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CHEMICAL AND PHARMACOLOGICAL STUDIES ON *PERESKIA BLEO* KUNTH. (CACTACEAE) AND *CHOISYA TERNATA* KUNTH. (RUTACEAE)

BY

IKARASTIKA RAHAYU ABDUL WAHAB

BEING A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACOGNOSY

AT

UNIVERSITY OF DUBLIN

TRINITY COLLEGE

UNDER THE SUPERVISION AND DIRECTION OF

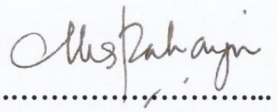
FABIO BOYLAN

B.Sc (Pharm), M.Sc. (Chem. Nat. Pro.), Ph.D. (Chem. Nat. Pro.)

MARCH 2013

DECLARATION

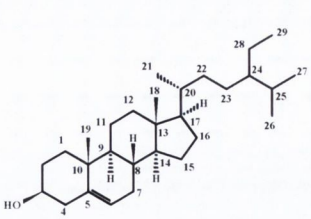
This thesis is submitted by the undersigned to the University of Dublin, Trinity College, for examination for the degree of Doctor of Philosophy. It has not been submitted for a degree at any other university. I myself carried out all the practical work except where duly acknowledged. This manuscript was written by me with the help of editorial advice from Dr. Fabio Boylan.



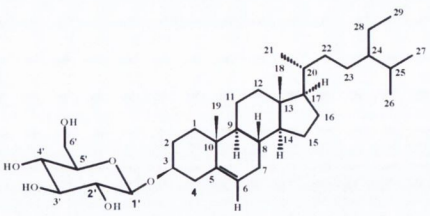
Ikarastika Rahayu Abdul Wahab

March 2013

COMPOUNDS ISOLATED FROM *PERESKIA BLEO* KUNTH. (CACTACEAE)



β-sitosterol



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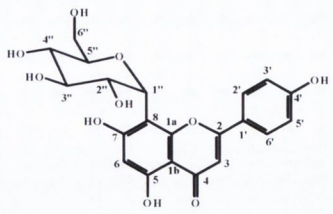

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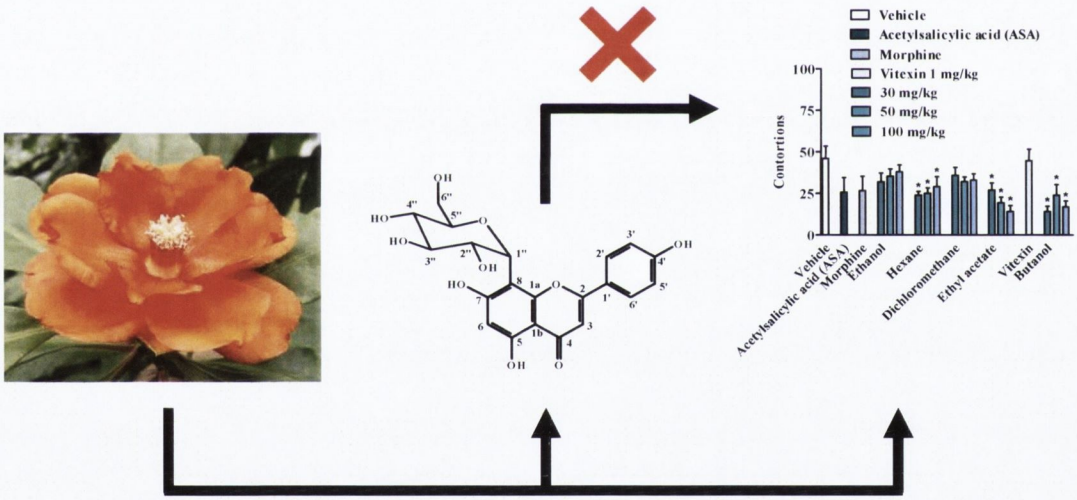
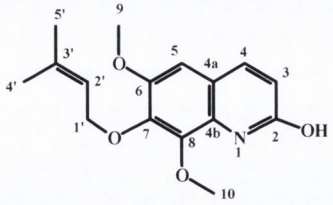
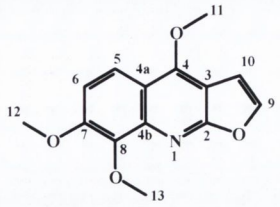


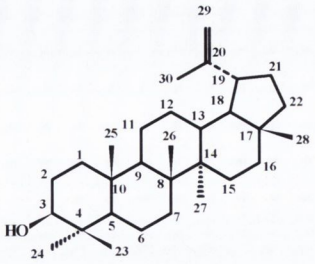
COMPOUNDS ISOLATED FROM *CHOISYA TERNATA* KUNTH. (RUTACEAE)



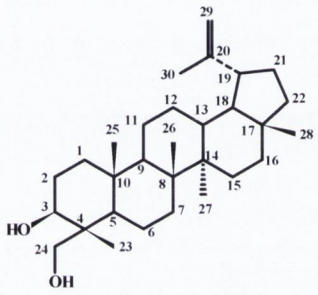
Choisyaternatine



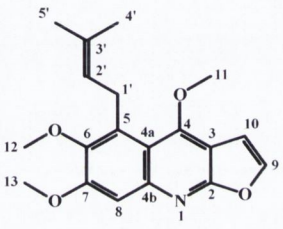
Skimmianine



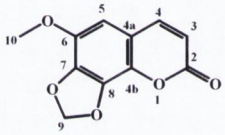
Lupeol



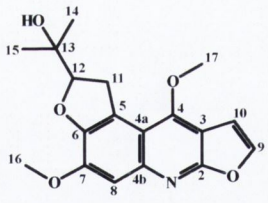
Lup-20(29)-en-3β,24-diol



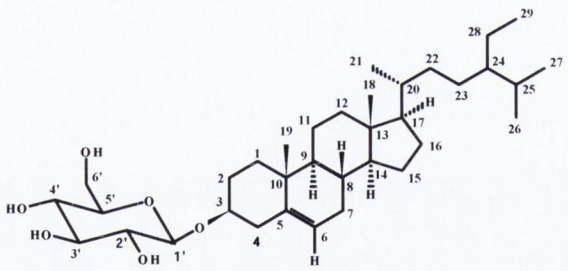
Tecleamaniensine A



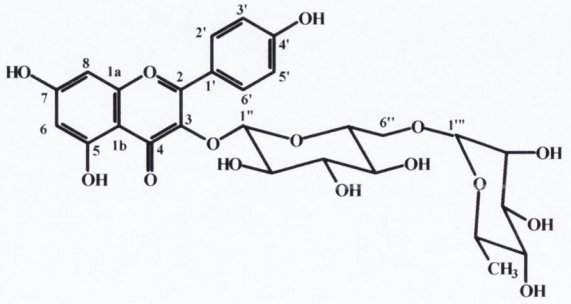
6-methoxyl-7,8-methylenedioxycoumarin



Choisyine

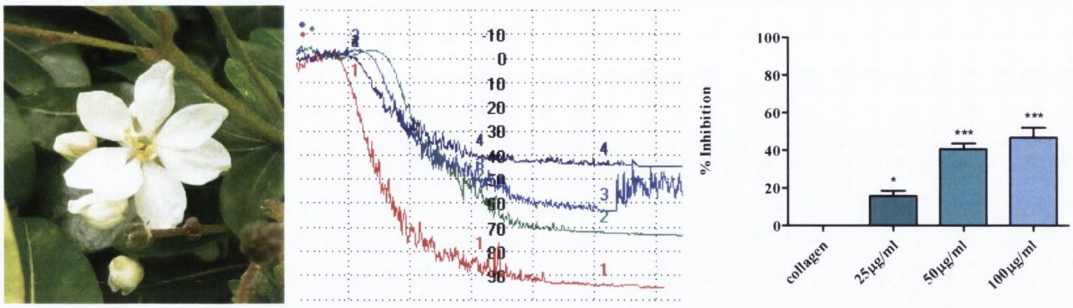


β-sitosterol glucoside

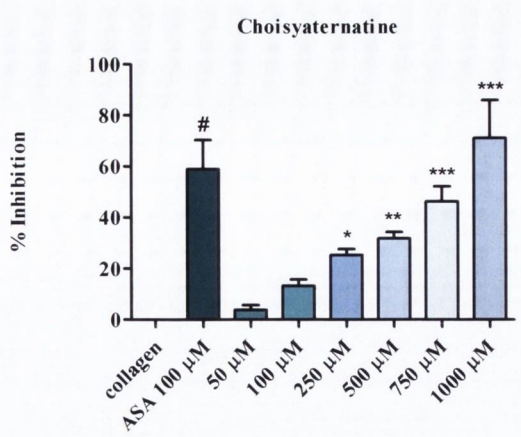


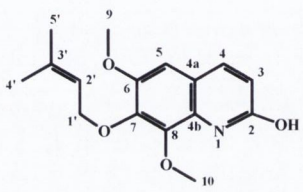
Kaempferol-3-*O*-rutinoside

MAIN PHARMACOLOGICAL FINDINGS FOR *CHOISYA TERNATA* KUNTH. (CACTACEAE)

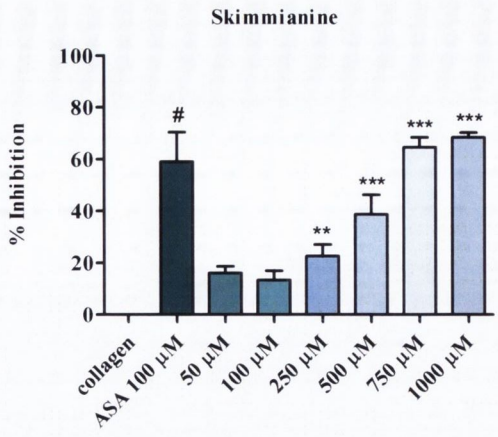


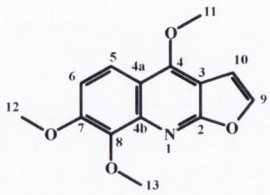
Effects of crude hexane extract on platelet aggregation. Light aggregometer. A) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + crude hexane extract 25 µg/ml; 3) collagen + crude hexane extract 50 µg/ml and 4) collagen + crude hexane extract 100 µg/ml.
B) Quantitative analysis of the inhibitory effect on collagen-induced platelet aggregation of crude extract with all concentrations tested. Data are expressed as mean ± S.D. *p<0.05 vs collagen; ***p<0.001 vs collagen.





IC_{50} = 698 µM





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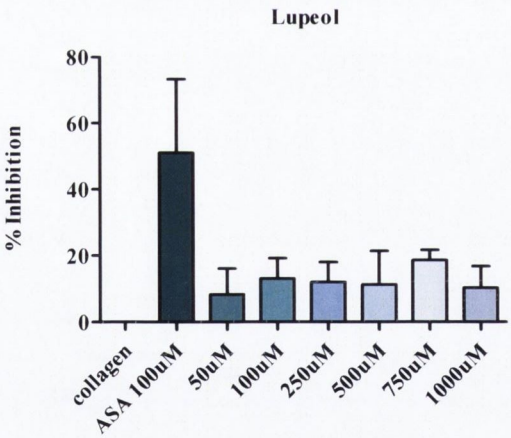
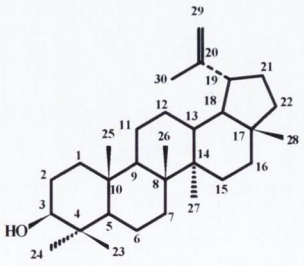
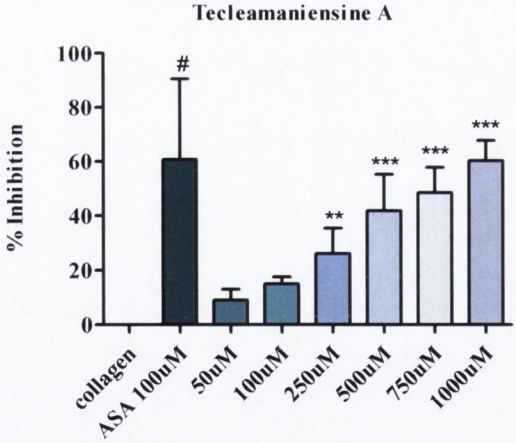
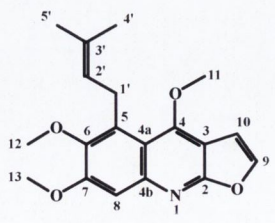


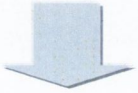


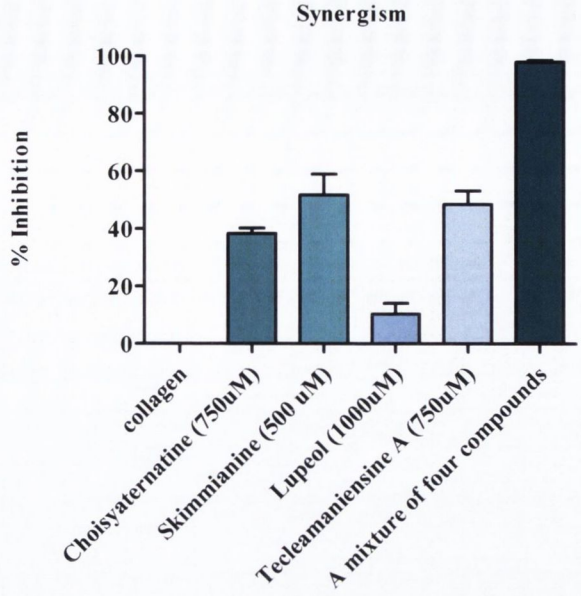
IC_{50} > 1000 µM





IC_{50} = 719 µM





However, lupeol (12), which has no action *per se*, intensifies the activity of the mixture, explaining at least in part, the great activity observed for the hexane extract.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisor, Dr. Fabio Boylan, who has stimulated my interest in pharmacognosy, particularly in phytochemistry. His ready advices, supports, patience and constructive critics of the project are gratefully acknowledged.

My gratitude would also be due to collaborators; Dr. Patricia Dias Fernandes at Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Brazil and Dr. Maria Jose Santos-Martinez at the School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin. Their coorporation and kindness in doing experiments, sharing experience and supports through allowing me to use facilities throughout this study are really appreciated.

My appreciation also goes to the Ministry of Higher Education, Malaysia, Universiti Malaysia Kelantan, Malaysia, technicians in the School of Pharmacy; Mr. Joseph Reilly and Mr. Brian Talbot, in the School of Chemistry; Dr. John O'Brien, Dr. Martin Feeney and Dr. Thomas McCabe, and also colleagues in Ireland and Malaysia, who direct or indirectly involved in collecting and analyzing data.

Last but not least, this thesis is dedicated to my family, especially my husband, Nik Azmil Nik Hisham and my son, Nik Affan Ikmil Nik Azmil, who are always with me during my ups and downs to complete the project. Last but not least, my mum, Hanisah Lazim, dad, Abdul Wahab Ngah, and brothers, Muhamad Bakhtiar and Muhammad Hafiz. Their love and support truly inspired me.

PRESENTATIONS AND PUBLICATIONS RELATED TO THIS THESIS

Poster presentations:

1. Cytotoxicity evaluation and antinociceptive activity of *Pereskia bleo* extracts. 33rd Joint Schools of Pharmacy, Research Seminar 2011, Royal College of Surgeon, Dublin, Ireland. April 2011. Ikarastika Rahayu Abdul Wahab and Fabio de Sousa Menezes.

2. Alkaloids of *Choisya ternata* Kunth. (Rutaceae) and their human platelet activity. International Conference on Natural Products Research, Grand Hyatt, New York, United States of America. July 2012. Ikarastika Rahayu Abdul Wahab, Nikki Siu Hai Wong, Maria Jose Santos-Martinez, Fabio Boylan.

3. Evaluation of antinociceptive activity of *Pereskia bleo* Kunth. International Conference on Natural Products Research, Grand Hyatt, New York, United States of America. July 2012. CC Guilhon, IRA Wahab, F Boylan, PD Fernandes.

Publications:

1. Antinociceptive activity of *Pereskia bleo* Kunth. (Cactaceae) leaves extracts. Accepted by the *Journal of Ethnopharmacology*. Ikarastika Rahayu Abdul Wahab, Carolina Carvalho Guilhon, Patricia Dias Fernandes and Fabio Boylan

2. Choisyaternatine, a new alkaloid isolated from *Choisya ternata*. Published in *Planta Medica*. Ikarastika Rahayu Abdul Wahab, Nikki Siu Hai Wong, Fabio Boylan

LIST OF ABBREVIATIONS

%	percentage
µl	microlitre
µm	micrometer
µM	micromolar
^{13}C	carbon (NMR)
^{1}H	proton (NMR)
Ace. A	acetic acid (antinociceptive assay)
AA	antioxidant activity (antioxidant assay)
Ar. A	arachidonic acid (antiplatelet assay)
A_B	blank absorbance
ADP	adenosine diphosphate
A_N	negative control absorbance
ANOVA	one-way analyses of variance
A_S	sample absorbance
ASA	acetylsalicylic acid
AUC	area under curve
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
br	broad
CC	column chromatography
CCDC	Cambridge Crystallographic Data Centre
cm	centimetre
COSY	Correlation Spectroscopy
COX	cyclooxygenases enzyme
d	doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC	effective concentration
EI-MS	electron ionisation mass spectroscopy
ELISA	enzyme-linked immunosorbent assay
et al.	'and others'
etc.	'and the rest'
ext.	extract
FE	flavonoid equivalent
FT-IR	Fourier Transform Infrared Spectroscopy
g	gram
GAE	gallic acid equivalent
GAEs	gallic acid equivalents
GC-MS	gas chromatography-mass spectroscopy
GI	gastrointestinal
h	hour
HCl	hydrochloric acid
HETCOR	heteronuclear correlation spectroscopy
HMBC	Heteronuclear Multiple-Bond Correlation Spectroscopy
HPLC	high performance liquid chromatography

HSQC	Heteronuclear Single-Quantum Correlation Spectroscopy
i.p.	Intraperitoneal injection
IB	increase in baseline
IC	inhibition concentration
J	coupling constant
kg	kilogram
L	litre
LOX	lipoxygenase enzyme
M	molar
m	multiplet (NMR)
MeOH	methanol
mg	milligram
MHz	megahertz
min	minutes
ml	mililitre
mm	milimetre
mp	melting point
n/d	not determined
NaOAc	Sodium acetate
NaOMe	Sodium methoxide
nm	nanometre
NMR	Nuclear Magnetic Resonance Spectroscopy
NSAIDs	non-steroidal anti-inflammatory agents
Ø	internal diameter
°C	degree celsius
OD	optical density
PAF	platelet activating factor
PBS	phosphate buffer saline
PGs	prostanglandins
ppm	parts per million
PRP	platelet-rich plasma
QE	quercetin equivalent
ROS	reactive oxygen species
rpm	rotation per minute
s	singlet (NMR)
S.D.	standard deviation
sp	species
t	time
t	triplet (NMR)
TLC	thin layer chromatography
TPC	total phenolic content
UV-Vis	Ultraviolet–Visible Spectroscopy
v/v	volume per volume
w/v	weight per volume
β	beta
γ	gamma
δ	delta
p	para

ABSTRACT

ABSTRACT

Phytochemical and pharmacological investigations of *Pereskia bleo* (Kunth) DC (Cactaceae) and *Choisya ternata* Kunth. (Rutaceae) fractions were carried out.

The chemical evaluation of *P. bleo* fractions resulted in the isolation and identification of two unreported compounds for the species, β-sitosterol glucoside (8) and vitexin (9) which were isolated from the dichloromethane and ethyl acetate fractions, respectively. β-sitosterol (1) was isolated from the hexane fraction. This is the first report of the occurrence of vitexin, a *C*-glucosylflavonoid in *Pereskia.* This finding is relevant in chemotaxonomy as it confirms the trend of Cactaceae being able to synthesize *C*-glycosylflavonoids, posing Cactaceae and Didieraceae with similar status both morphologically and chemotaxonomically.

Based on its popular uses among Malaysians, the fractions were evaluated for antioxidant, antinociceptive and anti-inflammatory activities. The results showed moderate pharmacological activity in all assays and can justify its use in Malaysian folk medicine as a drug to treat rheumatism, among other inflammatory conditions. The moderate analgesic activity of the ethyl acetate fraction is not due to the presence of vitexin.

The chemical evaluation of *Choisya ternata* fractions led to the isolation and identification of a new isoprenyl quinoline alkaloid, choisyaternatine (10) from the hexane fraction of *C. ternata* Kunth. (Rutaceae) leaves, together with the known compounds; skimmianine (11), lup-20(29)-en-3β-ol (lupeol) (12), lup-20(29)-en-3β,24-diol (13) and tecleamaniensine A (14). Four compounds; skimmianine (11), 6-methoxy-7,8-methylenedioxycoumarin (15), choisyine (16) and β-sitosterol glucoside (8) were isolated from the dichloromethane fraction while kaempferol-3-*O*-rutinoside (17) was isolated from the ethyl acetate fraction. Compounds 12, 13, 14 and 8 are reported for the first time in this species, while compound 10 is being described for the first time.

The structures of isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. The structure of choisyaternatine (10) was further confirmed based on X-ray analysis.

The hexane fraction, choisyaternatine (10), skimmianine (11), lupeol (12), tecleamaniensine A (14) and choisyine (16) were evaluated for their antiplatelet activity using whole human blood. Choisyaternatine, skimmianine and tecleamaniensine A only showed moderate inhibition of collagen-induced platelet aggregation in human platelets with the IC_{50} values of 698, 564 and 719 μM, respectively. The hexane fraction exhibited a good antiplatelet aggregation activity with the IC_{50} value in the range of 50-100 μg/ml,

while a mixture of the four isolated compounds from the hexane fraction showed inhibition almost 99%.

Additional studies are needed to investigate their action on the main platelet aggregation inducers involved in this process and therefore to elucidate the possible mechanism of action of these compounds.

INTRODUCTION

1. INTRODUCTION

Natural products have provided an endless supply of active compounds as source of medicines. An increasing number of natural products is being used in the traditional medicinal systems in many countries and alternative medicines for treatment of various diseases are getting more popular in the west. Traditional medicines, particularly herbal remedies, have been used for thousands of years in maintaining health and, in recent years, as an alternative or as a complement to modern medicine. This interest in drugs of plant origin is due to several reasons, such as conventional medicine can be inefficient or abusive. Incorrect use of synthetic drugs can also result in side effects and other problems. A large percentage of the world's population does not have access to conventional pharmacological treatment and folk medicine and ecological awareness suggest that natural products can be harmless (Rates, 2001).

Various ethnic groups around the world have acquired the use of a particular plant, through traditional beliefs and practices, from their elders, ancestors or by an indigenous people (Heinrich *et al.*, 2004). This later could direct researchers in the search for a new drug for the treatment of various diseases. However, certain considerations must be taken into account when it comes to the selection of a suitable plant for a pharmacological study as each ethnic group has its own concepts of health and illness, as well as different healthcare systems (Elisabetsky and Posey, 1986).

Besides the method described in the last paragraph, the search for potential plants as drugs could also be done through chemotaxonomic approach. Knowledge that a particular group of plants contain a certain class of natural products may be used to predict that taxonomically related plants may contain structurally similar compounds. This is highly useful when the chemistry and biological activity of a compound are well described and compounds with similar chemical structure are needed for further biological testing (Heinrich *et al.*, 2004).

Natural phytochemicals derived from dietary sources or medicinal plants have gained significant recognition in the potential management of several human clinical conditions (Neergheen *et al.*, 2009). Such discovery has led to the isolation of early drugs such as morphine which is still in use. Isolation and characterization of active compounds from medicinal plants continue today. More recently, drug discovery techniques such as chromatographic analysis have been applied to the standardization of herbal medicines in order to elucidate their analytical marker compounds (Balunas and Kinghorn, 2005).

Phytochemists prepare extracts from plant materials, subject these extracts to biological screening, and commence the process of isolation and characterization of the active compound(s) responsible for the pharmacological activity.

Plants have been an abundant source of highly effective phytochemicals which offer great potential in the fight against various ailments. According to The American Society of Pharmacognosy, pharmacognosy is defined as the study of the physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources (Balunas and Kinghorn, 2005). Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads.

Ethnophamacology has already played an important role in the development of conventional medicine and is likely to play a more significant role in the years to come. About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use, 252 drugs considered as basic and essential by the World Health Organisation (WHO) of which 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001).

According to the WHO, about three-quarters of the world's population relies upon traditional remedies, mainly herbs, for health care of its people and also to cure various ailments. The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs of plant origin in developing countries (Vulto and Smet, 1998). Plants can be used as therapeutic resources in several ways. They can be used as herbal teas or other homemade remedies, when they are considered as medicinal plants. They can be used as crude extracts or 'standardised enriched fractions' in pharmaceutical preparations, such as tinctures, fluid extracts, powder, pills and capsules, when they are considered as phytopharmaceutical preparations or herbal medicines (Rates, 2001).

The herbal medicine also sometimes called "traditional or natural medicine" existed in one way or another in different cultures and civilizations since ancient times and has been originally discovered through the study of traditional cures and folk knowledge of indigenous people (Gilani, 2005). However, the folkloric use of a plant can vary in different geographical origins due to the fact that the plants growing in different climatic conditions may have a different chemical composition of active principles (Gilani, 2005).

1.1 Medicinal plants and human health in Malaysia

Medicinal plants have traditionally been used in a variety of local herbal preparations by various ethnicities in many countries in the world, including Malaysia. However, the use of herbal medicines in Malaysia is not scientifically validated and often derived from traditional belief and experience. One of the commonest problems faced by local herbal industries is the inadequate standardisation and quality control in the preparation of herbal medicines (Nor Azah *et al.*, 1999).

In many developing countries, traditional medicine is still the mainstay of health care, and most of the drugs and cures used come from plants, traditional medicines were exploited for use in pharmaceutical products for new and effective remedies, or for use in treating many common and some uncommon conditions. Furthermore, Eastern countries, such as China and India, already have a well-established herbal medicines industry and Latin American countries have been investing in research programs in medicinal plants and the standardisation and regulation of phytomedicinal products, following the example of European countries, such as France and Germany (Rates, 2001).

The potential use of higher plants as a source of new drugs is still poorly explored. From the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties. In most cases, only pharmacological screening or preliminary studies have been carried out (Payne *et al.*, 1991).

1.2 Ethnopharmacology in Malaysia

Ethnopharmacology can be defined as "the interdisciplinary scientific investigation of biologically active substances utilized by humans" which combines aspects of botany, natural products chemistry, conventional pharmacology, pharmacognosy and medicine (Balunas and Kinghorn, 2005).

Malay Archipelago consists of the following countries: Brunei, Indonesia, Malaysia and the Philippines (Figure 1.1). These countries are known as reservoirs of herbal medicines. The complete pharmacological investigation on these plants needs to be performed as certain plants may contain active chemical constituents that after tests confirm their popular use by the population (Bakar *et al.*2000).

Figure 1.1: Malay Archipelago on the world map

Focusing on Malaysia, the number of plant species used for medical purposes could be much higher than reported. The knowledge on the indigenous use of plants was mostly passed on orally from one generation to another and has largely remained undocumented. In 1966, Burkill in his extensive compilation of the economic products of the Malay Peninsula recorded that not less than 1300 plants have been used in traditional medicine (Jantan, 2004).

Among Malaysians, the factors found to influence the use of herbal medicines are identified as gender, ethnicity, age and perceived health status (Aziz and Tey, 2009). The wide consumption of medicinal plants among its people has encouraged the researchers in Malaysia to engage in ethnopharmacological research. The approach taken by many research groups gradually shifted from pure phytochemical screening to include biological screening which involved subjecting plant extracts or isolates to various bioassays to confirm their biological activities. In the past five decades, medicinal plant research in Malaysia has been carried out mainly by researchers from government-funded universities and research institutes with little involvement of industries and multinationals. The Centre for Natural Products Research (CENAR), University of Malaya, for example, is very active in pursuing research on natural products from plant and animal source for medicinal purposes, as food additives and supplements, and as allelochemicals. Although natural products screening programmes are still actively ongoing in these institutions, there is yet a serious effort to embark on a systematic drug discovery programme at national level (Jantan, 2004).

Currently, the herbal product market in Malaysia is experiencing a tremendous growth. Malaysia consumes RM 1.2 billion (around 300 million euros) worth of imported herbal products annually (Jantan, 2004). The fact that, one in three Malaysian adults in

urban areas uses herbal medicines has highlighted the substantial health-economic implications medicinal plants use for Malaysia. Indeed, the herbal medicines industry was worth USD 2.5 billion in 2010 and the industry grew faster than the general economy at more than 15-20% per year (Aziz and Tey, 2009). However, most of them are not satisfactorily provided with information on their ingredients, indications, dosage, pharmacology, contraindications and possible adverse effects associated with prolonged use. There is concern for the lack of standardization of herbal preparations to guarantee their safety, quality and efficacy. Thus, there is an urgent need to conduct scientific research to provide experimental evidence for safety, efficacy and quality of herbal medicines while also investigating the plants as sources for new lead structures for drug development (Jantan, 2004).

In response to the increased use of medicinal plants by the Malaysian population, the government in its 7th Malaysian Plan had identified several strategies to enhance the development of the local herbal industry. In 1995, Forest Research Institute of Malaysia (FRIM) was given the mandate to set up a National Committee on Medicinal Plants (NCMP) which was comprised of representatives from the universities, research institutions, government agencies and the local herbal industry. Furthermore, in the 8th Malaysia Plan, the government had allocated research funds under the Intensification of Research in Priority Areas (IRPA) mechanisms to embark on research programmes to put some of the more widely used medicinal plants under close scientific and clinical scrutiny.

At present, five research groups comprising scientists from various academic and research institutions are actively involved in all aspects of herbal research on the abovementioned medicinal plants as follows: effective processing of medicinal plants for optimum production of herbal extracts; qualitative and quantitative analyses of herbal extracts for standardization and quality control purposes; pharmacognostic studies to develop monographs of medicinal plants for standard reference; *in vitro* and *in vivo* studies to determine biochemical, immunological, toxicological and pharmacological effects of herbal extracts; toxicological studies of herbal preparations; pharmacokinetic studies of herbal preparations; development of herbal medicines into modern pharmaceutical forms; clinical trials on standardized herbal preparations for various medical indications (Jantan, 2004).

In this study, a plant cultivated and widely used in Malaysia for the treatment of several conditions has been selected. *Pereskia bleo* is used for the treatment of cancer and as analgesic and anti-inflammatory for the maintenance of rheumatism. Several previous

works looked at the cancer-related diseases, but none took attention to its use as analgesic and anti-inflammatory agents. Besides, the Irish-cultivated plant, *Choisya ternata* was used as a tonic and appetizer, while its infusion has been used as antispasmodic and 'stimulative' agents by the native Mexican people. These plants are being studied in order to assess whether the pharmacological activities that have been reported for them is in fact justifiable.

1.3 AIMS OF THE STUDY

The main aim of this study is to provide scientific basis for the reputed efficacy of the Malaysian traditional medicinal plant *Pereskia bleo* Kunth. (Cactaceae), in the maintenance of rheumatism as possessing analgesic and anti-inflammatory activities and the cultivated Irish plant, *Choisya ternata* (Rutaceae) (a producer of skimmianine and other quinoline alkaloids) as an antiplatelet aggregant agent.

The specific aims are:

1. To isolate, purify and identify chemical constituents from *Pereskia bleo* and *Choisya ternata*,
2. To evaluate the antioxidant activity of *P. bleo* and *C. ternata* extracts by the use of DPPH, total phenolics and flavonoid equivalent content,
3. To evaluate the antinociceptive and anti-inflammatory activities of *P. bleo* extracts and isolates through formalin-induced, acetic acid-induced and hot plate models,
4. To evaluate the collagen-induced antiplatelet activity of *C. ternata* extracts and isolates using platelets from human blood.

PERESKIA BLEO KUNTH.

(CACTACEAE)

2. *PERESKIA BLEO* KUNTH. (CACTACEAE)

2.1 LITERATURE REVIEW

2.1.1 *Pereskia* sp. (Cactaceae)

Figure 2.1: *Pereskia* sp.

Scientific classification

Kingdom	:	Plantae
Division	:	Angiosperms
Order	:	Caryophyllales
Family	:	Cactaceae
Subfamily	:	Pereskioideae
Genus	:	*Pereskia*

The order Caryophyllales consist of Aizoaceae, Amaranthaceae, Basallaceae, Cactaceae, Chenopodiaceae, Didieraceae, Nyctaginaceae, Phytolaccaceae, Portulacaceae, Caryophyllaceae and Molluginaceae. According to Hunt (1999), Cactaceae is a dicotyledonous angiosperm family of about 2260 total accepted taxa: 1306 species, 301 accepted heterotypic subspecies, 582 provisionally accepted species and hybrids, 71 provisionally accepted heterotypic subspecies. Plants of this family are found in dry, arid and rocky and degraded terrains throughout North and South America. They are also

present in the Mediterranean basin, Middle-East, South Africa, India, Thailand and Australia. On the basis of vegetative and floral morphology, four major subfamilies have been described in the Cactaceae family: Pereskioideae, Cactoideae, Maihuenioideae and Opuntioideae (Anderson, 2001; Gibson and Park, 1986; Barthlott and Hunt, 1993). Cactaceae also bears morphological similarities with Didieraceae (Richardson, 1978). More specifically, Didieraceae, a family endemic in the Madagascar Island, bears similarity with the tribe Pereskiaeae in Cactaceae (Rauh, 1975). They are economically important as ornamentals; the fruit of *Opuntia* is used as food and the peyote (*Laphophora williamsii*) is a well-known hallucinogen (Maffei *et al.*, 1997). Several genera and species of this family produce alkaloids (Raffauf, 1996).

The genus *Pereskia* is named after Nicolas Fabre de Peiresc, a 16th century French botanist, which also has been given its own subfamily Pereskioideae. Members of this genus (Figure 2.1) are usually referred to as lemon vines, rose cacti or leaf cacti. *Pereskia* ecology and physiology has not been well studied (Edwards and Donoghue, 2006). Its species are found throughout a range of drier forest, tropical climates and scrub environments (Leuenberger, 1986). They are found from southern Mexico to Colombia and Venezuela and throughout the eastern part of Brazil south to northern Uruguay and Argentina. Several types are native to the Andes in Peru and Bolivia, though not on the Pacific side (Anderson, 2001). *Pereskia* species have been naturalized in the Caribbean, the West Indies and West Africa (Anderson, 2001; Leuenberger, 1986) and have been described as relatively mesic-loving, drought-deciduous plants (Edwards, 2006).

In a recent survey, Edwards and Donoghue (2006) characterized basic eco-physiological parameters and climate preferences of seven species, and found that *Pereskia* species are actually most commonly found in semi-arid and seasonally very dry environments. They are also not strictly 'drought deciduous'.

2.1.1.1 Morphology of *Pereskia* sp.

The genus *Pereskia* traditionally comprises seventeen species of leafy shrubs and trees that have long been thought to represent the ancestral cacti that do not look much like other types of cacti (Anderson, 2001; Leuenberger, 1986). They are leafy cacti with substantial leaves and thin stems (Edwards and Donoghue, 2006; Anderson, 2001; Leuenberger, 1986).

According to Anderson (2001) and Leuenberger (1986), species of *Pereskia* generally resemble other types of plants, such as wild roses. *Pereskia* species are shrubs or herbs that grow to a meter in height, climbing plants or arborescent species can reach 5 to 20 meters. They are hermaphrodite, non-lactiferous and without coloured juice with large, bright green, privet-like leaves and clusters of long spiny stems developing from areoles. The plants are very peculiar vegetative form with non-succulent, fleshy and hairy well developed leaves, with one-veined or pinnately veined entire lamina.

Close examination shows the distinctive floral cup of the cactus family. The flowers are very showy, zygomorphic arrangement may appear alone or in clusters. They generally resemble roses and reach a diameter of 1 to 5 cm. Colours of the flower depend on each species and vary from white, yellow to magenta or red. They have large shiny black seeds while fruits are ordinarily spherical, from 2 to 5 cm in diameter, and are wine red when ripe (Leuenberger, 1986; Watson and Dallwitz, 1992; Anderson, 2001).

They have fibrous wood, a simple stem cortex without stomata and poorly developed stem dermal layers with non-succulent tissues. Its succulent stems are derived from woody, non-succulent trees with C-3 photosynthesis, as opposed to other growth forms (e.g. an herbaceous, succulent CAM plant) (Watson and Dallwitz, 1992; Edward *et al.*, 2005).

Secretory cavities present, or absent. When present, they are accompanied with latex or with mucilage (but mucilage cells are much commoner). Cork cambium is present; initially superficial. Primary vascular tissue comprises a ring of bundles. Cortical bundles present, or absent. Medullary bundles present, or absent. Internal phloem is absent. Secondary thickening developing from a conventional cambial ring is present. 'Included' phloem is absent. Xylem is without fibre tracheids, with libriform fibres and vessels (usually), or without vessels. Vessel end-walls are simple, or reticulately perforated (rarely). Wood parenchyma is typically paratracheal. Sieve-tube plastids are P-type (Watson and Dallwitz, 1992; Edwards *et al.*, 2005).

2.1.1.2 Chemotaxonomy of *Pereskia* (Cactaceae)

A survey of leaves and thornes of 22 species of Cactaceae showed the presence of several classes of flavonoids such as flavones (apigenin, baicalein), flavonols (quercetin, kaempferol, isorhamnetin, flavonol-3-methyl-ethers), flavanones (naringenin and its dimethyl-4',7-ether) and flavanonols (taxifolin and aromadendrin). Flavonols quercetin and kaempferol were identified in spines extract of *P. aculeata* while no flavonoids have

been found in its leaves extract. A close related species to *Pereskia*, the *Opuntia* sp. (Opuntioideae), several types of flavonoids were identified. Quercetin, kaempferol and isorhamnetin were found as major constituents in O. *basilaris, O. galapageia, O. leuchotricha, O. lindheimeri* and *O. quimillo* stems extracts, together in *O. basilaris, O. lindheimeri* and *O. quimillo* spines extracts. Besides, methyl-3-quercetin were also found in *O. basilaris* and *O. leuchotricha* stems extracts, *O. lindheimeri* and *O. quimillo* stems and spines extracts and *O. galapageia* spines extract. Flavanonols taxifoline and aromadendrine were only identified in *O. basilaris* stems extract and *O. quimillo* stems and spines extracts (Burret *et al.*, 1982).

There are several reports on molecular systematics among Caryophyllales and Cactaceae particularly. According to Smith and Winter (1996), Crassulacean-Acid-Metabolism (CAM) is found in all genera in the Cactaceae family. All species from *Epiphyllum, Hylocereus, Nopalxochia, Opuntia, Pereskiopsis, Rhodocactus* and *Zygocactus* genera in the family were classified as Cyt because the labelling density in the chloroplasts was slightly higher than that in other organelles, but much lower than that in the cytosol. Furthermore, the activity of NAD-ME in Cyt species also was higher than that of NADP-ME or they were approximately the same. Such large differences in activities between NADP-ME and NAD-ME were not found in other species. The Cyt species also have substantial pyruvate, Pi dikinase (PPDK) activity, suggesting that PPDK in the cytosol is active and probably plays a functional role such as a substantial role in CAM function (Kluge and Osmond, 1971; Osmond and Holum, 1981). In plants, PPDK is a key enzyme in C4 photosynthesis, catalyzing the conversion of pyruvate to phosphoenolpyruvate (PEP) which is the substrate for primary carbon dioxide fixation by PEP carboxylase (PEP Case) (Hatch and Slack, 1968; Kondo *et al.*, 2000).

A study on pigment chemistry by Clement et al (1994) have shown that all Caryophyllales families including Cactaceae (except Caryophyllaceae and Molluginaceae, produce betalain pigments instead of anthocyanins with the clade formed by Cactaceae, Portulacaceae, Basellaceae, Didiereaceae and Halophytum was named the 'succulent' clade by Manhart and Rettig (1994). Another study of phylogeny of the order at inter- and infrafamilial levels using partial sequences of the *matK* plastid gene by Cuénoud *et al.* (2002) showed that all Cactaceae samples (*Opuntia, Pereskia, Quiabentia, Rhipsalis* and *Tacinga*) are grouped together with *Portulaca* (bootstrap, BS < 50%), based on *matK* analysis. A combination of these *matK* sequences with those from plastid *rbcL* and *atpB* genes and nuclear 18S rDNA showed that the betalain-producing families are not monophyletic (Clement and Mabry in press).

The above result was supported by a phylogenetic relationships study in the Cactus family (Cactaceae) which based on a evidence from *trnK/matK* sequences. The *trnK* intron (*trnK/matK*) the *matK* gene and two flanking introns in the Cactaceae comprise almost 2600 base pairs (bp) with a data set consisting of 2577 aligned sites and 286 informative characters, proved to be fairly successful in resolving relationships among the major clades in the family Cactaceae. The three species of *Pereskia* used in the study; *P. guamacho, P. zinniflora* and *P. stenatha* do not form a monophyletic group, but a basal grade. The analysis indicated that *Pereskia* sp. formed a distinct clade with maximum parsimony (mp) trees just one step longer. Hence, neither paraphyly nor monophyly of *Pereskia* can be determined based on the present data (Nyffeler, 2002).

In contrast, Butterworth *et al.* (2005) reported that *Pereskia* does not form a monophyletic group in this phylogeny and may be paraphyletic. (Leuenberger, 1986) has grouped investigated *Pereskia* species into seven groups; Group 1: *P. aculeata*, Group 2: *P. lychnidiflora*, Group 3: *P. horrida, P. diaz-romeroana, P. weberiana*, Group 4: *P. bleo*, Group 5: *P. stenantha, P. bahiensis, P. grandifolia, P. sacharosa, P. nemorosa*, Group 6: *P. guamacho, P. aureiflora*, Group 7: *P. zinniiflora, P. portulaciflora, P. quisqueyana.* Although statistical support is moderate, a single clade unites the Caribbean species of *Pereskia* (*P. zinniiflora, P. portulaciflora, P. quisqueyana*) with *P. bleo* (from Colombia) and *P. guamacho* (from Venezuela. Also, included in this clade is *P. aureiflora* (from Brazil) which is sister to *P. guamacho*. The remaining species form two moderately to well-supported clades such as *P. stenantha, P. bahiensis, P. grandifolia, P. sacharosa, P. nemorosa.*

Also, a recent phylogenetic study by Edwards *et al.* (2005) showed that *Pereskia* is a paraphyletic assemblage of species at the base of the cacti and confirms that their leafy habit and nonsucculet stems were not secondarily derived. Furthermore, Edwards and Donoghue (2006) suggested that there are ecological or physiological differences between *Pereskia* species and the core cacti. Certainly, the core cacti are superior to their leafy relatives both at storing water and in using it efficiently. The core cacti are so successful in extreme drought habitats precisely because of their anatomical specializations.

2.1.1.3 Ethnobotanical uses of *Pereskia* sp.

The genus is not of great economic importance. The most common usage being as hedges, easily transplanted and quickly grow into an impenetrable thicket, as well as flowering prolifically. Being more tolerant of moisture than more succulent cacti, they can

be used as rootstock for grafting of *Zygocactus* to create miniature trees (Anderson, 2001, Leuenberger, 1986).

2.1.1.4 Phytochemical studies of *Pereskia* sp.

Flavonols are present in Cactaceae family, with kaempferol found in abundance in Pereskiaeae, while *C*-glycosylflavonoids were absent (Richardson, 1978). According to Salt and co-workers (1987), Cactaceae is a predominantly Δ^7-sterol producing family and all species in the family also produce Δ^5-sterols.

The genus *Pereskia* was found to contain tyramine, and phenethylamine (Anderson, 2001; Leuenberger, 1986). Hegnauer (1989) reported that chemotaxonomically, the family possesses a number of secondary metabolites including betalains. This was supported by a pigment chemisty study by Cuénoud *et al.* (2002) which reported that all Caryophyllales families (except Caryophyllaceae and Molluginaceae, produce betalain pigments instead of anthocyanins. Besides, a wide array of alkaloids (mainly tyramine and phenethylamine), triterpenes (some only present in this family), sterols, mucilage and a few phenols are also present, while tannins are absent in green tissues (Maffei *et al.*, 1997).

P. grandifolia Haw. extracts were reported to contain oleanolic acid saponin (triterpene saponin) (Sahu *et al.*, 1974). Quercetin and kaempferol were also detected in its leaves (Richardson, 1978). In *P. aculeata*, the dominant sterol is β-sitosterol (73%), followed by 24ξ-methylcholesterol (19%), stigmasterol (6.3%) and cholesterol (2.5%) (Salt *et al.*, 1987).

Traces of mescaline were identified and quantified in *P. corrugata*, *P. tampicana* and *Pereskiopsis scandens* through a combination of the use of thin layer chromatography (TLC), high performance TLC and gas-liquid chromatography (Doetsch *et al.*, 1980).

2.1.1.5 Pharmacological studies of *Pereskia* sp.

The hexane extract of *Pereskia grandifolia* possessed a remarkable cytotoxic effects against KB cells with IC_{50} values of 5.0 µg/ml, while ethyl acetate extract showed high cytotoxic effect against KB and MCF-7 cells with IC_{50} values of 16.0 µg/ml and 20.0 µg/ml, respectively (Malek *et al.*, 2009a). A study of a relative cytotoxicity effect of *P. grandifolia* methanol extract on Saos-2 cells showed hypoxia condition increased the IC_{50}

values of the extract. No drastic differences in cytotoxicity was observed at low concentration of extract (<50 µg/ml), but higher in the nomoxic condition (Liew *et al.*, 2012).

β-sitosterol, vitamin E, phytone, 2,4-ditert-butylphenol (3) and a mixture consisting of 2,4-ditert-butylphenol, methyl palmitate, methyl oleate and methyl stearate, was isolated from the active ethyl acetate extract of *P. grandifolia*. Compound 3 possessed a remarkable cytotoxic activity against KB cell with IC_{50} value of 0.81 µg/ml (Malek *et al.*, 2009b). An acute toxiciy study on ICR mice using crude methanol extract of *P. grandifolia* showed a LD_{50} values of > 2500 mg/kg (the highest dose), indicating that the extract did not cause any acute toxicity and mortalities (Sim *et al.*, 2010).

In the antimicrobial activity against both Gram-positive and Gram-negative bacteria using agar disc diffusion assay, the ethyl acetate extract of *Pereskia grandifolia* showed modest antimicrobial activity against *P. aeruginosa* and *S. aureus*, with inhibition zones of 8.0 and 9.2 mm, respectively (Philip *et al.*, 2009).

2.1.2 *Pereskia bleo* Kunth. (Cactaceae)

Pereskia bleo (Kunth) DC (Cactaceae) commonly known as 'Jarum Tujuh Bilah' (in Malay) and 'Cak Sing Cam' (in Chinese) by the locals (Malek and Wahab, 2008), is a plant commonly used by the local community in Malaysia for its medicinal properties (Er *et al.*, 2007). *Pereskia bleo* (Figure 2.2) is a spiny shrub with distinct orange-red flowers and is claimed to treat a variety of illnesses including diabetes, hypertension and as natural remedy in cancer-related diseases. Moreover, its fruit extracts showed high antioxidant activity due to the presence of carotenoids (lutein) (Murillo *et al.*, 2010).

2.1.2.1 Morphology of *P. bleo*

Morphologically, *P. bleo* is hermaphrodite with well developed leaves and long spiny stems arranged in clusters. Leaves are simple, lamina entire with one-veined or pinnately veined and non-succulent (Figure 2.3). The flowers are very showy, orange-red in colour, zygomorphic arrangement which may appear alone or in clusters.

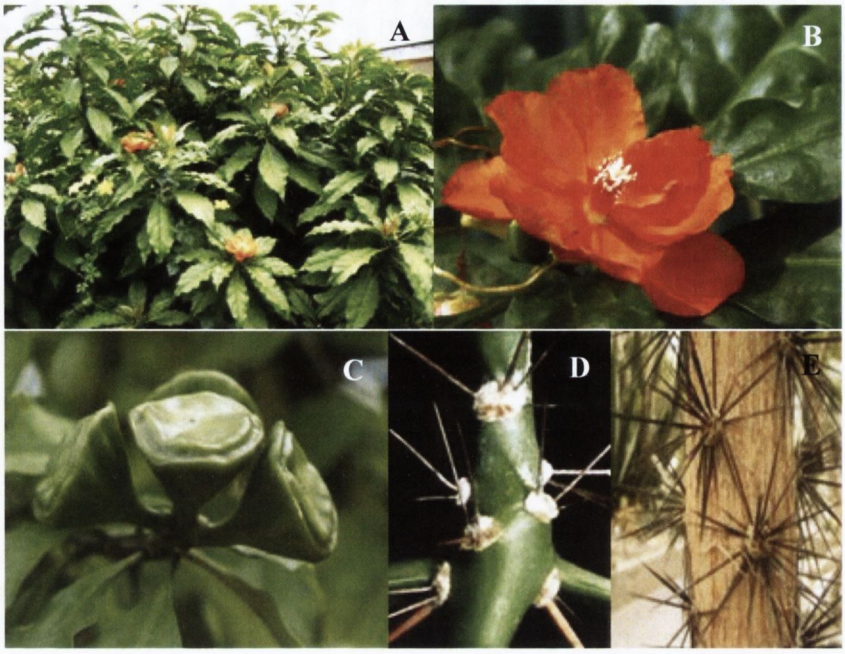


Figure 2.2*: Pereskia bleo* (Kunth) Cactaceae; the whole plant (A), the distinct orange flower (B), the spherical fruit (C), the succulent young spiny stem (D) and fibrous spiny stem (E) (Photos are courtesy of http://www.flickriver.com/photos/tags/pereskiableo/interesting/).

They generally resemble roses and reach a diameter of 1 to 5 cm. They have large shiny black seeds while fruits are ordinarily spherical with 2 to 5 cm diameter, and are wine red when ripe (Leuenberger, 1986; Watson and Dallwitz, 1992; Anderson, 2001).

Figure 2.3: A morphological drawing of *Pereskia bleo* (Kunth) DC (Cactaceae) (Edwards and Donoghue, 2006).

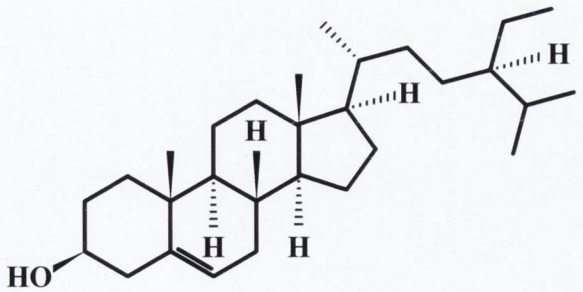
2.1.2.2 Ethnobotanical uses of *P. bleo*

As mentioned before, *P. bleo*, is a plant commonly used by the local community in Malaysia for its medicinal properties (Er *et al.*, 2007). *Pereskia bleo* is claimed to treat a variety of illnesses including diabetes, hypertension and as natural remedy in cancer-related diseases. It has also been used traditionally in Malaysia for the treatment of diseases associated with rheumatism, inflammation, gastric pain, ulcers and for revitalizing the body (Malek and Wahab, 2008; Malek *et al.*, 2009a; Goh, 2000; Malek *et al.*, 2009b; Er *et al.*, 2007). The leaves are generally consumed by the locals either eaten raw as vegetables or taken as a concoction brewed from fresh leaves (Malek and Wahab, 2008; Malek *et al.*, 2009b; Goh, 2000; Er *et al.*, 2007). It has been said that by drinking the tea made from mature leaves (6-7 pieces) everyday could prevent and cure the above mentioned diseases.

2.1.2.3 Phytochemical studies of *P. bleo*

The earliest phytochemical study of *Pereskia bleo* was performed by Doetsch and co-workers (1980) who reported the isolation of four alkaloids, namely 3,4-dimethoxy-β-phenethylamine, mescaline, 3-methoxytyramine and tyramine. Later, Malek and Wahab (2008) isolated four compounds from the ethyl acetate fraction of *P. bleo* which were identified as β-sitosterol (1), phytol (2), 2,4-di-tert-butylphenol (3) and vitamin E (4) (Figure 2.4).

1



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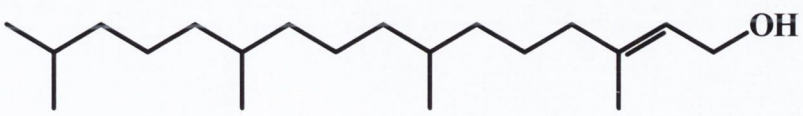

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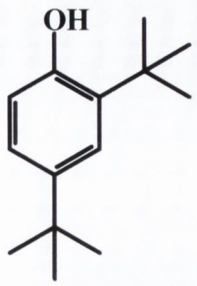

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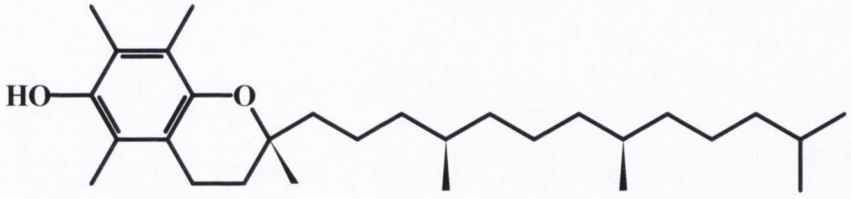
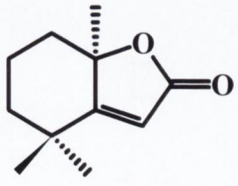


Figure 2.4: Compounds isolated from the ethyl acetate fraction; β-sitosterol (1), phytol (2), 2,4-di-tert-butylphenol (3) and vitamin E (4) (Malek and Wahab, 2008).

Furthermore, on repeated chromatographic purification of the active ethyl acetate extract, red viscous oil and white colored needles were obtained and identified as dihydroactinidiolide (5) and a mixture of sterols (white colored needles-GCMS analyses) consisted of β-sitosterol (1), campesterol (6), stigmasterol (7), besides the already known compounds for this species; phytol (2), 2,4-di-tert-butylphenol (3) and vitamin E (4) (Malek *et al.*, 2009a) (Figure 2.5).

5



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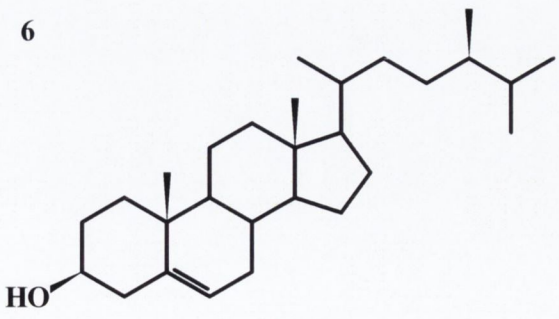

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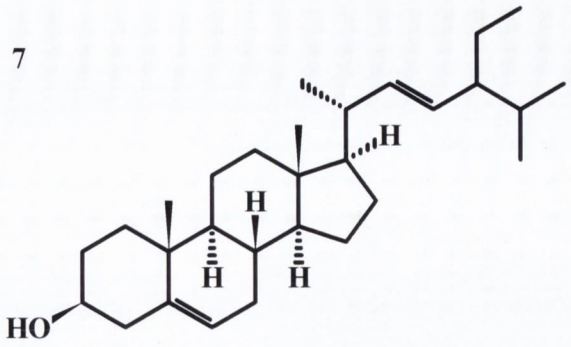


Figure 2.5: Compounds isolated from the ethyl acetate fraction; dihydroactinidiolide (5), campesterol (6) and stigmasterol (7), together with the same compounds isolated by Malek and Wahab, 2008 (Malek *et al.*, 2009b).

2.1.2.4 Pharmacological studies of *P. bleo*

There are several pharmacological reports on the extracts of *P. bleo*, mainly on cytotoxic and anti-proliferative activities (Malek and Wahab, 2008; Malek *et al.*, 2009a; Er *et al.*, 2007). Besides, antimutagenic and antimicrobial studies also showed promising results. In *in vitro* test of neutralization of viper venom (*Bothrops atrox*), haemorrhagic effect, the leaf and stem extracts of *P. bleo* showed no activity (Otero *et al.*, 2000).

The water extracts from the whole plant of *P. bleo* showed the IC_{50} (inhibitory concentration) of 100 µg/ml, CC_{50} (cell toxicity concentration) of 94 µg/ml and SI (selectivity index: CC_{50}/IC_{50}) of 0.94 on HIV-induced cytopathogenicity in MT-4 cells. The water extracts from the whole plant of *P. bleo* gave no effect on the inhibition of giant cell formation (Matsuse *et al.*, 1998). An investigation by Tan and co-workers (2005) reported that the methanol extract of *P. bleo* possessed cytotoxic effects against T-47D cells with EC_{50} of 2.0 µg/ml and a cell death was found to be apoptotic in nature, mainly *via* the activation of the caspase-3 and *c-myc* pathways. A more recent investigation by Er and co-workers (2007) indicated the aqueous and methanol extracts of the leaves of *P. bleo* do not have anti-proliferative effect against mice mammary cancer cells (4T1) or the normal mouse fibroblast cells (NIH/3T3) under optimal culture condition (with 10% Foetal Bovine Serum; FBS was used). The levels of apoptosis observed in these cells when treated with the extracts from leaves of *P. bleo* were found to be not statistically significant at all tested concentrations (10, 50, 200 and 300 µg/ml).

According to Malek and Wahab (2008), the crude methanol extract of *P. bleo* and its fractions (hexane, ethyl acetate and water) exert no damage to the MRC-5 normal cells (IC_{50} >100 µg/ml). The crude methanol extract and the ethyl acetate fraction of *P. bleo*

showed a high cytotoxic effect against KB cells with IC_{50} values of 6.5 and 4.5 µg/ml, respectively. The crude methanol extract showed only moderate activity on the MCF-7 cell line with IC_{50} value of 39.0 µg/ml. The high activity of the crude methanol extract was supported by the isolated compounds identified as phytol (2), 2,4-di-tertbutylphenol (3), vitamin E (4) and dihydroactinidiolide (5) (Malek *et al.*, 2009b). The water fraction was found to have no effect on all the cancer cell lines (IC_{50} >100 µg/ml in all cases). The remarkable inhibitory activity showed by the ethyl acetate extract is in consistence with those reported by Tan and co-workers (2005). Malek and co-workers (2009b) tried to justify in their paper that although 2,4-di-tertbutylphenol does not follow any possible biogenetic pathway, it is indeed a product isolated from the plant. They supported this statement by saying the same compound has been isolated from another plant and also by stating that they distilled again the solvent and continued to obtain the same compound. To my knowledge, I have to disagree with them as there is no possible way in nature a plant can possess the enzymatic machinery to have the tert-butyl unit synthesized. If the solvent was not the source of contamination, they might have the glassware used already contaminated or any other plausible explanation. A simple GC-MS analysis of the solvent or the washed glassware would have shown the authors the presence of the contamination.

A mutagenicity assay performed showed that in the absence of S-9 liver metabolic activation, the extract was not mutagenic up to the concentration of 165 µg/ml. However, in the presence of S-9 liver metabolic activation, the aqueous extract was mutagenic at all the concentrations tested (Er *et al.*, 2007). On the other hand, the aqueous extract of *P. bleo* generated mutagenic substances following metabolism by the liver enzymes.

The ethyl acetate extract also displayed a slightly lower cytotoxic activity against HCT 116 and MCF7 cells (IC_{50} 22.0 and 28.0 µg/ml, respectively). The hexane extract was found to exert a moderate effect on KB and MCF7 cells (IC_{50} 28.0 and 25.0 µg/ml, respectively). All the fractions demonstrated weak cytotoxic activity against CasKi cells compared to other cell lines. In addition, hexane, dichloromethane, ethyl acetate and methanol extracts also showed IC_{50} values over 30 µg/ml for MCF7, HT29 and CEM-SS cell lines after 72 hours incubation time (Wahab *et al.*, 2009). The results of the study are not in consistence with published data (Tan *et al.*, 2005) and that might have happened due to the different methods of extraction.

According to Malek and Wahab (2008), phytol (2) showed significant anti-tumor activity against P388 mouse lymphocytic leukemia cells, molt 4B lymphoid leukemia cells, HT-29 human colon cancer cells, MG-63 osteosarcoma cells and AZ-521 gastric cancer cells. The cytotoxic activity of Phytol was due to an induction of apotosis. Based on these

reported studies, it can be concluded that Phytol may be responsible for the remarkable cytotoxic effect of the ethyl acetate fraction against the KB cancer cell lines with the IC_{50} value of 7.1 µg/ml (Malek *et al.*, 2009a; Malek *et al.*, 2009b). 2,4-di-tert-butylphenol (3) possessed very remarkable cytotoxic activity against KB cells with an IC_{50} value of 0.81 µg/ml and strong cytotoxic effects against MCF7, A549 and CasKi cells with the IC_{50} values of 5.8, 6.0 and 4.5 µg/ml, respectively (Malek *et al.*, 2009b). Furthermore, α-tocopherol (4) also showed strong inhibitory activities with the IC_{50} velues of 6 µg/ml on both the CasKi and A549 cells and 7.5 µg/ml on the MCF7 cells, while dihydroactinidiolide (5) showed strong cytotoxic effect against HCT116 with the IC_{50} value of 5.0 µg/ml. β–sitosterol (1) and the mixture of sterols; β–sitosterol (1), campesterol (6) and stigmasterol (7) did not display any cytotoxic effects against the tested cell lines (Malek *et al.*, 2009b).

In my opinion, these results are hard to accept; especially when one thinks that *trans*-phytol, being a ubiquitous compound in nature found in all plant extracts as a product of chlorophyll degradation (either naturally or induced by isolation/extraction) could be the solely responsible for the observed effect. This would mean that all plants would have remarkable cytotoxic effect, which is not true.

According to Sim *et al.* (2010b), no significant adverse effects were observed in ICR mice following acute oral administration of *P. bleo* crude methanol extract at the highest concentration of 2500 mg/kg extract. Recent study by Liew *et al.*, 2012 showed that *P. bleo* methanolic extract exhibited only minimal cytotoxicity on Saos-2 cells in both normoxic and hypoxic conditions at concentration ranges tested (125, 250, 500, 750, 1000 and 1500 µg/ml). No significant effect was seen until at the highest concentration of extract.

An antioxidant study of *P. bleo* extracts using DPPH radical scavenging activity, reducing power and β-carotene assays exhibited that the ethyl acetate extract had the strongest antioxidant activity in the β-carotene bleaching assay. The high antioxidant activity of the ethyl acetate extract could be contributed by the isolated compounds identified as 2,4-di-tert-butylphenol, α-tocopherol, β-sitosterol and a mixture of sterol (campesterol, stigmasterol and β-sitosterol). These compounds were also reported to be the ones responsible for the highest phenolic content observed in the ethyl acetate extract of 40.12 mg of GAEs/g. The hexane extract showed the highest antioxidant activity in the DPPH assay with an EC_{50} value of 210 µg/ml as well as the highest in the reducing power assay (Sim *et al.*, 2010a).

Two studies of an antibacterial activity of *P. bleo* indicated that methanol, hexane and ethyl acetate extracts of *P. bleo*, exhibited moderate inhibition against *P. aeruginosa* with 9.8, 9.5 and 8.5 mm inhibition halo, respectively, at the concentration of 500 mg/ml. The hexane and ethyl acetate extracts of *P. bleo*, at the concentration of 500 mg/ml, also showed modest inhibition against *B. subtilis* with 8.2 and 7.8 mm inhibition halo, respectively (Philip *et al.*, 2009). Moreover, the dichloromethane and methanol extracts of aerial part of *P. bleo* were found inactive when tested against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Mycobacterium smegmatis, Candidia albicans, Pseudomonas aeruginosa* and *Helicobacter pylori* (Rüegg *et al.*, 2006).

As it can be noticed, there is no work to date in terms of investigating the anti-inflammatory activity of *P. bleo* leaves.

2.1.3 Pharmacological activity investigated in this study

2.1.3.1 Antioxidant activity

Medicinal herbs are known to contain antioxidants such as tocopherols, ascorbic acid, carotenoids and flavonoids (Shahidi *et al.*, 1992). The use of naturally occurring antioxidants extracted from plants and vegetables has been widely investigated (Ikawati *et al.*, 2001). Besides playing an important role in physiological systems, antioxidants have been used in the food industry widely (Wong *et al.*, 2006). These components in foods are readily oxidized by molecular oxygen and are a major cause of nutritional losses, off-flavour development and discolouration.

Many phenolic compounds in most medicinal plants such as phenolic acids and flavonoids, have been reported to possess potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antibacterial, antiviral or anti-inflammatory activities to a certain extent (Chung *et al.*, 1998; Cassidy *et al.*, 2000; Tapiero *et al.*, 2002). Their physiological and pharmacological functions may originate from their antioxidant properties. Previous studies on the antioxidant properties have shown a wide variety of natural phenolic compounds with various molecular structural features and possessing vastly different antioxidant activity (Shahidi and Naczk, 1995; Rice-Evans *et al.*, 1996; Cai *et al.*, 2006).

Vitamins C and E and carotenoids are antioxidants derived from the diet. Other group of compounds such as flavonoids also possesses antioxidant properties and may

account for part of the benefits associated with the consumption of fruits and vegetables. In plants, flavonoids commonly present as glycosides with a sugar moiety/more sugar moieties linked through an OH group (*O*-glycosides) or through carbon-carbon bonds (*C*-glycosides). Moreover, the number and position of the sugar moiety can cause different influences on the activity of the flavonoids (Pietta, 2000; Cai *et al.*, 2006). As antioxidants, flavonoids have been reported to be able to interfere with the activities of enzymes involved in reactive oxygen species generation, quenching free radicals, chelating transition metals and rendering them redox inactive (Heim *et al.*, 2002).

Reactive oxygen species (ROS: superoxide anion radicals, $O_2^{\bullet-}$; hydroxyl radicals, $OH^{\bullet}$, hydrogen peroxide, H_2O_2; and singlet oxygen, $^1O_2$) are the chemically reactive species, generated as byproducts of primary metabolic activities. Excess of ROS/free radicals damage enzymatic machinery, oxidize carbohydrates, proteins, lipids and DNA, and thus induce disease and cellular injury. In order to scavenge free radicals, reduce lipid peroxidation and prevent microbial degradation of food, synthetic antioxidants such as butylated hydroxylanisole (BHA) and butylated hydroxytoluene (BHT) have been widely used as food additives. However, due to increasing awareness among people for the use of safer compounds there has been a shift towards the use of natural compounds as antioxidants (Singh *et al.*, 2010).

Due to the importance of antioxidant activity in the treatment of several diseases, *P. bleo* extracts were evaluated using DPPH, total phenolics and flavonoid equivalent assays.

2.1.3.2 Antinociceptive and anti-inflammatory activities

Pereskia bleo extracts were also evaluated for its antinociceptive and anti-inflammatory activities based on its popular uses to treat rheumatism amongst local people in Malaysia.

Nociception can be defined as the detection of noxious stimuli and the subsequent transmission of encoded information to the brain. In contrast, pain is essentially a perceptual process that arises in response to such activity (Kidd and Urban, 2001). There are several types of pain such as physiological pain, acute and chronic inflammatory pain, neurogenic inflammation, neurophatic and inflammatory neuropathic pain (Backonja, 2003). In details, cutaneous and deep somatic tissues are innervated by primary afferent neurons that synapse with second-order neurons in the dorsal horn of the spinal cord.

Primary afferent neurons have three functions with respect to their role in antinociception; detection of noxious or damaging stimuli (transduction), passage of the resulting sensory input from peripheral terminals to the spinal cord (conduction) and synaptic transfer of this input to neurons within the dorsal horn (transmission) (Kidd and Urban, 2001).

On the other hand, inflammation is a dynamic process that is elicited in response to mechanical injuries, burns, microbial infections, and other noxious stimuli that may threaten the well-being of the host. This process involves changes in blood flow, increased vascular permeability, destruction of tissues via the activation and migration of leucocytes with synthesis of reactive oxygen derivatives (oxidative burst), and the synthesis of local inflammatory mediators, such as prostanglandins (PGs), leukotrienes and platelet-activating factors induced by phospholipase A2, cyclooxygenases (COXs) and lipoxygenase (LOX) (Wiart, 2006; Steele *et al.*, 2003).

It is useful to classify the animal models used in the nociception tests based on the physical characteristics of the stimuli (Le Bars *et al.*, 2001). Basically, tests consisting of long duration stimuli are related to pains of cutaneous or visceral origin with chemical stimuli being applied usually subcutaneously or intraperitoneally and the ones consisting of shorter duration stimuli are related to pain of cutaneous origin with physical stimuli (thermal, mechanical, electrical) (Le Bars *et al.*, 2001). The detection of noxious chemical, thermal, and mechanical stimuli all seem to be mediated by receptors located on damage-sensing neurons (nociceptors) (Wood and Heath, 2000).

The long duration stimuli (tonic pain) involve an irritant, algogenic chemical agent as the nociceptive stimulus. They involve a quantitative approach to the bahaviour observed after the application of a stimulus with a potency that is going to vary with time. The main types of behavioural test based on such stimuli use intradermal or intraperitoneal injections. The most commonly used substance for intradermal injections is formalin (37% solution of formaldehyde), other than carrageenan, hypertonic saline, ethylenediaminetetraacetic acid, Freund's adjuvant, capsaicin and bee sting. The number of licks or twitches of the paw per unit of time is used to measure the response. The injection later produces a biphasic behavioural reaction with the first phase occurring 3-5 minutes after the injection and the second phase occurring 20-30 minutes after the injection. In addition, the first phase is a sign of the direct stimulation of nociceptors (peripheral stimuli) while the second phase is involved with inflammatory pain response. Opioid analgesic tend to be antinocicpetive for both phases while NSAIDs seem to suppress only the second phase (Le Bars *et al.*, 2001).

The other model, the acetic acid-induced abdominal writhing is characterised by abdominal contractions, movements of the body, twisting of dorsal abdominal muscles and a reduction in motor activity and motor incoordination. The test is evaluated by the occurrence per unit of time of abdominal cramps resulting from the intraperitoneal injection of the algogenic agent such as diluted acetic acid or hydrochloric acid, acetylcholine, bradykinin, adrenaline, adenosine triphosphate, potassium chloride, tryptamine and ocytocin. The positive result, presented as no or less abdominal contortions corroborates with analgesic activity even though it has poor specificity due to the positive results showed by other than analgesic substances such as antihistamines, muscle relaxants and neuroleptics (Le Bars *et al.*, 2001).

Furthermore, in the hot plate test, a plate heated to a constant temperature produces two behavioural components that can be measured in terms of their reaction times such as paw licking and jumping. Both are considered to be supraspinally (central antinociceptive effect) integrated responses. As far as analgesic substances are concerned, the paw licking behaviour is affected only by opioids. On the other hand, the jumping reaction time is increased equally by less powerful analgesic such as acetylsalicylic acid, particularly when the temperature of the plate is 50°C or less (Le Bars *et al.*, 2001).

Through years, many non-steroidal anti-inflammatory agents (NSAIDs) and opioids are commonly prescribed drugs which are frequently used for the treatment of various painful conditions such as post-operative pain, inflammatory disorders and have considerable importance for potential use in cancer chemoprevention (Neubert *et al.*, 2010; Hussain *et al.*, 2012). NSAIDs particularly, have been developed as new drugs and marketed. These have given a big impact on the management of various inflammatory conditions such as rheumatism, arthritis and pain (Nguemfo *et al.*, 2007). Non-steroidal anti-inflammatory drugs (NSAIDs), represent a group of different chemical classes having only two common features: the absence of steroid structure, while pharmacologically, they share properties like analgesic, antipyretic and anti-inflammatory activities, that are the basis for their extensive clinical use (Hussain *et al.*, 2012).

NSAIDs reduce pain and inflammation by blocking the metabolism of arachidonic acid and inhibiting cyclooxygenase enzyme (COX), and thereby the production of prostaglandin (Nonato *et al.*, 2009; Kidd and Urban, 2001). The COX-1 isoform is implicated in homeostasis and the COX-2 is particulalrly implicated in inflammatory reactions and in promoting tumorigenesis (Hussain *et al.*, 2012). On the other hand, the consumption of steroid-based drugs as anti-inflammatory agents are not preferable due to

their multiple side effects (Nonato *et al.*, 2009). They may lead to stomach ulcers and other serious complications, such as upper gastrointestinal (GI) bleeding or perforation, bleeding nausea, diarrhea, hypersensitivity reactions and renal failure (Fiorucci *et al.*, 2001; Neubert *et al.*, 2010). To overcome these problems, NSAIDs nowadays, have been developed through various combinations with nitric-oxide and development of so-called nitro-aspirin. It allows greater antinociceptive and anti-inflammatory effects compared with their parent NSAIDs without damaging the GI tract (Kidd and Urban, 2001). Thus, the development of new nature-based drugs is really necessary, which is believed to have lesser side effects and could also be more affordable (Gilani and Rahman, 2005).

Due to the fact that *P. bleo* is traditionally used for the treatment of rheumatism, investigation of the extracts belonging to this plant in models of antinociception and inflammation is then evaluated. Rheumatoid arthritis (RA), a chronic inflammatory disorder, is characterized by pain and tenderness, not only in joints that are directly involved with the disease process but also in surrounding mainly normal tissue (Morris *et al.*, 1997). The results can also point out this plant for the treatment of other diseases related to inflammatory.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Samples of fresh leaves were collected by me in Kota Bharu, Kelantan in Malaysia in October, 2009. The leaves were then left to dry at room temperature. A specimen of *P. bleo* has been identified by Mr. Rafly Syamsir from the Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia and has been deposited in their herbarium under the number KL5729.

2.2.2 Phytochemical studies of *P. bleo*

2.2.2.1 General

The solvents used for the extraction, chromatographic procedures and reactions were analysis grade always, exception to ethanol, which was the commercial grade 96° GL. Activated charcoal (Sigma) was also used to remove chlorophyll, when needed (Sandusky *et al.*, 2006).

The extraction of dried leaves of *P. bleo* was performed using Soxhlet apparatus. The isolation of the compounds by column chromatography was achieved using silica gel 60 (70-230 mesh; Merck) through different gradients of the following organic solvents: hexane-ethyl acetate-methanol, in silica gel. The columns varied in length (ranging from 10-60 cm) and internal diameter (ranging from 1.0-3.5 cm).

The chromatographic analyses by thin layer chromatography (TLC) were performed using silica gel 60 F_{254} pre-coated plates (230-400 Mesh ASTM) (Merck) over aluminum, 20 cm × 20 cm plates on layer thickness 0.25mm. The eluents were prepared in concentration v/v and the visualization of the compounds was achieved by ultra-violet light (254 nm and 365 nm), followed by spraying with 10% sulphuric acid in ethanol. The presence of terpenes, phenolics and flavonoids was indicated by the formation of coloured spots after heating on a hot plate for 5-10 minutes. The evaporation of the solvents from the extracts and their fractions was performed by means of reduced pressure using a rotary evaporator in a heating bath (Buchi R-210).

The structural elucidation of the isolated compounds was performed by means of the analysis of their spectrums, mass spectra, H^1 Nuclear Magnetic Resonance (400 and 600 MHz), Carbon-13 Nuclear Magnetic Resonance (100 and 150 MHz). The NMR spectra were recorded on a BRUKER TOPSPIN 2.1 NMR System with deuterated chloroform, deuterated methanol or deuterated dimethyl sulfoxide as solvents. Chemical shifts are reported as δ (ppm) values, and the coupling constants are given in Hz. Both uni- and bi-dimensional techniques NMR spectra (^{1}H-^{1}H COSY, HMBC, HMQC) were also measured by BRUKER TOPSPIN 2.1 NMR System using standard Bruker pulse sequences.

Melting points were determined with SMP-1 Stuart Scientific, UK. Optical rotations $[\alpha]_D^{20}$ were measured in a 1 decimeter tube using an Alltech AA-55 polarimeter from Optical Activity Ltd. UV analysis was performed with Cary 300 Scan UV-Visible spectrophotometer and Varian-CaryWinUV program. The FT-IR spectroscopy was recorded in KBr discs on a Perkin-Elmer Spectrum 100 FT-IR Spectrometer (Perkin-Elmer Spectrum).

Electrospray ionization (ESI) mass spectroscopy data was collected using a TOF-MS (Time of Flight-Mass Spectrometer, LCT Premier) instrument supplied by Waters Corp. using Leucine Enkephalin (Leu-Enk) as internal lock mass reference. All data, including simulated isotope patterns and molecular weight calculation were processed using Masslynx v4.0 (Waters Corp.) data analysis software. An infusion of standard

sodium formate (HCOONa), 10% formic acid / 0.1M NaOH/acetonitrile, 1:1:8, v/v/v) solution was used, producing a mass calibration between 100-3000. Samples were introduced into the MS via a Waters Alliance 2690 HPLC with autosample mode and a solvent flow rate of 200 µl/min, whilst a Leu Enk solution (10 µg/ml in acetonitrile / 0.1% TFA in water, 50:50, v/v) was coinjected via a micropump at 2 µl/min. The ESI settings were set with the nebuliser gas and desolvation gas at 60 and 500 L/h, respectively. Source and desolvation gas temperature were set at 100°C and 300°C respectively. The ion polarity for all MS scans were recorded in positive or negative mode with voltage of the capillary tip set between 2.5-3 kV, sample cone at 30 v, extraction cone at 3 V, scan time at 0.9 sec and interscan delay set at 0.1 sec.

EI mass spectra were acquired using a GCT Premier Micromass time of flight mass spectrometer (tof). The instrument was operated in positive mode. Heptacosa (+) was used as an internal lock mass. Masses were recorded over a range of 100-600 m/z. Operating conditions were as follows; detector voltage 3000 V, source temperature 200°C, filament energy 70 eV, trap current 99 uA. MassLynx 4.0 software was used to carry out the analysis.

2.2.2.2 Isolation and purification of extracts

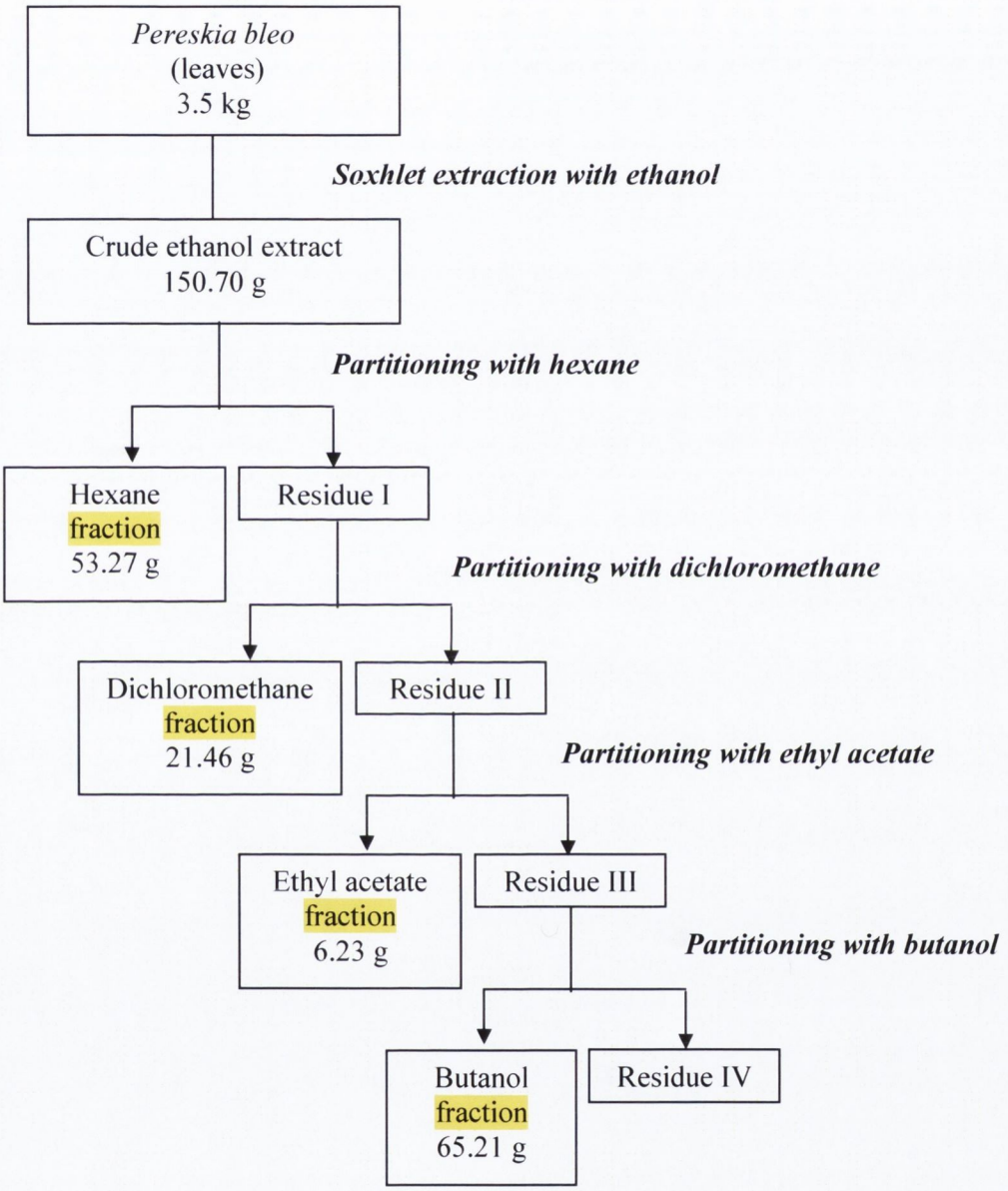
- **Crude ethanol extract and its fractions**

Three and a half kilos of *P. bleo* leaves were extracted using soxhlet apparatus with ethanol for 72 hours. The crude ethanol extract of *P. bleo* leaves was then treated overnight with activated charcoal to eliminate chlorophyll. The extracts were then filtered through Whatman filter paper (24.0 cm) and concentrated with rotary evaporator. The crude ethanol extract obtained (150.7 g) was then submitted to a liquid-liquid partition using hexane, dichloromethane, ethyl acetate and butanol (exhaustive extraction) yielding 53.27g, 21.46 g, 6.23 g and 65.21 g of fractions, respectively (Scheme 2.1).

Part of the hexane fraction of *P. bleo* (16 g) had the purification of its compounds performed by column chromatography (Ø column: 3.0 cm) using hexane-ethyl acetate-methanol (to flush the silica column) in gradients of increasing polarity as mobile phase and silica gel (65 g, 70-230 mesh, Merck) as stationary phase, yielding 202 fractions. The fractions eluted from the chromatographic column were analysed by thin layer chromatography and the similar fractions were then grouped, giving a total of 10 junctions.

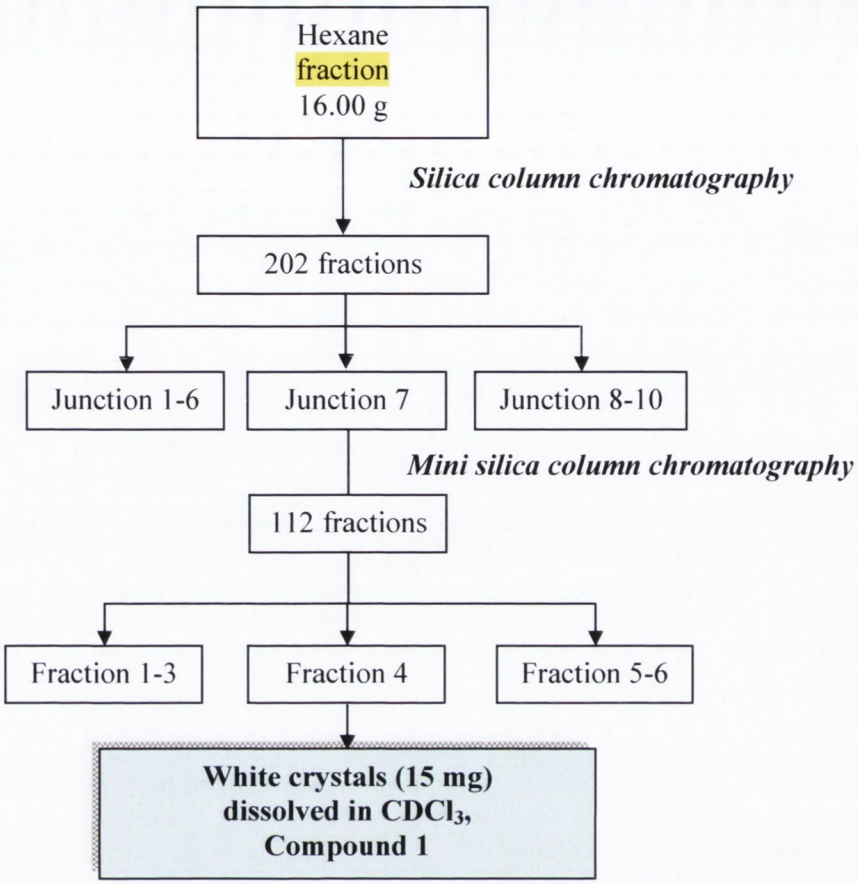
Junction 7 (0.7722 g) was further fractionated through a mini column (Ø column: 0.5 cm, 10 ml) using silica gel (10 g, 70-230 mesh) as stationary phase and hexane-ethyl

acetate-methanol in gradients of increase polarity as mobile phase, yielding 112 fractions and were combined to have six junctions. White crystals were obtained from junction 4 (mobile phase: hexane – ethyl acetate, 8:2) (Scheme 2.2).



Scheme 2.1: Preparation of ethanol extract and its fractions with hexane, dichloromethane, ethyl acetate and butanol from the leaves of *P. bleo*.

- **Analysis of the hexane fraction**

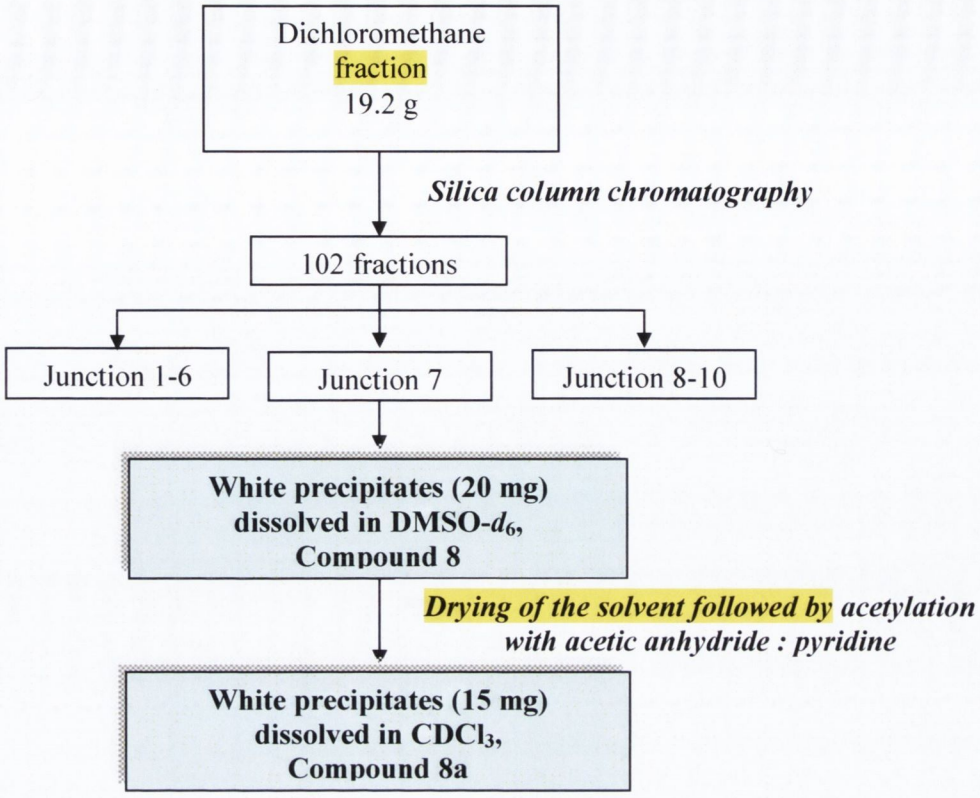


Scheme 2.2: Fractionation of the hexane fraction from *P. bleo* leaves yielded white crystals compound 1.

- **Analysis of the dichloromethane fraction**

Part of the dichloromethane fraction of *P. bleo* (19.2 g) was submitted to a chromatographic fractionation in column (Ø column: 3.5 cm) using hexane – ethyl acetate – methanol in gradients of increasing polarity as mobile phase and silica gel (60 g, 70-230 mesh) as stationary phase, yielding ten fractions. The TLC visualization and profiling were done as described previously (Section **2.2.2.1)**. Similar fractions were then re-united. White precipitates were obtained from junction 7. After rinsing with methanol, the crystals were dissolved in DMSO-d_6 and sent for NMR analysis (Scheme 2.3).

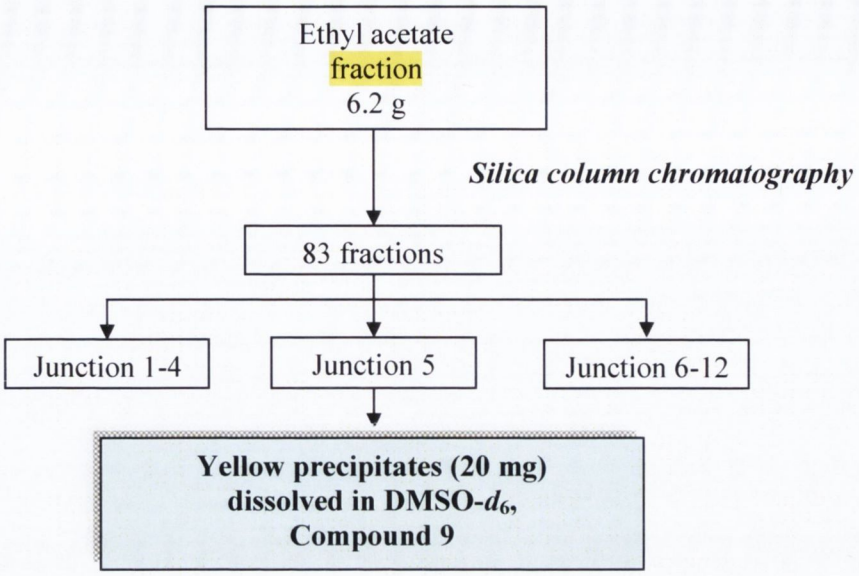
Compound 8 was freeze-dried and part of the compound (15 mg) was acetylated overnight with acetic anhydride (Ac_2O : 8 drops -pyridine : 2 ml) at room temperature. Crystallization of the product from methanol gave white precipitates. The precipitates were dissolved in deuterated chloroform and sent for NMR (Scheme 2.3).



Scheme 2.3: Fractionation of the dichloromethane fraction from *P. bleo* leaves yielded white precipitates compound 8.

- **Analysis of the ethyl acetate fraction**

Part of the ethyl acetate fraction of *P. bleo* (6.2 g) was submitted to a chromatographic fractionation in column (Ø column: 3.5 cm) using chloroform – ethyl acetate – methanol in gradients of increasing polarity as mobile phase and silica gel (60 g, 70-230 mesh) as stationary phase, yielding 83 fractions that were combined into 12 junctions. The TLC visualization and profiling were done as described previously. Similar fractions were then re-united. Yellow precipitates were obtained from junction 5. After rinsing with chloroform, the crystals were dissolved in DMSO-d_6 and sent for NMR analysis (Scheme 2.4).



Scheme 2.4: Fractionation of the ethyl acetate fraction from *P. bleo* leaves yielded yellow precipitates compound 9.

2.2.3 Pharmacological studies of *P. bleo* extracts and an isolated compound

As stated in the description of *P. bleo* and its ethnopharmacological uses, the plant can be eaten raw. If people ingest the raw leaves and observe pharmacological activities, we expect that any compound within the array of compounds in the plant could be active. This is the reason why we decided to work with organic extracts of different polarities to try and track in which range of polarity we would find significant activity.

2.2.3.1 Antioxidant activity

The antioxidant activity assays were performed on crude ethanol, hexane, dichloromethane, ethyl acetate and butanol fractions of *P. bleo* (leaves). The antioxidant activity was investigated using three different assays: DPPH free radical scavenging activity microassay using a 96-well plate, quantification of total phenolics and determination of total flavonoid equivalents.

- **DPPH free radical scavenging activity assay**

Radical scavenging activity of plant crude extract and its fractions against stable DPPH (1,1-diphenyl-2-picrylhydrazyl, Sigma-Aldrich) was determined spectrophotometrically according to a method adapted from Mensor *et al.* (2001). *Pereskia bleo* extract and fractions (crude ethanol, hexane, dichloromethane, ethyl-acetate and butanol partitions) were successively diluted in ethanol (250, 125, 50, 25, 10 and 5 µg/ml). Tanakan 40 mg oral solution (commercial *Ginkgo biloba* extract - Egb 761) was used as positive control in this test. Egb 761 was the standard chosen because the antioxidant activity evaluated in this study was assessed only using plant extracts, not pure compounds. In a 96-well plate, these samples (100 µl/well) received, in triplicate, 100µl of DPPH (172 µM in ethanol). The plate was then incubated in a light-free area for 30 minutes. When DPPH reacts with an antioxidant compound, which can donate hydrogen radical, it is reduced. The changes in colour from deep violet to light yellow were measured at 544 nm on a microplate reader (BMG Labtech). The extracts solutions (100 µl) plus ethanol (100 µl) was used as a blank, while DPPH (100 µl, 172 µM) plus ethanol (100 µl) was used as a negative control. The percentage of antioxidant activity was then calculated using the Equation 2.1 below.

$$AA\% = 100 - \{ [(A_S - A_B) \times 100] / A_N \} \qquad \textbf{Equation 2.1}$$

Where, AA % is the antioxidant activity expressed as percentage of discolouration, A_S is the absorbance of the sample, A_B is the absorbance of the blank (ethanol and crude extract) and A_N is the absorbance of the negative control (DPPH solution plus ethanol).

- **Total phenolic content assay**

The total phenolic content of the extracts was determined using the Folin Ciocalteau assay with slight modifications according to the method by Gursoy *et al.* (2009). Gallic acid was used as a standard and had the calibration curve plotted. The solutions of gallic acid were prepared in methanol with final concentrations of 500, 250, 125, 50, 25, 10 and 5 µg/ml. The blank solution consisted of 4 µl extract solution, 184 µl distilled water and 12 µl sodium carbonate solution (Na_2CO_3) (2%).

In this assay, 4 µl of the extracts (400 µg/ml) were added to 180 µl of distilled water and 4 µl of Folin Ciocalteau reagent. The sample was prepared in four replicates

using 96 well plate. After adding the reagent, the plate was shaken vigorously and after 3 minutes, 12 µl of aqueous sodium carbonate (2%) solution was added. The mixtures were allowed to stand in the dark for 2 hours with intermittent shaking. The absorbance reading was measured at 595 nm.

The phenolic content of the extracts was determined according to the gallic acid standard curve and the results were expressed as µg of gallic acid equivalents per mg of extract. The absorbance value after subtraction of blank was translated into total phenolic content, using the Equation 2.2 below.

Total Phenolic Content = (y - 0.062) / 0.0004, R^2 = 0.9967 **Equation 2.2**

- **Flavonoid equivalent assay**

The determination of the flavonoid equivalent of the extracts was performed according to the method by Gursoy *et al.* (2009). Quercetin was used as a standard and had its calibration curve plotted. The solutions for quercetin were prepared in methanol giving final concentrations of 500, 250, 125, 50, 25, 10 and 5 µg/ml. A blank solution consisted of 100 µl extract solution and 100 µl methanol without aluminium (III) chloride.

In this assay, 100 µl of the extracts (400 µg/ml) were added to 100 µl of 2% aluminium (III) chloride in methanol. The sample was prepared in four replicates using 96 well plate. The absorbance reading was measured at 405 nm after ten minutes.

The flavonoid content of the extracts was determined according to the quercetin standard curve and the results were expressed as µg quercetin equivalents per mg of extract, using the Equation 2.3 below.

Flavonoid Equivalent = (y - 0.2357) / 0.0078, R^2 = 0.9919 **Equation 2.3**

- **Statistical analysis**

All experimental groups in the DPPH radical scavenging assay were in triplicate. Data was analyzed using GraphPad Prism 5.0 software. The IC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R^2 \geq 0.90$). The results are presented as means±S.D. Statistical significance between groups was calculated by analyses of variance (ANOVA), followed by Bonferroni's test. *P*-values less than 0.05 ($p<0.05$) were used as the significant level.

2.2.3.2 Antinociceptive activity

All experiments described in this section were carried out by Dr. Fernandes in her lab in Rio de Janeiro – Brazil (Laboratório de Farmacologia do Óxido Nítrico - Instituto de Ciências Biomédicas, Departamento de Farmacologia Básica e Clínica, Universidade Federal do Rio de Janeiro). I provided the extracts and the isolate and she provided raw data back. The results were statistically worked and conclusions were drawn by me in Ireland.

- **Animals**

The antinociceptive assays were run according to the methods described by Matheus and co-workers (2005). These assays were performed at Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Brazil. All experiments were performed with male Swiss mice (20-25 g) obtained from Instituto Vita Brazil. Animals were maintained in a room with controlled temperature 22 ± 2°C for 12 hours: 12 hours light/dark cycle with free access to food and water. Twelve hours before each experiment, animals received only water, in order to avoid food interference with substances absorption.

Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazillian College of Animal Experimentation (COBEA) and approved by the Ethical Committee for Animal Research (Biomedical Science Institute/UFRJ; #ICBDFBC-015). Initially, *P. bleo* extracts were dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution at 100 mg/ml and administered by oral gavages at doses of 30, 50, and 100 mg/kg which were diluted with phosphate buffer saline (PBS), in a final volume of 0.1 ml (mice). Positive control groups were composed by animals that received morphine (Merck Inc.) or acetylsalicylic acid (ASA). The negative control group received the PBS, containing the same amount of DMSO.

- **Formalin-induced model**

The procedure used is similar to the method described by Gomes and co-workers (2007). Animals received the injection of 20 µl of formalin (2.5%, v/v) into the dorsal surface of the left hind paw. Immediately, the time that the animal spent licking the injected paw was recorded. The nociceptive response develops in two phases: the first 5 minutes after formalin injection (first phase, neurogenic pain response) and 15-30 minutes after formalin injection (second phase, inflammatory pain response). The animals were

pre-treated with oral doses of the extracts, vehicle, or morphine 60 minutes before administration of formalin.

- **Acetic acid-induced abdominal writhing model**

Mice were treated according to Pinheiro and co-workers (2010). Briefly, the total number of writhing that followed intraperitoneal administration of 2% (v/v) acetic acid (Ac. A) was recorded over a period of 20 minutes, starting 5 minutes after Ac. A injection. Mice were pre-treated with *P. bleo* extracts or vehicle, 60 minutes before administration of Ac. A. Positive control group was received by the reference drug morphine (5 mg/kg).

- **Hot plate model**

Mice were treated according to the method described by Franzotti and co-workers (2000). The animals (n=5) were placed on a hot plate (Insight Equipment, Brazil) set at 55 ± 1°C. Reaction time was recorded when the animals licked their fore- and hind-paws and jumped at several intervals of 30 minutes after oral administration of 30 and 100 mg/kg of *P. bleo* extracts, or reference drug (morphine, 5 mg/kg, subcutaneously). Baseline was considered as the mean of reaction time obtained at 60 and 30 minutes before administration of extracts or morphine and was defined as normal reaction of animal to the temperature. Increase in baseline (%) was calculated using the Equation 2.4 below.

Increase in baseline (%) = [(reaction time x 100) / baseline] – 100 **Equation 2.4**

Antinociception was quantified as area under curve (AUC) of responses from 30 to 120 min after oral drug administration. The Equation 2.5 below, based on the trapezoid rule, was used to calculate the AUC.

AUC = 30 x IB [(min 30) / 2 + (min 60) + ... + (min 120) / 2],
where IB is the increase in baseline (in %) **Equation 2.5**

- **Acute toxicity**

Acute toxicity was determined following the experimental model described by Lorke (1983). A single oral dose of *P. bleo* extracts (500 mg/kg) was administered to a group of ten mice (five males and five females). Behaviour parameters including convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, and food and water intake were observed over a period of 14 days.

After this period, animals were killed by cervical dislocation, stomachs were removed, an incision along the greater curvature was made, and the number of ulcers (single or multiple erosion, ulcer or perforation) and hyperaemia were counted. Lethal dose was not determined due to small available quantity of the extracts and isolated substances.

- **Statistical analysis**

All experimental groups for the antinociceptive evaluation were composed of 5 animals. The results are presented as the mean ± S.D. Statistical significance of the differences between the mean values of tested groups was calculated by analyses of variance (ANOVA), followed by Bonferronis's test using Graphpad Prism 5.0 software. *p*-values less than 0.05 ($p<0.05$) were used as the significant level.

2.3 RESULTS AND DISCUSSION

2.3.1 Phytochemical studies of *P. bleo*

2.3.1.1 Isolation and purification of extracts

- **Analysis of the hexane fraction**

β-sitosterol (1)

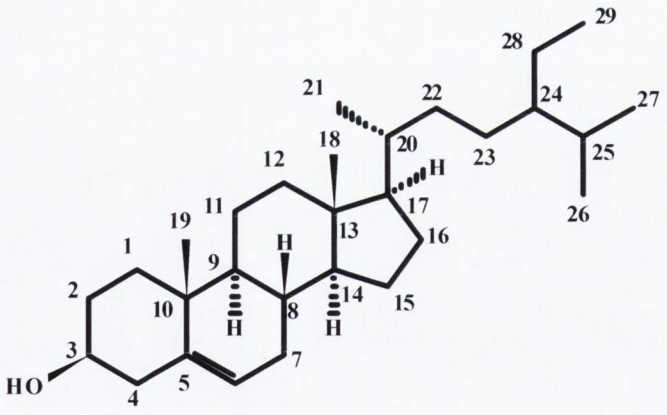


Figure 2.6: β-sitosterol (1)

White colourless needle; R_f: 0.36 (hexane:ethyl acetate; 8:2); m.p: 124-126°C; $[\alpha]_D^{20}$ -30.3 (CH_2Cl_2); UV (MeOH) λ_{max}: 244; IR ν_{max}: 3323, 2934, 2865, 1463, 1375, 1240, 925, 801 cm^{-1}; ESI-MS for $C_{29}H_{50}O$ m/z; 415 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 0.72 (3H, *s*, H-18), 0.83 (3H, *d*, *J*=6.9 Hz, H-27), 0.84 (3H, *d*, *J*=6.9 Hz, H-26), 0.85 (3H, *t*, *J*=7.4 Hz, H-29), 0.87 (3H, br, *s*, H-19), 1.03 (3H *d*, *J*=6.5 Hz, H-21), 3.55 (1H, *m*, H-3), 5.38

(1H, br, *s*, H-6); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 11.9 (C-18), 12.0 (C-29), 18.8 (C-21), 19.0 (C-27), 19.4 (C-19), 19.9 (C-26), 21.1 (C-11), 23.1 (C-28), 23.5 (C-13), 24.3 (C-15), 26.0 (C-23), 28.3 (C-16), 29.1 (C-25), 29.7 (C-8), 31.7 (C-7), 31.9 (C-2), 33.9 (C-22), 36.2 (C-20), 36.5 (C-10), 37.3 (C-1), 39.8 (C-12), 42.3 (C-4), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.8 (C-14), 71.8 (C-3), 121.8 (C-6), 140.7 (C-5).

Compound 1 was isolated as a white precipitate (81 mg) with a molecular formula of $C_{29}H_{50}O$ as determined from ESI-MS analysis 415 $[M+H]^+$. Comparison of the melting point obtained for β-sitosterol (m.p. 124-126°C) with the one published (m.p. 127-128°C) (Malek and Wahab, 2008) and co-cromatography by TLC using β-sitosterol standard left no doubt that we isolated the same compound. The co-chromatography together with the specteroscopic analyses, these findings supported the assignment of the structure of compound 1 to be β-sitosterol glucoside (Figure 2.6). The spectral data on β-sitosterol agreed with that reported by Malek and Wahab (2008).

- **Analysis of the dichloromethane fraction**

β-sitosterol glucoside (8)

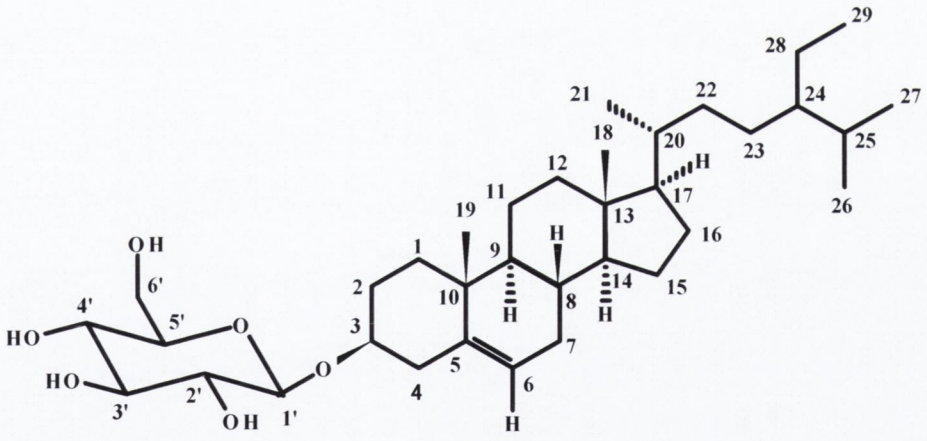


Figure 2.7: β-sitosterol glucoside (8)

White amorphous powder; R_f: 0.84 (ethyl acetate:methanol; 8:2); m.p: 284-288°C, UV (MeOH) λ_{max}: 242; IR ν_{max}: 3379, 2933, 2867, 1462, 1366, 1255, 926, 800 cm^{-1}; ESI-MS for $C_{35}H_{60}O_6$ m/z; 599 $[M+Na]^+$; ^{1}H-NMR (DMSO-d_6, 600 MHz) δ_H: 0.66 (3H, *s*, H-18), 0.79 (3H, *d*, *J*=6.7 Hz, H-27), 0.82 (3H, *d*, *J*=6.7 Hz, H-26), 0.84 (3H, *t*, *J*=7.6 Hz, H-29), 0.92 (3H, *d*, *J*=6.5 Hz, H-21), 0.97 (3H, *s*, H-19), 2.36 (2H, *t*, *J*=1.6 Hz, H-4), 3.58 (1H, *m*, H-3), 5.33 (1H, br, *s*, H-6), glucoside moiety : 2.91 (1H, *m*, H-2'), 3.03 (1H, *m*, H-4'), 3.04 (1H, *m*, H-5'), 3.13 (1H, *m*, H-3'), 3.65 (2H, *m*, H-6'), 4.23 (1H, *d*, *J*=7.8 Hz, H-1'), 4.42

(1H, *s*, OH-6'); ^{13}C-NMR (DMSO-d_6, 150 MHz) δ_C: 11.6 (C-29), 11.8 (C-18), 18.6 (C-21),18.9 (C-27), 19.1 (C-19), 19.7 (C-26), 20.7 (C-11), 22.6 (C-28), 23.9 (C-15), 25.6 (C-23), 27.9 (C-16), 29.2 (C-2), 31.3 (C-7), 31.4 (C-8), 33.3 (C-22), 36.2 (C-10), 35.4 (C-20), 36.9 (C-1), 38.3 (C-12), 39.1 (C-4), 41.8 (C-13), 45.1 (C-24), 49.6 (C-9), 55.4 (C-17), 76.9 (C-3), 121.1 (C-6), 140.4 (C-5), 56.2 (C-14), 28.7 (C-25), glucoside moiety : 61.1 (C-6'), 70.1 (C-4'), 73.4 (C-3'), 76.8 (C-2'), 76.8 (C-5'), 100.8 (C-1').

Compound 8 was isolated as a colourless amorphous powder and formed froth after mixing with DMSO (37 mg) with a molecular formula of $C_{35}H_{60}O_6$ as determined from ESI-MS analysis 599 $[M+Na]^+$. Co-chromatography with β-sitosterol glucoside standard and NMR analysis proved this compound to be β-sitosterol glucoside (Figure 2.7). The spectral data on β-sitosterol glucoside agreed with that reported by Khatun *et al.* (2012).

The ^{1}H NMR spectrum of β-sitosterol glucoside exhibited a broad singlet signal at δ_H 5.33 that related to H-6 while a proton, multiplet at δ_H 4.42, showed a carbinolic proton signal related to the proton at position three. A number of multiplet signals either at or between δ_H 1.02 – 1.81 explained different methylene and methine protons in the structure. The protons of its sugar moiety resonated at δ_H 3.03- 4.23. Two doublets at δ_H 0.92 (J=6.5 Hz) and 0.82 (J=4.8 Hz) integrated three protons each, were associated to H-21, H-26 secondary methyl while a triplet at 0.84 (J=7.6 Hz) was associated to H-29 methyl protons. H-18 and H-19 tertiary methyl proton signals appeared as singlets at δ_H 0.66 and 0.97, respectively. The anomeric C-1' proton appearing at δ_H 4.23 as a doublet (J=7.8 Hz) is related to the presence of one sugar unit. A signal, appearing as multiplet at δ_H 3.04 was due to proton at position five of the sugar moiety, while four carbinolic protons which resonated as multiplets at δ_H 2.91, 3.03, 3.13 and 3.65 were associated with C-2', 4', 3' and 6', respectively.

The ^{13}C NMR and DEPT-135 spectra showed the presence of thirty-five carbons in the molecule, presented as fourteen methine, twelve methylene, six methyl and three quartenary carbons. Six of these signals were due to sugar moiety. The remaining twenty-nine carbon signals were due to the aglycone moiety. The olefinic carbons at δ_C 140.4 and δ_C 121.1 correspond to the endocyclic double bond between carbons at positions five and six. The anomeric signal resonated at δ_C 100.8 while the carbinolic carbon signal and oxygenated methylene carbon of the sugar moiety were observed at δ_C 76.8 and δ_C 61.1, respectively.

The COSY-45 plot showed the vicinal couplings between the protons and the correlation of δ_H 2.91 (H-2') with 4.23 (H-1'), δ_H 3.03 (H-4') with 3.13 (H-3') and δ_H 3.65 (H-6') with 3.04 (H-5'). The HSQC spectrum confirmed the protons that are attached to

their carbons identified at position 4, 6, 1', 2', 3', 4' and 6'. Other relationships between proton and carbon resonance can also be seen amongst the methyl groups. Long distance relationships between proton and carbon resonances can be observed in HMBC spectrum. A three bond correlation of δ_H 4.42 (H-3) with the anomeric carbon δ_C 100.8 (C-1') and that of anomeric proton δ_H 4.23 (H-1') with δ_C 76.9 (C-3) confirmed the point of attachment of the sugar moiety with the aglycone. The proton resonance at δ_H 0.66 (H-18) is correlated to carbon signals at δ_C 41.8 (C-13), 55.4 (C-17) and 56.2 (C-14), while a signal at δ_H 0.97 (H-19) is correlated to carbon signals at δ_C 36.9 (C-1), 36.2 (C-10), 49.6 (C-9) and 140.4 (C-5).

Furthermore, the other methyl protons at δ_H 0.92 (H-21), 0.82 (H-26), 0.79 (H-27), 0.84 (H-29) showed strong correlations to δ_C 33.3 (C-22) and 55.4 (C-17), δ_C 22.6 (C28) and 28.7 (C-25), δ_C 19.7 (C-26), 28.7 (C-25) and 45.1 (C-24), δ_C 22.6 (C-28) and 45.1 (C-24), respectively. Proton resonances δ_H 5.33 (H-6) is related to both carbon signal at δ_C 31.3 (C-7) and 31.4 (C-8). The anomeric proton resonance of the sugar moiety at δ_H 4.23 (H-1') is related to δ_C 76.8 (C-2'). The complete NMR spectral data are summarized in Table 2.1 below.

Table 2.1: Comparison of ^{1}H and ^{13}C NMR data for compound 8 (600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C- NMR, DMSO-d_6) and β-sitosterol glucoside ($CDCl_3$ and CD_3OD).

	Present study		Literature data*	
Position	**^{1}H NMR, δ_H (ppm) (J in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^{1}H NMR**	**^{13}C NMR**
1		36.9		36.9
2		29.2		29.1
3	3.58 (1H, *m*)	76.9	3.13	78.6
4	2.36 (2H, *t*, *J*=1.6 Hz)	39.1	2.14	42.1
5		140.4		139.9
6	5.33 (1H, br, *s*)	121.1	5.09	121.5
7		31.3		31.4
8		31.4		31.5
9		49.6		49.8
10		36.2		36.3
11		20.7		20.2
12		38.3		38.2

13		41.8		41.9
14		56.2		56.4
15		23.9		23.8
16		27.9		27.8
17		55.4		55.7
18	0.66 (3H, br, *s*)	11.8	0.62	11.3
19	0.97 (3H, br, *s*)	19.1	0.94	19.1
20		35.4		35.7
21	0.92 (3H, *d*, *J*=6.5 Hz)	18.6	0.84	18.7
22		33.3		33.5
23		25.6		25.6
24		45.1		45.5
25		28.7		28.7
26	0.82 (3H, *d*, *J*=6.7 Hz)	19.7	0.75	18.7
27	0.79 (3H, *d*, *J*=6.7 Hz)	18.9	0.73	18.4
28		22.6		22.6
29	0.84 (3H, *t*, *J*=7.6 Hz)	11.6	0.77	12.3
1'	4.23 (1H, *d*, *J*=7.8 Hz)	100.8	4.11	100.7
2'	2.91 (1H, *m*)	76.8	3.14	73.2
3'	3.13 (1H, *m*)	73.4	3.14	76.2
4'	3.03 (1H, *m*)	70.1	3.14	69.9
5'	3.04 (1H, *m*)	76.8	3.06	75.6
6'	3.65 (2H, *m*)	61.1	2.94	61.4

***Khatun *et al.* (2012)**

Comparison of the melting point obtained for β-sitosterol glucoside (m.p. 284-288°C) with the one published (m.p. 272-274°C) (Khatun *et al.*, 2012) and co-cromatography by TLC using β-sitosterol glucoside standard left no doubt that we isolated the same compound. Together with the specteroscopic analyses, these findings supported the assignment of the structure of compound 8 to be β-sitosterol glucoside.

Peracetylation following the methodology described by Faiz *et al.* (2001) allowed us to confirm the identity of this molecule. β-sitosterol glucoside tetraacetate is an acetylated derivative obtained during the acetylation process of β-sitosterol glucoside using acetic anhydride and pyridine.

β-sitosterol glycoside tetraacetate (8a)

White amorphous powder; R_f: 0.5 (hexane:ethyl acetate; 7:3); mp: 136-142°C, IR ν_{max}: IR ν_{max}: 2865-2961, 1739-1749, 1215 cm^{-1}; ESI-MS for $C_{43}H_{68}O_{10}$ m/z; 397 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ_H: 0.69 (3H, *s*, H-18), 0.84 (3H, *d*, *J*=6.9 Hz, H-27), 0.85 (3H, *t*, *J*=7.9 Hz, H-29), 0.87 (3H, *d*, *J*=6.9 Hz, H-26), 0.95 (3H, *d*, *J*=6.4 Hz, H-21), 1.01 (3H, *s*, H-19), 2.03 (3'-CH_3), 2.04 (6'-CH_3), 2.07 (4'-CH_3), 2.09 (6'-CH_3), 3.51 (1H, *s*, H-3), 3.69 (1H, *m*, H-5'), 4.13 (2H, *dd*, *J*=2.3, 12.2 Hz, H-6'), 4.62 (1H, *d*, *J*=8.0 Hz, H-1'), 4.98 (1H, *t*, *J*=8.8 Hz, H-3'), 5.09 (1H, *t*, *J*=9.8 Hz, H-4'), 5.23 (1H, *t*, *J*=9.5 Hz, H-2'), 5.38 (1H, br, *s*, H-6); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ_C: 37.0 (C-1), 29.5 (C-2), 79.9 (C-3), 38.8 (C-4), 140.2 (C-5), 122.0 (C-6), 31.8 (C-7), 31.7 (C-8), 50.0 (C-9), 36.57 (C-10), 20.9 (C-11), 39.6 (C-12), 42.2 (C-13), 56.6 (C-14), 24.1 (C-15), 28.1 (C-16), 55.9 (C-17), 11.7 (C-18), 19.2 (C-19), 35.9 (C-20), 18.6 (C-21), 33.8 (C-22), 25.9 (C-23), 45.7 (C-24), 29.3 (C-25), 19.7 (C-26), 18.9 (C-27), 22.9 (C-28), 11.8 (C-29), 99.5 (C-1'), 71.4 (C-2'), 72.8 (C-3'), 68.4 (C-4'), 71.5 (C-5'), 61.9 (6'-CH_2), 20.6 (2'-CH_3), 20.6 (3'-CH_3), 20.6 (4'-CH_3), 20.6 (6'-CH_3), 169.2 (2'-C=O), 170.2 (3'-C=O), 169.3 (4'-C=O), 170.6 (6'-C=O).

The principle of the acetylation process is to substitute the active hydroxyl groups (particularly in the sugar moiety) in the molecule with acetyl groups (CH_3CO-) to obtain an acetate (Bledzki *et al.*, 2008). Hence, the type of sugar presence in the molecule could be identified. The substituted acetyl groups will appear in the proton NMR spectrum at δ_H 2-3 ppm. In this molecule, its ^{1}H NMR spectrum showed four peaks at δ_H 2.03, 2.04, 2.07 and 2.09 ppm corroborating to the four hydroxyl groups at position 2', 3', 4' and 6' of the glucose moiety that have been acetylated. Four C=O signals in the ^{13}C NMR spectrum at δ_C 169.2, 169.3, 170.2 and 170.6 confirm the presence of four acetyl carbons. The spectral data on β-sitosterol glucoside tetraacetae agreed with that reported by Faiz *et al.* (2001).

Table 2.2: Comparison of ^{1}H and ^{13}C NMR data for compound 8a (600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C- NMR, $CDCl_3$) and β-sitosterol glucoside tetraacetate ($CDCl_3$).

	Present study		Literature data*	
Position	**^{1}H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^{1}H NMR**	**^{13}C NMR**
1		37.0		37.2
2		29.5		29.5
3	3.51(1H, *s*)	79.9	3.46	80.1

4		38.8		38.9
5		140.2		140.5
6	5.38 (1H, br, *s*)	122.0	5.33	122.2
7		31.8		32.0
8		31.7		31.9
9		50.0		50.2
10		36.6		36.8
11		20.9		21.1
12		39.6		39.8
13		42.2		42.4
14		56.6		56.8
15		24.1		24.3
16		28.1		28.2
17		55.9		56.1
18	0.69 (3H, *s*)	11.7	0.65	11.9
19	1.01 (3H, *s*)	19.2	1.00	19.4
20		35.9		36.1
21	0.95 (3H, *d*, *J*=6.4 Hz)	18.6	0.93	18.8
22		33.8		33.9
23		25.9		26.2
24		45.7		45.9
25		29.3		29.2
26	0.87 (3H, *d*, *J*=6.9 Hz)	19.7	0.86	19.1
27	0.84 (3H, *d*, *J*=6.9 Hz)	18.9	0.81	19.8
28		22.9		23.1
29	0.85 (3H, *t*, *J*=7.9 Hz)	11.8	0.85	11.9
1'	4.62 (1H, *d*, *J*=8.0 Hz)	99.5	4.57	99.7
2'	5.23 (1H, *t*, *J*=9.5 Hz)	71.4	4.94	71.6
3'	4.98 (1H, *t*, *J*=8.8 Hz)	72.8	5.17	72.9
4'	5.09 (1H, *t*, *J*=9.8 Hz)	68.4	5.07	68.7
5'	3.69 (1H, *m*)	71.5	3.65	71.8
6'	4.13 (2H, *dd*, *J*=2.3, 12.2 Hz)	61.9	4.09	62.2
2'	2.04 (CH_3)	20.6 (CH_3)	169.2 (C=O)	
	*2.00	20.6	169.3	
3'	2.03 (CH_3)	20.6 (CH_3)	170.2 (C=O)	

	*1.98	20.6	170.4
4'	2.07 (CH_3)	20.6 (CH_3)	169.3 (C=O)
	*2.03	20.7	169.5
6'	2.09 (CH_3)	20.6 (CH_3)	170.6 (C=O)
	*2.07	20.7	170.7

***Faiz *et al.* (2001)**

- **Analysis of the ethyl acetate fraction**

Vitexin (9)

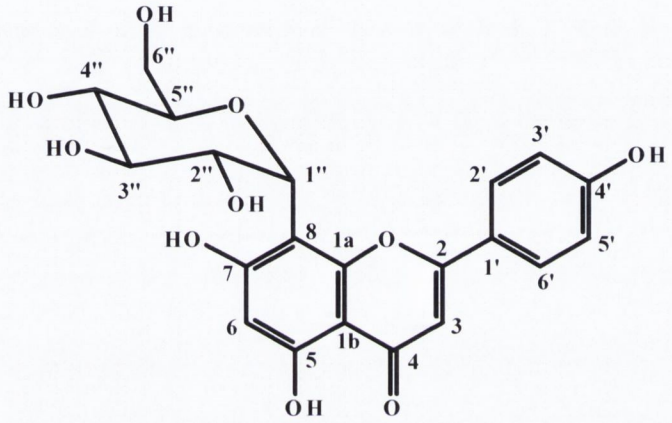


Figure 2.8: Vitexin (9)

Yellow precipitate; R_f: 0.76 (ethyl acetate:methanol; 8:2); mp: 258-260°C; $[\alpha]_D^{20}$ -7.5 (*c* 1.4mg/ml, MeOH); UV (MeOH) λ_{max}: 270, 329; IR ν_{max}: 3361, 1650, 1611, 1505, 1430 cm^{-1}; ESI-MS for $C_{21}H_{20}O_{10}$ m/z; 431 $[M-H]^-$; ^{1}H-NMR (DMSO-d_6, 600 MHz) δ_H: 6.29 (1H, *s*, H-6), 6.79 (1H, *s*, H-3), 6.90 (2H, *d*, *J*=8.7 Hz, H-3', 5'), 8.03 (2H, *d*, *J*=8.7 Hz, H-2', 6'), glucoside moiety : 3.26 (1H, *m*, H-5"), 3.53 (1H, *m* , H-6"b), 3.77 (1H, *m*, H-6"a), 3.85 (1H, *m*, H-2"), 4.69 (1H, *d*, *J*=7.8 Hz, H-1"); ^{13}C-NMR (DMSO-d_6, 150 MHz) δ_C: 98.1 (C-6), 102.5 (C-3), 104.0 (C-1b), 104.6 (C-8), 155.6 (C-5), 160.3 (C-1a), 162.3 (C-7), 164.9 (C-2), 182.7 (C-4), 115.8 (C-3', 5'), 121.6 (C-1'), 128.9 (C-2', 6'), 161.3 (C-4'), glucoside moiety : 61.3 (C-6"), 70.5 (C-4"), 70.8 (C-2"), 73.5 (C-1"), 78.7 (C-3"), 81.8 (C-5").

Compound 9 was isolated as a yellow precipitate (24 mg) with a molecular formula of $C_{21}H_{20}O_{10}$ as determined from EI-MS analysis 431 $[M-H]^-$. The UV spectrum of 9 had absorption maxima at 270 and 331 nm, suggesting the presence of a flavonoid skeleton (Harborne and Williams, 2000; Fossen and Andersen, 2006). Its IR spectrum showed the bands at 3361 (hydroxyl group), 1650 (carbonyl of group), 1611, 1505, 1430 (aromatic

double bond) cm^{-1}. The spectral data on vitexin agreed with that reported by Vázquez *et al.* (2002).

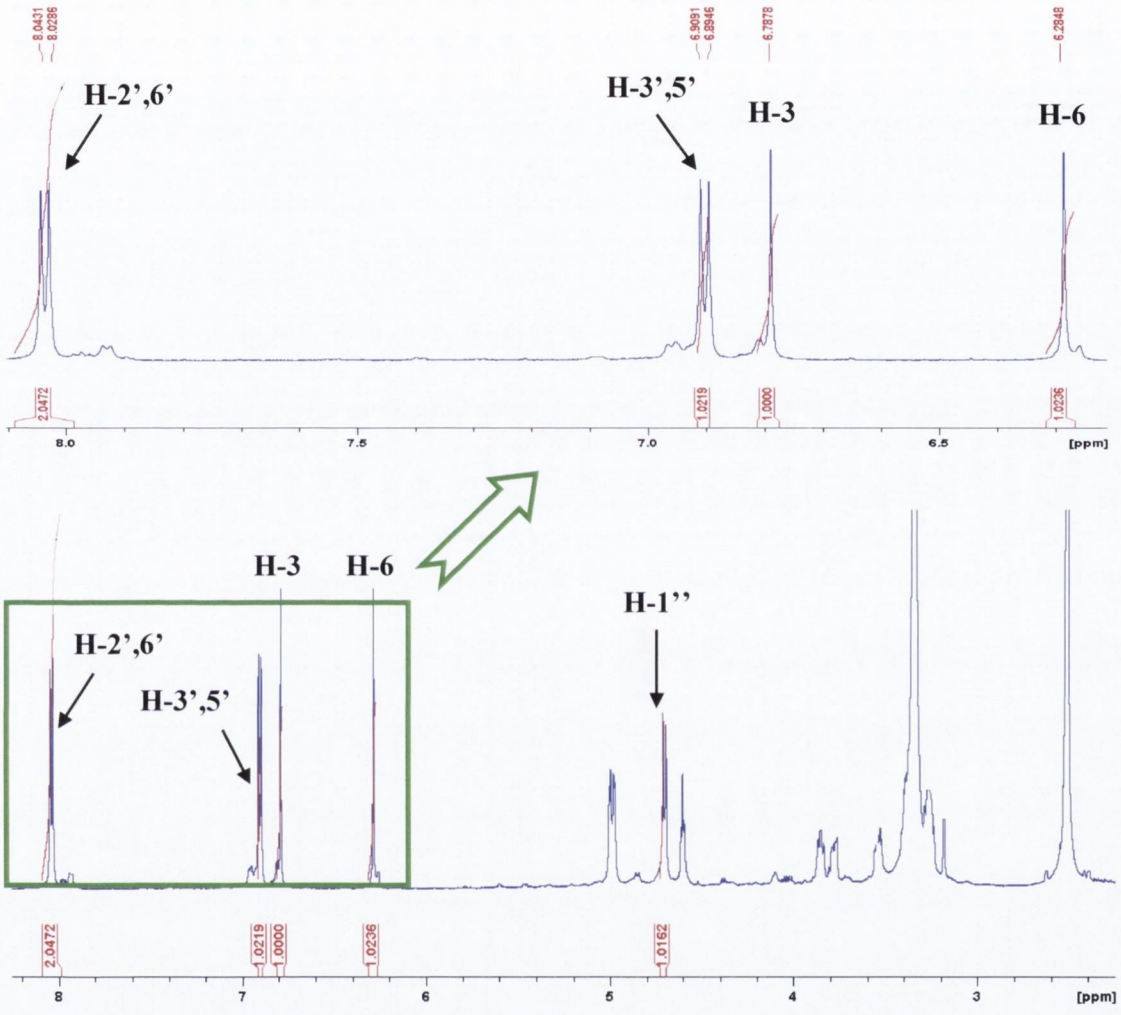


Figure 2.9: The overall ^{1}H NMR spectrum of compound 9 and amplification region showing the doublets of AA'BB' system coupling of H-2',6' with H-3',5' of ring B and singlets of H-3 and H-6.

In ^{1}H NMR spectrum (Figure 2.9), two pseudo-doublet signals at δ_H 8.03 (J=8.7 Hz, H-2', 6') and δ_H 6.90 (J=8.7 Hz, H-3', 5') suggests the typical splitting AA'BB' pattern of flavonoid skeleton in the ring B. In addition, a singlet signal, integrated as one proton, was detected at δ_H 6.79, characteristic of a proton at the position three. A singlet at δ_H 13.18 belongs to the hydroxyl group at position five. The second hydroxyl could be located at the position seven as in the apigenin derivatives. The signal of δ_H 6.29 is expected from the proton located in position six of the ring A. The typical signal of a *beta* anomeric proton is

detected as doublet at δ_H 4.69 (J=7.8 Hz, H-1") and other protons of β-glucose are detected as a multiplet at δ_H 3.53~3.85 ppm.

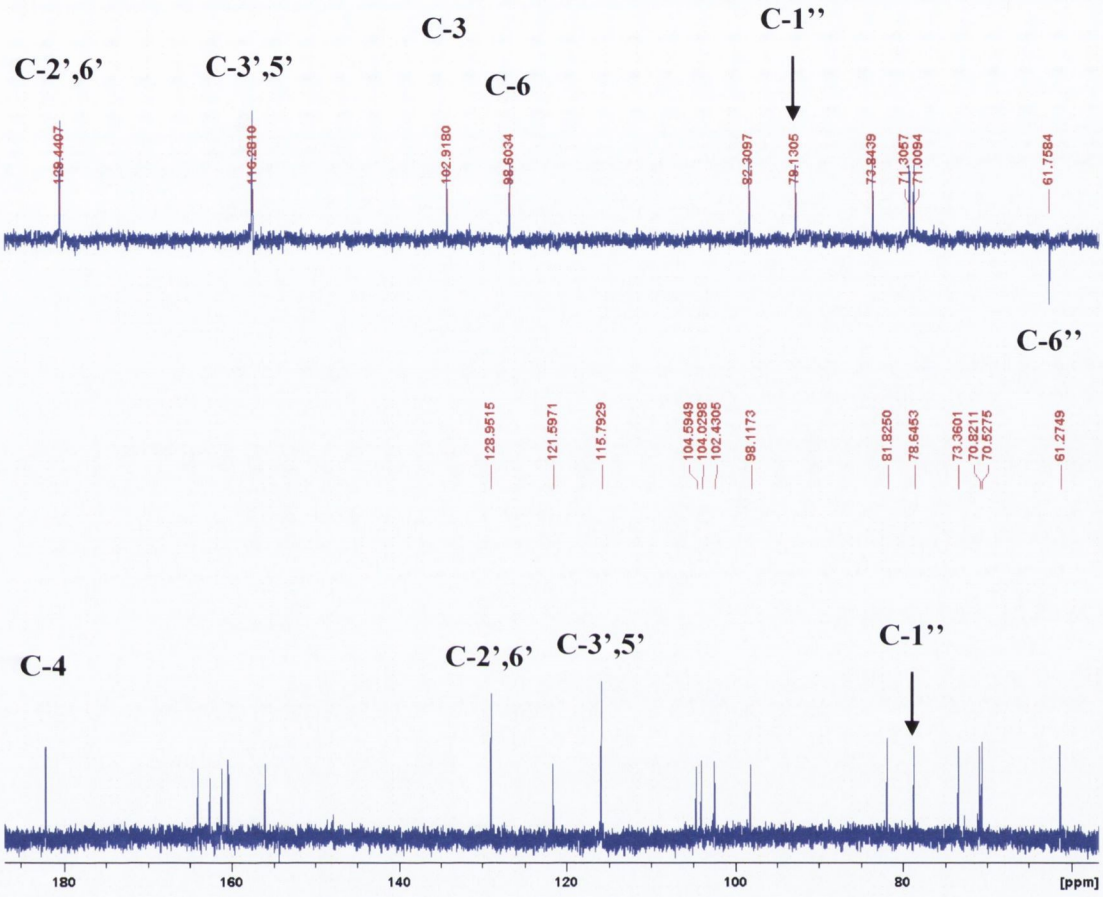


Figure 2.10: The overall ^{13}C (below) and DEPT-135 (above) overall spectrum of compound 3 show the presence of one methylene group of the sugar moiety and ten methine groups, including four from the sugar moiety.

The ^{13}C NMR spectrum (Figure 2.10) shows the presence of twenty-one carbons, associated to fifteen carbons belonging to a flavonoid unit and six carbons belonging to a sugar moiety of hexose between δ_C 60 and 82 ppm. The carbonyl carbon was shown at δ_C 182.7 and the fourteen aromatic carbons are detected at δ_C 98.1 to 164.9. DEPT-135 spectrum (Figure 2.10) shows the presence of nine methine and one methylene that belongs to the sugar moiety.

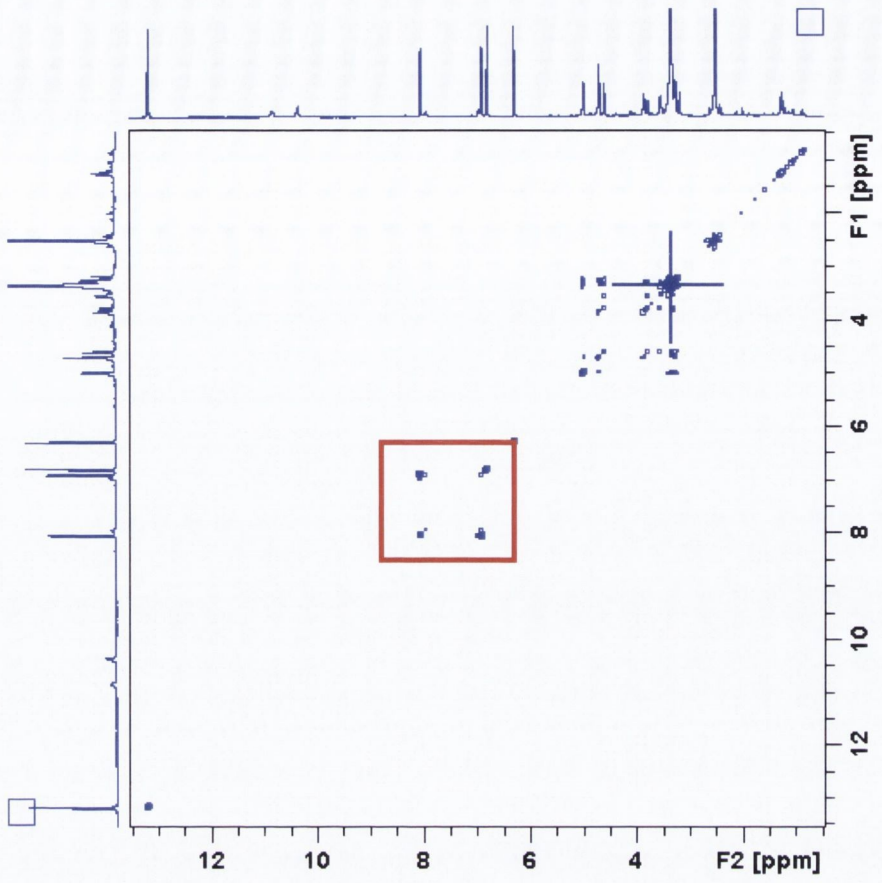


Figure 2.11: ^{1}H-^{1}H COSY-45 spectrum of compound 3 shows coupling of the doublet belonging to H-2', 6' with H-3', 5'.

The ^{1}H-^{1}H COSY-45 spectrum (Figure 2.11) shows that the doublet at δ_H 8.03 (H-2', 6') is coupled to that at δ_H 6.90 (H-3', 5'), forming an AA'BB' system in ring-B of a flavonoid, indicating the *ortho* coupling (J=8.7 Hz).

HSQC plot (Figure 2.12) shows the anomeric proton from a sugar moiety (H-1") at δ_H 4.69 coupling with an anomeric carbon of glycoside at δ_C 73.5 which confirmed the *C*-glycosilation. As the compound is *C*-glycoside linked, six carbon signals of the sugar moiety are confirmed at δ_C 81.8 (C-5"), 78.7 (C-3"), 73.5 (C-1"), 70.8 (C-2"), 70.5 (C-4") and 61.3 (C-6"). The complete NMR spectral data are summarized in Table 2.3 below.

HMBC spectrum was not available due to small amount of available sample. However, the structure of compound 9 was further confirmed by comparison with literature data of vitexin and isovitexin.

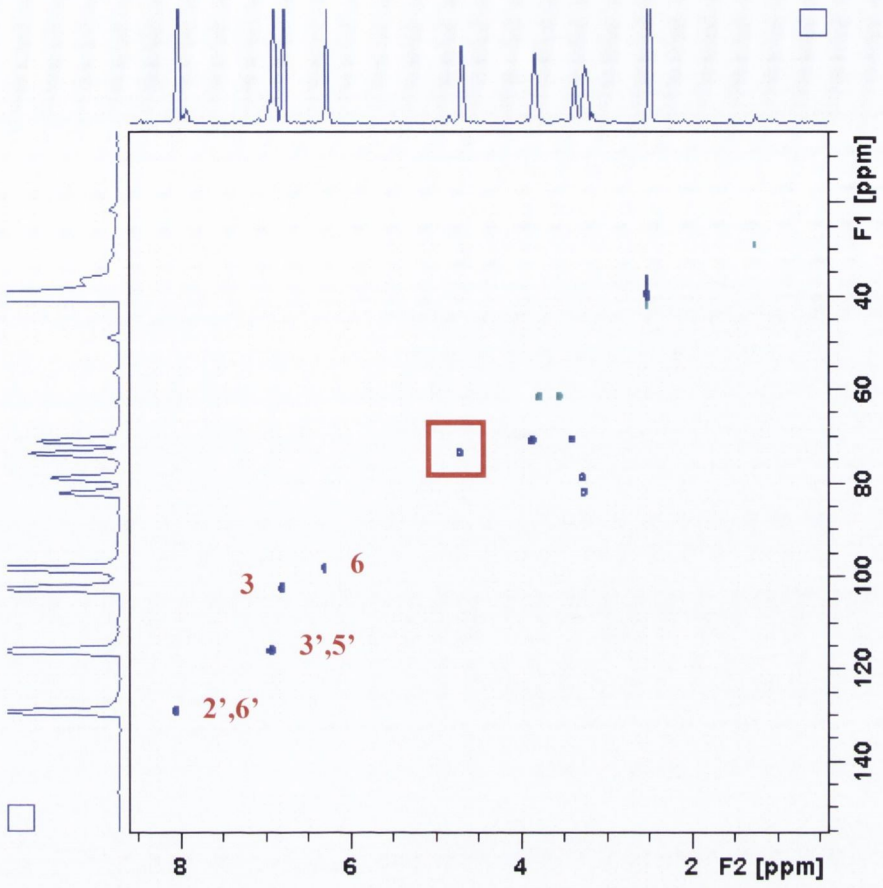


Figure 2.12: HSQC spectrum of compound 3 showing the protonated carbons in the molecule.

Table 2.3: Comparison of ^{1}H and ^{13}C NMR data for compound 9 (600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C- NMR, DMSO-d_6) and vitexin (DMSO-d_6).

	Present study		Literature data*	
Position	**^{1}HNMR, δ_H (ppm) (J in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^{1}HNMR**	**^{13}C NMR**
1a		160.3		160.4
1b		104.0		103.9
2		164.9		163.9
3	6.79 (1H, *s*)	102.5	6.78	102.4
4		182.7		182.1
5		155.6		155.9
6	6.29 (1H, *s*)	98.1	6.27	98.1
7		162.3		162.5
8		104.6		104.6

1'		121.6		121.6
2'	8.03 (1H, *d*, *J*=8.7 Hz)	128.9	8.01	128.9
3'	6.90 (1H, *d*, *J*=8.7 Hz)	115.8	6.88	115.7
4'		161.3		161.0
5'	6.90 (1H, *d*, *J*=8.7 Hz)	115.8	6.88	115.7
6'	8.03 (1H, *d*, *J*=8.7 Hz)	128.9	8.01	128.9
1"	4.69 (1H, *d*, *J*=7.8 Hz)	73.5	4.68	73.3
2"	3.85 (1H, *m*)	70.8	3.83	70.7
3"		78.7		78.6
4"		70.5		70.5
5"	3.26 (1H, *m*)	81.8	3.24	81.8
6"a	3.77 (1H, *m*)	61.3	3.75	61.2
6''b	3.53 (1H, *m*)		3.51	

***Vásquez *et al.* (2002)**

Comparison of the melting point obtained for vitexin (m.p. 258-260°C) with the one published (m.p. 262-264°C) (Vásquez *et al.*, 2002) and co-cromatography by TLC using vitexin standard left no doubt that we had the same compound. Together with the specteroscopic analyses, these findings supported the assignment of the structure of compound 9 to be vitexin (Figure 2.8).

According to Richardson (1978), flavones appeared to be absent while flavonols were found in each of the families. Flavonols quercetin and kaempferol were identified in both *P. bleo* and *P. grandifolia* examined. Numerous morphological and chemical studies over the past few decades have firmly established the taxonomic position of the Cactaceae in the Caryophyllales. The genus *Pereskia*, with its wooden stems and succulent leaves is considered to be the most archaic genus, subdivisions and taxonomic relationships between the other genera are difficult to establlish (Salt *et al.*, 1987).

C-glycosylflavonoids were commonly found in Caryophyllales. However, that type of compounds were found to be absent in the Cactaceae family. Cactaceae was found to be similar morphologically to Didieraceae family (especially the plants from Pereskiaeae) although they were deemed to be different chemotaxonomically. The tribe Pereskiaeae contained kaempferol while the family Didieraceae contained *C*-glycosylflavones (Richardson, 1978). *P. bleo* has been previously examined for *C*-glycosylflavonoids with the results being negative (Gupta *et al.*, 2005). In contrast, four taxa of the Didieraceae

family were found to contain unidentified *C*-glycosylflavones together with quercetin (50%) and myricetin (25%) (Richardson, 1978).

However, in this present study, vitexin has been isolated from the ethyl acetate extract of *P. bleo.* This is the first report of the occurrence of a *C*-glucosylflavonoid in *Pereskia bleo* and in Pereskiaeae. In 2002, Gupta and co-workers described the isolation of vitexin in *Opuntia dillenii*, a plant from the family Cactaceae. This was the first time a *C*-glusosylflavonoid was isolated from this family.

The isolation of vitexin from *Pereskia bleo* confirms the trend of Cactaceae being able to synthesize *C*-glucosylflavonoids and therefore has significance in chemotaxonomy by posing Cactaceae and Didieraceae with similar status both morphologically and chemotaxonomically. To finally confirm this trend and to emphasize the chemotaxonomic importance, other species of *Pereskia* as well as other genera in Cacteceae must be analyzed to show the occurrence of *C*-glycosylflavonoids. This can be easily done by monitoring their ethyl acetate extracts using bi-dimendional NMR techniques such as HSQC.

Another species from the same family, *Opuntia* sp. is known for a great number of potentially active nutrients and their multifunctional properties. Their pear fruits and cladodes are a great choice for the production of health-promoting food and food supplements (Feugang *et al.*, 2006). Alkaloid mescaline, tyramine and *N*-methyltyramine were identified in *Opuntia ficus-indica* extracts with *N*-methyltyramine reported to be the main amine in the whole plant extract from *O. clavata.* Tyramine and its derivatives *N*-methyltyramine and 3-methoxytyramine were identified as the most prominent compounds in *Opuntia* sp. (Stintzing and Carle, 2005; De Vries and Moyna, 1971).

Purification of the butanol fraction from the methanol extract of *Opuntia ficus-indica* var. *saboten* yielded three compounds identified as isorhamnetin-3-*O*-(6''-*O*-*E*-feruloyl)neohesperidoside, (6*R*)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-*O*-β-*D*-glucopyranoside (opuntiside A) and (6*S*)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-*O*-β-*D*- glucopyranoside (opuntiside B), together with 15 known compounds; quercetin, kaempferol, quercetin-3-*O*-methylether, 2,3-dihydrokaempferol, isorhamnetin-3-*O*-glucoside, 2,3-dihydroquercetin, coumaric acid, kaempferol-7-*O*-glucoside, ferulic acid, isorhamnetin-3-*O*-neohesperidoside, isorhamnetin-3-*O*-rutinosyl-4'-*O*-β-*D*-glucoside, isorhamnetin-3-*O*-(2,6-dirhamnosyl)glucoside, zataroside-A, n-butyl-β-*D*-fructopyranoside and 4-*O*-glucosyl-mapic acid (Saleem *et al.*, 2006).

In another investigation on a dried and milled cladode extract of *Opuntia dillenni*, isorhamnetin-3-glucoside, isorhamnetin-3-galactoside, quercetin-3-rhamnoside, myricetin,

vitexin and orientin were isolated (Gupta *et al.*, 2002). Quercetin, kaempferol and isorhamnetin were reported as major compounds in cladodes of *O. basilaris*, *O. leucotricha*, *O. lindheimeri* and *O. quimillo* with methyl-3-quercetin identified for the first time (Burret *et al.*, 1981). In the spines and the cladode pericarp of *O. leucotricha*, methyl-3-kaempferol, 3-hydroxyflavonenes taxifolin (dihydroquercetin) and aromadendrine (dihydrokaempferol) were also identified.

In another study of *O. dillenii*, several chemical constituents were reported from its ethanol extract identified as opuntioside, isorhamnetin-3-rutinoside, opuntiol, manghaslin, *p*-hydroxybenzoic acid, 1-heptanecanol, ferulic acid, 3,4-dihydroxybenzoic acid, vanillic acid, 3,3'-dimethylquercetin, malic acid, kaempferol-7-*O*-β-glucopyranoside, 3-*O*-methylquercetin-7-*O*-β-*D*-glucopyranoside, rutin, ethyl-3,4-dihydroxybenzoic acid, 4-ethoxy-6-hydroxy-methyl-α-pyrone and kaempferol-7-O- β-*D*-glucopyranosyl (1→4)- β-*D*-glucopyranoside (Qiu *et al.*, 2002; Qiu *et al.*, 2003).

It is also worth to mention that usually sitosterol is found together with other steroids, mainly stigmasterol. In this plant, it is possible to isolate sitosterol alone without any other free steroid. This finding is also in accordance with studies performed by Salt and co-workers (1987) in relation to the steroidal composition of Cactaceae plants.

2.3.2 Pharmacological activity of *P. bleo* extracts

2.3.2.1 Antioxidant activity

- **DPPH free radical scavenging activity**

Table 2.4 shows the antioxidant activity (%) of each plant fraction measured by DPPH at absorbance of 544 nm at various concentrations tested; 250, 125, 50, 25, 10 and 5 µg/ml, while Table 2.5 shows the activity of the positive control *Ginkgo biloba* extract tested using the same protocols as the extracts. The EC_{50} values of radical scavenging activity tested for *P. bleo* extract and fractions obtained from the equation of linear regression at 544 nm ranged from 148.570±9.17 to 431.167±8.35 µg/ml (Table 2.6).

Furthermore, Table 2.7 and Figure 2.14 show equivalent values (µg/µg DPPH) ranging between 2.185±9.17 and 6.341±8.35. The crude ethanol extract possessed the highest antioxidant activity with the equivalent value of 2.185±9.17. The dichloromethane fraction showed the second highest activity, followed by the hexane, ethyl acetate and butanol fractions with equivalent values of 2.799±9.26, 3.060±2.93, 4.002±8.28 and 6.341±8.35, respectively. All extract and fractions were statistically different when compared to the positive control, *G. biloba* extract (EC_{50} value 38.225±0.46, equivalent to

0.562±0.46). Figure 2.13 shows the antioxidant activity of *P. bleo* extract and fractions obtained from equation of linear regression.

Table 2.4: Antioxidant activity (%) of *P. bleo* extract and fractions measured after reaction with DPPH, absorbance measured at 544 nm (values of antioxidant activity±standard deviation of the replicates).

conc.	Antioxidant activity (%) of the extract and fractions measured at 544 nm				
	crude ethanol extract	hexane fraction	dichloromethane fraction	ethyl acetate fraction	butanol fraction
250	55.205±0.58	50.797±1.53	49.943±1.32	49.460±4.094	45.027±2.44
125	50.013±1.36	46.353±2.14	48.653±1.53	38.553±2.807	38.490±2.24
50	47.157±1.36	42.840±0.77	44.543±2.65	38.673±1.96	38.130±2.11
25	42.567±1.78	39.740±0.49	39.040±1.73	37.190±3.41	36.823±3.51
10	42.170±1.44	39.23±1.74	38.123±2.39	35.713±3.17	35.880±2.75
5	39.44±1.19	37.73±2.29	36.940±3.17	36.503±4.36	34.807±1.72

The results are presented as means±S.D., n=3. Statistical significance between groups was calculated by analyses of variance (ANOVA), followed by Bonferroni's test. *P*-values less than 0.05 ($p<0.05$) were used as the significant level when compared to the positive control *Ginkgo biloba* extract (Egb 761).

Table 2.5: Antioxidant activity (%) of the positive control, *G. biloba* extract (Egb 761) measured after reaction with DPPH, absorbance measured at 544 nm (values of antioxidant activity±standard deviation of the replicates).

conc.	Antioxidant activity (%) of the positive control *G. biloba* extract (Egb 761) measured at 544 nm			Mean	S.D.
250	95.88	96.28	96.66	96.273	0.39
125	80.63	82.76	81.95	81.780	1.08
50	65.91	67.72	66.57	66.733	0.92
25	50.52	50.95	50.78	50.750	0.22
10	30.72	30.75	30.56	30.677	0.10
5	15.84	15.81	15.68	15.777	0.09

Values were expressed as mean and standard deviation (n=3).

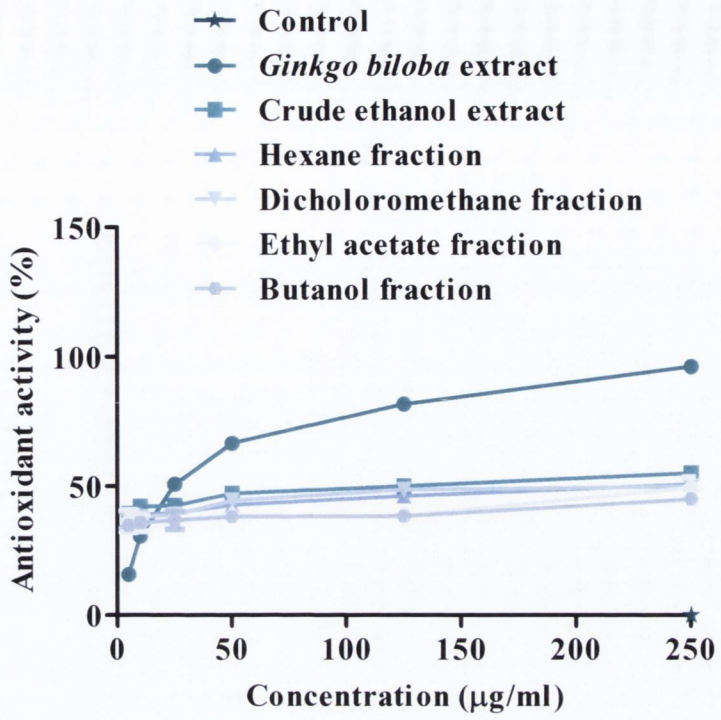


Figure 2.13: Antioxidant activity of *P. bleo* leaves extracts and *G. biloba* extract obtained from equation of linear regression at 544 nm.

Table 2.6: EC_{50} values of the *P. bleo* extracts and positive control, *G. biloba* extract obtained from the equation of linear regressions at 544 nm.

Extract and fractions	EC_{50} values (µg/ml)
G. biloba extract	38.225±0.46
crude ethanol extract	148.570±9.17
hexane fraction	208.104±2.93
dichloromethane fraction	190.346±9.26
ethyl acetate fraction	272.137±8.28
butanol fraction	431.167±8.35

Values were expressed as mean ± standard deviation (n=3). The EC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R^2 \geq 0.90$). $p<0.05$) were used as the significant level when compared to the positive control *G. biloba* extract (Egb 761).

Table 2.7: EC_{50} equivalent the *P. bleo* extracts and positive control, *G. biloba* extract in relation to DPPH (µg/µg DPPH) with DPPH being used at 68 µg/ml.

Extract and fractions	EC_{50} equivalent (µg/µg DPPH) (544 nm)
G. biloba extract	0.562±0.46
crude ethanol extract	2.185±9.17
hexane fraction	3.060±2.93
dichloromethane fraction	2.799±9.26
ethyl acetate fraction	4.002±8.28
butanol fraction	6.341±8.35

Values were expressed as mean ± standard deviation (n=3). The EC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R^2 \geq 0.90$). $p<0.05$) were used as the significant level when compared to the positive control *G. biloba* extract (Egb 761).

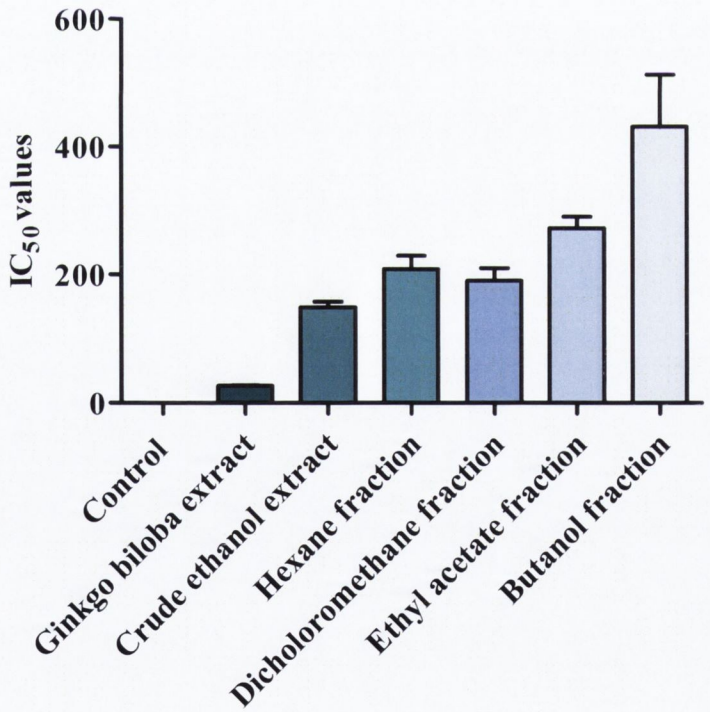


Figure 2.14: EC_{50} values of the crude ethanol extract, hexane, dichloromethane, ethyl acetate and butanol fractions of *P. bleo* in the DPPH radical scavenging activity assay. The results are presented as means±S.D., n=3, $p<0.05$ significantly different compared to the positive control *G. biloba* extract (Egb 761).

Many antioxidants (scavengers) are plant based molecules which play an important role in protecting plants that are exposed to sunlight and are under severe oxygen stress. Antioxidants can also play an important role in human health due to the inability of the biologic defense mechanism to operate properly under severe oxygen stress (Ahmad and Beigh, 2009). Experimental evidence suggests that free radicals and reactive oxygen species (ROS) or reactive nitrogen species (RNS), have been involved in more than 100 diseases, including aging, hardening of the arteries and heart-related disease, diabetes, cancer, tissue injury, skin and gastric ulcer (Policegoudra *et al.*, 2007; Ahmad and Beigh, 2009). There has been an increase interest globally to identifying antioxidant compounds that are pharmacologically potent and have low or no side effects for use in preventive medicine and the food industry (Ali *et al*, 2008).

The use of DPPH provides an easy and rapid way to evaluate antioxidant activity (Silva *et al.*, 2005). Any substance that can donate a hydrogen radical (antioxidant) to the solution of DPPH can reduce the stable free radical and change the color of its solution from violet to pale yellow (Milardović *et al.*, 2006). In the spectroscopic method, the result of antioxidant efficiency is expressed as EC_{50} determined as the concentration of substrate that causes 50% loss in absorbance (DPPH activity) (Milardović *et al.*, 2006).

In this study, the DPPH scavenging activity assay had been used to evaluate the radical scavenging activity of the extract and fractions prepared with *P. bleo* leaves. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time (Yamaguchi *et al.*, 1998). The previous antioxidant activity study by Wahab *et al.* (2009) and Sim *et al.* (2010a) showed that the hexane extract of *P. bleo* was the most effective DPPH radical scavenger (37.55%) and exhibited the lowest EC_{50} value of 210 µg/ml, respectively. Ethyl acetate and dichloromethane extracts were less effective free radical scavenger (16.1%). In the present study, the most effective extract using the same assay was the crude ethanol extract with equivalent value of 2.185±9.17 (µg/µg DPPH). At the highest concentration of extract (250 µg/ml), the scavenging activity was still quite low at 55.205±0.58%. The dichloromethane and hexane fractions also showed low activities at this concentration with values of 49.943±1.32% (equivalent value of 2.799±9.26 µg/µg DPPH) and 50.797±1.53% (equivalent value of 3.060±2.93 µg/µg DPPH). Meanwhile, at the same concentration of extract, the ethyl acetate fraction showed the antioxidant activity at 49.460±4.094% (equivalent value of 4.002±8.28 µg/µg DPPH), followed by the lowest one, butanol fraction at only 45.027±2.44% (equivalent value of 6.341±8.35 µg/µg DPPH).

Although there was a previous report by Wahab and co-workers (2009) discussing the DPPH radical scavenging activity for *P. bleo* fractions, the results were rather confusing. They affirmed as well that 2,4-ditert-butylphenol, the adduct discussed before, was responsible for the observed pharmacological activity. We decided to repeat this assay in this study together with other two methodologies to assess the antioxidant profile to point us out to the more prominent extracts from *P. bleo*.

All extracts tested were statistically different from the positive control used, *Ginkgo biloba* extract. In both studies we can see the absence of an extract producing good or very good antioxidants, at least being detected by DPPH.

The isolation of one compound will not exactly reflect the overall action (Leong and Shui, 2002), especially in this case where we could not find any good antioxidant activity in the extracts. Moreover, the oxidation process is influenced by several factors such as temperature, light, air, physical and chemical properties of the substrates, and the presence of oxidation catalysts or itiators (Maisuthisakul *et al.*, 2007). Besides, differences in polarity (and thus different extractability) are identified as another reason why antioxidant activity of the extracts differ (Maisuthisakul *et al.*, 2007).

- **Total phenolic content assay**

The total phenolic content of the extracts was determined using the gallic acid as a standard and had the callibration curve plotted (Figure 2.15), using the absorbance values obtained with its solutions from 5 to 500 µg/ml (Table 2.8).

Table 2.8: Absorbance values (595 nm) for gallic acid (standard) at various concentrations.

Absorbance values of gallic acid	
Gallic acid concentration (µg/ml)	**Abs. 595 nm**
5	0.060±0.020
10	0.063±0.004
25	0.072±0.004
50	0.081±0.004
125	0.108±0.004
250	0.158±0.005
500	0.237±0.005

Values were expressed as mean ± standard deviation (n=3)

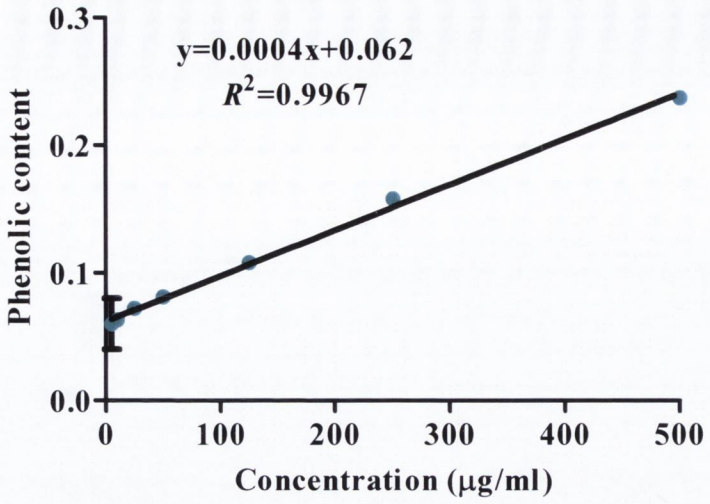


Figure 2.15: The callibration curve of gallic acid used as a standard reference in the total phenolic content assay.

Table 2.9 shows the total phenolic content of each extract and fractions of *P. bleo*. The dichloromethane fraction of *P. bleo* showed the highest total phenolic content among its fractions with 877.500±0.007 µg GAEs/mg, followed by butanol, hexane, crude ethanol and ethyl acetate fractions with 827.500±0.007, 469.175±0.007, 444.175±0.007 and 119.175±0.007 µg GAEs/mg, respectively. According to Sim *et al.* (2010a), the highest phenolic content in the ethyl acetate extract of 40.12 mg of GAEs/g could be explained by the presence of a compound identified as 2,4-di-tert-butylphenol together with α-tocopherol, β-sitosterol and a mixture of sterol (campesterol, stigmasterol and β-sitosterol). As already discussed the first compound is not a natural product and has been identified as such by mistake.

Table 2.9: Total phenolic content (TPC) of the extract and fractions of *P. bleo*.

Extract and fractions	TPC±S.D. (µg GAEs/mg extract)
crude ethanol extract	444.175±0.007
hexane fraction	469.175±0.007
dichloromethane fraction	877.500±0.007
ethyl acetate fraction	119.175±0.007
butanol fraction	827.500±0.007

Values were expressed as mean ± standard deviation (n=3), calculated based on the equation of the gallic acid callibration curve.

These results for *P. bleo* are difficult to explain. Its dichloromethane fraction was the higher in phenolic molecules. Using this solvent for the extraction, the presence of phenolics, especially polyphenols would not be expected. What probably happened was an interference of other molecules, not necessarily phenolics giving a false positive results. Studies of antioxidant activity using medicinal plants are much more complex than they look like making conclusions should be carefully studied.

Phenolics present in plants have received a huge attention because of their potential antioxidant activities. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids presented in the Folin-Ciocalteau reagent. However, it should be noted that some amino acids, proteins, organic acids, sugars and aromatic amines could react with this reagent (Prasad *et al.*, 2009). Phenolic compounds such as flavonoids, phenolic acids and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also possess diverse biological activities as anti-inflammatory, antiatherosclerotic and anticarcinogenic activites (Gursoy *et al.*, 2009).

- **Flavonoid equivalent assay**

The determination of the flavonoid equivalent in the extract and fractions were performed using quercetin as a standard and had its calibration curve plotted (Figure 2.16), based on the absorbance values obtained with its solutions from 5 to 500 µg/ml (Table 2.10).

Table 2.10: Absorbance values (405 nm) of quercetin (standard) at various concentrations.

Absorbance values of quercetin	
Quercetin concentration (µg/ml)	**Abs. 405 nm**
5	0.204±0.005
10	0.260±0.004
25	0.371±0.005
50	0.627±0.008
125	1.280±0.004
250	2.434±0.005
500	3.994±0.011

Values were expressed as mean ± standard deviation (n=3)

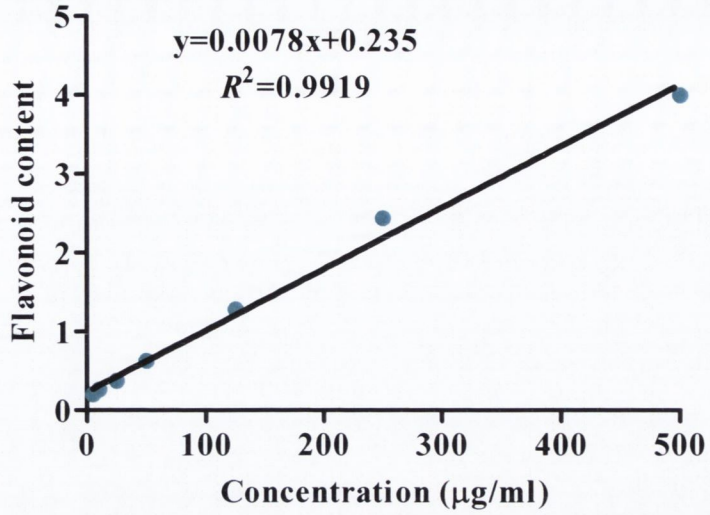


Figure 2.16: The callibration curve of quercetin used as a standard reference in the flavonoid equivalent assay.

Table 2.11: Flavonoid equivalents (FE) of the extract and fractions using the equation of the callibration curve.

Extract and fractions	FE±S.D. (µg QEs/mg extract)
crude ethanol extract	32.775±0.002
hexane fraction	20.275±0.002
dichloromethane fraction	32.325±0.002
ethyl acetate fraction	45.275±0.002
butanol fraction	72.600±0.002

Values were expressed as mean ± standard deviation (n=3), calculated based on the equation of the quercetin callibration.

Briefly, the butanol fraction of *P. bleo* showed the highest flavonoid equivalent amongst its extracts with 72.600±0.002 µg QEs/mg, followed by ethyl acetate, crude ethanol, dichloromethane and hexane fractions with 45.275±0.002, 32.775±0.002, 32.325±0.002 and 20.275±0.002 µg QEs/mg, respectively (Table 2.11). The presence of flavonoid compounds in the ethyl acetate, including vitexin, could contribute to the high activity in this assay.

There could be differences among the tests ran by different extracts. This difference may be attributed to differences in extraction and hydrolysis times and temperatures, or the fore mentioned preharvest factors such as climate, geography, or agronomic practices (Andarwulan *et al.*, 2010) and also various absorption abilities of each type of flavonoid compound as the number and position of the sugar moiety caused different influences on the activity of the flavonoids (Peterson and Dwyer, 1998; Cai *et al.*, 2006). Preparation of the flavonoid-contained food for consumption can also lead to some loss in flavonoid content. Peeling and skinning may lead to the reduction of the total flavonoid content. Flavonoids are relatively stable compounds. Although resistant to heat, oxygen and moderate degrees of acidity, kitchen preparation will cause some flavonoid losses (Peterson and Dwyer, 1998).

- **Correlation analysis between the DPPH antioxidant activity, the total phenolic content and flavonoid equivalent**

From the analysis of Table 2.12 and Figure 2.17, it can be seen that there was no distinct correlation between these studied parameters (total phenolic content, flavonoid equivalent and DPPH scavenging activity) for *P.bleo* extracts.

Table 2.12: Correlation between studied parameters DPPH antioxidant activity with the total phenolic and flavonoid equivalent of the extract and fractions.

Extract and fractions	Absorbance (µg/µg DPPH) 544nm	TPC±S.D. (µg GAEs/mg extract)	FE±S.D. (µg QEs/mg extract)
crude ethanol extract	2.185±9.17	444.175±0.007	32.775±0.002
hexane fraction	3.060±2.93	469.175±0.007	20.275±0.002
dichloromethane fraction	2.799±9.26	877.500±0.007	32.325±0.002
ethyl acetate fraction	4.002±8.28	119.175±0.007	45.275±0.002
butanol fraction	6.341±8.35	827.500±0.007	72.600±0.002

Values were expressed as mean ± standard deviation (n=3). The EC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R^2 \geq 0.90$). $p<0.05$) were used as the significant level compared to the positive control *G. biloba* extract.

P. bleo leaves extract and fractions possessed a low antioxidant activity according to DPPH radical scavenging assay, but a high total phenolic quantity and a low flavonoid equivalent content. Among all of its fractions, its crude ethanol extract showed the highest activity with equivalent value of 2.185±9.17. However, its dichloromethane and butanol fractions showed the highest contents of total phenolics and flavonoid equivalents with 877.500±0.007 µg GAEs/mg and 72.600±0.002 µg QEs/mg, respectively. The highest phenolic content observed in the dichloromethane fraction was not expected and could be due to error during the experiments. The second highest phenolic content was observed in the butanol fraction. This is in agreement with the highest flavonoid content observed in the flavonoid equivalent assay for this same extract.

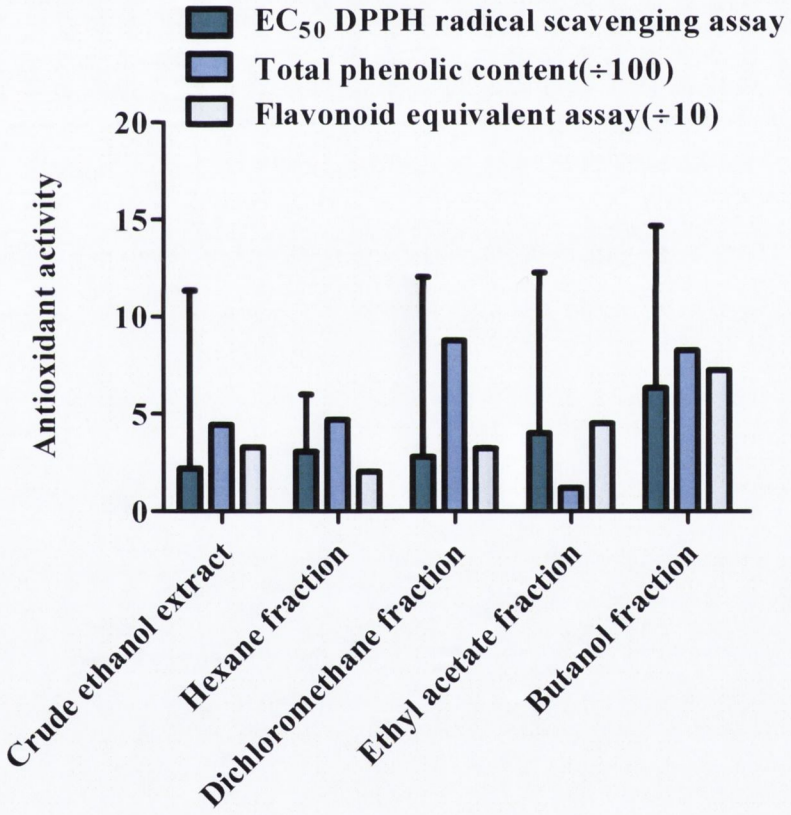


Figure 2.17: Correlation analysis between the DPPH radical scavenging activity with the total phenolic content (÷100) and flavonoid equivalent (÷10) of the extract and fractions. Values were expressed as mean ± standard deviation (n=3). Statistical significance between groups was calculated by two way analyses of variance (ANOVA). $p<0.05$ significantly different in all interaction between the three antioxidant assays used and the extracts.

It is well known that the antioxidant activity of plant materials is well correlated with their content in phenolic compounds (Gursoy *et al.*, 2009). However, in this study, the lowest DPPH radical scavenging activity exhibited by the butanol fraction could be explained by the absence of smaller molecules that could approach the DPPH free radical and donate the hydrogen radical to stabilize it. The antioxidants in the butanol fraction could possibly be complex phenolics. Furthermore, the poor correlation showed by *P. bleo* extracts could be due to the lack of an important quantity of phenolics, together with the possibility of false positive reaction in the tested methods (Prior and Cao, 1998; Wojdylo, 2007).

There are many methods to determine antioxidant capacity. The methods differ in terms of their assay principles and experimental conditions; consequently, different methods evaluate the presence of antioxidants with different antioxidant mechanisms (Prior and Cao, 1998). Generally, the reduction of scavenging effect of the extracts on DPPH radicals increased with the increase in extract concentrations.

Predicting that this plant could synthesize phenolic molecules, it is reasonable to suggest its use in diseases where one of the causes could be oxidative impair. The high total phenol content in an ethanol extract from *O. ficus-indica* var. *saboten* was reported as the one that contributed to the high radical scavenging activity towards superoxide and hydroxyl anions (Qiu *et al.*, 2002). Furthermore, Kuti (2004) has reported that a high antioxidant effect of the cactus fruits was due to the major flavonoids compounds quercetin, kaempferol and isorhamnetin.

P. bleo has been used traditionally in Malaysia to treat rheumatism and the extracts were also evaluated for their antinociceptive and anti-inflammatory activity. The inflammatory diseases were reported to have a close relationship with antioxidant activity with respect to scavenging ROS (Fu *et al.*, 2010). The reactive oxygen species (ROS) and reactive nitrogen species (RNS, e.g. nitric oxide, $NO^{\bullet}$) have been reported to play important role in the pathogenesis with its intensive production being associated with inflammation, generally resulted from oxidative stress (Valko *et al.*, 2007; Novoselova *et al.*, 2009; Fu *et al.*, 2010). NO, which is derived from L-arginine is an important mediator of nociception and also capable of inducing analgesia by analgesic compounds, including NSAIDs and natural products. Furthermore, it also a crucial signalling molecule that plays an important role in acute and chronic pain states of both central and peripheral levels.

2.3.2.2 Antinociceptive and anti-inflammatory activities

In this study, *P. bleo* extracts and vitexin isolated from the ethyl acetate fraction have been assayed for peripheral and central antinociception in three models. The results show that the extracts when given orally produce dose-related and significant antinociceptive activity according to the assessment of the formalin-induced, acetic acid-induced writhing and hot plate methods.

- **Formalin-induced model**

The first test, formalin test, is an established model of chemically-induced nociception (Ferreira *et al.*, 2006). The crude ethanol, hexane, dichloromethane and ethyl acetate fractions of *P. bleo* (30, 50 and 100 mg/kg) produced inhibition of formalin-induced biphasic pain responses (formalin 2.5%) (neurogenic and inflammatory pain) in mice (Figure 2.18). First phase occurs until 5 minutes after injection and second phase occurs between 15 and 30 minutes after formalin injection. During the early phase, all the extracts were not able to decrease the time of licking with similar results to the pattern showed by the vehicle. The analgesic effect of the extracts occurred predominantly during the second phase in case of all extracts, at 100 mg/kg, showing inhibition in licking time when compared to the control. Thus, all the extracts reduced formalin-induced pain at the late phase with the percentage of inhibition reaching 68.97% for hexane fraction, followed by dichloromethane, crude ethanol, ethyl acetate and butanol fractions with 62.07, 41.38, 37.93 and 24.14%, respectively.

It is well known that drugs that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit the late phase (Tjølsen *et al.*, 1992). In the present study, the extracts inhibited the licking time only in the second phase (inflammatory phase), therefore, the effect observed with the extract suggested that the antinociceptive action would be related with peripheral mechanisms. The response in the second phase suggested a possible inhibition and/or liberation of inflammatory mediators such as bradykinin, histamine, sympathomimetic amines, tumor necrose factor-α and interleukins, released in the mice paw or directly blocking their receptors. These mediators are also the target of action of most non-steroidal anti-inflammatory drugs (NSAIDs). Considering the inhibitory property of the hexane fraction in the inflammatory phase, it suggests that this fraction contains active principles acting peripherally.

- **Acetic acid-induced abdominal writhing model**

The abdominal writhing test is a visceral pain model and used to screen and assess the antinociceptive or anti-inflammatory properties of new analgesic agents (Alves *et al.*, 2012; De Souza *et al.*, 2009). It involves differents nociceptive mechanisms, such as the sympathetic system, metabolites and opioid mechanisms and could lead to an increase level of cyclooxygenase and lipoxygenase products in peritoneal fluids as well as the release of many inflammatory mediators such as substance P and bradykinin (Huo *et al.*, 2007; Ikeda *et al.*, 2001).

In the acetic acid-induced abdominal writing assay, the extracts of *P. bleo* were found to reduce the number of contortions after intraperitoneal (*i.p.*) injection of acetic acid. The number of writhing and stretching was recorded and permitted to express the percentage of inhibition. Hexane, ethyl acetate and butanol fractions (30, 50 and 100 mg/kg) reduced the number of contortions of acetic acid-induced pain in mice, with the butanol fraction (30 mg/kg; inhibition of 69.1% / 100 mg/kg; inhibition of 56.4%) and ethyl acetate fraction (50 mg/kg; inhibition of 56.4% / 100 mg/kg; inhibition of 69.1%) showing the lowest number of contortions, followed by the other doses of the butanol, ethyl acetate and hexane fraction s (Figure 2.19). Particularly in the ethyl acetate fraction, it is clearly showed that the fraction acts in a dose-dependent manner, which is an increasing trend in activity with the increase of concentration.

Although the writhing test demonstrates good sensitivity, in some cases, the test shows poor specificity because the abdominal writhing response can be suppressed by muscle relaxants and other types of drugs which could lead to misinterpretation of the results (Le Bars *et al.*, 2001).

- **Hot plate model**

The extracts were also tested at doses of 30, 50 and 100 mg/kg on the supra-spinal model (central antinociceptive effect), the hot plate test, which is demonstrated as area under curve (AUC) (Figure 2.20). In comparison to the vehicle at doses of 30 and 50 mg/kg, all the extracts were not able to achieve higher AUC than morphine (internal standard). Meanwhile, with a dose of 100 mg/kg, the butanol fraction was able to achieve 31.82% higher AUC than morphine.

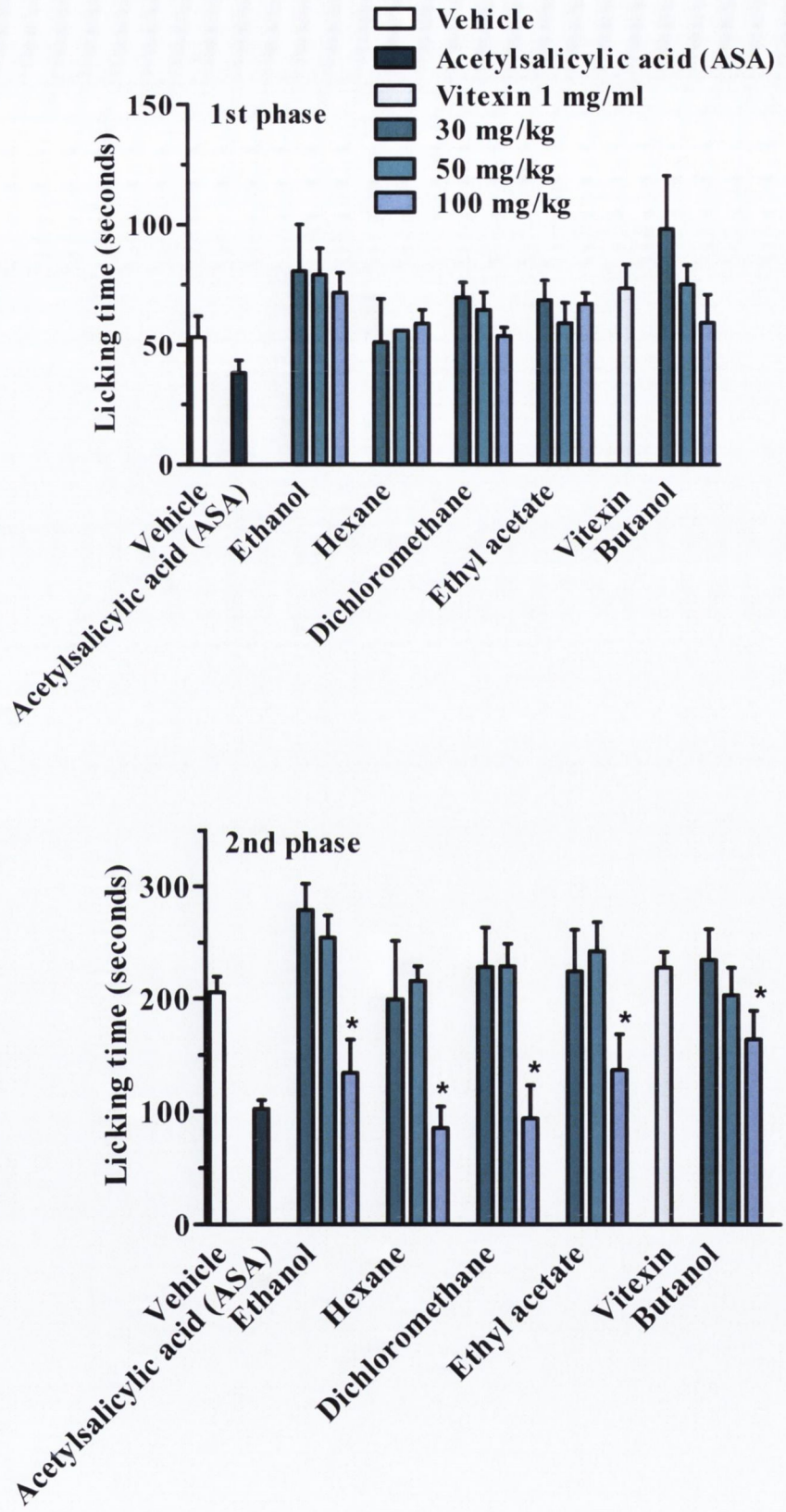


Figure 2.18: Effects of the crude ethanol, hexane, dichloromethane and ethyl acetate fractions of *P. bleo* in the formalin-induced test. Animals were pre-treated by oral

administration of extracts or vehicle (control). The results are presented as means ± S.D., n=5, $p<0.05$ significantly different compared to control, 0-5 min. first phase, 15-30 min. second phase.

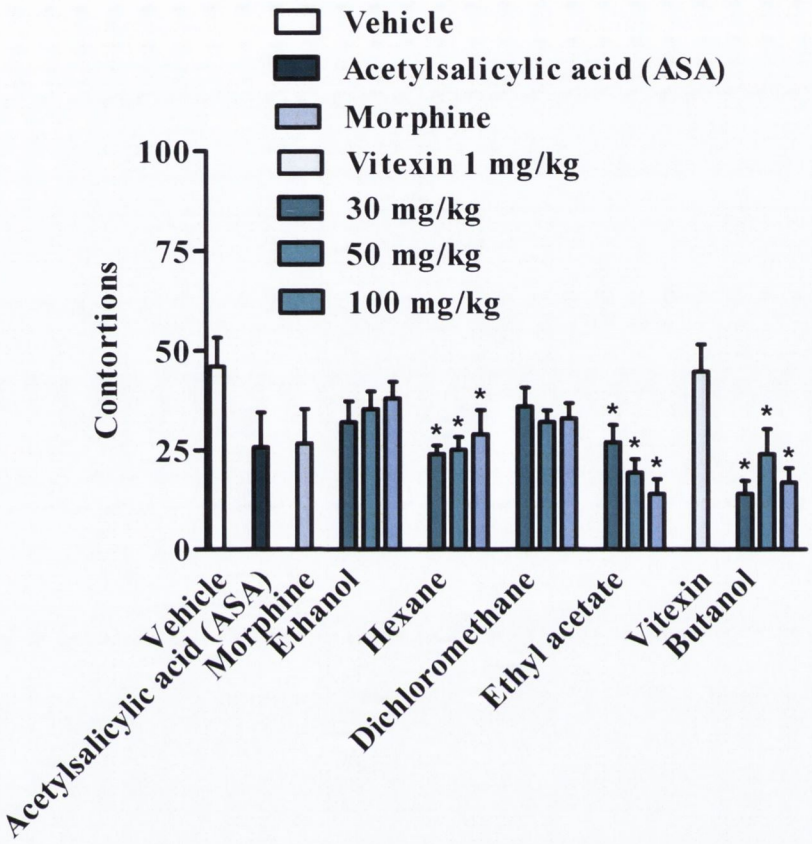
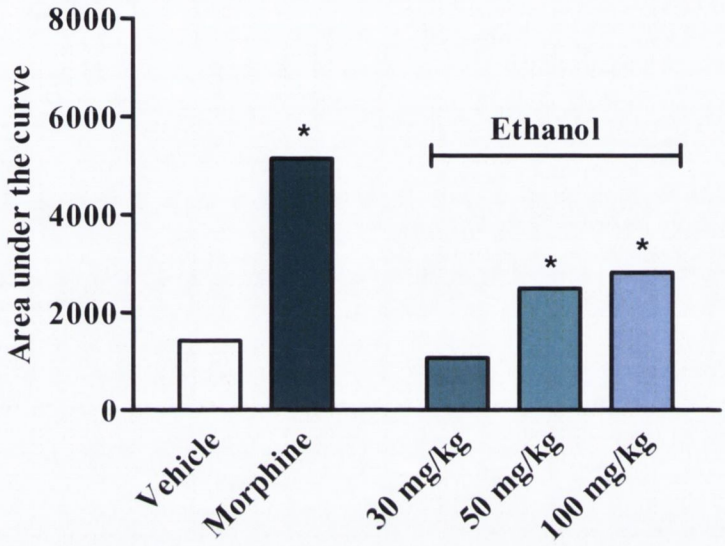
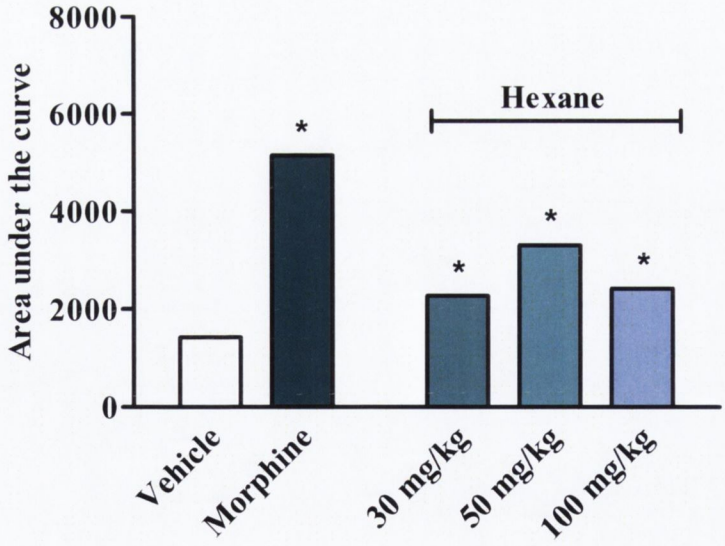


Figure 2.19: Effects of *P. bleo* extract and fractions on acetic acid-induced abdominal writhing. Animals were pretreated by oral administration of crude ethanol, hexane, dichloromethane and ethyl acetate fractions or vehicle (control). The results are presented as means ± S.D., n=5, $p<0.05$ significantly different compared to control. *$p<0.05$ ANOVA followed by Bonferroni's test when comparing extract-treated group with vehicle-treated group.

A large number of natural alkaloids, triterpenes and flavonoids with antinociceptive and anti-inflammatory activities has been reported (Mills and Bone, 2000; Rao *et al.*, 2003). Flavonoids also may inhibit the cyclooxygenase and/or the 5-lipoxygenase pathways of arachidonate metabolism (Harborne and Williams, 2000). In the present study, the known compound (unreported to this species) isolated from the ethyl acetate fraction, vitexin was also tested at the same dose as that received with the ethyl acetate fraction of the crude ethanol extract (1 mg/kg) as it was found in the ethyl acetate fraction using the

same models. Vitexin only achieved AUC of of 1900 (data not shown) when compared to morphine with AUC of 5000. In this study, if 100 mg/kg of the ethyl acetate fraction produced 69.1% pharmacological response, taking into account that 1 mg was vitexin, we tested vitexin at 1 mg/kg to assess if it would have a similar response.





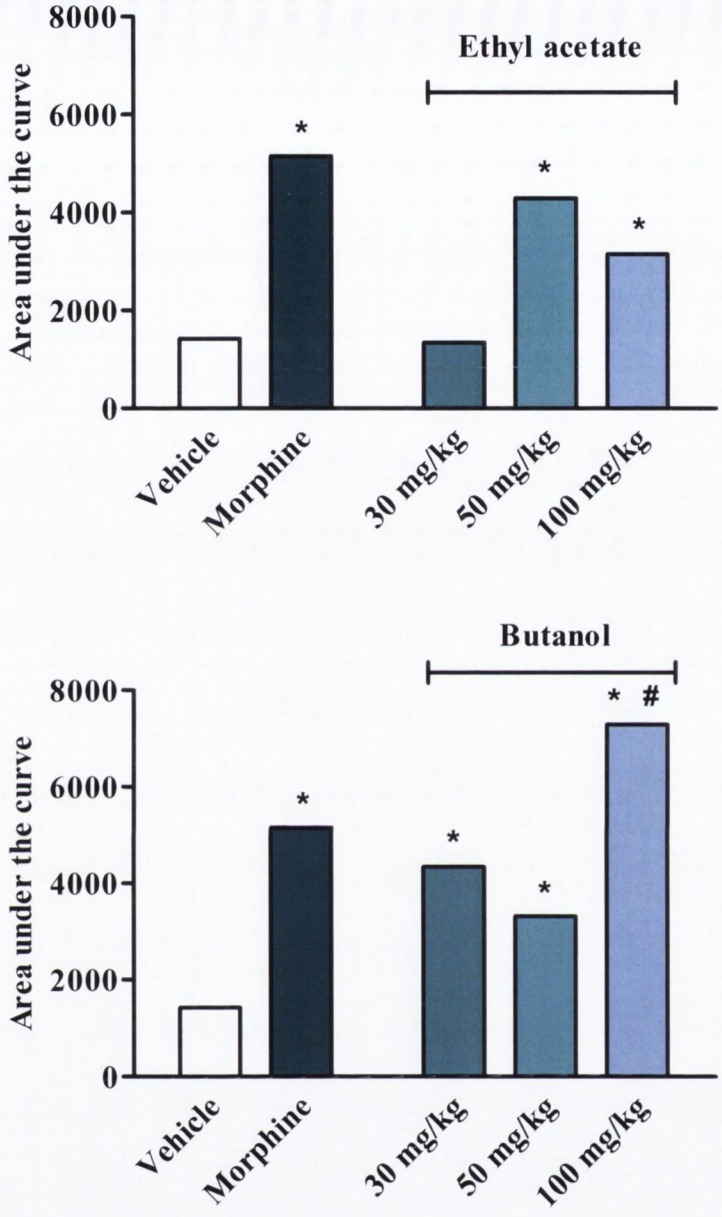


Figure 2.20: Effects of *P. bleo* extract and fractions in the hot plate model. Animals were pre-treated by oral administration crude ethanol, hexane, dichloromethane and ethyl acetate fractions (100 mg/kg) and morphine (5 mg/kg). The results are presented as means ± S.D., n=5 of area under the curve calculated by the Prism 5.0 software. $p<0.05$ significantly different compared to control. * $p<0.05$ when comparing a treated group with a vehicle-treated group, $^{\#}$ $p<0.05$ when comparing a treated group with morphine-treated group.

The result demonstrates for example that vitexin alone is not the responsible for the antinociceptive activity of the ethyl acetate fraction in the acetic acid-induced writhing test.

On the other hand, a previous study by Gorzalczany *et al.* (2011) has proved that vitexin is the most active compound isolated from *Urtica circularis*. In the acetic acid-induced writhing model, at dose of 10 mg/kg *i.p.*, vitexin produced a significant inhibition of 91% abdominal contortions in mice. This high inhibition could probably due to the higher concentration used when compared to the one used in this study (1 mg/kg). If the same concentration was used here, it would be misleading the results as the concentration of the vitexin found in the ethyl acetate fraction was slightly lower than 1%.

The good activity of the hexane and dichloromethane fractions in the formalin-induced and acetic acid-induced writhing assays could be due to the high concentrations of β-sitosterol (yielding 10%) and β-sitosterol glucoside (yielding 7.5%) in the extracts, respectively. Although the active principle responsible for the antinociceptive of the *P. bleo* extracts remains to be investigated, a number of scientific reports on the isolated compounds, β-sitosterol and β-sitosterol glucoside supported that they may responsible to the activity observed (Villaseñor *et al.*, 2002).

β-sitosterol and β-sitosterol glucoside are known as the major phytosterols in most higher plants (Ju *et al.*, 2004; Villaseñor *et al.*, 2002; Maridass and Ramesh, 2010). Previous reports showed that they possess numerous biological activity including analgesic, anti-inflammatory and anticomplement activities, anthelminthic and antimutagenic activities, exhibiting antipyretic, antineoplastic, immune-modulating and blood sugar controlling effects, apoptosis-promoting effects and may prevent colon, prostate and breast cancer. Besides, they also act as immunomodulatory agents and were used in the treatment of pulmonary tuberculosis and modulate the immune system in cases such as chronic viral infection, allergy and rheumatoid arthritis (Villaseñor *et al.*, 2002; Ju *et al.*, 2004; Maridass and Ramesh, 2010; Saeed *et al.*, 2010; Jayaprakasha *et al.*, 2009).

Focusing on the antinociceptive and anti-inflammatory activities, Villaseñor and co-workers (2002) and Saeed and co-workers (2010) reported β-sitosterol and β-sitosterol glucoside as analgesic constituents. The acetic acid-induced writhing test showed that β-sitosterol and β-sitosterol glucoside decreased the number of contortions induced by acetic acid by 70% and 73%, respectively at a dose of 100 mg/kg mice. This was also supported by another study by Backhouse and co-workers (2008). The hot plate method confirmed their analgesic activities, as β-sitosterol and β-sitosterol glucoside exhibited a 300% and 157% increase in pain tolerance, respectively.

Although the doses of *P. bleo* extracts (100 mg/kg) used were higher than established analgesic and anti-inflammatory drugs, it must be taken into account that *P. bleo* was always used as a plant extract. The extract is composed of by different molecules

at different concentrations. Another important observation is that all the extracts were administered orally. Influence of pH in the stomach and lipo-solubility may interfere with their absorption in the gastrointestinal tract limiting their bioavailability in reaching blood and tissue (Matheus *et al.*, 2005; Rinaldi *et al.*, 2009). Even though, with all described factors, important activities were observed. Thus, it can be suggested that the plant could be a very inexpensive source of a new analgesic and anti-inflammatory drugs in relation to rheumatism (its main use in Malaysia).

This study demonstrated that the extracts of this plant possess moderate analgesic activity in a dose-dependent manner when tested in the above explained models and suggested that they and/or their active principles might represent potential therapeutic options for the treatment of pain related diseases. The doses used in this study are in accordance with the dose taken by an average man of 70 kg (Pinheiro *et al.*, 2010), in terms of tea drinking per day, as this plant can be consumed as water infusion. The antinociceptive effects showed in this study support the ethnomedical use of this plant to treat rheumatism and inflammations, and contribute to the knowledge of the chemical composition in natural products.

In order to evaluate the possibility of extracts to develop a toxic effect after oral administration, mice received 500 mg/kg of extracts. After 14 days of a single administration, the treated mice did not show behavioural alterations and no lesion or bleedings in stomachs were observed (data not shown). This suggests that the extracts have no toxicity, which indicates therapeutic safety for the use of pharmacology.

2.4 CONCLUSIONS

The phytochemical investigation of *Pereskia bleo* provided three known compounds isolated and identified from its fractions; hexane, β-sitosterol (1), dichloromethane, β-sitosterol glucoside (8) and ethyl acetate, vitexin (9). Vitexin, a *C*-glycosylflavonoid, isolated from the ethyl acetate fraction is the first *C*-glycosylflavonoid reported for this plant. The occurrence of *C*-glycosylflavonoids in Pereskiae, including in this species is reported for the first time and could have chemotaxonomic importance, as discussed.

In the DPPH radical scavenging activity test, the crude ethanol extract exhibited the highest antioxidant activity followed by the dichloromethane, hexane, ethyl acetate and butanol fractions with equivalent values (μg/μg DPPH) ranging between 2.185±9.17 and 6.341±8.35. However, when compared with the positive control, *Ginkgo biloba* extract, extract and fractions showed no statistically significant activity in the radical scavenging

activity assay (DPPH). In the total phenolic content test, the dichloromethane fraction of *P. bleo* showed the highest total phenolic content followed by butanol, crude ethanol and ethyl acetate fractions ranging between 119.175±0.007 and 877.500±0.007 µg GAEs/mg. On the other hand, the butanol fraction of *P. bleo* showed the highest flavonoid equivalent amongst its extracts, followed by ethyl acetate, crude ethanol, dichloromethane and hexane fractions ranging between 20.275±0.002 and 72.600±0.002 µg QEs/mg. No correlation was seen between the three parameters (DPPH scavenging activity, total phenolic content and flavonoid equivalent) used in this study.

The antinociceptive evaluations of all the extracts showed good results in the three models tested: formalin-induced, acetic acid-induced abdominal writhings and hot plate. Hexane fraction showed moderate effect in the formalin-induced test which could be explained by the presence of β-sitosterol, ethyl acetate fraction showed the lowest number of contortions in the acetic acid-induced test, while butanol fraction showed higher AUC than morphine in the hot plate test. Vitexin was found not be responsible for the antinociceptive activity of the ethyl acetate fraction at the concentration it appears in the extract. Nevertheless, further investigation are in course to evaluate the mechanism of action by which ethanol, ethyl acetate and butanol fraction from *P. bleo* leaves develop central antinociceptive activity.

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CHOISYA TERNATA KUNTH.

(RUTACEAE)

3. *CHOISYA TERNATA* KUNTH. (RUTACEAE)

3.1 LITERATURE REVIEW

3.1.1 *Choisya* sp. (Rutaceae)

Figure 3.1: *Choisya* sp.

Scientific classification

Kingdom	:	Plantae
Division	:	Angiosperms
Order	:	Rutales or Sapindales
Family	:	Rutaceae
Subfamily	:	Rutoideae
Genus	:	*Choisya*

Family in Order Rutales (Thorne, 1992) or Sapindales (Cronquist, 1988) consist of Flindersioideae, Aurantioileae, Rutoideae, Spathelioideae, Toddalioideae, Anacardaceae, Burseraceae, Cneoraceae, Meliaceae, Ptaeroxylaceae and Simaroubaceae (Waterman, 2007; Chase *et al.*, 1999). The Rutaceae is a predominantly tropical family of trees and shrubs consisting of genera with importance in horticulture, silviculture, herbal medicine, essential oil, food and spice. The family is subdivided into four subfamilies; Citroideae (Aurantioideae), Dictyolomatoideae, Flindersioideae and Rutoideae (Chase *et al.*, 1999). It is considered to consist of about 1815 species distributed between approximately 161 genera (Waterman, 1975; Salvo *et al.*, 2008), and has been investigated morphologically,

molecularly and biochemically and the latter revealed diversity of secondary chemical compounds (Salvo *et al.*, 2008).

Choisya (**Error! Reference source not found.**) is a rutaceous genus native to North America, initially studied by Gray in 1888, Standley in 1923, and later by Muller in 1940 (Creche *et al.*, 1993). The genus contains seven species; *C. ternata* Kunth, *C. neglecta* Muller, *C. dumosa* Gray, *C. mollis* Standley, *C. arizonica* Standley, *C. palmeri* Standley and *C. katherinae* Muller (Dreyer *et al.*, 1972).

3.1.1.1 Morphology of *Choisya* sp.

The genus *Choisya* is based on *Choisya ternata* which was first described in 1823 by the German botanist Kunth when he classified the plants harvested in North America by Humboldt and Bonpland. This species is one of the members of the family Rutaceae which is grouped with Simaroubaceae, Meliaceae, Cneoraceae and Ptaeroxylaceae, due to the absence of resin ducts in the bark, woody rays and leaf veins and presence of triterpenoid compounds (Cronquist, 1988), subfamily Rutoideae, tribe Xanthoxyleae, subtribe Choisyinae. It is fructified infrequently and pollen is often defective. According to Desai (1955), *C. ternata* may be a hybrid, however, some seeds were described by Boudouresques in 1895 (Creche *et al.*, 1993; Muller, 1940). A study of *rbcL* data analysis from some members in the Order Sapindales showed that Simaroubaceae was a sister group of Rutaceae, which recently was also supported by combined *rbcL* data and *atpB* matrices (Chase *et al.*, 1999).

It has estipulate leaves, seldom more than two ovules per carpel, a nectary disk, usually no more than twice as many stamens as sepals or petals and a superior ovary (Chase *et al.*, 1999).

3.1.1.2 Ethnobotanical uses of *Choisya* sp.

The only ethnobotanical uses of this genus so far, has been reported for *C. ternata*, which acted as tonic, appetizer, and also having an antispasmodic and 'stimulative properties'. Previously, in 1895, Boudoresques performed the first pharmacological test on himself (Creche *et al.*, 1993).

3.1.1.3 Phytochemical studies of *Choisya* sp.

A wide structural diversity of secondary metabolites occur in plants belonging to the Rutaceae, particularly *Choisya* species, most noteworthy alkaloids, limonoids, flavonoids, coumarins (notably furano- and pyrano-coumarins) and volatile oils (Waterman, 1973, 1975; Gray and Waterman, 1978).

The chemical diversity of alkaloids in the Rutaceae is correlated with biosynthetic pathways derived from aromatic amino acid precursors, anthranilic acid, tyrosine, tryptophan, and histidine (Waterman, 1973; Tillequin, 2007). The biosynthetic pathways could involve different aromatic acid precursors, and in some cases, two amino acid precursors at the same time (Tillequin, 2007). Anthranilic acid derivatives include numerous chemical series either completely or largely restricted to the Rutaceae, exemplified by quinolones, furoquinolines, acridones, carbazoles and related heterocyclic systems (Tillequin, 2007).

Alkaloids with a basic quinoline skeleton are present as the main compounds in the family (O'Donnell *et al.*, 2006). Both furoquinolines and acridones occur widely and are apparently specific to this family (Creche *et al.*, 1993). Neither of them have ever been reported elsewhere in the Angiospermae (Waterman, 1973). An abundant furoquinoline alkaloid, skimmianine is by far the most common and has been found in at least 38 of the 50 Rutaceous genera (Waterman, 1973). Other alkaloids reported to occur in *Choisya* sp. are evoxine (*O*-isoprene furoquinolines), kokusaginine and choisyine (Waterman, 1975). Acridones are less widespread but occur in at least 13 genera with arborinine being the most common. By contrast, Dreyer *et al.* (1972) have suggested that pentacyclic triterpenes are apparently uncommon in the Rutaceae, noting the presence of lupeol in *Choisya* HBK (all of the Rutoideae) (Waterman, 1973).

The closely related species, *C. arizonica* Standl. and *C. mollis* Standl., which grow in the canyons of mountains ranges of Southeastern Arizona, have now been shown to contain lupeol, limonin, skimmianine, kokusaginine and choisyine as well as the coumarins xanthyletin and 7-isopentenyloxy-8-isopentenylcoumarin. The presence of the alkaloid evoxine and friedelin, a triterpene, was detected in both *C. arizonica* and *C. ternata* extracts (Dreyer *et al.*, 1972).

3.1.1.4 Pharmacological studies of *Choisya* sp.

To date, there is only one pharmacological activity reported for *Choisya* sp., showing that leaves of *C. ternata* have antispasmodic properties (Salvo *et al.*, 2008). More

pharmacological activities were reported for compounds also found in *C. ternata* such as alkaloids evoxine, kokusaginine, skimmianine, platydesminium, pentacyclic triterpene lupeol and its derivatives and sterol β-sitosterol glucoside. A new minor compound named ternanthranin, isolated from *C. ternata* essential oil exhibited high antinociceptive activity (significant at 0.3 mg/kg) in a dose dependent manner (Radulović *et al.*, 2011).

However, there are several plant extracts of Rutaceae species reported to possess pharmacological activity and hence there is an interest in the isolation and characterization of the active principles present in Rutaceae plants (O'Donnell *et al.*, 2006).

3.1.2 *Choisya ternata* Kunth (Rutaceae)

Choisya ternata Kunth. (Rutaceae) (**Error! Reference source not found.**) is a bushy shrub with the maximum height of 2 meters. It is widely cultivated as an ornamental shrub (Dreyer *et al.*, 1972), with persistent leaves and white flowers resembling those of orange trees. Its English name is 'Mexican orange' because it originates from the central and southern mountains of Mexico (Creche *et al.*, 1993). It is widely cultivated in its native country and also in the warmer parts of Europe and America. It is commonly known as 'Hierba del Clavo', 'Flor del Clavo', 'Clavillo' and 'Clavo de Olor' among local people and since it is a very striking ornamental, it is exceedingly popular (Creche *et al.*, 1993; Muller, 1940).

3.1.2.1 Morphology of *C. ternata*

Choisya ternata is an aromatic shrub, leaves are sub-pinnately trifoliolate, the leaflets are broad, margins are entire and acute or rounded, terminally or laterally in the axils of the leaves. Leaves are green-yellow or yellow during summer and green during fall season. The flowers are fragrant with a sweet smell, large, white and perfect with 4 or 5 petals, and in cyme inflorescence of several flowers. Corolla shape is rotate or stellate with a superior ovary position. In 2005, Teodoridis and Kvaček compared *Choisya ternata* HBK, which is widely cultivated in warmer parts of North America and Europe and *Choisya arizonica* Standl with the genus *Chaneya* Wang *et* Manchester. The two species are different from *Chaneya*, particularly in relation to their floral diagrams. The stamens are in two whorls, the fruit consists of 2 capsules (rarely 1 or 3 or more) and differences of the venation of petals. However, Mulller (1940) reported that the inflorescence of subgenus *Choisya* matches well that of *Chaneya*.

Neverthesless, the discrepancies mentioned above rule out a possibility that *Chaneya* is very close to *Choisya*. None of the Rutaceae has accrescent perianth parts persistent of the fruits. A close correspondence with the representatives of *Picrasma* sp. from the family Simaroubaceae, in relation to the presence of oil cells commonly spread in the Rutaceae showed that both families (Rutaceae and Simaroubaceae) are the groups most similar to *Chaneya* (Teodoridis and Kvaček, 2005).

Figure 3.2*: Choisya ternata* Kunth (Rutaceae); the flower (A), the leaves (B) and the aerial parts of the plant (C).

3.1.2.2 Ethnobotanical uses of *C. ternata*

The first pharmacological use for this plant was in 1895. Boudoresques was the first to discover the medicinal effect of the aqueous extracts of leaves by experimenting on himself, which later he found them tonic and appetizing. Later, in 1923, Standley reported that Mexican people employed infusion of leaves for their antispasmodic and 'stimulative properties' (Creche *et al.*, 1993). *C. ternata* was later included in the Pharmacopoeia of Mexico (Respaud *et al.*, 1997).

3.1.2.3 Phytochemical studies of *C. ternata*

Previous phytochemical studies have shown that *C. ternata* contains numerous quinoline alkaloids identified as skimmianine, choisyine, evoxine, kokusaginie, dictamnine and lunacrine (Sejourne *et al.*, 1981, Creche *et al.*, 1993), which are derived from

anthranilic acid. The biosynthetic pathway of furoquinolines has been studied in different Rutaceae, including *C. ternata*, by Grundon *et al.* (1974), and the general scheme given in previous studies by Petit-paly and co-workers in 1989 and 1993 fits with *C. ternata* (Creche *et al.*, 1993). The furoquinoline alkaloid dictamine has been shown to be a precursor of the dioxygenated alkaloid, particularly skimmianine (Collins *et al.*, 1972).

Besides, two dihydrofuroquinoline alkaloids, balfourodinium and platydesminium, have been isolated from the leaves extracts (Sejourne *et al.*, 1981; Rideau *et al.*, 1979; Creche *et al.*, 1987; Montagu *et al.*, 1986). In addition to that, 7-isopentenyloxy-γ-fagarine has been isolated and eight new members of the furoquinoline family have been synthesised from this plant (Boyd *et al.*, 2007) (**Error! Reference source not found.**).

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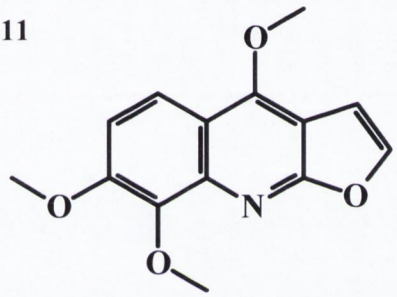

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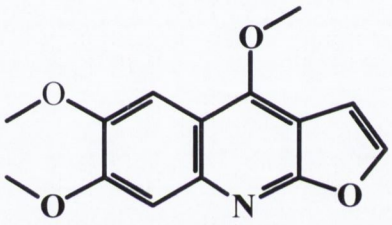

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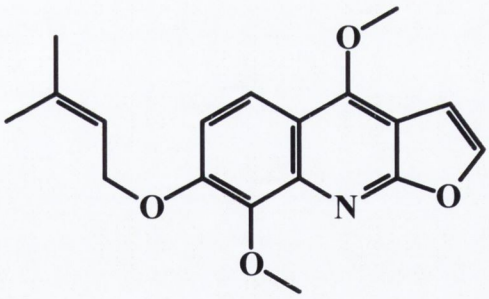

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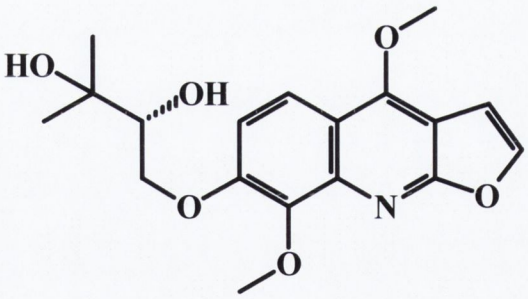

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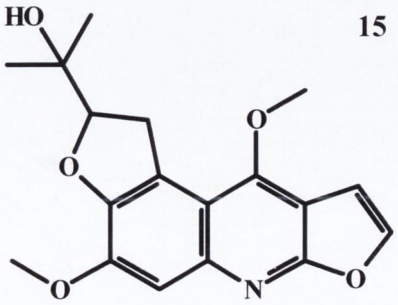

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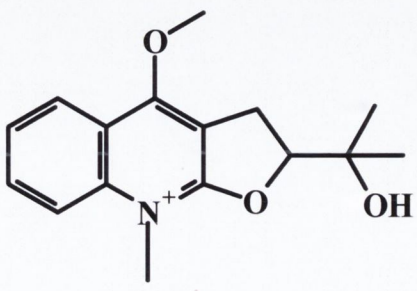

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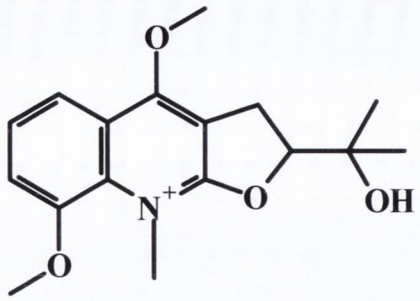


Figure 3.3: Compounds isolated from *C. ternata* extracts; skimmianine (11), kokusaginine (12), 7-isopentenyloxy-γ-fagarine (13), evoxine (14), choisyine (15), platydesminium cation (16) and balfourodinium cation (17) (Boyd *et al* ., 2007).

Frolova and Kuzovkov (1963) synthesised several choisyine derivatives, which later were identified as isochoisyine, ethoxychoisyine, norchoisyine, dihydrochoisyine, tetrahydrochoisyine, anhydrochoisyine, isohydrochoisyine, noranhydrochoisyine, acetylnoranhydrochoisyine and acronidine.

Bohlmann and co-workers (1972) isolated coumarins; xanthyletin, suberosine and dehydro-osthol from the roots of *C. ternata* while Gray and Waterman (1978) isolated coumarins from the stems or branches extract (consisting of mevalonate-derived side chains) and xanthyletin. Dreyer and co-workers (1972) detected triterpenes; limonin and friedelin from *C. ternata* leaves extracts and reported that pentacyclic triterpenes are not common (Chandler and Hooper, 1979; Creche *et al.*, 1993). Later, Tea *et al.* (1975) isolated flavonoids; astragaline and kaempferol-3-□-rhamnoglucoside from the leaves and petals (Creche *et al.*, 1993).

The first essential oil composition evaluation of *C. ternata* in 1997, characterised eighteen components with α-phellandrene (64-71%) as the major component (Respaud *et al.*, 1997). Recently, in depth evaluation of the essential oil composition from leaves of *C. ternata* led to the identification of a major compound, sabinene (35.1%) and new minor compound (ternanthranin) with potential antinociceptive activity. As the new compound was a minor compound in the essential oil, its synthesis has been performed to allow pharmacological evaluation as well as unequivocal structure determination (Radulović *et al.*, 2011).

3.1.2.4 Pharmacological studies of *C. ternata*

To date, there is limited pharmacological data for *C. ternata* extracts. However, compounds occurring in this plant have been reported to show pharmacological effects. According to Berezhinskaya in 1970, the antispasmodic properties of the leaves of *C. ternata* are reported, probably due to evoxine, which has sedative, hypnotic and antispasmodic effects (Salvo *et al.*, 2008), while Kovalenco in 1946 reported that kokusaginine showed ganglioplegic effects. In addition, in 1972, Polietsvev and co-workers reported that the main tertiary alkaloid, skimmianine, acted as an antidiuretic and hypothermic agent (Creche *et al.*, 1993). Balfourodinium has been found to produce a slight spasmolytic effect on isolated rat duodenum (Foussard-Blanpin, pers. Comm.) (Creche *et al.*, 1993). Besides, this compound and platydesminium were also reported to display cytotoxic and antimicrobial activities (Creche *et al.*, 1987). The anti-platelet activity of one of the major compounds of *Choisya* sp., skimmianine has been previously reported. According to Chen and co-workers (2000), skimmianine at 100 μg/ml (equivalent to 386 μM) inhibited collagen-induced platelet aggregation (around 66%) in washed rabbit platelets.

3.1.3 Pharmacological activity investigated in this study

3.1.3.1 Antioxidant activity

The antioxidant activity studies for *C. ternata* used the same rationale as the ones for *P. bleo* described in Section **2.1.3.1,** Chapter 2 of this thesis.

3.1.3.2 Antiplatelet activity

Based on the previous report for skimmianine, it is worth to test the compounds isolated from this species, especially alkaloids, for their antiplatelet activity using human blood. Skimmianine at 100 μg/ml (equivalent to 386 μM) inhibited collagen-induced platelet aggregation (around 66%) in washed rabbit platelets (Chen *et al.*, 2000). Human platelets are small with dimensions of approximately 2.0-4.0 by 0.5 μm, derived from megakaryocytes and typically circulate for 10 days and contain hormones and proteins for blood to coagulate. Their shape and small size enables the platelets to be pushed to the edge of vessels, placing them in the optimum location required to constantly survey the integrity of the vasculature (Harrison, 2005)

The multifunctional role of platelets in pathophysiology is well established (Surin *et al.*, 2008). In addition to haemostasis and thrombosis, platelets are also involved in maintaining of vascular tone, clot retraction, vessel constriction and repair, immune process mainly in fighting microbial infection, inflammation process including promotion of atherosclerosis, host defence and tumour angiogenesis and metastasis (Harrison, 2005; Katoh, 2009; Broos *et al.*, 2011) (Figure 3.4).

When the blood vessel wall damage due to external or internal injury, platelets undergo a highly regulated set of functional responses including adhesion, spreading, release reactions, aggregation, exposure of a procoagulant surface, microparticle formation and clot retraction. All these platelet responses function to rapidly form a haemostatic condition and block the site of damage to prevent blood loss by sticking to each other to perform larger clump and releasing chemicals that can cause blood vessels to narrow. When there is a defect in any of these functions and/or platelet number, haemostasis is usually impaired and there may be an associated increased risk of bleeding. On the other hand, a marked increase in platelet number or reactivity can lead to inappropriate thrombus formation. Arterial thrombi can also develop within atherosclerotic lesions resulting in stroke and myocardial infarction (Harrison, 2005).

Recent studies showed that platelet aggregation disorders play an important role particularly in the development of cardiovascular diseases and are the major complications of atherosclerosis and arterial thrombus diseases such as stroke and myocardial infarction (Amrani *et al.*, 2009; Sobotková *et al.*, 2009).

Moreover, the reactive oxygen species (ROS) and reactive nitrogen species (RNS, e.g. nitric oxide, NO) that play important role in oxidative stress processes also have been reported as important mediators inhibiting platelet aggregation. The release of NO from □-arginine by vascular endothelium is controlled by powerful mechanism. NO inhibits platelet adhesion, aggregation, recruitment and cause disaggregation of platelets both *in vivo* and *in vitro* (Chou *et al.*, 1999; Alonso and Radomski, 2003).

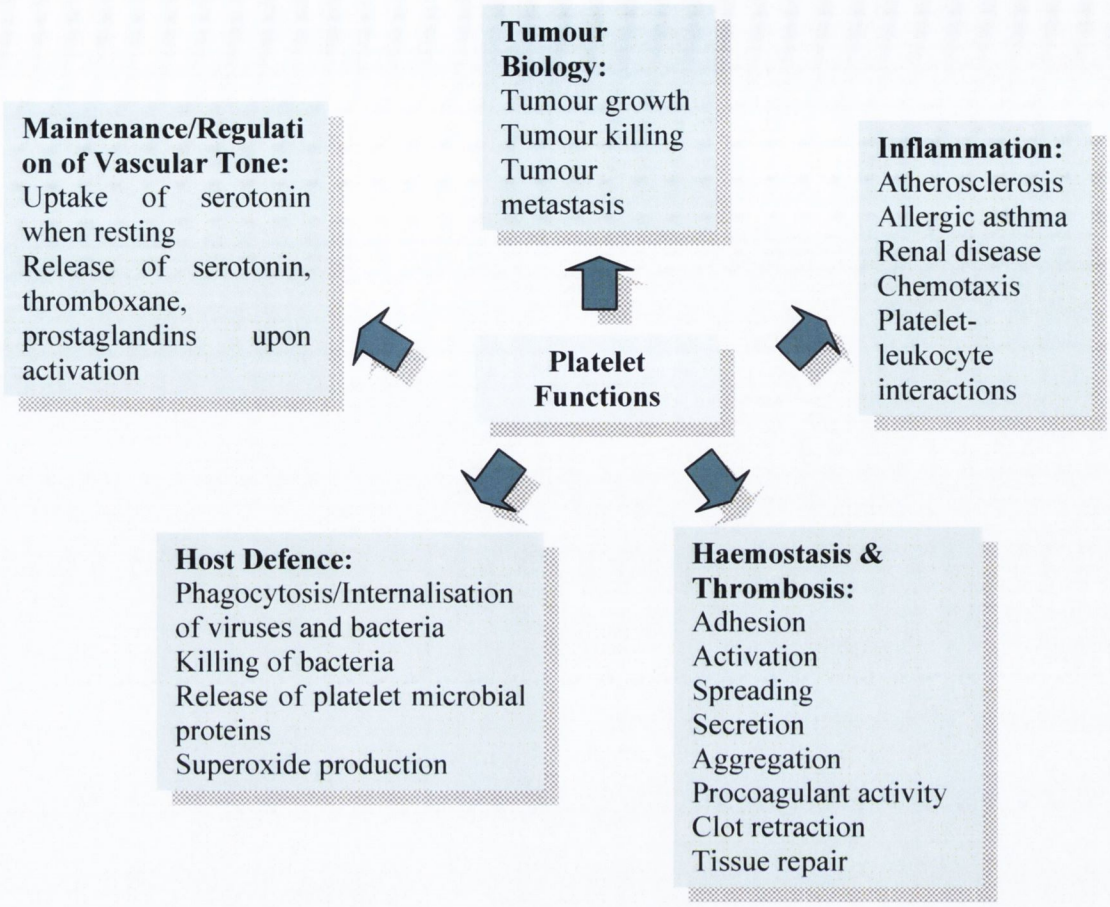


Figure 3.4: The multifunction of the platelets in many pathophysiological processes (Harrison, 2005).

The activation of platelets is regulated and modulated by numerous relatively well-characterized factors which further could cause aggregation, induced by the action of endogenous agonist such as collagen, arachidonic acid (Ar. A), adenosine diphosphate (ADP), platelet activating factor (PAF) and thrombin (Moharam *et al.*, 2010), as well as serotonin and thromboxane A_2 (Sobotková *et al.*, 2009). There are several platelets function tests with platelet aggregometry becoming the standard since it was developed in the early 1960s.

In the present study, aggregometry platelet function test was used to measure the platelet responsiveness to collagen. Collagen, one of the strongest inducer of platelets, is a matrix protein that has various structural characetristics and functions. It consists of 3 chains of amino acids arranged as a triple helix and it is synthesised by a wide variety of cells such as macrophages, smooth muscle cells, endothelial and epithelial cells, keratinocytes and fibroblasts (Surin *et al.*, 2008). It is considered as one of the most potent

vessel wall components in the initiation of platelet adhesion and aggregation (Broos *et al.*, 2011).

However, several problems in platelet function tests have been identified. A test such as aggregometry is poorly standardized with different laboratories using different agonists as well as different ranges of concentrations, other than the quality, handling, temperature and age of the blood sample which also could cause significant artifacts in platelet analysis. Therefore, ensuring sample quality is of utmost importance before any platelet function testing is performed (Harrison, 2005).

The search for potent antiplatelet drugs is continuously carried out in most laboratories, basically to substitute the known acetylsalicylic acid (aspirin). This is because aspirin may cause gastric erosion and gastric ulcers and patients may present with anemia and gastric haemorrhage (Moharam *et al.*, 2010). Hence, the search for a new natural antiplatelet agents is encouraged. This can be done by targeting platelet aggregation using different inducers. A modulation of platelet activity, particularly by inhibition of platelet-collagen interaction can lead to the discovery of natural products with this activity (Amrani *et al.*, 2009; Surin *et al.*, 2008).

Due to the lack of antiplatelet studies on *C. ternata* and a good collagen-induced antiplatelet activity showed by one of the compounds (skimmianine) also found in this plant, an antiplatelet activity was carried out using its hexane extract (the one rich in similar alkaloids) and its isolated compounds.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Leaves of *C. ternata* Kunth. were collected from the Trinity College Botanical Garden, Dartry, Dublin in October 2008 (living plant accession number 19850023) and its voucher specimen (SW 10-52) was deposited in the Herbarium of Trinity College, Dublin. The sample was identified by Dr. Steve Waldren from the Botany Department, Trinity College, Dublin.

3.2.2 Phytochemical studies of *C. ternata*

3.2.2.1 General

The solvents used for the extraction, chromatographic procedures and reactions, chromatographic analyses by the thin layer chromatography (TLC), the NMR procedure and analyses, melting points, optical rotations, UV-Vis and FT-IR spectroscopy analyses, as well as EI and ESI spectroscopy analyses are the same as explained in Section **2.2.2.1**

The extraction of dried leaves of *C. ternata* was performed using Soxhlet apparatus. The purification of the compounds by column chromatography was realised using silica gel 60 (70-230 mesh; Merck) or Sephadex LH-20 (Lipophilic, 25-100 μ, Sigma) through different gradients of the following organic solvents: hexane-ethyl acetate-methanol in silica gel and methanol when Sephadex was used.

Through the TLC, the presence of terpenes, phenolics and flavonoids was indicated by the formation of coloured spots after heating on a hot plate for 5-10 minutes and the Dragendorff reagent is used to detect the presence of alkaloids.

Preparative silica gel 60 PF_{254} plates (0.5 mm) were also used in few separations. These plates were activated at 120°C for 2 hours before use. The presence of band(s) was observed under ultra-violet light (254 nm and 365 nm). When the visualization could not be achieved by UV light, a small end of the plate was sprayed with universal reagent, 10% sulphuric acid in ethanol. The bands containing the separated compounds were then scrapped off the plate. Scrapped silica bands with compound(s) were washed in methanol, stirred and filtered by means of vacuum filtration.

Chromatotron (Model 7294; Harrison Research) was ran with rotor silica gel GF (Analtech) in various sizes (2, 4 and 6 mm), equipped with the FMI Lab Pump, using various flow rate; 2-4 ml/min, 6-8 ml/min and 8-10 ml/min for the 2,4 and 6 mm silica rotor, respectively, using chloroform : methanol (9:1) as mobile phase. The fractions eluted from the chromatographic chromatotron were analysed by thin layer chromatography and the similar fractions were then grouped.

Crystal data was collected by a Rigaku Saturn 724 CCD Diffractometer. A suitable crystal was selected and mounted using inert oil on a 0.3mm diameter glass fiber tip and placed on the goniometer head in a 123K N_2 gas stream. The data was collected using Crystalclear-SM 1.4.0 software. Data integration, reduction and correction for absorption and polarization effects were all performed using Crystalclear-SM 1.4.0 software. Space group determination, structure solution and refinement were obtained using Bruker Shelxtl* Ver. 6.14 software.

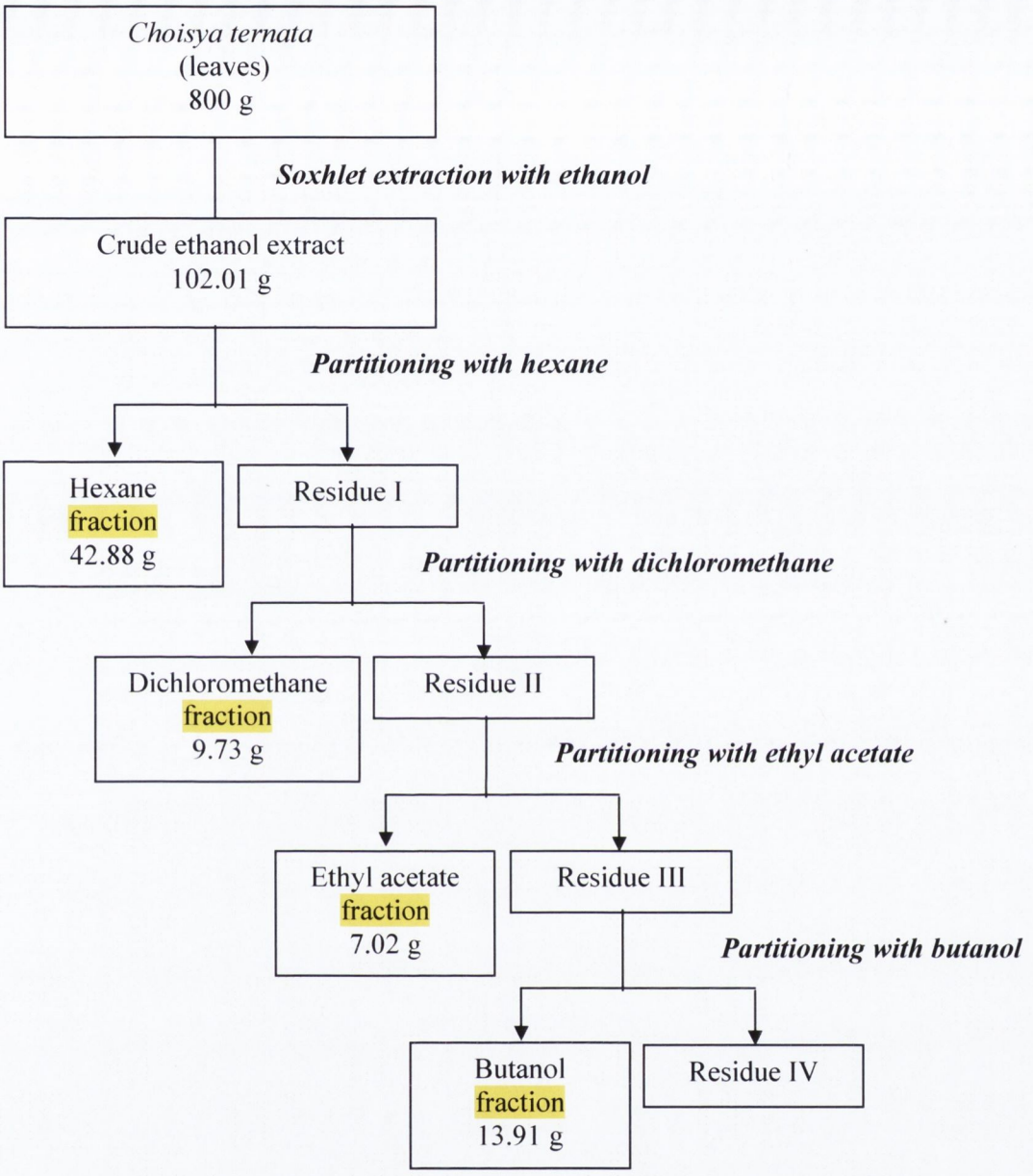
The structure was solved with Direct Methods using the SHELXTL program and refined against IF^2I with the program XL from SHELX-97 using all data. Non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were placed into geometrically calculated positions and refined using a riding model. The Software Reference Manual, version 5.625; Bruker Analytical X-Ray Systems Inc.: Madison, WI, 2001 and Sheldrick, G. M. SHELXTL were used for data collection, processing, structure solution and refinement. The crystal data from X-ray analysis was deposited in the Cambridge Crystallographic Data Centre (CCDC).

3.2.2.2 Isolation and purification of extracts

- **Crude ethanol extract and its fractions**

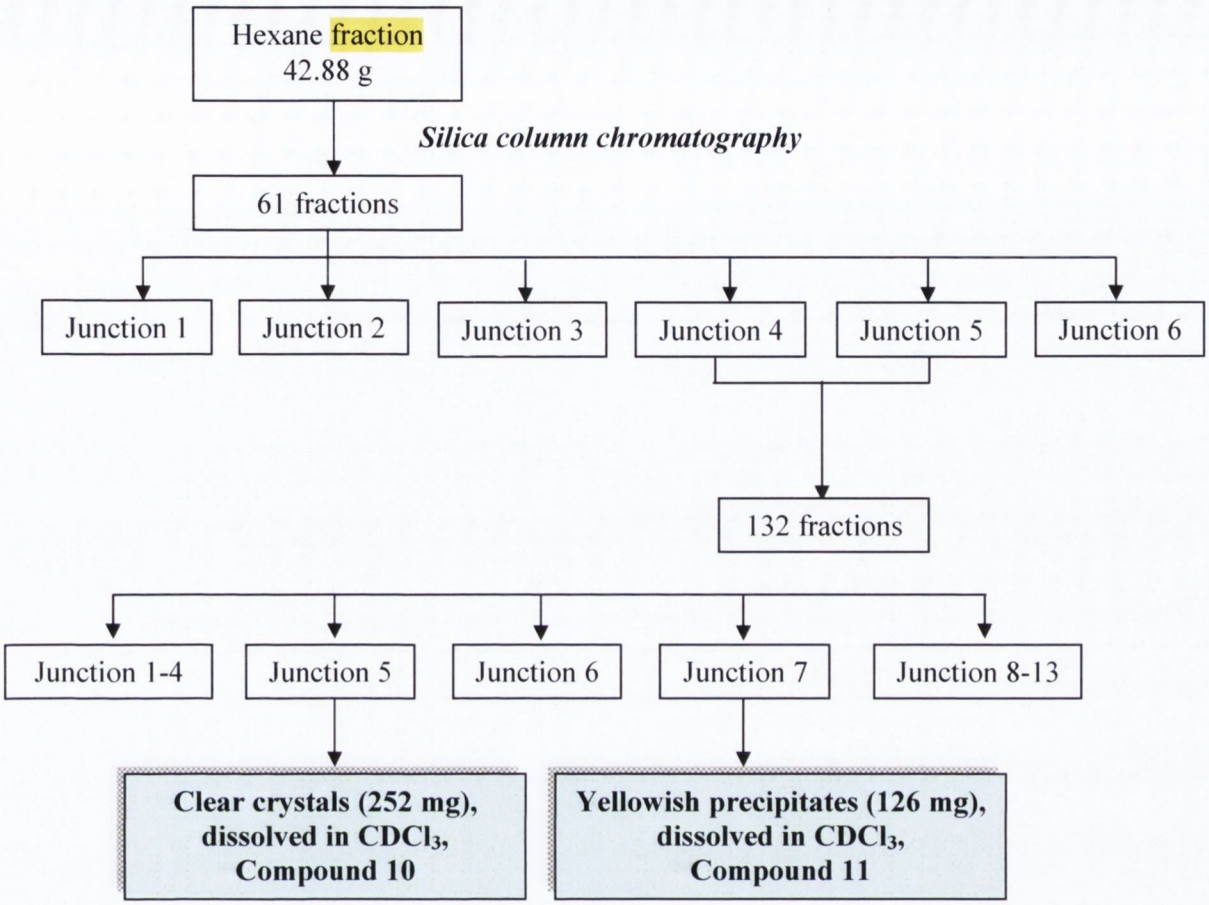
800 g ground dried leaves of *C. ternata* were extracted using soxhlet apparatus with ethanol for 72 hours. The crude ethanol extract of *C. ternata* leaves was then treated overnight with activated charcoal (Sigma) to eliminate chlorophyll. The extracts were then filtered through Whatman filter paper (24.0 cm) and concentrated with rotary evaporator.

The crude extract (102.01 g) was subjected to liquid-liquid partition yielding hexane (42.88 g), dichloromethane (9.73 g), ethyl acetate (7.02 g) and butanol (13.91 g) fractions (**Error! Reference source not found.**). The hexane fraction was subjected to a column chromatography (36 cm, Ø: 7.5 cm) over silica gel (185g, 70-230 mesh, Merck) eluted with hexane:ethyl acetate:methanol gradient system to give 6 junctions. Junction 4 and 5 (12.31g) were combined and subjected to another CC (85 cm, Ø: 3.5cm; Si gel: 60 g, 70-230 mesh, Merck) eluted with the same mobile phase to give 13 junctions. Junction 5 and 7 and 11 were continuously washed with methanol to give a clear crystalline solid **10** (252 mg) and yellowish precipitate skimmianine **11** (126 mg), respectively (**Error! Reference source not found.**).



Scheme 3.1: Preparation of an ethanol extract and its fractions with hexane, dichloromethane, ethyl acetate and butanol from the leaves of *C. ternata*.

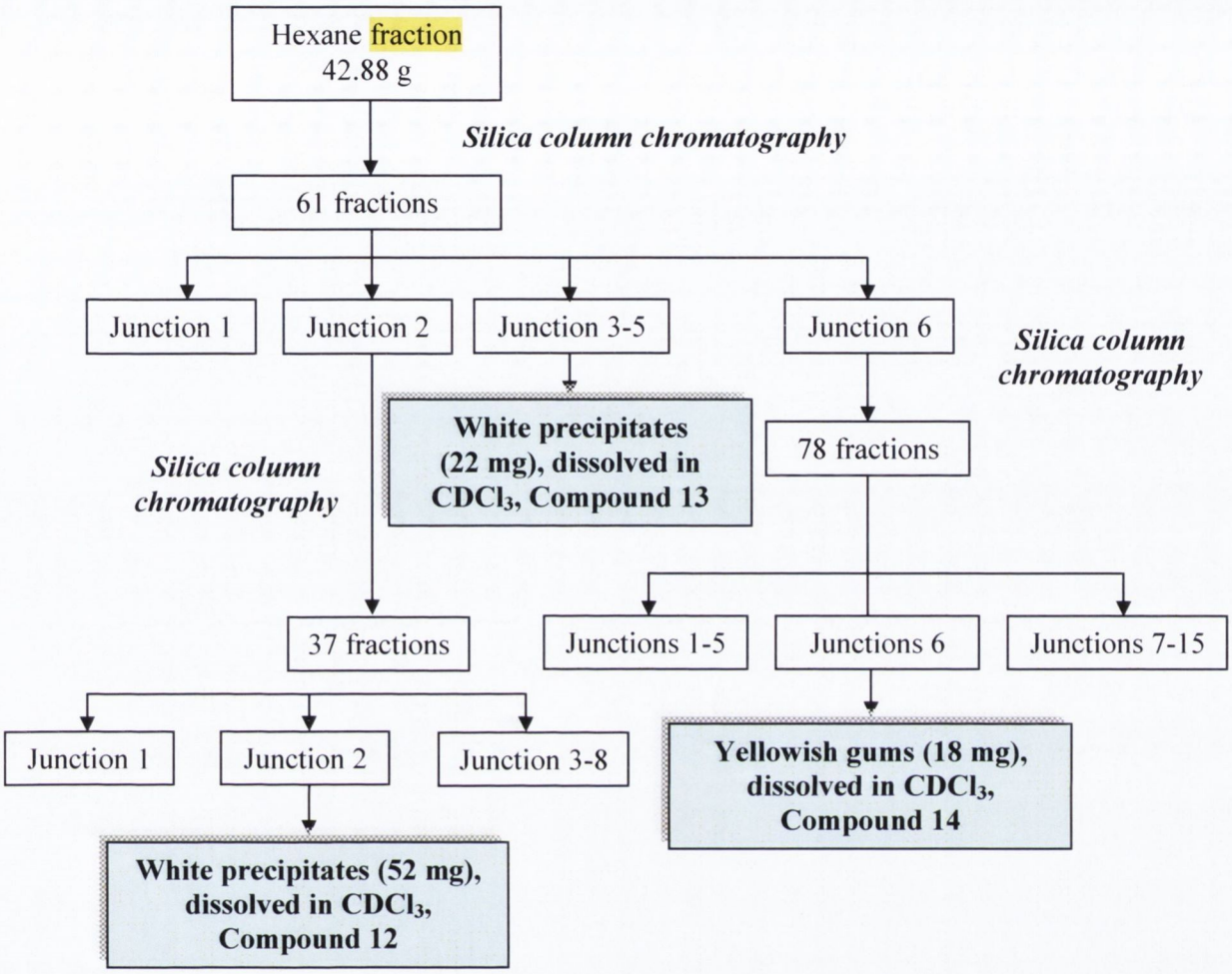
- **Analysis of the hexane fraction**



Scheme 3.2: Isolation and purification of the hexane fraction from *C. ternata* leaves, yielded clear crystals of compound 10 and yellowish precipitates of compound 11.

Junction 2 was purified by CC (85 cm, Ø: 3.5 cm; Si gel: 60 g, 70-230 mesh, Merck) eluted with the same mobile phase as above to give 8 junctions. Junction 2 was washed continuously with methanol to give a white precipitate lupeol **12** (52 mg). Junction 3-5 was observed to contain precipitate and was continuously cleaned with methanol to give a white amorphous precipitate lup-20(29)-en-3β,24-diol **13** (22 mg). Junction 6 from the first column chromatography was subjected to another CC (85 cm, Ø: 2.5 cm) over silica gel (30 g, 70-230 mesh, Merck) eluted with hexane:ethyl acetate:methanol gradient system to give 15 junctions. Junction 6 was observed to contain a yellowish gum tecleamaniensine A **14** (18 mg) (

Scheme 3.3).



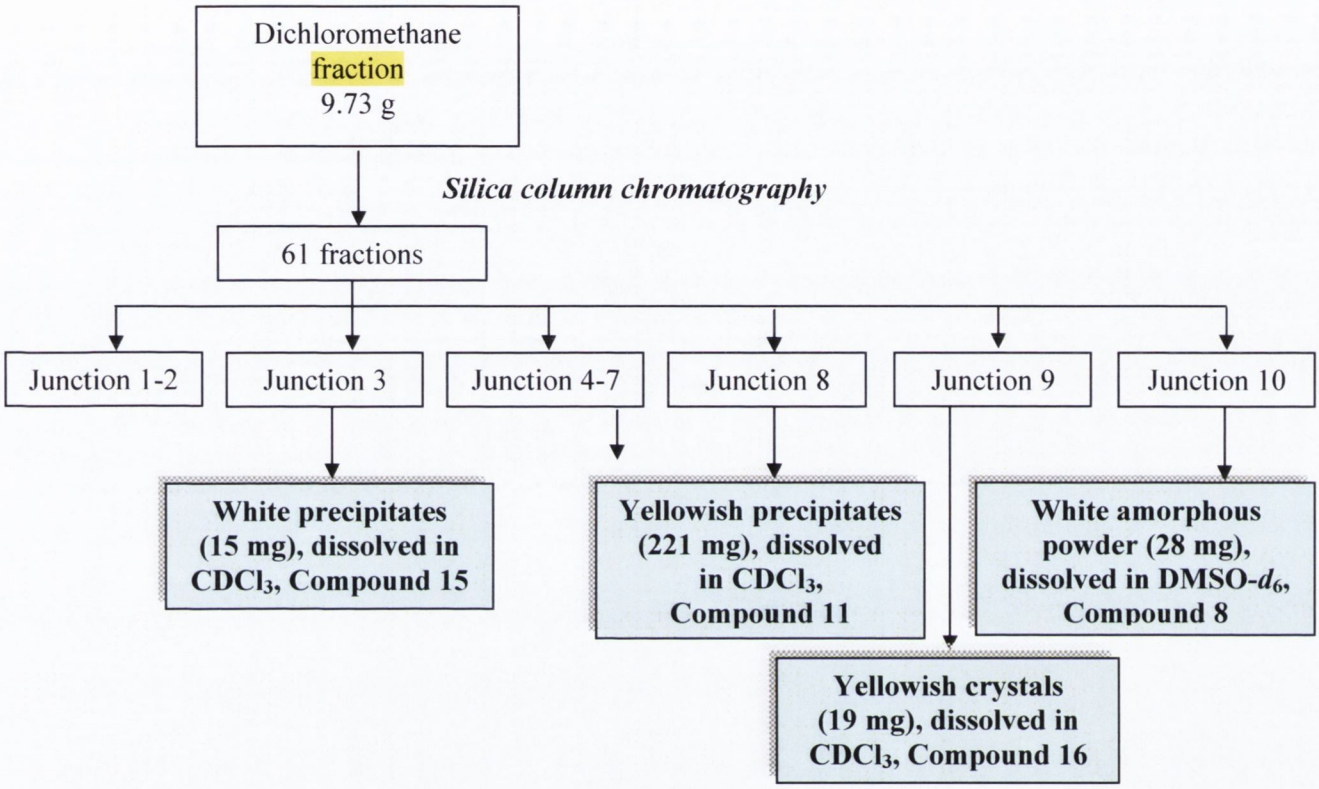
Scheme 3.3: Isolation and purification of the hexane fraction from *C. ternata* leaves yielded white precipitates of compound 12, 13 and yellowish gums of compound 14.

- **Analysis of the dichloromethane fraction**

The dichloromethane fraction (9.73 g) was also subjected to a column chromatography (85 cm, Ø: 3.5cm) over silica gel (65 g, 70-230 mesh, Merck) and eluted with hexane:ethyl acetate:methanol in gradients of increasing polarity as a mobile phase to give 10 junctions. White precipitates were collected from Junction 3, after washing with methanol to give coumarin **15** (15 mg). Yellowish precipitates and crystals collected from Junction 8 and 9 to give the same compound as obtained from the hexane fraction, skimmianine **11** (221 mg) and choisyine **16** (19 mg), respectively. Finally, white

amorphous powder was collected from Junction 10, after washing with methanol to give β-sitosterol glucoside **8** (28 mg) (

Scheme 3.4).



Scheme 3.4: Isolation and purification of the dichloromethane fraction from *C. ternata* leaves yielded white precipitates of compound 15, yellowish precipitates of compound 11, yellowish crystals of compound 16 and white amorphous powder of compound 8.

- **Analysis of the ethyl acetate fraction**

Part of the ethyl acetate fraction of *C. ternata* (1.567 g) had the isolation of its compounds performed by column chromatography (Ø column: 3.0 cm) using chloroform - ethyl acetate-methanol in gradients of increasing polarity as a mobile phase and silica gel (65 g, 70-230 mesh, Merck) as a stationary phase, yielding 123 fractions. The fractions eluted from the chromatographic column were analysed by thin layer chromatography and the similar fractions were then grouped, giving a total of 6 junctions.

Junction 4 and 5 were combined and chromatographed by chromatotron (rotor thickness: 2 mm) using chloroform - methanol (9:1) as a mobile phase yielding 100 fractions. The fractions eluted were analysed by thin layer chromatography and similar fractions were grouped, giving a total of 6 junctions. Junction 1 and 3 from the above process were further purified using preparative plate (silica gel 60 PF_{254} 0.5 mm; 20x20 cm) to obtain 4 (band 1, 2, 3 and 4) and 2 (band 1 and 2) fractions, respectively. Bands 1 and 2 from the Junction 1 were combined together with band 2 from the Junction 3 (78.3 mg), and subjected to another silica column (Ø column: 3.5 cm) using chloroform – methanol in gradients of increasing polarity as mobile phase, to give 10 junctions. Later, Junction 6 was subjected to a pipette column (Ø column: 10 cm) using chloroform – methanol (9:1) as mobile phase yielding 4 junctions. Junction 3 was observed to have a yellow precipitate and was rinsed several times with chloroform to achieve a cleaner pale yellow precipitate, identified as kaempferol-3-*O*-rutinoside **17** (15 mg) (

Scheme 3.5).

3.2.3 Pharmacological studies of *C. ternata* extract and isolated compounds

3.2.3.1 Antioxidant activity

The antioxidant activity was performed on crude ethanol extract, hexane, dichloromethane, ethyl acetate and butanol fractions of the *C. ternata* leaves using the same method as described in Section **2.2.3.1**.

3.2.3.2 Antiplatelet activity

This assay was kindly supervised by Dr. Maria Jose Santos-Martinez and performed in the lab she shares with Dr. Marek Radomski at the School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin.

- **Blood collection and human platelet isolation**

Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for 14 days prior to the study as previously described (Radomski and Moncada, 1983). Briefly, platelet-rich plasma (PRP) was obtained by centrifugation at

250 g for 20 min and diluted with Tyrode's salt solution at final concentration of 250,000 platelets/µl. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 15,000 rpm for 10 minutes at room temperature.

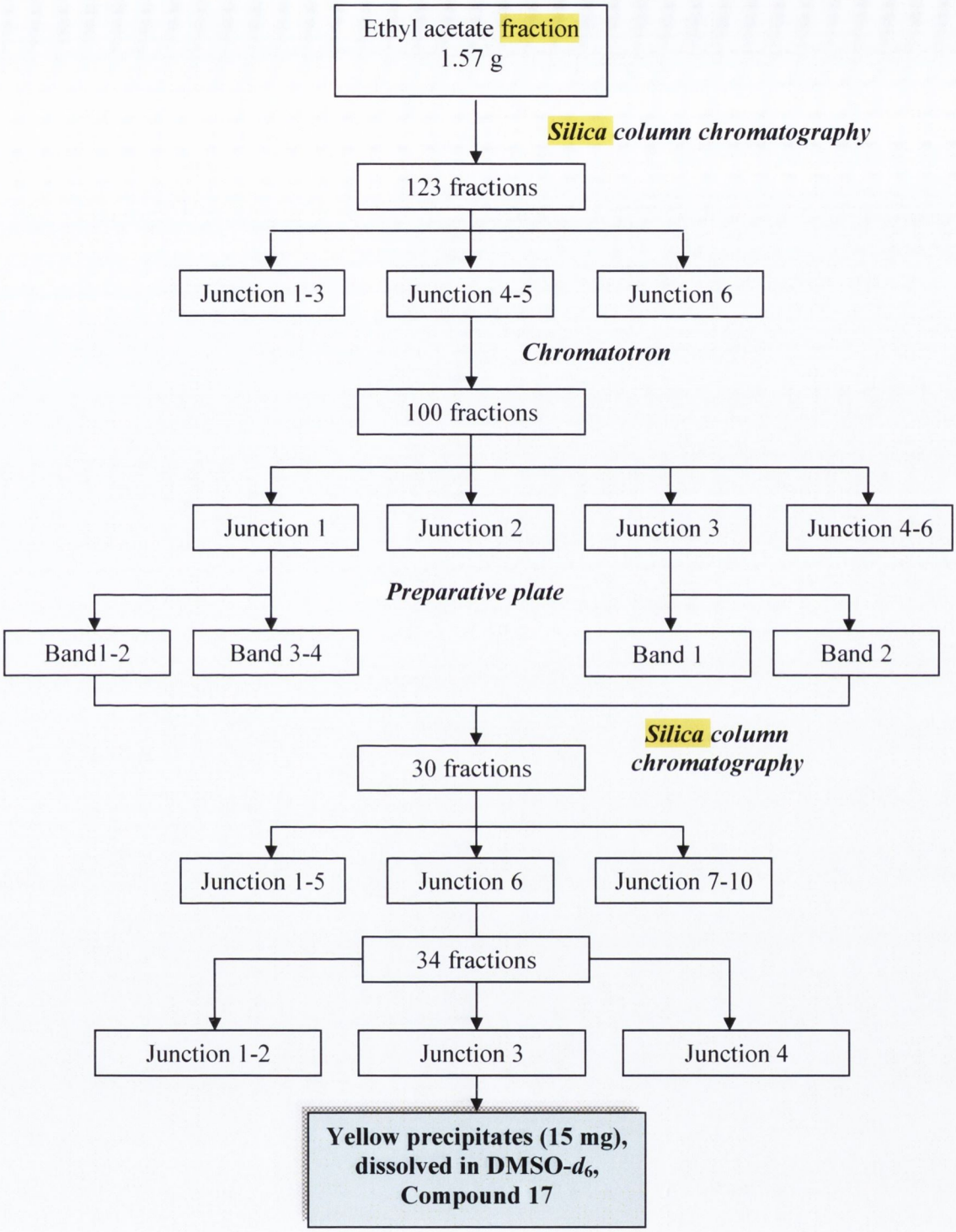
- **Platelet aggregation monitored by light aggregometry**

Platelet aggregation was measured by a light aggregometry as previously described (Jurasz *et al.*, 2001, Radomski *et al.*, 2005, Medina *et al.*, 2006). The ability of the hexane fraction and compounds 10, 11, 12, 14 and 16 to inhibit platelet aggregation induced by collagen in PRP was measured using an eight-channel Platelet Aggregation Profiler® Model PAP-8E (Bio/Data Corporation, Brennan & Company, Ireland). Platelet samples (PRP) were incubated with stirring at 900 r.p.m. for 5 min at 37°C with vehicle (0.4 % of DMSO) or with extract or test compounds at various concentrations (extract: 25, 50 and 100 µg/ml; test compounds: 50, 100, 250, 500, 750 and 1000 µM). The cyclooxygenase inhibitor acetylsalicylic acid (ASA) was used as internal control at 100 µM; IC_{50} from previous study (Jones *et al.*, 2009). Platelet aggregation was initiated by collagen (2 µg/ml) and the results were recorded as percentage of light transmission, where 100% transmission (PPP) was taken as maximal aggregation induced by collagen.

Synergism test was run based on the above described method using a mixture of compounds 10, 11, 12 and 14 to compare their activity with the hexane fraction from where the four compounds were isolated.

- **Statistical analysis**

In experiments using a light aggregometer, data from at least three independent experiments were analyzed using GraphPad Prism 5.0 software. All means are reported with standard deviation. Paired Student's t-test, One-way analyses of variance (ANOVA) and Dunnett's multiple comparisons post test were performed where appropriate. Statistical significance was considered when $p<0.05$. The IC_{50} (concentration which inhibit 50% of platelet aggregation) was calculated from the dose-response curves using GraphPad Prism 5.0 software.



Scheme 3.5: Isolation and purification of the ethyl acetate fraction from *C. ternata* leaves yielded yellow precipitates of compound 17.

3.3 RESULTS AND DISCUSSION

3.3.1 Phytochemical studies of *C. ternata*

3.3.1.1 Isolation and purification of extracts

- **Analysis of the hexane fraction**

6,8-Dimethoxy-7-(3-methylbut-2-enyloxy)quinolin-2-ol (choisyaternatine) (10)

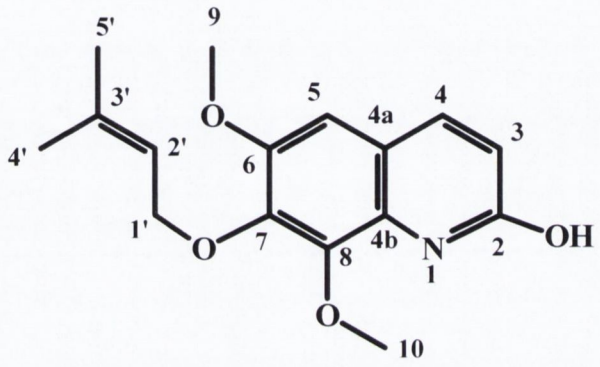


Figure 3.5: Choisyaternatine (10)

Colourless crystalline solid; R_f: 0.44 (hexane:ethyl acetate; 6:4); mp: 88-92°C; $[\alpha]_D^{20}$ 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 241, 300, 340; IR ν_{max}: 3083, 2942, 1715, 1563, 1406, 1272 cm^{-1}; EI-MS for $C_{16}H_{19}NO_4$ m/z; 290 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 1.72 (3H, *s*, H-4'), 1.78 (3H, *s*, H-5'), 3.90 (3H, *s*, OCH_3-9), 4.04 (3H, *s*, OCH_3-10), 4.65 (2H, *d*, *J*=7.3 Hz, H-1'), 5.57 (1H, *t*, *J*=7.3 Hz, H-2'), 6.35 (1H, *d*, *J*=9.4 Hz, H-3), 6.68 (1H, *s*, H-5), 7.63 (1H, *d*, *J*=9.4 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 17.9 (C-4'), 25.8 (C-5'), 56.3 (C-9), 61.7 (C-10), 70.3 (C-1'), 103.6 (C-5), 114.4 (C-4a), 115.1 (C-3), 119.9 (C-2'), 139.3 (C-3'), 141.7 (C-8), 142.9 (C-4b), 143.5 (C-4), 144.9 (C-7), 150.7 (C-6), 160.6 (C-2).

Compound 10 was isolated as a colourless crystalline solid (252 mg) with a molecular formula of $C_{16}H_{19}NO_4$ as determined by EI-MS analysis of the protonated molecular ion 290 $[M+H]^+$. The UV spectrum of 10 had absorption maxima at 241, 300 and 340 nm, suggesting the presence of a quinoline moiety (Yang *et al.*, 2010). Its IR spectrum showed absorptions at 3083 (C-H aromatic), 2942 (OH stretch carboxylic), 1563 (aromatic ring), 1406 (C-C stretch aromatic), 1272 (C-O stretch carboxylic) cm^{-1}.

The ^{1}H NMR spectroscopic data of 10 (Table 3.1) are closely comparable with those of prenyl-quinoline and quinolone derivatives isolated by Brown and co-workers (1980). The ^{1}H NMR spectrum (Figure 3.6) clearly shows the presence of aromatic, methoxyl and methyl protons. The presence of two methyl groups that appeared as singlets at δ_H 1.72 and 1.78 might correspond to the isoprenyl group in the molecule. Two singlets at δ_H 3.90 and 4.04 are characteristic of methoxyl groups in a quinoline skeleton. Three doublets at δ_H 4.65 (J=7.3 Hz), 6.35 (J=9.4 Hz) and 7.63 (J=9.4 Hz) show that each of them has one neigbouring proton, while a triplet at δ_H 5.57 (J=7.2 Hz) corroborates that there are to two neighbouring protons belonging to the prenyl group. Signals at δ_H 6.35 and 7.63 in the aromatic region suggest that these are the methine protons of the typical quinoline skeleton.

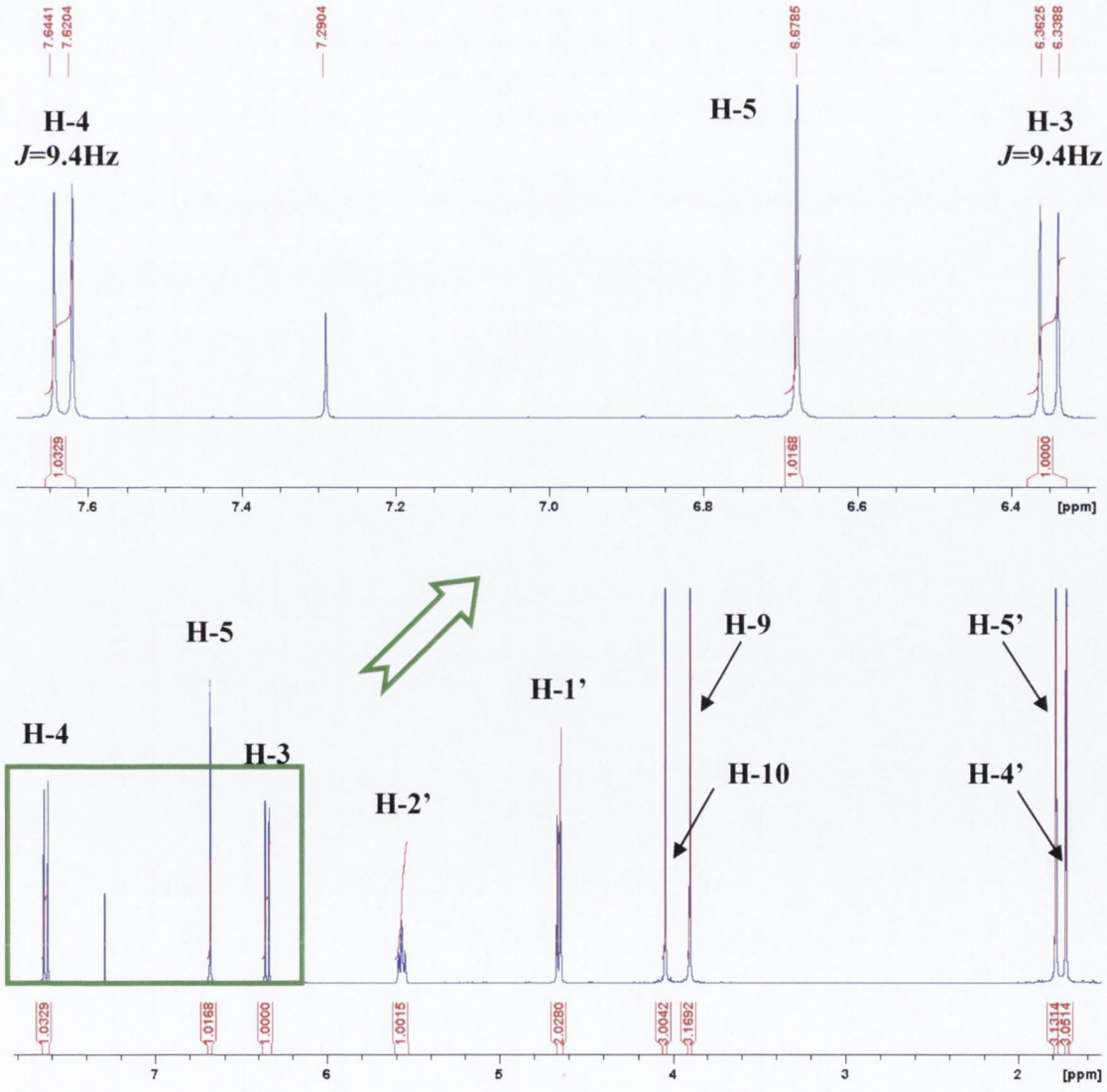


Figure 3.6: The overall ^{1}H NMR spectrum of compound 10 and amplification region showing the *ortho* coupling between δ_H 6.35 and 7.63. The signal at δ_H 4.65 integrated as two protons suggests that this proton belongs to the isoprenyl group.

In the ^{13}C NMR spectrum (Figure 3.7), in addition to the nine carbon signals of the quinoline skeleton and two methoxyl carbons, there are two methyl signals at δ_C 25.8 and 17.9 belonging to the *gem*-dimethyl carbons. The only methylene signal at δ_C 70.3 and two upfield methine carbons at δ_C 119.9 and 139.3 belong to the oxyprenyl moiety. The high frequency signal at δ_C 160.6 belongs to the carbon attached to a more electronegative atom such as O (oxygen) which is always present in a typical quinoline structure. According to the basic quinoline skeleton, it can be concluded that the most downfield carbon would be at position 2.

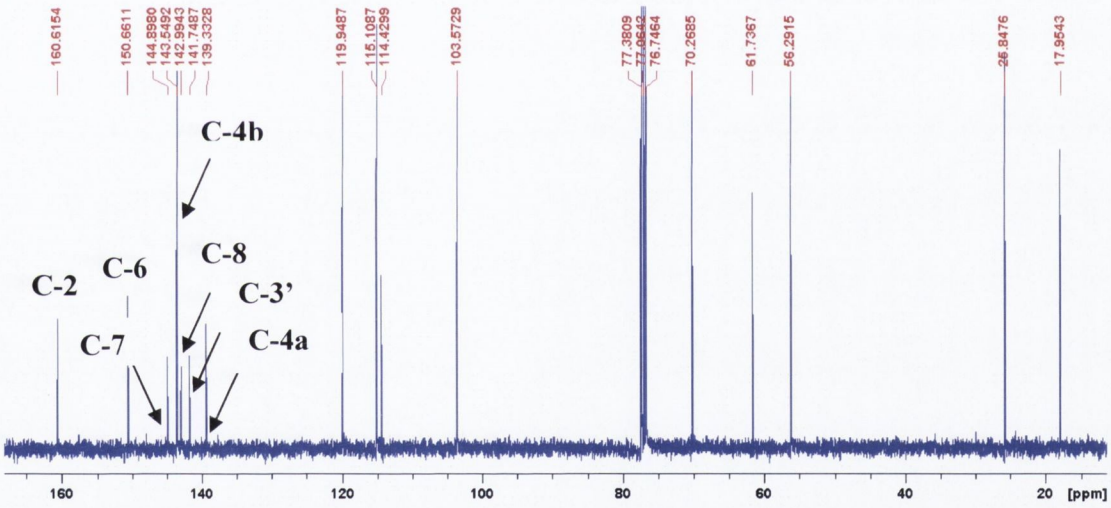


Figure 3.7: ^{13}C NMR spectrum shows the two methyl carbons of the isoprenyl group, together with two methoxyl, four methine and aromatic carbons.

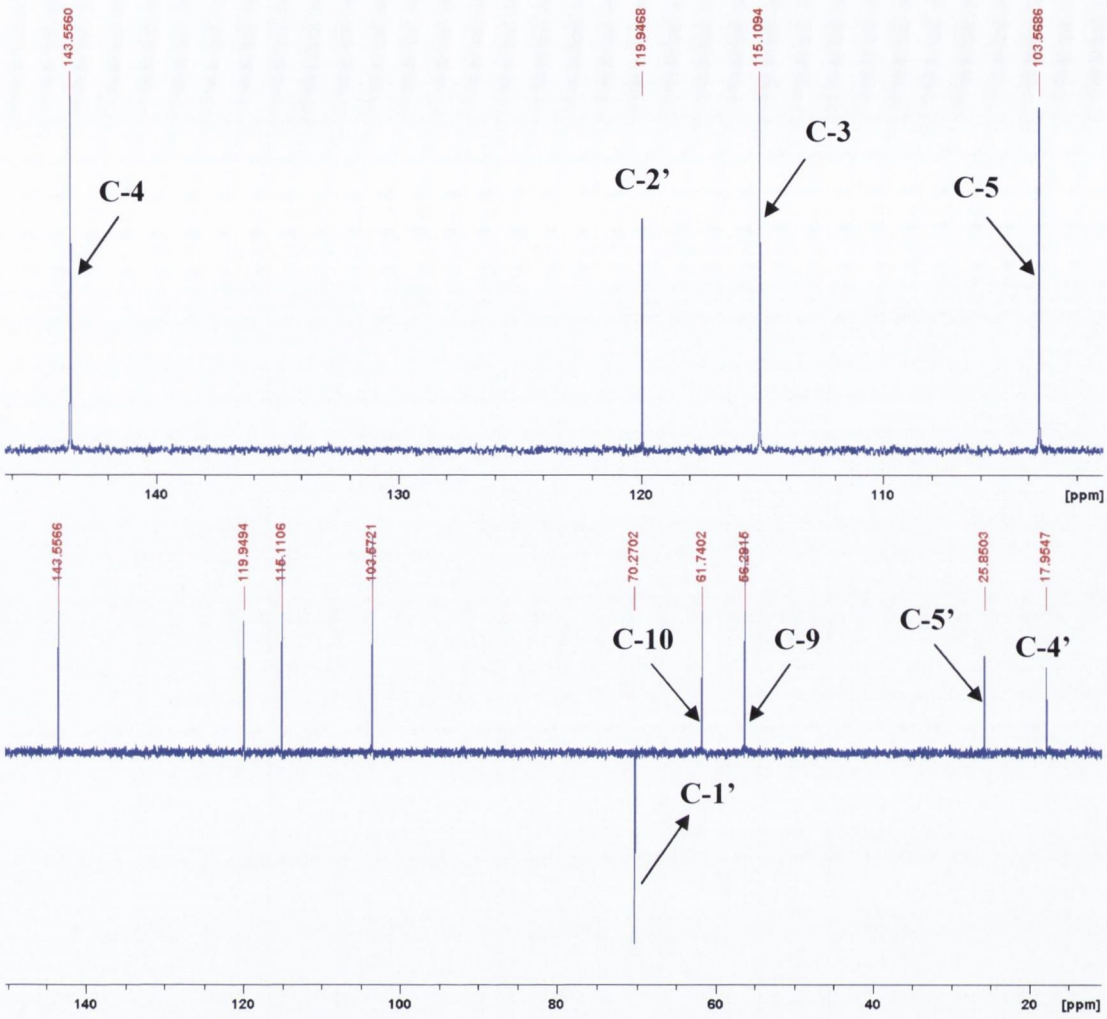


Figure 3.8: DEPT-90 (above) and -135 (below) spectra of compound 10 clearly show the presence of four methine groups and only one methylene group.

Dept-135 and -90 spectra (Figure 3.8) exhibit the presence of a methylene carbon of the prenyl group which is supported by a signal at δ_H 4.65 that appeared as doublet (J=7.3 Hz) at δ_C 70.3 and four methine carbons at δ_C 103.6, 115.1, 119.9 and 143.5 with three belonging to the aromatic ones. A quarternary carbon at δ_C 144.9 found in a downfield area suggests that it is attached to an O atom, which relates to the prenyl group.

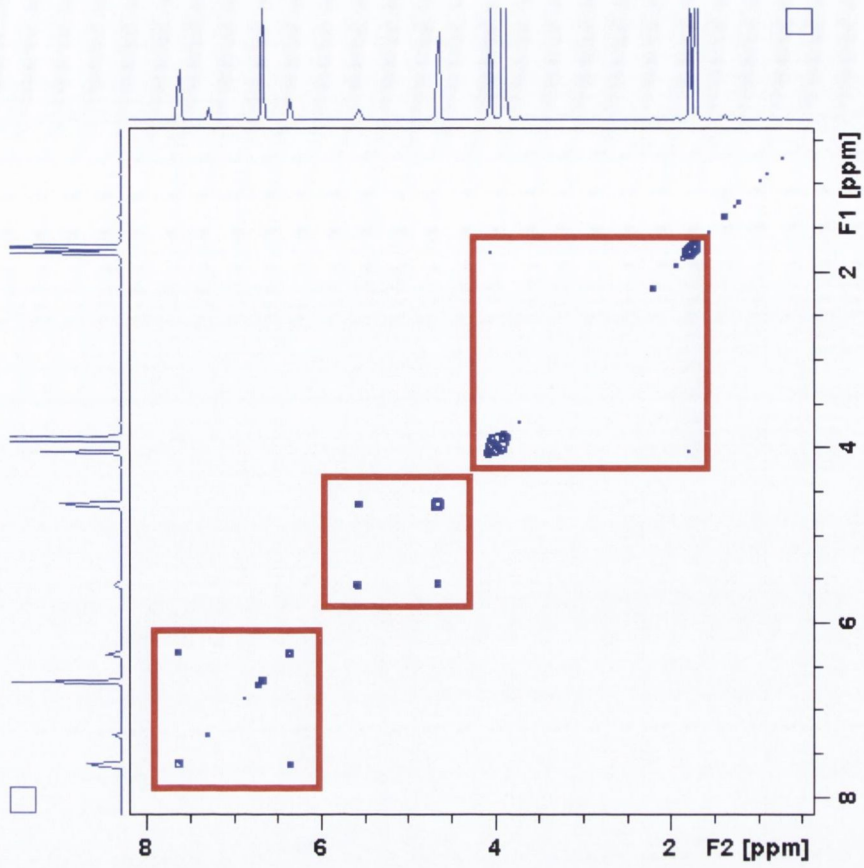


Figure 3.9: ^{1}H-^{1}H COSY spectrum of compound 10 shows coupling of the protons at δ_H 3.90 with 1.78, δ_H 4.65 with 5.57 and δ_H 6.35 with 7.63.

The assignation of all proton and carbon NMR signal was made possible only by use of 2D-NMR (^{1}H-^{1}H COSY and HETCOR spectra) as well as comparison with literature data. The ^{1}H-^{1}H COSY spectrum (Figure 3.9) shows a small coupling between the protons resonance at δ_H 4.65 with 5.57 and between the proton at δ_H 6.35 with δ_H 7.63.

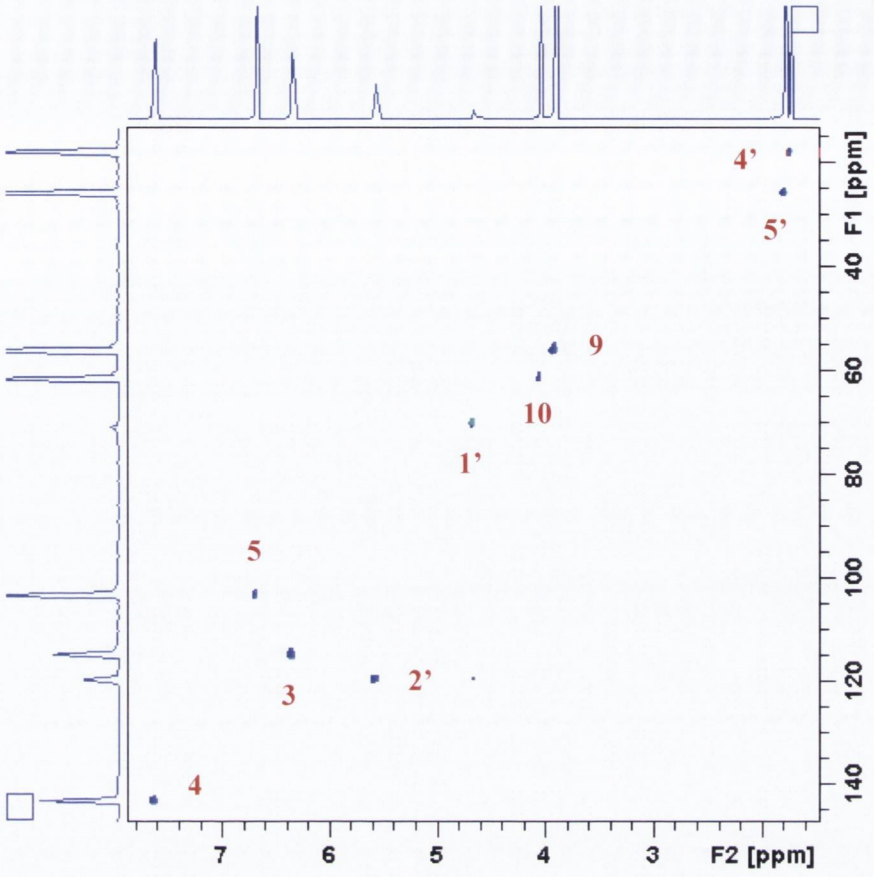


Figure 3.10: An amplification region of HSQC spectrum of compound 10 showing the protonated aromatic, methyl and methoxyl carbons.

HSQC spectrum of compound 10 (Figure 3.10) shows the protonated aromatic carbons; δ_H 5.57 / δ_C 119.9, δ_H 6.35 / δ_C 115.1, δ_H 6.68 / δ_C 103.6, δ_H 7.63 / δ_C 143.5, as well as protonated methyl carbons; δ_H 1.72 / δ_C 17.9, δ_H 1.78 / δ_C 25.8 and methoxyl groups; δ_H 3.90 / δ_C 56.3, δ_H 4.04 / δ_C 61.7.

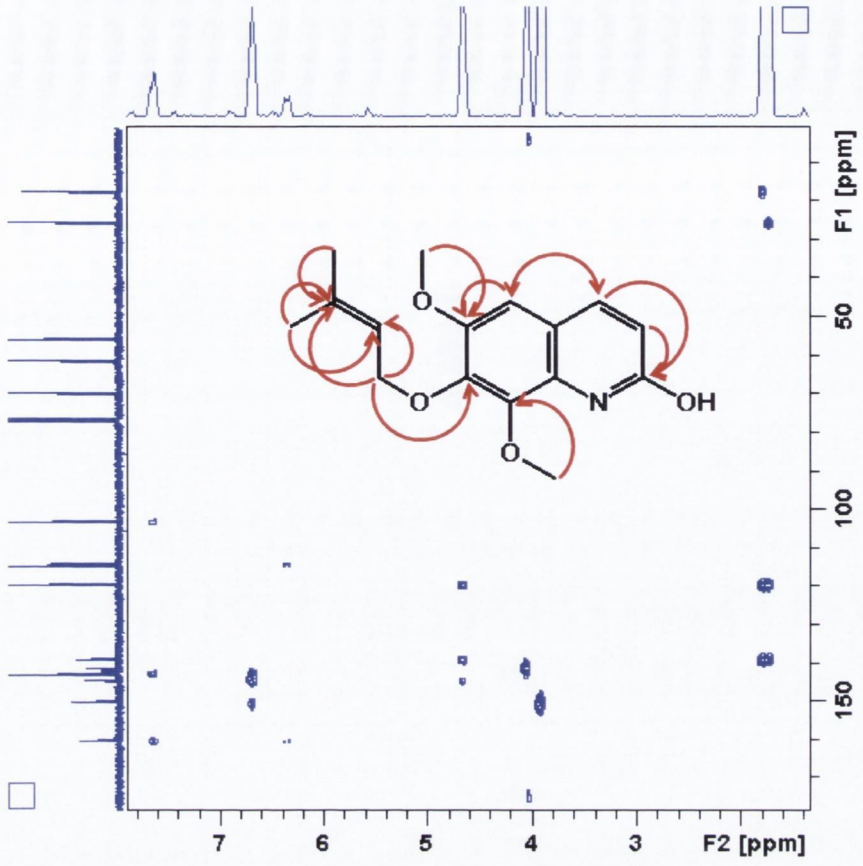


Figure 3.11: HMBC spectrum of compound 10 exhibits strong correlations between protons and carbons of the prenyl and aromatic groups, as well as the methoxyl group.

Further assignments based on long distance relationships were performed by the analysis of the HMBC spectrum (Figure 3.11). The spectrum shows strong correlation between the methyl protons at δ_H 1.72 and 1.78 with carbons at δ_C 119.9 and 139.3. Resonance at δ_H 4.65 shows interactions with resonance at δ_C 119.9, 139.3 and 144.9, methoxyl protons at δ_H 3.90 correlates with the one at δ_C 150.7, while methoxyl protons at δ_H 4.04 correlate with signal at δ_C 141.7. Aromatic signals at δ_H 6.68 strongly interact with aromatic carbons at δ_C 143.5 and δ_C 150.7, the aromatic signal at δ_H 7.63 correlates with aromatic carbons at δ_C 160.6 and δ_C 103.6 and aromatic proton at δ_H 6.35 correlates with signal at δ_C 160.6 (C-2). The complete NMR spectral data are summarized in Table 3.1 below by comparing the prenyl group with tecleamaniensine A and the quinoline skeleton with skimmianine.

Table 3.1: Comparison of ^{1}H and ^{13}C NMR data for compound 10 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C- NMR, $CDCl_3$) with tecleamaniensine A (^{1}H NMR, $CDCl_3$) and skimmianine (^{13}C NMR, $CDCl_3$).

	Present study		Literature data	
Position	**^{1}H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}CNMR, δ_C (ppm)**	**^{1}H NMR#**	**^{13}C NMR***
1				
2		160.6		163.2
3	6.35 (1H, *d*, *J*=9.4 Hz)	115.1		
4	7.63 (1H, *d*, *J*=9.4 Hz)	143.5		
4a		114.4		113.0
4b		142.9		145.8
5	6.68 (1H, *s*)	103.6		
6		150.7		
7		144.9		
8		141.7		
9	3.90 (3H, *s*, OCH_3)	56.3		
10	4.04 (3H, *s*, OCH_3)	61.7		
1'	4.65 (2H, *d*, *J*=7.3 Hz)	70.3	4.02	65.5
2'	5.57 (1H, *t*, *J*=7.3 Hz)	119.9	5.21	121.5
3'		139.3		139.9
4'	1.72 (3H, *s*)	17.9	1.66	18.3
5'	1.78 (3H, *s*)	25.8	1.82	25.7

#Magadula *et al.* (2008) and *Brown *et al.* (1980)

Crystallography data of 10

A piece of clear crystal compound 10 was further analysed using X-ray crystallography providing the crystal data shown below (Table 3.2) which confirms the proposed structure for compound 10 (Figure 3.12). Crystallographic data of compound 1 has been deposited in the Cambridge Crystallographic Data Center as supplementary publication number CCDC 865486. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK, (Fax: +44/1223/336033 or email: deposit@ccdc.cam.ac.uk)

Crystal Data:

$$^{a}R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|,\ wR_2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma w(F_o^2)^2]^{1/2}.$$

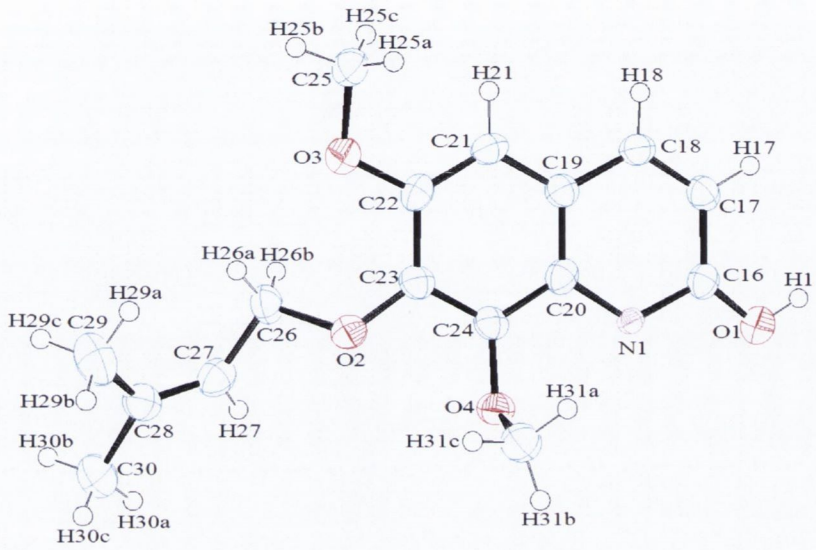


Figure 3.12: The confirmed skeleton obtained by X-ray crystallography analysis of clear crystals of compound 10.

Table 3.2: Structure of the compound 10 isolated from the hexane fraction of *C. ternata* leaves.

Structure compound 10	
Molecular formula	$C_{16}H_{19}NO_4$
MW, g/mol	289
System	Monoclinic
Space group	P2(1)/c
Z	1
a, Å	14.811(3)
b, Å	12.559(3)
c, Å	7.7975(16)
α	90°
β	97.45(3)°
γ	90°
U, Å^3	1438.25(5)

ρ, g/cm^3	1.336
T	150 K
μ (Mo Kα), mm^{-1}	0.096
Number of reflections with	11100
$^aR_1$	0.1253
$wR2$ [$I > 2\sigma(I)$]	0.3341
Gof	1.693
CCDC	865486

These findings confirm the assignment of the structure of compound 10 is a new compound and was named as 6,8-Dimethoxyl-7-(3-methylbut-2-enyloxy)quinolin-2-ol that we propose to be named choisyaternatine (Figure 3.5).

Based on the structure, it can be seen that the compound does not follow the standard anthranilic acid pathway biosynthesis, where usually alkaloids bear a methoxyl group at position four. In this compound, methoxyl groups are in positions six and eight. Lack of oxygenation in position four could be caused by bacterial dioxygenase enzymes in a biosynthetic precursor and parent furoquinoline alkaloid, dictamnine (Boyd *et al.*, 2007).

A tentative biosynthetic route for choisyaternatine is given in Figure 3.13 below. Quinoline alkaloids arise by extension of the carbonyl group of anthranilic acid by acetate (malonate), followed by cyclisation. The formed compound has a 2,4 dihydroxy quinoline structure. This structure can suffer an enzymatic reduction at position four, followed by enzymatic oxidations at positions six, seven and eight. The 2, 6, 7, 8 tetrahydroxyquinoline reacts with isoprenyl pyrophosphate at position seven - the more susceptible for this substitution. The prenylated derivative undergoes reduction with *S*-adenosylmethionine (SAM: as the methyl donor) to transform the hydroxyl groups in positions six and eight into methoxyl groups.

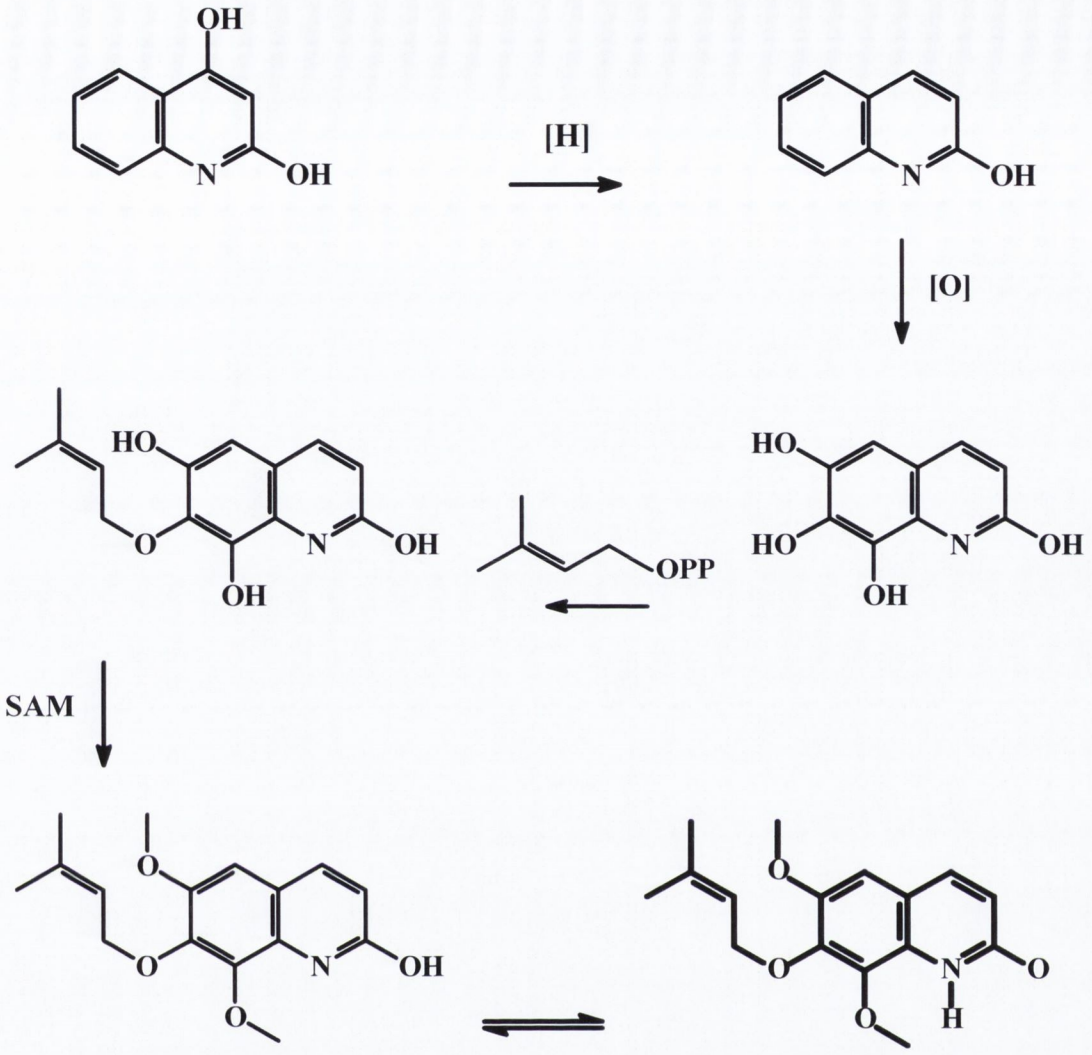


Figure 3.13: A tentative biosynthetic pathway for the formation of choisyaternatine.

Skimmianine (11)

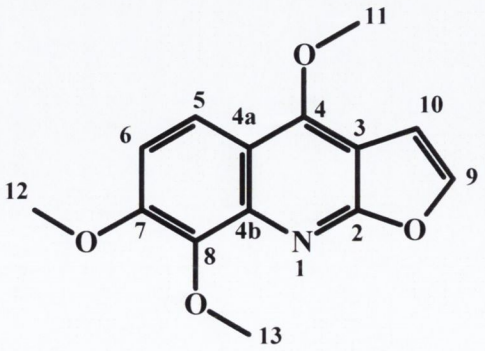
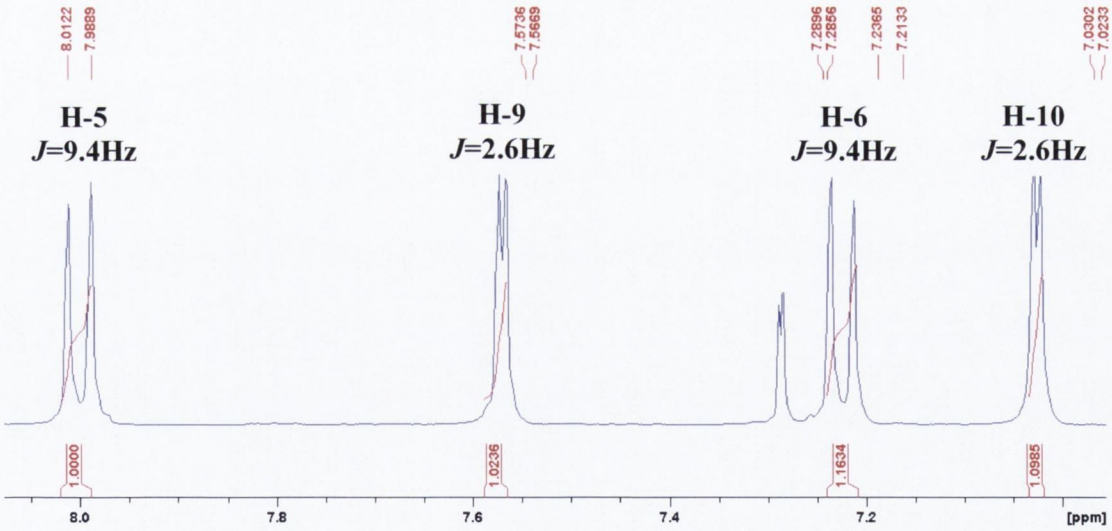


Figure 3.14: Skimmianine (11)

Yellowish precipitate; R_f: 0.51 (chloroform:methanol:water; 9:1:1); mp: 170-175°C; $[\alpha]_D^{20}$ 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 258, 321, 334; IR ν_{max}: 2975, 2845, 1618, 1578 cm^{-1}; ESI-MS for $C_{14}H_{13}NO_4$ m/z; 260 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 4.02 (3H, *s*, OCH_3-12), 4.10 (3H, *s*, OCH_3-13), 4.40 (3H, *s*, OCH_3-11), 7.01 (1H, *d*, *J*=2.6 Hz, H-10), 7.21 (1H, *d*, *J*=9.4 Hz, H-6), 7.56 (1H, *d*, *J*=2.6 Hz, H-9), 7.98 (1H, *d*, *J*=9.4 Hz, H-5); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 56.3 (C-12), 58.5 (C-11), 61.2 (C-13), 101.5 (C-4a), 104.2 (C-10), 111.4 (C-6), 114.3 (C-3), 117.8 (C-5), 140.9 (C-8), 141.3 (C-7), 142.9 (C-9), 51.7 (C-4b), 156.7 (C-4), 163.9 (C-2). This data is in fair agreement to that reported by Cordoso-Lopes *et al.*, (2010) and Brown *et al.* (1980).

Compound 11 was isolated as colourless needle-like crystals (126 mg isolated from the hexane fraction, 221 mg isolated from the dichloromethane fraction) with a molecular formula of $C_{14}H_{13}NO_4$ as determined by ESI-MS analysis of its protonated ion 260 $[M+H]^+$. The UV spectrum of 2 had absorption maxima at 258, 321, 334 nm, suggesting the presence of a furoquinoline moiety (Magadula *et al.*, 2008). Its IR spectrum showed absorptions at 2975 (N-H stretch), 2845 (C-H stretch), 1618 (aromatic ring) and 1578 (C-C stretch aromatic) cm^{-1}.



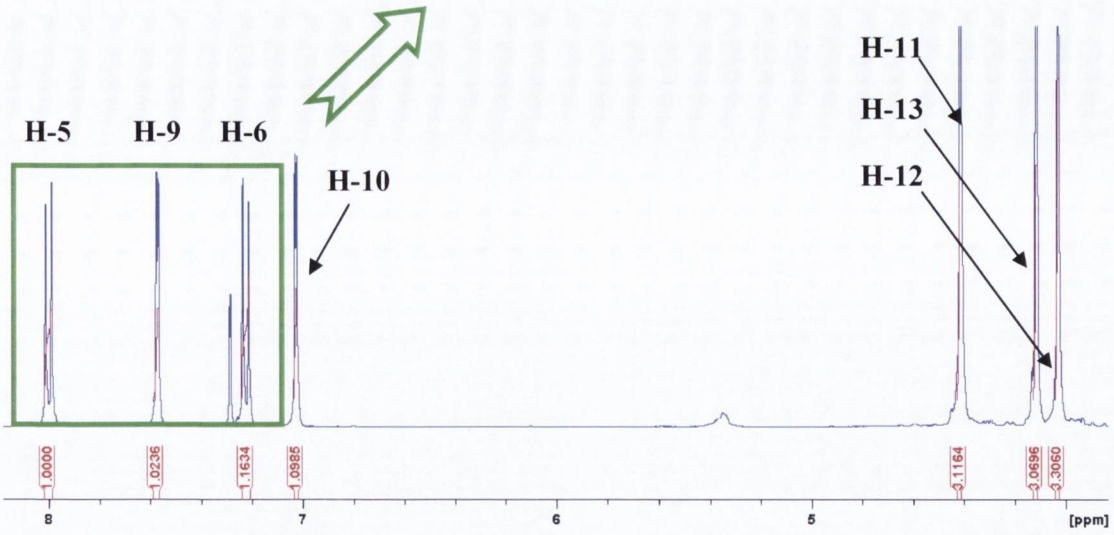


Figure 3.15: The overall ^{1}H NMR spectrum of compound 11 and an amplification region of *ortho* couplings between δ_H 7.01 and 7.56, and between δ_H 7.21 and 7.98.

The ^{1}H NMR spectroscopic data of 11 (Table 3.3) are closely comparable with those of furoquinoline derivatives isolated by Brown and co-workers (1980). Based on the knowledge of the previous isolated furoquinoline-type alkaloid, the ^{1}H NMR spectrum (Figure 3.15) shows the presence of nine aromatic protons and two protons of the furan ring. Referring to the coupling constants the two sets of doublets at δ_H 7.01 and 7.56 with J=2.6 Hz each are signals corresponding to the olefinic protons H-10 and H-9 in the furan ring, respectively, and doublets at δ_H 7.21 and δ_H 7.98 with J=9.4 Hz each, correspond to H-6 and H-5 in the aromatic ring, respectively. The presence of only two aromatic protons indicated that all other carbons were substituted. Following the biosynthetic pathway of an alkaloid derived from anthranilic acid, we suggest the main methoxyl substitution to be placed at position four. Three singlets integrated as three protons found deshielded at δ_H 4.02, 4.10 and 4.40 suggest that the protons belong to methoxyl groups and correspond to H-12, H-13 and H-11, respectively.

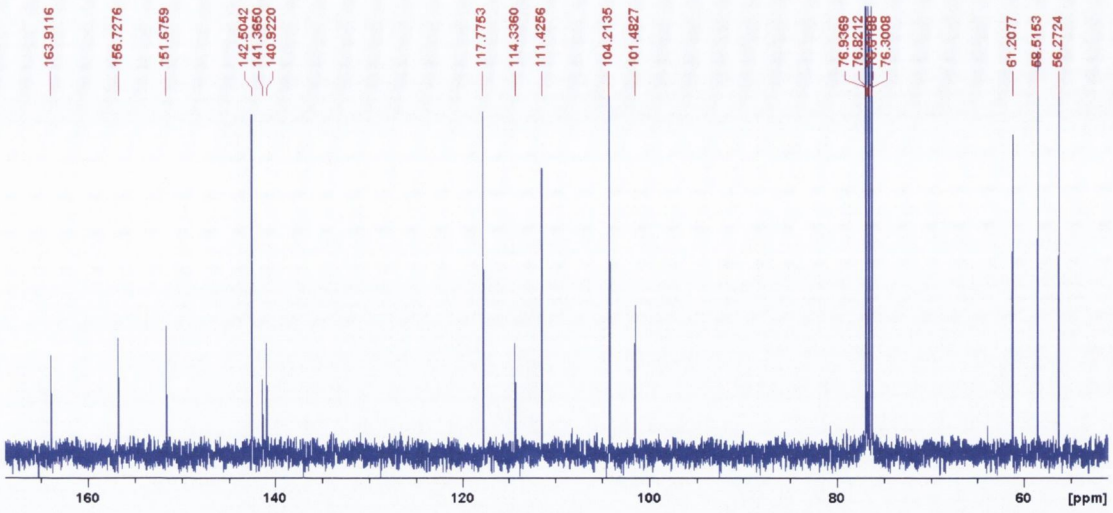
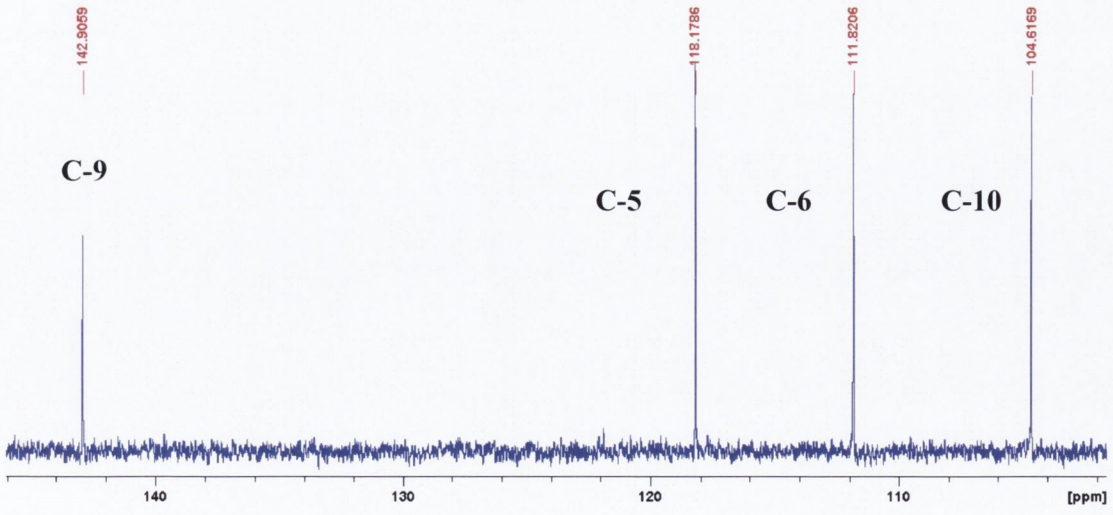


Figure 3.16: The overall ^{13}C NMR spectrum of compound 11.

The ^{13}C NMR spectrum (Figure 3.16) shows the presence of fourteen carbon resonances, including six aromatic carbons with two quarternary carbons at δ_C 101.5 and 151.7. Other quarternary carbons are observed at δ_C 114.3 and 163.9, suggest that they are attached to a furan ring with signal at δ_C 163.9 being deshielded. This happens as this particular carbon is close enough to the electronegative atom O (oxygen) of the furan ring. Signals at δ_C 114.3 and 163.9 could be assigned to C-3 and C-2, respectively.



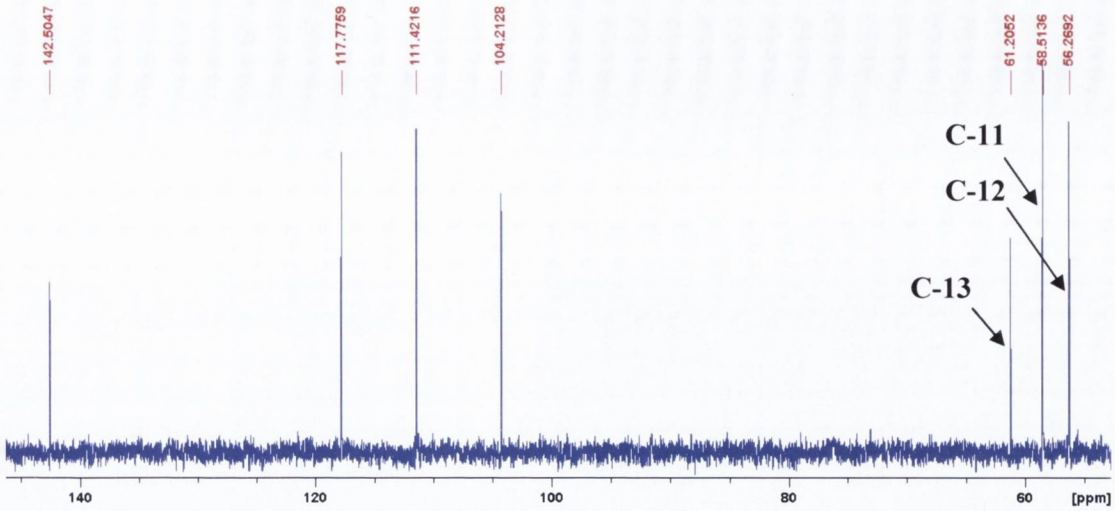


Figure 3.17: DEPT-90 (above) and -135 (below) spectra of compound 11 show the presence of only methoxyl and methine groups.

The presence of the four methine and three methoxyl groups is strongly supported by the DEPT-135 and 90 spectra (Figure 3.17).

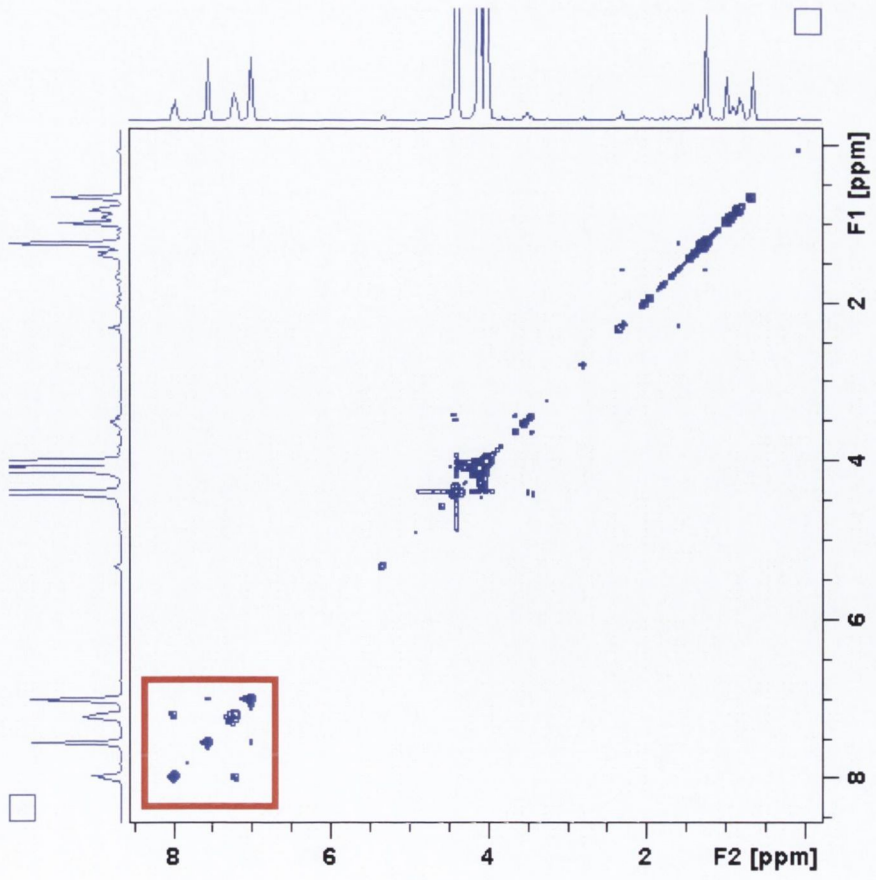


Figure 3.18: ^{1}H-^{1}H COSY spectrum of compound 11 shows strong couplings between signals at δ_H 7.01 and 7.56, and δ_H 7.21 and 7.98.

^{1}H-^{1}H COSY spectrum (Figure 3.18) supports the assignment for H-9 and H-10 of the furan ring, based on the coupling between the resonances at δ_H 7.01 (H-10) and δ_H 7.56 (H-9), while coupling between the signals at δ_H 7.21 and 7.98 supports the assignment for H-6 and H-5 of the aromatic ring, respectively.

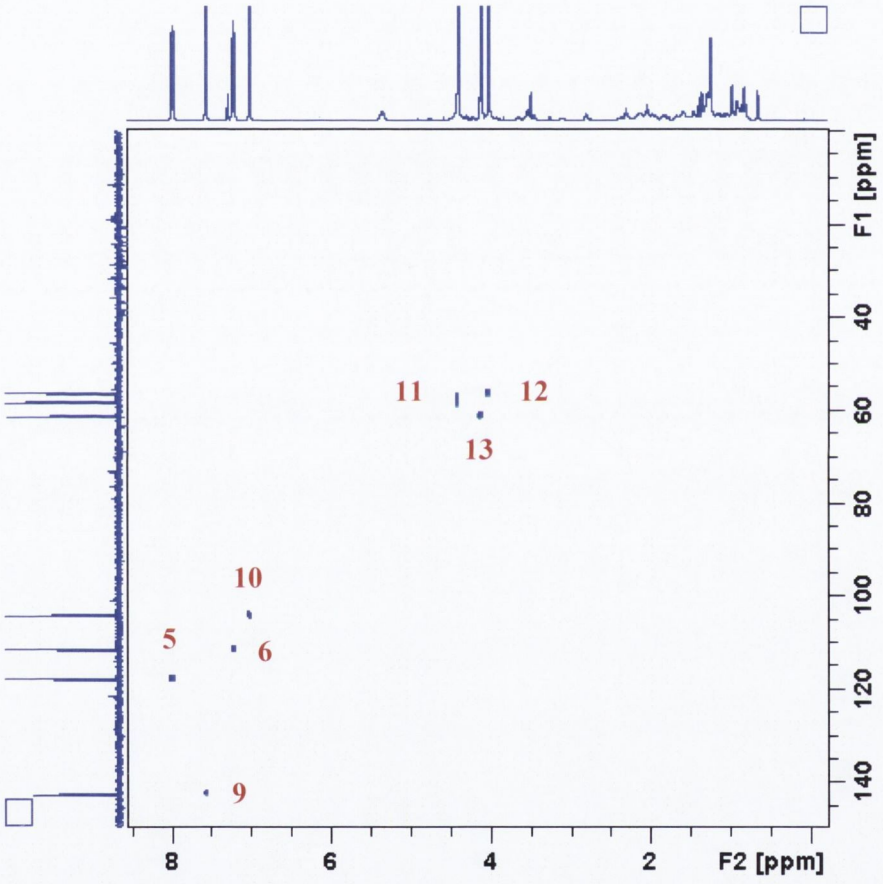


Figure 3.19: HSQC spectrum of compound 11 showing the protonated carbons.

The HSQC spectrum (Figure 3.19) shows the relationships between the signals at δ_H 7.01 (H-10), 7.56 (H-9), 7.21 (H-6) and 7.98 (H-5) and their carbon signals at δ_C 104.2, 142.9, 117.8 and 111.4, respectively. Furthermore, the methoxyl proton signals at δ_H 4.02 (H-12), 4.10 (H-13) and 4.40 (H-11) also show correlations to their carbon signals δ_C 56.3, 61.2 and 58.5, respectively. The complete NMR spectral data were summarized in Table 3.3 below.

Table 3.3: Comparison of ^{1}H and ^{13}C NMR data of compound 11 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C- NMR, $CDCl_3$) and skimmianine ($CDCl_3$).

	Present study		Literature data	
Position	**^{1}H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_H (ppm)**	**^{1}H NMR#**	**^{13}C NMR***
1				
2		163.9		164.6
3		114.3		115.0
4		156.7		157.2
4a		101.5		102.1
4b		151.7		152.2
5	7.98 (1H, *d*, *J*=9.4 Hz)	117.8	7.95	118.2
6	7.21 (1H, *d*, *J*=9.4 Hz)	111.4	7.17	112.2
7		141.3		141.6
8		140.9		141.0
9	7.56 (1H, *d*, *J*=2.6 Hz)	142.9	7.54	143.0
10	7.01 (1H, *d*, *J*=2.6 Hz)	104.2	7.00	104.6
11	4.40 (3H, *s*, OCH_3)	58.5	4.39	59.0
12	4.02 (3H, *s*, OCH_3)	56.3	4.06	56.9
13	4.10 (3H, *s*, OCH_3)	61.2	3.97	61.7

#Cordoso-Lopes *et al.* (2010) and *Brown *et al.* (1980)

These findings support the assignment of the structure of compound 11 to be skimmianine (

Figure 3.14).

Lup-20(29)-en-3β-ol (Lupeol) (12)

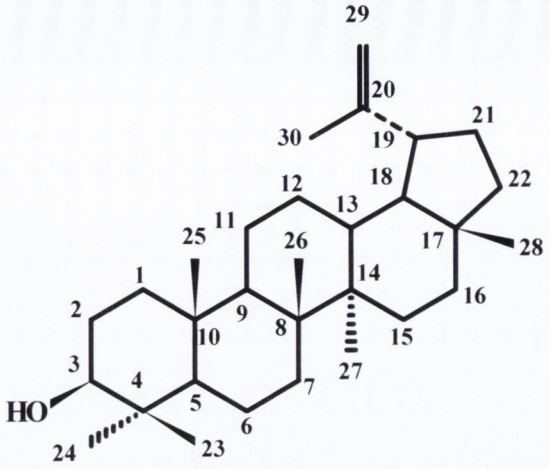


Figure 3.20: Lupeol (12)

White precipitate; R_f: 0.59 (hexane:ethyl acetate; 6:4); mp: 214-217°C; $[\alpha]_D^{20}$ +28.9 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 243; IR ν_{max}: 3318, 2920, 2850, 1453, 878 cm^{-1}; ESI-MS for $C_{30}H_{50}O$ protonated molecular ion m/z; 427 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 0.72 (1H, *m*, H-5), 0.78 (3H, *s*, CH_3-24), 0.81 (3H, *s*, CH_3-28), 0.85 (3H, *s*, CH_3-25), 0.97 (3H, *s*, CH_3-27), 0.99 (3H, *s*, CH_3-23), 1.05 (3H, s, CH_3-26), 1.28 (1H, *m*, H-9), 1.36 (2H, *m*, H-16), 1.39 (2H, *m*, H-7), 1.41 (2H, br, *m*, H-11), 1.62 (2H, *m*, H-2), 1.70 (3H, *s*, CH_3-30), 1.94 (2H, *m*, H-21), 2.39 (1H, *m*, H-19), 3.18 (1H, *dd*, *J*=11.0, 5.3 Hz, H-3); 4.59 (2H, *m*, H-29b), 4.71 (2H, *d*, *J*=2.4 Hz, H-29a); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 14.6 (C-27), 15.4 (C-24), 15.9 (C-26), 16.1 (C-25), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.2 (C-12), 27.4 (C-2), 27.5 (C-15), 28.0 (C-23), 29.9 (C-21), 34.3 (C-7), 35.6 (C-16), 37.2 (C-10), 38.1 (C-13), 38.9 (C-4), 40.0 (C-22), 40.8 (C-8), 42.8 (C-14), 43.0 (C-17), 47.9 (C-19), 48.3 (C-18), 50.4 (C-9), 55.4 (C-5), 79.0 (C-3), 109.4 (C-29), 150.9 (C-20). This data is in agreement to that reported by Agarwal and Rangari. (2003). The complete NMR spectral data is summarized in
Table 3.4 below.

Table 3.4: Comparison of ^{1}H and ^{13}C NMR data for compound 12 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C- NMR, $CDCl_3$) and lupeol ($CDCl_3$).

	Present study		Literature data*	
Position	**^{1}H NMR, δ_H (ppm)**	**^{13}C NMR, δ_C (ppm)**	**^{1}H NMR**	**^{13}C NMR**

	(*J* in Hz)			
1		38.7		38.7
2	1.62 (2H, *m*)	27.4	1.60	27.4
3	3.18 (1H, *dd*, *J*=11.0, 5.3 Hz)	79.0	3.19	79.0
4		38.9		38.9
5	0.72 (1H, *m*)	55.4	0.68	55.3
6		18.3		18.3
7	1.39 (2H, *m*)	34.3	1.39	34.3
8		40.8		40.8
9	1.28 (1H, *m*)	50.4	1.27	50.4
10		37.2		37.2
11	1.41 (2H, *m*)	20.9	1.41	20.9
12		25.2		25.2
13		38.1		38.1
14		42.8		42.9
15		27.5		27.5
16	1.36 (2H, *m*)	35.6	1.39	35.6
17		43.0		43.0
18		48.3		48.3
19	2.39 (1H, *m*)	47.9	2.38	48.0
20		150.9		150.9
21	1.94 (2H, *m*)	29.9	1.92	29.9
22		40.0		40.0
23	0.99 (3H, *s*)	28.0	0.97	28.0
24	0.78 (3H, *s*)	15.4		15.4
25	0.85 (3H, *s*)	16.1	0.83	16.1
26	1.05 (3H, *s*)	15.9		15.9
27	0.97 (3H, *s*)	14.6	0.95	14.6
28	0.81 (3H, *s*)	18.0		18.0
29a	4.71 (1H, *d*, *J*=2.4 Hz)	109.4	4.69	109.3
29b	4.59 (1H, *m*)		4.56	
30	1.70 (3H, *s*)	19.3		19.3

*** Agarwal and Rangari (2003)**

Comparison of the melting point obtained for lupeol (m.p. 214-217°C °C) with the one published (m.p. 215-216°C) (Agarwal and Rangari., 2003) and co-cromatography by TLC using lupeol standard left no doubt that we isolated the same compound. Together with the specteroscopic analyses, these findings support the assignment of the structure of compound 12 to be lupeol (Figure 3.20).

Lup-20(29)-en-3β,24-diol (13)

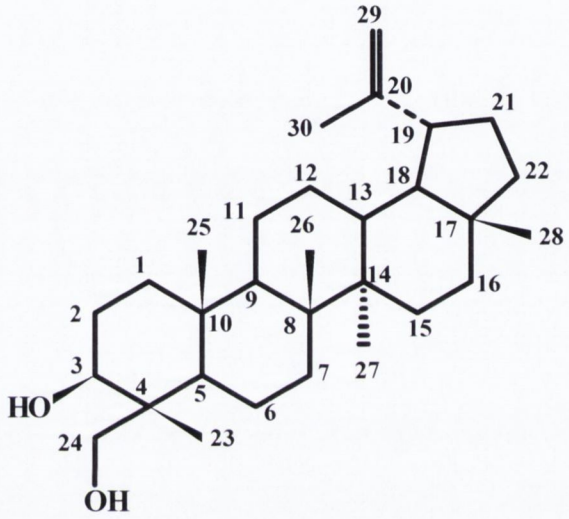


Figure 3.21: Lup-20(29)-en-3β,24-diol (13)

White precipitate; R_f: 0.57 (hexane:ethyl acetate; 6:4); mp: 250-252°C; $[\alpha]_D^{20}$ +9.09 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 244; IR ν_{max}: 3320, 2916, 2848, 1462, 1266, 718 cm^{-1}; ESI-MS for $C_{30}H_{50}O_2$ protonated molecular ion m/z; 443 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 0.72 (1H, *m*, H-5), 0.79 (3H, *s*, H-25, CH_3), 0.82 (3H, *s*, H-28), 0.97 (3H, br, *s*, H-27), 0.99 (3H, *s*, H-23), 1.06 (3H, *s*, H-26), 1.28 (1H, *m*, H-9), 1.36 (2H, *m*, H-16), 1.39 (2H, *m*, H-7), 1.42 (2H, *m*, H-11), 1.63 (2H, *m*, H-2), 1.71 (3H, br, *s*, H-30), 1.94 (2H, *m*, H-21), 2.41 (1H, *m*, H-19), 4.10 (3H, *s*, H-24), 4.59 (2H, *m*, H-29b), 4.72 (2H, *d*, *J*=2.4 Hz, H-29a); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 14.6 (C-27), 15.9 (C-25), 16.1 (C-26), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.1 (C-12), 27.4 (C-15), 27.5 (C-2), 27.9 (C-23), 29.7 (C-21), 34.3 (C-7), 35.6 (C-16), 37.2 (C-10), 38.1 (C-13), 38.7 (C-1), 38.9 (C-4), 40.0 (C-22), 40.8 (C-8), 42.8 (C-14), 43.0 (C-17), 48.0 (C-19), 48.3 (C-18), 50.5 (C-9), 55.3 (C-5), 63.1 (C-24), 79.0 (C-3), 109.3 (C-29), 150.9 (C-20). This data are in agreement to that reported by Tanaka *et al.* (1988) and Mahato and Kundu (1994).

Compound 13 was isolated as a white precipitate (22 mg) with a molecular formula of $C_{30}H_{50}O_2$ as determined by ESI-MS analysis of its protonated molecular ion 443 $[M+H]^+$. The UV spectrum of 4 had an absorption maximum at 244 nm, suggesting the absence of α, β unsaturated carbonyl group. Its IR spectrum showed the absorptions at 3320 (hydroxyl group), 2916 (C-H stretch, olefinic), 2848, 1462 (C=C), 1266, 718 cm^{-1}. The complete NMR spectral data is summarized in Table 3.5 below.

Table 3.5: Comparison of ^1H and ^{13}C NMR data for compound 13 (400 MHz for ^1H-NMR and 100 MHz for ^{13}C- NMR, $CDCl_3$) and lup-20(29)-en-3β,24-diol ($CDCl_3$).

	Present study		Literature data	
Position	**^1H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^1H NMR#**	**^{13}C NMR***
1		38.7		38.5
2	1.63 (2H, *m*)	27.5		27.8
3		79.0		80.9
4		38.9		42.8
5	0.72 (1H, *m*)	55.3		55.9
6		18.3		18.4
7	1.39 (2H, *m*)	34.3		34.9
8		40.8		40.9
9	1.28 (1H, *m*)	50.5		50.5
10		37.2		38.0
11	1.42 (2H, *m*)	20.9		21.2
12		25.1		25.1
13		38.1		36.9
14		42.8		42.8
15		27.4		27.4
16	1.36 (2H, *m*)	35.6		35.6
17		43.0		43.0
18		48.3		48.0
19	2.41 (1H, *m*)	48.0		48.3
20		150.9		150.9
21	1.94 (2H, *m*)	29.7		29.9
22		40.0		40.0

23	0.99 (3H, *s*)	27.9	0.99	22.4
24	4.10 (2H, *s*)	63.1	4.18	64.5
25	0.79 (3H, *s*)	15.9	0.79	15.9
26	1.06 (3H, *s*)	16.1	1.02	16.7
27	0.97 (3H, br, *s*)	14.6	0.94	14.6
28	0.82 (3H, *s*)	18.0	0.87	18.0
29a	4.72 (1H, *d*, *J*=2.4 Hz)	109.3	4.69	109.4
29b	4.59 (1H, *dd*, *J*=2.4, 1.3 Hz)		4.65	
30	1.71 (3H, br, *s*)	19.3	1.68	19.3

#Tanaka *et al.* (1988) and *Mahato and Kundu (1994)

Comparison of the melting point obtained for lup-20(29)-en-3β,24-diol (m.p. 250-252°C) with the one published (m.p. 249-250°C) (Tanaka *et al.*, 1988) and co-cromatography by TLC using donated standard left no doubt that we isolated the same compound. Together with the specteroscopic analyses, these findings support the assignment of the structure of compound 13 to be lup-20(29)-en-3β,24-diol (Figure 3.21).

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5-[3-Methylbut-2-enyl]-4,6,7-trimethoxylfuro[2,3-b]quinoline, tecleamaniensine A (14)

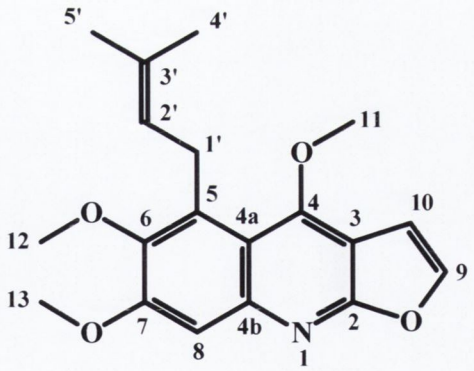


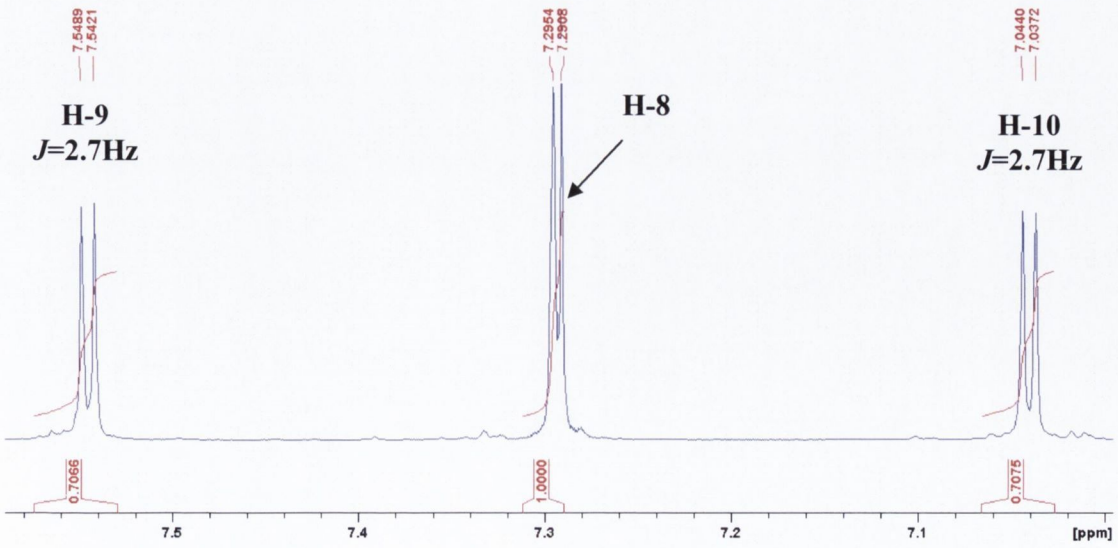
Figure 3.22: Tecleamaniensine A (14)

Yellowish gum; R_f: 0.75 (chloroform:methanol:water; 9:1:1); mp: 120-125°C; $[\alpha]_D^{20}$ 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 252, 320, 333; IR ν_{max}: 2930, 2856, 1607, 1576, 1356, 1255, 1063 cm^{-1}; ESI-MS for $C_{19}H_{21}NO_4$ m/z; 328 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 1.69 (3H, *d*, *J*=1.3 Hz, H-4'), 1.86 (3H, *d*, *J*=0.8 Hz, H-5'), 3.85 (3H, *s*, OCH_3-12), 4.00

(3H, *s*, OCH_3-13), 4.06 (2H, *d*, *J*=6.2 Hz, H-1'), 4.35 (3H, *s*, OCH_3-11), 5.25 (1H, *t*, *J*=6.3 Hz, H-2'), 7.04 (1H, *d*, *J*=2.7 Hz, H-10), 7.29 (1H, *s*, H-8), 7.55 (1H, *d*, *J*=2.7 Hz, H-9); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 18.2 (C-4'), 25.8 (C-5'), 27.5 (C-1'), 55. 7 (C-13), 59.4 (C-11), 61.0 (C-12), 103.5 (C-3), 105.3 (C-10), 106.2 (C-8), 113.0 (C-4a), 124.9 (C-2'), 130.4 (C-5), 130.9 (C-3'), 142.2 (C-9), 145.8 (C-4b), 145.9 (C-6), 154.9 (C-7), 159.1 (C-4), 163.2 (C-2). This data is in agreement to that reported by Magadula *et al.* (2008).

Compound 14 was obtained as a yellowish gum (18 mg). The ESI-MS suggested a molar mass of its protonated molecular ion m/z 328 $[M+H]^+$, which is in accordance to the molecular formula of $C_{19}H_{21}NO_4$. The UV spectrum shows signals at 252, 320, 333 nm suggesting a furoquinoline alkaloid skeleton (Magadula *et al.*, 2008). Its IR spectrum showed the absorptions at 2930 (C-H aromatic), 2856 (OH stretch carboxylic), 1576 (aromatic ring), 1356 (C-C stretch aromatic), 1255 (C-O stretch carboxylic) cm^{-1}. Knowing that the previous isolated compound is a furoquinoline alkaloid, the assignment for compound 14 will follow the one of compound 11.

From the ^{1}H NMR spectrum (Figure 3.23), it can be seen that compound 14 is similar to the quinoline alkaloid compound 2 with an isoprenyl group. Two singlets δ_H 1.69 and 1.86 are assigned to the two methyl groups at position 4' and 5' in compound 14, respectively. A singlet proton resonance at δ_H 4.35, integrating for three protons is characteristic of a methoxyl group at position four in a furoquinoline alkaloid. The presence of a pair doublets at δ_H 7.04 and 7.55 with *J*=2.7 Hz each, characteristic of the coupling in the furan ring. A doublet at δ_H 4.06 (*J*=6.2 Hz), integrating as two protons corresponds to the only methylene group of the prenyl group.



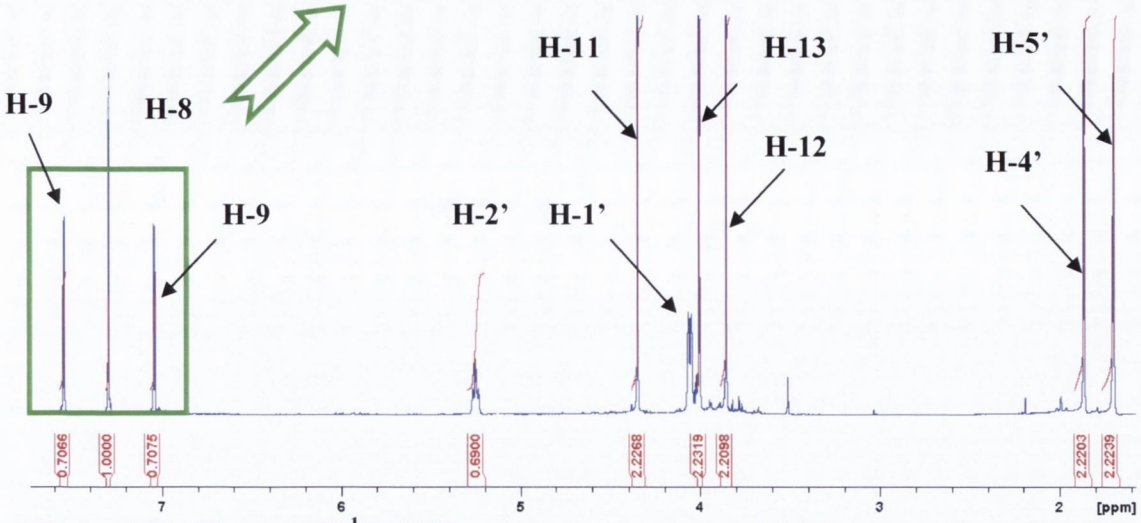


Figure 3.23: The overall ^{1}H NMR spectrum of compound 14 shows a pair of doublets at δ_H 7.04 and 7.55 of the furan ring other than a pair of methyl and three methoxyl signals.

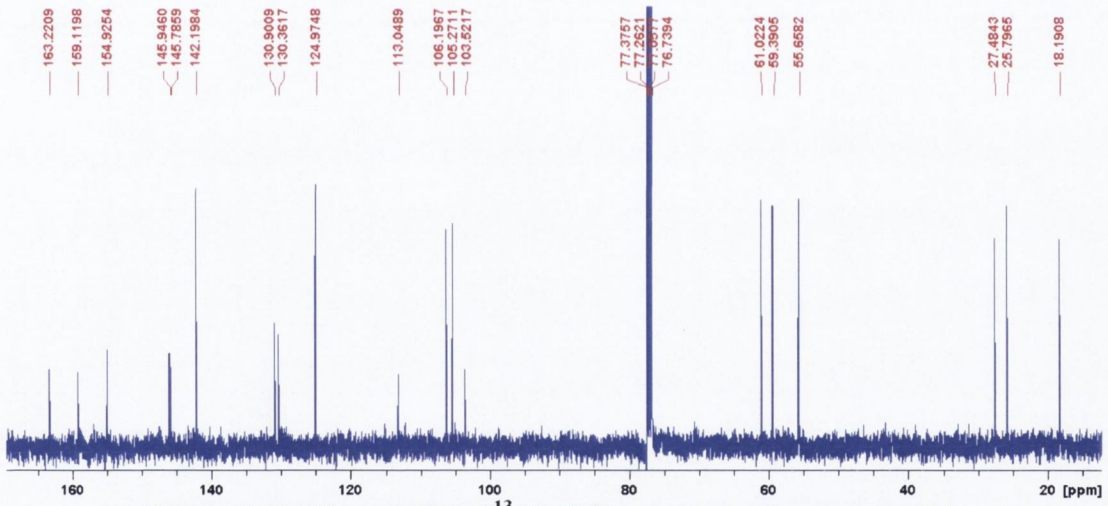
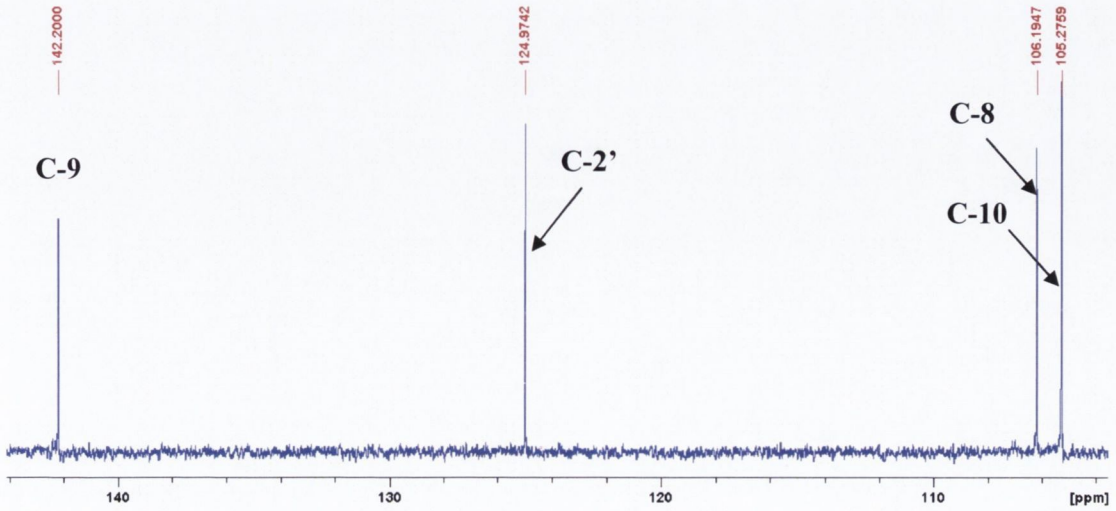


Figure 3.24: The overall of ^{13}C NMR spectrum of compound 14.



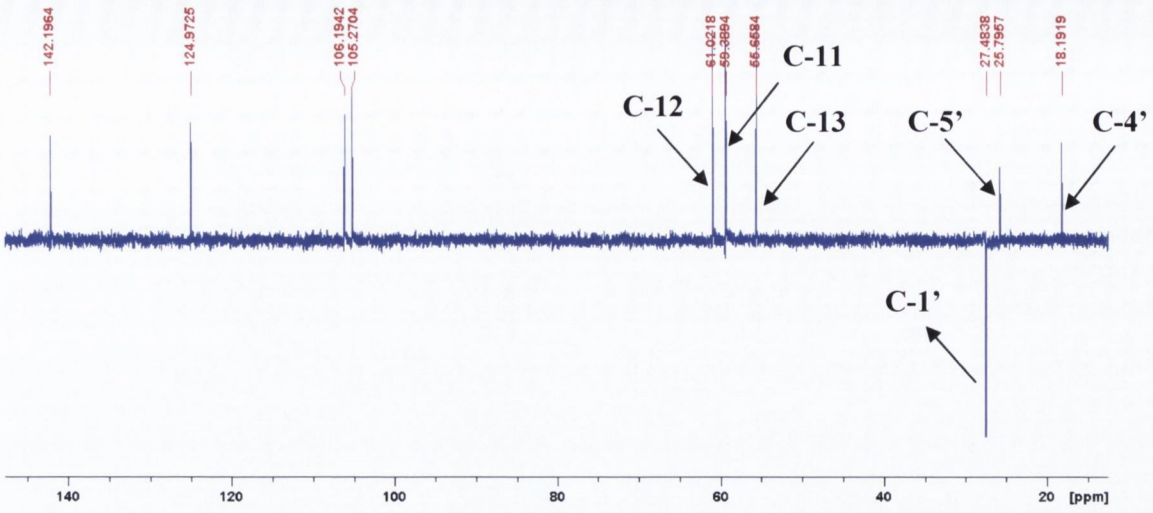


Figure 3.25: DEPT-90 (above) and -135 (below) spectra of compound 14 exhibit the presence of one methylene group together with four methine, three methoxyl and two methyl groups.

According to the ^{13}C NMR (Figure 3.24), DEPT-135 and -90 spectra (Figure 3.25), in addition to the eleven carbon signals of the furoquinoline nucleus and three methoxyl carbon resonances, there are five carbon signals at 27.5 (C-1'), 124.9 (C-2'), 130.9 (C-3'), 18.2 (C-4') and 25.8 (C-5') that corresponds to a 3-methylbut-2-enyl group. Figure 3.25 above shows the presence of a signal of methylene group at δ_H 27.5.

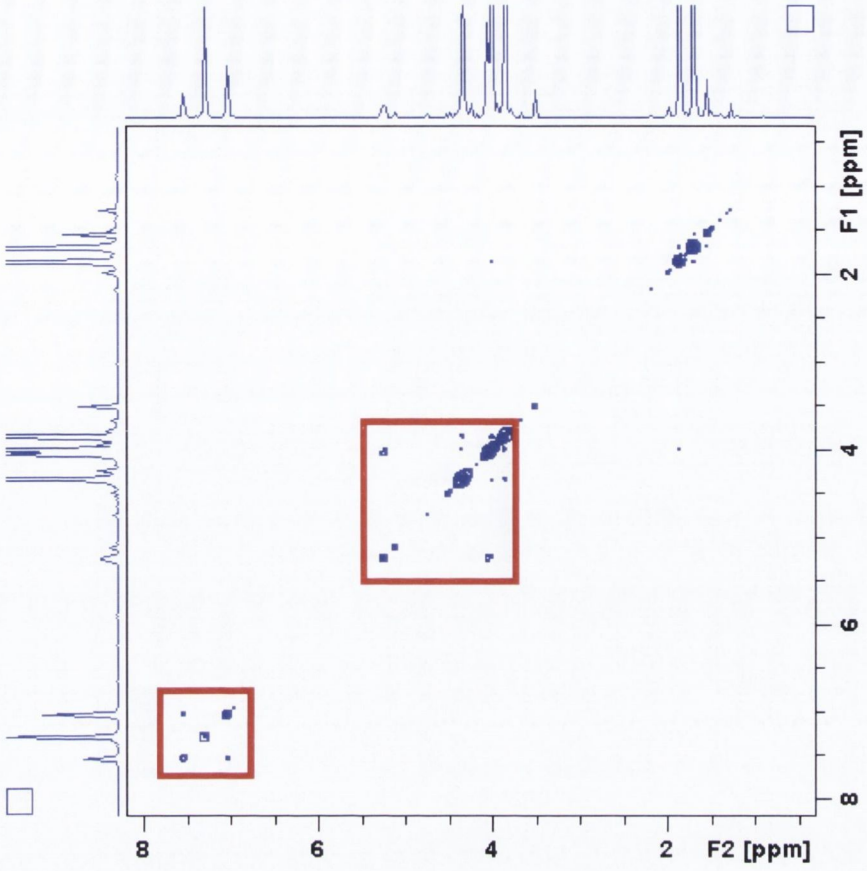


Figure 3.26: ^{1}H-^{1}H COSY spectrum of compound 14 shows coupling of the H-9 with H-10 of the furan ring and H-1' with H-2' of the prenyl group.

The ^{1}H-^{1}H COSY spectrum (Figure 3.26) shows strong correlations between protons at δ_H 7.04 (H-10) and 7.55 (H-9) of the furan ring, and between δ_H 4.06 (H-1') and 5.25 (H-2') of the prenyl group.

The HSQC spectrum (Figure 3.27) shows a correlation between protons and their carbons resonances; methyl groups at δ_H 1.69 / δ_C 25.8, δ_H 1.86 / δ_C 18.2, methoxyl group at δ_H 3.85 / δ_C 61.0, δ_H 4.00 / δ_C 55.7, δ_H 4.35 / δ_C 59.4, and the methine group at δ_H 7.04 / δ_C 105.3, δ_H 7.29 / δ_C 106.2, δ_H 7.55 / δ_C 142.2. The complete NMR spectral data are summarized in Table 3.6.

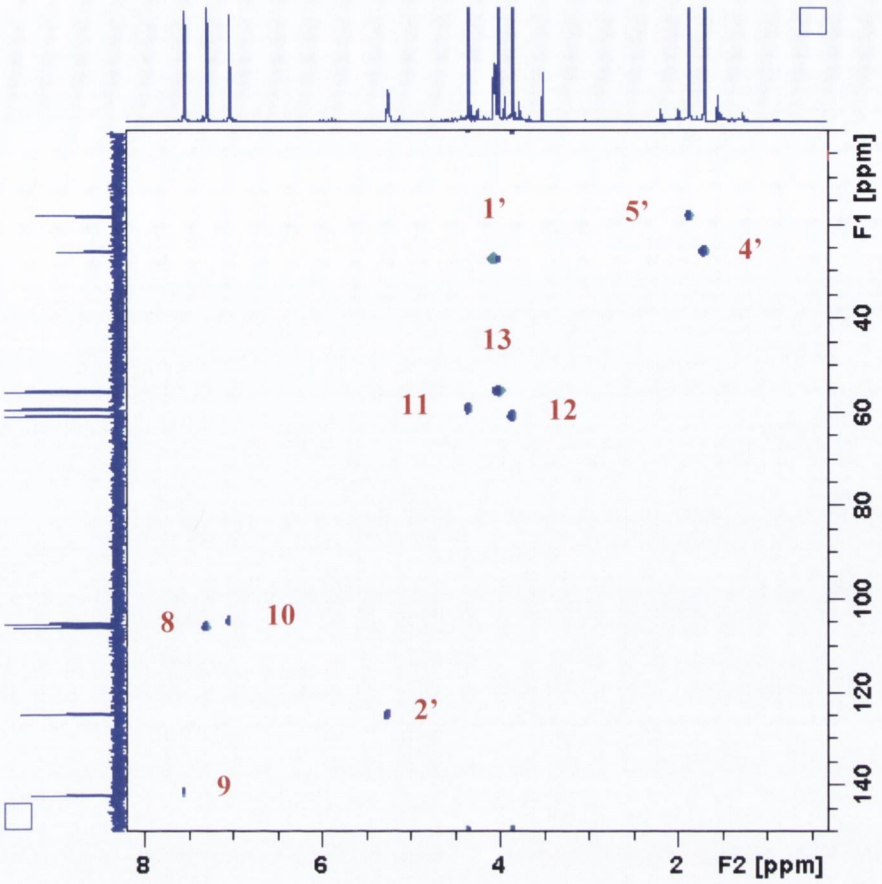


Figure 3.27: An amplification region of HSQC spectrum of compound 14 showing protonated carbons.

Table 3.6: Comparison of ^{1}H and ^{13}C NMR data of compound 14 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C- NMR, $CDCl_3$) and tecleamaniensine A ($CDCl_3$).

	Present study		Literature data*	
Position	**^{1}H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^{1}H NMR**	**^{13}C NMR**
1				
2		163.2		163.2
3		103.5		103.5
4		159.1		159.2
4a		113.0		113.0
4b		145.8		145.8
5		130.4		130.3
6		145.9		145.9
7		154.9		154.9

8	7.29 (1H, *s*)	106.2	7.27	106.1
9	7.55 (1H, *d*, *J*=2.7 Hz)	142.2	7.51	142.2
10	7.04 (1H, *d*, *J*=2.7 Hz)	105.3	7.01	105.2
11	4.35 (3H, *s*, OCH_3)	59.4	4.31	59.4
12	3.85 (3H, *s*, OCH_3)	61.0	3.81	60.9
13	4.00 (3H, *s*, OCH_3)	55.7	3.96	55.6
1'	4.06 (2H, *d*, *J*=6.2 Hz)	27.5	4.02	27.6
2'	5.25 (1H, *t*, *J*=6.3 Hz)	124.9	5.21	124.9
3'		130.9		130.9
4'	1.69 (3H, *d*, *J*=0.8 Hz)	18.2	1.66	18.1
5'	1.86 (3H, *d*, *J*=1.3 Hz)	25.8	1.82	25.8

***Magadula *et al.* (2008)**

These findings support the assignment of the structure of compound 14 to be tecleamaniensine A (Figure 3.22).

- **Analysis of the dichloromethane fraction**

6-methoxyl-7,8-methylenedioxycoumarin (15)

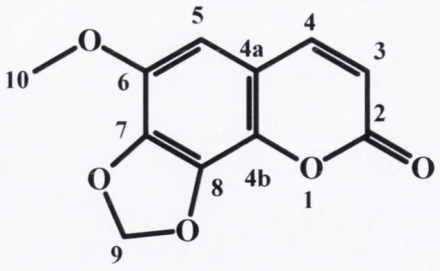


Figure 3.28: 6-methoxyl-7,8-methylenedioxycoumarin (15)

White precipitate; R_f: 0.83 (chloroform:methanol:water; 9:1:1); mp: 222-224°C; $[\alpha]_D^{20}$ 0.0 (MeOH); UV (CH_2Cl_2) λ_{max}: 330; IR ν_{max}: 3420, 2926, 1717 (α-pyrane ring), 1582, 1455 (aromatic C=C), 1321, 1120 (carboxyl group) cm^{-1}; EI-MS for $C_{11}H_8O_5$ m/z; 221 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ_H: 3.87 (3H, *s*, OCH_3-10), 6.23 (2H, *s*, OCH_2O-9), 6.33 (1H, *d*, *J*=9.6 Hz, H-3), 7.04 (1H, *s*, H-5), 7.95 (1H, *d*, *J*=9.6 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ_C: 56.8 (C-10), 103.9 (C-9), 106.2 (C-5), 113.8 (C-3), 114.6 (C-4a), 132.6 (C-4b),

134.6 (C-8), 138.0 (C-6), 140.9 (C-7), 145.0 (C-4), 159.5 (C-2). This data is in agreement to that reported by Saeed and Sabir (2008).

Compound 15 was obtained as a colourless precipitate (15 mg) with a molecular formula of $C_{11}H_8O_5$ as determined from EI-MS analysis of its protonated molecular ion 221 $[M+H]^+$. The UV spectrum showed signal at 330 nm suggesting a coumarin skeleton. Its IR spectrum showed absorptions at 3420, 2926, 1717 (α-pyrane ring), 1582, 1455 (aromatic C=C), 1321, 1120 (carboxyl group) cm^{-1}.

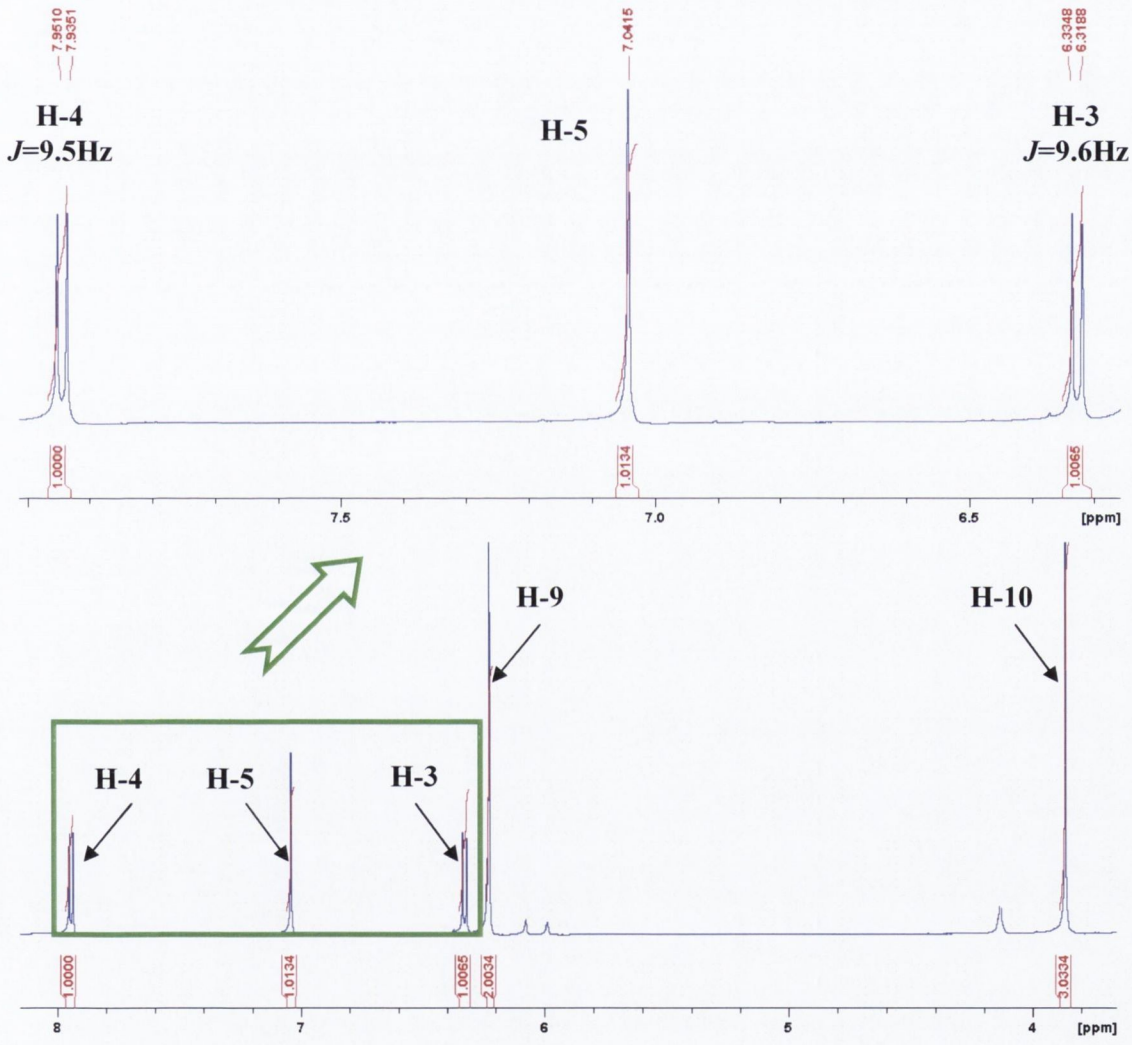


Figure 3.29: The overall ^{1}H NMR spectrum of compound 15 shows a typical coumarin chemical shift with protons at position 3 and 4.

The ^{1}H NMR spectrum (Figure 3.29) shows the presence of a methoxyl group, a singlet integrated for three protons at δ_H 3.87, and methylenedioxy group, a singlet integrated for two protons at δ_H 6.24. Another singlet at δ_H 7.04 characteristic of an

aromatic proton at position five, while a pair of doublets with *ortho* coupling at δ_H 6.33 (J=9.6 Hz) and 7.95 (J=9.5 Hz), correspond to the proton at position four and three of a α-pyran ring system, respectively.

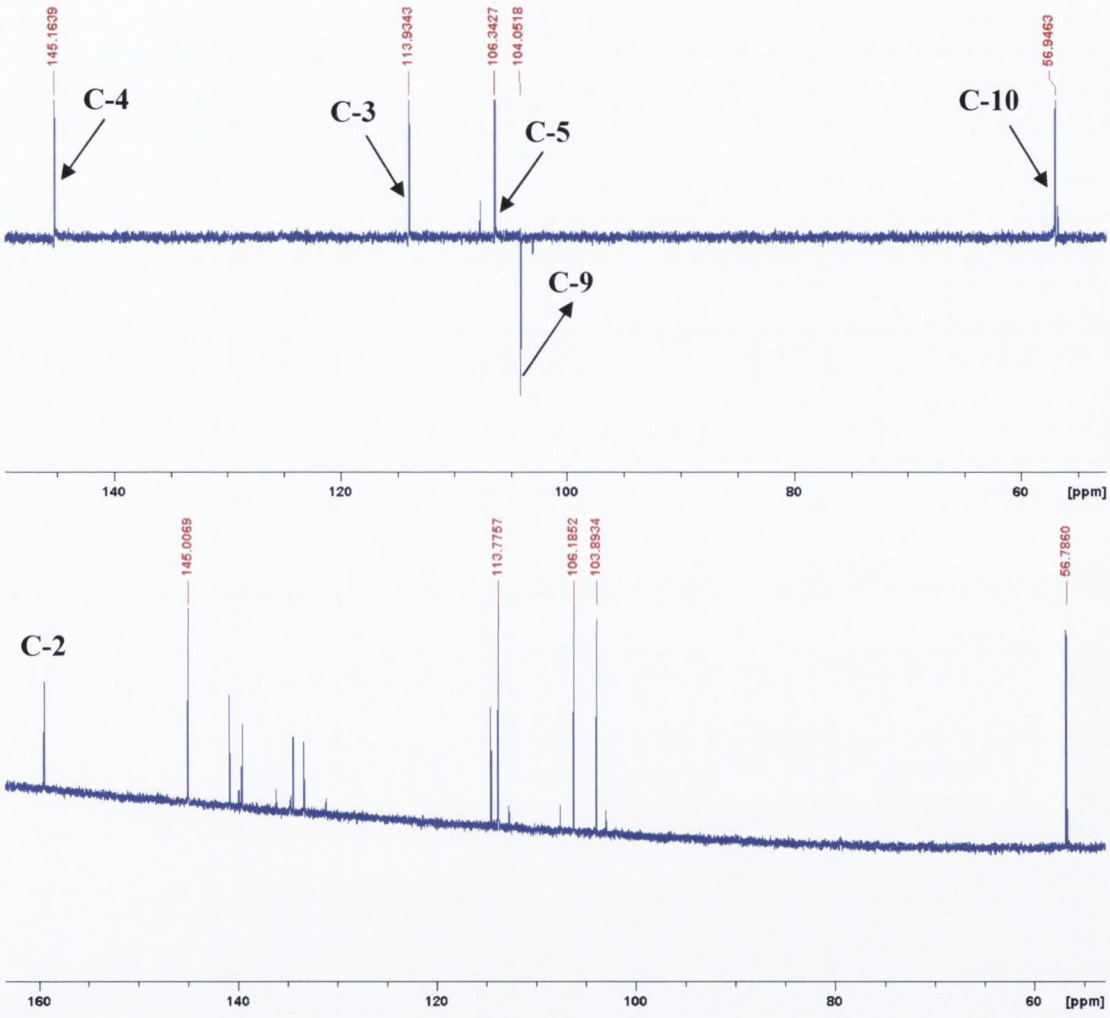


Figure 3.30: The overall ^{13}C (below) and DEPT-135 (above) NMR spectra of compound 15 show the presence of three methine, one of each methylene and methine groups.

The ^{13}C NMR spectrum (Figure 3.30) clearly showed the presence of a carboxyl group at δ_C 159.5, while DEPT-135 spectrum (Figure 3.30) showed the methoxyl carbon at δ_C 56.8, methine carbons at δ_C 106.2, 113.8 and 145.0, and methylene carbon of methylenedioxy group at δ_C 103.9. According to the skeleton, this methylenedioxy group is at position nine. The most deshielded carbon at δ_C 159.5 is the typical characteristics of C=O at position 2 of a coumarin. The quarternary carbons at δ_C 134.6 and 140.9 are found

slightly downfield suggest that they are attached to the methylenedioxy group at position eight and seven, respectively.

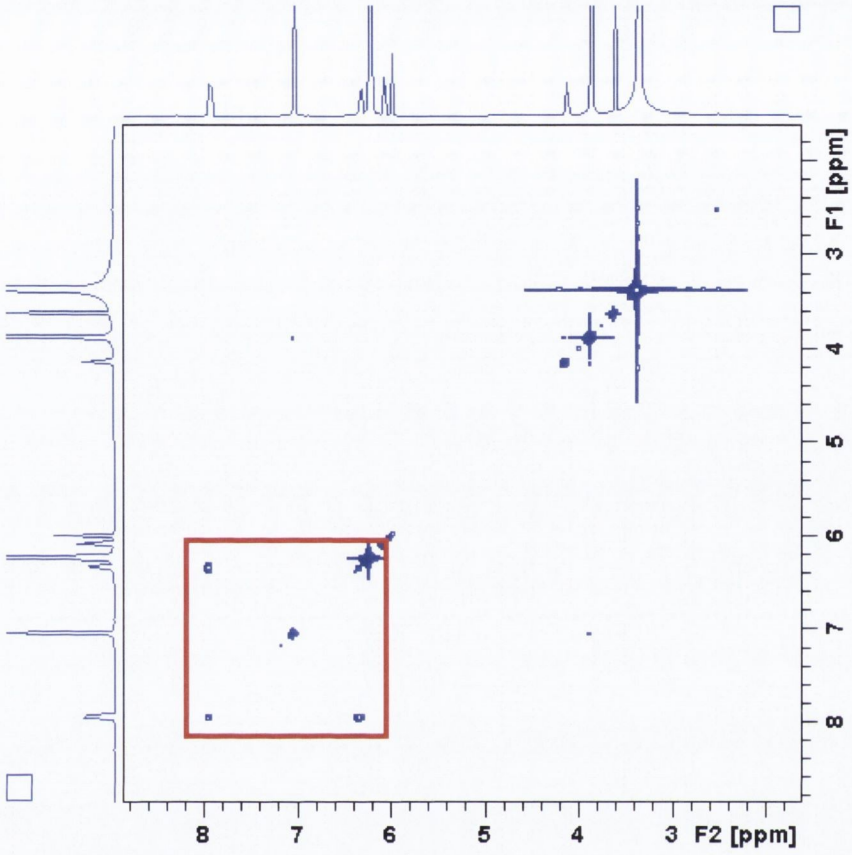


Figure 3.31: ^{1}H-^{1}H COSY spectrum of compound 15 shows couplings between δ_H 6.33 with 7.95 and between δ_H 7.04 with methoxyl protons at δ_H 3.78.

The ^{1}H-^{1}H COSY spectrum (Figure 3.31) shows a strong correlation between resonances at δ_H 6.33 and 7.95 relative to the *ortho* coupling at position three and four. The position of the methoxyl group at position six is confirmed by the detection of a weak coupling between the signals at δ_H 3.87 (H-10) and δ_H 7.04 (H-5).

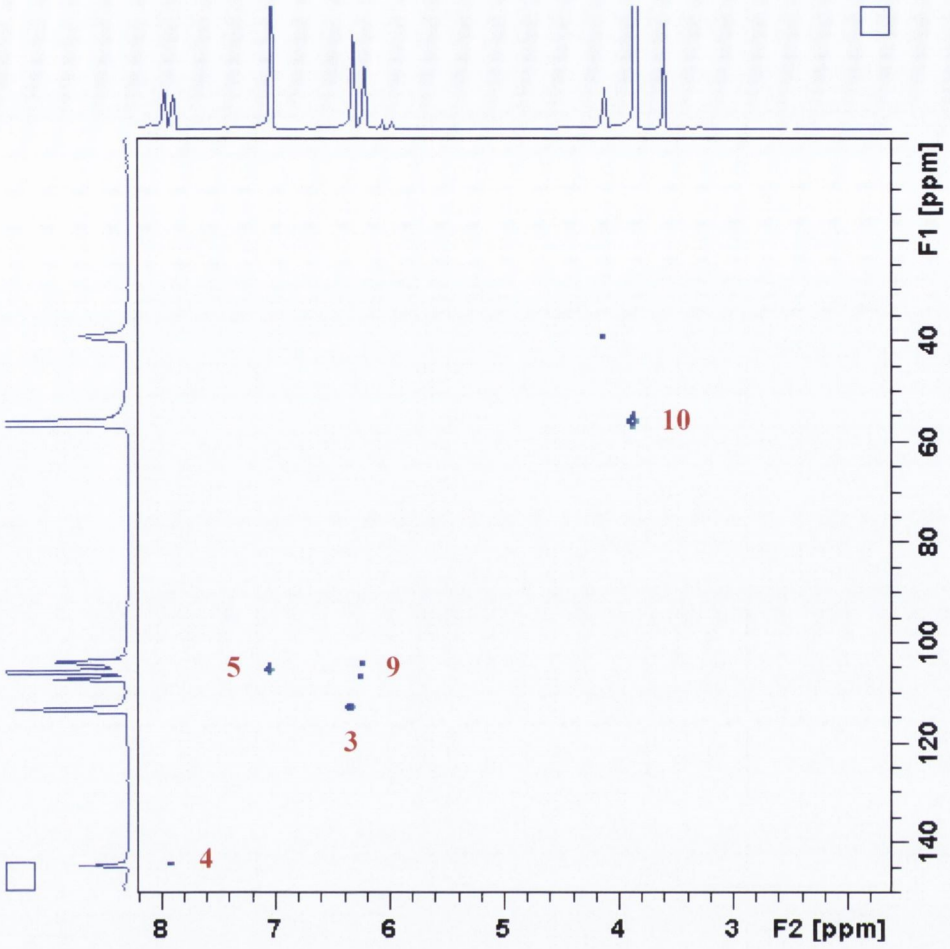


Figure 3.32: HSQC spectrum of compound 15 showing protonated carbons.

The HSQC spectrum (Figure 3.32) of this compound clearly assigned the protonated carbons; δ_H 3.87 / δ_C 56.8, δ_H 6.23 / δ_C 103.9, δ_H 6.33 / δ_C 113.8, δ_H 7.04 / δ_C 106.2 and δ_H 7.95 / δ_C 145.0. The complete NMR spectral data are summarized in Table 3.7 below.

Table 3.7: Comparison of ^{1}H and ^{13}C NMR data of compound 15 (600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C- NMR, DMSO-d_6) and 6-methoxyl-7,8-methylenedioxycoumarin ($CDCl_3$).

	Present study		Literature data*	
Position	**^{1}H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^{1}H NMR**	**^{13}C NMR**
1				
2		159.5		160.1

3	6.33 (1H, *d*, *J*=9.6 Hz)	113.8	6.25	112.7
4	7.95 (1H, *d*, *J*=9.6 Hz)	145.0	7.56	143.5
4a		114.6		113.4
4b		132.6		132.5
5	7.04 (1H, *s*)	106.2	6.59	109.1
6		138.0		138.4
7		140.9		139.1
8		134.6		134.5
9	6.23 (2H, *s*)	103.9	6.18	103.6
10	3.87 (3H, *s*, OCH_3)	56.8	3.92	56.4

***Saeed and Sabir (2008)**

Chemical shifts appear to be inconsistent when the comparison of our data is made with the literature data, but this can be explained by the two different solvents used to acquire the spectra. DMSO is known to cause important shifts.

These findings support the assignment of the structure of compound 15 to be 6-methoxyl-7,8-methylenedioxycoumarin (Figure 3.28).

Choisyine (16)

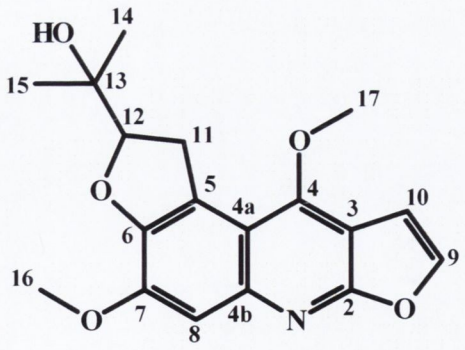
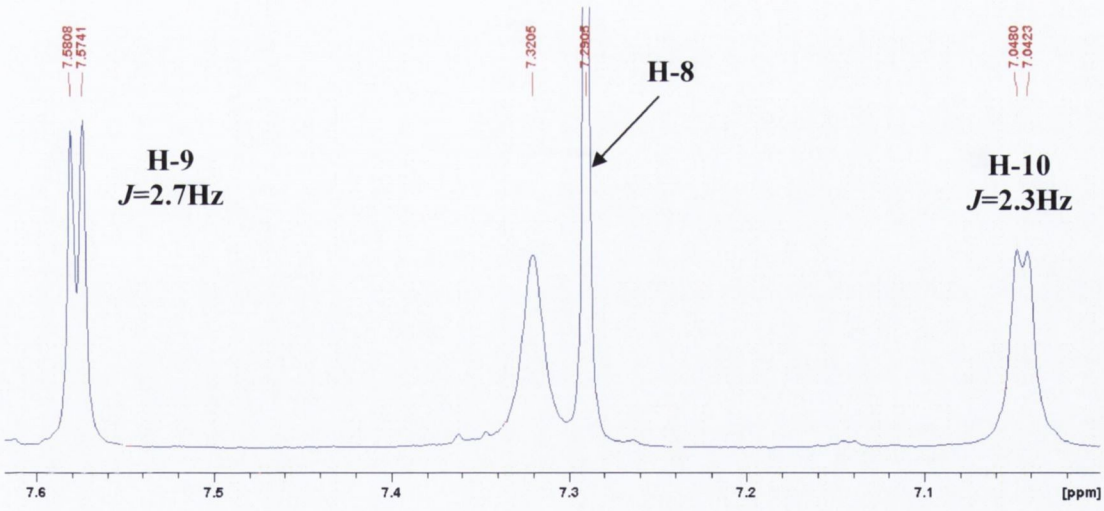


Figure 3.33: Choisyine (16)

Yellowish needle-like crystal; R_f: 0.33 (chloroform:methanol:water; 9:1:1); mp: 188-190°C; $[\alpha]_D^{20}$ 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 258, 318, 352; IR ν_{max}: 3284, 2934, 1622, 1460, 1266, 951 cm^{-1}; ESI-MS for $C_{18}H_{19}NO_5$ m/z; 330 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400

MHz) δ_H: 1.33 (3H, *s*, H-14), 1.46 (3H, *s*, H-15), 3.77 (2H, *m*, H-11), 4.02 (3H, *s*, OCH_3-16), 4.39 (3H, *s*, OCH_3-17), 4.84 (1H, *t*, *J*=9.6 Hz, H-12), 7.04 (1H, *d*, *J*=2.3 Hz, H-10), 7.32 (1H, br, *s*, H-8), 7.57 (1H, *d*, *J*=2.3 Hz, H-9); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C: 24.2 (C-14), 26.6 (C-15), 33.8 (C-11), 55.9 (C-16), 58.9 (C-17), 71.9 (C-13), 90.6 (C-12), 102.7 (C-3), 104.8 (C-10), 106.2 (C-8), 111.4 (C-4a), 118.8 (C-5), 142.0 (C-4b), 142.6 (C-9), 146.6 (C-6), 148.6 (C-7), 156.9 (C-4), 162.4 (C-2). This data is in fair agreement to that reported by Johns *et al.* (1967) and Brown *et al.* (1980).

Compound 16 was isolated as colourless needle-like crystals (19 mg) with a molecular formula of $C_{18}H_{19}NO_5$ as determined from ESI-MS analysis of its protonated molecular ion 330 $[M+H]^+$. The UV spectrum of 16 has absorption maxima at 258, 318, 352 nm, suggesting the presence of a furoquinoline moiety (Yang *et al.*, 2010; Magadula *et al.*, 2008). Its IR spectrum showed the absorptions at 3284 (N-H stretch), 2934 (C-H stretch), 1622 (aromatic ring), 1460 (C-C stretch aromatic) and 1266 (C-O stretch) cm^{-1}. Knowing that this compound could be another furoquinoline alkaloid, the assignment for the furoquinoline skeleton is compared to the ones of compound 11 and 14.



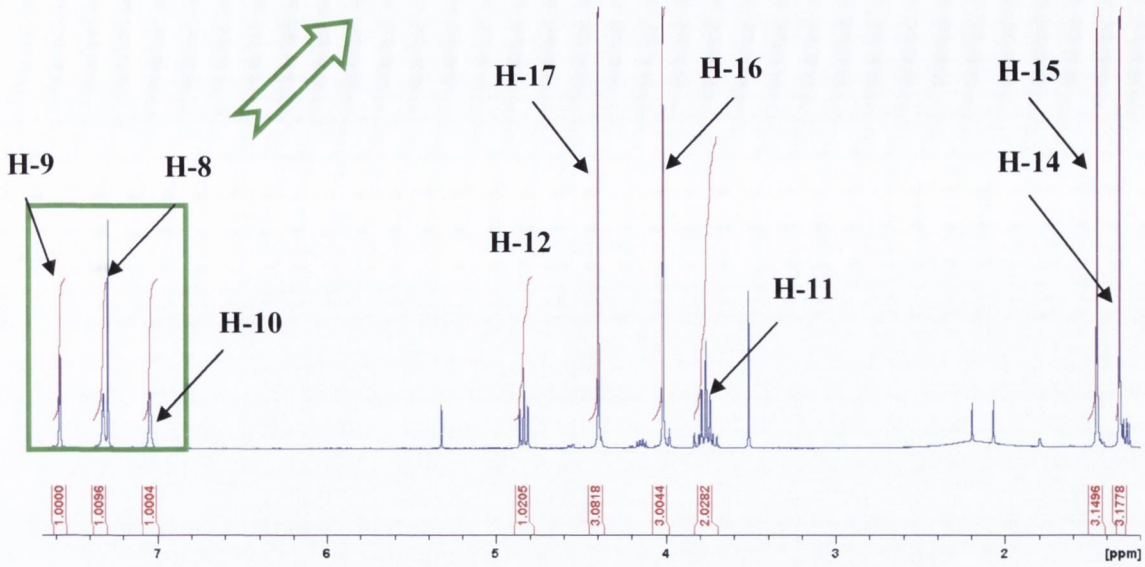


Figure 3.34: The overall ^{1}H NMR spectrum of compound 16 and an amplification region showing the coupling of H-9 and H-10 of the furan ring and a broad singlet belonging to the hydroxyl group.

The ^{1}H NMR spectroscopic data of 16 (Table 3.8) are closely comparable with those of furoquinoline derivatives isolated by Brown and co-workers (1980). The ^{1}H NMR spectrum (Figure 3.34) suggests the presence of two *C*-methyl groups that appeared as singlets at δ_H 1.33 and 1.46, which are found to be slightly deshielded, suggesting their proximity to an electronegative atom O (oxygen), perhaps from a hydroxyl group. This is supported by a singlet at δ_H 3.51, characteristic of a hydroxyl group. Two singlets integrated as three protons at δ_H 4.02 and 4.39 characteristic of methoxyl groups with the lower field signal are assigned to the methoxyl group at position four in accordance with the chemical shift data recorded for alkaloids of this group. The presence of a pair of doublets at δ_H 7.04 (J= 2.3 Hz) and 7.57 (J=2.3 Hz) correspond to the methines of the furan ring on the position ten and nine, respectively. A multiplet at δ_H 3.77 corroborates with the only methylene group neighbouring the methine proton of the hydroxy-isopropyl group. Finally, the last singlet at δ_H 7.32 is assigned to C-8 as this is the only non substituded position left.

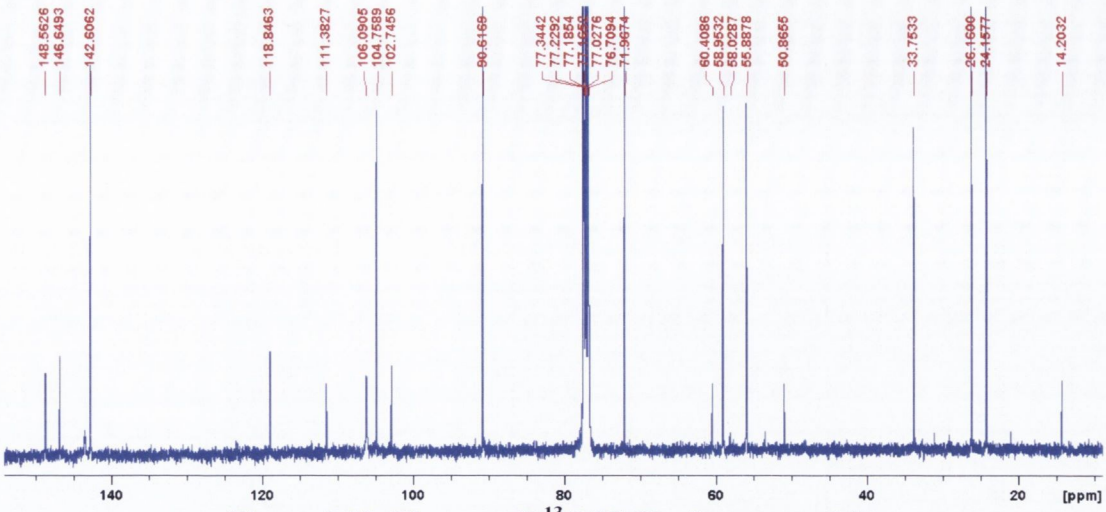


Figure 3.35: The overall ^{13}C NMR of compound 16

In the ^{13}C NMR spectrum (Figure 3.35), in addition to the nine carbon signals of the quinoline skeleton and two methoxyl carbons at δ_C 55.9 and 58.9, there are three carbon signals at δ_C 24.2, 26.6 and 71.9 that correspond to a hydroxy-isopropyl group. The resonance at δ_C 71.9 in the aliphatic region is found to be slightly deshielded, which is explained by the proximity to a more electronegative atom, -O. Three signals that represent methine group at δ_C 90.6, 104.8 and 142.6 correspond to C-12, C-10 and C-9. The signal of C-9 is found to be more downfield because of the effects caused by its position next to an electronegative atom.

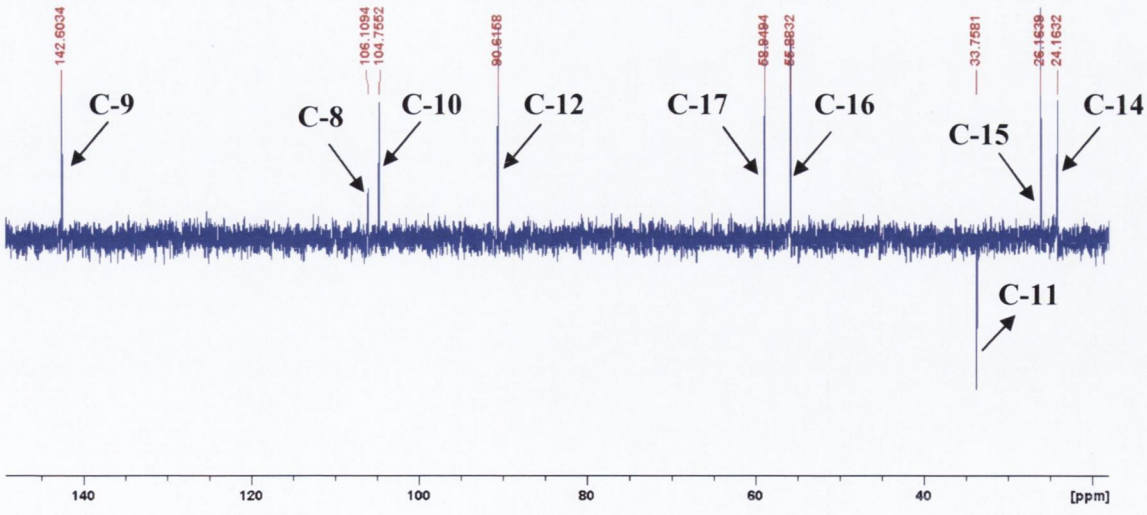


Figure 3.36: DEPT-135 spectrum of compound 16 shows the presence of two methyl, two methoxyl, one methylene and four methine groups.

Based on the DEPT-135 spectrum (Figure 3.36), the presence of methylene protons of aliphatic ring is found at δ_C 33.8 (C-11). Quarternary carbons at δ_C 146.6 (C-4) and 156.9 (C-6) explain their condition that attach to an atom O of the aromatic ring. Besides, the spectrum also clearly shows the presence of two methyl and methoxyl, together with four methine groups.

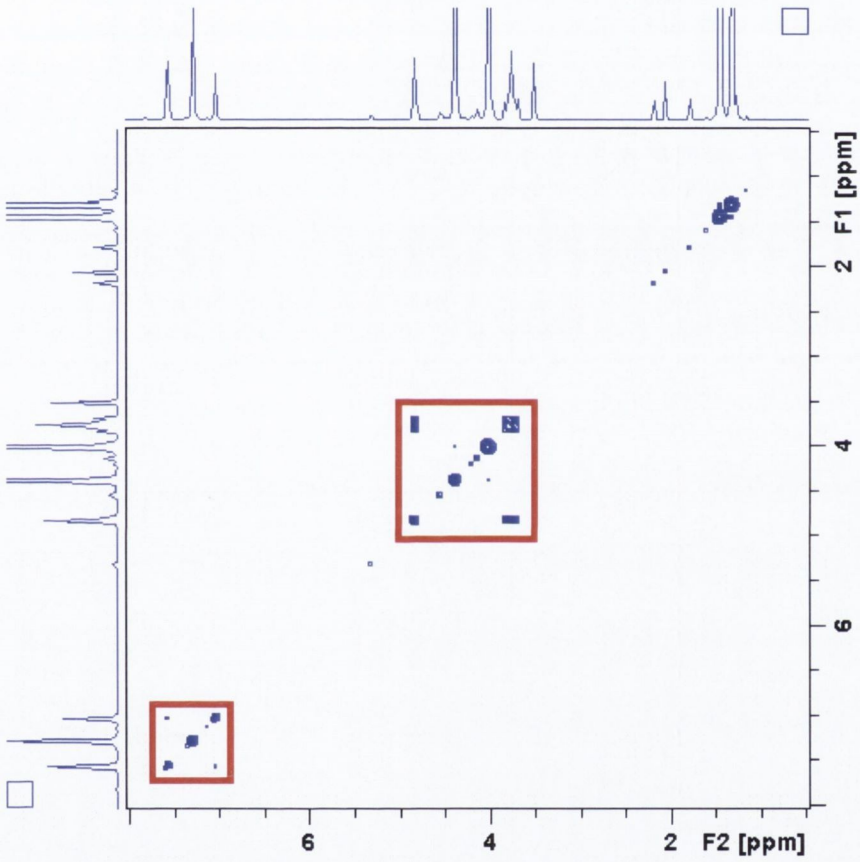


Figure 3.37: ^{1}H-^{1}H COSY spectrum of compound 16 shows strong couplings between H-9 and H-10 of the furan ring and between methylene protons of H-11 and H-12 of the hydroxy-isopropyl group.

The ^{1}H-^{1}H COSY spectrum (Figure 3.37) shows a coupling between the proton resonances at δ_H 7.57 (H-9) and 7.04 (H-10) of the furan ring, and between the methylene proton at δ_H 3.77 (H-11) and 4.84 (H-12) of the hydroxy-isopropyl group.

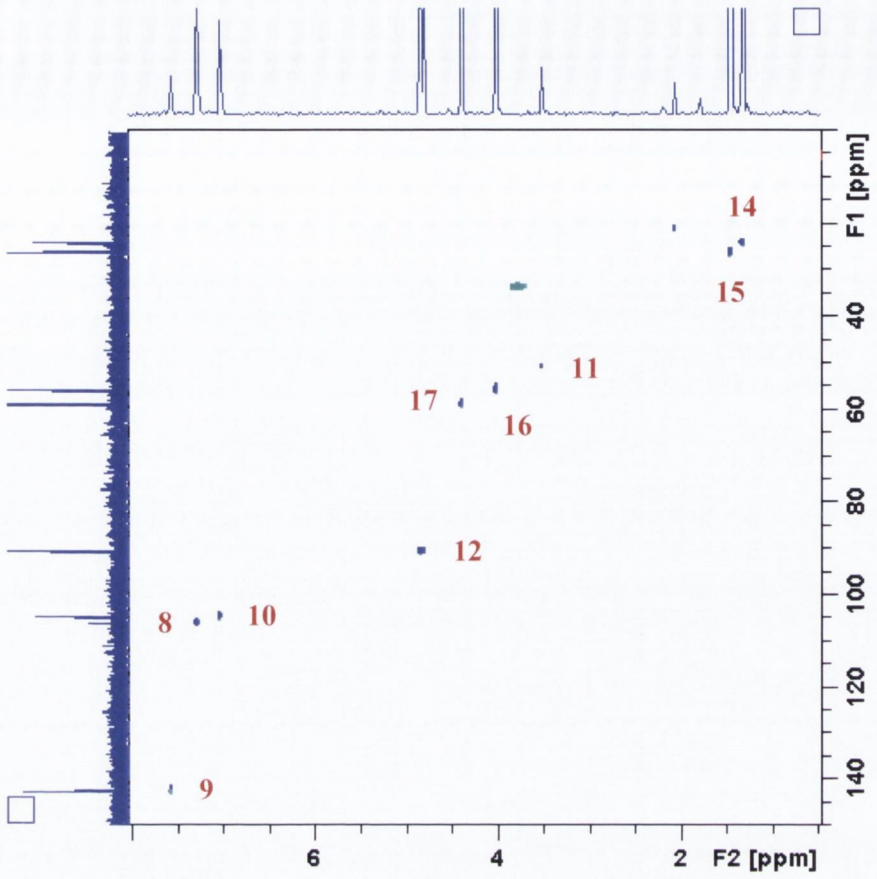


Figure 3.38: HSQC spectrum of compound 16 showing the protonated carbons of the methyl, methoxyl, methylene and methine carbons of both furan ring and the hydroxy-isopropyl group.

HSQC spectrum (Figure 3.38) allows further assignment of all protonated carbons. The two methyl signals at δ_H 1.33 and 1.46 are coupling to carbons at δ_C 24.2 (C-14) and 26.6 (C-15), respectively, while the two methoxyl protons; δ_H 4.02 and 4.39 show correlations with carbons at δ_C 55.9 (C-16) and 58.9 (C-17), respectively. Four methine protons at δ_H 4.84, 7.04, 7.32 and 7.57 correlate to carbons at δ_C 90.6 (C-12), 104.8 (C-10), 106.2 (C-8) and 142.6 (C-9), respectively. The complete NMR spectral data are summarized in Table 3.8 below.

Table 3.8: Comparison of ^1H and ^{13}C NMR data of compound 16 (400 MHz for ^1H-NMR and 100 MHz for ^{13}C- NMR, $CDCl_3$) and choisyine ($CDCl_3$).

	Present study		Literature data	
Position	**^1H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^1H NMR#**	**^{13}C NMR***

1				
2		162.4		163.8
3		102.7		93.6
4		156.9		156.7
4a		111.4		101.7
4b		142.0		138.9
5		118.8		117.6
6		146.6		141.6
7		148.6		141.0
8	7.32 (1H, *s*)	106.2	7.12	106.8
9	7.57 (1H, *d*, *J*=2.3 Hz)	142.6	7.50	142.6
10	7.04 (1H, *d*, *J*=2.3 Hz)	104.8	6.95	104.6
11	3.77 (2H, *m*)	33.8	3.70	33.9
12	4.84 (1H, *t*, *J*=9.6 Hz)	90.6	4.77	90.7
13		71.9	2.50	72.0
14	1.33 (3H, *s*)	24.2	1.29	24.4
15	1.46 (3H, *s*)	26.6	1.42	26.1
16	4.02 (3H, *s*, OCH_3)	55.9	3.91	55.8
17	4.39 (3H, *s*, OCH_3)	58.9	4.29	58.9

[#]Johns *et al.* (1967) and *Brown *et al.* (1980)

These findings strongly suggested the structure of compound 16 to be choisyine (Figure 3.33).

β-sitosterol glucoside (8)

Compound 8 was isolated as a white precipitate (28 mg) with a molecular formula of $C_{35}H_{60}O_6$ as determined from ESI-MS analysis of its pseudo molecular ion 599 $[M+Na]^+$. Comparison of the melting point obtained for β-sitosterol glucoside (m.p. 274-276°C) with the one published (m.p. 272-274°C) (Khatun *et al.*, 2012) and co-cromatography by TLC using β-sitosterol glucoside standard left no doubt that we isolated the same compound. Together with the specteroscopic analyses, these findings supported the assignment of the structure of compound 8 to be β-sitosterol glucoside.

- **Analysis of the ethyl acetate fraction**

Kaempferol-3-O-rutinoside (17)

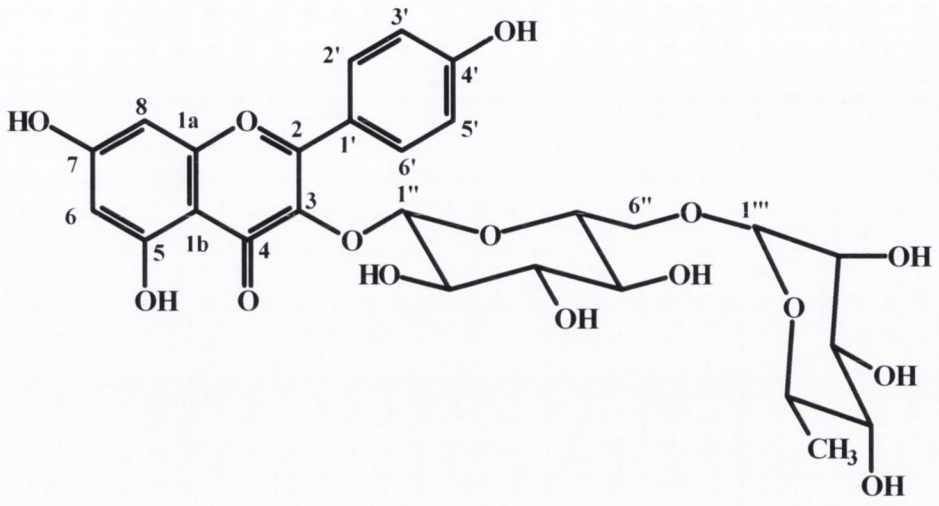


Figure 3.39: Kaempferol-3-*O*-rutinoside (17)

Yellow amorphous powder; R_f: 0.52 (chloroform:methanol, 7:3); mp: 177-179°C; $[\alpha]_D^{20}$ - 0.07 (*c* 0.0020, MeOH); UV (MeOH) λ_{max}: 266, 299, 350; IR ν_{max}: 3400, 1710, 1670, 1510, 1441 cm^{-1}; ESI-MS for $C_{27}H_{30}O_{15}$ pseudo molecular ion, m/z; 617 $[M+Na]^+$; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ_H: 1.00 (3H, *d*, *J*=6.12 Hz, H-6''' rham), 3.05-3.43 (10H, m, remaining rutinosyl protons, except for H-1'''), 3.69 (2H, *d*, *J*=9.7 Hz, H-6'' glu), 4.39 (1H, *d*, *J*=1.8 Hz, H-1''' rham), 5.32 (1H, *d*, *J*=7.44 Hz, H-1'' glu), 6.20 (1H, *d*, *J*=1.5 Hz, H-6), 6.41 (1H, *d*, *J*=1.5 Hz, H-8), 6.91 (1H, *d*, *J*=8.8 Hz, H-3', 5'), 7.99 (1H, *d*, *J*=8.8 Hz, H-2', 6'); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ_C: 18.2 (C-6''' rham), 67.4 (C-6'' glu), 68.7 (C-5'''), 70.4 (C-2'''), 70.8 (C-3'''), 71.1 (C-4''), 72.3 (C-4'''), 74.6 (C-2''), 76.2 (C-5''), 76.8 (C-3''), 94.3 (C-8), 98.0 (C-6), 101.2 (C-1'''), 101.9 (C-1''), 104.2 (C-1b), 115.6 (C-3', 5'), 121.3 (C-1'), 131.3 (C-2', 6'), 133.6 (C-3), 156.9 (C-2), 157.2 (C-1a), 160.4 (C-4'), 161.6 (C-5), 164.0 (C-7), 177.7 (C-4). This data is in agreement to that reported by (Sang *et al.*, 2002, Harput *et al.*, 2004).

Compound 17 was isolated as a yellow amorphous powder (20 mg) with a molecular formula of $C_{27}H_{30}O_{15}$ as determined by ESI-MS analysis of its pseudo molecular ion 617 $[M+Na]^+$. The UV spectrum of 9 has absorption maxima at 266, 299 and 350 nm, suggesting the presence of a flavonoid skeleton (Fathiazad *et al.*, 2006). Furthermore, the range of the wavelength arising from the flavonols suggests that glycosilation occurs at a

hydroxyl group bound to the C-3 (Harput *et al.*, 2004). The flavonoid identity is supported by coumpond 17 TLC plate observed as dark spot against a fluorescent green background when observed in UV light (254 nm) and appeared as dark yellow in 365 nm UV light, which is intensified after spraying with sulphuric acid.

This suggests that this compound could be a flavonoid, possibly kaempferol (Marston *et al.*, 2006). Its IR spectrum showed absorptions at 3400 (hydroxyl group), 1710 (C=O group), 1670 (C=C group), 1510, 1441 (aromatic ring) cm^{-1}.

The spectral data of 17 are comparable to that of rutin as it possessed a similar glycoside moiety structure (more than one sugar) but differs in the aglycon part. The aglycon was identified as the flavonol kaempferol and the high molecular weight of 594 g/mol suggests that this flavonol has more than one sugar moiety, when compared to its aglycon moiety.

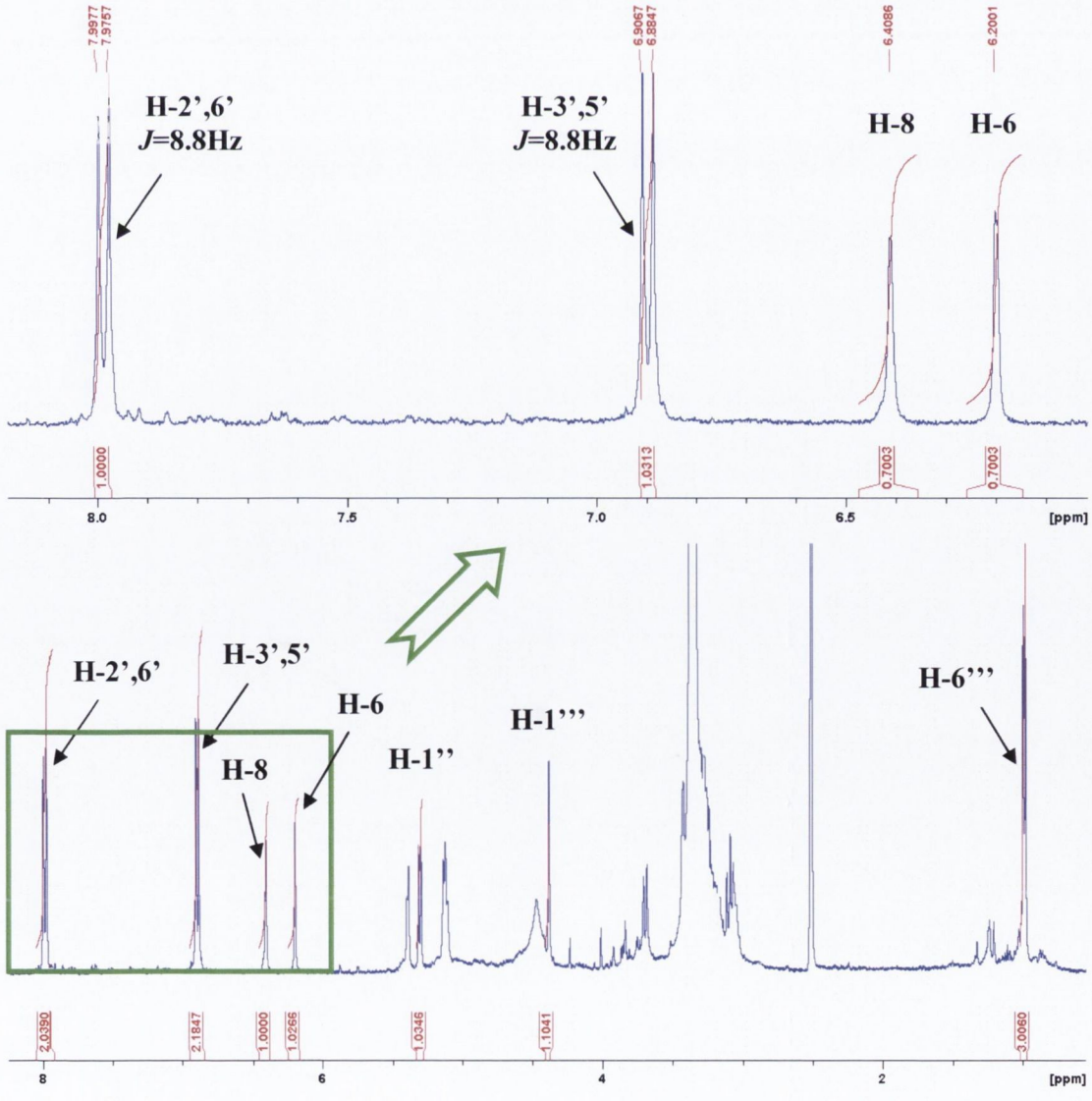


Figure 3.40: Overall of ^{1}H NMR spectrum and an amplification region of a possible AA'BB' coupling in the ring B of a flavonol.

The ^{1}H NMR spectrum (Figure 3.40) shows the important signals of the kaempferol unit by the presence of doublets at δ_H 6.20 (J=1.5 Hz) and 6.41 (J=1.5 Hz), assigned to methine protons on positions six and eight of the ring A, respectively. The signals for aromatic ring B appear at δ_H 7.99 and 6.91 with J=8.8 Hz (pseudo doublets) each, suggesting the AA'BB' (H-2', 6' and H-3', 5', respectively) system in the ring. The chemical shifts and coupling constants for these positions are also characteristic for the flavonol kaempferol (Fossen and Markham, 2006). The presence of the two anomeric proton signals at δ_H 5.32 (H-1'' glu) and 4.39 (H-1''' rham) is indicative of its disaccharidic structure. Considering the chemical shifts and coupling constants, compound 9 is possibly composed of β-□-glucopyranose and α-□-rhamnopyranose units. The secondary methyl signal at δ_H 1.00 supports the presence of a rhamnosyl unit. The multiplets appeared at δ_H 3.05-3.43 are identified as remaining rutinosyl protons.

The ^{13}C NMR spectrum (Figure 3.41) shows 15 carbons of an aglycon moiety in the aromatic region and 12 carbons of the glycoside moiety. The only methyl carbon at δ_C 18.2 is indicative of the methyl group of rhamnose at position six. The anomeric carbon attached to the rutinoside moiety is identified at δ_C 101.9 (C-1''). The most downfield resonances at δ_C 177.7, 164.0, 161.6 and 160.4 are characteristic of a carbonyl group on position four and hydroxyl groups position C-7, C-5 and C-4', respectively.

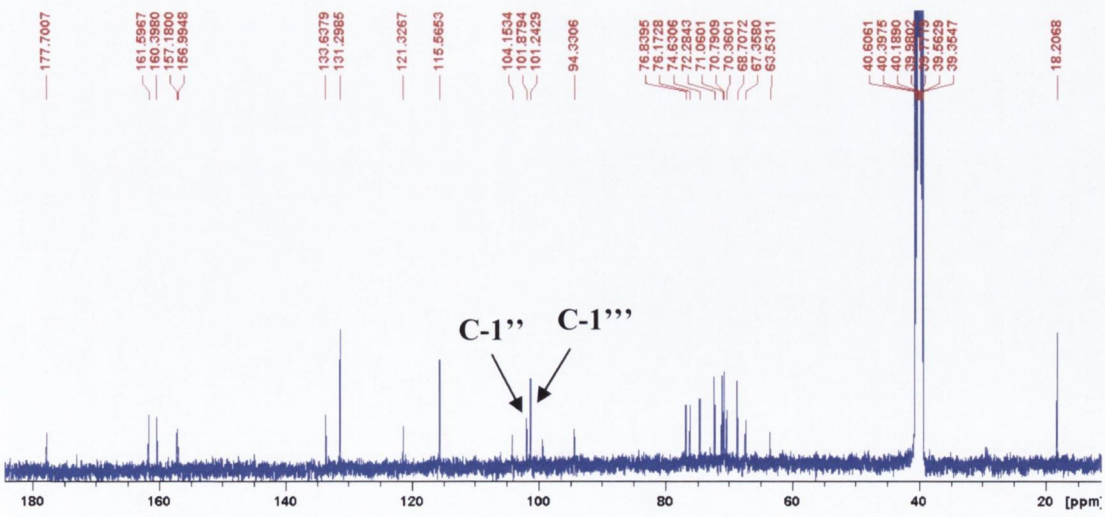


Figure 3.41: ^{13}C NMR spectrum of compound 17 shows the glycoside moiety carbons, as well as the ones of the aromatic ring kaempferol.

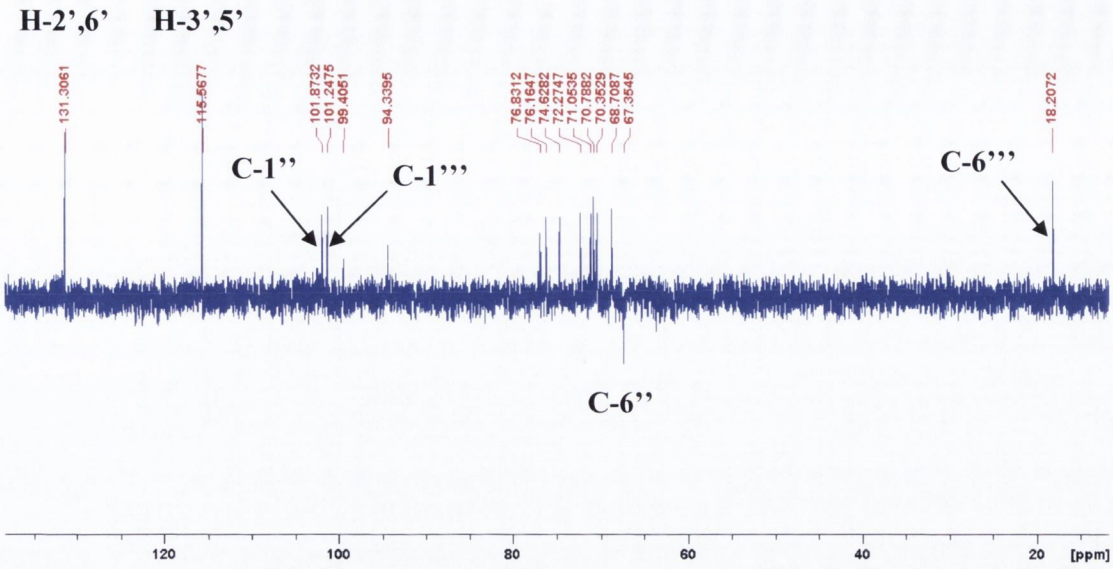


Figure 3.42: DEPT-135 spectrum of compound 17

The DEPT-135 spectrum (Figure 3.42) confirmed the presence of methyl and methylene carbons at δ_C 18.2 (C-6''' rham) and 67.4 (C-6'' glu), respectively. The other sixteen resonances identified belong to methine carbons at δ_C 98.0 (C-6), 94.3 (C-8), 131.3 (C-2'), 115.6 (C-3'), 115.6 (C-5'), 131.3 (C-6'), 101.9 (C-1"), 74.6 (C-2"), 76.8 (C-3"), 71.1 (C-4''), 76.2 (C-5"), 101.2 (C-1'''), 70.4 (C-2'''), 70.8 (C-3'''), 72.3 (C-4''') and 68.7 (C-5''').

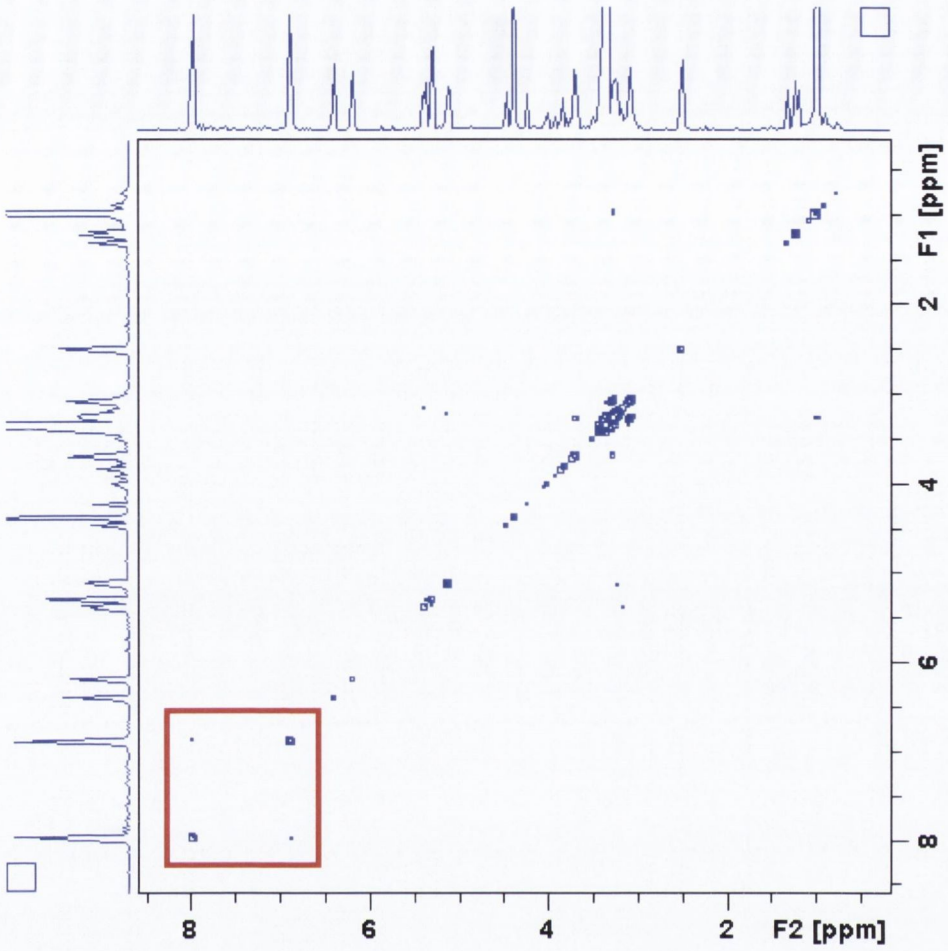


Figure 3.43: ^{1}H-^{1}H COSY spectrum of compound 17 shows the coupling of resonances at δ_H 7.99 and 6.91.

The ^{1}H-^{1}H COSY spectrum (Figure 3.43) of this compound shows the important coupling of a flavonol; a AA'BB' coupling (J=8.8 Hz) (pseudo doublet) system of the ring B characteristic of H-2', 6' and H-3', 5' at δ_H 7.99 and 6.91, respectively.

Furthermore, the HSQC spectrum (Figure 3.44) exhibits correlations of all protonated carbons of the methyl group at δ_H 1.00 / δ_C 18.2, anomeric glucose (δ_H 5.32 / δ_C 101.9) and rhamnose (δ_H 4.39 / δ_C 101.2) moieties, together with protonated carbons of the ring B at δ_H 6.91 / δ_C 115.6 and δ_H 7.99 / δ_C 131.3, and protonated carbons of the ring A at δ_H 6.20 / δ_C 98.0 and δ_H 6.41 / δ_C 94.3.

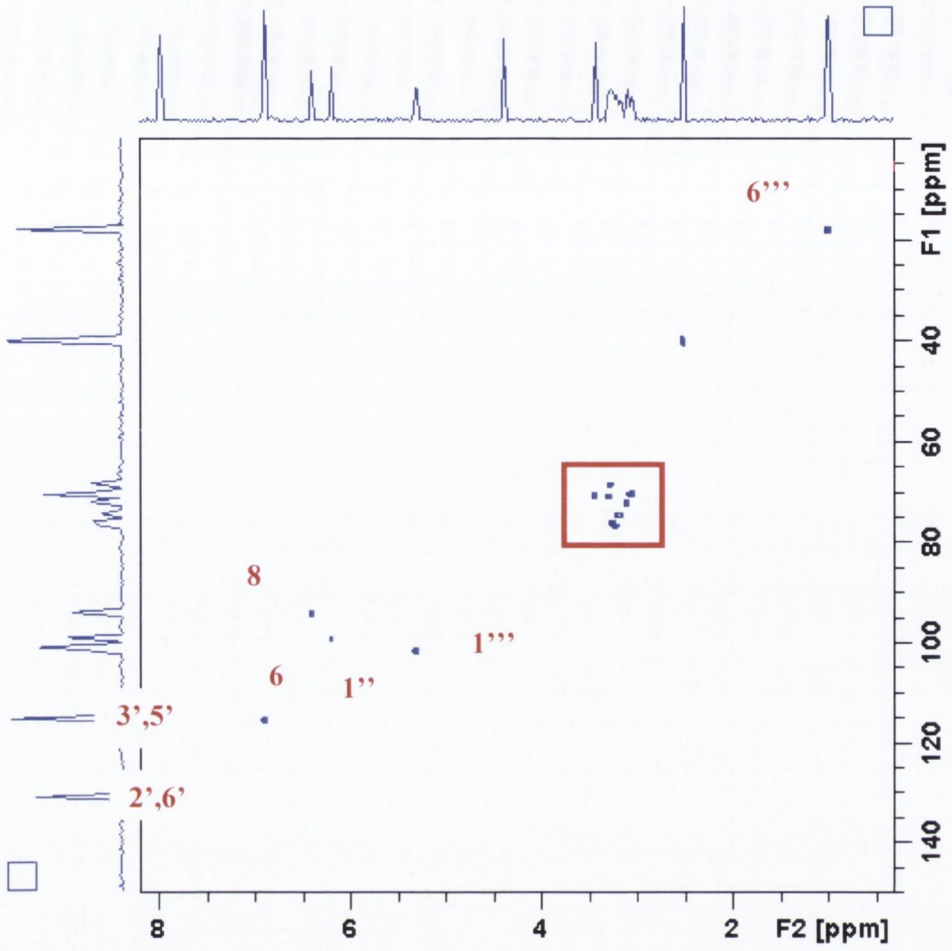


Figure 3.44: HSQC spectrum of compound 17 shows protonated carbons, including the ones from the glycoside moiety.

Downfield shifts of C-6'' in the ^{13}C NMR spectrum and HMBC correlation between H-1''' (δ_H 4.39) and C-6'' (δ_C 67.4) indicate that rhamnose is linked to C-6'' of glucose, confirming the rutinose unit as the sugar moiety (Figure 3.45). The attachment of the rutinose moiety to the aglycone is determined from the correlations between H-1'' (δ_H 5.32) and C-3 (δ_C 133.6) of the aglycone. Besides, there are also correlation between signals at δ_H 7.99 (H-2', 6') with δ_C 156.9 (C-2) and between signals at δ_H 6.91 (H-3', 5') with δ_C 121.3 (C-1'). The complete NMR spectral data were summarized in Table 3.9.

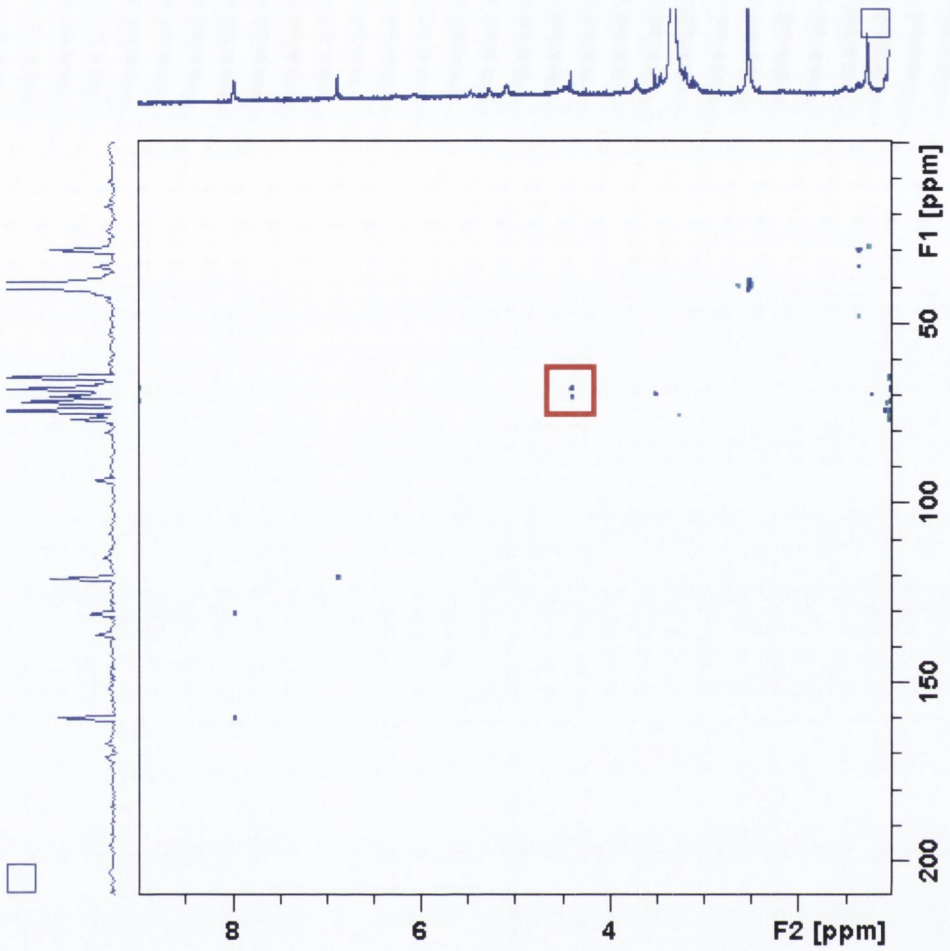


Figure 3.45: HMBC spectrum of compound 17 shows the correlation between signals at δ_H 4.39 (H-1''') and δ_C 67.4 (C-6'').

Table 3.9: Comparison of ^{1}H and ^{13}C NMR data of compound 17 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C- NMR, DMSO-d_6) and kaemperol-3-*O*-rutinoside (CD_3OD).

	Present study		Literature data*	
Position	**^{1}H NMR, δ_H (ppm) (*J* in Hz)**	**^{13}C NMR, δ_C (ppm)**	**^{1}H NMR**	**^{13}C NMR**
1a		157.2		159.5
1b		104.2		105.7
2		156.9		158.6
3		133.6		135.6
4		177.7		179.5
5		161.6		163.0
6	6.20 (1H, *d*, *J*=1.5 Hz)	98.0	6.21	100.2

7		164.0		166.4
8	6.41 (1H, *d*, *J*=1.5 Hz)	94.3	6.40	95.1
1'		121.3		122.9
2'	7.99 (1H, *d*, *J*=8.8 Hz)	131.3	8.07	132.5
3'	6.91 (1H, *d*, *J*=8.8 Hz)	115.6	6.90	116.2
4'		160.4		161.2
5'	6.91 (1H, *d*, *J*=8.8 Hz)	115.6	6.90	116.2
6'	7.99 (1H, *d*, *J*=8.8 Hz)	131.3	8.07	132.5
1''	5.32 (1H, *d*, *J*=7.4 Hz)	101.9	5.15	104.8
2''		74.6		75.9
3''		76.8		78.3
4''		71.1		72.4
5''		76.2		77.3
6''	3.69 (2H, *d*, *J*=9.7 Hz)	67.4	3.54	68.7
1'''	4.39 (1H, *d*, *J*=1.8 Hz)	101.2	4.54	100.4
2'''		70.4		71.6
3'''		70.8		72.2
4'''		72.3		74.0
5'''		68.7		69.8
6'''	1.00 (3H, *d*, *J*=6.1 Hz)	18.2	1.14	18.0

***Sang *et al.* (2002)**

Flavonol *O*-glycosides make up one of the largest classes of flavonoids including kaempferol-*O*-glycoside. Kaempferol could undergo glycosilations at the positions 3,5,7,4' (Valant-Vetschera *et al.*, 2006). Therefore, the most usual position for a sugar attachment in flavonols is at the 3-hydroxyl (Williams, 2006).

The number of possible combinations is enormous and depends on the hydroxylation and methoxylation pattern of the aglycone, the number and nature of sugars and their position of attachment through hydroxyl groups to the aglycone, the nature of the sugar linkage to the aglycone and different interglycosidic linkages, and whether the sugars are in the pyranose or furanose form.

The various flavonoid classes can be recognised by their UV spectra and UV spectral characteristics of individual flavonoids including the effects of the number of aglycone hydroxyl groups, glycosidic substitution pattern and nature of aromatic acyl

groups (Harborne and Williams, 2000, Fossen and Andersen, 2006b, Fossen and Andersen, 2006a). The application of UV has been used widely in analyses of flavonoids as these polyphenolic compounds reveal two characteristic UV absoption bands with maxima in the 240 to 285 and 300 to 550 nm range (Fossen and Andersen, 2006a).

The use of UV shift reagents has proven to be very useful for determining the substitution patterns of hydroxyl groups by methoxyl or *O*-glycosyl in many flavonoids (Fossen and Andersen, 2006b). From the structure, it can be seen that four positions of hydroxyl groups (3, 5, 7, 4') have possibilities to be substituted by sugars. The UV spectral analysis was used to identify which hydroxyl group has been substituted. Figure 3.46 exhibits the UV spectrum of a methanolic solution of kaempferol-3-*O*-rutinoside showing three major absorption bands at 276, 299 and 350, which confirmed the flavonol structure (Fathiazad *et al.*, 2010, Song *et al.*, 2007). Bathochromic shifts with $AlCl_3$ (Figure 3.47, Figure 3.48) and NaOAc (Figure 3.49, Figure 3.50) were related to 5-hydroxyl and 7-hydroxyl functions, respectively, while bathochromic shifts with NaOMe (Figure 3.51) supported the presence of 4'-hydroxyl. This fact indicated that the 3-hydroxyl was the one substituted.

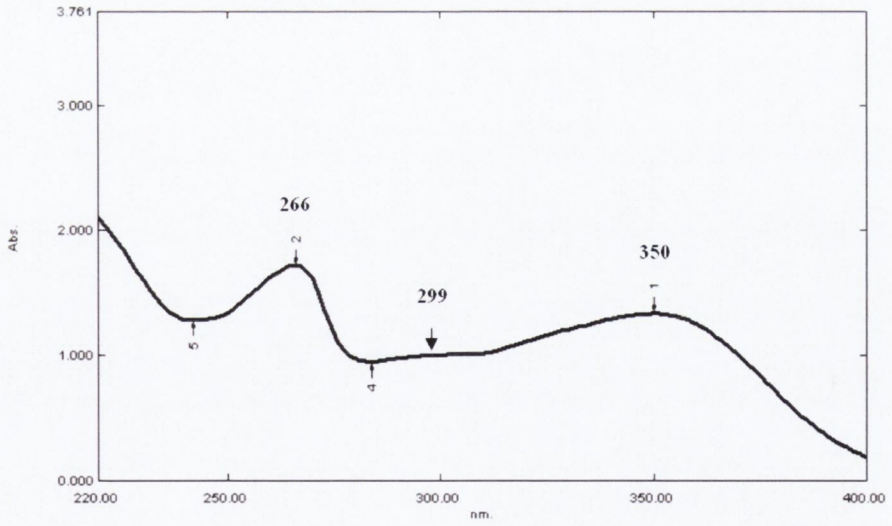


Figure 3.46: The UV spectrum of the methanolic solution of compound 17 exhibits the three major absorption bands corresponding to flavonol structure.

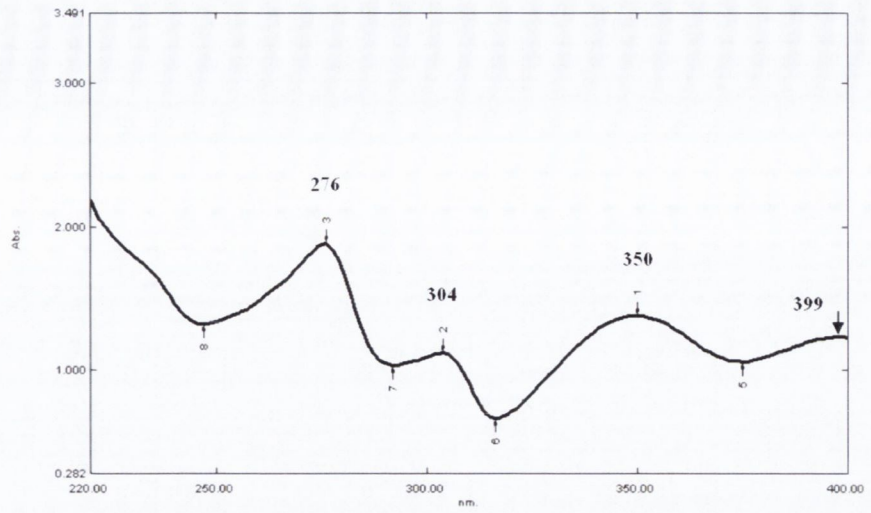


Figure 3.47: The bathochromic shift of compound 17 using $AlCl_3$ shifting reagent.

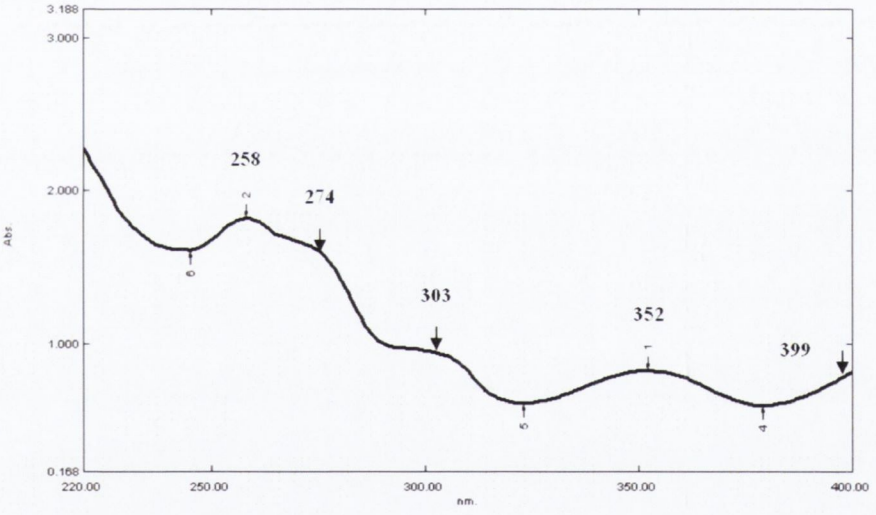


Figure 3.48: The bathochromic shift of compound 17 using $AlCl_3$/HCl shifting reagent.

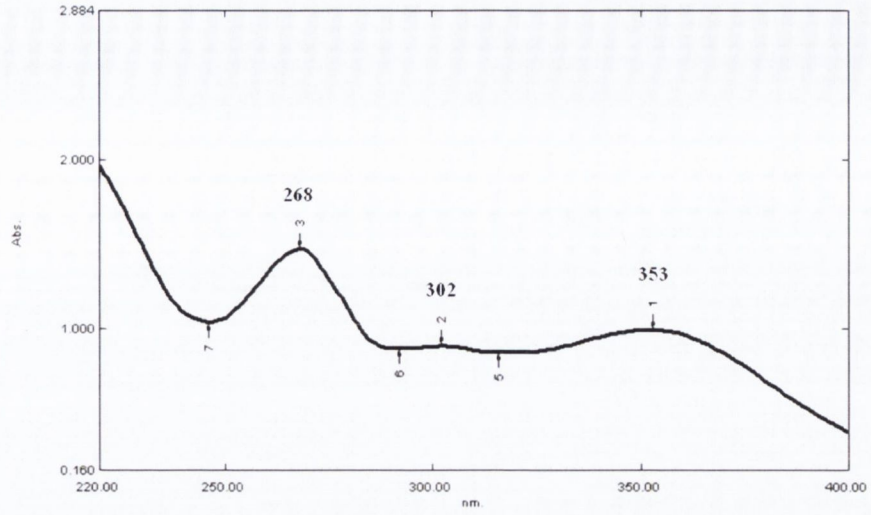


Figure 3.49: The bathochromic shift of compound 17 using NaOAc shifting reagent.

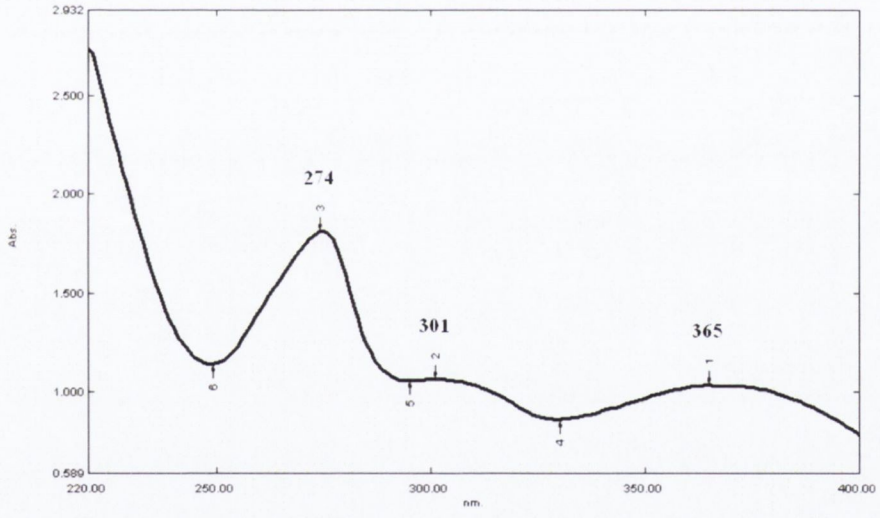


Figure 3.50: The bathochromic shift of compound 17 using NaOAc/H_3BO_3 shifting reagent.

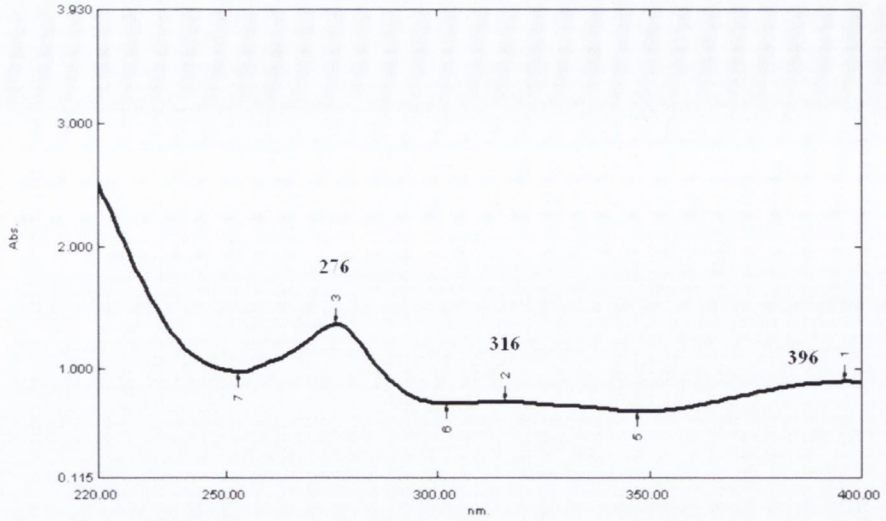


Figure 3.51: The bathochromic shift of compound 17 using NaOMe shifting reagent.

Table 3.10: The UV-visible absoption bands for compound 17 in methanol and their shifts with different reagents.

Shifting reagents					
MeOH	**$AlCl_3$**	**$AlCl_3$/HCl**	**NaOAc**	**NaOAc/H_3BO_3**	**NaOMe**
266	276	258	268	274	276
299	304	303	302	301	316
350	350	352	353	365	396
	399				

Table 3.10 shows absorption bands of compound 17 using different shifting reagents. The shifts showed that the flavonoid is a flavonoid glycoside with free hydroxyl groups at positions 5, 7 and 4' (shifts with $AlCl_3$, NaOAc and NaOMe, respectively). From the above analysis, the hydroxy group at position three of the aglycon was confirmed as being the one substituted with α-□-rhamnopyranosyl-(1→6)-β-□-glucopyranoside. These findings support the assignment of the structure of compound 17 to be kaempferol-3-*O*-rutinoside (Figure 3.39).

The previous phytochemical studies of *C. ternata* showed quinoline alkaloids (skimmianine, choisyine, evoxine, kokusaginine, dictamnine and lunacrine) which derived from the anthranilic acid biosynthetic pathway (Sejourne *et al.*, 1981, Creche *et al.*, 1993, Grundon *et al.*, 1974), together with dihydrofuroquinoline alkaloids (balforodinium and platydesminium, including their salts) and 7-isopentenyloxy-γ-fagarine (Sejourne *et al.*,

1981, Rideau M, 1979, Creche *et al.*, 1987, Boyd *et al.*, 2007). Besides, (Bohlmann, 1972, Gray and Waterman, 1978) isolated coumarins xanthyletin, suberosine and dehydro-osthol were isolated from the roots and whole stems, (Dreyer *et al.*, 1972) isolated triterpenes limonin and friedelin from the leaves extracts and pentacyclic triterpenes were reported as not common in this species while (Tea *et al.*, 1975) isolated flavonoids astragaline and kaempferol-3-*D*-rhamnoglucoside from the leaves and petals extracts.

In this study, we report the occurrence of a new quinoline alkaloid in the Plant Kingdom, choisyaternatine (10), isolated from the hexane fraction of *C. ternata*, together with four compounds isolated for the first time in this species; pentacyclic triterpenes lupeol (12) and lup-20(29)-en-3β,24-diol (13), furoquinoline alkaloid tecleamaniensine A (14) from the hexane fraction and sterol β-sitosterol glucoside (8) from the dichloromethane fraction. Moreover, a quinoline alkaloid skimmianine (11) has also been isolated from the hexane fraction and two known compounds of the species, isolated from the dichloromethane fraction; 6-methoxyl-7,8-methylenedioxycoumarin (15) and choisyine (16), and one from the ethyl acetate fraction, kaempferol-3-*O*-rutinoside (17).

Choisya ternata H.B.K. (Rutaceae), has been placed close to the genus *Platydesma* in the classification of Engler and Prantl (Johns *et al.*, 1967). The family Rutaceae is known to be prolific in the production of alkaloids bearing 3-prenyl-2-quinolones, furo (2,3-b)-, pyrano (2,3-b)- and pyrano (3,2-C-quinoline) systems (Shobana *et al.*, 1989).

According to Waterman (1975), Engler, in his first edition in 1896, proposed seven sub-families for the Rutaceae. Three of them, Rutoideae, Toddalioideae and Aurantioideae, are subject to marked morphological variation; the validity of placing them together in one family seems to be universally accepted (Waterman, 1975). In 1973, Hegnauer has proposed a classification of Rutaceae alkaloids based on their biosynthesis. However, some of the quinolines and quinazolines do not follow the biosynthetic inter-relationships between alkaloids that he classified together. Most alkaloids in this family are formed by the initial condensation of anthranilic acid and a 2-carbon (acetate) unit to give rise to the quinoline ring system, mainly in the form of a 2-quinolone. Simple 2-quinolones have been recorded from four sub-families; Aurantioideae, Rutoideae, Spathelioideae and Toddalioideae, with oxygenation at C-4 and most are *N*-methylated with differentiation occurring by varying substitution on the aromatic ring. In this study, the only quinoline alkaloid isolated from *C. ternata* (sub-tribe Choisyinae, Tribe Ruteae, Sub-family Rutoideae) is choisyaternatine (10) that showed that the oxygenation happens at C-6, with a substitution of *O*-prenyl group at C-7. This pattern of oxidation and prenylation is quite common in *Flindersia* sp. (sub-family Flinersioideae), *Balfourodendron* sp., *Helietta* sp.,

Ptelea sp., *Vepris* sp. and *Teclea* sp. (sub-family Toddalioideae). In addition, three furoquinoline alkaloids were isolated from *C. ternata*; skimmianine (11), tecleamaninesine A (14) and choisyine (16). The substitution in the benzenoid ring happens at C-4.

C-prenylated alkaloids are not common in Rutaceae. Only few reports showed the occurrence of this type of alkaloids. The occurrence of 3-prenylated-2-quinolones identified as preskimmianine was once reported in *Vepris louisii* (Toddalioideae) and 3-dimethylallyl-4-methoxy-2-quinolone was identified in *Fagara xanthoxyloides* (Fish and Waterman, 1973; Eshiett and Taylor, 1968). A prenylated C-5 furoquinoline, tecleaverdoornine was reported in *Teclea verdoorniana* (Toddalioideae) (Dagne *et al.*, 1988) and recently, tecleamaniensine A has been reported occurred in *Teclea amaniensis* (Magadula *et al.*, 2008). The latter was also isolated from *C. ternata* (Rutoideae) in this study. With these findings, the presence of *C*-prenylation could be suggested as a good chemotaxonomic link between Rutoideae and Toddalioideae.

3.3.2 Pharmacological activity of *C. ternata* extract and isolates

3.3.2.1 Antioxidant activity

- **DPPH free radical scavenging activity assay**

Table 3.11 shows the antioxidant activity (%) of each plant extract measured by DPPH at absorbance of 544 nm at various concentrations tested; 250, 125, 50, 25, 10 and 5 µg/ml, while Table 3.12 shows the activity of the positive control *Ginkgo biloba* extract tested using the same protocols as the extracts. The EC_{50} values of radical scavenging activity tested for *C. ternata* extracts obtained from the equation and R^2 of linear regressions at 544 nm ranging between 5.937±1.21 and 414.619±6.52 µg/ml (Table 3.13).

Meanwhile, Table 3.14 and Figure 3.53 show equivalent values (µg/µg DPPH) ranging between 0.087±1.21 and 6.097±6.52. The ethyl acetate fraction possessed the highest antioxidant activity with the equivalent value of 0.087±1.21. Butanol fraction showed the second highest activity, followed by the crude ethanol, dichloromethane and hexane fractions with equivalent values of 0.386±2.12, 1.409±4.92, 2.266±8.79 and 6.097±6.52, respectively. Among all, the ethyl acetate and butanol fractions exhibited significant radical scavenging activity while the crude ethanol extract, hexane and dichloromethane fractions showed no significant activity when compared to the positive control, *G. biloba* extract (EC_{50} value 38.225±0.46, equivalent to 0.562±0.46). Figure 3.52 shows the antioxidant activity of *C. ternata* extracts obtained from equation of linear.

Table 3.11: Antioxidant activity (%) of each plant extract and fractions measured after reaction with DPPH, absorbance measured at 544 nm (values of antioxidant activity±standard deviation of the replicates).

conc.	Antioxidant activity (%) of the extract and fractions measured at 544 nm				
	crude ethanol extract	hexane fraction	dichloromethane fraction	ethyl acetate fraction	butanol fraction
250	76.527±3.44	43.131±2.68	64.878±1.54	98.296±2.11	96.932±2.50
125	50.448±2.00	37.083±4.11	44.324±2.91	97.195±1.06	93.212±3.47
50	44.178±1.73	34.347±1.73	33.945±2.53	92.675±1.59	64.498±3.05
25	38.562±2.38	30.887±2.03	31.893±2.11	85.043±2.35	52.605±2.93
10	34.240±3.93	31.105±2.43	30.332±1.94	53.480±2.55	31.836±2.05
5	37.246±1.44	34.287±1.71	29.910±0.85	41.976±1.89	29.689±3.49

The results are presented as means±S.D., n=3. Statistical significance between groups was calculated by analyses of variance (ANOVA), followed by Bonferroni's test. *P*-values less than 0.05 ($p<0.05$) were used as the significant level compared to the positive control *Ginkgo biloba* extract (Egb 761).

Table 3.12: Antioxidant activity (%) of the positive control, *G. biloba* extract (Egb 761) measured after reaction with DPPH, absorbance measured at 544 nm (values of antioxidant activity±standard deviation of the replicates).

conc.	Antioxidant activity (%) of the positive control *G. biloba* extract (Egb 761) measured at 544 nm			Mean	S.D.
250	95.88	96.28	96.66	96.273	0.39
125	80.63	82.76	81.95	81.780	1.08
50	65.91	67.72	66.57	66.733	0.92
25	50.52	50.95	50.78	50.750	0.22
10	30.72	30.75	30.56	30.677	0.10
5	15.84	15.81	15.68	15.777	0.09

Values were expressed as mean and standard deviation (n=3)

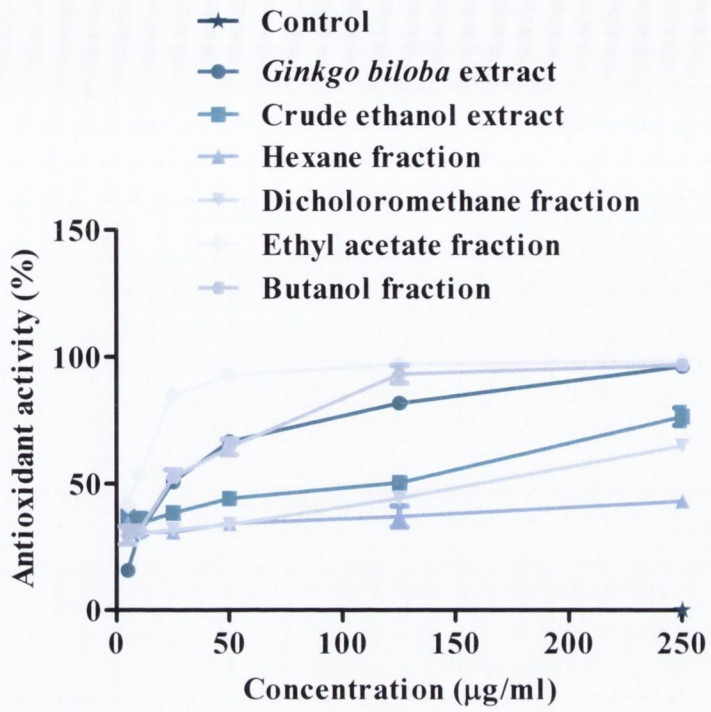


Figure 3.52: Antioxidant activity of *C. ternata* leaves extracts and *G. biloba* extract obtained from equation of linear regressions at 544 nm.

Table 3.13: EC_{50} values of the *C. ternata* extracts and positive control, *G. biloba* extract obtained from the equation of linear regressions at 544 nm.

extracts	EC_{50} values (µg/ml)
G. biloba extract	38.225±0.46
crude ethanol extract	95.790±4.92*
hexane fraction	414.619±6.52*
dichloromethane fraction	154.062±8.79*
ethyl acetate fraction	5.937±1.21
butanol fraction	26.268±2.12

Values were expressed as mean ± standard deviation (n=3). The EC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R^2 \geq 0.90$). $p<0.05$) was used as the significant level compared to the positive control *G. biloba* extract (Egb 761). *$p<0.0001$ significance (no statistical different).

Table 3.14: EC_{50} equivalent of the *C. ternata* extracts and positive control, *G. biloba* extract in relation to DPPH (µg/µg DPPH) with DPPH being used at 68 µg/ml.

Extract and fractions	EC_{50} equivalent (µg/µg DPPH) (544 nm)
G. biloba extract	0.562±0.46
crude ethanol extract	1.409±4.92*
hexane fraction	6.097±6.52*
dichloromethane fraction	2.266±8.79*
ethyl acetate fraction	0.087±1.21
butanol fraction	0.386±2.12

Values were expressed as mean ± standard deviation (n=3). The EC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R2 \geq 0.90$). $p<0.05$) was used as the significant level compared to the positive control *G. biloba* extract (Egb 761). *$p<0.0001$ significance (no statistical different).

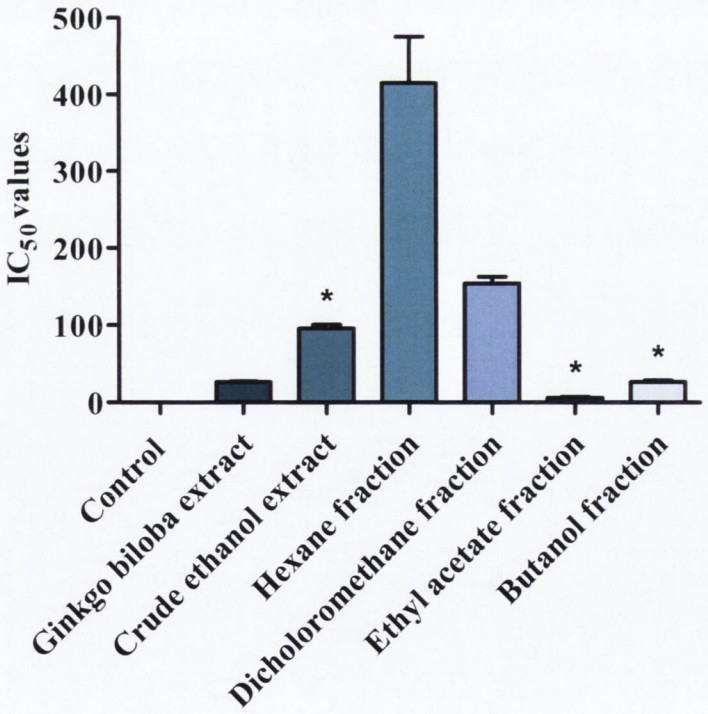


Figure 3.53: EC_{50} values of the crude ethanol extract, hexane, dichloromethane, ethyl acetate and butanol fractions of *C. ternata* in the DPPH radical scavenging activity assay. The results are presented as means±S.D., n=3, $p<0.05$ significantly different

compared to the positive control *G.. biloba* extract (Egb 761). *p<0.0001 significance (no statistical different).

Using this method, it can be seen that the ethyl acetate fraction showed the highest activity at 0.087±1.21 (µg/µg DPPH), At the highest concentration of extract (250 µg/ml), the scavenging activity was very high at 98.296±2.11%, followed by the butanol (96.932±2.50%), crude ethanol (76.932±3.44%) and dichloromethane (64.878±1.54%) fractions. However, at the highest concentration of extract (250 µg/ml), the scavenging activity of the hexane fraction was quite low 43.131±2.68

A previous antioxidant study using the DPPH assay showed that kaempferol-3-*O*-rutinoside isolated from the crude ethanol extract of *Cassia auriculata* exhibited a moderate antioxidant activity - EC_{50} value of 28.5±1.2 (Juan-Badaturuge *et al.*, 2011). In this study, the activity showed by the extract containing this compound (EC_{50} value of 5.937±1.21) was higher than the one found by Juan-Badaturuge in his study using another plant that also sources the same compound. This could be explained by the synergistic activity with other compounds in our butanol extract. Previous studies demonstrated that the interaction of a potential antioxidant with the DPPH depends on its structural conformation, among other factors. The number of the DPPH molecule that are reduced seems to be correlated with the number of available hydrogen group (Brand-Williams *et al.*, 1995). It is strongly suggested that the DPPH free radical abstracts the phenolic hydrogen of the electron donating molecule and this could be the general mechanism of the scavenging action of antiperoxidative flavonols (Ratty and Das, 1988).

- **Total phenolic content assay**

The total phenolic content of the extracts was determined using the gallic acid as a standard and had the callibration curve plotted (Figure 3.54), using the absorbance values obtained with its solutions from 5 to 500 µg/ml (Table 3.15).

Table 3.15: Absorbance values (595 nm) for gallic acid (standard) at various concentrations.

Absorbance values of gallic acid	
Gallic acid concentration (µg/ml)	**Abs. 595 nm**
5	0.060±0.020

10	0.063±0.004
25	0.072±0.004
50	0.081±0.004
125	0.108±0.004
250	0.158±0.005
500	0.237±0.005

Values were expressed as mean ± standard deviation (n=3)

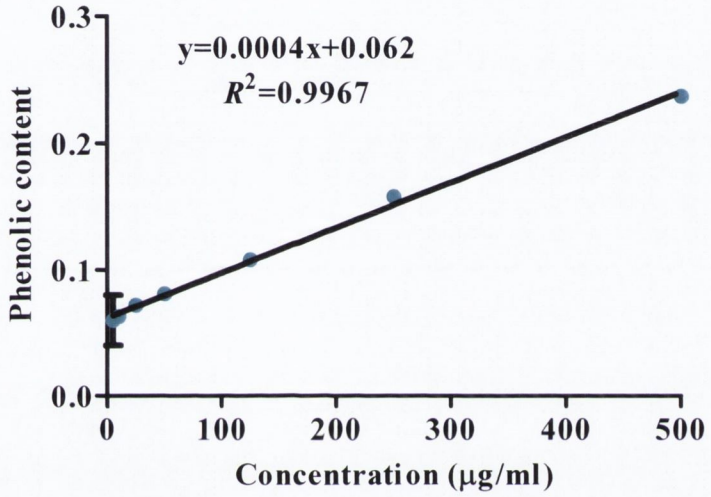


Figure 3.54: The callibration curve of gallic acid used as a standard reference in the total phenolic content assay.

Table 3.16: Total phenolic content (TPC) of the extract and fractions of *C. ternata*.

Extract and fractions	TPC±S.D. (µg GAEs/mg extract)
crude ethanol extract	260.818±0.007
hexane fraction	269.734±0.007
dichloromethane fraction	307.901±0.007
ethyl acetate fraction	318.401±0.007
butanol fraction	290.484±0.007

Values were expressed as mean ± standard deviation (n=3), calculated based on the equation of the gallic acid callibration curve

Table 3.16 shows the total phenolic content of each extract of *C. ternata.* The ethyl acetate fraction of *C. ternata* showed the highest total phenolic content among its fractions with 318.401±0.007 µg GAEs/mg, followed by the dichloromethane, butanol, hexane and crude ethanol extract, respectively. Phenolic compounds such as flavonoids are considered to be major contributors to the antioxidant capacity of plants (Gursoy *et al.*, 2009). Hence, the high phenolic content in the ethyl acetate extract might be due to this type of compound.

- **Flavonoid equivalent assay**

The determination of the flavonoid equivalent in the extract and fractions were performed using quercetin as a standard and had its calibration curve plotted (Figure 3.55), based on the absorbance values obtained with its solutions from 5 to 500 µg/ml (Table 3.17).

Table 3.17: Absorbance values (405 nm) of quercetin (standard) at various concentrations.

Absorbance values of quercetin	
Quercetin concentration (µg/ml)	**Abs. 405 nm**
5	0.204±0.005
10	0.260±0.004
25	0.371±0.005
50	0.627±0.008
125	1.280±0.004
250	2.434±0.005
500	3.994±0.011

Values were expressed as mean ± standard deviation (n=3)

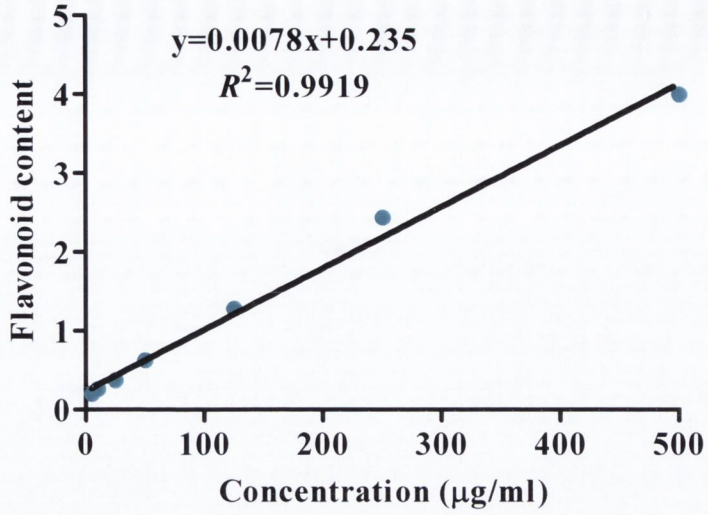


Figure 3.55: The callibration curve of quercetin used as a standard reference in the flavonoid equivalent assay.

Table 3.18: Flavonoid equivalents (FE) of the extract and fractions using the equation of the callibration curve.

Extract and fractions	FE±S.D. (µg QEs/mg extract)
crude ethanol extract	30.635±0.002
hexane fraction	33.339±0.002
dichloromethane fraction	26.292±0.002
ethyl acetate fraction	70.181±0.002
butanol fraction	31.501±0.002

Values were expressed as mean ± standard deviation (n=3), calculated based on the equation of the quercetin callibration.

As it can be seen from Table 3.18, the method used to determine the total flavonoid content was based on the equation of the callibration curve of quercetin. Briefly, the ethyl acetate fraction of *C. ternata* showed the highest flavonoid equivalent amongst its extracts with 70.181±0.002 µg QEs/mg, followed by the hexane, butanol, crude ethanol and dichloromethane fractions, respectively. As mentioned above, a flavonol was isolated from this extract. The high flavonoid content of the ethyl acetate fraction supported its high activity observed in the DPPH assay.

- **Correlation analysis between the DPPH antioxidant activity, the total phenolic content and flavonoid equivalent**

From the analysis of Table 3.19 and Figure 3.56, it can be seen that there is a good correlation between these studied parameters (total phenolic content, flavonoid equivalent and DPPH scavenging activity) for *C. ternata* extracts.

Table 3.19: Correlation between studied parameters DPPH antioxidant activity with the total phenolic and flavonoid equivalent of the extract and fractions.

Extract and fractions	Absorbance (µg/µg DPPH) 544nm	TPC±S.D. (µg GAEs/mg extract)	FE±S.D. (µg QEs/mg extract)
crude ethanol extract	1.409±4.92*	260.818±0.007	30.635±0.002
hexane fraction	6.097±6.52*	269.734±0.007	33.339±0.002
dichloromethane fraction	2.266±8.79*	307.901±0.007	26.292±0.002
ethyl acetate fraction	0.087±1.21	318.401±0.007	70.181±0.002
butanol fraction	0.386±2.12	290.484±0.007	31.501±0.002

Values were expressed as mean ± standard deviation (n=3). The EC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($R^2 \geq 0.90$). $p<0.05$) were used as the significant level compared to the positive control *G. biloba* extract (Egb 761). *$p<0.0001$ significance (no statistical different).

C. ternata leaves extracts possessed not only a very high antioxidant activity in the DPPH radical scavenging assay, but also a high content of total phenolics and flavonoids. Among all of its extracts, its ethyl acetate fraction showed a very good correlation between the three assays which exhibited not only the highest activity in the DPPH assay at 0.087±1.21, but also the highest content of the total phenolics and flavonoid equivalent with 318.401±0.007 (µg GAEs/mg extract) and 70.181±0.002 (µg QEs/mg extract), respectively.

As described by Bors *et al.* (1990), there are three structural features that are important determinants for the antioxidant potential of flavonoids; the ortho 3',4'-dihydroxy structure in the B ring, the 2,3-double bond in conjuction with the 4-oxo group in the C ring or electron delocalization and the presence of a 3-OH group in C ring and 5-OH group in the A ring. Among them, the 3-OH group is the most significant determinant of electron-donating activity.

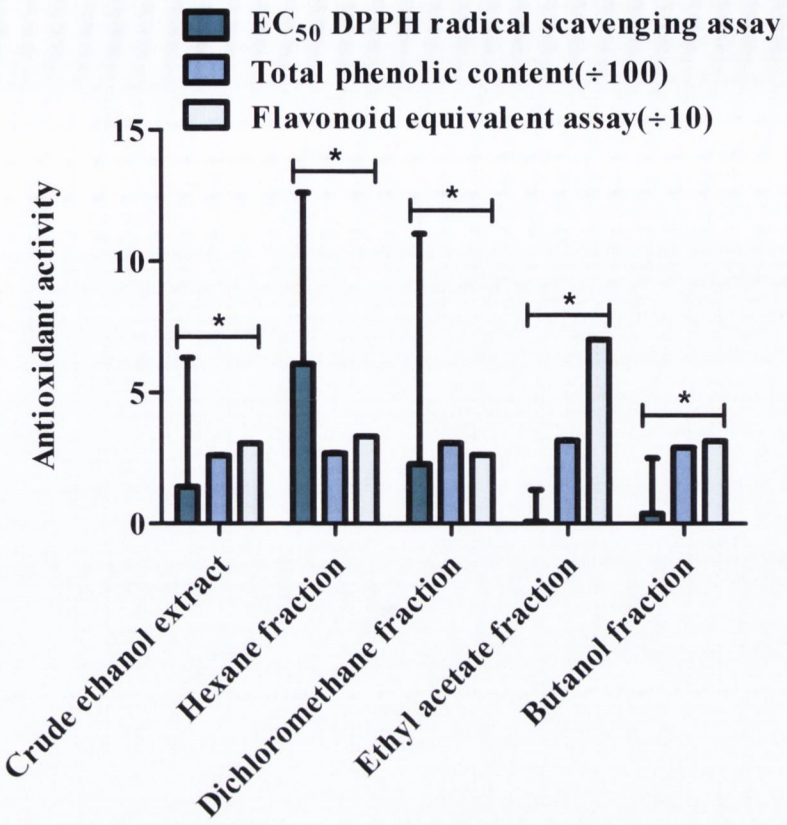


Figure 3.56: Correlation analysis between the DPPH radical scavenging activity with the total phenolic content (÷100) and flavonoid equivalent (÷10) of the extract and fractions. Values were expressed as mean ± standard deviation (n=3). Statistical significance between groups was calculated by two way analyses of variance (ANOVA). $p<0.05$ significantly different in all interaction between the three antioxidant assays used and the extracts. *$p<0.0001$ significance (no statistical different).

3.3.2.2 Human platelet activity

The hexane fraction, from where the alkaloids came from (except for compound 16 isolated from the dichloromethane fraction), and compounds 10, 11, 12, 14 and 16 were assayed for antiplatelet activity. The percentage of inhibition of collagen-induced platelet aggregation at various concentrations for the hexane fraction, choisyaternatine (10), skimmianine (11), lupeol (12), tecleamaniensine A (14) and choisyine (16) are shown in Figure 3.59.

First we tested the ability of the hexane fraction to inhibit platelet aggregation. As shown in Figure 3.57, the extract was able to significantly inhibit collagen-induced platelet aggregation in a concentration-dependent manner. Therefore, the antiplatelet activity of the

alkaloids isolated from the hexane fraction (10,11,12,14) and an alkaloid isolated from the dichloromethane fraction (16), was subsequently investigated.

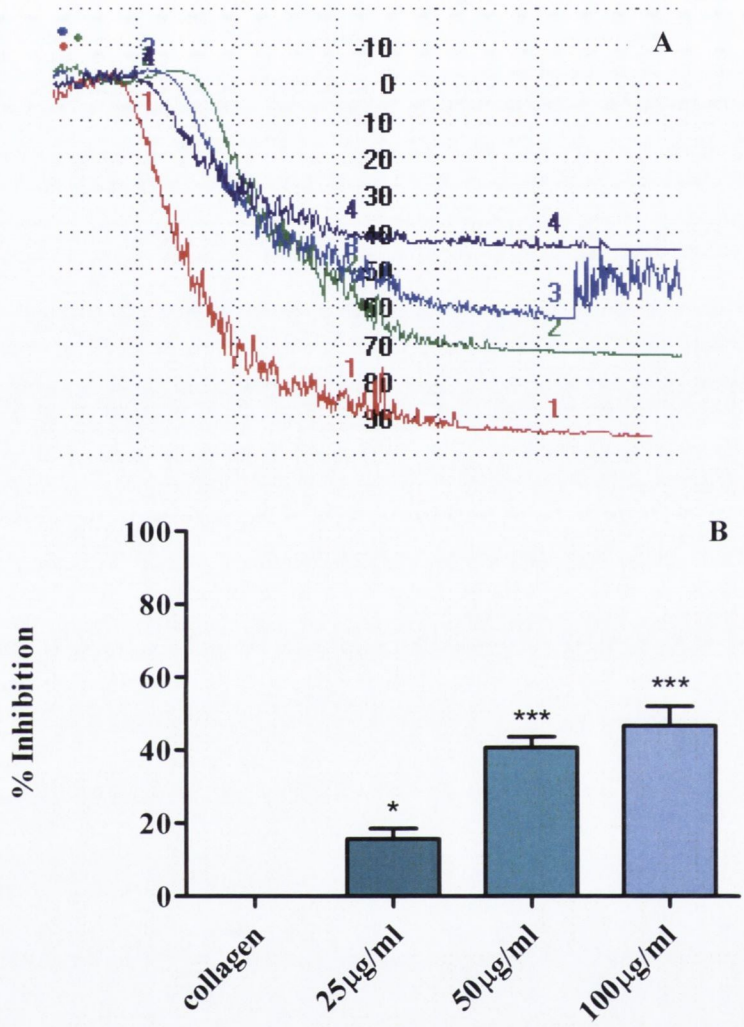


Figure 3.57: Effects of the hexane fraction on platelet aggregation.
A) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + hexane fraction 25 µg/ml; 3) collagen + hexane fraction 50 µg/ml and 4) collagen + hexane fraction 100 µg/ml.
B) Quantitative analysis of the inhibitory effect on collagen-induced platelet aggregation of the hexane fraction with all concentrations tested. Data are expressed as mean ± S.D. *p<0.05 vs collagen; ***p<0.001 vs collagen.

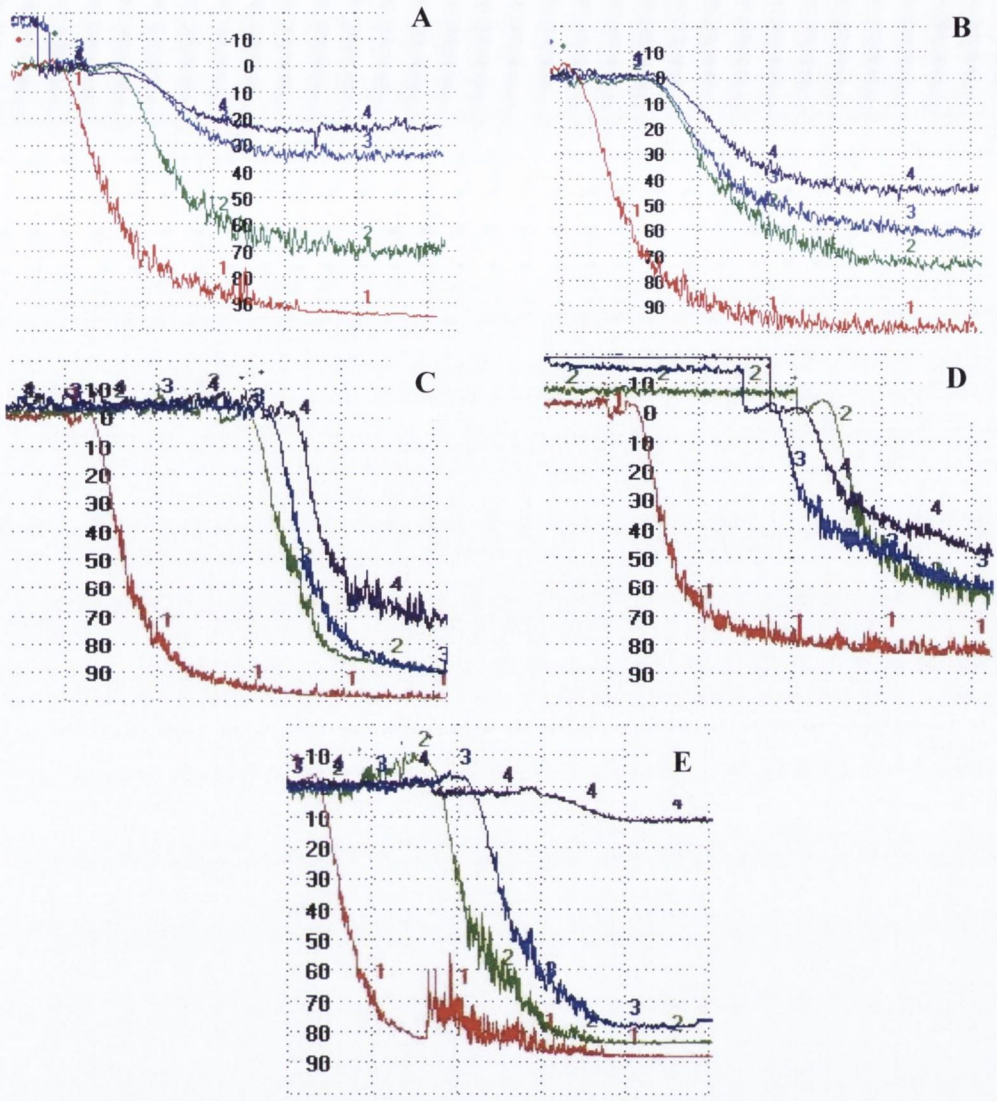


Figure 3.58: Effects of choisyaternatine (10), skimmianine (11), lupeol (12), tecleamaniensine A (14) and choisyine (16) on platelet aggregation.

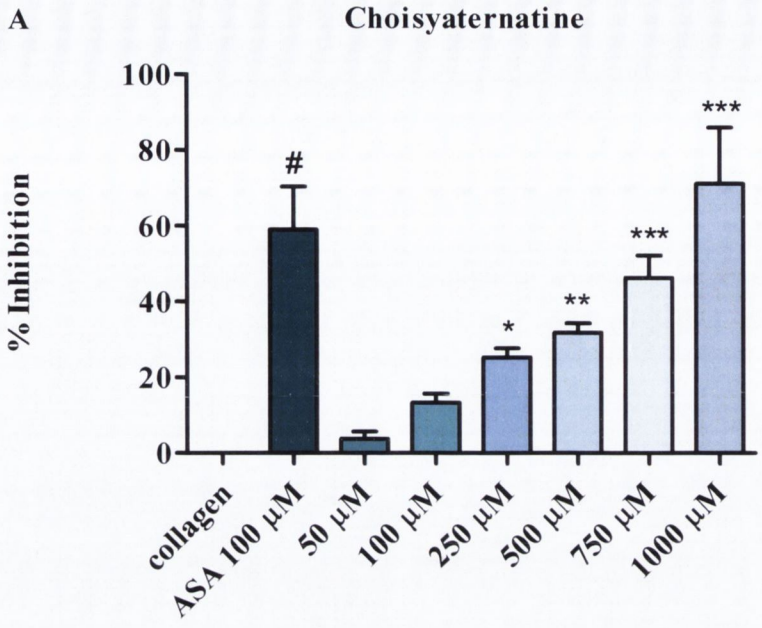
A) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + choisyaternatine 250 µM; 3) collagen + choisyaternatine 500 µM and 4) collagen + choisyaternatine 750µM.

B) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + skimmianine 250 µM; 3) collagen + skimmianine 500 µM and 4) collagen + skimmianine 750µM.

C) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + lupeol 250 µM; 3) collagen + lupeol 500 µM and 4) collagen + lupeol 750µM.

D) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + tecleamaniensine A 250 µM; 3) collagen + tecleamaniensine A 500 µM and 4) collagen + tecleamaniensine A 750µM.

E) Representative traces from aggregometry studies in human platelet rich plasma (PRP): 1) collagen; 2) collagen + choisyine 250 µM; 3) collagen + choisyine 500 µM and 4) collagen + choisyine 750µM.


A
Choisyaternatine
% Inhibition
100
80
60
40
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collagen
ASA 100 µM
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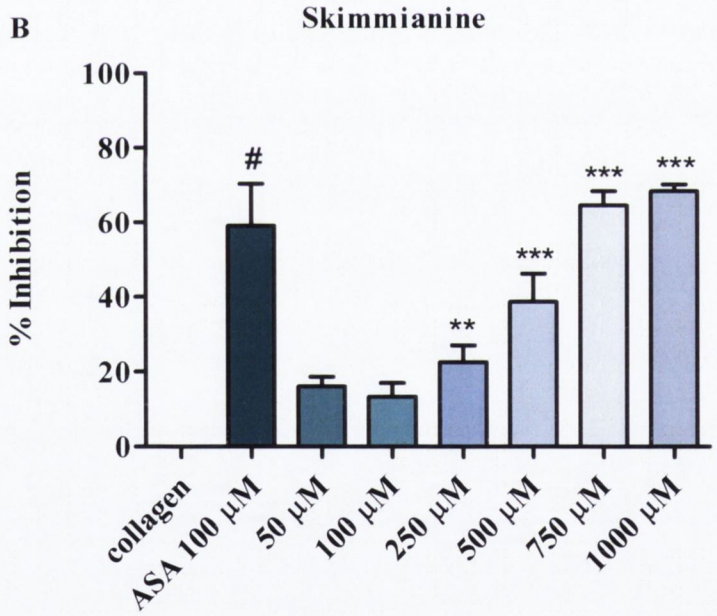
500 µM
750 µM
1000 µM


B
Skimmianine
% Inhibition
100
80
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collagen
ASA 100 µM
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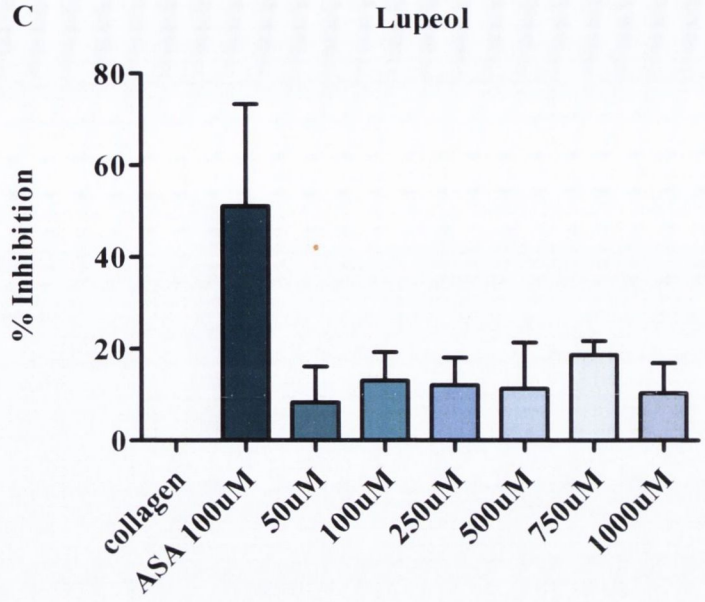
Lupeol
% Inhibition
80
60
40
20
0
collagen
ASA 100uM
50uM
100uM
250uM
500uM
750uM
1000uM

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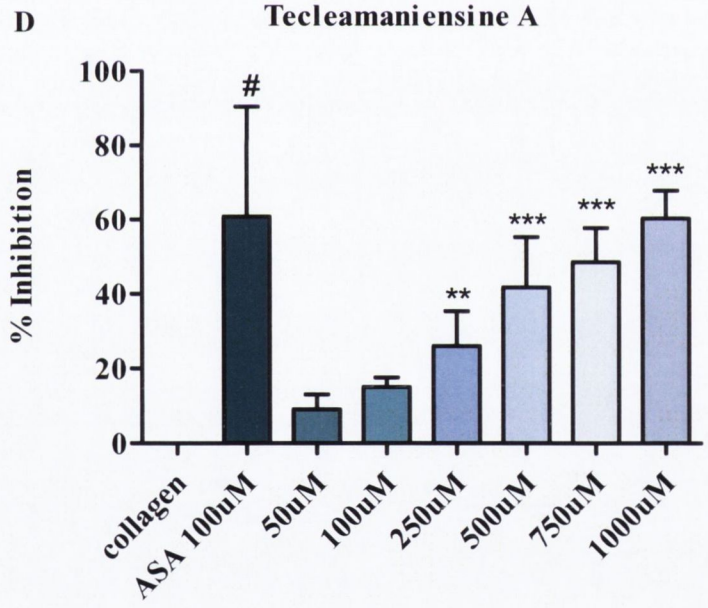
Tecleamaniensine A
% Inhibition
100
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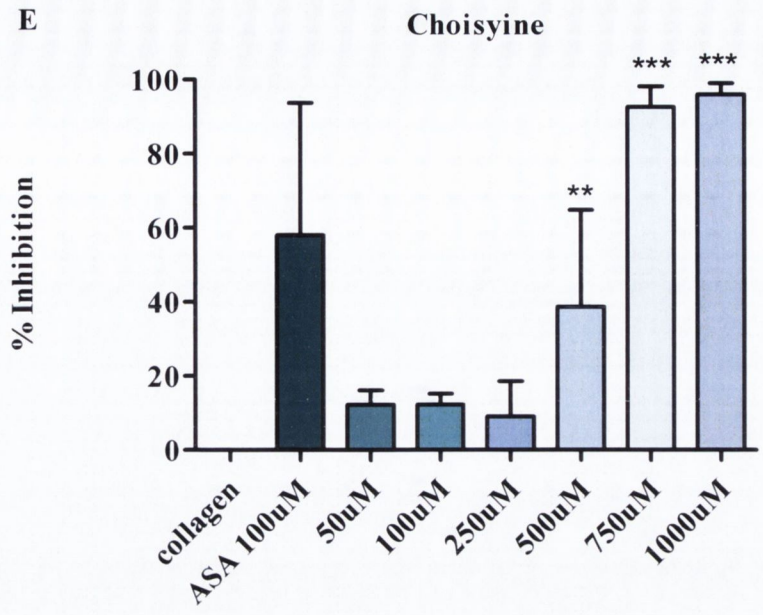

Figure 3.59: Quantitative analysis of the inhibitory effect on collagen-induced platelet aggregation of (A) choisyaternatine, (B) skimmianine, (C) lupeol, (D) tecleamananiensine A and (E) choisyine. Data are expressed as mean ± S.D. *$p<0.05$ vs collagen; **$p<0.01$; ***$p<0.001$ vs collagen. Acetylsalicylic acid (ASA) is included as internal control (# $p<0.05$ vs collagen).

Among all the isolated alkaloids reported in this study, only skimmianine has been previously reported as the one that inhibited platelet aggregation. Skimmianine at 100 µg/ml (equivalent to 386 µM) inhibited collagen-induced platelet aggregation (around 66%) in washed rabbit platelets (Chen *et al.*, 2000). Here we show, for the first time, that this compound is also able to inhibit collagen-induced platelet aggregation in human platelets, together with choisyaternatine (10), lupeol (12), tecleamaniensine A (14) and choisyine (16) (

Figure 3.58 A-E). The new compound choisyaternatine, as well as tecleamaniensine A, showed a significant inhibition of collagen-induced platelet aggregation in a concentration dependent manner from 50-1000 µM. The concentrations which inhibit 50% of platelet aggregation (IC_{50}) were found to be 698, 564 and 719 µM for choisyaternatine, skimmianine and tecleamaniensine A, respectively. The IC_{50} for the hexane extract was much lower than these four compounds, at 50-100 µg/ml, while no significant inhibition showed by triterpene lupeol (Figure 3.59 A-D).

Knowing that the synergism of the compounds in the extract could contribute to its higher activity, a mixture of choisyaternatine (700 µM), skimmianine (560 µM), lupeol (>1000 µM) and tecleamaniensine A (719 µM) was tested using the same procedure. The

results were able to show that the mixture of these four compounds had a higher percentage of platelet inhibition when compared to their individual activities at the same concentration (Figure 3.60). Furthermore, the effect showed by the mixture is higher that the hexane fraction, with the percentage of inhibition for the mixture was 99% compared to only 50% for the extract. This could be possibility due to the interaction with other compounds present in the hexane extract, together with the four tested compounds.

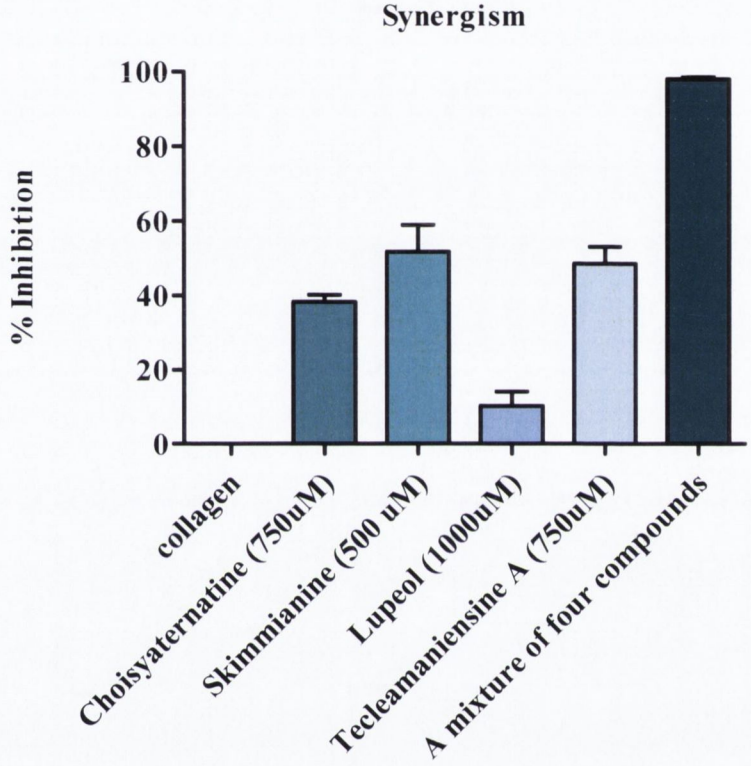

Figure 3.60: Quantitative analysis of the inhibitory effects on collagen-induced platelet aggregation of a mixture of choisyaternatine (10), skimmianine (11), lupeol (12) and tecleamaniensine A (14). Data are expressed as mean ± S.D. *p<0.05 vs collagen; **p<0.01; ***p<0.001 vs collagen. Acetylsalicylic acid (ASA) is included as internal control (# p<0.05 vs collagen). Control consists in 2 µL DMSO + 2 µg/ml collagen.

In addition, choisyine (16), the alkaloid isolated from the dichloromethane fraction (other than skimmianine) showed the highest percentage of platelet inhibition with the IC_{50} value of 533 µM (Figure 3.59 E). The result indicates that the dichloromethane fraction could possibly possess a higher activity as both skimmianine (11) and choisyine (16) were the main compound in this fraction. It is worth to compare the platelet aggregation activity shown by the hexane fraction with other fractions of the same plant.

Isolated compounds from *Ruta graveolens* (Rutaceae), mainly furoquinoline alkaloid skimmianine and dictamnine showed inhibitory activity of platelet aggregation induced by collegen (10 µg/ml). At the highest concentration of 100 µg/ml, skimmianine and dictamnine inhibited the platelet aggregation at 45.9±17.4 and 84.4±6.2, respectively. The higher antiplatelet activity was shown by the quinoline alkaloid graveolinine (100 µg/ml) with 72.6% of inhibition of collagen-induced platelet aggregation (Wu *et al.*, 2003).

Lupeol, isolated from *Garcinia hombroniana*, showed a moderate inhibition of whole human blood platelet-induced by collagen (2 µg/ml) with 59.0±3.3% at 100 µg/ml (Saputri and Jantan, 2012), while the one that was isolated from the root of *Petasites formosanus* showed a weak inhibition against washed rabbit platelets induced by collagen (10 µg/ml) with only 3.1±2.1% at 20 µg/ml (Wu *et al.*, 1999). In this study, at the highest concentration of 1000 µM, percentage of inhibition of lupeol was only 10% (collagen 2 µg/ml) which could be due to its poor solubility in DMSO.

α- and β-amyrin, pentacyclic triterpenes, isolated from *Ardisia elliptica* were found to be more potent than aspirin (positive control) in inhibiting collagen-induced platelet aggregation. The IC_{50} value of β-amyrin was found to be 5 µg/ml (10.5 µM) while that aspirin was found to be only 11 µg/ml (62.7 µM). The result showed β-amyrin to be six times more active than the aspirin in inhibiting platelet using rabbit blood. Furthermore, a standard of α-amyrin showed to be three times more active than the aspirin at IC_{50} value of 9.1 µg/ml (21.3 µM). A mixture of α- and β-amyrin obtained from *Protium heptaphyllum* exhibited collagen-induced platelet aggregation with IC_{50} value of 38.4 µg/ml, which was higher than the one found in *Ardisia elliptica* (Ching *et al.*, 2010). In another study of *Protium heptaphyllum*, a mixture of α- and β-amyrin inhibited human platelet aggregation, induced by collagen (10 µg/ml) with the IC_{50} value of 90.0 µM (Aragao *et al.*, 2007).

The discrepancy found between the previous and the present study may be due to the different samples and experimental conditions (concentration of collagen, incubation time *etc.*) For example, the previous studies by Chen *et al.* (2000) used 10 µg/ml of collagen and the one by Ching *et al.* (2010) as well (data not shown in the paper), used the different concentration than the one used in this study (2 µg/ml).

However, additional studies are needed to investigate the action of these compounds with the other platelet mediators involved in the aggregation process. It is also worth to try and elucidate the mechanism of action of these compounds. As a matter of comparison only (as we don't know yet the mechanism of action of our fraction and its isolated compounds), the effect of the established antiplatelet agent acetylsalicylic acid (ASA, 100 µM) was also evaluated as an internal control (Figure 3.59 A-E).

3.4 CONCLUSIONS

A new isoprenyl quinoline alkaloid in the Plant Kingdom, choisyaternatine (10) was isolated from the hexane fraction of *C. ternata* Kunth. (Rutaceae) leaves, together with known compounds; skimmianine (11), lup-20(29)-en-3β-ol (lupeol) (12), lup-20(29)-en-3β,24-diol (13) and tecleamaniensine A (14). The other four known compounds, skimmianine (11), 6-methoxyl-7,8-methylenedioxycoumarin (15), choisyine (16) and β-sitosterol glucoside (8), were isolated from the dichloromethane fraction while kaempferol-3-*O*-rutinoside (17) was isolated from the ethyl acetate fraction. Compounds 12, 13, 14 and 8 are reported for the first time in this species. The structures of isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. The structure of choisyaternatine (10) was further confirmed by X-ray data analysis.

In the DPPH radical scavenging activity test, the ethyl acetate fraction exhibited the highest antioxidant activity followed by the butanol, crude ethanol, dichloromethane and hexane fractions with equivalent values (μg/μg DPPH) ranging between 0.087±1.21 and 6.097±6.52. Among all, only the ethyl acetate and butanol fractions exhibited significant radical scavenging activity when compared to the positive control, *Ginkgo biloba* extract. In the total phenolic content test, the ethyl acetate fraction of *C. ternata* showed the highest total phenolic content followed by dichloromethane, butanol, hexane and crude ethanol extract ranging between 260.818±0.007 and 318.401±0.007 μg GAEs/mg. Similarly, the ethyl acetate fraction of *C. ternata* showed the highest flavonoid equivalent amongst its extracts, followed by hexane, butanol, crude ethanol and dichloromethane fractions ranging between 26.292±0.002 and 70.181±0.002 μg QEs/mg. A strong correlation can be seen between these three parameters, while the ethyl acetate fraction showed the highest DPPH scavenging activity, total phenolic content and flavonoid equivalent.

The hexane fraction, choisyaternatine (10), skimmianine (11), lupeol (12), tecleamaniensine A (14) and choisyine (16) were evaluated for their antiplatelet activity using whole human blood. In this study, skimmianine and choisyaternatine only showed a moderate collagen-induced platelet aggregation activity in human platelets with the IC_{50} value of 564 and 698 μM, respectively, while choisyine (16), isolated from the dichloromethane fraction showed lower IC_{50} value of 533 μM. Lupeol showed no activity at all concentrations tested. A mixture containing all isolated compounds from the hexane fraction was able to inhibit aggregation at 99% using the same concentration of collagen to induce aggregation as previous.

Additional studies are needed to investigate the contribution of the main platelet mediators involved in this process and elucidate the mechanism of action of these compounds and other extracts including their isolates.

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CONCLUDING REMARKS

4. CONCLUDING REMARKS

It can be summarized that the aims of the study were successfully achieved using the two medicinal plants named *Pereskia bleo* Kunth. (Cactaceae), collected in Malaysia and *Choisya ternata* Kunth. (Rutaceae), collected in Ireland.

From this study, two known compounds were isolated for the first time from *Pereskia bleo*; β-sitosterol glucoside and vitexin. The isolation of vitexin, a *C*-glycosylflavonoid, bears important chemotaxonomic implications as this confirms the trend of Cactacea in being able to synthesize *C*-glycosylflavonoids. In addition, the results of antinociceptive and anti-inflammatory activities showed that fractions of this plant possess moderate pharmacological activity in all assays and can justify its use in Malaysian folk medicine as a drug to treat rheumatism, among other inflammatory conditions. Vitexin, one of the constituents of the ethyl acetate fraction was proven not to be responsible for the observed activity due to its concentration in the plant. However, its presence could justify the evaluation of *P. bleo* in models related to cardiac conditions as vitexin isolated from *Crataegus oxycantha* (Hawthorn) is reported as one of the molecules responsible for such activity.

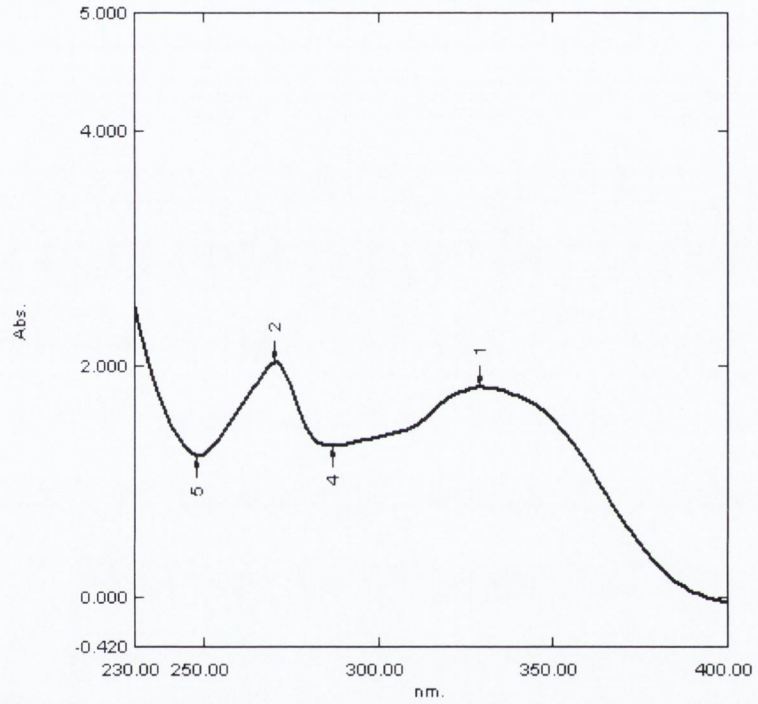
Also, a total of five compounds were isolated for the first time from *Choisya ternata*. Among them, a new natural product, choisyaternatine (10) is described for the first time. As the hexane fraction of this plant showed an outstanding antiplatelet activity (similar to the one of aspirin), full evaluation of its constituents must be performed in future. It is worth to try different platelet aggregators with them, at different doses to achieve the correct mechanism of action by which they are inhibiting platelet aggregation.

APPENDICES

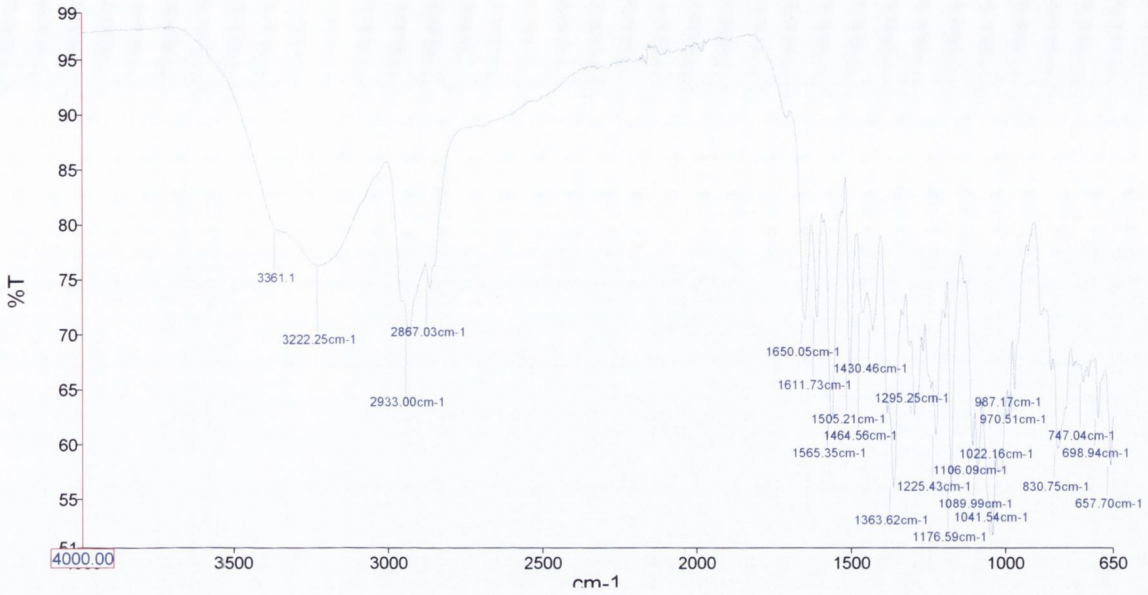
5. APPENDICES

5.1 SPECTROSCOPY DATA

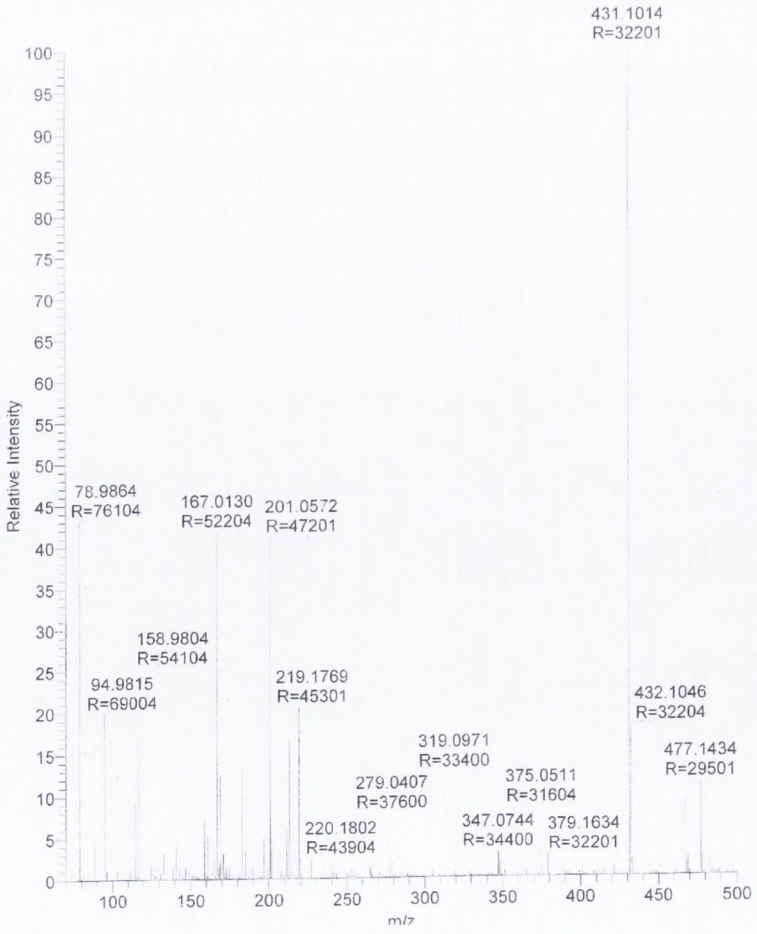
5.1.1 *Pereskia bleo*



Appendix 5.1: The UV spectrum of compound 9, vitexin, showing peaks at λ_{max} 270 and 329

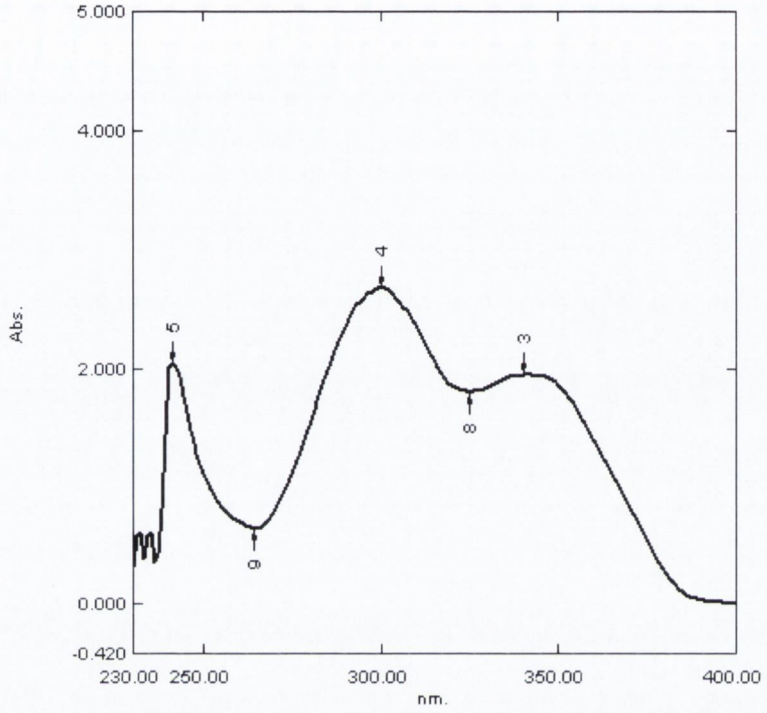


Appendix 5.2: The IR spectrum of compound 9, vitexin

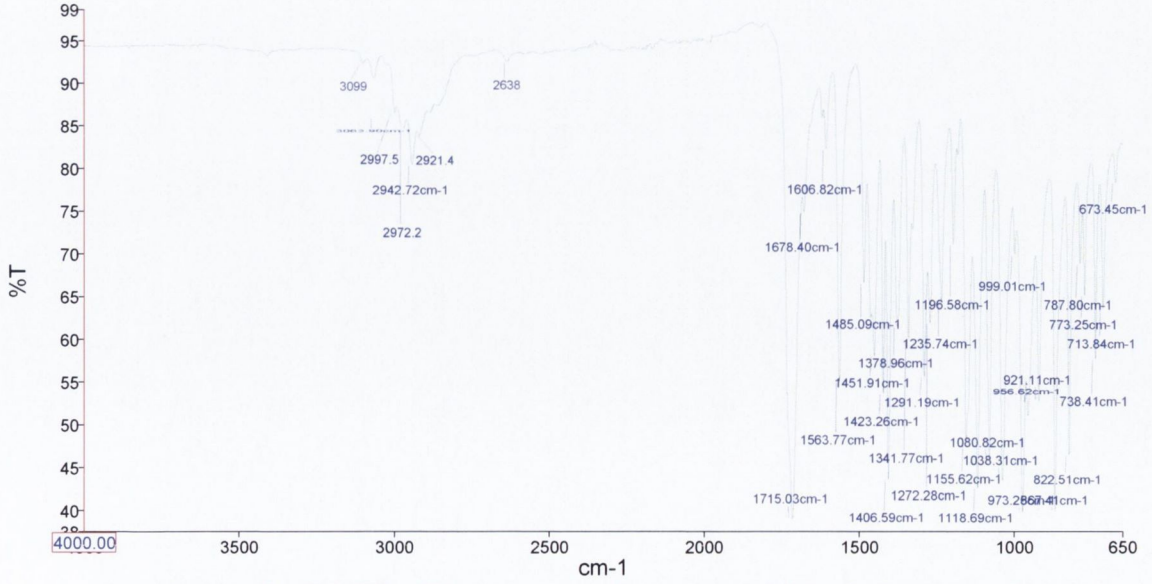


Appendix 5.3: The ESI-MS of compound 9, vitexin in the negative mode m/z 431 $[M-H]^-$

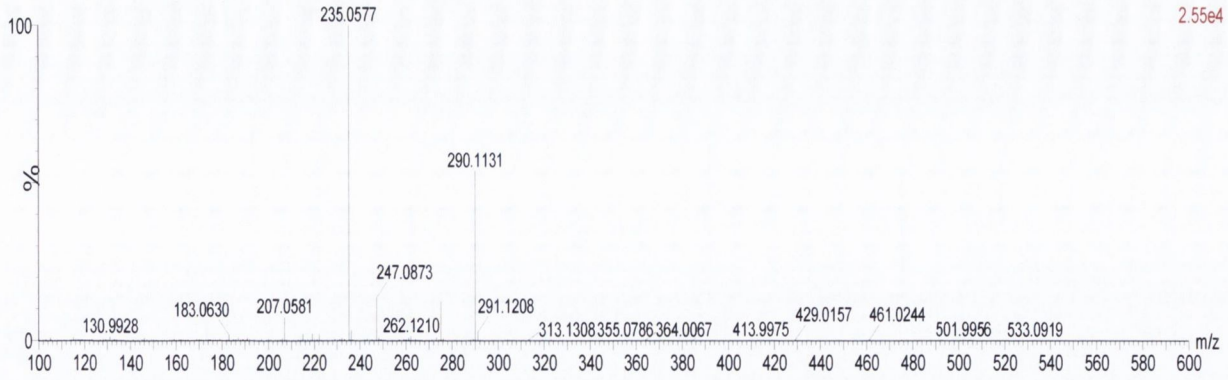
5.1.2 *Choisya ternata*



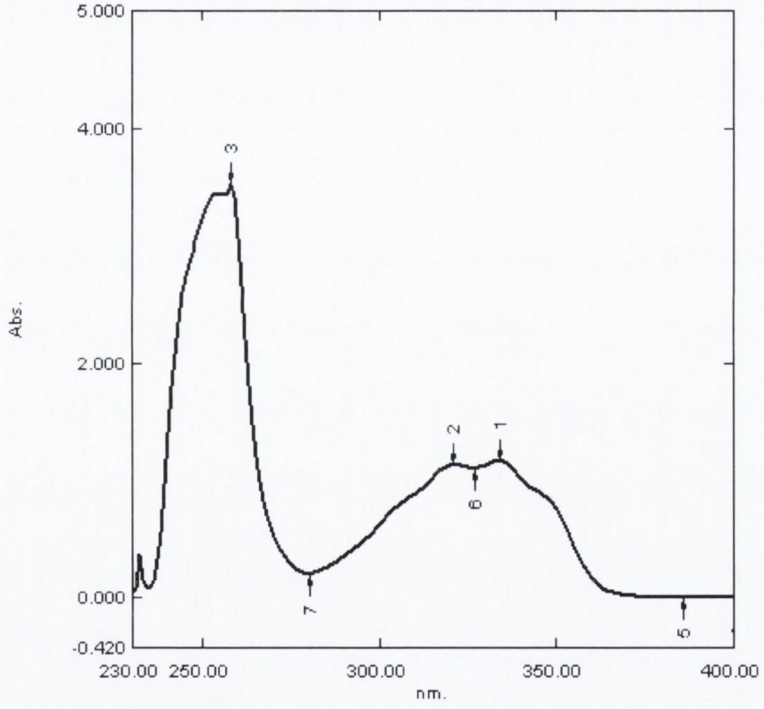
Appendix 5.4: The UV spectrum of compound 10, choisyaternatine, showing peaks at λ_{max} 241, 300 and 340



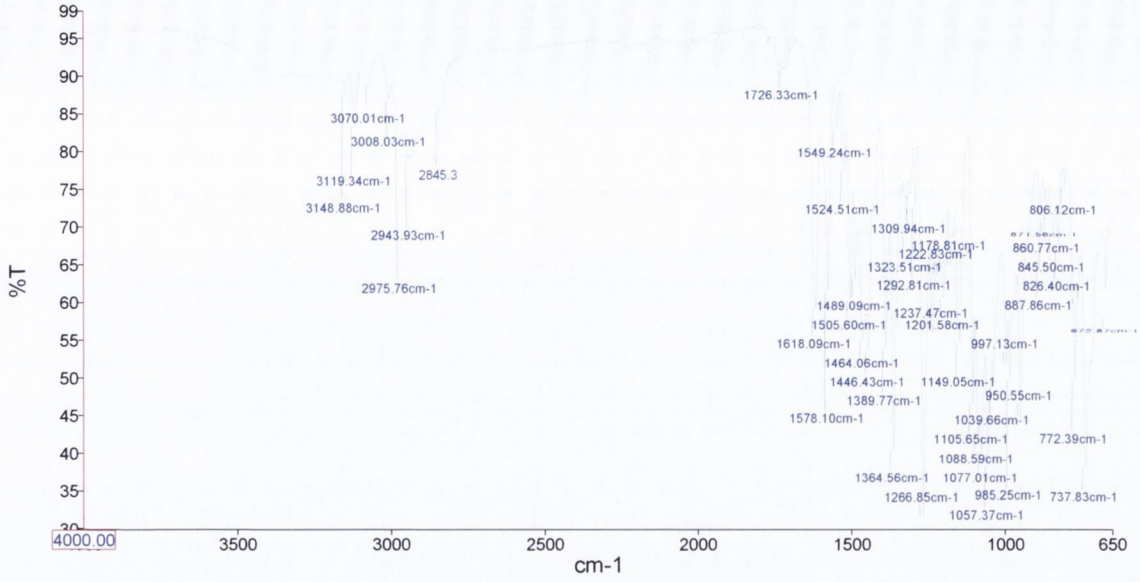
Appendix 5.5: The IR spectrum of compound 10, choisyaternatine



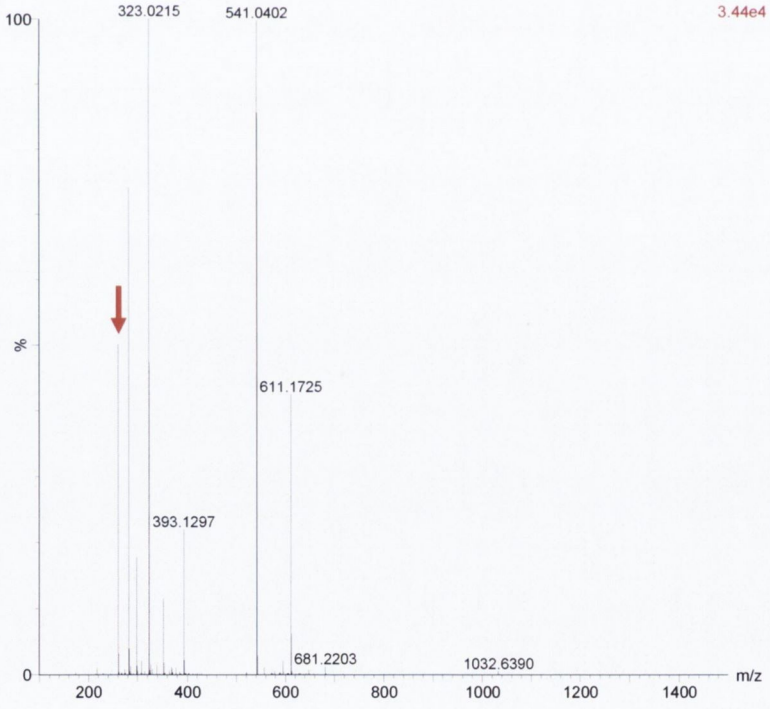
Appendix 5.6: EI-MS of compound 10, choisyaternatine in the positive mode m/z 290 $[M+H]^+$



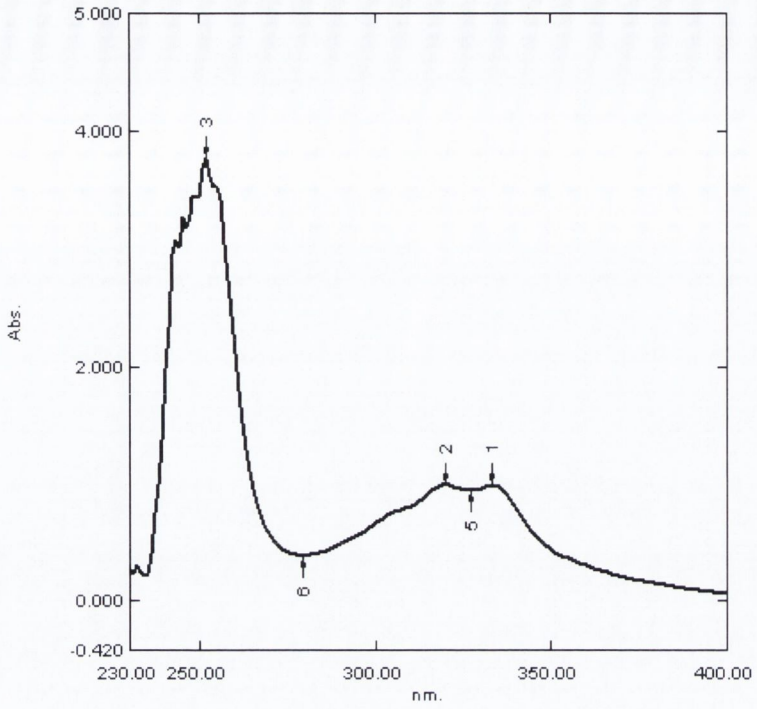
Appendix 5.7: The UV spectrum of compound 11, skimmianine, showing peaks at λ_{max} 258, 321 and 334



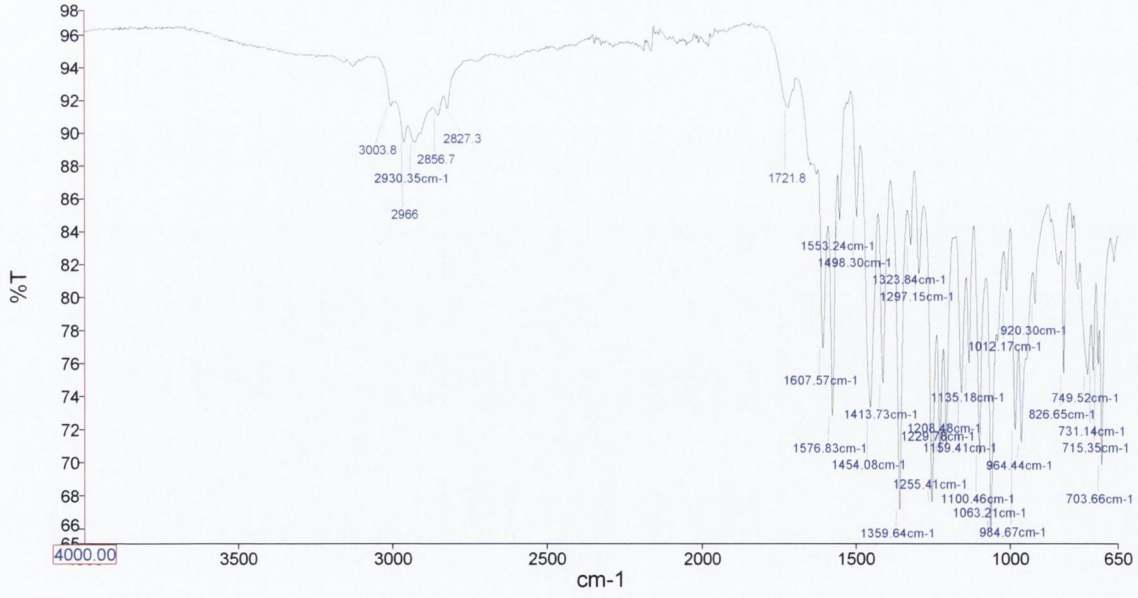
Appendix 5.8: The IR spectrum of compound 11, skimmianine



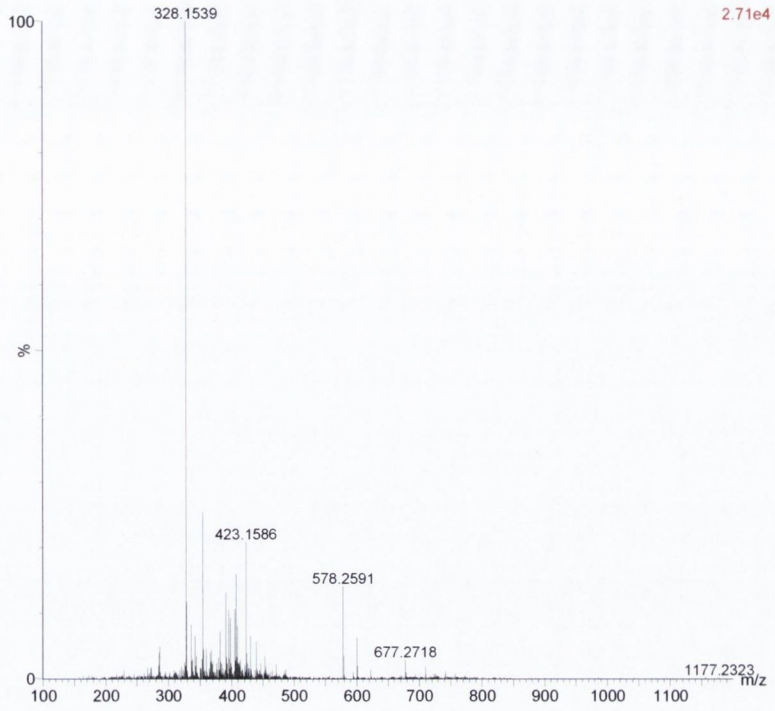
Appendix 5.9: The ESI-MS of compound 11, skimmianine in the positive mode m/z 260 $[M+H]^+$



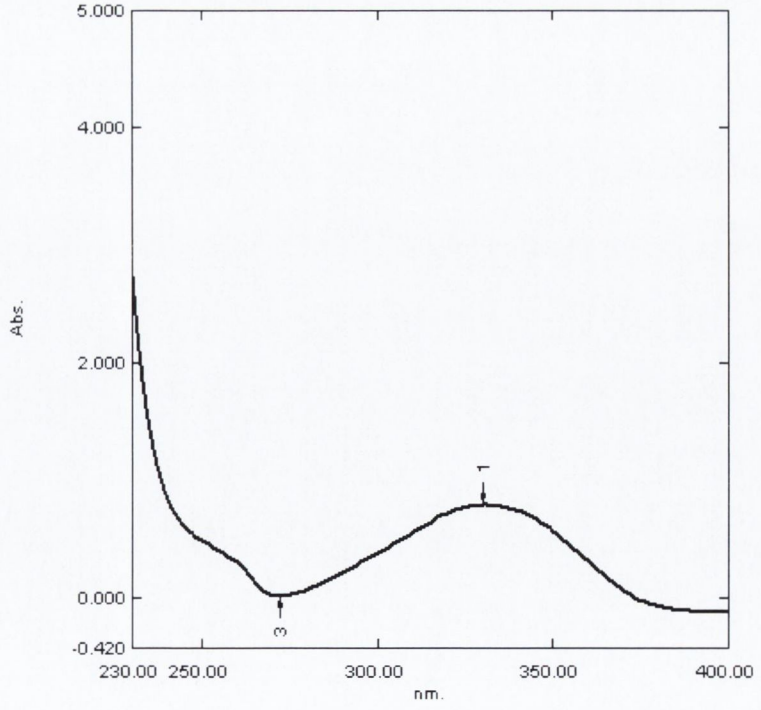
Appendix 5.10: The UV spectrum of compound 14, tecleamaniensine A, showing peaks at λ_{max} 252, 320 and 333



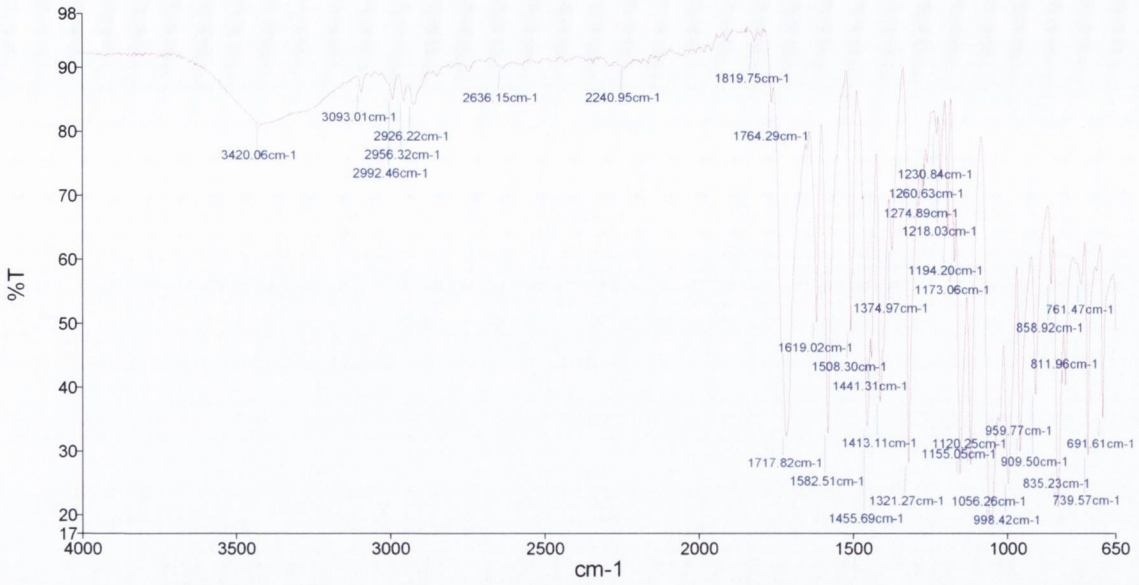
Appendix 5.11: The IR spectrum of compound 14, tecleamaniensine A



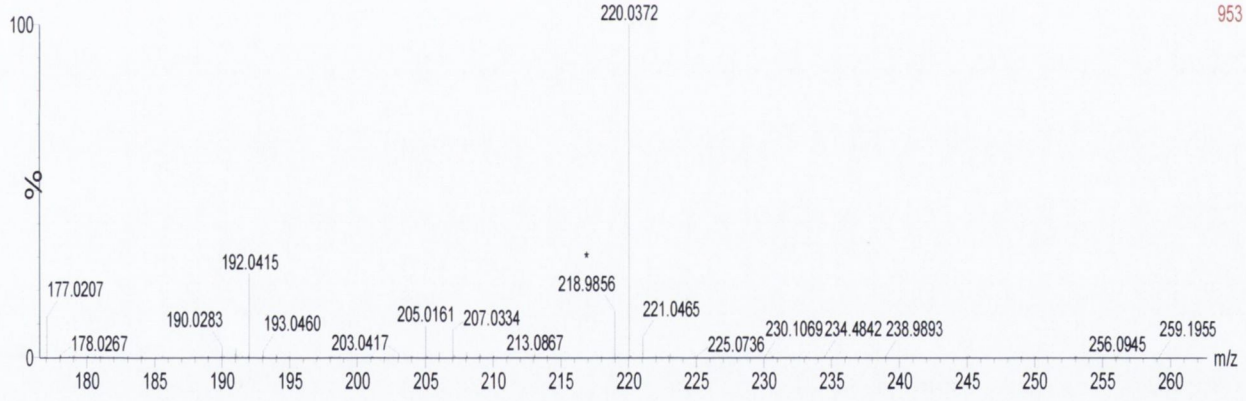
Appendix 5.12: The ESI-MS of compound 14, tecleamaniensine A in the positive mode m/z 328 $[M+H]^+$



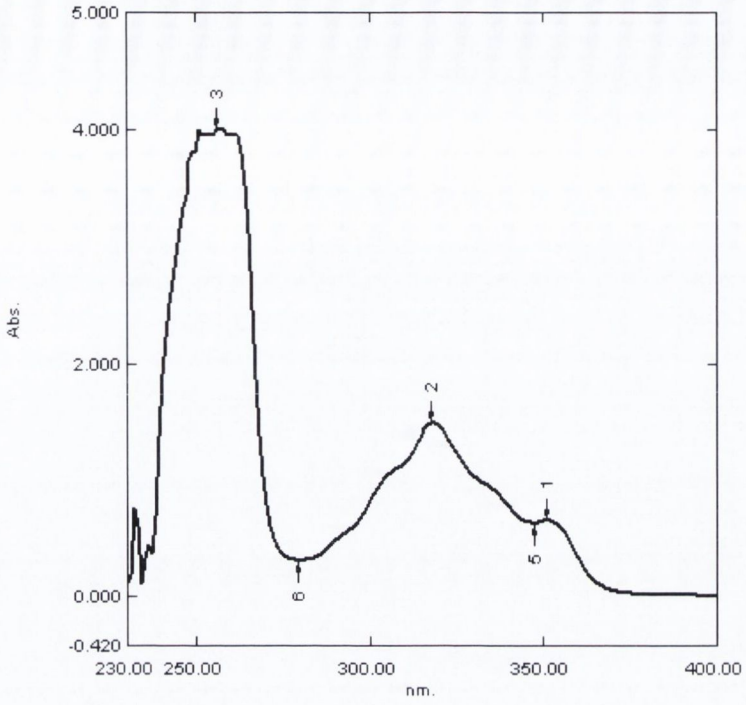
Appendix 5.13: The UV spectrum of compound 15, 6-methoxy-7,8-methylenedioxy coumarin, showing a peak at λ_{max} 330



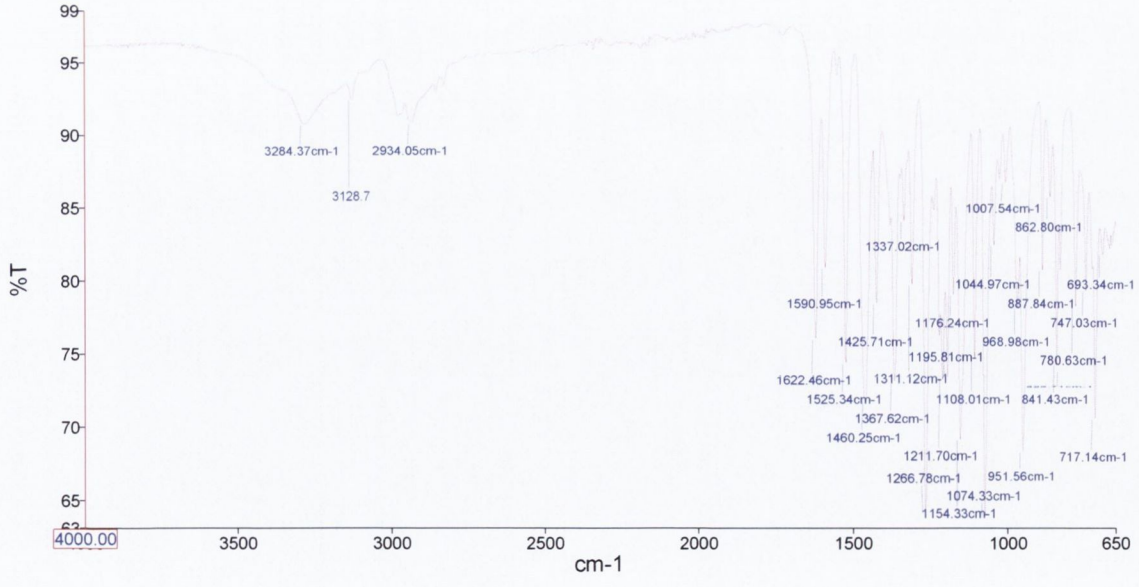
Appendix 5.14: The IR spectrum of compound 15, 6-methoxy-7,8-methylenedioxycoumarin



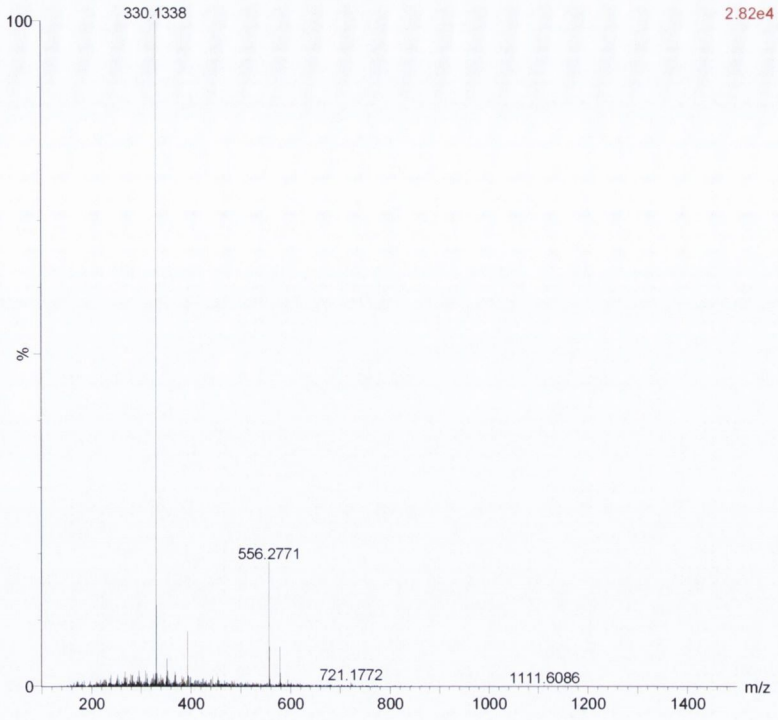
Appendix 5.15: The EI-MS of compound 15, 6-methoxy-7,8-methylenedioxy coumarin in the positive mode m/z 221 $[M+H]^+$



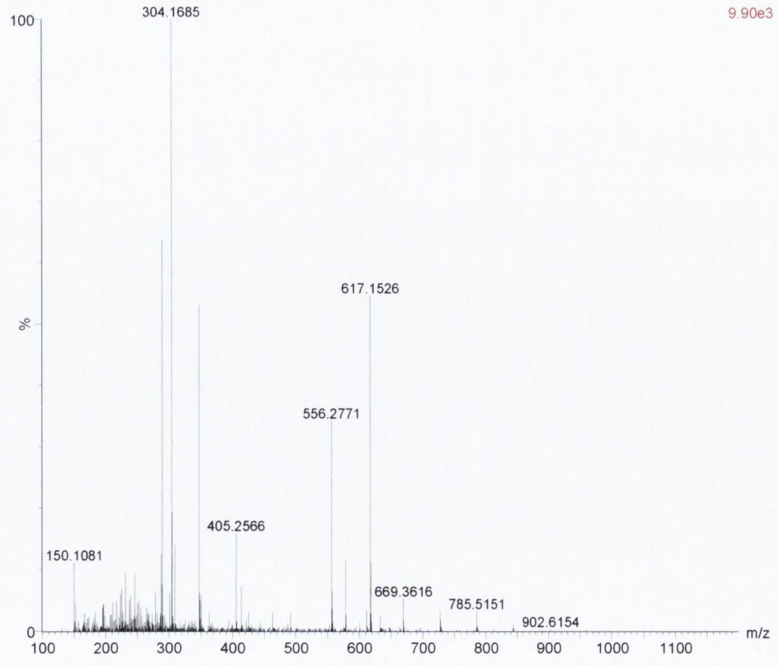
Appendix 5.16: The UV spectrum of compound 16, choisyine, showing peaks at λ_{max} 258, 318 and 352



Appendix 5.17: The IR spectrum of compound 16, choisyine



Appendix 5.18: The ESI-MS of compound 16, choisyine in the positive mode m/z 330 $[M+H]^+$



Appendix 5.19: The ESI-MS of compound 17, kaempferol-3-*O*-rutinoside in the positive mode m/z 617 $[M+Na]^+$

5.2 PRESENTATIONS AND PUBLICATIONS RELATED TO THIS THESIS

Cytotoxicity evaluation and antinocicpetive activity of *Pereskia bleo* (Cactaceae) extracts

Poster presentation on the 33rd Joint Schools of Pharmacy, Research Seminar 2011, Royal College of Surgeon, Dublin, Ireland. April 2011.

Abdul Wahab IR[1,2] and Menezes FS[1]
[1]School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Ireland & [2]Universiti Malaysia Kelantan, Malaysia

Medicinal plants have traditionally been used in a variety of local herbal preparations by various ethnicities in many countries in the world. *Pereskia bleo* Kunth. (Cactaceae) is a medicinal plant traditionally used in Malaysian folk medicine for the treatment of cancer-related diseases and rheumatism besides other inflammatory diseases (1,2,3,4). Extracts of this plant have been prepared using solvents of different polarities. These extracts were then submitted to their cytotoxicity evaluation against HCC1143 cell line, using both MTT and Neutral Red methodologies. They have also been tested in relation to their antinociceptive activity through the following methods: writhing inhibition, formalin test and hot plate. The dichloromethane extract obtained from leaves of *P. bleo* was the more active one with IC_{50} values of 56.95µg/mL (neutral red assay) and 61.99µg/mL (MTT assay). Ethyl acetate extract showed a very good antinociceptive activity inhibting the contortions induced by acetic acid in mouse (70%) which means a good potential to possess compounds working as a peripheral analgesics. This extract was also the second best in the hot plate model (60% increase in the area under the curve when compared with no treatment), a model that points to the central analgesic activity. The best extract in this model was the butanol extract showing more than 400% increase in the area under the curve when compared with non treated mice. This was even higher than mice receiving morphine. The results showed by the extracts are in accordance to their traditional use and stimulated the chemical study to isolate compound(s) that could be the responsible for such activities.

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3. Goh K.L. Advanco Press, Malaysia. 2000; 142.
4. Er H. M., En-Hsiang C. and Radhakrishnan A. K. *Journal of Ethnopharmacology*. 2007; 113: 448-456.

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DOI: 10.1055/s-0032-1320957

Alkaloids from *Choisya ternata* and their human antiplatelet activity

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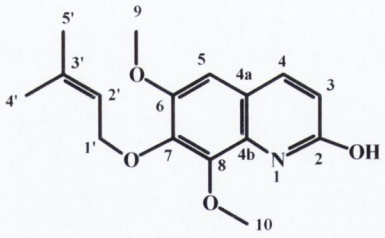
Congress Abstract

One novel alkaloid was isolated from the hexane extract of *Choisya ternata* Kunth. (Rutaceae) leaves, choisyaternatine (1), together with the known alkaloids tecleamaniensine A (2) and skimmianine (3). From the dichloromethane extract, skimmianine (3) and choisyine (4) were also isolated. Tecleamaniensine A is reported for the first time in this species. The structures of isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. The structure of choisyaternatine was further confirmed based on X-ray data analysis. Anti-platelet activity of hexane extract, choisyaternatine and skimmianine was also evaluated.



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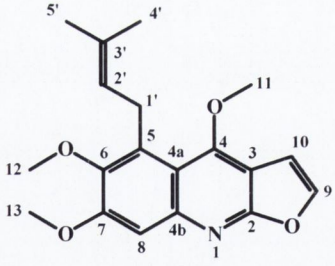

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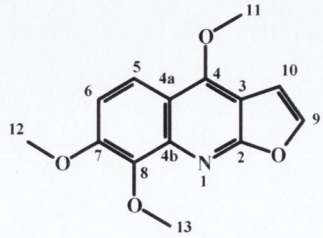

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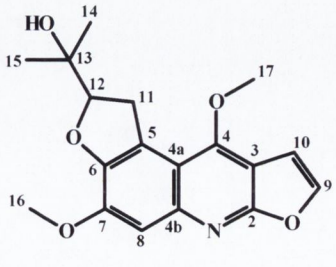

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Evaluation of antinociceptive activity of *Pereskia bleo* Kunth

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Congress Abstract

Our aim was to identify the antinociceptive effect for *P. bleo* leaves as they are popularly used in Malaysia for the treatment of rheumatism, among other conditions. Plant material was collected in Malaysia (2009). An ethanol extract (E) was prepared from leaves (1.4g) and submitted to liquid liquid partition with: hexane [H], ethyl acetate [EA], and butanol [B]. Antinociceptive activity was evaluated by the hot plate test (calculated as area under the curve, AUC) at the doses 30, 50, and 100mg/kg. The mechanism of action was evaluated pre-treating the animals with naloxone (1mg/kg, N). The protocol for the use of animals received the number ICBDFBC015. Statistical analyses were performed by ANOVA and Bonferroni's test (* or # $p<0.05$ in relation to vehicle or fraction-treated group, respectively).

Results:

mg/kg	Vehicle	Morphine	E	H	EA	B
30	1,425±159	5,153±581*	1,071±113*	2,268±233*	1,340±141	4,341±450*
50	1,425±159	5,153±581*	2,495±244*	3,306±344*	4,293±401*	3,318±365*
100	1,425±159	5,153±581*	2,826±314*	2,415±256*	3,153±336*	7,292±741*
100 + N	1,219±113	133±42#	1,808±231#	256.5±301#	172.5±55#	1,146±129#

Conclusions:

Fractions from *P. bleo* demonstrated central antinociceptive effect mediated, at least in part, by opioid system. Financial support: CNPq, FAPERJ, and Instituto Vital Brazil.

Antinociceptive activity of *Pereskia bleo* Kunth. (Cactaceae) leaves extracts

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ABSTRACT

Ethnopharmacological relevance: Local communities in Malaysia consume *Pereskia bleo* Kunth. (Cactaceae) leaves as raw vegetables or as a conconction and drink as a tea to treat diabetes, hypertension, rheumatism, cancer-related diseases, inflammation, gastric pain, ulcers and for revitalizing the body.
Aim of the study: To evaluate antinociceptive activity of the extracts and vitexin, isolated for the first time in this species, in three analgesic models; formalin-induced, acetic acid-induced abdominal writhing and hot plate.
Materials and methods: Three and a half kilos of *P. bleo* leaves were extracted using Soxhlet apparatus with ethanol for 72 h. The crude ethanol extract was treated with activated charcoal overnight and subjected to a liquid-liquid partition yielding hexane, dichloromethane, ethyl acetate and butanol extracts. All extracts, including the crude ethanol and vitexin isolated from the ethyl acetate partition were tested for peripheral and central anti-nociceptive activity using formalin test, acetic acid-induced abdominal writhing and hot plate test, besides having their acute toxicity assays performed.
Results: The phytochemical analyses resulted in the isolation of vitexin (1), β-sitosterol glucoside (2) and β-sitosterol (3) isolated from the ethyl acetate, dichloromethane and hexane extracts, respectively. This is the first time vitexin and β-sitosterol glucoside were isolated from this species. The anti-nociceptive activities for all extracts were only moderate. The butanol extract showed stronger activity in the hot plate test, when compared to morphine (5 mg/kg). Vitexin, which was isolated from the ethyl acetate extract did not show any activity in all models used when tested alone at the same concentration as it appears in the extract.
Conclusion: This study showed that all the extracts possess moderate antinociceptive activity. Vitexin is not the compound responsible for the antinociceptive effect in the ethyl acetate extract.. Further investigations are needed to identify the compound(s) that might be responsible for the antinociceptive activity in this plant.

Choisyaternatine, a novel compound isolated from *Choisya ternata*

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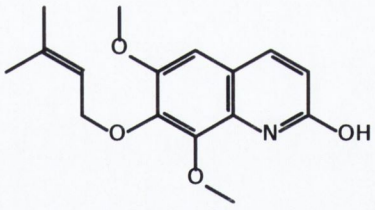
Abstract

One novel compound was isolated from the hexane extract of *Choisya ternata* Kunth. (Rutaceae) leaves, choisyaternatine (1), together with the known compounds tecleamaniensine A (2), Lup-20(29)-en-3β-ol (lupeol) (3), Lup-20(29)-en-3β,24-diol (4), β-sitosterol glucoside (5) and skimmianine (6), plus the following known compounds from the dichloromethane extract, choisyine (7) and 6-methoxy-7,8-methylenedioxycoumarin (8). Compounds 2-5 are reported for the first time in this species. The structures of isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. The structure of choisyaternatine was further confirmed based on X-ray data analysis.

Keywords

Choisya ternata, Rutaceae, choisyaternatine, alkaloids, triterpenes



A novel compound from *Choisya ternata* Kunth. (Rutaceae); choisyaternatine

Journal of Ethnopharmacology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

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Anti-nociceptive activity of *Pereskia bleo* Kunth. (Cactaceae) leaves extracts

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ABSTRACT

Ethnopharmacological relevance: Local communities in Malaysia consume *Pereskia bleo* Kunth. (Cactaceae) leaves as raw vegetables or as a concoction and drink as a tea to treat diabetes, hypertension, rheumatism, cancer-related diseases, inflammation, gastric pain, ulcers, and for revitalizing the body.

Aim of the study: To evaluate anti-nociceptive activity of the extracts and vitexin, isolated for the first time in this species, in two analgesic models; formalin-induced licking and acetic acid-induced abdominal writhing.

Materials and methods: Three and a half kilos of *P. bleo* leaves were extracted using Soxhlet apparatus with ethanol for 72 h. The crude ethanol extract was treated with activated charcoal overnight and subjected to a liquid–liquid partition yielding hexane, dichloromethane, ethyl acetate and butanol extracts. All extracts, including the crude ethanol and vitexin isolated from the ethyl acetate partition were tested for peripheral anti-nociceptive activity using formalin test and acetic acid-induced abdominal writhing, besides having their acute toxicity assays performed.

Results: The phytochemical analyses resulted in the isolation of vitexin (1), β-sitosterol glucoside (2) and β-sitosterol (3) isolated from the ethyl acetate, dichloromethane and hexane extracts, respectively. This is the first time vitexin and β-sitosterol glucoside are isolated from this species. The anti-nociceptive activities for all extracts were only moderate. Vitexin, which was isolated from the ethyl acetate extract did not show any activity in all models tested when used alone at the same concentration as it appears in the extract.

Conclusion: This study showed that all the extracts possess moderate anti-nociceptive activity. Vitexin is not the compound responsible for the anti-nociceptive effect in the ethyl acetate extract. Further investigations are needed to identify the compound(s) that might be responsible for the anti-nociceptive activity in this plant.

1. Introduction

In Malaysia, the knowledge on the indigenous use of plants was mostly passed on orally from one generation to another and has largely remained undocumented. In 1966, Burkill in his extensive compilation of the economic products of the Malay Peninsula recorded that not less than 1300 plants have been used in traditional medicine (Jantan, 2004).

Pereskia bleo (Kunth) DC (Cactaceae), commonly known as Jarum Tujuh Bilah (in Malay) and Cak Sing Cam (in Chinese) by the locals (Abd. Malek and Abd. Wahab, 2008) is a plant commonly used by the local communities in Malaysia due to its medicinal properties (Er et al., 2007). *P. bleo* is a spiny shrub with distinct orange–red flowers and is claimed to treat a variety of illnesses including diabetes, hypertension and as natural remedy for cancer-related diseases. It has also been used traditionally in Malaysia for the treatment of diseases associated with rheumatism, inflammation, gastric pain, ulcers and for revitalizing the body (Abd. Malek and Abd. Wahab, 2008; Er et al., 2007; Abd. Malek et al., 2009; Goh, 2000). The leaves are usually consumed by the locals, either raw as vegetables or as a concoction after having them brewed (Abd. Malek and Abd. Wahab, 2008; Er et al., 2007; Goh, 2000). Popularly, there is a belief that by drinking tea made from the mature leaves (6–7 pieces) everyday, one could prevent and cure the above mentioned diseases.

The earliest phytochemical study was by Doetsch et al. (1980) who reported the isolation of four alkaloids, 3,4-dimethoxy-β-phenethylamine, mescaline, 3-methoxytyramine and tyramine from *P. bleo*. Later, in 1978, Richardson reported that kaempferol and quercetin were detected in leaf extracts of *P. bleo*. No

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C-glycosylflavonoids were detected in all 10 species of Cactaceae studied. Abd. Malek and Abd. Wahab (2008) isolated four compounds from the ethyl acetate fraction of *P. bleo* which were identified as phytol, β-sitosterol (1), 2,4-di-*tert*-butylphenol and vitamin E.

Furthermore, on repeated chromatographic purification of the active ethyl acetate fraction yielded dihydroactinidiolide and a mixture of sterols (campesterol, stigmasterol and β-sitosterol), besides 2,4-di-*tert*-butylphenol, vitamin E and phytol (Abd. Malek et al., 2009).

There are several pharmacological reports on the extracts of *P. bleo*, mainly on cytotoxic and anti-proliferative activities (Abd. Malek and Abd. Wahab, 2008; Abd. Malek et al., 2009; Er et al., 2007). To date, there is no report on the pharmacology of pain and inflammation for this species.

Several plant-based compounds were reported to have significant analgesic properties with very little adverse effects, which have been used as substitute therapeutics to treat nociception (Pinheiro et al., 2010). Due to the fact that *P. bleo* is traditionally used for the treatment of rheumatism, investigation of the extracts belonging to this plant in models of anti-nociception is justified in this present study using formalin-induced test, acetic acid-induced abdominal writhing and hot plate test.

2. Materials and methods

2.1. Reagents and chemical analyses

All reagents were purchased from Sigma-Aldrich (Dublin, Ireland). Melting points were recorded in °C on a SMP-1 Stuart Scientific, UK. Optical rotations $[\alpha]_D^{20}$ were measured in a 1 dm. tube using an Alltech AA-55 polarimeter from Optical Activity Ltd. UV spectra were performed with Cary 300 Scan UV–Visible spectrophotometer and Varian–CaryWinUV program. The FT-IR spectroscopy was recorded in KBr discs on a Perkin–Elmer Spectrum 100 FT-IR Spectrometer (Perkin–Elmer Spectrum). EI mass spectra were measured on Waters Micromass GCT Premier Mass spectrometer and Mass Lynx V4.1 software. The HPLC-MS analyses were performed on ThermoScientific LTQ Orbitrap.

The ^{1}H and ^{13}C NMR spectra were recorded on a BRUKER TOPSPIN 2.1 NMR System (^{1}H NMR: 400 and 600 MHz, ^{13}C NMR: 125 MHz) spectrometers, using standard Bruker pulse sequences, as well as both uni- and bi-dimensional techniques NMR spectra (^{1}H–^{1}H COSY, HMBC, HMQC).

Column chromatography was performed using silica gel 60 (70–230 mesh; Merck). For thin layer chromatography (TLC), aluminum sheets of silica gel 60 F_{254}, (230–400 mesh ASTM, 20 cm × 20 cm, layer thickness: 0.25 mm, Merck) were used for analytical purposes and the compounds were visualized under ultraviolet light. Solvents for extraction and chromatography were analytical grade, exception to ethanol, which was the commercial grade.

2.2. Plant material

Samples of dried leaves of *P. bleo* (3.5 kilos) were collected in Kota Bharu, Kelantan in Malaysia in August, 2009. The plant has been identified by Mr. Rafly Syamsir from the Chemistry Department, Faculty of Sciences, University of Malaya, Kuala Lumpur, Malaysia where a voucher specimen (KL5729) has been deposited.

2.3. Extraction and isolation

Three and a half kilos of *P. bleo* leaves were extracted using Soxhlet apparatus with ethanol for 72 h. The crude ethanol extract of *P. bleo* leaves was then treated with activated charcoal for overnight to eliminate chlorophyll.

Part of the ethyl acetate extract of *P. bleo* (6.2 g) was submitted to a chromatographic fractionation in column (∅ column: 3.5 cm) using chloroform – ethyl acetate – methanol in gradients of increasing polarity as mobile phase and silica gel (70–230 mesh) as stationary phase, yielding 12 fractions. Yellow precipitates were obtained from fractions 5 and identified as vitexin (1). The purification of compounds from the dichloromethane extract of *P. bleo* (19.2 g) was performed by chromatographic fractionation using hexane – ethyl acetate – methanol in gradients of increasing polarity as mobile phase yielding 10 fractions. White precipitates were obtained from junction 8 which were identified as β-sitosterol glucoside (2). Part of the hexane extract of *P. bleo* (16 g) was chromatographed by column chromatography using hexane – ethyl acetate – methanol in gradients of increasing polarity as mobile phase and silica gel (70–230 mesh, Merck) as stationary phase, yielding 202 fractions. Further chromatographic procedures led to the isolation of white crystals (mobile phase: hexane—ethyl acetate, 8:2), identified as β-sitosterol (3).

This is the first report of vitexin (1) and β-sitosterol glucoside (2) occurring in *P. bleo*. The assignment of all proton and carbon 13 NMR signals was further confirmed by use of 2D –NMR (^{1}H-^{1}H COSY and HETCOR spectra) as well as comparison with literature data (Vázquez et al., 2002; Akhtar et al., 2010).

2.4. Animals

The anti-nociceptive assays were run according to the methods described by Matheus et al. (2005). These assays were performed at Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Brazil. All experiments were performed with male Swiss mice (20–25 g) obtained from Instituto Vita Brazil. Animals were maintained in a room with controlled temperature 22 ± 2°C for 12 h: 12 h light/dark cycle with free access to food and water. Twelve hours before each experiment, animals received only water, in order to avoid food interference with substances absorption. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethical Committee for Animal Research (Biomedical Science Institute/UFRJ; #ICBDFBC-015). Initially, *P. bleo* extracts were dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution at 100 mg/ml and administered by oral gavages at doses of 30, 50, and 100 mg/kg, in a final volume of 0.1 mL (mice). Positive control groups were composed by morphine (Merck Inc.) or acetylsalicylic acid (ASA). The negative control group received the vehicle phosphate buffer saline (PBS), containing the same amount of DMSO.

2.5. Formalin-induced test

The procedure used is similar to the method described by Gomes et al. (2007). Animals received the injection of 20 μl of formalin (2.5%, v/v) into the dorsal surface of the left hind paw. Immediately, the time that the animal spent licking the injected paw was recorded. The nociceptive response develops two phases: the first 5 min after formalin injection (first phase, neurogenic pain response) and 15–30 min after formalin injection (second phase, inflammatory pain response). The animals were pre-treated with oral doses of the extracts, vehicle, or morphine 60 min before administration of formalin.

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2.6. Acetic acid-induced abdominal writhing

Mice were treated according to Pinheiro et al. (2010). Briefly, the total number of writhing following intraperitoneal administration of 2% (v/v) acetic acid (AA) was recorded over a period of 20 min, starting 5 min after AA injection. Mice were pre-treated with *P. bleo* extracts or vehicle, 60 min before administration of AA. Positive control group was composed by the reference drug morphine (5 mg/kg). The difference in response between this test and the previous one is that although both tests show peripheral anti-nociception, the former indicates the possibility of anti-inflammatory action due to its second phase that is mediated by the release o inflammatory mediators.

2.7. Acute toxicity

Acute toxicity was determined following the experimental model described by Lorke (1983). A single oral dose of plant extracts (500 mg/kg) was administered to a group of 10 mice (five males and five females). Behaviour parameters including convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, and food and water intake were observed over a period of 15 days. After this period, animals were killed by cervical dislocation, stomachs were removed, an incision along the greater curvature was made, and the number of ulcers (single or multiple erosion, ulcer or perforation) and hyperaemia were counted. Lethal dose was not performed due to small quantity of the extracts and isolated substances.

2.8. Statistical analysis

All experimental groups for the anti-nociceptive evaluation were composed of five animals. The results are presented either as the mean ± S.D. Statistical significance between groups was calculated by analyses of variance (ANOVA), followed by Bonferronis's test. *p*-values less than 0.05 ($p < 0.05$) were used as the significant level.

3. Results and discussion

Natural products exhibiting analgesic properties are of great interest recently. In this present study, two unreported compounds; vitexin and β-sitosterol glucoside have been isolated from the ethyl acetate and dichloromethane extracts, besides a known compound, β-sitosterol found abundantly in the hexane extract (Fig. 1). Numerous morphological and chemical studies over the past few decades have firmly established the taxonomic position of the Cactaceae in the Caryophyllales (Salt et al., 1987).

Compound 3 was previously isolated from the ethyl acetate extract of *P. bleo* as reported by Abd. Malek and Abd. Wahab (2008), Abd. Malek et al. (2009), while compound 2, was unreported for the species until now. Besides, this is the first report of the occurrence of a *C*-glucosylflavonoid, compound 1 (vitexin) in *P. bleo* and in the entire subfamily Pereskiaeae and family Cactaceae. According to Richardson (1978) and Gupta (2005), *C*-glycosylflavonoids were found to be absent in the Cactaceae family, particularly in this species. The importance of finding this compound in *P. bleo* also lies in the fact that *C*-glucosylflavonoids have several interesting pharmacological activities (Quílez et al., 2010; Kim et al., 2005) and this could partially explain and justify few of the popular uses for this plant.

In this present study, *P. bleo* extracts and vitexin isolated from the ethyl acetate extract have been assayed for peripheral and central anti-nociception in three models. The results show that the extracts when given orally produce dose-related and significant anti-nociceptive activity according to assessment of the formalin and acetic acid-induced.

The first test, formalin test is an established model of chemically-induced nociception (Ferreira et al., 2006). The crude ethanol, hexane, dichloromethane and ethyl acetate extracts of *P. bleo* (30 and 100 mg/kg) produced inhibition on formalin-induced biphasic pain responses (formalin 2.5%) (neurogenic and inflammatory pain) in mice (Fig. 2). First phase occurs until 5 min after injection and second phase occurs between 15 and 30 min after formalin injection. During the early phase, all the extracts were not able to decrease the time of licking, similarly with the pattern showed by the vehicle. The analgesic effect of the extracts occurred predominantly during the second phase with all extracts, at 100 mg/kg, showing inhibition in licking time when compared with the control. Thus, all the extracts reduced formalin-induced pain at the late phase with the percentage of inhibition reaching 65% for hexane extract, followed by dichloromethane, crude ethanol, ethyl acetate and butanol extracts with 55, 37.5, 37.5 and 25%, respectively.

The early phase is short-lived and initiates immediately after injection, being characterized by C-fiber activation due to peripheral stimuli. The late phase is a longer, persistent period caused by local tissue inflammation and also by functional changes in the dorsal horn of the spinal cord (Gorzalczany et al., 2011). In this present study, the extracts inhibited the licking time only in second phase; therefore, the effect observed with the extract suggested that the anti-nociceptive action would be related with peripheral mechanisms. The response in the second phase suggested a possible inhibition and/or liberation of inflammatory mediators such as bradykinin, histamine, sympathomimetic amines, tumour necrosis factor-α and interleukins, released in the mice paw or directly blocking their receptors. These mediators are also the target of action of most non-steroidal anti-inflammatory drugs (NSAIDs). Moreover, the formalin injection could cause a characteristic biphasic response which initiated by direct stimulation of nociceptors and lead to an activation of C-fibers (Ferreira et al., 2006).

The abdominal writhing test has been used in many other researches to screen and assess the analgesic or anti-inflammatory properties of new substances (Alves et al., 2012). It involves different nociceptive mechanisms, such as the sympathetic system, cyclooxygenase and their metabolites and opioid mechanisms (Huo et al., 2007).

In the acetic acid-induced abdominal writhing assay, the extracts of *P. bleo* were found to reduce the number of contortions after i.p. injection of acetic acid. The number of writhing and

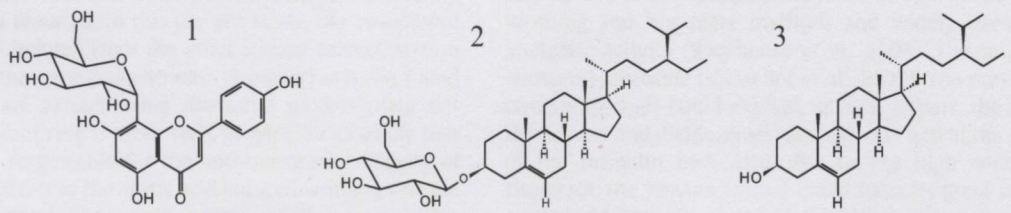

Fig. 1. Vitexin (1), β-sitosterol glucoside (2) and β-sitosterol (3), isolated from the ethyl acetate, dichloromethane and hexane, respectively.

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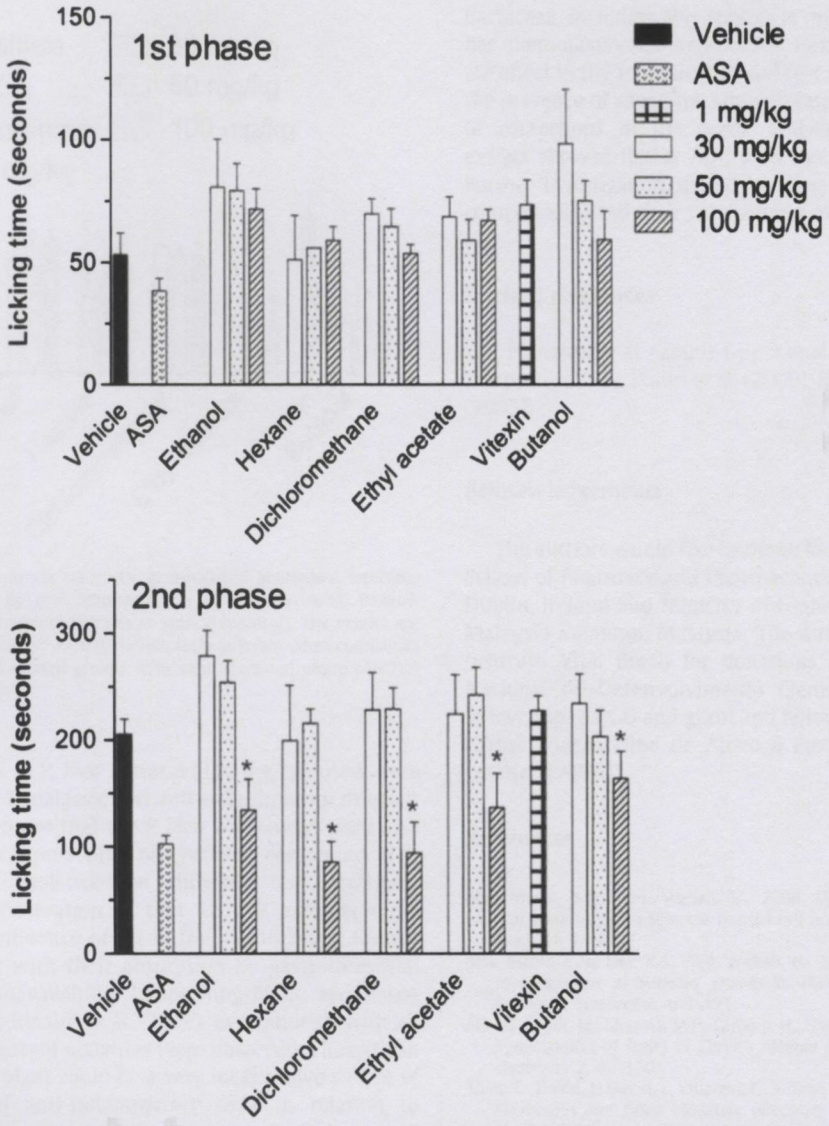

Fig. 2. Effects of the crude ethanol, hexane, dichloromethane and ethyl acetate extracts of *P. bleo* in the formalin-induced test. Animals were pre-treated by oral administration of extracts or vehicle (control). The results are presented as means ± S.D., $n=5$, $p<0.05$ significantly different when compared to vehicle-treated group, 0–5 min. first phase, 15–30 min. second phase.

stretching was recorded and permitted to express the percentage of protection. All the extracts (30 and 100 mg/kg) reduced the number of contortions of acetic acid-induced pain in mice, with the butanol extract (30 mg/kg – inhibition of 69.1% and 100 mg/kg – inhibition of 56.4%) and ethyl acetate extract (50 mg/kg – inhibition of 56.4% and 100 mg/kg – inhibition of 69.1%) showing the lowest number of contortions, followed by other doses of the butanol, ethyl acetate and hexane extracts (Fig. 3). The ethyl acetate extract shows the anti-nociceptive activity in the acetic acid-induced abdominal writhing assay in a dose dependent manner.

A large number of different kinds of natural alkaloids, triterpenes and flavonoids with anti-nociceptive and anti-inflammatory activities has been reported. In this present study, the unreported known compound isolated from the ethyl acetate extract, vitexin was also tested at the same concentration (1 mg/kg) as it was found in the ethyl acetate extract using the same models (data not shown). No significant results were seen, proving for example that vitexin is not the responsible for the anti-nociceptive activity of the ethyl acetate extract in the acetic acid induced writhing test. On the other hand, vitexin has been proved as the most active compound isolated from *Urtica circularis*. In the acetic acid-induced writhing model, at dose of 10 mg/kg i.p., vitexin produced a significant inhibition of 91% abdominal constrictions in mice (Gorzalczany et al., 2011). This high inhibition could probably due to the higher concentration used when compared to the one used in this present study (1 mg/kg). If the same concentration used, it could be misleading the result as the concentration of the vitexin found in the ethyl acetate was only 1 mg/kg.

β-sitosterol, previously was also reported to have analgesic and anti-inflammatory effects (Saeed et al., 2010), therefore it can be one of the molecules responsible for the activity showed by the hexane extract in the formalin-induced model. β-sitosterol was also proved to increase pain tolerance in both acetic acid-induced writhing and hot plate methods and widely used to evaluate analgesic activity (Backhouse et al., 2008). The same applies to sitosterol glucoside (Vilaseñor et al., 2002). The presence of these two compounds could explain, at least in part, the good activity the hexane and dichloromethane extracts had in the second phase of the formalin test. Also, due to the high concentration of sitosterol, the hexane extract could have its good activity in the acetic acid-induced abdominal writhing assay, at least in part explained.

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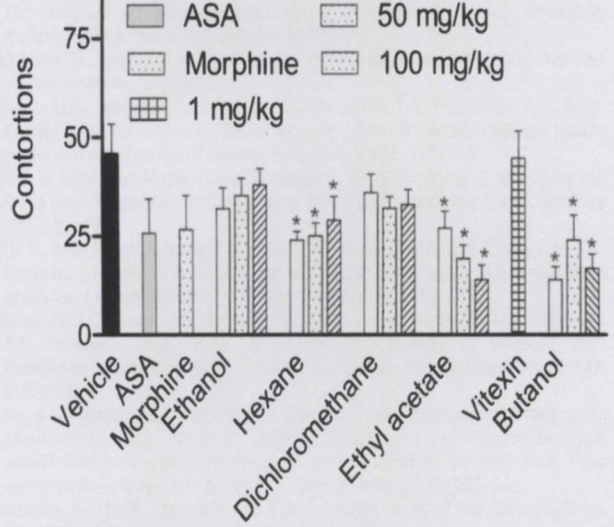

Fig. 3. Effects of *P. bleo* extracts on acetic acid-induced abdominal writhing. Animals were pretreated by oral administration of crude ethanol, hexane, dichloromethane and ethyl acetate extracts or vehicle (control). The results are presented as means ± S.D., $n=5$, * $p < 0.05$ significantly different when comparing fractions, morphine or ASA-treated groups with vehicle-treated group (ANOVA followed by Bonferroni's test).

Although the doses of *P. bleo* extracts (100 mg/kg) used were higher than established analgesic and anti-inflammatory drugs, it must be taken into account that the *P. bleo* was always used as a plant extract. It is not a pure drug or synthetic compound. It is formed by different molecules at different concentrations. Another important observation is that all the extracts were administered orally. Influence of pH in the stomach and liposolubility may interfere with their absorption by gastrointestinal tract limiting their bioavailability in reaching blood and tissue (Matheus et al., 2005; Rinaldi et al., 2009). Even though with all described factors, important activities were observed. Thus, it can be suggested that the plant could be a very inexpensive source of a new analgesic and anti-inflammatory drug in relation to rheumatism.

This study demonstrated moderate analgesic activity in a dose-dependent manner when tested in the above explained models and suggesting that the extract and/or its active principles might represent potential therapeutic options for the treatment of pain related diseases. The doses used in this study are in accordance with the dose taken by an average man with 70 kg (Pinheiro et al., 2010), in terms of tea drinking per day, as this plant is consumed as water infusion. The anti-nociceptive effects showed in this study supports the ethnomedical use of this plant to treat rheumatism and inflammations, and contribute to the knowledge of the chemical composition in natural products.

In order to evaluate the possibility of extracts develop toxic effect after oral administration, mice received 500 mg/kg of extracts. After 15 days of a single administration, treated mice did not show behavioral alterations and no lesion or bleedings in stomachs were observed (data not shown). This confirms that the extracts have low toxicity profiles, which indicate therapeutic safety for the pharmacologically active doses.

4. Conclusion

Vitexin (1), a *C*-glycosylflavonoid isolated for the first time from *P. bleo* is not the responsible for the anti-nociceptive active of the ethyl acetate extract. The occurrence of *C*-glycosylflavonoids in the presence of sitosterol, ethyl acetate showed the lowest number of contortions in the acetic acid-induced test, while butanol extract showed higher AUC than morphine in the hot plate test. Further investigation should be done to identify the responsible compound(s) and their mechanisms of actions.

Uncited references

; Franzotti et al. (2000), Gupta et al. (1996), Murillo, Meléndez-Martínez (2010), Philip et al. (2009), Rüegg et al. (2006), Tan et al. (2005).

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Isolated from *Choisya ternata*

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Abstract

One novel compound was isolated from the hexane extract of *Choisya ternata* Kunth. leaves, choisyaternatine (**1**), together with the known compounds tecleamaniensine A (**2**), lup-20(29)-en-3*β*-ol (lupeol) (**3**), lup-20(29)-en-3*β*,24-diol (**4**), *β*-sitosterol glucoside (**5**), and skimmianine (**6**), plus the following known compounds from the dichloromethane extract, choisyine (**7**) and 6-methoxy-7,8-methylenedioxycoumarin (**8**). Compounds **2–5** are reported for the first time in this species. The structures of isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. The structure of choisyaternatine was further confirmed based on X-ray data analysis.

Key words

Choisya ternata · Rutaceae · choisyaternatine · alkaloids · triterpenes

Choisya ternata Kunth. (Rutaceae) is a bushy shrub with a maximum height of 2 meters. It is an ornamental, with persistent leaves and white flowers resembling those of orange trees. Its English name is "Mexican orange" because it originated from the central and southern mountains of Mexico. It is widely cultivated in its native country and known as Hierba del Clavo, Flor del Clavo, Clavillo, and Clavo de Olor. There was not much ethnobotanical knowledge of this species. However, in 1895, Boudoresques discovered the first pharmacological test by experimenting with the effects of the aqueous extract of the leaves on himself, which he later found to be tonic and appetizing. In 1923, Standley reported that Mexican people employed an infusion of the leaves for their antispasmodic and "stimulative" properties [1].

A wide structural diversity of secondary metabolites occur in plants belonging to the Rutaceae, particularly *Choisya* species, most noteworthy of which are the alkaloids, derived from anthranilic acid and tyrosine, limonoids, and coumarins [2], while pentacyclic triterpenes are not common [3]. Several phytochemical studies have shown that *C. ternata* contains numerous quinoline alkaloids identified as skimmianine, choisyine, evoxine, kokusaginie, dictamnine, and lunacrine [1,4]. Besides, two dihydrofuroquinoline alkaloids, balfourodinium and platydesminium, have been isolated from leaf extracts [4–6]. In addition to that, 7-isopentenyloxy-γ-fagarine has been isolated and eight new members of the furoquinoline family have been synthesized from this plant [7].

Recently, an in-depth evaluation by our group of the essential oil composition from the leaves of *C. ternata* led to the identification of a new minor compound (ternanthrin) with potential antinociceptive activity. As the new compound was a minor compound in the essential oil, its synthesis has been performed to allow pharmacological evaluation as well as unequivocal structure determination [9].

As part of our ongoing search for molecules with structural and biological diversity from traditional medicine, a phytochemical investigation of the nonvolatile extracts from the leaves of *C. ternata* has been performed, which led to the isolation of a novel quinoline alkaloid, choisyaternatine (**1**), new compounds to the species, tecleamaniensine A (**2**), lupeol (**3**), lup-20(29)-en-3*β*,24-diol (**4**), and *β*-sitosterol glucoside (**5**), together with known compounds to the species, skimmianine (**6**), choisyine (**7**), and 6-methoxy-7,8-methylenedioxycoumarin (**8**). Compound **1** has been isolated from the Plant Kingdom for the first time and its structure was further confirmed using X-ray single crystal diffraction crystallography experiments.

Compound **1** (choisyaternatine) was isolated as a colorless crystalline solid (252 mg) with a molecular formula of $C_{16}H_{19}NO_4$ as determined from EIMS analysis $[M + H]^+$ 290.1361. The UV spectrum of **1** had an absorption maxima at 229, 299, 342 nm, suggesting the presence of a quinoline moeity [10]. Its IR spectrum showed the absorptions at 3083 (C–H aromatic), 2942 (OH stretch carboxylic), 1563 (aromatic ring), 1406 (C-C stretch aromatic), and 1272 (C–O stretch carboxylic) cm^{-1}.

The ^1H NMR spectroscopic data of **1** (© **Table 1**) were closely comparable with those of prenyl-quinoline and quinolone derivatives isolated by Brown and coworkers [11]. The ^1H NMR spectrum suggested the presence of two methyl groups that appeared as singlets at δ_H 1.72 and 1.78, which correspond to the oxyprenyl group in the molecule. Two singlets at δ_H 3.90 and 4.04 are characteristic of methoxy groups at C-6 and C-8 in a quinoline alkaloid. A singlet at δ_H 6.68 corresponds to H-5, while three doublets at δ_H 4.65, 6.35, and 7.63 correspond to protons of H-1′, H-3, and H-4, respectively.

In the ^{13}C NMR spectrum, in addition to the nine carbon signals of the quinoline skeleton and two methoxy carbons, there are five carbon signals at δ_C 70.3 (C-1′), 119.9 (olefinic carbon of C-2′), 139.3 (C-3′), 25.8 (C-4′), and 17.9 (C-5′) that correspond to the oxyprenyl moiety, with the two latter ones at 25.8 and 17.9 comprising the *gem*-dimethyl carbons. The resonance of C-2 is found to be slightly deshielded at δ_C 160.6, corroborating to its condition as being attached to more electronegative atoms, O and N. Based on Dept-135, the presence of methylene protons at C-1′ can be assured (δ_H 4.65 that appeared as a doublet $J = 10.92$). The protonated aromatic C atoms (C-3, C-4, and C-5) are identified by the characteristic doublets of H-3 and H-4 at δ_H 6.35 and 7.63, respectively, and a singlet of H-5 at δ_H 6.68.

The assignation of all proton and carbon NMR signals was made possible only by the use of 2D-NMR (^1H-^1H COSY and HETCOR spectra) as well as comparison with literature data. The ^1H-^1H COSY spectrum showed a small coupling between the olefinic protons resonance of H-1′ (δ_H 4.65) and H-2′ (δ_H 5.57), and between the protons H-3 (δ_H 6.35) and H-4 (δ_H 7.63). The olefinic proton H-2′ (δ_H 5.57) showed no correlations with the methoxy protons of C-6 (δ_H 3.90) and C-8 (δ_H 4.04). The resonance of H-5

Carbon	δ_H (ppm)	δ_C (ppm)	DEPT	HMBC*
1			C	
2		160.6	C	
3	6.35 (H, d, J = 14.22)	115.1	CH	C-2
4	7.63 (H, d, J = 14.22)	143.5	CH	C-2, C5
4a		114.4	C	
4b		142.9	C	
5	6.68 (H, s)	103.6	CH	C-4, C-6
6		150.7		
	3.90 (3H, s, OCH_3)	56.3	CH_3	C-6
7		144.9	C	
8		141.7		
	4.04 (3H, s, OCH_3)	61.7	CH_3	C-8
1′	4.65 (2H, d, J = 10.92)	70.3	CH_2	C-2′, C-3′, C-7
2′	5.57 (H, t)	119.9	CH	
3′		139.3	C	
4′	1.72 (3H, s, CH_3)	25.8	CH_3	C-2′, C-3′
5′	1.78 (3H, s, CH_3)	17.9	CH_3	C-2′, C-3′

Table 1 ^1H, ^{13}C and HMBC NMR data of compound **1** (600 MHz for ^1H- NMR and 125 MHz for ^{13}C-NMR, $CDCl_3$).

* Long-range ^1H-^{13}C correlation, protons to carbons of HMBC

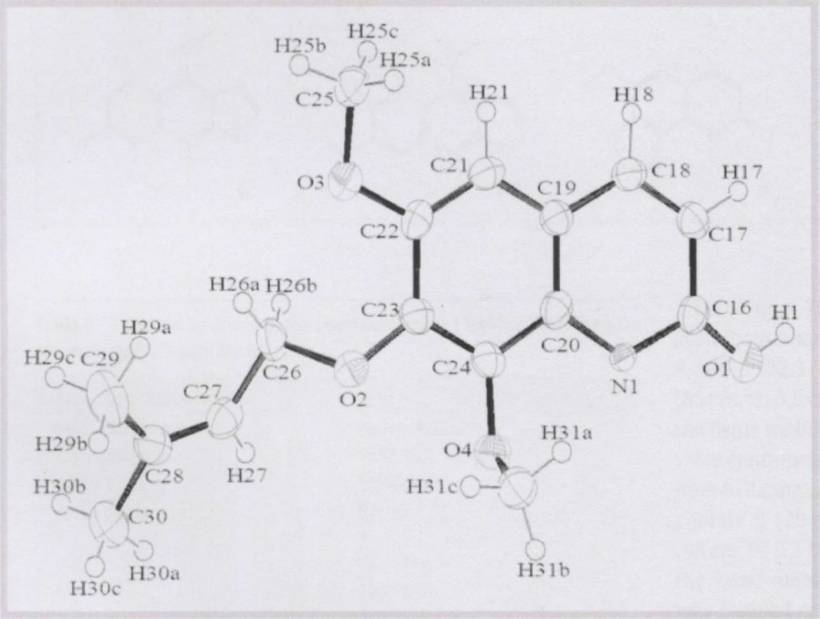

Fig. 1 Ortep representation of the X-ray crystal structure of compound **1** with thermal elipsoids set at 50% probability.
Crystal data: $^aR_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, $wR_2 = [\Sigma w (F_o^2 - F_c^2)^2/\Sigma w (F_o^2)^2]^{1/2}$.

(δ_H 6.68) is not coupled with any other proton resonances. HSQC spectrum allowed the assignment of all protonated carbons as shown in **Table 1**.

Further assignments based on a long-distance relationship were performed by HMBC analysis, which showed a strong correlation between the methyl protons (H-4′ and H-5′; 1.72 and 1.78 respectively), and C-2′ (δ_C 119.9) and C-3′ (δ_C 139.3). H-1′ (δ_H 4.65) shows interactions with C-2′ (δ_C 119.9), C-3′ (δ_C 139.3), and C-7 (δ_C 144.9). The methoxy protons at the position C-6 (δ_H 3.90) correlates with C-6 (δ_C 150.7), while methoxy protons at C-8 (δ_H 4.04) correlated with C-8 (δ_C 141.7). H-5 (δ_H 6.68) strongly interacts with C-4 (δ_C 143.5) and C-6 (δ_C 150.7). H-4 (δ_H 7.63) correlates with C-2 (δ_C 160.6) and C-5 (δ_C 103.6), while H-3 (δ_H 6.35) correlates with C-2 (δ_C 160.6). These findings strongly suggested the structure of compound **1** as shown in **Fig. 1**, which was later named choisyaternatine after crystallographic analysis (**Table 2**).

In addition, four known compounds were isolated for the first time in this species from the hexane extract, tecleamaniensine A, lupeol, lup-20(29)-en-3β,24-diol, and β-sitosterol glucoside, along with three known compounds from the dichloromethane extract, skimmianine (also isolated from the hexane extract), choisyine, and 6-methoxy-7,8-methylenedioxycoumarin. Their structures (**Fig. 2**) were identified by comparison of the NMR spectroscopic data and the physical data with those previously reported in the literature [11–17].

Materials and Methods

Leaves of *C. ternata* Kunth. were collected from the Trinity College Botanical Garden, Dartry, Dublin in October 2008 (living plant accession number 19850023) and its voucher specimen (SW 10–52) was deposited in the Herbarium of Trinity College, Dublin.

Eight hundred grams of ground dried leaves of *C. ternata* were extracted using a Soxhlet apparatus with ethanol (1.5 L) for 72 h.

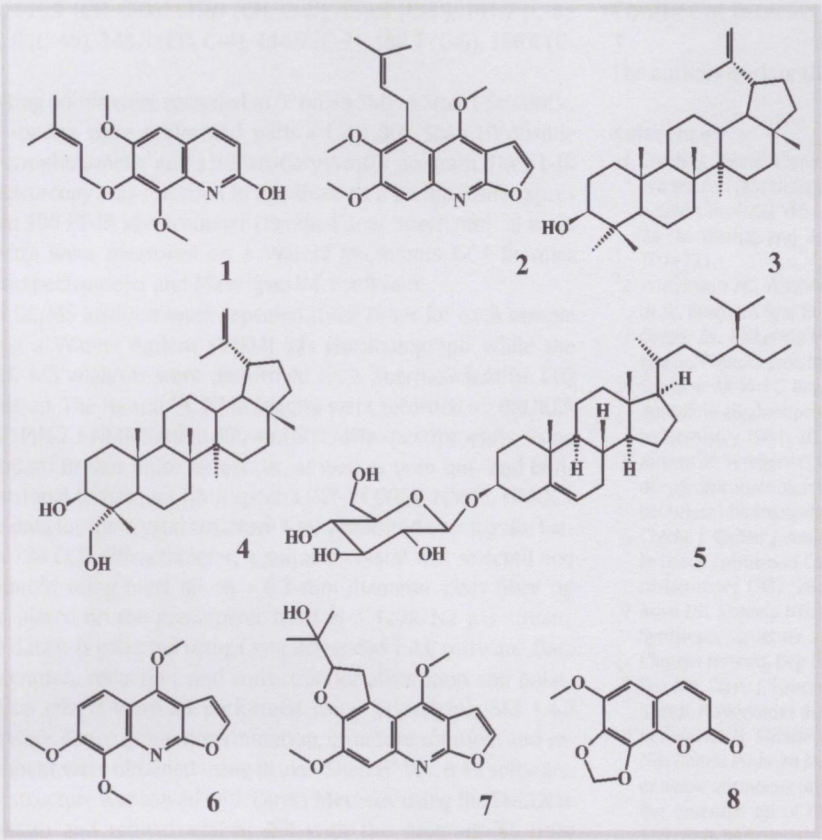

Fig. 2 Compounds isolated in the present study; choisyternatine (**1**), tecleamaniensine A (2), lupeol (**3**), lup-20(29)-en-3β,24-diol (**4**), β-sitosterol glucoside (**5**), skimmianine (**6**), choisyine (**7**), and 6-methoxy-7,8-methylenedioxycoumarin (**8**).

Table 2 Proposed structure of the novel compound **1** isolated from the hexane extract of *C. tenata* leaves.

Structure compound 1	
Molecular formula	$C_{64}H_{76}N_4O_{16}$
MW, g/mol	1157.29
System	Monoclinic
Space group	P2(1)/c
Z	1
a, Å	14.811(3)
b, Å	12.559(3)
c, Å	7.7975(16)
α	90°
β	97.45(3)°
γ	90°
U, Å^3	1438.25(5)
ρ, g/cm^3	1.336
T	150 K
μ (Mo Kα), mm^{-1}	0.096
Number of reflections with	11100
$^aR_1$	0.1253
wR2 [$I > 2\sigma(I)$]	0.3341
Gof	1.693
CCDC	865486

The crude ethanol extract of the *C. ternata* leaves was then treated with activated charcoal (Sigma) overnight to eliminate chlorophyll.

The crude extract (102.01 g) was subjected to a liquid-liquid partition yielding hexane (42.88 g), dichloromethane (9.73 g), ethyl acetate (7.02 g), and butanol (13.91 g) extracts. The hexane extract was chromatographed over a silica gel column (36 cm, Ø: 7.5 cm/185 g, 70–230 mesh; Merck) eluted with a hexane : ethyl acetate : methanol gradient system to give 6 fractions. Fractions 4 and 5 (12.31 g) were combined and subjected to another CC (85 cm, Ø: 3.5 cm; Si gel: 60 g, 70–230 mesh; Merck) eluting with the same mobile phase to give 13 fractions. Fractions 2, 5, and 11 were continuously cleaned with methanol yielding white precipitate **4** (22 mg), a clear crystalline solid **1** (252 mg), and white precipitate **5** (28 mg), respectively. Fraction 3 was purified by CC (85 cm, Ø: 3.5 cm; Si gel: 60 g, 70–230 mesh; Merck) eluting with the same mobile phase as above to give 8 fractions. Fraction 2 was cleaned continuously with methanol to give white precipitate **3** (52 mg). Both fractions 7 and 8 were observed to contain yellowish precipitate that after cleaned with methanol, yielded yellowish precipitate **6** (126 mg) and yellowish gum **2** (18 mg), respectively. Furthermore, a dichloromethane extract (9.73 g) was also subjected to a column chromatography (85 cm, Ø: 3.5 cm) over silica gel (65 g, 70–230 mesh; Merck) eluted with a hexane : ethyl acetate : methanol gradient system to give 10 fractions. White precipitates were collected from fraction 3, after cleaning with methanol, to give white precipitate **8** (15 mg). Yellowish precipitates and crystals collected from fractions 8 and 9 gave **6** (221 mg) and **7** (19 mg), respectively.

6,8-Dimethoxy-7-(3-methylbut-2-enyloxy)quinolin-2-ol (choisyaternatine) (**1**): Colorless crystalline solid; R_f: 0.44 (hexane : ethyl acetate; 6 : 4); m.p.: 88–92 °C; UV (CH_2Cl_2) λ_{max} (log ε) 229, 299, 342; IR ν_{max}: 3083, 2942, 1715, 1563, 1406, 1272 cm^{-1}; EIMS for $C_{16}H_{19}NO_4$ *m/z*; 289 [M]$^+$; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.72 (3H, s, CH_3-4′), 1.78 (3H, s, CH_3-5′), 3.90 (3H, s, OCH_3-C-6), 4.04 (3H, s, OCH_3-C-8), 4.65 (2H, d, J = 10.92, H-1′), 5.57 (H, t, H-2′), 6.35 (H, d, J = 14.22, H-3), 6.68 (H, s, H-5), 7.63 (H, d, J = 14.22, H-4); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 17.9 (CH_3, C-5′), 25.8 (CH_3, C-4′), 56.3 (OCH_3, C-6), 61.7 (OCH_3, C-8), 70.3 (CH_2, C-1′), 103.6 (CH, C-5), 114.4 (C-

4a), 115.1 (CH, C-3), 119.9 (CH, C-2′), 139.3 (C-3′), 141.7 (C-8), 142.9 (C-4b), 143.5 (CH, C-4), 144.9 (C-7), 150.7 (C-6), 160.6 (C-2).

Melting points were recorded in °C on an SMP-1 Stuart Scientific. UV spectra were performed with a Cary 300 Scan UV-Visible spectrophotometer and a Varian-CaryWinUV program. The FT-IR spectroscopy was recorded in KBr discs on a Perkin-Elmer Spectrum 100 FT-IR spectrometer (Perkin-Elmer Spectrum). EI mass spectra were measured on a Waters Micromass GCT Premier mass spectrometer and Mass Lynx V4.1 software.

The GC/MS analyses were repeated three times for each sample using a Waters Agilent 6890 N gas chromatograph while the HPLC-MS analyses were performed on a ThermoScientific LTQ Orbitrap. The ^{1}H and ^{13}C NMR spectra were recorded on BRUKER TOPSPIN 2.1 NMR System 400 and 600 MHz spectrometers, using standard Bruker pulse sequences, as well as both uni- and bidimensional techniques NMR spectra (^{1}H-^{1}H COSY, HMBC, HMQC). The data for the crystal structure **1** was collected on a Rigaku Saturn 724 CCD diffractometer. A suitable crystal was selected and mounted using inert oil on a 0.3-mm diameter glass fiber tip and placed on the goniometer head in a 123K N2 gas stream. The data was collected using Crystalclear-SM 1.4.0 software. Data integration, reduction, and correction for absorption and polarization effects were all performed using Crystalclear-SM 1.4.0 software. Space group determination, structure solution, and refinement were obtained using Bruker Shelxtl* Ver. 6.14 software. The structure was solved with Direct Methods using the SHELXTL program and refined against $IF^{2}I$ with the program XL from SHELX-97 using all data. Non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were placed into geometrically calculated positions and refined using a riding model. The Software Reference Manual, version 5.625; Bruker Analytical X-Ray Systems, Inc.: Madison, WI, 2001 and Sheldrick, G.M. SHELXTL were used for data collection, processing, structure solution, and refinement. The crystal data from X-ray analysis was deposited to the Cambridge Crystallographic Data Centre (CCDC).

Crystallography data of **1**: Crystallographic data of compound **1** has been deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 865486. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK, (Fax: +44/1223/336033 or email: deposit@ccdc.cam.ac.uk)

Column chromatography was performed using silica gel 60 (70–230 mesh; Merck). For thin-layer chromatography (TLC), aluminium sheets of silica gel 60 F_{254}, (230–400 Mesh ASTM, 20 cm × 20 cm, layer thickness 0.25 mm; Merck) were used for analytical purposes and the compounds were visualized under ultraviolet light. Solvents for extraction and chromatography were analytical grade, except for ethanol, which was commercial grade. All reagents were purchased from Sigma-Aldrich unless otherwise stated.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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