ANTIMICROBIAL POTENTIAL OF MEDICINAL PLANT EXTRACT TOWARDS MULTI DRUG RESISTANT MICROBIAL STRAIN

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DECEMBER 2006

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LAPORAN AKHIR PENYELIDIKAN "ANTIMICROBIAL POTENTIAL OF MEDICINAL PLANT EXTRACT TOWARDS MULTI DRUG RESISTANT MICROBIAL STRAIN"

Merujuk kepada perkara di atas, bersama-sama ini disertakan 3 (tiga) naskah Laporan Akhir Penyelidikan bertajuk " Antimicrobial Potential Of Medicinal Plant Extract Towards Multi Drug Resistant Microbial Strain" oleh kumpulan Penyelidik dari Fakulti Farmasi untuk makluman pihak puan.

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Yang benar,

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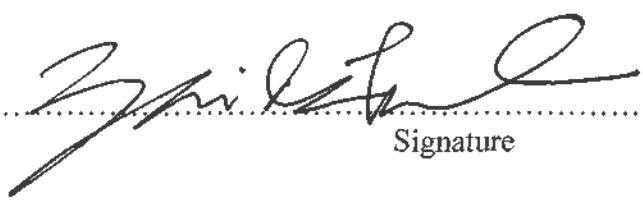

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ABSTRACT

Aim: Some traditional Malay medicinal plants were selected and evaluated for the antimicrobial activity potential against sensitive strains of *Staphylococcus aureus* and *Eschericia coli* and multidrug resistant strains of methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta lactamase *E.coli* (RESCO). **Method:** The ground dried leaves or seeds were extracted with 70 % aqueous methanolic solution for two days. The solution was subsequently filtered and dried *in vacuo* using a rotary evaoparator. The dried methanolic extract was later dissolved in 2 % DMSO and subjected to antimicrobial evaluation by means of microwell plate dilution method. **Conclusion:** All the traditional Malay medicinal plants inhibited the growth of *Staphylococcus aureus* strains with inhibition index between 0.36 and 0.84. The leaves of *Cassia alata* and seeds of *Cassia surattensis* exhibited moderate activity against both the MRSA and ESBL *E.coli* strains. All the *Lycopodium* species evaluated inhibited the growth of *Staphylococcus aureus* strain with inhibition index ranging from 0.61 to 1.10. Three of the *Lycopodium* species inhibited the growth of the MRSA strain.

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CHAPTER I

INTRODUCTION

For the past years, one of the principal areas of medical concern has been the emergence and transmission of multi-drug resistant (MDR) microorganisms in humans. The wide use of antibiotics has increased the number of resistant bacteria in the environment. Gantz *et al.* (1999) mentioned that for many years, there were serious concerns globally regarding the emerging antimicrobial resistance of numerous bacteria. Resistance is the degree to which a disease or disease-causing organism remains unaffected by antibiotics. The resistance organisms towards multiple antimicrobial were initially found in hospitals whereby the antimicrobial agents were used most extensively among the patients, but resistance is currently found almost as frequently in the community.

Examples of resistance bacteria are *Streptococcus pneumoniae, Enterococcus* species and *Mycobacterium tuberculosis*. Recently, quinolone resistance *Escherichia coli*, *Gonococci*, and *Campylobacter*, multidrug resistant *Salmonella typhimurium* and extended broad spectrum beta lactamase (ESBL) producing organism which were mainly *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) and methicillin resistance *Staphylococcus aureus* (MRSA) have all been recognized. Infection due to the mentioned MDR pathogen, present a major therapeutic concern as the choice of antibiotics is extremely limited. For the time being, vancomycin is the only

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drug effective against MRSA (Ariffin *et al*, 2004) while imipinem and ciprofloxacin are drugs of choice to combat ESBL producing organisms (Norazah *et al*, 2002).

Okeke *et al.* (2005) stated that resistant infections adversely affect mortality. For example, gastrointestinal, respiratory, sexually transmitted and nosocomial infections were leading causes of disease and death in the developing world, and management of all these conditions had been critically compromised by the appearance and rapid spread of resistance. The cost in treating the resistance infection had also increased. This happened when infections become resistant to first-line antimicrobials, therefore the treatment had to be switched to third-line drugs, which were nearly always much more expensive and sometimes more toxic.

The antimicrobial era has reached a level where the incidences of resistance microorganisms existing is increasing, while the development of new drugs and other alternatives are somehow, decreasing. Therefore there is a global urgency for the development of MDR pathogens drugs.

Throughout the history of mankind, nature's gift of herbal medicines has always been useful in treating various types of infectious diseases. Surprisingly, the role of these plant materials is still important as therapeutic remedies, even in the developing countries. They provide a large source of unexplored therapeutic drugs. As they are still considered to be the source of drugs for the majority population of the world, it is necessary to search for plants with potential antimicrobial activities. Research in this area is very important as there are increasing demands in searching alternative ways to

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combat against the increasing number of diseases caused by bacterial which are exhibiting drug resistance.

In parallel with the above mentioned global drug discovery and the highly emergence of multi-resistant microorganism obstacles, it is of great interest to carry out a screening on Malaysian plants in order to determine its antimicrobial properties. Ali *et al.* (2001) suggested that in order to overcome the growing problem of antimicrobial resistance, the researchers would be able to design and develop potentially new drugs from herbal remedies used in the traditional folk medicine. The herbal remedies used in the traditional folk medicine still provide a large source of unexplored therapeutic drugs. The used of medicinal plants still play a vital role to cover the basic health needs in the developing countries. For example, Heinrich *et al.* (2004) reported the used of bearberry (*Arctostaphylos*, uva-ursi), garlic (*Allium sativum*) and tree tea (*Malaleuca alternifolia*) which are known as broad-spectrum antimicrobial agents (cited by Rios and Recio, 2005).

Therefore, the objective of this study is to determine the potential antibacterial properties of selected plant extracts against sensitive and resistant Gram-negative and Gram-positive clinical isolates. In this research, the major interest is to investigate the antibacterial properties of plant extracts against pathogenic sensitive, *Escherichia coli* and *Staphylococcus aureus* and resistance strains extended-spectrum beta-lactamase (ESBL) and methicillin-resistance *Staphylococcus aureus* (MRSA).

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CHAPTER II

LITERATURE REVIEW

2.1 Multi-Drug Resistant (MDR)

Henry (2000) stated that MDR has become a very important aspect in pharmacotherapeutics since the cases of infectious diseases resistant towards antibiotic treatments are increasing (Elgorashi and Staden, 2004). Therefore, the urge of searching new drugs to overcome this problem has becoming a concern (Coates *et al*, 2002).

At first, one of the most important pathogens that can cause harm to human by bringing many infections had been proved to be fatal or susceptible to antimicrobial substances. Bramley *et al* (1989) and You *et al* (1999) stated that, unfortunately, as the antibiotic use has elevated, the resistant strain of *Staphylococcus aureus* (*S. aureus*), which is the MRSA has rapidly emerged (Kim *et al*, 2004).

MRSA was isolated in the early 1960's. According to Tsuchiya *et al* (1996), the strain, (which has the resistance mechanism due to penicillin-binding protein (PBP) 2' production) is resistant towards methicillin, β-lactamases and many other antibiotics (Kim *et al*, 2004). Shovein and Young (1992) reported that MRSA has also spread to

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North America in the late 1960's with the first reported outbreak occurring in Boston (Granitto, 1998).

Eventually, a new glycopeptides antibiotic called vancomycin was developed and seems to be the only drug effective to combat the MRSA (Granitto, 1998). However, in 1997, it was reported that an isolate of *S. aureus* had reduced susceptibility towards vancomycin in Japan (Hiramatsu *et al,* 1997). It is anticipated that glycopeptides resistance will be more frequent within a few years. Therefore, potentially new antimicrobial agents are needed to treat MRSA.

There are several mechanisms of resistance towards the β-lactam antibiotics, but the production of β-lactamase enzyme to break the β-lactam ring of the antibiotic, is the most significant one. This extended-spectrum β-lactamases (ESBLs) hydrolyze the expanded-spectrum cephalosporins and also monobactams (Morris *et al*, 2003). However other choices of antibiotics such as carbapenem and cephamycins are still available where both antibiotics categories were not affected or inactivated by the hydrolyzing enzymes (Maglio *et al*, 2004).

Karas *et al* (1996) and Paterson *et al* (2001) stated that there were several reports related to incidences where serious infections with ESBLs producing pathogens failed to be treated by β-lactams (Maglio *et al*, 2004). According to Knothe *et al* (1983), the first ESBL was reported in Germany in year 1983 (Morris *et al*, 2003). Most of the ESBLs are produced by the Enterobacteriaceae family members even though there are also other

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families of Gram-negative bacterial that produce those (Morris *et al*, 2003). Philippon *et al* (1989) and Shen *et al* (2001) wrote that the predominant host species for ESBLs from the Enterobacteriaceae family are *E. coli* and *K. pneumoniae* (cited by Morris *et al*, 2003).

Generally, microorganisms have exhibited various levels of drug resistance as a result of indiscriminate the use of antibiotics to treat infectious diseases (Cowan, 1999). Due to this alarming increase in the rate of infections with antibiotic resistant microorganisms in this era, the interest and effort to discover new antimicrobial agents has been significantly rising (Davies, 1994). One of the approaches for search of biologically and pharmacologically active substance to treat various ailments is the systemic screening of plants, which have been the sources of many therapeutic agents (Elgorashi and Staden, 2004).

2.2 The Importance of Medicinal Plants

For thousand of years plants have been a source of medicinal agents in virtually all cultures to treat diseases all over the world. Furthermore, an increasing dependence on the use of medicinal plants in the industrialized societies has been identified to the development of several modern drugs and chemotherapeutics, from these plants and also from traditionally, rural herbal remedies (Nair *et al*, 2005).

Due to the serious problem of MDR, ethno pharmacologists, botanists, microbiologists, natural-products chemists and pharmacists, are searching all over the world for

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phytochemicals which can be used to treat infectious diseases (Cowan, 1999). Rios and Recio (2005) reviewed the number of articles published on the antimicrobial activity of medicinal plants in Pubmed during the period between 1994 and 1996 totaled to 115 and increased for the following decades between 1995 and 2004 to 307 which was almost double to the previous number. The specific study of the activity of a plant or principle against a concrete pathological microorganism has been done by Machado *et al.* (2003) towards methicillin resistant *Staphylococcus aureus* (cited by Rios and Recio, 2005).

According to Farombi (2003), higher plants have always been important in maintaining human health and well-being since even the ancient time (cited by Nair *et al*, 2005). Eventually, plants are able to produce a wide range of bioactive molecules as a source of developing different types of medicines. These plants consists many secondary metabolites such as tannins, terpenoids, alkaloids, flavanoids, glycosides and various oils which has been found to have important antimicrobial properties (Ezeifeka *et al,* 2004). Khan *et al* (1985) stated that the compounds maybe localized in certain parts of the plant, whether in the glands or even all over the body and it was also observed that different chemical substances are obtained in members of the same species but in different areas (cited by Ezeifeka *et al,* 2004).

Obviously, plant preparations exhibited lower number of adverse reactions cases when compared to modern conventional pharmaceutics. In addition, plants are well known to be cheaper and this eventually encourages both the public and national health care

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institutes to consider plant medicines as an alternative to the chemically synthetic drugs (Nair *et al.,* 2005).

2.3 Description of Plants

Cassia alata (Caesalpiniaceae) leaves are pinnately compound, 30 to 40 cm long, with 6-12 pairs of broad oblong leaflets. The plant grows in waste places, often along streams, banks and in swamps. In traditional medicine, it is well known to treat ringworm in many countries (Ivan, 2003). Antimicrobial, laxative, wound healing and analgesic properties have been reported for the leaves (Khan *et al*, 2001). In vitro, the leaves exhibited high activity against various species of dermatophytic fungi but low activity against non-dermatophytic fungi (Ibrahim and Osman, 1995).

Current reports have indicated that methanolic extracts of the *Melastoma malabathricum* (Melastomataceae) shows antiviral properties against HSV-1 and Poliovirus. In addition, it is also found to be cytotoxic on murine and human cell lines activities. In traditional medicine, the leaves are used to treat diarrhea, dysentery, ulcers, piles and gastric ulcer (Devehat *et al*, 2002).

Azadirachta indica, commonly known as "Neem" has been extensively used in India as traditional Ayurvedic and folklore medicine for the treatment of various diseases. The extract from bark, leaves, fruits, oil and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children. The biological activities and medicinal properties of "Neem' has recently been reported (Bandyopadhyay *et al*, 2002).

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Astragali radix (Hwang-gi) is the root of Astragalus which belongs to Leguminoceae family. *Astragali* root is one of the oldest and most frequently used crude drugs for oriental medicine in Korea, China, Japan and other Asian countries and is well known to strengthen the most defense system as a tonic. It has also reported that it has effects on circulation and immune system, and enhanced the cell metabolism in vitro. However, the mechanism of action of this plant is still unknown (Lee, 2005).

Fructus schisandrae (Magnoliaceae) contains volatile oils (including α-pinene, β-pinene, camphene, myrcene, α-terpinene and others). It is indicated for enduring cough and deficiency wheezing. Besides, it constrains the lung and enriches the kidney, generates body fluids and constrains sweating (Xu and Wang, 2002).

The well-known *Eurycoma longifolia* (Simaroubacea) is one of the most popular folk medicines for antipyretic, antimalarial and restorative activities in Southeast Asia and is known to be a promising natural source of biologically active compounds. Some of the constituents have been known to possess anti-amoebic, cytotoxic, anti-tumoral and plasmodicidal activities. In particular, the quassinoids are one of the major bioactive groups in this plant (Jiwajinda *et al*, 2002).

The botanical medicines *Centella asiatica* has been used for decades, both topically and internally to enhance wound repair. Scientific studies are now beginning to validate efficacy and explore mechanisms of action for these botanicals (MacKay and Miller, 2003). This extract is used effectively in the treatment of cheloids, leg ulcers, phlebitis,

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slow-healing wounds, leprosy, surgical lesions, striae distensae and cellulites (Hausen, 1993).

Selaginella species (Selaginelaceae) are known as the lower ferns which possess true vascularized stems, leaves and roots. Silva *et al.* (1995) described several *Selaginella* species used in traditional medicine in various countries to treat a variety of diseases such as cancer, cardiovascular problems, skin diseases and urinary tract infections. Extract from some *Selaginella* species have shown anti-inflammatory, antimutagenic, antispasmodic, cytotoxic and immunostimulant and RNA reverse-transcriptase inhibitory activities. However, only a few studies on the bioactive components of species in this genus have been performed. A previous investigation on S. *doederleinii* demonstrated that the cytotoxic activity against L929 murine carcinoma cells was correlated to its lignan constituents.

Moss species are small green plants growing in dense clusters in damp, shady places. These nonvascular plants have an ancient lineage. Borel *et al.* (1993) showed that out of nine mosses extracts, the CH_2Cl_2 extract of *Dicranum scoparium* was found to possess pronounced antimicrobial activity against *Bacillus cereus, Bacillus stearothermophilus, Bacillus subtilis, Staphylococcus aureus, and Escherichia coli*. Dicranin structure was found to be responsible for most of the biological activity. The strongest antimicrobial effect was observed against *Streptococcus faecalis* (disc diffusion assay). In contrast to the CH_2Cl_2 extract of *D. scoparium*, dicranin was inactive against *E. coli*.

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According to Evans (2002), *Lycopodium* (Lycopodiacea) consists about 50 % of fixed oil, which consists mainly of the glycerides of lycopodiumoleic acid. *Lycopodium clavatum* is one of the original Hahnemann remedies favored for treatment of dyspepsia. The mother tincture is derived from the crushed spores of the plants. It was claimed to be effective in digestive imbalances originating from emotional and stressed states. Takayama *et al.* (2002) illustrated seven new lycopodium alkaloids from *Lycopodium serratum* Thunb. Huperzine A is a potential therapeutic agent found in this plant for the treatment of Alzheimer's disease that acts as acetyl cholinesterase inhibitors.

2.4 Antimicrobial Susceptibility Testing (AST) and Minimum Inhibitory Concentration (MIC50) Determination Test

In the AST and MIC_{50} determination test, the methods used have their own advantages and disadvantages, which can possibly affect the outcome result. For example, the agar-well diffusion method results are time consuming and not applicable for bactericidal studies. However, in broth dilution test such as microdilution plate method, it is useful in determining the MIC and the minimal bactericidal concentration (MBC) of an antimicrobial agent (Tortora *et al*, 2002). The minimum concentration of antibiotics that inhibited 50 % of the isolates tested is known as MIC_{50} (Kim *et al*, 2004).

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