ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *Orthosiphon stamineus* BENTH. WITH EMPHASIS ON ROSMARINIC ACID CONTENT

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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *Orthosiphon* *stamineus* BENTH. WITH EMPHASIS ON ROSMARINIC ACID CONTENT

by

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LIST OF SYMBOLS

SYMBOLS

˚C	Degree Celsius
μg	Microgram
cm	Centimetre
EC_{50}	Half maximal effective concentration
M	Molarity
mg	milligram
mL	Milliliter
nm	Nanometre
pK_a	Acid dissociation constant
R_f	Retention factor

LISTS OF ABBREVIATIONS

ABBREVIATIONS

ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BHTQ	Tetrabutylated hydroquinone
CAE	Caffeic acid equivalent
CFU	Colony forming unit
$CHCl_3$	Chloroform
DLAP	Double layer agar plate
DMSO	Dimethylsulfoxide
GLC	Gas-liquid chromatography
HCl	Hydrochloric acid
HNO_2	Nitrous acid
HPLC	High performance liquid chromatography
LA	Lactic acid
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MPN	Most probable number
MS	Mass spectrometry
PC	Paper chromatography
TLC	Thin layer chromatography

AKTIVITI ANTIMIKROB DAN ANTIOKSIDAN *Orthosiphon stamineus* BENTH. DENGAN PENEKANAN PADA KANDUNGAN ASID ROSMARINIK

ABSTRAK

Orthosiphon stamineus Benth. merupakan tanaman herba yang biasa ditanam dan mudah didapati di Malaysia. Tanaman ini digunakan dalam perubatan tradisional untuk mengubati pelbagai penyakit. Dalam kajian ini, lima jenis ekstrak *O. stamineus* diperolehi melalui nisbah berbeza metanol gred analitikal (0, 25, 50, 75 dan 100%) dan air suling diuji terhadap sembilan jenis bakteria makanan secara *in vitro* menggunakan kaedah resapan cakera. *V. parahaemolyticus* didapati paling rentan terhadap ekstrak *O. stamineus* melalui kaedah ini. *V. parahaemolyticus* kemudian diuji dengan lebih lanjut menggunakan kaedah kepekatan perencatan minimum (MIC) dan kepekatan bakteriosid minimum (MBC). Daripada ujian MIC, *V. parahaemolyticus* adalah paling rentan terhadap 50 % dan 75 % ekstrak metanol. Proses pecahan ekstrak metanol 50 % menghasilkan enam pecahan, dengan pecahan ke-5 mempunyai aktiviti antibakteria yang paling tinggi terhadap *V. parahaemolyticus*. Ekstrak metanol 50 % dan 75 % serta pecahan ke-5 memberikan nilai MIC terendah 1.56 mg/ mL. Daripada semua ekstrak kasar, pecahan dan sampel kawalan, hanya pecahan ke-5, kloramfenikol dan asid laktik memberikan nilai MBC. Ujian analisis kandungan fenolik untuk ekstrak kasar menunjukkan bahawa 50 % ekstrak metanol mempunyai kandungan fenolik tertinggi. Analisis HPLC ekstrak kasar dan pecahan menunjukkan bahawa pecahan ke-5, diikuti oleh ekstrak metanol 50 % mengandungi kepekatan asid rosmarinik tertinggi. Ekstrak metanol 50 % didapati mempunyai aktiviti penyingkiran radikal bebas tertinggi dalam ujian penyingkiran radikal bebas DPPH, juga mengandungi kepekatan asid rosmarinik

yang tinggi antara ekstrak-ekstrak kasar. Ini menunjukkan bahawa kehadiran asid rosmarinik dapat menyingkirkan radikal bebas dengan baik. Kesan penghambatan ekstrak kasar metanol 50 % kemudian dinilai dengan lebih lanjut dalam sampel udang, dengan mempertimbangkan jumlah bakteria aerobik dan jumlah *V. parahaemolyticus*. Ekstrak diuji secara individu serta dengan kombinasi 1 % asid laktik untuk menentukan keberkesanan ekstrak. Keputusan menunjukkan bahawa ekstrak metanol 50 % individu sebanyak 2 % dan 5 %, asid laktik secara individu dan kombinasi asid laktik-ekstrak mengurangkan jumlah bakteria aerobik dalam sampel udang berbanding dengan udang yang tidak dirawat selama masa kajian keseluruhan 21 hari. Untuk *V. parahaemolyticus*, jumlah bacteria pada hari ke-21 menunjukkan bahawa tidak ada perbezaan statistik antara semua rawatan dengan kawalan, kecuali untuk 75: 25 nisbah 5 % ekstrak: 1 % asid laktik. Keputusan eksperimen ini menunjukkan bahawa ekstrak *O. stamineus* mengandungi aktiviti antimikrob untuk melanjutkan tempoh simpanan dan ini membuktikan potensinya untuk kajian lanjut.

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *Orthosiphon stamineus* BENTH. WITH EMPHASIS ON ROSMARINIC ACID CONTENT

ABSTRACT

Orthosiphon stamineus Benth. is a commonly planted herbal plant and is easily available in Malaysia. This herb is used in traditional medicine to treat a wide range of diseases. In this research, five types of *O. stamineus* extracts obtained through different ratios of analytical grade methanol (0, 25, 50, 75 and 100 %) and distilled water were tested against nine strains of food-borne bacteria *in vitro* using disc diffusion assay. From this assay, *V. parahaemolyticus* was found to be the most susceptible bacteria. *V. parahaemolyticus* was then subjected to further tests of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). From MIC test, *V. parahaemolyticus* was most susceptible to 50 % and 75 % of methanolic extracts. The fractionation of 50 % methanolic extract yielded six fractions, with fraction 5 possesses highest antibacterial activity against *V. parahaemolyticus*. These 50 % and 75 % methanolic extracts as well as fraction 5 gave the lowest MIC values of 1.56 mg/mL. Of all the crude extracts, fractions and the control samples, only fraction 5, chloramphenicol and lactic acid gave MBC values. Total phenolic assay analysis for crude extracts showed that 50 % methanolic extract possesses highest phenolic content. HPLC analysis of the crude extracts and fractions showed that fraction 5, followed by 50 % methanolic extracts contains the highest rosmarinic acid concentration. The 50 % methanolic extract was found to possess the highest free radical scavenging activity in DPPH free-radical scavenging assay, which also contains high concentrations of rosmarinic acid among the crude extracts, suggests that the presence of rosmarinic acid contributes tremendously to

the extracts' free radical scavenging activity. The inhibitory effect of the 50 % methanolic crude extracts was then further evaluated on shrimp samples, taking into account of the total aerobic bacteria and *V. parahaemolyticus* counts. The extracts were tested alone and in combination with 1 % lactic acid to determine the effectiveness of the extract. Results demonstrated that the 50 % methanolic extracts alone at 2 % and 5 %, lactic acid alone and the combination of extract-lactic acid reduced the total aerobic bacteria counts in shrimp samples compared to the non-treated shrimps for the whole study duration of 21 days. For *V. parahaemolyticus* counts, the counts on the 21st day shows that there is no statistical difference among all the treatments with the control except for the 75 : 25 ratio of 5 % extract: 1 % lactic acid. This suggests that the application of *O. stamineus* extract possesses antimicrobial activity to prolong shelf life of deshelled shrimps and this warrants further studies.

CHAPTER 1 INTRODUCTION

Nowadays, consumers are more aware and informed of the ingredients, nutritional values as well as the preservatives present in the food they consume due to the widespread of information in the various types of media and they are also more educated compare to our forefathers. Consumers want their food to be healthy, natural and unadulterated to reap the full potential of the nutrients and various diseases-preventing compounds offered by our mother nature. At the mean time, natural foods without additional processing are susceptible to various kinds of spoilage and thus reduce the margin of profit of the manufacturers. A good solution to this war is natural preservatives, especially those derived from plant. Therefore, the interest in developing new types of effective and nontoxic antimicrobial compounds is increasing due to concerns about the safety of food containing synthetic preservatives (Shan *et al*., 2007). Consequently, interest in using natural antibacterial compounds, such as extracts of spices and herbs for preserving food, has become increasingly popular, as consumers these days demand for natural products, free of artificial additives (Suhaj, 2006).

These challenges paved the way for scientists to conduct more researches on natural alternative preservatives. The overuse of chemical preservative agents has pressured the food industry to either eliminate all the preservatives added to food stuffs or find a safer and natural alternative preservative to lengthen the shelf life of foods (Nychas, 1995). Besides, there has been reports on the resistance of certain microorganisms towards some preservative agents used currently (Piper *et al*., 1998).

Since ancient time, long before mankind discovered the existence of microorganisms, the thought that certain plants possess healing potential was well accepted. The healing and preservative properties they contained are what we currently describe as antimicrobial properties. Herbs and plant-based products have been widely used then by mankind to treat and prevent illnesses and improve well-being. Plant materials such as barks, leaves, roots and flowers have been used traditionally for their therapeutic effect valued by our forefathers since long time ago. Plants contain numerous biologically active compounds, many of which have been documented to possess antimicrobial properties (Cowan, 1999). Furthermore, new compounds inhibiting microorganisms such as benzoin and emetine also have been isolated from plant. According to Cox (1994), the antimicrobial compounds from plants may inhibit bacteria by different mechanisms than the presently used antibiotics and probably have clinical value in treating resistant microbial strains.

A lot of countries have been working on changing their folk medicines or natural flora into formal applications. For instance, Ríos *et al.* (1991) screened 140 medicinal plants used in the Mediterranean region as anti-infection agents and subsequently one of them, *Helichrysum stoechas*, was chosen to be studied in detail, with the isolation and identification of the active principles as well as the following determination of the spectra and potency of the isolated compounds. Other examples of such scientific researches include studies of medicinal plants of Thailand by Wannissorn *et al.* (2005) and India by Jeevan Ram *et al.* (2004).

Previous researches too have shown that a lot of plant extracts, especially herbs and spices, are rich in phenolic secondary metabolites, and some have antimicrobial activity (Lin *et al.*, 2005). For example, curcumin isolated from turmeric (*Curcuma longa*) is effective against *Staphylococcus aureus*, *Staphylococcus albus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Jayaprakasha *et al*., 2005). There were a number of researches done on the use and effectiveness of herbal extracts for their antimicrobial activity besides turmeric. Among the herbs studied for their antibacterial effects against food-borne pathogens are coriander (*Coriandrum sativum*) (Delaquis *et al*., 2002), cinnamon (*Cinnamomum zeylanicum*) (Burt, 2004), clove (*Syzygium aromaticum*) (Daferera *et al*., 2000), tea tree oil (*Melaleuca alternifolia*) (Ponce *et al*., 2003), oregano (*Origanum vulgare*) as well as sage (*Salvia officinalis*) (Marino *et al*., 2001).

Malaysia is recognized to be one of the mega diversity countries where a variety of organisms, animals and high level plants can be found. With a total land area of about 33,000 million hectares (where 58 % is under tropical rainforest), Malaysia has more than 20,000 plant species with 2,000 species have been reported to have medicinal properties (Wan Hassan, 2007). A variety of the plants species have been employed traditionally by the local society as the medicine to heal illness or consumed as a booster for health and energy. Herbal products are well accepted by communities here as a component for treating diseases, aromatherapy and even incorporated into foods. Wan Hassan (2007) reported that the current Malaysian market for herbal products is estimated to worth RM 4.55 billion and is growing at 15 % annually but 90 % of the raw materials were imported as only about 50 of the local species are being used and even less were scientifically evaluated for their medicinal properties. Therefore, Malaysia has very high potential to discover new novel plant compounds that could be used to treat various ailments.

Nevertheless, information on antimicrobial activity by *Orthosiphon stamineus* is not available although antimicrobial activity in plants for medicinal purposes was intensively researched and comprehensively reviewed (Ríos & Recio, 2005). Therefore, this research was done to find an alternative preservative agent from this herbal plant that could be used to preserve foods in food industry. This research is viable as this plant (*O. stamineus*) is found planted or wild grown abundantly throughout this country and the region such as Vietnam, Thailand, the Philliphines and Indonesia. Moreover, in this study, the whole plant comprised of young shoots and stems in addition to the leaves were used, thus reducing the amount of agricultural waste, increasing farmers' income and maximizing the yield of the active compound. In addition, powder of the whole *O. stamineus* plant is also easily obtained because of its availability as herbal tea.

The information obtained from this research such as the types of effective extract, the concentration needed to halt the growth or kill the bacteria could be documented internationally so that further researches could be done. Besides, this research could promote this herb to the world and help the farmer to increase their income.

1.1 Research objectives

This study was undertaken to determine the potential of selected *Orthosiphon stamineus* (Misai Kucing) extracts to extend the shelf life and promote safety in the food industry. This was achieved by investigating the *in vitro* as well as the food model system antimicrobial activity of methanolic and water extracts of *O. stamineus* against selected food-borne bacteria.

The more specific objectives of this research are:

1. To evaluate the antimicrobial activity of *Orthosiphon stamineus* herbal extracts against selected food pathogens *in vitro* and antioxidant activity of the extracts *in vitro*.

2. To quantitate the concentration of rosmarinic acid in crude extracts and partially purified extracts.

3. To study the effectiveness of the herbal extract as antimicrobial agent in deshelled shrimps stored in refrigerated condition.

CHAPTER 2 LITERATURE REVIEW

2.1 *Orthosiphon stamineus* Benth.

In this study, *Orthosiphon stamineus* Benth. herbal plant extracts were used as a source of antimicrobial agent. Plate 2.1 shows the picture of the flower of the plant.

Plate 2.1 : *Orthosiphon stamineus* Benth. (Misai Kucing)

2.1.1 Distribution of O. stamineus

Orthosiphon stamineus or *Misai Kucing*, is also known as *Kumis Kucing*, *Remujung*, *Ruku Hitam*, Java Tea, Cat Whiskers and Kidney Tea Plant among other names. This plant is a native of Southeast Asia and Northern Australia (Wan Hassan, 2007). In Malaysia, *O. stamineus* is commonly seen in many home gardens and in the wild, as well as growing along the forest edges, roadsides, and wastelands. This plant is relatively easy to be cultivated, widely distributed and is becoming one of plantation crops in tropical countries such as Thailand, Indonesia, the Phillipines, Brunei and Malaysia due to its medicinal properties.

2.1.2 Description of O. stamineus

Orthosiphon stamineus Benth. is of the Lamiaceae family. It is a perennial herb which can grow up to about 1.5 m in height. The stems are short, quadrangle in shape and reddish in colour and it branches profusely bearing simple, lightly-hairy, green leaves arranged in opposite pairs. The leaves are simple, opposite, with ovate-oblong-lanceolate blades and serrated margins. Each leaf is about 1.5-4.5 cm wide, 5-10 cm long. The inflorescences are borne on the terminal apices of the branches. They are vertical and can measure up to 16 cm in length. The bracts are green, minute caudiform in shape with two bracts normally hold a cluster of five flowers. The flowers are hermaphroditic, bell-shaped, creamy-white to bluish in colour with long exerted filaments making them look like cat's whiskers. The flower is a compound flower in the bunch that came out at the end of the branching. The fruits are oblong-ovoid brownish nutlets, each about 1.5 to 2 mm long. The leaves can be harvested about 4 months after planting or just before flowering by clipping the branches and subsequently thereafter at 8 to 10 weeks intervals (Wan Hassan, 2007).

2.1.3 Active constituents and biological activities of Orthosiphon species

This herb has been used for years for treating the urinary tract and kidney ailments. *O. stamineus* is believed to promote the elimination of fluids, nitrogenous substances, and sodium chloride. It has been used in Malaysia and Indonesia for centuries in treating kidney and bladder ailments (Wan Hassan, 2007). In Southeast

Asia, it is mainly used for the treatment of eruptive fever, epilepsy, hepatitis, gallstone, rheumatism, hypertension, renal calculus and syphilis (Akuwoah *et al*., 2004a).

O. stamineus, contains twenty phenolic compounds, including two flavonol glycosides, nine lipophilic flavones and nine caffeic acid derivatives, such as rosmarinic acid and 2, 3-dicaffeoyltartaric acid (Sumaryono *et al*., 1991). The main components of *O. stamineus* leaves and extracts are the pharmacologically active polyphenols (the polymethoxylated flavonoids such as eupatorine and sinesetine and caffeic acid derivatives) (Olah *et al*., 2003).

Other active constituents present are listed below:

Beta-caryophyllene, alpha-humulene, caryophyllene-epoxide, eupatorin, sinensetin, scutellarine tetramethyl ethers, salvigenin, 7,3’,4’-tri-O-methylluteolin, 5-hydroxy-6,7’,3',4’-tetramethoxyflavone, 6-hydroxy-5,7’,4'-trimethoxyflavone, tetramethyl ethers, salvigenin, ladanein, 2,3-dicoffeoyltartrate, rosmaric acid, 2-caffeoyltartrate, terpenoids. diterpene ester, orthosiphole A to E (diterpene dibenzoyl diacetyl ester of primarane type), neoorthosiphol A and B orthosiphole F to J, staminol A and B, norstaminol A orthochromene A (benzochromene), triterpene saponins; aglycone hederagenin, orthosiphole A to E, (diterpene dibenzoyl diacetyl ester of primarane type), neoorthosiphol A and B, orthosiphole A and B, staminol A and B, norstaminol A, octhochromene A (benzochromene), aglycone hederagenin (triterpene saponins), alpha- & beta-carotene, crptoxantin, beta-zeacarotene, neo-beta-carotene, alpha-carotene oxide, vomo ofoliol, aurantiamide acetate, oleanolic acid, ursolic acid, betulinic acid, beta-sitosterol (Lyckander & Malterud, 1996; Malterud *et al*., 1989; Ohashi *et al*., 2000a; Zhari *et al*., 2003).

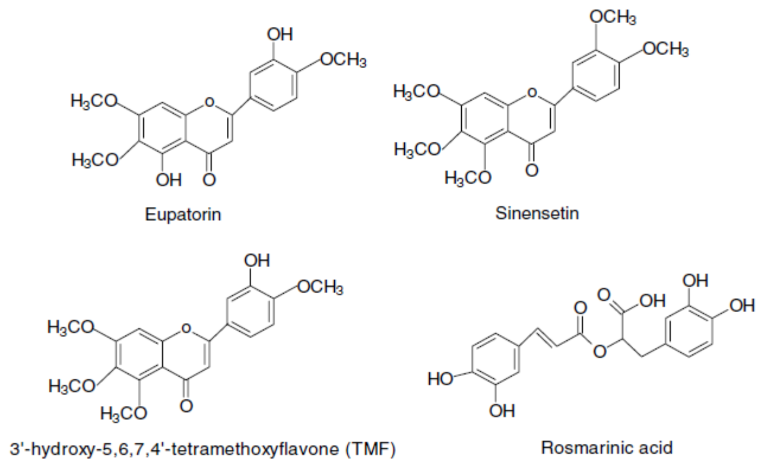

Figure 2.1 Chemical structures of some of the active constituents present in *Orthosiphon stamineus* (Akuwoah *et al*., 2004a)

2.1.4 Previous researches

Lamiaceae family, which has over 5000 species, has been one of the most key contributors of medicinally important and culinary species. They are aromatic and have also yielded essential oils commercially. Several species such as the *Orthosiphon* sp. accumulate rosmarinic acid and other caffeic acid derivatives (Gurib-Fakim, 2006). Rosmarinic acid (Figure 2.1) is important in pharmaceutical because of its non-specific complement activation and inhibition of leukotrienes which has anti-inflammatory effect.

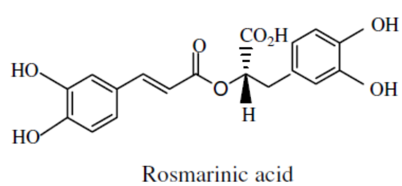

Figure 2.2 Chemical structure of rosmarinic acid (Gurib-Fakim, 2006)

Rosmarinic acid has a number of biological activities such as anti-inflammatory, antimicrobial, antioxidant and anitiviral (Petersen & Simmonds, 2003). The authors added that the existence of rosmarinic acid in medicinal plants, herbs and spices has valuable and health promoting property as in plants, this caffeic acid derivative is supposed to act as essentially accumulated defence compound.

There have been researches done related to the diuretic activity of *Orthosiphon* extract. Diuretic effects were reported in human, such as increased diuresis and elimination of chlorides and urea when the lyophilized aqueous extract was ingested. It was reported that there are no influences recorded for 12 and 24 hrs urine output or on the sodium excretion for any of drugs when tested under standardized conditions in a placebo controlled double-blind crossover model (Doan *et al*., 1992). In clinical studies with healthy volunteers in Thailand, the extract of *Orthosiphon* was shown to cause the increase of the citrate and oxalate excretion (Nirnoy & Muangman, 1991).

Even though an elevated level of oxalate may increase the risk of kidney stones formation, the increased citrate output helped prevent stone formation. Studies have been done on a hyro-alcohol extract of *Orthosiphon* for diuretic activities in rats which pharmacological evaluations revealed that they led to an increase in urine flow with increment of urinary sodium excretion (Beaux *et al.*, 1999).

Besides the diuretic property, *Orthosiphon stamineus* also demonstrated anti-hypertensive activities. Ohashi *et al*. (2000b) reported that the chloroform-soluble portion of the water decoction of this plant showed an inhibitory effect on the contractile responses on rat thoracic aorta smooth muscle stimulated with KCl beforehand. Methylripariochromene A was isolated from the leaves and studied on several pharmacological reactions related to hypertensive activity (Matsubara *et al*., 1999). The authors found that methylripariochromene A possesses some activities related to decreasing blood pressure as well as diuretic action.

Furthermore, this plant also possesses antimicrobial property. Amzad Hossain *et al.* (2008) reported that the essential oils, methanolic extract and derived fractions of methanolic extract of *O. stamineus* showed great potential of anti-fungal activity such as mycelia growth inhibitor against *Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium solani*, *Colletotricum capsici* and *Phytophthora capsici* in the range of 49.3 - 70.3 % and minimum inhibitory concentration of 500 to 1000 µg/mL.

2.2 Discovery of plant-derived drugs and food preservatives

It is estimated that there are 250,000 to 500,000 species of plants on Earth (Borris, 1996). Plants have provided mankind with all their needs such as of shelter, clothing, food, flavours and fragrances as well as medicines. A fair percentage (1 to 10 %) of plants is used as foods by both humans and other animal species. Moerman (1996) reported that probably even more are used for medicinal purposes. Plants have been the backbone of sophisticated traditional medicine systems such as the Ayurvedic, Unani and Chinese (Gurib-Fakim, 2006). These systems of medicine have contributed to some important drugs relevant to these modern days. These plant-based systems continue to play a vital role in health care and people still continue to rely on traditional medicines systems for their health care as they are cheaper compared to modern western medicines or when modern medicines are not accessible.

Plants' contributions to the medicinal field are largely due to the activity of plant-derived drugs. Plant drugs or phytomedicines or phytopharmaceuticals are 'plant-derived medicines that contain a chemical compound or more which are usually mixtures of chemical compounds that act individually or in combination on the human body to prevent disorders and to restore or maintain health' (Gurib-Fakim, 2006). The author further added that among the well-known medicinal products originating from this region are such as *Croton tiglium* (purging croton), *Duboisia hopwoodii* (pituri), *Eucalyptus globulus* (bluegum), *Melaleuca alternifolia* (tea tree), *Myristica fragrans* (nutmeg), *Piper methysticum* (kava kava), *Strychnos nuxvomica* (strychnine), *Styrax benzoin* (benzoin) and *Syzygium aromaticum* (cloves).

Traditional medicine often targets restoring balance by using chemically complex plants, or by creating a concoction by mixing together several different plants to be made into a medicine to take full advantage of a synergistic effect or to improve the chances of an interaction with a relevant molecular target. It is probably that the vast knowledge of herbal remedies used in traditional cultures were developed through trial and error over a long period of time, and that the discovered cures were passed down from one generation to another. Previously, the people who used traditional remedies may not understand the scientific rationale behind their usage of miracle cures, but they know from personal experience that certain medicinal plants can be highly effective if used at the proper doses.

Given that now we comprehend better how the body functions, therefore, we can understand the healing powers of plants and their potential as multi-functional chemical entities for treating complicated health conditions. Medicinal plants usually contain mixtures of various chemical compounds that may act individually or synergistically to improve health. For instance, Gurib-Fakim (2006) acknowledged that 'a single plant may contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as an antioxidant and venotonics, anti-bacterial and anti-fungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being'.

These days, the search for new molecules has changed its course where the science of ethnobotany and ethnopharmacognosy are being used as guide to lead the chemist towards different sources and classes of compounds (Gurib-Fakim, 2006). It was estimated that 14-28 % of higher plant species are used medicinally and 74 % of pharmacologically active plant derived components were discovered after following up on traditional uses of the plants (Ncube *et al.*, 2008). Some interesting outcomes have been documented with the use of a mixture of natural products to treat diseases, particularly on the synergistic effects and polypharmacological application of plant extracts (Gibbons, 2003). The development of pharmaceuticals starts with identification of active principles, detailed biological assays and dosage formulations. These new drugs will then have to go through clinical studies to establish its safety, efficacy and pharmacokinetic profile. If the plant extracts were to be accepted as valid medicinal agents, then they will have to go through biological evaluation to ensure their efficacy and safety. Numerous plants have been used due to their antimicrobial properties have been investigated and documented by a number of researchers worldwide.

Besides their therapeutic properties and mainly used for in the field of medicine, plant extracts nowadays are even used in the food industry. Originally, spices and herbs were added to foods to improve or change their taste. However, in recent years, consumers have become more aware and concern regarding the processed foods that they buy and consume. Therefore, demands for natural and preservative-free foods are rising. This poses a challenge to the food industry to produce foods that are safe and stable, using either natural or no preservatives at all (Brul & Coote, 1999).

Edible, medicinal and herbal plants and the essential oils derivatives and isolated compounds possess significant numbers of secondary metabolites that are known to retard or inhibit the growth of bacteria, moulds and yeasts (Burt & Reinders, 2003). Food safety is usually achieved and increased by the addition of the antimicrobials that could significantly retard or prevent microbial spoilage on food products (Davidson, 1997). Since natural antimicrobials have been identified in herbs and spices, the interest in using natural antibacterial compounds, such as extracts of spices and herbs for preserving food, has become increasingly popular, as consumers today ask for products that are natural, as well as free of synthetic additives (Suhaj, 2006).

Plant extracts, especially herbs and spices, are loaded with phenolic secondary metabolites, and several contain antimicrobial activity (Lin *et al*., 2005). For example, the essential oil fractions of leaves of rosemary, sage, basil, marjoram and thyme, as well as the bulbs of onions and garlic were found to possess antimicrobial compounds (Gutierrez *et al*., 2008). The above mentioned herbs were considered as food condiments to improve the taste of foods and have been consume for centuries. It is only lately that scientists are studying the potential of these herbs to be used to preserve foods due to demands from health-conscious consumers.

An application of herbs and spices in food model system was demonstrated by Lin *et al.* (2004). The authors mixed in a ratio of 75 % oregano and 25 % cranberry aqueous extracts and found them to be effective in inhibiting the growth of *L. monocytogenes* in cod fillets stored at 4 ˚C. Moreover, the antimicrobial efficacy was further enhanced when combined with lactic acid. The authors added that the partial hydrophobic nature of phenolic constituents in oregano/cranberry mixture facilitates accumulation and attachment to the bacterial cytoplasmic membrane, where inhibitory effects possibly lead to cell death. In addition, extracts that possess phenolics at high concentrations could decrease the pH due to proton donation and cell membrane disruption.

2.3 Antimicrobial

2.3.1 Antimicrobial agents

Antimicrobial agent is a substance that kills or inhibits the growth or prevents damages due to the action of infectious microorganisms and according to Baron *et al*. (1994), also comprises of antibacterial, antifungal, antiprotozoal, antibelminthic and antiviral agents. The usage and discovery of modern antibiotics by Sir Alexander Flemming was once regarded as one of the biggest discoveries in the 20th century since it was effectively saving many lives then against bacterial infections. Ever since antibiotics were discovered, researches on antimicrobial compounds from plants had been sidelined.

However, the emergence of bacterial resistance to all known antibiotic classes were globally reported and started to gain the attention of many people for the need of new antibacterial sources (Ojala *et al*., 2000). Antibiotics were once produced by fungi and bacteria but now most were synthetically produced. Eloff (2000) cited the reasons for the emergence of bacterial resistance towards antibiotics being misuse, abuse and over-prescription of the antibiotics.

The demand of new antimicrobial agents due to increasing resistance of microorganisms to antibiotics has made scientists considered plants as a new source for the discovery of novel antimicrobial agents (Rangasamy *et al*., 2007). The increasing researches done on antimicrobial compounds from plants have been conducted for several reasons such as (Lewis and Elvin-Lewis, 1995; Borris, 1996; Cowan, 1999):

1) Several of the phytochemicals prescribed by physicians as antimicrobial agents have been clinically tested
2) Public awareness on the safety of current antibiotics available in the market
3) Rapid rate of plant extinction within the past 20 years has attracted the interests of scientists who worried that potential useful phytochemicals which could not be synthesized chemically is at risk of being lost irretrievably

Researches have been conducted to validate the traditional therapeutic value of plants recognized in many different communities to treat bacterial infections as well as to discover their potential antimicrobial activities. For example, Grosvenor *et al.* (1995) screened 124 plants from the Riau province of Indonesia and reported that of the 124 plants, 121 species showed inhibitory activity against *S. aureus* while 42 species inhibited the growth of *E. coli*.

Various compounds synthesized by plants are medically useful as some of them possess antimicrobial properties. The antimicrobial compounds found in plants may inhibit bacterial growth by different mechanisms from antibiotics and therefore have significant value in the treatment of bacterial infections (Eloff, 1998a). Therefore, researchers are now focusing on discovering new antimicrobial agents of the antimicrobial compounds from plants (figure 2.3).

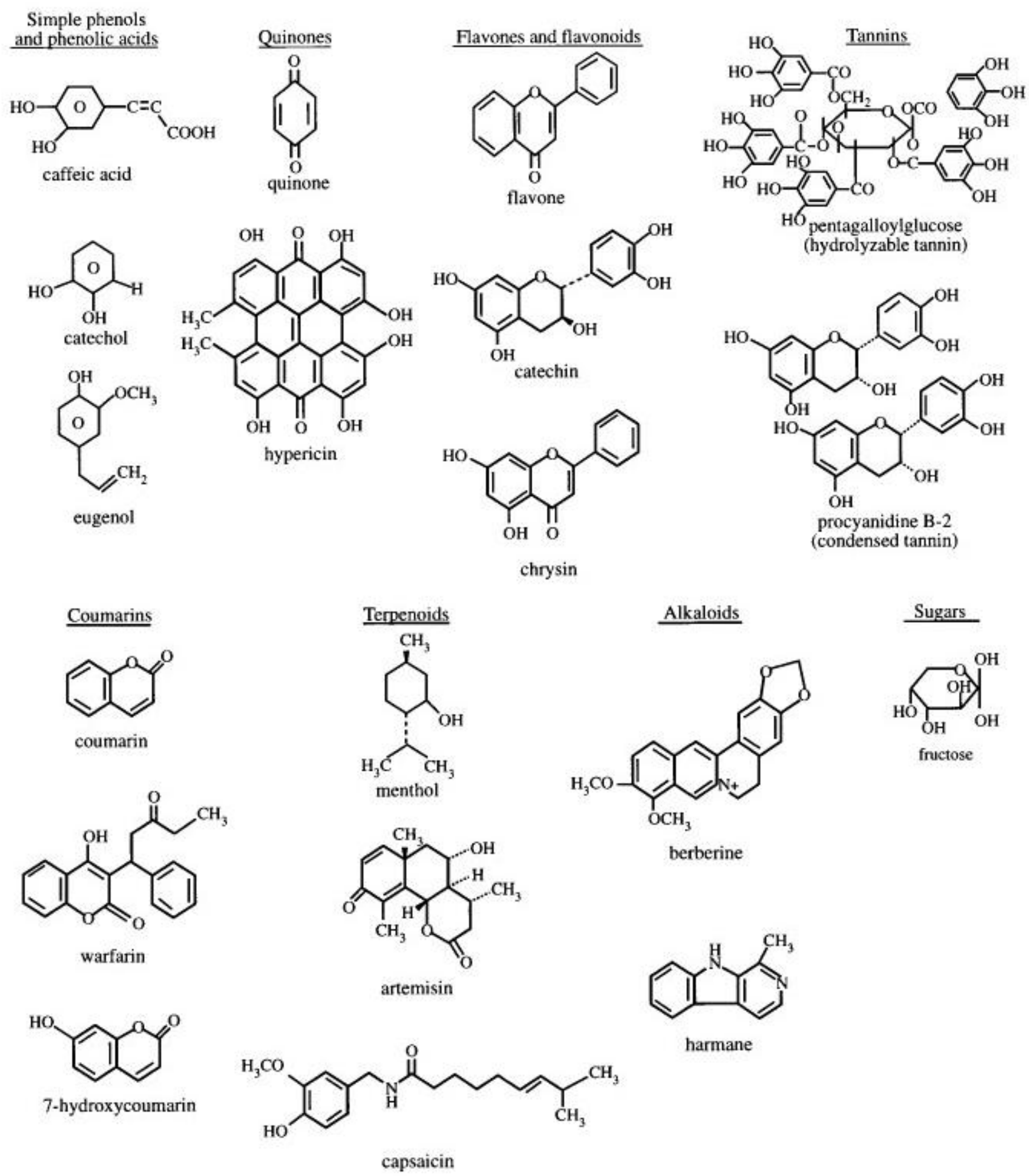

Figure 2.3 Structure of common antimicrobial plant chemicals (Cowan, 1999)

Another major group of medicines, the anti-tumour/ anti-cancer drugs, are being searched in the plant kingdom with native remedies from remote parts of the world are being enthusiastically looked into for possible cures. Early work on the American mandrake *(Podophyllum peltatum)* showed that its resinous constituents, which had been used as a drastic purgative, contained substances effective in the cure of venereal warts and podophyllin paint is still used for this and similar conditions and further research work showed that some of the constituents were cytotoxic and antitumour (Fairbairn, 1971). Another herb that had been researched for its antitumor activity is *Vinca rosea,* a semitropical periwinkle. This herb has been long used by many native societies as a cure for diabetes. Research found that it could diminish production of white blood cells and, hence, *Vinca rosea* was screened for anti-cancer activity as certain cancers (leukemias, Hodgkin's disease etc.) are accompanied by an overproduction of white blood cells. About sixty alkaloids were isolated by Svoboda and co-workers and at least two of these, vincaleukoblastine (vinblastine) and leurocristine (vincristine), have been shown to be effective against certain forms of cancer clinically (Fairbairn, 1971).

2.3.2 Antimicrobials in foods

Food antimicrobials or preservatives are chemical compounds added to or present in foods that function to retard, inhibit or inactivate spoilage microorganisms or killing them. In Malaysia, the usage of preservative agents in food is regulated by Food Act 1983 and Food Regulations 1985. According to Food Regulations 1985, Part V: Food Additive and Nutrient Supplement, "food additive" means ‘any safe substance that is intentionally introduced into or on a food in small quantities in order to affect the food's keeping quality, texture, consistency, appearance, odour, taste, alkalinity, or acidity, or to serve any other technological function in the manufacture, processing, preparation, treatment, packing, packaging transport, or storage of the food, and that results or may be reasonably expected to result directly or indirectly in the substance or any of its by-products becoming a component of, or otherwise affecting the characteristic of the food, and includes any preservative, colouring substance, flavouring substance, flavour enhancer, antioxidant and food conditioner, but shall not include nutrient supplement, incidental constituent or salt'.

In food production, it is important that appropriate measures are taken to ensure the safety and stability of the product during its whole shelf-life. Consumers' requirements of higher quality, preservative-free, safe but mildly processed foods with extended shelf-life may mean foods have to be preserved at higher pH and treated at mild-pasteurisation rather than sterilisation temperatures (Brul & Coote, 1999). The authors further added that legislation has restricted the use and allowed levels of some currently accepted preservatives in different foods which created problem to the industry as susceptibility of some microorganisms to most currently used preservatives is falling.

2.3.3 Commonly used chemical preservatives for foods

Foods are a good substrate for the growth of microorganisms and the unwanted growth of microorganisms on foods can lead to food poisoning and spoilage. Therefore, the use of antimicrobial agents in foods are meant to increase the safety of food for consumption as well as prolonging their life-span by killing or inhibiting the growth of the microorganisms present in the foods. According to Adams and Moss (2003), the addition of chemical to food is not something new but has been practised throughout history. Even though some people would regard the addition of chemicals to food synonymous with food adulteration, many are actually useful and allowed.

Preservatives are defined as 'substances capable of inhibiting, retarding or arresting the growth of microorganisms or of any deterioration resulting from their presence or of masking the evidence of any such deterioration' (Adams & Moss, 2003).

Preservatives may be microbicidal which kills the target organisms or they may be microbistatic in which they simply prevent the microorganisms from growing. Therefore, high level of an antimicrobial is lethal to microorganism while the lower concentrations of preservatives generally allowed in foods tend to be microbistatic. Hence, chemical preservatives are only useful in controlling low level of contaminants.

Preservatives also do not include substances (antioxidants) such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which act by inhibiting chemical reaction that limit shelf-life such as the control of rancidity or oxidative discolouration of foods (Adams & Moss, 2003).

2.3.3 (a) Organic acids

The acidity and alkalinity of an environment plays a very important effect on the activity and stability of macromolecules such as enzymes and hence, the growth of and metabolism of microorganisms are influenced by pH. Generally, bacteria and yeast grow fastest in the pH range of 6.0-8.0 and 4.5-6.0 whereas filamentous fungi at pH 3.5-4.0 (Adams & Moss, 2003). The usage of organic acids are usually limited to foods with pH less than 5.5 as most of the organic acids have pK_a between 3 to 5 (Doores, 1993).

Acetic acid (pK_a 4.75) and lactic acid (pK_a 3.86) are produced microbiologically even though food grade acetic acid can be derived petrochemically and at times used as an alternative to vinegar. They can be added in formulated products such as pickles and sauces. Lactic acid is used in the concentration between 0.3 to 7.5 % depending on the target microorganisms (Ibrahim *et al.*, 1996). Diluted lactic acid (1-2 %) has been shown to reduce the surface microflora of raw meat by between 1 and 3.5 log cycles (Adams & Moss, 2003). They are usually present in sufficient amount to have an effect on flavour and on product pH, hence, potentiating their own action by increasing the proportion of undissociated acid present.

Benzoic acid (pK_a 4.19) occurs naturally in cranberries, cherry barks tea, and anise but is prepared synthetically for food use. It is only effective in acid foods as it is a relatively strong acid (pK_a 4.19) and its antimicrobial activity is mainly in the undissociated form. Therefore, it is mainly used to inhibit the growth of spoilage yeasts and moulds. The ability of the undissociated molecule to interfere with membrane energetics and functions is very important as growth inhibition has been shown to parallel closely the inhibition of amino acid uptake in whole cells and membrane vesicles.

Sorbic acid (pK_a 4.8), found naturally in the berries of the mountain ash, is an unsaturated fatty acid, 2, 4-hexadienoic acid. It is effective against yeasts, moulds and catalase-positive bacteria but less active against catalase-negative bacteria, hence its use as selective agent in media for lactic acid bacteria and clostridia as well as functioning as fungal inhibitor in lactic fermentations.

Propionic acid (pK_a 4.9), occurs in a number of plants and also produced by activity of propionibacteria in certain cheeses. This acid is used as mould inhibitor in cheese and baked products where it also inhibits rope-forming bacilli.

2.3.3 (b) Nitrite

Nitrite is inhibitory to a range of bacteria. It has long been used to cure meats where it is also responsible for their characteristic colour and flavour. Its most important use is its ability to to inhibit spore-forming bacteria such as *Clostridium botulinum* which survived heat process applied to many cured meats (Adams & Moss, 2003).

Bacterial inhibition by nitrite increases with decreasing pH which proposes that nitrous acid (HNO_2, pK_a 3.4) is the active ingredient. Nitrite acts by inhibiting the germination and outgrowth of heated spores and by reacting with components in the product to form other inhibitory compounds. According to Ibrahim *et al*. (1996), the drawback of the use of nitrite is the reaction of nitrite with secondary amines, which forms *N*-nitrosamines, especially at low pH, can be carcinogenic.

2.3.3 (c) Sulfur dioxide

Sulfur dioxide is a colourless gas that dissolves in water readily to establish a pH-dependant equilibrium. The unionised forms of sulfur dioxide have the best antimicrobial activity as they can readily penetrate the cells (Adams & Moss, 2003). It is a reducing agent and can break disulfide linkages in protein and interfere with redox reactions as well as forming additional compounds with pyrimidine bases in nucleic acids, sugars and other key metabolic intermediates.

2.3.4 Antimicrobial agents from plants

Plant-derived substances have lately garnered great interest due to their versatile applications. It is known that plant cells do synthesized various compounds which are not necessarily used for growth or propagation but for keeping predators away and their continual survival. Generally, a plant composition can be divided into two categories of chemical compounds which are the primary metabolism products such as protein, fats and carbohydrates as well as the secondary metabolism products such as flavanoids, alkaloids and phenol compounds.

It is the secondary metabolism products that possess the antimicrobial agents. The secondary metabolites serve as plant defense mechanisms against attacks by microorganisms, insects, and herbivores (Table 2.1). Metabolites such as terpenoids, give plants their odors while quinones and tannins are responsible for plant pigment. A lot of compounds are responsible for plant flavour, for example, the terpenoid capsaicin from chili peppers, as well as some of the herbs and spices used by humans to season food possesses valuable medicinal compounds (Cowan, 1999).

Antimicrobial compounds found naturally in healthy plants are such as alkaloids, flavones, glycosides, phenolic compounds, diene and organic acids whereas injured or infected plants have phenolic compounds, isocyanate, sulfoxide and phytoalexin (López-Malo *et al*., 2000). All these natural compounds can be found in barks, flowers, leaves stems as well as the fruits and possess wide spectrum activity against microorganisms. Therefore, there is a possibility for plant extracts to be used as food preservative agents.

2.3.5 Antimicrobial susceptibility testing

The antimicrobial susceptibility test is commonly used to determine the efficacy of potential antimicrobials from biological extracts against a number of different microbial species. The antimicrobial susceptibility tests are used to screen plant extracts for antimicrobial activity as well as to determine the effectiveness of an antimicrobial against selected microorganisms by determining its minimum inhibitory concentration (MIC).

According to EUCAST (2003), clinical research *in vitro* susceptibility tests are particularly important if an organism is suspected to belong to a species that has shown resistance to frequently used antimicrobial agents and in comparing new and existing microbial agents.