ANTIBACTERIAL ACTIVITY OF NEEM (*AZADIRACHTA INDICA*) EXTRACTS AGAINST MULTIPLE DRUG RESISTANT (MDR) BACTERIA

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Report submitted in partial fulfillment of the requirements for the award of Bachelor of Applied Science (Honor) in Industrial Chemistry

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January 2012

ABSTRACT

This study concerned with the evaluation of antibacterial activity of four crude extracts of three parts (leaf, root and bark) of Neem (*Azadirachta Indica*) obtained by four solvents (chloroform, acetone, cold and hot ethanol). The antibacterial activity of these extracts was assessed against two multiple-drug-resistance bacteria strains, namely *E. coli* and *S. aureus* by the conventional well diffusion method. For the antibacterial bioassay, four concentrations (0.1, 0.2, 0.4 and 0.8%) of each solvent and part extract solutions were prepared; DMSO was used to solubilize the extract in water. 1% of DMSO was used as control. All extracts were active against both test organisms *E. coli* and *S .aureus*, the root acetone displayed overall more potent activity and reaches 52.5% and 51.88% respectively with concentration of 0.8% and the lowest activity observed with bark hot ethanol at concentration of 0.8%. Bioactive groups such as alkaloids and sesquiterpene lactones were screened by thin layer chromatography; and the results obtained were negative for alkaloids and positive for sesquiterpene lactones. The experimental results obtained from this study suggest that Neem (*Azadirachta Indica*) extracts are promising as natural antibacterial and this may warrant further research to determine the bioactive compound (s).

ABSTRAK

Kajian ini berkenaan dengan penilaian aktiviti antibakteria oleh Neem (*Azadirachta Indica*) bagi empat ekstrak mentah dan tumbuhan itu dibahagi kepada tiga bahagian (daun, akar dan kulit kayu) yang diperolehi oleh empat pelarut (kloroform, aseton, etanol sejuk dan etanol panas). Aktivit antibakteria pati ini telah dinilai terhadap dua jenis bakteria dadah rintangan beberapa terikan, iaitu *E. Coli* and *S. Aureus* oleh kaedah resapan serta konvensinal.Untuk bioassay antibakteria, empat kepekatan (0.1, 0.2, 0.4 dan 0.8%) telah disediakan; DMSO telah digunakan untuk keterlarutan cabutan di dalam air. Kawalan menggunakan 1% DMSO untuk perbandingan. Semua ekstrak aktif terhadap kedua-dua ujian organisma *E. Coli* and *S. Aureus*, sample akar yang diekstrak daripada aseton mempaparkan aktiviti keseluruhan lebih tinggi dan mencapai 52.5% dan 51.88% dengan kepekatan 0.8% masing masing dan aktiviti terendah yang diperhatikan adalah sample balak yang diekstrak daripada etanol panas dengan kepekatan 0.8% . Kumpulan bioaktif seperti alkaloid dan sesquiterpene laktones bahantara telah ditayangkan oleh Thin Layer Chromatography dan keputusan yang diperolehi adalah negatif untuk alkaloid dan positif untuk sesquiterpene laktones. Keputusan uji kaji yang diperolehi daripada kajian ini mencadangkan bahawa ekstrak Neem (*Azadirachta Indica*) adalah cerah sebagai antibakteria semulajadi dan ini boleh menjamin penyelidikan lanjutan bagi menentukan komponen bioaktif (s).

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°C	Degree Celsius
$	Dollar
g	Gram
h	Hour
µL	Microliter
mg	Milligram
mL	Milliliter
%	Percentage

LIST OF ABBREVIATIONS

A.hydrophila	*Aeromonas Hydrophila*
AIDS	Acquired Immunodeficiency Syndrome
C. alata	*Crescentia Alata*
C. albicans	*Candida albicans*
DMSO	Dimethyl Sulfoxide
E. coli	*Escherichia coli*
EHEC	Enterohaemorrhagic Escherichia coli
HC	Haemorrhagic Colitis
H_2O	Water
H_2SO_4	Sulfuric Acid
HUS	Haemolytic-Uremic Syndrome
KI	Calcium Iodide
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
P. aeruginosa	*Pseudomonas Aeruginosa*
Shigella sp.	*Shigella* Species
Salmonella sp.	*Salmonella* Species
S. aureus	*Staphylococcus aureus*
TLC	Thin Layer Chromatography
TTP	Thrombocytopenic Purpura
US	United State

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Natural products are important for the latest years. Historically, the 'golden age' of natural product occurred between approximately 1950 through 1970, and during this time the successful isolation of potent antibiotic compounds was routinely achieved through a sequential process. Natural productsfall into several different categories that are steroids from marine animal, plant, and fungal sources, alkaloids from plants and some bacteria, proteins, amino acids, and antibiotics from microbes, pigments from microbes and plants, pyrimidines and purines from microbes, and terpenes, carbohydrates, fats, macromolecular productand miscellaneous compounds from all sources, including terrestrial animals. The advantages for the natural products are antibiotics, immune suppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigrane agents, herbicides, antiparasitic agents and ruminant growth promoters, and bioinsecticides, anticancer agents and etc (Strege, 1999).

Azadirachta Indica, a Meliaceae family tree, locally known as 'Neem' in Pakistan, is widely used in the ethno veterinary medicine system for the treatment of indigestion, tick infestation and toxemia (Iqbal et al., 2010). Previous study also shown that is has been used in India for many years in the treatment of several diseases in medicine and dentistry (Polaquini, 2006). More than 140 compounds have been isolated

from different parts of neem. There are six parts of the Neem (*Azadirachta Indica*) tree which are leaves, flowers, seeds, fruits, roots and bark (Subapriyaet al., 2006). The centuries old healing system, Ayurvedic medicine, has utilized thesetimeless Neem (*Azadirachta Indica*) formulations as a mainstay of Ayurvedic pharmacy. Its medicinal qualities are outlined in the earliest 'Sanskrit' writings that states uses of various parts of Neem (*Azadirachta Indica*) treat bacterial, fungal, and viral infections and to boost the immune system. It is also usefulness as a natural non-toxic insecticide among other fascinating properties increases it phenomenal applications (Dasgupta et al, 2004). Neem (*Azadirachta Indica*) helps support a strong immune system and is used in cases of inflammatory skin conditions. Traditionally Neem (*Azadirachta Indica*) has been used for skin and blood purifying conditions. Neem (*Azadirachta Indica*) not only helps in curing diseases, but it also provides us with the strength of fighting diseases by enhancing our immunity (Bhowmik et al., 2010).

According to the previous study, different parts of the Neem (*Azadirachta Indica*) have different medical uses. Neem (*Azadirachta Indica*) barks are used in production of some dental-care products like toothpaste. Itsleaves possess excellent medicinal properties. In addition to it is usefulness in Pest management and Disease control they can also be fed to livestock when mixed with other fodder. Neem (*Azadirachta Indica*) leaves can also be used to protect stored woolen and silk clothes from insects. Neem (*Azadirachta Indica*) fruits are bitter, purgative, antihemorrhodial and anthelmintic (vermifuge) in nature. The flowers are used in vitiated conditions of pitta (balancing of the body heat) and kapha (cough formation). Neem (*Azadirachta Indica*) seeds are also described as anthelmintic, antileprotic (cures or prevents leprosy) and antipoisonous.Neem (*Azadirachta Indica*) oil, derived from crushing the seeds, is antidermatonic, a powerful vermifuge and is bitter in taste. It has a wide spectrum of action and is highly medicinal in nature. As oil used in aromatherapy, it has been effective in the treatment of head lice in children, especially where tea tree has failed to clear up the condition (Bhowmik et al., 2010). However, this study we are testing about the antibacterial activities of the *Neem (Azadirachta Indica)*extract. According to the

previous study, the stem bark possesses anti-tumour and interferon inducing activities and other plant parts have been reported to have antibacterial, antifungal, antimalarial and anticancer effects. Regarding Neem (*Azadirachta Indica*) oil, it has been reported to have anti-fertility activity and stimulate cell mediated immune responds (Sairam et al., 2000).

Neem (*Azadirachta Indica*) has been reported to have antibacterial and antifungal effect. It has been shown to be active against pathogenic bacteria such as *Staphylococcus aureus and Salmonella typhi*and against various pathogenic fungi belonging to the genera *Trichophyton, Epidermophyton, Microsporum, Geotrichium* and *Candida.* In addition, Neem (*Azadirachta Indica*) leaf extract was found to be active against a number of viruses such as small pox, chicken pox, fowl pox, poliomyelitis, herpes viruses etc (Sairam et al., 2000). According to the previous study, Neem (*Azadirachta Indica*) contains flavonoids, which are reported to be antiviral and anti-inflammatory (Fabry et al., 1998).

1.2 PROBLEM STATEMENT

1. One part of the problem is the bacteria and other microbes cause infections are remarkably resilient and have developed several ways to resist antibiotics and other antimicrobial drugs.
2. Another part of the problem is due to increasing use, and misuse of existing antibiotics in human and veterinary medicine and in agriculture

CHAPTER 2

LITERATURE REVIEW

2.1 NATURAL PRODUCTS

Natural products are important for the latest years. Historically, the 'golden age' of natural product occurred between approximately 1950 through 1970, and during this time the successful isolation of potent antibiotic compounds was routinely achieved through a sequential process. Natural product fall into several different categories that are steroids from marine animal, plant, and fungal sources, alkaloids from plants and some bacteria, proteins, amino acids, and antibiotics from microbes, pigments from microbes and plants, pyrimidines and purines from microbes, and terpenes, carbohydrates, fats, macromolecular product and miscellaneous compounds from all sources, including terrestrial animals (Strege, 1999).

Natural product has many advantages in medical use such as immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigrane agents, herbicides, antiparasitic agents and ruminant growth promoters, and bioinsecticides. Natural products have had a profound impact upon both chemical biology and drug discovery. For example, in cancer research, the natural products actinonin,

geldanamcyin,rapamycin, trapoxin, fumagillin and their analogs have been used to identify and study important new potential therapeutic targets. Natural products such as the anthracyclines, *arabino*-nucleosides, camptothecins, taxoids and vinca alkaloids have also been of enormous clinical utility (Shang and Tan, 2005). Natural products continue as a source for innovation in drug discovery by playing a significant role in the discovery and understanding of cellular pathways that are an essential component in the drug discovery process. In many cases, natural products provide compounds as clinical/marketed drugs, or as biochemical tools that demonstrate the role of specific pathways in disease and the potential of finding drugs. Numerous reviews have been written that describe the importance of compounds derived from microbes, plants and animal sources to treat human diseases (Gullo et al., 2006). Although almost half of the best-selling pharmaceuticals are natural or closely related to natural product , there remains tremendous potential for the identification of new medicinal compounds from these sources, since it has been estimated that only a small percentage of compounds from biological sources have been investigated for this purpose (Strege, 1999).

2.2 NEEM (*AZADIRACHTA INDICA*)

Medicinal plants are part and parcel of human society to combat diseases, from the dawn of civilization (Biswas et al., 2002). Indian Neem tree, *Azadirachta Indica* A. Juss, a Meliaceae family tree, contains at least 35 biologically active principles and is widely grown all over the tropics (Dasgupta et al., 2004). Neem is an evergreen tree, cultivated in various parts of the Indian subcontinent .The sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness' and hence is considered as 'Sarbaroganibarini'. The tree is still regarded as 'village dispensary' in India. The importance of the neem tree has been recognized by the US National Academy of Sciences, which published a report in 1992 entitled 'Neem – a tree for solving global problems' (Biswas et al., 2002). In previous study also shown that is has been used in India for many years in the treatment of several diseases in medicine and dentistry

(Polaquini et al., 2006) *Azadirachta Indica,* traditionally employed intensively as folklore remedy for a wide spectrum of diseases in India. *Azadirachta Indica* has a wider arrays of uses than any other herb. The first recorded use of Neem (*Azadirachta Indica)* is attributed to the ancient East Indian 'Harrappa Culture' which added the plant to dozens of health and beauty aids 4500 years ago. The centuries old healing system, Ayurvedic medicine, has utilized these timeless Neem (*Azadirachta Indica)* formulations as a mainstay of Ayurvedic pharmacy. Its medicinal qualities are outlined in the earliest 'Sanskrit' writings that states uses of various parts of *Azadirachta Indica* to treat bacterial, fungal, and viral infections and to boost the immune system. Also its usefulness as a natural non-toxic insecticide among other fascinating properties increases it phenomenal applications. Numerous scientific reports validate the traditional uses of Neem (*Azadirachta Indica)* in both the maintenance of general health and skin care. Practically every parts of *Azadirachta Indica* which are leaf, bark, fruit, flowers, oil, and gum have been reported to be associated with various remedial properties (Dasguptaet al., 2004).

According to the previous study, different parts of the Neem (*Azadirachta Indica*) have different medical uses. Neem (*Azadirachta Indica*) bark is used in production of some dental-care products like toothpaste (Bhowmik et al., 2010). Neem stem bark extract also shows lethal effect in three common snail species Biomphalariapfeifferi, Bulinustruncatus and Lymnaeanatalensis and against fish, Aphyosemon Giardneri (Biswas et al., 2002). Neem (*Azadirachta Indica*) leaves possess excellent medicinal properties. In addition to it is usefulness in Pest management and Disease control they can also be fed to livestock when mixed with other fodder. Neem (*Azadirachta Indica*) leaves can also be used to protect stored woolen and silk clothes from insects. In previous study also report that leaves of the tree are used for anti-inflammatory, anxiolytic, anti-androgenic, anti-stress, humoral and cell-mediated immuno-stimulant, anti-hyperglycemic, liver-stimulant, anti-viral, and anti-malarial treatments (Bhattacharyya and Sharma,2005). Neem (*Azadirachta Indica*)fruits are bitter, purgative, antihemorrhodial and anthelmintic (vermifuge) in nature. The flowers are

used in vitiated conditions of pitta (balancing of the body heat) and kapha (cough formation). Neem (*Azadirachta Indica*) seeds are also described as anthelmintic, antileprotic (cures or prevents leprosy) and antipoisonous. Neem (*Azadirachta Indica*) oil, derived from crushing the seeds, is antidermatonic, a powerful vermifuge and is bitter in taste. It has a wide spectrum of action and is highly medicinal in nature. As oil used in aromatherapy, it has been effective in the treatment of head lice in children, especially where tea tree has failed to clear up the condition (Bhowmik et al., 2010).

Present study report that although leaves of neem have been reported as the main sources of the active compounds obtainable from the plant, the fruits and seeds appear to be more important. Neem seed contains 35–45% oil, and this is usually extracted by means of organic solvents, the more commonly used ones being acetone, ethanol, methanol and petroleum ether (Lale and Abdulrahman, 1999).

Chemical investigation on the products of the Neem tree was extensively undertaken in the middle of the twentieth century. Since the early report by Siddiqui in 1942 on the isolation of nimbin, the first bitter compound isolated from Neem oil, more than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural diversity of these compounds. The compounds have been divided into two major classes: isoprenoids and others. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and Csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. The details of the chemistry of various compounds falling under these groups have already been reviewed. Only a few compounds whose bioactivity has been studied are presented here (Biswas et al., 2002).

However, this study we tested about the antibacterial activities of the Neem (*Azadirachta Indica*) extract. According to the previous study, the stem bark possesses anti-tumour and interferon inducing activities and other plant parts have been reported to have antibacterial, antifungal, antimalarial and anticancer effects. Regarding Neem (*Azadirachta Indica*) oil, it has been reported to have anti-fertility activity and to stimulate cell mediated immune responds (Sairam et al., 2000). Neem products have been shown to exhibit a wide rangeof effects that are potentially useful for malaria control and include antifeedancy, ovicidal activity, fecundity suppression, insect growth regulation and repellency (Okumu et al., 2007).

The literature also shows that the extract from *Azadirachta Indica*is a powerful inhibiting agent against the increase and the establishment of microorganisms that cause infectious diseases in the mouth cavity. Clinical studies have shown that the extract decreases the dental plaque index, whereas in vitro studies have demonstrated that the formation of the bacterial plaque has been positively affected. Certain anti-plaque traits may be due to the fibrous nature of these sticks, which may mechanically cause plaque removal. The plant, however, may contain anti-plaque chemotherapeutical agents. Neem is neither toxic nor does it have any mutagenic properties. Besides containing long-known bactericidal traits, it also seems to have anti-inflammatory, astringent, antiseptic, anti-ulcer, antiviral, antihyperglycaemia and immunostimulant properties (Polaquini et al., 2006).

2.3 ANTIBACTERIAL ACTIVITY OF PLANT EXTRACT

In many parts of the world there is a rich tradition in the use of herbal medicine for the treatment of many infectious diseases. Because of the side effect and the resistance that pathogenic micro-organisms build against the antibiotics, much recent attention has been paid to extracts and biologically active compounds isolated from plant

species used in herbal medicine (Essawi and Srour, 2000). Plants are among the most important common sources of potentially valuable new drugs. There is, therefore, an urgent need to investigate the biological properties of additional medicinal plants in order to develop new drugs (Kone et al., 2004).Medicinal plants may offer a new source of antibacterial agents for use. In many parts of the world medicinal plants are used for antibacterial, antifungal, and antiviral activities (Essawi and Srour, 2000).

In previous study reported that the use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils, as well as in tannin. The antimicrobial properties of plants have been investigated by a number of researchers worldwide, especially in Latin America. In Argentina, a research tested 122 known plant species used for therapeutic treatment. It was documented that among the compounds extracted from these plants, twelve inhibited the growth of *Staphylococusaureus*, ten inhibited *Escherichia coli*, and four inhibited *Aspergillus niger* and also reported that the most potent compound was one extracted from *Tabebuiaimpetiginosa*. The antimicrobial properties of compounds obtained from *Parthenumargenta-tum* against *Candida albicans, Torulopsis, Hansemula, Klebsiellapneu-moniae* and *Pseudomonas aeruginosa* was detected. Work done was observed that the substances extracted from nine known plants in Uruguai did not show any activity against *C. albicans* and *Saccharomyces cerevisiae*, but inhibited the growth of *Bacillus subtilis*, *E. coli* and *P. aeruginosa* (Nascimentoet al., 2000).

Previous study also showed that the powdered leaves and fruits of *Rhuscoriaria* and the other *Rhus* species were reported to possess

antibacterial properties and used by the local people for wound healing in the Black Sea region (Sokmen et al., 1999).

Previous study reported that the West Bank and Gaza Strip (Palestine) herbal medicine is used to treat various diseases including gastrointestinal diseases, urinary tract infections, infertility, and cutaneous abscesses. From the study, 15 plants which had been described in herbal books and medicinal folklore were screened for their antibacterial activity (Table 2.1) (Essawi and Srour, 2000).

Table 2.1: Ethnobotanical data of studied plants

Species/voucher specimens	**Part used**	**Popular uses**
Artemisia herbal (408)	Aerial	Activates the function of the liver
		Heals rash and joints inflammations
		Helps in rheumatoid arthritis
		Acts as antiseptic
		Helps people with diabetes
Nigella sativa (412)	Seeds	Used for cough, especially whooping cough
		Treats asthma and cold
		Treats stomach disorders and headaches
		Treats skin infection
		Treats leprosy
Matricariachamomilla (413)	Aerial	Used as antifungal and antibacterial
		Activates the circulatory system
		Acts as antiseptic
		Provides relief from cold and tonsillitis, and reduces fever
		Treats inflammations of the urinary tract system

Species/voucher specimens	Part used	Popular uses
Pimpinellaanisum (402)	Seeds	Helps in headaches and cold
		Used as insecticides
		Decreases coughing and chest pain
Inula viscosa (411)	Aerial	Stated in literature that they are used in herbal medicine, but no specification
Thymus vulgaris (416)	Aerial	Whooping cough
		Emphysema
		Intestinal diseases
		Treats ulcers of the stomach and the duodenum
Thymus origanium (445)	Aerial	Whooping cough
		Emphysema
		Intestinal diseases
		Treats ulcers of the stomach and the duodenum
Salvia officinalis (401)	Aerial	Treatment for stomach pains
		Helps in pulmonary inflammations
		Used for hepatitis
		Treats intestinal infections
		Shows an antigermal effect, especially the leaves of the plant
		Helps patients suffering from tuberculosis
Rosmarinusofficinalis (407)	Aerial	Acts as antirheumatic
		Increases blood outflow in the menstrual cycle
Teucrium polium (444)	Aerial	Leaves decoctions used for stomach pains
Foeniculumvulgare (432)	Aerial	Treatment of soreness of the eyes
	Seeds	Treatment of whooping cough and asthma
		Treats gastrointestinal disorders
		Treats urinary tract infections
Commiphora opobalsamum(405)	Aerial	Used to reduce pain sensation and

Species/voucher specimens	Part used	Popular uses
		increases stool excretion and urine outflow
Calaminthaofficinalis (430)	Aerial	Settles gas and indigestion
		An expectorant, good for cough and cold remedy
		Heals respiratory infections
Malva sylvestris (422)	Aerial	Treats cutaneous abscesses
		Treats inflammation of tonsils and oropharynx
		Treats asthma, diarrhoea
Majoranasyriaca (403)	Aerial	Heals pulmonary inflammation
		Used for whooping cough
		Removes pain and heals infection in the stomach

Source: (Essawi and Srour, 2000).

In present study is using two types of bacteria to test the antibacterial activity of Neem (*Azadirachta Indica*) extracts. The bacteria that are using are *E. coli and Staphylococcus aureus.* In previous study report thatprevious investigation revealed that water extract from *C. alata* leaves contained potential antifungal agent against *Candidia albicans* and antibacterial agent against *E. coli* for the treatment of opportunistic infections in patients afflicted with Acquired Immunodeficiency Syndrome (AIDS) (Somchit et al., 2003). Another previous study, *E. coli* is an efficient source for recombinant protein production (Phue and Shiloach, 2004).Producing of *E. coli* may cause different human diseases, from mild diarrhoea to haemorrhagic colitis, hemolytic-uraemic syndrome, and thrombotic thrombocytopenic purpura, especially among children, the elderly and others with underdeveloped immunity (Sanchez et al.,2007). There is also a study report that extracts from Chinese chives and cassia reduced the count of *Escherichia coli* and other bacteria during storage of juices, milk and meat (Alzoreky and Nakahara, 2003). Enterohaemorrhagic *Escherichia coli* (EHEC), in

particular, serotype O157:H7, have increasingly emerged as pathogens that cause significant human diseases, including diarrhoea , haemorrhagic colitis (HC) , and occasionally complications such as haemolytic–uremic syndrome (HUS) and thrombocytopenic purpura (TTP) Many risk factors for HUS during an infection of *E.coli* O157:H7 have been discussed (Voravuthikunchai et al., 2004).

Staphylococcus aureus is an important opportunistic pathogen that causes a variety of diseases in humans and animals. In cattle, *Staphylococcus aureus* is the most frequently isolated pathogen causing clinical or subclinical mastitis worldwide (Zarzosaet al., 2008). Author reported that *Staphylococcus aureus* is one of the most common gram-positive bacteria causing food poisoning. Its source is not the food itself, but the humans who contaminate foods after they have been processed.Gram-negative bacteria are represented by *Escherichia coli*, which belong to the normal flora of humans. However, an enterohemmoragic strain of *E. coli* has caused serious cases of food poisoning, and preservatives to eliminate its growth are needed (Rauha et al., 2000).

Previous study showed that there is another types of medicinal plants having antibacterial activity such as *Entadaabyssinica* (stem bark), *Terminaliaspinosa* (young branches), *Harrisoniaabyssinica* (roots), *Ximeniacaffra* (roots),and *Spilanthesmauritian a* (roots and flowers) (Fabry et al., 1998).