STUDY OF ANTIMICROBIAL AND DNA-BINDING ACTIVITIES OF ALKALOIDS FROM THE LEAVES OF *RUTA ANGUSTIFOLIA* (L.) PERS

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science in Pharmaceutical Chemistry

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OCTOBER 2013

ABSTRACT

Significant finding in searching of new drugs from Rutaceae alkaloids has led to an important strategy to overcome the problems of resistance and side effects associated with conventional antibiotics. In this study, the leaves of *Ruta angustifolia* (L.) Pers. were extracted and the alkaloidal extracts were fractionated by using column chromatography. Through bioassay-guided isolation, combined fraction of $R3^3$ and $R4^8$, fraction RC-8 and Rd-10 yielded a total of three antimicrobial active alkaloids. These isolated alkaloids were then identified by means of their Thin Layer Chromatography profile, melting point and maximum wavelength for UV absorption in methanol ($UV\lambda_{max}$.MeOH) in comparison with that of authentic alkaloids. The identification was further confirmed by their ^1H NMR and ^{13}C NMR spectroscopic data. The alkaloids were characterized as acridone, furoquinoline and 4-quinolone with trivial names arborinine, skimmianine and graveoline respectively. The antimicrobial activity of arborinine and graveoline was tested against *Staphylococcus aureus*, *Enterococcus fecalis*, *Helicobacter pylori*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* of ATCC strains. Broth microdilution assay of both alkaloids gave Minimum Inhibitory Concentration (MIC) values ranging from 250 µg/ml to 1000 µg/ml. Minimum Bactericidal Concentration (MBC) values were recorded at 1000 µg/ml and more than 1000 µg/ml. Antibacterial combination effects between graveoline and either erythromycin or vancomycin were studied against *S. aureus*, *E. fecalis* and *E. coli* by means of Fractional Inhibitory Concentration (FIC) index. The tested combinations against *S. aureus* resulted in synergistic and additive effects with FIC indices of 0.57 to 0.75, respectively. All tested combinations against *E. fecalis* and *E. coli* resulted in additive effect with FIC indices ranged from 0.5 to 2.0. Antifungal combination between arborinine and ketoconazole against *C. albicans* showed additive interaction with FIC index of 0.75. Bacterial DNA-binding properties of the three alkaloids were investigated against double-stranded DNA with various restriction enzymes. The investigation revealed that the alkaloids mostly affect the cleavage activity of the restriction enzymes which contains 5'-TpA sequence rather than 5'-ApT sequence in their recognition pattern and potential crosslink sites under UV exposure. These isolated alkaloids were screened to possess antimicrobial activity through DNA synthesis inbition mechanism and might need to be combined with other agent to enhance its inhibitory effects.

ملخص البحث

قاد الاكتشاف الهام لقلويدات الـRutaceae في البحث عن أدوية جديدة إلى استراتيجيات هامة للتغلب على المقاومة الآثار الجانبية المرتبطة بالمضادات الحيوية التقليدية. في هذه الدراسة تم استخلاص أوراق نبات *Ruta angustifolia* (L.) Pers. وتجزئته بالطرق الكروماتوغرافية القياسية. من خلال العزل الموجه بالفعالية الحيوية، الجزء المجمع من $R3^{3}$ و $R4^{8}$، الجزء RC-8 و Rd-10 قادت إلى ثلاثة قلويدات فعالة كمضادات حيوية. أُجري لهذه القلويدات المعزولة اخيبارات الذاتية بواسطة كروماتوغرافية الطبقة الرقيقة، درجة الانصهار و الامتصاص الأعظمي للاشعة فوق البنفسجية في المتانول ($UV\lambda_{max}$-MeOH) بالمقارنة مع قلويدات مشابهة موثّقة. كما تم تأكيد الذاتية أيضاً بأطياف الـ^{1}H NMR و الـ^{13}C NMR وبذلك تم التأكيد على أن المركبات هي acridone، furoquinoline و 4-quinolone المسمى arborinine، skimmianine و graveoline على الترتيب. الفعالية المضادة للمكروبات تم اختبارها للـarborinine و graveoline ضد *Staphylococcus aureus, Enterococcus fecalis, Helicobacter pylori, Escherichia coli, Pseudomonas aeruginosa* و *Candida albicans* من سلالات ATCC. المعايرة التمديدية للمرق Broth microdilution assay لكلا القلويدين أعطت التركيز المثبط الأدنى (MIC) بقيمة تتراوح بين 250 µg/ml إلى 1000 µg/ml. التركيز الأدنى القاتل للبكتيريا (MBC) تم تقديره بـ1000 µg/ml وأكثر من 1000 µg/ml. تم مقارنة قيم الـMIC والـMBC للمركبات بتلك القيم الخاصة بالمضادات الحيوية المعيارية المسماة norfloxacin، ciprofloxacin، vancomycin، erythromycin و ketoconazole. تأثيرات المشاركة المضادة للبكتيريا للـgraveoline والـerythromycin أو الـvancomycin تم

اختبارها ضد *S.aureus, E. Fecalis* . و *E. Coli* بواسطة قرينة الـ Fractional Inhibitory Concentration (FIC). كل المشاركات المختبرة أعطت نتائج تأثيرات مؤازرة بقرينة الـFIC تراوحت بين 0.75 و 1.02. تأثيرات المشاركة المضادة للفطريات للـarborinine و الـketoconazole تم اختبارها ضد *C. albicans* وأظهرت تفاعلاً تآزرياً بقرينة FIC تساوي 0.5. خصائص الارتباط بالـDNA البكتيري للقلويدات الثلاثة تم دراستها ضد الـ double-stranded DNA مع تطبيق عدة restriction enzymes. تبين من الدراسة أن هذه القلويدات الثلاثة على الأغلب تؤثر على فعالية التقطيع للـrestriction enzymes التي تحتوي على تسلسل 5'- TpA بدلاً من تسلسل 5'-ApT في نموذج التعرف الخاص بهم والمواقع التشعبية المحتملة تحت التعرض للأشعة فوق البنفسجية. كخلاصة، نستنتج أن الفعالية المضادة للمكروبات لهذه القلويدات المعزولة هي من خلال آلية تثبيط اصطناع الـDNA وقد تحتاج إلى المشاركة مع مركبات أخرى لتعزيز التأثير المثبط.

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 thesis for the degree of Master of Science in Pharmaceutical Chemistry

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DECLARATION

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

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4/10/2013 ..... Date

This thesis is dedicated to my parents who have supported me all the way since the beginning of my studies. Also, this thesis is dedicated to my husband who has been a great source of motivation and inspiration. Finally, this thesis is dedicated to all those who believe in the richness of learning

ACKNOWLEDGEMENTS

In the name of Allah the Most Beneficent and the Most Merciful.

First and above all, I praise Allah for providing me this opportunity and granting me the capability to proceed successfully. This thesis appears in its current form due to the assistance and guidance of several people. I would therefore like to offer my sincere thanks to all of them.

Above all, this thesis would not have been possible without the help, support and patience of my principal supervisor, Assistant Professor Dr. Norazian Mohd Hassan, not to mention her advice and unsurpassed knowledge in botany and phytochemisty. The good advice, support and friendship of my second supervisor, Asst. Prof. Dr. May Khin Soe has been invaluable on both an academic and a personal level, for which I am extremely grateful.

I also would like to express my deepest gratitude to all postgraduate students of Kulliyyah of Pharmacy that have been so cooperative and helpful whenever I was facing difficulties. And also to all staff and lab assistants who were very helpful throughout this research study, credits should be given to them. Thank you.

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LIST OF ABBREVIATIONS

DCM	Dichloromethane
DNA	Deoxyribonucleic acid
FIC	Fractional Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
TLC	Thin Layer Chromatography
^{1}H NMR	Nuclear Magnetic Resonance of Proton
^{13}C NMR	Nuclear Magnetic Resonance of Carbon

LIST OF SYMBOLS

d	Doublet
dd	Double doublet
m	Multiplet
ppm	Part per million
s	Singlet
t	Triplet
J	Coupling constant (Hz)
δ	Chemical shift (ppm)
µg/ml	microgram per millilitre

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CHAPTER 1

INTRODUCTION

1.1 PLANTS AS POTENTIAL SOURCE FOR ANTIMICROBIAL AGENTS

The emergence of new infectious diseases, the recovery of several infections that appeared to have been controlled and increasing incidences of drug-resistant pathogens have accelerated a number of researches and exploitation of drugs originated from plant of natural sources (Cleidson, et al., 2007). There are great potentials to discover new bioactive products from tropical forests as it holds many varieties of plants (Heinrich, et al., 2006). In Peninsular Malaysia for instance there are over six thousand species of tropical plants spreading among 550 genera and around 1,300 species are of medicinal value (Muhammad and Mustafa, 1994).

These plants were proven to be a potential new drug source based on the practically limitless of novel mechanism-based *in vitro* bioassays. It has been estimated that 14% to 28% of higher plant species are traditionally used as medicine and 74% of those plants are pharmacologically active (Ncube, Afolayan and Okoh, 2007). Verification of ethnomedicine theory showed that pure bioactive compounds isolated from plants which have long-term history of utilization by human are likely to be safer than those isolated from plants without human use history. In some cases, plants provided unlimited source of significant and complex chemical structures in which they would never be the subject of establishment of synthetic programme. Thus, the patented active compounds derived from plants can be assured (Daniel and Norman, 2001). Moreover, plant bioactive components showed a significant role of

being combined with other antibiotics with the advantages of mitigating many side effects, better patient tolerance, cheaper and being renewable in nature. In addition, higher plants also represent a potential source of novel antibiotic prototypes (Iwu, Duncan and Okunji, 1999; Shariff, 2001; Sumitra and Kalpna, 2011).

The biologically active natural compounds helped in surviving the increasing number of bacterial resistance towards currently existing antibiotics. Bacteria developed the resistance mechanisms through drug inactivation or modification, alteration of target site, alteration in the metabolic pathway or reduced drug accumulation (Katzung, 2004). In the environment, the bacteria create a biofilm in which they are protected. This biofilm usually created by nosocomial bacteria that grow on wounds, scar tissues and medical implants or devices which consequently results in infections of the urinary tract, central nervous system, eyes, ears, skin and musculoskeletal system (Raja, Gajalakshmi and Raja, 2010).

In 2004, more than 70% of pathogenic bacteria were estimated to be resistant to at least one of the drugs commonly used in anti-infectious therapy (Katz, et al., 2006). Among Gram-positive organisms, the most important resistant pathogens are *Staphylococcus aureus*, *Enterococcus* and *Streptococcus* species in which they are responsible for half of the hospital-associated infections (Balaban and Dell' Aqua, 2005). On the other hand, Gram-negative pathogens such as *Klebsiella*, *Escherichia*, *Enterobacter*, *Serratia*, *Salmonella* and *Pseudomonas* species contributed to more than 60% of sepsis cases in hospitals. They represent a serious problem in patients hospitalized with cancer, cystic fibrosis and burns, which causing death in 50% of the cases (Raja, et al., 2010).

Bioactive compounds were basically derived through several inter-acting metabolic pathways, preferably through secondary metabolic pathway which also depends on both primary processes such as photosynthesis and respiration for basic carbon compounds (Meena, 2009). They were also probably evolved from plants as their chemical defense against predation or infection (Satish, Raghavendra and Raveesha, 2008). The bioactive compounds such as alkaloids, flavanoids, tannins, phenolics, saponins and glycosides were studied abundantly on their antimicrobial activity. The efficacy as antimicrobial agents was imparted when they are pathogenic or symbiotic in human body. Amazingly in either way the bioactive compounds were able to regulate host-microbe interaction in favour of the host (Meena, 2009).

The efficiency in strategies for drug discovery combined with high-throughput screening techniques allows screening millions of phytochemicals per year (Gunnar, 2004). Most of the screening proved that plants are the potential source of antimicrobial agents. Around 25% of prescription medicine owes their origin to plant source (Heinrich, et al., 2006). Another important factor for the interest in plant antimicrobials is the rapid rate of plant species extinction. There is a feeling among natural product chemists and microbiologists alike that the multitude of potentially useful phytochemical structures which could be synthesized chemically is at risk of being lost permanently (Das, Tiwari and Shrivastava, 2009). Therefore, the identification of bioactive compounds in plants, their isolation, purification and characterization of active ingredients from crude extracts by various analytical methods are crucially important.

1.2 DESCRIPTIONS OF *RUTA ANGUSTIFOLIA* (L.) PERS.

Ruta angustifolia is belongs to the family of Rutaceae and the genus of *Ruta* (rue). It is originated from southern, Mediterranean Europe and Eurasia. The species was initially identified by Swedish biologist, Carolus Linnaeus in 1805 and later by Christiaan Hendrik Persoon (Wikipedia, 2010).

(a) (b)

Figure 1.1: *Ruta angustifolia* (L.) Pers. (a) The whole plant (b) The leaves (http://www.mrkumai.blogspot.com)

R. angustifolia (Figure 1.1) is an erect, small shrub reaching 1.5 m in heights. The whole plant has a strong, heavy, unpleasant smell and bitter, acrid, pungent taste owing to its volatile oil. The leaves are tripinnate and alternately arranged; leaflets lanceolate to obovate-lanceolate, tip blunt, base tapered, 8-20 mm long, 2-6 mm wide with entire margin, veins are not obvious and light-green in colour. It is important that the plant has ample moisture and well-drained soil. In Malay, it is called 'aruda' or 'garuda' (Najihah, et al., 2009).

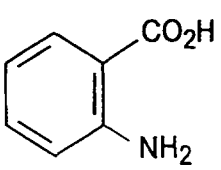
Traditional used of this plant includes paralysis, coughs and stomachache treatments. The leaves are heated and placed inside the ear to treat earache. It was also considered to be an important means of protection against supernatural evil in many parts of the world. Study also shows a high correspondence of the main medicinal uses

to the pharmaceutically demonstrated properties of the plant with the ability to improve circulation, treat rheumatism, treat infections and inflammation, to relieve pain, and remove parasites among others (Elia, 2003).

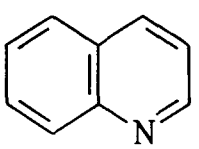
The Rutaceae plant is a rich source of several acridones, quinoline alkaloids, coumarins and immidazole alkaloids (Khalid, et al., 2000). Reported phytoconstituents of this plant include rutoside, rutaverine, arborinine, rutin, elemol, pregeijerene, geijerene, furocoumarins, bergapten, xanthotoxin, fagarine, graveolinine and skimmianine (Koh, Chua and Tan, 2002). The presence of quinoline nucleus in this plant promotes a pharmacologically active compounds with antiasthmatic, antibacterial and antifungal activities (Nadaraj, Selvi and Raju, 2006).

1.3 THE ALKALOIDS OF *RUTA* SPECIES

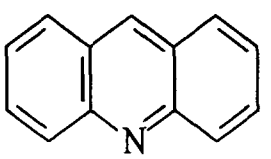
Alkaloids in the *Ruta* species are completely to be anthranillic acid derived alkaloids particularly which possess quinoline and acridine skeletons (Figure 1.2)



Anthranillic acid **(1)**



Quinoline **(2)**



Acridine **(3)**

Figure 1.2: Amino acid precursor of *Ruta* alkaloid, anthranillic acid **(1)** which give rise to quinoline **(2)** and acridine **(3)** type of alkaloids (Alessandra, et al., 2005).

These include 2- and 4-quinolones, furoquinolines, acridones and related heterocyclic systems. The high chemical diversity of quinolone alkaloids contributes to the isolation of pharmacologically active new alkyl-, aryl-, and alkylarylquinoline/ones.