ANTIMICROBIAL EFFECT OF *Baccaurea angulata* FRUIT EXTRACTS ON HUMAN PATHOGENIC MICROORGANISMS

BY

LAILUMA MOMAND

A thesis submitted in fulfilment of the requirement for the degree of Master in Health Sciences

Kulliyyah of Allied Health Science
International Islamic University Malaysia

DECEMBER 2014

ABSTRACT

The research application for drugs and food supplements derived from plants extracts have increased in recent years. Plant extracts and their constituents are recognized to be safe, either because of their traditional use without any documented detrimental impact or because of dedicated toxicological studies. On the other hand, human resistant pathogenic microorganisms increase worldwide mortality and morbidity. Due to emerging resistant and low susceptible strains to antibiotics, search for new alternative antimicrobials is raised significantly. Medicinal plants had been investigated and had been considered as potential sources of antimicrobial agents. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of medicinal plants have not been adequately evaluated. Thus, a systematic investigation was undertaken to screen for antibacterial activity from Baccaurea angulata (BA). The purpose of this study was to explore bacteriostatic and bactericidal effect of methanol, ethanol and aqueous extracts of three parts (whole fruit, fruit skin and berry) of BA fruit in vitro against tested pathogenic microorganisms. Total 9 extracts from three parts of BA fruit (whole fruit, fruit skin and berry) were tested against Gram positive (Staphylococcus aureus, Streptococcus pneumoniae, Staphylococcus epidermidis, and Clostridium botulinum) Gram negative bacteria (Escherichia coli, Pseudomonas aeruginosaa, Klebsiella pneumoniae and Salmonella paratyphi A), and fungus (Candida albicans), using disk diffusion, agar well diffusion and microdilution methods. Results show that BA fruit extracts have potential antimicrobial properties against all tested microorganisms, it is various between three parts (whole fruit, fruit skin, and berry), three solvents (methanol, ethanol and aqueous), different method (agar well diffusion, disk diffusion and microdilution method) and different aforementioned pathogens. The highest inhibitory activity was observed in ethanol extract of fruit skin using agar well diffusion against Streptococcus pnumoniae (37.0±1.0 mm) at the concentration of 1,000.0 μg/mL. Among tested Gram negative bacteria K. pnumoniae was the most susceptible bacterium which showed MIC and MBC using microdilution method at the concentration of 7.8 and 15.6 μg.mL respectively.

خلاصة البحث

تطبيق البحث المختصّ بالأدوية والمكملات الغذائية المستمدة من مستخلصات نباتية قد شهد ارتفاعا خلال السنوات الماضية. المستخلصات النباتية ومكوناتها تعتبر آمنة سواء بسبب استخداماتها التقليدية دون وجود آثار ضارة أو بسبب وجود بحوث متعلقة بالسموم مختصة بها. من جهة أخرى، البكتيريا المرضية المقاومة للإنسان تسبب زيادة في معدل الوفيات والأمراض. نظرا لظهور سلالات مقاومة وأقل عرضة للمضادات الحيوية فإنّ البحث عن مضادات ميكروبات بديلة قد ارتفع بنسبة عالية. تمّ التحقيق حول النباتات الطبية باعتبارها مصدرا ممكنا لمضادات الميكروبات. بالرغم من أنّ مئات النباتات تمّ اختبارها كمضادات للبكتيريا إلا أنّ البقية لم يتمّ التحقيق فيها بعد. لهذا السبب، تمّ عمل تحقيق منهجي. الهدف من هذه الدراسة هو *Baccaurea angulata* (BA) لفحص الامكانيات المضادة للبكتيريا الخاصة بالنبات اكتشاف القدرة البكتيريوستاتيكية والبكتيريسيدية للنبات عن طريق مستخلصات بالميثانول، الايثانول والماء لثلاث أجزاء من الفاكهة في المختبر ضدّ بكتيريا ضارة مختبرة. تسعة مستخلصات من ثلاثة أجزاء من الفاكهة تمّ اختبارها الفاكهة كاملة، القشرة واللب ضدّ بكتيريا من الغرام الايجابي Staphylococcus aureus, Streptococcus pneumoniae, Staphylococcus epidermidis, and Clostridium botulinum وبكتيريا الغرام السلبي Escherichia coli, Pseudomonas aeruginosaa, Klebsiella pneumoniae and Salmonella paratyphi A وفطريات Candida albicans. عن طريق الانتشار بالقرص،الانتشار بواسطة لوحة أغار والتخفيف الجزئي. تشير النتائج إلى أنّ مستخلصات فاكهة تمتلك خاصية ضدّ بكتيرية ضدّ كل البكتيريا المستخدمة وتختلف عند كل مستخلص (اللب، الفاكهة كاملة، القشور)، طريقة الاستخلاص (ميثانول، ايثانول، ماء)، والطرق الأخرى (الانتشار بالقرص،الانتشار بواسطة لوحة أغار والتخفيف الجزئي) والبكتيريا الضارة المستخدمة التي سبق ذكرها. أعلى نسبة تثبيط تمت ملاحظتها عند مستخلص الايثانول لقشور الفاكهة باستخدام طريقة الانتشار بواسطة لوح أغار ضد بكتيريا Streptococcus pnumoniae(37.0±1.0 mm) بنسبة بتركيز 1000.0 ميكروغرام/مل. كانت الأكثر عرضة وتأثرا والتي أظهرت K. pnumoniae من بين بكتيريا غرام السلبية بتركيز 7.8 و 15.6بالترتيب باستخدام الانتشار الجزئي MIC، MBC

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 Biomedical Science (Microbiology).

……..
Ridhwan Abdul Wahab
Supervisor

……..
Robaiza
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 Biomedical Science (Microbiology).

……..
Salehhuddin Hamdan
External Examiner

……..
Muhammad Ibrahim
Internal Examiner

This dissertation was submitted to the Department of Biomedical Science and is accepted as a fulfilment of the requirement for the degree of Master of Biomedical Science (Microbiology)

……..
Mohammad Syaiful Bahari Abdul Rasad
Head, Department of Biomedical Science

This dissertation was submitted to the Kulliyyah of Allied Health Science and is accepted as fulfilment of the requirement for the degree of Master of Biomedical Science (Microbiology)

……..
Wan Azdie Bin Mohd Abu Bakar
Dean, Kulliyyah of Allied Health Science

DECLARATION

I hereby declare that this dissertation 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.

Lailuma Momand

Signature……. Date ……..**.....**

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DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

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Signature Date

I dedicate my dissertation work to our ALLAH (S.W.T.) the almighty, and beloved prophet Muhammad peace be upon him. A special feeling of gratitude to my friend Maryam and my loving children Hamid, Maryam, Amina, Bilal and khalid

ACKNOWLEDGMENTS

Firstly, praise is due to almighty ALLAH S.W.T with His compassion and mercifulness to help me to complete this research, and may His peace and blessings be upon our beloved prophet Muhammad (S.A.W).

I would like to express much gratitude to my supervisors Dr. Ridhwan Abdul Wahab and Dr. Robaiza Zakeria for their guidance, support, and advice through this study. Also I am thankful to entire research laboratories members of Allied Health Science IIUM and USM those who assisted me during this research. May Allah S.W.T bless them and their families. I am also grateful to my colleagues for their contribution and guidance. And finally I express my thanks to my family those who really were patient and allowed me to continue this study.

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LIST OF ABBRIVIATIONS

BA	*Baccaurea angulata*
MIC	Minimum inhibitory concentration
MBC	Minimum inhibitory concentration
WHO	World health organization
EGCG	Epigallocatechin gallate
MRSA	Methicillin resistance s. aureus
CHD	Congestive heart disease
ESBL	extended-spectrum-β lactamase
CDC	Control diseases cantor
UTI	Urinary tract infection
MW	Methanol extract of whole fruit
MS	Methanol extract of fruit skin
MB	Methanol extract of fruit berry
EW	Ethanol extract of whole fruit
ES	Ethanol extract of fruit skin
EB	Ethanol extract of fruit berry
DW	Aqueous extract of whole fruit
DS	Aqueous extract of fruit skin
DB	Aqueous extract of fruit berry
CDC	Canter for disease control
VISR	vancomycin-intermediate *S. aureus*
AST	Antimicrobial sensitivity test
NCCLs	National Committee for Clinical Laboratory Standard
TTC	Triphenyl *tetrazolium chloride*
NFGNB	Nonfermentative Gram-negative bacteria

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Humans are constantly infected with pathogenic microorganisms, which use extensive strategies to continue their existence (Kraiczy and Würzner, 2006). Pathogenic bacteria increase the incidence of resistant strains worldwide, and thus become an important cause of mortality and morbidity. Nosocomial and community acquired infections are both caused by emerging resistant strains to antimicrobials. (Oskay et al., 2010). High cost in the discovery of antibiotics, and high side effect and toxicity of chemotherapeutic agent for the treatment of diseases caused by pathogens limit therapeutic options to combat pathogenic microorganisms (Savoia, 2012).

Treatment of infectious diseases is becoming more difficult due to emerging resistance and low susceptibility of strains to antibiotics (Wendakoon et al., 2012).

Medicinal plants contain compounds that are capable of providing health benefits by initiating certain physiological actions in the body. These plants have thus been used for treatment of infectious diseases (Oskay et al., 2009). Combination of these compounds with antibiotics for synergistic purpose may show adverse reaction, such adverse reaction is often due to synthetic antibiotics that are combined with medicinal plants. (Joshi et al., 2009). So far, the mechanisms of action of medicinal plants have not been identified in the majority of cases, but these mechanisms are involved in countless therapeutic responses as well as in many important biological functions (Djeussi et al., 2013). Almost all medicinal plants are useful chemotherapeutants, and important source of natural drug, natural pesticide and bio fertilizer (Jahan et al., 2010).

According to an estimation made by the World Health Organization (WHO), around 80% of people in developing countries prefer medicinal plants than drugs for their simple health problems (Dubey et al., 2012). Different parts of medicinal plants are used as raw drugs, and demonstrated numerous medicinal properties (Mahesh and Satish, 2008). The aims of researching on antimicrobial activity of medicinal plant is to reduce synthetic antimicrobial agent in pharmaceutical industry, resistance to antimicrobials and treatment of pathogens caused by resistant microorganisms (Mahady, 2005). The level of antibiotics that inhibits bacterial growth, but not causes bacterial disruption is called the minimum inhibitory concentration (MIC), while the higher concentration that kills the bacteria is called minimum bactericidal concentration (MBC). Almost all antibiotics possess both bactericidal and bacteriostatic effects, depending upon the concentration of the antibiotics (Yamamoto, 2003). In the current study higher concentration of *B. angulata* fruit extracts mostly revealed higher antimicrobial activity compare to the lower concentrations.

1.2. STATEMENT OF THE PROBLEM AND SIGNIFICANCE OF THE STUDY

Infectious diseases are often difficult to treat due to the fact that pathogenic bacteria are constantly resisting the effect of most or all potent antimicrobials (Mohana et al., 2008). When bacteria become resistant to first and second-line antibiotics, a change to more expensive third and fourth-line antibiotics is usually done. However, using these last two groups of antibiotics are often restricted in low income community due to their high cost. Therefore infectious diseases caused by bacteria that are resistant to the first two groups of antibiotics are no longer treatable (Sibanda and Okoh, 2007).

Antibiotic therapy increases the growth of fungal microbiota, which is one of the common side effects of antimicrobial (Noverr et al., 2004). The notable incidence of resistant strains of medically important bacteria increases constant demand for new and effective antimicrobial agents. Plants are novel sources of medicines as they have the ability to synthesize many potential chemical substances with therapeutic properties. Phytochemical constituents of medicinal plants are affordable, and rarely have side effects than manufactured antimicrobials (Savoia, 2012). In fact, some seed-bearing *plants* have been found to be an important alternative to manage resistance of human pathogens (Satish et al., 2008).

As it has long been known that plants possess antimicrobial activities, many studies on screening and testing of numerous plants have been undertaken and published to explore their antimicrobial activities. The remarkable compounds in medicinal plants have been thought as a protective agents against microbial pathogens, and therefore, they received more attention from pharmacists and scientists, due to their promising antimicrobial potential (Savoia, 2012). Hence, the antimicrobial importance of plants has been tested both *in vivo* and *in vitro* (Adegoke et al., 2011). Only around 2% of medicinal plants from around the world have been examined for their biological and pharmacological properties and sustainable numbers of new antibiotics have been reported from natural and semi synthetic resources (Narayan, 2010). As an extension to the existing studies on medicinal plant, this study investigated the antimicrobial activity of three parts of *B. angulata* fruit namely whole fruit, fruit skin and berry. The medicinal values in the fruit extract of *B. angulata* have not been tested previously.

1.3. RESEARCH OBJECTIVES

This study intended to

1. Screen antimicrobial property of *B. angulata* using different methods namely disk diffusion, agar well diffusion, and microdilution methods.
2. Evaluate bacteriostatic and bactericidal effects of *B. angulata* (whole fruit, fruit skin, and berry) fruit extracts on selected pathogenic microorganisms
3. Compare bacteriostatic and bactericidal effects of nine extracts of whole fruit, fruit skin and berry using methanol, ethanol and aqueous

1.4. HYPOTHESIS

Plant extracts of *B. angulata* using different solvents and different methods are expected to have antimicrobial effect on some human pathogenic microorganisms.

1.5. SCOPE OF THE STUDY

The scope of the present study was to evaluate the antimicrobial potential of plant extracts from whole fruit, fruit skin, and berry of *B. aungulata* using different organic solvents namely methanol 100%, ethanol 100% and aqueous and different method agar well diffusion, disk diffusion and microdilution methods.

CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

Euphorbiaceae is the fourth major family of the angiosperms, containing more than 300 genera and almost 7,500 species (Uduak and Kola, 2010). *B. angulata* (Figure 2.1) is a species that belongs to the family Euphorbiaceae (Lim, 2012) native to Borneo island of Malaysia locally known as belimbing dayak or belimbing hutan. The soft whitish part of this fruit (berry) (Figure 2.1.B) is edible, while the red part (skin) (Figure 2.1 C) is sour, and usually cooked by the rural communities. The uniform red coloured skin may be due to flavonoid contents (Jauhari et al., 2013: Ibrahim et al., 2013). This tree prefers shadow and grows well in sandstone and lateritic soils (Lim, 2012). The antimicrobial activity of genus of Euphorbia, which belongs to the family Euphorbiaceae, has been carried out against many bacteria. Ethanol, acetone, and aqueous extracts of *Euphorbia fruticosa* and methanol extracts of *Euphorbia macroclada* demonstrate antimicrobial effect against *Staphylococcus aureus* (Rojas et al., 2008). The antimicrobial properties of these plants make them therapeutically efficient against several infectious diseases (Girish and Satish, 2008).

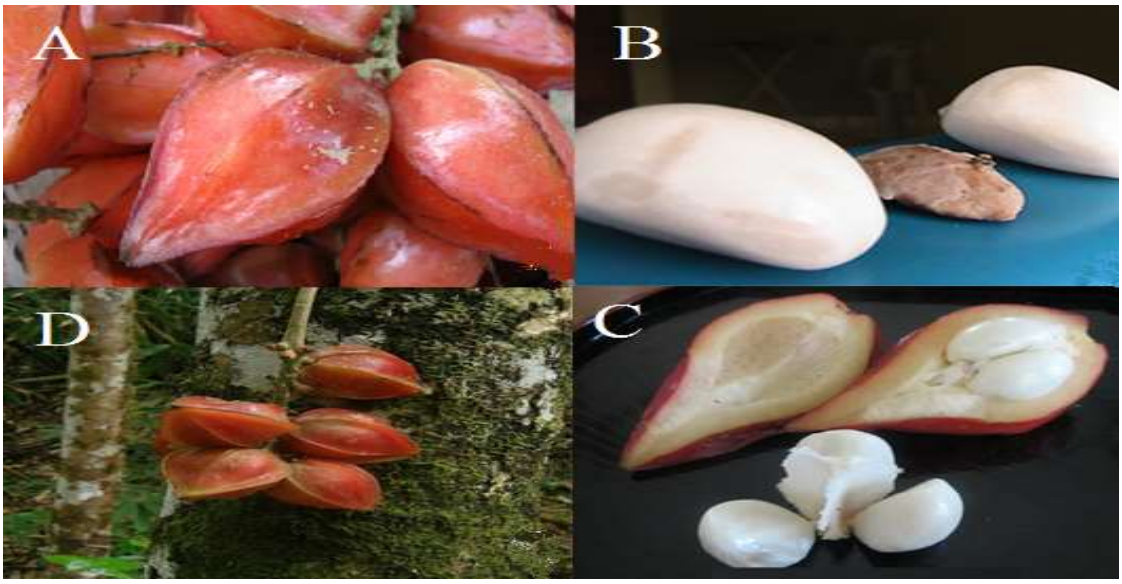

Figure 2.1 *B. angulate* fruit. A: whole fruit, B: berries and seed, C: fruit skin and berry D: *B. angulata* plant

Besides *E. fruticosa* and *E. macroclada*, *Euphorbia hirta* is another member of the family Euphorbiaceae. Dysentery, asthma, and chronic bronchial infections can be treated using aqueous extract of *E. hirta* (Rojas et al., 2008).

Acalypha indica also belongs to the family Euphorbiaceae. This species is a common weed in many parts of Asia including Pakistan, India, SriLanka, and some African countries. This plant is traditionally used as an expectorant for the treatment of asthma and pneumoniae. Besides, antimicrobial and anti-inflammatory effects of these species also have been evaluated by Shanmugapriya (2012). Hyaluronidase enzyme which causes tissue damage is produced by *S. pneumoniae*. The inhibitory effect of this enzyme by *Rubus fruticosus* fruit, which subsequently decreases the incidence of skin diseases, has been reported (Riaz et al., 2011). Medicinal plants contain secondary metabolites such as alkaloids, steroids, tannins, and phenolic compounds. These compounds are synthesised and deposited in specific parts or in all parts of the plants (Jadeja et al., 2005). Green tea is a popular beverage in Chinese

traditional medicine. Antimicrobial activity of green tea as well as its hypocholesterolemic and hypoglycaemic effects have been reported. This plant contains a large amount of polyphenols, which may have positive effects on lifestyle-related diseases such as obesity and diabetes (Cunha et al., 2013). Moreover, these compounds do not only demonstrate antibacterial effect against many pathogenic bacteria such as *S. maltophili*a and *Shigella* sp*p* but synergistic effect of phenol group with some antibiotics has also been found. For example, synergy of a natural phenol (flavan-3-ol) such as epigallocatechin gallate (EGCG) to ampicillin has been found in the treatment of methicillin resistant *S. aureus* (MRSA) (Betts, 2012). *Tragia tennifolia* Benth, which belongs to the family of Euphorbiaceae, is used by Ghanaians as a traditional medicine. This plant increases the effect of medication against diabetes, and thus there can decrease the dosage of antidiabetic drug especially when this plant is taken as juice (Koffuor et al., 201). The role of primary metabolites is totally different from the role of secondary metabolites. Primary metabolites are essential for cell division, respiration, reproduction, and storage of energy in plant (Bourgaud et al., 2001). Carbohydrates, lipids, proteins, chlorophyll, and nucleic acids are available in plants and they are responsible for the primary metabolism and survival of plant cells (Briskin, 2000), while the role of secondary metabolites.in plant was first defined by Kossed in 1891. He found out that secondary metabolites are different from primary metabolites, which can fight against many pathogenic microorganisms (Sansinenea and Ortiz 2011). These metabolites were named 30 years later by Czapek who states that secondary metabolites are derived from metabolism of nitrogen. Improvement of chromatography analysis in the middle of 20^{th} century allows the recovery of more secondary metabolites molecules. Secondary metabolites defend plants against insects, and they protect plant leaves

from serious damage due to ultraviolent light (Bourgaud et al., 2001: Kliebenstein, 2004). These metabolites have several beneficial effects on human health including antibacterial, anticarcinogenic, antithrombotic, and vasodilatory (Wendakoon et al., 2012). Around one and half of all registered drugs before 2007 were obtained from natural products and their synthetic derivatives (Kennedy and Wightman, 2011). For example, acetyl salicylate or aspirin derivative of salicylate (Bourgaud et al., 2001) is obtained from bark of willow tree, which possess anti-inflammatory, analgesic, and antipyretic medicinal effects (Fuster and Sweeny, 2011). Digitoxin and digoxin are cardiac glycosides; digitoxin is derived from either *Digitalis purpurea* (foxglove) or *Digitalis lanata*, while digoxin is derived from *D. lanata* only. Both derivatives have been used for several decades for the treatment of congestive heart disease (CHD) (Mashour et al., 1998). Quinine has been used for more than three centuries in medicine as the compound exhibits a vital role for the treatment of malaria (Stork et al., 2001). Quinine is derived from the bark of *Cinchona calisaya Cinchona calisaya*, and *Cinchona succirubra.* In Brazil, these plants are named as quinas, from which the name quinine is derived (Neto et al., 2003).

2.2. TRADITIONAL USE OF MEDICINAL PLANTS IN MALAYSIA

Medicinal and nutritious fruit are abundantly available in Malaysia. The plenty availability of medicinal plant in this geographic area is due to their prolong exposure to sunlight through the year (Ibrahim et al., 2013). Natural products have showed many medicinal properties worldwide (Koshy et al., 2009). Around 121 clinically valuable drugs are derived from medicinal plants, and the majority of their compounds have been identified from folk or traditional use. Malaysia is one of those countries that have many natural resources (Jamal et al., 2011). The traditional use of seed

producing plants reported from Peninsular, Sabah and Sarawak Malaysia. Among 35,000 medicinal plants, around 1,200 species are used traditionally in Peninsular and 2,000 species in Sabah and Sarawak (Koshy et al., 2009). *B. angulata* plant that belong to the Euphorbiaceae family are used as food by the rural community in Sarawak Malaysia as well as in treatment of infectious diseases such as diarrhoea, dysentery, and skin infections (Uduak and Kola, 2010).

2.3. ANTIMICROBIAL EFFECT OF BIOLOGICAL ACTIVE COMPOUNDS

The therapeutic importance of medicinal plants is often due to combination of secondary metabolites (phytochemicals). Many researches have shown the essential function of these compounds for growth and life of plants, although previously the important role of these compounds for plants was poorly understood (Briskin, 2000). There are thousands of known phytochemicals, some of which are alkaloids, allicin, anthocyanins, biflavonoids, carotenoids, coumestans, flavan-3-ols, flavonoids, hydroxycinnamic acids, indoles, isoflavones, lignans, lignins, lutein, lycopene, monophenols, monoterpenes, organosulfides, phenolic acids, phytosterols, saponins, stylbenes, triterpenoids and xanthophylls. Generally, these phytochemicals are categorised into three groups namely (a) terpenes or terpenoids, (b) alkaloids, and (c) phenolic compounds (Devappa et al., 2011: Petti and Scully, 2009). The antimicrobial activity in extract of a medicinal plant is not by phytochemicals themselves, but they can inhibit bacterial resistance by blocking the efflux pumps or other mechanisms. The efflux pump inhibitors from natural sources can be administered with the antibiotic to decrease the degree of resistance of the bacteria to the drugs, and subsequently decrease the acquired resistance or reduce the emergence of resistant

pathogens (Adwan et al., 2011). In the next section, phenolic compounds and flavonoids (a class of phenolic compounds) are discussed in general.

2.3.1. Phenolic Compounds

Plants are natural sources of phenolic compounds. These compounds are found in leaves, bark, fruit, and wood or in a specific organ or tissues of medicinal plants (Nitiema et al., 2012). Phenol compounds are derivatives of benzene classified to more than 10 classes based on their chemical structures (Visioli et al., 2000). These derivatives consist of one aromatic ring and one or more hydroxyl group (Figure 2.2). They range from quite simple compounds to highly polymerised constituent. Several thousand molecules of phenol compounds are found in medicinal plants that have anti-inflammatory, antioxidant, and antimicrobial activities (Nitiema et al., 2012). The antioxidant activity of phenolic acid is found to be much higher than protective effect of vitamin C and protective effect of vitamin E against reactive oxygen in tissues (Chua et al., 2011). Gallic acid (Figure 2.2 A) found in plant is a type of phenolic compound with protective property against human pathogenic bacteria (*S. aureus* and *Corynebacterium accolens*) and fungus (*C. albicans*). Antibacterial and antifungal activities of methyl gallate (Figure 2.2 B) against Gram-positive and Gram-negative bacteria as well as against *C. albicans* have also been found (Karamaæ et al., 2006).