Czech University of Life Sciences in Prague

Faculty of Agrobiology, Food and Natural Resources

Department of Quality of Agricultural Products

Antimicrobial activity of essential oils from *Eucalyptus deglupta*

Diploma Thesis

Author: Rafael Sumar Valdivia

Supervisor: Ing. Pavel Nový, Ph.D.

Declaration

I hereby declare that this M.Sc. thesis entitled "Antimicrobial activity of essential oils from *Eucalyptus deglupta* is my independent research work, carried out under the guidance of my supervisor. All scientific literature and all other information sources used in it have been cited in the text and acknowledged in the bibliography.

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Rafael Sumar Valdivia

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Antimicrobial activity of essential oils from *E.deglupta*

Summary

Plant essential oils are known to exert potent antibacterial and antifungal activities against broad spectrum microorganisms. This activity has previously been confirmed for various *Eucalyptus* spp. In this study, the antimicrobial activity of leave essential oil of *Eucalyptus deglupta* Blume collected in the Independent State of Samoa, was evaluated using the broth microdilution method *in vitro*. The essential oil composition was analyzed using gas chromatography-mass spectrometry. The antibacterial activity of the essential oil tested against potentially pathogenic gram-positive and gram-negative bacteria *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Klebsiella pneumoniae*.

The chemical analysis revealed *trans-nerolidol* and *α-pinene* as the predominant constituents of the oil with the content of 61.92 % and 11.02 %, respectively.

No minimum inhibitory concentrations were obtained by the antimicrobial assy. The *E. deglupta* essential oil showed only very weak inhibitory effect against the bacteria tested with ≤ 50 % inhibition of the bacterial growth whereas *B. cereus* was the most sensitive organism and E.faecalis was the most resistant one. The low antimicrobial activity can be explained by the total absence of eucalyptol, an antimicrobial compound typical for *Eucalyptus* spp.

Keywords:

Eucalyptus deglupta; essential oil; antimicrobial; antibacterial

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List of Abbreviations

MIC	Minimum Inhibitory Concentration
GC	Gas chromatographer
GC-MS	Gas chromatography–mass spectrometry
Zdi	Zone diameter of inhibition
CFU	Colony forming Units
ATCC	American Type Culture Collection
SCFE	Supercritial fluid extraction
UAE	Ultrasound assisted extraction
MAE	Microwave assisted extraction
MHG	Microwave hydrodiffusion and gravity
MSD	Microwave steam distillation
MSDf	Microwave steam diffusion
WHO	World health organization

1 Introduction

Paleontology provides more and more data to confirm the fact that infectious diseases and their treatment have always been inseparable phenomena of human life, so in the history of mankind and civilizations is known how people looked and inquired the possibility of finding new ways to cure. Based on the "trial and error" method the man found many plants that were effective against infections and gradually built a pharmacopoeia book for the treatment of infectious processes based on rudimentary knowledge. That is why humans always viewed the nature as a friend in this way to find solutions.

Essential oils of some aromatic plant species have been used since antique in flavor and fragrance, as a condiment or spice, and to repeal insect or protect stored products. Nowadays, they are used in many fields all over the world and have become an integral part of everyday life. They are used in industries for the production of soaps, perfumes and toiletries. Many of them are also used in traditional medicine for various purposes. Investigations on the evaluation of the biological activities of essential oils of some medicinal plant species have revealed that some of them exhibited interesting activities such as insecticidal. Moreover, active principles of essential oils with antibacterial or antifungal activity were also isolated and identified (Oyedeji, Olawore, Ekundayo, & Koenig, 1999).

E.deglupta is one of the examples of medicinal plant in the South West Pacific region in the tropics, little known for its benefits in natural medicine. It belongs to the family *Myrtaceae* native from Sulawesi (Indonesia) and Mindanao (Philippines) eastward to New Britain (Papua New Guinea). There are a few reports of antimicrobial activity of *E.deglupta* essential oil reported against some species (Cimanga et al., 2002), therefore, there is high probability of identifying more bacterial species sensitive to this essential oil . However, this aromatic plant species is used in some traditional medicine but the effects of this plant are still poorly known. Thus the leaves from *E.deglupta* tree located in Independent State of Samoa were collected to verify the antimicrobial activity from this traditional plant.

2 Objectives of work

The aim objective of this research is to evaluate in vitro antimicrobial activity of *E.deglupta* essential oil obtained from the leaves of this tree located in the South West Pacific region in the tropics, Independent State of Samoa, against various potentially microbial species.

In this context, the specific objectives of this study are:

- Determine the Minimum Inhibitory Concentration (MIC) of the *E.deglupta* essential oil against selected bacteria using broth microdilution method.
- Examine the chemical composition of the oil

Hypothesis

The antimicrobial activity of *E.deglupta* has already been reported against some species, therefore, there is high probability of identifying more bacterial species sensitive to this essential oil.

3 Literature review

3.1 Traditional plants

Traditional plants have been used and identified through the history of mankind. They have a variety of chemical compounds that can be synthesize and be used to play important biological functions as well as to defend against predators. The uses of these plants as medicine is recognized as an effective way to treat disease and discover future medicines. Undeveloped societies and people that can't afford buying the latest pharmaceuticals in the market, use these plants for their primary healthcare. Also in some countries, they continue giving a traditional use as in rituals and spiritual events. Now a days these plants has got more attention because antibiotics are more resistant to antibiotics, are much easier to be found, and are now available from natural-food stores and herbal providers. Therefore self-medication with these substances are a better natural alternative from conventional medical treatments.

3.1.1 A cure for disease

From the plagues of ancient times to the pandemic viruses of today, infectious diseases have played an important role in the discovery and manufacture of medicines that were used and still using today. When the first groups of human's settlements into sedentary agricultural people and started to form villages and the relationship between small towns began to be consolidated, human populations became big enough to maintain direct life cycle bacterial and viral infections. It is in these first cities that the now common diseases of humans began to appear (Chandler & Fox, 1974). According to Dobson & Carper "many of the first pathogens to infect humans evolved from diseases of domestic animals. Measles, for example, is closely related to two other morbillivirus - canine distemper and rinderpest (a disease of cattle) - whereas smallpox probably evolved from cow-pox" (Dobson & Carper, 1996). Even though written descriptions of many early disease sprout are almost impossible to decode, rabies is known from scripts in Babylonian tombs from approximately the twenty-third century B.C., while smallpox and tuberculosis were fully described in Chinese manuscripts from approximately A.D. 1000 (Dobson & Carper, 1996).

Like McNeill mentions, “Hippocrates (460-377 B.C.) was probably the first person to record diseases with enough precision for them to be identified today as malaria, mumps, diphtheria, tuberculosis, and perhaps influenza. Interestingly, none of Hippocrates's records indicate the presence of smallpox, measles, or bubonic plague in ancient Greece”. In fact he “suggests that the size of ancient Greek and Egyptian cities may not have been large enough to continuously sustain measles and smallpox infections, and these pathogens may have died out” (McNeill, 1976).

All disease outbreaks, regardless of their cause, were called plagues. Not much was known about their identity or cause, but their association with unpleasant odors gave rise to the popular belief that they were caused by vapors in the air or miasmas. Religious fatalism was another popular explanation (Cunningham, 1992). However, the precursors of microbial discovery can be traced to Fracastoro, a medical doctor, who wrote in 1546 that infection or contagion passes from one to another in very small imperceptible particles which he called "spores" that could transmit infection by direct or indirect contact or even without contact over long distances (Rosati, 2001). In his writing, the "spores" of diseases may refer to chemicals rather than to any living entities. In the 17th century, utilizing hand-made microscopes, Antony Van Leeuwenhoek observed and described Fracastoro’s speculative particles which would eventually be labeled bacteria, protozoa, and yeast (Rosati, 2001). More recently the last major field in infectious disease pathology that must be mentioned is virology. Although this is essentially a 20th century development, the seeds were sewn for the first by Dmitri Ivanovsky in 1892, who in his article described a non- bacterial pathogen infecting tobacco plants (Long, 1962).

3.1.2 History of traditional plants

In search for a cure for their diseases, people looked for drugs in nature. The beginning of the use of medicinal plants were instinctive, as in the case of animals. Several samples of evidence indicates that medicinal plants represent the oldest and most prevalent form of medication (Halberstein, 2005). In view of the fact that at the time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how

it could be utilized as a cure, everything was based on experience. The first records were found in fossils from Mesopotamia about 2600 B.C. This showed that among the substances that were used, the oils of *Cedrus species* (Cedar), *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice),*Commiphora species* (Myrrh) and *Papaver somniferum* (Poppy juice) were the most used for that times, all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Gurib-Fakim, 2006).

In several ancient cultures botanical products were ingested for bio medically curative and physiotherapeutic purposes, as in old Greek for example. The Greeks have made worthy contribution in pharmaceutical sciences, especially in phytopharmaceuticals. Aristotle has described 500 crude drugs used in the cure of different pathological conditions (Chatard, 1908). Hippocrates (460-337 BC) is considered as the father of allopathic medicine. He formulated the first scientific medical paradigm of treatment (Sykiotis, Kalliolias, & Papavassiliou, 2006). He proposed that a large number of pathological conditions were due to disturbance in the normal physiology of human systems. The treatment was, therefore, based on the causes of the diseases to normalize the imbalance body systems. He has pointed out nearly 400 samples of medicinal substances from plant origin. Theophrastus (370-287 BC), a Aristotle's student, has also mentioned 500 crude drugs in his book (Khan, 2014).

In the other hand, Egyptians are credited with developing an elaborate and effective pharmacological curing materials obtained from natural resources. The Egyptians doctors prescribed a serial of sedatives, analgesics, gastrointestinal remedies, and medicines for urinary tract disease and the common cold. Plant extracts were prepared and taken internally, applied topically and administered by fumigation and vapor inhalation. The Egyptians are also accredited with early medicinal use of the wine, castor oil, marijuana, opium, mints, and beer made from barley and wheat. Oakes (2003) point out that "the Egyptians where the first people to use a number of drugs that modern studies have proved would have been medicinally effective". In time, the causes for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants usage gradually abandoned the empirical framework and became based on explicatory facts (Kelly, 2010).

Following those developments, additional discoveries of useful medicinal plants resulted from experimentations in several early historic cultures in China, India, Tibet, North Africa, Central and South America. The herbal specialist was recognized as a powerful and influential character in these communities (Halberstein, 2005). It was not until the 19th century that man began to isolate the active principles of medicinal plants and one particular landmark was the discovery of quinine from Cinchona bark by the French scientists Caventou and Pelletier. Such discoveries led to an interest in plants from the new world and expeditions began to be so popular to the almost impenetrable jungles and forests in the quest for new medicines.

Prior to World War II, a series of natural products isolated from higher plants were well known in the clinical field, and a number are still in use today. However the antibiotic era dawned during and after World War II due to the antibacterial effect of a whole series of natural products isolated from species of *Penicillium, Cephalosporium,* and *Streptomyces*. In the post-war years there were relatively few discoveries of new drugs from higher plants with the notable exception of reserpine from the *Rauwolfia* species that proclaim the age of the tranquilizers and also vinblastine and vincristine from *Catharanthus roseus* which were effective in cancer chemotherapy. Despite these discoveries, the impact of phytochemistry on new drug development decrease and inevitably the innovative pharmaceutical industry turned to synthetic chemicals. (Phillipson, 2001).

Marjorie Murphy states that currently, of the one-quarter to one-half of all pharmaceuticals distributed in the United States having higher-plant origins, very few are intended for use as antimicrobials, since we have relied on bacterial and fungal sources for these activities (Murphy, 1999). According to her, with the arrival of the antibiotics in the 1950s, the use of plant derivatives as antimicrobials has been virtually nonexistent (Murphy, 1999). Nonetheless, now this use is calling the attention of scientists as they notice that the effective life lapse of any antibiotic is limited. In fact, conventional medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics become useless and viral diseases remain intractable to this type of drug. (Murphy, 1999). This interest in plant antimicrobials is also generated by the increasing rate of plant species extinction. There is a feeling among natural-products alike that the multitude of potentially

useful phytochemical structures which could be synthesized chemically. This is at risk of being lost irretrievably (Lewis & Elvin-Lewis, 1995).

The majority of individuals still trust on their traditional method for their everyday healthcare needs. It is also a fact that one quarter of all medical prescriptions are formulations based on substances derived from plants or plants-derived synthetic analogs, and according to the WHO, 80 % of the world's population especially from developing countries, believe on medicinal plants for their healthcare (Gurib-Fakim, 2006). As a result, disease control using natural plant products, including essential oils, holds a good promise which are either safer and do not causes any toxicological effect on the environment.

3.1.3 Antimicrobial effects of plants

All plants containing active compounds are important. The beneficial effects of medicinal plant materials typically are from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites, which are synthesized and deposited in specific parts or in all parts of the plant (CIOCAN & BĂRA, 2007). These compounds are more complex and unique and are found in certain species, genus or family, but heterogeneity of secondary compounds is found in wild species. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct. The plants secondary products may perform their action by resembling endogenous metabolites, ligands, hormones, or neurotransmitters and therefore, have beneficial medical effects on humans due to similarities in their potential target sites. (CIOCAN & BĂRA, 2007).

Moreover, presences of volatile monoterpenes or essential oils in plants provide an important defense strategy to the plants, particularly against herbivorous insect pests and pathogenic fungi. These volatile terpenoids also play a vital role in plant interactions and serve as attractants for pollinators (Batish, Singh, Kohli, & Kaur, 2008).

3.1.4 Uses of traditional plants in the medical area

To mention some examples of the most used higher plants with microbiological agents we have, the Willow tree (*Salix caroliniana*) that is a major component of aspirin, Poppy flower (*Papaver somniferum*) a source of opium, morphine and codeine that is use for relieve pain. Foxglove plant (*Digitalis lanata*) contains digoxin, is a heart medicine used in small doses as a cardiac stimulant. Aloe plant (*Aloe Vera*) source of *aloin* used in dermatologic and other topical medicines especially in cosmetics as an emollient and for the treatment of burns. Cinchona tree (*Cinchona Officinalis*) anti-malarial drug quinine is extracted from the bark used as a tonic, and the Dogbane plant (*Rauwolfia serpentina*) reserpine drug obtained from the bark used in the treatment of hypertension, as an antihypertensive, tranquilizing (Halberstein, 2005). In addition, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinum macrocarpon*) are very well known to treat urinary tract infections, While species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as effective against a large variety of microorganisms. That being said, it has been generally the essential oils of these plants rather than their extracts that have had the greatest use in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal, as well as on the skin (Ríos & Recio, 2005).

3.2 Essential Oils

3.2.1 General Approaches

According to the European Pharmacopoeia 7th edition, Essential Oils are defined as: “Odorant product, generally of a complex composition, obtained from a botanically defined plant raw material, either by driving by steam of water, either by dry distillation or by a suitable mechanical method without heating. An essential oil is usually separated from the aqueous phase by a physical method that does not lead to significant change in its chemical composition”.

Essential oils are volatile, natural, complex compounds characterized by a strong odor extracted from aromatic plant materials. They could be biosynthesized in different plant

organs as secondary metabolites such as, flowers, herbs, leaves, fruits, bark, seeds, wood, rhizome, roots, and be stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes. (Asbahani et al., 2015). Due to the various processes and the multiple parameters implicated, essential oils have lots of compounds with many structures and functional groups. In the table below we can see examples of them found in essential oils (table 1).

Table 1 Examples of compounds found in essential oils (Do, Hadji-Minaglou, Antoniotti, & Fernandez, 2015) .

Compound	**Essential Oil**
Menthol	Mint
Linalool	Lavender, cardamom
Thymol	Thyme
Eugenol	Clove
Carvone	Caraway
α-vertivone, β-vetivone	Vetiver
Benzoic acid	Almond
Cinnamic acid	Cinnamon
Citral	Lemon
Cinnamic aldehyde	Cinnamon
Geranyl acetate	Geranium
Linalyl acetate	Lavender
Limonene	Orange, lemon
Pinene	Geranium, star anise
Caryophyllene	Clove

They could be extracted by different methods. Due to their hydrophobic nature and their density often lower than water, they are generally lipophilic, soluble in organic solvents, and water immiscible. They could be separated from the aqueous phase by decantation. However, their extraction yields vary depending on species and organs. They remain, however, very low (about 1%), which makes them highly valuable rare substances. Among the plant species, only 10% contain essential oils and are called aromatic (Svoboda, 2003), that's why we can

presume such oils are called "essential," away to the flavor and perfume industry they are the *quinta essentia*, as chemists of the middle ages called them (Macginitie, 1961). According to Bruneton they could be found in genres in which are order in a small number of families: *Lamiaceae, Lauraceae, Asteraceae, Rutaceae, Myrtaceae, Poaceae, Cupressaceae* and *Piperaceae* (Bruneton, 1999).

3.2.2 Essential oil secretion

Essential oils are biosynthesized, accumulated and stored in specialized histological structures, the secretory gland confirmed that there are two types of secretory glands: those located on the plant surfaces with exogenous secretion and those located inside the plant in internal organs with endogenous secretion. They are also located in the cytoplasm of some secretory cells in one or more plant organs (Bouwmeester, Davies, & Toxopeus, 1995). We can distinguish different types (table 2).

Table 2 Secretory structures specialized in accumulation and stockage of essential oils. (Asbahani et al., 2015).

Secretory structures	Description	Organ plant	Example	Botanic family
External secretory tissue				
Epidermis papillae	Conical epidermal secretory cells	Flower	*Rosa damascene*	*Rosaceae*
		Petals	*Convallaria majalis*	*Asparagaceae*
Secretory bristles or glandular trichomes	Terminal cells of trhicomes secreting Essential oils	Stem	*Pelargonium sp.*	*Geraniaceae*
		Leaves	*Salvia sp., Mentha sp.*	*Lamiaceae*
Internal secretory tissue				
The *schizogenous* or secretory pocket	Intercellular space filled with the cells secretions	Epicarp of fruit	*Citrus sp.*	*Rutaceae* *Myrtaceae*
Secretory canals	Small canals formed of aggregated secreting cells throughout the plant	Stem	*Petroselinum sp.* *Pimpinella sp.* *Daucus sp.*	*Apiaceae*
Intracellular secretory cells	Cells specialized in the Essential oils accumulation inside their vacuoles	Stem	*Cinnamomum ceylanicum*	*Lauraceae*
		Leaves	*Laurus nobilis*	
		Rhizome	*Acorus calamus*	

3.2.3 Chemical composition

Essential oils are very complex natural mixtures which contains about 20-60 components at pretty different concentrations (Betts, 2001; Pichersky, 2006). Although there are some exceptions, generally the main group is composed of terpenes and terpenoids mostly odorless or with low global odor distribution. On the other hand, in small amounts but not less important are the aromatic and aliphatic constituents, responsible for their aromatic characteristics of the essential oil all characterized by low molecular weight. (Table 3).

Some examples of aromatic compounds:

Aldehyde: *Benzaldehyde, Cinnamaldehyde, Butyraldehyde, Propionaldehyde.*
Acids: *Acetic, Palmitic*
Alcohol: *Linalool, Geraniol, Menthol.*
Phenols: *Anethole, Eugenol*
Esters: *Linalyl acetate, Geranyl acetate*
Ketones: *Thujone*
Other esters, nitrogenous derivatives, sulfides, thioethers, thioesters.

Considering that essential oil as an odorous characteristic product and classifying its composition based on this property, we can say that an essential oil is a mixture of substances consisting mainly of an integrated basis for terpene hydrocarbons. In lower concentration we found (in not too much high number) volatile chemicals that are primarily responsible for the overall odor of essential oil. Finally we have a lot of substances at very low concentration having the characteristic of rounding up the global fragrance (Ortuño, 2006).

Part of the group of substances responsible for rounding and giving the odor profile of the essential oil can vary depending on weather conditions, geographical origin, plant variety, age, etc. establishing significant differences between oils of different components origins. Other essential oils are not related to its fragrance (waxes, acids, etc.) but they can be important for certain applications as they act as preservatives, antibiotics or fixers of the fragrance (Lock de Ugaz, 1994). Moreover are those unintentional components that may be included in the essential oil, depending on the method used or the conditions the plant was.

The terpenes form structurally and functionally different classes. It is originally hydrocarbons found in oil of *turpentine* and were mainly composed of alkenes, so were given the name of terpenes. Later on, it was found that not all were alkene even hydrocarbons, nevertheless they contain aldehydes, ketones, esters, etc. In this sense all terpene derivatives are denoted, functionalized or not, as terpenoids (Guerrero & Nuñez, 1991). All terpenes or terpenoids (which are also called isoprenoids) have the common characteristic of being molecules that can be formed by joining at ones several molecules of isoprene (*2-methyl-1, 3-butadiene*). As isoprene has 5 carbon atoms, all terpenoids present in the molecule a number of carbons multiple of 5 (except for those who may lose a carbon atom by different processes). Thus *limonene*, terpene majority in the essential oil of lemon (with 10 carbon atoms), has a molecular structure that can be formed by 2 molecules of isoprene, talking of a monoterpene in terms of its classification.(Bakkali, Averbeck, Averbeck, & Idaomar, 2008).

The principal terpenes are the monoterpenes (C_{10}) and sesquiterpenes (C_{15}), but hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (C_{40}) also exist and can be found. The monoterpenes are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures. (Bakkali et al., 2008).

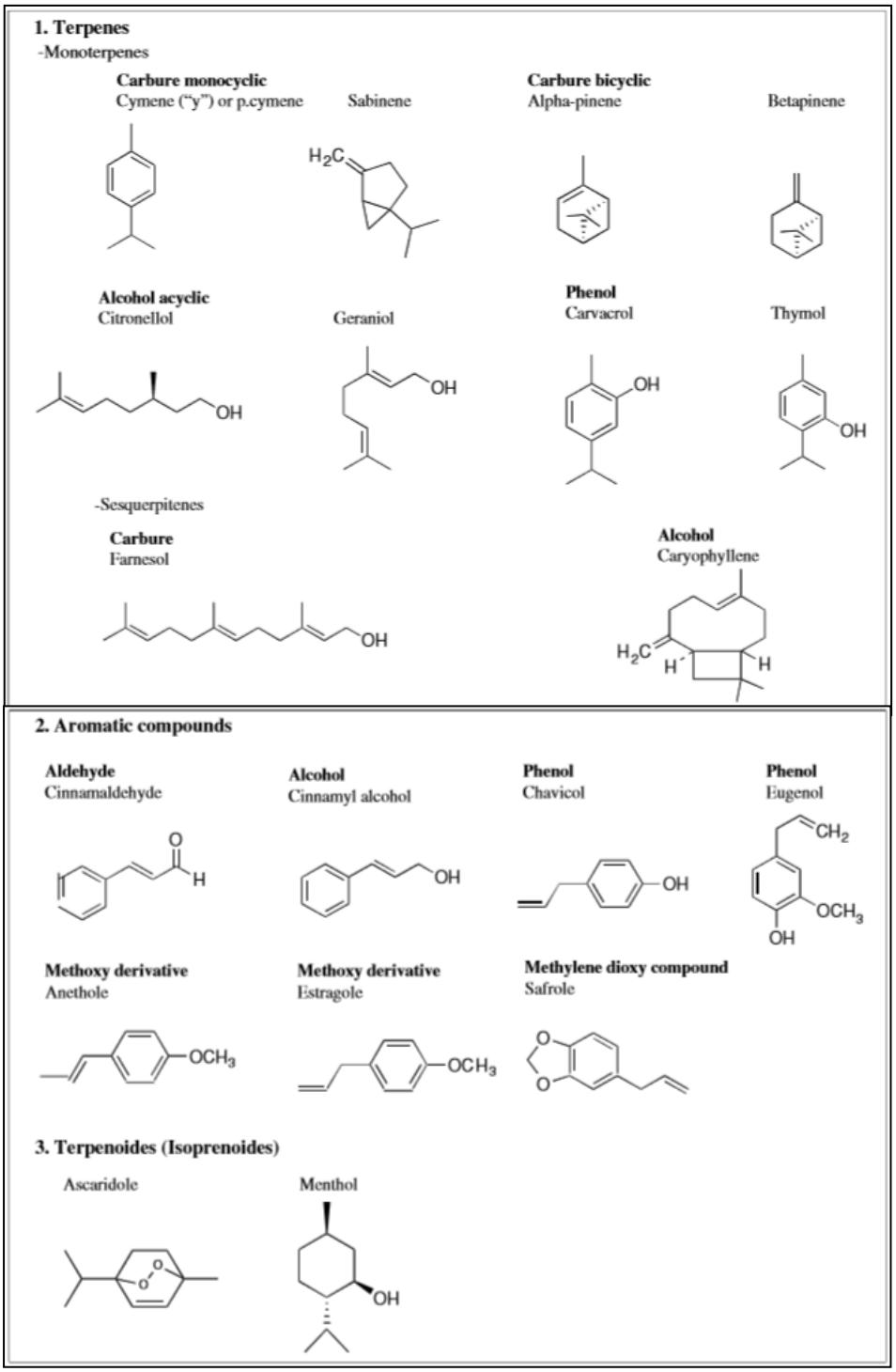

Figure 1 Chemical structures of selected components of essential oils. (Bakkali et al., 2008)

3.2.4 Uses

Essential oils are natural materials widely used in many fields all over the world and have become an integral part of everyday life. They have been used since antique in flavor and fragrance, as condiments or spices and to repeal insects or protect stored products.

Medical Field:

In the medical field they are used thanks to their biocidal activity and medicinal properties. Furthermore, they could also provide a pleasant feeling of psychic comfort for patients given their pleasant aroma (Burt, 2004; Tiwari et al., 2009).

Pharmaceutical Industry:

In the pharmaceutical field, they are included in the composition of many dosage forms like capsules, ointments, creams, syrups, suppositories, aerosols and sprays. (Asbahani et al., 2015).

Food Industry:

Food industry also presents a growing demand for essential oils because of their important applications as food preservatives (Burt, 2004), innovation in food packaging and the fight against pathogens generating dangerous food poisoning. Numerous studies have shown the efficiency of this oils in low doses in the fight against bacterial pathogens encountered in food industry and meat product (Oussalah, Caillet, Saucier, & Lacroix, 2007).

Likely, there was an increased public concern about the use of antibiotics in livestock feed because the emergence of antibiotic resistant bacteria and their possible transmission from livestock's to humans. In fact, in the European Union, use of synthetic antibiotics, health and growth promoters as additives in livestock feed has been prohibited since 2006 (Castanon, 2007). In this context, essential oils were shown to be an interesting alternative because of their well-known and well documented antimicrobial activity. Essential oils contain

components with biocide and antiviral properties that can be used as substitutes of synthetic drugs in livestock (Varona, Kareth, Martín, & Cocero, 2010).

Cosmetics Industry:

This industry employs essential oils in the production of cosmetics, soaps, cologne, perfume and makeup.

Industrial deodorizers:

In recent times has been increased the consumption of essences to suppress the unpleasant odor of some industrial products such as rubber, plastics and paints. The paint industry used lemon as biodegradable solvent. The same technique is used for toys. In textile, odor concealer in mordant treatments before and after dyeing. In stationery, fragrances to impregnate notebooks, cards, toilet paper, facial tissues, etc.

Biocides and insecticides:

Essential oils possess a wide range of desirable pest management. They provide an important defense strategy to the plants, particularly against herbivorous insect pest and pathogenic fungi. The interests in essential oils for this industry primarily are due to their fumigant and contact insecticidal activities, the less stringent regulatory approval mechanism of exploitation, safer and absence of toxicological effect on the environment as synthetic pesticides (Barton, 1999).

3.2.5 Essential oils extraction methods

3.2.5.1 Conventional and classic methods

These are many methods based on water distillation by heating to recover the essential oil from plant.

Hydro-distillation:

The most simple and old method. It consists in putting the material plant (dried and miller) immersed directly in the water inside a distillation bowl and boiling it. At atmospheric pressure and during boiling process, water and essential oil molecules form a heterogeneous mixture which reach its boiling temperature at a lower point close to 100°C while for essential oils components is very high. The mixture then is distilled simultaneously as if they were one compound. The benefit of the water is that it is immiscible with the majority of the terpenes molecules of the essential oils and thus, after condensation, it could be easily separated from water by simple decantation.

Steam-distillation:

In the steam distillation process, it's carried out the selective steam of the volatile compound of a mixture formed by esters and others "no volatiles". This is achieved by injecting steam occurring outside of the distillation bowl which pass directly to the plant matter, being called the "stripping steam", but in fact its function is not to drag the volatile component, its function is to condense to form another immiscible phase that will transfer its latent heat to the mixture to be distilled to achieve its evaporation. In this case there would be the presence of two immiscible phases along distillation (organic and aqueous), therefore each liquid will behave as if the other was not present. That is to say, each one produce its own vapor pressure and corresponds to the pure liquid to a reference temperature (Wankat, 2006). The most important condition for such distillation can be applied, if both, the volatile component and an impurity of water could be soluble in water, because distillate product (volatile) produce two phases upon condensation, allowing separation of the product and water easily. The total system

pressure is the sum of the vapor pressures of the components of the organic mixture and water. However, the mixture to be distilled is a hydrocarbon with some oil, being oil vapor pressure considered negligible as it is very small. “The disadvantage with this methods are that the properties of original plant material are subjected to the combined effects of heat and acid, and are subject to chemical modifications (hydrolysis, hydrations, cyclization and deprotonations). This essential oil obtained differs significantly from the original essence, especially if boiling is long and pH is low” (Faborode & Favier, 1996).

Organic solvent extraction:

The plant (dried and milled sample) is macerated in an organic solvent, then the extract is concentrated by filtering and removing the solvent under reduced pressure. This technique avoids alterations and chemical manipulation by cold extraction. The remaining solvent must be separated at low temperature but not always is easy and 100% removed (Ortuño, 2006). The disadvantage of this method is that the extracts obtained by organic solvents contain polluted residues that could contaminate foods and fragrance to which they are added.

3.2.5.2 Innovative essential oils extraction methods

The handicap of conventional techniques is related with the thermolability of essential oils components which suffer chemical alterations due to the high applied temperatures. The quality of extracted oil is consequently extremely damaged particularly if the extraction time is long. It is important that extraction methods could maintain the chemical composition and natural proportion at its original state. That’s why new extraction techniques are continuously trying to reduce extraction times, energy consumption, solvent use and CO_2 emissions (Asbahani et al., 2015).

Supercritical fluid extraction (SCFE):

Supercritical fluid extraction is a unit operation which exploits the solvent power of supercritical fluids in conditions above its critical temperature and pressure. Free extracts can

be obtained using supercritical fluid solvent and the extraction is faster than with conventional extraction organic solvents. These advantages are due to the high volatility of supercritical fluids (gases under normal environmental conditions) and upgraded transport properties (high diffusivity and low viscosity). Using carbon dioxide in particular treatment is moderate temperature and can achieve high selectivity of valuable micro-components in natural products (Del Valle & Aguilera, 1999). CO_2 selectivity is also suitable for the extraction of essential oils. The extraction efficiency improves significantly when the sample is pretreated with compressed CO2, decreasing the amount of waxes in the extract. In this case CO_2 is the most widely solvent used for essential oils extraction. (Herrero, Cifuentes, & Ibañez, 2006).

Some others modern's techniques are used for extracting methods, the most mentioned are:

Ultrasound assisted extraction of essential oils (UAE):

It is applied as an alternative extraction or to assist in extraction processes volatile plant components, including essential oils. The compositional ratio of the extracts and the performance of these depends on the temperature at which reactions can take place and the process solvent or solvent mixtures used (Filgueiras, Capelo, Lavilla, & Bendicho, 2000).

Microwave assisted extraction (MAE):

This method offers benefits such as a considerable reduction of time and energy consumption.

Microwave hydro diffusion and gravity (MHG):

Propose that the mass transfer and heat during the process occurs in the same direction, from inside the material outward. This allows the essential oil and water in situ of the material, to be extracted (hydro diffusion) and are separated by gravity (Bousbia et al., 2009).

Microwave steam distillation (MSD) and Microwave steam diffusion (MSDf):

MSD is more used for the extraction of respectively, orange peel essential oils and dry Lavender flower. This innovative method prove to be more effective offering important

advantages like better sensory properties (better reproduction of natural fresh fruit aroma of the citrus essential oil) without causing considerable changes in the volatile oil composition.
MSDf It is based on the same principle as for the MSD except that vapors flow through the plant material down. This method proved to be more efficient in terms of kinetic of extraction, energy saving and cleanliness, quality of the extracts and waste water reduction. Microwave steam diffusion is a green, cleaner, environmentally friendly and an economic procedure (Sahraoui, Vian, Bornard, Boutekedjiret, & Chemat, 2008).
The only general disadvantage of all this Innovative essential oils extraction methods are development, the high cost of the equipment's, their installations and their maintenance operations.

3.3 Characteristics of *E.deglupta*

E.deglupta is the only species of Eucalyptus that is found in the northern hemisphere, its natural habitat.

3.3.1 Taxonomy description of *E.deglupta*

Commonly known as the rainbow eucalyptus, Mindanao gum, or rainbow gum. The generic name comes from the ancient Greek *eu* = "good, fairly" and *kalyptos* = "covered, covering".

Figure 2: *E.deglupta*, Source: Ing. Pavel Nový.

Scientific classification of following taxonomical way:

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Myrtales*
Family: *Myrtaceae*
Genus: *Eucalyptus*
Species: *E.deglupta*

3.3.2 Origin and distribution

E.deglupta has a natural distribution from Sulawesi (Indonesia) and Mindanao (Philippines) eastward to New Britain (Papua New Guinea). It is one of the few Eucalyptus species not occurring in Australia. It is widely planted throughout the humid tropics, where it is one of the most important eucalypts.

3.3.3 Botanical description

E.deglupta is a huge evergreen tree of up to 60 and approx. to a maximum of 75 meters tall, bole generally of good form, 50-70% of the tree height, up to 240 cm in diameter, sometimes with buttresses 3-4 m high; bark smooth, yellow, brown, and purple, but green after flaking, that's why it's called "rainbow tree" because of its beautiful cortex of various colors; twigs 4-sided, often with 4 longitudinal wings. Juvenile leaves opposite (Figure 2), ovate to lanceolate; adult leaves opposite to subopposite, rarely alternate, shortly petiolate, held almost horizontal on branches, ovate to ovate-lanceolate or acuminate, thicker than juvenile leaves, 7.5-15 max. 20 cm x 5-7.5 max. 10 cm (Orwa, Muta, Kindt, Jamnadass, & Simons, 2009).

Figure 3: Young leaves of *E.deglupta* by Forest & Kim Starr.
https://en.wikipedia.org/wiki/Eucalyptus_deglupta

The flowers 3-7 umbels in terminal or axillary panicles 5-20 x 5-18 cm; pedicels terete or slightly angular, about 5 mm long; young buds small, green with double opercula; developed buds pale green or cream, globular, apiculate, 0.2-0.4 x 0.2-0.5 mm, operculum hemispherical, apiculate and wider than long; flowers with many white to pale yellow stamens 2-10 mm long (Figure 3), strongly reflexed in the unopened bud; anther dehiscing by separate slits. Fruit pedicellate, hemispherical, with 3-4 valves, thin, deltoid, exserted to 2 mm, making the capsule appear globular, 3-5 x 3-5 mm (figure 4), and disc very narrow; mature fruits brown to dark brown, containing 3-12 well-formed seeds per valve; seeds minute, brown, flattened, with a small terminal wing (Eldridge & Davidson, 1993).

Figure 4: *E.deglupta* flowers, Hawai by Forest & Kim Starr.
https://en.wikipedia.org/wiki/Eucalyptus_deglupta

Figure 5: *E.deglupta* (capsules forming) Hawai by Forest & Kim Starr.
https://en.wikipedia.org/wiki/Eucalyptus_deglupta

3.3.4 Habitat and growth

E.deglupta requires full overhead light for development, and dense stands are commonly found along rivers where it has colonized newly formed trees and non-stagnant river flats. It is also found on sites that have been cleared or disturbed in some way, for example, by landslides, volcanic eruptions, or shifting cultivation. *E.deglupta* generally reproduces in pure stands. Occasionally, however, it forms an association with *Octomeles sumatrana*, an

aggressive secondary species. As stands pass maturity, they are invaded by primary forest species such as *Pometia pinnata, Dracontomelum mangiferum, Celtis spp.,* and *Pterocarpus indicus* (Orwa et al., 2009).

E.deglupta is the only species of Eucalyptus that is adapted to lowland and lower montane rainforest habitats. It does not grow naturally in areas with a pronounced dry season but occurs in those where the annual rainfall is very high and the monthly rainfall usually exceeds 150 mm. Because of this, it is widely planted throughout the wet tropics. *E.deglupta* does not withstand prolonged flooding, is highly sensitive to fires and, although it may grow in cool environments, it does not tolerate frost (Jacobs, 1981).

3.3.5 Chemical composition

A study of *E.deglupta* leaves collected in Nigeria reported that the essential oil leaves gave a spicy volatile oil 0.2% yield. The dominant constituents were α -pinene (24.7%), *β-pinene* (1.8%), *p-cymene* (3.4%), *limonene* (4.1%), *α-terpineol* (4.1%), *β-caryophyllene* (5.9%), *aromadendrene* (1.2%) and *E-nerolidol* (34.8%). The essential oil of the plant was characterized by a high amount of sesquiterpenoids (48%) in which *E-nerolidol* dominated (Oyedeji et al., 1999). Another studied was made of fresh leaves from DR Congo yielded 0.15% of essential oil, with as main compounds 1,8-*cineole* (35.7%), *cryptone* (25.4%), *myrtenol* (7.4%), *β-Phellandrene* (7,2%), *β-terpineol* (6.3%) and *globulol* (3.1%) (Cimanga et al., 2002).

Difference in yields of both studies could be attributed to some factors such as climate, nature of the soil, age of the tree, time of collection, mode of extraction, etc. These data shows that the essential oil of the same plant but different location can vary in specific qualitative and quantitative composition (Cimanga et al., 2002) .

3.3.6 Antimicrobial Activity of Essential oil from *E.deglupta*

According to the available literature, the *E.deglupta* plant species have some compounds with antimicrobial properties as shown in a study report from essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. As they mention *E.deglupta* oil were active against 13 bacteria (59%) out of 22 (table 4).

Table 3 Antimicrobial activity of essential oils from some plant species (Cimanga et al., 2002).

Microorganisms	Essential oils from some plant species (zone diameter of inhibition in mm)			
	I	II	III	IV
B. subtilis 1	18	22	15	14
B. subtilis 2	16	20	-	12
Citrobacter sp. 1	15	12	16	12
Citrobacter sp. 2	16	18	14	22
C. diversus 1	12	20	12	-
C. diversus 3	13	22	-	9
E.coli 1	-	12	8	16
E.coli 2	9	10	14	-
K. oxytoca 1	16	15	14	12
K. oxytoca 2	15	12	17	12
K. pneumoniae 2	18	10	16	16
K. pneumoniae 3	15	16	15	10
P. mirabilis 2	-	-	-	-
P. mirabilis 3	-	-	-	-
P. vulgaris 2	24	27	14	-
P. vulgaris 3	24	26	-	-
P.aeruginosa 1	8	16	-	-
P.aeruginosa 3	-	15	8	8
S.aureus 2	12	30	14	14
S.aureus 3	-	18	-	-
S. typhimurium 1	-	10	13	13
S. typhimurium 2	14	-	12	12
S. flexneri 2	-	14	15	15
S. flexneri 3	-	15	15	15

I. *Eucalyptus alba;* II. *Eucalyptus camadulensis;* III. *Eucalyptus citridora;* IV. *E.deglupta*

4 Materials and Methods

4.1 Plant Material

The leaves of *E.deglupta* were used in this study as plant material. They were collected and identified by Dr. Pavel Nový on the Savai´i Island of the Independent State of Samoa near the Gataivai village on September 22, 2015. The leaves were dried in a shade and stored at a dry place till the extraction. The voucher specimen is deposited at the Department of Crop Sciences and Agroforestry, Faculty of Tropical Agrisciences, Czech University of Life Sciences Prague.

The Independent State of Samoa, is a sovereign state in Polynesia, encompassing the western part of the Samoan islands in the South Pacific Ocean, which is the reason of its tropical climate and immense biodiversity. The specimen were gathered on September 22 of 2015, with an average temperature for that day of 28 °C (82.4 °F), wind of 13 km/h E, humidity of 59% and air pressure of 1015 hPa. The climate is tropical, with a rainy and hot season from November to April and a cool and dry season from May to October. Rainfall in August 2015 for Samoa was below average to well below average. Drier than normal conditions were mainly experienced in the northern parts of the islands. Rainfall deficiency was justified and indicated by the dry conditions of certain weeds and grasses observed along the island of Savaii. The island group is frequently hit by tropical cyclones between December and March, due to its positioning in the South Pacific Ocean.

4.2 Essential oil extraction

49.63 g of dried plant material from *E.deglupta* was obtained after finely milling the leaves using Grindomix GM 100 (Retsch, Germany). The material was then mixed with distillated water in a spherical glass vessel. Extraction was performed using a Clevenger-type *apparatus* (Figure 5), for 3 hours. The extraction yielded 223 μL of the essential oil. The oil was separated by decantation, dried with anhydrous sodium sulfate and stored in a glass vial at 4 °C.

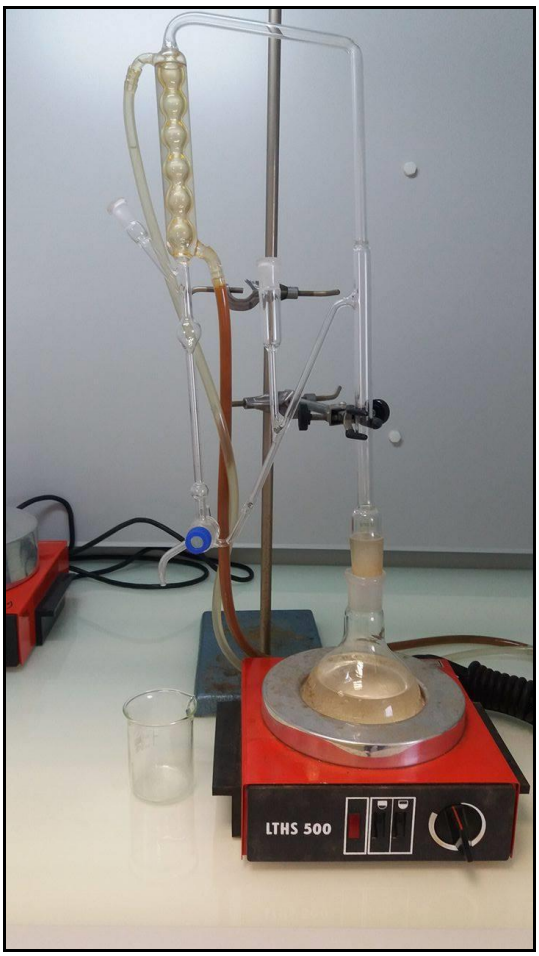

Figure 6: Clevenger-type apparatus. Source: Sumar, R., 2016.

4.3 Chemical Analysis using GC-MS

The essential oil was analyzed on a gas chromatographer Agilent 7890A coupled with Agilent MSD5975C single quadrupole mass detector (both from Agilent, Santa Clara, FL, USA). The GC was equipped with nonpolar column HP-5MS (30 m x 250 µm x 0.25 µm) from Agilent (Santa Clara, FL, USA). Essential oil was diluted in GC grade n-hexane in the ratio 5:1000.
1 µL of sample was injected into the injector heated to 250°C in the split ratio of 12:1. Helium was used as a carrier gas with flow rate 1 mL/min. GC temperature program set to initial 60°C, held constant for 3 min, then increase at rate 3°C/min up to 231°C and held 10 min. Total time of the analysis was 70 min. The ionization energy was set to 70 eV and the data

was acquired at full scan mode. The identification was based on the comparison of mass spectra and retention times with the National Institute of Standards and Technology Library (NIST, USA) as well as with the literature (Adams, 2007). The content of individual compounds was expressed as relative percentage according to peak areas.

4.4 Microorganisms

Antimicrobial activity was evaluated for 8 bacterial strains of the American Type Culture Collection (ATCC) stored in the laboratory of the faculty Tropical Agri-Science of Czech University Life Science Prague and obtained from (Oxoid, Basingstoke, United Kingdom). Bacterial strains were selected as representatives of both classes, four gram-positive and four gram-negative bacteria.

Gram –positive bacteria strains:

- *Bacillus cereus* (ATCC 11778)
- *Enterococcus faecalis* (ATCC 29212)
- *Staphylococcus epidermidis* (ATCC 12228)
- *Staphylococcus aureus* (ATCC 29213)

Gram-negative bacteria strains:

- *Escherichia coli* (ATCC 25922)
- *Klebsiella pneumoniae* (ATCC 700603)
- *Pseudomonas aeruginosa* (ATCC 27853)
- *Salmonella enteritidis* (ATCC 13076)

4.4.1 Cultivation media and Inoculum preparation

Microorganisms were grown on (MHB) Mueller-Hinton broth with TBS (Tris-buffered saline) and inoculated with the corresponding strains and refrigerated at 4°C.

Media

- Medium for inoculum preparation (pure MH broth)
- Medium for the antimicrobial assay (MH broth supplemented with Tris Buffered Saline)
- For *E.faecalis* we prepare special medium MH broth supplemented with Tris Buffered Saline) enriched with 1% of glucose.

Inoculum

- Overnight culture (in MH, incubated overnight at 35+/-2°C)
- Inoculum standardization (adjustment of the bacterial suspension to the density to 0,5 McFarland)
- Inoculation using a multi-blot inoculator to get final bacterial concentration of approx. 5 x 10^5 CFU in the inoculated wells.

The control of tested bacteria's was checked with the following antibiotic: T*etracycline* (Sigma-Aldrich, Prague, Czech Republic).

4.5 Antimicrobial Assay

In vitro antimicrobial activity of essential oil of *E.deglupta* was examined by the broth microdilution method using 96-well microtiter plates (CLSI, 2012). Ten two-fold serial dilutions of essential oil were prepared for each strain. Its concentrations ranged from 0.004 to 2.048 µl/ml. The microtiter plates were incubated at 35°C for approximately 24 hours with a final volume of each well of 100 µl. Finally, the samples were checked for the minimum inhibitory concentrations (MICs). The growth of microorganisms was determined with

Multiscan Ascent Microplate Reader (Thermo Fisher Scientific, Waltham, USA) at 405 nm which observed the turbidity in the sample and calculated based on the density of the growth control. All samples were tested in triplicate in three independent experiments. Results were expressed as the lowest concentrations that resulted in >= 80% reduction in bacterial growth compared to the oil-free growth control.

Figure 7: Densitometer. Source: Sumar, R., 2016.

5 Results

The yield of the *E.deglupta* essential oil extracted by hydrodistillation was 0.4 (% w/w). It was a yellowish oil, still liquid when refrigerated (4°C). Total of 24 components were identified in the oil using GC-MS (Table 5). The antimicrobial assays showed no significant activity of the oil at the concentrations tested (Table 4).

5.1 Antimicrobial Assay

Essential oil from *E.deglupta* leaves showed no antimicrobial activity against eight microorganisms representatives of both classes, four gram-positive and four gram-negative bacteria at concentration ranging from 0.004 to 2.048 µl/ml (Table 4), according to the lowest concentrations that resulted in > 80% reduction in bacterial growth compared to the oil-free growth control. For the antibiotic *(Tetracycline*) control, results showed an antimicrobial activity against the eight microorganisms ranging from 0.125 to 64 µg/ml showing to be more effective in gram-positive bacteria, specifically the *B.cereus* who showed that was the most sensitive bacteria against the antibiotic with a minimum inhibitory concentration of 0.5 µg/ml (Table 4). The detailed percentage inhibitory effect of *E.deglupta* essential oil against gram-positive and gram-negative is presented in Figure 9 and Figure 10, respectively. *B.cereus* was the most sensitive species showing 50% inhibition at concentration 2.048 µl/ml (Table 4; Figure 9).

Table 4 Antibacterial activity of *E.deglupta* essential oil

Minimum Inhibitory Concentration				
Bacteria		***E.deglupta*** **MIC (µl/mL)**	***E.deglupta*** **IC50 (µl/mL)**	**ATB (*Tetracycline*) (µg/mL)**
Gram Positive Bacteria	*B.cereus*	-	2.048	0.5
	E.faecalis	-	-	8
	S.aureus	-	-	1
	S.epidermidis	-	-	16
Gram Negative Bacteria	*E.coli*	-	-	8
	K.pneumoniae	-	-	16
	S.enteritidis	-	-	4
	P.aeruginosa	-	-	4
-, not active (>2.048 µl/ml).				

5.2 GC-MS Analysis

The GC-MS revealed total of 24 identified compounds in the *E.deglupta* essential oil which represent 89.87 % of the total oil composition. The main major components were identified as (E)-Nerolidol (61.92 %) followed by α-pinene (11.02 %). Detailed chemical composition is listed in (Table 5) and typical chromatogram is shown in Figure 6.

Table 5 Chemical composition of *E.deglupta* essential oil

RI[a]	**Component**	**Content (%)**[b]
938	α-Pinene	11.02
952	Camphene	0.29
979	β-Pinene	0.73
989	Methyl heptenone	1.22
1032	D-Limonene	1.31
1114	Fenchol	0.23
1141	L-pinocarveol	0.25
1167	Borneol	0.36
1191	α-Terpineol	1.25
1287	Bornyl acetate	0.27
1377	α-Copaene	0.25
1419	β-Caryophyllene	0.89
1509	β-Bisabolene	1.47
1513	γ-Cadinene	0.34
1524	δ-Cadinene	0.92
1533	Cadine-1,4-diene	0.34
1570	(E)-Nerolidol	61.92
1584	Globulol	0.93
1599	(Z,E)- α-Bergamotol	0.35
1629	τ-Muurolol	1.84
1642	Cubenol	0.36
1684	α-Bisabolol	1.20
1686	Levomenol	1.78
1723	Farnesol	0.66
	Total	**89.87**

[a] RI = retention indices relative to n-alkanes on HP-5MS capillary column (30m× 0.25 mm, 0.25 μm)

[b] peak area relative to total peak area in %

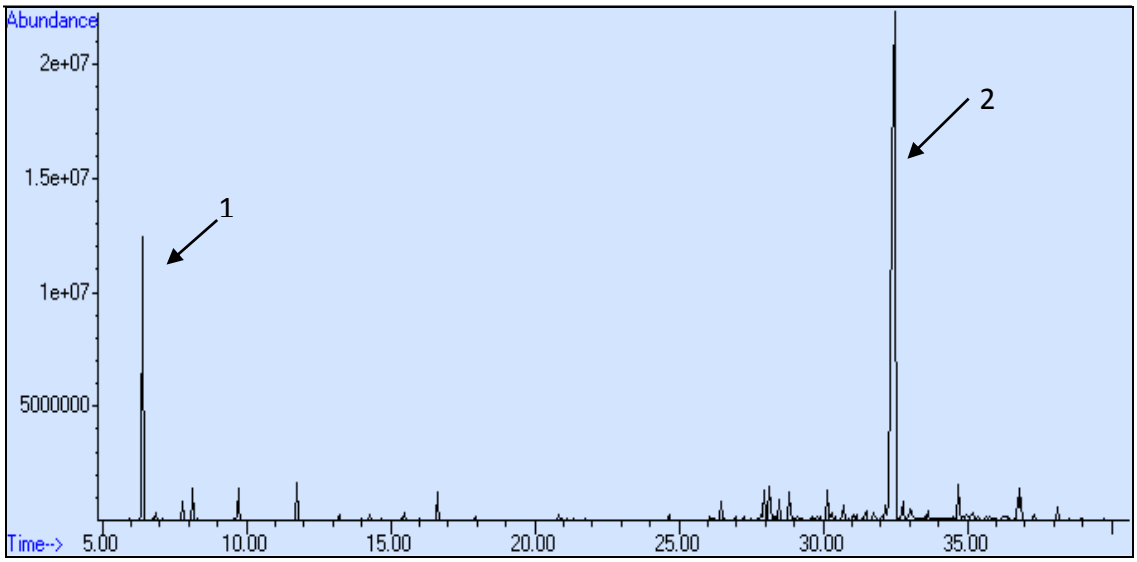

Figure 8: GC-MS chromatogram of *E.deglupta* essential oil. 1*: α-Pinene* (11.02 %); 2: *(E)-Nerolidol* (61.92 %)

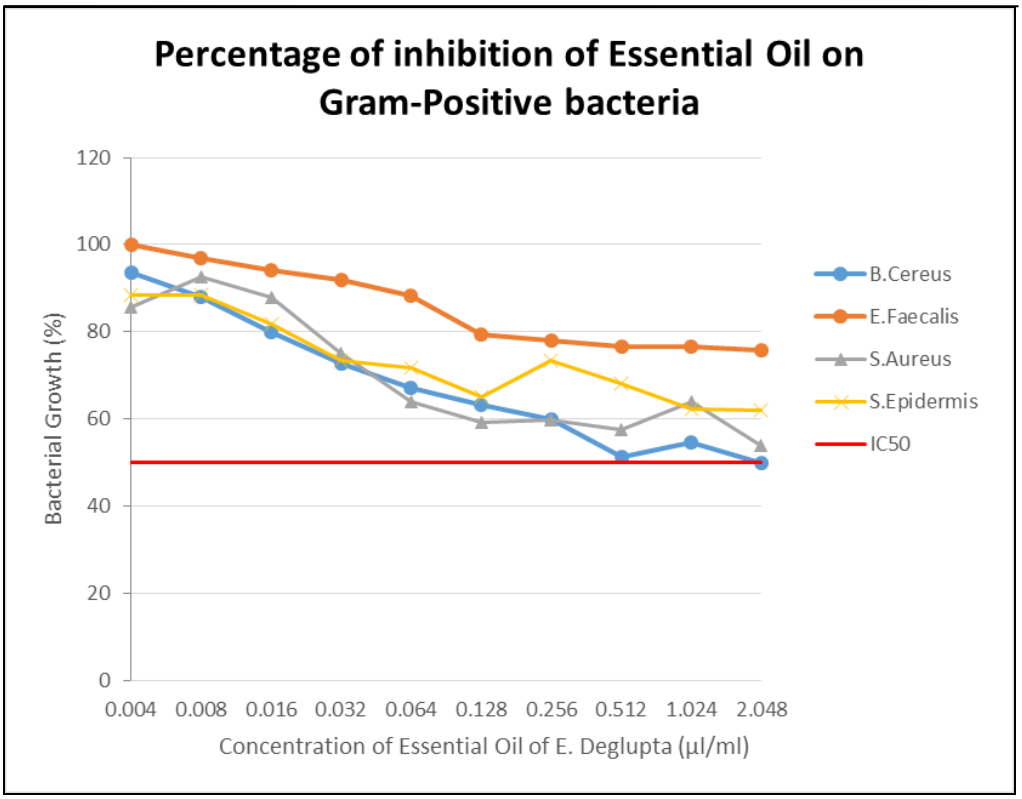

Figure 9 Percentage of inhibition of Essential oil from *E.deglupta* leaves on gram-positive bacteria (Source: Sumar, R., 2016)

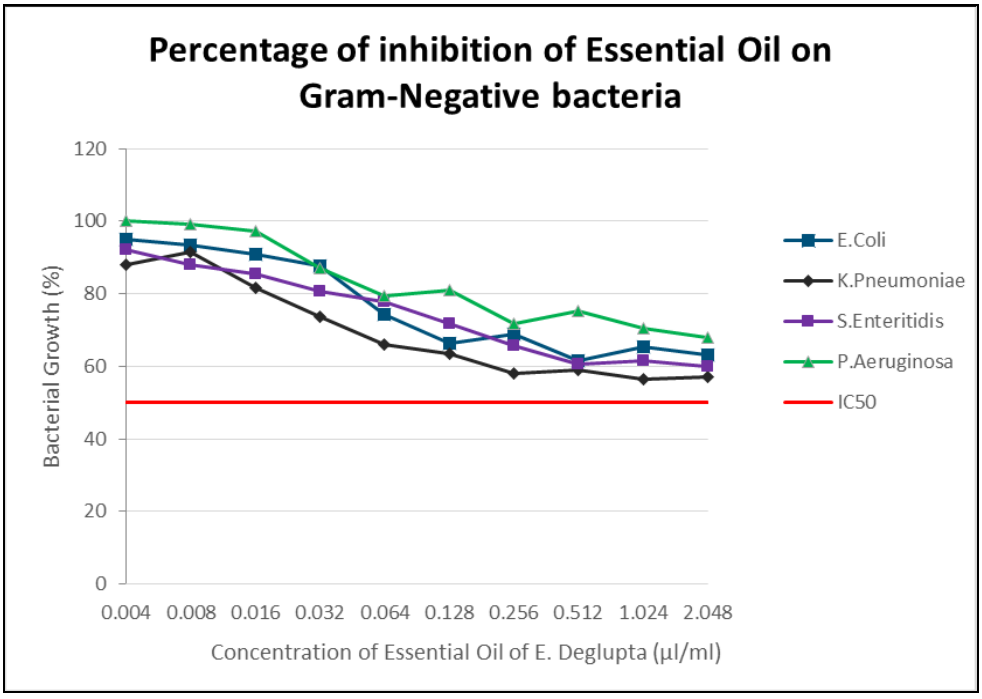

Figure 10 Percentage of inhibition of Essential oil from *E.deglupta* leaves on gram-negative bacteria. (Source: Sumar, R., 2016)

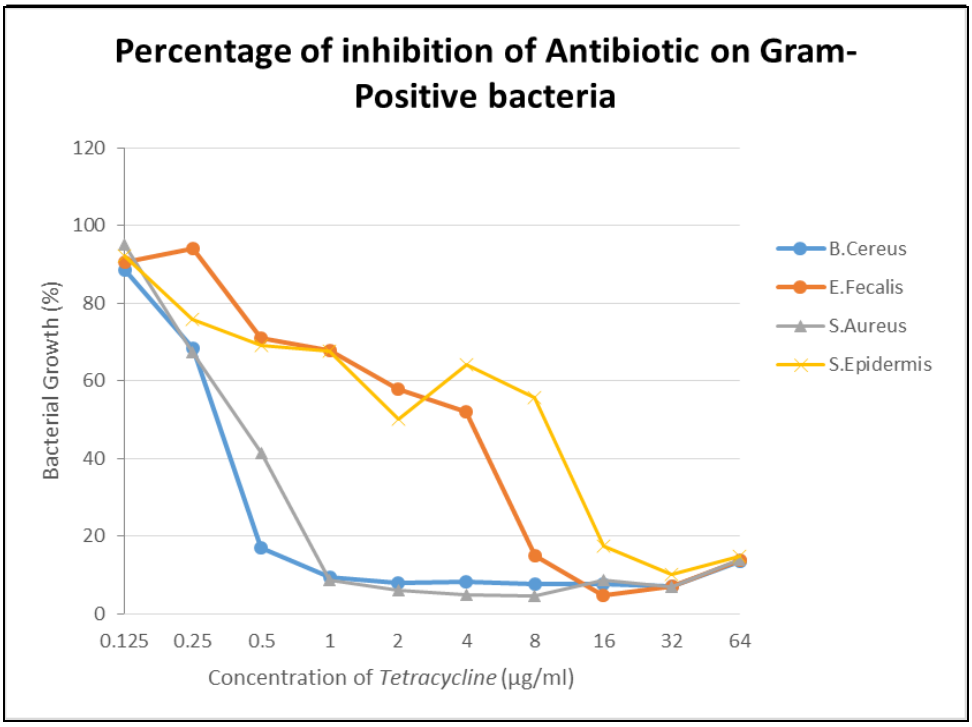

Figure 11 Percentage of inhibition of Antibiotic (Tetracycline) on gram-positive bacteria. (Source: Sumar, R., 2016)

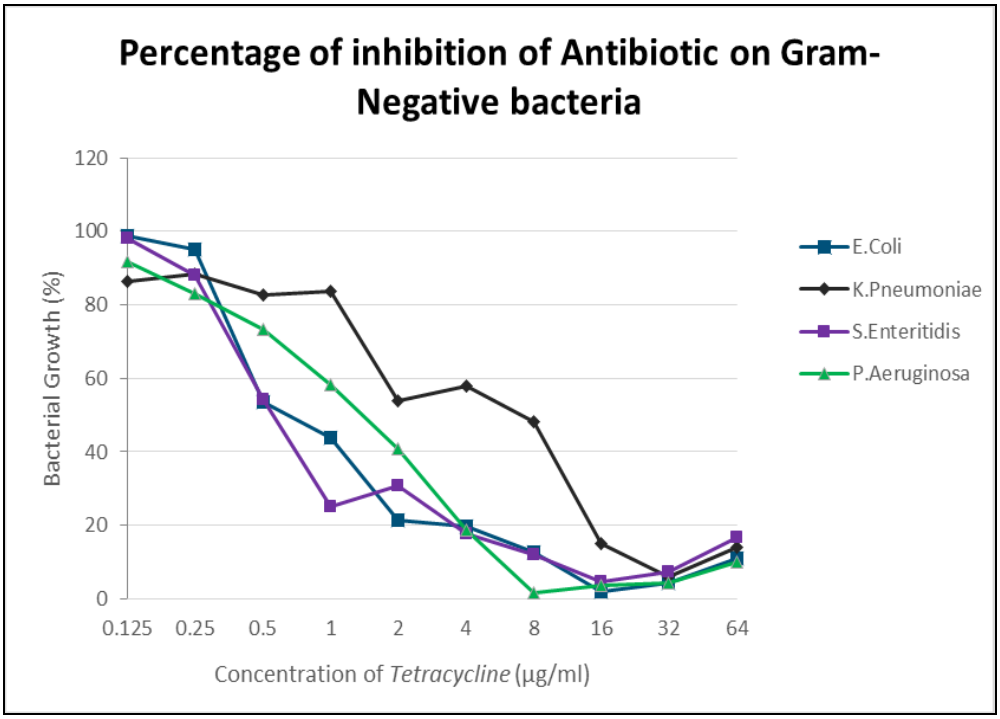

Figure 12 Percentage of inhibition of Antibiotic (Tetracycline) on gram-negative bacteria. (Source: Sumar, R., 2016)

6 Discussion

Although the results showed that there were no antimicrobial activity expressed MIC, there were a slight inhibition activity from the essential oil, being more effective against gram-positive bacteria, whereas *B.cereus* was the most sensitive one, showing up to 50% inhibition at highest concentration of 2.048 µl/ml (Figure 9). The most resistant gram-positive bacteria was *Enterococcus faecalis* with only 24 % of inhibition in the highest concentration 2.048 µl/ml (Figure 7). In the case of the gram-negative bacteria the more sensitive to the essential oil was the *K.pneumoniae* with a 43% of inhibition in the highest concentration 2.048 µl/ml (Figure 8). The most resistant gram-negative bacteria was the *P.auriginosa* with 32% of inhibition with the highest concentration 2.048 µl/ml (Figure 8).

In accordance to Cimanga et al. (2002), essential oil of *E.deglupta* leaves are mostly compound of 1,8-*cineole* > 30% that is a good indicator for antimicrobial activity. In our results, compound of the 1,8-*cineole* was absent. 1,8- *cineole* or also called *Eucalyptol*, is considered to be the most quantitatively important constituent of commercial Eucalyptus oils, due to its medicinal property. The main major components were *trans-nerolidol* (61.92 %) and *α-pinene* (11,02 %). Oyedeji et al. (1999) in accordance with our results, showed in his investigation that their main constituents of the oil were α-*pinene* (24.7%), *β-pinene* (1.8%), *ρ-cymene* (3.4%), *limonene* (4.1%), *α-terpineol* (4.1%), *β-caryophyllene* (5.9%), *aromadendrene* (1.2%) and *E-nerolidol* (34.8%). The essential oil of the plant was characterized by a high amount of *sesquiterpenoids* (48%) in which *E-nerolidol* dominated. The non-detection of 1,8-*cineole* is significant. In contrast, Cimanga et al. (2002) showed that the oil main constituents were 1,8-*cineole* >30% , *α-pinene* >5% and *cryptone* >20%. According to Janssen et al. (1987). , the composition of the essential oil influence its antimicrobial activity in the following factors; the botanical source, the provenience of the plant material, the time of harvesting (time of the day, stage of the development), the plant material used (fresh or dried), the isolation technique, the age of the plant, the quantity of the oil used for the test, etc. These are some factors that are implicated in the great variation of the activity of the essential oil.

There are several studies that have been aimed to encourage and to create options that improve the quality of life or make people life easier and even more, to exploit natural wealth and in some native countries support the idea of creating new natural alternatives treatment to the conventional. *Eucalyptus,* belongs to *Myrtaceae* family and more than 300 species of this genus contain volatile oil in their leaves. Less than 20 of these have ever been exploited commercially for the production of essential oils rich in 1,8-*cineole* (more than 70%) by pharmaceutical and cosmetic industries (Pino, Marbot, Quert, & García, 2002). Leaf extracts of *Eucalyptus* have also been approved as food additives (Takahashi, Kokubo, & Sakaino, 2004). In fact, for many years, essential oils have involved interest as a source of natural products and also are considered in the medical field were they are used thanks to their biocidal activity and medicinal properties (Burt, 2004; Tiwari et al., 2009).

To our knowledge, there are few reports about antimicrobial and antifungal activity of *E.deglupta*. Cimanga et al. (2002) reports, showed positive results that indicates that the oil has antimicrobial activity against 13 out of 22 microorganisms tested from essential oil of *E.deglupta* leaves, that were evaluated by the diffusion- agar method. The microorganisms were clinical isolates from different pathologic medium from patients diagnosed as having various infections. There were four out of eight microorganisms evaluated in the current study, *E.coli* 1 (feces) exhibited a 16 mm zdi and *E.coli* 2 (urine) was inactive. *K.pneumoniae* 2 (abces) showed 16 mm zdi and *K.pneumoniae* 3 (urine) reported 10 mm zdi. *P.aeruginosa* 1 (abces) was inactive and *P.aeruginosa* 3 (urine) exhibited 8 mm zdi. *S.aureus* 2 (abces) showed 14 mm zdi while *S.aureus* 3 (abces) was inactive. Results from this study are partially in accordance with our results, showing resistant against almost half of the microorganisms tested.

Previous studies used different methods for analysis in their researches (Cimanga et al., 2002; O. Oyedeji, Ekundayo, Olawore, Adeniyi, & Koenig, 1999) that can cause higher value of MICs. They used agar-diffusion method which is according to Cos et al. (2006) not suitable for testing non-polar samples because of solubility, volatility and diffusion characteristics in agar. Also the method of extracting the oil from the leaves might cause difference in the yield and oil compounds. In our study we use the broth microdilution method because it allows to

determine MIC a large number of bacteria to be tested relatively quickly. Also because *E.deglupta* essential oil has not been evaluated by this method before.

In our study essential oil reveals a slight inhibition in high concentrations, it showed that *B.cereus* had the greatest, inhibition, showing up to 50% in the highest concentration of 2.048 µl/ml. According to previous study (Cos et al., 2006) also *E.deglupta* showed a MIC of 10 mm zdi against *B.cereus* strain. *S.aureus* exhibited inhibition of 46% in a concentration of 2.048 µl/ml and according to O. Oyedeji et al. (1999) studies, the essential oil showed a MIC of 10 mm, 11 mm for *S.aureus* strain. *S.epidermidis* and *E.faecalis* two gram-positive bacteria were first time tested in this study, both shows no MIC but despite the results, there was a low inhibition of 32% and 28% respectively in a concentration of 2.048 µl/ml. In O. Oyedeji et al. (1999) investigations, *E.coli* exhibited a MIC of 10 mm, 16 mm and 11 mm respectively, in contrast of our study that the oil was no active against this bacteria, however it show a low inhibition of 37% in 2.048 µl/ml of concentration. *P.aeruginosa* showed 9 mm and 12 mm of MIC according to O. Oyedeji et al. (1999) investigations and differ in our study that showed not MIC but very low inhibition of 32% in 2.048 µl/ml of concentration. In Cimanga et al. (2002) research, *K.pneumoniae* registered a MIC of 16 mm and 10 mm respectively while in our study *K.pneumoniae* was no active, but show low inhibition at 2.048 µl/ml of concentration with 43%. *S.enteritidis* was the only gram-negative bacteria for the first time tested and showed no MIC with a low inhibition of 40% with 2.048 µl/ml of concentration.

In previous studies *P.aeruginosa,* the most resistant clinical bacteria, showed against *E.deglupta* essential oil that was the most resistant among all the bacteria tested. According to Cimanga et al. (2002), from the two *P.aeruginosa* tested, *P.aeruginosa* 1 (abces) was inactive and *P.aeruginosa* 3 (urine) exhibited 8 mm zdi showing a high resistant to the diffusion method. In our study, results are in accordance against this bacteria. *P.aeruginosa* showed a poor 37% of inhibition, thus it was not the most resistant bacteria. *E.faecalis* which was tested in our study for the first time against *E.deglupta* oil, exhibited a 24 % of inhibition in the highest concentration 2.048 µl/ml.

Although in our study there is no MIC at all, we can achieve a MIC by using a higher concentration than we use for the study (2.048 µl/ml), but we must be cautious with the

dosage concentration for medical purpose because of its toxicity according to Batish et al. (2008). In higher-than-normal doses, this oil can be hazardous via ingestion, skin contact, or inhalation. It can have acute health effects on behavior, respiratory tract, and nervous system. Therefore should be further toxicity testing, specifically with compound of *E.deglupta* essential oil in order to determine the permissible limits for the application of this oil in the medical and pharmaceutical area without any adverse effect applications.

Results reported here are not in a good agreement with the antimicrobial activity of *E.deglupta* essential oil reported in previous research. In fact, as Janssen affirms four factors are especially important when testing essential oils: the assay technique; the growth medium; the micro-organism and the essential oil. (Janssen et al., 1987).

This research reported a 0.4% (w/w) yield of *E.deglupta* oil. In previous literature, we found difference compared to those yields previously reported for the same aromatic plants such as Eucalyptus species collected in other geographic areas in the world. In Cimanga et al. (2002) study, it was reported a 0.15% (w/w) yield from the leaf of a *E.deglupta* collected in Kinshasa, capital of the Democratic Republic of Congo between February and December 1989. In another investigation, Oyedeji et al. (1999), *E.deglupta* collected in Nigeria gave a volatile oil 0.2% (w/w) yield. We could attributed these difference to some factors such as climate, nature of the sol, age of the tree, time of collection (season recollected), mode of extraction, properties of the soil, etc. (Oyedeji et al., 1999).

7 Conclusion

The antimicrobial activity of the leave essential oil derived from *Eucalyptus deglupta* against a set of gram-positive and gram-negative bacteria was evaluated *in vitro* and the chemical composition of the oil was analyzed by GC-MS.

The aims of this work were fulfilled. However, the hypothesis was confirmed only partially, since the antibacterial activity observed was very poor against all bacteria tested.

The chemical analysis revealed the absence of eucalyptol, the antimicrobial compound commonly present in Eucalyptus spp. which can explain the low antibacterial effect of *E. deglupta* essential oil.

8 Bibliography

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