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THE ANTIBACTERIAL EFFECT OF SELECTED MEDICINAL PLANT EXTRACTS AGAINST MULTI-DRUG RESISTANCE *Staphylococcus aureus* (MRSA) USING 2 DIMENSIONAL ELECTROPHORESIS (2-DE)

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biotechnology)

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JULY 2010

ABSTRACT

Plant parts from 15 species were collected for anti-MRSA screening. Crude extraction was carried out using ethanol and water. The yield of the ethanol extracts ranged from 5.2% (w/w) to 38% (w/w) while for water soluble extracts, the yield ranged from 4.5% (w/w) to 26.7% (w/w). The screenings for anti-MRSA activity were performed in 96-well microtiter plate using micro broth dilution method. The reference strain used was ATCC33591, and Vancomycin was used as the positive control. Out of 30 crude extracts which were screened for anti-MRSA activity, 17 extracts were found to be active. The MIC and MBC level obtained was as low as 0.3 µg/µL for 3 crude extracts, namely the ethanol extract of *Macaranga gigantea,* and both ethanol and water soluble extracts of *Quercus infectoria*. These 3 extracts were screened for the presence of major heavy metal elements: Mercury (Hg), Lead (Pb), Arsenic (As) and Cadmium (Cd). These elements were the most common major heavy metal elements screened in herbal products in the market. The water soluble extract of *Q. infectoria* and the ethanol extract of *M. gigantea* passed the permissible level according to Malaysia Poison Act 1952. These 2 extracts were tested for animal toxicity by administration to mice and were evaluated at oral gavage dosages of 0, 10, 200, 1000, 2000 and 5000 mg/kg bodyweight. From this test, the water soluble extract of *Q. infectoria* showed 0% mortality for all dosages. For ethanol extract of *M. gigantea*, death of mice were first seen at dose of 2000 mg/kg bodyweight with 10% mortality and followed by 30% mortality of mice in dose of 5000 mg/kg bodyweight. No death in mice was observed in lower level of dosages. However, median lethal dose (LD_{50}) for ethanol extract of *M. gigantea* is higher than 5000 mg/kg bodyweight. Based on it being not toxic to mice and the MIC obtained, the water soluble extract of *Q. infectoria* was selected for general profiling by HPLC for a general chemical fingerprint. Then, 2D PAGE profiling of treated and non-treated MRSA with the water soluble extract of *Q. infectoria* were performed to investigate any differences of protein expression. By using the pI 3-10 gel setting, 2D-PAGE gel images obtained detected some 157 protein spots in non-treated MRSA while there were only 13 protein spots detected in treated MRSA. This finding also reveals the potential use of proteomics *in-vitro* in the evaluation of the response to anti-MRSA agent in terms of quantification of protein expression after the treatment. This study also suggests that the water soluble extract of *Q. infectoria* is a potential anti-MRSA candidate to be explored further for antibiotic development in the future.

ملخص البحث

وقد تم جمع أجزاء النبات من 15 نوعا لفحص لمكافحة هذه الجرثومة. وتم استخراج الخام من استخدام الايثانول والماء. تراوحت العائد من مقتطفات الايثانول بين 5.2 ٪ (وزن/وزن) إلى 38 ٪ (وزن/وزن) وحين لمقتطفات الذوبان في الماء تتراوح العائد ما بين 4.5 ٪ (وزن/وزن) إلى 26.7 ٪. وأجرى التصوير الشعاعي لمكافحة هذه الجرثومة الفعالة في لوحة 96 ميكروتتر الجيد باستخدام طريقة التخفيف الجزئي الصغرى. وكان يستخدم لسلالة المرجعية وحين تستخدم فانكوميسين كالتحكم الإيجابي. ومن 30 مقتطفات الخام التي تمّ فحصها لمكافحة هذه الجرثومة الفعالة، وجدت 17مقتطفات منها فعالة. وحصول درجة MIC وMBC كانا على مستوى أدني من 0.3 ميكروغرام/ميكرولتر لمدة ثلاث مقتطفات الخام، وهي استخراج الايثانول من (*Macaranga gigantea*) و الايثانول و مقتطفات الذوبان في الماء معا من (*Quercus infectoria*). لقد فحصت هذه ثلاث مقتطفات لوجود العناصر المعدنية الثقيلة الرئيسية: الرصاص (Hg)، والزئبق (Pb)، والزرنيخ (As)، والكادميوم (Cd) (سي دي). وكانت هذه العناصر هي معظم العناصر المعدنية الثقيلة الرئيسية فحصت في المنتجات العشبية في السوق. استخراج المياه الذائبة من (*Q. infectoria*) واستخراج الايثانول من (*M. gigantea*) مرت على المستوى المسموح بها وفقا لقانون السموم ماليزيا سنة 1952م. اختبرت هاتان مقتطفتان لسمية الحيوانية من الإدراة للفئران و قيّمت عن طريق الجرعات الشفوية من 0، 10، 200، 1000، 2000، 5000 ملغم/كغم من وزن الجسم. من هذا الاختبار، أظهرت استخراج المياه الذائبة من (*Q. infectoria*) بـــ 0 ٪ وفيات لجميع معدل الجرعات. و لاستخراج الايثانول من (*M. gigantea*) شوهدت وفاة الفئران أول مرة في جرعة من 2000 ملغم/كغم وزن الجسم مع 10 ٪ وفيات و تليها بـــ 30 ٪ وفيات من الفئران في جرعة من 5000 ملغم/كغم من وزن الجسم. ولم يلاحظ أي وفاة في الفئران في مستوى أدنى من الجرعات. ومع ذلك، ، جرعة مميتة متوسطة (LD50) لاستخراج الايثانول من (*M. gigantea*) أعلى من 5000 ملغم/كغم من وزن الجسم. استنادا إلى كونها ليست سامة للفئران و حصل MIC، واختارت استخراج المياه الذائبة من (*Q. infectoria*) لتحديد ملامح عامة من (هـــ بـــ لـــ ك) للبصمات الكيميائية العامة. ثم أجريت 2د صفحة للمعالجة وغير المعالجة (م ر س ي) مع استخراج المياه الذائبة من (*Q. infectoria*) للتحقيق في أي الاختلافات في التعبير البروتيني. باستخدام بي 3-10 وضع ج ي ل، وكشفت 2د-صفحة ج ي ل عن بعض 157 بروتين في غير المعالجة (م ر س ي)، في حين كانت 13 بروتين نقاط فقط مكشوفة في معالجة (م ر س ي). هذا الاستنتاج يكشف أيضا احتمال استخدام البروتينات في المختبر في تقييم الاستجابة لمكافحة عامل هذه الجرثومة (م ر س ي) من حيث القياس الكمي للتعبير البروتيني بعد العلاج. تقترح هذه الدراسة أيضا إلى أن استخراج المياه الذائبة من (*Q. infectoria*) هو مرشح لمكافحة هذه الجرثومة (م ر س ي) يمكن أن يتم استكشاف المزيد من المضادات الحيوية التنمية في المستقبل.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion it confirms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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This thesis was submitted to the Kulliyyah of Science and is accepted as fulfilment of the requirement for the degree of Master of Science.

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DECLARATION

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

Nor Datiakma Mat Amin

Signature……... Date……

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Affirmed by Nor Datiakma Mat Amin

…… ……..

Signature Date

ACKNOWLEDGEMENTS

In the name of God, Most Gracious. Most Merciful, Alhamdulillah, with Allah's permission I have completed the preparation of the thesis, which His help enables me to develop and prepare myself with knowledge of science & technology which is essential for the betterment of humankind.

My deepest gratitude and respect to my kind supervisory committee Assoc. Prof Dato' Dr. Syed Zahir Idid, Assoc. Prof Dr. Hamzah, Dato' Dr. Marzalina and Dr. Saeid Reza Doust Jalali for their advises, guides and support. Not forgotten, my appreciation to Dr Huzwah for her advice at the beginning of my study.

To my beloved family, my father, mother, family in-laws for their understanding and constant support throughout my study. For my husband Mohd Hamizi and my children, Deanna & Haziq, thank you for your love and patience with me in preparing this thesis.

Last but not least, I would like to express my thanks and gratitude to my colleagues in Drug Discovery Centre (DDC) especially to Ms. Aruna, Pn. Noraliza and Pn. Anee Suryani for their assistance, Pn. Nashatul Zaimah and Seed Technology Laboratory, Pn. Mastura Mohtar, Hj Abdul Rashih Ahmad and colleagues from Medicinal Plant Division, FRIM, for their good co-operation in completing this study.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

µg	Microgram
µL	Microlitre
2D-PAGE	2 Dimensional Polyacrylamide Gel Electrophoresis
As	Arsenic
Ca-MRSA	Community-associated MRSA
Cd	Cadmium
Cr	Chromium
Cu	Copper
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitiol
Fe	Ferum
FELDA	Federal Land Development Authority
FRIM	Forest Research Institute of Malaysia
GC	Gas Chromatography
Hg	Mercury
HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography–Photodiode Array Detection
HPLC-UV	High-Performance Liquid Chromatography–Ultra Violet
HPLC-MS	High-Performance Liquid Chromatography–Mass Spectrometry
IAA	Iodoacetamide
IEF	Isoelectric focusing
kg	Kilogram
LC-MS	Liquid Chromatography Mass Spectrometry
M	Molar
m	Metre
MALDI-TOF-MS	Matrix-Assisted Laser Ionization-Time of Flight Mass Spectrometry
MetRSA	Methicillin-Resistant *S. aureus*,
mg	Miligram
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal Inhibitory Concentration
mL	Milliliter
min	Minutes
mm	Millimeters
Mn	Manganese
MRSA	Multi-drug Resistance *Staphylococcus aureus*
ng	nanogram
Ni	Nickel
^{o}C	Degree Celcius
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development

Pb	Lead
rpm	Rotation per minutes
SDS-PAGE	Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis
Se	Selenium
TLC	Thin-Layer Chromatography
t_R	Retention time
UV	Ultra Violet
VRE	Vancomycin Resistance Enterococci
ng	Nanogram

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

There are increasing resistances of microorganisms to many current antimicrobial agents in the treatment of infectious diseases and there is a need to develop alternative drugs. One bacterium cited as the most common cause of nosocomial infections is the Multi-drug Resistance *Staphylococcus aureus* (MRSA) (Hardy, Hawkey, Gao and Oppenheim, 2004). The increasing prevalence of this super bug has led to a demand for new antibiotics that could be used to combat the infection.

Currently, Vancomycin is amongst the effective drug available to treat serious MRSA infection. Its use however is restricted due to its potential of causing nephrotoxicity effects besides being slow in action. Therefore, the need to explore and develop new anti-infective agents is urgently required.

Appreciating the fact that the Malaysian rainforest is a repository of valuable phytochemicals yet to be discovered, a study to search for potential anti-MRSA agent was initiated. The use of plants in traditional healthcare system is a common and popular practice in Malaysia, especially among the indigenous communities.

1.2 RESEARCH JUSTIFICATION

This study was carried out in an attempt to seek new antibiotics from plants with medicinal values for use in the treatment of MRSA infection.

1.2.1 Plant Candidate Selection

The plant candidates for this study were selected based upon their inhibitory and cidal activity through literature search and/or from their ethno botanical history in the treatment of bacterial infection (Strobel and Daisy, 2003). Random screening was also performed to seek for anti-MRSA candidates.

1.2.2 Solvent Selection

As described by Cowan (1999), methanol, ethanol and water are the common solvents used for active component extraction. Therefore, ethanol and water were chosen as the extractants in this study in view that the potential plant candidates identified to be further develop as commercialised medicinal plant product. The extractants' toxicity in bioassay is also highlighted by Eloff (1998) where water is non-toxic and ethanol is much safer than methanol.

1.2.3 Anti-MRSA Screening

The anti-MRSA screening assay is the key for this study. For this purpose, Minimal Inhibitory Concentration (MIC) test was performed to determine the crude extracts' efficacy against MRSA. MIC is considered as the "gold standard" for determining the susceptibility of organisms to antimicrobials and therefore used to judge the performance of all other methods of susceptibility testing (Andrews, 2001).

MIC is determined by using the twofold serial dilution as described by the National Committee for Clinical Laboratory Standards (NCCLS, 2006) at concentration ranging from 5 μg/μL to 0.0391 μg/μL in 96 well microtiter plates. Oxacillin was used to determine the susceptibility breakpoint of *S. aureus* and

vancomycin was included as positive controls. The negative controls of this anti-MRSA assay were bacteria suspension and extract-broth mixture.

Plant compounds are normally categorized as anti-microbial on the basis of susceptibility tests that produce Minimal Inhibitory Concentrations (MICs) in the range of 0.1 to 1 µg/µL (Tegos, Stermitz, Lomovskaya and Lewis, 2002; Gibbons, 2004). Concentration of 5 µg/µL has been set as the cut-off concentration for crude extracts of plant species, which is 5 times higher than that of pure compound taking into account the diluted quantity of active compound present in the crude extract.

The MRSA strain used in this study was ATCC33591 acquired from American Type Culture Collection and supplied by Anti-infective Laboratory, Program of Medicinal Plants, Division of Biodiversity and Biotechnology, FRIM. Strain ATCC33591 is the MRSA research material derived from American Type Culture Collection Bioresource Centre (Methicillin-Resistant and Methicillin-Sensitive Research Materials, 2009). Microbe strains from ATCC are preferably as they are widely used and well-characterized (Cos, Vlietinck, Berghe and Maes, 2006). In addition, MRSA also refers to isolates of *S. aureus* with an MIC of oxacillin ≥ 0.004 µg/µL (Swenson, Skov and Patel, 2007b; Mo and Wang, 1997).

1.2.4 Heavy Metal Test

Heavy metals were extracted from the samples using dry ashing and digestion with Nitric Acid. Four major heavy metal screened for were Lead (Pb), Arsenic (As), Cadmium (Cd) and Mercury (Hg) which are the common major elements screened in herbal products in the market. Heavy metals are a known contaminant or adulterant of many traditional remedies (Obi, Akunyili, Ekpo and Orisakwe, 2006). This test was conducted to check whether any sample collected at the particular location was

contaminated with heavy metal elements and thus, to exclude the contaminated sample from further screening. The safety level of heavy metal elements were compared to levels given in the Malaysian Poison Act 1952.

1.2.5 Animal Toxicity Study

This study was undertaken to provide data on the safety focusing on the oral acute toxicity of selected extract which had passed the previous studies given orally to albino mice. It is important to carry out toxicity studies on the crude extracts coming from medicinal plants even though the medicinal plants have been used or consumed for decades. Therefore, based on this, the short term toxicity study to determine the safety level of the selected plant extracts were assayed using mice in order to highlight any unrecognized toxic manifestation.

Research was conducted based on Guideline 420 (Acute Oral Toxicity-Fixed Dose Procedure) of Organisation for Economic Co-operation and Development (OECD). Mice were orally gavaged with selected plant extracts for seven consecutive days and mortality of the mice were observed for 14 days. According to Guideline 420, females were used for this because they are generally slightly more sensitive compared to male. Testing on male only required when the knowledge of the toxicological properties of the test chemical indicates that males are likely more sensitive than female. Therefore, justification on the need of conducting acute toxicity study on male should be provided.

According to the guideline, a minimum of 5 animals of one sex are used for each dose level investigated study and considered sufficient for the acute toxicity evaluation and minimum. Maximum dose volume of 5000 mg/kg of bodyweight used as the limit dose for this study and should not be exceeded. For reasons of animal

welfare concern, replication of experiment is not required (Organisation for Economic Co-operation and Development [OECD], 2001).

1.2.6 HPLC Profiling Study

HPLC profiling will stand as a general chemical fingerprint of the crude extract. This chemical fingerprint will serve as quality identification of the selected crude extract and thus can be used for comparison for the next extraction of the selected crude extract in the future if required.

1.2.7 Proteomics Analysis: 2D-PAGE Profiling

2D-PAGE profiling will be performed as a preliminary study in order to observe *in-vitro* protein expressions which could lead to knowledge of the mechanism of action. In this study, 2D-PAGE profiles were developed for non-treated and treated MRSA with the selected crude extract. Proteins separated were visualised by using silver staining method. Silver staining is more sensitive compare to other staining methods where the detection limit is as low as 0.1 ng protein per spot (Berkelman and Stenstedt, 1998). The image of protein maps will be analyzed using PDQuest version 7.3.0 software and the image of gels from the non-treated and treated organism will be compared in terms of numbers of protein spots detected in the pI 3-10 range.

1.3 RESEARCH OBJECTIVES

1.3.1 Main Objective:

i. To determine anti-MRSA potential of selected medicinal plants.

1.3.2 Sub-Objectives:

i. To determine the efficacy of the ethanol and water soluble extracts of medicinal plants on MRSA by using Minimal Inhibitory Concentration (MIC) tests.

ii. To perform heavy metal tests of selected medicinal plant species showing best activity from anti-MRSA assay.

iii. To perform acute animal toxicity study of selected crude extracts showing best activity from the anti-MRSA assay.

iv. To perform a general HPLC profile of one selected crude extract which shows the best result from animal toxicity study.

v. To perform 2-D PAGE analysis of treated and non-treated MRSA with selected crude extract showing the best result selected from animal toxicity study.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram positive coccus with "grape-like clusters" morphology. This bacterium is spherical in shape and grows in golden-yellow colonies. *S. aureus* is a facultative anaerobe and is commonly found on the skin of healthy people and are ordinarily harmless inhabitant of the human nasal tract. *S. aureus* is a very adaptable organism and an opportunistic pathogen. It can live in a wide variety or stressful environments (Dyke, 2003).

S. aureus develops resistance to antibiotics very fast. Antibiotics could be defined as natural chemotherapeutic agents that inhibit or abolish the growth of micro-organisms, such as bacteria, fungi, or protozoa, i.e. they are anti-bacterial, anti-fungal, or anti-protozoan.

S. aureus causes a variety of infections ranging from skin lesions (boils, styes) to more serious infections such as pneumonia, mastitis, phlebitis and meningitis. Most importantly it is a major cause of hospital (nosocomial) infections where it survives in an environment rich in antibacterial agents.

Newborns and nursing mothers have a higher tendency to be infected with staphylococcal infections. Other susceptible individuals are those infected by influenza, chronic bronchopulmonary disorders such as cystic fibrosis and pulmonary emphysema, leukemia, neoplasms, those who had undergone transplants, those with internal prostheses or other foreign bodies, those suffering from burns, chronic skin

disorders, those with surgical incisions, or suffering from diabetes mellitus, and people with indwelling intravascular plastic catheters (Gotz, 2004).

2.2. MULTIDRUG-RESISTANT *S. aureus* (MRSA)

The resistant strain of *S. aureus* to antibiotics was first called Methicillin Resistance *Staphylococcus aureus* (MRSA). This strain was commonly cited as a major hospital-acquired pathogen responsible for a wide range of infections (Cespedes, Miller, Quagliarello, Vavagiakis, Klein and Lowy, 2002; Gibbons, 2004; Hardy et al., 2004). However, the acronym MRSA is somewhat misleading because the semisynthetic β-lactam methicillin is no longer used to treat *S. aureus* infections.

The term MRSA is synonymous with Multidrug-Resistant *S. aureus* because many nosocomial MRSA strains are resistant to most commonly used antibiotics. Vancomycin is the last available drug to which this organism had remained uniformly sensitive until recent reports of low-level glycopeptide resistance and, very recently, the transfer of high-level vancomycin resistance from *Enterococcus* to *S. aureus*. Although new drugs, including linezolid and synercid, have recently been introduced to treat MRSA infections, there is a worrying lack of novel drugs in the pipeline (Foster, 2004).

Nosocomial infection is the terminology to describe infection that develops within a hospital or is produced by micro-organisms acquired during hospitalization, and whose incubation period begins in the hospital. Major types of nosocomial infections are Urinary Tract Infections, Surgical Wound Infections, Nosocomial Pneumonia and Nosocomial Bacteremia.

Bacteremia or blood poisoning or toxemia is the presence of bacteria in the blood. Hospital patients who are catheterized or who have been treated surgically have

a significantly higher rate of infection (Foster, 2004). Patients at the burn unit, the elderly and infants or any individual with a compromised or damaged immune system have a higher risk of the infection of MRSA.

Starting from 1989, hospitals have reported a rapid increase in vancomycin resistance in enterococci (VRE) (Rubin, Harrington, Poon, Dietrich, Greene and Adil, 1999). The situation became more serious when the first strain of *Staphylococcus aureus* with reduced susceptibility to vancomycin was reported from Japan in 1997. Soon thereafter, a report of two additional cases from the United States was published (Hiramatsu, Hanaki, Ino, Yabuta, Oguri and Tenover, 1997*)*.

Malaysia is not being excluded from MRSA cases and these bacteria are an important nosocomial pathogen in Malaysia. In 1985-1986, Lim (1998) conducted a survey in 14 hospitals and the prevalence rate was 10 to 25 percent. Putucherry, Chen and Dugdale (1972) reported the infection prevalence in local university to be as much as 3 percent in 1971. In 1991, the prevalence of MRSA isolates reported in a tertiary hospital in Malaysia was even higher at 35 percent (Cheong, Tan, Wong, Zainudin and Rahman, 1994). The consistent reports of the prevalence of MRSA indicate that MRSA is spreading in Malaysia and its prevalence as an endemic nosocomial pathogen is increasing.

However, since 1990, the situation has worsen as MRSA infections have been increasingly recognized in the community, and MRSA strains isolated from patients with community-associated cases have been called community-associated MRSA (Ca-MRSA) (Eady and Cove, 2003; Xiao, Galiana, Pedriera, Mowszowicz., Christophersen, Machiavello, Lope, Benaderet, Buela, Vicentino, Albini, Bertaux, Constenla, Bagnulo, Llosa, Ito and Hiramatsu*,* 2005).

The early reports of Ca-MRSA have been found in United States since 1980 and Canada since 1986 (Moellering, 2006; Hawks, Barton, Conly, Nicolle, Barry and Ford-Jones, 2007). The transmission mode of the MRSA is by contact with patients discharged from hospitals and spreading the infection to others by sharing items contaminated with MRSA. There is also the possibility of the bacteria arising though the *de novo* development of community strains. However, to date, no community acquired related infection has been documented in Malaysia (Chong, Chow, Afra, Zamberi, Nyi, Farida and Norlijah, 2006).

Most Ca-MRSA infections involve the skin and soft tissue infection. However, severe and sometimes fatal infections have been observed, including sepsis, necrotizing pneumonia, purpura fulminans, pyomyositis and necrotizing fascitis, even in healthy patients (Hawkes et al., 2007).

The prevalence of MRSA in both settings will continue to increase in time due to the constant selective pressure from antibiotic use (Gardam, 2000). This issue also emphasizes the importance of finding alternatives to existing antibiotics. The constant evolution of bacterial pathogens and the changing nature of healthcare needs will create opportunities for significantly improved agents (Shahidi Bonjar, 2004; Chavan, Joshi and Patwardhan, 2006).

2.3 ANTIBIOTIC

Antibiotic is a chemical substance produced by microorganisms that kill or inhibits the growth of another microorganism (Madigan, Martinko and Parker, 1997). Antibiotics are product of secondary metabolism. Most of antibiotics are natural products or semi-synthetic derivatives (Walsh, 2003).