CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Institute of Tropics and Subtropics

# *In vitro* evaluation of tropical and subtropical plant extracts for the control of honeybee pathogens

Master thesis

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2008

## Certification

I, Tomáš Čermák, declare that this thesis, submitted in partial fulfilment of the requirements for the degree of Master of Science, in the Institute of Tropics and Subtropics, Czech University of Life Sciences Prague, is wholly my own work unless otherwise referenced or acknowledged.

April 30, 2008

Tomáš Čermák

## Acknowledgement

This research was financially supported by NAZV project No.72144

I would like to thank my thesis supervisor doc. Ing. Ladislav Kokoška, Ph.D. (Department of Crop Sciences and Agroforestry in Tropics and Subtropics, Institute of Tropics and Subtropics, Czech University of Life Science Prague) for his thoughtful suggestions and patient guidance on the substantive aspects of this research and to Ing. Jaroslav Flesar (Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague) for his enduring management and cooperation during the analytical part of this research.

I am also grateful to my family and friends, for their emotional and financial support they gave me during all my studies.

## Abstract

In frame of this work, fifteen dichlormethan/methanol extracts from several plant species, namely rosemary (*Rosmarinus officinalis*), mango (*Mangifera indica*), annato (*Bixa orellana*), green olive (*Olea europaea*), khat (*Catha edulis*), bay laurel (*Laurus nobilis*), turmeric (*Curcuma longa)*, caper (*Capparis spinosa*), pomegranate (*Punica granatum*), tea (*Camellia sinensis*), guava (*Psidium guajava),* ginger (*Zingiber officinale*), cider gum (*Eucalyptus gunnii),* lemon gum (*Eucalyptus citriodora*), and myrtle (*Myrtus communis*), were tested *in vitro* for antimicrobial activity against different strains of *Paenibacillus larvae*, causal agent of American foulbrood in honey bees (*Apis mellifera* L.). Extrats were prepared from different parts of the plants by using a soxhlete apparatus. Minimal inhibitory concentration (MIC) was evaluated by the broth microdilution method. Antimicrobial screening showed that all plants tested in this study inhibited growth of at least one of the examined strains at concentration ≤ 256 μg/ml. Highly significant *in vitro* antibacterial action exhibited extract from aerial part of *M. communis*, inhibiting growt of three strains of *P. larvae* with MICs ranging from 2 to 4 μg/ml. Significant antibacterial action possessed also extracts of *R. officinalis* (MICs 16-32 μg/ml), *L. nobilis* (MICs 16-32 μg/ml), and *E. gunnii* with MIC values ranging from 16 to 32 μg/ml. *Myrtus communis* proved to be the most effective *in vitro* extract against *P. larvae*. Therefore, it may be a promising natural alternative to traditional antibiotics, meriting further tudies on its field applicability for American Foul Brood (AFB) control.

***Key words:*** American Foul Brood, antimicrobial activity, MIC, broth, microdilution method

# Abstrakt

V rámci této práce byla studována *in vitro* antimikrobiální aktivita patnácti (dichlormethan/methanol) extraktů vybraných rostlin: rozmarýna lékařská (*Rosmarinus officinalis*), mangovník indický (*Mangifera indica*), oreláník barvířský (*Bixa orellana*), olivovník evropský (*Olea europaea*), kata jedlá (*Catha edulis*), vavřín vznešený (*Laurus nobilis*), kurkuma dlouhá (*Curcuma longa)*, kapara trnitá (*Capparis spinosa*), marhaník granátový (*Punica granatum*), čajovník čínský (*Camellia sinensis*), kvajáva hrušková (*Psidium guajava),* zázvor lékařský (*Zingiber officinale*), blahovičník gunnův (*Eucalyptus gunnii*)*,* blahovičník citrónový (*Eucalyptus citriodora*), a myrta obecná (*Myrtus communis*) proti kmenům *Paenibacillus larvae,* který u včely medonosné (*Apis mellifera* L) způsobuje onemocnění včelího plodu, včelí mor. Rostlinný materiál byl extrahován v aparatuře Soxhletova typu. Minimální inhibiční koncentrace (MIC) byly stanoveny pomocí bujónové mikrodiluční metody. Antimikrobiální aktivita byla prokázána u všech rostlinných extraktů v koncentraci ≤ 256 μg/ml, v závislosti na testovaném kmeni *P. larvae*. Nejvýraznější inhibiční účinek *in vitro* se projevil v případě extraktu nadzemní části *M .communis,* který prokázal inhibici 3 kmenů *P. larvae* (MIC 2- 4 μg/ml). Významný ihibiční efekt byl prokázán též u extraktu *R. officinalis* (MIC 16-32 μg/ml), *L. nobilis* (MIC 16-32 μg/ml) a *E. gunnii* (MIC 16-32 μg/ml). *M. communis,* jakožto rostlina s vysokým antibakteriálním účinkem proti všem testovaným kmenům *P. larvae* by mohla v budoucnu najít uplatnění v boji proti moru včelího plodu.

***Klíčová slova:*** mor včelího plodu, léčivé rostliny, antimikrobiální aktivita, MIC, bujón mikrodiluční metoda

## List of Abbreviations

| | |
|---|---|
| AFB | American Foul Brood |
| EFB | European Foul Brood |
| MIC | minimum inhibitory concentration |
| OTC | Oxytetracycline |
| ATCC | American Type Culture Collection |
| DMSO | dimethyl sulfoxide |
| TBS | Tris-buffer saline |

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# 1 Foreword

Honey bees play a vital role in the environment by pollinating both wild flowers and many agricultural crops as they forage for nectar and pollen, in addition to producing honey and beeswax. The essential and valuable activities of bees depend upon beekeepers maintaining a healthy population of honey bees, because like other insects and livestock, honey bees are subject to many diseases and pests. The apiculture industry plays an important role in generating employment and in increasing family income in the rural areas of the world. Many developing countries are trying to improve the quality of their honey products but they frequently encounter the main obstacle in apiculture; control of diseases and pests of honey bees.

Some of diseases and pests are more deleterious to bee colonies than others. The most virulent disease around the world is American foulbrood, which causes considerable economic losses to beekeepers. During the past decade there has been an increase in the number of AFB cases in Europe and the disease has given serious problems in many parts of Czech Republic.

At first, the population of an infected colony is not noticeably decreased and only a few dead larvae or pupae may be present. The disease may not develop to the critical stage where it seriously weakens the colony until the following year, or it may advance rapidly and seriously weaken or kill the colony the first season. American Foul Brood (AFB) caused by the spore-forming bacterium *Paenibacillus larvae* (White). The only known host of this bacterium is the honeybee *Apis mellifera*. In the field the disease is detected by inspection, and a positive diagnosis is based on clinical symptoms. In an infected colony, spores from *P. larvae* can be isolated from honey, wax, pollen, and hive walls (GOCHNAUER, 1981).

It has been reported that the *P. larvae* spores can remain infective for at least 35 years (HASEMAN, 1961). The disease spreads when spores are transported on drifting bees, hive parts, clothing, and contaminated pollen or honey. The examination of honey for spores may therefore be of value in tracing disease outbreaks, and there have been a number of studies using honey for this purpose. The detection of these inapparent or latent infections would identify sources of pathogens which may cause fully developed disease in these hives or spread of infection to other hives (STEINKRAUS & MORSE, 1992).

A great deal of controversy exists concerning the feeding of antibiotics to colonies for the prevention of AFB. It has been shown that preventive Oxytetracycline hydrochloride (OTC) treatments effectively mask all symptoms, with the consequent risk of spreading the disease by the movement of infected materials around apiaries. In the USA, the only antibiotic that can be legally used is OTC; although sodium sulfathiazole had previously been used for autumn applications, it is no longer approved because the drug is stable in honey for several years (OLDROYD ET AL., 1989).An inappropriate use of antibiotics may lead to antibiotic resistance of *P. larvae* strains and honey contaminated with residues of these drugs may reach the market place. Strains of *P. larvae* resistant to OTC and to sodium sulfathiazole have been reported in Poland, Germany and Argentina (GLINSKI & RZEDZICKI, 1977).

Recently, natural compounds such as essential vegetable oils and fatty acids have been reported to be effective for limiting the growth of *P. larvae* strains *in vitro*. Further studies are needed in order to determine their effectiveness, appropriate doses and mode of application in honeybee colonies. The use of essential oils and fatty acids for the control of AFB would represent a safe alternative to antibiotics (FELDLAUFER ET AL., 1993).

Thus we decided to test 15 selected plants used traditionally for the treatment of various infections, and evaluated them for potential antibacterial activity, in order to confirm their popular use and to compare their efficiency with antibiotics agents.

# 2 Introduction

Although all pollinators are susceptible to diseases, a large proportion of pollinator disease research has focused on bee populations, specifically the honey bee. There has been a dramatic increase in diseases associated with honey bees due to increased international commerce and trade.

The honey bee is a colonial insect. As such it is often necessary to look at the colony as a whole to determine damage by disease or pests. However, the beekeeper must be careful not to assume all conditions leading to population decline or reduced honey production are the result of disease. Colonies can be slightly damaged by pesticides, for example, and/or nutritional deficiencies. It is important, therefore, for the beekeeper to be as informed in diagnosing bee disease and pest problems.

Bees have two distinct life forms (brood and adult) and most diseases are specific to either one stage or the other.

## 2.1 Honey bee diseases

### 2.1.1 *Brood diseases*

**American Foul Brood disease**

Beekeepers in temperate and subtropical regions around the world generally regard AFB as possibly the most destructive microbial disease affecting bee brood (GOODWIN & VAN EATON, 1999). The disease did not originate in, nor is it confined to, the Americas. It is widely distributed wherever colonies of *Apis mellifera* are kept. In tropical Asia, where sunlight is abundant and temperatures are relatively high throughout the year, the disease seldom causes severe damage to beekeeping operations. The disease is contagious and the pathogenic bacterium can remain dormant for as much as and more than 50 years. Therefore, beekeepers and extension specialists throughout Asia should be acquainted with the symptoms of this disease and know how to cope with it should the need arise (BAILEY & BALL, 1991).

AFB is caused by grampositive, spore-forming bacterium, *Paenibacillus larvae*, which only affects bee brood; adult bees are safe from infection. At the initial stage of colony infection, only a few dead older larvae or pupae will be observed. Subsequently, if remedial action is not taken, the disease will spread within the colony and can quickly spread to other colonies in the apiary as a result of robbing, drifting workers, or contamination caused by the beekeeper's hive manipulations. In the same way the pathogen agent can spread to other apiaries. Natural transfer mainly takes place within a radius of 1 km around the apiary. Often spores enter the bee colonies via foreign honey. Commercially available honey may be highly contaminated; therefore, special attention should be paid near honey processing enterprises and waste disposal sites (GOODWIN & VAN EATON, 1999).

**European Foul Brood disease**

As with American Foul Brood disease, the name of this bacterial bee brood disease is inappropriate. The range of distribution of European Foul Brood (EFB) disease is not confined to Europe alone and the disease is found in all continents where *Apis mellifera* colonies are kept. Reports from India indicate that *A. cerana* colonies are also subject to EFB infection. The damage inflicted on honey bee colonies by the disease is variable. EFB is generally considered less virulent than AFB; although greater losses in commercial colonies have been recorded in some areas resulting from EFB (KAAL ET AL., 1992).

The pathogenic bacterium of EFB is *Mellissococcus pluton*. It is lanceolate in shape and occurs singly, in chains of varying lengths, or in clusters. The bacterium is grampositive and does not form spores. While many strains of *M. pluton* are known, all are closely related (STEINKRAUS & MORSE, 1992).

**Chalkbrood disease (Ascosphaerosis)**

In Asia, Chalkbrood is rarely considered to be a serious honey bee disease, although in Japan the disease has been reported to cause problems to beekeepers. In temperate America and Europe, however, cases have occurred in which Chalkbrood has caused serious damage to beekeeping; therefore, Asian beekeepers should be aware of this problem (HEATH, 1982).

Chalkbrood is a disease caused by the fungus *Ascosphaera apis*. As its name implies, it affects honey bee brood. This fungus only forms spores uring sexual reproduction. Infection by spores of the fungus is usually observed in larvae that is three to four days old. The spores are absorbed either via food or the body surface (HEATH, 1982).

**Sacbrood disease**

Is perhaps the most common viral disease of honey bees. In Asia, at least two major types have been recorded. Sacbrood disease that affects the common honey bee *Apis mellifera* and the Sacbrood disease of the Asian hive bee *A. cerana*. A new type of Sacbrood virus has recently been reported in Asian colonies of *A. cerana*. It is highly probable that the virus is native to the continent and that it has been with the Asian hive bees over the long period of its evolution. Since its first discovery in Thailand in 1981, it has been found in association with *A. cerana* in India, Pakistan, Nepal, and perhaps all other countries in Asia within the honey bee's range of distribution. Several reports indicate that nurse bees are the vectors of the disease. Larvae are infected via brood-food gland secretions of worker bees (AKRATANAKUL, 1986).

### 2.1.2 *Adult bee diseases*

**Nosema disease (Nosemosis)**

Nosema disease is generally regarded as one of the most destructive diseases of adult bees, affecting workers, queens and drones alike. Seriously affected worker bees are unable to fly and may crawl about at the hive entrance or stand trembling on top of the frames. The bees appear to age physiologically: their life-span is much shortened and their hypopharyngeal glands deteriorate, the result is a rapid dwindling of colony strength. Other important effects are abnormally high rates of winter losses and queen supersedures. In climates with pronounced long periods of flight restrictions or no flight opportunities even for a day, the infection easily reaches a severe stage that visibly affects the strength of the colony. Less obvious infection levels in other climates often go undetected (BAILEY & BALL, 1991). The damage caused by Nosema disease should not be judged by its effect on individual colonies alone as collectively it can cause great losses in apiary productivity (MOELLER, 1978).

The disease is caused by the protozoan *Nosema apis*, whose 5 to 7 mm spores infest the bees, are absorbed with the food and germinate in the midgut. After penetration into the gut wall the cells multiply forming new spores that infect new gut cells or can be defecated. The nutrition of the bees is impaired, particularly protein metabolism (ROBINSON, 1985).

### 2.1.3 *Pests and predators*

**Varroa mite (Varroasis)**

This mite is a native parasite of *A. cerana* throughout Asia. Since the initiation of beekeeping development projects with *A. mellifera* on the continent, it has been reported as causing damage in both temperate and tropical Asia. The overall effect of varroa infestation is to weaken the honey bee colonies and thus decrease honey production, often seriously. Occasionally in *A. mellifera*, and more frequently in *A. cerana*, heavy infestation may cause absconding (AKRATANAKUL, 1986). Today this parasite is found throughout the world, except for Australia and New Zealand South Island. In temperate Asia, most beekeepers agree that varroa damage is a constraint to the success of beekeeping operations with *A. mellifera*, while in tropical Asia success is limited by the loss of *A.cerana* colonies through absconding, which is far less serious and frequent than damage to *A. mellifera*. Most treatment descriptions are for *A. mellifera*. Occasional removal of *A. cerana* male brood combs and keeping the hive in healthy condition are the way of prevention of varroosis for *A. cerana* ( WIENANDS & MADEL, 1985).

*Varroa destructor* (previously confused with *Varroa jacobsonii*) is quite large, as compared with other mite species, and can be seen with the unaided eye. The shape of the adult female is distinctive: observed from above, the width of the body is clearly seen to be greater than the length, i. e. about 1.6 x 1.1 mm. The mite is reddish brown in colour and shiny and the body is dorsoventrally flattened covered with short hairs (setae). Adult females *of V. destructor* are found inside brood cells or walking rapidly on comb surfaces. Individual mites are often seen clinging tightly to the body of adult bees, mostly on the abdomen, where the segments overlap, between the thorax and the abdomen and at the ventral entry. Adult males, and the immature stages of both sexes (egg, protonymph and deuteronymph), are not commonly seen outside the brood cells. All immature stages of the parasite live inside the brood cells. They can be observed when infested cells are opened and the brood is carefully removed. The immature mites are bright white and the adult females are brown, while male mites are smaller than females and are rarely seen since they are only found inside brood cells (BAILEY & BALL, 1991).

**Tropilaelaps mite**

Modern beekeeping with *Apis mellifera* in tropical and subtropical Asia frequently encounters problems caused by infestation with *Tropilaelaps spp.* The mite is a native parasite of the giant honey bee *A. dorsata*, widely distributed throughout tropical Asia, and whenever *A. mellifera* is kept within the range of distribution of *A. dorsata*, mite infestation of the colonies cannot be avoided. Thus, in Thailand beekeepers consider *Tropilaelaps* to be a more serious pest than varroa-mites, even though it may be easier to control. Dual parasitism of *A. mellifera* colonies by both parasites sometimes occurs, the population of *Tropilaelaps* often being greater than that of varroa, as the *Tropilaelaps* mite can almost completely prevent multiplication of the *varroa* mite (WIENANDS & MADEL, 1985).

*Tropilaelaps* mites are much smaller than varroa mites, although the trained unaided eye can still see them. The adult female mite is light reddish-brown in colour, with an oval-shaped body about 0.96 mm in length and 0.55 mm in width. The mite's entire body is covered with short setae. A red streak running longitudinally on the ventral surface of the adult female, the fusion of her epigynial and anal shields may be perceived through a strong magnifying glass. When the mites are present in a honey bee colony in large numbers, they can be observed walking rapidly on the surface of the comb. They are rarely found on adult bees. In all its immature stages, the mite lives within the brood cells of the bees, feeding on the brood's haemolymph. Fertilized adult females enter the cells before they are capped to lay their eggs. The stages of development of the mite are as follows: egg, six-legged larva, protonymph, deutonymph, adult. Adult males of *Tropilaelaps* do not feed, their chelicerae (the organs originally used for piercing the bees' integument) having been modified to transfer sperm as with the varroa mite. The life cycle of the mite is well synchronized with that of the host bee (CROMROY, 1985).

**Tracheal mite (Acarapidosis)**

This mite, *Acarapis woodi*, infests the tracheal system of adult bees, queens, workers and drones, which are all equally susceptible to its attacks. Since it was first reported in *Apis mellifera* colonies in Europe in 1921, opinions regarding the extent of the damage it can cause to honey bee colonies have varied (DE JONG, 1981). Reports from India and Pakistan indicate that the tracheal mite caused severe losses of *A. cerana* colonies. However, the mite's range of distribution in Asia has not been firmly established, and many of the reported losses of *A. cerana* were later shown to have been inflicted by *Apis iridescent virus* and not by tracheal mites. After the first appearance of the *Acarapis* mite in North America it has led to increasing damage; therefore, beekeepers in Asia should remain vigilant (BAILEY, 1964). *A. woodi* is a very small mite (0.1 m) species that lives and breeds within the thoracic tracheae of adult bees . The mite penetrates through the stigma (spiracles) into the first trachea pair of the thorax of 10-day old honey bees. There it lays eggs at intervals of a few days. After the deutonymph stage, male offspring emerge after around 12 days and females after 13 to 16 days (BROTHER, 1968).

## 2.2 Antimicrobial activity of higher plants

Higher plants produce hundreds to thousands of diverse chemical compounds with different biological activities. These compounds have an important ecological role. They can work as pollinator attractants and as chemical defenses against insects, herbivores and microorganisms. In order to adapt to environmental insults, plants produce a vast number of natural products that have antimicrobial potential. These include isoflavonoids, indoles, phytosterols, polysaccharides, sesquiterpenes, alkaloids, glucans, tannins, vitamins, trace minerals, and many other phytochemical substances. Some members of above mentioned groups of plant compounds have previously been found to be active against pathogenic microorganisms (HAMBURGER & HOSTETTMANN, 1991).

The identification of effective natural compounds will increase the number of available chemotherapeutic agents and reduce the threat of resistance in parasite and pathogen populations by providing alternatives with greater public acceptance. Identification of compounds with activity against more than one organism may reduce in the total amount of chemicals required for parasite and pathogen management (CALDERONE, SHIMANUKI & ALLEN-WARDELL, 1994).

Natural plant extracts may play a significant role in the management of honeybee parasites and pathogens. The translation of the results of *in vitro* studies into a successful management strategy requires the resolution of several complex problems. These include the demonstration of *in vitro* activity at levels that are not toxic to honeybee larvae, pupae and adults, and that do not affect the flavour of the honey. The development of an adequate delivery system to ensure the consumption of extracts in food supplements by adult bees is also important. Typically, the consumption of supplements containing extracts at concentrations above 0.5% is greatly reduced. Additionally, formulations must be competitive with naturally-occurring nectar and pollen flows (HEATH, 1982).

## 2.3 Control of honey bee disease with natural products

The lack of any effective control agent for chalkbrood has resulted in an increased interest in the investigation of alternative control strategies.

Evaluation of plant extracts for the control of honeybee pathogens and parasites is limited. Several studies have shown essential oils to be effective in controlling bee diseases such as American Foulbrood disease (CARPANA ET AL., 1996), tracheal mite (*Acarapis woodi*), *Varroa jacobsoni* and Chalkbrood (HIGES ET AL., 1998). (COLIN ET AL., 1989), using *in vitro* tests, demonstrated fungicidal activity of essential oils of *Thymus vulgaris*, *Satureja montana* and *Origanum vulgare* against Chalkbrood. Field trials demonstrated that application of *Satureja montana* resulted in a reduction in the number of Chalkbrood-infected larvae observed at entrances of infected colonies. (CALDERONE, SHIMANUKI & ALLEN-WARDELL, 1994) tested 8 plant extracts for their activity against *A. apis* and found that cinnamon oil completely inhibited the growth of *A. apis* at 100 ppm for 168 h. Bay oil, citronellal, clove oil, origanum oil and thymol inhibited all growth at 1,000 ppm for 168 h. Camphor inhibited all growth at 10,000 ppm for 168 h. and α-terpinene inhibited all growth for 72 h. at 10,000 ppm. Several compounds reduced growth in a dose-dependent manner at levels below their threshold values.

## 2.4 Selected plants

### 2.4.1 *Bixa orellana* L.

**Family:** Bixaceae

**Common names:** Achiote, achiotec, achiotl, achote, annatto, urucu, beninoki, bija, eroya, jafara, kasujmba-kelling, kham thai, onoto, orleanstrauch, orucu-axiote, rocou, roucou, ruku, roucouyer, unane, uruku, urucum, urucu-üva

**Origin and geographic distribution:** Native to the tropical American area*,* *B. orellana* is found in largest quantities from Mexico to Ecuador, Brazil, and Bolivia.

**Description:** Shrub or bushy tree which ranges from 3 to 10 meters in height. Its glossy, ovate leaves are evergreen with reddish veins; they have a round, heart-shaped base and a pointed tip. With a thin, long stem, the leaves are between 8 and 20 cm long and 5 and 14 cm wide. The twigs are covered with rust colored scales when young and bare when older. Bixa's flowers are pink, white, or some combination, and are 4 to 6 cm in diameter. From the flower protrudes a striking two-valved fruit, covered either with dense soft bristles or a smooth surface. These round fruits, approximately 4 cm wide, appear in a variety of colors: scarlet, yellow, brownish-green, maroon, and most commonly bright red. When ripe, they split open and reveal a numerous amount of small fleshy seeds, about 5 mm in diameter and covered with red-orange pulp, the embryo of which is poisonous (HONYCHURCH, 1980).

**Medicinal action and uses:** Traditionally, the crushed seeds are soaked in water that is allowed to evaporate. A brightly colored paste is produced which is added to soups, cheeses, and other foods to give them a bright yellow or orange color. Annatto seed paste produced in South America is exported to North America and Europe, where it is used as a food coloring for margarine, cheese, microwave popcorn and other yellow or orange foodstuffs. Many times, this natural food coloring replaces the very expensive saffron in recipes and dishes around the world. Annatto paste is also used as a natural dye for cloth and wool and is sometimes employed in the paint, varnish, lacquer, cosmetic and soap industries (MORTON, 1981).

Throughout the rainforest, indigenous tribes have used annatto seeds as body paint and as a fabric dye. It has been traced back to the ancient Mayan Indians, who employed it as a principal coloring agent in foods, for body paints, and as a coloring for arts, crafts, and murals. Although mostly only the seed paste or seed oil is used commercially today, the rainforest tribes have used the entire plant as medicine for centuries. A tea made with the young shoots is used by the Piura tribe as an aphrodisiac and astringent, and to treat skin problems, fevers, dysentery, and hepatitis. The leaves are used to treat skin problems, liver disease, and hepatitis. The plant has also been considered good for the digestive system. The Cojedes tribe uses an infusion of the flowers to stimulate the bowels and aid in elimination as well as to avoid phlegm in newborn babies. Traditional healers in Colombia have also used annatto as an antivenin for snakebites. The seeds are believed to be an expectorant, while the roots are thought to be a digestive aid and cough suppressant (SCHULTES & RAFFAUF, 1990).

Today in Brazilian herbal medicine, a leaf decoction of annatto is used to treat heartburn and stomach distress caused by spicy foods, and as a mild diuretic and mild laxative. It is also used for fevers and malaria, and, topically, to treat burns. Annatto is a common remedy in Peruvian herbal medicine today, and the dried leaves are called achiotec. Eight to ten dried leaves are boiled for 10 minutes in 1 liter of water for this popular Peruvian remedy. One cup is drunk warm or cold 3 times daily after meals to treat prostate disorders and internal inflammation, arterial hypertension, high cholesterol, cystitis, obesity, renal insufficiency, and to eliminate uric acid. This decoction is also recommended as a vaginal antiseptic and wound healer, as a wash for skin infections, and for liver and stomach disorders. Curanderos (herbal healers) in the Peruvian Amazon squeeze the juice from the fresh leaves and place it in the eye for inflammation and eye infections, and they use the juice of 12 fruits taken twice daily for 5 days to "cure" epilepsy (HONYCHURCH, 1980).

**Antimicrobial activity:** The extract (5 mg/ml) produced antimicrobial action in agar diffusion assays against standard strains of grampositive bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus faecalis* while exerting only slight action against *Escherichia coli, Serratia marcescens, Candida utilis,* and *Aspergillus niger*. The zones of inhibition obtained against the susceptible bacteria were 15-17 min while those obtained in assays with Chloramphenicol and phenol positive controls were 12-18 nm and 10-28 mm, respectively. The minimum inhibitory concentration (MIC) of the extract was 4-16 mg/ml while its bactericidal action (MBC) was exerted at higher doses (16-64 mg/ml). The extract therefore, appears to possess a narrow spectrum of antimicrobial activity, being effective against only the grampositive bacteria used in our study (IROBI, MOOYOUNG & ANDERSON, 1996).

**Chemical composition:** Analysis of annatto seeds indicates that they contain 40% to 45% cellulose, 3.5% to 5.5% sucrose, 0.3% to 0.9% essential oil, 3% fixed oil, 4.5% to 5.5% pigments, and 13% to 16% protein, as well as alpha- and beta-carotenoids and other constituents. Annatto oil is extracted from the seeds and is the main source of pigments named bixin and norbixin, which are classified as carotenoid. Annatto contains also bixaghanene, bixein, bixol, crocetin, ellagic acid, ishwarane, isobixin, phenylalanine, salicylic acid, threonine, tomentosic acid, and tryptophan. (SCHULTES & RAFFAUF, 1990).

### 2.4.2 *Camellia sinensis* L. Kuntze

**Family:** Theaceae

**Common names:** Tea, assam, black tea, broken orange pekoe pannings, ch'a, darjeeling, dust, flowery orange pekoe, green tea, gunpowder, hyson, iced tea, imperial, keemum, lapsung souchong, leaf pekoe, oolong, orange pekoe, pekoe souchong, pekoe tip, sencha, souchong, tsocha, twanky, women's-tobacco, young hyson

**Origin and geographic distribution:**. Native to China, spread to India and Japan, then to Europe and Russia, arriving in the New World in the late 17$^{th}$ century

**Description:** Small evergreen tree to 16 m tall, usually pruned back to shrubs in cultivation, with strong taproot giving rise to a surface mat of feeders with endotrophic mycorrhizae; leaves alternate, exstipulate, lanceolate to obovate, up to 30 (usually 4-15) cm long, 2-5 (7-12) cm broad, pubescent, sometimes becoming glabrous, serrate, acute or acuminate; flowers 1-3, in axillary or subterminal cymes, deflexed, 2-5 cm broad, aromatic, white or pinkish, actinomorphic, sepals and petals 5-7, pedicels 5-15 mm long, stamens numerous, ovary 3-5 carpellate, each carpel 4-6 ovulate, capsules depressed-globose, brownish, lobate, to 2 cm broad, valvate, with 1-3 subglobose seeds in each lobe; approximately 500 seeds per kg.

**Medicinal action and uses:** The infusion, once recommended in China as a cancer cure, contains some tannin, suspected of being carcinogenic. Chinese regard tea as antitoxic, diuretic, expectorant, stimulant, and stomachic (LEUNG, 1980). Tea, considered astringent, stimulant and acts as a nervine or nerve sedative, frequently relieving headaches. It may also cause unpleasant nerve and digestive disturbances. The infusion is also recommended for neuralgic headaches. According to Leung, tea is reportedly effective in clinical treatment of amebic dysentery, bacterial dysentery, gastroenteritis, and hepatitis. It has also been reported to have antiatherosclerotic effects and vitamin P activity. Teabags have been poulticed onto baggy or tired eyes, compressed onto headache or used to bathe sunburn. The plant has a folk reputation as analgesic, antidotal, astringent, cardiotonic, carminative, CNS-stimulant, demulcent, deobstruent, digestive, diuretic, expectorant, lactagogue, narcotic, nervine, refrigerant, stimulant and stomachic; used for bruises, burns, cancer, cold, dogbite, dropsy, dysentery, epilepsy, eruptions, fever, headache, hemoptysis, hemorrhage, malaria, ophthalmia, smallpox, sores, toxemia, tumors, and wounds (DUKE & ATCHLEY, 1984).

**Antimicrobial activity:** The antibacterial activity of the methanol and aqueous extract of *Camellia sinensis* on *Listeria monocytogenes* were investigated using agar-gel diffusion, paper disk diffusion and microbroth dilution techniques. The results obtained showed that methanol and water extract exhibited antibacterial activities against *Listeria monocytogenes*. The leave extract produced inhibition zone ranging from 10.0-20.1 mm against the test bacteria. The methanol extracts of the test plant produces larger zones of inhibition against the bacteria than the water extract. The minimum inhibitory concentration (MIC) for the methanol and water leave extract was 0.26 mg/ml and 0.68 mg/ml respectively (FERRARI, MONTESANO & SENATORE, 2001).

**Chemical composition:** Fresh leaves from Assam contain 22.2% polyphenols, 17.2% protein, 4.3% caffeine, 27.0% crude fiber, 0.5% starch, 3.5% reducing sugars, 6.5% pectins, 2.0% ether extract and 5.6% ash. Per 100 g, the leaf is reported to contain 293 calories, 8.0 g $H_2O$, 24.5 g protein, 2.8 g fat, 58.8 g total carbohydrate, 8.7 g fiber, 5.9 g ash, 327 mg Ca, 313 mg P, 24.3 mg Fe, 50 mg Na, 2700 ug beta-carotene equivalent, 0.07 mg thiamine, 0.8 mg riboflavin, 7.6 mg niacin, and 9 mg ascorbic acid. Another report tallies 300 calories, 8.0 g $H_2O$, 28.3 g protein, 4.8 g fat, 53.6 g total carbohydrate, 9.6 g fiber, 5.6 g ash, 245 mg Ca, 415 mg P, 18.9 mg Fe, 60 mg Na, 8400 ug beta-carotene equivalent, 0.38 mg thiamine, 1.24 mg riboflavin, 4.6 mg niacin, and 230 mg ascorbic acid. Yet another gives 299 calories, 8.1 g $H_2O$, 24.1 g protein, 3.5 g fat, 59.0 g total carbohydrate, 9.7 g fiber, 5.3 g ash, 320 mg Ca, 185 mg P, 31.6 mg Fe, 8400 ug beta-carotene equivalent, 0.07 mg thiamine, 0.79 mg riboflavin, 7.3 mg niacin, and 85 mg ascorbic acid (DUKE & ATCHLEY, 1984). Leaves also contain carotene, riboflavin, nicotinic acid, pantothenic acid and ascorbic acid. Caffeine and tannin are among the more active constituents. (FERRARI, MONTESANO & SENATORE, 2001) Ascorbic acid, present in the fresh leaf, is destroyed in making black tea. Malic and oxatic acids occur, along with kaempferol, quercitrin, theophylline, theobromine, xanthine, hypoxanthine, adenine, gums, dextrins and inositol. Chief components of the volatile oil (0.007-0.014% fresh weight of leaves) is hexenal, hexenol and lower aldehydes, butyraldehyde, isobuteraldehyde, isovaleraldehyde, as well as n-hexyl, benzyl and phenylethyl alcohols, phenols, cresol, hexoic acid, n-octyl alcohol, geraniol, linalool, acetophenone, benzyl alcohol and citral. (LEUNG,1980).

### 2.4.3 *Capparis spinosa* L.

**Family:** Capparaceae

**Common names:** Caper, cappero, alcaperro, caper berry, caper bud, caperbush, caper fruit, kapari, smooth caper, spiny caper, tapèra

**Origin and geographic distribution:** Capers can today be found growing wild all over Mediterranean and are frequently cultivated (e.g., in France, Spain, Italy and Algeria; furthermore, Iran, Cyprus and Greece produce significant amounts); their origin is, though, supposed in the dry areas of Western or Central Asia

**Description:** Caper plants are small shrubs and may reach about one meter upright. However, uncultivated caper plants are more often seen hanging, draped and sprawling as they scramble over soil and rocks. The caper's vegetative canopy covers soil surfaces which helps to conserve soil water reserves. Leaf stipules may be formed into spines. Flowers are born on first-year branches.

**Medicinal action and uses:** Capers are said to reduce flatulence and to be anti-rheumatic in effect. In ayurvedeic medicine capers (Himsra) are recorded as hepatic stimulants and protectors, improving liver function. (INOCENCI, ALCATRAZ & CALDERON, 2002) Capers have reported uses for arteriosclerosis, as diuretics, kidney disinfectants, vermifuges and tonics. Infusions and decoctions from caper root bark have been traditionally used for dropsy, anemia, arthritis and gout. Capers contain considerable amounts of the anti-oxidant bioflavinoid rutin. Caper extracts and pulps have been used in cosmetics, but there has been reported contact dermatitis and sensitivity from their use (RODRIGO ET AL., 1992).

**Antimicrobial activity:** *Capparis spinosa* has a very good antibacterial activity against grampositive and negative bacteria as well as moderate to good antifungal activity against *C albicans and A. flavus* (MAHASNEH, 2002).

**Chemical composition:** The plant contains the flavonoids rutin, kaempferol-3-glucoside, kaempferol-3-rutinoside, and kaempferol-3-rhamnorutinoside (RODRIGO ET AL., 1992). Other components contained within the plant include quercetin 3-O-glucoside, quercetin 3-O-glucoside-7-O-rhamnoside, a new flavonoid quercetin 3-O-(6-alpha-L-rhamnosyl-6-beta-D-glucosyl)-beta-D-glucoside and 2 novel (6S)-hydroxy-3-oxo-alpha-ionol glucosides (ÇALIS, KURUÜZÜM-UZ & LORENZETTO, 2002).

### 2.4.4 *Catha edulis* Forssk

**Family:** Celastraceae

**Common names:** Bushman's tea, umHlwazi, iQgwaka, khat

**Origin and geographic distribution:** Catha is found in woodlands and on rocky outcrops. It is scattered in KwaZulu-Natal and Eastern Cape, mostly from the mistbelt, moving inland. It is also found in the Western Cape, Mpumalanga, Swaziland, Mozambique and through to tropical Africa and the Arab countries.

**Description:** Catha edulis is a shrub to small tree growing up to 10 m tall. The stem is usually straight and slender, with a narrow crown. The bark is light grey, becoming darker. It is rough and often cracked. The young stems are pinkish in colour. The leaves of this tree are opposite and are hanging. They have a leathery texture and are shiny bright green on the upper surface and paler beneath. The leaf margins are strongly serrated. Leaf stalks are short and pinkish in colour. They are 10 mm long and in late summer split to release the narrowly winged seeds (POOLEY, 1993).

**Medicinal action and uses:** The plant is widely used against respiratory diseases (KIPLE. & CONEÈ, 2001). In tropical Africa and Arab countries it provides the habit-forming stimulant found in the leaves. The leaves are brewed as tea or chewed for this purpose. The effects include wakefulness and hyperexcitability, and suppressed hunger. In South Africa, this plant is regarded as a drug, since the drug cathinone, which is extracted from it, is listed in the Drug Act. It is however not widely used in this country, except by some groups of people from the Eastern Cape. The wood of Bushman's tea is also used for a number of purposes. It is hard and fine-grained, and therefore is good for firewood and furniture. The bark is also used as an insect repellent and the stem for fence poles (VAN WYK, VAN OUDTSHOORN & GERICKE, 1997).

**Antimicrobial activity:** The isolated compounds from *Catha edulis* were identified as 22b-hydroxytingenone and tingenone by various spectral methods. Both compounds exhibited significant activities against *B. subtilis, S. aureus,* and *S. durans* (MIC 0.6 mg/ml). Both compounds were also found to be more potent against *Mycobacterium* species (MIC was 5.0 mg/ml for both compounds) than the positive controls, streptomycin and isonicotinic acid hydrazide. However, both compounds; were found to be inactive against the gramnegative bacteria *E Coli* and the fungus *C. albican* (JAYASURIA, 1988).

**Chemical composition:** Fresh khat leaves (100 g) contains approximately 36 mg cathinone, 120 mg norpseudoephedrine and 8 mg norephedrine. More than 30 other minor compounds (e.g., cathinine, cathidine, eduline, ephedrine) have been isolated from khat leaves (VAN WYK, VAN OUDTSHOORN & GERICKE, 1997). Khat contains khatamines (phenylpropyl and phenylpentenylamines) in amounts that vary according to the origin, type, and quality of the product. Khat leaves and twigs contain large amounts of tannins (up to 14% of dry weight) (HALBACH, 1972).

### 2.4.5 *Curcuma longa* L.

**Family:** Zingiberaceae

**Common names:** Curcuma, turmeric, ukon, goeratji, kakoenji, koenjet, kondin, kunir, kunyit, oendre, rame, renet, temu kuning, temu kunyit, tius

**Origin and geographic distribution:** Because of ancient trade, the origin of Turmeric cannot accurately be reconstructed, probably South East Asia or South Asia.

**Description:** Curcuma longa is a perennial plant with roots or tubers oblong-palmate and deep orange inside. Leaves about 2 feet long, lanceolate, petioled, tapering at each end, smooth, of a uniform green. Flowers are dull yellow, three or five together surrounded by bracteolae (ROSS, 2001). It is propagated by cuttings from the root. In fresh state, the roots have an aromatic and spicy fragrance, which by drying gives way to a more medicinal aroma.

**Medicinal action and uses:** Turmeric has long been used in Asian traditional medicine as a stomach tonic and blood purifier and for the treatment of liver ailments, skin diseases and wound healing. It also was used externally, to heal sores and as a cosmetic. In Thailand, turmeric rhizomes have been use to treat dizziness, gonorrhea and peptic ulcers and as an appetite stimulant, carminative, astringent and antidiarrheal. Externally the rhizome is used to treat insect bites, ringworm, wounds, bleeding and the teeth and gums (GHAZANFAR, 1994). In Thailand, turmeric is one of the most important folk remedies, with official sanction for use. Among the herbs often classified as spices, Turmeric is one of the best-researched for pharmacological effects. Today, it is considered potentially beneficial in treating or reducing symptoms associated with a wide range of health conditions, due to its antioxidant, antitumor, anti-inflammatory, and antibacterial effects (ROSS, 2001).

**Antimicrobial activity:** *Curcuma longa* rhizome extracts were evaluated for antibacterial activity against pathogenic strains of grampositive (*Staphylococcus aureus, Staphylococcus epidermidis*) and gramnegative (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium*) bacteria. Essential oil was found to be most active and its activity was compared to standard antibiotics gentamycin, ampicillin, doxycycline and erythromycin in these strains. Only the clinical isolate of *S. aureus* showed more sensitivity towards essential oil fraction than the standard strain (IYENGAR, RAMA & BAIRY, 1995). The use of essential oil from turmeric as a potential antiseptic in prevention and treatment of antibacterial infections has been suggested (SINGH, 1999).

**Chemical composition:** Turmeric contains up to 5% essential oils and up to 3% curcumin, a polyphenol. It is the active substance of turmeric and it is also known as C.I. 75300, or Natural Yellow 3. The systematic chemical name is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. It can exist at least in two tautomeric forms, keto and enol. The keto form is preferred in solid phase and the enol form in solution (GHAZANFAR, 1994).

### 2.4.6 *Eucalyptus citriodora* Hook.

**Family:** Myrtaceae

**Common names:** Shito babit zaf, kafur limuni, lemon gum, lemon-scented eucalypt, lemon-scented iron gum, spotted gum, kalintusi, citriodora, mkaratusi

**Origin and geographic distribution:** Native to Queensland, Australia, *E. citriodora* is commonly planted throughout the tropics, in the Mediterranean area and in the Malesian area, mainly in Malaysia.

**Description:** Evergreen tree 24–40 m high with tall straight trunk 0.6–1.3 m in diameter and thin, graceful crown of drooping foliage. Bark smooth, gray, peeling off in thin irregular scales or patches and becoming mottled, exposing whitish or faintly bluish inner layer with powdery surfaces appearing dimpled. Twigs slender, slightly flattened, light green, tinged with brown.

Leaves alternate, narrowly lance-shaped, 10–20 cm long, 1–2.5 cm wide, apically acuminate, basally acute, entire, glabrous, thin, light green on both surfaces, with many fine parallel straight veins and with vein inside edge (MORTON, 1981).

**Medicinal action and uses:** Bark may contain up to 12% tannin. Kenyans favor the honey produced by this species. Reported to be antiseptic and fumigant (LEWIS & ELVIN-LEWIS, 1977). Cubans place the leaves under the sheets of fever patients and inhale the steam from boiled leaves for cold and various pulmonary problems. Cubans also poultice the leaves onto ulcers, wounds, and other skin ailments. Guatemalans decoct the leafy shoots for coughs. Orally administered leaf extracts in rabbits artifically diabetic, produced temporary hypoglycemia and reduced the blood sugar levels. Myrtillin, in the leaf extract, is said to induce a temporary (MORTON, 1981).

**Antimicrobial activity:** *E. citriodora* oil was checked against clinically important bacterial strains (WATT & BREYER-BRANDWIJK, 1962). The oil was generally active against all the bacterial strains studied except *P. aeruginosa* which was the most resistant bacterial strain studied. *P. aeruginosa* is problematic as it has intrinsic resistance to several antibiotics and a capability to acquire resistance during antibiotic therapy. The pattern of antibacterial activity varied with the increase in the concentration of oil and decrease in the concentration of solvent. The oil showed maximum activity at the concentration of 20 - 40 µl against *E. coli, S. aureus, P. mirabils, P. aeruginosa, P. vulgarit, S. typhimurium, E. aerogenes, P. testosteroni, A. fecalis, B. cereus, C. freundii.* Amongst the gramnegative strains studied, the oil was highly active against *P. mirabilis* and *A. fecalis*. The oil was active against *S. aureus* amongst the grampositive (BECK ET AL., 1988).

**Chemical composition:** Leaves may contain oil with 65.5% citronellal, 12.2% citronellol, and 3.6% isopulegol, hairy leaves contain more oil with 86.6–90.1% citronellal, 4.6–6.0% citronellol, and 0.7–0.8% isopulegol, α-pinene, β-pinene, and isovaleric aldehyde are also recovered. Bark contains cca 9% tannin (MORTON, 1981). The young leaf is reported to contain citric-, glutaric-, malic-, quinic-, shikimic- (carcinogenic), and succinic-acids. Leaves and fruits test positive for flavonoids and sterols (ATAL & KAPUR, 1982).

## 2.4.7 *Eucalyptus gunnii* Hook. f.

**Family:** Myrtaceae

**Synonyms***:* Cider gum

**Origin and geographic distribution:** Native to Southern Australia and Tasmania

**Description:** In its natural habitat it can reach up to 100 feet (30 m) tall. The bark is smooth green and white. Juvenile leaves are leathery, glaucous blue and rounded to oblong to one to two inches (2.5-5 cm) wide. The adult leaves are greenish to 4 inches (10 cm) long and narrowly to broadly lanceolate. When leaves are crushed they are heavily (GRIEVE, 1984).

**Traditional uses:** Eucalyptus leaves are a traditional aboriginal herbal remedy. The essential oil found in the leaves is a powerful antiseptic and is used all over the world for relieving coughs and colds, sore throats and other infections (CHEVALLIER, 1996). The essential oil is a common ingredient in many over the counter cold remedies. An essential oil obtained from the leaves is antiseptic. The essential oil obtained from various species of eucalyptus is a very powerful antiseptic, especially when it is old, because ozone is formed in it on exposure to air. It has a decided disinfectant action, destroying the lower forms of life. The oil can be used externally, applied to cuts, skin infections etc., it can also be inhaled for treating blocked nasal passages, it can be gargled for sore throat and can also be taken internally for a wide range of complaints. Some caution is advised, however, because like all essential oils, it can have a deleterious effect on the body in larger doses (GRIEVE, 1984).

**Biological activity:** There are no reports on biological activity of *E. gunnii*

**Chemical composition:** The major components of *E. gunnii* were 1,8-cineole, α-pinene and aromadendrene (LEWIS & ELVIN-LEWIS, 1977).

### 2.4.8 *Laurus nobilis* L.

**Family:** Lauraceae

**Common names:** Bay laurel, true laurel, sweet bay, grecian laurel

**Origin and geographic distribution:** Probably Asia Minor. Today, the laurel tree grows all over the Mediterranean. Turkey is one of the main exporters.

**Description:** A broadly conical tree, this species grows up to 40 ft (12 m) high and 30 ft (9 m) wide, but is generally smaller in cultivation. Its glossy, dark green leaves are smooth and leathery and in Classical times were used to make the victor's 'crown of laurels'. It produces small, star-shaped, fragrant yellow flowers in late spring to early summer, followed by small, round, green berries that ripen to dark purplish black in fall (autumn). This tree is particularly suited to clipping and shaping (GRIEVE, 1984).

**Medicinal action and uses:** The bay tree has a long history of folk use in the treatment of many ailments, particularly as an aid to digestion and in the treatment of bronchitis and influenza (CHEVALLIER, 1996). It has also been used to treat various types of cancer (DUKE & AYENSU, 1985). The fruits and leaves are not usually administered internally, other than as a stimulant in veterinary practice, but were formerly employed in the treatment of hysteria, amenorrhoea, flatulent colic etc. (LEUNG,1980). Another report says that the leaves are used mainly to treat upper respiratory tract disorders and to ease arthritic aches and pains (CHEVALLIER, 1996). It is settling to the stomach and has a tonic effect, stimulating the appetite and the secretion of digestive (DUKE & AYENSU, 1985). The leaves are antiseptic, aromatic, astringent, carminative, diaphoretic, digestive, diuretic, emetic in large doses, emmenagogue, narcotic, parasiticide, stimulant and stomachic. The fruit is antiseptic, aromatic, digestive, narcotic and stimulant. An infusion has been used to improve the appetite and as an emmenagogue. The fruit has also been used in making carminative medicines and was used in the past to promote abortion. A fixed oil from the fruit is used externally to treat sprains, bruises etc., and is sometimes used as ear drops to relieve pain. The essential oil from the leaves has narcotic, antibacterial and fungicidal properties (CHEVALLIER, 1996).

**Antimicrobial activity:** *Laurus nobilis* was investigated for activity against *Acinetobacter baumanii, Aeromonas veronii, Candida albicans, Enterococus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens* and *Staphylococcus aureus*, using an agar dilution method. Laurus inhibited all organisms at concentrations of less than or equal to 2.0% (v/v) (HAMMER, CARSON & RILEY, 1999).

**Chemical composition:** The fruit contains up to 30% fatty oils and about 1% essential oils (terpenes, sesquiterpenes, alcohols and ketones). The leaves contain about 1.3% essential oils, consisting of 45% eucalyptol, 12% terpenes, 3-4% sesquiterpenes, 3% methyleugenol and other α- und β-pinenes, phellandrene, linalool, geraniol and terpineol (DUKE & AYENSU, 1985).

### 2.4.9 *Mangifera indica* L.

**Family:** Anacardiaceae

**Common names:** Mango, an lo kuo, anbah, manga agaci, manga, mangot fil, mangot, manguier, mamuang, aangga, merpelam, pelem

**Origin and geographic distribution:** The mango is native to southern Asia, especially Burma and eastern India. It spread early on to Malaya, eastern Asia and eastern Africa. Mangos were introduced to California in 1880.

**Description:** Large evergreen tree to 20 m tall with a dark green, umbrella-shaped crown (NAIK & GANGOLLY, 1950). Trunk stout, 90 cm in diameter; bark brown, smoothish, with many thin fissures; thick, becoming darker, rough and scaly or furrowed; branchlets rather stout, pale green and hairless. Inner bark light brown and bitter. A whitish latex exudes from cut twigs and a resin from cuts in the trunk. Leaves alternate, simple, leathery, oblong-lanceolate, 16-30 x 3-7 cm, on flowering branches, up to 50 cm on sterile branches, curved upward from the midrib and sometimes with edges a little wavy. Young leaves red, aging to shiny dark green above, lighter below, with yellow or white venation (SAMSON, 1986).

**Medicinal action and uses:** Dried mango flowers, containing 15% tannin, serve as astringents in cases of diarrhea, chronic dysentery, catarrh of the bladder and chronic urethritis resulting from gonorrhea. The bark contains mangiferine and is astringent and employed against rheumatism and diphtheria in India. The resinous gum from the trunk is applied on cracks in the skin of the feet and on scabies, and is believed helpful in cases of syphilis. Mango kernel decoction and powder (not tannin-free) are used as vermifuges and as astringents in diarrhea, hemorrhages and bleeding hemorrhoids. The fat is administered in cases of stomatitis. Extracts of unripe fruits and of bark, stems and leaves have shown antibiotic activity (GANGOLLY ET AL., 1957). In some of the islands of the Caribbean, the leaf decoction is taken as a remedy for diarrhea, fever, chest complaints, diabetes, hypertension and other ills. A combined decoction of mango and other leaves is taken after childbirth (MORTON, 1987).

**Antimicrobial activity:** The mango seed kernel ethanol extract (MKE) showed an antimicrobial activity against grampositive and gramnegative bacteria. MICs against gramnegative bacteria were varied among species. MICs of the MKE against *E. coli*, *Salmonella* sp., *Klebsiella aerogenes* and *Aeromonas hydrophila* were higher than 1000 ppm. MICs of the MKE, against both *Campylobacter jejuni* and *Yersinia enterocolitica* were 100 ppm. The MKE showed remarkable antimicrobial activity against grampositive strains and MICs of the MKE against *Staphylococcus aureus*, *Bacillus* sp., *Clostridium* sp. and *Listeria monocytogenes* varied in the range between 50 and 500 ppm. The MICs of the MKE, against 8 species of lactic acid bacteria (LAB) and *Staphylococcus aureus*, were higher than 1000 ppm in MRS agar and TS agar, respectively (KABUKI, NAKAJIMA & ARAI, 2000).

**Chemical composition:** Citric acid and related compounds are responsible for the sour taste (SAMSON, 1986). Several terpenes (ocimene, myrcene, limonene), aldehydes and esters have been found in the dried unripe fruits. Further more, unripe mangoes contain proteolytic enzymes. In ripe mangoes, volatile compounds (40 to 70 ppm) are ocimene, limonene, α-terpineol, 3-carene, β-selinene and myrcene. The yellow colour is due to about 30ppm β-carotene (CAMPBELL & MALO, 1976).

### 2.4.10 *Myrtus communis* L.

**Family:** Myrtaceae

**Common names:** Myrtle, myrte, mürt, mirto, gaelic miortal, mirtia

**Origin and geographic distribution:** The plant grows abundantly in the North Western to Eastern Mediterranean

**Description:** Evergreen shrubs or small trees, growing to 5 m tall. The leaves are entire, 3-5 cm long, with a pleasantly fragrant essential oil. The star-like flowers have five petals and sepals, and an amazingly large number of stamens. Petals are usually white, with globose blue-black berries containing several seeds. The flowers are pollinated by insects, and the seeds dispersed by birds that feed on the berries (CHIEJ, 1984).

**Medicinal action and uses:** The leaves are aromatic, balsamic, haemostatic and tonic (UPHOF, 1959). Recent research has revealed a substance in the plant that has an antibiotic action CHIEJ, 1984). The active ingredients in myrtle are rapidly absorbed and give a violet-like scent to the urine within 15 minutes (BOWN, 1995). The plant is taken internally in the treatment of urinary infections, digestive problems, vaginal discharge, bronchial congestion, sinusitis and dry coughs (CHEVALLIER, 1996). In India it is considered to be useful in the treatment of cerebral affections, especially epilepsy. Externally, it is used in the treatment of acne (the essential oil is normally used here), wounds, gum infections and haemorrhoids. The leaves are picked as required and used fresh or dried. An essential oil obtained from the plant is antiseptic. It contains the substance myrtol this is used as a remedy for gingivitis. The oil is used as a local application in the treatment of rheumatism. The fruit is carminative. It is used in the treatment of dysentery, diarrhoea, haemorrhoids, internal ulceration and rheumatism (BOWN, 1995).

**Antimicrobial activity:** The antibacterial activity of methanol crude extract of *Myrtus communis* was evaluated against 10 laboratory strains of microorganisms, including 6 grampositive (*Staphylococcus aureus, Micrococcus luteus, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Listeria monocytogenes*) and 4 gramnegative bacteria (*Escherichia coli, Proteus* *vulgaris, Pseudomonas aeruginosa* and *Campylobacter jejuni*). The crude extract inhibited the growth of all tested bacteria except *C. jejuni*. Essential oil was also active against the tested bacteria, and *M. luteus* showed the highest level of sensitivity (MIC 1:1600). The presence of antibacterial activity in different fractions and essential oil indicates that the extract possesses different compounds, which have different activities (SHAHLA ET AL., 2001).

**Chemical composition:** The dichlormethan/methanol extract of *M. communis* aerial part exhibited the most promising results during our anti-*P. larvae* activity screening of tropical and subtropical plant extracts, we decided to establish its *P. larvae* inhibiting potential more in details. YADEGARINIA ET AL. (2006) suggested that the antibacterial activity of *M.communis* was probably due to their major components, which was obtained by gas chromatography/mass spectrometry (GC–MS) analysis of the essential oils. Chemical analysis of the oil components resulted in the identification of 32 components (Table 1). The major components were α-Pinene (29.1%), Limonene (21.5%), 1,8-Cineole (17,9%), Linalool (10.4%), Linalyl acetate (4.8%) and α-Terpineole (3.17%).There is considerable variability in the composition of oil from different locations (CHIEJ, 1984).

**Table 1** *Chemical composition of (Myrtus communis) essential oil* (YADEGARINIA ET AL., 2006)

| No. | Compound | % |
|---|---|---|
| 1. | Isobutyl isobutyrate | 0.8 |
| 2. | α-Thujene | 0.3 |
| 3. | α-Pinene | 29.1 |
| 4. | Sabinene | 0.6 |
| 5. | Myrcene | 0.2 |
| 6. | δ-3-Carene | 0.2 |
| 7. | p-Cymene | 0.3 |
| 8. | Limonene | 21.5 |
| 9. | 1,8-Cineole | 17.9 |
| 10. | (E)-Ocimene | 0.1 |
| 11. | γ-Terpinene | 0.6 |
| 12. | Terpinolene | 0.3 |
| 13. | Linalool | 10.4 |
| 14. | α-Campholenal | 0.03 |
| 15. | trans-Pinocarveole | 0.07 |
| 16. | δ-Terpineole | 0.09 |
| 17. | Terpinene-4-ol | 0.5 |
| 18. | α-Terpineole | 3.17 |
| 19. | trans-Carveole | 0.4 |
| 20. | cis-Carveole | 0.07 |
| 21. | Geraniol | 1.1 |
| 22. | Linalyl acetate | 4.8 |
| 23. | Methyl geranate | 0.2 |
| 24. | α-Terpinyl acetate | 1.3 |
| 25. | Neryl acetate | 0.09 |
| 26. | Methyl eugenol | 1.6 |
| 27. | β-Caryophyllene | 0.2 |
| 28. | α-Humulene | 0.2 |
| 29. | Spathulenol | 0..07 |
| 30. | Caryophylleneb epoxide | 0.1 |
| 31. | Humulene epoxide II | 0.08 |
| 32. | Acetocyclohexane dione(2) | .4 |

### 2.4.11 *Olea europaea L.*

**Family:** Oleaceae

**Common names:** Green olive, king-of-trees, manzanillo, mission, pickling olive, queen, ripe olive, sevillano

**Origin and geographic distribution:** Cultivation of the olive tree is known in the Eastern Mediterranean since five millennia. Whether the plant really stems from these regions or is a native to Central Asia is subject to debate.

**Description:** Olea europaea is a small, ever green tree, averaging 20 feet or more inheight. It has many thin branches with opposite branchlets and shortly-stalked, opposite, lanceolate leaves about 2 1/4 inches long, acute, entire and smooth, pale green above and silvery below. The bark is pale grey and the flowers numerous, small and creamywhite in colour. The dark purple fruit is a drupe about 3/4 inch long, ovoid and often pointed, the fleshy part filled with oil. The thick, bony stone has a blunt keel down one side. It contains a single seed (BOWN, 1995).

**Medicinal action and uses:** The oil from the pericarp is cholagogue, a nourishing demulcent, emollient and laxative. Eating the oil reduces gastric secretions and is therefore of benefit to patients suffering from hyperacidity. The oil is also used internally as a laxative and to treat peptic ulcers. It is used externally to treat pruritis, the effects of stings or burns and as a vehicle for liniments. Used with alcohol it is a good hair tonic and used with oil of rosemary it is a good treatment for dandruff (GRIEVE, 1984). The oil is also commonly used as a base for liniments and ointments. The leaves are antiseptic, astringent, febrifuge and sedative. A decoction is used in treating obstinate fevers, they also have a tranquillising effect on nervous tension and hypertension. Experimentally, they have been shown to decrease blood sugar levels by 17 - 23%. Externally, they are applied to abrasions. The bark is astringent, bitter and febrifuge. It is said to be a substitute for quinine in the treatment of malaria. In warm countries the bark exudes a gum-like substance that has been used as a vulnerary (BOWN, 1995).

**Antimicrobial activity:** The antimicrobial activity was screened using grampositive (*Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*) and gramnegative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*) and fungi (*Candida albicans, Cryptococcus neoformans*). Olives revealed a wide range of antimicrobial activity. *C. albicans* was resistant to all the analyzed extracts (TUCK & HAYBALL, 2002).

**Chemical composition:** The desired flavour of olive oil is dominated by aldehydes (hexanal and 2-hexenal). Furthermore, higher aldehydes, primary alcohols (mainly C6 compounds like hexanol, 2-hexene-1-ol, 3-hexene-1-ol) and their acetic acid esters contribute to the characteristic olive oil aroma. Lastly, hemiterpenoid volatiles were found (3-methyl butanal, 4-methoxy-2-methyl-butanethiol, ethyl esters of 2- and 3-methyl butyric acid) (BOWN, 1995).

### 2.4.12 *Psidium guajava* L.

**Family:** Myrtaceae

**Common names:** Guava, goiaba, guayaba, djamboe, djambu, goavier, gouyave, goyave, goyavier, perala, bayawas, dipajaya jambu, petokal, tokal, guave, guavenbaum, guayave, banjiro, goiabeiro, guayabo, guyaba, goeajaaba, guave, goejaba, kuawa, abas, jambu batu, bayabas, pichi, posh, enandi.

**Origin and geographic distribution:** Guava has been cultivated and distributed by man, by birds, and sundry 4-footed animals for so long that its place of origin is uncertain, but it is believed to be an area extending from southern Mexico into or through Central America. It is common throughout all warm areas of tropical America and in the West Indies (since 1526), the Bahamas, Bermuda and southern Florida where it was reportedly introduced in 1847.

**Description:** A small tree to 33 ft (10 in) high, with spreading branches, the Guava is easy to recognize because of its smooth, thin, copper-colored bark that flakes off, showing the greenish layer beneath and also because of the attractive, "bony" aspect of its trunk which may in time attain a diameter of 10 in (25 cm) (SMITH ET AL., 1992). Young twigs are quadrangular and downy. The leaves, aromatic when crushed, are evergreen. The fruit, exuding a strong, sweet, musky odor when ripe, may be round, ovoid, or pear-shaped, 2 to 4 in (5-10 cm) long, with 4 or 5 protruding floral remnants (sepals) at the apex with thin, light-yellow skin, frequently blushed with pink.

**Medicinal action and uses:** Guava fruit is still enjoyed as a sweet treat by indigenous peoples throughout the rainforest, and the leaves and bark of the guava tree have a long history of medicinal uses that are still employed today (MORTON, 1987). The Tikuna Indians decoct the leaves or bark of guava as a cure for diarrhea. In fact, an infusion or decoction made from the leaves and/or bark has been used by many tribes for diarrhea and dysentery throughout the Amazon, and Indians also employ it for sore throats, vomiting, stomach upsets, for vertigo, and to regulate menstrual periods. Tender leaves are chewed for bleeding gums and bad breath, and it is said to prevent hangovers (if chewed before drinking). Indians throughout the Amazon gargle a leaf decoction for mouth sores, bleeding gums, or use it as a douche for vaginal discharge and to tighten and tone vaginal walls after childbirth. A decoction of the bark and/or leaves or a flower infusion is used topically for wounds, ulcers and skin sores. Flowers are also mashed and applied to painful eye conditions such as sun strain, conjunctivitis or eye injuries (SMITH ET AL., 1992). Commercially the fruit is consumed fresh or used in the making of jams, jellies, paste or hardened jam, and juice. In Peruvian herbal medicine systems today the plant is employed for diarrhea, gastroenteritis, intestinal worms, gastric disorders, vomiting, coughs, vaginal discharges, menstrual pain and hemorrhages, and edema. A decoction is also recommended as a gargle for sore throats, laryngitis and swelling of the mouth, and used externally for skin ulcers, and vaginal irritation and discharges (BOWN, 1995).

**Antimicrobial activity:** The antibacterial activity of guava (Psidium guajava) extracts against foodborne pathogens were determined on *Listeria monocytogenes*, *Staphylococcus aureus, Escherichia coli O157:H7, Salmonella Enteritidis, Vibrio parahaemolyticus* and *Bacillus cereus,* and on five food spoilage bacteria: *Pseudomonas aeroginosa, P. putida, Alcaligenes faecalis* and *Aeromonas hydrophila*. Guava extracts showed higher antimicrobial activity against grampositive bacteria compared to gramnegative bacteria except for *V. parahaemolyticus, P. aeroginosa* and *A. hydrophila*. None of extract showed antimicrobial activity against *E. coli O15:H7* and *Salmonella Enteritidis*. The minimum inhibitory concentration (MIC) of ethanol extracts of guava showed the highest inhibition for *L. monocytogenes JCM 7676* (0.1 mg/mL*), S. aureus* JCM 2151 (0.1 mg/mL), *S. aureus* JCM 2179 (0.1 mg/mL) and *V. parahaemolyticus* IFO 12711 (0.1 mg/mL) and the lowest inhibition for *Alcaligenes faecalis* IFO 12669, *Aeromonas hydrophila* NFRI 8282 (4.0 mg/mL), and *A. hydrophila* NFRI 8283 (4.0 mg/mL).

**Chemical composition:** Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus (80 mg of vitamin C in 100 g of fruit) and contains appreciable amounts of vitamin A as well (JIMENEZ-ESCRIG ET AL., 2001). Guava's main plant chemicals include: alanine, alpha-humulene, alpha-hydroxyursolic acid, alpha-linolenic acid, alpha-selinene, amritoside, araban, arabinose, arabopyranosides, arjunolic acid, aromadendrene, ascorbic acid, ascorbigen, asiatic acid, aspartic acid, avicularin, benzaldehyde, butanal, carotenoids, caryophyllene, catechol-tannins, crataegolic acid, D-galactose, D-galacturonic acid, ellagic acid, ethyl octanoate, essential oils, flavonoids, gallic acid, glutamic acid, goreishic acid, guafine, guavacoumaric acid, guaijavarin, guajiverine, guajivolic acid, guajavolide, guavenoic acid, guajavanoic acid, histidine, hyperin, ilelatifol D, isoneriucoumaric acid, isoquercetin, jacoumaric acid, lectins, leucocyanidins, limonene, linoleic acid, linolenic acid, lysine, mecocyanin, myricetin, myristic acid, nerolidiol, obtusinin, octanol, oleanolic acid, oleic acid, oxalic acid, palmitic acid, palmitoleic acid, pectin, polyphenols, psidiolic acid, quercetin, quercitrin, serine, sesquiguavene, tannins, terpenes and ursolic acid (BECKSTROM-STERNBERG & DUKE, 1994).

### 2.4.13 *Punica granatum* L.

**Family:** Punicaceae

**Common names:** Chinese apple, dalima, granada, grenade, grenadine, melograno, pomegranate

**Origin and geographic distribution:** native from Iran to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa and Europe.

**Description:** An attractive shrub or small tree, to 20 or 30 ft (6 or 10 m) high, the pomegranate is much-branched, more or less spiny, and extremely long-lived, some specimens at Versailles known to have survived two centuries. It has a strong tendency to sucker from the base (MORTON, 1987). The leaves are evergreen or deciduous, opposite or in whorls of 5 or 6, short-stemmed, oblong-lanceolate, 3/8 to 4 in (1-10 cm) long, leathery. Showy flowers are home on the branch tips singly or as many as 5 in a cluster. They are 1 1/4 in (3 cm) wide and characterized by the thick, tubular, red calyx having 5 to 8 fleshy, pointed sepals forming a vase from which emerge the 3 to 7 crinkled, red, white or variegated petals enclosing the numerous stamens. Nearly round, but crowned at the base by the prominent calyx, the fruit, 2 1/2 to 5 in (6.25-12.5 cm) wide, has a tough, leathery skin or rind, basically yellow more or less overlaid with light or deep pink or rich red. The interior is separated by membranous walls and white spongy tissue (rag) into compartments packed with transparent sacs filled with tart, flavorful, fleshy, juicy, red, pink or whitish pulp (technically the aril). In each sac, there is one white or red, angular, soft or hard seed (HOQUE, BARI & INATSU, 2007). The seeds represent about 52% of the weight of the whole fruit. (MORTON, 1987).

**Medicinal action and uses:** The juice of wild pomegranates yields citric acid and sodium citrate for pharmaceutical purposes. Pomegranate juice enters into preparations for treating dyspepsia and is considered beneficial in leprosy. The bark of the stem and root contains several alkaloids including isopelletierine which is active against tapeworms. Either a decoction of the bark, which is very bitter, or the safer, insoluble Pelletierine Tannate may be employed. Overdoses are emetic and purgative, produce dilation of pupila, dimness of sight, muscular weakness and paralysis. Because of their tannin content, extracts of the bark, leaves, immature fruit and fruit rind have been given as astringents to halt diarrhea, dysentery and hemorrhages. Dried, pulverized flower buds are employed as a remedy for bronchitis (YEUNG, 1985). In Mexico, a decoction of the flowers is gargled to relieve oral and throat inflammation. Leaves, seeds, roots and bark have displayed hypotensive, antispasmodic and anthelmintic activity in bioassay (MORTON, 1987).

**Antimicrobial activity:** Extract of *Punica granatum* (leaves, flowers) showed broad-spectrum antimicrobial activity with inhibition zones ranging from 4 to 34 mm. The most resistant organisms were *Escherichia coli, Candida albicans, Pseudomonas fluorescens, Bacillus subtilis* ATCC 6683*, and Enterobacter faecalis* ATCC 29212, and the most susceptible species were *Proteus vulgaris* ATCC 6997*, Salmonella typhimurium* CCM 5445*, Staphylococcus epidermidis* ATCC 12228*, and Serratia marcescens* CCM 583, respectively. The MICs of active extract ranged from 8 to 14.2 mg/mL while the MBCs were 14.2 to 24.4 mg/mL (OSKAY & SARI, 2007).

**Chemical composition:** Punica granatum is an important source of phenolic compounds, with anthocyanins being one of the most important, especially the 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin. These components along with gallagyl-type tannins, ellagic acid derivatives, and other hydrolysable tannins could contribute in some way to the antioxidant activity of pomegranate juice (FADAVI, 2005).

### 2.4.14 *Rosmarinus officinalis* L.

**Family:** Lamiaceae

**Common names:** Rosemary, romarin, osmarini rosmarino, alecrim, romero

**Origin and geographic distribution:** Rosemary was one of the plants that, according to the Capitulare de villis, was grown in medieval monasteries. However, its poor resistance to freezes limited its popularity, especially in regions north of the Alps. Today, rosemary is cultivated in nearly all countries around the Mediterranean Sea, furthermore in England, the US and Mexico.

**Description:** The evergreen leaves of this shrubby herb are about 1 inch long, linear, revolute, dark green above and paler and glandular beneath, with an odour pungently aromatic and somewhat camphoraceous. The flowers are small and pale blue. Much of the active volatile principle resides in their calyces. There are silver and goldstriped varieties, but the green-leaved variety is the kind used medicinally (CHIEJ, 1984).

**Medicinal action and uses:** Rosemary is commonly grown in the herb garden as a domestic remedy, used especially as a tonic and pick-me-up when feeling depressed, mentally tired, nervous etc. (WATT & BREYER-BRANDWIJK, 1962). Research has shown that the plant is rich in volatile oils, flavanoids and phenolic acids, which are strongly antiseptic and anti-inflammatory (GRIEVE, 1984). Rosmarinic acid has potential in the treatment of toxic shock syndrome, whilst the flavonoid diosmin is reputedly more effective than rutin in reducing capillary fragility. Rosmarol, an extract from the leaves, has shown remarkably high antioxidant activity. The whole plant is antiseptic, antispasmodic, aromatic, astringent, cardiac, carminative, cholagogue, diaphoretic, emmenagogue, nervine, stimulant, stomachic and tonic. An infusion of the flowering stems made in a closed container to prevent the steam from escaping is effective in treating headaches, colic, colds and nervous diseases. A distilled water from the flowers is used as an eyewash. The leaves can be harvested in the spring or summer and used fresh, they can also be dried for later use. This remedy should not be prescribed for pregnant women since in excess it can cause an abortion (MORTON, 1987). An essential oil distilled from the stems and leaves is often used medicinally, that distilled from the flowering tops is superior but not often available. The oil is applied externally as a rubefacient, added to liniments, rubbed into the temples to treat headaches and used internally as a stomachic and nervine (ROSS, 2001). The essential oil is used in aromatherapy (CHIEJ, 1984).

**Antimicrobial activity:** The dried extracts of the whole plant of Rosmarinus officinalis were tested in vitro against 12 bacterial species and strains by the agar diffusion method. *Bacillus brevis* FMC3, *Bacillus megaterium* DSM32, *Bacillus subtilis* IMG22, *Bacillus subtilis* ATCC 10, *Micrococcus luteus* LA 2971, *Mycobacterium smegmatus* RUT, *Escherichia coli* DM, *Listeria monocytogenes* SCOTT *A*, *Staphylococcus aureus* ATCC 25923, *Streptococcus thermophilus, Pseudomonas fluorescens,* and *Yersinia enterocolitica* O:3 P 41797 were used. All the extracts of the leaves of *Rosmarinus officinalis* showed various inhibitory effects (7-16 mm/20 mul inhibition zone), except the acetone extract against *Yersinia enterocolitica* (MARYOUK ET AL., 2006).

**Chemical composition:** The leaves contain about 1 to 2.5% essential oil. 1,8-cineol (30%), camphor (15 to 25%), borneol (16 to 20%), bornyl acetate (max. 7%), α-pinene (max. 25%) and others contribute to the complex taste (BECKSTROM-STERNBERG & DUKE, 1994).

## 2.4.15 *Zingiber officinale* Roscoe

**Family:** Zingiberaceae

**Common names:** Ginger, juany, gingembre, Ingwer, zenzero, shoga, gengibre

**Origin and geographic distribution:** Ginger seems to originate from Southern China. Today, it is cultivated all over tropic and subtropical Asia (50% of the world's harvest is produced in India), in Brazil, Jamaica (whence the best quality is exported) and Nigeria, whose ginger is rather pungent, but lacks the fine aroma of other provenances.

**Description:** The common cooking ginger is an herbaceous perennial with upright stems and narrow medium green leaves arranged in two ranks on each stem. The plant gets about 4 ft (1.2 m) tall with leaves about 3/4 in (1.9 cm) wide and 7 (17.8 cm) long. Ginger grows from an aromatic tuberlike rhizome (underground stem) which is warty and branched. The inflorescence grows on a separate stem from the foliage stem, and forms a dense spike, to 3 in (7.6 cm) tall. The bracts are green with translucent margins and the small flowers are yellow green with purple lips and cream colored blotches (ROSS, 2001). Most gingers in cultivation are sterile cultivars grown for the edible rhizome, and the flower is rarely seen (THEILADE, 1996).

.**Medicinal action and uses:** The medical form of ginger historically was called "Jamaica ginger"; it was classified as a stimulant and carminative and used frequently for dyspepsia and colic. It was also frequently employed to disguise the taste of medicines. Ginger is on the Food and Drug Administrations (FDA) generally recognized as safe list, though it does interact with some medications, including warfarin. Ginger is contraindicated in people suffering from gallstones as the herb promotes the release of bile from the gallbladder (CHIEJ, 1984). Ginger may also decrease joint pain from arthritis, though studies on this have been inconsistent, and may have blood thinning and cholesterol lowering properties that may make it useful for treating heart disease (DUKE & AYENSU, 1985). The characteristic odor and flavor of ginger root is caused by a mixture of zingerone, shoagoles and gingerols, volatile oils that compose about one to three percent of the weight of fresh ginger. In laboratory animals, the gingerols increase the motility of the gastrointestinal tract and have analgesic, sedative, antipyretic and antibacterial properties (CHEVALLIER, 1996).

**Antimicrobial activity:** The oil, together with petroleum ether, dichloromethane and methanol extracts, were assayed for antibacterial activity. The essential oil, petroleum ether and dichloromethane extracts exhibited antibacterial activity against *Bacillus substilis, Escherichia coli, Staphylococcus aureus* and *Sarcina* sp. However, none of the extracts was active against *Pseudomonas aeruginosa* (CHAIRGULPRASERT, PRASERTSONGSKUN & WICHAPORN, 2005).

**Chemical composition:** Ginger contains up to 3% of an essential oil that causes the fragrance of the spice. The main constituents are sesquiterpenoids with (-)zingiberene as the main component. Lesser amounts of other sesquiterpenoids (β-sesquiphellandrene, bisabolene and farnesene) and a small monoterpenoid fraction (β-phelladrene, cineole, and citral) have also been identified (LEUNG & FOSTER, 1996). The pungent taste of ginger is due to nonvolatile phenylpropanoid-derived compounds, particularly gingerols and shogaols (ROSS, 2001).

# 3 Objectives

## 3.1 The main objective

The aim of this work was to compare *in vitro* antibacterial effects of dichlormethane/methanol extracts from 15 plants, namely *Rosmarinus officinalis, Mangifera indica, Bixa orellana, Olea europaea, Catha edulis, Laurus nobilis, Curcuma longa, Capparis spinosa, Punica granatum, Camelia sinensis, Psidium quajava, Zingiber officinale, Eucalyptus gunnii, Eucalyptus citriodora* and *Myrtus communis* against three strains of bee pathogenic bacterium *Paenbacillus larvae*, the causal agent of American Foul Brood Disease.

## 3.2 Specific objectives

Summarization of information on botany, origin and geographical distribution, medicinal action and uses, antimicrobial activities and chemical composition of these plants.

Determination of minimum inhibitory concentration (MIC) of methanol/dichlormethan extracts from different parts of selected plants using the broth microdilution method.

Selection of prospective plants as potential source of inhibitor compounds for further study.

# 4 Experimental part

## 4.1 Material and methods

### 4.1.1 Chemicals and instruments

**Chemicals**

| | |
|---|---|
| Ethanol 96% pharm. | Lach-Ner, s.r.o., Neratovice, CZ |
| Dichlormethan | Lach-Ner, s.r.o., Neratovice, CZ |
| Methanol | Lach-Ner, s.r.o., Neratovice, CZ |
| Dimethyl sulfoxid (DMSO) p.a. | Lach-Ner, s.r.o., Neratovice, CZ |
| Tris- buffer saline pH 7.6 (TBS) | Sigma-Aldrich, Prague, CZ |
| 1,8 Cineol | Sigma-Aldrich, Prague, CZ |

**Antibiotics**

| | |
|---|---|
| Oxytetracycline | Sigma-Aldrich, Prague, CZ |
| Tylosine tartrate | Sigma-Aldrich, Prague, CZ |

**Cultivation media**

MYPGP aerob. broth (pH 7.3 ± 0.2)

| | | |
|---|---|---|
| Composition: | Yeast extract 1.5 g/l | Oxoid, Basingstoke, UK |
| | Mueller – Hinton broth 1,0 g/l | Oxoid, Basingstoke, UK |
| | Glucose 0,2 g/l | Sigma-Aldrich, Prague, CZ |
| | $K_2HPO_4$ 0,3 g/l | Sigma-Aldrich, Prague, CZ |
| | Sodium pyruvate 0,1 g/l | Sigma-Aldrich, Prague, CZ |
| | Nalidix acid 0,6 g/l | Oxoid, Basingstoke, UK |

The required quantity was weighted on the analytical scales (KERN 770) and dissolved in distilled water, heated if necessary. Then it was placed into penicillin bottles of 20 ml volume, which were sealed, sterilized by autoclaving and stored in a fridge.

Sterilization

All prepared media, TBS and laboratory material were sterilized in the autoclave at 120°C and 1.2 MPa for 30 minutes or single-use sterile needles, syringes, microtitration plates (96 flat-bottomed wells) and test tubes were used.

**Laboratory instrumens**

| | |
|---|---|
| Homogenisator Grindomix GM 100 | Retsch GmbH, Haan, GER |
| Evaporator Büchi Rovapor 205 | Büchi Labortechnik AG, Flawil, CH |
| Multiscan Ascent Microplate Photometer | Thermo Fisher Scientific, Waltham, USA |
| Densitometer Densi-La-Meter | Lachema, a.s., Neratovice, CZ |
| Analytical balance KERN 770 | Kern & Sohn GmbH, Balingen, GER |
| Pipettes (single-, 12-channel, volume 0.1 – 1 ml) | Eppendorf AG, Hamburg, GER |

## 4.2 Plant material and extract preparation

### 4.2.1 Plant material

Plant material samples were obtained from the greenhouses of Institute of Tropics and Subtropics (ITS) of the Czech University of Life Sciences Prague (CULS Prague). Data of the plants species selected for the study (botanical names, families and common are summarized in Table 2.)

**Table 2** *Botanical data of tested plants*

| Spicies | Family | Common name |
|---|---|---|
| *Rosmarinus officinalis* L. | Lamiaceae | Rosemary |
| *Mangifera indica* L. | Anacardiaceae | Mango |
| *Bixa orellana* L. | Bixaceae | Anatto |
| *Olea europaea* L. | Oleaceae | Green olive |
| *Catha edulis* Forssk | Celastraceae | Khat |
| *Laurus nobilis* L. | Lauraceae | Bay Laurel |
| *Curcuma longa* L. | Zingiberaceae | Turmeric |
| *Capparis spinosa* L. | Capparaceae | Caper |
| *Punicum granatum* L. | Punicaceae | Pomegranate |
| *Camelia sinensis* Kuntze | Theaceae | Tea |
| *Psidium quajava* L. | Myrtaceae | Guava |
| *Zingiber officinale* Roscoe | Zingiberaceae | Ginger |
| *Eucalyptus gunnii* Hook. F. | Myrtaceae | Cider Gum |
| *Eucalyptus citriodora* Hook | Myrtaceae | Lemon Gum |
| *Myrtus communis* L. | Myrtaceae | Myrtle |

### 4.2.2 Exctract preparation

Air dried material was finely ground to powder, using a homogenisator (Restsch Grindomix, 10 000 turn per minute). Fifteen grams of sample were weighted in filter paper and placed in a 500 ml soxhlet glass timble. The extraction was carried out using dichlormethan/methanol (1:1) as solvent (500 ml of sample) at the solvent boiling point for 24 h. After extraction, the solvent was evaporated by vacuum evaporator (Rotavapor R-200) at 40° C and weighted. The residue for each extract obtained after evaporation is given in Table 3.

**Table 3** *Yiealds of selected plant extracts( in 500ml dichlormethan/methanol, 1:1) after evaporization*

| Species | Part tested | Yeld (g) | Yeld (%) |
|---|---|---|---|
| *Rosmarinus officinalis* L. | aerial parts | 1.44 g | 9.60 |
| *Mangifera indica* L. | leaves | 1.76 g | 11.73 |
| *Bixa orellana* L. | leaves | 2.58 g | 17.20 |
| *Olea europaea* L. | leaves | 3.14 g | 20.93 |
| *Catha edulis* Forssk | leaves | 1.25 g | 8.33 |
| *Laurus nobilis* L. | leaves | 2.91 g | 19.4 |
| *Curcuma longa* L. | roots | 1.78 g | 11.86 |
| *Capparis spinosa* L. | aerial parts | 1.46 g | 9.73 |
| *Punica granatum* L. | rind | 1.39 g | 9.26 |
| *Camellia sinensis* Kuntze | leaves | 2.47 g | 18.26 |
| *Psidium guajava* L. | leaves | 1.86 g | 12.4 |
| *Zingiber officinale* Roscoe | roots | 1.45 g | 9.66 |
| *Eucalyptus gunnii* Hook. f. | leaves | 3.07 g | 20.46 |
| *Eucalyptus citriodora* Hook | leaves | 2.58 g | 17.2 |
| *Myrtus communis* L. | aerial parts | 1.79 g | 11.93 |

It is advisable to extract the plants and to evaporate the extracts at low temperature in order not to destroy any thermo-labile antimicrobial agents present in extract. Therefore, sterilization of the extracts by autoclaving or other strenuous methods should be avoided as well.

**Extract stock solution**

On analytical balance (KERN 770) 0.128mg of each crude evaporated extract was weighed and dissolved in 0.5 ml of DMSO to create a stock solution of 25.6 mg/ml for testing in antimicrobial assay.

## 4.3 Microorganisms

Microbial strains used in assays are known to cause AFB disease. Isolates of *P. larvae* were sampled from those received by Bee Research Institut at Dol, Czech Republic from January-March, 2008. These isolates were derived from infected bees and hive materials collected by apiary inspectors and beekeepers in Czech Republic. The following strains were used: *P. larvae* 17/08, h3 and *P. larvae* 15/08, f4 .All other used bacteria obtained from the American Type Culture Collection (ATCC) (*P. larvae* ATCC 9545). All strains were grown and tested in MYPGP broth at 37°C for 48 hours.

**Susceptibility test**

The susceptibility of bacteria to Oxytetracyclin and Tylosine tartrate were checked as antibiotic controls. The antibiotics were disolved in 5% DMSO in TBS pH 7.6.

## 4.4 Antimicrobial assay

In vitro antimicrobial activity was determined by the broth microdilution method (JORGENSEN, TURNIDGE & WASHINGTON, 1999) using microtiter plates (96 flat-bottomed wells), modified according to the recommendations recently proposed for more effective assessment of anti-infective potential of natural products (COS ET AL., 2006). Two-fold dilutions (ten) of each extract were prepared in MYPGP broth in concentrations ranging from 256 to 1 µg/ml. Each well was inoculated with 1 µl of bacterial suspension at a density of $10^7$ CFU/ml. Microplates were incubated at 37 °C for 48 h. Growth of microorganisms was observed as turbidity determined by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific ) at 405 nm. Minimum inhibitory concentrations (MICs) were calculated based on the density of the growth control and were the lowest extract concentrations that resulted in 80% reduction in growth compared with that of the extract-free growth control. Final concentrations of DMSO did not exceed 1% in any sample tested. All samples were tested in triplicate

# 5 Results and discussion

The results of all plant extracts tested showed antibacterial activity against one or more bacteria tested in this study at the concentration 256 μg/ml or below. Rosemary (*Rosmarinus officinalis*), bay laurel (*L. nobilis*), guava (*P. guajava*), ginger (*Z. officinale*) cider gum (*E. gunnii*) and myrtle (*M. communis*) showed maximum activity against all the bacterial species tested. On the other hand, mango (*M. indica*), annato (*B. orellana*), green olive (*O. europaea*), khat (*C. edulis*), turmeric (*C. longa)*, caper (*C. spinosa*), pomegranate (*P. granatum*), tea (*C. sinensis*), lemon gum (*E. citriodora*) failed to inhibit some of the tested strains. The results indicated significant differences between the strains tested; however all of them were sensitive to the potent plant extracts. *P. larvae* (15/08, f4) were inhibited by 14 extracts followed by *P. larvae* (17/08, h3) by 12 extracts and the most resistant strain *P. larvae* (ATCC 9545) was inhibited by 8 extracts. Experimental results have shown that wild strains tested in this study were more susceptible than ATCC one.

The inhibitory effect of extract dilutions on microbial growth varies from one microorganism to another but the MICs for fifteen extracts ranged from 2 to 256 μg/ml (Table 4). Among all plants tested in this study, the greatest *in vitro* antibacterial action exhibited extract from aerial part of *M. communis*, inhibiting growth of three strains of *P. larvae* with MICs ranging from 2 to 4 μg/ml. Significant antibacterial action possessed also extracts of *R. officinalis, L. nobilis* and *E. gunnii* with MIC values ranging from 16 to 32 μg/ml. The use of these plants and their extracts for inhibition effect on many bacterial strains has already been reported by several authors.

BARATTA ET AL. (1998) tested the antibacterial and antifungal activities of essential oil a commercial sample of *R. officinalis* where R-pinene, 1,8-cineole, camphor, R-terpineol were detected as a major compounds. The results showed the low activity except against *S. aureus*. Other chemotypes of *R. officinalis* with R-pinene, verbenone, bornyl acetate as a major substances exhibited a nonsignificant activity, against grampositive (*S. aureus*, and *S. epidermidis*) and gramnegative (*E. coli*, *P. aeruginosa*). On the other hand, the third chemotype characterized by myrcene, 1,8-cineole and camphor expressed insecticidal properties and *in vitro* antifungal activity against *A. apis*. In general, methanol extracts exhibited very low antimicrobial activities compared to the essential oils. The results of this antimicrobial screening showed low activity against *S. aureus* whereas the rest of the extracts were inactive against other microorganisms.

The inhibiton effect against *P. larvae* in our study is consistent with results from earlier studies, where essential oil of *R.officinalis* from the aerial parts exhibited activities against *P. larvae* (ALIPPI ET AL., 1996).

In study of ÖZCAN & ERKMEN (2001), *L. nobilis* essential oil from leaves possessed previously antibacterial activity *in vitro*, inhibiting *S. typhimurium, B. cereus, S. aureus, E. faecalis, E. coli, Y. enterocolitica, S. cerevisiae, C. rugosa, R. oryzae* and *A. niger*, what is in correspondence with results obtained in this research, where the leave extract demonstrated good inhibition of all bacterial strains tested.

Previous phytochemical studies on *L. nobilis* confirmed the presence of 1,8-cineole in high amount, which was previously described as strong antimicrobial agent (COX, MANN & MARKHAM, 2001). Based on these researches one of our hypothesis was 1,8-cineole has significant anti-*P. larvae* activity which was also decided to test and the results are stated below.

In our study, *in vitro* inhibitory test against *P. larvae* showed significant activity of the leaf extract from *E. gunnii,* whilst *E. citriodora* was found to be inactive. In former research *E. globulus* was found to be effective against *P. larvae* (ALIPPI ET AL., 1996). Based on the close chemotaxonomical relationship between species within genus Eucalyptus we aasume that some of major compounds, such as 1,8 cineole, limonene, *p*-cymene and α-pinene (ATAL & KAPUR, 1982), could be responsible for anti-*P. larvae* activity. On the other hand some of them, such as limonene, *p*-cymene and α-pinene, had been previously shown to exhibit a low antibacterial activity (KNOBLOCH ET AL., 1989; CHALCHAT ET AL., 1997)

As far as other results achieved in this study are considered, the extract of *P. guajva* leaves and *Z. officinale* roots possessed a wide antibacterial spectrum because they inhibited the growth of all *P. larvae* strains with MICs ranging from 64 to 256 μg/ml. Another group of 9 extracts including *M. indica, B. orellana, C. edulis*, *O. europaea, P.granatum, C. longa*, *C. spinosa*, *C. sinensis* and *E. citriodora* were not active against all bacteria strains tested.

The results of tests with antibiotic positive standards showed that all *P.larvae* strains were highly susceptible to Oxytetracycline and Tylosin tartrate (MIC values ranging from 0.03125 to 0.015625 μg/ml).

Since the dichlormethan/methanol extract of *M. communis* aerial part exhibited the most promising results during our anti-*P. larvae* activity screening of tropical and subtropical plant extracts, we decided to establish its *P. larvae* inhibiting potential more in detail. For this reason, new samples (A,B,C) from three individual plants were obtained in the greenhouses of ITS of the CULS Prague and subsequently submitted to the procedure as described in Material and Methods section.

In this study, the dichlormethan/methanol extracts of *M. communis* were highly effective (Table 5). The MIC values of the extract sample B against all bacteria were 2 μg /ml followed by sample A and C with MICs ranging from 4 to 8 μg/ml. Earlier studies of YADEGARINIA ET AL. (2006) suggested that the leaf essential oil was probably responsible for antibacterial activity of *M. communis*. The folowing chemical analysis performed by gas chromatography-mass spectrometry (GC–MS) resulted in the identification of 32 compounds, whereas α-pinene (29.1%), limonene (21.5%), 1,8-cineole (17.9%), linalool (10.4%), linalyl acetate (4.8%) and α-terpineole (3.17%). This major components in the essential oil of leaves appear also in most solvent leave extracts and in the same order of abundance. Previously, the high amount of monoterpenes hydrocarbons such as α-pinene, has been reported to be the cause of the antifungal activity of oil from *Pistacia lentiscus* (Anacardiaceae) (MAGIATIS ET AL., 1999). Limonene, the most abundant constituent of the oil, was known to exhibit antibacterial activity (SASS ET AL., 2008). Another monoterpene alcohol, linalool, is reported to have a wide range of antibacterial and antifungal activity by (PATTNAIK ET AL., 1997). Research on the antimicrobial actions of monoterpenes suggests that they diffuse into and damage cell membrane structures (SIKKEMA, DE BONT & POOLMAN, 1995). In fact it seems, as described previously by (SASS ET AL., 2008), that essential oils containing terpenoids are more active against grampositive organisms than against gramnegative ones.

**Table 4** *Minimum inhibitory concentrations (µg/ml) of dichlormethan/methanol extracts of selected plants tested by broth microdilution method.*

| Species or reference compound | Plant part | Microorganisms [a] | | |
|---|---|---|---|---|
| | | pl 1 | pl 2 | pl 3 |
| *Rosmarinus officinalis* | aerial parts | 16 | 16 | 32 |
| *Mangifera indica* | leaves | NA | 128 | 256 |
| *Bixa orellana* | leaves | NA | 64 | 256 |
| *Olea europaea* | leaves | NA | 256 | NA |
| *Catha edulis* | leaves | 32 | 64 | NA |
| *Laurus nobilis* | leaves | 16 | 16 | 32 |
| *Curcuma longa* | roots | 256 | 64 | NA |
| *Capparis spinosa* | aerial parts | 32 | 32 | NA |
| *Punica granatum* | rind | 256 | NA | NA |
| *Camellia sinensis* | leaves | 256 | 256 | NA |
| *Psidium guajava* | leaves | 64 | 128 | 256 |
| *Zingiber officinale* | roots | 64 | 32 | 256 |
| *Eucalyptus gunnii* | leaves | 16 | 16 | 32 |
| *Eucalyptus citriodora* | leaves | 64 | 32 | NA |
| *Myrtus communis* | aerial parts | 4 | 2 | 4 |
| Oxytetracyclin [b] | | 0.03125 | 0.03125 | 0.015625 |
| Tylosine tartrate [b] | | 0.03125 | 0.03125 | 0.015625 |

[a] pl1 = *Paenibacilus larvae* (17/O8, h3); pl2 = *Paenibacilus larvae* (15/O8, f4); pl3= *Paenibacilus larvae* ( ATCC 9545)

[b] Oxytetracycline and Tylosine tartrate were used as antibiotic positive reference standarts for bacteria.

NA – no activity (MIC > 256 µg/ml)

**Table 5** Verification of the minimum inhibitory concentrations (μg/ml) of the most effective plant (*Myrtus communis*) by broth microdilution method

| Species | Plant part | Microorganisms [a] | | |
|---|---|---|---|---|
| | | **pl 1** | **pl 2** | **pl 3** |
| *Myrtus communis A* | aerial parts | 4 | 4 | 4 |
| *Myrtus communis B* | aerial parts | 2 | 2 | 2 |
| *Myrtus communis C* | aerial parts | 4 | 4 | 8 |

[a] pl1 = *Paenibacilus larvae* (17/O8, h3); pl2 = *Paenibacilus larvae* (15/O8, f4); pl3= *Paenibacilus larvae* (ATCC 9545)

According to results achieved in this study, where the aerial parts extract of *M. communis* showed promising activity inhibiting all microbial strains tested (ranging from 2 to 8 μg/ml) it is possible to expect that the strong antimicrobial action could be caused by presence one of the major compound 1,8 cineole, which have been previously detected in the plant and has been known to exhibit strong antibacterial activity against *E. coli*, *P. aeruginosa*, *S. typhi*, *S. aureus*, *R. leguminosarum*, *B. subtilis* and other bacteria (COX, MANN & MARKHAM, 2001). This natural organic compound is colorless liquid cyclic ether and a monoterpenoid which comprises up to 90% of the essential oil of some species of Eucalyptus hence the well known common name of the compound – eucalyptol (BOLAND, BROPHY & HOUSE, 1991; FLORIS & CARTA, 1990). It is also known by a variety of synonyms: 1,8-cineol, limonene oxide, cajeputol, 1,8-epoxy-p-menthane, 1,8-oxido-p-menthane, 1,3,3-trimethyl-2-oxabicyclo [2,2,2] octane, cineol, cineole.

Since this compound has also been identified in various parts of the most effective plants in our study, namely rosemary (*R. officinalis*), bay laurel (*L. nobilis*) and cider gum (*E. gunnii*) (KILIC ET AL., 2004), there was an assumption that 1,8-cineole could be responsible for the detected anti-*P. larvae* activity of extracts derived from these plants.

Based on these facts we have decided to confirm our assumption In this study, we have used determination of antimicrobial activity by the broth microdilution method (JORGENSEN, TURNIDGE & WASHINGTON, 1999) using microtiter plates (96 flat-bottomed wells), modified according to the recommendations recently proposed for more effective assessment of anti-infective potential of natural products (COS ET AL., 2006). The 1,8 cineole sample was carried out from a starting concentration of 64 μg/ml.

However, in following tests the results demonstrated week activity of the 1,8-cineole (Sigma-Aldrich, Prague, CZ) against *P. larvae*. MICs were observed in our study over the ranges of 64 μg/ml. Obtained results of this testing has not confirmed above mentioned proposed hypothesis that 1,8-cineole is not necessarily responsible for the greatest share of the total anti-*P. larvae* activity.

Thus, the involvement of the less abundant constituents should be considered. In addition, the activity could be attributed to the presence of minor components such as neryl, known to exhibit an antibacterial activity or a synergistic effect between all components (ONAWUNMI, YISAK & OGUNLANA, 1984; HINOU, HARVALA & HINOU, 1989; KNOBLOCH ET AL., 1989; ZAKARYA, FKIH-TETOUANI & HAJJI, 1993; FLORIS & CARTA, 1990). This finding is in good agreement with DELLACASSA ET AL., (1989) and CHITCHAT ET AL., (1997). These authors have also suggested that the antibacterial activity of some essential oils and particularly that of Eucalyptus species, are related to the presence of some favourable classes of compounds such as alcohols, aldehydes, alkenes, esters and ethers. However, a pronounced difference found in the activity of the extracts from *M. communis, L. nobilis, R.officinalis* and *E.gunnii* containing the same major constituents 1,8-cineole and α-pinene in almost equal amounts could be due to the presence of some unfavourable components in high amount in the second oil than in the first one. This is consistent with the results from ZAKARYA, FKIH-TETOUANI & HAJJI (1993) for the antibacterial activity of some eucalyptus essential oils.

# 6 Conclusions

This study is the report on the activity of plant extracts as inhibitors of the most destructive microbial disease *P.larvae.* Antimicrobial study of fifteen plants, all of them selected based on their relevant antimicrobial inhibition, has provided various extracts with strong activity against several microbial strains. According to results obtained in our study, dichlormethane/methanol extracts of *M. communis, L. nobilis, R. officinalis*, and *E.gunnii* showed significant antibacterial action. Their main component, 1,8-cineol, resulted inactive, evidencing that other compounds and/or complex interactions between the components of the extracts play an important role.

Based on our results of *in vitro* tests we suggest that this plant extracts have strong potential as natural alternatives to synthetic antibiotics in the control of American Foul Brood, lessening considerably the risk of antibiotic residues in the world honey supply.

Although our study yielded several promising plant extracts for control of *P. larvae* on honeybees, more effort is needed to enhance their incorporation in integrated disease management strategies. Urgent further steps might be: the elucidation of active ingredients of the selected plants, optimization of application methods and the study of detrimental effects on bees within a longer time scale and in realistic conditions.

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# 8 Appendices

**Appendix 1.** *Bixa orellana*

**Appendix 2.** *Camellia sinensis*

**Appendix 3.** *Capparis spinosa*

**Appendix 4.** *Catha edulis*

**Appendix 5.** *Curcuma longa*

**Appendix 6.** *Eucalyptus citriodora*

**Appendix 7.** *Eucalyptus gunni*

**Appendix 8.** *Laurus nobilis*

**Appendix 9.** *Magnifera indica*

**Appendix 10.** *Myrtus communis*

**Appendix 11.** *Olea europea*

**Appendix 12.** *Psidium guajava*

**Appendix 13.** *Punica granatum*

**Appendix 14.** *Rosmarinus officinalis*

**Appendix 15.** *Zingiber officinalis*