة للتكاثر باستخدام اختباري .LC-MSو ODC) مستويات البروتين بطريقة لوري، وتحليل الجينات الرئيسية المشاركة في أيض البوليامينات بالإضافة إلى تحديد آلية موت الخلايا من ،qPCR باستخدام (SMOXو OAZ1و SSATو واختبارات نشاط الكاسبيز 3 و8 و9. بعد الفحص ،Annexin V خلال تحليل دورة الخلية، واختبار بناءً على أعلى تأثير سُمّي خلوي، حيث *E. scaber*و *S. aromaticum* الأولي، تم اختيار كل من عند 177.6 و88.8 ميكروغرام/مل بعد 48 ساعة على التوالي. أظهرت IC50 أظهرا أقل قيم

كما تم رصد ،($p < 0.05$) المستخلصات تثبيطًا كبيرًا لنمو الخلايا، وانخفاضًا في محتوى البروتين S. aromaticum مستويات منخفضة من البوليامينات داخل المستخلصين: 0.585 نانومول/ملغم لـ تسببت المعالجة في تثبيط تخليق البوليامينات من خلال خفض .E. scaber و0.192 نانومول/ملغم لـ أظهر .($p < 0.05$) *SMOX*و *SSAT*و *OAZ1* وزيادة تعبير ($p < 0.05$) *ODC* تعبير عن Annexin V بينما كشف اختبار ،G2/M وتراكمًا في G0/G1 تحليل دورة الخلية توقفًا في زيادة في الخلايا التي تمر بمرحلة الأبوبتوس المتأخر، كما أظهرت اختبارات الكاسبيز زيادة في نشاط كاسبيز-*S.* وزيادة كبيرة في كاسبيز-9 بفعل *E. scaber* مع تنشيط كاسبيز-8 بشكل بارز بواسطة ،3 كمستخلصات *E. scaber*و *S. aromaticum* تُظهر هذه الدراسة فعالية .*aromaticum* واعدة للوقاية الكيميائية من سرطان عنق الرحم، وتدعم النتائج إمكانيتهما في تثبيط تكاثر الخلايا، وخفض مما يشير إلى ،SiHa محتوى البوليامين، وتحفيز الاستماتة، والتسبب في توقف دورة الخلية في خلايا إمكاناتهما كمرشحين علاجيين قائمين على الاستماتة للوقاية والعلاج من سرطان عنق الرحم.

APPROVAL PAGE

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DECLARATION

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

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To my family, whose endless love and encouragement have been the cornerstone of my journey through this pursuit. To my beloved Harrison, your unwavering support, love, and faith in me have illuminated every step of this path. For all that you have given, I dedicate this achievement to you.

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LIST OF SYMBOLS

g	gram
mg	Milligram
µg/ml	Microgram per millilitre
µm	Micrometre
mM	Millimolar
nM	Nanomolar
nmol/l	Nanomole per litre
nmol/mg	Nanomole per milligram
pH	Potential of hydrogen
°C	degree Celsius
%	percentage
% v/v	Volume per volume
^{3}H	Tritium
^{14}C	Carbon-14
L	litre
min	minute
rpm	Revolutions per min
ml	millilitre
µl	microlitre
nmol/ µl	Nanomole per microlitre
w/v	weight per volume
nm	nanometre
h	hour
s	second
ΔΔCt	Double delta cycle threshold
V	Volt
dH_2O	Distilled water

LIST OF ABBREVIATION

ACTB	actin beta
AIDS	Acquired immunodeficiency syndrome
ANOVA	analysis of variance
APAO	Acetylpolyamine oxidase
ATPase	Adenosine triphosphatase
AZ	antizyme
AZIN	antizyme inhibitor
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra-large
BP	benzo[a]pyrene
BSA	Bovine Serum Albumin
BSC	biological safety cabinets
CDK	Cyclin Dependent Kinase
CDKIs	Cyclin Dependent Kinase Inhibitors
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
CO_2	carbon dioxide
Ct	Cycle threshold
Cq	Quantification cycle
DC	dead cells
DCP	Division of Cancer Prevention
DDNP-CIP	Discovery and Development of Natural Products for Cancer Interception and Prevention
DF	Dilution factor
DFMO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	diphenyl-2-picrylhydrazyl
E2F	E2 promoter-binding factor
eIF-5A	eukaryotic initiation factor-5A

ERK	extracellular signal-regulated protein kinase
ESI	electrospray ionization
ESR1	Erythrocyte sedimentation rate
FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
FRAP	ferric reducing antioxidant power
FSC	forward-scatter light
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC-MS	gas chromatography-mass spectroscopy
GCMS	GC-MS Analysis The instrument used for Gas Chromatography – Mass Spectrometry
GOI	genes of interest
GPC1	glypican-1
GSTII	glutathione S-transferase II
Go	Gap zero
G1	Gap 1
G2/M	Gap 2/Mitosis
HepG2	human liver cancer cell line
HER2	human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HPLC	high performance liquid chromatography
HPV	human papillomavirus
IC_{50}	50% Inhibition concentration
IIUM	International Islamic University Malaysia
iPROMISE	Integrative Pharmacogenomics Institute
KAHS	Kulliyyah of Allied Health Sciences
LC-MS	Liquid Chromatography-Mass Spectrometry
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor-kappa B
NH3	ammonia

NIST	National Institute of Standards and Technology
NISTII	NIST library
NSAIDs	nonsteroidal anti-inflammatory drugs
NTC	no template control
NCI	National Cancer Institute
OAZ1	ornithine decarboxylase antizyme 1
OD	optical density
ODC	ornithine decarboxylase
PA	polyamines
PAO	polyamine oxidase
PBS	phosphate buffer saline
PDA	photodiode array
PI	propidium iodide
PLP	pyridoxal phosphate
PM1	Particulate matter (aerodynamic diameter of <1.0 μm)
PPE	personal protective equipment
pRb	Retinoblastoma protein
PS	phosphatidylserine
PTS	Polyamine transport system
p53	Tumour protein p53
qPCR	quantitative polymerase chain reaction
RFU	relative florescence unit
RG	reference genes
RIS	RNA integrity score
RNA	ribonucleic acid
ROS	reactive oxygen species
RP-HPLC	Reversed-phase HPLC
RT-qPCR	Real-time Expression Analysis
S phase	Synthesis
SAM	S-adenosyl methionine
SAMDC	S-adenosylmethionine decarboxylase
SAT1	spermine/spermidine N1-acetyltransferase 1
SD	standard deviation

SDG	Sustainable Development Goals
SEBs	surrogate endpoint biomarkers
SLC3A2	solute carrier family 3 member 2
SLC7A2	solute carrier family 7 member 2
SLC22A16	solute carrier family 22 member 16
SMO	spermine oxidase
SMOX	spermine oxidase
SPDS	spermidine synthase
SPDSYN	spermidine synthase
SPMS	spermine synthase
SPMSYN	spermine synthase
SSAT	spermidine/spermine-N-acetyltransferase
TBE	Trypan Blue Exclusion
Tm	Melting temperature
UiTM	Universiti Teknologi MARA
VC	viable cells
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer poses a significant global health challenge, with its prevalence and impact anticipated to rise. Addressing this challenge worldwide is imperative to enhance outcomes and alleviate the burden it places on individuals, families, and healthcare systems. Cervical cancer ranks among the top 10 cancers in terms of both incidence and mortality worldwide. In Malaysia, it is the second most prevalent cancer among women aged 15 to 44 (Mustafa *et al.*, 2022; Romli *et al.*, 2019). The World Health Organization (WHO) aims to eliminate cervical cancer as a public health concern by 2030 through reducing both incidence and mortality rates (Mastura *et al.*, 2019). Despite these efforts, survival rates vary across ethnic groups, with Malays having the lowest survival rate, followed by Indians and Chinese. Early detection significantly enhances survival rates, emphasizing the importance of timely screening and intervention programs like the Pap smear and HPV vaccination.

Squamous cell carcinoma and adenocarcinoma are the two main types of cervical cancer. While persistent HPV infection remains the primary risk factor, cervical cancer is considered a complex and gradual process involving multiple factors (Rahmat *et al.*, 2021). These factors include lack of HPV vaccination, certain sexual behaviours (such as early sexual activities, multiple partners, or unprotected intercourse), acquired immunodeficiency diseases (like HIV/AIDS or immunosuppressive therapy), smoking, history of sexually transmitted infections, low socioeconomic status, prolonged use of oral contraceptives, and multiple childbirths (Muhamad *et al.*, 2015). HPV has over 200 genotypes, categorized into high-risk, putative high-risk, and low-risk groups, with high-risk types posing a greater likelihood of cervical cancer development later in life (Y. Liu *et al.*, 2022; Puteh *et al.*, 2011).

Reducing mortality and incidences of cervical cancer through chemoprevention involves implementing strategies aimed at inhibiting, delaying, or reversing the process of carcinogenesis. Chemoprevention approaches can target various stages of cervical cancer development, from preventing initial cellular changes to inhibiting the progression of precancerous lesions to invasive cancer (Mohan *et al.*, 2022; Parveen *et al.*, 2023). One key aspect of chemoprevention is the use of natural or synthetic chemical agents that interfere with the biological pathways involved in cervical cancer development. These agents may include phytochemicals found in plants, such as polyphenols and flavonoids, as well as synthetic compounds like nonsteroidal anti-inflammatory drugs (NSAIDs) and retinoids (Drețcanu *et al.*, 2021; Murata *et al.*, 2023). By targeting specific molecular pathways implicated in cervical carcinogenesis, these agents can help prevent the initiation and progression of cancerous changes in cervical cells.

Another important strategy in preventing cervical cancer is the use of vaccines targeting high-risk strains of human papillomavirus (HPV), the primary etiological agent of the disease (Morosetti *et al.*, 2017). HPV vaccines, such as Gardasil and Cervarix, are highly effective in preventing new HPV infections and reducing the incidence of cervical lesions associated with high-risk HPV types (Roy *et al.*, 2023). However, while these vaccines offer substantial prophylactic benefit, they do not treat existing HPV infections or pre-cancerous lesions in individuals already infected. Moreover, HPV vaccination uptake and awareness in Malaysia remain suboptimal, particularly among certain socio-economic and rural populations. This results in a clinical gap for women who are unvaccinated, already infected, or who develop persistent lesions despite prior vaccination. In this context, chemoprevention remains a relevant and complementary strategy—particularly for high-risk or underserved groups—by providing therapeutic options that target molecular pathways involved in cervical carcinogenesis. In addition to vaccination, chemopreventive approaches may involve lifestyle modifications and behavioural interventions aimed at reducing associated risk factors. These include promoting healthy sexual behaviours, delaying sexual debut, reducing the number of sexual partners, encouraging smoking cessation, and enhancing screening efforts such as regular Pap smear testing (Choi *et al.*, 2023; Islam *et al.*, 2023).

A comprehensive approach to chemoprevention involves integrating multiple strategies, including the use of chemical agents, vaccines, and lifestyle modifications, to target different stages of cervical cancer development and reduce both the incidence and mortality associated with the disease. By addressing the underlying biological mechanisms and risk factors involved in cervical carcinogenesis, chemoprevention offers a promising avenue for reducing the burden of cervical cancer on public health.

Polyamines, such as putrescine, spermidine, and spermine, play crucial roles in cellular processes involved in cervical cancer development. Dysregulation of polyamine metabolism has been associated with carcinogenesis, making them potential targets for chemoprevention strategies. By targeting polyamine pathways, chemopreventive agents can modulate cell growth, proliferation, and apoptosis, thereby inhibiting the initiation and progression of cervical cancer (Igarashi *et al.*, 2020). Additionally, emerging research suggests that certain natural compounds found in Malaysian herbs may interact with polyamines, offering potential pathways for novel chemopreventive approaches. Integrating polyamine-targeted interventions into cervical cancer chemoprevention efforts could provide valuable insights into the development of more effective prevention strategies, potentially reducing the incidence and mortality of cervical cancer worldwide (Y. Chen *et al.*, 2018; J. Yang *et al.*, 2018).

Malaysian herbs are known for their abundance of bioactive compounds, which have been shown to possess antimicrobial, antioxidative, and anti-inflammatory properties, contributing to their numerous health benefits. The use of traditional Malaysian medicinal plants dates back centuries, as evidenced by records in Malay Medical Manuscripts documenting their application in treating various diseases (Mohd Afeendi *et al.*, 2017). However, the interaction between polyamines and Malaysian herbs in cervical cancer chemoprevention remains a relatively unexplored area. Understanding how these natural compounds interact with the polyamine pathway could provide valuable insights into developing innovative preventive and therapeutic strategies. By elucidating the mechanisms through which polyamines and Malaysian herbs exert their effects, this research aims to contribute to the advancement of more effective cervical cancer prevention strategies. These findings may not only expand our understanding of cervical cancer biology but also offer a basis for novel, sustainable,

and culturally relevant prevention approaches, particularly in regions where Malaysian herbs are commonly used.

While most previous *in vitro* chemoprevention studies on cervical cancer have focused heavily on HeLa cells—a widely used cervical adenocarcinoma model—these cells may not fully represent the heterogeneity of cervical squamous cell carcinoma, which is the predominant histological subtype (A. Sharma *et al.* 2019; Kurnia *et al.* 2021; Ansor *et al.* 2023). In contrast, the SiHa cell line, derived from squamous cell carcinoma and harbouring integrated HPV-16 DNA, provides a more relevant model for studying HPV-associated squamous cervical carcinogenesis (Y. Li *et al.* 2015). However, SiHa cells remain underexplored in chemopreventive studies, especially in relation to polyamine-targeted intervention using traditional herbs. Therefore, this study addresses an important gap in the literature by investigating the polyamine-modulating and antiproliferative potential of selected Malaysian herbs specifically in SiHa cells, offering new findings into alternative preventive strategies.

The interaction between polyamines and Malaysian herbs in cervical cancer chemoprevention presents a promising yet underexplored avenue for novel therapeutic interventions. Polyamines, including putrescine, spermidine, and spermine, are essential for cell growth, proliferation, and differentiation. However, their overaccumulation in cancer cells contributes to tumour progression by promoting uncontrolled proliferation, evading apoptosis, and supporting angiogenesis and metastasis. Targeting polyamine metabolism has gained increasing attention in cancer research as it offers a unique approach to disrupting cancer cell survival. Unlike traditional chemotherapies that broadly target proliferating cells, polyamine-targeted strategies specifically interfere with metabolic pathways essential for tumour growth, potentially reducing cytotoxic effects on normal tissues.

Incorporating the polyamine pathway into chemoprevention strategies represents a novel avenue for cervical cancer treatment, particularly since polyamine dysregulation is linked to HPV oncogenesis (Harbison *et al.*, 2022). High-risk human papillomavirus (HPV) infections drive cervical carcinogenesis by modulating host cell metabolism, including polyamine synthesis. The viral oncoproteins E6 and E7 (both

encoded by HPV) are known to upregulate ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, leading to increased polyamine levels that sustain the transformed phenotype of cervical cancer cells (Basukala *et al.*, 2020; Del Nonno *et al.*, 2011). Moreover, elevated polyamine synthesis in HPV-infected cells has been associated with enhanced DNA stability, reduced oxidative stress, and increased resistance to cell death, further contributing to tumour progression (Morosetti *et al.*, 2017). As a result, targeting the polyamine pathway through natural bioactive compounds may provide multiple advantages—disrupting a critical metabolic pathway while selectively sensitizing cancer cells to apoptosis.

Recent studies have highlighted the role of polyamine inhibitors such as difluoromethylornithine (DFMO) in reducing cancer cell proliferation by inhibiting ODC activity, thereby lowering intracellular polyamine levels (Laukaitis & Gerner, 2011). However, DFMO primarily exerts a cytostatic effect rather than a cytotoxic one, meaning it halts cancer cell growth rather than inducing cell death. The limited efficacy of DFMO as a standalone treatment is partly due to compensatory mechanisms that upregulate polyamine transport and alternative biosynthetic pathways to sustain intracellular polyamine levels, allowing cancer cells to survive despite growth inhibition (Sholler *et al.*, 2018). This limitation underscores the need for complementary approaches, such as combining DFMO with natural compounds that influence polyamine metabolism through multiple mechanisms, including apoptosis induction.

Despite the growing body of evidence supporting polyamine-targeted therapies, few studies have investigated the potential of Malaysian herbs in modulating this pathway, particularly in the context of cervical cancer. Integrating traditional herbal medicine with polyamine-targeted strategies presents a culturally relevant and sustainable approach to chemoprevention (Gamble *et al.*, 2019; R. Liu *et al.*, 2021). By bridging the gap between traditional herbal knowledge and molecular cancer research, this study lays the foundation for future investigations into the potential of Malaysian herbs as novel polyamine-targeting agents. Polyamine metabolism has been increasingly recognised as a metabolic vulnerability in various cancers, including cervical cancer, as it plays a central role in sustaining tumour cell proliferation, survival, and resistance to apoptosis (Schultz *et al.* 2025). Therefore, targeting this pathway

offers a promising and specific therapeutic approach that goes beyond general cytotoxic mechanisms.

Moreover, polyamine-targeted chemoprevention aligns with the principles of personalised medicine, which seeks to tailor preventive and therapeutic strategies according to an individual's unique biological, genetic, and metabolic profile. Since polyamine levels and enzyme activity can vary across patients due to differences in tumour biology or host metabolism, identifying specific bioactive compounds from Malaysian herbs that can modulate this pathway offers the potential to customise interventions for greater efficacy (Long *et al.* 2024; Murray-Stewart *et al.* 2018). For instance, a patient with high ornithine decarboxylase (ODC) expression may benefit from a polyamine-targeted strategy that suppresses ODC activity. Such precision-based approaches could enhance treatment effectiveness, minimise unnecessary side effects, and ultimately contribute to the advancement of more individualised, low-toxicity cancer prevention strategies.

Thus, incorporating polyamine pathway modulation into cervical cancer research represents a novel and promising approach in chemoprevention. Leveraging the bioactive potential of Malaysian herbs to target polyamine metabolism not only offers a culturally relevant and sustainable healthcare option but also provides a natural, low-toxicity alternative to conventional therapies. Several phytochemicals have demonstrated efficacy against cervical cancer *in vitro* and *in vivo*. Notably, curcumin from Curcuma longa, 6-gingerol from Zingiber officinale Roscoe, and flavonoids such as catechin, quercetin, kaempferol, and luteolin from *Clinacanthus nutans* have shown potential through antiproliferative, pro-apoptotic, and anti-inflammatory actions (A. Sharma *et al.* 2019; Murata *et al.* 2023; Zaid *et al.* 2022). Furthermore, chemical classes like monoterpene hydrocarbons, oxygenated monoterpenes, and sesquiterpenes found in Malaysian ginger (*Z. officinale*) also exhibit promising anticancer properties. Future research should focus on validating the efficacy of these herbal extracts in preclinical models and identifying the key molecular targets involved in their polyamine-modulating effects. Through such integrative approaches, polyamine-targeted chemoprevention could become a viable strategy for reducing the burden of cervical cancer, particularly in high-risk populations.

1.2 PROBLEM STATEMENT

Cervical cancer remains one of the most prevalent malignancies among women worldwide, particularly in low- and middle-income countries such as Malaysia. Despite the implementation of HPV vaccination and screening programmes, the incidence and mortality rates of cervical cancer remain concerning due to limitations such as incomplete vaccine coverage, low public awareness, and the inability of vaccines to treat pre-existing infections or precancerous lesions. Moreover, current treatment modalities like surgery, radiotherapy, and chemotherapy often come with significant side effects and are not universally accessible or affordable (Choi *et al*. 2023).

In the search for alternative and complementary strategies, chemoprevention has emerged as a promising approach, especially for high-risk and underserved populations. Natural products, particularly medicinal herbs traditionally used in Malay medicine, offer great potential due to their accessibility, cultural acceptance, and pharmacologically active compounds. However, the mechanistic understanding of how these herbs influence cancer-related molecular pathways—specifically the polyamine biosynthesis pathway, which is known to be dysregulated in many cancers including cervical cancer—is still limited. While studies have explored the anticancer potential of several Malaysian herbs, there is a lack of scientific evidence focusing on their role in modulating polyamine-related gene expression and inducing apoptosis in cervical cancer cells. Furthermore, the selection of herbs based on traditional knowledge has not been systematically validated using molecular and cellular approaches.

Therefore, this study addresses a critical knowledge gap by investigating the phytochemical composition, antiproliferative activity, effects on polyamine metabolism, and the mechanism of cell death induced by Syzygium aromaticum and *Elephantopus scaber* in human cervical cancer (SiHa) cells. The findings are expected to contribute to the development of culturally relevant, plant-based chemopreventive strategies aligned with global cancer prevention goals.

1.3 PURPOSE OF THE STUDY

The purpose of the study is to investigate and elucidate the potential therapeutic mechanisms underlying the use of Malaysian herbs in the prevention and treatment of cervical cancer. Cervical cancer is a significant global health concern, particularly in regions like Malaysia, where it ranks as the second most common cancer among women in the 15 to 44 age group, and identifying effective chemopreventive strategies is crucial for reducing its incidence and associated morbidity. Malaysian herbs have long been recognized for their medicinal properties and are a rich source of bioactive compounds, offer a unique resource for exploring novel chemopreventive agents. Meanwhile, the potential of polyamines as regulators of cellular processes and their implication in carcinogenesis make them an intriguing target for chemoprevention strategies. This study seeks to specifically explore the role of Malaysian herbs as cervical chemoprevention agent and its impact on polyamines pathways which also involves in numerous cellular processes.

Polyamines play pivotal roles in cell proliferation, differentiation, and apoptosis, making them intriguing candidates for investigation in the context of cancer. Previous research has hinted at the potential interactions between polyamines and bioactive compounds in herbs, but there is a dearth of comprehensive research in this area. By systematically examining how polyamines are influenced by Malaysian herbs and how these changes impact the growth and survival of cervical cancer cells, we aim to contribute to a better understanding of the molecular and cellular mechanisms underlying the chemopreventive properties of these herbs. The findings of this study are expected to have significant implications for the development of novel, nature-based therapies for cervical cancer prevention. Understanding the role of polyamines and their interactions with Malaysian herbs can potentially lead to the development of more targeted and effective chemopreventive strategies. Ultimately, this research aspires to bridge the gap between traditional Malaysian herbs and modern biomedical science, opening new direction for the development of complementary or alternative therapies for cervical chemoprevention, and potentially other types of cancer as well. As such, this study represents an important step toward improving the prevention and management of cervical cancer.

1.4 RESEARCH OBJECTIVE

1.4.1 Main Objective

The aim of this study is to investigate the potential of selected Malaysian herbs as potential chemopreventive agents in human cervical cancer cells via polyamine pathway.

1.4.2 Specific Objectives

1. To investigate antiproliferative effects of selected Malaysian herbs aqueous extracts on human cervical cancer cell line.
2. To determine the phytochemical compounds, present in aqueous extracts of selected Malaysian herbs.
3. To examine the effects of *Syzygium aromaticum* and *Elephantopus scaber* aqueous extracts on polyamine activities in human cervical cancer cell line.
4. To identify the effects of *S. aromaticum* and *E. scaber* aqueous extracts on cell cycle profile of human cervical cancer cell line.
5. To define mechanism of cell death induced by *S. aromaticum* and *E. scaber* aqueous extracts on human cervical cancer cell line.

1.5 RESEARCH QUESTIONS

1. What are the antiproliferative effects of selected Malaysian herbs' aqueous extracts on the human cervical cancer cell line?
2. What are the phytochemical compounds present in the aqueous extracts of selected Malaysian herbs?
3. What are the effects of *S. aromaticum* and *E. scaber* aqueous extracts on polyamine activities in the human cervical cancer cell line?

4. What are the effects of *S. aromaticum* and *E. scaber* aqueous extracts on the cell cycle profile of the human cervical cancer cell line?
5. What are the mechanisms of cell death induced by *S. aromaticum* and *E. scaber* aqueous extracts on the human cervical cancer cell line?

1.6 RESEARCH HYPOTHESIS

1. H_O: There are no significant antiproliferative effects of selected Malaysian herbs' aqueous extracts on human cervical cancer cell lines.
   H_1: Selected Malaysian herbs' aqueous extracts exhibit significant antiproliferative effects on human cervical cancer cell lines.
2. H_O: There are no significant phytochemical compounds present in the aqueous extracts of selected Malaysian herbs.
   H_1: There are significant phytochemical compounds present in the aqueous extracts of selected Malaysian herbs.
3. H_O: There is no significant difference in polyamine activities between the untreated and treated cells with *S. aromaticum* and *E. scaber* aqueous extracts.
   H_1: *S. aromaticum* and *E. scaber* aqueous extracts significantly alter polyamine activities in the human cervical cancer cell line compared to untreated cell.
4. H_O: *S. aromaticum* and *E. scaber* aqueous extracts do not significantly alter the cell cycle profile of human cervical cancer cell lines.
   H_1: *S. aromaticum* and *E. scaber* aqueous extracts significantly alter the cell cycle profile of human cervical cancer cell lines.
5. H_O: There is no significant difference in the mechanism of cell death between the untreated and treated cells with *S. aromaticum* and *E. scaber* aqueous extracts.
   H_1: *S. aromaticum* and *E. scaber* foot aqueous extracts induce cell death through a specific mechanism that differs significantly from the untreated cells.

1.7 SIGNIFICANCE OF THE STUDY

Developing an optimized aqueous extraction method for these herbs is valuable not only for this study but also for the broader field of herbal medicine. The cytotoxicity screening of Malaysian herbs on cervical cancer cells contributes to the identification of bioactive compounds with potential anticancer properties. Investigating the antiproliferative effects and associated signalling pathways provides insights into the mechanisms by which these herbs inhibit cancer cell growth. Meanwhile, examining the effects of Malaysian herbs on polyamine pathways in cervical cancer cells contributes to our understanding of the molecular mechanisms underlying cancer development and progression through this pathway. The study also adds to the scientific validation of traditional herbal remedies used in Malaysia, potentially supporting the integration of traditional medicine into mainstream healthcare practices.

Moreover, exploring the effects of herb treatment on different phases of the cell cycle provides insights into the specific stages at which these herbs exert their maximum impact. Defining the mechanisms of cell death induced by the aqueous extract of Malaysian herbs contributes to our understanding of how these herbs exert their anticancer effects. Given the global impact of cervical cancer, especially in developing regions, the research has the potential to contribute to global health initiatives by identifying cost-effective and accessible treatment options. The findings from the study could lay the foundation for the development of novel therapeutic strategies and interventions in cervical cancer treatment, potentially leading to the formulation of herbal-based pharmaceuticals. Overall, the research has the potential to advance scientific knowledge, contribute to the development of alternative cancer treatments, and provide insights that may have practical implications for healthcare and cancer therapy, both in Malaysia and globally.

CHAPTER TWO

LITERATURE REVIEW

2.1 THE GLOBAL HEALTH BURDEN ON CANCER

There is an urgent need to address the escalating burden of cancer, with global incidences projected to reach 24.3 million cases by 2030. Currently, the World Health Organization (WHO) reports cancer as the third leading cause of mortality, particularly prevalent in low and middle-income countries due to late diagnosis and inadequate access to quality treatment (Siegel *et al.*, 2023). In Malaysia, cancer ranks as the fourth most common cause of death, and projections suggest that its incidence will double by the year 2040, rising from 43,837 cases recorded in 2020 (Lazam *et al.*, 2023). Despite awareness of several lifestyle factors contributing to cancer, this public health crisis demands significant attention, aligning with the third Sustainable Development Goals (SDG) aimed at preventing unnecessary suffering and premature death from preventable diseases (Hamilton & Coleman, 2023; J. Zhao *et al.*, 2023).

Furthermore, statistics reveal cervical cancer as the ninth leading cause of mortality globally and the second most common cancer among women of childbearing age in Malaysia. The primary cause of cervical cancer is human papillomavirus (HPV), alongside factors such as smoking and HIV infection. Among the 200 types of HPV infection, types 16 and 18 are the most carcinogenic, accounting for over 70% of cervical cancer cases worldwide (Nelson & Mirabello, 2023; Park *et al.*, 2019). Data from Malaysia indicates higher incidence rates among Malays (49%) compared to Chinese (42%) and Indians (9%) per 100,000 population. Additionally, the 5-year survival rate is lowest among Malays (59.2%) compared to Indians (69.5%) and Chinese (73.8%) (Mustafa *et al.*, 2022; Nahrawi *et al.*, 2020). This discrepancy is further underscored by the observation that older age groups are more susceptible to cervical cancer compared to younger cohorts, highlighting the importance of preventive measures such as school health programs and HPV vaccination initiatives implemented since 2010.

2.1.1 Cervical Cancer: Treatments and Limitations

Cervical cancer typically presents no symptoms in its early stages; however, as it progresses and spreads to other areas of the body, symptoms such as bleeding, pelvic pain, and dyspareunia may manifest. Prevention strategies for cervical cancer primarily involve vaccination during preadolescence, regular screening for women, and comprehensive education for girls. Screening methods, including Papanicolaou tests and HPV genotyping, particularly targeting high-risk strains like 16 and 18, are essential for early detection (Babakanrad *et al.*, 2023). Despite advancements, lower survival rates are associated with factors such as advanced age and late-stage diagnosis (Arechkik *et al.*, 2022). Treatment for cervical cancer depends on the disease stage and extent of metastasis, typically involving surgery, chemotherapy, radiotherapy, or hysterectomy. Each treatment modality carries its own set of side effects; for example, chemotherapy may lead to hair loss, nausea, and vomiting, while radiotherapy can cause skin irritation, fatigue, and diarrhoea (Leshcheva *et al.*, 2022; Robinson *et al.*, 2023). Despite medical advancements, challenges persist in understanding the disease's etiology, pathogenesis, and diagnostic methods.

Cervical cancer pathogenesis is strongly associated with persistent infection by high-risk human papillomavirus (HPV) types, which are now considered a necessary cause of nearly all cervical cancer cases (Lee *et al.* 2022). The viral oncogenes E6 and E7 play a central role by inactivating tumour suppressor proteins p53 and pRb, respectively, leading to genomic instability, unchecked cellular proliferation, and evasion of apoptosis. This viral interference creates a microenvironment conducive to carcinogenesis and progression from normal epithelium to cervical intraepithelial neoplasia (CIN) and ultimately invasive cervical carcinoma. The long latency period between HPV infection and the development of cervical cancer provides a unique window for preventive interventions, including both vaccination and chemoprevention approaches (Roy *et al.* 2023; Sun *et al.* 2021).

The two most common types of cervical cancer are squamous cell carcinoma and adenocarcinoma. While persistent infection with HPV types constitutes the primary risk factor for cervical cancer, it is also considered a multifactorial and stepwise

carcinogenic process, involving factors such as lack of HPV vaccination, sexual behaviours (early onset of sexual activity, multiple sexual partners, or absence of protection during sexual intercourse), acquired immunodeficiency diseases (resulting from HIV/AIDS or immunosuppressive therapy), smoking, a history of sexually transmitted infections (such as herpes, syphilis, chlamydia, and gonorrhoea), low socioeconomic status, long-term use of oral contraceptives (exceeding 5 years), and multiple childbirths (Chan *et al.*, 2023; Mustafa *et al.*, 2022; Shrestha *et al.*, 2018). Achieving the elimination of cervical cancer is feasible, given the cervix's accessibility and the availability of advanced screening tests such as the Pap smear for early detection, alongside interventions such as HPV vaccination programs.

2.2 CHEMOPREVENTION IN CERVICAL CANCER

Cancer chemoprevention involves the use of natural or synthetic chemical agents to suppress, reverse, or prevent malignant cell transformation or uncontrolled cell growth. Phytochemicals, bioactive compounds found in non-nutritive plants, serve as major chemopreventive agents, acting through various molecular pathways such as anti-inflammatory effects, antioxidant activity, hormonal signalling modulation, immune response enhancement, oncogenic pathway inhibition, and DNA repair mechanism modulation (Y. Chen *et al.*, 2018; Corral & Wallace, 2020; Tuli *et al.*, 2022). These bioactive phytochemicals offer promising therapeutic benefits due to their non-toxic effects, leading to extensive research focused on natural products (M. Singh *et al.*, 2012). Notable agents studied in cervical cancer chemoprevention include retinoids, β-carotene, folate, vitamin C, difluoromethylornithine (DFMO) as a polyamine synthesis inhibitor, indole-3-carbinol as an adduct reducer, and nonsteroidal anti-inflammatory drugs (NSAIDs) (Madka *et al.*, 2023; Singh *et al.*, 2016).

Although HPV immunisation is effective in preventing new infections, particularly those involving high-risk oncogenic strains like HPV 16 and 18, it does not treat existing infections or pre-cancerous lesions. Moreover, vaccination coverage and awareness in Malaysia remain suboptimal, especially among rural and underserved populations (Rahmat *et al.* 2021; Keane *et al.* 2021). As a result, there remains a clinical

gap for women who are already infected, unvaccinated, or who develop persistent lesions despite vaccination. Therefore, chemoprevention offers a crucial complementary strategy—targeting early carcinogenic processes and providing therapeutic benefits even after HPV infection has occurred.

Chemoprevention strategies are categorized into primary, secondary, or tertiary prevention. Primary chemoprevention targets the general population or individuals at increased cancer risk, while secondary prevention focuses on patients with premalignant lesions such as cervical intraepithelial neoplasia (CIN). Tertiary prevention aims at individuals who have undergone potentially curative therapy but remain at risk for disease recurrence. These strategies utilize blocking or suppressing agents to inhibit tumorigenesis initiation or decrease the proliferative capacity of initiated cells during promotion and progression stages (Chen *et al.*, 2022; George *et al.*, 2021; Landis-Piwowar & Iyer, 2014). The role of polyamine pathways, directly or indirectly associated with oncogenes, in determining chemopreventive agent effectiveness underscores the importance of further elucidating their significance in cervical cancer.

Designing chemoprevention trials involves identifying high-risk cohorts, selecting appropriate medications, conducting phase I to III clinical studies, and incorporating surrogate endpoint biomarkers (SEBs). High-risk cohorts in cervical cancer include individuals with confirmed carcinogenic HPV infections or those engaging in risky sexual behaviour. Randomized controlled trials ensure the efficacy and safety of chemopreventive agents, with SEBs providing insights into their effects. SEBs, such as polyamines and their precursors, serve as intermediate measures of cervical chemoprevention, aiming to reduce carcinogenesis incidences. Difluoromethylornithine (DFMO), an established polyamine synthesis inhibitor, is used as a chemoprevention agent, with polyamine levels serving as SEBs detectable through urine and plasma samples.

However, challenges persist in cervical cancer treatment, including high toxicity from chemotherapeutic drugs and HPV vaccination program indifference. Elevated polyamine levels in cancer cells are often associated with increased survivability and

proliferation, leading to metastasis and recurrence. Dai *et al.* (2017) suggested a polyamine-deficient diet for cancer patients to inhibit tumour growth. Nonetheless, understanding the molecular mechanisms of polyamines in cervical cancer remains uncertain. YuChen *et al.* (2018) and YiHong Tian *et al.* (2021) demonstrated spermidine-induced growth inhibition and apoptosis in cervical cancer cells, while intrinsic metabolic polyamine expression in cervical cancer cells promotes proliferation, angiogenesis, and metastasis. Further research is essential to clarify polyamine fluctuations' role as SEBs in cervical cancer chemoprevention strategies.

Table 2.1 Cervical squamous carcinoma preventive interventions in the natural history of cervical cancer development from progressive cervical intraepithelial neoplasia (CIN)

Progression of cervical cancer	Normal	CIN 1	CIN 2/ 3	Invasive cancer
Occurrence of cervical diseases caused by HPV	70% develop cervical cancer		50% develop into CIN 3	
Types of HPV	High-risk HPV: 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70		Low risk HPV: 6,11, 42, 43, and 44	
Risk factors	High-risk HPV infection	Sexually transmitted diseases	Early coitus, multiple sex partner	Long-term use of contraceptives
Treatment of cervical cancer	Radiotherapy	Cisplatin-based chemotherapy		Radical hysterectomy
Type of prevention	**Primary**	**Secondary**		**Tertiary**
	HPV vaccination	Early screening (pap-smear) and treatment of pre-cancerous lesions		Diagnosis and treatment of invasive cervical cancer and palliative care
Chemoprevention agents in cervical cancer	Natural products (contain retinoids, β-carotene, folate, and vitamin C)	Polyamines synthesis inhibitor (DFMO)	Adduct reducers (indole-3-carbinol)	NSAIDs
Design of chemoprevention trials	Identify high risk cohorts	Suitable medication	Include phase I, II, and III trials	Include use of SEB

SEB, surrogate endpoint biomarkers; DFMO, α-difluoromethylornithine; NSAIDs, non-steroidal anti-inflammatory drugs

2.3 NATURAL PRODUCTS AS CHEMOPREVENTIVE AGENTS

The development of natural products as chemopreventive agents involves a systematic and rigorous process to ensure their efficacy and safety. The National Cancer Institute (NCI) has outlined a comprehensive protocol to facilitate this development, focusing on critical steps such as bioactive compound identification, preclinical evaluation, clinical trials, and eventual regulatory approval and integration into public health practices (Flaherty *et al.*, 2020). The initial step in this process involves the identification of bioactive compounds with potential chemopreventive properties. High-throughput screening of natural product libraries is employed to discover compounds exhibiting activity against specific cancer-related pathways. Programs such as the NCI's Discovery and Development of Natural Products for Cancer Interception and Prevention (DDNP-CIP) aim to uncover safe and non-toxic natural agents for cancer prevention, highlighting the importance of early-stage identification and evaluation (Harkenrider *et al.*, 2020).

Following the discovery phase, these compounds undergo preclinical evaluation to assess their efficacy and safety. This step includes *in vitro* studies on cancer cell lines and *in vivo* experiments using animal models to elucidate mechanisms of action and determine optimal dosing regimens. The DDNP-CIP program emphasizes the development of robust high-throughput screening methods and specific assays to identify non-toxic agents effectively (George *et al.*, 2019). Importantly, many plant-derived compounds, particularly those used in traditional medicine, have demonstrated excellent safety profiles in both experimental and clinical settings. Malaysian herbs such as *Syzygium aromaticum* (clove) and *Elephantopus scaber* have been widely used in ethnomedicine for their antimicrobial, antioxidant, and anti-inflammatory properties (Mohd Afeendi *et al.* 2017). Studies have shown that aqueous extracts of these herbs exert cytotoxic effects selectively on cancerous cells without significantly affecting normal cells, indicating a high therapeutic index and favourable safety margin (S. Sharma 2023; Chugh *et al.* 2018; Aiza *et al.* 2020).

Safety assessments remain a cornerstone in the development of chemopreventive agents. Although herbal medicines are often perceived as safe due to their natural origin, increasing evidence shows that they may contain adulterants or bioactive compounds that can cause adverse reactions, particularly when consumed in high doses or over extended periods (Roziman *et al.* 2024). For instance, data from Malaysia revealed that many adulterated traditional herbal medicine (THM) products originated from abroad, with some linked to adverse reactions involving cardiac, respiratory, and reproductive systems—especially when spiked with steroids or other pharmaceuticals (Baihaqi and Levita 2021). Furthermore, studies have reported hepatotoxicity and nephrotoxicity associated with certain herbal plants commonly used in Asia, particularly when consumed at high doses or for prolonged durations (Philips *et al.* 2020). While the majority of adverse effects were mild and involved a small portion of users, the potential for harm underscores the need for rigorous toxicological evaluation.

In Malaysia, several herbs traditionally used in ethnomedicine have undergone acute and sub-chronic toxicity studies to determine their safety margins. For example, *Clinacanthus nutans* polysaccharide extract, when administered orally up to 3,000 mg/kg, did not induce observable acute toxicity, though mild liver enzyme elevation was noted at doses ≥500 mg/kg/day over 14 days (Chia *et al.* 2025). Likewise, repeated oral administration of *Elephantopus scaber* extract up to 2000 mg/kg showed no significant toxicity in rats (Ho *et al.* 2012), and *Syzygium aromaticum* extract exhibited a high safety margin in both *in vitro* and *in vivo* studies (Saeed *et al.* 2017). These findings suggest that many Malaysian herbs are safe within defined therapeutic ranges, but careful dose optimisation remains essential.

Moreover, the belief that "natural means safe" has been challenged by increasing reports of herb-drug interactions and potential toxicity. As many herbal compounds can act as agonists or antagonists of drug-metabolising enzymes, understanding their pharmacokinetics is vital for preventing adverse events, particularly in patients on concurrent conventional therapies (Kim *et al.* 2013). Therefore, safety assessments not only include toxicological profiling but also awareness of potential interactions and adulteration risks. To support safe chemopreventive application, identifying the

optimum dose that balances efficacy and safety is essential. The therapeutic window must be carefully established through controlled *in vitro* and *in vivo* studies. This process ensures the selection of the most suitable herbal candidates for further clinical testing and public health integration. With increasing demand for herbal-based health products, especially in developing countries, such assessments play a critical role in ensuring quality, safety, and efficacy (Ariffin *et al.* 2021; Baihaqi and Levita 2021).

Compounds showing promise in preclinical testing are then advanced to clinical trials, which are conducted in three phases. Phase I focuses on safety and dosage, Phase II evaluates efficacy and side effects, and Phase III compares the new agent with standard treatments. Clinical trials ensure that only agents demonstrating significant benefits with acceptable safety profiles progress further. The NCI's Division of Cancer Prevention (DCP) provides funding and support to encourage research into novel agents with potential chemopreventive effects (Grkovic *et al.*, 2020; Rybak *et al.*, 2023).

The final stage involves regulatory approval and public health integration. After successful clinical trials, regulatory bodies thoroughly review the new agent to ensure its safety and efficacy. Once approved, strategies are developed to incorporate the agent into public health policies, including guidelines for specific populations and long-term monitoring. Programs such as the DDNP-CIP play a pivotal role in advancing research and integrating these findings into cancer prevention strategies (Grkovic *et al.*, 2020; Stefanska *et al.*, 2012; Tracy *et al.*, 2016). By following this structured approach, researchers can ensure that natural products developed as chemopreventive agents are both safe and effective. These protocols contribute significantly to the advancement of cancer prevention, reducing the incidence of cancer through the targeted use of natural compounds.

To ensure optimal outcomes, dose standardisation and herb selection must be prioritised. As efficacy and toxicity are highly dose-dependent, identifying the minimum effective concentration that elicits chemopreventive activity without harming normal cells is crucial (Ismail *et al.* 2022; Lee *et al.* 2021). Additionally, among the vast repertoire of Malaysian herbs, selection based on mechanistic evidence—such as modulation of polyamine metabolism, antioxidant pathways, and caspase activation—

can help identify the most promising candidates for further translational and clinical research. Integration of these safe, culturally relevant herbal extracts into preventive care may enhance accessibility and acceptance, particularly in resource-limited settings where cervical cancer burden remains high.

2.3.1 Terpenoids-rich natural products as chemoprevention agent

Terpenoids, also known as terpenes, exhibit significant chemopreventive potential against cervical cancer due to their ability to modulate key cellular pathways involved in carcinogenesis (Vemula *et al.*, 2019). Their anti-inflammatory and antioxidant properties help counteract oxidative stress and chronic inflammation, both of which contribute to cervical cancer progression (Parveen *et al.*, 2023; W. Yang *et al.*, 2020). Additionally, terpenoids exert anti-proliferative effects by interfering with cancer cell growth and division, inducing cell cycle arrest, and suppressing oncogenic signalling pathways. Their pro-apoptotic activity enhances programmed cell death in HPV-transformed cervical cancer cells by regulating intrinsic and extrinsic apoptotic pathways, including mitochondrial dysfunction and death receptor activation (Chen *et al.* 2018; Singh *et al.* 2016).

Studies have demonstrated that different classes of terpenoids, such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes, exhibit strong cytotoxic effects against cervical cancer cells by targeting polyamine metabolism, inhibiting angiogenesis, and modulating tumour suppressor and oncogene expression (Imran *et al.*, 2020; Siraj *et al.*, 2021). Many Malaysian herbs, including clove (*Syzygium aromaticum*), elephant's foot (*Elephantopus scaber*), *ulam raja* (*Cosmos caudatus*), hibiscus (*Hibiscus rosa-sinensis*), *bunga telang* (*Clitoria ternatea*), and cinnamon (*Cinnamomum spp.*), are rich sources of terpenoids with chemopreventive properties. These herbs have been reported to influence polyamine biosynthesis, disrupt cancer cell proliferation, and induce apoptosis in cervical cancer models. Given their diverse pharmacological actions, terpenoid-rich Malaysian herbs present a promising natural strategy for cervical cancer prevention and therapy.

Terpenoids exert their chemopreventive effects on cervical cancer through multiple mechanisms, targeting key hallmarks of cancer progression. One major mode of action involves the regulation of polyamine metabolism, which is often dysregulated in HPV-induced cervical cancer. By inhibiting ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, terpenoids help reduce intracellular polyamine levels, thereby suppressing cancer cell proliferation and enhancing apoptosis (S. H. Sharma *et al.* 2017; Afshar *et al.* 2017; D. J. Kim *et al.* 2017). Additionally, terpenoids modulate key signalling pathways such as PI3K/Akt/mTOR, MAPK/ERK, and NF-κB, which are critical for cervical cancer cell survival, growth, and resistance to therapy (Pandey *et al.*, 2025). Their ability to downregulate pro-survival proteins, such as Bcl-2 and survivin, while upregulating pro-apoptotic factors, such as Bax, cytochrome c release, and caspase activation, reinforces their role in promoting apoptosis and sensitizing cervical cancer cells to chemotherapeutic agents (El-Baba *et al.*, 2021; Silva *et al.*, 2024).

Furthermore, terpenoids contribute to chemoprevention by disrupting the tumour microenvironment, particularly through the inhibition of angiogenesis and inflammation. By downregulating vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), terpenoids reduce blood vessel formation and limit cancer cell invasion and metastasis (Kumar *et al.*, 2016; Simha *et al.*, 2017). Their anti-inflammatory effects, mediated by the suppression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), further prevent the chronic inflammatory responses that support cervical carcinogenesis (H. Sun *et al.* 2021). Collectively, these mechanisms highlight the therapeutic relevance of terpenoid-rich Malaysian herbs in targeting multiple cancer-promoting pathways, making them valuable candidates for cervical cancer prevention and treatment (Ismail *et al.*, 2023; Zaid *et al.*, 2022).

2.4 ROLES OF POLYAMINES IN CELLULAR FUNCTIONS

2.4.1 Overview of Polyamine Pathways and its Physiological Functions

In normal physiological conditions, polyamine metabolism is regulated by a complex network of biosynthesis, catabolism, and transport system that mainly occurs in the cytoplasm where every mechanism involves several gene-related enzymes. PA and their derivatives have application in the clinical diagnosis and treatment of cancer as their concentration was commonly elevated in blood plasma and urine of cancer patients as well as determination of the success of therapy.

2.4.1.1 Polyamine synthesis

The protagonists of polyamine biosynthesis include the production three characters: putrescine, spermidine, and spermine the most abundant types of polyamines. The key rate-limiting enzyme for polyamine biosynthesis is ornithine decarboxylase (ODC) where ornithine that produced from arginase, by-product of urea cycle is then catalysed by ODC to generate putrescine. To produced spermidine and spermine from putrescine, S-adenosyl methionine (SAM) produced from methionine act as the precursor just like ornithine undergo decarboxylation with the aid of SAM decarboxylase to become decarboxylated SAM (providing the aminopropyl group) and react with putrescine and enzymatic transfer reaction of spermidine synthase and spermine synthase producing spermidine and spermine respectively.

To be catalytically active, ODC (non-dimer) requires co-factor (pyridoxal 5'-phosphate, PLP) to become homodimer. To maintain normal cellular proliferation, regulation of polyamine synthesis is imposed by antizyme (AZ1) by inactivating ODC activity and enhancing its degradation when combined. AZ1 decrease the production of putrescine by three mechanisms; (1) disrupting the active ODC homodimer; (2) targeting ODC monomer for ubiquitin-independent degradation by the 26S proteasome; and (3) inhibit polyamine transporter that mediated the polyamine uptake into cells.

Conversely, another responsible regulator of ODC activity is antizyme inhibitor (AZIN) where it is a positive regulator of polyamine levels by binding to AZ1 and suppressing its ODC-inhibitory effects whenever there is drought of bioavailability of polyamines occur. Thus, both AZ1 and AZIN are part of opposing autoregulatory circuits designed to maintain optimal levels of polyamines within a cell.

The activity of ODC in cancer cells are reportedly to be consistently increased in many types of cancer such as neuroblastoma, liver, cervical, breast, lung and prostate cancers which directly related with high occurrence and progression of malignancy (He *et al.*, 2017; Svensson *et al.*, 2008; Wang & Jiang, 2014). Its availability also influenced by variety of stimuli such as hormones, growth factors, oncogenes, and free polyamines. Elevated levels of ODC expression are associated with cancer development and excess accumulation of polyamines has a cytotoxic effect on cells resulting from gene amplification, mutations, or altered transcriptional regulation (Zhu *et al.*, 2023). Moreover, dysregulated cancer cells may also display increased stability of ODC protein, leading to its accumulation and higher enzymatic activity. Reduce level of AZ1 in cancer cells on the other hand occur due to alterations in transcriptional regulation and post-translational modification in AZ1 protein, such as phosphorylation or ubiquitination, which can impact its stability or binding to ODC thus increase polyamine synthesis (Qiu *et al.*, 2017). Targeting these dysregulated components has been explored as a potential therapeutic strategy for inhibiting polyamine synthesis and treating cancer.

2.4.1.2 Polyamine catabolism

Polyamine levels in cells can be controlled through their breakdown, facilitated by three enzymes: spermidine/spermine-N-acetyltransferase (SSAT), polyamine oxidase (PAO), and spermine oxidase (SMO). SSAT acetylates spermidine and spermine, forming N-acetyl spermidine and N-acetyl spermine, respectively, which are further metabolized by PAO through oxidative deamination. SMO directly oxidizes spermine to spermidine, while spermidine converted to putrescine can be degraded into ammonia (NH3) and carbon dioxide (CO2). SSAT expression is tightly regulated to maintain

polyamine homeostasis, crucial for cell function. Increased SSAT activity reduces the metabolic flow of free polyamines induced by various stimuli, contributing to cellular damage in several cancer types. Furthermore, SMO and PAO promote oxidative changes, generating reactive oxygen species (ROS) detrimental to cancer cells, ultimately leading to cell death (Chan *et al*. 2015; Pathak *et al.* 2020; Wang *et al*. 2011).

2.4.1.3 Polyamine transport system (PTS)

There are several explanations of cellular polyamine transport system (PTS) through active transportation depending on the saturation, temperature, and energy level as they cannot adopt the passive transport through plasma membrane due to protonated state at physiological pH. The major theory is the normal transport of PA is mediated by endocytosis by knocking-out of caveolin-1 as its absence positively regulates polyamine internalization. The endocytosis process requires transporters or solute carriers; SLC3A2, SLC7A2, and SLC22A16 where polyamines bind to the polyamine binding proteins. SLC3A2 acts as a regulatory subunit and interact with SLC7A2 serves as the transport subunit to form a high-affinity transporter that facilitates the uptake of putrescine and acetylated polyamines into the cells. SLC22A16 transporter on the other hand involves in the transport of spermine and plays a role in maintaining intracellular PA levels and preventing PA accumulation.

Additionally, investigation on other mechanism that regulates PTS suggested that secretion of glutathione S-transferase II (GSTII) from knocking out of caveolin-1 have encouraged higher spermidine transport in human cancer cells therefore promotes the polyamine levels into the cells (Uemura *et al*. 2010). Moreover, other theory suggests that heparan sulphate and glypican-1 (GPC1) co-ordinately transport spermine. Spermine interacts with the heparan sulphate group in GPC1 on the cell surface and enters into the cell. Spermine is then released through nitrogen oxide mediated oxidation to act on cells. Alternatively, it is thought that polyamines are transported into cells through an unrecognized transporter driven by membrane potential. Polyamine penetrates the cell through the plasma membrane. Then, the accumulated polyamines are subsequently localized in polyamine isolation vesicles, which relies on the vacuoles

ATPase pH gradient and proton exchange, illustrating the two-step mechanism of polyamine transport and vesicle chelation. Investigation and further studies in combating carcinogenesis through chemoprevention by targeting PTS have yet been fully elucidated.

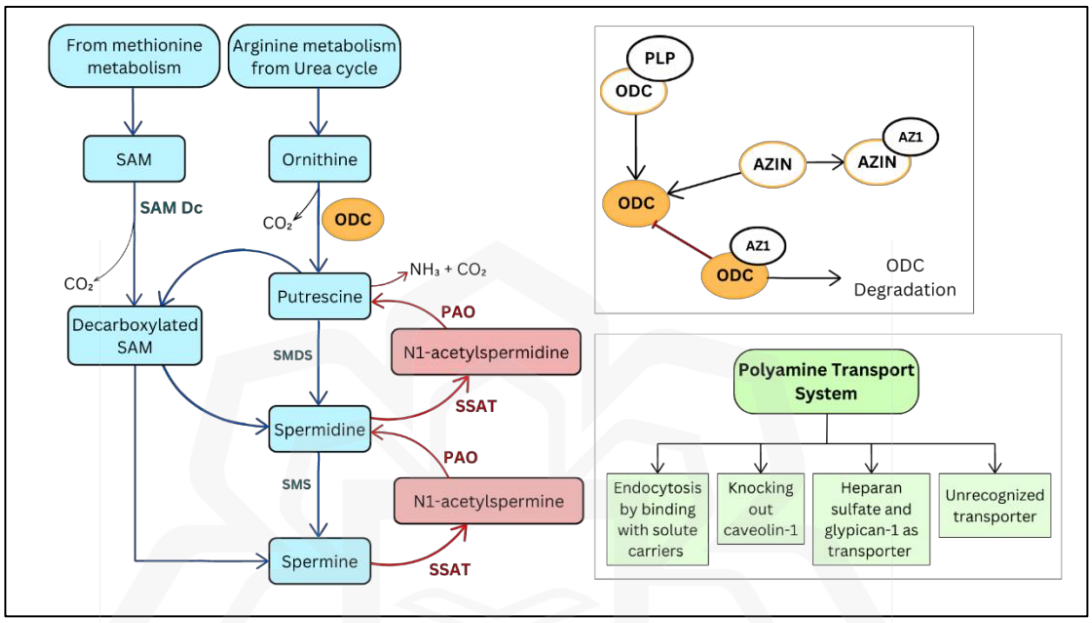


Figure 2.1 Polyamine pathway through polyamine synthesis, catabolism, and transport system (Maryam *et al.*, 2023)

2.4.1.4 Role of Polyamines in Cancer Development and Progression

Polyamines are classified as small organic cations which are essential for normal cell growth and proliferation. In healthy cells, sufficient cellular polyamine levels are accomplished by a precise proportion between cellular uptake of polyamines from diet, biosynthesis and intestinal microorganisms. Three most prevalent aliphatic compounds of polyamines present in the cells of living organisms are putrescine, spermine and spermidine as they involved in a lot of physiological roles such as growth, development and tissue repair (Akinyele and Wallace 2022). Figure 2.1 depicted the polyamine pathway or metabolism that involves biosynthesis, catabolism and transport that occur in cellular cytoplasm where every mechanism requires several gene-related enzyme.

The main pathway of polyamine biosynthesis is the decarboxylation of ornithine catalysed by ornithine decarboxylase (ODC) to generate putrescine. After that, spermidine and spermine are produced by the enzymatic transfer reaction of spermidine synthase (SPDSYN) and spermine synthase (SPMSYN) respectively. Whereas, spermidine and spermine can also be produced from enzymatic reaction of decarboxylated S-adenosyl methionine that catalysed by S-adenosyl methionine decarboxylase (SAM Dc) from S-adenosyl methionine.

However, in the case of high bioavailability of cellular polyamines (PA), healthy normal cells will be able to control the imbalance by degrading the production of those three types of PA. The catabolism of reducing the availability of putrescine could be accomplished through inactivation of ODC activity and enhancing its degradation by antizyme (AZ), the regulator in PA homeostasis. Whereby, spermine and spermidine can be degraded with enzyme N1-Acetylpolyamine oxidase (APAO) and spermidine/spermine N-1 acetyltransferase (SSAT). Dysregulation of PA metabolism can be observed in many types of cancers where PA accumulates at high levels in tissues undergoing rapid growth and its biosynthesis were found to be correlated with the development of several pathological states of cancer. Therefore, anti-cancer drugs had been developing since 1960s targeting polyamine pathway because of its potential as cancer treatment (Casero *et al.* 2018).

Increasing levels of polyamines have often been associated with colon, breast, prostate, lung and skin cancers (Evageliou *et al*. 2016; Harbison *et al.* 2022; Corral and Wallace 2020). In neoplastic cells, loss of polyamine homeostasis occurs and is accompanied by dysregulated proliferation involving upregulated biosynthesis, downregulated catabolism, and increased uptake. Occurrence of interruption of polyamines level in cancer patients usually can be detected through high expression of PA in urine and blood. When malignant tumour grows in size, new blood vessels will also increase in number (angiogenesis) that resulted in limited oxygen supply and give hypoxic internal microenvironment in both normal and tumour cells. The synthesis of intracellular PA will then be limited; thus cells will increase its absorption of PA from surrounding to compensate the depletion. Linsalata *et al*. (2014) suggested that,

avoiding or limiting dietary intake of polyamines may reduce the likelihood of cancer development especially in the high-risk population.

Based on these observations and the unequivocal necessity for polyamines in tumours growth, the polyamine pathway is a suitable target for chemoprevention and chemotherapeutics (Long *et al.* 2024). Another study done by Wu *et al.* (2018) on the role of dietary polyamines in preventing colorectal cancer has strongly suggested that a compelling interaction was identified between dietary polyamine group and treatment in relation to cancer recurrence. Due to the significant relationship between polyamines and cancer development, several efforts were made to analyse the polyamine content of common food in order to reduce the dietary intake of polyamine in recovering cancer patient and also as chemo preventive strategy among high-risk group of getting cancer.

2.4.1.4.1 Role of Polyamines in Gene Expression

Polyamines also play critical roles in regulating gene expression in both cancer cells and normal cells, but their effects can vary significantly depending on the cellular context and the specific genetic and epigenetic alterations present in cancer cells. In normal cells, polyamines regulate gene expression by influencing chromatin structure and function. They can interact with histones, the proteins that package DNA into chromatin, to modulate chromatin compaction and accessibility to transcription factors and RNA polymerase (Islam *et al*., 2022; Lu *et al*., 2022). Additionally, polyamines can directly bind to specific DNA sequences, known as polyamine-responsive elements, to regulate the expression of target genes involved in cell growth, differentiation, and apoptosis. For example, studies have shown that polyamines can stimulate the expression of genes involved in cell cycle progression and DNA replication, promoting cell proliferation in normal cells.

Meanwhile, dysregulation of polyamine metabolism in cancer cells is a common feature, leading to altered gene expression patterns that contribute to tumourigenesis

and tumour progression. Cancer cells often exhibit increased polyamine synthesis and uptake, resulting in elevated intracellular polyamine levels compared to normal cells. This dysregulated polyamine metabolism can drive aberrant gene expression programs that support cancer cell growth, survival, and metastasis (Galiana-Roselló *et al.*, 2020; Tang *et al.*, 2023). Research has revealed several mechanisms by which polyamines influence gene expression in cancer cells. For instance, polyamines can interact with oncogenes and tumour suppressor genes to modulate their expression and activity, thereby promoting cancer cell proliferation and survival. Additionally, polyamines can regulate the expression of genes involved in angiogenesis, invasion, and metastasis, facilitating tumour progression and spread (Brel *et al.*, 2011; Syatkin *et al.*, 2020a).

Furthermore, polyamines have been implicated in the regulation of epigenetic modifications, such as DNA methylation and histone acetylation, which can alter gene expression patterns in cancer cells. Studies have shown that polyamines can affect the activity of enzymes involved in epigenetic regulation, leading to changes in chromatin structure and gene transcription that contribute to cancer development and progression (Stewart *et al.*, 2022; Zeyen *et al.*, 2022). Overall, the roles of polyamines in regulating gene expression in cancer cells versus normal cells are complex and multifaceted. While polyamines play essential roles in both contexts, dysregulated polyamine metabolism in cancer cells can lead to aberrant gene expression patterns that drive tumorigenesis and tumour progression. Understanding the molecular mechanisms underlying the effects of polyamines on gene expression in cancer cells is crucial for identifying novel therapeutic targets and developing strategies to selectively target cancer cells while sparing normal cells.

2.4.1.4.2 Role of Polyamines in Cellular Proliferation

Polyamines are essential for promoting normal cell proliferation by facilitating processes such as DNA synthesis, cell cycle progression, and mitotic spindle assembly. Polyamines interact with various cellular components involved in these processes, including enzymes responsible for DNA replication and repair, as well as proteins that regulate cell cycle checkpoints. Additionally, polyamines influence the activity of

signalling pathways that control cell growth and proliferation, such as the mTOR pathway. Research has shown that polyamines, particularly spermidine, are required for the efficient progression through the cell cycle and for maintaining the proliferative capacity of normal cells (J. Zhu *et al.*, 2022). Furthermore, polyamines have been implicated in the regulation of other cellular processes that influence cell proliferation in cancer cells, such as angiogenesis, invasion, and metastasis. Polyamines can stimulate the expression of genes involved in these processes, as well as interact with signalling pathways that control cell migration and invasion.

Conversely, dysregulated polyamine metabolism is a hallmark of cancer cells, marked by increased synthesis and uptake, which drives uncontrolled proliferation. Elevated polyamine levels in cancer cells foster aberrant cell cycle progression and hinder apoptosis, contributing to tumour growth and progression. Research has provided insights into the mechanisms by which polyamines influence cell proliferation in cancer cells. One key mechanism involves the interaction of polyamines with the eukaryotic initiation factor-5A (eIF-5A) protein, which plays a crucial role in protein synthesis and cell growth. Polyamines are required for the activation of eIF-5A through a process known as hypusination, which is essential for its function in promoting the translation of specific mRNAs encoding proteins involved in cell proliferation and survival. Dysregulated polyamine metabolism in cancer cells can lead to aberrant eIF-5A activation, resulting in the dysregulation of protein synthesis and the promotion of tumour growth (Coni *et al.*, 2020, 2023; Schultz *et al.*, 2018).

Beyond mere proliferation, polyamines also exert their influence over cellular signalling pathways implicated in cancer progression. These molecules serve as clandestine intermediaries, covertly modulating the activity of various signalling molecules and pathways to promote tumour growth and survival. For instance, polyamines have been implicated in the activation of the mTOR pathway, a central regulator of cellular growth and metabolism frequently dysregulated in cancer. By tweaking the knobs and levers of signalling cascades like mTOR, polyamines effectively tip the scales in favour of tumour progression, steering cancer cells toward a path of relentless proliferation and survival (Akinyele & Wallace, 2022; Jiang *et al.*, 2007; Novita *et al.*, 2021).

Furthermore, polyamines play a pivotal role in angiogenesis, the process by which new blood vessels form to supply tumours with oxygen and nutrients. In the tumour microenvironment, polyamines aid in the construction of a complex network of blood vessels to fuel the growth of proliferating cancer cells. By promoting angiogenesis, polyamines ensure that tumours receive a steady influx of vital resources, allowing them to flourish and expand within the body (L. Gao *et al.*, 2020; Y. Zhang *et al.*, 2022).

Polyamines emerge as formidable architects of cancer development and progression, wielding their influence over a myriad of cellular processes critical to tumour growth, survival, and metastasis. By manipulating cell proliferation, signalling pathways, angiogenesis, metastasis, and immune evasion, polyamines play a role in molecular events that brings cancer cells toward malignancy. Understanding the intricate roles of polyamines in cancer biology holds promise for the development of novel therapeutic strategies aimed at disrupting these malignant machinations and curbing the relentless progression of cancer.

2.4.2 Polyamines Contents Classification in Plants Origin

Dietary intake plays a crucial role in regulating intracellular polyamine concentrations. Consuming foods rich in polyamines can lead to an increase in their levels within the body, which may influence various cellular processes, including cell proliferation and survival. For cancer patients, this is particularly concerning, as elevated polyamine availability has been associated with enhanced tumour growth, increased cell proliferation, and resistance to apoptosis, all of which can contribute to disease progression. Studies have shown that tumour cells exhibit an increased dependency on polyamines to sustain their rapid growth and metabolic demands (Casero *et al.*, 2018; Chin *et al.*, 2022). Therefore, excessive dietary polyamine intake may further fuel cancer cell survival, making dietary control an important consideration for patients undergoing treatment or at risk of tumour recurrence.

Given the well-established role of polyamines in cancer progression, it is advisable for cancer patients to monitor and, if necessary, limit their consumption of foods high in polyamines. A diet low in polyamines may help regulate intracellular levels, potentially slowing tumour progression and improving patient outcomes. Several dietary strategies have been proposed to manage polyamine intake, including adopting low-polyamine diets or combining dietary interventions with polyamine-inhibiting drugs, such as difluoromethylornithine (DFMO), which suppresses ornithine decarboxylase (ODC) activity and polyamine biosynthesis (Schultz *et al.*, 2021; Sholler *et al.*, 2015; Y. Zhang *et al.*, 2022). The combination of dietary polyamine restriction with pharmacological intervention may provide a more effective approach to controlling polyamine metabolism in cancer therapy.

To better assess dietary sources of polyamines, Muñoz-Esparza *et al.* (2021) proposed a classification system for polyamine content in plant-derived foods. According to their guidelines, polyamine levels can be categorized into low, high, and very high concentrations. A low polyamine content is defined as having a total polyamine concentration of less than 23.2 nmol/mg, while high and very high levels exceed this threshold (Table 2.2). Such classifications are useful in guiding dietary recommendations, particularly for cancer patients who may benefit from reducing their intake of polyamine-rich foods to limit tumour-promoting effects.

Table 2.2 Classification of polyamines level in plant origin

Classification	**Putrescine (nmol/mg)**	**Spermine (nmol/mg)**	**Spermidine (nmol/mg)**	**Total PA (nmol/mg)**
Low	<11.3	<6.9	<4.9	<23.2
High	<113.4	<68.9	<49.4	<231.7
Very high	>113.4	>68.9	>49.4	>231.7

2.5 TARGETING CELL DEATH AND CELL CYCLE PROGRESSION IN CERVICAL CANCER

Cervical cancer progresses due to disruptions in cell cycle regulation and apoptosis, enabling malignant cells to proliferate uncontrollably while evading programmed cell death. These two processes play a critical role in maintaining cellular homeostasis, as cell cycle checkpoints control whether a cell proceeds with division, repairs DNA damage, or undergoes apoptosis. When these mechanisms become dysfunctional, cancerous cells gain a survival advantage, making them highly resistant to treatment. Understanding the molecular alterations underlying these pathways is essential for developing effective chemopreventive strategies to slow or prevent tumour progression.

The cell cycle is controlled by a network of regulatory proteins, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs), which ensure the proper progression of cells through different phases (Arisan *et al.*, 2014). In cervical cancer, this regulation is often disrupted due to the overexpression of CDKs and cyclins and the downregulation of tumour suppressors, allowing continuous cell division. For example, CDK4 and CDK6, which govern the G1/S phase transition, are frequently upregulated in cervical carcinoma, while p21^WAF1, p27^KIP1, and p16^INK4A, which normally inhibit CDK activity, are often silenced. This imbalance promotes unchecked proliferation and disease progression (Y. Li *et al.*, 2015; Tavakolian *et al.*, 2020). Furthermore, in HPV-infected cells, the E7 oncoprotein degrades retinoblastoma protein (pRb), releasing E2F transcription factors that push the cell into the S phase, bypassing normal regulatory control. Meanwhile, E6 promotes the degradation of p53, preventing DNA damage-induced apoptosis. These molecular changes drive genomic instability and persistent proliferation, contributing to cervical carcinogenesis (Harbison *et al.*, 2022).

Uncontrolled proliferation alone is not sufficient for cancer progression—evading apoptosis is equally crucial for cervical cancer cells to thrive. Apoptosis, a tightly regulated process of programmed cell death, is primarily mediated by two pathways: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor-mediated) pathway (Bhoora *et al.*, 2022). In cervical cancer, several apoptotic regulators

are altered, including Bcl-2 family proteins, caspases, and inhibitors of apoptosis proteins (IAPs) (Y. Chen *et al.*, 2018). Survivin, a key IAP that inhibits caspase activation, is overexpressed in cervical cancer, leading to treatment resistance and enhanced cancer cell survival. Additionally, pro-apoptotic genes like BAX and BID are often suppressed, further shifting the balance in favour of cell survival (L.-M. Zhu *et al.*, 2021). This imbalance allows cervical cancer cells to resist natural cell death mechanisms and persist in the body.

Therapeutic strategies that target cell cycle dysregulation and apoptosis resistance may enhance the effectiveness of chemopreventive interventions. Several natural compounds and chemotherapeutic agents have been shown to restore apoptosis sensitivity and induce cell cycle arrest in cervical cancer cells (Delgado *et al.*, 2023). For instance, isoliensinine, a plant-derived alkaloid, inhibits the AKT/GSK3α pathway, triggering apoptosis and slowing cell cycle progression. Additionally, re-activation of tumour suppressor genes such as RASSF1A, frequently silenced by promoter hypermethylation, has been associated with restoring apoptotic function in cervical carcinoma. These findings highlight the potential of targeting cell cycle checkpoints and apoptotic regulators in the prevention and treatment of cervical cancer (Izbicka *et al.*, 2010).

One of the main targets for chemopreventive agents is the G1/S checkpoint, which controls the transition from growth to DNA synthesis. Under normal conditions, pRb binds to E2F transcription factors, preventing premature cell cycle progression. However, in HPV-infected cells, E7 degrades pRb, leading to continuous cell proliferation (Zhao *et al.*, 2016). Similarly, E6 targets p53, preventing apoptosis and further promoting tumour progression. This dysregulation results in accumulated genetic mutations and tumour initiation (Tavakolian *et al.*, 2020). Natural bioactive compounds, such as those from clove (*Syzygium aromaticum*) and elephant's foot (*Elephantopus scaber*), have been shown to downregulate cyclins (Cyclin D1, Cyclin E), inhibit CDKs, and upregulate CDK inhibitors (p21, p27), thereby inducing G1/S and G2/M cell cycle arrest (Mostafa *et al.*, 2023). By delaying the cell cycle at these critical checkpoints, these bioactive compounds restrict cancer cell proliferation and reduce tumour growth potential.

Apoptosis resistance is another defining feature of cervical cancer, allowing cells to evade death signals despite accumulating DNA damage. The intrinsic apoptosis pathway, regulated by Bcl-2 family proteins, is often disrupted, with Bcl-2 and Bcl-xL overexpressed, while pro-apoptotic proteins like Bax and Bak are suppressed (Kurnia *et al.*, 2021; Lin *et al.*, 2014). This shift in balance promotes survival and resistance to therapy. Similarly, IAPs such as survivin are highly expressed in cervical cancer, further preventing caspase activation and suppressing apoptosis (Li *et al.*, 2019). Studies have shown that natural compounds like eugenol, flavonoids, and terpenoids from Malaysian herbs can induce apoptosis by upregulating pro-apoptotic proteins (Bax, NOXA, PUMA), downregulating anti-apoptotic proteins (Bcl-2, survivin), and activating caspases (-3, -8, -9) (Alexiou *et al.*, 2019). Notably, *E. scaber* extract has been reported to activate caspase-8, suggesting involvement in extrinsic apoptosis, whereas *S. aromaticum* extract predominantly triggers caspase-9 activation, indicating mitochondria-mediated apoptosis.

Targeting cell cycle regulation and apoptosis pathways in cervical cancer is a crucial strategy for chemoprevention. The ability of bioactive compounds to restore cell cycle checkpoints and induce apoptosis provides a valuable alternative to conventional therapies (Zhou *et al.* 2016). Furthermore, the involvement of polyamine metabolism in both cell proliferation and apoptosis resistance highlights the significance of integrating polyamine-targeted interventions into cervical cancer treatment strategies (Yi *et al.* 2015). Natural compounds from Malaysian herbs, hold promise in modulating polyamine metabolism, disrupting uncontrolled proliferation, and restoring apoptosis sensitivity, making them strong candidates for chemopreventive applications in cervical cancer (Atiya *et al.* 2011; Davis *et al.* 2014; Samoylenko *et al.* 2011).

2.6 MALAYSIAN HERBS AS POTENTIAL CHEMOPREVENTIVE AGENTS

Natural products, contain reservoirs of bioactive compounds like phytochemicals, offers various potential of therapeutic interventions, including anti-inflammatory, antioxidant, and hepatoprotective effects, alongside potent anticancer properties. Approximately 25% of recently approved anticancer drugs trace their origins to natural products, spanning microbes, plants, marine environments, slime moulds, and various living organisms, following extensive clinical evaluations for efficacy and safety. Medicinal plants, some mentioned in the Quran and utilized in traditional remedies, are undergoing rigorous scrutiny as potential chemopreventive agents through *in vitro* and *in vivo* research efforts. These herbs and spices, embedded in historical and cultural contexts, hold immense promise for biomedical research endeavours, offering a treasure trove of chemo preventive leads worthy of exploration (Kim *et al.* 2016; Ismail *et al.* 2022).

The introduction of natural products into chemoprevention strategies hinges on their ability to modulate polyamine metabolism within cancer cells, depriving intracellular polyamine synthesis. Accessible, cost-effective, and with a documented history of safe use, herbs and spices represent prime candidates for chemoprevention due to their diverse bioactive compounds. Certain herbs have demonstrated selective toxicity against cancer cells while exerting protective effects on normal cells, underscoring their potential as safe and efficacious chemopreventive agents.

Herbs and fruits mentioned in the Quran, such as olive, ginger, banana, and fig, have garnered attention for their anti-carcinogenic, anti-proliferative, and anti-angiogenesis properties against various cancer types, including cervical, breast, liver, and colorectal cancers (Radiah *et al.* 2019). Additionally, herbs like onion, fenugreek, wild thyme, and beetroot exhibit cytotoxicity against liver, breast, and cervical cancer cells, as evidenced by *in vitro* studies. These findings underscore the potential of Quranic herbs as invaluable resources for cancer chemoprevention, offering novel avenues for therapeutic exploration and intervention (H. Zaid *et al.* 2012; Kamarubahrin *et al.* 2020).

Moreover, Islamic herbal medicine traditions advocate for cancer prevention and treatment through natural products, as referenced in the Quran and Hadith. Dietary interventions and the use of natural products hold significant sway in combating cancer, with the Quran serving as a source of inspiration and guidance for therapeutic endeavours. Scholars emphasize the importance of early cancer detection and prevention through dietary modifications and the targeted use of natural remedies with known chemopreventive properties. Through the integration of Quranic herbs and Islamic medicinal wisdom, innovative approaches to cancer prevention and treatment are poised to emerge, heralding a new era of holistic and culturally resonant healthcare practices.

Malaysian herbs hold significant promise as potential chemoprevention agents, owing to their rich diversity of bioactive compounds and traditional medicinal uses. These herbs, deeply ingrained in Malaysian culture and culinary practices, offer a treasure trove of natural remedies with potential anticancer properties. Research indicates that certain Malaysian herbs exhibit cytotoxic effects against cancer cells while sparing normal cells, highlighting their selective anticancer activity (Ismail *et al.*, 2022; Razali *et al.*, 2020). Moreover, the affordability, accessibility, and widespread use of these herbs make them appealing candidates for integration into chemoprevention strategies. By harnessing the therapeutic potential of Malaysian herbs, researchers aim to unlock novel avenues for cancer prevention and treatment, offering hope for more effective and culturally relevant approaches to combating cancer in Malaysia and beyond.

The comprehensive exploration of Malaysian herbs as potential chemopreventive agents yielded promising results, with 13 out of 107 Malaysian herbs (as listed by Malaysian Agricultural Research and Development Institute, MARDI) identified to possess chemo-preventive effects on various cancers. These herbs *include Baru cina (Artemisia asiatica), bawang merah (Allium cepa), bawang putih (Allium sativum), belalai gajah (Clinacanthus nutans)*, cardamom *(Elettaria cardamomum), Hibiscus rosa sinensis*, *daun kaduk (Piper sarmentosum*), curcumin (*Curcuma longa), lengkuas (Alpinia galanga), lidah buaya (Aloe barbadensis), pudina (Mentha arvensis), and temu lawak (Curcuma zanthorrhiza*) (Maryam *et al.* 2023).

In line with this, the selection of 11 specific herbs in this study—*Syzygium aromaticum (Cengkih), Elephantopus scaber(Tapak Sulaiman), Cosmos caudatus Kunth (Ulam Raja), Hibiscus rosa-sinensis (Bunga Raya), Clitoria ternatea L. (Kacang Telang), Cinnamomum verum* (Cinnamon), *Curcuma zedoaria (Temu Putih), Ficus deltoidea (Mas Cotek), Strobilanthes crispus (Pecah Kaca), Cinnamomum tamala* (Bay Leaves), and *Myristica fragrans* (Nutmeg)—was based on their historical use in traditional Malay medicine, their reported pharmacological properties, and their cultural relevance as locally available herbs in Malaysia (Ismail *et al.* 2022).

Many of these herbs have been cited in classical Malay medical manuscripts for their roles in promoting women's reproductive health, detoxification, immune enhancement, and anti-inflammatory effects. Additionally, these herbs are widely accessible, cost-effective, and commonly consumed in the Malaysian diet either as food condiments or herbal remedies, making them suitable candidates for population-wide chemopreventive strategies (Mohd Afeendi *et al.* 2017; Ali *et al.* 2021). The inclusion of both commonly studied herbs *(H. rosa-sinensis, Curcuma zedoaria*) and lesser-studied traditional plants (*E. scaber, S. crispus*) further supports a holistic screening approach to identify novel candidates targeting cancer-related pathways, including the polyamine biosynthesis pathway in cervical carcinogenesis (Nurani *et al.* 2021; Rasul *et al.* 2023; Ade *et al.* 2025).

Notably, *in vitro* studies revealed the efficacy of these herbs against a spectrum of cancer cell lines, including lung, colon, neuroblastoma, hepatocellular carcinoma, mammary gland, melanoma, breast, leukaemia, liver, prostate, skin, and hypo-pharyngeal carcinoma. Additionally, certain herbs, such as *A. asiatica*, exhibited significant suppression of ornithine decarboxylase (ODC) expression in mouse skin cancer models, while others like Allium cepa and *E. cardamomum* inhibited ODC activity in colon cancer and hepatocellular carcinoma, respectively. These findings underscore the potential of Malaysian herbs in modulating polyamine metabolism and warrant further investigation into their chemopreventive mechanisms against cancer. Nonetheless, numerous Malaysian herbs with potential anti-cancer properties remain unexplored regarding their impact on polyamine pathways.

2.6.1 Clove (*Syzygium aromaticum*)



Figure 2.2 Clove (*Syzygium aromaticum*)

The flower buds of *S. aromaticum* belonging to family Myrtaceae is an aromatic flower buds used as a spice, food preservative and many medicinal purposes worldwide including in Malaysia (Zari *et al.*, 2021). Besides its uses in cooking, *S. aromaticum* has been reported to exhibit numerous therapeutic potential which are antimicrobial, antioxidant, antiviral, analgesic, insecticidal and free radical scavenging activities (Das *et al.*, 2018). Eugenol, a phytogenic bioactive component of *S. aromaticum* and a type of phytochemical has antimutagenic, antigenotoxic and anti-inflammatories properties. *S. aromaticum* has also been found to be able to reduce polyamines in cancer cells.

In different concentrations range with various histological types, eugenol was found to induce cytotoxicity and inhibit the growth of cancer cell lines including HL-60 (leukaemia), U-937 (lymphoma), HepG2 (hepatocellular carcinoma), 3LL (lung carcinoma) and SNU-C5 (colon cancer) (Das *et al.*, 2018). According to (Bezerra *et al.*, 2017), although the mode of action of eugenol varies in different cancer cell lines, it was found to induce apoptotic cell death because the treated cell lines display DNA fragmentation, reactive oxygen species (ROS) production, loss of mitochondrial transmembrane potential, reduction in anti-apoptotic protein Bcl-2, Bax translocation, cytochrome C release and caspase-9 and -3 activation. However, the effect of *S.*

aromaticum on polyamine pathway in cervical cancer cells has not been fully delineated.

Aqueous extract of *S. aromaticum* was effective in reducing the incidence of hyperplasia, dysplasia and carcinoma in situ in benzopyrene induced lung carcinoma in mice (Ali *et al.*, 2019). It has also been found that the aqueous extract of *S. aromaticum* demonstrated the inhibitory effects on breast cancer *in vitro*. The *S. aromaticum* extract inhibited the growth and proliferation of MDA-MB-231, MCF7 and T47-D in a dose-dependent manner (Al-Sharif *et al.*, 2013). This extract also has capabilities to inhibit the enzymatic activity of the Matrix Metalloproteinase (MMP) (Kim *et al.*, 2019).

The other study done by (H. Liu *et al.* 2014a) indicated that the *S. aromaticum* flower bud shows anti-cancer effect in ovarian cancer cell lines, SKOV3 and OV277. In addition, the *S. aromaticum* extract was found to reduce expression of MCP-1 and RANTES which are major determinants of macrophage recruitment at tumour sites in ovarian cancer cells (Tossetta and Marzioni 2022). Study done by Ali *et al.* (2014) revealed that eugenol-rich fraction of *S. aromaticum* reverses biochemical changes in liver cirrhosis and inhibits hepatic cell proliferation. This fraction has been found to inhibit liver cell proliferation as demonstrated by reduced [3H]-thymidine uptake. Eugenol, an active compound of *S. aromaticum*, is reported to suppress oxidative stress and inflammation reduction in deposition and resolution of liver cirrhosis therefore to decrease cell proliferation in liver cells.

While in study conducted by Ali *et al.* (2019) the chemo preventive potential of aqueous infusion of *S. aromaticum* during benzo[a]pyrene (BP)-induced lung carcinogenesis in strain A mice demonstrated significant reduction in the number of proliferating cells after treatment with *S. aromaticum* infusion. Moreover, an increased number of apoptotic cells was also noted in these BP-induced lung lesions following *S. aromaticum* treatment. Western blotting analysis revealed that *S. aromaticum* infusion upregulates the expression of pro-apoptotic proteins p53 and Bax and downregulates the expression of antiapoptotic protein Bcl-2 in the precancerous stages. Expression of caspase 3 and its activation by *S. aromaticum* infusion were evident from a very early stage of carcinogenesis (eighth week). *S. aromaticum* infusion was also found to

downregulate the expression of some growth-promoting proteins, viz, COX-2, cMyc, Hras. The observations signify the chemo preventive potential of *S. aromaticum* in view of its apoptogenic and anti-proliferative properties (Salah *et al.* 2022; Nayanna *et al.* 2024; Kubatka *et al.* 2017).

2.6.2 Elephant's foot (*Elephantopus scaber*)



Figure 2.3 Elephant's foot (*Elephantopus scaber*)

Elephantopus scaber, commonly known as *Tutup Bumi* or elephant's foot, has gained attention for its potential as chemoprevention agent. Extensive research has explored the phytochemical composition of *E. scaber* and its bioactive compounds that contribute to its anticancer properties. *E. scaber* is rich in phytochemicals that exhibit various biological activities, including anticancer effects. There are several phytochemical compounds isolated in *E. scaber* such as triterpenoids, sesquiterpene lactones, sesquiterpene phenols, steroids, flavones, anthraquinones, peptides, lipid compounds and aryl compounds where the plants' part that usually being used are leaves and roots. The major phytoconstituents that showed anticancer activity of *E. scaber* are two types of sesquiterpene lactones which are deoxyelephantopin and isodeoxyelephantopin. These compounds have been shown to possess antioxidant, anti-inflammatory, and anticancer properties, making *E. scaber* a promising candidate for chemoprevention.

Research has elucidated the mechanisms through which *E. scaber* exerts its anticancer effects. The phytochemicals present in *E. scaber* have been found to inhibit cancer cell proliferation, induce apoptosis, and suppress tumour growth. Additionally, *E. scaber* has demonstrated anti-angiogenic properties, which are crucial for limiting tumour vascularization and metastasis. The chemopreventive potential of *E. scaber* lies in its ability to target multiple pathways involved in carcinogenesis. By modulating signalling pathways associated with cell proliferation, apoptosis, and metastasis, *E. scaber* shows promise in preventing cancer initiation and progression. Its phytochemical constituents play a pivotal role in mediating these effects and offer a natural alternative for cancer prevention strategies.

2.6.3 Ulam raja (Cosmos caudatus)

Ulam raja or *Cosmos caudatus*, a herb of the family Asteraceae is one of the most recognized traditional salad in Southeast Asia and have been widely used as traditional medicine because of its health benefits. Previous studies reported that *C. caudatus* have high antioxidant capacity, antidiabetic activity, antihypertensive properties, anti-inflammatory responses, bone protective effect, antimicrobial activity and anticancer properties (Umar *et al.* 2025; Ramadhan *et al.* 2018). There are a lot of interests in the nutritional and medicinal function of this herb in the last 10 years. *C. caudatus* was found to contain rich source of bioactive compounds for instance quercetin, chlorogenic acid, catechin and ascorbic acids.

Ahda *et al.* (2023) purported those high antioxidants activity of this herb was associated with the ability to reduce oxidative stress because they found the *C. caudatus* ethanol extract showed high 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and ferric reducing antioxidant power (FRAP) activity on cervical cancer cells (HeLa). Since the progression of cancer caused by oxidative stress and affecting further DNA alteration, high free radical scavenging potential of *C. caudatus* can impede the DNA damage through mutations and structural alteration as well as demote oxidative stress that leads to increase cancer cells proliferation (Safitri *et al.* 2020).



Figure 2.4 *Ulam raja* (*C. caudatus*) planted in MyUlam Garden, Kulliyyah of Allied Health Sciences, IIUM.

Although *C. caudatus* had lower cytotoxicity on HeLa, it had showed potent dose dependent cytotoxicity, apoptosis and anti-tumourigenicity on human colorectal carcinoma cell (HCT 116) (Ahmed and Shahidan 2021). In relation of specific phenolic compound that also present in *C. caudatus* with HCC, chlorogenic acid was found to stimulate the induction of activation of extracellular signal-regulated protein kinase (ERK)1/2 and suppression of expression of matrix metalloproteinase (MMP)-2 and MMP-9 that are responsible as central regulator of cell proliferation (Ahda *et al.*, 2023). On top of that, chlorogenic acid, quercetin and catechin which present in other natural products were found to have important role in polyamine pathway by targeting arginase inhibition and supressing the production of ornithine decarboxylase (ODC) (Liu *et al.* 2011; Endringer *et al.* 2009).

2.6.4 Hibiscus *(Hibiscus rosa sinensis*)



Figure 2.5 *Hibiscus rosa sinensis* plant

Hibiscus rosa sinensis or *Bunga Raya* is also known as the national flower of Malaysia belonging to the Malvaceae family. This plant did not show any signs of toxicity at higher doses according to histological analysis in most extracts from all parts of plants including leaves, stem or flower. There are various phytochemicals with medicinal effects present in this plant namely flavonoids, tannins, terpenoids, saponins, glycosides and alkaloids which have been uses traditionally for treating wounds, inflammation, fever and coughs, diabetes, infections caused by bacteria and fungi, hair loss and gastric ulcers (Ade *et al.* 2025). Specifically, the edible flower of this plant contains several other nutrients and various flavonoids including nitrogen, fat, crude fibre calcium, phosphorus, iron, quercetin, malvalic acid and kaempferol. It was also suggested that although the flower colour differed, the phytochemical constitutions were very similar.

The pharmacological activity for anti-cancer in different extract of *H. rosa sinensis* was found in many cancer cell lines which are B16F10 melanoma cells, HeLa cell, MCF-7, lung carcinoma cell (NCI-H23), colorectal cancer cell (HT-29), leukemic cancer cells (K-562) and oral cancer cell (Ade *et al.* 2025; Rasul *et al.* 2023). Sharma *et al.* (2017) investigated the role of gentisic acid in the chemo preventive activity of *H. rosa sinensis* essential oil extract on two-stage skin carcinogenesis in mouse. They reported that gentisic acid not only shown to possess antioxidant properties by scavenging free radicals but also caused inhibition of ODC activity and cyclooxygenase activity in *in vivo* experiment model which also supported by Lu *et al*. (2022).

Moreover, in *in silico* docking performed to know the binding energies of bioactive molecules from this plant were found to be quercetin, kaempferol, myricetin and rutin against the human epidermal growth factor receptor 2 (HER2) and oestrogen receptor (ESR1) proteins in breast cancer treatment. This concluded this plant extract could be used as an inhibitor of HER2 and ESR1 for the treatment of breast carcinoma in the future (Agrawal *et al.*, 2021).

2.6.5 Bunga Telang (Clitoria ternatea)



Figure 2.6 *Bunga Telang (Clitoria ternatea)* planted in MyUlam Garden, Kulliyyah of Allied Health Sciences, IIUM

C. ternatea or butterfly pea flower belongs to Fabaceae family is an indigenous plant to the Southeast Asia. Since a long time ago, this herb plant has been eaten as salad and traditional medicine because of its health benefits. The various parts of the plant have been scientifically evaluated for various pharmacological activities including antioxidant, antibacterial, analgesic, antidepressant, anticonvulsant, hypoglycaemic, anxiolytic and cytotoxic activities (Deepika *et al.* 2023).

There are various phytoconstituents having bioactivities found in butterfly pea flower which are alkaloids, saponins, anthocyanins, terpenoids, epicatechin, gallic acid, flavonoids, kaempferol glycosides, chlorogenic, apigenin, caffeic, ferulic, cinnamic acid and phytosterol (Deepika *et al.*, 2023). The aqueous and methanol extracts of *Bunga telang* have been experimented for its cytotoxicity towards many cancer cell lines including hormone-dependent breast cancer cell line (MCF-7), non-hormone-

dependent breast cancer cell line (MDA-MB-231), human ovary cancer cell line (Caov-3), human cervical cancer cell line (Hela), human liver cancer cell line (HepG2) and human foreskin fibroblast cell line (Hs27) which had demonstrated anti-proliferative activities in cancer cells (Alshamrani *et al.* 2022). Spandana *et al.* (2024) had reported that its major phytochemicals of potent therapeutic value are kaempferol, myricetin, p-hydroxycinnamic acid, chlorogenic acid, quercetin, beta-sitosterol, tannic acid etc.

Cahyono *et al*. (2024) had also proposed that there are four phytochemicals from *C. ternatea* which responsible as inhibitors of two cell cycle checkpoints proteins; Cyclin Dependent Kinase (CDK)-2 and CDK-6 in Cyclin pathway including kaempferol, myricetin, quercetin and p-hydroxycinnamic acid. CDKs are specific serine/threonine kinases that contribute to the cell growth and cell cycle progression by phosphorylating and inactivating Retinoblastoma (Rb) protein. Therefore, the association of dysregulated hyperactivity of CDKs are use as indicator to the uncontrolled cell growth that characterizes the cancer in which they lack the ability to bind inhibitor and ultimately become resistance.

Additionally, high concentration of amino acid namely cyclotides have been isolated from *C. ternatea* and other various plants because of its wide range of bioactivities. Previous studies have demonstrated the *in vitro* cell-based assays that cyclotides have significant cytotoxicity activities by disrupting the cancer cell membrane integrity in human lung cancer cell line (A549) and doxorubicin-resistant human breast cancer cell line (MCF-7/ADR) (Ullah *et al*. 2019). Sen *et al.* (2013) also summarized those five types of cyclotides (CT4, CT7, CT12, CT10 and CT2) have the potential to be used in chemo sensitization for treating cancer that is notable in A549 cell.

2.6.6 Cinnamon (*Cinnamomum verum*)



Figure 2.7 Cinnamon (*Cinnamomum verum*)

Cinnamon, derived from the bark of several species of Cinnamomum trees, has garnered attention for its potential chemopreventive properties due to its rich composition of phytochemicals. Various *in vitro* and *in vivo* studies have explored the anti-cancer effects of cinnamon and its bioactive compounds, shedding light on its promising role in cancer prevention (Sudan *et al.*, 2013). Cinnamon contains numerous phytochemicals, including cinnamaldehyde, cinnamic acid, eugenol, and coumarin, among others, which exhibit diverse biological activities. These compounds have been shown to possess antioxidant, anti-inflammatory, anti-proliferative, and apoptotic properties, all of which are crucial in combating cancer development and progression.

In vitro studies have demonstrated the potential of cinnamon extracts and its phytochemicals in inhibiting the growth and proliferation of various cancer cell lines (Tsai *et al.*, 2016). For example, cinnamaldehyde, the main component of cinnamon oil, has been shown to induce apoptosis and cell cycle arrest in cancer cells, including those derived from the colon, breast, prostate, and leukaemia. Additionally, cinnamon extracts have been found to suppress the expression of pro-inflammatory cytokines and enzymes involved in carcinogenesis, thereby exerting anti-inflammatory effects that contribute to cancer prevention.

Moreover, *in vivo* studies using animal models have provided further evidence of cinnamon's chemopreventive effects. Administration of cinnamon extracts or its bioactive compounds has been shown to inhibit tumour growth, reduce tumour volume, and enhance survival rates in various cancer models. These effects have been attributed to the modulation of signalling pathways involved in cell proliferation, apoptosis, angiogenesis, and metastasis (Khan *et al.*, 2023; Perng *et al.*, 2016). The mechanisms underlying the chemopreventive effects of cinnamon and its phytochemicals are multifaceted. One key mechanism involves the regulation of oxidative stress and inflammation, which are implicated in cancer initiation and progression. Cinnamon compounds act as scavengers of free radicals, thereby reducing oxidative damage to DNA and other cellular components. Additionally, they inhibit the production of inflammatory mediators and suppress the activity of transcription factors involved in inflammation-associated carcinogenesis (Shanmugam *et al.*, 2022).

Furthermore, cinnamon and its constituents have been shown to modulate signalling pathways that regulate cell proliferation and apoptosis. By targeting key molecules involved in these processes, such as cyclins, caspases, Bcl-2 family proteins, and NF-κB, cinnamon compounds exert anti-proliferative and pro-apoptotic effects on cancer cells while sparing normal cells. Moreover, cinnamon has been reported to inhibit angiogenesis, the process by which tumours acquire a blood supply for growth and metastasis, through the downregulation of pro-angiogenic factors like VEGF (YLiu *et al.*, 2017; Sudan *et al.*, 2013). Cinnamon and its phytochemicals possess significant chemopreventive potential against cancer through their diverse biological activities. *In vitro* and *in vivo* studies have provided compelling evidence of their anti-cancer effects, highlighting cinnamon as a promising natural agent for cancer prevention and therapy. However, further research is warranted to fully elucidate the mechanisms of action and therapeutic efficacy of cinnamon in cervical cancer.

2.7 CONCEPTUAL SUMMARY AND STUDY JUSTIFICATION

Figure 2.8 illustrates the comprehensive rationale for this study, beginning with the public health challenge of cervical cancer. Despite the availability of HPV immunisation, its preventive function is limited to new infections and does not address existing infections or lesions. Furthermore, low awareness and uptake in Malaysia contribute to an unmet need in cervical cancer prevention. Conventional treatments such as surgery, chemotherapy, and radiotherapy remain effective but are often associated with high toxicity, side effects, and economic burden. This highlights the necessity for alternative or complementary strategies. In this context, chemoprevention emerges as a promising approach, especially for early-stage or high-risk populations. By employing natural agents to prevent, delay, or reverse cancer progression, chemoprevention offers safer and more affordable interventions.

Among various molecular targets, the polyamines pathway is of particular interest due to its association with cancer cell proliferation, survival, and metastasis. Although difluoromethylornithine (DFMO) is an established inhibitor of this pathway, there remains a research gap in exploring locally available Malaysian herbs as alternative, plant-based modulators of polyamine metabolism. Thus, this study investigates 11 Malaysian herbs through *in vitro* experiments, focusing on cytotoxicity, polyamine-related gene expression, and apoptosis/cell cycle mechanisms. By targeting a clinically relevant biochemical pathway and addressing a national public health concern with indigenous resources, this study justifies the development of herb-based chemopreventive agents as a complementary strategy for cervical cancer control in Malaysia.

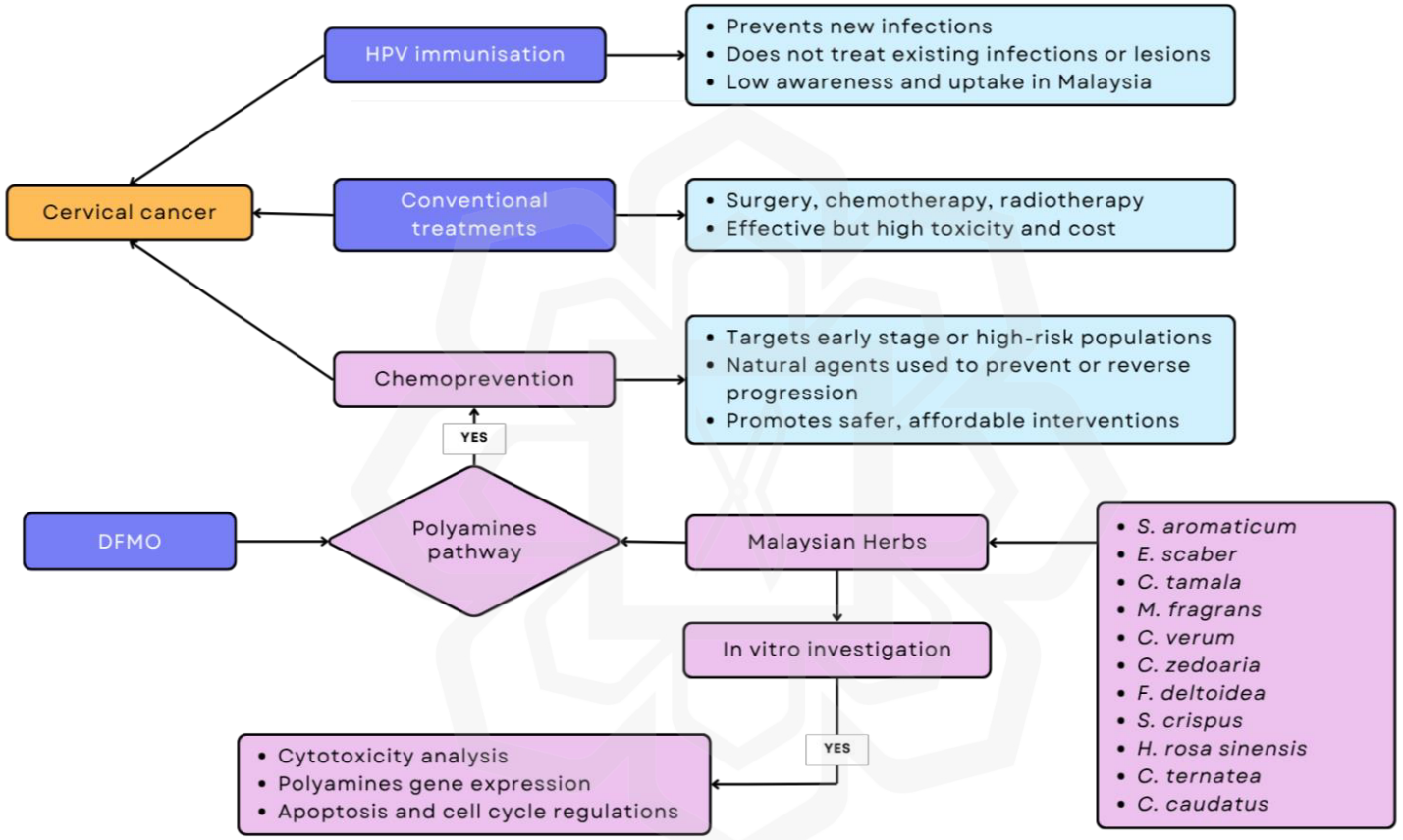


Figure 2.8 The research gap and rationale for *in vitro* chemopreventive investigation of Malaysian herbs via the polyamines pathway.

CHAPTER THREE

MATERIALS AND METHODS

This section consisted of four main sections including materials, general cell culture methods, experimental methods, and statistical data analysis. The focus of experimental methods presented the principle and protocols of each experiments performed according to the research objectives and questions (cell viability and cytotoxicity assays, polyamine gene expression assay, and methods in cell death investigation). The flow chart of the study was as presented in the figure below.

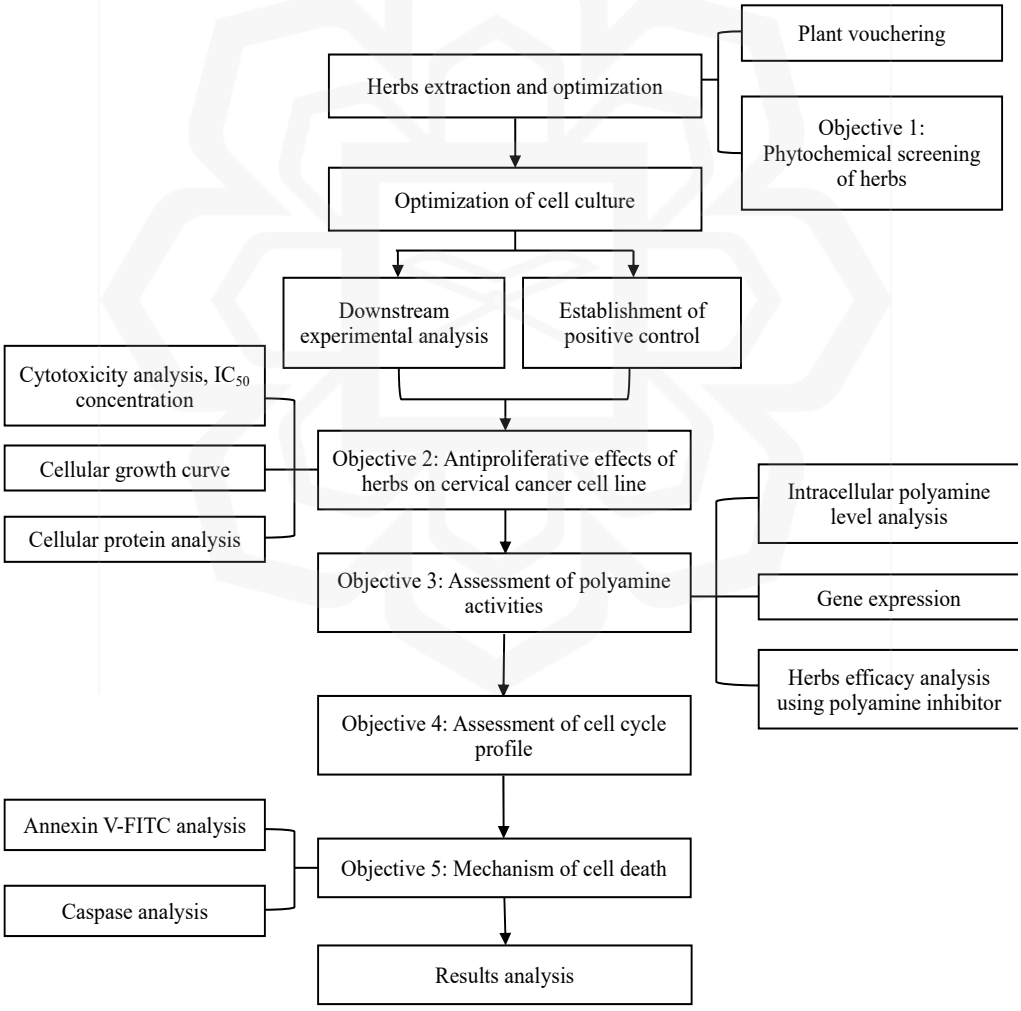


Figure 3.1 Flow chart of the experimental method

3.1 MATERIALS

3.1.1 Cell lines

3.1.1.1 SiHa cell

The cervical squamous cell carcinoma, SiHa, was selected for *in vitro* studies was obtained by the courtesy of Dr Yusmazura Zakaria, Department of Biomedicine, School of Health Sciences, Universiti Sains Malaysia (Health Campus) where it was purchased from the American Type Culture Collection (ATCC®HTB-35TM). The epithelial-type cell line was isolated from a 55-year-old female, Japanese patient with squamous cell carcinoma. SiHa cells were cultured in complete growth media consisted of Dulbecco's Modified Eagle Medium (DMEM) (with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate, liquid, sterile-filtered) with 10% (%v/v) of fetal bovine serum (FBS) and 1% (%v/v) of penicillin-streptomycin (Pen-strep). SiHa cells were observed daily to ensure they are healthy and growing as expected and routinely passaged when their growth was at 75 to 85% of confluency every two to four days. For experimental procedure, the cell lines were seeded at the density of 5.0×10^4 cells/cm^2. Stock of cryopreserved SiHa cells that contain freezing media were kept in liquid nitrogen. Figure 3.2 showed the image of SiHa cells using microscope at 10x magnification using Evos XL Core Imaging System (Invitrogen).

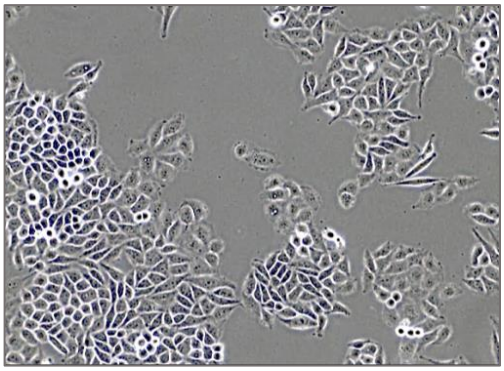


Figure 3.2 Morphological structure of SiHa cell line at 10x magnifications

3.1.1.2 HEK 293T cell

The human embryonic kidney (HEK 293T) cell line was generously provided by Dr. Tengku Muhamad Faris Syafiq from the Kulliyyah of Nursing, International Islamic University Malaysia (IIUM), and was procured from Merck KGaA (SigmaAldrich). This cell line, originally isolated from fetal human kidney tissue, expresses the large T antigen of simian virus 40 (SV40) and exhibits an adherent, dendritic-, and epithelial-like morphology. Due to these characteristics, HEK 293T cells were used as a non-cancerous comparison to assess the toxicity of selected Malaysian herb aqueous extracts. This comparison ensures that the extracts are cytotoxic to cancer cells without affecting normal cells. The culture conditions and incubation environment were the same as those used for the SiHa cell line. Figure 3.3 shows an image of HEK 293T cells.

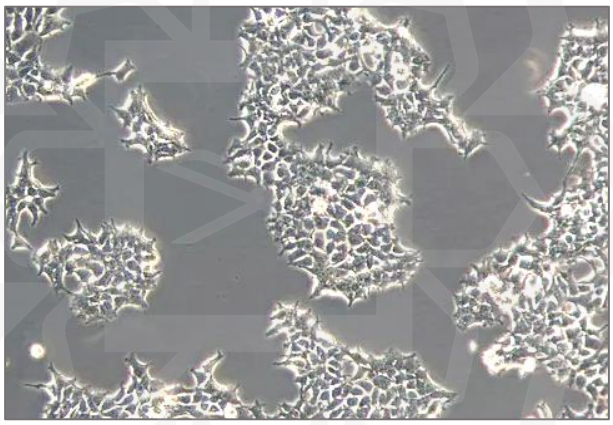


Figure 3.3 Morphological structure of Human Embryonic Kidney 293T cell line (Picture taken from https://www.sigmaaldrich.com/MY)

3.1.2 Drug

3.1.2.1 Etoposide

Etoposide is a chemotherapeutic drug that was used for establishment of positive control for experimental procedures in the study. Stock solution of etoposide was prepared as suggested by the manufacturer by dissolving the drug in 1% (%v/v) dimethyl sulfoxide (DMSO) and filtered using 0.22 μm pore nylon syringe filter. Stock solutions were then diluted with complete growth media as working stock at desired concentration before used according to the experimental methods.

3.1.2.2 Eflornithine (DFMO)

Eflornithine or commonly known as α-difluoromethylornihtine (DFMO) is a drug used in the pre-treatment on SiHa cell to target the rate-limiting enzyme in polyamine biosynthesis as irreversible inhibitor of ODC. DFMO was used in this study to determine cytotoxicity effects (using MTT assay) on cell line and followed by combination with selected herbs to determine the effect of DFMO on dosage reduction of herbs extract. DFMO were weighted according to desired final concentration and dissolved in serum-free culture media (DMEM), adjusted to pH 7.2-7.4 and stored at -20°C. Before use, the solution was sterilized by membrane filtration (0.22 μm pores).

3.1.3 Plant specimens

A total of 11 Malaysian herbs were selected and obtained to be screened for this study. Flower buds of clove (*Syzygium aromaticum*), bay leaves (*Cinnamomum tamala*), nutmeg seed (*Myristica fragrans*), and inner bark of cinnamon (*Cinnamomum verum*) was bought from Kuantan local hypermarket with the recent manufacturing date while fresh temu putih rhizome (*Curcuma zedoaria*), leaves part of Mas cotek (*Ficus deltoidea*), pecah kaca (*Strobilanthes crispus*) and Elephant's foot (*Elephantopus*

scaber), and hibiscus flower (*Hibiscus rosa sinensis*), were obtained from Kuantan, Pahang by local botanist. Butterfly pea flower (*Clitoria ternatea*) and *Ulam raja* leaves (*Cosmos caudatus*) were obtained from MyUlam Garden at Kulliyyah of Allied Health Sciences, IIUM Kuantan, Pahang.

3.1.3.1 Vouchering of Plant Specimens

Plant vouchering involves collecting, preserving, and documenting plant specimens for future reference. Plant specimens were collected with stem, branches, leaves, and flower together with necessary information, including collector's name, locality where the plant was collected, type of plant community, characteristics, and plant photos. Healthy and representative specimens were selected where excess soil and debris were removed. The plants were further processed by placing it between two sheets of newspaper and blotting paper to facilitate proper drying and pressed using two cardboards by applying uniform pressure. Dried herbs, pressed plant specimens and complete plant information was then submitted to Natural Medicinal Product Center, Herbarium, Kulliyyah of Pharmacy, IIUM for identification and authentication process.

All plant samples were initially selected based on ethnobotanical relevance, traditional use, and availability for preliminary phytochemical screening. However, vouchering and herbarium deposition were conducted only for five key herbs—*S. aromaticum, E. scaber, H. rosa-sinensis, C. caudatus, and C. ternatea*—which were shortlisted for further experimental analysis. These five herbs were prioritised for authentication and vouchering due to their reproducible extract quality, sustainable sourcing, and demonstrated bioactivity in initial screening. The remaining herbs were excluded from vouchering as they were not selected for downstream *in vitro* assays, thereby aligning with the practical scope and focus of the study. Vouchered specimens' number for these plants were tabulated in Table 3.1. The certificate of vouchered specimens' verifications was included in appendix IV.

Table 3.1 Plant specimens' details and voucher number

Local name	Scientific Name	Family Name	Voucher No.
Cengkih	*Syzygium aromaticum* (L.) Merr. & L.M. Perry	Myrtaceae	PIIUM 0300-1
Tapak Sulaiman	*Elephantopus scaber* L.	Asteraceae	PIIUM 0368
Bunga raya	Hibiscus rosa-sinensis L.	Malvaceae	PIIUM 0333
Ulam raja	Cosmos caudatus Kunth	Compositae	PIIUM 0334
Kacang Telang	Clitoria ternatea L.	Fabaceae	PIIUM 0335



Figure 3.4 *S. aromaticum* specimen used for this study



Figure 3.5 *E. scaber* specimen used for this study

3.1.3.2 Plant Samples Extraction Preparation and storage of Plant Samples Extract

All selected Malaysian herbs aqueous extracts were prepared for cytotoxicity screening on cervical squamous carcinoma cell line. Dried herbs (clove, bay leaves, nutmeg, and cinnamon) were washed with distilled water and dried using drying oven at 40°C. Meanwhile, fresh plant's part was washed twice using tap water and finally washed using distilled water. The plant part was dried using drying oven at the same temperature. Once dried, the herbs were pulverised to fine powder Electric Grain Mill Grinder (220V), at rotating speed of 30,000 rpm. The powder was then sieved using a stainless-steel mesh sieve (60 mesh size) to ensure uniform particle size, with crushing fineness ranging from 50 to 300 mesh.

To achieve higher extraction yields and greater phytochemicals extraction profiles, cosolvent was used to tune water solvent properties by mixing with ethanol. Addition of cosolvent can influenced the ability of water to solubilize more non-polar natural products following the method by Lajoie *et al.* (2022). The aqueous extracts were prepared with addition of cosolvent, ethanol. Three different water to ethanol proportion (%v/v) was used for cytotoxicity screening which are 100% water (1:0), 70% water (7:3), and 50% water (1:1). The addition of ethanol as a co-solvent was employed to improve the solubility and extraction efficiency of certain phytochemicals that are poorly soluble in water alone, such as flavonoids, terpenoids, and phenolic compounds. This approach is commonly used in herbal extraction to ensure a broader range of bioactive compounds is present in the extract for cytotoxicity assessment (Lajoie *et al.* 2022; C. Li *et al.* 2019).

The herbs were then macerated at a ratio of 1:2 (w/v) and continuously stirred in distilled water for 48 hours at 60°C. The solution was filtered twice; first using cotton cloth and second filtration using Whatman No.1 filter paper. The filtrate was then evaporated over steam bath (50-60°C) until the solution become more concentrated. The concentrates were stored in ultra-low temperature freezer at -80°C overnight. Then, frozen sample were lyophilized using freeze dryer for a week at set temperature and pressure of -60°C and 0.36 mbar respectively. Dried samples were kept at 4°C in the

dark until analysis. The method of aqueous extraction followed according to (Chebaro *et al.*, 2023; Ibrahim & Kebede, 2020).

The dried aqueous extracts of *S. aromaticum* were sent to Kulliyyah of Sciences, IIUM Kuantan for qualitative phytochemical analysis using gas chromatography-mass spectroscopy (GC-MS) while *E. scaber* extract was sent to Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA (UiTM) Puncak Alam, Selangor for Low chromatography/Mass spectrometry Quadrupole Time-of-Flight (LC-MS-QTOF) qualitative analysis. The polyamines content in the plants were quantified by high performance liquid chromatography (HPLC). The method was described in sub-section 3.3.2.

For experimental analysis, the lyophilized herb extract was weighed to prepare a concentrated stock solution, which was then dissolved in sterile distilled water until fully dissolved. The freshly prepared stock solution was centrifuged at 1500 rpm for 5 minutes to separate any sediments. The supernatant was sterilized using a membrane filter with 0.22 µM pores. This sterilized solution was then diluted to create various working concentrations for the cytotoxicity assay. Based on the cytotoxicity screening results, two herbs were selected for further investigation to assess their molecular and cell-targeted antiproliferative effects on human cervical cancer cells.

3.1.3.3 Phytochemical Profiling of Selected Malaysian Herbs

The qualitative phytochemical analysis was conducted to screen the phytochemical profiles of *S. aromaticum* and *E. scaberi*. This is to identify the main classes of bioactive compounds (tannins, saponins, flavonoids, alkaloids, phenols, glycosides, steroids, and terpenoids) present in the extracts following standard protocols.

3.1.3.3.1 GC-MS Analysis

The instrument used for Gas Chromatography – Mass Spectrometry (GCMS), Perkin Elmer with Elite-5MS capillary column. Start with sample preparation from crude plant extracts – dilution of sample. Diluted samples (1/100, v/v, in methanol) of 1 µL was injected in the split mode with a split ratio 100:1. To make lyophilized herbs extracts volatile, the solid powdered extracts were dissolved with non-polar solvent, methanol (Hashmi, 2013; Zygmunt & Namiesnik, 2003). Helium gas was used as carrier gas and was adjusted to column velocity flow of 1.0 ml/min. Other GC-MS conditions are ion-source temperature, 250 °C; interface temperature, 300 °C; pressure, 16.2 psi; out time, 1.8 mm; and 1 µl injector in split mode with split ratio 1:50 with injection temperature of 300 °C. The column temperature started at 36 °C for 5 min and changed to 150 V at the rate of 4 °C/min. The temperature was raised to 250 °C at the rate of 20 °C/min and held for 4 minutes. The total elution was 47.5 min. The relative percent amount of each component was calculated by comparing its average peak area to total areas.

MS solution software provided by supplier was used to control the system and to acquire the data. Identification of components was achieved based on their retention indices and interpretation of mass spectrum was conducted using the database of National Institute of Standards and Technology (NIST) – Mass Spectrometry Data Center NIST (NIST 23 GC Method/Retention Index Library). The database consists of more than 62,000 patterns of known compounds. The spectra of the unknown components of the herbs obtained were compared with the standard mass spectra of known components stored in NIST library (NISTII) (Njoku Ugochi Olivia *et al* 2021).

The identified compounds were confirmed and acknowledged by Science Officer, Mr Che Muhamad Arif Che Malek.

3.1.3.3.2 LC-MS-QTOF Analysis

The crude extract of the *E. scaber* plant underwent analysis and characterization using LC-MS-QTOF. A 20 mg sample was dissolved in 2 mL of 70% ethanol to achieve a working concentration of 10 mg/mL. The sample was vortexed for 60 seconds until fully dissolved, then further diluted to a 2 mg/mL concentration. It was then filtered through a 0.22 μm pore size syringe filter and centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred into a 50 μL insert before injection into the LC-MS-QTOF. The system used an Agilent 1200 liquid chromatography setup, featuring a binary pump, vacuum degasser unit, auto sampler, and a 6520-quadrupole time-of-flight mass spectrometer with an electrospray ionization (ESI) source. Chromatographic separation was conducted at 40°C using an Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT column (2.1 × 100 mm, 1.8 μm). The separation was performed in positive mode with (A) 0.1% formic acid in dH_2O and (B) 0.1% formic acid in acetonitrile. The gradient elution program was as follows: 0.00–18.00 min, 5–95% (B); 18 to 23 minutes, 95% (B); 23.01 minutes, 5% (B). The total run time was 30 minutes, with the LC condition re-equilibrated for 2 minutes before the next injection. The sample injection volume was 2 μL, and the mobile phase flow rate was set at 0.25 mL/min. The mass spectrometer operated in positive ESI mode with optimal settings of 325°C for gas temperature, 11 L/min for gas flow, and 35 psi for the nebulizer.

Data analysis was performed using Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA), and the chromatographic profiles were analyzed based on the accurate mass.

3.2 GENERAL CELL CULTURE METHODS

3.2.1 Aseptic Technique and Cell Culture Condition

All tissue culture activities and handling of cell were performed in Class II laminar airflow biological safety cabinets (BSC). Complete personal protective equipment (PPE) was worn including laboratory gown, sterile gloves, face mask, and safety shoes in tissue culture laboratory. All surfaces and items to be used in BSC were sprayed and wiped with 70% ethanol before and after lab works. The pipette tips, bottles, microcentrifuge tubes, and distilled water were sterilized by autoclaved at 121°C for 15 minutes while all cell culture consumables purchased were gamma irradiated at the point of manufactured. Sterilization of solutions and drugs prior to use were autoclaved or filtered using 0.22 μm syringe filters if solution is heat sensitive. The solutions were kept at 4°C, drugs were aliquoted into 1 ml tubes and stored at -20°C, and herb extract were prepared fresh. The solutions were pre-warmed to 37°C in water bath before use.

Both SiHa and HEK293T cells were cultured in complete growth media consisted of Dulbecco's Modified Eagle Medium (DMEM) (with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate, liquid, sterile-filtered) with 10% (%v/v) of fetal bovine serum (FBS) and 1% (%v/v) of penicillin-streptomycin (Pen-strep) in T75 tissue culture flask. The cells were observed daily to ensure they are healthy and growing as expected and routinely passaged when their growth was at 70 to 80% of confluency every two to four days. Moreover, when new complete culture media were prepared, the media were incubated for 2 days to observe any contamination in the media before use for cell culture. The cells were maintained in incubator set at 37°C, with an atmosphere of 5% CO_2 and 95% humidified air. The humidity was regulated by sterilized water tray containing 1% (w/v) copper II sulphate placed at the bottom of incubator to minimize bacterial and fungal growth. Regular checks were conducted to ensure no signs of bacterial or fungal contamination within the incubator.

3.2.2 Recovery of Cell from Frozen Stock

Prior to thawing the cells, culture media were pre-warmed, and all necessary consumables (such as pipette tips, serological pipettes, centrifuge tubes, tissue culture flasks, etc.) were prepared within the biosafety cabinet (BSC) hood. The cells had been stored frozen at -80°C in a ultra-low temperature freezer. Thawing was conducted rapidly within one minute at 37°C. The resulting defrosted cell suspension, which included cryoprotectant agents (10% DMSO and 90% FBS), was then transferred to a 15ml sterile centrifuge tube. To avoid osmotic shock, 5ml of fresh culture media was gradually pipetted into the centrifuge tube containing the cell suspension in a dropwise manner. The cell pellets obtained after centrifugation were dispersed by tapping and new complete media were added. The cell suspension was then transferred to tissue culture flask and incubated.

3.2.3 Routine subculture of Cells

Before initiating sub-culturing, a daily examination of the cells under a light microscope was conducted to verify their health and confirm optimal growth rates. The cells exhibited attachment to the flask bottom, displayed an elongated shape, and refracted light around the membrane. Split ratios were employed to ensure the cells were appropriately prepared for experiments on specific days. The standard split ratio guideline involved selecting a flask with 70-80% confluence. For instance, a 1:3 split should be performed when the flask reaches 70 to 80% confluency, making it ready for an experiment in 2 to 3 days. Similarly, a 1:5 split required 4 to 5 days until reaching 70 to 80% confluency. Cells acquired from sub-culturing will be cryopreserved to prevent loss due to contamination, minimize genetic alterations in continuous cell lines, and forestall aging. Following cell harvesting and the acquisition of the cell pellet, the pellet was dispersed by gently tapping the centrifuge tube. Subsequently, 1 ml of cryoprotectant agent was added to the cells, and the mixture was promptly transferred to appropriately labelled cryovials indicating the cell line, passage number, and date. These cryovials were then placed in the Mr. Frosty™ freezing container, which was initially kept at -80°C overnight before being transferred to a cryovials container. The

freezing container facilitates a controlled cooling rate of 1°C per minute, optimizing the success of cell cryopreservation and subsequent recovery.

3.2.4 Handling Contamination of Cell Culture

In the event of contamination, cleaning procedures involved using a diluted bleach solution, sprayed with 70% (v/v) ethanol, and set the incubator at 160°C (4 hours) to sterilize all the components within the incubator (Memmert GmbH incubator). This incubator equipped with automatic sterilization process control for hot air sterilization of appliance, including sensors and fan rotor. All contaminated flasks with cells were discarded to prevent cross-contamination with other flasks. The solutions such as PBS and distilled water were sterilized again using autoclave. The diagnosis of contamination in cell culture were performed by two different ways; using naked eyes and under light microscopy Table 3.2.

Table 3.2 The diagnosis and treatment suggestion of tissue culture contaminations (adapted from Ian Cree Kurbacher *et al.*, 2011)

Method	Contamination	Symptoms	Treatments
Viable	Bacteria	- Muddy medium - Yellowish medium	- Antibiotics - Reject culture
	Fungi	- Cottonwool like clusters - Thread-like structures	- Anti-mycotic - Reject culture
Light microscopy	Bacteria	- Mobile microorganisms, overlaying cells or floating	- Antibiotics - Reject cultures
	Yeast	- Necklace-shaped cell formation, budding next to cells	- Anti-mycotic - Reject culture
	Mycoplasma	- Hyper-vacuolations - Leopard cells - Hyper-granulations	- Antibiotics - Reject culture

3.2.5 Harvesting Protocol of Cell Culture

Cells were harvested for a different experiment and subsequently seeded at a desired concentration of 5 x 10^4 cells/cm^2. The flask containing cells was removed from the incubator, sprayed, and wiped with 70% ethanol before being transferred into a BSC hood. The media were discarded using a serological pipette. To eliminate the presence of serum and maintain cell osmolarity, 2 ml of PBS solution was added into the flask, and this PBS wash was performed twice. Pre-warmed trypsin (1 ml) was introduced into the flask and incubated for 1 to 5 minutes to facilitate cell detachment followed by gentle tapping. Subsequently, 2 ml of fresh media were added to halt trypsinization, and the contents were transferred into a centrifuge tube. The flask was washed with an additional 2 ml of PBS transferred into the tube to collect any remaining cells. The cell suspension was then centrifuged at 1500 rpm for 5 minutes. After discarding the supernatant, the cell pellet was dispersed in 1 ml of fresh media. For cell counting, 10-50 µl of the cell suspension was aliquoted into a microtube.

3.2.6 Cell Counting using Hemocytometer

Cell counting was conducted using a hemocytometer, with cells being stained using Trypan Blue dye. The protocol for the Trypan Blue Exclusion assay was detailed in sub-chapter 3.3.1.1. To ensure sterility, the hemocytometer and coverslip were initially treated with 70% alcohol and delicately wiped. Subsequently, the coverslip was positioned to completely cover the counting chamber on the hemocytometer. A volume of 10 µl of the cell suspension and dye mixture was carefully pipetted to saturate the chambers beneath the coverslip, allowing the cell suspension to be drawn out by capillary action. The configuration of the counting grids on the hemocytometer was illustrated in Figure 3.6. Inverted microscope (Evos XL Core - Invitrogen) was used by focusing the gridlines of the hemocytometer at 10x magnification. Live unstained cells were counted using hand tally counter. Counting system wherein cells are tallied only when they fall within a square or along the right-hand or bottom boundary line was implemented. The tallying process was replicated twice, and the resulting average was considered the final concentration of the cells. The formula for cell counting was presented in Table 3.3.

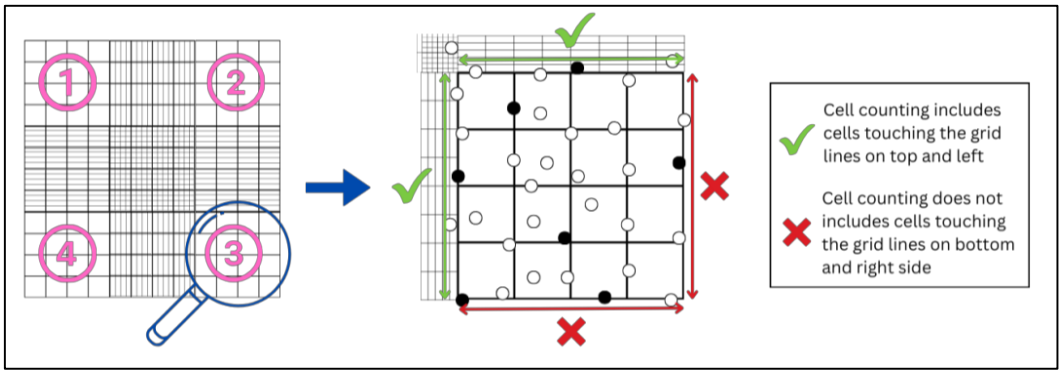


Figure 3.6 Cell counting guidelines

Table 3.3 Cell counting calculation formula

Cell counting formula	
Total no. of cells (TC)	= viable cells (VC) + dead cells (DC)
Average number of cell per grid square (Avg)	$= \frac{VC}{No.of\ grid}$
Percentage of viable cells	= (VC/TC) x 100%
Dilution factor (DF)	$= \frac{Final\ volume}{Volume\ of\ cells}$
Cell concentration (viable cell/ml)	= Avg x DF x 10^4
Total no. of cells	= Cell concentration x Volume of sample

3.3 EXPERIMENTAL METHODS

3.3.1 Cell Viability Assay

3.3.1.1 Trypan Blue Exclusion (TBE) Assay

3.3.1.1.1 Principle of TBE assay

TBE assay operates on the fundamental principle that live cells with intact cell membranes prevent the entry of certain dyes, such as trypan blue, while dead or dying cells with compromised membranes allow the dye to penetrate and stain them blue. This assay relies on the selective uptake of trypan blue, a vital dye that cannot normally enter viable cells due to their intact membranes. By staining cells with trypan blue and examining them under a microscope, researchers can distinguish between live (unstained) and dead (blue-stained) cells, enabling the quantification of cell viability within a population. Following staining, viable (unstained) and non-viable (stained) cells are counted using a hemocytometer or an automated cell counter. The percentage of viable cells is then calculated based on the ratio of unstained to stained cells, providing a measure of cell viability (as described in sub-section 3.2.6).

3.3.1.1.2 Protocol of TBE assay

The cell pellet was collected according to the procedure outlined in sub-section 3.2.5. Afterward, 1 ml of PBS was added to the dispersed pellet. A 10 µl sample of the cell suspension was then mixed with 10 µl of trypan blue. The remaining cell suspension was later proceeded with extraction of acid fraction for polyamines quantification and protein extraction for Lowry's assay. Subsequently, 10 µl of this mixture was placed onto the hemocytometer. Both live (unstained) and dead (stained) cells were counted as per the instructions in sub-section 3.2.6. This entire process followed the guidelines

provided by Strober (2015). The TBE assay was used to observed growth inhibitory impact of Malaysian herbs aqueous extracts on cervical cancer cells. After 48 hours of incubation, treatments were administered, and cells were harvested and counted at seven time points: 0, 24, 48, 72, 96, 120, and 144 hours. A growth curve was plotted, showing cell numbers against these time points. Differences in cell growth between untreated and treated cells were then compared.

3.3.1.2 MTT Assay

3.3.1.2.1 Principle of MTT Assay

In this study, the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was employed to evaluate the cytotoxic effects of natural products on cervical cancer cells compared to an untreated group. The MTT assay measures cellular metabolic activity by converting a water-soluble dye into an insoluble formazan, indicating cell viability and proliferation. Mitochondrial activity, reflected through the conversion of MTT into violet-coloured formazan crystals, correlates with the number of viable cells. Higher formazan levels signify more living cells. The concentration of formazan was quantified using a microplate reader, measuring optical density (OD). The reduction in cell number, corresponding to 50% growth inhibition (IC_{50}), was determined by comparing treated cells to the untreated control after applying various herbs extracts and drug concentrations.

3.3.1.2.2 Protocol of MTT Assay

The MTT assay involves four key steps: cell plating, treatment addition, MTT stock addition, and formazan absorbance measurement. After harvesting and counting, cells were plated in a 96-well flat-bottomed plate at a density of 5×10^4 cells/cm^2 (100 ul) according to the plate layout (see Figure 3.7). The cells were incubated at 37°C for 24h

to allow for attachment before treatment addition. To avoid edge effects, the edge wells were filled with 100 µl of sterile distilled water (BL). Column 2, with complete media but no cells, served as the background (0). Column 3, the vehicle control (VC), contained 90% cell suspension and 10% solvent used for the treatments to assess compound-specific changes in cell viability without solvent toxicity. Column 4 had negative control (C), complete media with cells but no treatment. Increasing concentrations of the treatment were added to columns 5 to 11. This protocol was performed according to Tolosa *et al.* (2015).

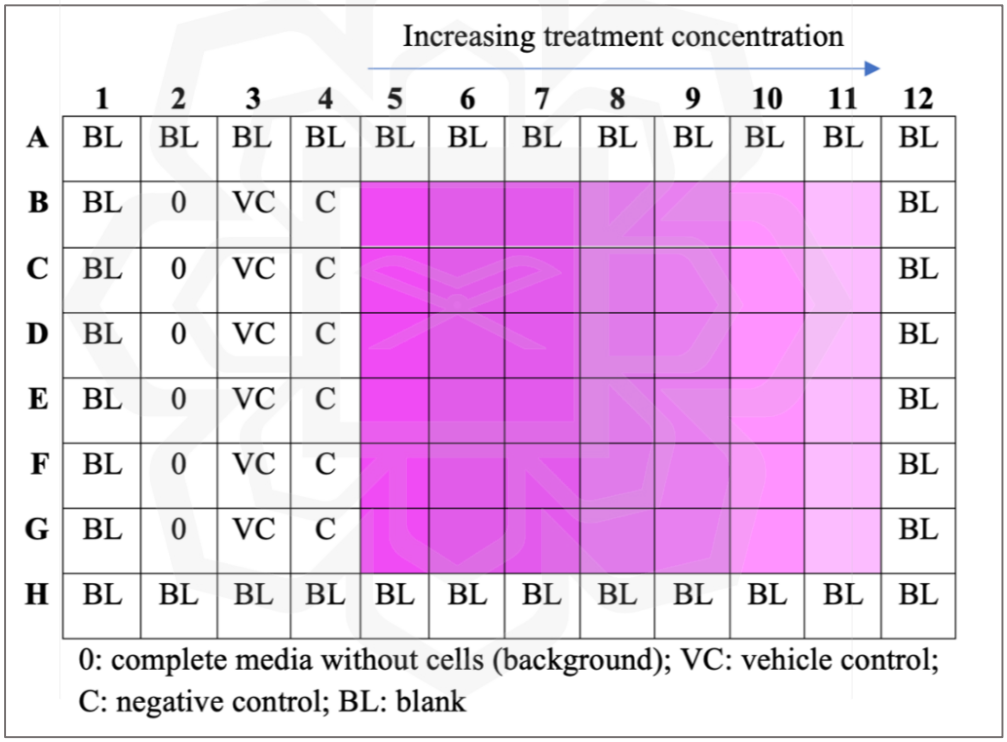


Figure 3.7 MTT Plate Layout

S. aromaticum and *E. scaber* aqueous extracts, and etoposide were diluted to create a range of treatment concentrations, from low to high. After treatment, cells were incubated for 24, 48, and 72 hours. At each time point, without removing the existing media, 10 µl of pre-warmed MTT stock solution (5 mg/ml) was added to each well, excluding the edge wells. MTT solution addition was performed in the dark. Then, the

plate was covered with aluminium foil and incubated for 3 hours to form formazan crystals. The media were then carefully removed using a needle and syringe, ensuring the crystals were undisturbed. Next, 100 µl of DMSO was added to each well dissolve the crystals. The plate was covered again with aluminium foil and placed on orbital shaker and the plate was incubated for 20 minutes at room temperature. Absorbance was measured at 570 nm (test) and 620 nm (reference) using a microplate reader. The experiments were performed three times with three replicates each. Cell viability percentage was calculated using the provided formula below. The graph of cell viability (%) against increasing herbs concentration was plotted.

$$Percentage\ of\ cell\ viability\ (\%) = \frac{OD\ treatment - OD\ background}{OD\ control - OD\ background} \times 100$$

3.3.2 Polyamines Quantification in Herbs Extract and Cells

Analyzing polyamines (PA) in biological samples requires high sensitivity and specificity due to their typically low concentrations. High-performance liquid chromatography (HPLC) is commonly employed for PA analysis because it provides sensitive and reliable separation. HPLC necessitates pre-column derivatization for effective detection. In this experiment, the benzoylation method was used, as it yields stable reaction products, and the available facilities at IIUM included a photodiode array (PDA) detector for HPLC. A calibration curve for three polyamine standards (putrescine, spermidine, and spermine) was developed using concentrations ranging from 0 to 5.0 nmol/µl, ensuring that the correlation coefficient (R^2) values exceeded 0.95. The PA quantification process started with obtaining the acid fraction from herb extracts and cells at various time points. Following the preparation of PA standard solutions, pre-column derivatization via benzoylation was performed. Finally, HPLC analysis was carried out using benzoylated polyamines.

3.3.2.1 Extraction of Acid Fraction from Herbs Extract

Lyophilized herb extracts, prepared as described in sub-section 0, were each weighed to 1 gram and placed into 15 ml centrifuge tubes. To these, 10 ml of ice-cold 0.2M perchloric acid was added, and the mixture was vortexed for 1 minute until fully dissolved. Ice-cold perchloric acid was used to ensure the stability of the polyamines during the extraction and prevent unwanted side reactions such as oxidation and therefore more accurate extraction process. The solution was then incubated on ice for 1 hour. Subsequently, the acid-soluble polyamines were centrifuged at 4200 rpm for 15 minutes at 4°C. The supernatant was pipetted into 1 ml microcentrifuge tubes and stored at -20°C until the benzoylation process. This procedure followed the method outlined by Lokesh *et al.* (2019).

3.3.2.2 Extraction of Acid Fraction from Cells

The remaining cell suspension, harvested as described in sub-chapter 3.3.1.1, was centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed, leaving the cell pellets. Then, 200 to 300 µl of ice-cold 0.2M perchloric acid was added to the cell pellets, vortexed for 1 minute, and incubated on ice for 20 minutes to extract the cellular acid fraction. The mixture was centrifuged again at 1500 rpm for 5 minutes. The supernatant, containing the acid fraction, was transferred to new microcentrifuge tubes, and was kept at -20°C until derivatization. The remaining cell pellets were processed for protein extraction using Lowry's Assay. This acid fraction extraction method followed the protocol of Zhaolai Dai *et al.* (2014).

3.3.2.3 Preparation of Polyamines Standard Solutions

Standard solutions of polyamines were prepared to establish the calibration curve, which was then used to quantify polyamines from herb extracts and cells based on the curve equation. Working stock solutions of putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride at 5 nmol/µl were prepared, followed by the preparation of seven different standard dilutions. The standards were weighed and dissolved in 10 ml of 0.2M perchloric acid solution The standard concentrations were 0 to 5.0 nmol/200ul for all three standards.

3.3.2.4 Pre-column Derivatization of Polyamines by Benzoylation

The extracted herb, cell, and standard samples were thawed, and 500 µl of each sample was transferred to 15 ml centrifuge tubes. Then, 10 µl of 10 ppm 1,7-diaminoheptane, an internal standard, was spiked into each sample to enable relative recovery samples and ensure accurate quantification in a single run. After allowing the samples to rest for 15 minutes at room temperature, 1 ml of 2% (v/v) benzoyl chloride was added, and the samples were vortexed for 30 seconds. To adjust the pH of the samples to a more basic level, 1 ml of 2N sodium hydroxide (NaOH) was added and vortexed for another 30 seconds. This moderate alkaline medium is necessary for the deprotonation of biogenic amines (pH 8.0 to 9.6). The samples underwent derivatization for 20 minutes at room temperature. The reaction was stopped by adding 1 ml of 2% (w/v) L-proline aqueous solution and leaving it for 15 minutes. Subsequently, 1 ml of chloroform was added to each sample, and they were vigorously vortexed for 1 minute. After centrifuging the samples at 4200 rpm for 10 minutes at 4°C, the clear organic phase, denser than the aqueous phase, was collected at the bottom of the tubes and transferred into new microcentrifuge tubes. The organic phase was then dried over a water bath at 60°C. Dried samples were stored at -20°C until analysis. This protocol was modified and optimized based on the methods of Atiya *et al.* (2011) and Takahashi *et al.* (2018).

3.3.2.5 Polyamines Quantification by High Performance Liquid Chromatography (HPLC)

The dried benzoylated polyamines were dissolved in 350 µl of HPLC grade methanol, vortexed, and sonicated until fully dissolved. The samples were then filtered through a 0.45 µm nylon syringe filter and transferred into HPLC vials. Reversed-phase HPLC (RP-HPLC) was employed on a Perkin Elmer system equipped with a PDA detector using a C-18 narrow-bore column (2.1 × 200 mm, 5 µm particle size Hypersyl, Hewlett-Packard, USA). 1 µl of samples was injected using a mobile phase consisting of 80% methanol and 20% deionized water through an autosampler. The mobile phases ran at a flow rate of 1 ml/min over 7 minutes with an additional 1 minute for column cleanup for each sample, and the column temperature was maintained at 35°C. Chromatographic separation of polyamines was optimized using a wavelength of 240 nm. Chromera Microsoft software was utilized for analyzing the peak areas on the absorbance graph against retention time.

3.3.3 Total Protein Quantification

The acid-insoluble fraction from cell pellets, as described in sub-chapter 3.3.2.2, was further processed using the Lowry assay method to quantify cellular protein (Lowry *et al.*, 1951). Each sample received 500 µl of 0.3M sodium hydroxide (NaOH) and was incubated overnight to dissolve the protein. The samples were then stored at -20°C until analysis. A standard curve was created by preparing increasing concentrations of Bovine Serum Albumin (BSA) from 0 to 250 µg/ml in 0.3M NaOH solution.

Initially, each sample was diluted at ratios of 1:2 and 1:5 (v/v) with 0.3M NaOH solution. Then, 50 µl of protein samples from each dilution and standards were added in triplicate to a 96-well flat-bottomed microtiter plate. Next, 100 µl of Lowry reagent A (comprising 150 µl of 2% (w/v) sodium potassium tartrate, 150 µl 1% (w/v) copper sulphate, and 14.7 ml of 2% (w/v) sodium carbonate) was added and allowed to sit at room temperature for 10 minutes. Lowry reagent A contains copper II ions that bind to aromatic amino acid residues in proteins.

Meanwhile, Lowry reagent B (14 ml deionized water and 1 ml Folin & Ciocalteau's Phenol Reagent) was prepared, keeping it in the dark due to light sensitivity. Subsequently, 100 µl of Lowry reagent B was added to each well containing samples or standards. This reagent cleaves the copper-aromatic amino acid bonds, reducing the product to tungsten blue. The plate was then kept in the dark for 20 minutes to allow the color change to develop. The plates were read using a Tecan Sunrise microtiter plate reader set at 690 nm. A standard curve was generated by plotting the absorbance values of the standard protein samples against their respective concentrations, which was used to calculate the total protein present in the samples.

3.3.4 Real-time Expression Analysis (RT-qPCR)

The effect of two herbs, *S. aromaticum* and *E. scaber,* was tested by treating SiHa cells with the IC_{50} concentration of the herbs, and the changes in polyamine levels at different time points—0, 24, 48, 60, 72, and 96 hours of exposure—were observed. The cells were harvested at various time points for total RNA extraction, followed by conversion into complementary DNA (cDNA). The cDNA was then amplified and quantified quantitative polymerase chain reaction (qPCR). In this experiment, polyamine expression levels in the treated groups were detected and compared with the untreated group.

3.3.4.1 Cells treatment and acquisition

Cells were seeded in T75 flasks at a density of 5×10^4 cells/cm^2 for five different time points, with each time point including untreated, etoposide-, *S. aromaticum*-, and *E. scaber*-treated SiHa cells. Treatments were administered after 48 hours of incubation. Cells were harvested at specific time points: 0, 24, 48, 72, and 96 hours of exposure according to the protocols detailed in sub-section 3.2.5. Briefly, floating dead cells in the media were collected and combined with the attached cells from the flask. Once cell pellets were harvested, they were washed twice with ice-cold PBS solution and re-centrifuged at 1500 rpm for 5 minutes to completely remove any remaining culture

media. The supernatant was discarded, leaving only the cell pellets in the centrifuge tubes. The RNA from these cell pellets was then immediately extracted according to the manufacturer's protocol.

3.3.4.2 RNA Extraction

The RNA extraction was carried out using the Analytik Jena® (Germany) innuPREP DNA/RNA Mini Kit 2.0, following the manufacturer's instructions. Initially, a lysis solution was added to the cell pellet to break open the cell membrane and nuclear envelope, releasing cellular contents, including RNA, which was then incubated for 2 minutes. The cell pellet was resuspended by pipetting to ensure there were no clumps.

After that, the lysate was transferred to a spin filter D with a receiver tube and centrifuged at 1100 rpm for 2 minutes, separating genomic DNA from the sample. The spin filter D was then discarded. An equal amount of 70% ethanol was added to the filtrate and mixed by pipetting. The mixture was transferred to a spin filter R with a new receiver tube and centrifuged at the same speed for 2 minutes, allowing total RNA to bind to the spin filter R. Washing solution HS was added followed by washing solution LS to the spin filter R and centrifuged at the same speed and duration. After each centrifugation, the filtrate was discarded and replaced with a new receiver tube.

Next, an additional washing step with 80% ethanol was performed to enhance RNA purity, followed by a final centrifugation at the same speed for 3 minutes to remove ethanol traces. Finally, the spin filter R was placed in an elution tube, and 50 µl of RNase-free water was added, incubated for 1 minute, then centrifuged for 1 minute at the same speed. To check RNA purity, 1 µl of total RNA was pipetted onto a nanoplate reader.

3.3.4.3 RNA Quality Check

During the RNA quality check, three key results were expected: RNA concentration (ng/µl), A260/280 ratio, and A260/230 ratio. The A260/280 ratio was targeted to be between 1.8 and 2.1, with lower values indicating protein contamination. Similarly, the A260/230 ratio should also fall within the same range, with lower values suggesting contamination with organic materials such as phenol and TRIzol. The quality check was conducted using a TECAN Microplate Reader Infinite® M200 PRO with a NanoQuant plate insert. After the plate was wiped dry with 70% alcohol, 1 µl of total RNA was pipetted onto the NanoQuant plate. The absorbance ratios (260/280 and 230/260) were read, and the concentration of total RNA was quantified and recorded by the plate reader.

3.3.4.4 RNA Integrity Analysis

RNA integrity analysis was conducted using the QIAxcel® RNA Quality Control Kit v2.0, 1200 (QIAGEN GmbH, Germany) following the provided protocol. For sample preparation, 1 µl of total RNA was mixed with 1 µl of denatured buffer in PCR tubes. The mixture was spun, vortexed, and then transferred to a thermal cycler to run at 70°C for 2 minutes. Afterward, dilution buffer was added to a final volume of 10 µl, mixed well, and the PCR tubes were placed into the QIAxcel Advanced RNA Analysis System.

Each sample's peak calling was recorded, focusing on the 2:1 ratio of 28S to 18S bands, where the 28S band should be twice as intense as the 18S band, indicating high-quality RNA. A ratio of 2.0 denotes high-quality RNA. Partially degraded RNA appears smeared, lacks sharp RNA bands, and does not maintain the 2:1 ratio. Additionally, an RNA integrity score (RIS) from 0 to 10 was recorded, with 10 indicating fully intact RNA and less than 1 indicating completely degraded RNA.

3.3.4.5 cDNA Synthesis

The cDNA synthesis from total RNA was performed according to the protocol using the LunaScript® RT SuperMix Kit (Biolabs® Inc, New England, US). Firstly, the total RNA samples and the kit components were thawed, vortexed, and spun down. The cDNA synthesis reaction was prepared by mixing 4 µl of LunaScript® RT SuperMix, 1 µg of total RNA sample, and nuclease-free water was added to ensure the final volume of the mixture was 20 µl per reaction in PCR strip tubes. To ensure genomic DNA or carry-over products were absent from the total RNA, a no-RT (no reverse transcriptase) control reaction was performed, along with the no template control (NTC) to demonstrate a meaningful positive reaction. The reactions were incubated in a thermocycler according to the protocol: primer annealing (25°C for 2 minutes), cDNA synthesis (55°C for 10 minutes), and heat inactivation (95°C for 1 minute) in a single cycle. The synthesized cDNA was further used to perform quantitative PCR (qPCR).

3.3.4.6 Primer Design

The PCR primers for the targeted gene were designed using Primer BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI), adhering to guidelines for good primer design: GC content between 40-60%, length of 18-24 base pairs, sequences starting and ending with 1-2 GC pairs, and primer pairs having melting temperatures (Tm) within 5°C of each other. The sequences of the forward and reverse primers were further validated and synthesized by Macrogen Co. Four genes of interest (GOI) and two reference genes (RG) were selected: ornithine decarboxylase (*ODC*), spermine/spermidine N1-acetyltransferase 1 (*SAT1*), ornithine decarboxylase antizyme 1 (*OAZ1*), spermine oxidase (*SMOX*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and actin beta (*ACTB*), respectively. The primers sequences were tabulated in Table 3.4.

Table 3.4 The sequences of the primers

Target Gene	Primer sequence	
ODC	Forward	: 5' – AAAACATGGGCGCTTACAC – 3'
	Reverse	: 5' – TGGAATTGCTGCATGAGTTG – 3'
SAT1	Forward	: 5' – CACCCCTTTTACCACTGCCT – 3'
	Reverse	: 5' – TGCCAATCCACGGGTCATAG – 3'
OAZ1	Forward	: 5' – TCTCCCTCCACTGCTGTAGTAACC – 3'
	Reverse	: 5' – GTTGAGAATCCTCGTCTTGTCGTT – 3'
SMOX	Forward	: 5' – CCGCAGACTTACTTCCCCG – 3'
	Reverse	: 5' – GAGAGGGTCATCCGCACTG – 3'
GAPDH	Forward	: 5' – TGAGAACGGGAAGCTTGTCA – 3'
	Reverse	: 5' – TGGACTCCACGACGTACTCA – 3'
ACTB	Forward	: 5' – GTCATTCCAAATATGAGATGCGT – 3'
	Reverse	: 5' – GCTATCACCTCCCCTGTGTG – 3'

3.3.4.7 qPCR procedure

The cDNA amplification during each qPCR cycle was measured using dye-based quantitative PCR (qPCR) with SYBR® Green I, using Luna® Universal qPCR Master Mix (Biolabs® Inc, New England, US). Quantification cycle values (Cq) were used to determine the relative target abundance within the samples. The qPCR procedure was conducted according to the manufacturer's protocol. In brief, the cDNA synthesized from subsection 3.3.4.1.5 was diluted and used for the standard curve to determine the primer efficiency and gene expression activities in SiHa cells upon treatment with *S. aromaticum*, *E. scaber*, or etoposide. All kit components, including DNA samples, were first thawed at room temperature, and then placed on ice. All components were mixed by inversion, pipetting, or gentle vortexing. The total number of reactions was properly measured, thoroughly mixed by triturating, and the liquid was spun down by centrifugation. The components required per reaction were tabulated in the Table 3.5.

Table 3.5 The volume of each component required per qPCR reaction

Component	20 µl/reaction
Luna Universal qPCR Master Mix	5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
Template cDNA (≤ 1 µg)	variable
Nuclease-free water	to 20 µl

The assay mixture was aliquoted into qPCR tubes, and the DNA template was added while minimizing bubbles and maintaining consistent pipetting volumes. The tubes were sealed with flat, optically transparent caps and then briefly centrifuged at 3,000 rpm for 1 minute to remove bubbles and collect the liquid at the bottom. The thermocycling protocol was set up in the qPCR instrument using the SYBR/FAM scan mode setting. The settings were detailed in Table 3.5. The real-time PCR machine used was the CFX96 Touch™ Real-Time PCR Detection System.

Table 3.6 Real-time PCR protocol

Cycle Step	Temperature (°C)	Time (s)	Cycles
Initial denaturation	95	60	1
Denaturation	95	15	45
Extension	60	30 (+ plate read)	
Melt curve	60-95 (0.5°C increase)	5	1

3.3.4.8 Primer Efficiency Optimization and Designing qPCR standard curve

The qPCR standard curve was optimized to ensure primers efficiency and understand detection limit. This optimization is important to ensure robust, reproducibility, and sensitivity of qPCR assay. Following the protocol mentioned in sub-chapter 3.3.4.7, the concentrated DNA template (control- untreated SiHa cells upon 48 h incubation) was diluted from 1:1 to $1:10^6$ in serial dilution to make a standard curve. The primer efficiency was considered between 90 to 110%. The illustration of serial dilution was shown in Figure below.

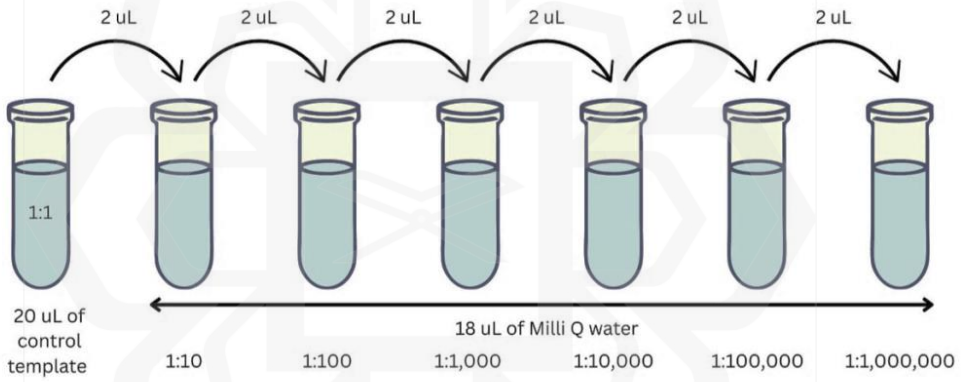


Figure 3.8 Serial dilution of control DNA template for Primer Efficiency

3.3.4.9 qPCR Data Analysis

The fold-change analysis was conducted using the comparative double delta Ct ($2^{-\Delta\Delta Ct}$) method. This relative quantification strategy calculates the gene expression levels between different samples by directly using the threshold cycles (Ct) from the qPCR system. First, the difference in Ct between the target and reference genes was calculated separately for treated and untreated cells using the ΔCt formula. Then, the differences between the ΔCt values of treated and untreated cells were calculated to obtain the ΔΔCt. Finally, the gene fold-change expression was determined.

$$\Delta Ct = Ct\ (GOI) - Ct\ (HKG)$$

$$\Delta\Delta Ct = \Delta Ct\ (Treated\ cells) - \Delta Ct\ (Untreated\ cells)$$

$$Fold\ difference\ in\ GOI\ relative\ to\ untreated = 2^{-(\Delta\Delta Ct)}$$

3.3.5 Cell Cycle Analysis Assay

The cell cycle is a series of events that a cell goes through as it grows and divides. Different stages of the cell cycle involve variations in the amount of DNA within a cell. Cell cycle analysis aims to distinguish and quantify cells at various stages of the cycle, such as the G1 (Gap 1), S (Synthesis), and G2/M (Gap 2 and Mitosis) phases.

3.3.5.1 Principle of Cell Cycle Analysis using Propidium Iodide

Cell cycle analysis using propidium iodide is useful as it allows the assessment of cell proliferation, identification of cell cycle arrest, and the characterization of cell cycle dynamics in response to various treatments or experimental conditions. The principle of cell cycle analysis using propidium iodide (PI) involves selectively staining cellular DNA at different stages of the cell cycle. PI, as a DNA-binding dye, binds to DNA and emits fluorescence upon intercalating with the DNA double helix. By analysing the fluorescence emitted by propidium iodide-stained cells using flow cytometry, researchers can distinguish and quantify cells in various phases of the cell cycle, providing valuable information about cell cycle dynamics and alterations in response to experimental conditions or treatments.

3.3.5.2 Protocol for Cell Cycle Analysis

3.3.5.2.1 Cell seeding, Treatment, and Sample Preparation

The cells were collected and quantified for concentration, as outlined in sections 3.3.5 and 3.3.5. Subsequently, SiHa cells, along with fresh media, were seeded into a T75 flask and incubated for 48 hours. Following this incubation period, treatments were introduced at the IC_{50} concentration determined from MTT assay results. The cells were treated with either *S. aromaticum*, *E. scaber*, or etoposide as a positive control, while another set remained untreated as a negative control. Harvesting of cells occurred at different time points (24, 48, and 72 hours). Upon obtaining cell pellets in centrifuge tubes, 1 ml of cold PBS was added to disperse the cells into a suspension. For cell counting using a hemocytometer, 10-20ul of cell suspension was transferred into microtubes to ensure an adequate cell count for subsequent cell cycle analysis. The cell suspension underwent centrifugation again to obtain a cell pellet. After discarding the supernatant, the cells were washed once more with PBS solution and the centrifugation process was repeated. Following removal of the supernatant, the cells were then subjected to cell fixation.

3.3.5.2.2 Cell Fixation

The cell pellet was gently vortexed continuously as 10 ml of 70% ice-cold ethanol was gradually added to the tube in a dropwise manner. The cells were fixed at -20°C overnight before the staining process. The counted cells were adjusted at concentration of 5 x 10^5 cells/ml.

3.3.5.2.3 Cell Staining

The staining buffer (50 µg/ml propidium iodide) and Ribonuclease A were prepared in advance and distributed into 1 ml tubes. These solutions were stored at -20°C until needed. The procedure for preparing the staining buffer can be found in the recipe section (refer to Glossary). Following fixation with ethanol, cells were centrifuged at 450 rpm for 5 minutes with a low brake setting. The supernatant was discarded, and the centrifuge cap was left open to facilitate ethanol evaporation. Subsequently, 1 ml of PBS was added to resuspend the cells, followed by vortexing for 1 minute. The cell suspension underwent centrifugation at the same speed and duration, and the supernatant was discarded. This process was repeated twice to eliminate residual ethanol. The cell pellet was then treated with 50 µl of RNase stock solution (final concentration 50 µg/ml) to enhance DNA resolution, considering that PI can also bind to double-stranded RNA. Following this, 200 µl of PI solution was added and thoroughly mixed. The samples were incubated in the dark at 37°C for 1 hour. Subsequently, the samples were transferred to a 96-well plate and acquired using the Guava High-throughput Flow Cytometer.

3.3.5.3 Protocol for Acquiring of DNA content using Flow Cytometry

The flow cytometry instrument was warmed up for 30 minutes before acquiring samples. During this time, a worklist was created using GuavaSoft® Software, noting the wells with samples along with the flow rate and the total number of events to acquire. The settings for acquired events, flow rate, and forward-scatter light (FSC) gain were configured to 5000 cells, 0.12 µl/s, and 548 V, respectively. The cell populations corresponding to different cell cycle phases were gated, with debris and clumps being eliminated from the analysis.

3.3.6 Mechanism of Cell Death Assay

3.3.6.1 Annexin V-FITC Assay

Apoptosis is a crucial cell death process that regulates normal development, tissue homeostasis, and certain disease mechanisms. In healthy cells, phosphatidylserine (PS) resides on the inner membrane surface. During apoptosis, PS translocate to the outer membrane. This PS exposure signals phagocytes to remove the dying cells, preventing the inflammatory response typical of necrosis. *In vitro*, externalized PS detection is facilitated by annexin V, which binds to PS with high affinity in the presence of calcium. This binding occurs before nuclear breakdown, DNA fragmentation, and the appearance of other apoptosis markers, making annexin V a reliable indicator of early apoptosis.

3.3.6.1.1 Protocol of Annexin V-FITC Assay

The Annexin-V analysis was performed using Calbiochem® Annexin-V FITC Apoptosis Detection Kit II (Merck KGaA, Darmstadt, Germany). The procedure for Annexin V-FITC assay includes cell incubation, harvest, cell number adjustment, cell acquisition, and finally analysing the sample using flow cytometer. The SiHa cells were incubated for 48 hours at a density of 5.0×10^4 cells/cm^2 in a T75 tissue culture flask. Following incubation, the cells were either left untreated or treated with *S. aromaticum*, *E. scaber*, or etoposide at the IC_{50} concentration determined from the MTT assay. The cells were incubated for an additional 48 hours before being harvested according to the protocol in sub- section 3.2.5.

Next, the harvested cells were washed three times with PBS, and the supernatant was discarded. The cell pellet was resuspended in 1 ml of binding buffer and adjusted to a concentration of 5.0×10^5 cells/ml to ensure optimal cell binding to Annexin V-FITC and subsequent PI staining. A 195 µl cell suspension was pipetted into a clean tube, and 5 µl of Annexin V-FITC was added. The solution was mixed and incubated

in the dark for 10 minutes at room temperature. The cell suspension was then washed with binding buffer and centrifuged at 1,000 rpm to pellet the cells, and the supernatant was discarded. Finally, the cell pellet was resuspended in 190 µl of binding buffer, 10 µl of PI was added, and the mixture was transferred into a 96-well round-bottom plate (200 µl per well) for analysis by flow cytometry.

3.3.6.1.2 Cells Acquisition and Analysis for Apoptosis using Flow Cytometry

The Guava® eacyCyte™ flow cytometer by Merck Millipore (KGaA, Darmstadt, Germany) was used for cells acquisition. The sample prepared in sub-section 3.4.7.1 was loaded into tray after warmed up for 30 minutes. The worklist was then created and the wells with samples were selected. A total of 3,000 events were acquired and medium flow rate of 0.59 µl/s. The voltages of PM1 and PM2 were adjusted so that the living cell population was positioned in the lower-left corner of PM1 versus PM2 dot plot. The identification of living cells, apoptotic, and necrotic cells were differentiated by the position of cells population on the dot plot as illustrated in Figure 3.9.

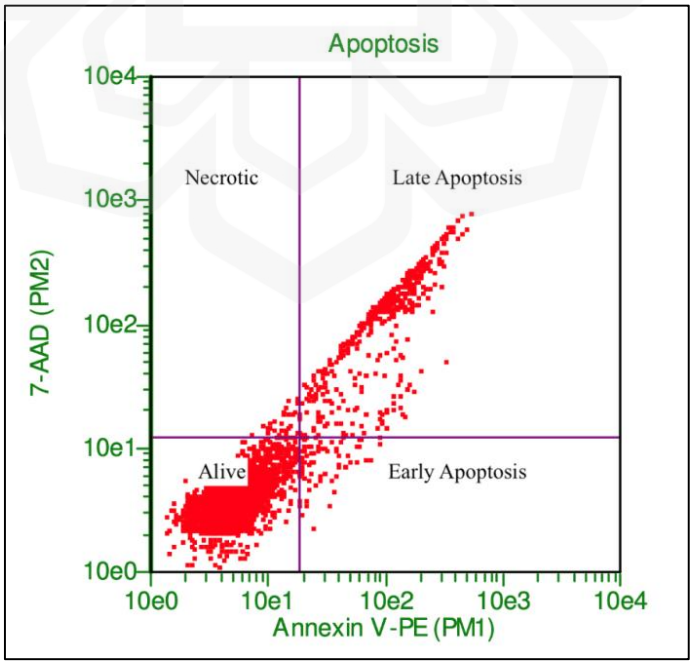


Figure 3.9 Identification of living cell population from apoptotic and necrotic cells

3.3.6.2 Caspase Activity Assay

During early stage of apoptosis, caspases, a distinctive group of cysteine proteases are activated and triggers the cell death. Caspases are classified into two; initiator (apical) caspases such as caspase-8, -9, and -10; and effector (executioner) caspases such as caspase-3, -7. The activation of effector caspases requires initiator caspases through an internal cleavage to separate the large and small subunits. This study investigated caspase activity within SiHa cells upon treatment with *S. aromaticum*, *E. scaber*, and etoposide as compared to untreated cells. Specific substrate is required to cleave active caspase that eventually forming a highly fluorescent product which then the fluorescent intensity can be measured by microplate reader. The Caspase-3 Caspase-8 and Caspase-9 Multiplex Activity Assay Kit was used for this investigation.

3.3.6.2.1 <u>*Protocol of Caspase assay*</u>

Caspase 3, Caspase 8 and Caspase 9 Multiplex Activity Assay Kit (Fluorometric), Abcam © 2023 was used to investigate the effects of *S. aromaticum* and *E. scaber* extracts on the key caspases involved in cell death of human cervical cancer cell line. First, SiHa cells were seeded at 5.0×10^4 cells/cm^2 into sterile black clear-bottom 96-well plate in duplicate. The cells were further incubated for 24 hours. The IC_{50} dose of *S. aromaticum* and *E. scaber* extracts were then added into wells. The cells were then further incubated for 24-, 48-, and 72-hours. At each specified timepoints, the caspase assay was performed according to the kit protocol. Caspase assay solution was added into each well (50 µl) and further incubated at room temperature for 30 minutes. The fluorescence changes were monitored using TECAN Infinite 200 PRO microplate reader. The fluorescence microplate reading the plate at RFU (relative fluorescence unit): Caspase 3: 535/620 nm (red), Caspase 8: 490/525 (green), and Caspase 9: 370/450 (blue).

3.4 STATISTICAL DATA ANALYSIS

All data were presented as the mean ± standard deviation (SD) of at least three independent experiments. The normality of the data distribution was assessed using the Kolmogorov-Smirnov test, with $p > 0.05$ indicating a normal distribution. Statistical analyses were performed using GraphPad Prism 9 and SPSS Version 25 software. For Objective 2 (antiproliferative effects), Objective 3 (effects on polyamine pathway), Objective 4 (cell cycle profile), and Objective 5 (mechanism of cell death), comparisons between treatment groups and the untreated control group were conducted using a two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test to identify significant differences. Results with a *p-value* < 0.05 were considered statistically significant. Where appropriate, additional post-hoc tests were applied to evaluate pairwise comparisons and ensure robust analysis of the dataset. Graphical representations of the data were generated using GraphPad Prism 9 for clarity and visualization of trends.

CHAPTER FOUR

RESULTS

Screening anti-proliferative compounds using *in vitro* systems is essential for assessing the potential of Malaysian herbs in cancer chemoprevention, ensuring the efficacy and safety of traditional herbs. Initially, cancer and normal cell lines are used to establish testing systems that evaluate the cytotoxicity and anti-proliferative effects of these herbs. After selecting effective Malaysian herbs, phytochemical screening is conducted to identify the bioactive compounds present in the extract. Following this, the optimal dosages (IC_{50}) are determined, and the herbs are used to treat cancer cells to study their molecular effects on intracellular polyamine levels, gene expression, and mechanisms of cell cycle regulation and apoptosis.

4.1 CYTOTOXICITY SCREENING OF SELECTED MALAYSIAN HERBS

Cytotoxicity screening was performed on 11 selected Malaysian herbs aqueous extracts using MTT assay. The study observed that six out of 11 types of Malaysian herbs had shown cytotoxic activity with half-maximal inhibitory concentration (IC_{50}) achieved which includes *Cengkih* (*Syzygium aromaticum*), *Tapak Sulaiman* (*Elephantopus scaber*), *Ulam raja* (*Cosmos caudatus* Kunth), *Bunga Raya* (*Hibiscus rosa-sinensis*), *Kacang Telang* (*Clitoria ternatea* L.), and cinnamon (*Cinnamomum verum*). Meanwhile, other five herbs; *Temu putih* (*Curcuma zedoaria*), *Mas cotek* (*Ficus deltoidea*), *Pecah Kaca* (*Strobilanthes crispus*), bay leaves (*Cinnamomum tamala*), and nutmeg (*Myristica fragrans*) demonstrated inactive cytotoxicity activity on SiHa cells where IC_{50} could not be achieved despite using different ratio of cosolvent (100% water, 70% water to 30% ethanol, or 50% water to 50% ethanol aqueous extract) at concentration up to 1,000 µg/ml. Table 4.1 demonstrated the cytotoxicity screening results of the herbs with IC_{50} values and three timepoints; 24-, 48-, and 72-h. The extract with the lowest IC_{50} value was selected for further analysis, as recommended by the NCI protocol, which considers extracts with IC_{50} values below 20 µg/mL to exhibit significant cytotoxic activity and potential anticancer properties (Grkovic *et al.*, 2020).

Table 4.1 Screening results on cytotoxicity activity of Malaysian herbs

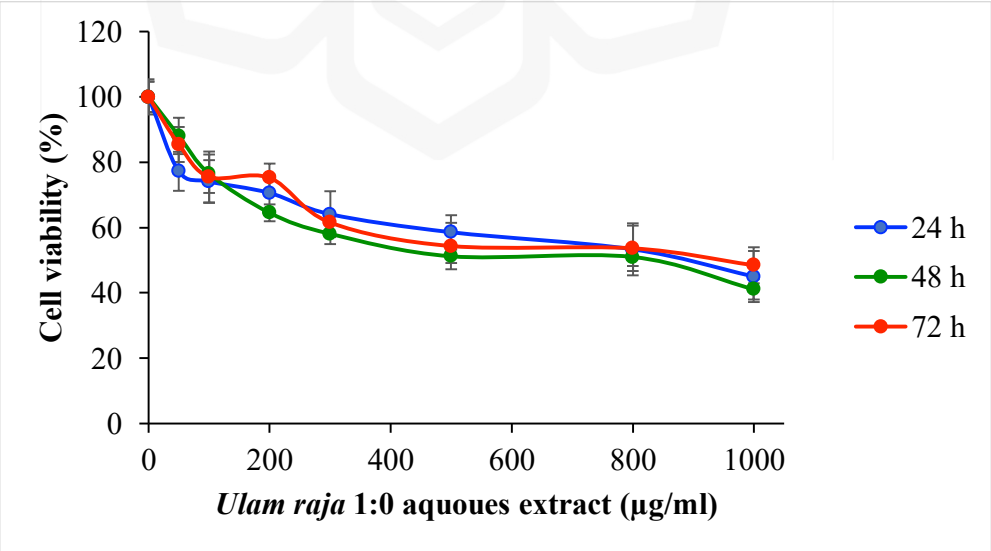
Common name (Scientific name)	**Extract ratio, %v/v**	**Cytotoxicity activity (IC_{50}) at different timepoints (μg/ml)**		
		24 h	**48 h**	**72 h**
Ulam raja (*Cosmos caudatus Kunth*)	1:0	880.2	820.9	942.6
	7:3	474.6	334.0	381.5
Bunga Raya (*Hibiscus rosa-sinensis L.*)	1:0	np	na	na
	7:3	np	na	853.5
Kacang Telang (*Clitoria ternatea L.*)	1:0	na	na	940.6
	7:3	827.1	716.4	402.3
Clove (*Syzygium aromaticum*)	1:0	316.8	177.6	169.6
	1:1	na	242.5	195.1
	7:3	478.9	220.6	189.4
Elephant's foot (*Elephantopus scaber*)	1:0	np	na	na
	7:3	149.5	88.8	56.3
	1:1	148.0	89.1	83.4
Cinnamon (*Cinnamomum verum*)	1:0	na	na	na
	7:3	np	na	na
	1:1	np	988.2	913.7
Temu putih (*Curcuma zedoaria*)	7:3	np	na	na
	7:3	np	na	na
Mas cotek (*Ficus deltoidea*)	1:1	np	na	na
	7:3	np	na	na
Pecah Kaca (*Strobilanthes crispus*)	1:1	np	na	na
	7:3	np	na	na
Bay leaves (*Cinnamomum tamala*)	1:1	np	na	na
	7:3	np	na	na
Nutmeg (*Myristica fragrans*)	1:1	np	na	na
	7:3	np	na	na

na: not achieved; np: not performed; 1:0 – 100% water extract; 7:3 – 70% water and 30% ethanol extract; 1:1 – 50% water and 50% ethanol extract. MTT assay was performed using seven different concentrations of the herbs aqueous extracts from 0 to 1,000 μg/ml.

In this study, 48-hour and 72-hour exposure periods were selected as the primary time points for cytotoxicity screening, as herbal extracts are generally known to exert their effects more gradually compared to synthetic agents. Many phytochemicals exhibit delayed onset of action due to complex cellular uptake and metabolism, which becomes more pronounced with extended exposure (Ibrahim and Kebede 2020; Kim *et al.* 2013). Therefore, to maximise the detection of potential antiproliferative effects, longer incubation times were prioritised. Since no significant cytotoxic activity ($IC_{50} \geq$ 1,000 µg/ml) was observed at either 48 or 72 hours for certain extracts, the 24-hour treatment was not pursued. A shorter exposure time was considered unlikely to yield meaningful results based on the trend of delayed efficacy associated with herbal compounds. Moreover, this approach also helped to minimise unnecessary wastage of reagents and consumables in line with good laboratory practice.

Ulam raja aqueous extract (1:0) was found to achieved IC_{50} on SiHa cell at 24-, 48-, and 72-hour treatment at 880, 820, and 942 µg/ml respectively. Meanwhile, at 7:3 extract ratio, IC_{50} was found lower which are 474.6, 334.0 and 381.5 µg/ml at 24, 48, and 72 h respectively. Referring to the IC_{50} of both 1:0 and 7:3 *Ulam raja* extracts, the potency of *Ulam raja* are inactive and weakly active respectively. The cell viability (%) against increasing concentration of *Ulam raja* extracts at 1:0 and 7:3 ratios were depicted in Figure 4.1 (a) and (b) respectively.

(a)



(b)

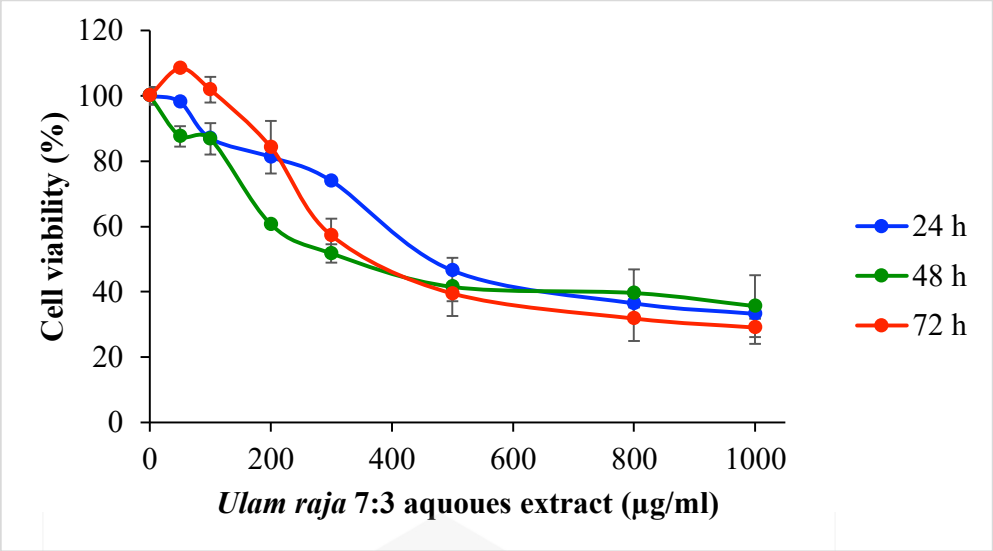


Figure 4.1 (a) and (b) The cytotoxicity of *Ulam raja* 1:0 and 7:3 aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 24, 48, and 72 hour timepoint.

The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.

Bunga Raya or hibiscus aqueous extract did not showed potency on SiHa cells except at 72 hours with 7:3 extract ratio at 853.5 µg/ml. Figure 4.2 illustrated the cytotoxicity activity of 7:3 Hibiscus aqueous extract. The cytotoxicity graph of hibiscus 1:0 extract was inserted in Appendix I: Supplementary Results.

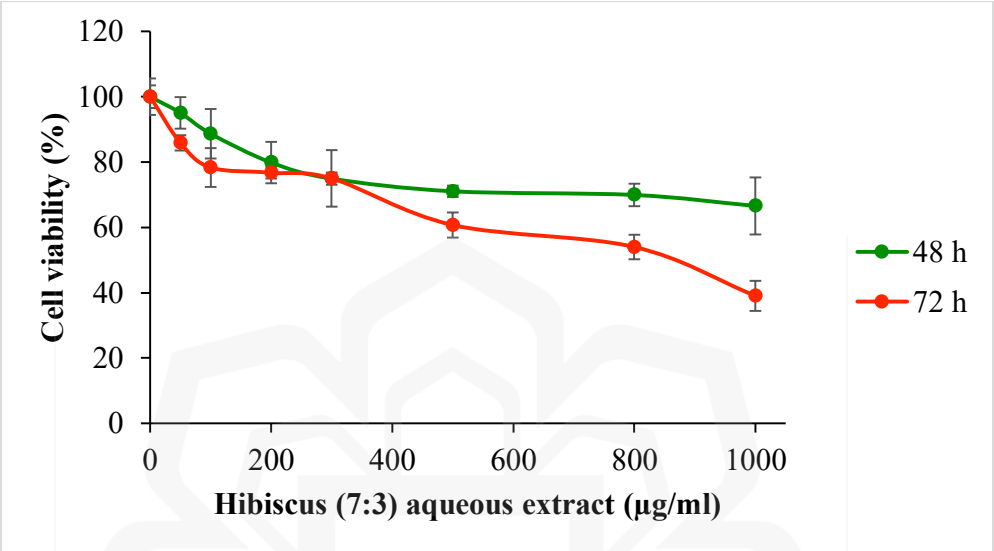


Figure 4.2 The cytotoxicity of hibiscus (7:3) aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 48 and 72 h timepoint.

The 24 h treatment was not performed. The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.

The anti-proliferative activity of *Kacang Telang* flower extract on SiHa cells varied depending on the ratio of water used in the extraction process. The extract prepared with 100% water demonstrated lower potency, with an IC_{50} of 940.6 µg/ml after 72 hours of treatment. In contrast, 7:3 ratio extract exhibited a higher cytotoxic effect on SiHa cells. The IC_{50} values were 827.1 µg/ml, 716.4 µg/ml, and 402.3 µg/ml after 24, 48, and 72 hours of treatment, respectively. Figures 4.3 (a) and (b) illustrate the cytotoxic activity of *Kacang Telang* extract on SiHa cells at these different time points.

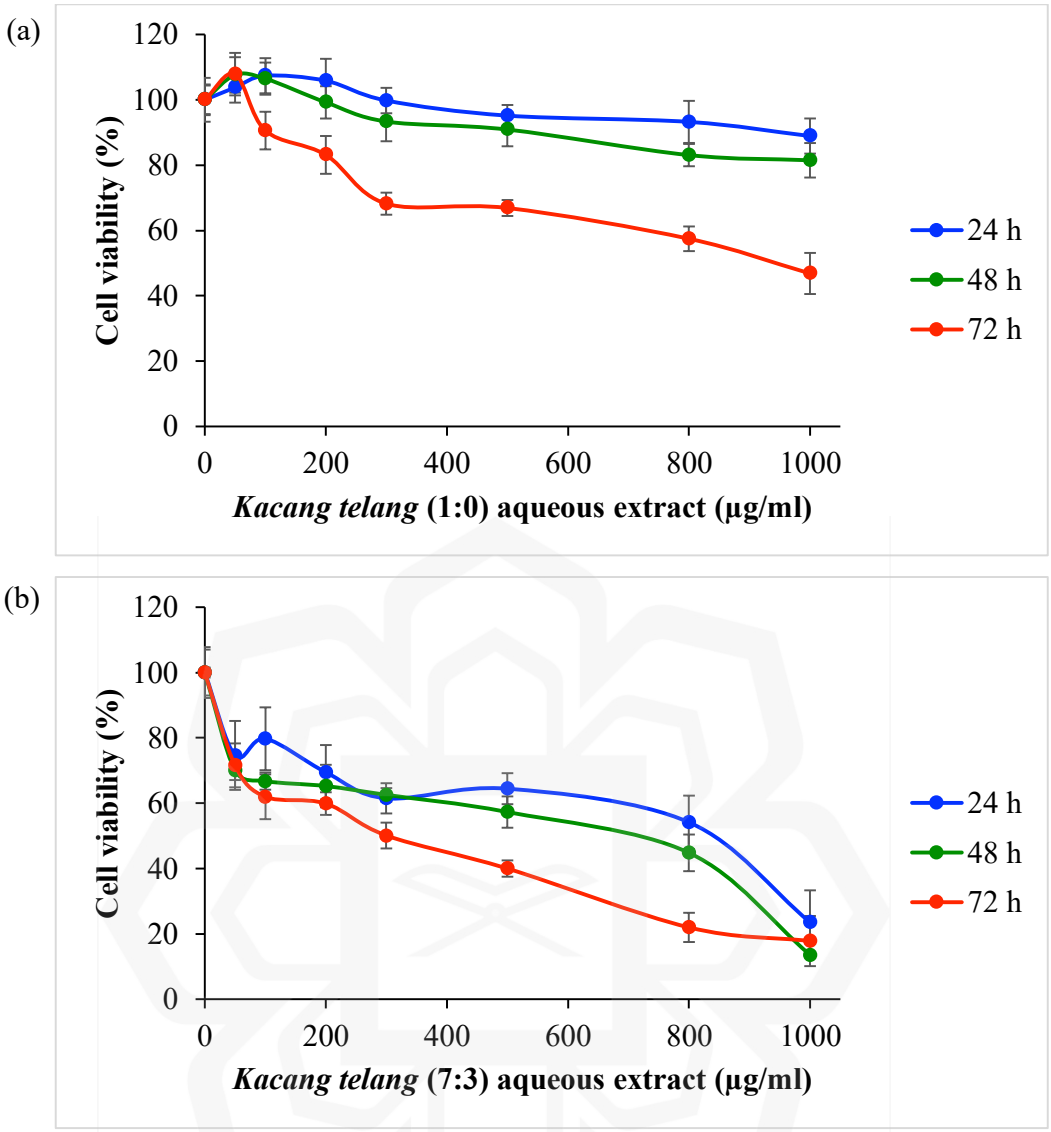


Figure 4.3 (a) and (b) The cytotoxicity of 1:0 and 7:3 *Kacang Telang* aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 24, 48, and 72 h timepoint. The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.

Next, the antiproliferative activity of cinnamon aqueous extract was investigated on three different waters to ethanol ratio which are 1:0, 7:3, and 1:1 on SiHa cells at concentration ranging from 0 to 1000 µg/ml. While both 1:0 and 7:3 ratio aqueous extracts showed no cytotoxicity on SiHa cells, the 1:1 ratio cinnamon aqueous extract achieved IC_{50} at 48 and 72 h treatment at 988.2 and 913.7 µg/ml, respectively. However, cinnamon extract showed inactive cytotoxicity according to NCI guidelines as IC_{50} concentration is more than 500 µg/ml. Figure 4.4 presented the cell viability against

different concentrations of 1:1 cinnamon aqueous extract while the cytotoxicity graphs of 1:0 and 7:3 cinnamon extract was attached in Appendix I: Supplementary Results.

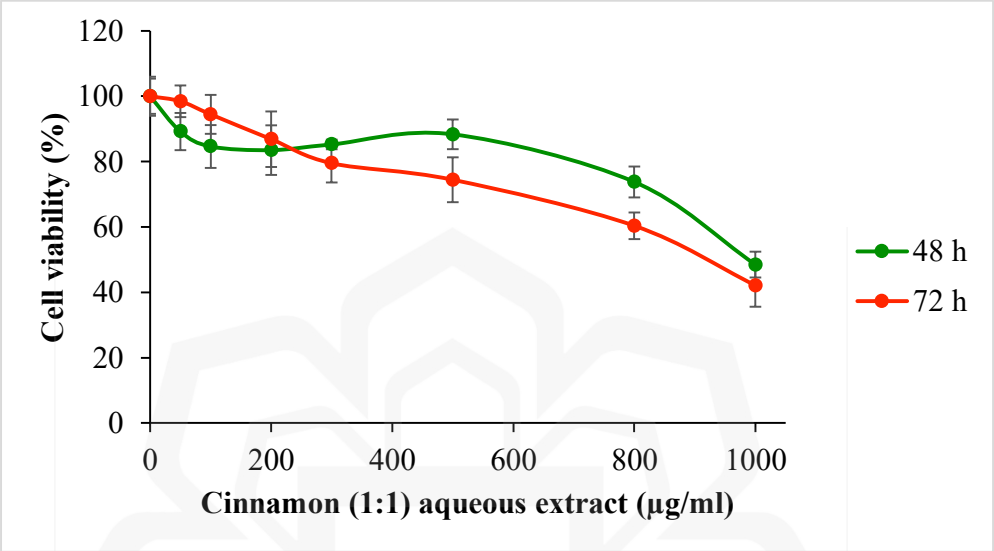


Figure 4.4 The cytotoxicity of 1:1 cinnamon aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 48 and 72 h timepoint. The 24 h treatment was not performed. The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.

Among the 11 Malaysian herbs tested for cytotoxicity using the MTT assay, *S. aromaticum* and *E. scaber* demonstrated the lowest IC_{50} concentrations against SiHa cells. Consequently, these two herbs were chosen for further investigation to explore their anticancer potential, specifically focusing on the molecular mechanisms involving polyamine pathways. Detailed cytotoxicity results for *S. aromaticum* and *E. scaber* are presented in sub-chapter 4.3.

4.2 THE PHYTOCHEMICAL PROFILING OF *S. AROMATICUM* AND *E. SCABER*

4.2.1 Clove (*Syzygium aromaticum*)

A qualitative analysis was conducted using gas chromatography-mass spectrometry (GC-MS) to identify the phytochemical compounds present in the aqueous *S. aromaticum* extract (100% water). The analysis revealed a prominent peak corresponding to eugenol, which accounted for 79.36% of the total peak area, indicating that eugenol is the major bioactive compound in the *S. aromaticum* extract. The other component present in *S. aromaticum* aqueous extract was Butylaldehyde, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl]-. The retention time and spectral characteristics of the detected peak were consistent with the reference standards for eugenol, further confirming its identity.

Figure 4.7 presents the GC-MS chromatogram of the aqueous *S. aromaticum* extract, displaying the relationship between the retention time and the peak area of eugenol. Additionally, Table 4.2 provides a detailed summary of the chromatographic data, including retention time, peak area percentage, and compound identification. The absence of additional significant peaks suggests that the extraction method used primarily isolated eugenol, with minimal interference from other phytochemicals or impurities.

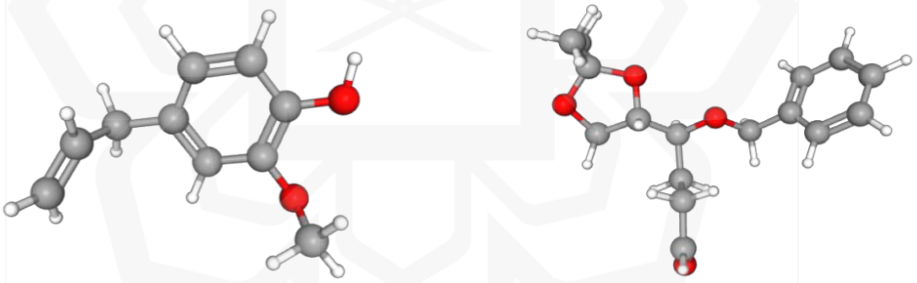
Eugenol is a phenolic compound widely recognized for its diverse pharmacological properties, including antioxidant, anti-inflammatory, and anticancer activities. Its high abundance in the extract convinces the potential efficacy of *S. aromaticum* as a natural chemopreventive agent. The high eugenol concentration in the aqueous extract highlights the efficiency of the maceration process employed in this study for extracting polar compounds. This method, using 100% water as a solvent, appears to selectively extract eugenol while minimizing the co-extraction of non-polar or less soluble compounds. The results provided a strong basis for further bioactivity

studies focused on eugenol as a key contributor to the therapeutic properties of *S. aromaticum*.

Table 4.2 Phytochemical profile of aqueous *S. aromaticum* extract using GC-MS analysis

Name of component	**Molecular formula**	**Molecular weight (g/mol)**	**RT (min)**	**Peak area (%)**
Eugenol	$C_{10}H_{12}O_2$	164.20	10.90	79.36
Butylaldehyde, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl]-	$C_{16}H_{22}O_4$	278.34	12.91	10.15
			12.82	7.23
			11.83	3.26

RT: retention time



(a) Eugenol (b) Butylaldehyde, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl]-

Figure 4.5 Chemical structure model of eugenol identified in *S. aromaticum*. Ball and stick chemical structure model were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov).

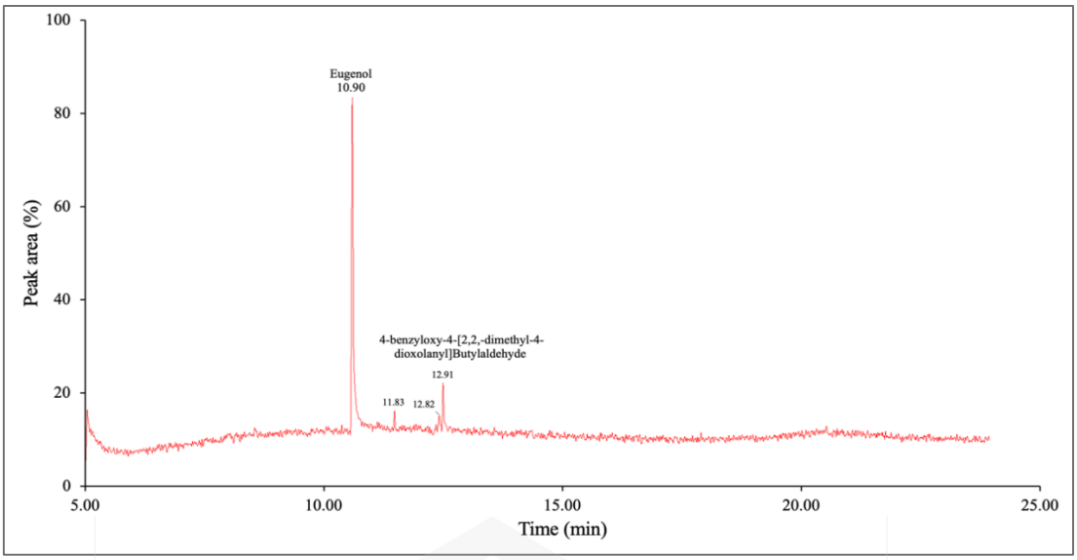


Figure 4.6 GC-MS chromatogram of *S. aromaticum* bud extract displaying its bioactive components

4.2.2 Elephant's foot (*Elephantopus scaber*)

The initial phytochemical analysis of the *E. scaber* aqueous extract (7:3 water-to-ethanol ratio) was performed using GC-MS; however, no detectable peaks were observed. Following consultation with my co-supervisor, Assoc. Prof. Dr. Deny Susanti, an expert in bioactive compounds, and further literature review, LC-MS in positive ionization mode was identified as a more suitable technique. This decision was based on the fact that LC-MS operates at a lower chromatographic separation temperature of 40°C, whereas GC-MS requires significantly higher temperatures of up to 250°C. Since many bioactive compounds in *E. scaber* are heat-sensitive, the lower operating temperature of LC-MS provided a more effective means of preserving and identifying these compounds. The LC-MS analysis successfully detected 32 bioactive compounds in the aqueous extract of *E. scaber*. These compounds were classified into several chemical groups, including flavonoids, alkaloids, terpenoids, and phenolic derivatives, each contributing to the plant's potential therapeutic properties. Compound identification was achieved by comparing the retention times, peak area percentages, height percentages, and mass spectral fragmentation patterns with reference data from the National Institute of Standards and Technology (NIST) library.

Eleven compounds were classified as flavonoids and phenolic compounds: Iretol – isoflavone, 8-C-Methylvelloquercetin 3,5,3'-trimethyl ether, 1-O-Feruloylglucose, Blumenol C glucoside, 2',4'-Dihydroxydihydrochalcone, Flavanone, 2',3',4',6'-Tetramethoxychalcone, (E)-4-stilbenol, Liquoric acid, 7-hydroxycoumarinyl-γ-Linolenate, 5-(6-Hydroxy-3,7-dimethyl-2,7-octadienyloxy)-7-methoxycoumarin. The alkaloid class included two compounds: Parsonsine and Leonuridine. Meanwhile, 10 compounds were classified as terpenoids including, Methylpicraquassioside A, Lepidine E, Ponalactone A, Molephantinin, Gibberellin A108, Osthol, Tagitinin F, Deoxymiroestrol, Absindiol, and Emmotin A.

Moreover, the fatty acid derivatives and hydroxylated compounds class, two compounds were identified: 10-tridecynoic acid and 18-hydroxy-9S,10R-dihydroxy-stearic acid. Two carotenoid derivatives and two amino acids were identified which are prasinoxanthin, annohexocin and L-Leucine, Quinacetol, respectively. Other bioactive compounds included are cis-β-D-Glucosyl-2-hydroxycinnamate, Citrusal, and 2-Methyl-3-phenyl-2-propenal. The predominance of flavonoids, terpenoids, and alkaloids highlights the plant's ability to modulate cancer-related pathways. Table 4.3 presented the phytochemicals identified in *E. scaber* aqueous extract through LC-MS system while Figure 4.9 presented the chromatographic results of *E. scaber*.

Table 4.3 LC-MS profile of phytochemicals identified and their distribution in *E. scaber* aqueous extract in positive ionization mode.

No	RT (min)	Compound classification	Identification	Molar mass (g/mol)	Chemical formula	Peak area (mV/sec)	METLIN
1	1.059	Flavonoids and phenolic compounds	Iretol - isoflavon	156.04	C7 H8 O4	335.07	43632
2	5.262		8-C-Methylvelloquercetin 3,5,3'-trimethyl ether	424.15	C24 H24 O7	425.16	51154
3	6.78		1-O-Feruloylglucose	356.11	C16 H20 O9	395.07	91926
4	7.974		Blumenol C glucoside	372.22	C19 H32 O7	373.22	95146
5	11.482		2',4'-Dihydroxydihydrochalcone	242.09	C15 H14 O3	243.1	52245
6	12.909		Flavanone	224.08	C15 H12 O2	225.09	43635
7	12.912		2',3',4',6'-Tetramethoxychalcone	328.13	C19 H20 O5	329.14	52144
8	12.932		(E)-4-stilbenol	196.09	C14 H12 O	197.1	53251
9	22.637		Liquoric acid	484.32	C30 H44 O5	507.31	89984
10	20.297		7-hydroxycoumarinyl-γ-Linolenate	422.25	C27 H34 O4	845.5	64760
11	13.693		5-(6-Hydroxy-3,7-dimethyl-2,7-octadienyloxy)-7-methoxycoumarin	344.16	C20 H24 O5	345.17	88854
12	7.134	Alkaloids	Parsonsine	439.22	C22 H33 N O8	462.21	68273
13	8.015		Leonuridine	348.14	C15 H24 O9	387.11	71500
14	9.372		Methylpicraquassioside A	412.14	C19 H24 O10	451.1	93067

No	RT (min)	Compound classification	Identification	Molar mass (g/mol)	Chemical formula	Peak area (mV/sec)	METLIN
15	11.31		Lepidine E	346.4	C20 H18 N4 O2	385.11	88987
16	11.484	Terpenoids and steroids	Ponalactone A	346.37	C19 H22 O6	385.1	67487
17	12.405		Molephantinin	360.16	C20 H24 O6	399.12	67765
18	12.88		Gibberellin A108	330.15	C19 H22 O5	331.15	87404
19	12.886		Osthol	244.11	C15 H16 O3	245.12	44022
20	12.889		Tagitinin F	348.16	C19 H24 O6	349.16	67799
21	13.758		Deoxymiroestrol	342.15	C20 H22 O5	343.15	72068
22	14. 539		Absindiol	266.15	C15 H22 O4	305.11	89096
23	18.865		Emmotin A	278.15	C16 H22 O4	317.11	53414
24	7.979	Fatty acids and lipid derivatives	10-Tridecynoic acid	210.16	C13 H22 O2	211.17	74260
25	13.727		18-hydroxy-9S,10R-dihydroxy-stearic acid	332.26	C18 H36 O5	371.22	36012
26	22.092	Carotenoids	Prasinoxanthin	600.42	C40 H56 O4	639.38	41451
27	24.458		Annohexocin	628.46	C35 H64 O9	667.42	95767
28	1.409	Amino acid	L-Leucine	131.09	C6 H13 N O2	132.1	233
29	2.294	Other bioactive compounds	Quinacetol	187.06	C11 H9 N O2	205.1	72704
30	3.398		cis-β-D-Glucosyl-2-hydroxycinnamate	326.1	C15 H18 O8	344.13	64166

No	RT (min)	Compound classification	Identification	Molar mass (g/mol)	Chemical formula	Peak area (mV/sec)	METLIN
31	6.639		Citrusal	260.11	C15 H16 O4	261.11	87196
32	12.921		2-Methyl-3-phenyl-2-propenal	146.07	C10 H10 O	147.08	87771

RT: retention time, METLIN: Metabolite LINk

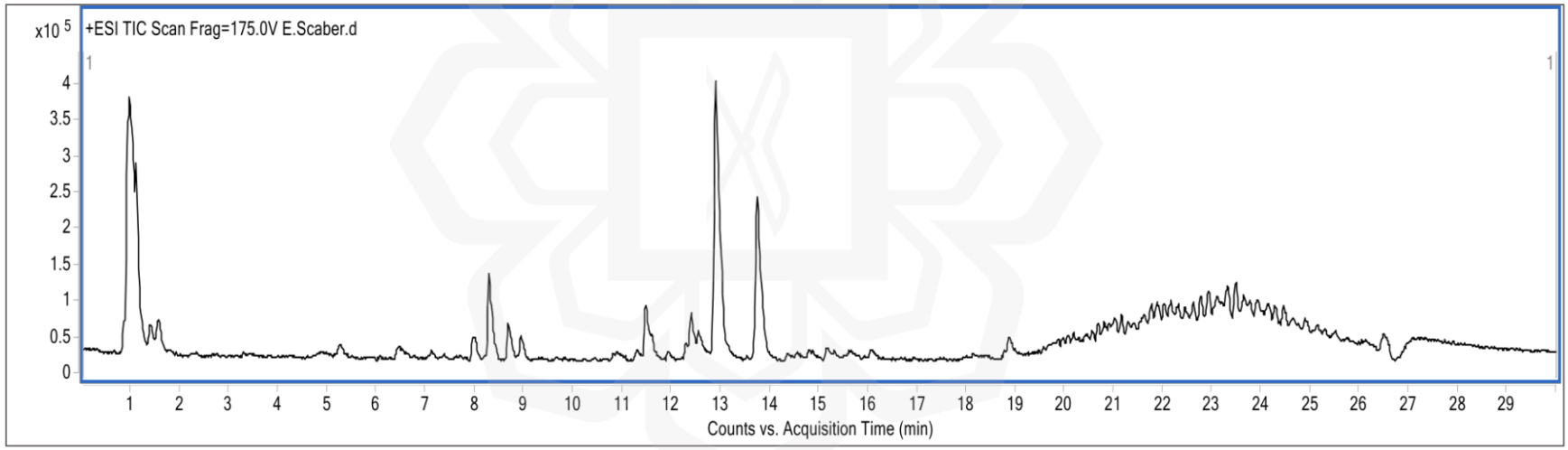


Figure 4.7 LC-MS report of *E. scaber* aqueous extract

4.3 CYTOTOXICITY EFFECTS AND CELL GROWTH PROFILE ON HUMAN CERVICAL CANCER CELL LINE

The antiproliferative activity of selected herbal extracts on a human cervical cancer cell line was evaluated using the MTT assay, while cell growth was assessed via the Trypan Blue Exclusion (TBE) assay, following the protocols outlined in subsections 3.3.1.2 and 3.3.1.1, respectively. Based on the cytotoxicity screening results presented in section 4.1, two herbs—*S. aromaticum* and *E. scaber*—were chosen for further investigation of their molecular effects on polyamine metabolic pathways and their potential as chemopreventive agents in cervical cancer. Their selection was based on the lowest IC_{50} values from the MTT assay, which demonstrated significant antiproliferative effects on SiHa cells.

This subsection also presents the establishment of a positive control using etoposide, a chemotherapeutic drug known for its effectiveness against cervical cancer cell lines. Additionally, the toxicity of *S. aromaticum* and *E. scaber* extracts on normal cell lines was assessed using the MTT assay.

4.3.1 Establishment of Positive Control

Positive control was required to ensure accurate biological responses. Etoposide was used as the positive control for SiHa cells, as it is a commonly used chemotherapy treatment for cervical cancer. If SiHa cells did not respond to etoposide treatment, it could indicate issues with the biology, plate, or sample preparation. The MTT assay was performed to assess the effect of positive control on SiHa cell proliferation and to determine the IC_{50} values. Figure 4.8 illustrates the effect of increasing concentrations of etoposide on SiHa cell viability. Table 4.4 presents the IC_{50} concentrations measured at 24, 48, and 72 hours of treatment. The IC_{50} dose at 48 hours (30 μg/ml) was selected for subsequent experiments to investigate its molecular effects in comparison to untreated, *S. aromaticum*-treated, and *E. scaber* -treated cells.

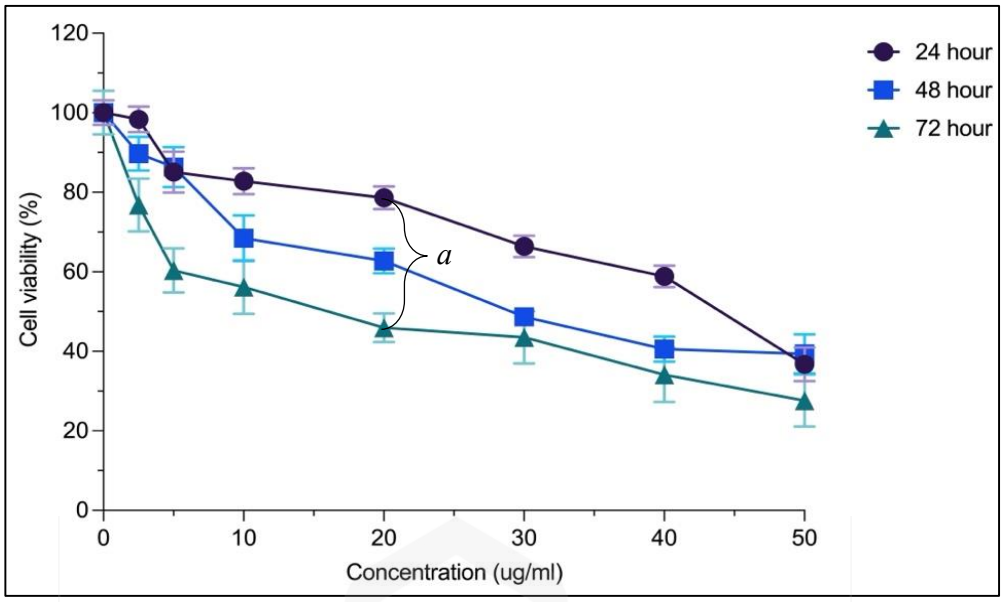


Figure 4.8 The cytotoxicity of etoposide on SiHa cells at concentration 0 to 50 µg/ml at 24, 48, and 72 h timepoint.

SiHa cells were seeded at a density of 5.0×10^4 cells/cm^2 in a 96-well plate and incubated for 24 hours. The medium was then replaced with fresh medium containing increasing concentrations of etoposide. The plates were further incubated for 24, 48, or 72 hours, and cell viability was assessed following the protocol described in subsection 3.3.1.2. Results are presented as mean ± S (n = 3), with three independent replicates conducted for each experiment. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test, revealing a significant difference between time points and concentrations (*a* indicates $p < 0.05$, representing a significant difference between 24 and 72 hours). Unlabelled data points indicate no significant differences.

Table 4.4 The IC_{50} values of etoposide at 24, 48, and 72 hours of exposure

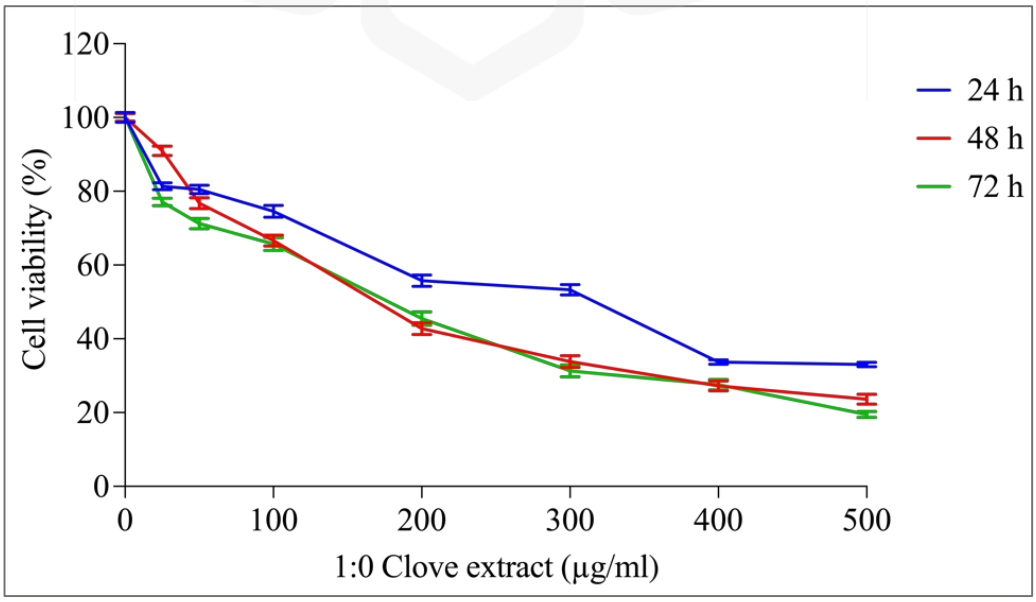
Timepoint (h)	**24**	**48**	**72**
IC_{50} value (µg/ml)	43.990	29.036	16.006

4.3.2 Antiproliferative Assessment of *S. aromaticum* and *E. scaber* on SiHa cells

At all three time points (24, 48, and 72 hours) following the addition of the MTT solution, SiHa cell viability decreased as the concentration of *S. aromaticum* aqueous extract increased. The IC_{50} values for the 100% aqueous *S. aromaticum* extract at 24, 48, and 72 hours were 316.80, 177.64, and 169.60 µg/ml, respectively, while the IC_{50} values for the 70% aqueous *S. aromaticum* extract at the same time points were 478.91, 220.58, and 189.35 µg/ml. The lowest IC_{50} value for *S. aromaticum* was observed with the 100% aqueous extract after 72 hours of treatment. Figure 4.9 and Table 4.5 illustrate the cytotoxic activity of the 100%, 70%, and 50% *S. aromaticum* aqueous extracts at increasing concentrations (0 to 500 µg/ml) and their respective IC_{50} values at 24, 48, and 72 hours. The results showed no significant differences between same concentrations at different timepoints for 1:0 and 7:3 ratio extract, as determined by one-way ANOVA with Tukey's multiple comparison statistical test.

Based on the IC_{50} values, the 100% aqueous *S. aromaticum* extract exhibited moderate cytotoxicity at 48 and 72 hours of treatment, while the 70% and 50% *S. aromaticum* extracts demonstrated similar cytotoxicity only at 72 hours of exposure. To further evaluate the potential of *S. aromaticum* as a chemopreventive agent for cervical cancer, a treatment concentration of 180 µg/ml was selected for subsequent experiments.

(a)



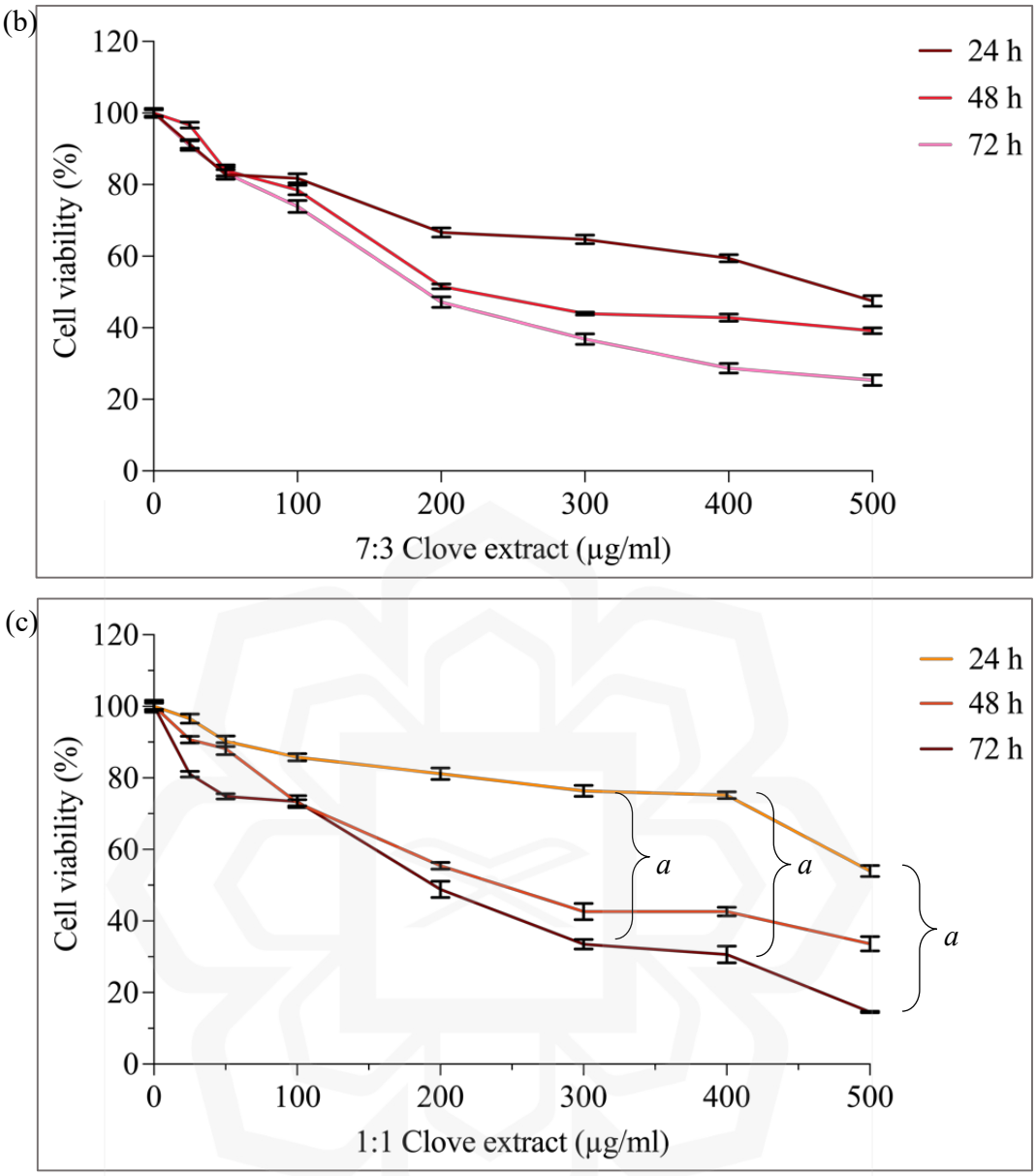


Figure 4.9 The anti-proliferative effects of 1:0 (a), 7:3 (b), and 1:1 (c) ratio aqueous *S. aromaticum* extracts on SiHa cells.

SiHa cells were seeded at a density of 5.0×10^4 cells/cm² in a 96-well plate and allowed to adhere for 24 hours. Following this, the culture medium was replaced with fresh medium containing varying concentrations of *S. aromaticum* extract. The treated cells were then incubated for 24, 48, or 72 hours, after which cell viability was evaluated according to the procedure outlined in subsection 3.3.1.2. Data are expressed as mean ± SD (n = 3), with each experiment conducted in triplicate. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test, demonstrating a significant difference between time points and concentrations (a denotes $p < 0.05$, indicating a significant difference between 24 and 72 hours). Data points without labels indicate no significant differences.

Table 4.5 IC_{50} values of 1:0, 7:3, and 1:1 ratio of *S. aromaticum* aqueous extract at 24, 48, and 72 hours of treatment

Treatment Duration (h)	**IC_{50} values of *S. aromaticum* Aqueous extract (µg/ml)**		
	1:0	**7:3**	**1:1**
24	316.80	478.91	NA
48	177.64	220.58	242.51
72	169.60	189.35	195.12

NA: not achieved

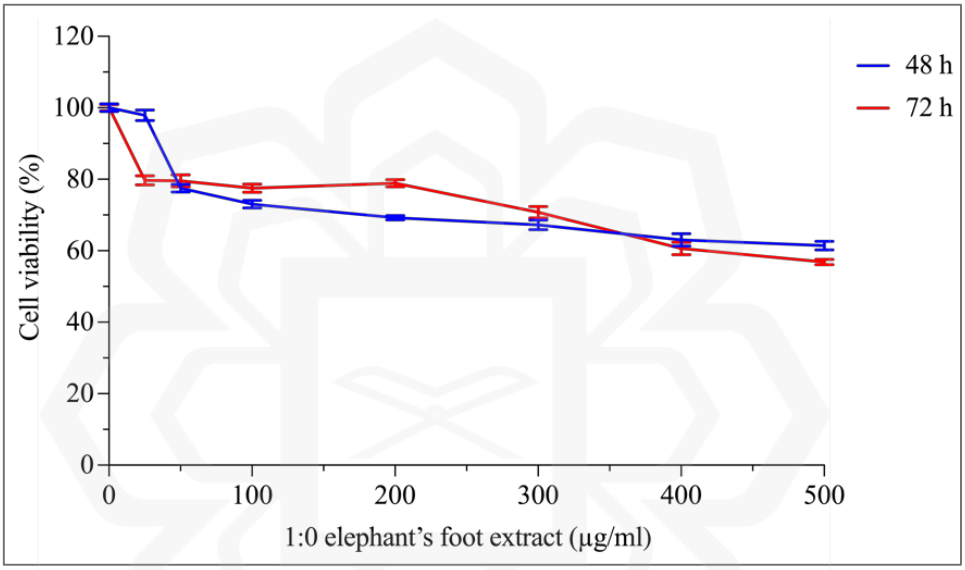
Figure 4.10 and Table 4.6 depict the cytotoxic activity of 1:0, 7:3, and 1:1 ratio of *E. scaber* aqueous extracts on SiHa cells, as well as the IC_{50} values for the extracts at increasing concentrations (0 to 500 µg/ml) at 24, 48, and 72 hours. The cytotoxicity of the 1:0 aqueous *E. scaber* extract was examined at 48 and 72 hours of treatment, but IC_{50} values were not achieved. The *E. scaber* aqueous extract (7:3 and 1:1 ratio extracts) showed moderate cytotoxic activity on SiHa cell. The IC_{50} values for the 7:3 aqueous *E. scaber* extract at 24, 48, and 72 hours were 149.53, 88.76, and 56.30 µg/ml, respectively, while the IC_{50} values for the 1:1 aqueous extract at the same time points were 147.99, 89.05, and 83.38 µg/ml. The lowest IC_{50} for *E. scaber* was recorded with a 7:3 water-to-ethanol extract (%v/v) after 72 h of treatment. For subsequent experiments, the treatment concentration for *E. scaber* was set at 90 µg/ml. The results showed no significant differences between same concentrations at different timepoints, as determined by one-way ANOVA with Tukey's multiple comparison statistical test.

Table 4.6 IC_{50} values of 100%, 70%, and 50% (v/v) *E. scaber* aqueous extract at 24, 48, and 72 hours of treatment

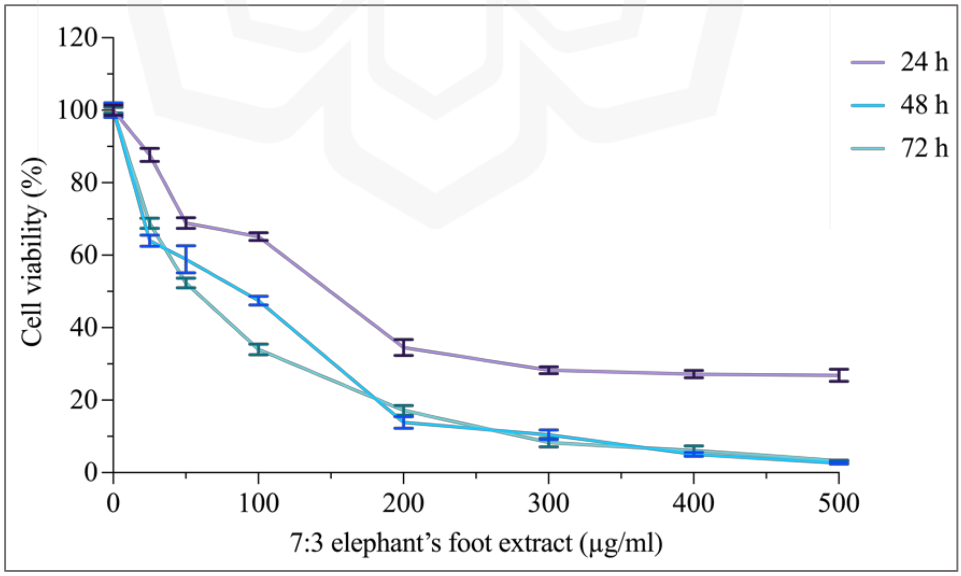
Treatment Duration (h)	**IC_{50} values of *E. scaber* aqueous extract (µg/ml)**		
	1:0	**7:3**	**1:1**
24	NP	149.53	147.99
48	NA	88.76	89.05
72	NA	56.30	83.38

NP: not performed, NA: not achieved

(a)



(b)



(c)

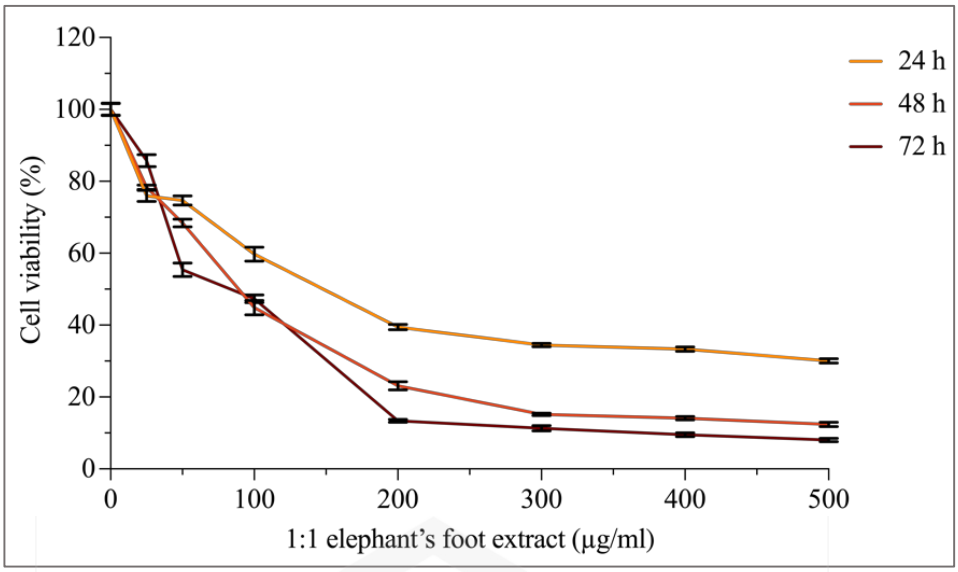


Figure 4.10 The cytotoxic effects of 1:0 (a), 7:3 (b), and 1:1 (c) aqueous extracts of *E. scaber* on SiHa cells.

4.3.3 Toxicity Assessment of *S. aromaticum* and *E. scaber* on Normal Cell Line, HEK 293t cell

The criteria of chemoprevention agents are to specifically target cancer cells by inhibit, prevent, or slow down the progression of cancer cells while protecting the normal cells. Thus, further investigation of *S. aromaticum* and *E. scaber* extract were conducted on human embryonic kidney (HEK 293t) cells to ensure there are no off-target effect caused by the herbs. Figure 4.11 and Figure 4.12 presented the cell viability of HEK293t cells upon treatment with 1:0 *S. aromaticum* extract and 7:3 *E. scaber* extracts, respectively. Results showed that both extracts showed no toxicity on normal cell line with increasing treatment concentration up to 500 µg/ml. The results showed no significant differences between same concentrations at different timepoints, as determined by one-way ANOVA with Tukey's multiple comparison statistical test on both *S. aromaticum*- and *E. scaber* -treated HEK293t cells.

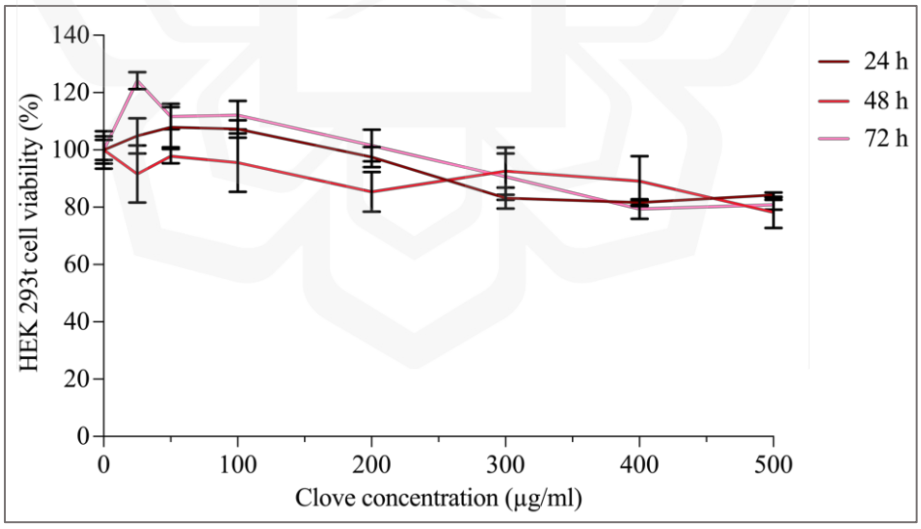


Figure 4.11 Antiproliferative investigation of 1:0 *S. aromaticum* aqueous extract on HEK 293t cell.

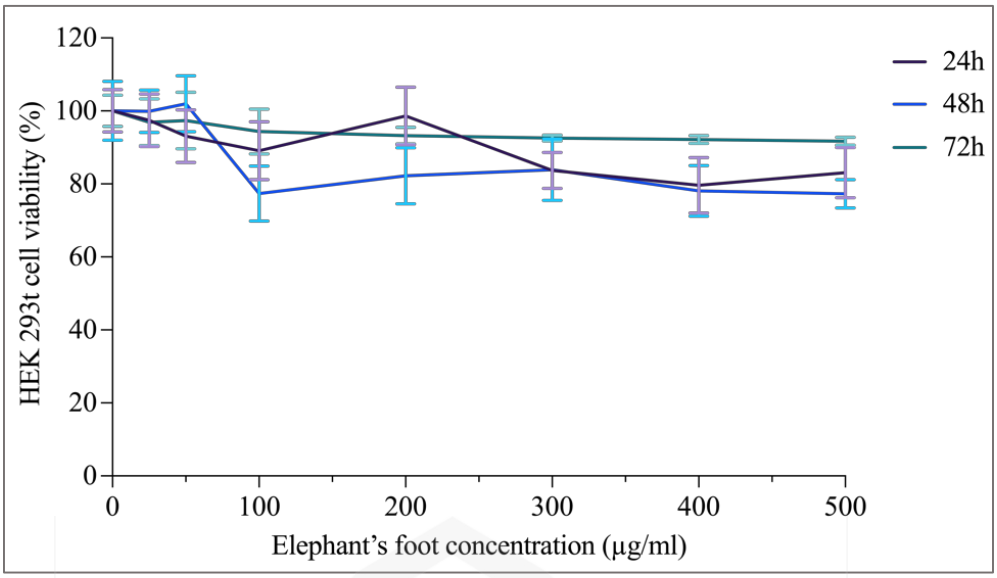


Figure 4.12 Antiproliferative investigation of 7:3 *E. scaber* aqueous extract on HEK 293t cell.

4.4 GROWTH INHIBITORY IMPACT OF *S. AROMATICUM* AND *E. SCABER* ON SIHA CELL GROWTH

The potency of *S. aromaticum* and *E. scaber* on the growth inhibition of SiHa cells was investigated using trypan blue exclusion (TBE) assay by treating the cells with IC_{50} of the herbs and the measurement were taken every 24 h over a period of 96 h. There were four treatment groups which includes negative control (untreated), positive control (etoposide), *S. aromaticum*-treated and *E. scaber* -treated group. Upon incubation for 48 h (cell doubling time) after seeding, the cells were treated according to the treatment groups. The cells were then harvested, counted and growth pattern of the cells were established. The effects *S. aromaticum* and *E. scaber* on the cell number of SiHa cells were presented in Figure 4.13.

In all treatment groups, cell numbers slightly increased after 24 hours of exposure, though this rise was not significantly different from the starting point at 0 hours (treatment initiation). At 96 h timepoint, there was significant reduction ($p<0.05$) on cell number only on etoposide-treated cells as compared to untreated cells. At 120 hours timepoint, *S. aromaticum* and *E. scaber* treatments showed a significant cell growth inhibition ($p<0.05$) in cell numbers compared to the untreated group, as determined by two-way ANOVA with Dunnett's multiple comparison test. Etoposide treatment resulted in an even more significant reduction in cell numbers compared to the untreated group ($p<0.001$). Prolonged treatment to IC_{50} values at 144 hours significantly inhibited SiHa cell growth relative to the untreated cells ($p<0.05$ on *S. aromaticum* and *E. scaber* groups and $p<0.001$ on etoposide-treated group). The study observed that extended treatment duration led to a further reduction in cell numbers compared to the untreated group. Additionally, the untreated group showed a significant cell number increase from 72 h to 120 h timepoints, followed by a reduction at 144 hours ($p<0.05$).

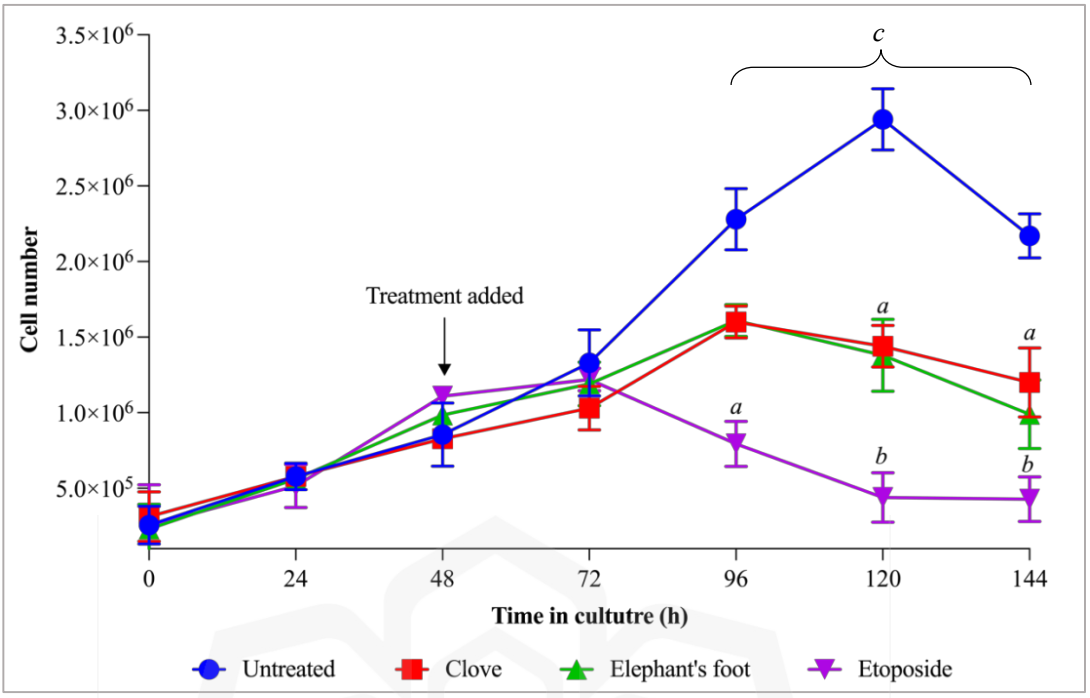


Figure 4.13 Growth inhibitory effects of SiHa cells upon treatment with *S. aromaticum* and *E. scaber*.

The results shown in mean±SD, n=4 with triplicates each experiment. Statistical analyses were performed using two-way ANOVA with Dunnet's multiple comparison test (*a* is $p<0.05$, *b* is $p<0.001$, and *c* is $p<0.0001$ when compared with untreated at the same timepoint).

On the other hand, TBE assay results demonstrated that *S. aromaticum* and *E. scaber* aqueous extracts had no adverse effects on the proliferation of HEK293t cells. The growth curve of treated HEK293t cells closely mirrored that of the untreated control, following the characteristic phases of lag, exponential (log), stationary, and decline (Figure 4.14). This consistent growth pattern indicates that the extracts did not disrupt the normal growth dynamics of HEK293 cells, showing their non-toxic nature toward normal human cells.

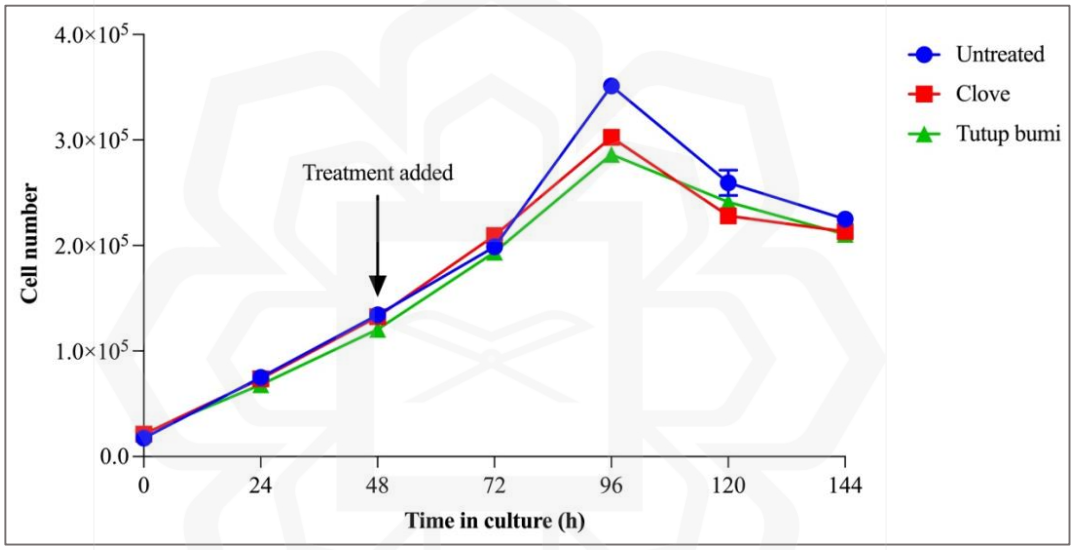


Figure 4.14 Growth inhibitory effects of HEK293t cells upon addition of *S. aromaticum* and *E. scaber* aqueous extracts

Figure 4.15 illustrates the impact of aqueous extracts from *S. aromaticum*, *E. scaber*, and the drug etoposide (positive control) on the viability of SiHa cells after 48 hours of incubation. Statistical analysis using two-way ANOVA with Dunnet's multiple comparison test found there was no significant difference in cell viability was observed between untreated and treated groups at 72 h timepoint. However, at 96, 120, and 144 h timepoints, all treatment groups showed a significant decrease in cell viability compared to the untreated group. Table 4.7 present the mean ± SD values of cell viability (%) across various timepoints and treatment groups. At 72 h timepoint, cell viability in treated groups was reduced by about 12 to 17% relative to untreated cells. At 96 h timepoint, the viability continued to decline, reaching 58.92 ± 1.53%, 48.75 ± 1.06%, and 38.17 ± 1.06% for *S. aromaticum*, *E. scaber*, and etoposide treatments, respectively. By 144 h timepoint, cell viability in the *S. aromaticum*- and etoposide-treated groups dropped to 17.75% and 15.33%, respectively, with *S. aromaticum*-treated cells showing a relatively higher viability of 38.08%.

The observations on SiHa cell viability on the treatments of *S. aromaticum* and *E. scaber* were in parallel with etoposide treated cells which support the potency of both herbs on cervical cancer cells. Moreover, these observations were also supported the results from growth inhibitory effects of SiHa cells upon treatment with the herbs. Both measurement of cell viability (%) and cell number are assessing the anti-cancer and anti-proliferative effects of the herb's treatment. Thus, the consistent decline in cell viability and cell number across all treatment groups suggests that *S. aromaticum* and *E. scaber* extracts exert significant cytotoxic effects on SiHa cells, comparable to the standard chemotherapeutic agent, etoposide. This reinforces their potential as natural chemopreventive agents capable of targeting cervical cancer cell proliferation. Further supporting this finding, the reduction in cell viability observed in the MTT assay aligns with the cell growth inhibition patterns seen in the TBE assay, indicating that the herbs not only impede metabolic activity but also limit overall cell proliferation. This suggests that the bioactive compounds present in the extracts disrupt key cellular processes necessary for cancer cell survival. Given that cancer cells rely on sustained proliferation for tumour progression, the ability of these herbs to suppress SiHa cell growth further strengthens their therapeutic relevance.

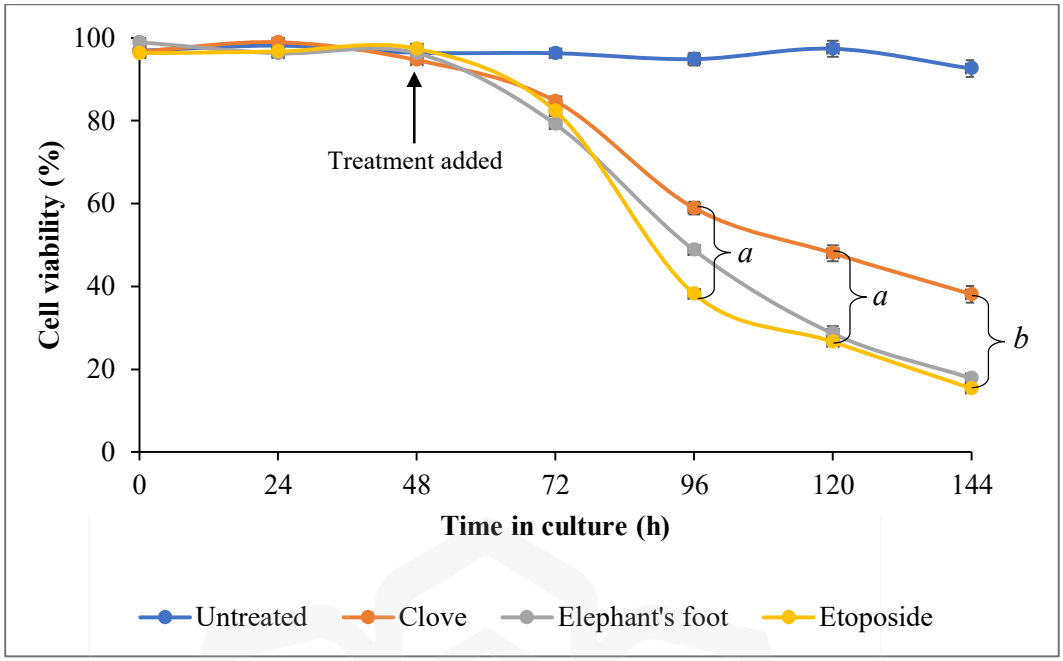


Figure 4.15 SiHa cell viability upon treatment with IC_{50} of *S. aromaticum*, *E. scaber*, and etoposide at 0 to 144 h timepoints.

The results shown in mean ± SD, n=4 with triplicates each experiment. Statistical analyses were performed using two-way ANOVA with Dunnet's multiple comparison test (*a* is $p<0.05$ and *b* is $p<0.001$ when compared with untreated at the same timepoint). Unlabelled data points indicate no significant difference.

Table 4.7 The mean ± SD of SiHa cell viability (%) at different timepoints and treatment groups

Time in culture (h)	**Treatment**			
	Untreated	***S. aromaticum***	***E. scaber***	**Etoposide**
0	96.95 ± 0.62	96.75 ± 0.71	99.00 ± 0.30	96.33 ± 0.62
24	98.17 ± 0.61	99.00 ± 0.30	96.33 ± 0.62	96.75 ± 0.71
48*	96.45 ± 0.71	94.67 ± 0.87	96.33 ± 0.62	97.42 ± 0.67
72	96.33 ± 0.62	84.75 ± 0.85	79.17 ± 1.04	82.33 ± 1.21
96	94.91 ± 0.92	58.92 ± 1.53	48.75 ± 1.06	38.17 ± 1.06
120	97.42 ± 0.67	48.00 ± 1.93	28.58 ± 1.80	26.58 ± 0.43
144	92.64 ± 0.85	38.08 ± 2.02	17.75 ± 1.12	15.33 ± 0.83

*Treatment added

4.5 QUANTIFICATION OF PROTEIN CONTENT IN SIHA CELL FOLLOWING TREATMENT WITH SELECTED MALAYSIA HERBS

Protein content was quantified using a modified Lowry assay, following the protocol in sub-section 3.3.3. Commonly used in chemopreventive studies of herbal compounds, the Lowry assay measures protein concentrations in biological samples, which aids in standardizing polyamine levels across samples in anticancer research. Since polyamines are linked to cell growth and proliferation, assessing their levels relative to protein concentration provides insights into cellular changes due to herbal treatments. Bovine serum albumin (BSA) was used as a standard, and sample protein concentrations were calculated by substituting absorbance values into a linear equation from the BSA standard curve. Figure 4.16 illustrates the Lowry assay's standard curve with absorbance at 690 nm plotted against BSA concentrations from 0 to 250 µg/ml. Intracellular protein variations in SiHa cells treated with *S. aromaticum*, *E. scaber* extracts, and etoposide are shown in Figure 4.17. The correlation between cell counts, following the untreated and treated groups with protein content was also evaluated, revealing how the treatments affect growth inhibition relative to cellular protein levels (Figure 4.18).

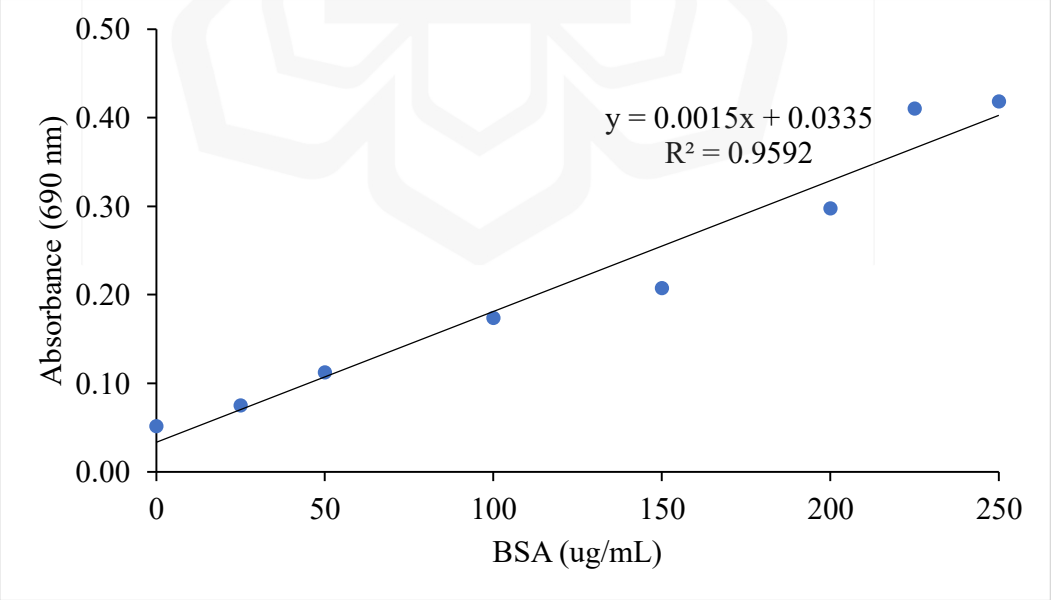


Figure 4.16 The standard curve of Lowry's Assay using bovine serum albumin (BSA)

4.5.1 Intracellular Protein Level Changes at Different Timepoints with different treatment groups

Figure 4.17 illustrate the effects of *S. aromaticum*, *E. scaber*, and etoposide on SiHa cell protein content over a 144-hour period. In the untreated control group, protein content steadily increased, reflecting normal cellular growth and protein synthesis. In contrast, the *S. aromaticum*-treated group displayed a significant reduction in protein content compared to the control at all time points at 96 h onwards, indicating *S. aromaticum* inhibited protein synthesis or enhance protein degradation in SiHa cells. Similarly, the *E. scaber* treatment also showed a marked decrease in protein content across all time points, suggesting it also impacts protein levels negatively. The etoposide-treated group exhibited a notable decrease in protein content, especially at later time points, aligning with etoposide's known cytotoxic effects that lead to reduced protein synthesis and cell death. Statistical analysis using two-way ANOVA with Dunnett's multiple comparison test confirmed significant differences ($p < 0.05$ or $p < 0.001$) between treated and untreated groups at various time points.

Collectively, these findings suggest that *S. aromaticum* and *E. scaber* extracts interfere with protein synthesis or promote protein degradation in SiHa cells, further reinforcing their potential as chemopreventive agents. The significant reduction in intracellular protein content observed after 48 hours of treatment aligns with the trends seen in cell viability and cell number assays, indicating a strong correlation between protein depletion and the overall inhibition of cancer cell proliferation. At the molecular level, protein synthesis is essential for maintaining key cellular processes, including cell cycle regulation, apoptosis, and DNA repair. A disruption in protein homeostasis can severely impact cancer cell survival, as tumour cells often require increased protein production to sustain their rapid growth and resistance to apoptosis. The observed decline in intracellular protein levels in SiHa cells following treatment with *S. aromaticum* and *E. scaber* suggests that these extracts may exert their anticancer effects by impairing essential biosynthetic pathways, thereby weakening the tumour's ability to sustain its proliferative and survival mechanisms.

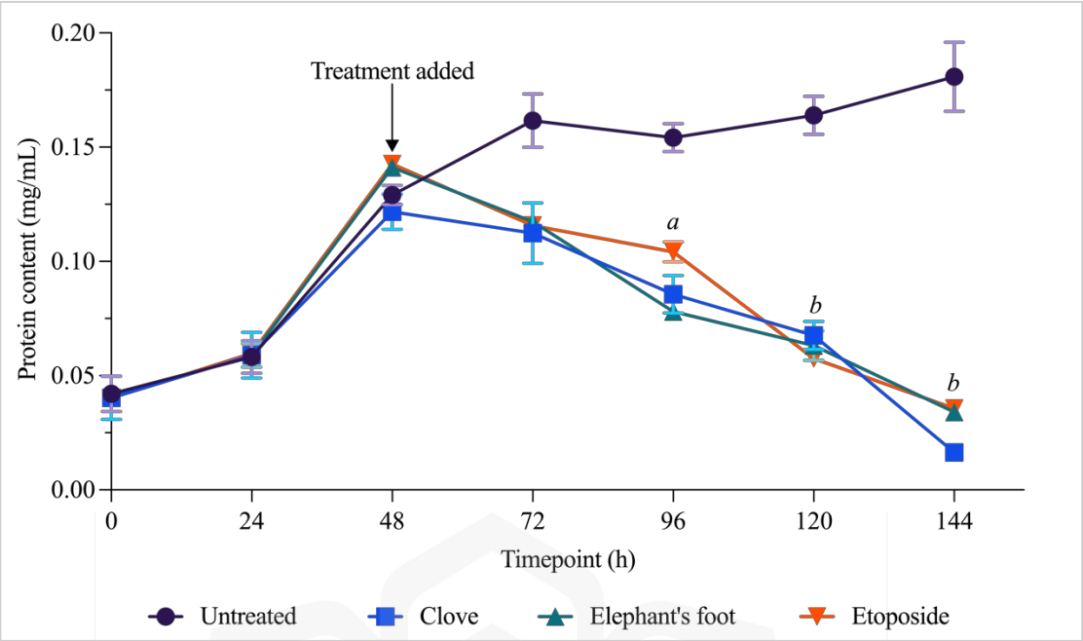


Figure 4.17 Total protein content in SiHa cells after treatment with *S. aromaticum*, *E. scaber*, and the positive control etoposide.

Data are presented as mean ± SD (n=4) with three replicates per experiment. Statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test (*a* is $p<0.05$, *b* is $p<0.001$, compared to untreated).

Figure 4.18 shows the correlation between intracellular protein content (nmol/mg) and cell number in SiHa cells subjected to treatments with *S. aromaticum*, *E. scaber*, etoposide, and the untreated control. The graph demonstrates a positive linear relationship between protein content and cell number across all treatment groups, suggesting that as cell number increases, total protein content also rises proportionally and vice versa. The correlation coefficients (R^2) are as follows: untreated = 0.9526, *S. aromaticum* = 0.9741, *E. scaber* = 0.9358, and etoposide = 0.9611. These high R^2 values indicate a strong positive correlation between protein content and cell number for all groups. This suggests that the increase in protein content observed in the untreated and treated groups is directly related to the increase in cell number and vice versa.

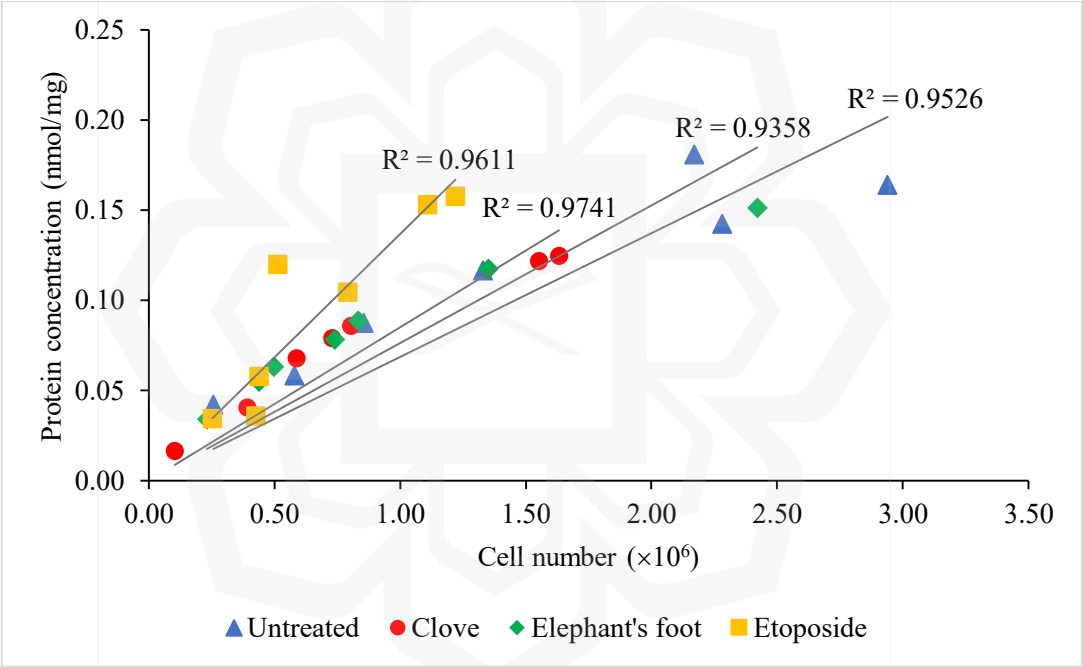


Figure 4.18 Correlation of intracellular protein content (nmol/mg) and cell number at different treatment groups.

4.6 THE EFFECTS OF SPECIFIC POLYAMINE PATHWAY INHIBITOR ON CERVICAL CANCER CELL LINE

4.6.1 Effect of short-term exposure to DFMO on human cervical cancer cell line

DFMO is known to inhibit the enzyme ornithine decarboxylase (ODC), which is crucial in polyamine synthesis—a process that supports cell growth and proliferation in cancer cells. Figure 4.19 (a) and (b) illustrate the impact of varying concentrations of DFMO on SiHa cell viability after 24 and 48 hours of exposure. In both time points, the general trend indicates that DFMO does not induce significant cytotoxicity at the tested concentrations. The cell viability remains relatively stable across the concentration range, suggesting that DFMO does not affect cell proliferation but caused cytostatic effect. This is evidenced by the plateauing of the curves at higher DFMO concentrations, suggesting that the cells have reached a state of growth arrest.

Additionally, the slight fluctuations in cell viability at certain concentrations may be attributed to experimental variability or inherent biological responses. However, the overall trend remains consistent, indicating that DFMO has a cytostatic effect on SiHa cells. These findings align with the known mechanism of action of DFMO, which inhibits polyamine synthesis, a critical process for cell proliferation and survival. By limiting the availability of polyamines, DFMO can effectively suppress cell growth without causing immediate cell death.

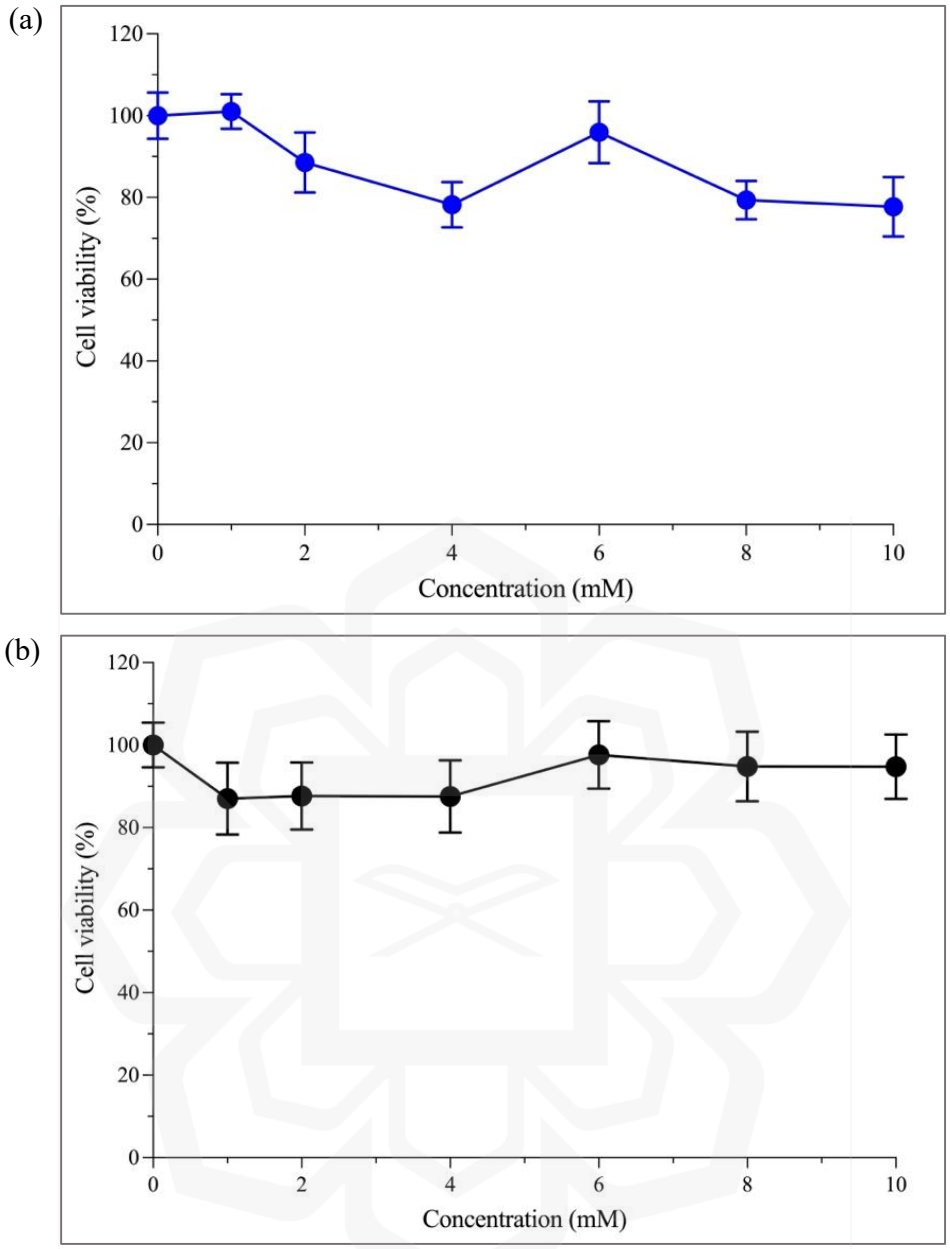


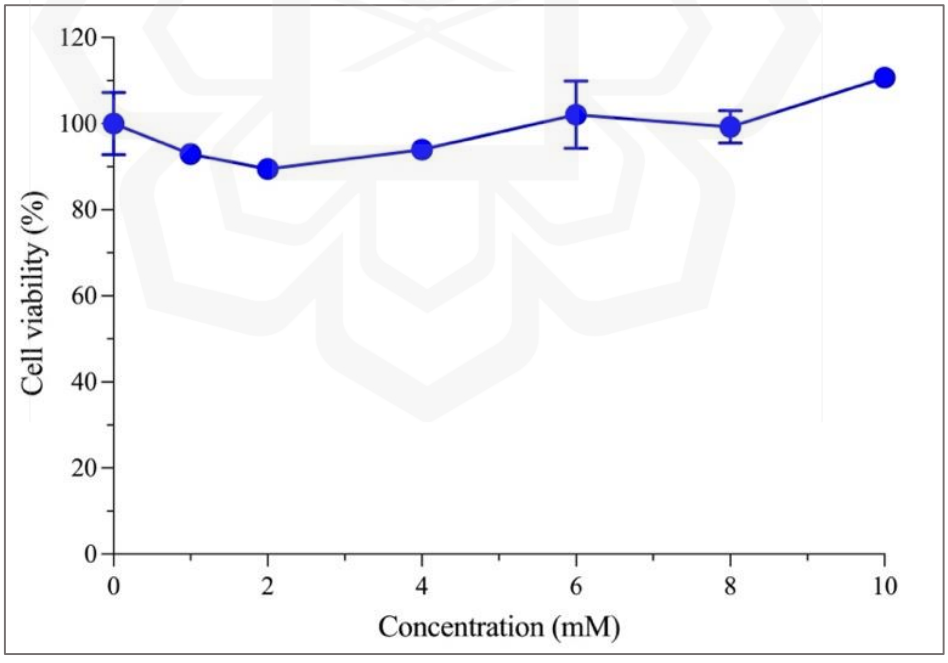
Figure 4.19 The effect of 24 h (a) and 48 h (b) exposure to DFMO in SiHa cells

Cells were seeded at a density of 5.0×10^4 cells/cm^2 in 96-well flat-bottomed plates and incubated for 24 h for attachment and growth. The medium was then replaced with medium containing increasing concentration of DFMO (0 to 10 mM) or vehicle (culture media without pen strep and FBS). The plates were then assayed for MTT assay per protocol (sub-section 3.3.1.2) at 24 and 48 h exposure. Results shown are mean±SD (n=3 with 6 replicates per experiment). Statistical analysis were performed using two-way ANOVA with Dunnet's multiple comparison test when compared between different concentrations of DFMO. Unlabelled data points indicate no significant difference.

4.6.2 The Effect of Long-Term Exposure to DFMO in Cervical Cancer Cell Line

Figure 4.20 illustrates the prolonged exposure of DFMO on SiHa cell viability, showing the percentage of viable cells at various DFMO concentrations (0 to 10 mM). Similar trends observed in shorter exposure durations, the data reveal that DFMO does not significantly induce cytotoxicity at the tested concentrations, as cell viability remains relatively consistent across the concentration range at 72 and 96 hours of treatment. This suggests that DFMO does not cause cell death but rather exhibits a cytostatic effect, inhibiting cell proliferation without inducing cytotoxicity. The plateauing of the viability curves at higher DFMO concentrations further supports this, indicating that the cells have likely entered a growth arrest state and suggesting that the cells had adapted to the polyamine-depleted environment and were in a dormant state.

(a)



(b)

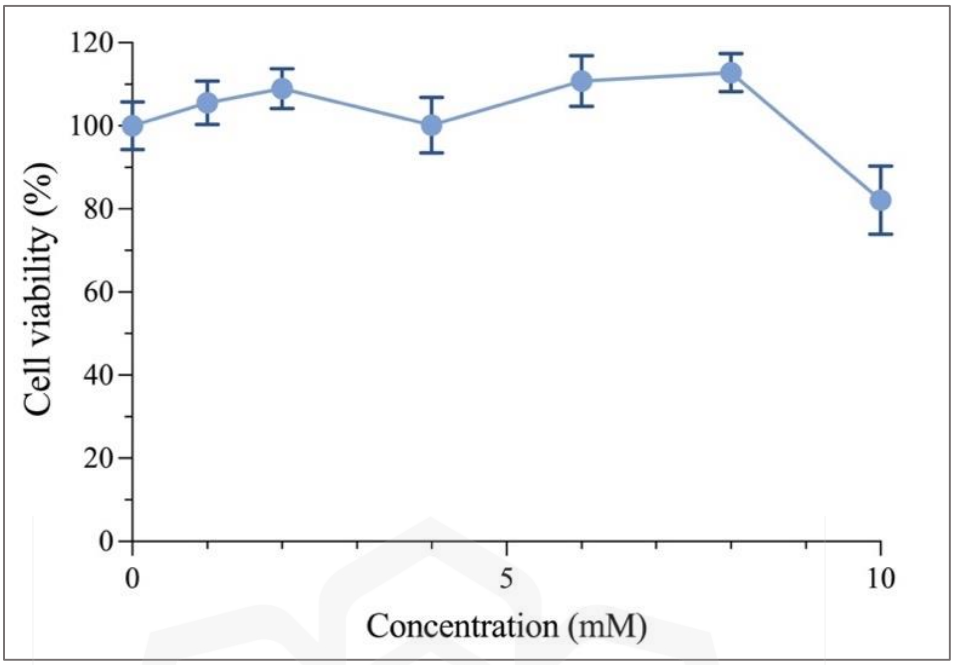


Figure 4.20 The effect of 72 h (a) and 96 h (b) exposure to DFMO in SiHa cells. Cells were seeded at a density of 5.0×10^4 cells/cm^2 in 96-well flat-bottomed plates and incubated for 24 h for attachment and growth. The medium was then replaced with medium containing increasing concentration of DFMO (0 to 10 mM) or vehicle (culture media without pen strep and FBS). The plates were then assayed for MTT assay per protocol (sub-section 3.3.1.2) at 24 and 48 h exposure. Results shown are mean ± SD (n=3 with 6 replicates per experiment). Statistical analysis were performed using two-way ANOVA with Dunnet's multiple comparison test when compared between different concentrations of DFMO. Unlabelled data points indicate no significant difference.

4.7 EFFECT OF HERBS ON POLYAMINE METABOLISM

4.7.1 Sensitization of Cytotoxicity Effect of selective Malaysian herbs with DFMO pre-treatment on SiHa cell

When DFMO pre-treatment is applied, polyamine levels within the cells decrease, leading to reduced cellular growth and metabolic activity. This depletion weakens the cells, making them more vulnerable to the cytotoxic compounds in the herbs. Consequently, the herbs (*S. aromaticum* and *E. scaber* aqueous extracts) can exert their chemopreventive effects more effectively at lower concentrations, as the cells are already in a compromised state due to inhibited polyamine synthesis.

4.7.1.1 Effect of DFMO on S. aromaticum induced Cytotoxicity in SiHa cells

MTT assay was conducted to evaluate the effect of DFMO pre-treatment on *S. aromaticum*-induced cytotoxicity in SiHa cells. SiHa cells were initially seeded at 5.0×10^4 cells/cm² in 96-well plates and incubated for 24 hours to allow attachment. Following this, the cells were pre-treated with 1.0 or 5.0mM DFMO for another 24 hours. They were then treated with varying concentrations of *S. aromaticum* aqueous extract (0 to 0.500 mg/mL). The experiment was repeated three times with three replicates each, and IC_{50} values from DFMO pre-treatment were compared to those without pre-treatment at 24, 48, and 72 hours.

Figure 4.21 and Figure 4.22 show the combined effects of DFMO and *S. aromaticum* extract on SiHa cell viability over time. Both figures indicate a synergistic effect, with DFMO and *S. aromaticum* extract together significantly reducing cell viability. In Figure 4.21, pre-treatment with 1.0 mM DFMO shows a marked decrease in cell viability as *S. aromaticum* extract concentration rises, enhancing *S. aromaticum*'s cytotoxicity. This suggests that even at a lower DFMO concentration, the inhibition of polyamine biosynthesis already plays a role in sensitizing the cells to *S. aromaticum* extract's cytotoxic effects. However, Figure 4.22 where cells were pre-treated with a

higher DFMO dose (5.0 mM), an even more pronounced reduction in cell viability was observed. The combination of 5.0 mM DFMO and IC_{50} of *S. aromaticum* extract induced the most substantial cytotoxic effect, suggesting that high-dose DFMO more effectively primes SiHa cells for enhanced sensitivity to *S. aromaticum* treatment. The significant reduction in cell viability in the high-dose DFMO group suggests that, beyond merely inducing a cytostatic effect, DFMO also creates a metabolic vulnerability in SiHa cells, making them more susceptible to apoptosis when challenged with *S. aromaticum* extract.

Additionally, the sharp decline in cell viability observed with the high-dose DFMO and *S. aromaticum* combination suggests a potential threshold effect, where sufficient polyamine depletion shifts the balance from growth arrest to enhanced apoptosis. This is particularly evident when comparing the 1.0 mM and 5.0 mM DFMO pre-treatments, where the higher DFMO concentration resulted in a greater magnitude of cell viability reduction. The findings suggest that while low-dose DFMO enhances the efficacy of *S. aromaticum* extract, higher doses provide an even more robust sensitization effect, reinforcing the potential therapeutic advantage of this combination.

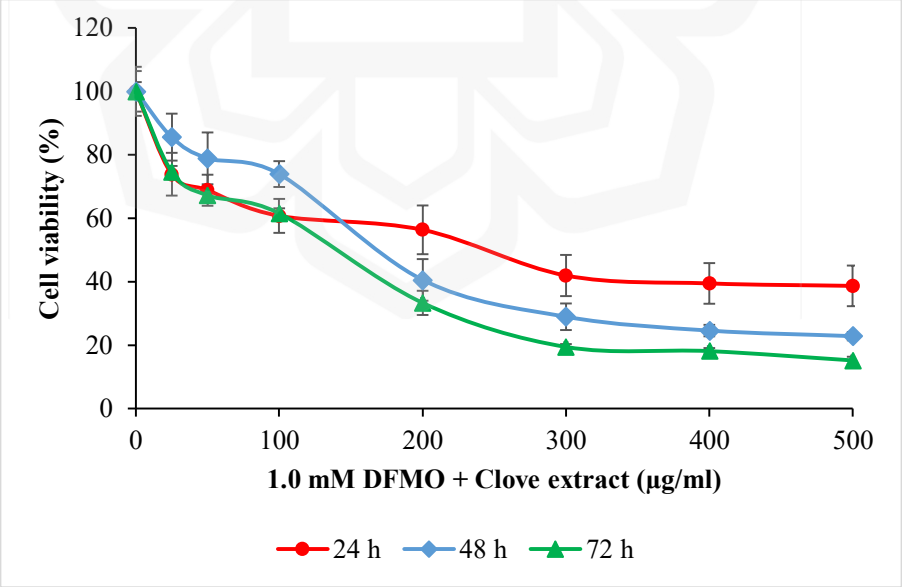


Figure 4.21 The SiHa cells viability with 1.0 mM pre-treatment of DFMO and *S. aromaticum* extract at increasing concentrations (0 to 500 µg/mL) at 24, 48, and 72 h.

Results shown are mean ± SD (n=3 with 6 replicates per experiment). Statistical analysis was performed using two-way ANOVA with Dunnet's multiple comparison

test when compared between timepoints and different concentrations of *S. aromaticum* extract. Unlabelled data points indicate no significant difference.

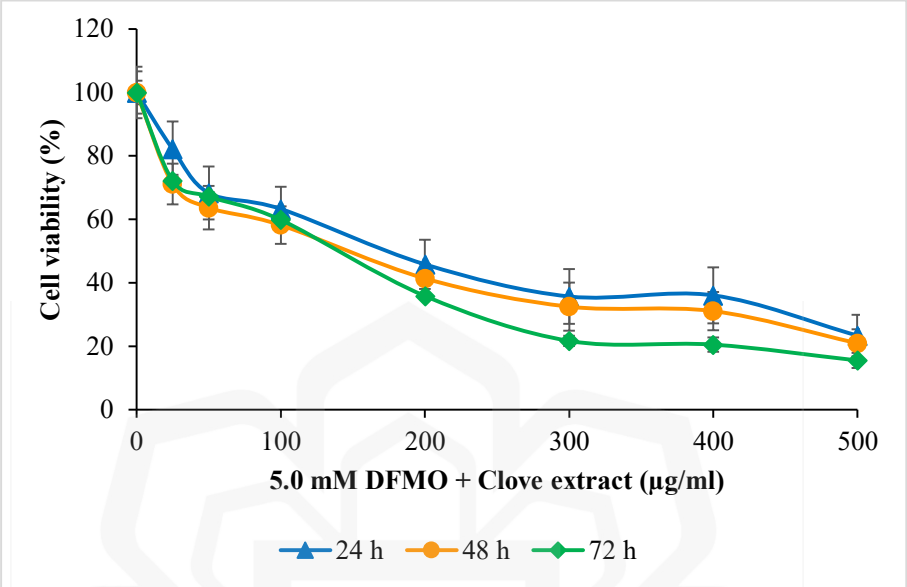


Figure 4.22 The SiHa cells viability with 5.0 mM pre-treatment of DFMO followed by treatment of *S. aromaticum* aqueous extract (0 to 500 µg/ml) at 24, 48, and 72 h.

Results shown are mean ± SD (n=3 with 6 replicates per experiment). Statistical analysis was performed using two-way ANOVA with Dunnet's multiple comparison test when compared between timepoints and different concentrations of *S. aromaticum* extract. Unlabelled data points indicate no significant difference.

Table 4.8 presents the cytotoxic activity (IC_{50} values) of *S. aromaticum* extract with and without DFMO pre-treatment. At 24 h of treatment, the IC_{50} values of *S. aromaticum* extract were significantly lower in both 1.0 mM and 5.0 mM DFMO pre-treatment groups compared to the group without pre-treatment ($p < 0.05$). At 48 h treatment, a significant reduction in IC_{50} values was only observed in the DFMO pre-treated groups at the concentration of 5.0 mM compared to the untreated group. At 72 h treatment, both DFMO pre-treated groups showed significant decrease in IC_{50} values as compared to cells without pre-treatment of DFMO ($p < 0.05$). Thus, additions of low and high dose of DFMO significantly improved the cytotoxicity activity of *S. aromaticum* extract on SiHa cells.

Table 4.8 The cytotoxicity activity, IC_{50} values (mg/ml) of *S. aromaticum* aqueous extract and pre-treatment of DFMO at 1.0 and 5.0 mM on cervical cancer cell, SiHa

Treatment	**Cytotoxicity activity, IC_{50} (mg/mL)**		
	24-h	**48-h**	**72-h**
S. aromaticum	316.8 ± 3.21	177.6 ± 2.53	169.6 ± 0.95
1 mM DFMO + *S. aromaticum*	244.3 ± 1.13[a]	171.7 ± 3.41	141.3 ± 1.52[a]
5 mM DFMO + *S. aromaticum*	175.8 ± 0.94[a]	148.2 ± 1.26[a]	130.2 ± 2.40[a]

[a] is *p*-value < 0.05. Ordinary one-way ANOVA with Tukey's multiple comparison test was performed to compare between DFMO pre-treated cells and without pre-treatment.

4.7.1.2 Effect of DFMO on E. scaber induced Cytotoxicity in SiHa cells

The combined effect of both low- and high-dose DFMO pre-treatment with *E. scaber* aqueous extract on SiHa cell viability is illustrated in Figure 4.23 and Figure 4.24. The results indicate that DFMO significantly enhances the cytotoxic potential of *E. scaber* extract, further reducing SiHa cell viability compared to treatment with the extract alone. This suggests that polyamine depletion through DFMO pre-treatment sensitizes SiHa cells, making them more vulnerable to the bioactive compounds in *E. scaber*.

Notably, at both low-dose (1.0 mM) and high-dose (5.0 mM) DFMO pre-treatment, a significant reduction in cell viability was observed, demonstrating that DFMO effectively improves the efficacy of *E. scaber* extract. The viability of SiHa cells showed a consistent pattern of decline across increasing concentrations of *E. scaber* extract, reinforcing its role as a potent chemopreventive agent. The enhanced reduction in cell viability following DFMO pre-treatment suggests that *E. scaber* extract may act synergistically with polyamine depletion, potentially by targeting multiple cellular pathways involved in cancer cell survival.

A comparison between Figures 4.23 and 4.24 highlights that while both low and high DFMO doses enhanced the extract's effects, a more pronounced reduction in cell viability was observed at the higher DFMO concentration (5.0 mM). This suggests that a higher degree of polyamine depletion further amplifies the cytotoxic response of *E. scaber* extract, possibly by increasing oxidative stress, disrupting cell cycle progression, or sensitizing cells to apoptosis. Interestingly, despite the overall significant reduction in SiHa cell viability, there were no substantial differences in cytotoxicity between different treatment durations (24, 48, and 72 hours) across both low and high DFMO pre-treatment conditions. This finding suggests that the combined effect of DFMO and *E. scaber* extract reaches its maximum potential early in the treatment period and remains sustained over time. The lack of significant time-dependent variations implies that once polyamine metabolism is sufficiently disrupted, SiHa cells may be unable to recover, regardless of treatment duration.

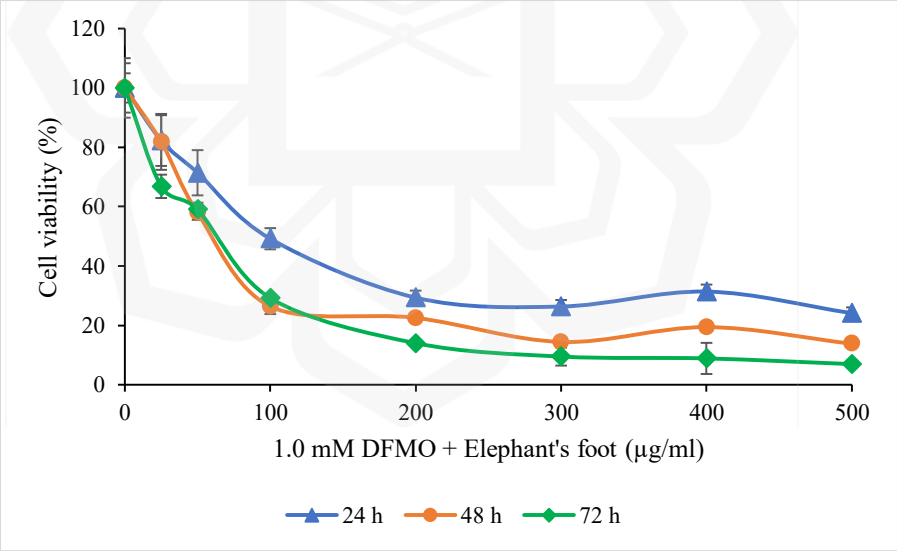


Figure 4.23 The SiHa cells viability with 1.0 mM pre-treatment of DFMO followed by treatment of *E. scaber* aqueous extract (0 to 500 µg/ml) at 24, 48, and 72 h. Results shown are mean ± SD (n=3 with 6 replicates per experiment). Statistical analysis was performed using two-way ANOVA with Dunnet's multiple comparison test when compared between timepoints and different concentrations of *S. aromaticum* extract. Unlabelled data points indicate no significant difference.

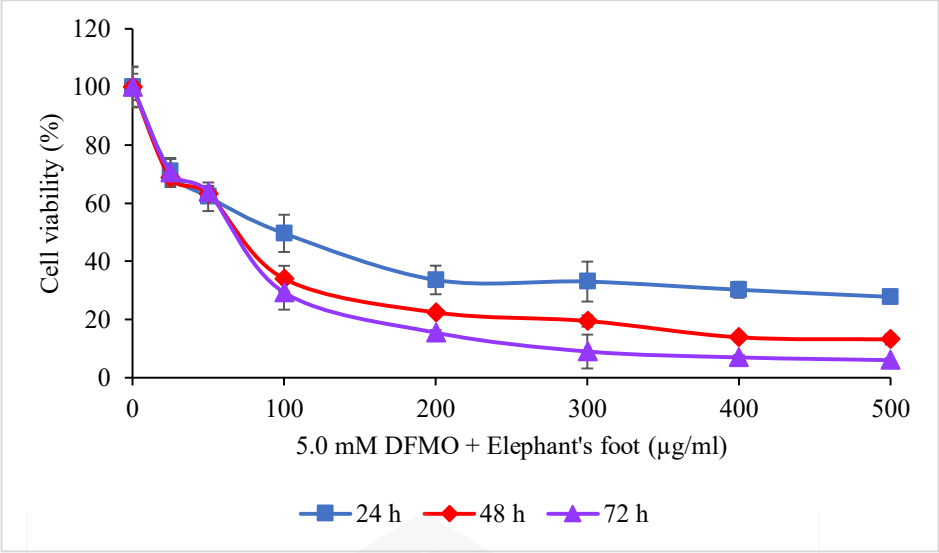


Figure 4.24 The SiHa cells viability with 5.0 mM pre-treatment of DFMO followed by treatment of *E. scaber* aqueous extract (0 to 500 µg/ml) at 24, 48, and 72 h.

Table 4.9 presents the differences in IC_{50} values of *E. scaber* extract with and without DFMO pre-treatment at 1.0 mM and 5.0 mM. The results show that pre-treatment with DFMO significantly lowered the IC_{50} of *E. scaber* in SiHa cells at all time points (24, 48, and 72 hours) compared to treatment without DFMO. Without DFMO pre-treatment, the IC_{50} values for *E. scaber* at 24, 48, and 72 hours were 149.5, 88.8, and 56.3 µg/ml, respectively. However, with 1.0 mM and 5.0 mM DFMO pre-treatment, the IC_{50} values decreased to 98.2 and 98.6 µg/ml at 24 hours, 62.6 and 72.7 µg/ml at 48 hours, and 65.1 and 70.1 µg/ml at 72 hours.

Interestingly, there was no significant difference between the IC_{50} values for 1.0 mM and 5.0 mM DFMO pre-treatment, indicating that both low and high doses of DFMO were equally effective in improving the potency of *E. scaber* extract against SiHa cells. These findings support the idea that DFMO enhances the effectiveness of *E. scaber*, further confirming their synergistic interaction in reducing SiHa cell viability.

Table 4.9 The cytotoxicity activity, IC_{50} values (μg/ml) of *E. scaber* aqueous extract with or without pre-treatment of DFMO on SiHa cells

Treatment	Cytotoxicity activity, IC_{50} (μg/mL)		
	24 h	48 h	72 h
E. scaber	149.5 ± 1.12	88.8 ± 1.25	56.3 ± 1.32
1 mM DFMO and *E. scaber*	98.2 ± 3.56[a]	62.6 ± 2.38[a]	65.1 ± 2.32[a]
5 mM DFMO and *E. scaber*	98.6 ± 2.41[a]	72.7 ± 1.45[a]	70.1 ± 0.90[a]

[a] is *p*-value < 0.05. Values showed mean ± SD (n=3 with 6 replicates per experiment). Ordinary one-way ANOVA with Tukey's multiple comparisons test were performed when compared between DFMO pre-treatment and without DFMO pre-treatment.

4.7.2 Effect of *S. aromaticum* and *E. scaber* on Intracellular Polyamine Level of SiHa cells

4.7.2.1 Polyamines Calibration Curves

Figure 4.25 is a calibration curve for three polyamines: putrescine, spermidine, and spermine. It shows the relationship between the concentration of each polyamine and its corresponding peak area in a chromatographic analysis. The R^2 values for each polyamine are very high (0.9739 for putrescine, 0.9586 for spermidine, and 0.9517 for spermine). This indicates a strong correlation between the concentration and peak area, suggesting that the calibration curve is reliable for quantitative analysis. This calibration curve used to quantify the amount of putrescine, spermidine, and spermine in unknown samples. By measuring the peak area of each polyamine in the sample chromatogram and comparing it to the calibration curve, the concentration of the polyamine in the sample determined. The HPLC chromatogram of polyamines standard and linear equation of each calibration curve presented in Appendix I: Supplementary Results.

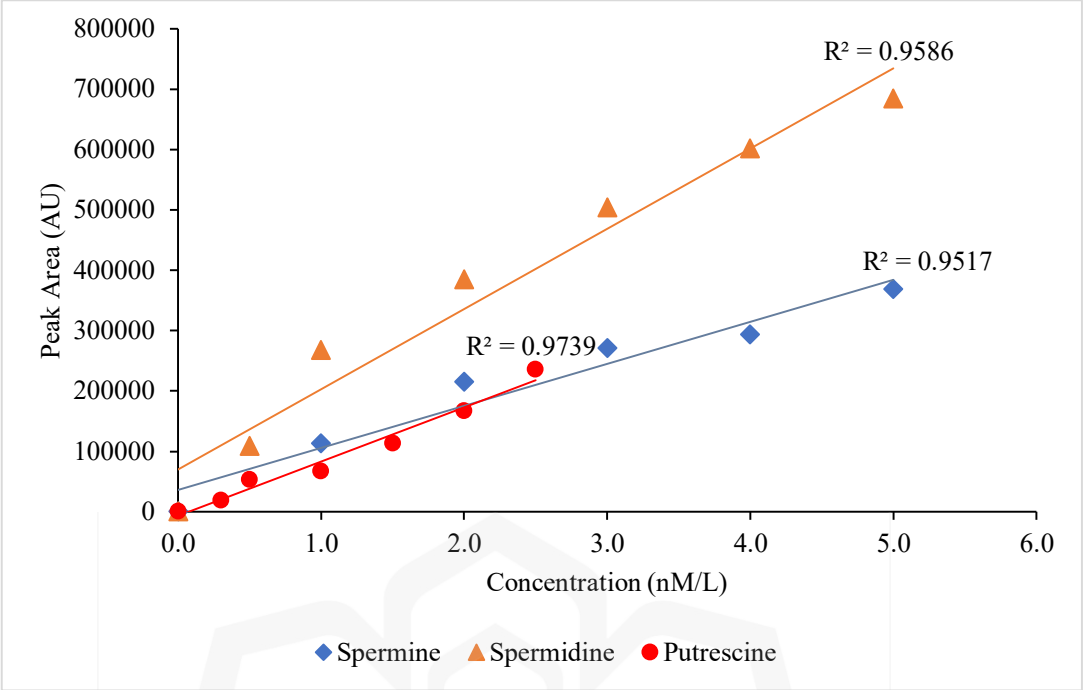


Figure 4.25 The calibration curve of Putrescine, Spermidine, and Spermine

4.7.2.2 Polyamines Contents in Selected Malaysian Herbs

Table 4.10 presents the polyamine content in selected Malaysian herbs as determined by HPLC analysis through pre-column derivatization of polyamines by benzoylation as described in sub-section 3.3.2. The polyamines quantified include spermine, spermidine, and putrescine. Overall, the results indicate that spermine is the predominant polyamine in all the analyzed herbs. Spermidine and putrescine were detected at below detection limits (Bdl) in most of the samples, suggesting that these polyamines may not be major components in these particular herbs. Among the herbs, Hibiscus showed the highest spermine content (0.634 ± 0.026 nmol/mg protein), followed by *Kacang Telang* (0.599 ± 0.022 nmol/mg protein) and *Ulam raja* (0.476±0.031 nmol/mg protein). *S. aromaticum* and *E. scaber* had similar and lowest spermine levels (0.168 ± 0.032 and 0.192±0.019 nmol/mg protein, respectively) as compared to other herbs. Spermidine only detected in *S. aromaticum* with value 0.418±0.012 nmol/mg protein while others were below detection limits. Putrescine was

not detected in all selected Malaysian herbs aqueous extracts. Overall, total polyamines content was found highest in *Kacang Telang* and lowest in *E. scaber*.

Table 4.10 Polyamines content in selected Malaysian herbs

Herbs	Polyamines content (nmol/mg protein)			
	Spermine	**Spermidine**	**Putrescine**	**Total PA**
S. aromaticum	0.168 ± 0.032	0.418 ± 0.012	Bdl	0.585 ± 0.044
E. scaber	0.192 ± 0.019	Bdl	Bdl	0.192 ± 0.019
Kacang Telang	0.599 ± 0.022	Bdl	Bdl	0.599 ± 0.022
Ulam raja	0.476 ± 0.031	Bdl	Bdl	0.476 ± 0.031
Hibiscus	0.634 ± 0.026	Bdl	Bdl	0.634 ± 0.026

Bdl: Below detection limits

4.7.2.3 Measurement of Intracellular Polyamines Level in SiHa cells Upon treatment with S. aromaticum and E. scaber

Figure 4.26 illustrates the effects of *S. aromaticum* and *E. scaber* extracts on intracellular polyamine levels in SiHa cells over seven time points (0, 24, 48, 72, 96, 120, and 144 hours). SiHa cells were seeded at a density of 5.0×10^4 cells/cm² in T75 flasks and incubated for 48 hours to allow proper cell attachment and growth. At the 48-hour time point, the IC_{50} concentrations of *S. aromaticum*, *E. scaber*, and etoposide (used as the positive control) were administered, while an untreated group was maintained as the control. Following treatment, cells were harvested at four additional time points: 72-, 96-, 120-, and 144-hours post-incubation start. Intracellular polyamine levels were quantified following the protocol detailed in sub-section 3.3.2.

The results showed that after 24 hours of treatment, there was no significant reduction in total intracellular polyamine levels in the treated groups compared to the untreated control. However, at 48, 72, and 96 hours of exposure, a significant reduction in polyamine levels was observed in all treated groups (*S. aromaticum*, *E. scaber*, and etoposide) compared to the untreated cells. This reduction demonstrates the ability of these treatments to disrupt polyamine biosynthesis and homeostasis, which is essential for the proliferation and survival of SiHa cells.

The decrease in polyamine levels was found to be consistent with a concomitant reduction in intracellular protein content across the treated groups. This finding suggests that the treatment-induced reduction in polyamine levels directly impacts the molecular functions and viability of SiHa cells. Given the role of polyamines in supporting cellular growth, proliferation, and metabolic activity, their depletion likely contributes to a loss of cellular function and viability in cancer cells treated with *S. aromaticum* and *E. scaber* extracts. Interestingly, the pattern of polyamine reduction observed in *S. aromaticum* and *E. scaber*-treated cells closely mirrored the effects seen with etoposide, a well-known chemotherapeutic agent. This comparison highlights the potential of these herbal extracts as effective chemopreventive or therapeutic agents capable of targeting critical metabolic pathways in cervical cancer cells.

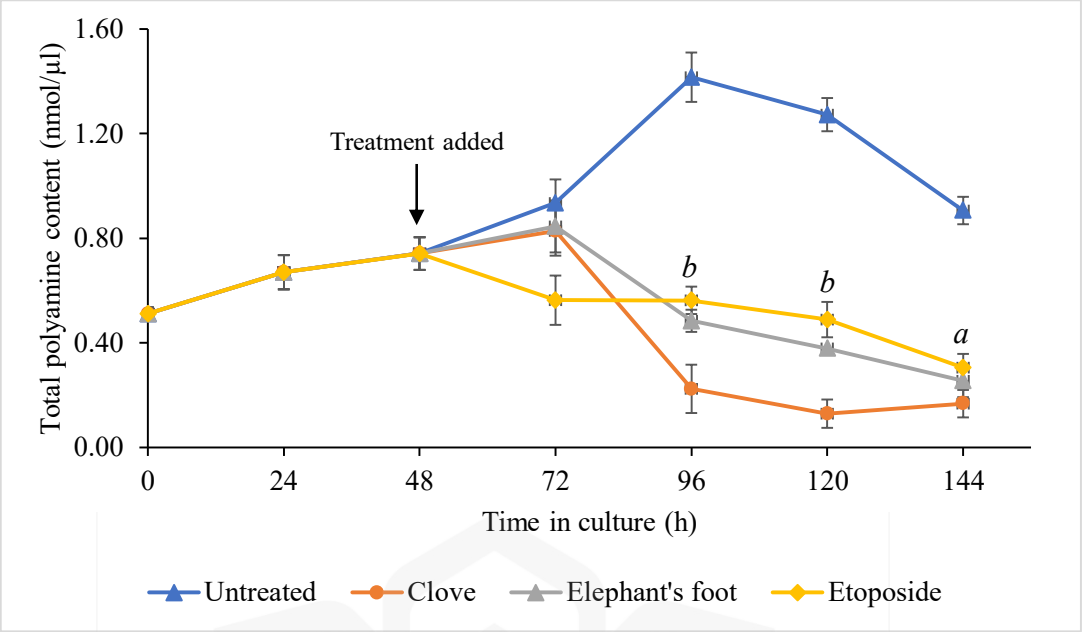


Figure 4.26 The effects of *S. aromaticum*, *E. scaber*, and etoposide (positive control) treatment on total polyamines content in SiHa cell.

Data are presented as mean ± SD (n=4) with three replicates per experiment. Statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test (*a* is $p<0.05$, *b* is $p<0.001$, compared to untreated)

4.7.3 Effect of *S. aromaticum* and *E. scaber* on polyamine gene expression

The potential chemopreventive effects of *S. aromaticum* and *E. scaber* on cervical cancer were further explored by examining their impact on the gene expression of polyamine (PA) pathways, specifically focusing on the metabolism and catabolism of PAs. Four target genes involved in maintaining cellular PA homeostasis were investigated: ornithine decarboxylase (*ODC*), spermidine/spermine N-acetyltransferase (*SSAT*), spermine oxidase (*SMOX*), and ODC antizyme1 (*OAZ1*). ODC regulates the conversion of putrescine, ensuring sufficient levels for normal cellular function, while SSAT and SMOX are involved in PA catabolism. *OAZ1*, on the other hand, acts as an inhibitor by promoting ODC degradation through complex formation with ODC when PA levels are elevated, thus reducing putrescine production. Treatments with *S. aromaticum* and *E. scaber* were administered to SiHa cells, with etoposide as a positive

control, to assess their effects on PA gene expression across six timepoints (0, 24, 48, 60, 72, and 96 hours). Gene expression changes, presented as fold-changes, were quantified using qPCR, with *β-actin* and *GAPDH* selected as reference genes for normalization of the target gene expressions. Statistical analysis was performed using two-way ANOVA followed by Dunnett's multiple comparison test to determine significant differences between the treatment groups and the untreated control.

4.7.3.1 qPCR Validation

In order to ensure the repeatability, accuracy, reproducibility, validity, and reliability of the gene expression results, this study strictly adhered to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). RNA was extracted with high integrity, and its quality and purity were verified using spectrophotometric analysis, ensuring acceptable A260/A280 and A260/A230 ratios. Gel electrophoresis was performed to confirm the integrity of ribosomal RNA bands, and only samples meeting the quality criteria were used for subsequent cDNA synthesis. Reverse transcription was carried out using equal amounts of RNA, typically 1 µg per reaction, to ensure consistency across samples.

Gene-specific primers were carefully designed and validated for specificity, with melt curve analysis and sequencing of PCR products used to confirm the absence of non-specific amplification or primer-dimer formation. Amplification efficiency for each primer pair was assessed using standard curves generated from serial dilutions of cDNA. All primer pairs demonstrated efficiencies within the acceptable range of 90–110%, ensuring reliable and accurate quantification of gene expression.

Normalization of gene expression was achieved by selecting suitable reference genes, such as GAPDH or ACTB, based on their stability under experimental conditions. Tools such as GeNorm and NormFinder were employed to confirm the stability of these reference genes, thereby ensuring reliable normalization of target gene expression data. All qPCR reactions were performed in technical triplicates to minimize

variability, and multiple biological replicates were included to enhance the robustness of the findings.

Negative controls, including no-template controls (NTCs) and reverse transcription-minus (RT-) controls, were incorporated into every qPCR run to confirm the absence of contamination and genomic DNA. The ΔΔCt method was employed to calculate relative gene expression levels, normalizing target gene expression to reference genes. Log-transformed data were used for statistical analysis to ensure normality and the validity of comparative results.

All essential experimental details, including RNA quality, primer sequences, amplification efficiency, reaction conditions, and controls, were documented comprehensively to ensure transparency and reproducibility. By adhering to these MIQE guidelines, the gene expression results obtained in this study are validated as robust, reliable, and suitable for publication.

4.7.3.2 Gene Expression Changes ODC at different timepoints after S. aromaticum and E. scaber treatment

Figure 4.27 shows that treatment with *S. aromaticum*, *E. scaber*, and etoposide significantly reduced *ODC* expression in SiHa cervical cancer cells compared to the untreated control. SiHa cells were seeded at a density of 5.0×10^4 cells/cm² in a T75 flask and cultured for 48 hours before treatment. Cells were then harvested at 0, 24, 48, 60, 72, and 96 hours. A total of 1 µg of RNA was extracted and converted into complementary DNA (cDNA) for qPCR analysis. Results are shown as mean ± SD (n=3), and statistical analysis was performed using a two-way ANOVA with Dunnett's multiple comparison test (a: $p<0.001$, b: $p<0.0001$, compared to untreated controls).

The most significant reduction in *ODC* expression was observed at 24 hours after treatment. *S. aromaticum* and *E. scaber* maintained a long-lasting effect, with *ODC* expression remaining significantly lower than in untreated cells up to 96 hours. Although there was a slight increase in *ODC* expression at 60 and 72 hours, the levels

were still significantly lower than in the untreated group. Similarly, etoposide, used as a positive control, strongly inhibited *ODC* expression at all time points.

These results indicate that *S. aromaticum* and *E. scaber* extracts effectively suppress *ODC* expression in SiHa cells, showing a response comparable to etoposide. The significant reduction in *ODC* expression from as early as 24 hours and its sustained suppression across all time points (24 to 96 hours) highlight the potential of these herbs in targeting polyamine metabolism in cervical cancer.

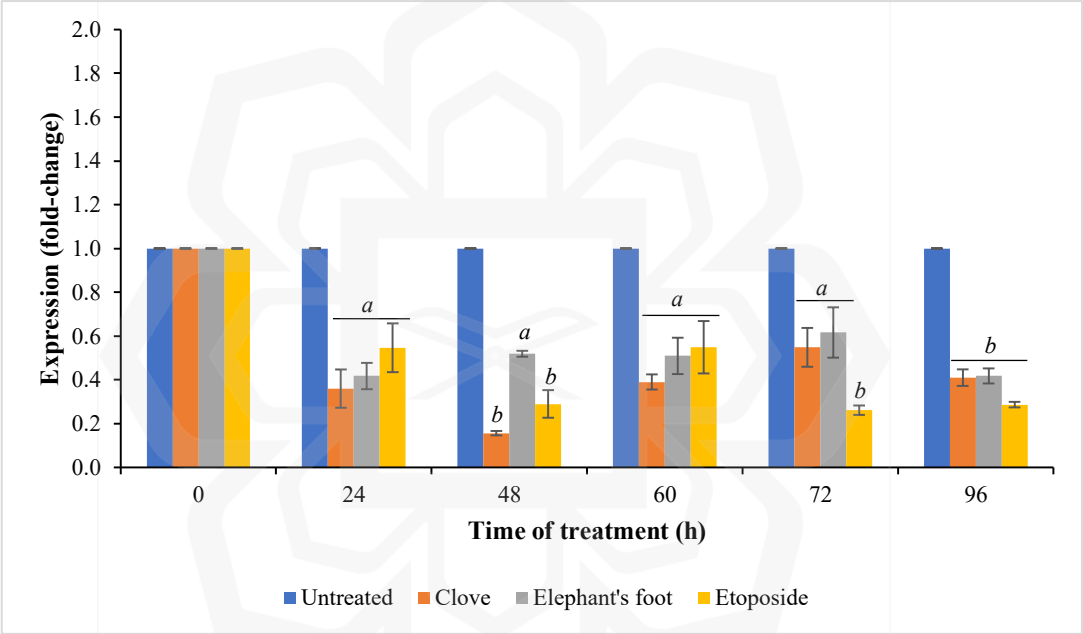


Figure 4.27 Changes in *ODC* expression following treatment with the IC_{50} concentrations of *S. aromaticum* and *E. scaber* in SiHa cells at six different timepoints.

4.7.3.3 Gene Expression Changes OAZ1 at different timepoints after S. aromaticum and E. scaber treatment

Figure 4.28 presented the changes of *OAZ1* expression level in response to treatment with *S. aromaticum*, *E. scaber*, and etoposide in SiHa cells. Initially, at 24 hours, *OAZ1* expression remained unchanged across all treatment groups, suggesting that early-stage responses to these compounds do not immediately trigger *OAZ1* modulation. However, after 48 hours of treatment, a significant upregulation of *OAZ1* was observed in all treatment groups, highlighting its potential role in polyamine homeostasis during prolonged exposure to these herbal extracts and etoposide.

At 60 hours, *OAZ1* expression began to decline, particularly in the etoposide-treated group, where the levels were no longer significantly different from those in the untreated cells. This suggests that the effects of etoposide on *OAZ1* expression may be transient, with the possibility of cellular adaptation or compensatory mechanisms dampening the initial upregulation. Meanwhile, *S. aromaticum* and *E. scaber* maintained a moderate *OAZ1* expression level, implying their sustained influence on polyamine metabolism.

By 72 hours, *OAZ1* expression surged dramatically, increasing more than fivefold in *S. aromaticum*- and etoposide-treated cells and twofold in *E. scaber*-treated cells compared to untreated controls. This peak may correspond to a heightened cellular response aimed at regulating polyamine metabolism and maintaining intracellular balance under continued exposure to these treatments. However, by 96 hours, a clear downregulation trend emerged in the *S. aromaticum* and etoposide-treated groups compared to their 72-hour expression levels, while *E. scaber* continued to sustain elevated OAZ1 expression.

The fluctuation in *OAZ1* expression throughout the treatment period suggests that SiHa cells undergo adaptive changes in response to the treatments, particularly in regulating polyamine metabolism. *OAZ1* is known to inhibit ornithine decarboxylase (*ODC*), the rate-limiting enzyme in polyamine biosynthesis, and its upregulation may serve as a feedback mechanism to counteract the depletion of polyamines induced by *S. aromaticum*, *E. scaber*, and etoposide. The persistent upregulation of *OAZ1* in the *E. scaber*-treated group at 96 hours suggests that this extract exerts a prolonged regulatory effect on polyamine metabolism compared to *S. aromaticum* and etoposide.

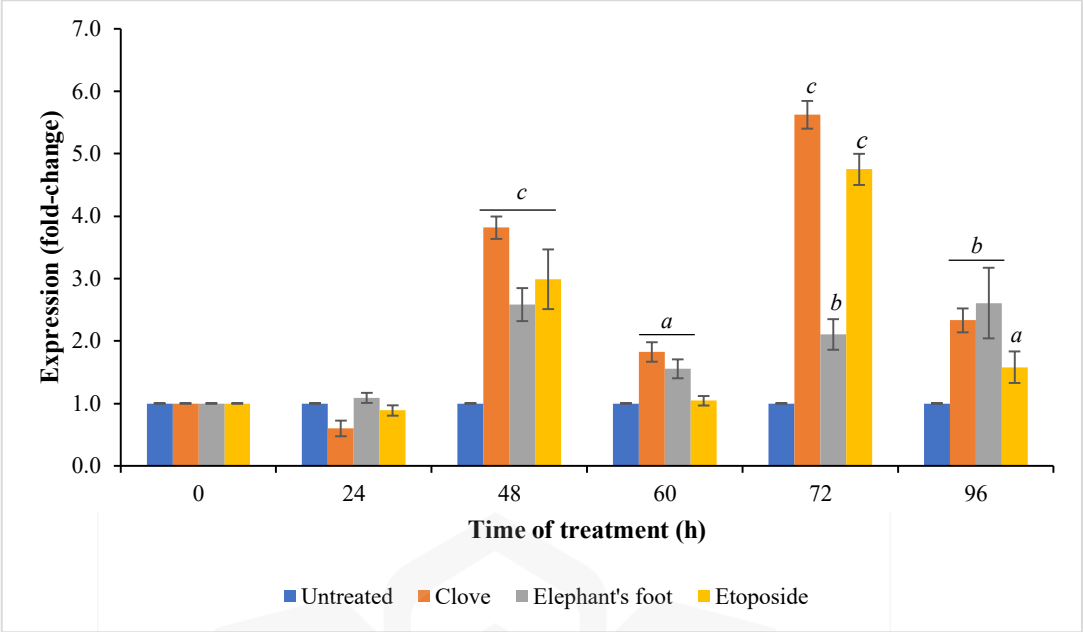


Figure 4.28 Changes in *OAZ1* expression after treatment with IC_{50} concentrations of *S. aromaticum* and *E. scaber* in SiHa cells across six timepoints.

Data are shown as mean ± SD (n=3). Statistical analysis was performed using a two-way ANOVA with Dunnett's multiple comparison test (*a* is $p<0.05$, *b* is $p<0.001$, and *c* is $p<0.0001$, compared with untreated controls).

4.7.3.4 Gene Expression Changes SSAT at different timepoints after S. aromaticum and E. scaber treatment

Figure 4.29 illustrates the time-dependent effects of untreated control, *S. aromaticum*, *E. scaber*, and etoposide treatments on the expression of *SSAT* in SiHa cervical cancer cells. At the 24-hour treatment timepoint, cells treated with *E. scaber* showed significant upregulation of *SSAT* ($p<0.05$) compared to untreated cells, while the other treatment groups showed no discernible changes. At 48 hours, *SSAT* expression in all treatment groups was significantly amplified compared to the untreated control, with *SSAT* expression in the *E. scaber*-treated cells remaining consistent. At the 60-hour timepoint, *SSAT* expression continued to be consistent in both the *S. aromaticum*- and *E. scaber*-treated groups, showing significantly higher levels compared to untreated cells.

However, *SSAT* expression in etoposide-treated cells decreased to levels similar to the untreated control, showing no significant difference. At the 72- and 96-hour timepoints, *SSAT* levels were overexpressed (p<0.0001) in all treated cells relative to untreated cells. These results indicate that treatment with *S. aromaticum* and *E. scaber* led to the upregulation of the *SSAT* gene, which is responsible for catabolism in the polyamine pathway, ultimately resulting in lower intracellular polyamine levels.

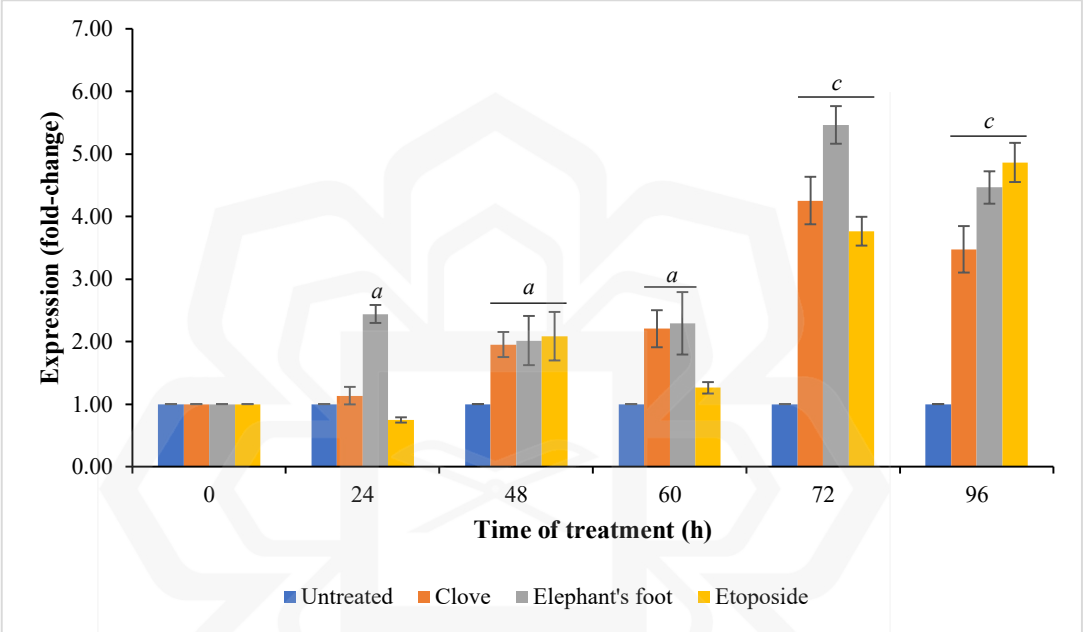


Figure 4.29 *SSAT* expression changes following treatment with IC_{50} concentrations of *S. aromaticum* and *E. scaber* in SiHa cells across six timepoints.

Data are presented as mean ± SD (n=3). Statistical analysis was conducted using a two-way ANOVA with Dunnett's multiple comparison test (*a* is $p < 0.05$, *b* is $p < 0.001$, *c* is $p < 0.0001$, compared with untreated controls).

4.7.3.5 Gene Expression Changes SMOX at different timepoints after S. aromaticum and E. scaber treatment

The expression of *SMOX* (spermine oxidase) was analyzed using qPCR to assess its changes in response to treatment. Figure 4.30 presents the *SMOX* expression levels after treatment with IC_{50} concentrations of *S. aromaticum* and *E. scaber* in SiHa cells at six different time points. After 48 hours of treatment, *SMOX* expression was significantly upregulated in *S. aromaticum*-, *E. scaber*-, and etoposide-treated cells compared to the untreated group.

Since *SMOX* plays a key role in the catabolism of polyamines, its significant upregulation indicates a reduction in intracellular polyamine levels in SiHa cells. Notably, the concurrent upregulation of *SSAT* further supports this finding, as *SSAT* and *SMOX* collectively enhance polyamine catabolism, thereby altering polyamine homeostasis. This increased bioavailability of polyamines for degradation aligns with the observed reduction in intracellular polyamine levels (as explained in sub-section 4.7.2.3), decreased intracellular protein levels (as presented in sub-section 4.5.1), and suppressed cell proliferation and reduced cell count following treatment with *S. aromaticum*, *E. scaber*, and etoposide.

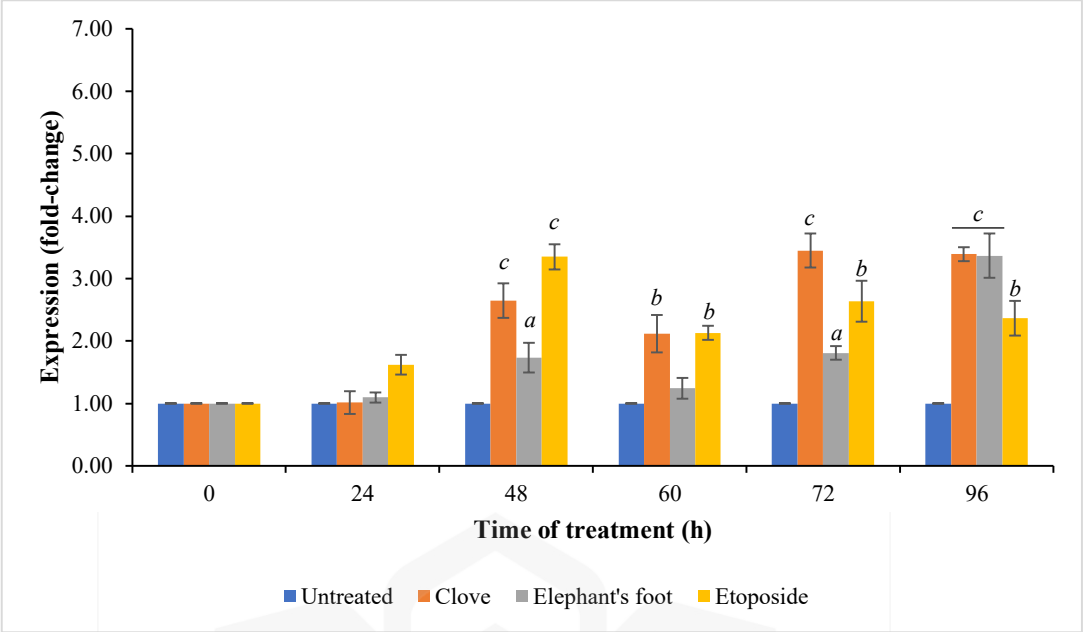


Figure 4.30 *SMOX* expression changes after treatment with IC_{50} concentrations of *S. aromaticum* and *E. scaber* in SiHa cells at six timepoints.

Results are shown as mean ± SD (n=3). Statistical analysis was performed using a two-way ANOVA with Dunnett's multiple comparison test (*a* is $p < 0.05$, *b* is $p < 0.001$, *c* is $p < 0.0001$, compared with untreated controls).

4.8 EFFECT OF *S. AROMATICUM* AND *E. SCABER* TREATMENT ON CELL CYCLE PROFILE IN CERVICAL SQUAMOUS CARCINOMA

4.8.1 Cell Cycle Analysis by Flow Cytometry

The effect of *S. aromaticum* and *E. scaber* aqueous extracts on cell cycle distribution in cervical squamous carcinoma (SiHa) cells was evaluated using flow cytometry with propidium iodide (PI) staining. The treatment of *S. aromaticum* and *E. scaber* on SiHa cells resulted in different distribution changes of cell cycle as compared to negative control through measurement of DNA content. Different period of exposures also resulted in different changes of cell phases' distribution. After 24 hours treatment, the cell phases' distribution analysis indicates significant arrest of cell cycle at G0/G1 and G2/M phase accumulation when SiHa cells treated with *E. scaber* and etoposide as

compared to untreated (Figure 4.31). No significant changes upon *S. aromaticum* treatment. The G0/G1 arrest suggests a reduction in cellular proliferation, as cells fail to replicate its DNA. Meanwhile, G2/M accumulation indicate a block before mitosis and prevent division for new cells generation.

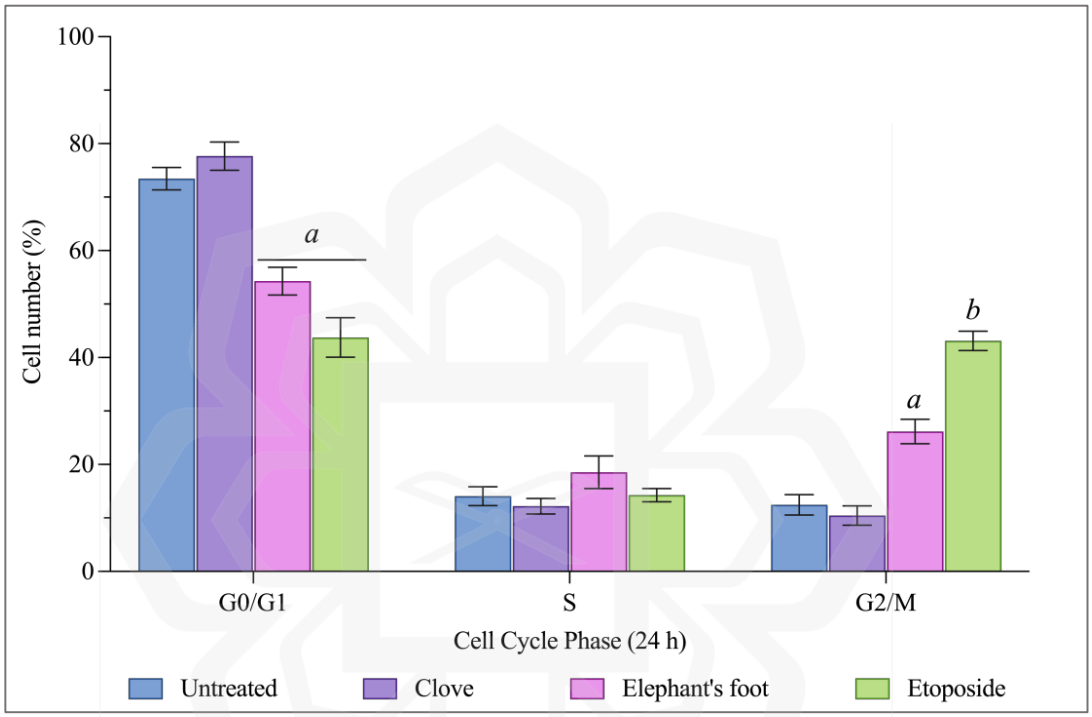


Figure 4.31 Cell cycle distribution analysis at 24-hour timepoint in untreated, *S. aromaticum*, *E. scaber*, and etoposide treated SiHa cells.

Results are shown as mean ± SD (n=3). Statistical analysis was performed using a two-way ANOVA with Tukey's multiple comparison test (*a* is $p < 0.05$, *b* is $p < 0.001$, compared with untreated controls).

Figure 4.31presents the cell cycle distribution of SiHa cells treated with *S. aromaticum*, *E. scaber*, and etoposide aqueous extracts for 24 hours, compared to untreated cells. Distinct variations were observed across the different cell cycle phases among the treated groups. At the G2/M phase, *S. aromaticum*-treated cells exhibited the lowest percentage (10.47%), suggesting minimal cell cycle arrest at this phase. In contrast, *E. scaber* and etoposide treatments induced a substantial accumulation of cells in the G2/M phase, with percentages of 36.93% and 34.86%, respectively, compared to

untreated cells (23.86%). This indicates that both *E. scaber* and etoposide significantly disrupt the cell cycle progression by arresting cells at the G2/M checkpoint, which is critical for ensuring proper DNA repair and preparation for mitosis. No significant differences were observed in the S phase across all treatment groups compared to the untreated cells. This indicates that the treatments did not substantially influence DNA synthesis at this timepoint, suggesting the S phase progression remained relatively unaffected during the 24-hour exposure.

A contrasting pattern was observed in the G0/G1 phase, where untreated cells showed 60.01% of cells in this phase. Both *E. scaber* and etoposide treatments significantly reduced the G0/G1 phase population to 42.94% and 42.72%, respectively. However, *S. aromaticum*-treated cells demonstrated the highest cell distribution in the G0/G1 phase at 77.68%, implying that *S. aromaticum* extract might inhibit the transition of cells from the G0/G1 phase to subsequent phases of the cell cycle. This unique effect of *S. aromaticum* suggests a potential mechanism by which *S. aromaticum* disrupts cancer cell proliferation at an earlier stage of the cell cycle compared to *E. scaber* and etoposide.

The observed differences in cell cycle distribution across the treatments highlight the distinct mechanisms of action of *S. aromaticum*, *E. scaber*, and etoposide. While *E. scaber* and etoposide prominently arrest cells at the G2/M phase, *S. aromaticum* appears to exert its effect by halting cells predominantly in the G0/G1 phase. This divergence emphasizes the complementary potential of these extracts in targeting different phases of the cell cycle, offering promising avenues for combination therapies. The ability of *S. aromaticum* and *E. scaber* to modulate cell cycle progression further reinforces their potential as chemopreventive agents in cervical cancer treatment.

Figure 4.32 presented the 48-hour treatment of *S. aromaticum*, *E. scaber*, and etoposide on cell cycle distribution of SiHa cells. Same as 24-hour observation, there were no significant different in S phase, but all treatments shared significant accumulation at G2/M phase compared to untreated cells. Moreover, significant arrest at G0/G1vphase were observed in all treatment as compared to untreated.

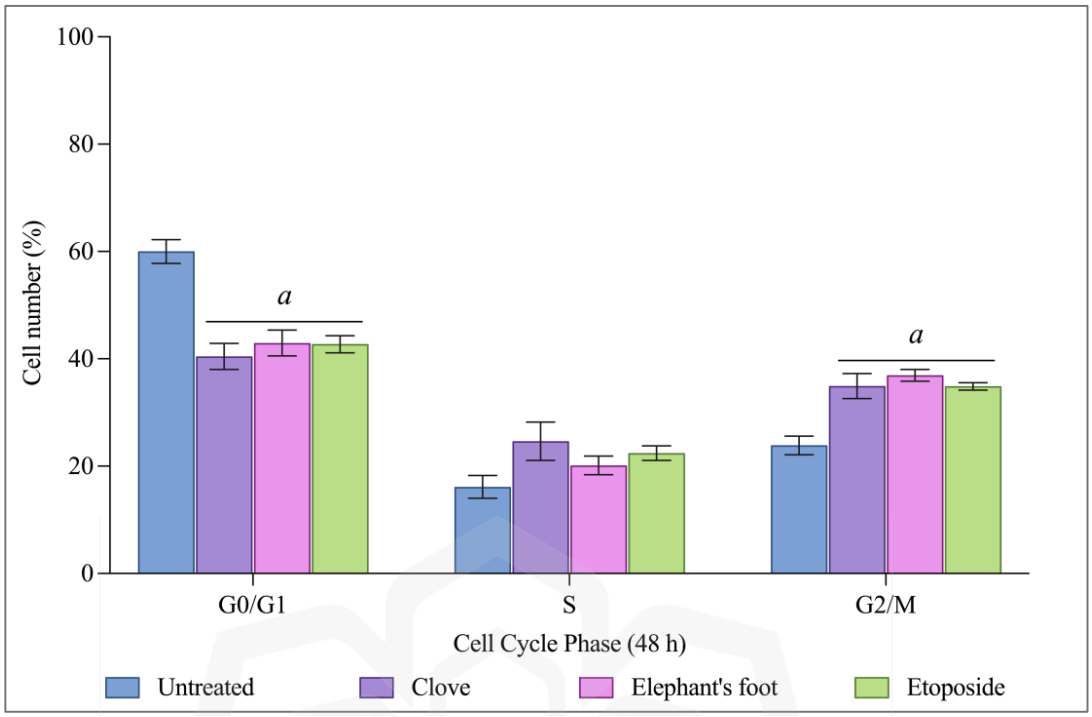


Figure 4.32 Cell cycle distribution analysis at 48-hour timepoint in untreated, *S. aromaticum*, *E. scaber*, and etoposide treated SiHa cells.

Figure 4.33 illustrates the cell cycle distribution of SiHa cells treated with etoposide, *S. aromaticum*, and *E. scaber* aqueous extracts for 72 hours, compared to untreated cells. The percentage of cells in the G2/M phase was significantly higher in all treated groups compared to the untreated group (31.16%). Etoposide treatment induced the highest G2/M phase accumulation (50.11%), followed by *E. scaber* (45.84%) and *S. aromaticum* (39.26%). This suggests that the treatments effectively arrested the cell cycle at the G2/M phase, a critical checkpoint for DNA repair and mitosis, highlighting their potential to inhibit cell division.

In contrast, the distribution of cells in the S phase showed variability among treatments. *S. aromaticum*-treated cells exhibited the highest percentage in the S phase (24.63%), suggesting active DNA synthesis despite the treatment, whereas etoposide-treated cells showed the lowest S phase population. This trend reflects the differing mechanisms by which these treatments influence DNA replication and cell cycle progression.

For the G0/G1 phase, a notable reduction in cell distribution was observed across all treated groups compared to untreated cells (49.56%). Etoposide-treated cells had the lowest G0/G1 phase population (30.81%), followed by *E. scaber* (33.73%) and *S. aromaticum* (36.13%). The significant reduction in G0/G1 phase cells indicates that the treatments push the cells out of the initial growth and resting phase, likely inducing cell cycle arrest in subsequent phases.

The observed shifts in cell cycle distribution demonstrate the capacity of *S. aromaticum* and *E. scaber* aqueous extracts to disrupt normal cell cycle progression in SiHa cells. These results align with the hypothesis that these treatments induce cell cycle arrest, particularly at the G2/M phase, thereby inhibiting cancer cell proliferation. Moreover, the differences in S phase and G0/G1 phase populations between treatments suggest distinct mechanisms of action, with *S. aromaticum* showing a relatively moderate effect compared to the robust impact of *E. scaber* and etoposide.

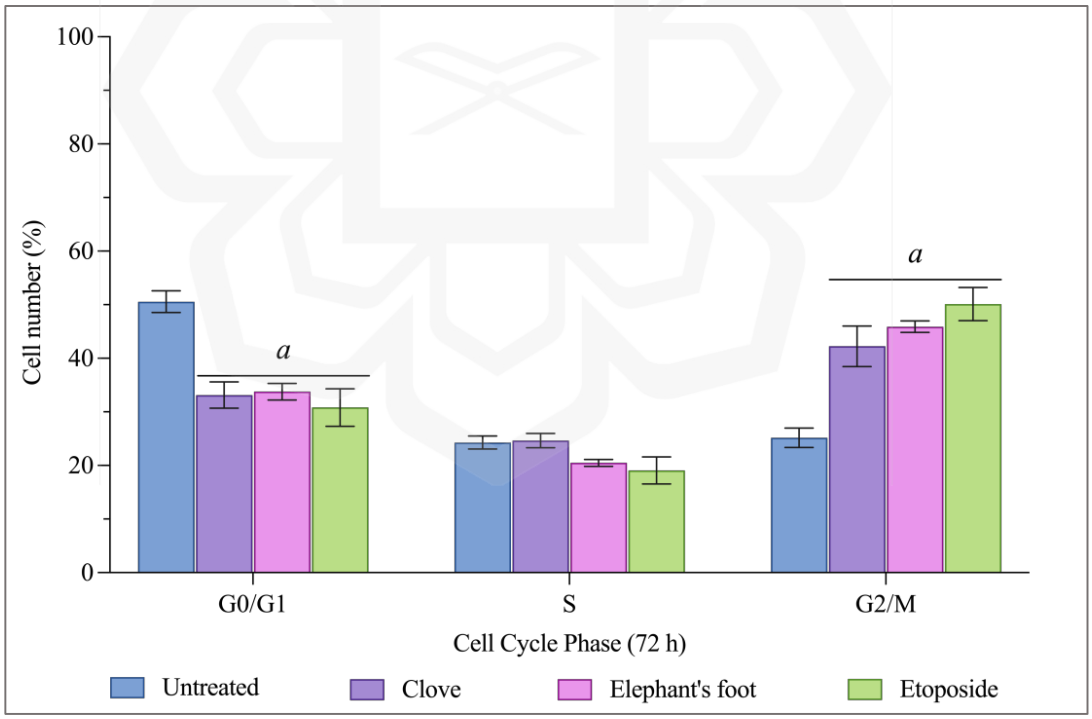


Figure 4.33 Cell cycle distribution analysis at 72-hour timepoint in untreated, *S. aromaticum*, *E. scaber*, and etoposide treated SiHa cells.

Results are shown as mean ± SD (n=3). Statistical analysis was performed using a two-way ANOVA with Tukey's multiple comparison test (*a* is $p < 0.05$ compared with untreated controls).

4.9 INDUCTION OF CELL DEATH MECHANISM BY *S. AROMATICUM* AND *E. SCABER* ON CERVICAL SQUAMOUS CARCINOMA

To investigate the mechanism of cell death induced by *S. aromaticum* and *E. scaber* aqueous extracts in cervical squamous carcinoma (SiHa) cells, two key assays were performed. The annexin V apoptosis assay was used to assess early and late apoptotic events, as well as necrosis, after 48 hours of treatment with the extracts. Additionally, caspase 3/7, 8, and 9 assays were conducted to evaluate the activation of apoptotic pathways. These experiments provide a detailed understanding of how *S. aromaticum* and *E. scaber* extracts induce apoptosis and activate critical apoptotic enzymes, highlighting their potential role in chemoprevention for cervical cancer.

4.9.1 Analysis of Early and Late Apoptotic Events Induced by *S. aromaticum* and *E. scaber* in SiHa cells

The investigation of early, late apoptotic, and necrotic cells following 48 hours of treatment with *S. aromaticum* and *E. scaber* aqueous extracts was assessed through the annexin V apoptosis assay. The results are presented in Figure 4.34. The observations revealed that the proportion of live cells was significantly reduced ($p < 0.0001$) in the *S. aromaticum*, *E. scaber*, and etoposide-treated groups compared to untreated cells. *S. aromaticum* and etoposide treatments significantly induced early apoptosis in SiHa cells at $p < 0.05$ and $p < 0.001$, respectively, whereas *E. scaber* treatment did not show a significant effect on early apoptosis. All treatments also significantly induced late apoptosis ($p < 0.0001$) in SiHa cells compared to the untreated group. Regarding necrosis, a significant increase in necrotic cells was observed in both *E. scaber* and etoposide treatments ($p < 0.0001$ and $p < 0.05$, respectively), but no significant increase was noted in *S. aromaticum*-treated cells. Two-way ANOVA followed by Dunnett's multiple comparison test was performed to assess the significant differences between the treatment groups and the untreated cells.

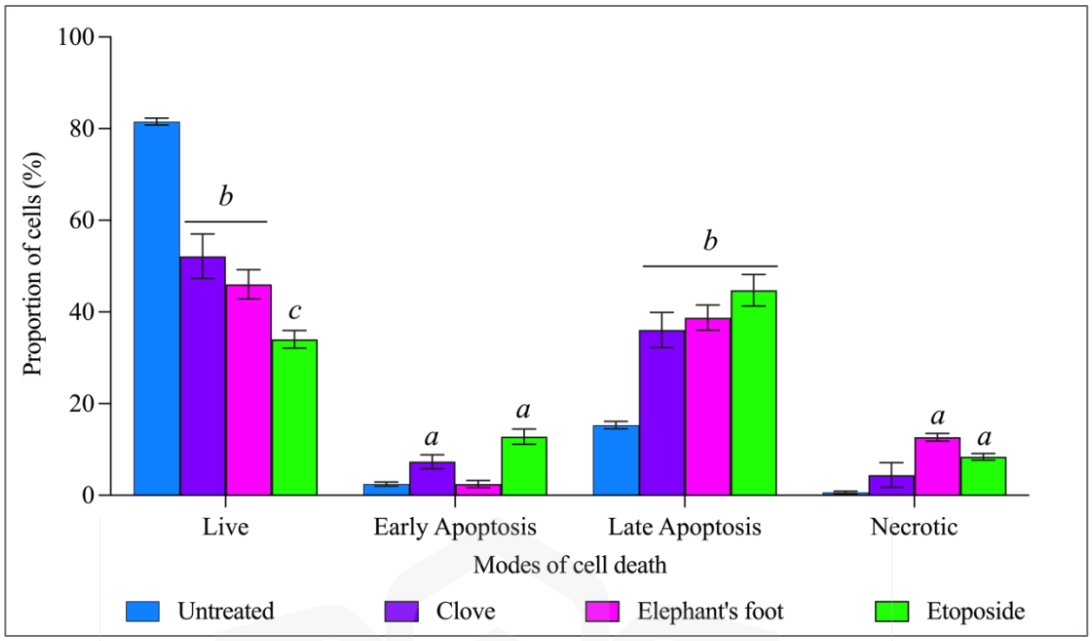


Figure 4.34 Annexin V apoptosis assay at 48 hour treatment of *S. aromaticum*, *E. scaber*, and etoposide in SiHa cell.

SiHa cells were seeded at 5.0×10^4 cells/cm^2 seeding density in T75 flask and incubated for 48 h prior to treatment of *S. aromaticum*, *E. scaber*, and etoposide. The cells were further incubated for 48 h before harvested following the protocol in sub-section 3.3.6.1. Statistical analysis was performed using a two-way ANOVA with Dunnett's multiple comparison test (*a* is $p < 0.05$, *b* is $p < 0.001$, *c* is $p < 0.0001$, compared with untreated controls).

4.9.2 Evaluation of Caspase Activation in Apoptotic Pathways

Figures 4.35, 4.36, and 4.37 present the activity of caspase 3, 8, and 9, respectively, upon treatment with *S. aromaticum* and *E. scaber*. All caspase analyses were performed following the manufacturer's protocol.

For the assays, SiHa cells were seeded at 5.0×10^4 cells/cm^2 in 96-well flat-bottom plates and incubated for 24 hours prior to treatment with the IC_{50} concentrations of *S. aromaticum* and *E. scaber* extracts. Caspase solutions were added to separate plates for each caspase, and measurements were taken at seven different time points (0,

12, 24, 48, 60, 72, and 96 hours). After adding the caspase solutions, the cells were incubated in the dark for 30 minutes, and fluorescence intensities were measured. The relative fluorescence units (RFU) were recorded using a microplate reader. The excitation and emission wavelengths for caspases 3, 8, and 9 were 535/620, 490/525, and 370/450 nm, respectively. The experiments were performed in triplicate, and results are presented as mean ± SD. Statistical analysis was conducted using Ordinary Two-way ANOVA with Dunnett's multiple comparison test (*a*: p-value< 0.05, *b*: p-value <0.001) compared to untreated controls.

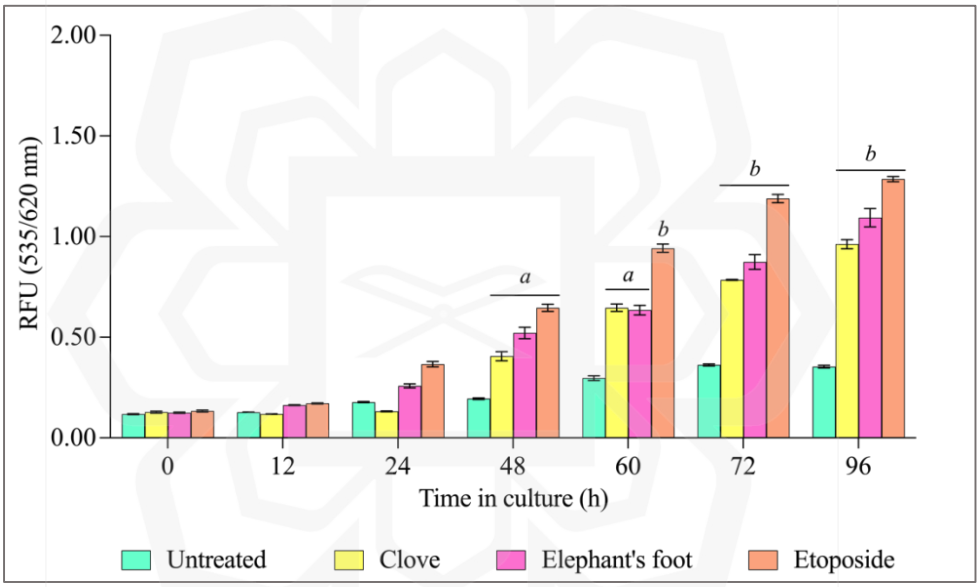


Figure 4.35 The activity of Caspase 3 upon treatment with *S. aromaticum* and *E. scaber*.

SiHa cells were seeded at 5.0×10^4 cells/cm^2 seeding density in black clear-bottom 96-well plate and incubated for 24 h prior to IC_{50} treatment of *S. aromaticum*, *E. scaber*, and etoposide. Statistical analysis was performed using a two-way ANOVA with Dunnett's multiple comparison test (*a* is p< 0.05, *b* is p< 0.001 compared with untreated controls).

A significant increase in caspase 3 activity was observed in treated cells (*S. aromaticum*, *E. scaber*, and etoposide) compared to the untreated control (Figure 4.31). At 48 hours, the caspase 3 levels in cells treated with *S. aromaticum*, *E. scaber* and etoposide as positive control were significantly higher ($p < 0.05$) than in the untreated group. This elevated activity persisted and further intensified at 60, 72 and 96 hours. Positive control showed highest elevation of caspase 3 activity. Notably, the magnitude of caspase 3 activation was slightly higher in cells treated with *E. scaber* compared to those treated with *S. aromaticum*, although the difference was minimal. This suggests that while both treatments induce significant apoptotic responses in SiHa cells, *E. scaber* may have a slightly stronger effect on caspase 3 activation.

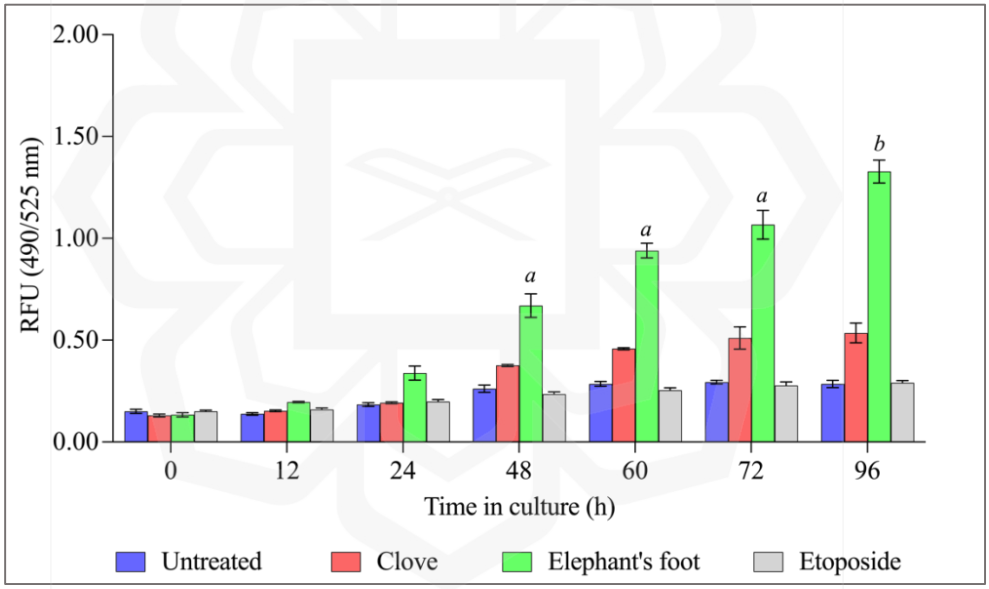


Figure 4.36 The activity of Caspase 8 upon treatment with *S. aromaticum* and *E. scaber*.

The treatment of *E. scaber* was found to significantly increased caspase 8 activity compared to the untreated control, highlighting the role of the extrinsic apoptotic pathway in the response to these agents (Figure 4.32). Treatment of *E. scaber* showed significant increase (*p*-value <0.05) in caspase 8 activities at 48, 60, and 72 hours but not treatment of *S. aromaticum*. Treatment with *E. scaber* also resulted in a highly significant increase in caspase 8 activity (*p*-value < 0.001) at 96-hour exposure,

suggesting a robust activation of the extrinsic apoptotic pathway. In contrast, treatment with *S. aromaticum* and etoposide did not show significant increase in caspase 8 activity. The observed differences indicate *E. scaber* activate caspase 8 and has a stronger pro-apoptotic effect through this pathway.

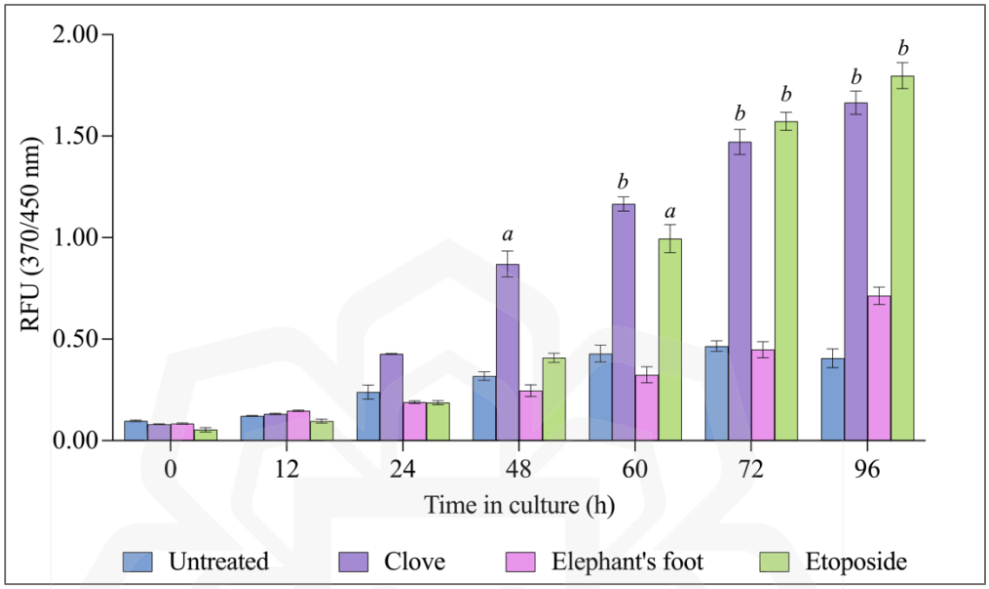


Figure 4.37 The activity of Caspase 9 upon treatment with *S. aromaticum* and *E. scaber*.

The activity of caspase 9, a critical initiator caspase in the intrinsic apoptotic pathway, was evaluated following treatment with *S. aromaticum* and *E. scaber*. *S. aromaticum* and etoposide treatments significantly increased caspase 9 activity compared to the untreated control, with clear differences in the timing and strength of activation between the two treatments. For *S. aromaticum*-treated SiHa cells, caspase 9 activity was highly significant as early as 48 hours ($p < 0.05$) and became progressively more significant at later time points, with $p < 0.001$ at 48, 60, 72, and 96 hours. Meanwhile, positive control of this study showed similar caspase 9 activation as *S. aromaticum* with significant increase starting from 60 to 96 hours of exposure. These pattern highlights a robust and sustained activation of the intrinsic apoptotic pathway, suggesting *S. aromaticum* and etoposide induces a strong pro-apoptotic response mediated through mitochondrial signalling.

CHAPTER FIVE

DISCUSSION

Cervical cancer is characterized by dysregulated molecular pathways that drive uncontrolled cell proliferation, evade apoptosis, and enhance tumour progression. A crucial aspect of cervical cancer pathogenesis is the disruption of cell cycle control and apoptosis regulation, often linked to the activity of high-risk human papillomavirus (HPV) oncoproteins E6 and E7 (Lee *et al.*, 2022; Muhamad *et al.*, 2015). These viral proteins inactivate tumour suppressors such as p53 and pRb, leading to unchecked cell cycle progression and reduced apoptotic sensitivity (Kanamori *et al.*, 2021). Additionally, metabolic alterations, including polyamine dysregulation, play a significant role in sustaining tumour growth and survival. Polyamines, essential for cellular functions such as DNA stabilization, RNA transcription, and protein synthesis, are frequently upregulated in cancer cells, including cervical cancer, providing an attractive target for intervention (Babbar & Gerner, 2011; Neborak *et al.*, 2022).

Given the central role of polyamine metabolism in cervical cancer progression, this study investigates the potential of Malaysian herbal aqueous extracts— *S. aromaticum* and *E. scaber*—as chemopreventive agents targeting the polyamine pathway. *In vitro* studies serve as a critical foundation for understanding the molecular effects of bioactive compounds on cervical cancer cells (SiHa cell), allowing for the identification of key regulatory pathways and mechanistic understanding into their therapeutic potential (Pauli *et al.*, 2017). By evaluating the effects of these herbs on cellular proliferation, polyamine biosynthesis, intracellular protein depletion, cell cycle distribution, and apoptosis induction, this study provides evidence supporting the use of natural compounds in cervical cancer prevention and treatment strategies.

Malaysian herbs consist of abundance of phytochemicals mainly polyphenols and flavonoids which found to induce toxicity to cancer cells while causing minimum toxicity to normal body cells (Radzishevska *et al.*, 2023). The main checklists for the natural products or in this study involve Malaysian herbs to be an effective chemopreventive agents are the ability of the herbs to prevent, delay, or suppress the

tumour. The principal goal of using Malaysian herbs or natural products was not only because of its bioactive compounds which specifically imparts cytotoxicity on cancer cells, but also at the same time protecting normal healthy cells from getting the effects of this drugs. There is multiple positive effect of the herbs clinically where it's not only aid in prevention of cancer recurrence, or spread of cancer in cancer patients, but also aid in protecting the individuals with high risk of cervical cancer (Mude *et al.*, 2022).

There was sufficient evidence from epidemiological and experimental studies to support the potential of *S. aromaticum* and *E. scaber* as chemopreventive agents as discussed in 2.6. However, to date the action of these two herbs on cervical cancer cell line was poorly investigated. The rationale of these herbs was that it was abundantly available and have been used as traditional treatment or ailments. The integration of traditional knowledge with modern scientific investigation provides a multiple advantage. Firstly, the use of traditionally recognized medicinal herbs offers a sustainable and cost-effective source of chemopreventive agents, particularly for low-resource settings (Ismail *et al.*, 2023). Secondly, the accumulated historical usage serves as a preliminary safety profile for these herbs, thereby reducing the initial barriers to their acceptance for therapeutic applications. Furthermore, leveraging locally available and culturally significant plants like *S. aromaticum* and *E. scaber* can promote community-based healthcare initiatives and support biodiversity conservation (Ali *et al.*, 2021; Ghani *et al.*, 2023).

By scientifically validating the traditional claims, this study not only highlights the relevance of ethnopharmacology but also leads the way for the discovery of novel bioactive compounds that could complement or replace conventional chemotherapeutic agents. This approach bridges the gap between traditional practices and modern drug discovery, reinforcing the value of natural products in cancer prevention and treatment. Therefore, the extensive investigation must be conducted on these two herbs to further elucidate its potential as chemopreventive agents.

In this study, SiHa cells were used as the cervical cancer cell line, while HEK 293T cells served as the normal cell line to investigate the toxicity effects of *S. aromaticum* and *E. scaber* on healthy cells. SiHa cells are known to harbour two copies of HPV-16, the most prevalent high-risk human papillomavirus genotype implicated in cervical carcinogenesis worldwide. HPV-16 is reported to contribute to 35.7% of cervical cancer infections in Malaysia and 5% globally (Sung *et al.*, 2021; Tan *et al.*, 2018). This makes SiHa cells a relevant model for studying the molecular mechanisms underlying cervical cancer progression and therapeutic interventions. This study was able to brings up the potential of Malaysian herbal extracts to disrupt the growth of cervical cancer cell lines leading to cell death with anti-proliferative effects while at the same time disrupt the polyamines metabolism of the cancer cells. Such interventions also offer additional benefits by targeting the metabolic vulnerabilities of cervical cancer cells while sparing normal cells, as demonstrated by the selective toxicity observed in SiHa cells compared to HEK 293T cells when *S. aromaticum* or *E. scaber* extracts were added to the normal cell line, as presented in section 4.3.3.

Furthermore, to justify the relevance of using HEK293t cells as a comparative model for SiHa cells, several key factors were considered beyond their similar epithelial-like morphology. HEK293t cells were chosen due to their shared ability with SiHa cells to undergo genetic modifications, their involvement in overlapping molecular pathways, and their established use in drug sensitivity studies. According to Snellenberg *et al.* (2014), both SiHa and HEK293t cells share a common apoptotic regulatory pathway involving PRDM14, NOXA, and PUMA. Since HEK293t cells retain functional apoptotic regulation, they provide a valid comparative model to study cancer cell apoptosis escape mechanisms in SiHa cells. HEK293t cells are also commonly used as a reference model for normal gene expression in studies investigating oncogenes and tumour suppressors (Sharma, 2023). A study by Wang *et al.* (2015) compared miR-373 expression levels in SiHa and HEK293t cells, which helped distinguish tumour-specific regulatory mechanisms from normal cellular functions. This further supports their relevance in cervical cancer research, particularly in identifying aberrant genetic and epigenetic alterations in SiHa cells (Mishra & Mishra, 2020; Wei *et al.*, 2018; Yang *et al.*, 2017). These studies highlight the importance of HEK293t cells as a baseline to assess the specificity of drug responses in

SiHa cells, ensuring that observed effects are cancer-specific rather than general cellular responses.

Polyamines play a crucial role in cellular processes, including DNA stabilization, RNA transcription, and protein synthesis. The polyamine biosynthesis pathway, involving key enzymes such as ornithine decarboxylase (*ODC*), spermidine synthase (*SPDS*), and spermine synthase (*SPMS*), is tightly regulated in normal cells to maintain homeostasis (Saunders & Wallace, 2010). However, in cancer cells, the polyamine pathway is frequently dysregulated, leading to increased levels of polyamines such as putrescine, spermidine, and spermine. These molecules promote cellular proliferation, inhibit apoptosis, and enhance genomic instability, which collectively support tumour growth and progression (Ghani *et al.*, 2023b).

In the context of cervical cancer, polyamines have been found to interact with HPV oncoproteins, particularly E6 and E7, which are pivotal in driving carcinogenesis. The HPV E7 protein inactivates retinoblastoma protein (pRb), facilitating cell cycle progression, while E6 promotes the degradation of p53, impairing the cell's ability to undergo apoptosis. Elevated polyamine levels further exacerbate these oncogenic effects by supporting uncontrolled cellular proliferation and by mitigating oxidative stress that would otherwise damage cancer cells. Targeting the polyamine pathway has been identified as a promising therapeutic strategy in cervical cancer. Inhibitors of ODC, such as difluoromethylornithine (DFMO), have demonstrated the ability to reduce polyamine levels, suppress tumour growth, and sensitize cancer cells to chemotherapeutic agents (Koomoa *et al.*, 2013). Natural compounds, including those derived from herbs, have shown potential in modulating the polyamine pathway. The use of *S. aromaticum* and *E. scaber* in this study is particularly significant, as these herbs may exert chemopreventive effects by altering polyamine metabolism in SiHa cells, thereby reducing their viability and promoting apoptosis.

5.1 INVESTIGATION OF ANTIPROLIFERATIVE EFFECTS OF SELECTED MALAYSIAN HERBS ON HUMAN CERVICAL CANCER

To determine the validity of the IC_{50} value obtained for etoposide in this study (30 µg/mL at 48-hour exposure in SiHa cells), it is essential to compare it with previous literature and assess its relevance in the experimental context. Various studies have reported different IC_{50} values for etoposide depending on factors such as cell line type, experimental conditions, drug exposure duration, and sensitivity of the cells to the chemotherapeutic agent. Recent findings by Reddy *et al.* (2024) and Çelebioğlu *et al.* (2022) reported IC_{50} values of 35.32 µg/mL and 33.67 µg/mL, respectively, for etoposide in SiHa cells at 48-hour exposure. These values closely align with the findings of the present study, further supporting the validity of the IC_{50} concentration obtained. Compared to earlier studies such as Lee & Lee (2000) and Yuan *et al.*, (2017), which reported lower IC_{50} values (10.04 µg/mL and 10 µM, respectively), and Jin *et al.* (2011), who observed IC_{50} values as low as 0.0195 µM (~0.0115 µg/mL), these discrepancies highlight the variability in etoposide sensitivity across different experimental setups. Similarly, Wasim & Chopra (2016) found an IC_{50} of 12 µM (~7.08 µg/mL) at 72-hour, demonstrating that exposure time influences drug potency.

The variations in IC_{50} values across studies can be attributed to differences in experimental conditions, including cell confluency, serum composition, medium pH, incubation duration, and passage number of the SiHa cells. Moreover, prolonged exposure can enhance drug uptake and induce secondary cellular responses, which may alter cytotoxicity outcomes (Li *et al.*, 2015; Wei *et al.*, 2018). Additionally, the genetic variability of SiHa cell populations used in different laboratories might contribute to variations in drug sensitivity (Hematulin *et al.*, 2018).

Despite these variations, the IC_{50} value obtained in this study (30 µg/mL at 48 hours) is well within the range reported in recent studies and is therefore considered valid. This concentration reliably inhibits 50% of SiHa cell viability under the experimental conditions used and serves as a crucial benchmark for subsequent cytotoxicity studies involving *S. aromaticum* and *E. scaber* extracts. Establishing etoposide as a positive control ensures the reliability of the experimental setup,

confirming that the observed effects of the herbal extracts can be accurately compared to a well-established chemotherapeutic agent. Establishing etoposide as a positive control not only confirms the reliability of the experimental setup but also ensures the reproducibility of the findings. The consistency of results across multiple replicates further strengthens the validity of the IC_{50} determination. Consequently, the IC_{50} value of etoposide obtained in this study serves as a crucial reference point, allowing direct comparisons between the cytotoxic effects of Malaysian herbal extracts and a well-established chemotherapeutic agent.

The cytotoxicity screening of 11 Malaysian herbs against SiHa cervical cancer cells was conducted to identify potent candidates with significant anti-proliferative effects. The herbs evaluated in this study included clove (*Syzygium aromaticum*), elephant's foot (*Elephantopus scaber*), *Ulam Raja (Cosmos caudatus Kunth), Bunga Raya (Hibiscus rosa-sinensis), Kacang Telang (Clitoria ternatea L.*), and Cinnamon (*Cinnamomum verum*), as well *as Temu Putih (Curcuma zedoaria*), *Mas Cotek (Ficus deltoidea), Pecah Kaca (Strobilanthes crispus*), Bay Leaves (*Cinnamomum tamala*), and Nutmeg (*Myristica fragrans*). Among these, only *S. aromaticum* and *E. scaber* demonstrated strong cytotoxicity and were selected for further investigation based on their low IC_{50} values at 24, 48, and 72 hours of treatment. The findings from this screening emphasize the selective cytotoxic potential of *S. aromaticum* and *E. scaber*, justifying their further investigation in this study. Their ability to reduce cancer cell viability while sparing normal cells aligns with the essential criteria for effective chemopreventive agents. Additionally, their phytochemical profiles, enriched with bioactive compounds, further support their role in modulating cancer cell metabolism, as discussed in sub-section 4.3 of the results.

According to the National Cancer Institute (NCI) guidelines, the cytotoxicity of natural products is classified into four categories: highly active ($IC_{50} \leq 20$ µg/ml), moderately active (IC_{50} 21–200 µg/ml), weakly active (IC_{50} 201–500 µg/ml), and inactive ($IC_{50} > 501$ µg/ml) (Grkovic *et al.*, 2020). In this study, both *S. aromaticum* and *E. scaber* fell into the category of moderate cytotoxicity, with IC_{50} values of 169.60 µg/ml and 56.30 µg/ml at 48 hours, respectively. These findings suggest that both herbs exhibit strong anti-proliferative effects and have potential as chemopreventive agents

for cervical cancer. In contrast, the remaining nine herbs exhibited either weak cytotoxicity or inactivity, with IC_{50} values exceeding 200 µg/ml. *Temu Putih (Curcuma zedoaria), Mas Cotek (Ficus deltoidea), Pecah Kaca (Strobilanthes crispus*), Bay Leaves (*Cinnamomum tamala*), and Nutmeg (*Myristica fragrans*) showed particularly low potency against SiHa cells, failing to reach the moderate cytotoxicity threshold. Meanwhile, *Ulam Raja, Bunga Raya, Kacang Telang*, and Cinnamon displayed weak cytotoxic activity, suggesting limited efficacy as anti-cervical cancer agents (results presented in sub-section 4.3).

These findings align with prior research on *S. aromaticum* aqueous extract. For instance, Li *et al.* (2019) reported an IC_{50} of approximately 150 µg/ml for *S. aromaticum* extract against human pancreatic ASPC-1 and human colon HT-29 cancer cells. Similarly, Mekky *et al.* (2024) observed a slightly higher IC_{50} of 250 µg/ml for *S. aromaticum* aqueous extract on liver cancer (HepG2) and breast cancer (MCF-7) cell lines. Such variations in IC_{50} values across studies may be attributed to differences in experimental conditions, cancer cell types, and the extract preparation methods.

The moderate cytotoxicity observed in this study supports the potential of *S. aromaticum* as an anti-cancer agent, with its efficacy likely attributed to its rich phytochemical profile. Eugenol, the primary bioactive compound in *S. aromaticum*, has been extensively reported for its anti-proliferative properties. It is known to induce apoptosis, inhibit cell cycle progression, and modulate molecular pathways involved in cancer progression, including the regulation of oxidative stress and inflammation. These mechanisms may synergistically enhance the anti-cancer potential of *S. aromaticum* extract. Furthermore, the time-dependent reduction in IC_{50} values observed in this study indicates that prolonged exposure to *S. aromaticum* extract enhances its cytotoxic effects on SiHa cells. This finding highlights the importance of treatment duration in maximizing the anti-proliferative efficacy of natural products. Future studies should explore whether combining *S. aromaticum* extract with conventional chemotherapeutic agents or other natural compounds could yield synergistic effects, thereby reduce the required therapeutic doses and minimizing potential side effects.

In addition to *S. aromaticum*, the current study also investigated the anti-proliferative and cytotoxic effects of *E. scaber* aqueous extract. Among the screened Malaysian herbs, *E. scaber* demonstrated potent cytotoxic activity with an IC_{50} value of 56.30 µg/ml at 72 hours, categorizing it as moderately active according to the National Cancer Institute (NCI) cytotoxicity guidelines. The differences in IC_{50} values between *S. aromaticum* and *E. scaber* suggests that the latter may possess higher potency in targeting cervical cancer cells, particularly the SiHa cell line. Previous studies corroborate the findings of this research, highlighting the rich phytochemical profile of *E. scaber*, which includes flavonoids, terpenoids, alkaloids, phenolic compounds, coumarins, and carotenoids. These bioactive compounds are well-known for their anti-proliferative and cytotoxic properties.

In previous investigations, *E. scaber* extract has shown significant cytotoxicity against various cancer cell lines, including hepatocellular carcinoma (HepG2), breast cancer (MCF-7), and colon cancer (HT-29), with IC_{50} values in the range of 50-70 µg/ml (Singh *et al.*, 2019). This suggests a broad-spectrum anti-cancer activity, possibly mediated by its ability to generate reactive oxygen species (ROS), disrupt mitochondrial integrity, and trigger caspase-dependent apoptosis. The findings of this study expand upon these earlier reports by demonstrating the efficacy of *E. scaber* extract specifically against SiHa cells, a cervical cancer cell line.

The time-dependent reduction in IC_{50} values observed for *E. scaber* extract suggests that prolonged exposure enhances its cytotoxic effects, similar to *S. aromaticum*. This could be attributed to the cumulative cellular damage caused by sustained interaction with the bioactive compounds. Notably, terpenoids such as molephantinin and flavonoids like 8-C-methylvelloquercetin, identified in the extract through LCMS analysis, have been associated with potent anti-cancer activity (Rajesh *et al.*, 2022). These compounds are known to inhibit key oncogenic pathways, including PI3K/Akt/mTOR and MAPK, which are critical for cancer cell survival and proliferation. The potent cytotoxic and anti-proliferative properties of *E. scaber* aqueous extract observed in this study, alongside its rich phytochemical profile, underscore its potential as a chemopreventive agent. These findings contribute to the

growing body of evidence supporting the therapeutic utility of *E. scaber* in cancer treatment and justify further in-depth pharmacological investigations.

Importantly, the potential toxicity of *S. aromaticum* and *E. scaber* extracts on normal cells was also assessed to evaluate their therapeutic safety profile. The findings revealed no significant reduction in cell viability when the extracts were tested on HEK 293t cells, a widely used normal human embryonic kidney cell line (Amuthan *et al.*, 2019; Waly *et al.*, 2011). This suggests that both extracts are selective in targeting cancer cells while sparing normal cells, making them promising candidates for chemoprevention or as complementary therapeutic agents. The observed selectivity could be attributed to the unique biochemical interactions of the phytochemicals with cancer-specific metabolic pathways. For instance, cancer cells often exhibit altered oxidative states and heightened metabolic demands, which may render them more susceptible to the pro-oxidative and anti-proliferative effects of the compounds. Additionally, the low polyamine content in both *S. aromaticum* and *E. scaber* extracts (Table 4.10) might contribute to their ability to disrupt the polyamine pathway, which is frequently upregulated in cancer cells to support rapid growth and division.

5.2 DETERMINATION OF PHYTOCHEMICAL COMPOUNDS PRESENT IN SELECTED MALAYSIAN HERBS.

In the determination of the phytochemical profiles of *S. aromaticum* and *E. scaber*, the identification of bioactive compounds with potential anticancer properties was reported in sub-section 4.2. Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) analyses were employed to comprehensively profile the phytochemicals present in these herbs.

The findings of this study corroborate previous research done by Aldabaan *et al.* (2024) and Mostafa *et al.*, (2023), identifying eugenol, an aromatic compound, as the major bioactive component in *S. aromaticum* extracts. Eugenol has been extensively documented for its diverse pharmacological properties, including antioxidant, anticancer, and antiproliferative activities. Its anticancer effects are largely attributed to

its ability to induce apoptosis, modulate cellular signalling pathways, and reduce oxidative stress, which collectively inhibit cancer cell proliferation and survival. Previous studies have demonstrated the effectiveness of *S. aromaticum* extract against various cancer cell lines, including breast carcinoma (MCF-7), cervical cancer (HeLa), lung cancer (A549), prostate cancer (DU 145), colon cancer (CaCo-2), and oesophageal cancer (SK-GT-04) (Abadi *et al.*, 2022; Taha *et al.*, 2020). However, this study is the first to explore the chemopreventive potential of *S. aromaticum* extract on the SiHa cervical cancer cell line, providing novel insights into its therapeutic relevance in combating human papillomavirus (HPV)-associated cervical cancers. The confirmation of eugenol as the primary active compound in *S. aromaticum* further reinforces its role as a potent natural chemopreventive agent.

The phytochemical analysis of *E. scaber* revealed the presence of various bioactive compounds, including flavonoids, terpenoids, phenolics, alkaloids, coumarins, and carotenoids Table 4.3. These groups of compounds are widely recognized for their anticancer and antiproliferative properties, which position *E. scaber* as a promising chemopreventive agent. The detected phytochemicals align with findings from prior studies, which identified *E. scaber* extract as a rich source of terpenoids and flavonoids (Marina, 2021). These compounds are known to exert their effects through mechanisms such as apoptosis induction, cell cycle arrest, reactive oxygen species (ROS) generation, and inhibition of angiogenesis. Evidence from previous experimental studies further supports the potential of *E. scaber* against various cancer cell lines, including liver (HepG2), colon (HT-29), and breast (MCF-7) cancer cells (Kabeer *et al.*, 2019; Shinde & Mendhulkar, 2023). However, this study adds to the existing body of knowledge by characterizing its chemopreventive potential on SiHa cells, highlighting its utility in targeting HPV-associated cervical cancers.

The comparison of the two herbs demonstrates the complementary nature of their bioactive compounds. While *S. aromaticum*'s phytochemical profile is dominated by eugenol, a single compound with well-documented anticancer properties, *E. scaber* exhibits a diverse spectrum of compounds that act synergistically to exert anticancer effects. This diversity suggests that *E. scaber* may provide a broader range of therapeutic mechanisms, whereas *S. aromaticum* offers targeted efficacy through a

dominant active compound. The findings from this study not only validate the traditional medicinal uses of *S. aromaticum* and *E. scaber* but also emphasize their potential as chemopreventive agents. The confirmation of these bioactive compounds provides a foundation for further pharmacological studies, including *in vivo* investigations and clinical trials, to explore their mechanisms of action and optimize their therapeutic applications in cervical cancer treatment.

5.3 ASSESSMENT ON THE EFFECTS OF *S. AROMATICUM* AND *E. SCABER* ON CELLULAR POLYAMINES ACTIVITIES OF CERVICAL CANCER

High levels of polyamines are linked to tumour progression, and reducing polyamine intake through diet is often recommended for cancer patients to limit tumour growth. In line with this strategy, the polyamine content of *S. aromaticum* and *E. scaber* extracts was analysed using HPLC. The results showed that both extracts contained low levels of polyamines. This finding supports their potential as natural remedies to target polyamine imbalance in cancer. The low polyamine content (total polyamines < 23.2 nmol/mg) of these herbs not only complements their antiproliferative effects but also in parallel with dietary approaches aimed at reducing polyamine levels in cancer patients.

Polyamines play a crucial role in cellular growth, proliferation, and survival, making them essential for normal cell function. However, their dysregulation has been strongly implicated in cancer progression, including cervical cancer, where increased polyamine biosynthesis supports rapid tumour growth and resistance to apoptosis. Given their significant role in tumour development, targeting polyamine metabolism has emerged as a promising strategy in cancer treatment and chemoprevention. In this study, the impact of polyamine modulation was further explored by evaluating the efficacy of the selected Malaysian herbs, *S. aromaticum* and *E. scaber*, in the human cervical cancer cell line, SiHa. These herbs were assessed for their ability to influence polyamine-related mechanisms, particularly in regulating polyamine biosynthesis and catabolism, which may contribute to their anti-cancer properties. Aberrant polyamine

metabolism in cancer cells often results in elevated intracellular polyamine levels, contributing to the enhanced survival and aggressiveness of these cells.

In this study, the effects of *S. aromaticum* and *E. scaber* aqueous extracts on the intracellular polyamine levels in SiHa cells were investigated. Results demonstrated a significant reduction in intracellular polyamines levels across all treatment groups, including the positive control, after 48 hours of treatment compared to untreated SiHa cells. This finding reinforces the potential of *S. aromaticum* and *E. scaber* as promising chemopreventive agents. The observed decrease in polyamines concentration suggests that these extracts may disrupt key regulatory mechanisms of polyamine metabolism (section 4.7.2.3). Specifically, the significant reduction in intracellular polyamine levels indicates decreased polyamine transport, reduced compensatory polyamine biosynthesis, and downregulation of rate-limiting enzymes involved in polyamine metabolism. These disruptions impair the tightly controlled polyamine homeostasis that cancer cells rely on, leading to inhibition of cellular growth, induction of cell death, and eventual suppression of tumour-promoting functions. Such findings highlight the therapeutic potential of targeting polyamine metabolism as a novel approach for cervical cancer management.

Previous studies also supported the findings of this research by validating the therapeutic potential of targeting polyamine metabolism in cancer cells. For instance, Zhang *et al.* (2023) emphasized that polyamine levels are tightly regulated in cancer cells to sustain their uncontrolled proliferation and survival. Elevated polyamine levels in cancer cells have been shown to promote tumour growth and progression by enhancing cell division, DNA stability, and angiogenesis. Disrupting this dysregulated polyamine metabolism presents a promising avenue for cancer therapy. Lian *et al.* (2022) demonstrated that interfering with polyamine biosynthesis and transport pathways can effectively induce apoptosis and suppress tumour growth across various cancer models, further highlighting the importance of this pathway in cancer biology.

S. aromaticum extract has been extensively studied for its anticancer properties, which are attributed to its high content of bioactive compounds such as eugenol. Eugenol is known to inhibit key metabolic pathways in cancer cells, including

polyamine biosynthesis and transport. Truzzi *et al.* (2023) investigated the effects of *S. aromaticum* extract on polyamine metabolism in breast and colon cancer cells. Their findings revealed that *S. aromaticum* extract significantly reduced intracellular polyamine levels by downregulating critical biosynthesis enzymes, such as ornithine decarboxylase (ODC), and suppressing polyamine transport. This reduction in polyamines led to a marked decrease in cell proliferation and enhanced apoptosis, underscoring the potential of *S. aromaticum* extract as a natural modulator of polyamine metabolism in cancer therapy.

In this study, *S. aromaticum* extract demonstrated similar effects on polyamine levels in SiHa cervical cancer cells, with significant reductions observed after treatment (sub-section 4.7.2.3). These findings align with the existing literature and further validate the role of *S. aromaticum* extract as a chemopreventive agent. By disrupting polyamine biosynthesis and reducing intracellular polyamine levels, *S. aromaticum* extract impairs key metabolic processes that cancer cells rely on for survival and growth. This dual action, combining polyamine modulation with its known antiproliferative effects, suggests the potential of *S. aromaticum* extract as a valuable component of natural product-based cancer therapies.

Similarly, compounds such as terpenoids and flavonoids found in *E. scaber* extract have been documented for their roles in downregulating enzymes critical to polyamine synthesis, such as ornithine decarboxylase (Jamehdor *et al.*, 2021; Long *et al.*, 2024; J. Wang *et al.*, 2017). While there have been several anticancer studies investigating the effects of *E. scaber* extracts on various cancer cell lines, no studies to date have specifically examined the role of these extracts in modulating polyamine metabolism in cancer cells. This reminds a significant gap in the current literature.

The findings of this research provide valuable insights into the chemopreventive potential of *E. scaber* by demonstrating its ability to reduce intracellular polyamine levels in SiHa cervical cancer cells. This novel contribution expands our understanding of the therapeutic applications of *E. scaber*, particularly in the context of targeting dysregulated polyamine metabolism—a hallmark of cancer progression. By bridging this knowledge gap, the present study lays the foundation for further exploration of *E.*

scaber as a potential modulator of polyamine pathways, which could lead to the development of innovative strategies for chemoprevention and cancer therapy.

It is reasonable to assert that this study could serve as a cornerstone for future investigations into the role of *E. scaber* in polyamine metabolism and its broader implications in chemopreventive studies. The findings not only align with prior research which supports the anticancer properties of *E. scaber* but also provide a strong basis for further exploration of Malaysian herbs as promising natural agents in cancer prevention and therapy.

To establish a direct correlation between polyamine metabolism and the gene expression of polyamine-regulating enzymes, this study examined the expression levels of key genes involved in polyamine biosynthesis and catabolism. The reduction in intracellular polyamine levels observed in SiHa cells following treatment with *S. aromaticum* and *E. scaber* extracts suggests a significant alteration in the polyamine regulatory network. This decrease in polyamines was accompanied by notable changes in gene expression patterns, specifically in ornithine decarboxylase (*ODC*), spermidine/spermine N1-acetyltransferase (*SSAT*), ornithine decarboxylase antizyme 1 (*OAZ1*), and spermine oxidase (*SMOX*), which play central roles in maintaining polyamine homeostasis. In the next molecular investigation into the efficacy of *S. aromaticum* and *E. scaber* on SiHa cells, the expression of key genes involved in polyamine metabolism—*ODC, OAZ1, SSAT*, and *SMOX*—was analysed in SiHa cells treated with *S. aromaticum* and *E. scaber* extracts, as well as etoposide (positive control), at various time points. The findings revealed significant modulation of these genes, thus explains the ability of *S. aromaticum* and *E. scaber* to effectively disrupt polyamine metabolism in cancer cells.

ODC, a rate-limiting enzyme in polyamine biosynthesis, was significantly downregulated following treatment with both herbal extracts. Since ODC is responsible for catalysing the conversion of ornithine into putrescine, its suppression directly limits the availability of polyamine precursors, leading to a reduction in intracellular polyamine content. The significant reduction in *ODC* expression after 24 hours of treatment with *S. aromaticum*, *E. scaber*, and etoposide is notable. *ODC* is a rate-

limiting enzyme in polyamine biosynthesis and plays a critical role in converting ornithine to putrescine, a precursor for spermidine and spermine (Casero *et al.*, 2018). Its downregulation upon treatment aligns with the observed decrease in intracellular polyamine levels, as reduced *ODC* activity limits the production of putrescine and subsequent polyamines. This finding suggests that both *S. aromaticum* and *E. scaber* may inhibit tumour progression by targeting polyamine biosynthesis early in the treatment process, similar to other studies reporting *ODC* inhibition as a critical step in reducing polyamine pools in cancer cells (Murali *et al.*, 2023). This finding is consistent with the observed decrease in polyamine levels, reinforcing the hypothesis that *S. aromaticum* and *E. scaber* extracts exert their cytotoxic effects by disrupting polyamine biosynthesis.

Additionally, the upregulation of *OAZ1*, a natural negative regulator of *ODC*, aligns with the suppression of *ODC* expression and further supports the reduction in polyamine biosynthesis. *OAZ1* functions by targeting *ODC* for degradation, thereby preventing excessive polyamine accumulation. Moreover, the increased expression of *OAZ1* at 48, 72, and 96 hours in all treatment groups further supports the downregulation of polyamine biosynthesis. *OAZ1* is a feedback regulator that inhibits *ODC* activity and promotes the degradation of *ODC* protein, thus reducing polyamine levels (Hyvönen *et al.*, 2022; Tavallali *et al.*, 2024). This upregulation indicates a compensatory mechanism activated by the treatments to regulate excessive polyamine levels, a phenomenon observed in previous studies on polyamine-targeting therapies (Syatkin *et al.*, 2020b). The significant increase in *OAZ1* expression following treatment suggests a reinforced regulatory mechanism aimed at limiting polyamine production and curbing uncontrolled cell growth.

Conversely, the upregulation of polyamine catabolic enzymes such as *SSAT* and *SMOX* suggests an enhanced breakdown of existing polyamines. *SSAT* plays a crucial role in polyamine catabolism by acetylating spermidine and spermine, marking them for degradation or export, whereas *SMOX* catalyzes the oxidation of spermine to produce spermidine and hydrogen peroxide, a byproduct that induces oxidative stress and contributes to apoptosis. The increased expression of these enzymes following treatment with *S. aromaticum* and *E. scaber* extracts indicates a shift towards polyamine

degradation, further reducing the intracellular polyamine pool and inhibiting cancer cell proliferation.

The elevated expression of *SSAT* (spermidine/spermine N1-acetyltransferase), particularly in the *E. scaber*-treated group as early as 24 hours, and in *S. aromaticum* and etoposide-treated groups after 48 hours, supports an enhancement in polyamine catabolism. *SSAT* acetylates spermidine and spermine, facilitating their conversion to putrescine or their export from the cell (Chin *et al.*, 2022). This catabolic shift aligns with the reduction in intracellular polyamine levels observed in this study, as polyamine degradation accelerates their depletion. Similar findings have been reported for other natural products, such as curcumin and berberine, which upregulate *SSAT* expression to decrease intracellular polyamines and induce apoptosis in cancer cells (Wu *et al.*, 2018).

Meanwhile, the upregulation of *SMOX* (spermine oxidase) after 48 hours of treatment across all groups indicates enhanced oxidative catabolism of spermine to spermidine and hydrogen peroxide. This process not only contributes to the depletion of polyamines but also generates reactive oxygen species (ROS), which may enhance the pro-apoptotic effects of the treatments (Miller-Fleming *et al.*, 2015). The simultaneous increase in *SMOX* and *SSAT* expression suggests a coordinated effort to deplete polyamines and disrupt cellular functions reliant on polyamine homeostasis.

These findings demonstrate that the reduction in intracellular polyamine levels is not merely a result of impaired protein synthesis but is closely linked to the modulation of polyamine-related gene expression. The downregulation of *ODC* and the concurrent upregulation of *SSAT, SMOX*, and *OAZ1* collectively contribute to polyamine depletion, thereby disrupting the biochemical environment necessary for cancer cell survival. This mechanism highlights the potential of *S. aromaticum* and *E. scaber* extracts as polyamine-targeting agents capable of inducing cytotoxicity in SiHa cells through a multifaceted approach—suppressing biosynthesis, enhancing catabolism, and reinforcing negative regulatory feedback.

The observed modulation of these genes aligns with the significant reduction in intracellular polyamine levels following treatment with *S. aromaticum*, *E. scaber*, and

etoposide. Downregulation of *ODC* and upregulation of *OAZ1* reduce polyamine biosynthesis, while increased expression of *SSAT* and *SMOX* enhances polyamine catabolism, leading to polyamine depletion. This dual regulation of biosynthesis and catabolism underscores the ability of these treatments to target polyamine metabolism comprehensively, disrupting the metabolic balance essential for cancer cell proliferation and survival. By focusing on polyamine gene expression and its direct link to intracellular polyamine content, this study provides a more comprehensive understanding of how these herbal extracts exert their antiproliferative effects on cervical cancer cells. These findings emphasize the therapeutic relevance of polyamine pathway modulation and underscore the importance of targeting polyamine metabolism as a viable strategy in cervical cancer chemoprevention.

In addition to that, sensitization of both *S. aromaticum* and *E. scaber* extract were investigated with additional treatment of difluoromethylornithine (DFMO). The combination treatment of *S. aromaticum* or *E. scaber* extracts with DFMO revealed a potential sensitization effect on SiHa cells, as indicated by the reduction in IC_{50} values for both combinations. Although the reduction in IC_{50} values was not statistically significant, the observed trends suggest a synergistic interaction between DFMO and the herbal extracts. DFMO, a well-established inhibitor of ornithine decarboxylase (ODC), exerts cytostatic effects by inhibiting the biosynthesis of polyamines, a critical pathway supporting cancer cell proliferation and survival (Schultz *et al.*, 2021). The combination of DFMO with *S. aromaticum* or *E. scaber* likely augments this inhibition, amplifying the disruption of polyamine metabolism and enhancing the cytotoxicity of the extracts.

The observed synergistic effect may be attributed to the complementary mechanisms of action of DFMO and the bioactive compounds in the herbal extracts. *S. aromaticum* contains eugenol, a compound known to induce oxidative stress and mitochondrial dysfunction in cancer cells, while *E. scaber* is rich in terpenoids and flavonoids, which have been shown to modulate apoptotic pathways and inhibit tumour growth (Alhosin *et al.*, 2020; H. Zaid *et al.*, 2017). When combined with DFMO, these bioactive compounds may enhance the downregulation of polyamine biosynthesis while simultaneously targeting other critical pathways, such as oxidative stress and apoptosis.

This dual action could explain the reduced cell viability observed in the combination treatments compared to the individual treatments alone.

The reduction in IC_{50} values were statistically significant, the data provides the potential of DFMO to enhance the efficacy of *S. aromaticum* and *E. scaber* as chemopreventive agents. Similar trends have been observed in studies combining DFMO with other natural compounds, such as curcumin and resveratrol, where the combination resulted in enhanced inhibition of cancer cell growth despite the modest effects of DFMO alone (Alexiou *et al.*, 2019). These findings indicate the potential for combination therapies to amplify the therapeutic effects of natural extracts, particularly when paired with drugs that target specific metabolic vulnerabilities in cancer cells.

5.4 ASSESSMENT OF CELL CYCLE PROFILE

Following the next objective, the treatment of SiHa cells with *S. aromaticum* and *E. scaber* aqueous extracts also resulted in distinct alterations in cell cycle distribution, as evidenced by DNA content analysis. Notably, the duration of exposure influenced these changes across different cell cycle phases. After 24 hours of exposure, both *E. scaber* and etoposide treatments induced a significant arrest at the G2/M phase compared to untreated cells. This observation aligns with previous research demonstrating that deoxyelephantopin, a major component of *E. scaber*, can cause G2/M phase arrest in various cancer cell lines including liver, breast, lung cancer cell lines (Mehmood *et al.*, 2017).

At the 48-hour mark, *E. scaber* and etoposide treatments led to an accumulation of cells in the G0/G1 phase, suggesting a shift in the cell cycle arrest point. This phase-specific arrest may be attributed to the modulation of cell cycle regulatory proteins by compounds present in the extracts. For instance, *S. aromaticum* extract has been reported to promote G0/G1 cell cycle arrest in human cancer cell lines by upregulating p21(WAF1/Cip1) expression and downregulating cyclin-dependent kinases (Liu *et al.*, 2014).

After 72 hours of treatment, all groups—including those treated with *S. aromaticum*, *E. scaber*, and etoposide—exhibited a pronounced increase in the G2/M phase cell population. This consistent G2/M arrest across different treatments emphasizes the potential of these extracts to interfere with cell cycle progression at critical checkpoints. Such interference can halt the proliferation of cancer cells, as the G2/M checkpoint is crucial for ensuring DNA integrity before mitosis. The ability of natural compounds to induce cell cycle arrest at specific phases has been documented as a mechanism for their anticancer effects (Bai *et al.*, 2020). These findings collectively suggest that *S. aromaticum* and *E. scaber* extracts can modulate cell cycle progression in SiHa cells, leading to phase-specific arrests that impede cancer cell proliferation. The observed effects are consistent with the known actions of bioactive compounds present in these plants, which have been shown to target cell cycle regulators and induce apoptosis in various cancer models.

Polyamines, including putrescine, spermidine, and spermine, play a critical role in supporting cancer cell proliferation and cell cycle progression. The depletion of intracellular polyamine levels observed in this study after treatment with *S. aromaticum* and *E. scaber* extracts was accompanied by changes in cell cycle distribution, particularly an accumulation of cells in the G2/M phase. Previous research has shown that polyamine depletion disrupts the G2/M checkpoint by inhibiting cyclin-dependent kinases (CDKs) and interfering with microtubule formation, both of which are essential for mitotic progression (Marverti *et al.*, 2013; Qanash *et al.*, 2023). The ability of these extracts to simultaneously reduce polyamine levels and induce cell cycle arrest shows their potential to target multiple vulnerabilities in cancer cells.

Polyamine biosynthesis is tightly linked to cell cycle regulation, with the expression of key polyamine metabolism genes, such as *ODC1* and *AMD1*, fluctuating across different phases of the cell cycle. These genes are upregulated during the S and G2/M phases to support the heightened metabolic demands of proliferating cancer cells (Pegg, 2018). The reduction in intracellular polyamine levels and the observed cell cycle arrest in this study suggest that *S. aromaticum* and *E. scaber* extracts may downregulate the expression of polyamine biosynthesis genes. Previous studies have shown that targeting polyamine metabolism can lead to the repression of these genes,

further reinforcing cell cycle arrest and reducing tumour cell viability (Kuruburu *et al.*, 2022; Schultz *et al.*, 2019). The ability of these extracts to influence both polyamine levels and cell cycle regulation confirms it's as chemopreventive agents.

5.5 INVESTIGATION OF MECHANISMS OF CELL DEATH BY *S. AROMATICUM* AND *E. SCABER* ON CERVICAL CANCER

Understanding how *S. aromaticum* and *E. scaber* extracts induce cell death in SiHa cells is essential for determining their potential as chemopreventive agents. Identifying the pathways involved provides clarity on their specific anti-cancer effects and supports their therapeutic application. Apoptosis can occur through two main pathways: the intrinsic pathway, which is mediated by mitochondrial signalling, and the extrinsic pathway, which is initiated by death receptors on the cell surface. In this study, the activation of caspase 9 following treatment with *S. aromaticum* extract and etoposide indicates that these treatments rely on the intrinsic pathway to induce cell death. On the other hand, *E. scaber* extract showed significant activation of caspase 8, which supports its role in initiating the extrinsic apoptotic pathway.

The annexin V apoptosis assay revealed significant effects of *S. aromaticum* and *E. scaber* aqueous extracts, as well as etoposide, on the induction of cell death in SiHa cells. The significant reduction in live cells across all treatment groups ($p < 0.0001$) confirms the cytotoxicity of these treatments, which aligns with the known antiproliferative properties of both extracts (Li *et al.*, 2020b; Nwodo *et al.*, 2016). *S. aromaticum* and etoposide treatments significantly induced early apoptosis in SiHa cells. This finding is also consistent with previous studies where the ability of *S. aromaticum* extract, particularly its active compound eugenol, to initiate apoptosis by disrupting mitochondrial membrane potential and activating caspase cascades (Liu *et al.*, 2014; Liu *et al.*, 2022). In contrast, *E. scaber* treatment did not show a significant effect on early apoptosis, suggesting that its mechanism of action may be more effective in promoting late apoptotic pathways rather than early-stage apoptotic events.

Meanwhile, all treatment groups, including *S. aromaticum*, *E. scaber*, and etoposide, significantly induced late apoptosis in SiHa cells. This observation validates the apoptotic potential of both herbal extracts. Sesquiterpene lactones like deoxyelephantopin, found in *E. scaber*, have been shown to induce apoptosis by activating reactive oxygen species (ROS) and caspase-3, which align with the results of this study (Y. Gao *et al.*, 2022; Leenutaphong *et al.*, 2022).

Nonetheless, a significant increase in necrotic cells was also observed in the *E. scaber* and etoposide-treated groups, whereas *S. aromaticum* treatment did not show a significant increase in necrosis. The necrotic effect of *E. scaber* could be attributed to its ability to generate oxidative stress at higher doses, leading to irreversible cellular damage and loss of membrane integrity (Bai *et al.*, 2020; Nurkhasanah *et al.*, 2015). On the other hand, the absence of significant necrosis in the *S. aromaticum*-treated group suggests that *S. aromaticum* extract predominantly induces apoptosis without causing extensive secondary necrotic effects, making it a promising agent with minimal off-target cytotoxicity.

The differential effects of *S. aromaticum* and *E. scaber*-treated SiHa cells on early apoptosis, late apoptosis, and necrosis as compared to untreated cells presented their unique mechanisms of inducing cell death in cervical cancer cells. The significant reduction in live cells and induction of apoptosis, particularly late apoptosis, aligns with their ability to modulate polyamine metabolism and disrupt cell cycle progression, as demonstrated in earlier objectives. These findings further confirms the potential of *S. aromaticum* and *E. scaber* as chemopreventive agents capable of selectively inducing apoptosis while sparing normal cells.

The activation of caspases, including caspase 3, 8, and 9, is a critical hallmark of apoptosis. Building on the findings from the Annexin V analysis, which revealed significant induction of apoptosis in SiHa cells following treatment with *S. aromaticum* and *E. scaber* extracts, the caspase assays provide further insights into the underlying mechanisms of cell death. The results demonstrate that both extracts effectively induce apoptosis through distinct pathways, with notable differences in their effects on caspase activity.

Caspase 3, a key executioner of apoptosis, plays a central role in dismantling cellular components during the final stages of programmed cell death. In this study, *S. aromaticum*, *E. scaber*, and etoposide significantly elevated caspase 3 activity in SiHa cells compared to untreated controls. The findings confirm their ability to induce apoptosis effectively. While both extracts showed similar levels of activation, *E. scaber* exhibited a slightly stronger effect, suggesting it may have a more pronounced pro-apoptotic activity. This observation aligns with previous research where the apoptosis-inducing properties of terpenoids and flavonoids, which are abundant in *E. scaber* and known to activate caspase-dependent pathways in human colorectal carcinoma cells (Chan *et al.*, 2015). Similarly, eugenol, the primary bioactive compound in *S. aromaticum*, has been shown to enhance caspase 3 activity, inducing apoptosis in various cancer cell lines, including cervical and colon cancer cells (Padhy *et al.*, 2022).

Caspase 8, which is associated with the extrinsic apoptotic pathway triggered by death receptors like Fas and TRAIL, showed a unique activation pattern. Treatment with *E. scaber* significantly increased caspase 8 activity at multiple time points, while *S. aromaticum* and etoposide did not show a comparable effect. The distinctive ability of *E. scaber* to activate the extrinsic pathway, likely through its terpenoid content, such as molephantinin, which has been reported to enhance death receptor signalling and promote caspase 8 activation (Leenutaphong *et al.*, 2022; Tam *et al.*, 2021). The selective activation of this pathway emphasizes the potential of *E. scaber* as a targeted chemopreventive agent capable of modulating extrinsic apoptotic mechanisms.

In contrast, the intrinsic apoptotic pathway, which is mediated through mitochondrial signalling and depends on caspase 9 activation, showed robust responses to *S. aromaticum* and etoposide treatments. Both induced significant and sustained activation of caspase 9 starting at 48 hours, suggesting a shared mechanism involving mitochondrial disruption. This process likely involves the release of cytochrome c and subsequent formation of the apoptosome complex, triggering caspase 9 activation. Previous findings also presented the same caspase analysis response on breast cancer cells (MCF7, T47-D and MDA-MB-231 cell lines), gingival squamous carcinoma, and HeLa cells treated with eugenol (Al-Sharif *et al.*, 2013; Fathy *et al.*, 2019; Issa *et al.*, 2024). *S. aromaticum*'s polyphenols and flavonoids are known to influence

mitochondrial membrane potential, facilitating cytochrome c release and enhancing caspase 9 activity (Al Wafai *et al.*, 2017; Liang *et al.*, 2015). These findings support the role of *S. aromaticum* as a potent inducer of intrinsic apoptosis, further validating its potential as a chemopreventive agent.

Together, these findings provide strong evidence for the chemopreventive potential of *S. aromaticum* and *E. scaber* extracts. The distinct activation of caspase 8 by *E. scaber* and caspase 9 by *S. aromaticum* supports their complementary mechanisms of action, which could be leveraged in combination therapies to enhance pro-apoptotic effects. Furthermore, the low cytotoxicity of both extracts on normal cells, as shown in earlier parts of this study, reinforces their safety and viability as natural therapeutic agents for cervical cancer. These results contribute to the growing evidence that plant-based extracts can offer effective and selective mechanisms to combat cancer progression.

5.6 THE RELATIONSHIP OF POLYAMINES REGULATION WITH CELL DEATH MECHANISM IN SIHA CELL WITH TREATMENT OF *S. AROMATICUM* AND *E. SCABER*

In this study, the aqueous extracts of *S. aromaticum* and *E. scaber* significantly downregulated *ODC* expression while upregulating *OAZ1, SSAT*, and *SMOX* genes, suggesting a strong inhibitory effect on intracellular polyamine synthesis and an increase in catabolism. This disruption leads to reduced polyamine pools within the cervical cancer cells, sensitising them to programmed cell death mechanisms. Polyamine depletion has been shown to increase cellular susceptibility to apoptosis by destabilising DNA, impairing mitochondrial membrane potential, and enhancing oxidative stress. Specifically, the upregulation of *SMOX* promotes the generation of reactive oxygen species (ROS), contributing to mitochondrial dysfunction and cytochrome c release—key initiators of the intrinsic apoptotic pathway (Chan *et al.* 2015). Consistent with this, *S. aromaticum* extract demonstrated significant activation of caspase 9, while *E. scaber* predominantly activated caspase 8, implicating both

intrinsic and extrinsic apoptotic mechanisms. Caspase 3 activity was increased in both treatments, confirming the execution phase of apoptosis.

This response aligns with the concept of ‘polyamine addiction’, where cancer cells exhibit a heightened dependency on elevated polyamine levels for their growth and survival (Long *et al.* 2024). Consequently, interventions that deplete polyamines or disrupt their homeostasis trigger metabolic stress and apoptosis selectively in cancer cells, sparing normal cells that maintain tightly regulated polyamine balance (Arisan *et al.* 2012; Ade *et al.* 2025). Importantly, these findings show the role of polyamine modulation not only as a downstream effect but as a strategic molecular target in cancer chemoprevention. The observed alterations in gene expression and caspase activity suggest that the aqueous extracts of *S. aromaticum* and *E. scaber* disrupt polyamine homeostasis in a manner that promotes cell death and suppresses tumour growth. Thus, by exploiting this metabolic vulnerability, these herbs demonstrate a dual function—impairing cell proliferation while simultaneously inducing apoptotic pathways—thereby validating their potential as safe and effective natural chemopreventive agents in the early intervention and prevention of cervical cancer progression.

CHAPTER SIX :

CONCLUSION

6.1 LIMITATIONS

While this study successfully demonstrated the chemopreventive potential of *S. aromaticum* and *E. scaber* aqueous extracts against cervical cancer cells (SiHa), several limitations must be acknowledged to provide context for the findings and guide future research.

This study was conducted exclusively *in vitro* using SiHa cells as a cancer model and HEK 293T cells as a normal cell line. Although these models are widely used, they do not fully replicate the complexity of the tumour microenvironment or systemic interactions within a living organism. The lack of *in vivo* validation limits the ability to assess pharmacokinetics, bioavailability, and potential off-target effects of these herbal extracts. Future studies involving *in vivo* models are necessary to establish their efficacy and safety in a biological system.

Additionally, while the phytochemical profiling of *S. aromaticum* and *E. scaber* extracts successfully identified key bioactive compounds, this study did not isolate or quantify the individual contributions of these compounds to the observed anticancer effects. Given the complex composition of *E. scaber*, which contains diverse bioactive compounds such as flavonoids, terpenoids, and polyphenols, attributing specific therapeutic effects to individual compounds remains a challenge. Further fractionation and bioactivity-guided studies are necessary to determine the precise roles of these compounds.

This study was also unable to assess polyamine uptake activity, which represents an important component of intracellular polyamine homeostasis. Polyamine uptake refers to the process by which cells actively transport polyamines from the extracellular environment to replenish or maintain intracellular polyamine levels, particularly when

biosynthesis is inhibited (Corral & Wallace, 2020). In many cancer cells, including cervical cancer, the polyamine transport system is upregulated to support rapid cell growth and survival (W. Zhao *et al.*, 2023). Evaluating this uptake mechanism would have further validated the effects of the extracts on total polyamine regulation. However, such assays typically require the use of radiolabelled polyamines (e.g., [^{3}H]-spermidine or [^{14}C]-putrescine) to accurately trace and quantify polyamine influx (Schultz *et al.*, 2025). Unfortunately, due to the lack of access to radiolabelled handling facilities and designated radiation laboratories within our research setting in Malaysia, this aspect of the study could not be conducted. This highlights a technical limitation that restricted the comprehensiveness of polyamine pathway analysis in this research.

Despite these limitations, the findings of this study provide a strong foundation for future research. Expanding the scope to include *in vivo* models, detailed compound isolation, broader mechanistic studies, and optimized combination therapies will further elucidate the chemopreventive potential of *S. aromaticum* and *E. scaber* and support their development as natural therapeutic agents.

6.2 FUTURE WORK

Building upon the findings of this study, future research should aim to address its limitations and expand the scope of investigation into the chemopreventive potential of *S. aromaticum* and *E. scaber* extracts. A crucial next step is to elucidate the precise molecular mechanisms by which these extracts modulate polyamine pathways and influence cervical cancer progression. Investigating the specific roles of key bioactive compounds, such as eugenol and terpenoids, will help determine their molecular targets and optimize their efficacy in cancer prevention and therapy. Additionally, further studies should explore the synergistic effects of combining these herbal extracts with conventional chemotherapeutic agents such as etoposide or polyamine inhibitors like DFMO (difluoromethylornithine). Understanding whether these combinations enhance therapeutic outcomes or reduce the required doses of chemotherapeutic drugs could provide valuable insights into safer and more effective cancer treatments.

The study should also be expanded to *in vivo* models to validate the efficacy, bioavailability, and safety of these herbal extracts in a biologically relevant system. Investigating their effects on tumour progression, metastasis, and immune modulation in animal models will provide a clearer understanding of their clinical applicability. Broader cancer cell line studies, including HPV-negative cervical cancer lines and other cancer subtypes, may also help generalize the findings and identify cancers that are most responsive to these treatments. Furthermore, beyond polyamine metabolism, the role of these extracts in other cellular processes, such as oxidative stress, autophagy, and DNA damage response, should be explored.

This could provide a more comprehensive understanding of the mechanisms involved in their anticancer effects. Advanced molecular techniques, including transcriptomics and proteomics, would allow for a more detailed analysis of the pathways influenced by these herbal extracts. Expanding combination therapy experiments to include other polyamine inhibitors or chemotherapeutic agents may also reveal more robust synergistic effects. Finally, to facilitate clinical translation, future studies should focus on the development of standardized formulations and dosing regimens for *S. aromaticum* and *E. scaber* extracts. Investigating their

pharmacokinetics, pharmacodynamics, and potential toxicity in clinical trials will be critical in determining their feasibility as part of integrative cancer therapies.

6.3 CONCLUSION

This study investigated the chemopreventive potential of selected Malaysian herbs, focusing primarily on *S. aromaticum* and *E. scaber*, in targeting polyamine pathways in human cervical cancer (SiHa) cells. The conclusions are summarised based on the specific research objectives:

1. Objective 1: To determine the phytochemical compounds present in aqueous extracts of selected Malaysian herbs.
   Phytochemical profiling using GC-MS and LC-MS revealed that *S. aromaticum* and *E. scaber* contain diverse bioactive compounds, including flavonoids, phenolic acids, terpenoids, and polyamine-modulating agents. These compounds are associated with antiproliferative and apoptotic activities.

2. Objective 2: To investigate the antiproliferative effects of selected Malaysian herbs' aqueous extracts on human cervical cancer cell line.
   MTT and TBE assays confirmed that *S. aromaticum* and *E. scaber* exert significant cytotoxic effects on SiHa cells, with IC_{50} values of 177.6 µg/mL and 88.8 µg/mL respectively after 48 hours. Both extracts significantly inhibited cell growth and reduced total intracellular protein levels ($p < 0.05$).

3. Objective 3: To examine the effects of *S. aromaticum* and *E. scaber* aqueous extracts on polyamine activities in human cervical cancer cell line.
   Quantitative assessment of endogenous polyamine levels indicated a significant reduction following treatment with both herbal extracts. *E. scaber* (0.192 nmol/mg) and *S. aromaticum* (0.585 nmol/mg) showed the lowest residual polyamine content, supporting their role in disrupting polyamine biosynthesis.

4. Objective 4: To identify the effects of *S. aromaticum* and *E. scaber* aqueous extracts on the cell cycle profile of human cervical cancer cell line.
   Cell cycle analysis demonstrated that both extracts induced G_0/G_1 arrest and G_2/M phase accumulation, which are critical events in halting uncontrolled cell proliferation.

5. Objective 5: To define the mechanism of cell death induced by *S. aromaticum* and *E. scaber* aqueous extracts on human cervical cancer cell line.
   Annexin V-FITC assays revealed an increase in late apoptotic cells following treatment.
   Caspase activity analysis indicated elevated caspase-3 activity for both extracts, while *E. scaber* selectively increased caspase-8 and *S. aromaticum* increased caspase-9 levels, suggesting the involvement of both intrinsic and extrinsic apoptosis pathways.

This study contributes valuable insight into natural product-based chemoprevention by demonstrating that *S. aromaticum* and *E. scaber* effectively modulate polyamine biosynthesis and induce apoptosis in cervical cancer cells. A key strength of this study lies in its focus on polyamine metabolism—an underexplored but promising therapeutic target in cancer treatment. The novel observation of gene modulation (*ODC* downregulation and upregulation of *OAZ1, SSAT*, and *SMOX*) adds mechanistic clarity to how these herbs exert their antiproliferative effects. Although the combinatorial effects with DFMO were preliminary, they provide early evidence of potential synergy that warrants further investigation. In conclusion, this research lays the groundwork for the development of safe, effective, and affordable herbal-based chemopreventive strategies targeting the polyamine pathway. Future studies incorporating in vivo models, pharmacokinetic assessments, and clinical validation are recommended to strengthen the translational potential of these findings in cervical cancer prevention and therapy.

REFERENCES

Abadi, A. V. M., Karimi, E., Oskoueian, E., Mohammad, G. R. K. S., & Shafaei, N. (2022). Chemical investigation and screening of anti-cancer potential of *Syzygium aromaticum* L. bud (*S. aromaticum*) essential oil nanoemulsion. *3 Biotech*, *12*(2), 49. https://doi.org/10.1007/s13205-022-03117-2

Ade, A., Isma, Z. S., Steven, G., Ajeng, M. F., Lince, D. N., Norma, N. A., Hiroki, T., & Shun, H. (2025). Phytochemistry, Antioxidant and Cytotoxic Activities of Hibiscus (Hibiscus Rosa-Sinensis) Extract on MCF-7 Breast Cancer Cells. *Indonesian Journal of Medical Chemistry and Bioinformatics*, 3(2). https://doi.org/10.7454/ijmcb.v3i2.1038

Afshar, A. S., Nematpour, F. S., Meshkani, M., & Khafi, A. (2017). Growth inhibition of human breast cancer cells and down-regulation of ODC1 and ADA genes by nepeta binaloudensis. *Revista Brasileira de Farmacognosia*, *27*(1), 84–90. https://doi.org/10.1016/j.bjp.2016.07.005

Agrawal, K. K., Murti, Y., Agrawal, N., & Gupta, T. (2021). In silico studies of bioactive compounds from hibiscus rosa-sinensis against her2 and esr1 for breast cancer treatment. *International Journal of Pharmaceutical Sciences and Nanotechnology (IJPSN)*, 14(6), 5665-5671.

Ahda, M., Jaswir, I., Khatib, A., Ahmed, Q. U., & Syed Mohamad, S. N. A. (2023). A review on *Cosmos caudatus* as A potential medicinal plant based on pharmacognosy, phytochemistry, and pharmacological activities. *International Journal of Food Properties*, 26(1), 344–358. https://doi.org/10.1080/10942912.2022.2158862

Ahmed, A. A., & Shahidan, W. Nazatul. S. (2021). Cosmos Caudatus: A Possible Drug Candidate for Oral Squamous Cell Carcinoma. *Current Bioactive Compounds*, 17(6). https://doi.org/10.2174/1573407216999200911120311

Akinyele, O., & Wallace, H. M. (2022). Understanding the Polyamine and mTOR Pathway Interaction in Breast Cancer Cell Growth. *Medical Sciences (Basel, Switzerland)*, *10*(3). https://doi.org/10.3390/medsci10030051

Al Wafai, R., El-Rabih, W., Katerji, M., Safi, R., El Sabban, M., El-Rifai, O., & Usta, J. (2017). Chemosensitivity of MCF-7 cells to eugenol: release of cytochrome-c and lactate dehydrogenase. *Scientific Reports*, *7*(1), 43730. https://doi.org/10.1038/srep43730

Aldabaan, N. A., Turakani, B., Mahnashi, M. H., Shaikh, I. A., Alhazmi, A. Y., Almasoudi, H. H., Abdulaziz, O., Khuwaja, G., Khan, A. A., Basavegowda, N., Dafalla, S. E., Muddapur, U. M., & Iqubal, S. M. S. (2024). Evaluation of antimicrobial, anticancer, antidiabetic, antioxidant activities and silver nanoparticles synthesized from Indian Clove- *Syzygium aromaticum* leaf extract. *Journal of King Saud University - Science*, *36*(4), 103142. https://doi.org/10.1016/j.jksus.2024.103142

Alexiou, G. A., Vartholomatos, E., Tsamis, K. I., Peponi, E., Markopoulos, G., Papathanasopoulou, V. A., Tasiou, I., Ragos, V., Tsekeris, P., Kyritsis, A. P., & Galani, V. (2019). Combination treatment for glioblastoma with temozolomide, dfmo and radiation. *Journal of B.U.ON.*, *24*(1), 397–404. https://www.scopus.com/inward/record.uri?eid=2-s2.0-85062388905&partnerID=40&md5=74cc51fc60b3bacc5c30afe28e0ec6e5

Alhosin, M., Razvi, S. S. I., Sheikh, R. A., Khan, J. A., Zamzami, M. A., & Choudhry, H. (2020). Thymoquinone and Difluoromethylornithine (DFMO) Synergistically Induce Apoptosis of Human Acute T Lymphoblastic Leukemia Jurkat Cells Through the Modulation of Epigenetic Pathways. *Technology in Cancer Research and Treatment*, *19*. https://doi.org/10.1177/1533033820947489

Ali, A. B., Razali, N. H., Xian, N. S., & Sung, C. Y. (2021). The Use of Herbal Therapy to Improve the Quality of Life among Cancer Patients in the Southern Region of Peninsular Malaysia. *Asian Pacific Journal of Cancer Prevention*, *22*(6). https://doi.org/10.31557/APJCP.2021.22.6.1857

Al-Sharif, I., Remmal, A., & Aboussekhra, A. (2013). Eugenol triggers apoptosis in breast cancer cells through E2F1/survivin down-regulation. *BMC Cancer*, *13*(1), 600. https://doi.org/10.1186/1471-2407-13-600

Alshamrani, S. M., Safhi, F. A., Mobasher, M. A., Saleem, R. M., Alharthi, A., Alshaya, D. S., & Awad, N. S. (2022). Antiproliferative effect of Clitoria ternatea ethanolic extract against colorectal, breast, and medullary thyroid cancer cell lines. *Separations*, 9(11), 331.

Amuthan, A., Devi, V., Shreedhara, C. S., Rao, V., & Lobo, R. (2019). Cytoprotective Activity of Neichitti (Vernonia cinerea) in Human Embryonic Kidney (HEK293) Normal Cells and Human Cervix Epitheloid Carcinoma (HeLa) Cells against Cisplatin Induced Toxicity: A Comparative Study. *Journal of Clinical and Diagnostic Research*. https://doi.org/10.7860/JCDR/2019/40242.12624

Arechkik, A., Lahlou, L., Obtel, M., Kharbach, A., & Razine, R. (2022). Cervical cancer in Morocco: A systematic review. In *Revue d'Epidemiologie et de Sante Publique* (Vol. 70, Issue 5). https://doi.org/10.1016/j.respe.2022.05.008

Ariffin, S. H., A Wahab, I., Hassan, Y., & Abd Wahab, M. S. (2021). Adulterated Traditional-Herbal Medicinal Products and Its Safety Signals in Malaysia. *Drug, Healthcare and Patient Safety*, Volume 13, 133–140. https://doi.org/10.2147/DHPS.S305953

Arisan, E. D., Obakan, P., Coker-Gürkan, A., Calcabrini, A., Agostinelli, E., & Unsal, N. P. (2014). CDK inhibitors induce mitochondria-mediated apoptosis through the activation of polyamine catabolic pathway in LNCaP, DU145 and PC3 prostate cancer cells. *Current Pharmaceutical Design*, *20*(2), 180–188. https://doi.org/10.2174/13816128113199990029

Atiya, M. A., Poortvliet, E., Strömberg, R., & Yngve, A. (2011). Polyamines in foods: development of a food database. *Food & Nutrition Research*, *55*(1), 5572. https://doi.org/10.3402/fnr.v55i0.5572

Babakanrad, E., Mohammadian, T., Esmaeili, D., & Behzadi, P. (2023). Cervical Cancer: A Review of Epidemiology, Treatments and Anticancer Drugs. *Current*

Cancer Therapy Reviews, *19*(3). https://doi.org/10.2174/1573394719666230207101655

Babbar, N., & Gerner, E. W. (2011). Targeting polyamines and inflammation for cancer prevention. *Recent Results in Cancer Research*, *188*, 49–64. https://doi.org/10.1007/978-3-642-10858-7_4

Bai, M., Chen, J.-J., Xu, W., Dong, S.-H., Liu, Q.-B., Lin, B., Huang, X.-X., Yao, G.-D., & Song, S.-J. (2020). Elephantopinolide A-P, germacrane-type sesquiterpene lactones from *Elephantopus scaber* induce apoptosis, autophagy and G2/M phase arrest in hepatocellular carcinoma cells. *European Journal of Medicinal Chemistry*, *198*, 112362. https://doi.org/10.1016/j.ejmech.2020.112362

Baihaqi, A. al, & Levita, J. (2021). Efficacy and Safety of Herbal Medicines in Asia. Jurnal Ilmiah Farmako Bahari, 12(2), 169–182. https://doi.org/10.52434/jfb.v12i2.1244

Basukala, O., Sarabia-Vega, V., & Banks, L. (2020). Human papillomavirus oncoproteins and post-translational modifications: generating multifunctional hubs for overriding cellular homeostasis. *Biological Chemistry*, *401*(5), 585–599. https://doi.org/10.1515/hsz-2019-0408

Bezerra, D., Militão, G., De Morais, M., & De Sousa, D. (2017). The Dual Antioxidant/Prooxidant Effect of Eugenol and Its Action in Cancer Development and Treatment. *Nutrients*, *9*(12), 1367. https://doi.org/10.3390/nu9121367

Bhoora, S., Pillay, T. S., & Punchoo, R. (2022). Cholecalciferol induces apoptosis via autocrine metabolism in epidermoid cervical cancer cells. *Biochemistry and Cell Biology*, *100*(5). https://doi.org/10.1139/bcb-2022-0049

Brel, V., Annereau, J.-P., Vispé, S., Kruczynski, A., Bailly, C., & Guilbaud, N. (2011). Cytotoxicity and cell death mechanisms induced by the polyamine-vectorized anti-cancer drug F14512 targeting topoisomerase II. *Biochemical Pharmacology*, *82*(12), 1843–1852. https://doi.org/10.1016/j.bcp.2011.08.028

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Cahyono, E., Handayani, T., Ab Azid, S. B. M., Alighiri, D., Zaky, A. M., Aisyah, S., & Permatasari, B. (2024). A Cytotoxic Activity of Clitoria ternatea Flower Tea with the Cinnamon and Lemongrass Oil Vapor against T47D Breast Cancer Cells. Biosaintifika: *Journal of Biology and Biology Educatio*n, 16(3).

Casero, R. A., Murray Stewart, T., & Pegg, A. E. (2018). Polyamine metabolism and cancer: treatments, challenges and opportunities. *Nature Reviews Cancer*, *18*(11), 681–695. https://doi.org/10.1038/s41568-018-0050-3

Çelebioğlu, N. H., Becit, M., Çağlayan, A., & Aydın Dilsiz, S. (2022). Effects of thymoquinone and etoposide combination on cell viability and genotoxicity in human cervical cancer hela cells. *İstanbul Journal of Pharmacy*, *52*(3), 258–264. https://doi.org/10.26650/IstanbulJPharm.2022.1105443

Chan, C. K., Supriady, H., Goh, B. H., & Kadir, H. A. (2015). *Elephantopus scaber* induces apoptosis through ROS-dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. *Journal of Ethnopharmacology*, *168*, 291–304. https://doi.org/10.1016/j.jep.2015.03.072

Chan, Y. M., Ismail, M. Z. H., & Khaw, W. F. (2023). Factors influencing the prevalence of cervical cancer screening in Malaysia: a nationwide survey. *BMC Women's Health*, *23*(1). https://doi.org/10.1186/s12905-023-02553-3

Chebaro, Z., Abdallah, R., Badran, A., Hamade, K., Hijazi, A., Maresca, M., Mesmar, J. E., & Baydoun, E. (2023). Study of the antioxidant and anti-pancreatic cancer activities of Anchusa strigosa aqueous extracts obtained by maceration and ultrasonic extraction techniques. *Frontiers in Pharmacology*, *14*. https://doi.org/10.3389/fphar.2023.1201969

Chen, S., Hu, Q., Tao, X., Xia, J., Wu, T., Cheng, B., & Wang, J. (2022). Retinoids in cancer chemoprevention and therapy: Meta-analysis of randomized controlled

trials. In *Frontiers in Genetics* (Vol. 13). https://doi.org/10.3389/fgene.2022.1065320

Chen, Y., Zhuang, H., Chen, X., Zheqi, S. H. I., & Wang, X. (2018). Spermidine-induced growth inhibition and apoptosis via autophagic activation in cervical cancer. *Oncology Reports*, *39*(6). https://doi.org/10.3892/or.2018.6377

Chia, T. Y., Gan, C.-Y., Gurjeet, K., Pike-See, C., Vikneswaran, M., Ashfaq, A., Bader, A., Sulaiman, M. A. A., Muhammad, H. S., Selvamani, N. N., Mohammed, H. A., & Edward, J. J. (2025). Acute and Subacute Oral Toxicity Assessment of The Polysaccharides Extracted from Clinacanthus nutans Leaves: A Preclinical Model for Drug Safety Screening. Tropical Life Sciences Research, 36(1), 245–276. https://doi.org/10.21315/tlsr2025.36.1.13

Chin, A., Bieberich, C. J., Stewart, T. M., & Casero, R. A. (2022). Polyamine Depletion Strategies in Cancer: Remodeling the Tumor Immune Microenvironment to Enhance Anti-Tumor Responses. *Medical Sciences (Basel, Switzerland)*, *10*(2). https://doi.org/10.3390/medsci10020031

Choi, S., Ismail, A., Pappas-Gogos, G., & Boussios, S. (2023). HPV and Cervical Cancer: A Review of Epidemiology and Screening Uptake in the UK. In *Pathogens* (Vol. 12, Issue 2). https://doi.org/10.3390/pathogens12020298

Coni, S., Bordone, R., Ivy, D. M., Yurtsever, Z. N., Di Magno, L., D'Amico, R., Cesaro, B., Fatica, A., Belardinilli, F., Bufalieri, F., Maroder, M., De Smaele, E., Di Marcotullio, L., Giannini, G., Agostinelli, E., & Canettieri, G. (2023). Combined inhibition of polyamine metabolism and eIF5A hypusination suppresses colorectal cancer growth through a converging effect on MYC translation. *Cancer Letters*, *559*. https://doi.org/10.1016/j.canlet.2023.216120

Coni, S., Serrao, S. M., Yurtsever, Z. N., Di Magno, L., Bordone, R., Bertani, C., Licursi, V., Ianniello, Z., Infante, P., Moretti, M., Petroni, M., Guerrieri, F., Fatica, A., Macone, A., De Smaele, E., Di Marcotullio, L., Giannini, G., Maroder, M., Agostinelli, E., & Canettieri, G. (2020). Blockade of EIF5A

hypusination limits colorectal cancer growth by inhibiting MYC elongation. *Cell Death and Disease*, *11*(12). https://doi.org/10.1038/s41419-020-03174-6

Corral, M., & Wallace, H. M. (2020). Upregulation of polyamine transport in human colorectal cancer cells. *Biomolecules*, *10*(4). https://doi.org/10.3390/biom10040499

Das, A., Harshadha, K., Dhinesh Kannan, S. K., Hari Raj, K., & Jayaprakash, B. (2018). Evaluation of therapeutic potential of Eugenol-A natural derivative of *Syzygium aromaticum* on cervical cancer. *Asian Pacific Journal of Cancer Prevention*, *19*(7). https://doi.org/10.22034/APJCP.2018.19.7.1977

Del Nonno, F., Pisani, G., Visca, P., Signore, F., Grillo, L. R., Baiocchini, A., Garbuglia, A. R., Sepe, S., Piacentini, M., & Falasca, L. (2011). Role and predictive strength of transglutaminase type 2 expression in premalignant lesions of the cervix. *Modern Pathology*, *24*(6), 855–865. https://doi.org/10.1038/modpathol.2011.40

Delgado, W. I., Contreras, R. C., Salazar, A. S., Pessoa, J., Mitre, A. I., García, C. V., Pérez, P. C., & Jacobo, H. N. J. (2023). A triple-drug combination induces apoptosis in cervical cancer-derived cell lines. *Frontiers in Oncology*, *13*. https://doi.org/10.3389/fonc.2023.1106667

Deepika, S., Sahu, J. K., Kumar, V., & Tamrakar, S. K. (2023). Phytochemicals and medicinal uses of Clitoria ternatea. *International Journal of Plant and Soil Science*, 35(18), 942-951.

Drețcanu, G., Iuhas, C. I., & Diaconeasa, Z. (2021). The involvement of natural polyphenols in the chemoprevention of cervical cancer. In *International Journal of Molecular Sciences* (Vol. 22, Issue 16). https://doi.org/10.3390/ijms22168812

El-Baba, C., Baassiri, A., Kiriako, G., Dia, B., Fadlallah, S., Moodad, S., & Darwiche, N. (2021). Terpenoids' anti-cancer effects: focus on autophagy. *Apoptosis*, *26*(9–10), 491–511. https://doi.org/10.1007/s10495-021-01684-y

Endringer, D. C., Pezzuto, J. M., & Braga, F. C. (2009). NF-κB inhibitory activity of cyclitols isolated from Hancornia speciosa. Phytomedicine, 16(11), 1064–1069. https://doi.org/10.1016/j.phymed.2009.03.022

Fathy, M., Fawzy, M. A., Hintzsche, H., Nikaido, T., Dandekar, T., & Othman, E. M. (2019). Eugenol Exerts Apoptotic Effect and Modulates the Sensitivity of HeLa Cells to Cisplatin and Radiation. *Molecules*, *24*(21), 3979. https://doi.org/10.3390/molecules24213979

Flaherty, K. T., Gray, R. J., Chen, A. P., Li, S., McShane, L. M., Patton, D., Hamilton, S. R., Williams, P. M., Iafrate, A. J., Sklar, J., Mitchell, E. P., Harris, L. N., Takebe, N., Sims, D. J., Coffey, B., Fu, T., Routbort, M., Zwiebel, J. A., Rubinstein, L. V., … Conley, B. A. (2020). Molecular Landscape and Actionable Alterations in a Genomically Guided Cancer Clinical Trial: National Cancer Institute Molecular Analysis for Therapy Choice (NCI-MATCH). *Journal of Clinical Oncology*, *38*(33), 3883–3894. https://doi.org/10.1200/JCO.19.03010

Galiana-Roselló, C., Aceves-Luquero, C., González, J., Martínez-Camarena, Á., Villalonga, R., De Mattos, S. F., Soriano, C., Llinares, J., García-España, E., Villalonga, P., & González-Rosende, M. E. (2020). Toward a Rational Design of Polyamine-Based Zinc-Chelating Agents for Cancer Therapies. *Journal of Medicinal Chemistry*, *63*(3), 1199–1215. https://doi.org/10.1021/acs.jmedchem.9b01554

Gamble, L. D., Purgato, S., Murray, J., Xiao, L., Yu, D. M. T., Hanssen, K. M., Giorgi, F. M., Carter, D. R., Gifford, A. J., Valli, E., Milazzo, G., Kamili, A., Mayoh, C., Liu, B., Eden, G., Sarraf, S., Allan, S., Giacomo, S. D., Flemming, C. L., … Haber, M. (2019). Inhibition of polyamine synthesis and uptake reduces tumor progression and prolongs survival in mouse models of neuroblastoma. *Science Translational Medicine*, *11*(477). https://doi.org/10.1126/scitranslmed.aau1099

Gao, L., Ge, C., Wang, S., Xu, X., Feng, Y., Li, X., Wang, C., Wang, Y., Dai, F., & Xie, S. (2020). The role of p53-mediated signaling in the therapeutic response of colorectal cancer to 9F, a spermine-modified naphthalene diimide derivative. *Cancers*, *12*(3). https://doi.org/10.3390/cancers12030528

Gao, Y., Nie, Z., Cao, H., Huang, D., Chen, M., Xiang, Y., Yu, X., & Zhang, S. (2022). Scabertopin Derived from *Elephantopus scaber* L. Mediates Necroptosis by Inducing Reactive Oxygen Species Production in Bladder Cancer *In vitro*. *Cancers*, *14*(23). https://doi.org/10.3390/cancers14235976

George, B. P., Chandran, R., & Abrahamse, H. (2021). Role of phytochemicals in cancer chemoprevention: Insights. In *Antioxidants* (Vol. 10, Issue 9). https://doi.org/10.3390/antiox10091455

George, T. J., Franke, A. J., Chakravarthy, A. B., Das, P., Dasari, A., El-Rayes, B. F., Hong, T. S., Kinsella, T. J., Landry, J. C., Lee, J. J., Monjazeb, A. M., Jacobs, S. A., Raben, D., Rahma, O. E., Williams, T. M., Wu, C., Coleman, C. N., Vikram, B., & Ahmed, M. M. (2019). National Cancer Institute (NCI) state of the science: Targeted radiosensitizers in colorectal cancer. *Cancer*, *125*(16), 2732–2746. https://doi.org/10.1002/cncr.32150

Ghani, R. A., Azalan, M. S., Nor Azahan Shah, A. M., Sujangi, N. H., Hassan, H., & Wallace, H. M. (2023a). Chemoprevention Effects Through Polyamines Modulation Induced by Clinacanthus nutans in Human Lung Adenocarcinoma Cells (A549). *Malaysian Journal of Medicine and Health Sciences*, *19*, 52–58. https://www.scopus.com/inward/record.uri?eid=2-s2.0-85180601874&partnerID=40&md5=16d4c6c58d0b6b7ac35431c3b878e51f

Ghani, R. A., Jamil, E. F., & Ibrahim, M. (2019). Polyamines Deficient Diet is a Complementary Strategy in Fighting Cancer: *Analysis on Selected Medicinal Fruits*, 8(3S2), 577-585.

Grkovic, T., Akee, R. K., Thornburg, C. C., Trinh, S. K., Britt, J. R., Harris, M. J., Evans, J. R., Kang, U., Ensel, S., Henrich, C. J., Gustafson, K. R., Schneider, J. P., & O'Keefe, B. R. (2020). National Cancer Institute (NCI) Program for Natural Products Discovery: Rapid Isolation and Identification of Biologically Active Natural Products from the NCI Prefractionated Library. *ACS Chemical Biology*, *15*(4), 1104–1114. https://doi.org/10.1021/acschembio.0c00139

Hamilton, A. C., & Coleman, H. G. (2023). Shifting tides: The Rising Tide of Early-onset Cancers Demands Attention. *BMJ Oncology*, *2*(1), e000106. https://doi.org/10.1136/bmjonc-2023-000106

Harbison, R. A., Pandey, R., Considine, M., Leone, R. D., Murray-Stewart, T., Erbe, R., Mandal, R., Burns, M., Casero, R. A., Seiwert, T., Fakhry, C., Pardoll, D., Fertig, E., & Powell, J. D. (2022). Interrogation of T Cell–enriched Tumors Reveals Prognostic and Immunotherapeutic Implications of Polyamine Metabolism. *Cancer Research Communications*, *2*(7), 639–652. https://doi.org/10.1158/2767-9764.CRC-22-0061

Harkenrider, M. M., Markham, M. J., Dizon, D. S., Jhingran, A., Salani, R., Serour, R. K., Lynn, J., & Kohn, E. C. (2020). Moving Forward in Cervical Cancer: Enhancing Susceptibility to DNA Repair Inhibition and Damage, an NCI Clinical Trials Planning Meeting Report. *JNCI: Journal of the National Cancer Institute*, *112*(11), 1081–1088. https://doi.org/10.1093/jnci/djaa041

He, W., Roh, E., Yao, K., Liu, K., Meng, X., Liu, F., Wang, P., Bode, A. M., & Dong, Z. (2017). Targeting ornithine decarboxylase (ODC) inhibits esophageal squamous cell carcinoma progression. *Npj Precision Oncology*, *1*(1), 13. https://doi.org/10.1038/s41698-017-0014-1

Hematulin, A., Meethang, S., Utapom, K., Wongkham, S., & Sagan, D. (2018). Etoposide radiosensitizes p53-defective cholangiocarcinoma cell lines independent of their G2 checkpoint efficacies. *Oncology Letters*. https://doi.org/10.3892/ol.2018.7754

Ho, W. Y., Yeap, S. K., Ho, C. L., Abdul Rahim, R., & Alitheen, N. B. (2012). Hepatoprotective Activity of Elephantopus scaber on Alcohol-Induced Liver Damage in Mice. Evidence-Based Complementary and Alternative Medicine, 2012, 1–8. https://doi.org/10.1155/2012/417953

Hyvönen, M. T., Smirnova, O. A., Mitkevich, V. A., Tunitskaya, V. L., Khomutov, M., Karpov, D. S., Korolev, S. P., Häkkinen, M. R., Pietilä, M., Gottikh, M. B., Vepsäläinen, J., Alhonen, L., Makarov, A. A., Kochetkov, S. N., Wallace, H. M., Keinänen, T. A., & Khomutov, A. R. (2022). Role of Polyamine-Induced

Dimerization of Antizyme in Its Cellular Functions. *International Journal of Molecular Sciences*, *23*(9), 4614. https://doi.org/10.3390/ijms23094614

Ibrahim, N., & Kebede, A. (2020). *In vitro* antibacterial activities of methanol and aqueous leave extracts of selected medicinal plants against human pathogenic bacteria. *Saudi Journal of Biological Sciences*, *27*(9), 2261–2268. https://doi.org/10.1016/j.sjbs.2020.06.047

Igarashi, K., Kawaguchi, K., Zhao, M., Han, Q., Tan, Y., Kiyuna, T., Miyake, K., Higuchi, T., Nelson, S. D., Dry, S. M., Li, Y., Yamamoto, N., Hayashi, K., Kimura, H., Miwa, S., Singh, S. R., Tsuchiya, H., & Hoffman, R. M. (2020). Recombinant methioninase combined with tumor-targeting salmonella typhimurium a1-r induced regression in a PDOX mouse model of doxorubicin-resistant dedifferentiated liposarcoma. *Anticancer Research*, *40*(5), 2515–2523. https://doi.org/10.21873/anticanres.14222

Imran, S., VarRuchi, S., Hardeep, S. T., Diwakar, A., Atul, S., Pritesh, V., Anil, K. S., & Anupam, B. (2020). Cancer Chemoprevention by Flavonoids, Dietary Polyphenols and Terpenoids. *Biointerface Research in Applied Chemistry*, *11*(1), 8502–8537. https://doi.org/10.33263/BRIAC111.85028537

Islam, A., Shaukat, Z., Hussain, R., & Gregory, S. L. (2022). One-Carbon and Polyamine Metabolism as Cancer Therapy Targets. In *Biomolecules* (Vol. 12, Issue 12). https://doi.org/10.3390/biom12121902

Islam, Md. R., Dhar, P. S., Akash, S., & Sharma, R. (2023). Public concerns about cervical cancer: outbreak, types, risk factors, etiology, stages, symptoms, prevention, and treatment. *Annals of Medicine & Surgery*, *85*(4). https://doi.org/10.1097/ms9.0000000000000521

Ismail, F., Zakaria, Y., Hassan, N. F. N., & Isa, M. L. M. (2022). Malaysian herbs as potential natural resources of anticancer drugs: From folklore to discovery. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, *30*(1). https://doi.org/10.35118/apjmbb.2022.030.1.06

Ismail, N., Rajaratinam, H., Che Ghazali, K., Mohd Salleh, S. F., Wan Ibrahim, W. S. F. A., Yahya, M. M., & Wan Zain, W. Z. (2023). The Phytochemical

Components of Kelantan Grown Annona Muricata Leaves and Its Anti-Proliferative Properties on Mcf-7 Breast Cancer Cells. *Sains Malaysiana*, *52*(10), 2785–2801. https://doi.org/10.17576/jsm-2023-5210-05

Issa, H., Loubaki, L., Al Amri, A., Zibara, K., Almutairi, M. H., Rouabhia, M., & Semlali, A. (2024). Eugenol as a potential adjuvant therapy for gingival squamous cell carcinoma. *Scientific Reports*, *14*(1), 10958. https://doi.org/10.1038/s41598-024-60754-8

Izbicka, E., Streeper, R. T., Yeh, I.-T., Pressley, O., Grant, M., Andrews, J. V. R., Kuhn, J., & O'Shaughnessy, J. (2010). Effects of α-difluoromethylornithine on markers of proliferation, invasion, and apoptosis in breast cancer. *Anticancer Research*, *30*(6), 2263–2269. https://www.scopus.com/inward/record.uri?eid=2-s2.0-77955075875&partnerID=40&md5=4cfe7e79629481f589925b9ecba11077

Jamehdor, S., Farivar, S., Sangtarash, M. H., Amini, R., Pajouhanfar, S., & Teimoori, A. (2021). The effects of quercetin on the gene expression of arginine metabolism key enzymes in human embryonic kidney 293 cells. *Jundishapur Journal of Natural Pharmaceutical Products*, *16*(2). https://doi.org/10.5812/JJNPP.101957

Jiang, R., Choi, W., Hu, L., Gerner, E. W., Hamilton, S. R., & Zhang, W. (2007). Activation of polyamine catabolism by N1, N11-diethylnorspermine alters the cellular localization of mTOR and downregulates mTOR protein level in glioblastoma cells. *Cancer Biology and Therapy*, *6*(10). https://doi.org/10.4161/cbt.6.10.4800

Kabeer, F. A., Rajalekshmi, D. S., Nair, M. S., & Prathapan, R. (2019). *In vitro* and *in vivo* antitumor activity of deoxyelephantopin from a potential medicinal plant *Elephantopus scaber* against Ehrlich ascites carcinoma. *Biocatalysis and Agricultural Biotechnology*, *19*, 101106. https://doi.org/10.1016/j.bcab.2019.101106

Kamarubahrin, A. F., Haris, A., Mohd Daud, S. N., Ahmad, N., Muhamed, N. A., & Shukor, S. A. (2020). Indigenous alternate fruit for dates: Critical review on the

selection criteria for local prophetic fruits in Malaysia. *Journal of Tropical Agriculture and Food Science*, 48(2).

Kanamori, Y., Finotti, A., Di Magno, L., Canettieri, G., Tahara, T., Timeus, F., Greco, A., Tirassa, P., Gasparello, J., Fino, P., Gambari, R., & Agostinelli, E. (2021). Enzymatic spermine metabolites induce apoptosis associated with increase of p53, caspase-3 and mir-34a in both neuroblastoma cells, SJNKP and the N-Myc-amplified form IMR5. *Cells*, *10*(8). https://doi.org/10.3390/cells10081950

Khan, M. F., Charbaji, A. M. Y., Baabbad, A. A., Almoutiri, N. D., & Wadaan, M. A. M. (2023). Investigating the role of Cinnamomum verum in zebrafish swim bladder development and anti-cancer activity in human lung cancer cell lines. *Arabian Journal of Chemistry*, *16*(12), 105361. https://doi.org/10.1016/j.arabjc.2023.105361

Kim, E. J. Y., Chen, Y., Huang, J. Q., Li, K. M., Razmovski-Naumovski, V., Poon, J., Chan, K., Roufogalis, B. D., McLachlan, A. J., Mo, S.-L., Yang, D., Yao, M., Liu, Z., Liu, J., & Li, G. Q. (2013). Evidence-based toxicity evaluation and scheduling of Chinese herbal medicines. *Journal of Ethnopharmacology*, 146(1), 40–61. https://doi.org/10.1016/j.jep.2012.12.027

Kim, D. J., Lee, M.-H., Liu, K., Lim, D. Y., Roh, E., Chen, H., Kim, S.-H., Shim, J.-H., Kim, M. O., Li, W., Ma, F., Fredimoses, M., Bode, A. M., & Dong, Z. (2017). Herbacetin suppresses cutaneous squamous cell carcinoma and melanoma cell growth by targeting AKT and ODC. *Carcinogenesis*, *38*(11), 1136–1146. https://doi.org/10.1093/carcin/bgx082

Koomoa, D.-L. T., Geerts, D., Lange, I., Koster, J., Pegg, A. E., Feith, D. J., & Bachmann, A. S. (2013). DFMO/eflornithine inhibits migration and invasion downstream of MYCN and involves p27Kip1 activity in neuroblastoma. *International Journal of Oncology*, *42*(4), 1219–1228. https://doi.org/10.3892/ijo.2013.1835

Kumar, G., Patlolla, J. M. R., Madka, V., Mohammed, A., Li, Q., Zhang, Y., Biddick, L., Singh, A., Gillaspy, A., Lightfoot, S., Steele, V. E., Kopelovich, L., & Rao, C. V. (2016). Simultaneous targeting of 5-LOX-COX and ODC block NNK-

induced lung adenoma progression to adenocarcinoma in A/J mice. *American Journal of Cancer Research*, *6*(5), 894–909. https://www.scopus.com/inward/record.uri?eid=2-s2.0-85006224612&partnerID=40&md5=589e9dc607d44d1f47391e7601101365

Kurnia, H. P., Puruhita, D. T., & Nazhif Haykal, M. (2021). Eugenol Nanoparticle Encapsulated Chitosan Enhances Cell Cycle Arrest in HeLa Human Cervical Cancer Cells. In *Systematic Reviews in Pharmacy* (Vol. 12, Issue 1).

Kuruburu, M. G., Bovilla, V. R., Leihang, Z., & Madhunapantula, S. V. (2022). Phytochemical-rich Fractions from Foxtail Millet (Setaria italica (L.) P. Beauv) Seeds Exhibited Antioxidant Activity and Reduced the Viability of Breast Cancer Cells *In vitro* by Inducing DNA Fragmentation and Promoting Cell Cycle Arrest. *Anti-Cancer Agents in Medicinal Chemistry*, *22*(13), 2477–2493. https://doi.org/10.2174/1871520622666220215122141

Lajoie, L., Fabiano-Tixier, A. S., & Chemat, F. (2022). Water as Green Solvent: Methods of Solubilisation and Extraction of Natural Products—Past, Present and Future Solutions. In *Pharmaceuticals* (Vol. 15, Issue 12). MDPI. https://doi.org/10.3390/ph15121507

Landis-Piwowar, K. R., & Iyer, N. R. (2014). Cancer Chemoprevention: Current State of the Art. *Cancer Growth and Metastasis*, *7*. https://doi.org/10.4137/cgm.s11288

Laukaitis, C. M., & Gerner, E. W. (2011). DFMO: Targeted risk reduction therapy for colorectal neoplasia. *Best Practice and Research: Clinical Gastroenterology*, *25*(4–5), 495–506. https://doi.org/10.1016/j.bpg.2011.09.007

Lazam, N. M., Shair, S. N., Asmuni, N. H., Jamaludin, A., & Yusri, A. A. (2023). Forecasting the incidence rates of top three cancers in Malaysia. *AIP Conference Proceedings*, *2500*. https://doi.org/10.1063/5.0110929

Lee, J. E., Chung, Y., Rhee, S., & Kim, T. H. (2022). Untold story of human cervical cancers: HPV-negative cervical cancer. *BMB Reports*, *55*(9). https://doi.org/10.5483/BMBRep.2022.55.9.042

Lee, Y. S., & Lee, T. H. (2000). Effect of Topotecan in Combication with Other Antitumor Drugs *in vitro*. *Korean Journal of Gynecologic Oncology and Colposcopy*, *11*(1), 83. https://doi.org/10.3802/kjgoc.2000.11.1.83

Leenutaphong, P., Tancharoen, S., Nararatwanchai, T., Phruksaniyom, C., Sarikaphuti, A., Palungwachira, P., & Chaichalotornkul, S. (2022). Induction of Human Oral Squamous Carcinoma Apoptosis by Derris scandens Benth and *Elephantopus scaber* Linn Extracts. *Natural Product Communications*, *17*(6). https://doi.org/10.1177/1934578X221107970

Leshcheva, M. Y., Astapenko, E. F., & Gabbasova, N. V. (2022). Topical aspects of etiology and prevention of cervical cancer. In *Opuholi Zenskoj Reproduktivnoj Sistemy* (Vol. 18, Issue 1). https://doi.org/10.17650/1994-4098-2022-18-1-97-102

Li, C., Xu, H., Chen, X., Chen, J., Li, X., Qiao, G., Tian, Y., Yuan, R., Su, S., Liu, X., & Lin, X. (2019). Aqueous extract of clove inhibits tumor growth by inducing autophagy through AMPK/ULK pathway. *Phytotherapy Research*, *33*(7), 1794–1804. https://doi.org/10.1002/ptr.6367

Li, J., Meng, Y., Wu, X., & Sun, Y. (2020). Polyamines and related signaling pathways in cancer. In *Cancer Cell International* (Vol. 20, Issue 1). https://doi.org/10.1186/s12935-020-01545-9

Li, Y., Xiao, S., Dan, L., & Xue, M. (2015). P16INK4A is required for cisplatin resistance in cervical carcinoma SiHa cells. *Oncology Letters*, *9*(3), 1104–1108. https://doi.org/10.3892/ol.2014.2814

Lian, J., Liang, Y., Zhang, H., Lan, M., Ye, Z., Lin, B., Qiu, X., & Zeng, J. (2022). The role of polyamine metabolism in remodeling immune responses and blocking therapy within the tumor immune microenvironment. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.912279

Liang, W.-Z., Chou, C.-T., Hsu, S.-S., Liao, W.-C., Shieh, P., Kuo, D.-H., Tseng, H.-W., Kuo, C.-C., & Jan, C.-R. (2015). The involvement of mitochondrial apoptotic pathway in eugenol-induced cell death in human glioblastoma cells.

Toxicology Letters, *232*(1), 122–132. https://doi.org/10.1016/j.toxlet.2014.10.023

Lin, J.-J., Yu, C.-C., Lu, K.-W., Chang, S.-J., Yu, F.-S., Liao, C.-L., Lin, J.-G., & Chung, J.-G. (2014). α-Phellandrene alters expression of genes associated with DNA damage, cell cycle, and apoptosis in murine leukemia WEHI-3 cells. *Anticancer Research*, *34*(8), 4161–4180.

Liu, Y.-C., Hsu, D.-H., Huang, C.-L., Liu, Y.-L., Liu, G.-Y., & Hung, H.-C. (2011). Determinants of the Differential Antizyme-Binding Affinity of Ornithine Decarboxylase. PLoS ONE, 6(11), e26835. https://doi.org/10.1371/journal.pone.0026835

Liu, H., Schmitz, J. C., Wei, J., Cao, S., Beumer, J. H., Strychor, S., Cheng, L., Liu, M., Wang, C., Wu, N., Zhao, X., Zhang, Y., Liao, J., Chu, E., & Lin, X. (2014). Clove Extract Inhibits Tumor Growth and Promotes Cell Cycle Arrest and Apoptosis. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics*, *21*(5), 247–259. https://doi.org/10.3727/096504014X13946388748910

Liu, R., Zhu, L.-L., Yu, C.-Y., Shuai, Y.-P., Sun, L.-L., Bi, K.-S., & Li, Q. (2021). Quantitative evaluation of the compatibility effects of aidi injection on the treatment of hepatocellular carcinoma using targeted metabolomics: A new strategy on the mechanism study of an anticancer compound in traditional chinese medicine. *World Journal of Traditional Chinese Medicine*, *7*(1), 111–119. https://doi.org/10.4103/wjtcm.wjtcm_86_20

Liu, Y., Liu, H., Sheng, B., Pan, S., Wang, Z. wei, & Zhu, X. (2022). The functions of lncRNAs in the HPV-negative cervical cancer compared with HPV-positive cervical cancer. In *Apoptosis* (Vol. 27, Issues 9–10). https://doi.org/10.1007/s10495-022-01761-w

Liu, Y.-H., Tsai, K.-D., Yang, S.-M., Wong, H.-Y., Chen, T.-W., Cherng, J., & Cherng, J.-M. (2017). Cinnamomum verum ingredient 2-methoxycinnamaldehyde: a new antiproliferative drug targeting topoisomerase I and II in human lung

squamous cell carcinoma NCI-H520 cells. *European Journal of Cancer Prevention*, *26*(4), 314–323. https://doi.org/10.1097/CEJ.0000000000000265

Long, Q., Zhou, W., Zhou, H., Tang, Y., Chen, W., Liu, Q., & Bian, X. (2024). Polyamine-containing natural products: structure, bioactivity, and biosynthesis. *Natural Product Reports*, *41*(4), 525–564. https://doi.org/10.1039/D2NP00087C

Lu, B., Wang, L., Ran, X., Tang, H., & Cao, D. (2022). Recent Advances in Fluorescent Methods for Polyamine Detection and the Polyamine Suppressing Strategy in Tumor Treatment. In *Biosensors* (Vol. 12, Issue 8). https://doi.org/10.3390/bios12080633

Madka, V., Patlolla, J. M. R., Venkatachalam, K., Zhang, Y., Pathuri, G., Stratton, N., Lightfoot, S., Janakiram, N. B., Mohammed, A., & Rao, C. V. (2023). Chemoprevention of Colon Cancer by DFMO, Sulindac, and NO-Sulindac Administered Individually or in Combinations in F344 Rats. *Cancers*, *15*(15). https://doi.org/10.3390/cancers15154001

Marina, S. (2021). Utilization of *Elephantopus scaber* as traditional medicine and its bioactivity. *GSC Biological and Pharmaceutical Sciences*, *15*(1), 112–118. https://doi.org/10.30574/gscbps.2021.15.1.0106

Marverti, G., Ligabue, A., Lombardi, P., Ferrari, S., Monti, M. G., Frassineti, C., & Costi, M. P. (2013). Modulation of the expression of folate cycle enzymes and polyamine metabolism by berberine in cisplatin-sensitive and-resistant human ovarian cancer cells. *International Journal of Oncology*, *43*(4), 1269–1280. https://doi.org/10.3892/ijo.2013.2045

Maryam, S. A., Radiah, A. G., & Wallace, H. M. (2023). The roles of polyamines in Malaysian herbs as a chemoprevention strategy: A scoping review. Asia Pacific Journal of Molecular Biology and Biotechnology, 82–95. https://doi.org/10.35118/apjmbb.2023.031.4.09

Mastura, M. S., Sharifah Azdiana, T. D., & Hasmah, H. (2019). Obstacles to Implementing the HPV Vaccine: Is it Worth Pursuing or Not? *Asian Pacific Journal of Cancer Care*, *4*(4). https://doi.org/10.31557/apjcc.2019.4.4.165-169

Mehmood, T., Maryam, A., Ghramh, H., Khan, M., & Ma, T. (2017). Deoxyelephantopin and Isodeoxyelephantopin as Potential Anticancer Agents with Effects on Multiple Signaling Pathways. *Molecules*, *22*(6), 1013. https://doi.org/10.3390/molecules22061013

Mekky, A. E., Emam, A. E., Selim, M. N., Abdelmouty, E. S., & Khedr, M. (2024). Antibacterial and antineoplastic MCF-7 and HePG-2 characteristics of the methanolic (80%) clove (*Syzygium aromaticum* L.) extract. *Biomass Conversion and Biorefinery*, *14*(15), 16787–16798. https://doi.org/10.1007/s13399-023-03862-1

Miller-Fleming, L., Olin-Sandoval, V., Campbell, K., & Ralser, M. (2015). Remaining Mysteries of Molecular Biology: The Role of Polyamines in the Cell. *Journal of Molecular Biology*, *427*(21), 3389–3406. https://doi.org/10.1016/j.jmb.2015.06.020

Mishra, R., & Mishra, S. (2020). Updates in bile acid-bioactive molecule conjugates and their applications. *Steroids*, *159*, 108639. https://doi.org/10.1016/j.steroids.2020.108639

Mohan, G. S., Swetha, M., Keerthana, C. K., Rayginia, T. P., & Anto, R. J. (2022). Cancer Chemoprevention: A Strategic Approach Using Phytochemicals. In *Frontiers in Pharmacology* (Vol. 12). https://doi.org/10.3389/fphar.2021.809308

Mohd Afeendi, M. S., Intan, A. S., Oman, F., Harun, M. P., Faridah, A. R., Sarifah, A., Siti Aidah, R., & Widowati, W. (2017). *Malay medical manuscripts: heritage from The garden of healing*. The Islamic Manuscript Association LTD.

Morosetti, G., Criscuolo, A. A., Santi. F., Perno, C. F., Piccione, E., & Ciotti, M. (2017). Ellagic acid and Annona muricata in the chemoprevention of HPV-related pre-neoplastic lesions of the cervix. *Oncology Letters*, *13*(3).

Mostafa, A. A.-F., Yassin, M. T., Al–Askar, A. A., & Al-Otibi, F. O. (2023). Phytochemical analysis, antiproliferative and antifungal activities of different *Syzygium aromaticum* solvent extracts. *Journal of King Saud University - Science*, *35*(1), 102362. https://doi.org/10.1016/j.jksus.2022.102362

Mude, H., Balapure, A., Thakur, A., Ganesan, R., & Ray Dutta, J. (2022). Enhanced antibacterial, antioxidant and anticancer activity of caffeic acid by simple acid-base complexation with spermine/spermidine. *Natural Product Research*, *36*(24), 6453–6458. https://doi.org/10.1080/14786419.2022.2038597

Muhamad, N. A., Kamaluddin, M. A., Adon, M. Y., Noh, M. A., Bakhtiar, M. F., Ibrahim Tamim, N. S., Mahmud, S. H., & Aris, T. (2015). Survival rates of cervical cancer patients in Malaysia. *Asian Pacific Journal of Cancer Prevention*, *16*(7). https://doi.org/10.7314/APJCP.2015.16.7.3067

Muñoz-Esparza, N. C., Costa-Catala, J., Comas-Basté, O., Toro-Funes, N., Latorre-Moratalla, M. L., Veciana-Nogués, M. T., & Vidal-Carou, M. C. (2021). Occurrence of Polyamines in Foods and the Influence of Cooking Processes. *Foods*, *10*(8), 1752. https://doi.org/10.3390/foods10081752

Murali, A., Kumar, S., Akshaya, S., & Singh, S. K. (2023). Drug repurposing toward the inhibition of RNA-dependent RNA polymerase of various flaviviruses through computational study. *Journal of Cellular Biochemistry*, *124*(1), 127–145. https://doi.org/10.1002/jcb.30352

Murata, M., Komatsu, S., Miyamoto, E., Oka, C., Lin, I., Kumazoe, M., Yamashita, S., Fujimura, Y., & Tachibana, H. (2023). Quercetin up-regulates the expression of tumor-suppressive microRNAs in human cervical cancer. *Bioscience of Microbiota, Food and Health*, *42*(1). https://doi.org/10.12938/bmfh.2022-056

Mustafa, W. A., Halim, A., Nasrudin, M. W., & Rahman, K. S. A. (2022). Cervical cancer situation in Malaysia: A systematic literature review. In *Biocell* (Vol. 46, Issue 2). https://doi.org/10.32604/biocell.2022.016814

Nahrawi, N., Mustafa, W. A., Nurul, S., & Kanafiah, A. M. (2020). Knowledge of human papillomavirus (HPV) and cervical cancer among Malaysia residents: A review. *Sains Malaysiana*, *49*(7). https://doi.org/10.17576/jsm-2020-4907-19

Neborak, E. V, Kaldybayeva, A. B., Bey, L., Malmakova, A. Y., Tveritinova, A. S., Hilal, A., Yu, V. K., Ploskonos, M. V, Komarova, M. V, Agostinelli, E., & Zhdanov, D. D. (2022). Anticancer Cytotoxic Activity of Bispidine Derivatives

Associated with the Increasing Catabolism of Polyamines. *Molecules*, *27*(12). https://doi.org/10.3390/molecules27123872

Neda, G. D., Rabeta, M. S., & Ong, M. T. (2013). Chemical composition and anti-proliferative properties of flowers of Clitoria Ternatea. *International Food Research Journal*, 20(3).

Nelson, C. W., & Mirabello, L. (2023). Human papillomavirus genomics: Understanding carcinogenicity. *Tumour Virus Research*, *15*, 200258. https://doi.org/10.1016/j.tvr.2023.200258

Novita, S. I., Setiawan, T., Seock, K. K., Toni, W. Y., Won, C. K., & Young, K. H. (2021). Metabolism and function of polyamines in cancer progression. *Cancer Letters*, *519*. https://doi.org/10.1016/j.canlet.2021.06.020

Nurkhasanah, Trisnamurti, K. C., Gunaryanti, R. D., & Widyastuti, T. (2015). The Screening Of Cytotoxic Fraction From *Elephantopus scaber* Linn against Human Cervical Cancer (Hela) Cells. *International Journal of Pharma Sciences and Research*, *6*(6).

Nwodo, J. N., Ibezim, A., Simoben, C. V., & Ntie-Kang, F. (2016). Exploring Cancer Therapeutics with Natural Products from African Medicinal Plants, Part II: Alkaloids, Terpenoids and Flavonoids. *Anti-Cancer Agents in Medicinal Chemistry*, *16*, 108–127.

Padhy, I., Paul, P., Sharma, T., Banerjee, S., & Mondal, A. (2022). Molecular Mechanisms of Action of Eugenol in Cancer: Recent Trends and Advancement. *Life*, *12*(11), 1795. https://doi.org/10.3390/life12111795

Pandey, P., Verma, M., Sanghvi, G., R., R., Joshi, K. K., V., K., Ray, S., Ramniwas, S., Singh, A., Lakhanpal, S., & Khan, F. (2025). Plant-derived terpenoids modulating cancer cell metabolism and cross-linked signaling pathways: an updated reviews. *Naunyn-Schmiedeberg's Archives of Pharmacology*. https://doi.org/10.1007/s00210-025-03937-y

Park, E., Kim, J.-Y., Choi, S., Kim, D. S., & Oh, Y. L. (2019). Carcinogenic risk of human papillomavirus (HPV) genotypes and potential effects of HPV vaccines

in Korea. *Scientific Reports*, *9*(1), 12556. https://doi.org/10.1038/s41598-019-49060-w

Parveen, S., Masood, S., Kumar, S., & Banerjee, M. (2023). Phytonanomedicine: A therapeutic approach for cervical cancer. In *OpenNano* (Vol. 13). https://doi.org/10.1016/j.onano.2023.100178

Pauli, C., Hopkins, B. D., Prandi, D., Shaw, R., Fedrizzi, T., Sboner, A., Sailer, V., Augello, M., Puca, L., Rosati, R., McNary, T. J., Churakova, Y., Cheung, C., Triscott, J., Pisapia, D., Rao, R., Mosquera, J. M., Robinson, B., Faltas, B. M., … Rubin, M. A. (2017). Personalized *In vitro* and *In vivo* Cancer Models to Guide Precision Medicine. *Cancer Discovery*, *7*(5), 462–477. https://doi.org/10.1158/2159-8290.CD-16-1154

Pegg, A. E. (2018). Introduction to the thematic minireview series: Sixty plus years of polyamine research. *Journal of Biological Chemistry*, *293*(48), 18681–18692. https://doi.org/10.1074/jbc.TM118.006291

Perng, D.-S., Tsai, Y.-H., Cherng, J., Kuo, C.-W., Shiao, C.-C., & Cherng, J.-M. (2016). Discovery of a novel anti-cancer agent targeting both topoisomerase I and II in hepatocellular carcinoma Hep 3B cells *in vitro* and *in vivo*: Cinnamomum verum component 2-methoxycinnamaldehyde. *Journal of Drug Targeting*, *24*(7), 624–634. https://doi.org/10.3109/1061186X.2015.1132221

Philips, C. A., Ahamed, R., Rajesh, S., George, T., Mohanan, M., & Augustine, P. (2020). Comprehensive review of hepatotoxicity associated with traditional Indian Ayurvedic herbs. World Journal of Hepatology, 12(9), 574–595. https://doi.org/10.4254/wjh.v12.i9.574

Puteh, S. E. W., Samsuddin, N. R., Hussain, S. N. A. S., Azhar, S., Aljunid, S. M., & others. (2011). HPV positivity and its' influencing factors among invasive cervical cancer women in Malaysia. *International Journal of Public Health Research*, *1*(1).

Qanash, H., Bazaid, A. S., Binsaleh, N. K., Patel, M., Althomali, O. W., & Sheeha, B. B. (2023). *In vitro* Antiproliferative Apoptosis Induction and Cell Cycle Arrest

Potential of Saudi Sidr Honey against Colorectal Cancer. *Nutrients*, *15*(15). https://doi.org/10.3390/nu15153448

Qiu, S., Liu, J., & Xing, F. (2017). Antizyme inhibitor 1: a potential carcinogenic molecule. *Cancer Science*, *108*(2), 163–169. https://doi.org/10.1111/cas.13122

Radzishevska, Y. B., Sukhina, Vasylyev, L. Y., Sukhin, V. S., Nemaltsova, V., Radzishevska, Y. a. K., & Boiko. (2023). Risk factors of local recurrence and distant metastasis and manifestations of toxicity during chemoradiation treatment of cervical cancer patients. *Ukrainian Journal of Radiology and Oncology*, *31*(2). https://doi.org/10.46879/ukroj.2.2023.175-189

Rahmat, F., Kuan, J. Y., Hajiman, Z., Shakrin, N. N. S. M., Roos, N. A. C., Mustapa, M., & Ahmad, A. (2021). Human Papillomavirus (HPV) Prevalence and Type Distribution in Urban Areas of Malaysia. *Asian Pacific Journal of Cancer Prevention*, *22*(9). https://doi.org/10.31557/APJCP.2021.22.9.2969

Rasul, H. O., Aziz, B. K., Ghafour, D. D., & Kivrak, A. (2023). Screening the possible anti-cancer constituents of Hibiscus rosa-sinensis flower to address mammalian target of rapamycin: an in silico molecular docking, HYDE scoring, dynamic studies, and pharmacokinetic prediction. *Molecular Diversity*, 27(5), 2273–2296. https://doi.org/10.1007/s11030-022-10556-9

Razali, N. H., Ali, A., Hua Gan, S., & Sen Lim, C. (2020). Prevalence of Traditional and Complementary Alternative Medicine's Use among Cancer Patients in South Peninsular Malaysia. *Asian Pacific Journal of Cancer Biology*, *5*(1). https://doi.org/10.31557/apjcb.2020.5.1.19-26

Reddy, G. K., Shivakumara, S., Reddy, K. R., & Eppakayala, L. (2024). Synthesis and biological evaluation of 1,3,4-oxadiazole Linked Thiazole-Isoxazole-Pyridines as anticancer agents. *Results in Chemistry*, *7*, 101248. https://doi.org/10.1016/j.rechem.2023.101248

Robinson, E. F., Darby, J. P., & Moulder, J. K. (2023). Cervical cancer screening: Missed opportunities in a one-track model. In *International Journal of Gynecological Cancer* (Vol. 33, Issue 4). https://doi.org/10.1136/ijgc-2023-004311

Romli, R., Shahabudin, S., Saddki, N., & Mokhtar, N. (2019). Cervical cancer and pap smear screening: Knowledge, attitude and practice among working women in northern state of Malaysia. *Medical Journal of Malaysia*, *74*(1).

Roy, V., Jung, W., Linde, C., Coates, E., Ledgerwood, J., Costner, P., Yamshchikov, G., Streeck, H., Juelg, B., Lauffenburger, D. A., & Alter, G. (2023). Differences in HPV-specific antibody Fc-effector functions following Gardasil® and Cervarix® vaccination. *Npj Vaccines*, *8*(1). https://doi.org/10.1038/s41541-023-00628-8

Roziman, N. S. M., Mustafa Din, W., Mahadi, Z., Islahudin, F., & Said, M. Md. (2024). Safe use elements of finished herbal products: insights from consumers and practitioners in Malaysia. *BMC Complementary Medicine and Therapies*, 24(1), 252. https://doi.org/10.1186/s12906-024-04546-7

Rybak, L. P., Alberts, I., Patel, S., Al Aameri, R. F. H., & Ramkumar, V. (2023). Effects of natural products on cisplatin ototoxicity and chemotherapeutic efficacy. *Expert Opinion on Drug Metabolism and Toxicology*, *19*(9), 635–652. https://doi.org/10.1080/17425255.2023.2260737

Saeed, T. A., Osman, O. A., Amin, A. E., & Badwi, S. M. A. el. (2017). Safety Assessment and Potential Anti-Inflammatory Effect of Ethanolic Extract of Syzygium aromaticum in Albino Rats. *Advances in Bioscience and Biotechnology*, 08(11), 411–420. https://doi.org/10.4236/abb.2017.811030

Safitri, A., Putri, A. S., Octavianty, T. D., & Sari, D. R. T. (2020). Metabolomic Profiles of Curcuma longa L and Cosmos caudatus Extracts and Their In-Silico Anti-cancer Activity. *Journal of Physics: Conference Series*, 1665(1), 012022. https://doi.org/10.1088/1742-6596/1665/1/012022

Saunders, F. R., & Wallace, H. M. (2010). On the natural chemoprevention of cancer. *Plant Physiology and Biochemistry*, *48*(7), 621–626. https://doi.org/10.1016/j.plaphy.2010.03.001

Schultz, C. R., Aleiwi, B., Zhou, X. E., Suino-Powell, K., Melcher, K., Almeida, N. M. S., Wilson, A. K., Ellsworth, E. L., & Bachmann, A. S. (2025). Design, Synthesis, and Biological Activity of Novel Ornithine Decarboxylase (ODC)

Inhibitors. *Journal of Medicinal Chemistry*, *68*(5), 5760–5773. https://doi.org/10.1021/acs.jmedchem.4c03120

Schultz, C. R., Bupp, C. P., Rajasekaran, S., & Bachmann, A. S. (2019). Biochemical features of primary cells from a pediatric patient with a gain-of-function ODC1 genetic mutation. *Biochemical Journal*, *476*(14), 2047–2057. https://doi.org/10.1042/BCJ20190294

Schultz, C. R., Geerts, D., Mooney, M., El-Khawaja, R., Koster, J., & Bachmann, A. S. (2018). Synergistic drug combination GC7/DFMO suppresses hypusine/spermidine-dependent eIF5A activation and induces apoptotic cell death in neuroblastoma. *Biochemical Journal*, *475*(2), 531–545. https://doi.org/10.1042/BCJ20170597

Schultz, C. R., Swanson, M. A., Dowling, T. C., & Bachmann, A. S. (2021). Probenecid increases renal retention and antitumor activity of DFMO in neuroblastoma. *Cancer Chemotherapy and Pharmacology*, *88*(4), 607–617. https://doi.org/10.1007/s00280-021-04309-y

Sen, Z., Zhan, X. K., Jing, J., Yi, Z., & Wanqi, Z. (2013). Chemosensitizing activities of cyclotides from Clitoria ternatea in paclitaxel-resistant lung cancer cells. Oncology letters, 5(2), 641-644.

Shanmugam, K., Sellappan, S., Alahmadi, T. A., Almoallim, H. S., Natarajan, N., & Veeraraghavan, V. P. (2022). Green synthesized zinc oxide nanoparticles from Cinnamomum verum bark extract inhibited cell growth and induced caspase-mediated apoptosis in oral cancer KB cells. *Journal of Drug Delivery Science and Technology*, *74*, 103577. https://doi.org/10.1016/j.jddst.2022.103577

Sharma, S. (2023). Pharmacological mechanism of indigenous herb Exacum lawii on cisplatin instigated toxicity in human embryonic kidney cells (HEK-293). *Phytomedicine Plus*, *3*(2), 100454. https://doi.org/10.1016/j.phyplu.2023.100454

Sharma, S. H., Thulasingam, S., & Nagarajan, S. (2017). Terpenoids as anti-colon cancer agents – A comprehensive review on its mechanistic perspectives.

European Journal of Pharmacology, *795*, 169–178. https://doi.org/10.1016/j.ejphar.2016.12.008

Shinde, A. S., & Mendhulkar, V. D. (2023). Anticancer activity of gold nanobioconjugates synthesized from *Elephantopus scaber* (linn.) leaf extract. *Journal of Cancer Research and Therapeutics*, *19*(Suppl 1), S250–S259. https://doi.org/10.4103/jcrt.JCRT_1043_20

Sholler, G. L. S., Ferguson, W., Bergendahl, G., Bond, J. P., Neville, K., Eslin, D., Brown, V., Roberts, W., Wada, R. K., Oesterheld, J., Mitchell, D., Foley, J., Parikh, N. S., Eshun, F., Zage, P., Rawwas, J., Sencer, S., Pankiewicz, D., Quinn, M., … Kraveka, J. M. (2018). Maintenance DFMO Increases Survival in High Risk Neuroblastoma. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-32659-w

Sholler, G. L. S., Gerner, E. W., Bergendahl, G., MacArthur, R. B., VanderWerff, A., Ashikaga, T., Bond, J. P., Ferguson, W., Roberts, W., Wada, R. K., Eslin, D., Kraveka, J. M., Kaplan, J., Mitchell, D., Parikh, N. S., Neville, K., Sender, L., Higgins, T., Kawakita, M., … Bachmann, A. S. (2015). A phase I trial of DFMO targeting polyamine addiction in patients with relapsed/refractory neuroblastoma. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0127246

Shrestha, A. D., Neupane, D., Vedsted, P., & Kallestrup, P. (2018). Cervical cancer prevalence, incidence and mortality in low and middle income countries: A systematic review. In *Asian Pacific Journal of Cancer Prevention* (Vol. 19, Issue 2). https://doi.org/10.22034/APJCP.2018.19.2.319

Siegel, R. L., Miller, K. D., Wagle, N. S., & Jemal, A. (2023). Cancer statistics, 2023. *CA: A Cancer Journal for Clinicians*, *73*(1), 17–48. https://doi.org/10.3322/caac.21763

Silva, da M. F., Lins, A. A., Gomes, M. C., Marinho, W. P. de J., Araújo, de R. S. A., Moura, de R. O., Zhan, P., Nascimento, I. J. dos S., & Silva-Júnior, da E. F. (2024). Anticancer Drug Discovery from Natural Compounds Targeting

PI3K/AKT/mTOR Signaling Pathway. *Current Medicinal Chemistry*, *31*. https://doi.org/10.2174/0109298673325229240928040758

Simha, A., Aziz, K., Braganza, A., Abraham, L., Samuel, P., Lindsley, K. B., Bordewijk, E. M., Ng, K. Y. B., Rakic, L., Mol, B. W. J., Brown, J. V. E. P. R., Crawford, T. J., van Wely, M., Grillo-Ardila, C. F., Torres, M., Gaitán, H. G., Metwally, M., Raybould, G., Cheong, Y. C., … O'Sullivan, R. (2017). De-intensified adjuvant (chemo)radiotherapy versus standard adjuvant chemoradiotherapy post transoral minimally invasive surgery for resectable HPV-positive oropharyngeal carcinoma. *Cochrane Database of Systematic Reviews*.

Singh, M., Modi, A., Narayan, G., & Singh, S. K. (2016). Benzothiazole derivatives bearing amide moiety: Potential cytotoxic and apoptosis-inducing agents against cervical cancer. *Anti-Cancer Drugs*, *27*(6). https://doi.org/10.1097/CAD.0000000000000357

Siraj, M. A., Islam, Md. A., Al Fahad, Md. A., Kheya, H. R., Xiao, J., & Simal-Gandara, J. (2021). Cancer Chemopreventive Role of Dietary Terpenoids by Modulating Keap1-Nrf2-ARE Signaling System—A Comprehensive Update. *Applied Sciences*, *11*(22), 10806. https://doi.org/10.3390/app112210806

Snellenberg, S., Cillessen, S. A. G. M., Van Criekinge, W., Bosch, L., Meijer, C. J. L. M., Snijders, P. J. F., & Steenbergen, R. D. M. (2014). Methylation-mediated repression of PRDM14 contributes to apoptosis evasion in HPV-positive cancers. *Carcinogenesis*, *35*(11), 2611–2618. https://doi.org/10.1093/carcin/bgu197

Stefanska, B., Karlic, H., Varga, F., Fabianowska-Majewska, K., & Haslberger, A. G. (2012). Epigenetic mechanisms in anti-cancer actions of bioactive food components - The implications in cancer prevention. *British Journal of Pharmacology*, *167*(2), 279–297. https://doi.org/10.1111/j.1476-5381.2012.02002.x

Stewart, T. M., Foley, J. R., Holbert, C. E., Klinke, G., Poschet, G., Steimbach, R. R., Miller, A. K., & Casero, R. A. (2022). Histone deacetylase-10 liberates

spermidine to support polyamine homeostasis and tumor cell growth. *Journal of Biological Chemistry*, *298*(10). https://doi.org/10.1016/j.jbc.2022.102407

Strober, W. (2015). Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology*, *111*(1). https://doi.org/10.1002/0471142735.ima03bs111

Sudan, R., Bhagat, M., Gupta, S., Chitrarakha, & Devi, T. (2013). Comparative analysis of cytotoxic and antioxidant potential of edible Cinnamomum verum (bark) and Cinnamomum tamala (Indian bay leaf). *Free Radicals and Antioxidants*. https://doi.org/10.1016/j.fra.2013.05.005

Sun, H., Zhang, L., Sui, B., Li, Y., Yan, J., Wang, P., Wang, Y., & Liu, S. (2021). The Effect of Terpenoid Natural Chinese Medicine Molecular Compound on Lung Cancer Treatment. *Evidence-Based Complementary and Alternative Medicine*, *2021*, 1–13. https://doi.org/10.1155/2021/3730963

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Svensson, K. J., Welch, J. E., Kucharzewska, P., Bengtson, P., Bjurberg, M., Påhlman, S., Ten Dam, G. B., Persson, L., & Belting, M. (2008). Hypoxia-Mediated Induction of the Polyamine System Provides Opportunities for Tumor Growth Inhibition by Combined Targeting of Vascular Endothelial Growth Factor and Ornithine Decarboxylase. *Cancer Research*, *68*(22), 9291–9301. https://doi.org/10.1158/0008-5472.CAN-08-2340

Syatkin, S. P., Neborak, E. V., Khlebnikov, A. I., Komarova, M. V., Shevkun, N. A., Kravtsov, E. G., Blagonravov, M. L., & Agostinelli, E. (2020a). The investigation of structure–activity relationship of polyamine-targeted synthetic compounds from different chemical groups. *Amino Acids*, *52*(2), 199–211. https://doi.org/10.1007/s00726-019-02778-3

Syatkin, S. P., Neborak, E. V, Khlebnikov, A. I., Komarova, M. V, Shevkun, N. A., Kravtsov, E. G., Blagonravov, M. L., & Agostinelli, E. (2020b). The

investigation of structure–activity relationship of polyamine-targeted synthetic compounds from different chemical groups. *Amino Acids*, *52*(2), 199–211. https://doi.org/10.1007/s00726-019-02778-3

Taha, Y. M., Abdulrahman, A.-A. A., Abdel-Fattah, A., & El-Sheikh, M. A. (2020). Bioactivity of *Syzygium aromaticum* (L.) Merr. & L.M.Perry extracts as potential antimicrobial and anticancer agents. *Journal of King Saud University - Science*, *32*(8), 3273–3278. https://doi.org/10.1016/j.jksus.2020.09.009

Takahashi, T., Takano, A., & Kakehi, J.-I. (2018). *Detection of Thermospermine and Spermine by HPLC in Plants* (pp. 69–73). https://doi.org/10.1007/978-1-4939-7398-9_6

Tam, T. D. T., Ngoc, T. T. B., Nga, N. T. H., Trinh, N. T. M., Thuoc, T. L., & Thao, D. T. P. (2021). Ethyl acetate extract of Elephantopus mollis Kunth induces apoptosis in human gastric cancer cells. *BMC Complementary Medicine and Therapies*, *21*(1), 273. https://doi.org/10.1186/s12906-021-03444-6

Tan, S. C., Ismail, M. P., Duski, D. R., Othman, N. H., & Ankathil, R. (2018). Prevalence and type distribution of human papillomavirus (HPV) in Malaysian women with and without cervical cancer: an updated estimate. *Bioscience Reports*, *38*(2). https://doi.org/10.1042/BSR20171268

Tang, J., Wu, X., Cheng, B., & Lu, Y. (2023). Identification of a polyamine-related signature and six novel prognostic biomarkers in oral squamous cell carcinoma. *Frontiers in Molecular Biosciences*, *10*. https://doi.org/10.3389/fmolb.2023.1073770

Tavakolian, S., Goudarzi, H., & Faghihloo, E. (2020). Cyclin-dependent kinases and CDK inhibitors in virus-associated cancers. *Infectious Agents and Cancer*, *15*(1), 27. https://doi.org/10.1186/s13027-020-00295-7

Tavallali, V., Rahmati, S., Bahmanzadegan, A., & Lasibi, M. J. M. (2024). Phenolic profile and evaluation of antimicrobial and anticancer activities of Calendula officinalis L. using exogenous polyamines application. *Industrial Crops and Products*, *214*, 118571. https://doi.org/10.1016/j.indcrop.2024.118571

Tolosa, L., Donato, M. T., & Gómez-Lechón, M. J. (2015). *General Cytotoxicity Assessment by Means of the MTT Assay* (pp. 333–348). https://doi.org/10.1007/978-1-4939-2074-7_26

Tracy, R. M.-S., Woster, P. M., & Casero, R. A. (2016). Targeting polyamine metabolism for cancer therapy and prevention. *Biochemical Journal*, *473*(19), 2937–2953. https://doi.org/10.1042/BCJ20160383

Truzzi, F., Whittaker, A., D'Amen, E., Valerii, M. C., Abduazizova, V., Spisni, E., & Dinelli, G. (2023). Spermidine-Eugenol Supplement Preserved Inflammation-Challenged Intestinal Cells by Stimulating Autophagy. *International Journal of Molecular Sciences*, *24*(4), 4131. https://doi.org/10.3390/ijms24044131

Tsai, K., Liu, Y.-H., Chen, T.-W., Yang, S.-M., Wong, H.-Y., Cherng, J., Chou, K.-S., & Cherng, J.-M. (2016). Cuminaldehyde from Cinnamomum verum Induces Cell Death through Targeting Topoisomerase 1 and 2 in Human Colorectal Adenocarcinoma COLO 205 Cells. *Nutrients*, *8*(6), 318. https://doi.org/10.3390/nu8060318

Tuli, H. S., Yerer, M. B., & Sak, K. (2022). Editorial: Current Aspects in Chemopreventive Strategies, Volume II. In *Frontiers in Pharmacology* (Vol. 13). https://doi.org/10.3389/fphar.2022.961334

Umar, A. H., Biringallo, C. S. N., Tuyuwale, P. I., Kila, A., Febyola, K. D., Syahruni, R., Hendrarti, W., Rafi, M., & Ratnadewi, D. (2025). Unveiling the Pharmacological Mechanism of Cosmos Caudatus Compounds as Lung Cancer Drug Candidates: Pharmacology Networking, Molecular Docking, and Experimental Validation. *Journal of Pharmaceutical Innovation,* 20(3), 81. https://doi.org/10.1007/s12247-025-09989-0

Ullah, A., Prottoy, N. I., Araf, Y., Hossain, S., Sarkar, B., & Saha, A. (2019). Molecular docking and pharmacological property analysis of phytochemicals from *Clitoria ternatea* as potent inhibitors of cell cycle checkpoint proteins in the cyclin/CDK pathway in cancer cells. *Computational Molecular Bioscience*, 9(3), 81-94.

Vemula, S., Gupta, M. K., Arva Tatireddygari, V. R., & Vadde, R. (2019). Pancreatic cancer chemoprevention. In *Theranostic Approach for Pancreatic Cancer* (pp. 245–261). Elsevier. https://doi.org/10.1016/B978-0-12-819457-7.00012-8

Waly, M. I., Al Moundhri, M. S., & Ali, B. H. (2011). Effect of Curcumin on Cisplatin- and Oxaliplatin-Induced Oxidative Stress in Human Embryonic Kidney (HEK) 293 Cells. *Renal Failure*, *33*(5), 518–523. https://doi.org/10.3109/0886022X.2011.577546

Wang, J., Li, T. M., Zang, L., Pan, X., Wang, S., Wu, Y., & Wang, G. (2017). Apigenin Inhibits Human SW620 Cell Growth by Targeting Polyamine Catabolism. *Evidence-Based Complementary and Alternative Medicine*, *2017*. https://doi.org/10.1155/2017/3684581

Wang, L. Q., Zhang, Y., Yan, H., Liu, K. J., & Zhang, S. (2015). MicroRNA-373 functions as an oncogene and targets YOD1 gene in cervical cancer. *Biochemical and Biophysical Research Communications*, *459*(3), 515–520. https://doi.org/10.1016/j.bbrc.2015.02.138

Wang, X., & Jiang, L. (2014). Effects of ornithine decarboxylase antizyme 1 on the proliferation and differentiation of human oral cancer cells. *International Journal of Molecular Medicine*, *34*(6), 1606–1612. https://doi.org/10.3892/ijmm.2014.1961

Wasim, L., & Chopra, M. (2016). Panobinostat induces apoptosis via production of reactive oxygen species and synergizes with topoisomerase inhibitors in cervical cancer cells. *Biomedicine and Pharmacotherapy*, *84*, 1393–1405. https://doi.org/10.1016/j.biopha.2016.10.057

Wei, W., He, Y., & Wu, Y.-M. (2018). Identification of genes associated with SiHa cell sensitivity to paclitaxel by CRISPR-Cas9 knockout screening. *International Journal of Clinical and Experimental Pathology*, *11*(4), 1972–1978.

Wu, Y. Y., Li, T. M., Zang, L. Q., Liu, B., & Wang, G. X. (2018). Effects of berberine on tumor growth and intestinal permeability in HCT116 tumor-bearing mice

using polyamines as targets. *Biomedicine and Pharmacotherapy*, *107*. https://doi.org/10.1016/j.biopha.2018.08.130

Yang, J., Li, L., Zeng, Z., Cao, C., Lyu, Y., Qin, Y., & Wang, Y. (2018). Inhibiting expression of polyamines regulator-1 can enhance the antitumor activity of dexamethasone on human cervical cancer cells. *Chinese Journal of Cancer Biotherapy*, *25*(7). https://doi.org/10.3872/j.issn.1007-385x.2018.07.009

Yang, W., Chen, X., Li, Y., Guo, S., Wang, Z., & Yu, X. (2020). Advances in Pharmacological Activities of Terpenoids. *Natural Product Communications*, *15*(3). https://doi.org/10.1177/1934578X20903555

Yang, Y., Chen, S., Zhang, Y., Lin, X., Song, Y., Xue, Z., Qian, H., Wang, S., Wan, G., Zheng, X., & Zhang, L. (2017). Induction of autophagy by spermidine is neuroprotective via inhibition of caspase 3-mediated Beclin 1 cleavage. *Cell Death & Disease*, *8*(4), e2738–e2738. https://doi.org/10.1038/cddis.2017.161

Yuan, Z., Chen, D., Chen, X., Yang, H., & Wei, Y. (2017). Overexpression of trefoil factor 3 (TFF3) contributes to the malignant progression in cervical cancer cells. *Cancer Cell International*, *17*(1), 7. https://doi.org/10.1186/s12935-016-0379-1

Zaid, A., Haw, X. R., Alkatib, H. H., Sasidharan, S., Marriott, P. J., & Wong, Y. F. (2022). Phytochemical Constituents and Antiproliferative Activities of Essential Oils from Four Varieties of Malaysian Zingiber officinale Roscoe against Human Cervical Cancer Cell Line. *Plants*, *11*(10), 1280. https://doi.org/10.3390/plants11101280

Zaid, H., Silbermann, M., Amash, A., Gincel, D., Abdel-Sattar, E., & Sarikahya, N. B. (2017). Medicinal Plants and Natural Active Compounds for Cancer Chemoprevention/Chemotherapy. In *Evidence-based Complementary and Alternative Medicine* (Vol. 2017). https://doi.org/10.1155/2017/7952417

Zari, A. T., Zari, T. A., & Hakeem, K. R. (2021). Anticancer Properties of Eugenol: A Review. *Molecules*, *26*(23), 7407. https://doi.org/10.3390/molecules26237407

Zeyen, P., Zeyn, Y., Herp, D., Mahmoudi, F., Yesiloglu, T. Z., Erdmann, F., Schmidt, M., Robaa, D., Romier, C., Ridinger, J., Herbst-Gervasoni, C. J., Christianson, D. W., Oehme, I., Jung, M., Krämer, O. H., & Sippl, W. (2022). Identification of histone deacetylase 10 (HDAC10) inhibitors that modulate autophagy in transformed cells. *European Journal of Medicinal Chemistry*, *234*. https://doi.org/10.1016/j.ejmech.2022.114272

Zhang, E., Ding, C., Li, S., Aikemu, B., Zhou, X., Fan, X., Sun, J., Yang, X., & Zheng, M. (2023). Polyamine metabolism patterns characterized tumor microenvironment, prognosis, and response to immunotherapy in colorectal cancer. *Cancer Cell International*, *23*(1), 96. https://doi.org/10.1186/s12935-023-02892-z

Zhang, Y., Zhang, T.-T., Gao, L., Tan, Y.-N., Li, Y.-T., Tan, X.-Y., Huang, T.-X., Li, H.-H., Bai, F., Zou, C., Pei, X.-H., Tan, B.-B., & Fu, L. (2022). Downregulation of MTAP promotes Tumor Growth and Metastasis by regulating ODC Activity in Breast Cancer. *International Journal of Biological Sciences*, *18*(7), 3034–3047. https://doi.org/10.7150/ijbs.67149

Zhao, J., Xu, L., Sun, J., Song, M., Wang, L., Yuan, S., Zhu, Y., Wan, Z., Larsson, S., Tsilidis, K., Dunlop, M., Campbell, H., Rudan, I., Song, P., Theodoratou, E., Ding, K., & Li, X. (2023). Global trends in incidence, death, burden and risk factors of early-onset cancer from 1990 to 2019. *BMJ Oncology*, *2*(1), e000049. https://doi.org/10.1136/bmjonc-2023-000049

Zhao, M., Zhang, L., Qiu, X., Zeng, F., Chen, W., An, Y., Hu, B., Wu, X., & Wu, X. (2016). BLCAP arrests G1/S checkpoint and induces apoptosis through downregulation of pRb1 in HeLa cells. *Oncology Reports*, *35*(5), 3050–3058. https://doi.org/10.3892/or.2016.4686

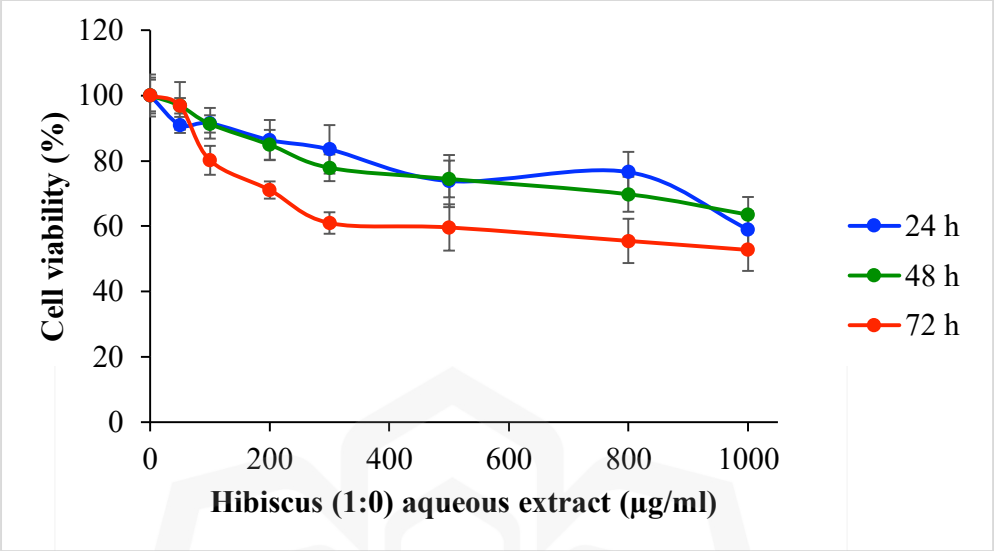
Zhao, W., Zheng, X., Tang, P. Y., Li, H., Liu, X., Zhong, J., & Tang, Y. (2023). Advances of antitumor drug discovery in traditional Chinese medicine and natural active products by using multi-active components combination. *Medicinal Research Reviews*, *43*(5), 1778–1808. https://doi.org/10.1002/med.21963

Zhu, J., Wang, H., & Jiang, X. (2022). mTORC1 beyond anabolic metabolism: Regulation of cell death. *Journal of Cell Biology*, *221*(12). https://doi.org/10.1083/jcb.202208103

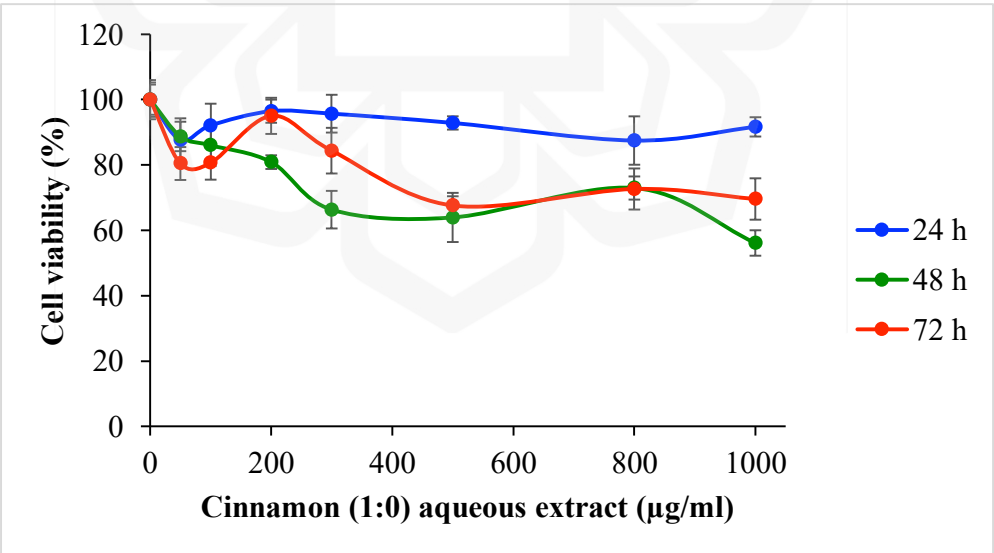
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Zhu, N., Zhu, L., Zhang, X., Huang, C., Xiang, W., & Huang, B. (2023). Triptolide attenuates irritable bowel syndrome via inhibiting ODC1. *BMC Gastroenterology*, *23*(1). https://doi.org/10.1186/s12876-023-02847-8

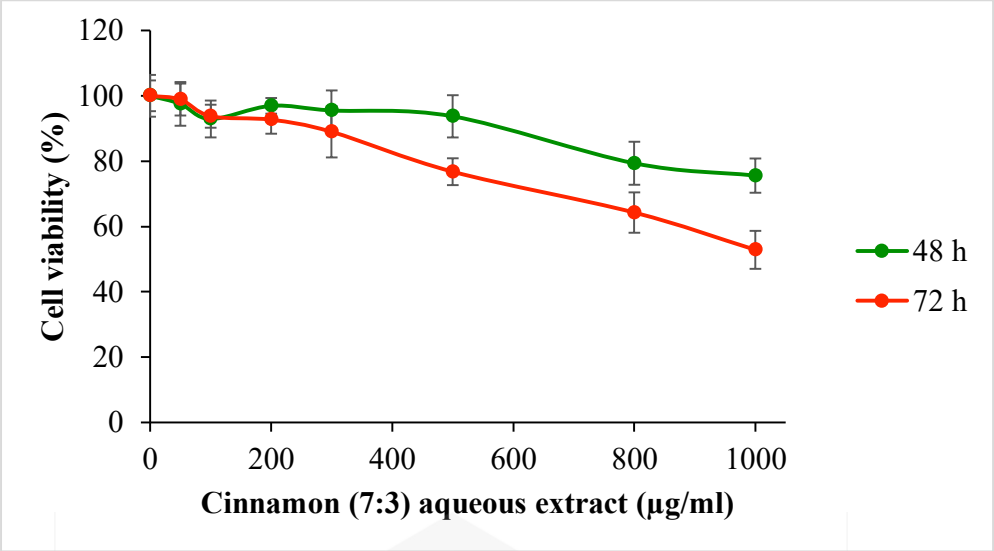
APPENDIX I: SUPPLEMENTARY RESULTS



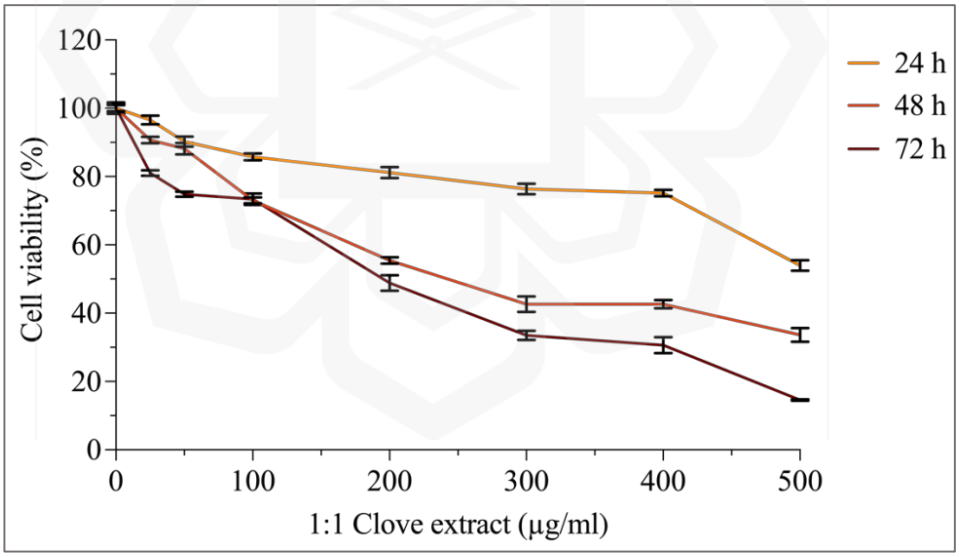
(A) The cytotoxicity of hibiscus (1:0) aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 24, 48, and 72 h timepoint. The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.



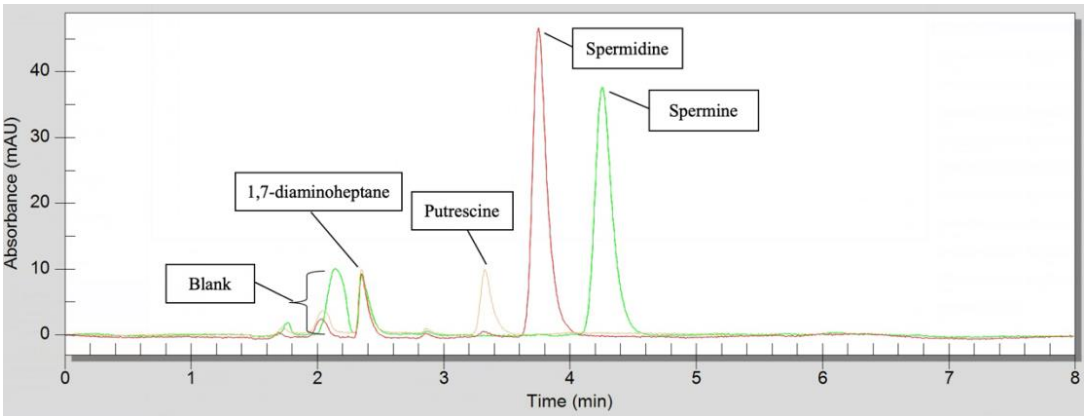
(B) The cytotoxicity of cinnamon (1:0) aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 24, 48, and 72 h timepoint. The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.



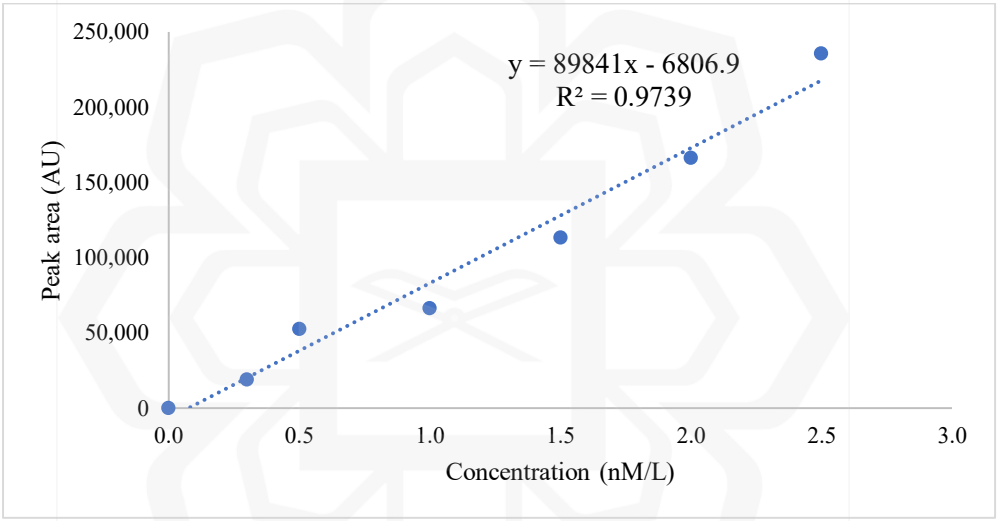
(C) The cytotoxicity of cinnamon (7:3) aqueous extract on SiHa cells at concentration 0 to 1000 µg/ml at 24, 48, and 72 h timepoint. The 24 h treatment was not performed. The results are presented as the mean ± SD (n=3), with three replicates conducted for each experiment.



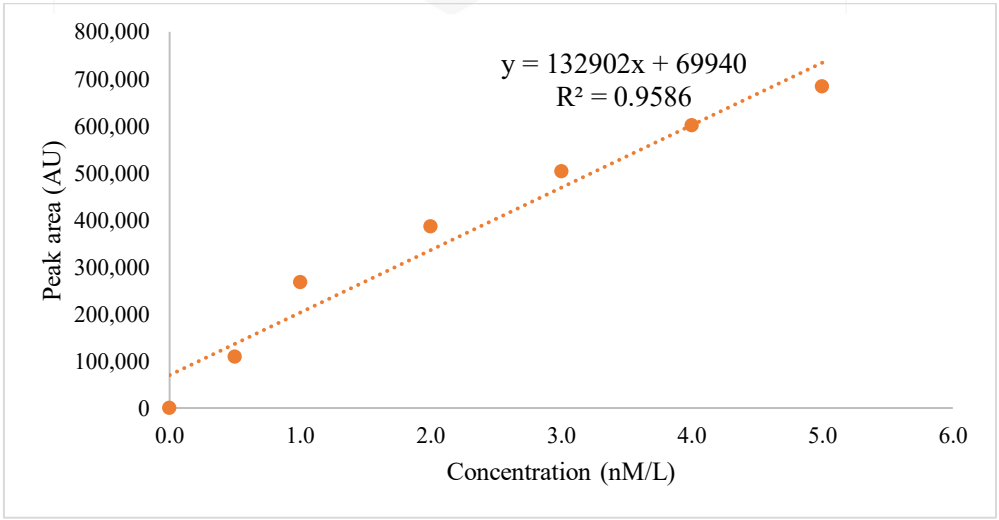
(D) The cytotoxic effects of 50% (v/v) aqueous clove extracts on SiHa cells.



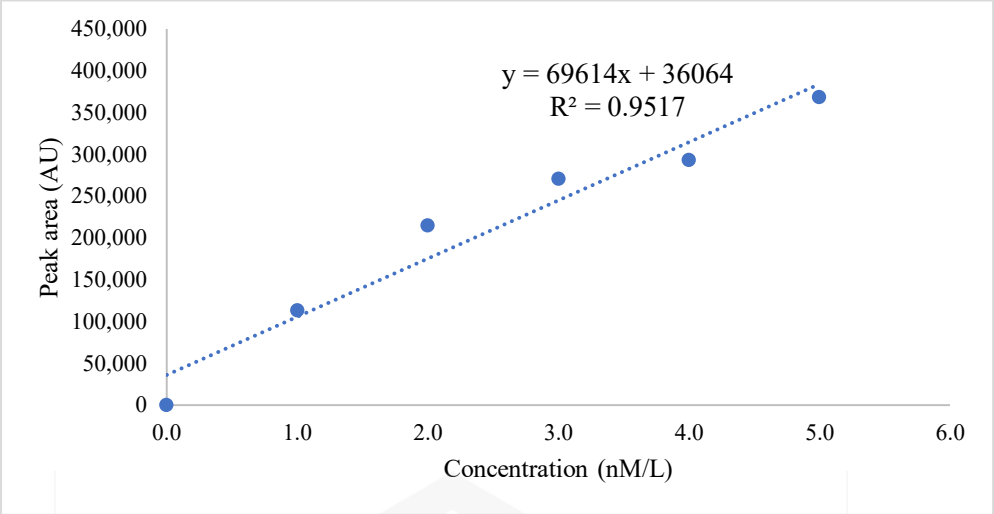
(E) Overlay of HPLC chromatogram of Polyamines Standard



(F) Calibration curve with linear equation of Putrescine

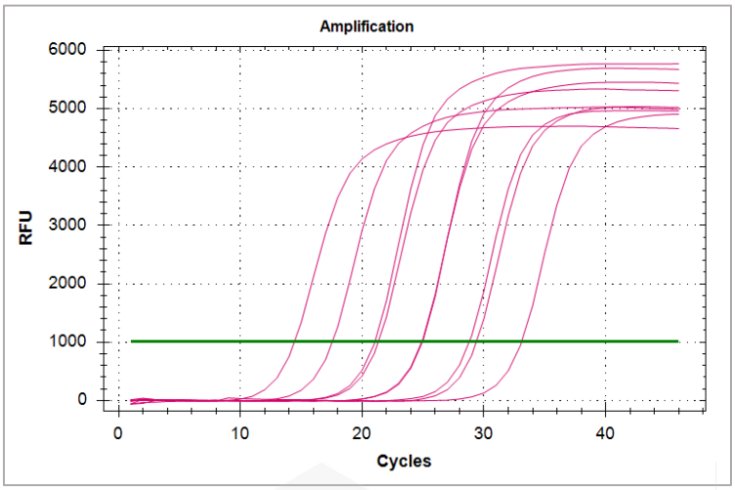


(I) Calibration curve with linear equation of spermidine

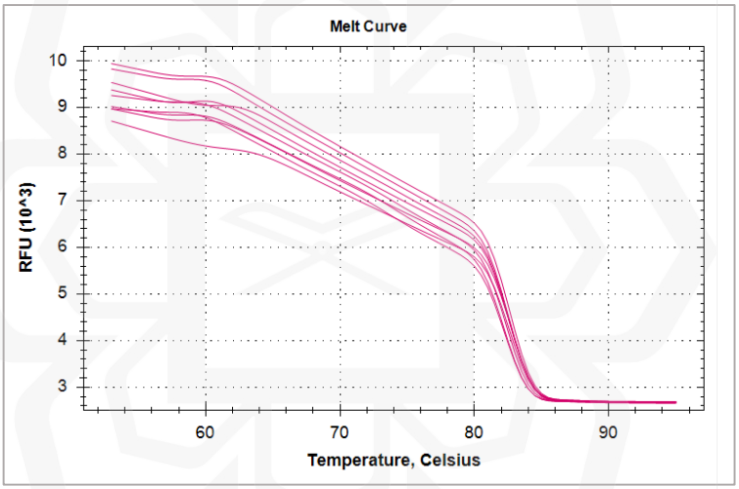


(J) Calibration curve with linear equation of spermine

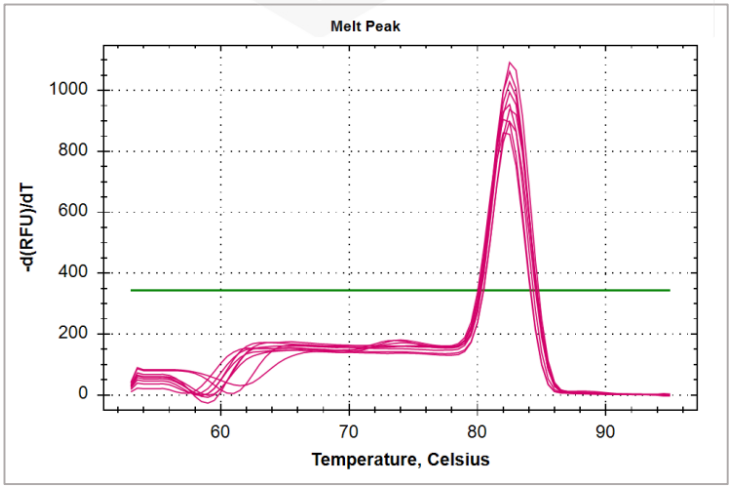
(H) Optimization of Primer Annealing Temperature Gradient Analysis



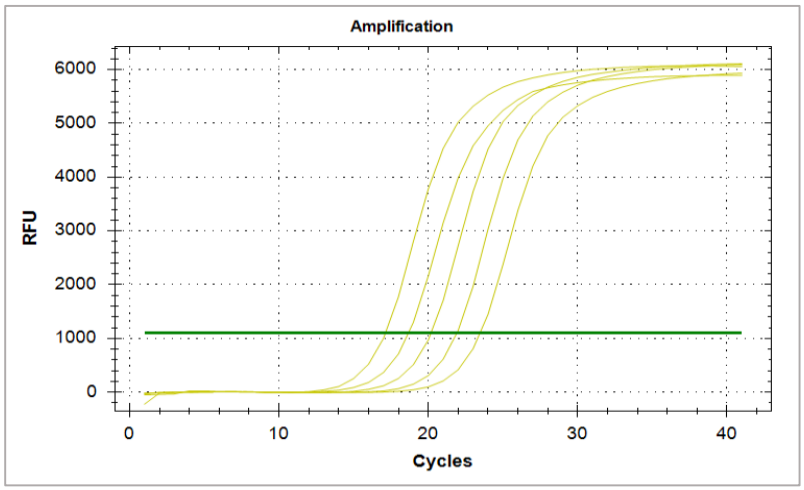
Amplification curve of *ACTB*



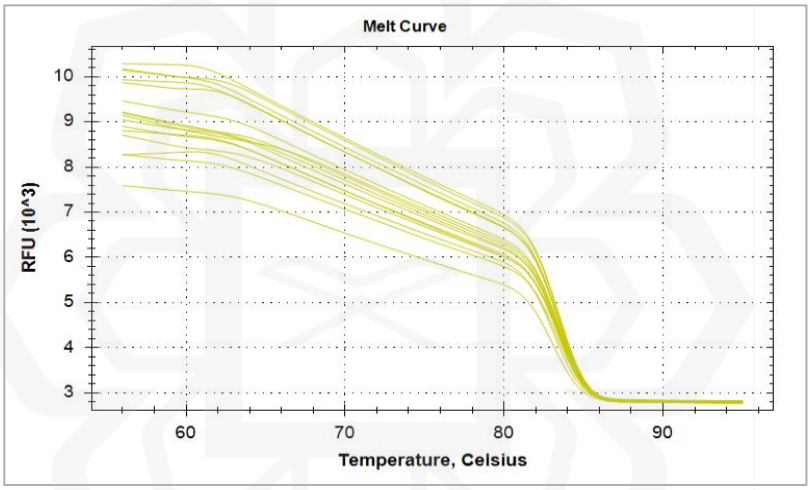
Melt curve of *ACTB*



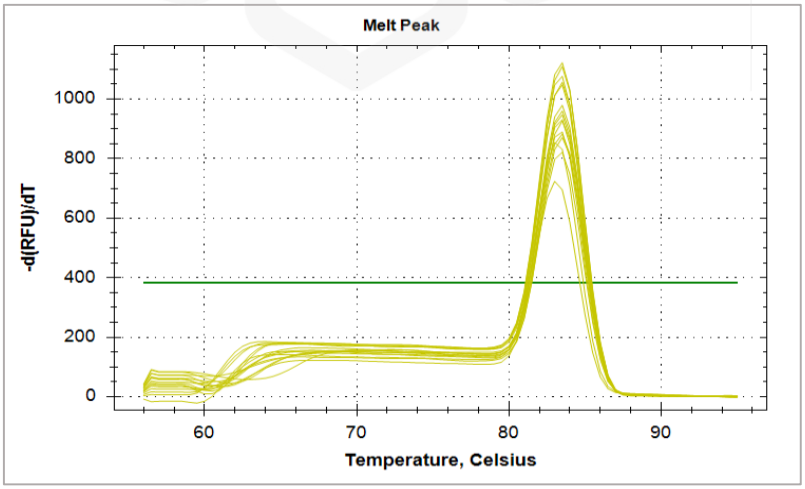
Melt peak of *ACTB*



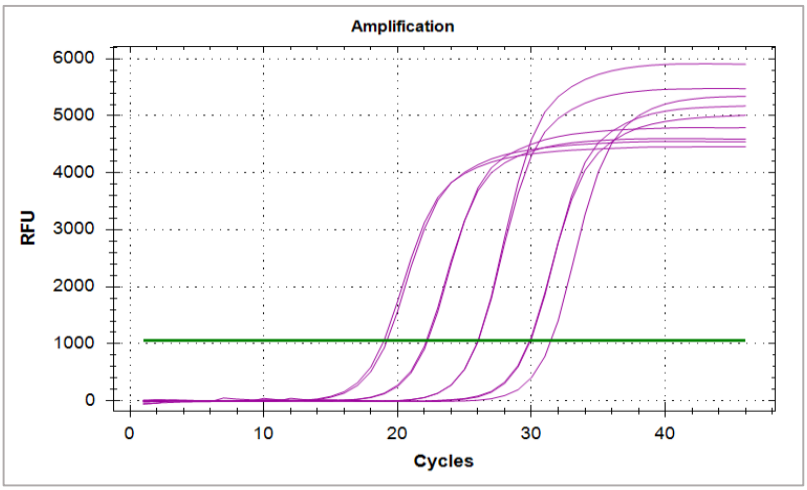
Amplification curve of *GAPDH*



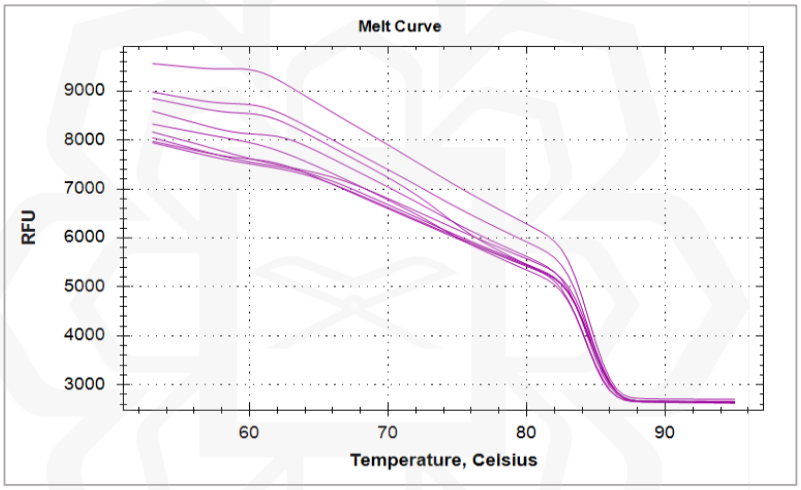
Melt curve of *GAPDH*



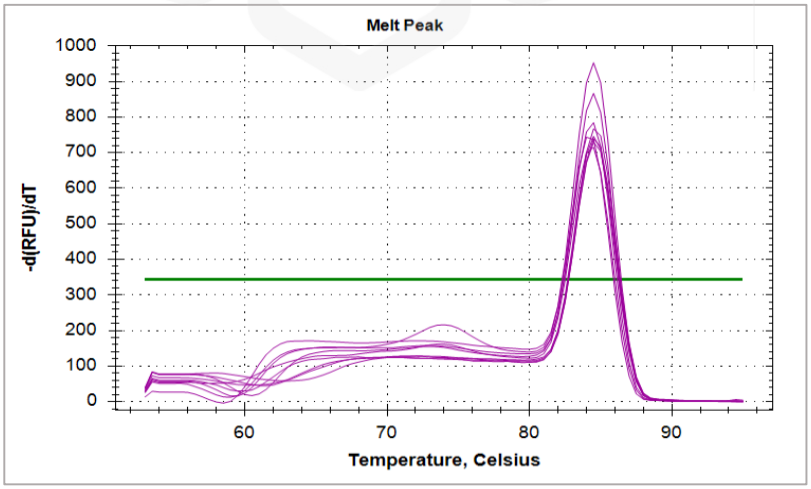
Melt peak of *GAPDH*



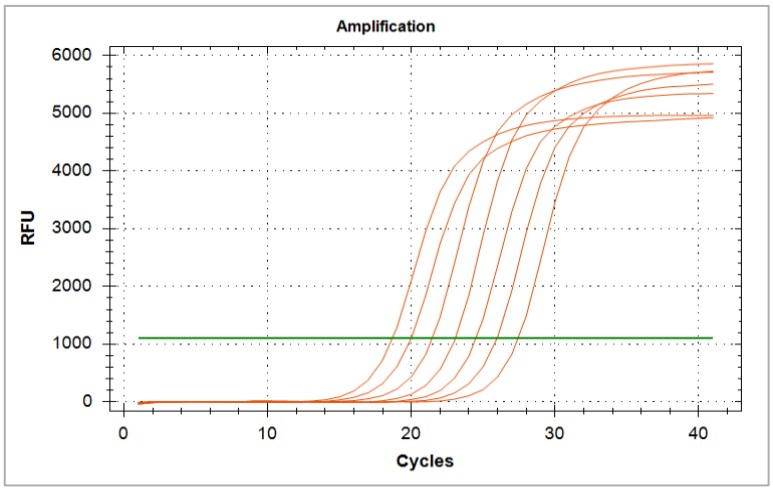
Amplification curve of *ODC*



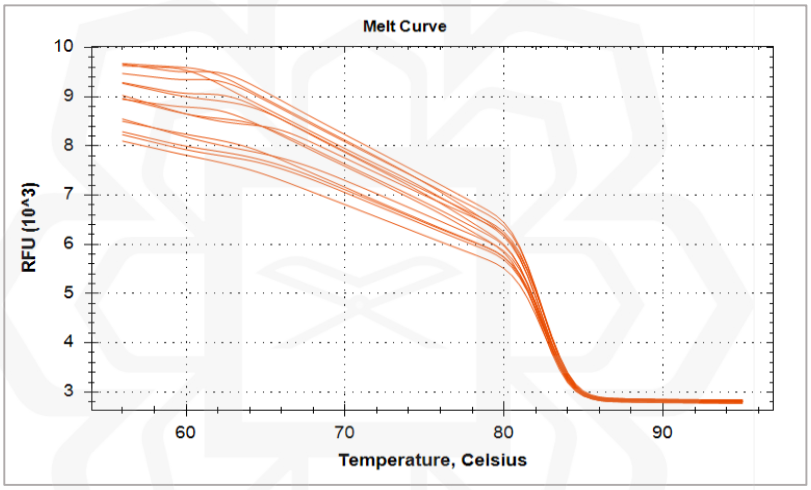
Melt curve of *ODC*



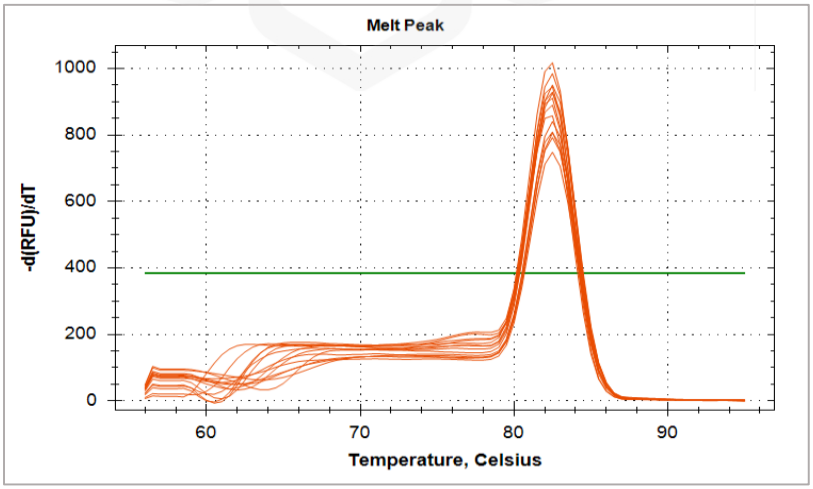
Melt peak of *ODC*



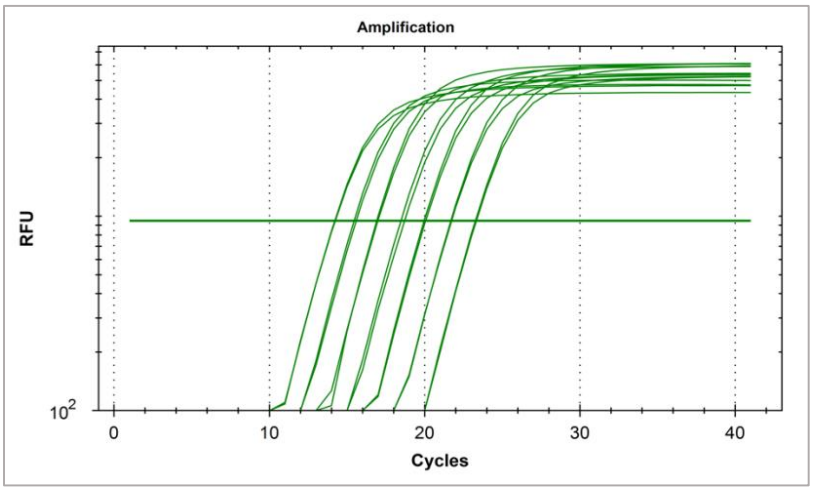
Amplification curve of *SSAT*



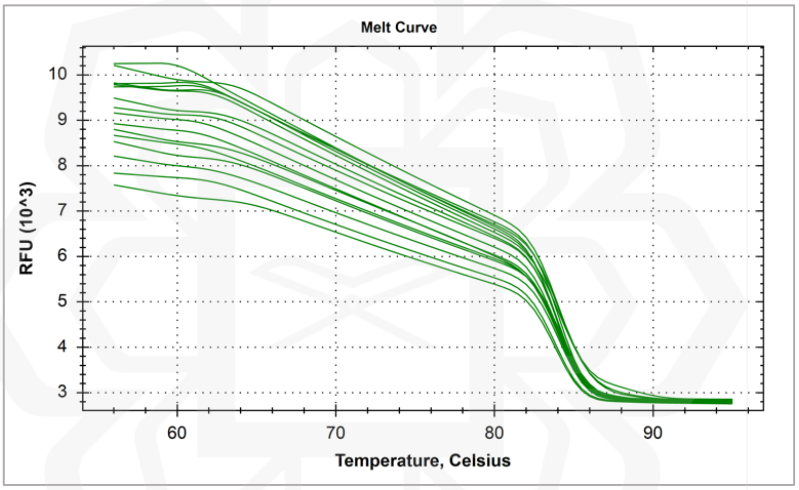
Melt curve of *SSAT*



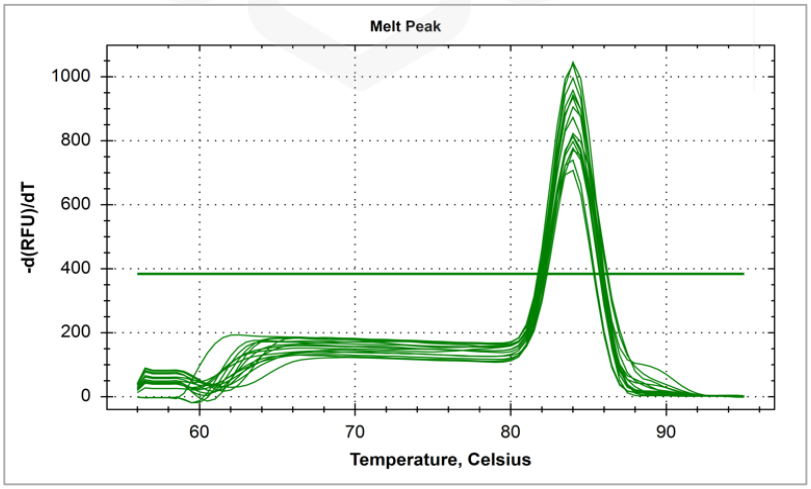
Melt peak of *SSAT*



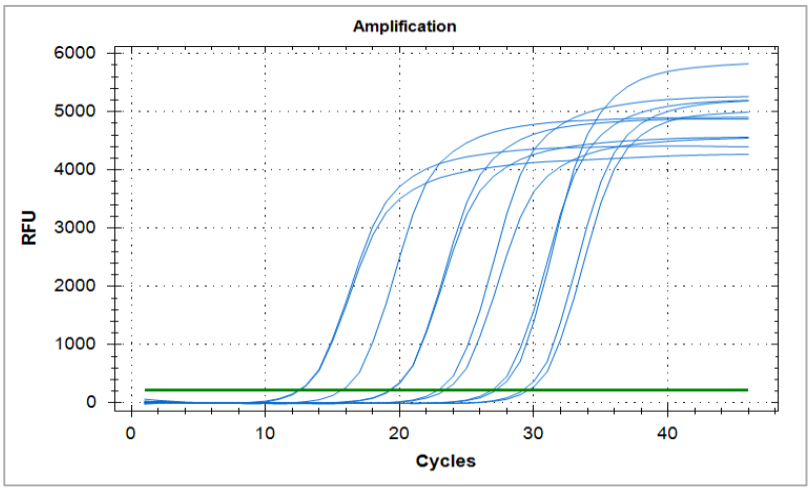
Amplification curve of *SMOX*



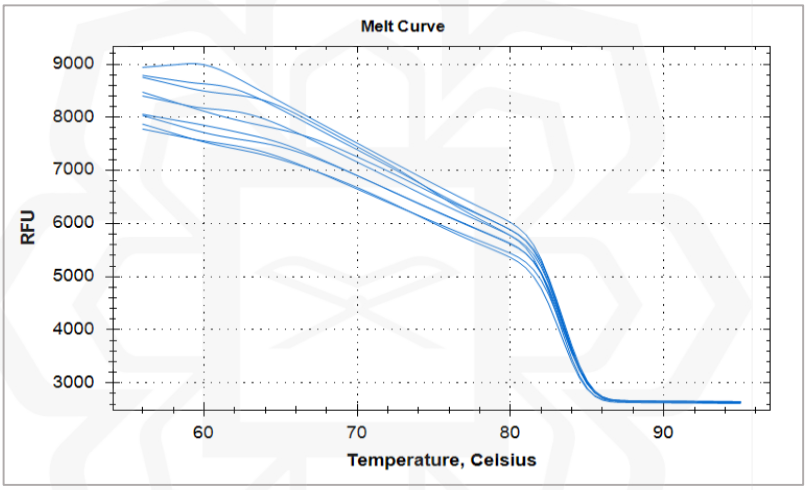
Melt curve of *SMOX*



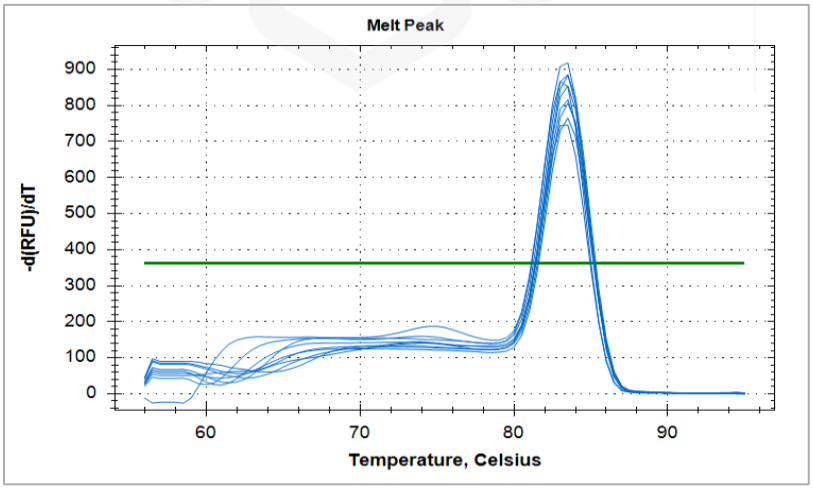
Melt peak of *SMOX*



Amplification curve of *OAZ1*

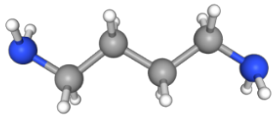
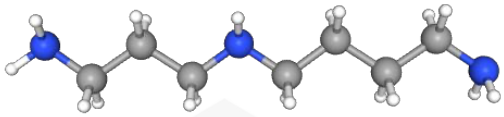
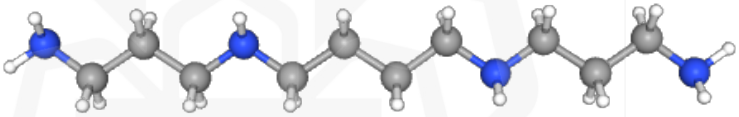
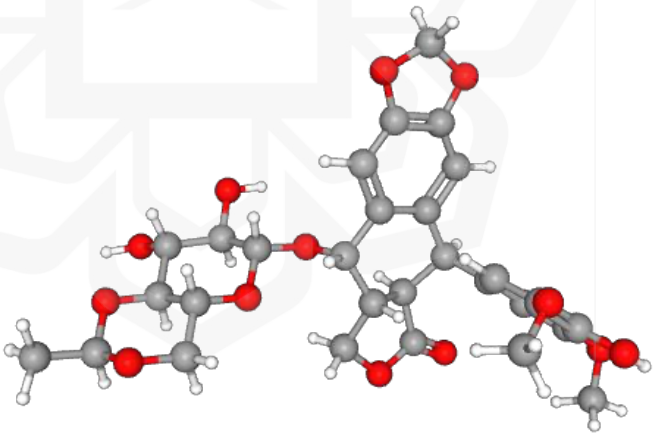
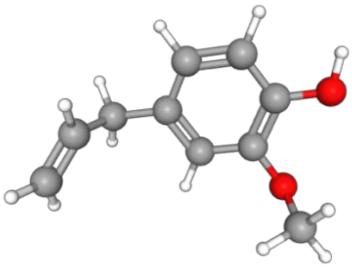


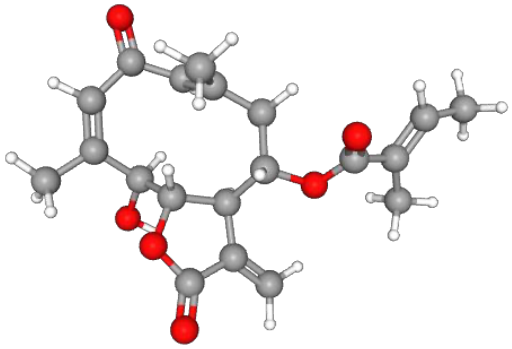
Melt curve of *OAZ1*



Melt peak of *OAZ1*

APPENDIX II: CHEMICAL STRUCTURES

Polyamines	
Putrescine	
Spermidine	
Spermine	
Positive control	
Etoposide	
Eugenol	

Molephantinin	

APPENDIX III: MATERIALS

1. Cell culture materials

DMEM High Glucose w/ L-Glutamine w/o Sodium Bicarbonate w/ Sodium Pyruvate	Biosera, France
Fetal Bovine Serum (South America)	Biosera, France
Phosphate Buffer Saline pH 7.40	
Trypsin-EDTA 1x in PBS	Biosera, France
Accutase® solution	Sigma-Aldrich, Germany
Dimethyl sulfoxide	Systerm
Trypan Blue Solution, 0.4%	Sigma-Aldrich, Germany
Neaubauer Hemocytometer	Quijing®

2. General disposable

25 cm^2 Tissue Culture Flask	SPL Lifesciences, KR
75 cm^2 Tissue Culture Flask	SPL Lifesciences, KR
Cell Scraper PS/LDPE Blade width 13mm	SPL Lifesciences, KR
Serological Pipette 10 ml	SPL Lifesciences, KR
Serological Pipette 5 ml	SPL Lifesciences, KR
Serological Pipette 1 ml	SPL Lifesciences, KR
50 ml Falcon tube	SPL Lifesciences, KR
15 ml Falcon tube	SPL Lifesciences, KR
Cryovials (2 ml)	SPL Lifesciences, KR
2.0 ml Micro centrifuge tube	Labcon, US
1.5 ml Micro centrifuge tube	Labcon, US

1.0 ml Micro centrifuge tube	Labcon, US
0.5 ml Micro centrifuge tube	Labcon, US
Sterile Nylon Syringe Filters, Pore: 0.22 µm	Microlab Scientific
10 ml Syringe Luer Lock without needle	Terumo, JP
Multi well Culture Plate, 6 wells	SPL Lifesciences, KR
Multi well Culture Plate, 12 wells	SPL Lifesciences, KR
Multi well Culture Plate, 24 wells	SPL Lifesciences, KR
Multi well Culture Plate, 96 wells	SPL Lifesciences, KR
Eclipse® 1000 µl Pipet Tips Bevel Point	Labcon, US
Eclipse® 200 µl Pipet Tips Bevel Point	Labcon, US
Eclipse® 100 µl Pipet Tips Bevel Point	Labcon, US
Eclipse® 10 µl Pipet Tips Ultrafine Point	Labcon, US
HPLC vials and caps	Agilent, US
Whatman No 1 filter paper (185 mm)	Cytiva, US
Nitrile Powder Free Exam Gloves	Confidenz, Malaysia

3. General chemicals

Perchloric acid 70%	Systerm, Malaysia
Sodium carbonate, anhydrous	Systerm, Malaysia
Copper II sulphate, pentahydrate	Systerm, Malaysia
Potassium sodium tartrate, tetrahydrate	Systerm, Malaysia
Potassium hydroxide pellets	Systerm, Malaysia
Sodium hydrogen carbonate	Systerm, Malaysia
Sodium carbonate decahydrate	Systerm, Malaysia
Sodium chloride	Systerm, Malaysia
Methanol	Systerm, Malaysia

Sodium hydroxide	Systerm, Malaysia
Folin & Ciocalteu's phenol reagent	Sigma-Aldrich, Germany
Chloroform	Sigma-Aldrich, Germany
Propidium Iodide (HPLC grade)	Sigma-Aldrich, Germany
Ribonuclease A from bovine pancreas	Sigma-Aldrich, Germany
Triton™ X-100	Thermo Fisher, US
Ethanol	Thermo Fisher, US
Isopropanol	Systerm, Malaysia
L-proline	Biochem
1,7-Diaminoheptane	Sigma-Aldrich, Germany
Dansyl chloride	Sigma-Aldrich, Germany
Benzoyl Chloride	Merck, Germany
Diethyl ether, HPLC grade	Sigma-Aldrich, Germany
4-(Dimethylamino)benzoyl chloride	Sigma-Aldrich, Germany
Putrescine dihydrochloride	Sigma-Aldrich, Germany
Spermine tetrahydrochloride	Sigma-Aldrich, Germany
Spermidine trihydrochloride	Sigma-Aldrich, Germany
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich, Germany

4. Test compounds and drugs

Etoposide	Sigma-Aldrich, Germany
DFMO	Sigma-Aldrich, Germany

5. Experimental Kits

Caspase 3, 8, and 9 Multiplex Activity Assay Kit (Fluorometric)	Abcam, UK
Annexin V-FITC Apoptosis Detection Kit II	Sigma-Aldrich, Germany
Innuprep RNA Mini kit 2.0	IST Innuscreen, DE
LunaScript® RT SuperMix Kit	NEB, USA
Luna® Universal qPCR Master Mix	NEB, USA

APPENDIX IV: SOLUTIONS AND MEDIA

1. **Complete growth media**

   From 500 ml of DMEM (with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate, liquid, sterile-filtered), add 10% (v/v) of fetal bovine serum (FBS) and 1% (v/v) of penicillin-streptomycin.

2. **MTT stock solution**

   Dissolve 5 mg of MTT powder in 1 mL of sterile PBS or culture medium, then filter-sterilize using 0.22 μm syringe filter and store at 4°C, protected from light.

3. **Lowry assay solution**

   Lowry assay standard solution:
   Make 0.5 mg/ml of Bovine Serum Albumin (BSA) working solution by mixing 25 mg of BSA powder to 50 ml of 0.3 M of NaOH solution.

   Sodium potassium tartrate 2% (w/v):
   2 g in 100 ml of distilled water. Store at 4°C

   Copper sulphate solution 1 % (w/v):
   1 g in 100 ml of distilled water and store at 4°C

   Sodium carbonate 2 % (w/v):
   20 g was added to 800 ml of distilled water and then made up to 1 l with more distilled water

   Solution A:
   Dissolve 2 g of sodium hydroxide (NaOH) and 10 g of sodium carbonate (Na_2CO_3) in 400 ml of distilled water, then adjust the volume to 500mL.

Solution B:

Dissolve 100 mg of potassium sodium tartrate and 50 mg of copper sulfate ($CuSO_4$) in 8 ml of distilled water in a plastic falcon tube, then adjust the volume to 10 ml.

4. **HPLC solution**

Standard solutions:

Weight the standard powder as calculated below, dissolve it in 10 ml of 0.2 M perchloric acid and stir. The concentration of working stock is 0.025 nmol/ul.

Standard	**Calculation of Working Stock**
Putrescine dihydrochloride	= 0.005 mol/L x 161.07 g/mol x 0.01 L = 0.008 g
Spermidine trihydrochloride	= 0.005 mol/L x 254.62 g/mol x 0.01 L = 0.013 g
Spermine tetrahydrochloride	= 0.005 mol/L x 384.19 g/mol x 0.01 L = 0.019 g

Sodium carbonate decahydrate:

For a 1 g/ml saturated solution, the crystals were added to 1/3 final volume of water and heated at 37°C for 1 h. The solution pH was adjusted to 10 with 3 M hydrochloric acid, then the volume adjusted with water to make up to the desired volume.

L-proline:

Dissolved 10 mg/ml in distilled water

1,7-diaminoheptane 1 mM stock:

1.3 mg in 10 ml of 0.2 M perchloric acid and store at -20°C

5.	**Cell cycle analysis solution**	*Propidium Iodide staining buffer*: For 10 ml of buffer add 500 µg of propdium iodide, 500 µg of ribonuclease A and 10 µl of Triton X-100 (0.1 % (v/v)) to distilled water. Store at 4°C. *Washing buffer – 1 % (w/v) BSA in PBS:* Add 0.1 g of BSA to 10 ml of PBS *Fixant – 70 % (v/v) ethanol:* 7 ml absolute ethanol to 3 ml of distilled water
6.	**DMSO solution (4%)**	Dilute 4 ml of 100% DMSO solution into 96 ml of autoclaved distilled water.
7.	**Etoposide stock solution (1 mM)**	Weight 5.88 mg of etoposide powder and dissolve in 10 ml of 4% DMSO solution.

APPENDIX V: PLANT SPECIES IDENTIFICATION



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KHALĪFAH - AMĀNAH - IQRA' - RAHMATAN LIL-ĀLAMĪN

KULLIYYAH OF PHARMACY

Our Ref. : IIUM/308/15/2/1/NMPC24-1/5
Date : 15 March 2024

Maryam Syahidah Binti Azalan
Kulliyyah of Allied Health Sciences
International Islamic University Malaysia
25200 Kuantan
Pahang.

Assalamu'alaikumWarahmatullahiWabarakatuh

Dear Maryam Syahidah,

PLANT SPECIES IDENTIFICATION

Voucher No.	Family Name	Scientific Name	Local Name
PIIUM 0368	Asteraceae	*Elephantopus scaber* L.	Tapak Sulaiman

Student's name : Maryam Syahidah Binti Azalan
Supervisor's name : Assoc. Prof. Dr. Radiah Abdul Ghani
Herbarium : Kulliyyah of Pharmacy
Person in-charged : Assoc. Prof. Dr.Norazian Mohd Hassan
Identified by : Dr. Shamsul Khamis

It should be noted that we are not involved in anything as a result of research conducted by the host.

UKMB VERIFIED 14 JUN 2024 Universiti Kebangsaan Malaysia Herbarium

Thank you. Wassalam.

Sincerely,

ASSOC. PROF. DR. NORAZIAN BINTI MOHD HASSAN
Kulliyyah of Pharmacy
International Islamic University Malaysia
Jalan Sultan Ahmad Shah
Bandar Indera Mahkota
25200 Kuantan
Pahang

DR. SHAMSUL KHAMIS
Botanist
Herbarium UKMB
Faculty of Science and Technology
The National University of Malaysia (UKM)
43600 Bangi
Selangor

Kulliyyah of Pharmacy
International Islamic University Malaysia Jalan Sultan Haji Ahmad Shah
Bandar Indera Mahkota, 25200 Kuantan
Pahang, Malaysia
Telephone: (+609) 570 4818 / 4816 / 4802 |Fax: (+609) 5716548

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KULLIYYAH OF PHARMACY

Our Ref. : IIUM/308/15/2/1/NMPC22-2/6
Date : 23 June 2022

UKMB
VERIFIED
28 JUN 2022
Universiti Kebangsaan Malaysia
Herbarium

Maryam Syahidah binti Azalan
Kulliyyah of Allied Health Sciences
International Islamic University Malaysia
25200 Kuantan
Pahang

Assalamu'alaikum Warahmatullahi Wabarakatuh

Dear Sr. Maryam,

PLANT SPECIES IDENTIFICATION

Kindly be informed that the plant specimen submitted for identification is confirmed as follows:

Voucher No.	Family Name	Scientific Name	Local Name
PIIUM 0300-1	Myrtaceae	*Syzygium aromaticum* (L.) Merr. & L.M. Perry	Cengkih

Student's name : Maryam Syahidah binti Azalan
Supervisor's name : Assoc. Prof. Dr. Radiah Abdul Ghani
Herbarium : Kulliyyah of Pharmacy
Person in-charged : Assoc. Prof. Dr. Norazian Mohd Hassan
Identified by : Dr. Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you and wassalam.

Sincerely,

ASSOC. PROF. DR. NORAZIAN BINTI MOHD HASSAN
Kulliyyah of Pharmacy
International Islamic University Malaysia
Jalan Sultan Ahmad Shah
Bandar Indera Mahkota
25200 Kuantan
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KULLIYYAH OF PHARMACY

Our Ref. : IIUM/308/15/2/1/NMPC22-2/7
Date : 23 June 2022

Maryam Syahidah binti Azalan
Kulliyyah of Allied Health Sciences
International Islamic University Malaysia
25200 Kuantan
Pahang

UKMB VERIFIED 28 JUN 2022 Universiti Kebangsaan Malaysia Herbarium

Assalamu'alaikum Warahmatullahi Wabarakatuh

Dear Sr. Maryam,

PLANT SPECIES IDENTIFICATION

Kindly be informed that the plant specimen submitted for identification is confirmed as follows:

Voucher No.	Family Name	Scientific Name	Local Name
PIIUM 0333	Malvaceae	*Hibiscus rosa-sinensis* L.	Bunga raya

Student's name : Maryam Syahidah binti Azalan
Supervisor's name : Assoc. Prof. Dr. Radiah Abdul Ghani
Herbarium : Kulliyyah of Pharmacy
Person in-charged : Assoc. Prof. Dr. Norazian Mohd Hassan
Identified by : Dr. Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you and wassalam.

Sincerely,

ASSOC. PROF. DR. NORAZIAN BINTI MOHD HASSAN
Kulliyyah of Pharmacy
International Islamic University Malaysia
Jalan Sultan Ahmad Shah
Bandar Indera Mahkota
25200 Kuantan
Pahang

DR. SHAMSUL KHAMIS
Botanist
Herbarium UKMB
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Selangor

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KULLIYYAH OF PHARMACY

Our Ref. : IIUM/308/15/2/1/NMPC22-2/8
Date : 23 June 2022

UKMB VERIFIED 28 JUN 2022 Universiti Kebangsaan Malaysia Herbarium

Maryam Syahidah binti Azalan
Kulliyyah of Allied Health Sciences
International Islamic University Malaysia
25200 Kuantan
Pahang

Assalamu'alaikum Warahmatullahi Wabarakatuh

Dear Sr. Maryam,

PLANT SPECIES IDENTIFICATION

Kindly be informed that the plant specimen submitted for identification is confirmed as follows:

Voucher No.	Family Name	Scientific Name	Local Name
PIIUM 0334	Compositae	*Cosmos caudats* Kunth	Ulam raja

Student's name : Maryam Syahidah binti Azalan
Supervisor's name : Assoc. Prof. Dr. Radiah Abdul Ghani
Herbarium : Kulliyyah of Pharmacy
Person in-charged : Assoc. Prof. Dr. Norazian Mohd Hassan
Identified by : Dr. Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you and wassalam.

Sincerely,

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International Islamic University Malaysia
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Bandar Indera Mahkota
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DR. SHAMSUL KHAMIS
Botanist
Herbarium UKMB
Faculty of Science and Technology
The National University of Malaysia (UKM)
43600 Bangi
Selangor

KULLIYYAH OF PHARMACY

Our Ref. : IIUM/308/15/2/1/NMPC22-2/9
Date : 23 June 2022

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Herbarium

Maryam Syahidah binti Azalan
Kulliyyah of Allied Health Sciences
International Islamic University Malaysia
25200 Kuantan
Pahang

Assalamu'alaikum Warahmatullahi Wabarakatuh

Dear Sr. Maryam,

PLANT SPECIES IDENTIFICATION

Kindly be informed that the plant specimen submitted for identification is confirmed as follows:

Voucher No.	Family Name	Scientific Name	Local Name
PIIUM 0335	Fabaceae	*Clitoria ternatea* L.	Kacang telang

Student's name : Maryam Syahidah binti Azalan
Supervisor's name : Assoc. Prof. Dr. Radiah Abdul Ghani
Herbarium : Kulliyyah of Pharmacy
Person in-charged : Assoc. Prof. Dr. Norazian Mohd Hassan
Identified by : Dr. Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you and wassalam.

Sincerely,

ASSOC. PROF. DR. NORAZIAN BINTI MOHD HASSAN
Kulliyyah of Pharmacy
International Islamic University Malaysia
Jalan Sultan Ahmad Shah
Bandar Indera Mahkota
25200 Kuantan
Pahang

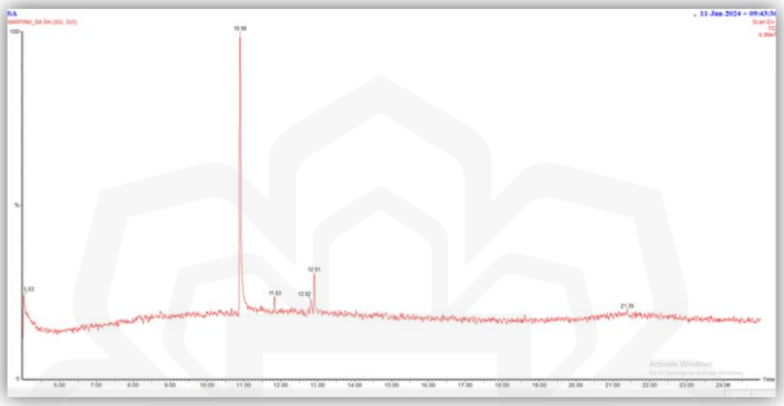
DR. SHAMSUL KHAMIS
Botanist
Herbarium UKMB
Faculty of Science and Technology
The National University of Malaysia (UKM)
43600 Bangi
Selangor

APPENDIX VI: GC-MS REPORT

QUALITATIVE REPORT

Client Name : Maryam Syahidah Binti Azalan
Sample Name : Clove
Analysis/ Services : Qualitative Analysis
Instruments : Gas Chromatography – Mass Spectrometry (GCMS)

CHROMATOGRAM



INTERPRATED DATA

Peak No.	Retention Time	Area	% Area	Expected Element
1	10.90	2914393.8	79.36	Eugenol
2	11.83	119599.8	3.26	Butylaldehyde, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl]-
3	12.82	265347.3	7.23	Butylaldehyde, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl]-
4	12.91	372807.3	10.15	Butylaldehyde, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl]-

Remark (if any): The chemical compounds were identified tentatively by matching their mass spectra data with those from the mass spectral search program for the NIST/EPA/NIH Mass Spectral Library (NIST MS Search version 2.0).

GC/MS PARAMETER

Brand	GC/MS Perkin Elmer	
Model	Clarus^{TM} 680/GC SQ8T/MS	
Column	Elite-5MS Capillary Column (Brand: Perkin Elmer)	
Temperature Program	Initial temperature: Ramp 1: Run Time: Mass Range:	40°C Hold 0 min 10°C/min to 250°C Hold 4 min 25 min 50 to 600 m/z

Prepared by;

Che Muhamad Arif Che Malek
Assistant Science Officer
Department of Chemistry
Kulliyyah of Science
International Islamic University Malaysia
Kuantan Pahang

APPENDIX VII: LC-MS REPORT

1. DEVELOPMENT OF LCMS QTOF QUALITATIVE METHOD

Instrument

The LC/MS-QTOF system used in this study comprised of an Agilent 1200 liquid chromatography system, equipped with a binary pump, a vacuum degasser unit, an auto sampler and 6520 quadrupole time of flight mass spectrometers with an electrospray ionization (ESI) source. Column used was Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm.

Chromatography and mass spectrometry method

Chromatographic separation was performed at 40°C using Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm with (A) 0.1% formic acid in dH_20 and (B) 0.1% formic acid in acetonitrile for positive mode. The gradient elution program was 0.00 – 18.00 min, 5 -95%(B); 18 to 23 minutes; 95% (B). 23.01 minutes;5% (B). The total run time is 30 minutes. The LC condition was re-equilibrated for 2 minutes before starting the new injection. The sample injection volume was set at 2 µl and the flow rate of the mobile phase was set at 0.25 mL/min.

Mass spectrometer was operated in positive electrospray ionization (ESI) mode with optimum gas temperature at 325°C, gas flow at 11 L/min and nebulizer at 35 psi, respectively.

Sample

The sample was provided by the customer. The sample (20 mg) was dissolved in 2 mL of 70% ethanol to produce the working concentration of 10 mg/mL. The sample was vortexed for 60 seconds until completely dissolved. Then the sample was further diluted to a concentration of 2 mg/mL. After that, the sample were filtered using 0.22 µm pore size syringe filter. The sample was centrifuged at 10,000 rpm for 15 minutes. The supernatant of solution was transferred into 50 µL insert before being injected into LC/MS-QTOF.

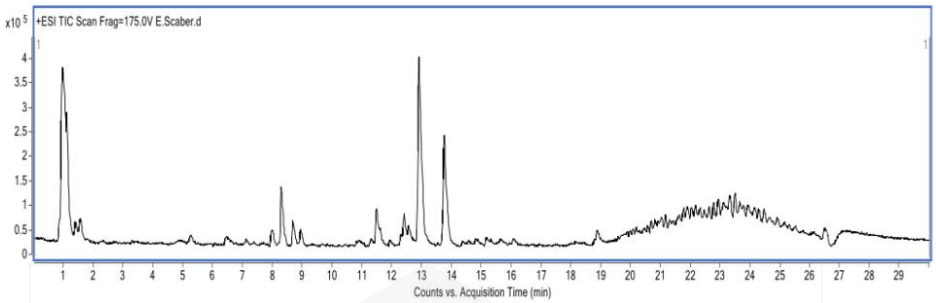
Data Analysis

Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA) was used for the data analysis (MS data (.d)). The chromatographic profiles were analyzed based on the accurate mass.

2. RESULTS

Chromatograms

1) E.Scaber



This report was

Prepared by,	Checked by,	Endorsed by,
Mohammad Zulfadhly Jan Jam Senior Asst. Research Officer iPROMISE, UiTM Date: 05.07.2024	Dr. Mohd Salleh Rofiee Fellow iPROMISE, UiTM Date: 09.07.2024	Prof. Dr. Hjh. Farida Zuraina Mohd Yusof Director iPROMISE, UiTM Date: 10.07.2024

APPENDIX VIII: GENE SEQUENCES VALIDATION

LIGO

macrogen singapore
(Ju Lin TAN)
Bukit Jalil, 57000 Kuala Lumpur Selangor
C-17-2, Jalil Link, 2, Jalan Jalil Perkasa 3
57000

HO00244215
Order Date :2023-07-04
Packing date :
Page :1/3

Oligo	ODC_F						
SEQ	5'-AAAACATGGGCGCTTACAC-3' (19mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
47	5805.8	5795.3	7	32	0.05	57.4	
vol. for 100pmol/ul	Purification		Modification				
320	MOPC				LOT No.	230704-029-F7	

Oligo	ODC_R						
SEQ	5'-TGGAATTGCTGCATGAGTTG-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
45	6203	6192.4	6.7	30	0.05	57.4	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC				LOT No.	230704-029-F8	

Oligo	SAT1_F						
SEQ	5'-CACCCCTTTTACCACTGCCT-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
55	5924	5914.8	5.6	30	0.05	63.1	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC				LOT No.	230704-029-F9	

Oligo	SAT1_R						
SEQ	5'-TGCCAATCCACGGGTCATAG-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
55	6102	6087.8	6.5	30	0.05	62.7	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC				LOT No.	230704-029-F10	

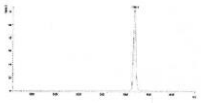
Humanizing Genomics macrogen

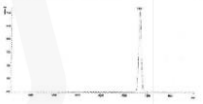
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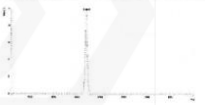
LIGO

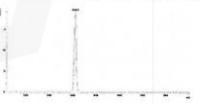
macrogen singapore
(Ju Lin TAN)
Bukit Jalil, 57000 Kuala Lumpur Selangor
C-17-2, Jalil Link, 2, Jalan Jalil Perkasa 3
57000

HO00244215
Order Date :2023-07-04
Packing date :

Oligo	OZA1_F						
SEQ	5'-TCTCCCTCCACTGCTGTAGTAACC-3' (24mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
54	7199.8	7188.5	7.1	30	0.05	65.3	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC					LOT No.	230704-029-F11

Oligo	OAZ1_R						
SEQ	5-GTTGAGAATCCTCGTCTTGTCGTT-3' (24mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
45	7340.8	7324.1	7.4	30	0.05	61.3	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC					LOT No.	230704-029-F12

Oligo	GAPDH_F						
SEQ	5-TGAGAACGGGAAGCTTGTCA-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
50	6206	6188.9	7	30	0.05	61.5	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC					LOT No.	230704-029-G1

Oligo	GAPDH_R						
SEQ	5-TGGACTCCACGACGTACTCA-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
55	6062	6045.8	6.4	30	0.05	63	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC					LOT No.	230704-029-G2

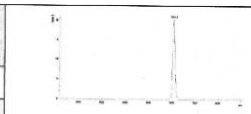
Humanizing Genomics macrogen

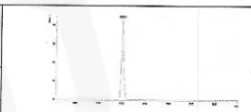
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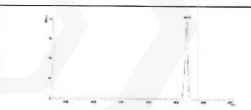
LIGO

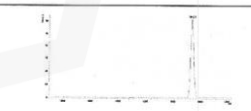
macrogen singapore
(Ju Lin TAN)
Bukit Jalil, 57000 Kuala Lumpur Selangor
C-17-2, Jalil Link, 2, Jalan Jalil Perkasa 3
57000

HO00244215
Order Date :2023-07-04
Packing date :
Page :3/3

Oligo	ACTB_F						
SEQ	5'-GTCATTCCAAATATGAGATGCGT-3' (23mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
39	7062.6	7041.1	7.8	30	0.05	56.5	
vol. for 100pmol/ul		Purification		Modification			
300		MOPC					LOT No. 230704-029-G3

Oligo	ACTB_R						
SEQ	5'-GCTATCACCTCCCCTGTGTG-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
60	6020	6005.8	5.8	30	0.05	63.5	
vol. for 100pmol/ul		Purification		Modification			
300		MOPC					LOT No. 230704-029-G4

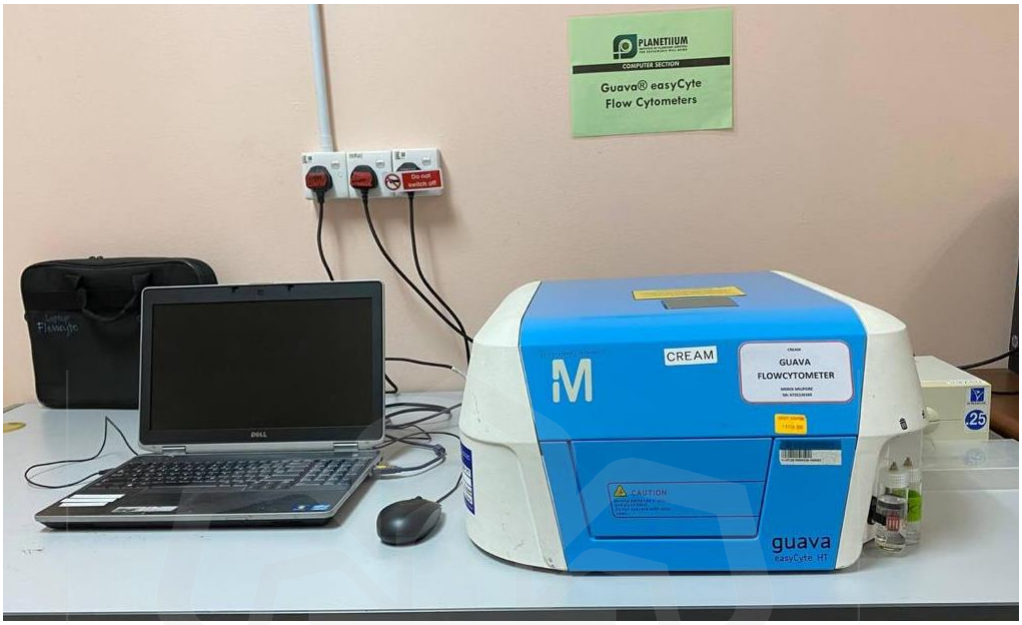
Oligo	SMOX_F						
SEQ	5'-CCGCAGACTTACTTCCCCG-3' (19mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
63	5684.8	5673	5.9	32	0.05	64.2	
vol. for 100pmol/ul		Purification		Modification			
320		MOPC					LOT No. 230704-029-G5

Oligo	SMOX_R						
SEQ	5'-GAGAGGGTCATCCGCACTG-3' (19mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
63	5853.8	5842	6.7	32	0.05	64	
vol. for 100pmol/ul		Purification		Modification			
320		MOPC					LOT No. 230704-029-G6

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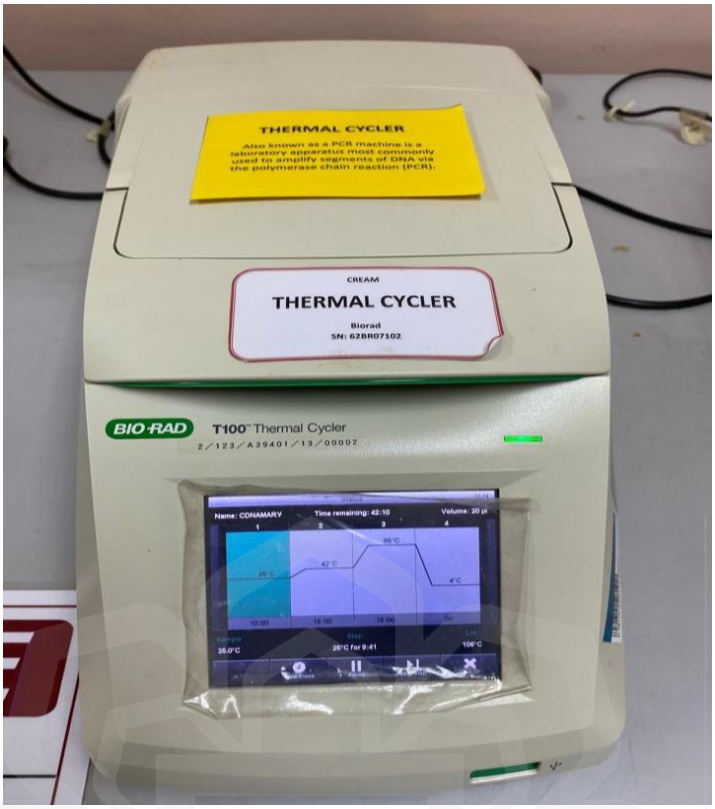
APPENDIX IX: LABORATORY FACILITIES



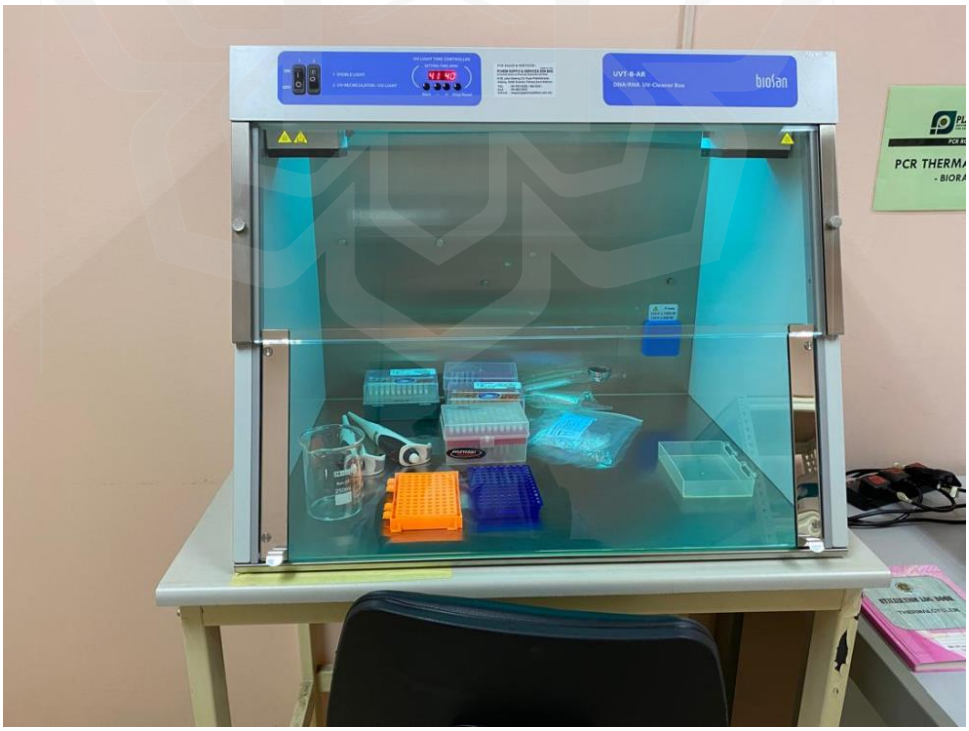
(1) Flowcytometer



(2) Real-time PCR



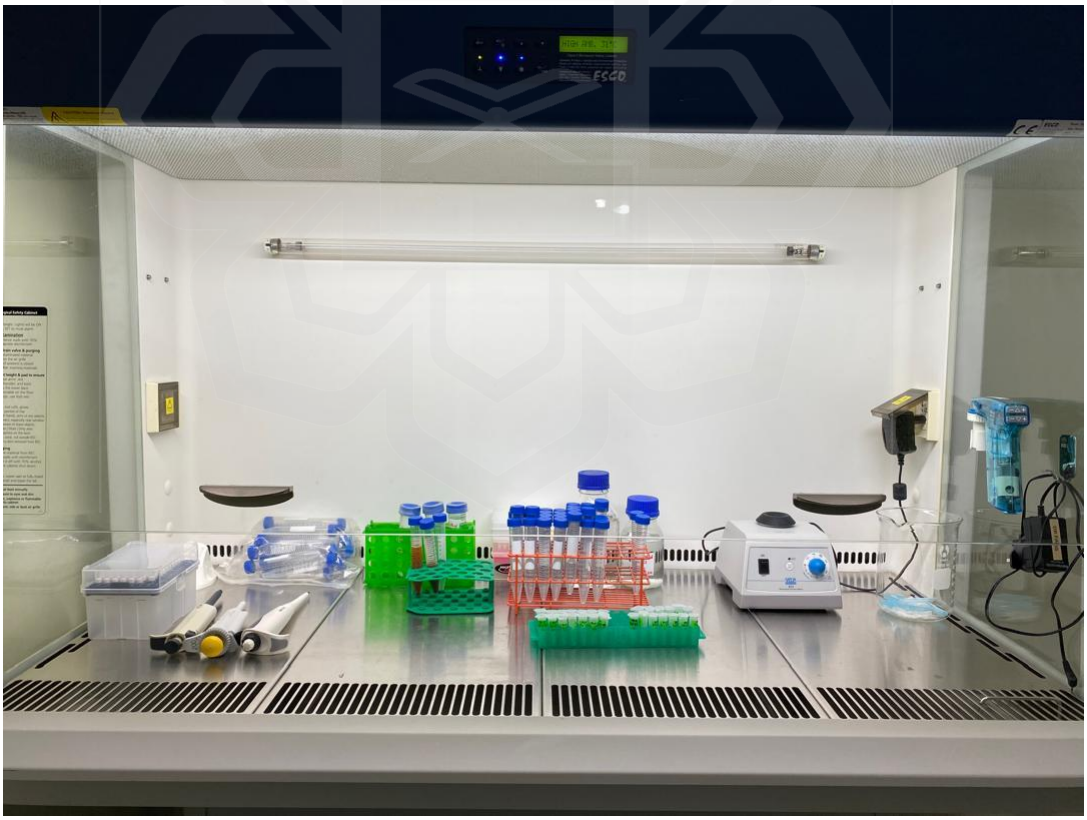
(3) Thermal cycle (for cDNA synthesis)



(4) PCR hood



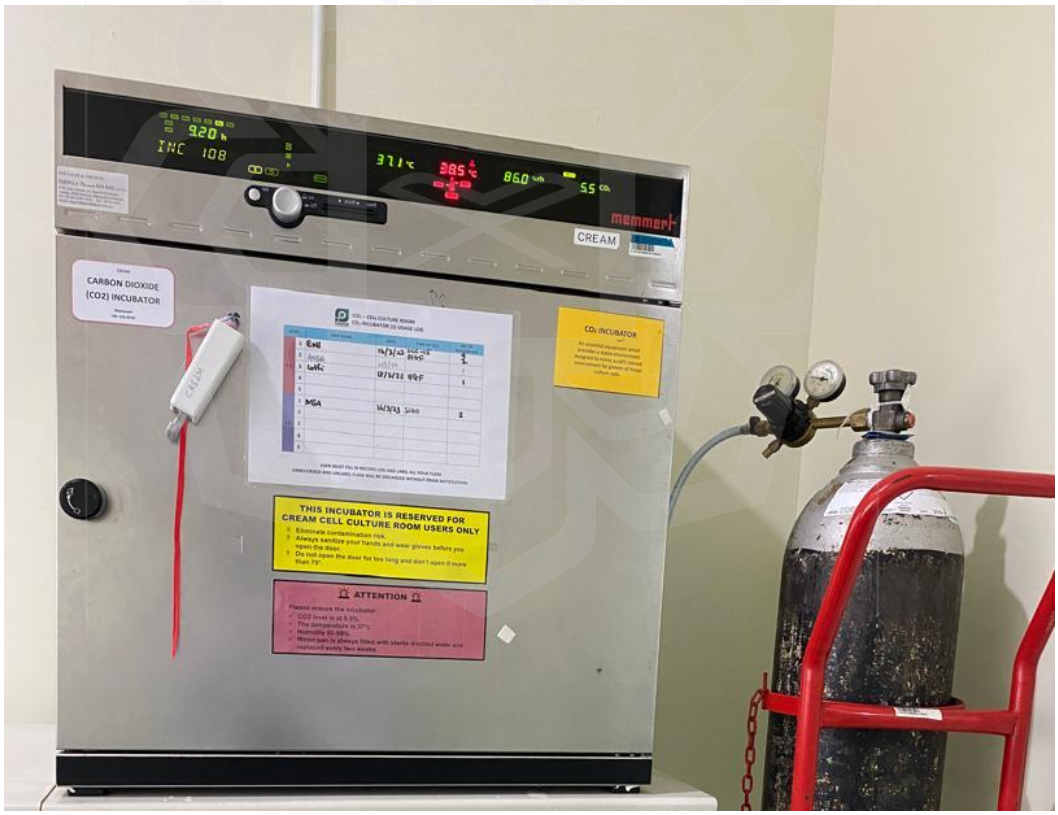
(5) HPLC



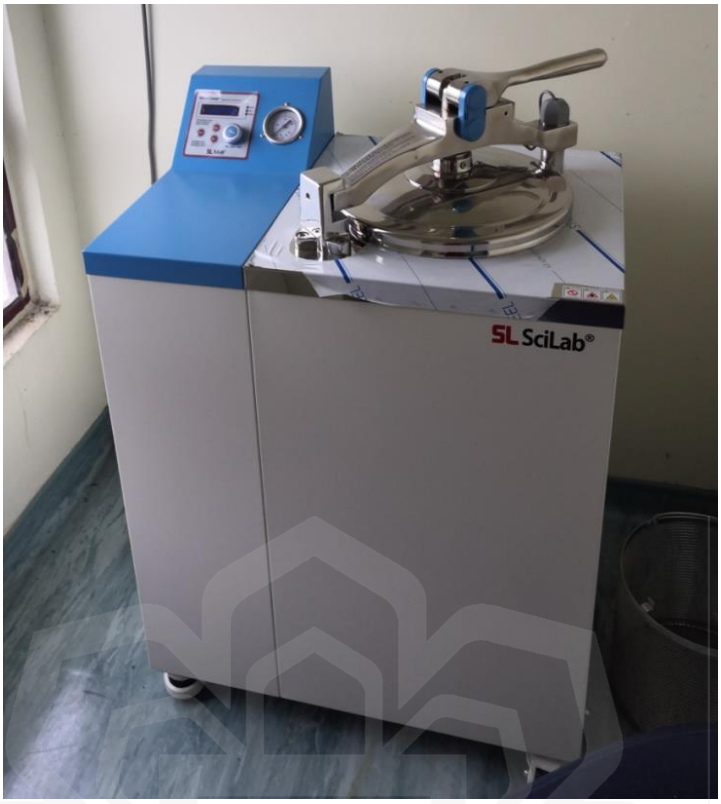
(6) Cell culture hood



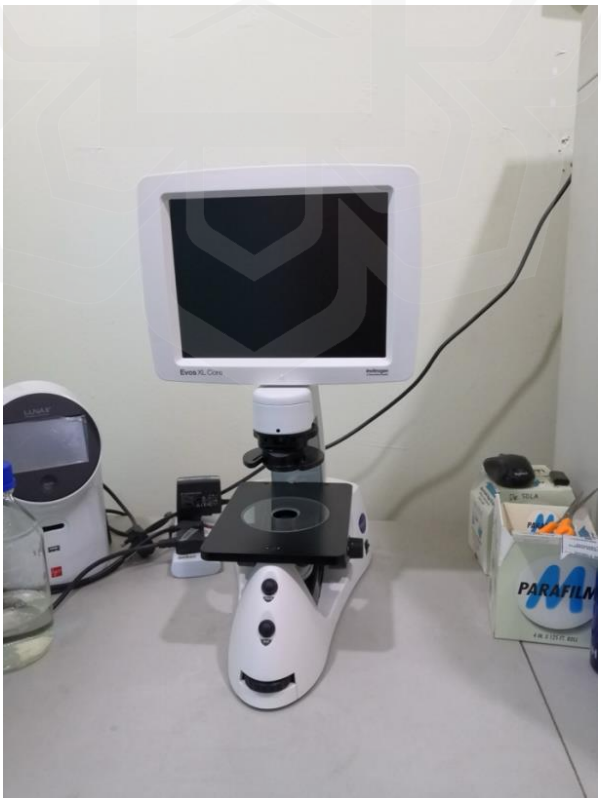
(7) Microplate reader



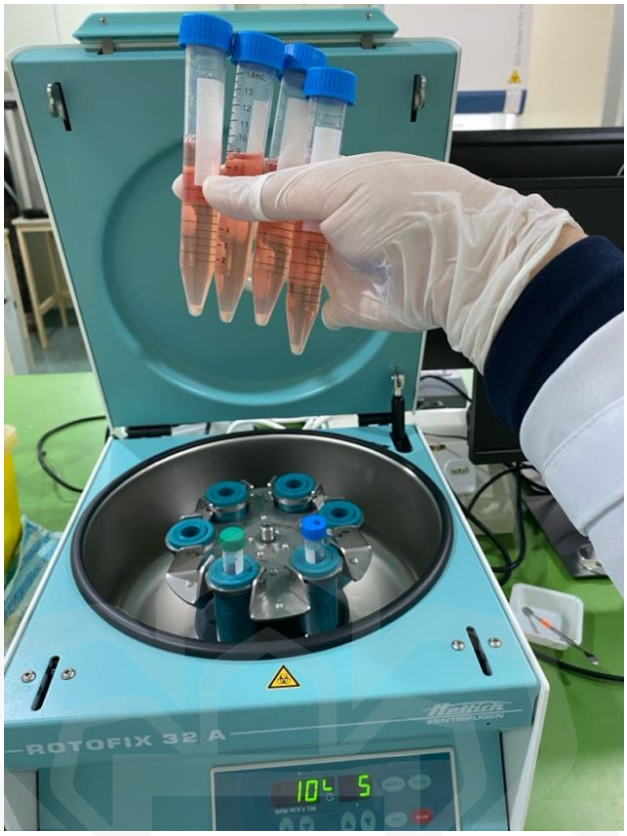
(8) Incubator



(9) Autoclave machine



(10) Invitrogen EVOS Microscope



(11) Centrifuge machine



(12) Liquid Nitrogen Tank

GLOSSARY

Apoptosis. A programmed cell death mechanism that eliminates damaged or unwanted cells, preventing abnormal cell proliferation and maintaining tissue homeostasis.

Benzoylation. A chemical process used to derivatize amines, such as polyamines, by attaching a benzoyl group. In this study, benzoylation was employed as a pre-column derivatization method for HPLC analysis of polyamines.

Caspase. A family of cysteine-aspartic proteases involved in initiating and executing apoptosis, inflammation, and other cellular processes.

Caspase 3. An executioner caspase that dismantles cellular components during the final stages of apoptosis.

Caspase 8. An initiator caspase in the extrinsic apoptotic pathway, activated by death receptors such as Fas and TRAIL.

Caspase 9. An initiator caspase in the intrinsic apoptotic pathway, activated by mitochondrial signalling and cytochrome c release. These assays measure the enzymatic activity of specific caspases to confirm apoptosis mechanisms.

Cervical Cancer. A malignant neoplasm originating in the cervix, often associated with persistent infection by high-risk human papillomavirus (HPV) types.

Chemoprevention. The use of natural or synthetic substances to prevent, delay, or reverse the initiation or progression of cancer. According to the National Cancer Institute (NCI), effective chemopreventive agents demonstrate minimal toxicity and significant efficacy in tumour suppression.

Cytotoxicity. The quality of being toxic to cells, often assessed through assays like MTT to determine a compound's ability to kill or inhibit cell growth.

DFMO (Difluoromethylornithine). A specific inhibitor of ornithine decarboxylase (ODC) that impairs polyamine biosynthesis, exerting cytostatic effects in cancer therapy.

Etoposide. A chemotherapeutic agent that inhibits topoisomerase II, leading to DNA damage and cell cycle arrest. Used as a positive control in this study, etoposide is a widely employed cancer treatment known for its pro-apoptotic effects.

Extrinsic Apoptotic Pathway. A cell death pathway triggered by the activation of death receptors, such as Fas and TRAIL, on the cell surface, leading to caspase 8 activation and apoptosis.

Flavonoids. Plant-derived polyphenolic compounds known for their antioxidant, anti-inflammatory, and anticancer effects.

Gene Expression. The process through which genetic information encoded in DNA is transcribed into RNA and translated into proteins or functional molecules.

HPLC (High-Performance Liquid Chromatography). An advanced analytical technique used to separate, identify, and quantify compounds in a mixture. In this study, HPLC was used for quantifying polyamines in treated and untreated cells.

HEK 293T Cells. Human embryonic kidney cells used as a normal cell line in cytotoxicity studies to evaluate the selective toxicity of treatments.

High-Risk Human Papillomavirus (HPV). A group of HPV genotypes, including HPV-16 and HPV-18, that are strongly associated with cervical carcinogenesis. HPV-16 is the most prevalent type globally and in Malaysia, contributing to cervical cancer progression.

IC_{50}. The half maximal inhibitory concentration of a compound, indicating the concentration required to reduce cell viability or activity by 50%.

Intrinsic Apoptotic Pathway. A cell death mechanism mediated by mitochondrial signals, leading to the release of cytochrome c and activation of caspase 9.

LC-MS (Liquid Chromatography-Mass Spectrometry). A sophisticated analytical technique for identifying and quantifying chemical compounds in biological samples, used for phytochemical profiling in this study.

Lowry Assay. A colorimetric assay used to quantify total protein concentration in a sample, based on the reaction of proteins with Folin-Ciocalteu reagent.

MTT Assay. A colorimetric assay that assesses cell viability by measuring mitochondrial activity in live cells, commonly used to evaluate cytotoxicity.

National Cancer Institute (NCI) Cytotoxicity Guidelines: Classification of natural product cytotoxicity based on IC_{50} values: Highly active: ≤ 20 μg/ml; Moderately active: 21–200 μg/ml; Weakly active: 201–500 μg/ml; and Inactive: > 501 μg/ml.

Ornithine Decarboxylase (ODC). A rate-limiting enzyme in the polyamine biosynthesis pathway, converting ornithine to putrescine, a precursor for spermidine and spermine.

OAZ1 (Ornithine Decarboxylase Antizyme 1). A regulatory protein that inhibits the activity of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis. OAZ1 also promotes the degradation of ODC, thereby reducing intracellular polyamine levels. Its upregulation serves as a feedback mechanism to maintain polyamine homeostasis, particularly in cancer cells where polyamine metabolism is often dysregulated.

Polyamines. Small organic cations (putrescine, spermidine, and spermine) essential for DNA stabilization, RNA transcription, protein synthesis, and cell proliferation. Dysregulated polyamine metabolism is a hallmark of cancer.

Polyamine Biosynthesis. The process of producing polyamines through enzymatic reactions involving ODC, spermidine synthase (SPDS), and spermine synthase (SPMS).

Polyamine Catabolism. The breakdown of polyamines via enzymes such as spermidine/spermine N1-acetyltransferase (SSAT) and spermine oxidase (SMOX), which regulate intracellular polyamine levels.

Phytochemicals. Natural bioactive compounds found in plants, such as flavonoids, terpenoids, phenolics, and alkaloids, with potential therapeutic effects.

Putrescine. The simplest polyamine, serving as a precursor for spermidine and spermine in polyamine biosynthesis. It is involved in cellular growth and proliferation.

qPCR (Quantitative Polymerase Chain Reaction): A molecular biology technique used to amplify and quantify DNA, allowing precise measurement of gene expression levels.

Reactive Oxygen Species (ROS): Chemically reactive molecules containing oxygen that can induce oxidative stress, contributing to cellular damage and apoptosis in cancer cells.

SiHa Cells. A human cervical cancer cell line derived from squamous carcinoma, characterized by the presence of two integrated copies of HPV-16, used to study cervical cancer mechanisms and therapeutic interventions.

SMOX (Spermine Oxidase). An enzyme involved in polyamine catabolism that oxidizes spermine into spermidine and generates hydrogen peroxide, contributing to oxidative stress and apoptosis.

Spermidine. A polyamine derived from putrescine and a precursor to spermine, essential for cellular growth and stability.

Spermine. A polyamine derived from spermidine, playing a critical role in stabilizing DNA structure and regulating cellular functions.

Spermidine/Spermine N1-Acetyltransferase (SSAT). An enzyme that acetylates spermidine and spermine, facilitating their degradation or export from cells, a critical process in polyamine catabolism.

Terpenoids. A diverse group of natural compounds derived from terpenes, known for their anticancer, anti-inflammatory, and antimicrobial properties.

TRAIL (Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand): A death receptor ligand that binds to TRAIL receptors on the cell surface, triggering the extrinsic apoptotic pathway.

TUNEL Assay: A laboratory technique used to detect DNA fragmentation, a hallmark of apoptosis.

PUBLICATIONS

Natural Product as Chemoprevention from *Maqasid Shariah* Perspective: A Narrative Review. Date of publication: 2023. Revelation and Science.

The roles of polyamines in Malaysian herbs as a chemoprevention strategy: A Scoping Review. Date of publication: 2023. Asian Pacific Journal of Molecular Biology and Biotechnology.

An Integrative Review on Female Reproductive Pathophysiology according to Islamic Perspective. Date of Publication: April 2024. Journal Al-Sirat.

Chemoprevention Effects Through Polyamines Modulation Induced by *Clinacanthus nutans* in Human Lung Adenocarcinoma Cells (A549). Date of Publication: 2023. Malaysian Journal of Medicine and Health Sciences.

Extended Abstract: The Cytotoxicity and Polyamines Regulation Induced by *Momordica Charantia* and *Gynura Procumbens* in Human Lung Adenocarcinoma Cells, A549. Accepted: 30th July, 2023. 3rd International Conference on Medical Science Technology (ICMST 2022).