ANTIMICROBIAL AND DNA-BINDING ACTIVITY OF ALKALOIDS FROM *GLYCOSMIS PENTAPHYLLA* (RETZ.) DC

BY

NURHAYA BINTI MD TAIB

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International Islamic University Malaysia

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ABSTRACT

Glycosmis pentaphylla (Retz.) DC. has been traditionally used to cure various illnesses and conditions such as fever, eczema and rheumatism. The present study was designed to evaluate the antimicrobial and DNA binding activities of the alkaloids isolated from the plant. Leaves and stem bark of *G. pentaphylla* were extracted by continuous extraction using hexane and acetone prior to acid base extraction. Screening of alkaloids available was done by Thin Layer Chromatography (TLC). Fractionation by column chromatography was employed to separate the extract into fractions of alkaloid compounds. Antimicrobial active alkaloids were screened by TLC Agar Overlay Assay against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 90028. The alkaloids were isolated by using column chromatography and were authenticated by comparing their TLC profile, maximum wavelength for UV absorption in methanol and melting point to that of authentic alkaloids. The identification of the alkaloids was further confirmed by their NMR spectral data. The antimicrobial activity of the alkaloids was determined quantitatively by means of their Minimum Inhibitory Concentration (MIC) and Minimum Microbiocidal Concentration (MMC) by using broth microdilution assay. Antimicrobial combination effects between the active alkaloids and selected antimicrobial agents, which were erythromycin and vancomycin was studied by cherkerboard assay and determined by their Fractional Inhibitory Concentration Index (FICI). DNA binding activity of the alkaloids was investigated by method using restriction enzymes and specially designed 1.5 kb DNA fragment. Three antimicrobial active alkaloids labeled as GP3-3, GP6-2 and GP11-2 were detected, isolated, authenticated and identified as arborinine, arborine and skimmianine respectively. Antimicrobial activity of arborinine and arborine were ranged between 250 µg/mL and 1000 µg/mL. Arborinine and arborine showed lowest MIC values of 250 µg/mL and 500 µg/mL respectively which were against *C. albicans*. Arborine displayed high MIC values against all other microbes while arborinine was weak against *S. aureus*, *E. coli* and *P. aeruginosa*. No synergistic effect was observed from the combination of the alkaloids with the selected antimicrobial agents against Gram positive, *S. aureus*, Gram negative *E. coli* and fungi, *C. albicans*. However, partial synergy was reported for all interactions between arborine and antimicrobial agents against *S. aureus* and interaction between arborinine and ketoconazole against *C. albicans*. Additive effect was only produced when arborine was combined with vancomycin while antagonism was observed for the interaction between arborine and ciprofloxacin when tested against *E. coli*. All alkaloids displayed photo activated DNA binding activity by strength in the order of arborine, arborinine and skimmianine.

ملخص البحث

وقد استخدمت *Glycosmis pentaphylla* (Retz) DCلعلاج مختلف الأمراض والأوضاع على نحو تقليدى مثل الحمى، والأكزيما، والروماتيزم. وقد تم تصميم هذه الدراسة لتقييم المضادة للميكروبات والأنشطة الملزمة ل DNA من القلويدات معزولة عن المصنع. وقد تم انتزاع أوراق الشجرة واللحاء الجذعية ل*G. pentaphylla* عن طريق استخراج مستمر استخداما الهكسان والأسيتون قبل استخراج على قاعدة حمضية. وقد تم فحص القلويدات المتوفرة بالطبقة الرقيقة اللونى(TLC). وقد تم توظيف التجزئة من قبل العمود اللونى لفصل استخراج فى أجزاء من مركبات قلويد. وقد تم فحص قلويدات نشطة مضادة للميكروبات بواسطة آجار تراكب الفحص لTLC خلاف المكورات العنقودية الذهبية ATCC 25923، *Escherichia coli* ATCC 25922و *Candida albicans* ATCC 90028 *Staphylacoccus aureus*. وتم عزل قلويدات باستخدام العمود اللونى وتمت المصادقة من خلال مقارنة الملف لTLC، والحد الأقصى لامتصاص UV فى الميثانول و نقطة الإنصهار إلى أن من قلويدات أصلية. وقد زاد تآكد التعرف على قلويدات بواسطة البيانات الطيفية لNMRلها. وتم تحديد نشاط مضادات الميكروبات من قلويدات كميا عن طريق التركير على الحد الأدنى للمثبطة Minimum Inhibitory Concentration (MIC)و التركير على الحد الأدنى لمبيد الأحياء الدقيقة Minimum Microbiocidal Concentration (MMC)باستخدام مرق فحصا لbroth microdilution assay. وتمت دراسة آثار مزيجة للميكروبات بين قلويدات نشطة والعوامل المختارة لمضادات الميكروبات التى كانت الاريثروميثين وفانكومايسين من خلال فحص الشطرنج وتم تحديدها من خلال مؤشر التركيز على التجزئة المثبطة Fractional Inhibitory Concentration Index (FICI).وتم تحقيق النشاط الملزم DNA من قلويدات بواسطة طريقة استخدام إنزيمات التقييد والمصممة شظية1.5كيلو بايت DNA بالخصوص. وتم الكشف والعزل والمصادقة عن ثلاثة قلويدات نشطة مضادة للميكروبات التى وصفت GP3-3، وGP6-3، GP11-2، وتم اعتبارها ب arborinine، وarborine، و skimmianine على التوالى. وتم التراوح لنشاط مضادات الميكروبات من arborinine، وarborine بين 250 ميكروغرام/مل و1000 ميكروغرام/مل. وأظهرت arborinine و arborine أدنى القيم لMIC من 250 ميكروغرام/مل و500 ميكروغرام/مل على التوالى، التى كانت ضد *C. albicans*. وعرض arborine القيم العالية لMIC ضد كل الميكروبات الأخرى بينما arborinineكان ضعيفا ضد *S. aureus*، و *E. coli*، و *P. aeruginosa*. ولم يلاحظ أى تأثير متناغم من مزيج من قلويدات مع عوامل مضادات الميكروباتضد

الغرام إيجابية .*S. aureus*، والغرام سلبية *E. coli*، والفطريات *C. albicans*. ومع ذلك، أفيد التآزر الجزئى لجميع التفاعلات بين arborine و عوامل مضادات الميكروبات ضد *S. aureus*، والتفاعل بين arborinine و ketoconazole ضد *C. albicans*.وقد أنتج التأثير المضاف فقط لما تم جمع بين arborine و فانكومايسين، و فى حين لوحظ العداء للتفاعل بين arborine و سيبروفلوكساسين عند اختباره ضد *E. coli*. وزودت صورة جميع قلويدات معروضة النشاط الملزم لDNA، من قبل قوة فى ترتيب arborine، arborinine، و skimmianine.

APPROVAL PAGE

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

……..
Norazian Mohd Hassan
Supervisor

……..
May Khin Soe
Co-Supervisor

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

……..
Siti Zaiton Mat So'ad
Internal Examiner

……..
Muhamad Zakaria
External Examiner

This dissertation was submitted to the Department of Pharmaceutical Chemistry and is accepted as a fulfilment of the requirement for the degree of Master of Science in Pharmaceutical Chemistry.

……..
Nik Mohd Idris Nik Yusoff
Head, Department of
Pharmaceutical Chemistry

This dissertation was submitted to the Kulliyyah of Pharmacy and is accepted as a fulfilment of the requirement for the degree of Master of Science in Pharmaceutical Chemistry.

…….
Siti Hadijah Shamsuddin
Dean, Kulliyyah of Pharmacy

DECLARATION

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

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Signature Date

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LIST OF ABBREVIATIONS

UV	Ultraviolet
TLC	Thin Layer Chromatography
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bacteriocidal Concentration
MMC	Minimum Microbiocidal Concentration
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
INT	Iodonitrotetrazolium
NMR	Nuclear Magnetic Resonance
ppm	part per million

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LIST OF SYMBOLS

g	gram
µg/mL	microgram per mililiter
OCH_3	Methoxyl
$N\text{-}CH_3$	N-methyl
C	Carbon
Hz	Hertz
≤	same or less than
≥	same or more than
<	less than
>	more than
^{1}H NMR	Proton NMR
^{13}C NMR	Carbon NMR
δ	Chemical shift
d	doublet
dd	doublet doublet
J	Coupling constant
t	triplet
m	multiplet
s	singlet

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CHAPTER ONE

INTRODUCTION

1.1 PLANTS AS POTENTIAL SOURCE OF ANTIMICROBIAL AGENTS

World-wide, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and well-being. They may become the base for the development of new drugs; either semi synthetic or synthetic, and lead to the treatment of various diseases and illnesses including the infectious disease which is the number one cause of death accounting for approximately one-half of all deaths in tropical countries (Iwu, Duncan and Okunji, 1999; Heilmann and Bauer, 1999). This phenomenon demonstrates the increased interest for research in antimicrobial activity of medicinal plants.

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Cowan (1999), listed two reasons on why would clinical microbiologists were interested in antimicrobial plant extracts. First, it is likely that phytochemicals will eventually developed into antimicrobial drugs prescribed by physicians as effective lifespan of any antibiotic is limited and new sources especially plants are also investigated. Second, the public became increasingly aware of problems of over prescription and misuse of tradition antibiotic. Moreover, many people want more autonomy over their medical care. As massive amount of plant compounds with unreliable purity is readily available from the herbs suppliers and natural food stores, self medication with these substances is routine. Thus the needs of investigating the substances and providing scientific information on the basis of the efficacy and safety of the substances are unavoidable.

A study on antibacterial agents from *Tragia involucrate,* a widely distributed shrub in India which is used for its different parts by the “Malaiali” tribe in Western

Ghat India to cure wound and skin problems revealed that the compounds isolated from the plant namely shellsol demonstrated efficient killing of wound causing bacteria; *Staphyllacoccus aureus*. The information on the antibacterial properties of the constituents explained some scientific basis to its usage in traditional medicine(Samy, Gopalakrishnakone, Houghton and Ignacimuthu, 2006). Two diterpenes of serrulatanes class served as the principal constituents for antibacterial activity of indigenous Australian medicinal plant, *Eremophila duttonii* F. Muell (Myoporaceae) and a hydrolated furanosesquiterpenes with mild antibacterial activity was identified and isolated following the screening by bioautography assay. It was reported that all isolated compounds from the same plant tested for the Minimum Inhibitory Concentration (MIC), showed appreciable activity against Gram positive organism tested except for one of the serrulate compound which is the serrulat-14-en-3,7,8,20-tetraol and a hydrolated furosesquiterpene compound; 4-hydroxy-4-methyl-1-(2,3,4,5-tetrahydro-5-methyl[2,3'-bifuran]-5-yl)pentan2-one against *Staphylacoccus epidermis* (ATCC 12228) with high MIC value of 1.5 mg/mL (Smith, Tucker, Watson and Jones, 2007).

Among plants constituents, alkaloids gained their reputation as common active constituents in medicinal plants and herbs. Though it is known that alkaloids appeared abundant; chemically and structurally various in types, in plants yet there is also some of its types which are still being discovered (Ong, 2006). The quinoline alkaloids are a group of alkaloids which having diverse structural types. The 4-oxygenated quinolone and 2-substituted 4-quinolone are the types of quinoline alkaloids that possess the chromophore structures which are similar to the pharmacophore of the conventional 4-quinolone antimicrobial agents. Therefore, it is presumed that these types of natural quinoline alkaloids could also share the same activity as the

conventional quinolone antimicrobial agents. Although these conventional agents demonstrate an excellent treatment against so many infectious diseases, the emergence of quinolone resistance against some microbes has been a disturbing feature of microbial infection (Shigemura, et al., 2003). There are an extensive numbers of Rutaceous species which have been reported to possess antimicrobial activity. In this study, the Rutaceous species namely *Glycosmis pentaphylla* (Retz.) DC. was investigated for its antimicrobial and DNA-binding activities.

1.2 DESCRIPTIONS OF *GLYCOSMIS PENTAPHYLLA* (RETZ.) DC.

Glycosmis pentaphylla Retz. DC., is a small tree or shrub classified under the genus *Glycosmis* within the tribe Clauseneae of Auratioideae subfamily of Rutaceae (Daniel, 2006). It is well distributed from India, Malaysia, and Southern China to the Philippines islands where it commonly found in tropical forest at low altitudes (Wang, Di, Yang, Li, Wang and Hao, 2006), Bangladesh, Sri Langka eastward to Myanmar, Sumatra, Java (Shams-Ud-Doha, Akter, Al Mahmud, Apu and Howlader, 2012) and West Australia. It occurs only in Eastern and Western Ghatt and Orissa in India whereas in Malaysia, this common village shrub is commonly found sprawling in orchards and rice fields in North Ipoh, Kedah, Perlis, Kelantan and Terengganu. This plant is locally known in Malaysia as Nerapan, Nyerapeh, Kenapeh, Terapeh and Terapai according to the locality where it was found (Corner, 1988) and commonly called as orange berry (English) (Sreejith, Praseeja and Asha, 2012; Shams-Ud-Doha, et al., 2012). The plant is portrayed to be small tree (1.5 m – 5 m) or shrub with green leaves that is native to its environment.

A detailed botanical description of this plant were found written in a book entitled Wayside Trees of Malaya by an English taxonomist Edred John Henry Corner

depicted this small shrub as;

> A sprawling, evergreen shrub up to 12 feet high: young leaves hoary. Leaves 6-14" long with 2-3 pairs of more or less upstanding leaflets and a terminal one, the crushed leaves with a lemony smell: leaflets 2 ½ - 8 x 1-2 ½", narrowly elliptic, tapered to each end, the apex rather blunt, with 8-14 pairs of rather inconspicuous side veins : leaflets-stalks 1-2" long. Flowers ¼-1/3" wide, white, not or slightly fragrant, in axillary panicles 206" long, generally several on a twig. Berries 1/3" wide, ripening white then translucent pink, slimy, edible, sweetish with a resinous taste.
>
> (Corner, 1988)

1.3 MEDICINAL USES OF *G. PENTAPHYLLA*

G. pentaphylla has been long known to possess medicinal property and widely used in traditional medicine as a therapeutic preparation against various illnesses according to locality. In several parts in India, the plant is used to cure cough, rheumatism, anaemia, jaundice and the leaves juice is used to treat fever, liver complaints, while leaves paste were applied on skin to treat eczema and skin disease. Meanwhile, roots are used to cure inflammation and stomach pain and also given to women after childbirth to aid increase the appetite (Sreejith, et al., 2012). The same treatment is also given to women after childbirth in Vietnam by giving them infusion of roasted leaves and root. In Vietnamese folk medicine, the leaves are considered appetitive and stomachic. In peninsular Malaysia, *Glycosmis*; including *G. parviflora* (Sims) Little, *G. puberula* Lindley ex Oliv, and *G. pentaphylla* (Retz.) DC. were used as the ingredients of various medicinal mixtures. An infusion of leaves and roots is given after childbirth as a protective medicine (Kamarudin & Abdul Latiff, 2002; Uddin, S. B., 2011).

(a)

(b)

Figure 1.1: *Glycosmis pentaphylla* (Retz.) DC.; (a) the aerial parts (b) clear view of the leaves

1.4 ALKALOIDS OF RUTACEOUS SPECIES

Alkaloids are nitrogen containing organic substances of natural origin with greater or lesser degree of basicity (Hesse, 2002). Alkaloids are commonly distributed among species of the Amarryllidaceae and Rutaceae families (Michael, 2005; Da Silva, Soares, Fernades and Vieria, 2007). It is well known to be toxic to human, yet exhibited a number of pharmacological activities which plants containing these compounds were acknowledged to have high medicinal value. It is believed that this plants product acted particularly in plant's defense mechanism against herbivores (Wink, Schmeller and Latz-Bruning, 1998), making its numerous presence in plants worthwhile as this toxic-compound-to-human and herbivores also seems to be toxic to insects, bacteria and fungi (Queener, et al., 1991). Rutaceous alkaloids occur in wide structural diversity.

The variety of chemical structure of these alkaloids is due to its biosynthetic pathways. Biosynthetically, the nitrogen atom which occurs within the heterocyclic ring origin of the compound is innately in the structure of the amino acids from which they came from or are the result of circularization of the given amino acids (Cseke, et al., 2010). The amino acids involved include various amino acids precursors, such as tryptophan, tyrosine, histidine and anthranillic acids **(1)** (Tillequin, 2007). The position of nitrogen atom in the ring structure of the compound and its origin lead to the formation of several structural nucleus or ring systems which imparts the nature of each molecule thus responsible for the satisfactory classification of alkaloids of various chemical structures (Saxena, 2007). Michael (2003; 2004) listed some available ring systems for classifying groups of alkaloids such as indolizidine and quinolizidine based system, also quinoline **(3),** quinazoline **(2)** and acridone based system. Others include phenylalanine group, pyrrolidine group, pyridine and