BIOASSAY-GUIDED FRACTIONATION AND IDENTIFICATION OF ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM *CALLISTEMON VIMINALIS* (GAERTN.) G. DON

by

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ABSTRAK

Penyaringan aktiviti antioksida ekstrak segar MeOH 80% daripada 30 spesies tumbuhan telah dilakukan dengan menggunakan ujian penyingkiran radikal bebas 2,2-difenil-1-pikrilhidrazil (DPPH). Empat tumbuhan daripada famili Myrtaceae (*Baeckea frutescens, Callistemon viminalis, Leptospermum flavescens* dan *Melaleuca alternifolia*) kemudian terpilih untuk proses pengekstrakan berperingkat menggunakan empat pelarut dengan kekutuban yang menaik. Minyak pati daripada empat tumbuhan ini juga disediakan dengan menggunakan proses penyulingan stim. Keputusan pengujian antioksidan terhadap ekstrak krud dan minyak pati mendapati ekstrak polar (MeOH 80% dan etil asetat) menunjukkan aktiviti penyingkiran radikal bebas yang lebih baik berbanding ekstrak tidak-polar dan ekstrak MeOH 80% *Callistemon viminalis* terpilih sebagai ekstrak yang paling berpotensi dengan nilai EC_{50} terendah (26.66 µg/ml). Aktiviti antioksidan ekstrak MeOH 80% dan MeOH 80% yang dipanaskan kemudian dibandingkan dan didapati ekstrak MeOH 80% yang dipanaskan menunjukkan nilai EC_{50} yang paling rendah (21.95 µg/ml). Ekstrak MeOH 80% yang dipanaskan kemudian terpilih untuk proses penyisihan dengan menggunakan teknik kromatografi kertas. Fraksi yang menunjukkan peratusan penyingkiran radikal bebas terbaik pada kepekatan akhir 125 µg/ml ialah FhM 2A1 (93.88 ± 0.10%). Nilai EC_{50} terbaik diperolehi daripada aglikon FhM 2A1 (6.18 µg/ml). FhM 2A1dan aglikon FhM 2A1 telah masing-masing dikenal pasti sebagai 7-metil-3,3'-diglikosida kuersetin and rhamnetin. Ujian antimikrob daripada ekstrak krud dan minyak pati *Callistemon viminalis* telah dilakukan

dengan menggunakan kaedah pembauran cakera dan penentuan nilai perencatan minimum (MIC). Ekstrak heksana telah terpilih sebagai ekstrak terbaik berdasarkan zon perencatan dan nilai MIC yang diperolehi terhadap bakteri ujian. Penyisihan telah dijalankan terhadap ekstrak ini dengan menggunakan kaedah kromatografi turus dan fraksi af3 kemudian terpilih sebagai fraksi terbaik berdasarkan nilai MIC. Setelah penyisihan semula dijalankan, refraksi df6 terpilih sebagai refraksi terbaik. Analisis GC-MS minyak pati, ekstrak heksana dan refraksi df6 telah mengenalpasti kehadiran sebatian eukaliptol, α-pinene, α-phelandrena dan D-limonena sebagai sebatian aktif antimikrob dalam semua sampel kajian.

BIOASSAY-GUIDED FRACTIONATION AND IDENTIFICATION OF ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM *CALLISTEMON VIMINALIS* (GAERTN.) G. DON

ABSTRACT

Antioxidant activities of fresh 80% MeOH extracts from 30 plant species (at 100 mg/ml) were screened using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test. Four plants from Myrtaceae family (*Baeckea frutescens, Callistemon viminalis, Leptospermum flavescens* and *Melaleuca alternifolia*) were selected to undergo subsequent extraction using four different solvents with ascending polarity. Essential oils of each plant were also prepared using steam distillation process. The results obtained from antioxidant test of crude extracts and essential oils concluded that polar extracts (80% MeOH and ethyl acetate) showed higher free radical scavenging activity as compared to non-polar extracts and 80% MeOH extract of *Callistemon viminalis* was selected as the most potent extract with the lowest EC_{50} value (26.66 µg/ml). Antioxidant activity of 80% MeOH and 80% heated MeOH was then compared and 80% heated MeOH extract showed the lowest EC_{50} value (21.95 µg/ml). 80% MeOH heated extract was then selected for fractionation process using paper chromatography technique. Fraction that showed the highest percentage of free radical scavenging activity at final concentration 125 µg/ml was FhM 2A1 (93.88 ± 0.10%). The best EC_{50} value was obtained from FhM 2A1 aglycone (6.18 µg/ml). FhM 2A1 and aglycone FhM 2A1 were identified as 7-methyl-3,3'-diglycosides quercetin and rhamnetin respectively. Antimicrobial test of crude extracts and essential oil of *Callistemon viminalis* were conducted using disc diffusion method and determination of Minimum Inhibitory

Concentration (MIC). Hexane extract was then selected as the best extract based on inhibition zones and MIC values obtained against tested bacteria. Fractionation of this extract was conducted using column chromatography technique and fraction af3 was selected as the best fraction based on its MIC value. After refractionation was conducted, refraction df6 was selected as the best refraction. GC-MS analysis of essential oil, hexane extract and refraction df6 have identified the presence of eucalyptol, α-pinene, α-phellandrene and D-limonene as active antimicrobial compounds in all analyzed samples.

CHAPTER 1
INTRODUCTION

1.1 Discovery of plant-derived drugs

1.1.1 Definition, history and examples of plant-derived drugs

Plant plays an important role in the human life as the main source of food, medicine, wood, oxygen producer and many more (Cowan, 1999). Plant contribution to the medicinal field is largely owing to the activity of plant derived drugs. Plant derived drugs term can be defined as biological active substances which are isolated or purified from plants.

Nowadays, 40% of all medicinal prescription in the United States (US) contains at least one plant derived drugs and physician in Europe routinely recommended to their patients herbs such as chamomile and coneflower. The use of plants as medicine by human can be traced back to the early pre-historic times and for a long time together with mineral and animal products, were the main sources of drugs (Rates, 2001). But then, booming of the usage of synthetic compounds was started when urea incidentally synthesized in the laboratory by Friedrich Wohler in 1828. Gradually, those synthesized compounds replaced the usages of plants as medicines. The discovery of antibiotic penicillin from *Penicillium notatum* mould then acted as the marking point of the rediscovery of plant derived drugs. Today, at least 25% of commercial drugs are plant derived drugs, such as aspirin, atropine, quinine, morphine, vincristine and vinblastine (Gilani and Atta-ur-Rahman, 2005).

Aspirin, which is consumed 80 million tablets each year in US only, originated from *Salix alba* (white willow). Analgesic activity of aspirin made it extremely useful in relieving fever, inflammation and pain. Anticoagulant activity of aspirin was also discovered and it is widely practiced to prevent strokes and heart attack in calibrated doses. Aspirin prevents aggregation of blood platelets and enhances the blood supply to the heart and brain. *Atropa belladona*'s atropine is used in alleviating pain and to treat palsy associated with Parkinson's disease. Quinine that is isolated from *Chinchona officinalis* and *Chinchona pubescens* bark is used to treat malaria. Morphine from *Papaver somniferum* capsules act as pain-killer.

However, wrong administration of morphine will also lead to addiction and casually fatal. Vinblastine and vincristine from *Catharanthus roseus* are used to treat Hodgkin's disease, choriocarcinoma, childhood leukemia and breast cancer (Sumner, 2000).

1.1.2 Importance of drugs discovery from plants

Nowadays rapid development is continuously to happen in the field of chemistry of medicinal research. Despite this rapid development, many plant derived drugs are still cannot be synthetically produced. Two reason stands behind the statements. Some compounds such as atropine and reserpine are still too expensive to be synthesized; and many useful drugs also still cannot be synthesized such as morphine, cocaine, ergotamine and digitalis (Sumner, 2000; Gilani and Atta-ur-Rahman, 2005). Thus, the isolation of plant derived drugs still holds important rules in drug discovery. Once plant derived drugs is isolated, then it can act as the lead compound which is a good starting point in developing new drug. It can allow the design and rational planning of the new drugs as well as biomimetic synthesis development and discovery of new biological activity not yet related to the known compounds (Hamburger and Hostettmann, 1991; Rates, 2001; Arya *et al*., 2002). One example is salicylic acid that originally synthesized to found replacement for phenol as antiseptic. Further finding then reported the antypiretic and antirheumatic activities (Sneader, 2005).

1.2 Selection of plants for drug discovery

There are vast numbers of plant species found on earth. The estimate number is up to 500 thousand species. From this number, about 10 thousands are reported to have medicinal usages and only 150 - 200 are commercially used. Thus the plant kingdom is really a potential source of medicinal properties (McChesney *et al.,* 2007). Many plants contain chemical compounds which are originally acted as defence agents against predator (for example cardiac glycosides from leaves of *Nerium oleander* protects the plant from herbivorous animals) or for protection (flavonoids act as UV protector of leaves) or for attraction (sesquiterpenoids with aromatic smell attract pollinators and dispersal organisms)

(Harborne, 2001). Some of those chemical compounds also have therapeutic value for human. Certain approach to isolate active compounds from plants must be employed in order to obtain the desired results.

Various screening approaches can be selected and are depending on the target diseases as well as on the available information about the plants. Figure 1.1 summarizes various approaches to bioassay-directed natural product drug development. Plants with ethnobotanical backgrounds are usually used for single-goal screening (using a specific bioassay technique) (Atta-ur-Rahman *et al*., 2001). Ethnobotany refers to the study of the use of plants used by human that associated with the traditional beliefs and cultural practices. The term includes the use of medicinal plants for treatment of diseases (Heinrich *et al.,* 2004). For example turmeric (*Curcuma longa*) has long been recognized to possess antimicrobial activity such as in Indian Ayurveda practices (Goel *et al*., 2008). Based on ethnobotanical data, curcumin was isolated from turmeric and possesses antimicrobial activity against *Staphylococcus aureus, S. albus, Bacillus cereus, B. subtilis, Escherichia coli* and *Pseudomonas aeruginosa* (Jayaprakasha *et al.,* 2005). Biorational studies correlate the data from natural history with ecological and evolutional theory (Scholes *et al.,* 2005). The fact that some plants are avoided being grazed by herbivores often means that the plant contains toxic. For example is *Nerium oleander* with its oleandrin (a cardiac glycoside) that causes lethal if it is ingested. Research conducted then found the usefulness of cardiac glycosides for treating heart failure and cardiac arrhythmia in human (Barbosa *et al.,* 2008). Chemotaxonomic study of plants can also be conducted since plant species within one particular taxa are usually assumed to have similar chemical properties (Bindseil *et al.,* 2001). For examples, Braca *et al.* (2003) studied biological activities of seven plants from *Licania* genus. While Goren *et al.* (2002) studied biological activities of *Tanacetum* genus. Basic knowledge on certain plant species found in literature also can be used as source of knowledge in discovery of plant derived drugs. Prior research conducted by Dat *et al.* (1992) regarding the diuretic activity of *Orthosiphon stamineus* (daun misai kucing) has inspired Olah *et al.* (2003) to isolate active compounds responsible for the diuretic activity. The last

method used in drug discovery is by random or blind collection. This method is useful when dealing with potential biodiversity in unstudied area. According to Farnsworth (1998) the discovery of vinblastine and vinscristine from *Catharanthus roseus* was through random sampling method.

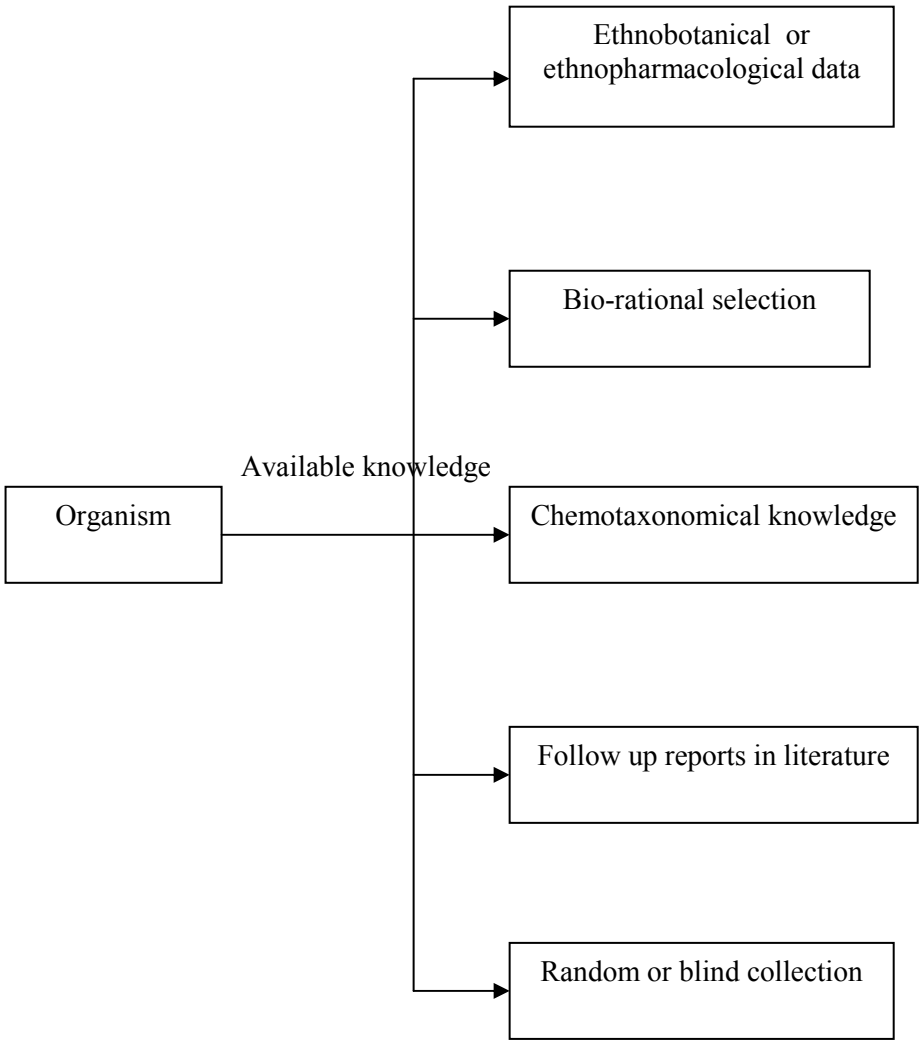

Figure 1.1 Approaches to bioassay-directed natural product drug development (Atta-ur-Rahman *et al.,* 2001).

1.3 The traditional therapeutic values of plants used in preliminary antioxidant screening

Free radical scavenging test was chosen for the preliminary screening of plant samples. Thirty plants species were used in the preliminary screening. Random collection approach was selected for the selection of plant samples. Table 1.1 shows various traditional applications of plant species used in this study. Methods of application of medicinal plants are related with the purpose of application. Internal application usually applied through raw consumption, inhalation, decoction, infusion and juice; while external application usually through poultices or compresses.

Table 1.1 Traditional usage of plant species used in the study

No.	Plant species	Traditional usage
1	*Acalypha australis* L.	This plant is used by the Indian as remedy for severe cough associated with bleedings from lungs and to treat tuberculosis. Leaves juice is also used to treat skin problems by the Indians (Ambasta *et al.,* 1986).
2	*Albizia saman* (Jacq.) F. Muell.	Leaves decoction is used to treat malaria (Kohler *et al.,* 2002).
3	*Amaranthus hybridus* L.	Roots decoction is used by the Cambodian as antipyretic and to treat rheumatism. Decoction of roots and leaves is employed as expectorant by the Malaysian. Leaves decoction is also believed to possess diuretic activity by the Indonesian and Malaysian. While the whole plant decoction is drunk to treat hepatitis by Malaysian and Philippines (Wiart, 2000). Decoction of this whole plant is also used to treat diarrhea by Malaysian while leaves and stems are crushed and applied to treat boils by the Malaysian and Indian (Ahmad and Raji, 1991).
4	*Baeckea frutescens* L.	Leaves decoction is used as antipyretic by the Chinese community in Southeast Asian countries. Leaves decoction is also employed as post-partum medicine by Malaysian and Indonesian (Chin, 1992). The leaves also acknowledged as one of ingredients of jamoo for body beauty care by the Indonesians (Riswan *et al.,* 1991).
5	*Barleria lupulina* Lindl.	The mixture of roots powder mixed with lime juice is used to treat ringworm in Indonesia. Malaysian drinks the leaves juice as laxative. Crushed leaves are applied to treat herpes by the Indonesian (Wiart, 2000).
6	*Callistemon viminalis* (Sol. Ex Gaertn) G. Don	Essential oil obtained from leaves are used as antihelminthic against hookworm and tapeworm (Garg and Kasera, 1982).
7	*Chamaesyce hyrta* (L.) Millsp.	The latex is used to treat conjunctivitis and ulcerated cornea (Chin, 1992).
8	*Chamaesyce thymifolia* (L.) Millsp.	Decoction of whole plant is used by the Malaysian and Indonesian to treat abdominal problem, diarrhea and dysentery. This plant is also used as diuretic agent by the Chinese (Chin, 1992).
9	*Cheilocostus speciosus* (J. Konig) C. Specht.	Tuber of this plant is used as anti-inflammatory and antihelminthic agents and to treat skin diseases and worms. Whole plants is also used as antidiabetic by the Indian (Vijayalakshmi and Sarada, 2008).
10	*Cinnamomum iners* Reinw. Ex. Blume	Roots decoction is used by the Malaysian as febrifuge, while barks decoction acts as carminative and purgative for the Indochinese (Chin, 1992).
11	*Cleome gynandra* L.	Decoction of roots and leaves is used as expectorant by the Malaysian. Crushed leaves are applied to treat herpes by the Indonesian. Whole plant decoction is used to treat hemorrhoid by Chinese (Wiart, 2000).

Table 1.1 Continued

No.	Plant species	Traditional usage
12	*Cyanthillium cinereum* (L.) H. Rob.	Leaves juice is used to stimulate labour and to hasten the expulsion of the placenta by the Indonesian. Decoction of whole plant is used to treat hepatitis by the Chinese and is used by the Indonesian, Malaysian, Chinese and Phillipines to treat bronchitis and asthma. Poultices of this plant are used to treat abscesses, boils and wounds by the Philippines (Chin, 1992). The entire plant is pounded and taken as a drink in the treatment of cancer (Zakaria and Mohamad, 1994).
13	*Desmodium triflorum* (L.) DC.	The decoction is used as antipyretic by the Chinese community in Southeast Asian countries. Roots decoction is used to treat dysentery by the Chinese, Philippines, Indochinese, Malaysian and Indonesian. Whole plant is used to treat rheumatism by the Chinese. Poultices of this plant are also used by the Malaysian, Philippines and Indonesian to treat skin problems (Chin, 1992).
14	*Eclipta prostrata* (L.) L.	Decoction of whole plant is used internally as post-partum medicine by the Indian. Pounded plant is applied externally to treat wounds contusions and burns by the Malaysian (Wiart, 2000). Whole plant decoction is drink to treat hepatitis (Zakaria and Mohamad, 1994).
15	*Emilia sonchifolia* (L.) DC.	Leaves decoction is used as antipyretic by the Chinese community in Southeast Asian countries. Tea made from leaves is used to treat dysentery by the Chinese, Philippines, Indochinese, Malaysian and Indonesian (Chin, 1992). While roots decoction is used by the Indonesian to cure dysentery, and crushed plants are applied to treat abscesses and burns (Wiart, 2000).
16	*Gynura bicolor* (Roxb. ex Wild.) DC.	Leaves poultices are used by the Vietnamese as antipyretic (Wiart, 2000).
17	*Gynura procumbens* (Lour.) Merr.	Leaves poultices are used by Vietnamese as antipyretic (Wiart, 2000).
18	*Hedyotis corymbosa* (L.) Lam.	Leaves poultices are applied by the Malaysian as antipyretic. While leaves decoction is used by the Chinese to treat various inflammations. Chinese also believes that this plant has anticancer activity (Chin, 1992).
19	*Leptospermum flavescens* Smith	Decoction of this plant is used to relieve discomfort in stomach by the Malaysian (Sulaiman *et al.,* 1991).
20	*Melaleuca alternifolia* Cheel	The aborigins use maceration of leaves under a warm mud compress as remedy for skin lesions or infected injuries (Budhiraja *et al.,* 1999). The oil from crushed leaves is inhaled to relieve coughs and colds (Shemesh and Mayo, 1991).
21	*Mimosa pudica* Mill.	Pounded leaves are applied by the Malaysian and Indonesian to reduce swelling. Leaves of this plant are soaked in coconut oil and placed on wounds and ulcers by the Philippines (Chin, 1992).

Table 1.1 Continued

No.	Plant species	Traditional usage
22	*Nothopanax scutellarium* (Burm. F.) Merr.	Decoction of leaves is used as anti-inflammatory activity by the Indonesian (Dalimartha, 1999).
23	*Oxalis corniculata* L.	Leaves poultices are applied by the Indonesian and Chinese as antipyretic. Juice of this plant is drunk by the Indonesian to relieve abdominal pain (Chin, 1992).
24	*Peperomia fraseri* C. DC.	Juice of this plant is drunk by Indonesian to relieve abdominal pain. While poultices are used by the Philippines to treat abscesses, boils and wounds (Chin, 1992). Whole plant decoction are drunk to treat rheumatism (Zakaria and Mohamad, 1994).
25	*Pluchea indica* (L.) Less.	Roots decoction of this plant is used by the Vietnamese and Malaysian as antipyretic and is drunk by the Vietnamese to treat rheumatism. The sap of this plant is ingested by Indonesian and Malaysian to cure dysentery. While decoction of leaves is used to wash scabies (Wiart, 2000). Crushed leaves is used to correct foul breath by the Philippines (Chin, 1992).
26	*Talinum fruticosum* (L.) Juss	Leaves and shoots are eaten by the Indian to treat diabetic (Ambasta *et al.,* 1986).
27	*Tradescantia spathacea* Sw.	Rosales-reyes *et al.* (2008) reported the use of this whole plant extract to treat cancer by the Mexican.
28	*Tridax procumbens* L.	The juice of its leaves possesses antiseptic, insecticidal and parasiticidal properties. It is also used to treat hemorrhage from cuts, bruises and wounds (Saxena and Albert, 2005).
29	*Urena lobata* L.	The Malaysian used the stem decoction to treat fever, trunk decoction to treat dysentery and crushed flower to treat boils (Ahmad and Raji, 1991).
30	*Zephyranthes candida* (Lindl.) Herb.	Decoction of leaves were used as antidiabetic by the Indian (Ambasta *et al.,* 1986).

1.4 *Callistemon viminalis*

1.4.1 Morphological description

Callistemon viminalis belong to Myrtaceae family. *Callistemon viminalis* is derived from Greek words *kallistos* (most beautiful) and *stema* (a stamen); while *viminalis* came from Latin word means a long flexible shoot or oosier (Gilman and Watson, 1993). So literally, *Callistemon viminalis* means most beautiful stamen from long flexible shoot. The common name weeping bottle brush is also derived from this definition. The fact is, *Callistemon viminalis* most notably by its beautiful scarlet blossoms during flowering time and the flower of *Callistemon viminalis* also produced large quantities of nectar, hence the tree is much sought after by birds and bees (Srivastava *et al.,* 2003).

Usual appearances of *Callistemon viminalis* is a shrub or small tree up to 8 m tall (Plate 1.1 A). The leaves of *Callistemon viminalis* are lanceolate 0.3 cm - 0.6 cm wide and 4 – 7 cm long. The flowers are borne in spikes 14 – 15 cm long with prominent red stamens 1.50 – 2.50 cm long (Plate 1.1 B). Petals are greenish or pale coloured, tiny and inconspicuous. Flowers mature into woody capsules (Gilman and Watson, 1993).

Callistemon viminalis is a plant native to Australia and now is widely spread to Asia, Europe and America. *Callistemon viminalis* is cultivated as ornamental tree and found to be useful in landscaping as a screen plant along river bank (for controlling erosion). *Callistemon vimnalis* also relatively adaptable to various soil condition and can grow well with limited maintenance (Sheat and Schofield, 1995).

Taxonomy of *Callistemon viminalis:*

Kingdom	: Plantae
Subkingdom	: Tracheobionta
Superdivision	: Spermatophyta
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Myrtales
Family	: Myrtaceae
Genus	: *Callistemon*
Species	: *Callistemon viminalis*

A

B

Plate 1.1: Morphology of *Callistemon viminalis*
A. Tree of *Callistemon viminalis*
B. Leaves and flower of *Callistemon viminalis*

1.4.2 Bioactivity studies of *Callistemon viminalis*

Although *Callistemon viminalis* is widely cultivated, the information on its biological properties is still scarce. Antihelminthic activities of leaf essential oil of *Callistemon viminalis* against earthworms, tapeworms and hookworms were reported by Garg and Kasera (1982) and Srivastava *et al.* (2003). While an aqueous extract of the flowers and leaves showed antibacterial activity against Gram-positive bacteria (Srivastava *et al.,* 2003). Lee *et al.* (2004) investigated fumigant toxicity of 42 essential oils from Myrtaceae family against *Sitophilus oryzae, Tribolium castaneum* and *Rhyzopertha dominica* and discovered that essential oil of *Callistemon viminalis* only showed moderate activity in killing those three stored-grain insects. Adonizo *et al.* (2006) studied anti-quorum sensing (anti-QS) activity of fifty plant species in Southern Florida against *Chromobacterium violaceum* and *Agrobacterium tumefaciens*. The study reported that the water and ethanol extracts of the inflorescence of *Callistemon viminalis* showed strong anti-QS activity against *C. violaceum* and *A. tumefaciens*, while the ethanol extract of the leaves of *Callistemon viminalis* showed anti-QS activity against *A. tumefasciens.*

1.5 Antioxidant

1.5.1 Definition of Antioxidant

Oxidation process occurs naturally in human body and defined as electron transfer from one atom to another. Since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP, oxidation is an essential part of aerobic life and human metabolism. But the problem may arise when electrons flow from oxidation process become unpaired and then subsequently generates free radicals, known as Reactive Oxygen Species (ROS), such as superoxide ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl (HO) and nitric oxide ($NO^{\bullet}$). Free radicals are very reactive and rapidly attack molecules in nearby cells (Pietta, 2000). Eventhough positive impact of ROS was also recorded such as involvement in phagocytosis, regulation of cell growth and intercellular signaling and synthesis of biologically important compounds (Halliwell, 1997), but their negative

influences also received special attention. Reactive oxygen species acts in damaging cell membranes and attacking proteins and DNA in tissues. Carcinogenesis may also be initiated through oxidatively-induced DNA damage. Repeated damage caused by ROS throughout the span of human life increases with time, and is a major cause of age-related cancers and other oxidatively-induced diseases (Reynertson, 2007).

Antioxidants are substances that when present in foods or body at low concentrations compared with that of an oxidizable substrate significantly delay or prevent the oxidation of that substrate (Saha *et al.,* 2004). Antioxidants will help to minimize oxidative damage as the most important approaches to the primary prevention of age-related diseases, since antioxidant terminate direct ROS attacks and radical-mediated oxidative reactions (Tepe and Sokmen, 2007). Antioxidants also can be described as acidic compounds (including phenols) that are used as preservative in foods, cosmetics and pharmaceutical preparations which can donate an electron or hydrogen atom to a peroxyl or alkoxyl radical and terminate lipid peroxidation and chain reaction (Aruoma, 2003).

Human then evolved with antioxidant systems to protect themselves against free radicals. Human defense systems include antioxidants produce naturally in the body (endogenous) and those obtain from the diet (exogenous). However, despite these defense antioxidant systems, some ROS are still escaped to cause oxidative damage. Therefore, dietary antioxidants are needed to protect the harmful action of ROS. Well established antioxidants derived from diet are vitamins A, C, E, polyphenols and carotenoids (Pietta, 2000). Current antioxidant research of free radicals also has confirmed that food with rich antioxidants play an essential role on the prevention of disease caused by oxidative stress. Therefore, plant derived antioxidants now receiving a special attention (Tepe *et al.,* 2005).

1.5.2 Classification of antioxidant based on mechanism of action

There are many types of antioxidant classification made. One of them, antioxidants is classified according to the nature of their mechanism of action. Gordon (1990) and Maisuthisakul *et al.* (2007) have divided antioxidants into two main types:

a. primary antioxidants or chain-breaking antioxidant; is compounds which can inhibit or retard oxidation by scavenging free radicals or by donation of hydrogen atoms or electrons which convert them to more stable products.
b. secondary antioxidants or preventive antioxidant; is that bind metal ions, scavenge oxygen, decompose hydroperoxides to non-radical species, absorb UV radiation or deactivate singlet oxygen.

1.5.3 Plant-derived antioxidants

Plant-derived antioxidants were discovered from different sources of plant parts and plant origin. The antioxidant hypothesis claims that antioxidants can prevent oxidative damages, and reduce the risks of chronic disease (Stanner *et al.,* 2004). The hypothesis is in line with efforts to discover natural antioxidants, especially plant-derived antioxidants.

Plant-derived antioxidants are mainly obtained from diets, such as vitamin A, C, E and carotenoids (Pietta, 2000). Apart from those vitamins, other substances in diet are also believed to contribute to the antioxidant action which might be beneficial to human health. Plant polyphenols or phenolic compounds are now widely studied by researchers due to complexity of their chemical nature and the ubiquitous occurrence in plant materials (Dimitrios, 2006). Phenolic compounds are range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to complex structure such as lignins, melanins and tannins. Flavonoids are the most common and widely distributed sub-group (Bravo, 1998; Soobrattee *et al.,* 2005). Phenolic compounds are commonly found in diet, most notably in vegetables, fruits and beverages (Luximon-Ramma *et al.,* 2003; Bahorun *et al.,* 2004; Luximon-Ramma *et al.,* 2005).

Phenolic compounds from vegetables were extensively studied and many commonly consumed vegetables reported to have strong antioxidant activity. *Cratoxylum formosum, Leucaena glauca, Momordica charantia, Sesbania grandiflora, Syzygium gratum* were studied by Maisuthisakul *et al.* (2007) and were reported to have strong antioxidant activity with total phenolic content > 40 mg GAE/g dry weight and EC_{50} values of DPPH free radical scavenging activities range from 0.23 to 0.55 µg/µg. *Ipomoea reptans* and *Solanum lycopersicum* (tomatoes) also have strong antioxidant activity (Toor and Savage, 2005; Dasgupta and De, 2007). Bahorun *et al.* (2004) reported strong antioxidant activity of *Brassica chinensis, Allium cepa, Artemisia vulgaris* and *Brassica oleracea.* Quercetin, myrcetin and kaempferol are reported as the main classes of flavonoids found abundance in these samples.

Fruits are important dietary sources of phenolic compounds. *Eugenia siamensis, Antidesma velutinum* and *Cleistocalyx operculatus* have high total phenolic content. D'Abrosca *et al.* (2007) reported strong antioxidant activity of apple (*Malus domestica*) that is rich with flavonoids compounds such as catechin, epicatechin, phloridzin and phloretin-2'-xyloglucoside. Mulberry fruits (*Morus alba*) was also reported to have strong antioxidant activity due to the presence of phenolic compounds, particularly anthocyanins (Bae and Suh, 2007).

Beverages such as fruit juices, tea, coffee and wines are also important sources of phenolics in human diet. High total phenolic contents reported from black tea, green tea, instant and ground coffee (Lakenbrink *et al.,* 2000; Khokhar and Magnusdottir, 2002; Luximon-Ramma *et al.,* 2005; Balasundram *et al.,* 2006). Tea in particular is rich with catechin including epicatechin, epicatechin 3-gallate, epigallocatechin and epigallocatechin-3-gallate, eventhough their composition varies from each type of teas (black, green and oolong tea) (Fraser *et al.,* 2007). Coffee is also rich of polyphenols such as caffeic, ferulic acid, *p*-coumaric acid and caffeoylquinic acid. The roasting process also increase the contents of Maillard reaction product that donate more to its antioxidant activity (Czerny *et al.,* 1999; Parras *et al.,* 2007). Pomegranate juice (*Punica granatum*) were also reported to be

rich of phenolic compounds, tannins and anthocyanins (Gil *et al.,* 2000). Antioxidant activity of wine is primarily contributed by its high anthocyanin contents (Rivero-Perez *et al.,* 2008). Dried *Hibiscus sabdariffa* flower that is drunk as red tea, is a rich source of polyphenols, especially anthocyanin and also vitamin C (Prenesti *et al.,* 2007).

Another important source of dietary phenolic compounds is obtained from spices that is used as food flavour. The cuisines of Asia, Southeast Asia and Mediterranean were considered to be healthier than Western diet, partly because of extensive uses of herbs and spices (Satia-Abouta *et al.,* 2002; Kaefer and Milner, 2008). Herbs and spices that often add flavour and aroma to cuisines are rich of volatile compounds from terpenes class which some of them also showed potent antioxidant activity. Cloves (*Syzygium aromaticum*) was reported to have an outstanding antioxidant activity that was correlated with phenolic compounds, in particular the volatile phenolic compounds (Ho *et al.,* 2008). Turmeric *(Curcuma longa*) is rich of curcumin and curcuminoids and was reported as a strong free radical scavenger (Fujisawa *et al.,* 2004; Meghana *et al.,* 2007). Coriander (*Coriandrum sativum)* is rich with well known antioxidants such as quercetin, caffeic acid, cineole, geraniol, borneol, eucalyptol, cinnamic acid, ferrulic acid and rutin (Melo *et al.,* 2005; Kaefer and Milner, 2008). Nutmeg (*Myristica fragrans*) was reported to be rich in eugenol, lauric acid, myrcene, myristicin and terpinen-4-ol. Chili pepper (*Capsicum annuum*) is rich of ascorbic acid, beta-carotene, caffeic acid, capsaicin, hesperidin, kaempferol and quercetin. While ginger (*Zingiber officinale)* was also reported to be rich with ascorbic acid, beta-carotene, caffeic acid, gamma-terpinene and isoeugenol (Suhaj, 2006). While Agbor *et al.* (2006) linked high free radical scavenging activity of peppers (*Piper nigrum* and *Piper guineense*) with their high phenolic contents.

1.6 Antimicrobial

1.6.1 Antimicrobial agents

Microorganisms can causes diseases to human being. Infectious diseases by bacterial action daily caused 50 thousands of premature deaths (Carey, 2004; Esterhuizen *et al.,* 2006). Thus, the control of microorganisms is crucial in prevention and curing of disease caused by their actions. Antimicrobial agent is a substance that kills or inhibits the growth or prevents damage due to the action of infectious microorganisms. Terms of antimicrobial agents comprises of antibacterial, antifungal, antiprotozoal, antihelminthic and antiviral agents (Baron *et al.,* 1994).

Antibacterial agents are closely associated with antibiotics. Antibiotics are substances produced by bacteria and fungi that inhibited the growth and killed other bacteria (Purohit *et al.,* 2003). While antifungal agent is defined as substances that kill or inhibit the growth of fungi (McDonnell, 2007). Antibiotics once produced by bacteria and fungi. But now most antibiotics are synthetically produced.

The use of antibiotics that once regarded as one of the biggest discovery in the 20th century is effective on saving many lives against bacterial infection. However, the emergence of bacterial resistance to all known antibiotics class are globally reported and started to gain the attention of many people for the need of new antibacterial source (Ojala *et al.,* 2000). Misuse, abuse and over-prescription of antibiotics are believed to be the reasons of emergence of resistance (Eloff, 2000; Peterson and Dalhoff, 2004). There are three mechanisms by which antibiotic resistance can occur: prevention of interaction of the drug with target; efflux of the antibiotics from the cell; and direct destruction or modification of the structure (Mendonca-Filho, 2006). New source of antimicrobial agents were then eventually lead to intensive research on antimicrobial compounds from plants.

1.6 .2 Plants as new source of antimicrobial compounds

Ever since antibiotic were discovered, the research on antimicrobial compounds from plants were no longer conducted. Due to increasing resistance of bacteria to antibiotics, there is an increasing demand of new antimicrobial agents from plants. Plants are considered a reliable source for the discovery of novel antimicrobial agents (Rangasamy *et al.,* 2007). The research on antimicrobial compounds from plants have been conducted for several reasons: 1) many phytochemicals were prescribed by physicians as antimicrobial agents; of which several are already being clinically tested; 2) the increasing of public awareness on the safety of current antibiotics; 3) the ascendance of the infection rate of human immunodeficiency virus (HIV) has encouraged the intensive investigation of plant derivatives which is effective but also cheap and accessible for use in underdeveloped countries with little access to expensive Western medicines; 4) rapid rate of plant extinction within the past 20 years has attract interest of chemist and microbiologist, whom believe that aggregation of potentially useful phytochemical structures which could not be synthesized chemically is at risk of being lost irretrievably (Lewis and Elvin-Lewis, 1995; Boris, 1996; Cowan, 1999).

Traditional therapeutic value of plants to treat bacterial infections has long been recognized in many different communities. Researches have also been conducted to validate the traditional therapeutic value of the plants as well as to discover its antimicrobial activity. For instance, Wiart *et al.* (2004) have reported a broad spectrum of antimicrobial activity of extracts of *Polyalthia laterifolia, Peristrophe tinctoria, Knema malayana, Solanum torvum, Celosia argentea, Eclipta prostrata, Dillenia suffruticosa, Piper stylosum* and *Rafflesia hasseltii.* While antimicrobial activity of *Cassia alata* against dermatophytic fungi *Trichophyton mentagrophytes* var. *interdigitale, T. mentagrophytes* var. *mentagorophytes, T. rubrum* and *Microsporum gypseum* were reported by Ibrahim and Osman (1995). Grosvenor *et al.* (1995) have screened 124 plant species from Riau province, Indonesia and reported that 121 species showed inhibitory activity against *S. aureus* while 42 species inhibited the

growth of *E. coli.* Table 1.2 shows a list of plants and antimicrobial compounds identified from it.

Table 1.2 Plant species and identified antimicrobial compounds (Cowan, 1999)

Common name	Scientific name	Compound	Class
Allspices	*Pimenta dioica*	Eugenol	essential oil
Apple	*Malus sylvestris*	Phloretin	Flavonoid derivatives
Aswagandha	*Withania somniferum*	Withafarin A	Lactone
Aveloz	*Euphorbia tirucalli*	-	Terpenoid
Barberry	*Barberis vulgaris*	Barberine	Alkaloid
Basil	*Ocimum basilicum*	Essential oils	Terpenoids
Bay	*Laurus nobilis*	Essential oils	Terpenoids
Betel pepper	*Piper betel*	Catechols, eugenol	Essential oils
Black pepper	*Piper nigrum*	Piperine	Alkaloid
Brazilian pepper tree	*Schinus terebinthifolius*	Terebinthone	Terpenoids
Burdock	*Arctium lappa*	-	Polyacetylene, tannins, terpenoids
Buttercup	*Ranunculus bulbosus*	Protoanemonin	Lactone
Cashew	*Anacardium pulsatilla*	Salicylic acids	Polyphenols
Chamomile	*Matricaria chamomilla*	Anthemic acid	Phenolic acid
Chapparal	*Larrea tridentate*	Nordihydroguaiaretic acid	Lignan
Chili peppers	*Capsicum annuum*	Capsaicin	Terpenoid
Clove	*Syzygium aromaticum*	Eugenol	Terpenoid
Coca	*Erythroxylum coca*	Cocaine	Alkaloid
Eucalyptus	*Eucalyptus globulus*	Essential oil	Terpenoids
Garlic	*Allium sativum*	Allicin	Sulfoxide
Gotu kola	*Centella asiatica*	Asiatocoside	Terpenoid
Green tea	*Camellia sinensis*	Catechin	Flavonoid
Hemp	*Cannabis sativa*	β-Resercyclic acid	Organic acid
Henna	*Lawsonia inermis*	Gallic acid	Phenolic acids
Papaya	*Carica papaya*	Latex	Mix of terpenoids, organic acids, alkaloids.
Peppermint	*Mentha piperita*	Menthol	Terpenoid
Periwinkle	*Catharanthus roseus*	Reserpine	Alkaloid
Thyme	*Thymus vulgaris*	Caffeic acid, thymol, tannins.	Terpenoid, phenolic alcohol, polyphenols.
Turmeric	*Curcuma longa*	Curcumin	Terpenoids

Various compounds synthesize by plant species are medicinally useful. Some of them are also known for having antimicrobial properties. The antibacterial compounds from plants may inhibit bacterial growth by different mechanisms as compared with antibiotics and have significant value in the treatment of bacterial infections (Eloff, 1998). Eventhough the exact antimicrobial actions of plant derived compounds was yet to be understood, but usually related with disruption of cell wall structure and outer membrane arrangement (Medeiros *et al.,* 2003). Table 1.3 summarizes major classes of antimicrobial compounds from plants and its mechanisms of action.

Table 1.3 Major classes of antimicrobial compounds from plants with its mechanism of action (Cowan, 1999)

Class	Subclass	Example(s)	Mechanism
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	Bind to adhesions, complex with cell wall, inactivate enzymes.
	Quinones	Hypericin	Bind to adhesions Complex with cell wall Inactivate enzymes
	Flavonoids	Chrysin	Inhibit HIV reverse transcriptase
	Flavones	Abyssinone	Membrane disruption
	Flavonols	Totarol	Bind to proteins
	Tannins	Ellagitannin	Bind to adhesins Enzyme inhibition Substrate deprivation Complex with cell wall Membrane disruption Metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (antiviral activity)
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine Piperine	Intercalate into cell wall and/or DNA
Lectins and polypeptides		Mannose-specific agglutinin Fabatin	Block viral or adsorption Form disulfide bridges

1.6.3 Essential oil

Essential oil is known as aromatic substances produced by specific plant species. The record of essential oils as medicine can be found from ancient times and is regarded as the most widely used natural products in many areas since many traditional folk medicines based mainly from plant materials. And the fact that most traditional medicine are consumed with hot water, or are extracted with hot water leads to the conclusion that some of the active components may be essential oils which can be partitioned into water by heating (Nakatsu *et al.,* 2000). Nowadays, essential oils are mainly used in food flavouring, perfume and pharmaceutical formulation due to their biological activity (Heravi and Sereshti, 2007)

Essential oil can be obtained from various plant materials, i.e. flowers, buds, seeds, leaves, twigs, barks, herbs, woods, fruits and roots (Burt, 2004). Plant families particularly rich in essential oil including Asteraceae, Lamiacae, Myrtaceae, Pinaceae, Rosaceae, Rutaceae and Apiaceae (Harborne, 1998). Essential oils and their components are relatively safe, wide acceptance by consumers and their multi-purpose functional usages (Feng and Zheng, 2007).

Essential oils comprise of many kinds or classes of molecules, including terpenoids, aromatics, cyclic and acyclic compounds, acetonides and sulfur- and nitrogen- containing compound (Nakatsu *et al.,* 2000). Terpenoids usually are the main component of essential oils which could be divided into two classes, the mono- and sesquiterpenes, which differ in their boiling point and the number of isoprene unit (Harborne, 1998).

The methods of extraction of essential oils from plants also significantly affect the chemical constituents and composition of the essential oils. Several methods that have been developed to extract essential oils are: cold press extraction, extraction of one oil with another, steam distillation, solvent extraction, supercritical fluid extraction, microwave oven extraction, solid phase extraction and fluorocarbon extraction (Nakatsu *et al.,* 2000).

1.6.4 Biological activity of essential oils

Many essential oils are applied externally to cure bacterial and fungal infections including boils, acne, gingivitis and vaginal candidacies. Tea tree oil, which originated from steam-distilled of native Australian plant *Melaleuca alternifolia* has been widely used as topical antiseptic in Australia for almost 80 years. Other biological activities of essential oils from various plants are summarizes in Table 1.4.

Table 1.4 Biological activity of essential oils

Biological activity	Common name	Source	References
Antimalaria activity	-	*Lippia javanica*	Manenzhe *et al.* (2004)
Antibacterial activity	Coriander	*Coriandrum sativum*	Delaquis *et al.* (2002)
	Cinnamon	*Cinnamomum zeylanicum*	Burt (2004)
	Oregano	*Origanum vulgare*	Marino *et al.* (2001)
	Rosemary	*Rosmarinus officinalis*	Daferera *et al.* (2000)
	Sage	*Salvia officinalis*	Marino *et al.* (2001)
	Clove	*Syzygium aromaticum*	Bauer *et al.* (2001)
	Thyme	*Thymus vulgaris*	Juliano *et al.* (2000)
	Tea tree oil	*Melaleuca alternifolia*	Ponce *et al.* (2003)
	Kanuka oil	*Leptospermum flavescens*	Porter and Wilkins (1998)
Antifungal activity	-	*Origanum compactum*	Bouchra *et al.* (2003)
	Tea tree oil	*Melaleuca alternifolia*	Hammer *et al.* (1998)
Antiinflammatory activity	Eucalyptol	*Eucalyptus globulus*	Santos *et al.* (2004)
	Baeckea oil	*Baeckea frutescens*	Tam *et al.* (2004)
		Casearia sylvestris	Esteves *et al.* (2005)
Antioxidant activity	-	*Achillea millefolium*	Candan *et al.* (2003)
	-	*Cylotrichium origanifolium*	Tepe *et al.* (2005)

1.7 Bioassay-guided fractionation

1.7.1 Definition of bioassay-guided fractionation

Bioassay-guided fractionation is a procedure of whereby extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated in a bioassay system and only active fractions are fractionated (Atta-ur-Rahman and Basha, 1988). Bioassay-guided fractionation method is commonly employed in drug discovery

research due to its effectiveness to directly linked the analyzed extract and targeted compounds using fractionation procedure that followed with certain biological activity.

For antioxidant research, Cho *et al.* (2003) have employed bioassay-guided fractionation procedure to isolate active compounds from 23 medicinal plants from Korea using Silica gel chromatography and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Successive fractionation technique followed by DPPH assay was leading to isolation of polyphenols such as procyanidin B-3, (+)-catechin, gallic acid, methyl gallate, quercetin, quercetin-3-*O*-β-D-glucoside, quercetin-3-*O*-β-galactoside, quercetin-3-*O*-rutinose and kaempferol which possessed strong DPPH free radical scavenging activity.

While for antimicrobial research, Si *et al.* (2006) used bioassay-guided fractionation procedure to isolate compounds that capable of inhibit the growth of several major food-borne pathogens (*E. coli, Salmonella typhimurium, Listeria monocytogenes, S. aureus* and *B. cereus*) from Chinese green tea extract. High speed counter current chromatography (HSCCC) method was developed for the separation and purification of active compounds, which led to the isolation of epicatechin gallate, epigallocatechin gallate, epicatechin and caffeine.

1.8 Problem statement

There are still very limited publications on the biological activities of four plant species from Myrtaceae family that are *Baeckea frutescens, Callistemon viminalis, Leptopspermum flavescens* and *Melaluca alternifolia*. The capability of plants to produce essential oils also added more interests due to the activity of the essential oil as antioxidant and antimicrobial agents.