LIPOSOMAL INCORPORATION OF *SATUREJA HORTENSIS L*. ESSENTIAL OIL AND *IN VITRO* ANTIMICROBIAL ACTIVITY ON ORAL MICROORGANISMS

by

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This thesis is dedicated to my family for
love and support throughout my life...

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ABSTRACT

LIPOSOMAL INCORPORATION OF *SATUREJA HORTENSIS L.* ESSENTIAL OIL AND *IN VITRO* ANTIMICROBIAL ACTIVITY ON ORAL MICROORGANISMS

The use of nanotechnology in the medical industry, involves the use of carrier systems to provide bioavailability, stability and to effectively deliver antimicrobial agents. The essential oil of *Satureja hortensis* L. a popular Turkish herb, is known to have high antimicrobial activity due to the presence of carvacrol, thymol and p-cymene. The aim of this study was to incorporate, characterize and determine the antimicrobial efficacy of *S.hortensis* essential oil, loaded liposomes. Microorganisms tested were: *Aggregatibacter actinomycetemcomitans* FDC Y4, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 25586, *S.mutans* and *C.albicans*. EO was extracted from *S.hortensis*, by hydrodistillation method and incorporated achieved by the Bangham method. Different types of loaded liposome (L1 and L2) formulations were prepared with phospholipid 85 G (P85G), cholesterol (CH) and stearylamine (SA) at different ratios, respectively, (P85G: CH: SA; 7:1:2 and 10:1:4). Liposomal formulations were characterized by Dynamic Light Scattering (DLS) and microscopic methods. The antimicrobial activity of L1 and L2 were examined by the Agar Well Diffusion Assay after 120 h incubation. Statistical analysis of results by way of Tukey, Anova and Dunnet tests. Both L1 and L2 showed antimicrobial effects against all test microorganisms. However, overall characterization and antimicrobial results, revealed L1 to have better properties for use against oral pathogens. A liposomal approach can be useful for containing volatile compounds that have antimicrobial effects on oral microorganisms.

Finally, liposomes incorporating *S.hortensis* L. EO, may be considered as a potential liposomal delivery system in the oral cavity in the future.

ÖZET

SATUREJA HORTENSIS L. TEMEL YAĞ ASİTLERİNİN LİPOZOM İLE BİRLEŞTİRİLMESİ VE *İN VİTRO* ORTAMDA AĞIZ MİKROORGANİZMALARI ÜZERİNDEKİ ANTİMİKROBİYAL AKTİVİTESİ

Sağlık endüstrisinde nanoteknolojinin kullanımı, taşıyıcı sistemlerin kullanımının dahil edilmesiyle biyoyararlılık, stabilite ve antimikrobiyal ajanların etkili bir şekilde taşınmasına olanak sağlamıştır. *Satureja hortensis L* uçucu yağları, yapısında bulunan carvacrol, thymol ve p-cymene' den dolayı yüksek antimikrobiyal aktiviteye sahip popüler bir Türk bitkisidir. Bu tez çalışmasının amacı, *S.hortensis L.* esansiyel uçucu yağları, lipozom ile birleştirmek, karakterize etmek, *S.hortensis L.* uçucu yağın ve yüklü lipozomların antimikrobiyal etkinliklerini belirlemektir.Test edilen mikroorganizmalar, *Aggregatibacter actinomycetemcomitans* FDC Y4, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 25586, *S.mutans* and *C.albicans*'tır. Bu çalışmada *S. hortensis L.* adlı bitkiden hidrodistilasyon methodu ile uçucu yağlar elde edilmiştir ve Bangham metodu ile birleşimi sağlanmıştır. İki farklı tip yüklü lipozom (L1 ve L2) formulleri farklı oranlarda fosfolipid 85 G (P85G), Kolesterol (CH) and Stearilamin (SA) kullanılarak sırasıyla hazırlanmıştır. Formüller Dinamik Işık Saçılımı (DLS) ve mikroskobik tekniklerle karakterize edilmiştir.L1 ve L2 nin antimikrobiyal aktiviteleri Agar kuyucuk metodu kullanılarak 120 saatin sonunda test edilmiştir. Tukey, Anova and Dunnet testleri kullanılarak sonuçlar istatistiksel olarak değerlendirilmiştir. Hem L1 hemde L2 test edilen mikroorganizmalara karşı antimikrobiyal etkinlik göstermiştir. Ancak, tüm karakterizasyon ve antimikrobiyal sonuçlar, L1 in ağız patojenleri üzerinde daha etkili olduğunu ortaya koymuştur. Uçucu bileşenleri içeren lipozomlar, ağız mikroorganizmaları üzerinde faydalı bir antimikrobiyal etkinliğe sahip olabilirler.

Sonuç olarak, *S. hortensis L.*uçucu yağlarını içeren liposomların potensiyel bir lipozomlu taşıyıcı sistem olarak ağız boşluğunda gelecekte kullanılabileceği düşünülmektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

AFM	Atomic Force Microscope
BHB	Brain Heart Broth
CH	Cholesterol
CHX	Clorhexidine gluconate
Cfu/ml	Colony forming units per mililiter
CO2	Carbon dioxide
DDS	Drug Delivery Systems
DODAB	Dioctadecyldimethylammonium Bromide
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
EO	Essential Oil
EPS	Extracellular Polymeric Substance
FAB	Fastidious Anaerobe Broth
GC/CIMS	Chemical Ionization Mass Spectrometry
GC-MS	Gas Chromatography equipped with Mass Spectrometry
h	hour
HSV-1	Herpex Simplex virus type-1
LMV	Large Unilamellar vesicles
LPS	Lipopolysaccharide
L1	P85G: SA: CHOL (7:1:2)
L2	P85G: SA: CHOL (10:1:4)
MASE	Microwave-Assisted Solvent Extraction
MBC	Minimum Inhibitory Concentration
MIC	Minimum Bactericidal Concentration
MLV	Multi Lamellar Vesicles
MS	Mass Spectrometry
NBGI	National Botanical Garden Of Iran
Naocl	Sodium Hypochlorite
OD	Optical Density
P	Page

PBS	Phosphate Buffered Saline
PLM	Polarize Light Microscope
PL85G	Phospholipon P85G
P90	Non-Hydrogenated Phospholipon
P90H	Hydrogenated Phospholipon
SA	Stearylamine
SDA	Saboaraud Dextrose Agar
SDB	Saboaraud Dextrose Broth
SFME	Solvent Free Micorwave Extraction
S.hortensis	Satureja Hortensis
SLN	Nisin Loaded Solid Nanoparticles
SUV	Small Unilamellar Vesicles
TIC	Total Ion Current
WHO	World Health Organizations
ZI	Zone Of Inhibition

1. INTRODUCTION

Oral health is an important worldwide issue and contributes to the quality of life. Oral health means being free of chronic mouth and facial pain, oral and throat cancer, oral sores, gum disease, tooth decay, tooth loss and other diseases and disorders that affect the mouth and oral cavity [1, 2].

There is substantial proof connecting poor oral health to chronic conditions and systemic diseases such as periodontal disease and diabetes [1, 2], cardiovascular diseases, renal, hepatic, pulmonary, rheumatoid arthritis, adverse pregnancy effects and osteoporosis [3]. The WHO (World Health Organization) has stated that upto 90 % of school children worldwide have dental caries and 5-20 % of middle-aged adults suffer from severe periodontal (gum) disease, which may result in tooth loss. The incidence of oral cancer ranges from 1 to 10 cases per 100.000 in most countries and 40-50% of people who are HIV-positive have oral fungal, bacterial and viral infections [4].

Since dental care is an important problem for many countries in the world, cheap and effective treatments or prevention methods have gained great interest worldwide [3, 4].

2. THEORETICAL BACKGROUND

2.1. ORAL DISEASES

Dental diseases affect the oral cavity most of which involve the teeth and surrounding supportive structures, collectively known as the periodontium (gingiva, ligaments, membrane, bone) (Figure 2.1) [5].

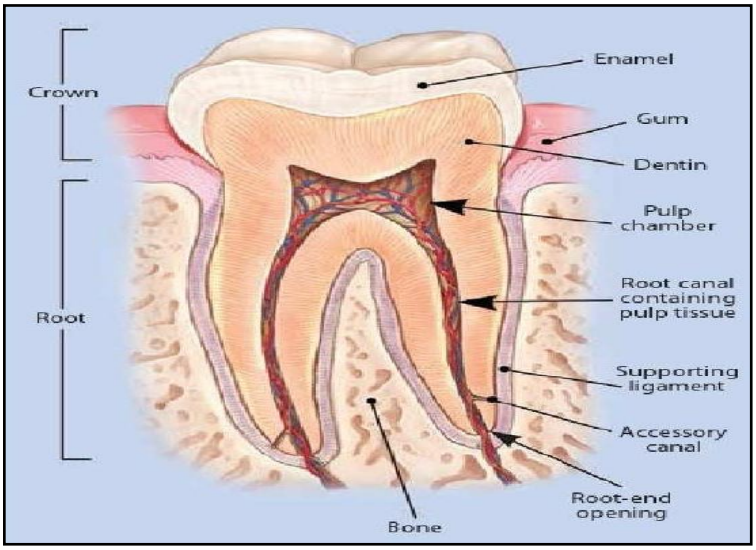

Figure 2.1. The anatomy of a tooth [5]

Dental pathology commonly influences both hard and soft tissues of oral cavity. Although both categories of disease start when microbes adhere to the tooth surface and produce dental plaque, their outcomes can vary. Figure 2.2 is a summary of the events leading to dental caries, periodontal diseases, and bone and tooth loss [5].

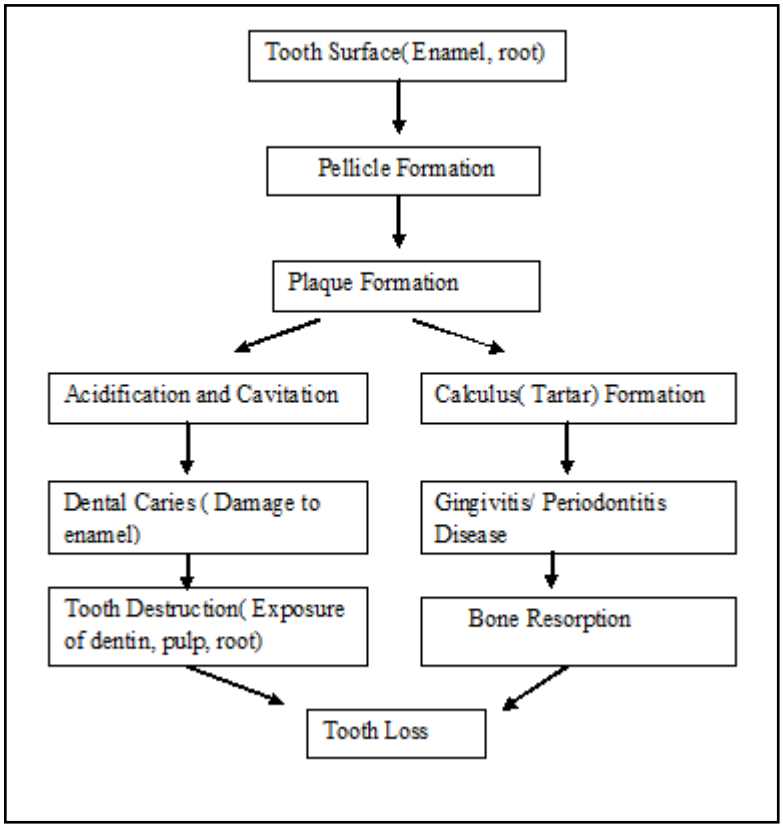

Figure 2.2. Events and Diseases Resulting in Tooth Loss [5]

2.1.1. Hard Tissue Disease: Dental Caries

Dental caries is the most common human disease. It is a complex mixed infection of the dentition that gradually destroys the enamel and often lays the background for the damage of deeper tissues. It occurs most often on tooth surfaces that are less accessible and harder to clean and on those that provide pockets or crevices where bacteria can cling. Caries commonly develops on enamel pits and fissures, especially those of the grinding surfaces, though they can also occur on the smoother crown surfaces and subgingivally on the roots.

Although they are not classified as life threatening diseases, dental caries are prevalent and costly problems for suppliers of health services. In order to decrease the prevalence of caries, an improved understanding of the role of the microorganisms in dental diseases is needed [6]. The tooth surface is covered with a biofilm slime layer consisting of millions of bacterial (cells) microcolonies, salivary polymers, fluid channels and complex communication systems [7]. This biofilm can easily reach a thickness of hundreds of cells

on the surfaces of the teeth. The formed biofilm, also called plaque, provides an excellent adhesion site for the colonization and growth of many bacterial species.

Over the years, several views have been put forth to explain how dental caries originate. At various times it has been believed that sugar, microbes and acid cause teeth to rot. Germ-free animals eventually displayed that no single factor can account for caries. Caries development occurs in many phases and requires multiple interactions involving the anatomy, physiology, diet, and bacterial flora of the host. The principal stages (Figure 2.3) in the formation of dental caries are pellicle formation, plaque formation, acid production and localization and enamel corrosion [5].

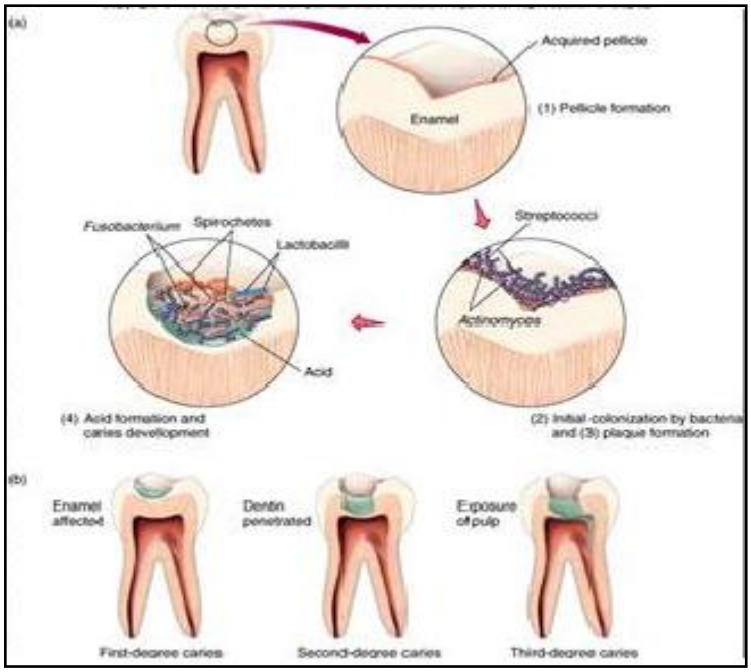

Figure 2.3. Stages in plaque development and cariogenesis. The macroscopic and microscopic appearance of plaque a. A microscopic view of pellicle and plaque formation, acidification and destruction of tooth enamel. b. Progress and degrees of cariogenesis [5]

2.1.2. Plaque Formation

Dental plaque is a biofilm community that accumulates through sequential and ordered colonization of multiple oral bacteria [8]. Three steps are involved in the development of dental plaque [6]. First, salivary molecules such as salivary proteins, believed to be

primarily secreted from *S. mutans* are attached to the enamel. This coats the enamel with a complex mixture of components that include glycoprotein, acidic proline-rich proteins, mucins, bacterial cell debris, exoproducts, and sialic acid [6]. Through this matrix, fluid channels form, that provide nutrients, oxygen, communication, genetic exchange, production of inhibitory factors and quorum sensing, which determines the metabolism and composition of the biofilm residents [7, 8]. Quarum sensing is a signaling system essential for genetic competence in *Streptococcus mutans*.[9]

Second, bacterial cells attach to this acquired pellicle via several specific cell-to-surface interactions [9]. The biofilm formation of 3 main primary colonizers, *S.mutans, S. sanguis* and *A. viscosus* [10], are influenced by a number of environmental factors, like osmolarity and pH of the oral cavity and carbon source [9]. These primary colonizers have an affinity for the matrix and their adhesion to it paves the way for the adherence and accumulation of other microorganisms.

During the third stage, other bacterial species (late colonizers) usually anaerobic bacteria such as *Prevotella* and *Porphyromonas* species [7], adhere to the primary colonizer by cell-to-cell interactions. In addition to cell-to-cell interactions, metabolic interactions and quorum sensing are also important for biofilm formation and development.

The exact process of these interactions is still unknown but it is believed that quorum sensing plays a role. Subsequent bacterial growth on tooth surface leads to the formation of a biofilm on the teeth, also called, dental plaque [9, 11]. The microorganisms live with one another in commensal or mutualistic symbiotic relationships [12]. Typically, anaerobic bacteria would succumb to high levels of oxygen, but with the redox reactions they are able to survive. This commensal relationship allows a mixture of aerobic and anaerobic bacteria to live in the same area. The formation begins by the adsorption of early colonizers onto an acquired pellicle through chemical processes [13].

Primary colonizer, *S. mutans* is a Gram-positive bacterium, non-motile, facultative anaerobe. *S. mutans* gives its name to a group of seven firmly connected species all together associated with the mutans streptococci. The original habitats of *S. mutans* are the mouth, pharynx, and intestine [14]. It can thrive in temperatures between 18-40°C.

S. mutans is a strong acid producer and hence causes an acidic environment creating the risk for cavities [15]. Virulence factor of *S. mutans* in cariogenicity is its ability to attach to the tooth surface and form a biofilm [16]. If mature plaque is not removed from sites that readily trap food, it usually evolves into a caries lession. The role of plaque in caries development is related directly to streptococci and lactobacilli, which produce acid as they ferment dietary carbohydrates. If this acid is immediately flushed from the plaque and diluted in the mouth, it has little effect. However, in the denser regions of plaque the acids can accumulate in direct contact with the enamel surfaces and lower the ph to below 5, which is acidic enough to begin dissolving the calcium phosphate of the enamel in that spot. This lesion remains localized in the enamel (first degree caries) and can be repaired with various inert material (fillings). Once degradation has reached the level of dentin (second-degree caries) tooth destruction speeds up, and the tooth can be rapidly destroyed. Exposure of the pulp is attended by severe tenderness and toothache and the chance of saving the tooth is diminished [5].

2.1.3. Soft-Tissue (Periodontal) Diseases

Periodontal diseases generally affect 97-100 % of the worldwide population, and have some manifestations by age 45. Most types are due to bacterial colonization and varying degrees of inflammation that occur in response to gingival damage. The most common predisposing condition occurs when the plaque becomes mineralized (calcified) with calcium and phosphate crystals. This process produces a hard porous substance called calculus above and below the gingiva, which can induce varying degrees of periodontal damage [5].

Calculus and plaque accumulating in the gingival sulcus causes abrasions in the delicate gingival membrane and the chronic trauma causes a pronounced inflammatory reaction. The damaged tissues become a portal of entry for a variety of bacterial residents. These include genera such as; *Actinobacillus, Porphyromonas, Bacteroides,* and *Fusobacterium* and numerous spirochetes. The anaerobic, gram-negative bacteria in these infections out numbers aerobes by 1 to 100. In response to the mixed infection, the damaged area becomes infiltrated by neutrophils and macrophages and later by lymphocytes, which cause further inflammation and tissue damage. The initial signs of gingivitis are swelling,

loss of normal contour, patches of redness and increased bleeding of the gingival spaces. Pockets of varying depth also develop between the tooth and the gingiva. If this condition persists, a more serious disease called Periodontitis results (Figure 2.4). This is the natural extention of the disease into the periodontal membrane and cementum. The deeper involvement increases the size of the pockets and can cause bone resorption severe enough to loosen the tooth in its sockets. If the condition is allowed to progress, the tooth can be lost [5].

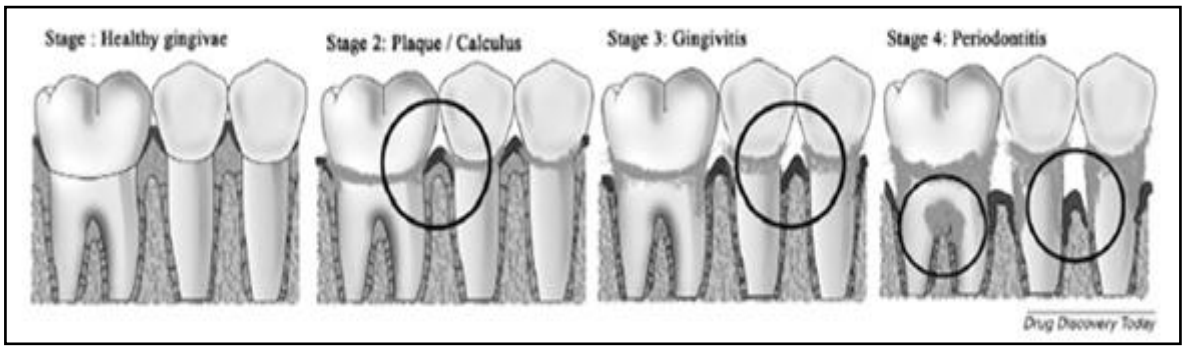

Figure 2.4. The image of disease progression from healthy gingiva to periodontitis [17]. Stage1. Healthy gum tissue, Stage 2. Plaque formation due to bacterial invasion, Stage 3. Bacterial toxins irritate gums and trigger host-mediated responses that lead to gingivitis, Stage 4. Destruction of gingiva and bone that support the tooth leading to periodontitis [17]

2.2. ROLE OF MICROORGANISMS IN ORAL DISEASE

Dental caries and periodontal diseases are among the most important treatable global oral diseases [4]. The relationship between humans and their microflora is complex. The mouth contains a diversity of surfaces for colonization, including the tongue, teeth, gingiva, palate, and cheeks and it provides numerous aerobic and anaerobic and micro-aerophilic microhabitants for the estimated 700 different oral species with which human coexist. The habitat of the oral cavity is warm, moist, and greatly enriched by the periodic infusion of food. In most humans, this association remains in balance with little adverse effect, but in people with poor oral hygiene, it teeters constantly on the brink of disease. Dental caries is a supra-gingival event [18]. While conversely, periodontal diseases are sub gingival cases

that have been related to anaerobic Gram-negative bacteria such as *Porphyromonas gingivitis, Actinobacillus, Prevotella, Fusobacterium sp* [19, 20].

2.3. ORAL MICROORGANISMS

The oral microbiota is complex and has some features that make it a unique habitat. Viruses, fungi, protozoa and bacteria colonize many oral sites including the tongue, oral mucous membranes, subgingival and supragingival tissues, and teeth [21, 22, 23]. Several microorganisms are present on the external surfaces of the human body and most of these commensal microbes are in harmony with the host. The oral microbiota, however, are the only part of the human body whose commensal microbes cause disease [4].

The human oral cavity is also called the human oral microbiome. This is because the human oral cavity can contain several environments at a given moment that could vary from tooth to tooth [4]. Additionally it has been estimated that the number of bacterial species that reside in the mouth is approximately 25,000 [5]. This is in contrast to the previously estimated 700 + species [6]. Studies have found that out of the 25,000 species that exist in the oral cavity, about 1000 species can exist as part of the dental biofilm ecosystem [7, 8]. 1000 species were identified with the help of developed molecular and microbial techniques [8].

Their composition varies in different sites in the oral cavity, e.g. a large and more diverse microbial load is present on the dorsum of the tongue. Most of these microbes are harmless, but under certain conditions, some can cause oral infections like caries or periodontal diseases [15].

2.3.1. Bacteria

Bacteria are the most common microorganisms of the oral cavity [24]. The oral cavity consists of different types of Gram positive and Gram negative, obligate aerobes and facultative and obligate anaerobes [9].

Bacterial species have been identified in the healthy and diseased sites of the oral cavity [9] by culture methods and culture-independent molecular methods. Approximately 36 phyla/divisions within bacterial domains have been determined in the mouth [25] and eight phyla that are found frequently in the mouth are *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Protebacteria, Spirochaetes, 'Synergistes'* and TM7. Amongst these, species of *Actinobacteria (Actinomyces naeslundii), Bacteroidetes (Porphyromonas gingivalis, Porphyromonas endodontalis, Tannerella forsythia, Prevotella intermedia, Prevotella nigrescens, Capnocytophaga ochracea, Capnocytophaga gingivalis), Firmicutes (Streptococcus mutans, S. mitis, S. sobrinus, S. oralis, S. gordonii, S. sanguinis), Fusobacteria (F. nucleatum), Protebacteria (Aggregatibcater actinomycetemcomitans*) and *Spirochaetes* (*Treponemes denticola* and *T. medium*) were shown to play a role in dental plaque formation and in plaque associated oral diseases [26].

2.3.2. Fungi

Yeasts are commensal microorganisms in the mouth [26]. *C. albicans* is the major causative agent of human oral fungal infections causing oral candidiasis. It is a major opportunistic pathogen of immunocompromised hosts, including AIDS patients, those undergoing chemotherapy, tissue transplants or patients with central venous catheter [27]. *C.albicans* colonizes many surfaces in the oral cavity such as tooth surfaces, tongue, and epithelial cell of the cheek [28].

2.3.3. Protozoa

Protozoa are not present in the normal oral cavity, they can only be seen in the absence of oral hygiene. For instance, *Entamoeba gingivalis* and *Trichomonas tenax* are protozoa species and can be found between the gingival pocket and teeth [29].

2.3.4. Viruses

In addition to bacteria, many kinds of viruses have been found in the mouth, such as *Herpes simplex* virus, Cytomegalo or *Epstein-Barr* virus. Although their prevalence has increased in the case of oral diseases such as periodontitis and periodontal abscesses, these viruses

have been encountered in healthy subjects [30, 31]. These viruses transmit through sexual contact [32] and breastfeeding [6, 32].

2.4. PREVENTION AND TREATMENT OF ORAL DISEASES

2.4.1. Mechanical Methods

Dental diseases can be controlled by careful mechanical oral hygiene [33, 34]. The main way of preventing oral diseases is adequate daily tooth brushing [35]. Daily removal of pre-formed biofilms by tooth brushing and flossing can stop the colonization of pathogenic bacteria [16, 28, 36, 37]. Despite its important role in the prevention of gingivitis and periodontitis, mechanical plaque control is not accurately practiced by most individuals [33, 38].

Further plaque formation causes the hardest plaque, which is named calculus or tartar. This calculus cannot be removed easily from the tooth surface by brushing techniques [39]. If tooth brushing cannot clean the area, mechanical scaling and root planning should be applied [40].

Previous studies have shown that periodontal therapeutic methods such as root planning and scaling have reduced inflammation and bleeding of the gingivia in the oral cavity [41, 42, 43]. With the development of technology, a lot of alternative methods are employed to kill periodontal pathogenic bacteria and to disrupt their biofilm matrix. Currently, the most important technique is photodynamic therapy [44]. This therapy includes the activity of solar light on pathogenic microorganisms, which are immersed by specific dyes [44].

2.4.2. Chemical Methods

A wide range of agents have been formulated into oral care products in order to enhance their plaque control potential [33, 34]. Scientists and dentists have been using systemic chemotherapeutic agents in combination with mechanical methods to prevent dental plaque efficiently. Chemical anti-plaque agents in mouth rinses and toothpastes reach the soft tissue surfaces, improving the control of biofilm growth on these surfaces [45].

Chlorhexidine (CHX) is the most conventional antiseptic agent in oral products. It is widely used in clinical practice as a skin and mucous membrane antiseptic and a disinfectant [40]. It is a biphenyl compound which has broad spectrum of activity against Gram-positive and Gram-negative bacteria and yeasts. It also decreases plaque, dental caries and gingivitis due to its cationic structure [40, 46]. CHX is bactericidal and at high concentrations causes lethal damage to the bacterial membrane. Conversely, at low concentrations could be bacteriostatic [40, 46]. The efficiency of the CHX influences the cell wall and damage the LPS layer and cytoplasmic membrane. Therefore, the leakage of the cell components cause death of bacteria at sub lethal concentrations, CHX can interfere with the metabolism of oral bacteria by inhibiting sugar transport and acid production in cryogenic *streptococci* [46] and various membrane functions in *Streptococci*, including enzymes responsible for maintaining an appropriate intracellular pH. The disadvantages of CHX is that it is a chemical based product and cannot be used long term (1 week), as it can cause discolouration of the teeth (yellowing).

Listerine is an antiseptic mouth rinse, which consists of a mixture of essential oils (menthol, thymol, methyl salicylate and eucalyptol) and ethanol (21 per cent) which helps dissolve the essential oils. Listerine was formulated as a surgical antiseptic in 1879. Now, however, it is consumed also for oral care. Listerine is a well-known efficient and anti-plaque and anti-gingivitis therapeutic solution.

The action of listerine was reported as bacterial cell wall destruction, bacterial enzyme inhibition and extraction of bacterial lipopolysaccharides [47]. Moreover, it kills the pre-formed accumulation and prevents accumulation of the pathogenic microorganisms [48, 49]. The disadvantages of Listerine are that it has an unpleasant taste with a burning sense on the mucosa. Studies indicate that prolonged consumption of this mouth rinse can induce some resistant species in the dental plaque and inconvenient oral pathogens [48].

Triclosan (5-chloro-2-(2, 4-dichlorophenoxy) phenol) is an oral care product and has a broad spectrum of antimicrobial, anti-inflammatory activity [50]. Many contemporary consumer and personal health-care products (deodorants, toothpastes, mouth washes), household items (plastics and textiles) include triclosan [50]. In the literature, triclosan demonstrated some anti-plaque and anti-gingivitis effects in a mixture with complementary

antimicrobial agents, such as zinc [50]. The bacteriostatic activity of triclosan was observed at concentrations ranging between 0.025 and 100 μg/mL., whereas bactericidal activity was observed at higher concentrations. At bactericidal concentrations, it destroys the bacterial cell wall functions, leading to leakage of the cellular contents and eradicates the bacteria species individually. Some studies demonstrated that gram negative bacteria species and some intestinal, dermal and environmental species can establish resistance to triclosan and some side effects, such as allergies, and toxicity in children [50].

Fluoride is synthesized chemically and present in low concentrations in drinking water, sea water and food. Many studies showed that fluoride has an anti-cryogenic effect [51, 52]. That is why tooth pastes and mouth rinse consisting of fluoride are used and sold worldwide and tap water is fluoridated in the USA and the UK [53]. However, there are many opposing ideas on the consumption of fluoride for human health. Antagonists support that fluoride is a toxic chemical; therefore, it should not be used in tap water, toothpastes, and mouth rinses [54]. The level of fluoride which is taken into the body is very critical. An adult can digest 0.042 mg/kg of fluoride whereas a child can digest 0.127 mg/kg fluoride daily [55]. Some studies have indicated that an intake of high levels of fluoride lead to some side effects, such as dental and skeletal fluorosis, immunotoxicity, carcinogenicity, genotoxicity, reprotoxicity, renal toxicity, and gastrointestinal tract toxicity [51, 55].

Sodium hypochlorite (NaOCl) is the most common household bleach and cleanser. It has been integrated into oral medicines to treat periodontal disease [56] It has been shown that 0.01 % NaOCl in many oral products may be helpful for prevention of dental plaque and oral biofilm [56, 57, 58]. However, high concentrations of sodium hypochlorite can cause irritation of the mucous membrane [56].

Povidone-iodine is a povidone (polyvinylpyrrolidone) polymer. Povidone-iodine has demonstrated sufficient action against certain microorganisms. While, it does not irritate the oral mucosa and tongue, or discolorize teeth [58], some studies have shown that mouth rinses that include 1% of povidone iodine do not kill plaque forming microorganisms [55, 59].

Antibiotics are synthetic and natural organic constituents that kill and restrict particular microorganisms at low concentrations. Many antibiotics that are widely used in dentistry, include: penicillin, metronidazole, doxycycline, tetracycline, clindamycin and amoxicillin [56]. However antibiotics have the risk of causing microbial resistance and have some reverse impacts such as gastrointestinal disturbances, headache, dizziness and hypersensitivity [60, 61].

With the many disadvantages of current methods [44, 61], alternative natural plant products are considered a good alternative to current therapies described [62].

2.4.3. Phytotherapy

2.4.3.1. Definition Of Phytotherapy And Its Applications

All natural extracts have a wide range of bioactive components such as lipids phytochemicals, flavors, fragrances, and including pigments (thymol, carvacrol, camphor, fenchone, thuyone, caryophyllene, logifolene, curcumenes, etc) [63]. Plant extracts are widely employed for different applications, exclusively as health promoting elements used in the formulation of food additives, nutraceuticals, pharmaceuticals and cosmetics [64]. Aromatic plants have been widely consumed since ancient times. However, over the last few decades researchers and scientists have been interested in investigating their biological activities and studying them for traditional treatment of many diseases [65- 69].

2.4.3.2. Phytotherapy In The Global Market

In rural areas of the world, plants are still being used as the main source of medicine therapy [70]. Approximately 80 % of the people use traditional medicines for treatment in developing countries [70, 71]. Since microorganisms are becoming increasingly resistant to therapeutic agents such as antibiotics this has led to interest in the discovery of novel anti-infective ingredients.

According to the WHO, traditional plant products have economical importance for pharmaceutical companies due to reduced cost of screening. There are approximately 121 plant based prescription drugs in the world [2, 72, 73] and the global market of these drugs have been predicted at $43 [2, 73] billion. In developing countries including Turkey, plant

based therapies also known as traditional medicine, are widely used due to their low cost and easy access [2].

2.4.3.3. Use Of Essential Oil

Essential oils are volatile, natural, complex compounds characterized by a strong odour [63]. They can be synthesized by aromatic plants and all plant organs, buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark where they are stored in secretary cells, cavities canals, epidemic cells or glandular trichomonas as secondary metabolites. They are liquid, seldom coloured, soluble in lipid organic solvents with generally a lower density than water [63, 74].

Essential oils are extracted from different aromatic plants generally from warm countries like Mediterranean and tropical countries where they represent an important part of the traditional pharmacopoeia [74]. They have been found to have aseptic, bactericidal, virucidal, and fungicidal properties [75-78].

In nature, essential oils play an important role in the protection of the plants as antibacterials, antivirals, antifungals, insecticides and against herbivores by reducing their appetite for such plants. In addition, they may attract some insects to favour disperison of pollens and seeds or repel undesirable predators [74].

At present, around there are 3000 known essential oils, 300 of which are commercially important specifically for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industry [79-83].

2.4.3.4. Essential Oil Extraction Techniques

There are several extraction methods for obtaining essential oils from different parts of plants. These methods provide extracts with different composition and yield [83]. These extraction methods include: water distillation (or hydro distillation), steam-distillation, cold pressing (expression), solvent extraction, supercritical carbon dioxide extraction, microwave-assisted solvent extraction (MASE) and solvent free microwave extraction (SFME) [81-91].

2.4.3.5. Chemical Composition Of Essential Oils

Essential oils are very complex natural mixtures which can contain approximately 20-60 components at varying concentrations. They are characterized by two or three major components at fairly high concentrations (20-70 %) compared to others components present in trace amounts. For example, carvacrol (30 %) and thymol (27 %) are the major components of the *Origanum compactum* essential oil [92]. Essential oil molecules are composed of mainly three atoms: carbon, hydrogen and oxygen bonded in a variety of ways. They are made of four major components namely terpenes, terpenoids, aromatic constituents and aliphatic constituents all having low molecular weight. Based on biosynthesis, these component molecules divide into two groups. First one, terpene hydrocarbons, (monoterpenes and sesquiterpenes) and terpenes modified by oxygen are called terpenoid. Second one is phenylpropane derivatives and they behave like terpenes. Also, phenylpropane derivatives organise into two groups; Aromatics and Aliphatics. They may exist in the form of alcohols, acids, esters, epoxides, aldehydes, ketones, amines, sulfides etc [93-97].

Terpenes are the main constitutents of essential oils in plants in nature and differ structurally and functionally with combinations of several 5-carbone-base (C5) units called isoprene. They are classified according to the number of isoprene units; monoterpenes (2 isoprene units), sesquiterpenes (3 isoprene units) , diterpenes (4 isoprene units), triterpenes (6 isoprene units) and tetraterpenes (8 isoprene units) [79, 97, 98]. The monoterpenes are formed from the coupling of two isoprene units (C_{10}). They are the most representative molecules constituting 90 % of the essential oils and allow a great variety of structures [98]. Also, Table 2.1 represents the functional groups in the essential oils.

The main 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. Terpenoids are the terpenes containing oxygen molecules or terpenes with functional group [97, 98]. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes, or ketones are called a terpenoid. Their building block is the hydrocarbon isoprene $CH_2=C(CH_3)-CH=CH_2$ terpene hydrocarbons therefore have molecular formulas $(C_5H_8)_n$.

Table 2.1. Monoterpenes and sesquiterpenes according to their functional groups [97, 98]

	Terpene and Terpenoids	
Functional group	Monoterpenes	Sesquiterpenes
Carbure	Acyclic: Myrcene, Ocimene Monocyclic: Terpinenes, p-Cymene, Phellandrenes Bicyclic: Pinenes, Camphene, Sabinene	Azulene, β-Bisabolene, Cadinenes, β-Caryophyllene, Logifolene, Curcumenes, Elemenes, Farnesenes, Zingiberene
Alcohol	Acyclic: Geraniol, Linalol, Citronellol, Lavandulol, Nerol Monocyclic: Menthol, α-Terpineol, Carveol Bicyclic: Borneol, Fenchol, Chrysanthenol, Thuyan-3-Ol	Bisabol, Cedrol, B-Nerolidol, Farnesol, Carotol, B-Santalol, Patchoulol, Viridiflorol
Aldehyde	Acyclic: Geranial, Neral, Citronellal	
Ketone	Acyclic: Tegetone Monocyclic: Menthones, Carvone, Pulegone, Piperitone Bicyclic: Camphor, Fenchone, Thuyone, Ombellulone, Pinocamphone, Pinocarvone	Germacrone, Nootkatone, Cis-Longipinan-2,7-Dione, β-Vetinone, Turmerones
Ester	Acyclic: Linalyl Acetate Or Propionate, Citronellyl Acetate Monocyclic: Menthyl Or α-Terpinyl Acetate Bicyclic: Isobornyl Acetate	
Ether	1,8-Cineole, Menthofurane	
Peroxyde	Ascaridole	
Phenol	Thymol, Carvacrol	

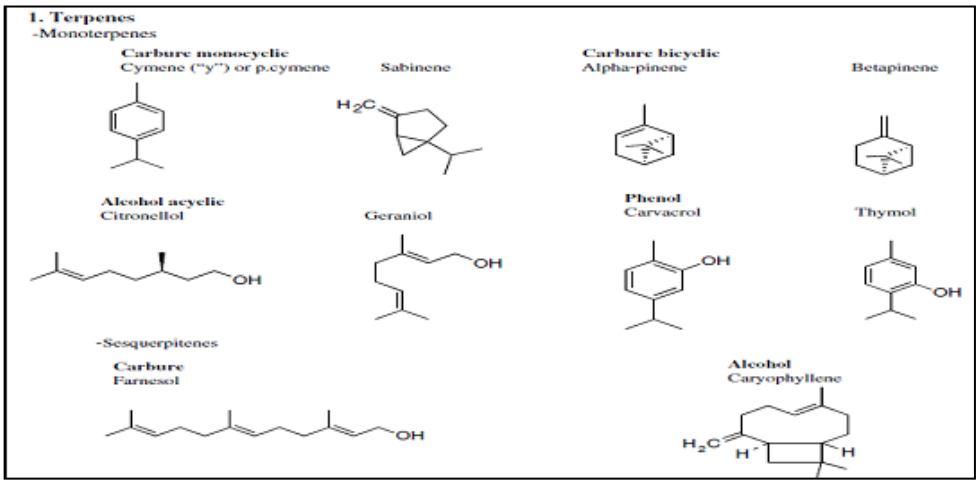

Figure 2.5. Chemical structures of some components of EO [97, 98]

Aromatic constituents derived from phenylpropane, are found less frequently than the terpenes. Aromatic compounds comprise of: Aldehyde, Alcohol, Phenols, Methoxy derivatives and Methylene [97, 98].

Aromatic constitutes of essential oils are derived from phenyl propane and they are present in plants at lower amounts than terpenes [97- 99]. Some plants containing aromatic compounds are cinnamon, clove, fennel, anise, etc. As monoterpenes and sesquiterpenes, naming of aromatic constituents depends on functional group that they contain (Table 2.2).

Aliphatic Compounds are extracted from plant by means of steam distillation methods. These compounds have a very low molecular weight such as hydrocarbons, acids (C3-C10), alcohols, aldehydes, acyclic esters or lactones [97, 99].

Nitrogeneous or sulphured components such as glucosinolates or isothiocyanate derivatives are also characteristic as secondary metabolites of diverse plants or of torrefiel, grilled or roasted products [79, 97].

Table 2.2. Aromatic constituents according to functional groups [97]

Functional group	
Alcohol	Cinnamic Alcohol
Aldehyde	Cinnamaldehyde
Phenol	Chavicol, Eugenol
Methoxy derivatives	Anethole, Elemicine,Estragole, Methyleugenol
Methylenedioxy compound	Apiole, Myristicine, Safrole

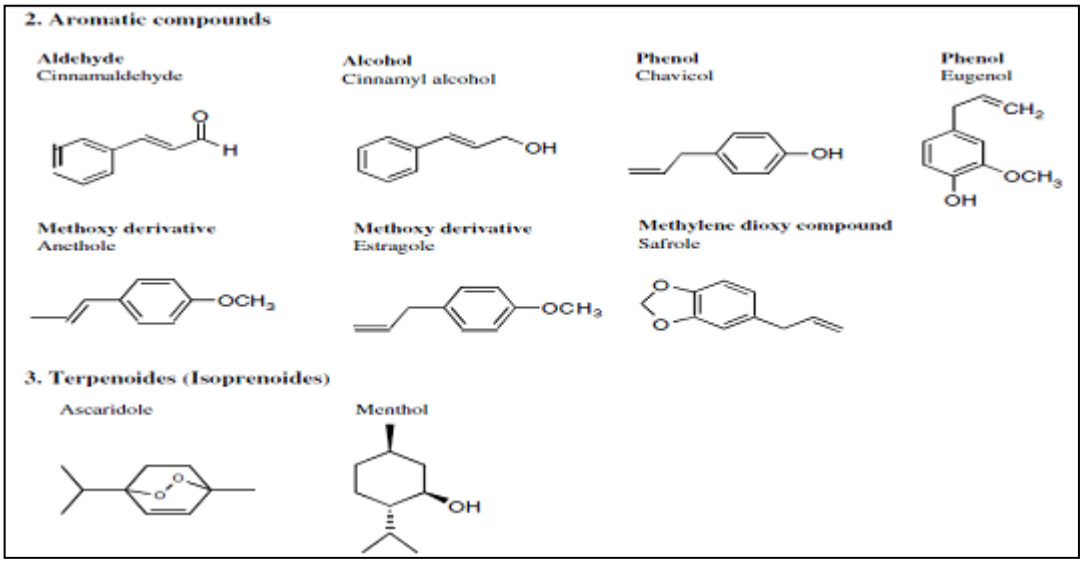

Figure 2.6. Chemical structures of some components of EO [97, 98]

2.4.3.6. Mechanisms Of Antimicrobial Activities Of Essential Oils

The components of essential oils, both major and minor, play an important role in its biological activities [94, 100]. Variation in the antimicrobial performance of the EO is related to differences in the volatile composition of the oil [95, 96, 97]. Several spices particularly garlic, ajowain, black pepper, clove, ginger, cumin and caraway are used extensively in the diet and medicine [96]. Studies have demonstrated that some essential oils(mint, lemon, bergamot EO of stems, leaves and flowers) can have high antimicrobial activity against a wide spectrum of both Gram (-) and Gram (+) pathogenic bacteria and fungal strains [97, 101, 102, 103, 104, 105, 106]. The antimicrobial activity is attributed to the phenolic content, which is present at very high percentages [107, 108]. An important characteristic of EOs and their components is their hydrophobicity, which enables them to enter between the lipids of the bacterial cell membrane and mitochondria, disturbing the structure and rendering them more permeable [109], thereby causing swelling. Increases in cytoplasmic membrane permeability appear to be a consequence of the loss of the cellular pH gradient, proton motive force and decreased ATP levels, resulting in the death of the cell [110, 111]. Leakage of ions and other cell contents can then occur [112]. Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents of, critical molecules such as ATP and ions lead to death [113]. It has been seen that the essential oil that has the strongest antibacterial properties

against pathogens, contain a high percentage of phenolic compounds such as carvacrol, eugenol, and thymol [63, 74, 101, 114, 115]. The biological precursor of carvacrol, p-cymene, is hydrophobic and causes an expansion of the cytoplasmic membrane. When combined with carvacrol in vitro, p-cymene incorporates into the cytoplasmic membrane, facilitating transport of carvacrol across the membrane [116]. Thus, the antimicrobial activity of carvacrol is increased by the presence of its precursor p-cymene, owing to the described synergistic effect [63].

Components of essential oils also appear to act on cell proteins embedded in the cytoplasmic membrane [117]. Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Cyclic hydrocarbons could act by two different mechanisms; lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and alter the lipid protein interaction or alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible [118].

Antimicrobial action of essential oils and their components have been generally thought against membrane structure and function [119]. Figure 2.7 shows the possible targets of essential oils on bacterial cell.

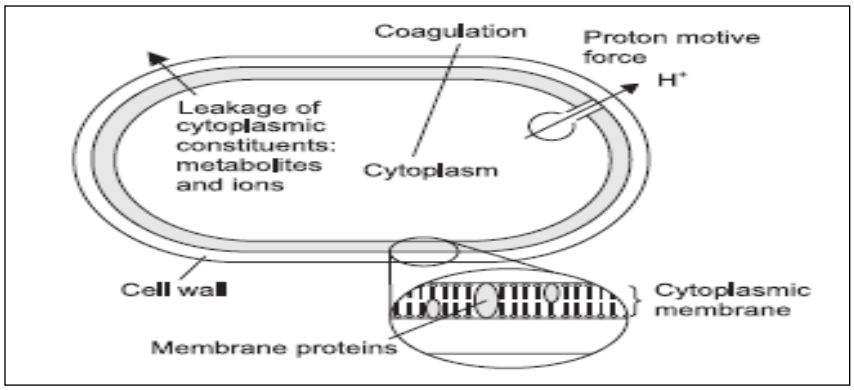

Figure 2.7. Targets of essential oil in the bacteria cells [99, 120]

EO acts on the bacterial cells by the cell wall, damaging the cytoplasmic membrane and membrane protein, causing leakage of cell contents, coagulation of cytoplasmic and depletion of the proton motive force [109, 113, 121].

2.4.3.7. Synergism And Antagonism Between The Components Of Essential Oils

The antibacterial activity of EO can be related to the chemical constituents of its component, the proportions in which they are present and their interactions [122, 123]. When the combined effect of the substances is higher than the sum of the individual effects, this is synergy. Antagonism is observed when a combination shows less effect compared to the individual applications [63].

Synergetic effects of some compounds, in addition to the major components in the EOs have been shown in some studies [99]. Applications of a certain combination of carvacrol-thymol can improve the efficacy of EOs against pathogenic microorganisms [124, 125]. Some studies have demonstrated that whole essential oil has a greater antimicrobial activity than the major components mixed [126]. This suggests that minor components are critical to the activity and may have synergetic effect [126]. The two structurally similar major components of oregano essential oil, carvacrol and thymol were found to give an additive effect when tested against Gram-positive and Gram-negative bacteria [127].

Synergism between carvacrol and its biological precursor acts on the cell wall. Even though p-cymene is a very weak antimicrobial agent, it destroys bacterial cell membranes at greater extent than carvacrol does. By this mechanism p-cymene probably enables carvacrol to be more easily transported in to the cell so that a synergetic effect is achieved when two are used together [116].

2.4.3.8. Satureja hortensis L.

The genus *Satureja (Lamiaceae)* is native to the Mediterranean region of Europe, western Asia, North Africa, The Canarian Islands and South America. The genus includes about 15 species of herbaceous perennial and subshrubs that can be found in Turkey. Five of them are endemic, including *S. aintabensis* [125, 128, 129, 130].

Figure 2.8. Images of *Satureja hortensis L* herbs [131]

Individual *Satureja* species (also known as summer savory) are locally named as ‘keklik otu’, ‘kılıç otu’, ‘firubu’,’çatlı’ or ‘kekik’ in the territory where they grow and are consumed as culinary or medical herbs in different regions of Turkey. Figure 2.8 represents the aerial parts of *Satureja* species [131]. In addition to summer savory's aromatic leaves, this fast-growing plant is spread all over with clouds of small pink or white flowers in spikes [128, 130]. It is used in the food industry as an aromatic and flavoring substance and is said to have anti-inflammatory antioxidant, antibacterial ,antifungal, fungicidal, antiviral, antispasmodic, and antidiarrhoel effects [80, 93, 94]. *S. hortensis* is also used as a muscle pain reliever, tonic for cramps, nausea and digestion by local people [68].

Savory species produce antimicrobial secondary metabolites, essential oils, either as a part of their normal program of growth and development or in response to pathogens attack or stress [132, 133, 134, 135]. Phytochemical studies on *S. hortensis* displayed that carvacrol and other monoterpene hydrocarbons in its essential oil, (flavonoids like apigenin and apigenin-4’-methyl ether and phenolic acids), were the main components of the aerial parts [136, 137]. The main constituents of the essential oil of *S.hortensis* are phenols, carvacrol and thymol as well as p-cymene, β-caryophyllene, linalool and other terpenoids [138]. The high percentage of carvacrol primarily accounts for its biological actions [137]. The chemical composition and yield of essential oil are influenced by many exogenous factors such as geographical origin, altitude, climate, soil make-up, variants of the species, part of

the plant used to extract oil, time of harvest, crushing intensity, distillation method, extraction time and duration of storage [107, 139, 140, 141]

2.4.3.9. ***Satureja*** **sp. Essential Oils**

Table 2.3. Literature studies on *Satureja* sp. EO

Satureja sp	**Collection Site and Date**	**Part Of The Plant**	**Drying Conditions**	**Extraction Method**	**Chemical Composition**	**Biological Activities**
S. biflora [137]	Mbeya,Tanzania, Flowering period , March, 2000	Aerial parts	Air-dried in the shade at room temperature	Hydro-distillation 3h	Monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpene hydrocarbons, others.	Antibacterial , Antimicrobial and Antifungal activities were tested by agar dilution technique.
S.masukensis [137]	Katumba-Mwakaleli, Rungwe district, Tanzania, March, 2000	Aerial parts	Air-dried in the shade at room temperature	Hydro-distillation 3h	Monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpene hydrocarbons, others.	Antibacterial Antimicrobial and antifungal activities were tested by agar dilution technique.
S.pseudosimensis [137]	Kawetere Mountain, Tanzania, March, 2000	Leaves and flowering tops	Air-dried in the shade at room temperature	Hydro-distillation 3h	Monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpene hydrocarbons, others.	Antibacterial antimicrobial and antifungal activities were tested by agar dilution technique.
Satureja hortensis L [139]	Nis, Malca, Serbia, The beginning of the flowering stage	Aerial parts	Air-dried in the shade at room temperature for 10 days	Hydro-distillation 3h	Oil was intensively yellow, strong and pleasant odor.	Antimicrobial Activity were tested by disc-diffusion and broth dilution assays
Satureja hortensis L [142]	National Botanical Garden of Iran (NBGI), June, 2006	Plant leaves	-	Plant leaves were Steam distilled for 60 min in a full glass apparatus, EO of leaves was extracted by hydrodistillation for 3h.	Carvacrol, thymol	Antifungal activity and inhibition of *A.parasiticus* growth.

Table 2.3. Literature studies on *Satureja* sp. EO (continued)

Satureja sp	**Collection Site and Date**	**Part Of The Plant**	**Drying Conditions**	**Extraction Methods**	**Chemical Composition**	**Biological Activities**
Satureja hortensis L [143]	Gaziler walley of Senkaya Erzurum, Turkey, 2003	Aerial parts (leaves, flowers, stems)	Dried in shade and powdered.	Soxhlet extraction for 72h at room temperature.	Essential oils	AntimicrobialActivity were tested by disc-diffusion and broth dilution assays
Satureja hortensis L [144]	West Mediterranean region of Turkey, in May, 2006.	Leaves and flowers	Air dried in shade for 2-4 days.	Steam extraction	Carvacrol,1,8-cineol/eucalyptol,camphorB-pinene,α-pinene,camphene	Antimicrobial Activity were tested by broth dilutionassays, Antibiofilm effects
Satureja montana L. Saturejacuneifolia [145]	Croatia, in 2002, during the flowering season	Aerial parts	Air-dried in shade	Hydro-distillation 3h	A-thujene, α-pinene, myrcene, α-terpinene, limonene, p-cimene,carvacrol,thymol	Antimicrobial Activity were tested by broth dilution assays
Satureja hortensis L [143]	Gaziler walley of Senkaya Erzurum, Turkey, 2003	Aerial parts (leaves, flowers, stems)	Dried in shade and powdered.	Soxhlet extractor for 72h at room temperature.	Essential oils	AntimicrobialActivity were tested by disc-diffusion and broth dilution assays
Satureja hortensis L [144]	West Mediterranean region of Turkey, in May, 2006.	Leaves and flowers	Air dried in shade for 2-4 days.	Steam extraction	Carvacrol,1,8-cineol/eucalyptol,camphorB-pinene,α-pinene,camphene	Antimicrobial Activity were tested by broth dilutionassays, Antibiofilm effects
Satureja montana L. Saturejacuneifolia [145]	Croatia, in 2002, during the flowering season	Aerial parts	Air-dried in shade	Hydro-distillation 3h	A-thujene, α-pinene, myrcene, α-terpinene, limonene, p-cimene,carvacrol,thymol	Antimicrobial Activity were tested by broth dilution assays
Satureja hortensis L [143]	Gaziler walley of Senkaya Erzurum, Turkey, 2003	Aerial parts (leaves, flowers, stems)	Dried in shade and powdered.	Soxhlet extractor for 72h at room temperature.	Essential oils	AntimicrobialActivity were tested by disc-diffusion and broth dilution assays

Table 2.3. Literature studies on *Satureja* sp. EO (continued)

Satureja sp	**Collection Site and Date**	**Part Of The Plant**	**Drying Conditions**	**Extraction Methods**	**Chemical Composition**	**Biological Activities**
Satureja hortensis L [144]	West Mediterranean region of Turkey, in May, 2006.	Leaves and flowers	Air dried in shade for 2-4 days.	Steam extraction	Carvacrol,1,8-cineol/eucalyptol,camphorB-pinene,α-pinene,camphene	Antimicrobial Activity were tested by broth dilutionassays, Antibiofilm effects
Satureja rechingeri [146]	Ilan, Iran, altitude 600 m, in August(beginning of flowering) and November(full flowering), 2004	Aerial Parts	Air-dried in shade	Hydro-distillation 3h	Carvacrol,p-cymene, α-cymene, α-thujone,etc.	-
Satureja mutica [147] *Satureja macrantha* *Satureja intermedia*	Khorassan, Azarbayejan, Ardebil, Full flowering stage	Aerial arts	Air-dried in shade	Hydro-distillation 3h	Carvacrol, thymol, p-cymene, limonene, etc.	-
Satureja boissieri [148] *Satureja coerulea* *Satureja icarica* *Satureja pilosa*	Adıyaman, 2001 Kırklareli, 2001 Gökçeada, 2001 Balıkesir, 2001	Aerial parts	Air-dried in shade	Hydro-distillation 3h	Carvacrol, p-cymene, γ-terpinene, borneol, limonene	Antimicrobial Activity were tested by broth dilution assays. and fungal spore inhibition assay
Satureja hortensis L [141]	Mersin, 2004	Aerial parts	Air-dried in shade	Hydro-distillation 3h	Essentia oils	Antimicrobial activity was tested
Satureja hortensisL.(1) *Satureja hortensisL.(2)* *Satureja macrantha* *Satureja cuneifolia Ten* *Satureja thymbra L.* *Satureja aintabensis* [149]	Malatya, 1999 K.maras, 2001 Erzurum ,1999 Izmir, K. M., 1995 Izmir, Kiraz, 2001 Gaziantep, 2001	Aerial parts	Air-dried in shade	Hydro-distillation 3h	Thymol, carvacrol, γ-terpinene, borneol, limonene, isothymol, Linalol, Myrcene, Terpinen-4-ol	Antimicrobial and antifungal Activity were tested by disc-diffusion and broth dilution assays

Table 2.3. Literature studies on *Satureja* sp. EO (continued)

Satureja sp	**Collection Site and Date**	**Part Of The Plant**	**Drying Conditions**	**Extraction Methods**	**Chemical Composition**	**Biological Activities**
Satureja hortensis L [150]	Erzurum, Turkey, 2007	Aerial parts	Air-dried in shade and grounded	Hydro-distillation 4h	Camphor,1,8-cineole, borneol, piperitone, sabinaketone	Insecticidal activity of EO was tested
Satureja hortensis L [79]	Isfahan, Iran, 1998	Aerial parts (leaves and flowers)	Air-dried in shade	Hydro-distillation 4h	Esssential oil	Analgesic activity and inflammatory activity
Satureja hortensis L. (Summer Savory) [151]	Portugal, in June, 1997	Except stalks the remaining parts	Air-dried	Supercritical fluid extraction	Carvacrol, γ-terpinene	Antioxidant activity
Satureja hortensis L [152]	Zabol, Iran,April, 2010	Aerial parts	Air-dried in shade	Hydro-distillation 4h and Supercritical fluid extraction	Essential oil	Optimization of process for EO components from *S.hortensis.*
Satureja thymbra [153]	Marmaris, Turkey, in May, 2009	Whole plant material	-	Hydro-distillation 4h and extracted with methanol at room temperature	Essential oil	Anticholinesterase and Antioxidant activity
Satureja hortensis L [154]	Erzurum, Turkey, in July, 2006	Aerial parts	Air-dried in shade and grounded	Soxhlet extraction for 72h and water distillation for 3h	Essential oil	Antifungal activity

According the literature review, *Satureja* species are well-known aromatic and medical herbs. Aromatic plants owing to their antiseptic properties, are used as spices and natural food preservatives, in the perfume industry, for aromatherapy and for different medical purposes. Among the aromatic plant species, the genus *Satureja L*. engages a special position. *Satureja* includes more than 30 species belonging to the family of Lamiceae. Also, distribution of the genus *Satureja* overlaps the region of southern and south-eastern Europe, Asia minor and Nothern Africa, with the predominance in the Mediterranean. It is known that the chemical composition and yield of EO are affected by several factors such as geographical position, altitude, climate, soil composition, drying method, extraction method.

Together with exogenous factors, the quality and quantity of EO are also affected by endogenous factors [155]. Many study concluded that drying of the aerial parts of *Satureja hortensis* in shady or dark places is more efficient, while extraction of EO by hydrodistillation is recommended. Beside this, many study showed that the highest content of phenolic compounds was in the oil isolated from material collected during the full flowering stage [147]. A literature review of studies on *Satureja* EOs including collection site, part of the plant, drying conditions, extraction methods, chemical compositions and biological activities are presented in Table 2.3.

2.5. LIPOSOMES

2.5.1. Definition, Formations and Classification of Liposomes

Liposomes were first investigated by Bangham and co-workers in 1965 at Cambridge University [156] and the first drug based on liposomes was presented to the market in 1996 [156, 157]. Liposomes are spherical microscopic dispersions with a thin lipid like membrane with an aqueous cavity at their centre. The cover is made of concentric biomolecular sheets (lamellae) composed of phospholipids, the main constituents of cell membranes. Phospholipids are amphipilic with a hydrophilic head and a lipophilic tail. In aqueous solutions, they resemble artificial cell membranes. The fatty acid tails are non polar and arranged towards the interior of the membrane, whereas the hydrophilic heads line up outward of the membrane. The number of the coating sheets may vary, leading to

typical sizes of 15-3500 nm, depending on the number of layers and are distinguished as small unilamellar vesicles (SUV) or large multilamellar vesicles (LMV) [122, 123].

Liposomes are largely related to lipid characteristics such as charge, size, saturation and length of the acyl chains and the characteristics of the encapsulated molecule itself (molecular mass, solubility, charge, hydrophobicity, etc). Other considerations are the method of vesicle production, drug encapsulation methodology, and the addition of molecules that are able to crosslink the membrane [158]. Effective liposomal encapsulation of small-molecular-mass molecules (most of the used therapeutic agents belong to this class of molecules) can be performed employing two different loading procedures. The first is passive loading, which is adding active substances into a liposome formulation during the preparation of the vesicle. The other is active loading where the active substance is added to preformed liposomes [159].

In terms of membrane permeability the size of the vesicle is very important. The most stable size of liposomes are between 100 and 200 nm in diameter and they display better drug retention than larger liposomes of the same composition. Saturation of the lipids influence membrane integrity, the release kinetics throughout the membranes increase with the degree of unsaturation of the acyl chains [160]. Cholesterol has also been found to increase the packing density of liposomes and to decrease the bilayer permeability [160, 161].

Liposomes encapsulate a part of the solvent, in which they freely diffuse (float) into their interior [145, 146]. Upon interaction with water, polar lipids self-assemble and form self-organized colloidal particles. A cross-section of a liposome (Fig 2.9) shows the hydrophilic heads of the amphipilic vesicle orienting towards the water compartment while the lipophilic tails orient away from the water towards the center of the vesicle, thus forming a bilayer. Consequently, water soluble compounds aggregate in the lipid section. Exclusively liposomes can encapsulate both hydrophilic and lipophilic materials [158].

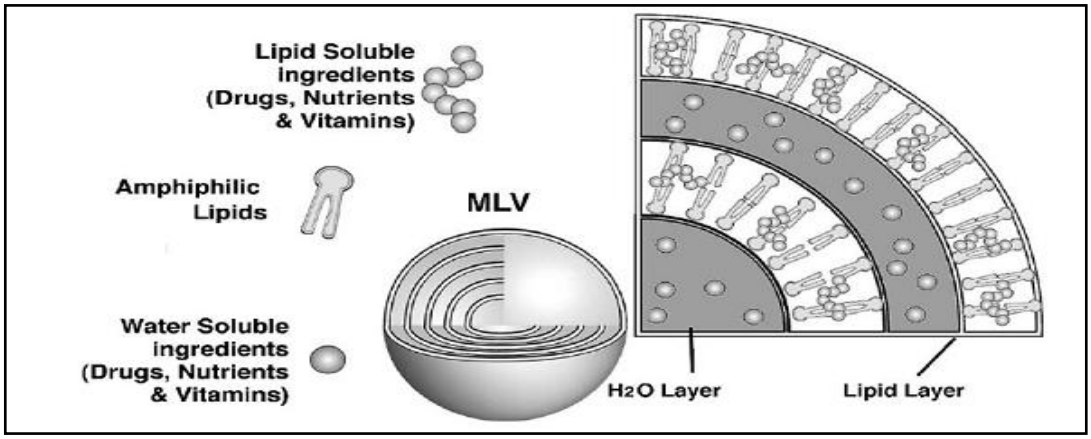

Figure 2.9. Cross-section of a liposome [153]

Liposomes are similar to the lipid membrane part of cells. Several biological processes in living organisms depend on the action of small uni-lammelar liposomes. Typical compositions involved phosphatidyl cholines, phosphatidyl ethanolamines, frequently containing negatively charged lipids such as phosphatidyl serine and phosphatidyl inositol. In addition, ceramides such as sphingomyelin, sterols (cholesterol, ergosterol, sitosterol, etc.) are also incorporated [162]. With respect to the number of lamellae and size of liposomes, small unilamellar vesicles (SUV) have a size < 0.1 μm, large unilamellar vesicles (LUV) have a size range of 0.1-1 μm, and large multilamellar vesicles can be up to 500 μm in diameter and contain hundreds of concentric bilayers [163]. Liposomes are composed of relatively biocompatible and biodegradable material, and they consist of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids. (Figure 2.9) [163].

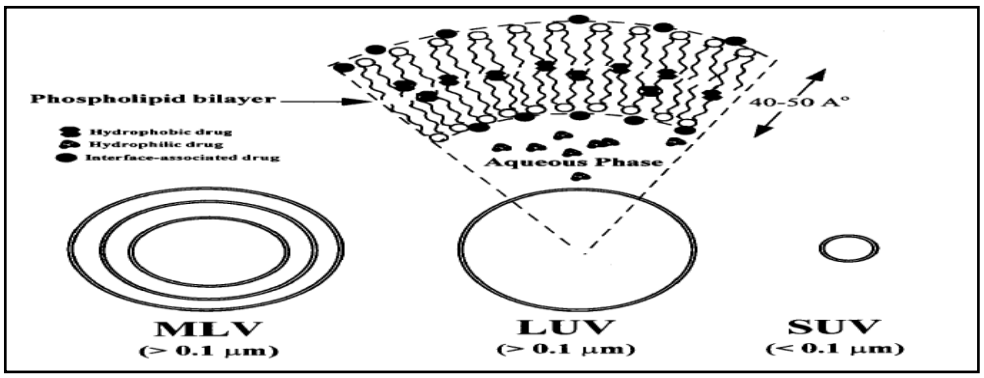

Figure 2.10. Type of liposomes depending on size and number of lamellae [167]

2.5.2. Liposome Applications

Liposome vesicles are particularly used as a model for drug delivery carriers in basic research, due to their composition and structure. Liposomes are used in applications ranging from drug and gene delivery to diagnostics, cosmetics, long-lasting immune contraception and the food industry, due to their biocompatibility, biodegradability and absence of toxicity [164].

2.5.3. Liposomes as Drug Delivery Carriers to Microbial Infections

Nanoencapsulation of bioactive compounds characterize a viable and efficient approach to increasing physical stability of EO's active substances [165]. While microcapsules may assure excellent protection for EOs against degradation or evaporation, they do not generally respond to antimicrobial activity against microorganisms; whereas, nanometric size delivery systems improve cellular fusion mechanisms. As a consequence, excellent antimicrobial activity can be observed against microbial pathogens [142]. This has been shown previously with the encapsulation of eugenol and carvacrol into nanometric surfactant micelles resulting in enhanced antimicrobial activity [158].

One of the most important properties of the oral mucosa is its selective permeability. This feature enables its utilization for systemic and local transfer of some drugs. The basic goal for liposomal vehicles carrying antimicrobial EO's is that active substance should be able to reach the target area in the right time, in the right concentration and at the right ratio [166]. Liposomal formulations have been used both to enhance absorption and to regulate release of incorporated drugs, thus localizing the effect of the drugs [167, 168]. Liposomes incorporating EO's have been shown as an appropriate instrument for successful application of local drug delivery systems in the oral cavity [160].

2.5.4. Antimicrobial Activity of EO incorporated Liposomes

The antimicrobial activity of many EOs is known to be due to the phenolic constituents of the EO [116]. Unfortunately, most natural compounds are biologically instable, poorly soluble in water and they distribute poorly to target sites. Currently, novel methods have

been introduced in order to improve their stability and their bioavailability, amongst which is the use of liposomal encapsulation [164].

Liposomes are useful drug delivery carriers for antimicrobial therapies owing to the potential to carry and deliver the entrapped EO across the cell membrane. Therefore, liposomal drug delivery carriers have been extensively studied as a carrier system which can increase the activity and safety of many drugs [77].

In the literature, liposomal incorporation of several plant EO and their in vitro antimicrobial activities were studied [77, 169, 170].

The effect of liposomal inclusion on the in vitro antiherpetic activity of *Artemisia arborescens L*. EO was investigated. In order to study the influence of liposome vesicle structure and composition on the antiviral activity of the vesicle-incorporated oil, Multilamellar (MLV) and Unilamellar (SUV) positively charged liposomes were prepared by the thin film hydration method and sonication. Also, liposomes included hydrogenated (P90H) and non-hydrogenated (P90) soy phosphatidylcholine. Then, antiviral activity was tested against *Herpes simplex* virus type-1 (HSV-1) by a quantitative tetrazolium-based colorimetric method. Results showed that *Artemisia* EO was successfully incorporated into the prepared liposomes. Antiviral assay results showed that that the liposomal incorporation of *Artemisia arbosencens* EO increased its in vitro antiherpetic activity especially when the liposomal vesicles were made with P90H. Conversely, no significant antiviral activity was observed between free and SUV-incorporated oil [77].

Another study demonstrated that liposomal incorporation of carvacrol and thymol isolated from EO of *Origanum dictamnus L*. had antimicrobial activity.. The chemical composition of the EO from *O. dictamnus* was determined by GC-MS. Then, carvacrol, thymol, p-cymene and γ- terpinen were identified as major constituents and isolated. These components were successfully encapsulated into phosphatidylcholine- based liposomes and then the possible enhancement of the antimicrobial activity was determined against four Gram positive and four Gram negative bacteria and three human pathogenic fungi, in addition to the food borne pathogen. All tested components presented antimicrobial activity after the encapsulation [169].

Incorporation of *Anethum graveolens* EO into liposomes was also studied in the literature. The influence of liposomes composition, size and lamellarity on the entrapment efficiency of EO was studied. For this purpose, MLV and SUV liposomes were prepared by the thin film hydration methods. The stability of liposome vesicles and vesicle dispersions were characterized for their oil content and average size distribution. Results showed the incorporation of *Anethum graveolens* EO in liposomes in good amounts when suitable formulation are used. The EO incorporated liposomes and its composition was approximately stable over one year, size distribution demonstrated also slight modification, especially SUV [170].

Also, in the literature interactions between cationic loaded liposomes and bacteria were investigated [171]. Liposomes were used as carriers for antimicrobial and anticancer agents in this study [171]. Small unilamellar DODAB vesicles (SUV) were prepared by ultrasonic dispersion. Susceptibilities of different bacteria species towards DODAB cationic vesicles were determined. Also, interaction between cationic liposomes and bacteria, the physical chemistry of the bactericidal action was determined [171]. Results showed that negatively charged cells were 100 % viable whereas positively charged cells did not survive. The results demonstrated a clear correlation between simple adsorption of entire vesicles generating a positive charge on the cell surfaces and cell death [171].

3. AIM OF THE PROJECT

The aim of this study was to assess the possibility of incorporating EO of *S. hortensis L.* into two novel liposome formulations prepared according to the Bangham method. Furthermore, to characterize these liposomes (mean size, zeta potential, poly dispersitive index using different microscopic techniques, (PLM and AFM) and evaluate their antimicrobial activity against oral pathogens using agar well diffusion assay.

4. MATERIALS

4.1. GROWTH MEDIA

Brain Heart Broth (BHB) (Salubrus, Massachusettes, USA), Fastidious Anaerobe Broth (FAB) (LabM, UK), Fastidious Anaerobe Agar (FAA)(LabM, UK) and Brain Heart Infusion (BHI) 5 % Sheep Blood Agar (Salubris, Massachusettes, USA) were employed for the cultivation of oral bacteria and Saboaraud Dextrose Agar (SDA) (Merck, New Jersey, USA) and Saboaraud Dextrose Broth (SDB) (Merck, New Jersey, USA) were used for yeast. All media were prepared according to the manufacturer's instructions.

4.2. CHEMICALS AND SOLUTIONS

4.2.1. Chemicals for Essential Oil Extraction

Anhydrous sodium sulphate powder (Sigma, Germany) and xylene (Sigma, Germany) were used for essential oil extraction.

4.2.2. Chemicals for Preparations of Liposomes

Phospholipon P85G (PL85G) (Lipoid, Germany), stearylamine (SA) (Sigma, Germany), cholesterol (CH) (Merck, Germany), chloroform (0.1%) (Sigma, Germany), ethanol (Sigma, Germany) were used for preparation of liposomes.

4.2.3. Chemicals and Solutions for Antimicrobial Tests

4.2.3.1. 1X Phosphate Buffered Saline (PBS) Solution (pH 7.4)

For the preparation of 1X PBS solution at pH 7.4 8.18 g of NaCl (Merck, Germany), 0.186 g of KCl (Appli.Chem, Germany), 1.136 g of Na_2HPO_4 (AppliChem, Germany) and 0.272 g of KH_2PO_4 (AppliChem, Germany) were dissolved in 800 ml of sterile distilled water. After adjusting the pH of the solution to 7.4, the final volume was completed to 1 liter. The solution was, then autoclaved.

4.2.3.2. Mc. Farland No: 0.5 Standard Solution

To prepare the standard solution, first of all stock solutions of 1 % barium chloride ($BaCl_2$) and 1 % sulfuric acid (H_2SO_4) were prepared as follows: 10 ml of 1 % $BaCl_2$ stock solution was prepared by dissolving 244 mg of $BaCl_2$ powder (AppliChem, Germany) in 10 ml of sterile distilled water. 100 ml of 1 % H_2SO_4 stock solution was prepared by taking 1.05 ml of H_2SO_4 (95-97 %) (Sigma, Germany) and adding it to 99 ml of distilled water.

0.5 McFarland standard was prepared by mixing 0.05 ml of 1.175 % Barium Chloride dehydrate ($BaCl_2$.2HO) with 9.95 ml of 1 % Sulfuric acid. The absorbance of this mixture was then measured at 600 nm (OD 600nm = 0.132) by microplate reader (Thermo Lab Systems, Germany).

4.2.3.3. Brain Heart Broth With 0.1 % Tween 80

Tween 80 is a nonionic emulsifier which facilitates the mixing of immiscible broth medium and essential oil. Brain heart broth (BHB) was prepared and sterilized. 50 µl of BHB brain heart broth was taken and discarded from 50 ml of BHB medium. 50 µl of Tween 80 (Sigma, Germany) was added to BHB to make a final concentration of Tween 80 0.1 % (Sigma, USA) in 50 ml of BHB.

4.2.3.4. Brain Heart Broth With 1 % Sucrose

Stock solution of 10 % sucrose solution was prepared by weighing 20 g of sucrose (Bio.Basic, Canada) and dissolving it in 200 ml of distilled water. BHB medium with 1 % sucrose was prepared as follows; 50 ml of 10 % sucrose solution was added into 450 ml of BHB, while passing the sucrose through 0.25 µm filters (Whatman, USA).

4.2.3.5. Brain Heart Broth With 0.1 % Tween 80 and 1 % Sucrose

50 ml of Brain Heart broth with 1 % sucrose was prepared and placed in a sterile falcon tube. Then 50 µl was discarded and replaced with 50 µl of Tween 80. This resulted in BHB medium with 0.1 % Tween 80 and 1 % sucrose.

4.2.3.6. Methanol

Methanol (≥99.6%, Sigma, USA) was used as a solvent. *S. hortensis* L. essential oil was dissolved in methanol and then diluted to different concentrations.

4.2.3.7. Plant Material

The aerial parts (flowers, leaves, stems, twigs, and roots) of *S. hortensis* L. herb were collected from the province of Erzurum at the end of the summer in August 2010. All the plant samples were stored in a dark and cool place until use.

4.2.3.8. Laboratory Equipments

Shaker (BS-T, Sartorius, Aubagne, France), Rotator (SB3, Stuart, Staffordshire, USA), Vacuum machine (GmbH, Ilmvac, Ilmenau, Germany), spectrophotometer (Mutiskan Spectrum, Thermo, Massachusetts, USA), Anaerobic workstation, (Don Whitley Scientific, UK), 50 ml falcon centrifuge (J-25 I, Beckman, Brea, USA), microcentrifuge (Sigma-Aldrich, Taufkirchen, Germany), autoclave (Hirayama, Saitama, Japan), laminar flow(HFsafe-1200, Heal Force, Shanghai, China), incubators (Memmert, ShellaB, Forest Grove, USA), and Malvern Zetasizer instruments Nano ZS (Worcestershire, United Kingdom), GC (Thermo Fisher Scientific, USA) Vortex (Heidolph,Germany), Clevenger apparatus (Inter Lab, Turkey), Atomic force microscopy (AFM) (Shimadzu, Japan), Polarized Light Microscopy (PLM) (Nikon, USA), Lyophilizator (Christ, Osterode, Germany), Vacuum incubator (Thermo Scientific, USA), Rotovapor, (Heidolph , Germany), Sonicator (Sonorex,Germany), Waterbath (OLS200, Grant, Cambridgeshire, England), Lab Scale (Ohous, UK), Extruder (Kanotz, USA), 20^{o}C Refrigerator (Beko, Turkey), Polycarbonate filter (Whatman, USA), turbidimetry (BIOLOG, Hayward CA) were used.

4.2.3.9. Laboratory Consumables

Elisa plates, pipettes, micropipettes, pipette tips, petri plates, plastic and glass erlenmeyers, beakers, eppendorfs, Bunsen burner, falcon tubes, pestle and mortar, paraffin, masks, napkins, scissors, standard ruler and gloves were used in different parts of the experiment.

5. METHODS

5.1. EXTRACTION OF *S. HORTENSIS L* ESSENTIAL OIL (EO) BY HYDRODISTILLATION

Figure 5.1. Clevenger apparatus for essential oil extraction

Leaves and flowers of *S.hortensis* L. were collected from the Eastern Anatolian region of Turkey in August 2010. Plant samples (aerial parts) were air-dried in a dark place at room temperature for 10 days. Following drying process, plant samples were stored for 1,5 months at room temperature. Then, the dried plant samples were transferred from Erzurum to Istanbul in order to use for the experimental analysis. Plants (Dried plant samples (3kg)) were crushed by pestle and mortar and a total of 120 g was placed into four 500 ml round bottom flasks (30g each) and 400 ml of sterile distilled water added to each flask. Plant parts including leaves, flowers, branches and roots were processed together. Plants were then subjected to hydrodistillation using Clevenger apparatus (Interlab, Turkey) for 3 h (Fig 5.1).

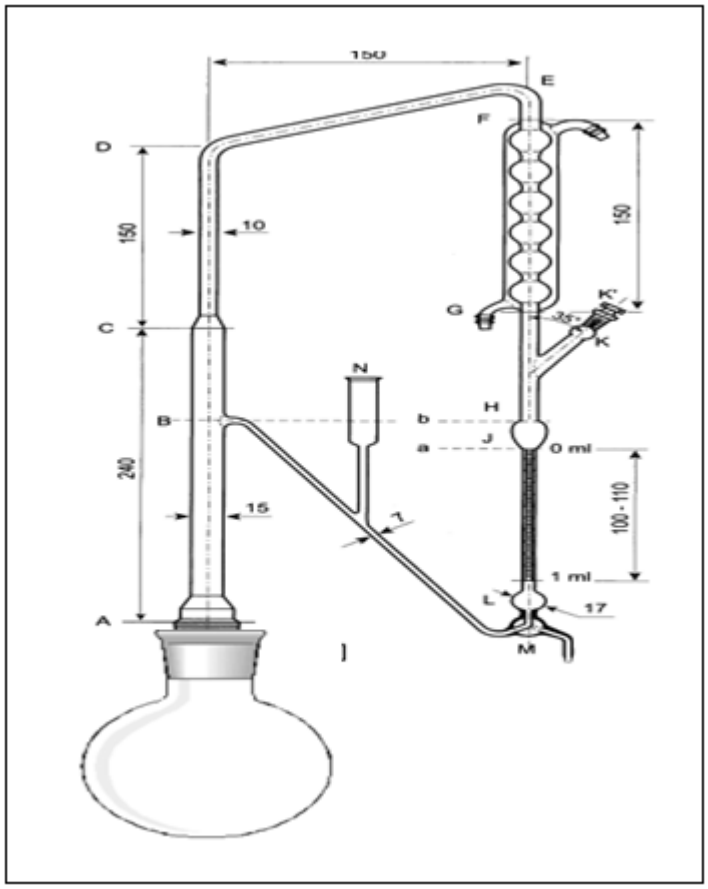

Figure 5.2. Schematic diagram of Clevenger apparatus (dimensions are shown in millimeters) [172, 173]

The mechanism of the Clevenger apparatus is shown in Fig 5.2. The plant sample was placed into the round bottom flask (I part). Distilled water was poured into the clevenger apparatus through the funnel 'N' until the water became equal at 'B' and 'H' points. 0.5 ml of xylene was added to the apparatus from the tube 'K' to entrap the extracted essential oil. When xylene is added, it flows on the water surface and during distillation, the little amount of extracted EO was collected in xylene After adding xylene, the 'K' part was closed with aluminum foil. Hydro-distillation was carried out for 3 hours in order to achieve maximum recovery of EO [138]. As soon as the distillation process was completed, essential oil (EO) was collected from the 'K2 tube by means of a glass pippet. Anhydrous sodium sulphate was then added to the mixture of essential oil, xylene, and water (H-J). Anhydrous sodium sulphate absorbed the residual water and xylene from the essential oil. Xylene also increases the yield of EO extract. EOs were then collected in glass vials, which were sealed with aluminum foil and stored in -20 $^{\circ}$C to be used later.

5.2. CALCULATION OF EO DENSITY AND YIELD

The relative density of the essential oil was determined using the gravimetric method [101] as follows: Mass of 10 ml distilled water was determined (m_3) using Lab Scale (Ohaus, UK). Then, 1 ml of distilled water was discarded, and mass of the remaining 9 ml was determined (m_1). 1ml essential oil was added into 9 ml sterile distilled water. Mass of this water and oil mixture (10 mL) was also determined (m_2). The relative density of the essential oil was then calculated according to the equation [101]:

$$d = \frac{m_2(mg) - m_1(mg)}{m_3(mg) - m_1(mg)}$$

Table 5.1. Explanation of Equation for Calculation of Essential Oil Density

d	Relative density of essential oil
m_1	Mass of 9 ml distilled water (mg)
m_2	Mass of 10 ml essential oil and distilled water mixture (mg)
m_3	Mass of 10 ml distilled water (mg)

Extraction yield was calculated as the of the weight of the crude extract to that of the raw material (120g) as follows :

$$\text{Extraction yield } (\%) = \frac{\text{weight of the essential oil extract (mg)}}{\text{weight of the original sample (mg)}} \times 100$$

5.3. SAMPLE PREPARATION FOR GC

Two hundred (200) µl *S. hortensis* L. extracted EO extract was dissolved in 800 µl hexane (≥ 85 %) (Sigma, USA) in order to dilute the EO. Following dilution with hexane, 200 µl of the sample solution was used for gas chromatography analysis.

5.4. GAS CHROMOTOGRAPHY ANALYSIS

A Thermo ISQ (Thermo Fisher Scientific, USA) GC-MS QP5050A system with CP-sil 5CB column (25mx0.25mm i.d.0.4 µ L film thickness) was used with helium as carrier gas. The GC oven temperature was kept at 60 °C and programmed to 260 °C at a rate of 5 °C/min, and was then kept constant at 260°C for 40 minutes. The split flow was adjusted to 50 ml/min. The injector temperature was at 250°C. In the mass spectrometry (MS) analysis of an electron impact source with the ionization energy set at 70 eV was used. A total ion current (TIC) chromatogram was produced by scanning between m/z 30 and 425. Identification of EO components were achieved from the TIC chromatogram, the mass spectra of the EO components were automatically searched using the in-house WILEY7 Library of the Essential Oil Constituents of the equipment [174, 175] by comparison of the data base including mass spectra of fragment of those chemicals [176, 177, 178]. Peaks were identified using GC/MS. The peaks were detected Mass spectral Data base having mass spectra of the chemicals [179].

5.5. PREPARATION OF LIPOSOME FORMULATIONS

Unloaded liposomes were prepared according to the thin film hydration method [156]. There are different liposome preparation method [180]. Liposomes containing stearylamine, cholesterol and phospholipids in varying amounts (Table 5.1) were dissolved in 40 ml of chloroform solvent (≥99.8% (chloroform + ethanol)) (Sigma, USA) containing 0.1 % ethanol in a 50 ml round bottomed flask [156]. All these ratios and substances were chosen according to thin film hydration method [156]. The solvent mixture was evaporated under reduced pressure (≤ 20 Bar nitrogen pressure) at room temperature using a rotary evaporator (Heidolph, Germany) to obtain a thin film. The flask was then incubated using the vacuum incubator (Thermo Scientific, USA) at 25°C for 24h. The thin film was hydrated with 40 ml sterile distilled water at room temperature. The film was then sonicated using a sonicator (Soniprep 150, MSE, Crowley) for 15 min. Then the unloaded liposomes were extruded by way of an extruder (LF-50) (Kanotz, USA) using a polycarbonate filter (200nm and 400nm; Whatman, Canada) in order to obtain a homogeneous and relatively smaller size dispersion under a nitrogen stream (Havas, Turkey). The extruder was connected to a nitrogen gas tube (Havas, Turkey). Finally, the

liposomes were freeze dried at – 80°C using a freeze drier (Christ, Osterode, Germany) and stored at -20°C, until further use.

Table 5.2. Different Liposome Formulations

	PHOSPHOLIPID (Type & Molarity)	**STEARLYAMINE Molarity(M)**		**CHOLESTEROL Molarity(M)**		**ESSENTIAL OIL**
LIPOSOME CODES	PL 85G (7M) (mg)	PL85G (10M) (mg)	SA (1M) (mg)	CH (2M) (mg)	CH (4M) (mg)	EO (mg)
UL1	19.25	Ab	1.075	3.10	Ab	Ab
UL 2	Ab	27.50	1.075	Ab	6.2	Ab
L1	19.25	Ab	1.075	3.10	Ab	911
L2	Ab	27.50	1.075	Ab	6.2	911

UL:Unloaded liposome
L: Loaded liposome
Ab: Absent (Not added)
M. Molarity

5.6. INCORPORATION OF EO INTO LIPOSOMES

Following preparation of unloaded liposomes (UL) by the thin film technique (Table 5.1) freeze dried liposomes were hydrated by dispersing in 5ml of sterile distilled water. One ml of the unloaded liposome dispersion was then placed in a sterile glass tube and an equal amount of EO was incorporated into the dispersed (hydrated) liposome by active loading. The final liposomal dispersion of EO was thoroughly vortexed until a milky appearance was obtained. Finally, liposomes (vesicles) containing EO were stored at 4°C until reuse.

5.7. CHARACTERIZATION STUDIES OF LIPOSOMES: MEAN PARTICLE SIZE, SIZE DISTRIBUTION, POLYDISPERSITY INDEX AND ZETA POTENTIAL

The EO loaded liposomes were characterized by way of zeta size determinations and zeta potential measurements, as they were extruded, in order to obtain homogenous particle size distribution. Small unilammelar vesicles always provide better properties for liposomal applications. The particle size and zeta potential of loaded liposomes were determined for both loaded and unloaded liposomes by Dynamic Light Scattering (DLS) (MALVERN,

USA). Mean particle sizes went in line with intensity-based data (DTS, Nano Application, MALVERN, USA). The mean particle size and size distribution together with poly-dispersity index data were obtained by DLS software and all data was statistically evaluated by Minitab 16 Statistical Software (Inova, UK). The intensity mean diameter of loaded liposomes size distribution was determined by photon correlation spectroscopy using Zeta sizer (Malvern instruments Ltd., USA) at 25°C with 90° scattering angle and statistically evaluated by Minitab 16 Statistical Software (Inova, UK). All experiments were carried out intriplicate.

5.8. MORPHOLOGICAL CHARACTERIZATION OF LIPOSOMES

5.8.1. Polarized Light Microscopy (PLM) Studies of Liposomes

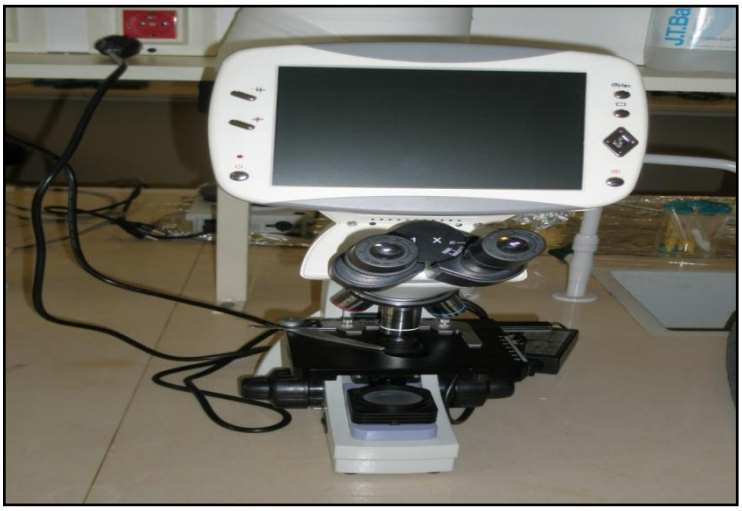

Figure 5.3. Polarized light microscope

Optical and light polarized micrographs were obtained by an optical microscope (Zeiss Axioplan 1, USA), at 25°C (Fig 5.3). A drop of hydrated liposome sample was placed onto a sterile glass slide and covered with a coverslip. Morphological observation were made using polarized light at 100 X magnification of the microscope using immersion oil, in order to confirm vesicular formation and determine vesicle type (Multilamellar vesicles (MLV), Small unilamellar vesicles (SUV), etc). Images were obtained using a bright-field with either the monochrome or coloured camera as appropriate. Axiovision software (Carl Zeiss Microscopy, Germany) was employed for image capture.

5.8.2. Atomic Force Microscopy (AFM) of Liposomes

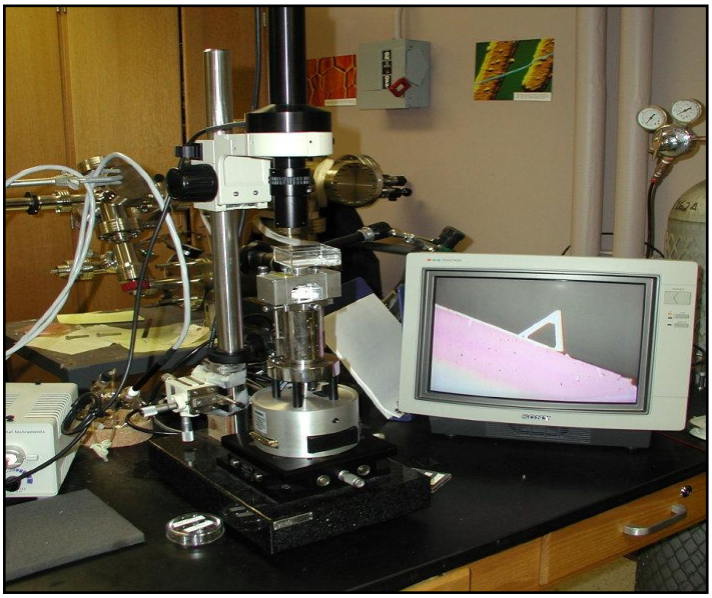

Figure 5.4. Atomic force microscope

AFM (Park Systems XE-100, Korea), was used to evaluate the physical characteristics (morphological and metrology) of the prepared liposomal formulations. Five µl of each liposome formulation was dropped onto individual glass slides and fixed by drying at room temperature. The AFM images of samples were obtained in noncontact mode at room temperature. Silicon nitride tips (Nano and More, Germany) were used with varying resonance frequencies (0.75, 6.5, 25, 35, 135, 200, 680, 1000, 1400 kHZ) at a linear scanning rate of 0.75 Hz. All measurements were taken using the Atomic Force Microscopy shown in Fig.5.4

5.9. TESTED MICROORGANISMS

The antimicrobial activity of *S.hortensis* L. EO loaded liposomes were tested against several oral microorganisms. Test microorganisms were selected based on their known association with oral infections. Species and strains studied were: *Aggregatibacter actinomycetemcomitans* FDC Y4, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 25586 which were kindly donated by Dr. Philip Bird, Queensland University, Australia. *Streptococcus mutans* and

Candida albicans were supplied from -80°C culture collection of Yeditepe University, Genetic and Bioengineering Department.

Test microorganisms were activated from -80°C stock culture by subculturing on Saboaraud Dextrose Agar (SDA) (Merck, New Jersey, USA) for *C. albicans* and both Brain Heart Broth (BHB) (Salubrus, Massachusettes, USA) and 5 % Brain Heart Infusion Sheep Blood Agar (Salubris, Massachusettes, USA) for all other test microorganisms. Yeast and bacteria were incubated aerobically at 37°C for 48 h and anaerobically at 37°C for 48-72 h in an anaerobic workstation (Don Whitley scientific, UK) containing10 % CO_2, 10 % H_2, and 80 % N_2 gas mixture (Havas, Turkey) respectively.

5.10. ANTI-MICROBIAL ACTIVITY TESTING

5.10.1. Agar Well Diffusion Assay

The antimicrobial activity of *S.hortensis L.* EO loaded liposomes (L1 and L2) were tested against six oral microorganisms and a mixed combined culture, by way of the agar well diffusion assay [168, 185]. Following 24 h aerobic incubation for *C. albicans* and 48 h for *S. mutans* at room temperature and 48 h anaerobic incubation for *F. nucleatum*, *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* at 37°C, a loopful of bacteria was inoculated into 2 ml of 1X phosphate buffered saline solution (pH 7.4) (Gibco, USA) and vortexed to obtain a homogenous suspension. A stock solution was prepared by adjusting the bacterial suspension to McFarland No. 0.5 ($2x10^8$cfu/ml) standard solution using a turbidity meter (BIOLOG, Hayward CA). A mixed culture was obtained by adding 100 µl of each bacterial stock solution together and adjusting the turbidity with PBS to reach a final suspension of $2x10^8$cfu/ml.

One hundred µl of the stock solution was inoculated onto the appropriate media with 15 µl of the (100 % concentration of EO with a density of 911 mg/ml) of free EO, unloaded liposomes (UL1 and UL2) and loaded liposomes (L1 and L2). Due to the hydrophobic nature of the free EO, 15 µl of 10 % (v/v) methanol solution (≥99.6%, Sigma, USA) was used as an emulsifier for free EO in order to increase its dissolving capacity. Furthermore, 0.1 % Tween 80 was added to loaded liposomes as an emulsifier in order to increase the

liposome surface zeta potential, stability of the liposome and penetration rate across the biological membrane due to its surfactant structure [168]. Negative and positive controls were used on each plate. Negative controls included sterile distilled water, unloaded liposomes and 10 % methanol. Positive controls were free EO and chlorhexidine gluconate (CHX) (0.12 mg/ml), (Kloroben, Drogsan, Turkey). A schematic diagram of the experimental design is shown in Figure 5.6.

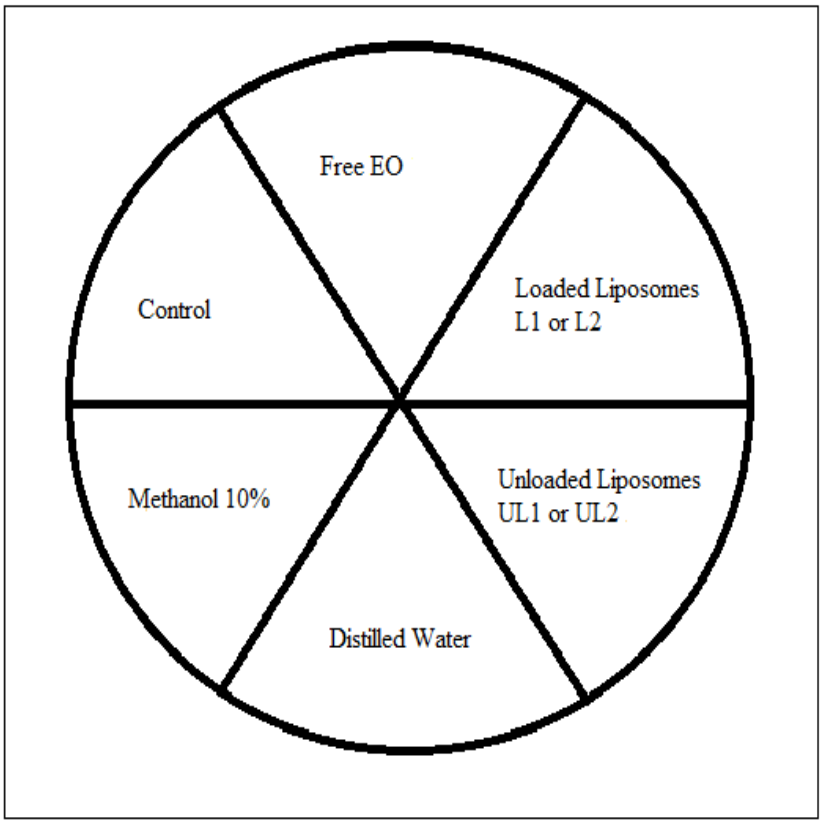

Figure 5.6. Schematic diagram of agar well diffusion assay
Free EO at 100% concentration, liposomes (L1 and L2), Negative controls: sterile distilled water, unloaded liposomes (UL1 and UL2) and 10% methanol. Positive Control: free EO and Chlorhexidine gluconate (CHX) (0.12 % v/v)

Plates were incubated at appropriate temperatures and tested against selected oral microorganisms upto 120 hrs. This time was chosen according to bacterial growth requirements (*e.g. P. gingivalis* 72 h) and the slow releasing nature of liposomes.

Following incubation, inhibition zones were measured including the diameter of wells and recorded over a period of up to 5 days to test for sustained release from the loaded liposomes.

5.11. STATISTICAL ANALYSIS

Statistical analysis was conducted with the statistical programme; Minitab 16 Statistical Software (Inova, UK). All experiments were performed in triplicate. Statistical significance was determined using one-way analysis of variance at 95 % confidence level. Descriptive statistics, including the mean ± standard deviation (SD) were calculated. To determine whether the mean were statistically different from each other a Tukey's pair wise comparison test was used at the chosen level of significance at $P < 0.05$. The statistical significance of killing and inhibition of bacterial growth were determined. Comparison of results between groups beside Anova, for multiple comparisons used Dunnet's multiple comparison tests. P values of 0.05 were considered significantly different.

6. RESULTS

In this study, the preparation, incorporation, characterization and antimicrobial effect of two liposomes incorporating *S.hortensis* L EO were investigated.

6.1. EXTRACTION OF ESSENTIAL OIL BY HYDRODISTILLATION

Distillation of the aerial parts of *S.hortensis* L. gave a volatile, yellow EO with a strong odor. The relative density of the EO extracted by the hydrodistillation method was calculated by the gravimetric method and found as 911 at room temperature [101]. Extraction yield was calculated as 7.5 % (w/w).

6.2. CHEMICAL COMPOSITION OF ESSENTIAL OIL (GC-MS)

GC-MS data shows the identification of the components listed according to their elution on the 5CB-column. In total, 52 components were identified (Table 6.1). GC-MS analysis revealed that the most abundant components were notably carvacrol (41.65 %), thymol (10.29 %), and monoterpenic hydrocarbons, p-cymene (16.16 %), α-Terpinene (4.91 %) as phenolic monoterpene, and others such as γ-Terpinene (27.44 %), β-caryophyllene (2.94 %). Moreover, Germacrene-D (0.08 %), Ledene (0.57 %), α-Cadinene (0.94 %), p-Cymen-2-ol (0.19 %), Junipene (0.03 %), Sabinene (0.28 %) and α-Phellandrene (0.49 %) were other components found in minimal concentrations.

Table 6.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS)

No	**R.t[a](min)**	**Components[b]**	**Peak Area (%)**
1	4.27	α-Phellandrene	0.49
2	4.41	α-Terpinene	4.91*
3	4.56	p-Cymene	16.16 *
4	4.67	Sabinene	0.28
5	4.77	(E)-β-Ocimene	0.16
6	5.05	ɣ-Terpinene	27.44*
7	5.29	cis-Sabinene hydrate	0.1
8	5.29	trans-Sabinene hydrate	0.1
9	5.36	Ethanone	0.06
10	5.43	p-Tolualdehyde	0.14
11	5.50	Ocimene	0.15
12	5.68	Terpinolene	0.04
13	5.83	Trans-4-Thujonol	0.09
14	6.05	Tran-2-caren-4-ol	0.04
15	6.25	1-Terpineol	0.03
16	7.20	Endo-Borneol	0.12
17	7.29	Terpine4-ol	0.65
18	7.56	β-fenchol	0.12
19	8.23	P-Cymenol	0.3
20	9.07	Carvacrol Methyl Ether	0.11
21	9.28	Carvacrol	41.65*
22	9.44	Thymol	10.29*
23	9.94	Cymenol	0.23
24	10.23	α-Copaene	0.05
25	10.68	α-Copaene	0.1
26	10.81	β-bourbonene	0.09
27	10.98	β-caryophyllene	2.94 *
28	11.72	Germacrene-D	0.08
29	11.92	Aromadendrene	0.32
30	12.11	α-caryophyllene	0.13
31	12.46	ɣ-Cadinene	0.42
32	12.75	α-Amorphene	0.42
33	12.83	α-Muurolene	0.04
34	12.90	Germacrene-d	0.15
35	13.05	Ledene	0.57
36	13.15	α-Muurolene	0.3
37	13.22	β-Bisabolene	0.77
38	13.43	α-Cadinene	0.94
39	13.54	Pentalene	0.04
40	13.70	α-Copaene	0.12

[a] Retention time (in minutes)

[b] Components listed in order of elution from a GC-MS column

* Indicates highest antimicrobial components of *S.hortensis* L. according to literature [143-154]

Table 6.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS) (continue)

No	R.t[a](min)	Components[b]	Peak Area (%)
41	14.27	Isoaromadendrenepoxid	0.03
42	14.37	(+) spathulenol	0.15
43	14.44	(-)-Caryophyllene oxide	0.19
44	15.02	Iospathulenol	0.03
45	15.11	α-Cadinol	0.04
46	15.27	Junipene	0.03
47	16.50	Aromendrene	0.06
48	16.79	Cyslohexene	0.06
49	17.16	Phenyltrimethysilone	0.11
50	17.29	p-cymen-2-ol	0.19
51	17.37	Sulfurous acid	0.05
52	17.92	Len-1-ol	0.03

[a] Retention time (in minutes)

[b] Components listed in order of elution from a GC-MS column

* Indicates highest antimicrobial components of *S.hortensis* L. according to literature [143-154]

6.3. PREPARATION AND CHARACTERIZATION OF LIPOSOMES

Two unloaded liposomes (UL1 and UL2) were prepared at different ratios of phospholipid 85 G (PL85G), cholesterol (CH) and stearylamine (SA) (Table 6.2). The liposomes were then loaded (L1 and L2) with extracted *S. hortensis* EO.

Results of the characterization studies (molar ratio, size, size distribution, zeta potential, poly dispersitive index (PDI) and appearance) of unloaded liposomes (UL1, UL2) and loaded liposomes (L1, L2) are shown in Table 6.2.

Table 6.2. Liposome formulations, their compositions, measurements for particle size, zeta potential and PDI values

Code	Composition	Molar Ratio	Average diameter (nm)	Zeta potential (mV)	PDI	Observations
UL1	PL 85 G:SA:CH	7:1:2	146.9 ± 2.8	34.8 ± 2.2	0.39 ± 0.01	Milky dispersion
UL2	PL 85G:SA:CH	10:1:4	147.8 ± 2.1	46. 5 ± 1.8	0.30 ± 0.01	Milky dispersion
L1	PL85 G:SA:CH +EO	7:1:2	412.5 ± 13.9	-36.7 ± 0.7	0.41 ± 0.02	Milky dispersion
L2	PL 85G:SA:CH +EO	10:1:4	2171 ± 151.0	-29.9 ± 1.7	0.16 ±0.07	Milky dispersion

Values represent the mean ±standard deviation of three experiments
UL: Unloaded liposomes
L: Loaded liposomes
PDI: Poly dispersive index
CH: Cholesterol
SA: Stearylamine
PL85 G: Phospholipid 85 G

The zeta potentials for UL1 and UL2 formulations were cationic (positive) 34.8 ± 2.2 mV and 46.5 ± 1.8 mV, respectively. Following loading with EO, it was observed that the zeta potential of the vesicles became anionic -36.7± 0.7 mV for L1 and -29.9 ± 1.7 for L2 loaded liposomes. Statistical analysis comparing the zeta potential of liposomes UL1 with UL2 and loaded liposomes L1 with L2 revealed a significant difference ($p<0.05$).

All liposome vesicles were extruded using polycarbonate filters (200nm and 400nm) to obtain homogeneous and relatively smaller size dispersion under a nitrogen stream. The obtained particle sizes were between 146.9 ± 2.8 - 147.8 ± 2.11 nm for unloaded liposomes (UL1 and UL2) while the diameter size of loaded liposomes were observed as L1: 412.5 ± 13.93 nm and L2: 2171 ± 151.0 nm, respectively.

Even the mean particle size of unloaded liposomes, UL1 (146.9 ± 2.8 nm) and UL2 (147.8 ± 2.11 nm) were not significantly different ($p>0.05$), when they loaded with EO their particle size increased to 412.5 ± 3.93 nm and 2171 ± 151.0 nm for L1 and L2, respectively (Figure 6.1 and 6.2). The data showed that EO loaded liposomes, L1 and L2, had significantly different mean particle sizes ($p<0.05$).

Furthermore, following loading with EO, PDI values for both L1 and L2 loaded liposome formulations according to DLS analysis were 0.41 ± 0.02 and 0.16 ±0.07, respectively. However, the particle size of L2 (2171 ± 151.0 nm) was seen to be much larger than that of L1 (412.5 ± 13.93 nm). The observed larger size was due to the aggregation of liposomes in the L2 formulation. According to the literature, a small PDI value (lower than 0.1) indicates a homogenous population of particles, while a PI above 0.3 indicates a high degree of heterogeneity [167, 168].

The PDI values of the UL1 and UL2 unloaded liposomes were not significantly different ($p<0.05$), upon loading with EO, L1 and L2 liposomes displayed significantly different PDI values ($p> 0.05$).

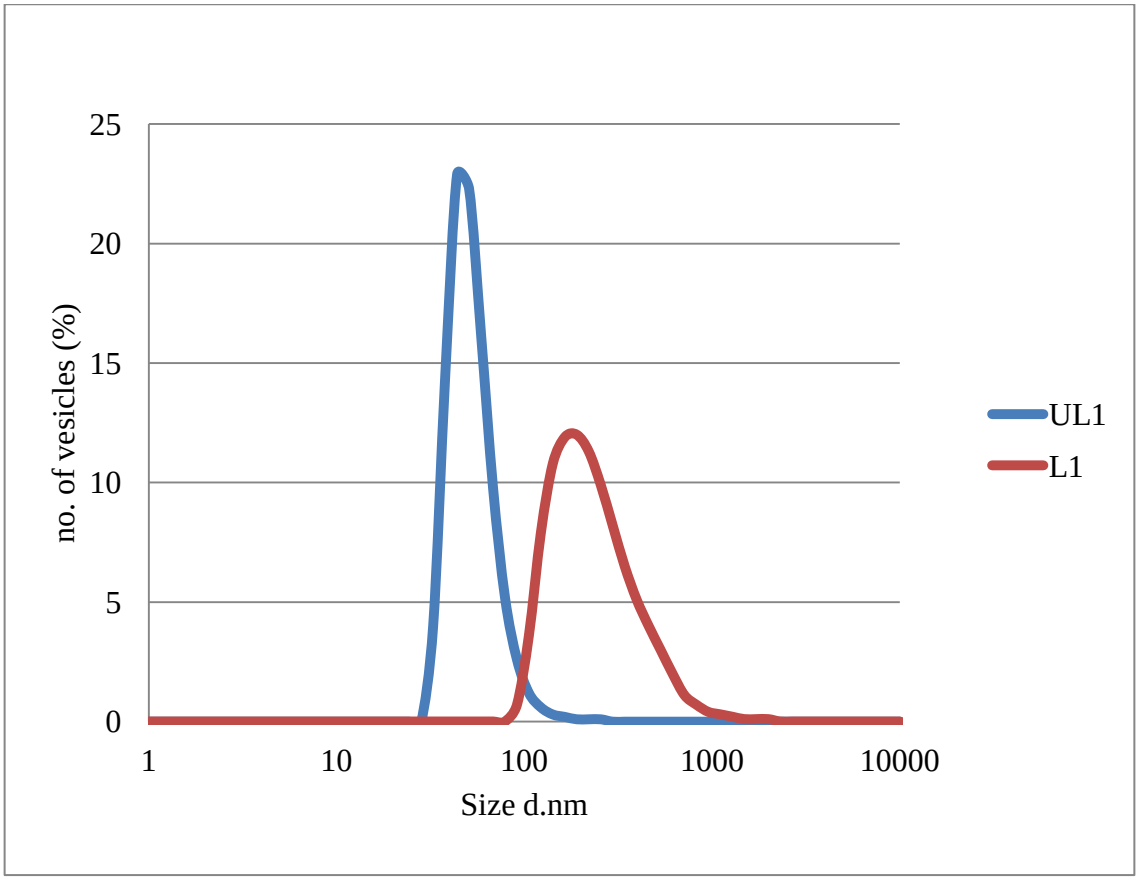

Figure 6.1. DLS Analysis of unloaded (UL1) and Loaded Liposomes (L1)

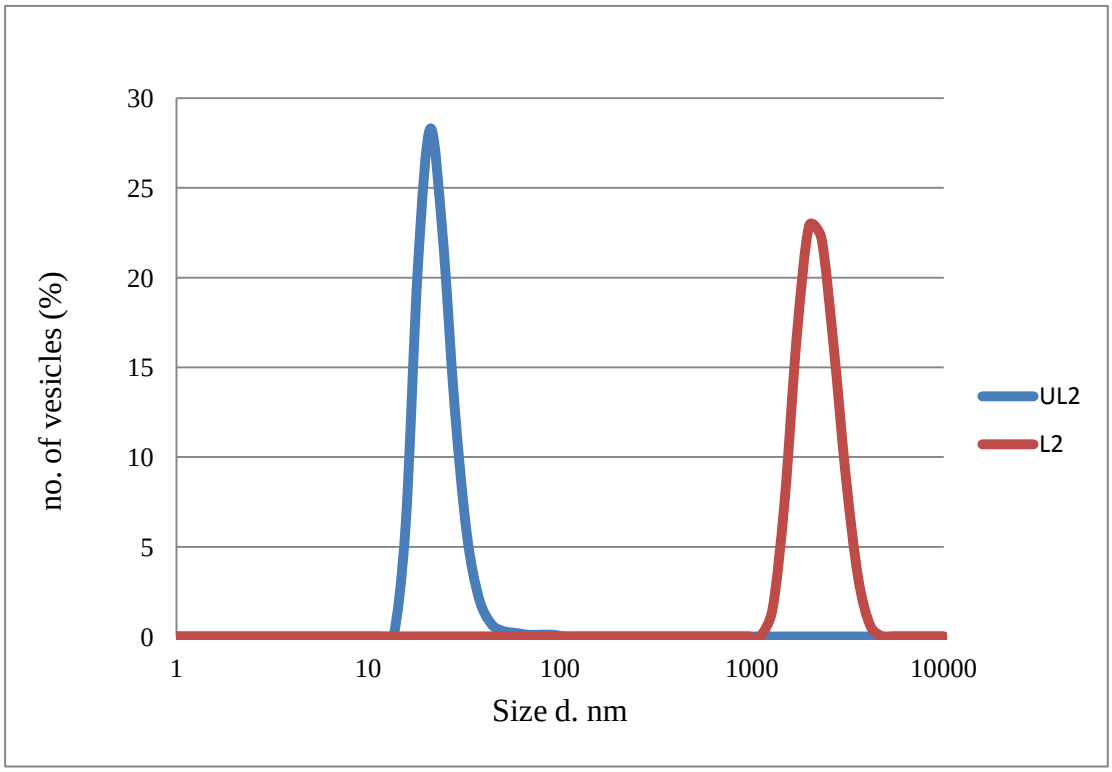

Figure 6.2. DLS Analysis of unloaded (UL2) and Loaded Liposomes (L2)

6.4. MORPHOLOGICAL CHARACTERIZATION OF LIPOSOMES

Morphological characterization of unloaded liposomes (UL1, UL2) and the loaded liposomes (L1 and L2) were examined by polarized light microscopy (PLM), and atomic force microscopy (AFM).

6.4.1. Polarized Light Microscopy

The images of unloaded and loaded liposomes are shown in Figures 6.3.a and 6.3.b, respectively. Images of polarized light microscopy confirmed that the EO was successfully loaded into the vesicles and as expected loading with EO increased the size of liposomes.

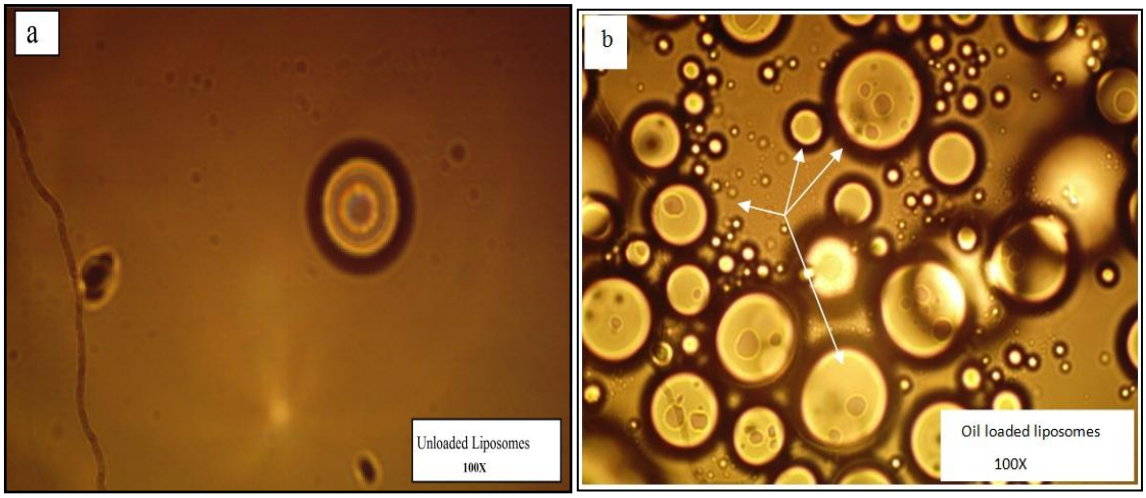

Figure 6.3. Polarized Light Microscopy images of a. UL1 at 100X
b. L1 at 100X

6.4.2. Atomic Force Microscopy

Since light microscopy is unable to provide comprehensive information about the lipid bilayers, AFM was carried out for more detailed information. Because of the deposition of large aggregates, some samples were diluted before being applied onto the slide. AFM images provided a three dimensional view of liposomes and are presented in Figures 6.4a and 6.4b.

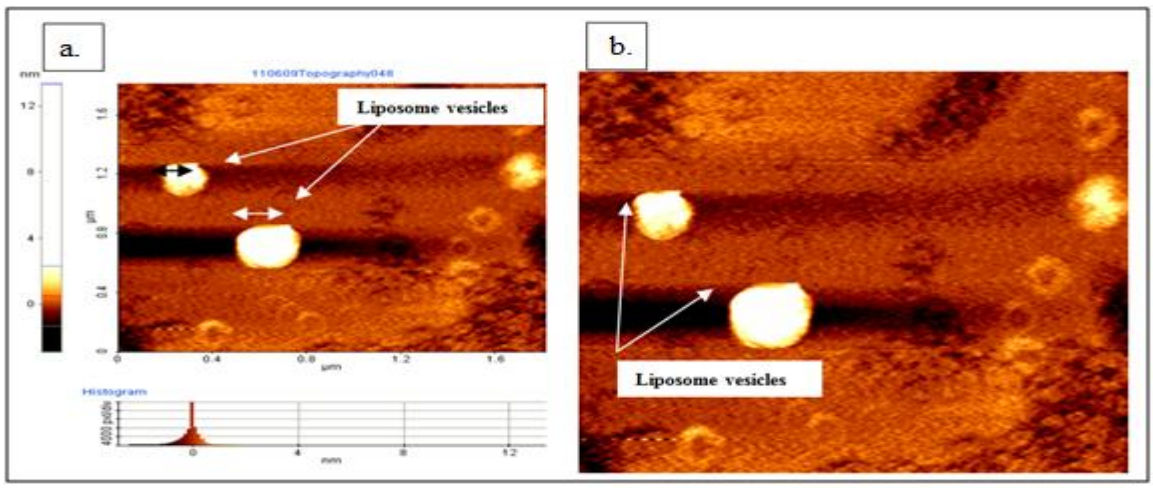

Figure 6.4. 3D Images of Loaded liposomes L1 (Topography of vesicles)

Loaded liposomes showed homogenous, spherical shape and intact bilayer structure vesicles. No difference was observed between AFM images for L1 and L2 vesicles.

According to the results of the liposome characterization studies L1 (7:1:2) molar ratio displayed better properties than L2 (10:1:4) loaded liposomes (Table 6.2) in terms of size, PDI and zeta potential.

6.5. ANTIMICROBIAL ACTIVITY TESTING

6.5.1. Antimicrobial Activity By Agar Well Diffusion Assay

The antimicrobial activity of loaded liposomes (L1 and L2) (50 % v/v) were examined after 120 hrs of incubation chosen according to bacterial growth requirements (*e.g P. gingivalis* 72 h) and the slow releasing nature of liposomes. The antimicrobial activity results of 120 h are presented in Table 6.3 and Figures 6.5.

Table 6.3. Antimicrobial activity of L1 and L2 against oral microorganisms following 120 h incubation

Zone of Inhibitions (ZI's) (mm)		
Test microorganism	**L1**	**L2**
S.mutans	19.0 ± 0.0	16.0 ± 0.0
F.nucleatum	15.3 ± 0.5	21.0 ± 0.0
P.gingivalis	23.3 ± 0.5	20.0 ± 0.0
P.intermedia	16.0 ± 0.0	13.2 ± 0.0
A.a	17.0 ± 0.0	16.6 ± 1.1
C.albicans	30.0 ± 0.0	30.0 ± 0.0
*Mix**	13.0 ± 0.0	12.0 ± 0.0

* Mixed-species of *A. actinomycetemcomitans. P. gingivalis, P. intermedia, F. nucleatum, S. mutans, A.a*
Values are calculated as the mean ±standard deviation of three experiments.
L1: P85G:SA:CHOL(7:1:2)
L2:P85G:SA:CHOL(10:1:4)

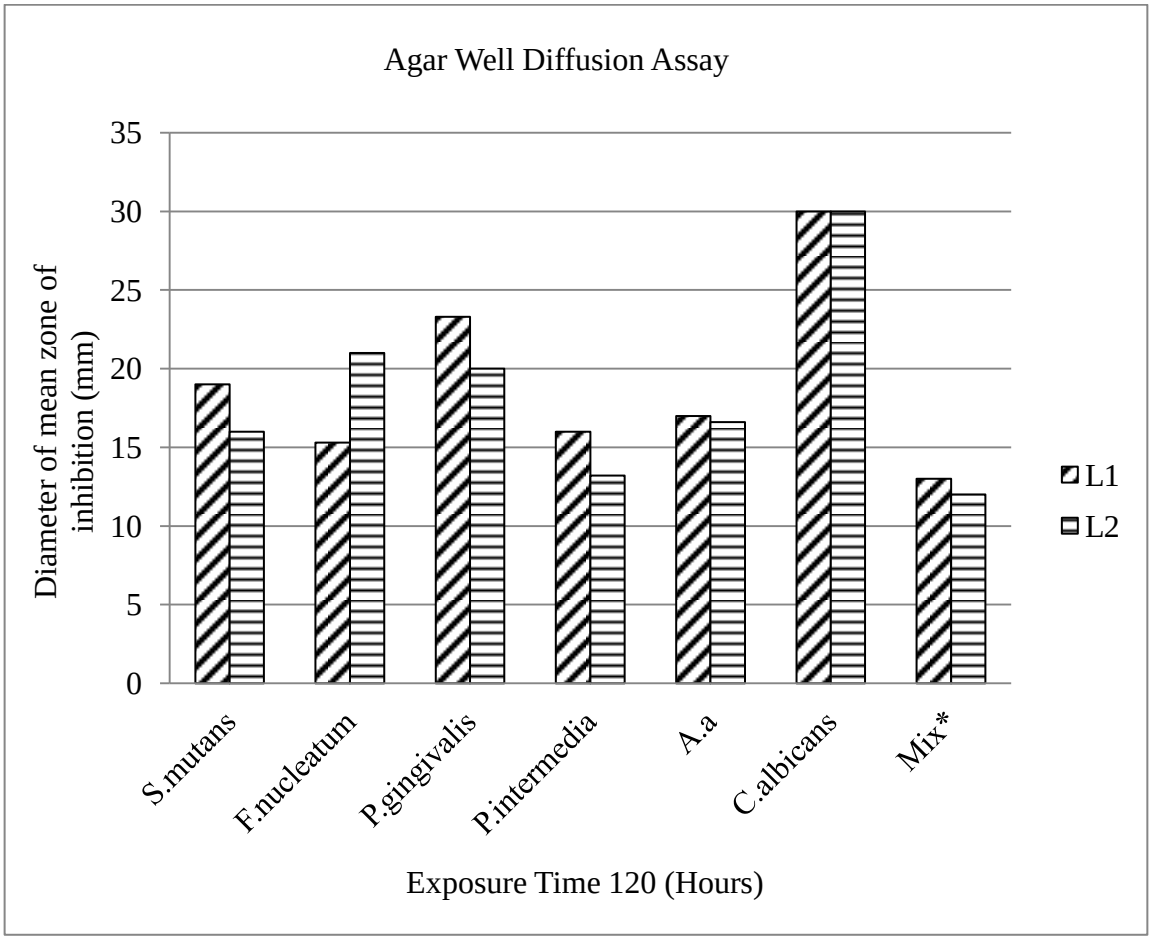

Figure 6.5. Inhibition zones of loaded liposomes L1 and L2 on test microorganisms

Free essential oil was used as a positive control in order to confirm its antimicrobial activity against all microorganisms. Strong, moderate and weak antimicrobial activity of free EO was determined. The highest antimicrobial activity of free EO (control) was observed with the largest ZI against *A.a* (56 mm) after 120 h. Following *A.a*, free EO displayed very strong antimicrobial activities against *C. albicans* (48 mm), *S. mutans* (39 mm) and *P. gingivalis* (36 mm), respectively. A strong antimicrobial activity was observed with *P. intermedia* and *F. nucleatum*, while a moderate one was seen against the mixed culture (18 mm).

After EO encapsulated into liposomes, antimicrobial assay results showed that best antimicrobial activity for both L1 and L2 were observed against *C. albicans* with both liposomes showing a ZI of 30 mm. Conversely, the weakest antimicrobial response was observed against the mixed culture for both L1 and L2 (13.0; 12.0), respectively.

Following the very strong response against *C. albicans,* L1 displayed a strong antimicrobial efficacy against *P. gingivalis* with 23.3 mm, while moderate activities were observed against *S. mutans* (19 mm), *A.a* (17 mm) and *P. intermedia* (16 mm), respectively. A weak response against *F. nucleatum* (15.3 mm) was observed. Statistical comparison of the antimicrobial activity between species displayed no significant antimicrobial effect ($p>0.05$) with L1 between *P.intermedia and F.nucleatum*. On the other hand, a significant difference ($p<0.05$) in comparison between *S.mutans, P.gingivalis, C.albicans* and *A. a.* species was observed.

Following *C.albicans* (30 mm) L2 showed its second strongest antimicrobial effect against *F. nucleatum* (21mm) and moderate responses against *P. gingivalis* (20 mm), *S. mutans* (16 mm) and *A.a* (15.5 mm). A weak response was observed for *P. intermedia* (13.2 mm). Comparison between individual species revealed the antimicrobial activity of L2 loaded liposomes on *F.nucleatum*, *A.a*, *S.mutans* and *P.gingivalis* to show no significant difference ($p>0.05$). The difference between antimicrobial effects of *C.albicans* and mix of microorganisms was significant ($p<0.05$).

Comparison between the efficacies of L1 and L2, revealed no statistical significance for all microorganisms ($p>0.05$).

7. DISCUSSION

7.1.1. CHEMICAL COMPOSITION OF ESSENTIAL OIL (GC-MS)

Plants are a good source for use as drugs for the treatment of many ailments and diseases. They naturally manufacture many different chemical components as a major part of their defense mechanism. Most of these chemical substances are desirable as they are widely used to protect living organisms from unwanted pathogens such as insects and microorganisms [169, 170]. Therefore, phytotherapy provides unexplored natural resources for the development of potential new drugs that can be used in various areas including dentistry and medicine [169, 170]. *S.hortensis L.* from the Lamiaceae family is one of the plants with a high treatment potential, due to its diverse chemical composition which includes; flavonoids, mono-, di-, and sesquiterpenoids, flavones, triterpenoids, and steroids [143, 171].

This study extracted and characterized the EO from dried aerial parts of the plant *S. hortensis L.*, by hydrodistillation method. The chemical composition of the volatile oil was qualitatively and quantitatively analyzed by GC-MS and the most abundant antimicrobial components were notably carvacrol (41.65 %), thymol (10.29 %), and monoterpenic hydrocarbons, p-cymene (16.16 %) [142, 169, 170, 171]. These components make the EOs of *Satureja* species amongst the strongest plant EOs with regards to their antimicrobial properties which have been confirmed and extended in this study [172, 173].

Several factors including; location, climate, geography, genotype, geology, seasonal changes of growth, harvest and experimental conditions are known to affect the chemical composition of plant extracts [174]. These effects have been observed between chemical compositions from different *Satureja* species in different areas of the world [175]. For example, EO extracted from *S. montana* collected from central parts of Dalmatia displayed carvacrol (84 %) as their major components [142]. In Iran, *S. rechingeri* was identified with fifty three components and carvacrol (56.1%), p-cymene (14.0%), and α-thujone (4.7%) were the main components [144]. *S. montana* has shown a high content of phenolic carvacrol (45.7%) and its minor components as p-cymene (12.6%), ɣ-terpinene (8.1%) and

oxygenated constituents carvacrol methyl ether (11.0%), borneol (4.8%), thymol (3.9%) and thymol methyl ether (2.3%). In contrast, *S. cuneifolia* contains the hydrocarbon β-cubebenesesquiterpene (8.7%) and shows a low percentage of carvacrol and thymol compared to *S. montana* [151].

Studies on different *Satureja* species in Turkey have also shown variations in their components. Carvacrol has been observed as the main constituent in *S. icarica*, *S. pilosa,* and *S. boissieri* EO [171]. *S. coerulea* included some sesquiterpenes [177]. 80 compounds were identified in the EO of *S. biflora* and included spathulenol (11.9 %), α-bisabolol oxide-B (8.77%), terpinen-4-ol (7.12%), linalol (6.03%) and bornyl actateta (4.75%) [151]. *S. hortensis* EO extracts have also shown similar qualitative components but quantitative variations amongst different strains [169, 172, 178].

Carvacrol and thymol are isomeric compounds containing (1-methylethyl) an oraphenol group in their structure. The only and main variation between these two phenolic derivatives is in the position of the hydroxyl group on their phenol rings [176]. These changes in position affect their biological activity. This occurs because of the difference in attachment to the cell membrane [179].

Many reports have shown that, carvacrol, thymol, and p-cymene have antimicrobial properties [171] and are well known growth inhibitors against a diverse range of microorganisms and fungi [172]. Minor components such as p-cymene, have also been seen to interact with other components in the EO, thereby affecting its antimicrobial potential [177]. It has been reported that p-cymene alone is an less effective antimicrobial agent. However, its combination with other components has shown synergistic activity, resulting in the destabilization of the microbial membrane [175]. Conversely, reports on the role of p-cymene as an antimicrobial agent have shown an antagonistic effect between phenolic monoterpenes, thymol, carvacrol, and p-cymene in *S. hortensis* EO [174].

This study used the whole essential oil rather than its individual components to utilize the synergistic effects of the diverse major and minor components present in its EO.

7.2. PREPARATION AND CHARACTERIZATION OF LIPOSOMES

7.2.1. Liposomes for the Incorporation of EO of *S. hortensis L.*

Although numerous methods for preparing liposomes have been published [180] only a limited number have been used in application. The first requirement for production is that the method should be simple and reproducible. According to Bangham et al., in 1965, it was found that the thin film hydration method was the simplest and suitable method [156, 157]. This study used the thin film method for successful preparation of liposomes.

The characteristics of liposomes, (Size and Zeta potential, PDI) were strongly influenced by the type of lipids used for preparation [179]. In our study, liposome formulations were prepared with phospholipid 85 G, stearylamine and cholesterol in different ratios as primary substances [156]. According to Bangham et al., the lipids (PL85 G, CH and SA) were dissolved in 5 ml choloroform including 0.1% ethanol [140]. The liposomes were prepared in two different molar ratios; PL85 G: SA: CHOL were used in a ratio of 7:1:2 and 10:1:4 , respectively.

The incorporation of *S. hortensis L*. EO into lipid vesicles as a model drug is based on the function of the lipid composition and may be attributed to their electrostatic and hydrophobic interactions [181]. The combination of liposomes and EO may have hydrophobic interactions and an antimicrobial effect on cell surfaces by non-specific interactions with target microorganisms due to the charge of the membranes.

Since, eukaryotic and bacterial cells possess negatively charged surfaces, this study prepared positively charged liposomal vesicles in order to provide the best liposome surface –bacterial cell interaction. This was accomplished by the use of stearylamine which induces a positive charge to liposome surfaces. However, the EO has strong negative charge due to the lipid structure of the oil. However, the charge liposomes have better attraction to microorganism’s cell surface than neutral liposome. Positively charged liposomes have previously been shown to be suitable for drug delivery to the oral cavity, due to their affinity to the anionic cell membrane [182]. In one study, positively charged liposomes with 1, 2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) was used as a

drug delivery system to the oral cavity. However, it was not found suitable enough for application to the oral cavity, since saliva constituents may interact with liposomes and cause a sudden drop in turbidity of saliva in oral cavity [182, 183].

This study also used cholesterol for liposome production, which is known to cause changes in the degree of head group dissociation and probably in the interaction with lipophilic compounds. Cholesterol molecules orientate with its steroid nucleus among the fatty acyl chains of phospholipid molecules and its hydroxyl group faces on the water side. Thus, cholesterol is often added to liposomes to improve their in vivo and in vitro stability [3, 184].

7.3. CHARACTERIZATION OF LIPOSOMES

Prepared liposomes were evaluated according to their mean particle size, size distribution analysis, zeta potential parameters and PDI values. Also microscopic techniques were performed for characterization in terms of surface morphology of liposomes (vesicles).

7.3.1. Mean particle size, size distribution, zeta potential and PDI

The data statistically showed that L1 loaded liposomes had significantly different mean particle sizes than L2 loaded liposomes ($p < 0.05$) (appendix p. 99). The reason behind homogenization of liposome suspensions is to decrease the size of the liposomes, as well as to keep the size distribution at a minimum, thereby improving the macroscopic appearance of the suspension and also improving the physical stability of the system in terms of sedimentation or floating [184].

Temperature and lipid composition are some of the variables that influence liposome size. Because the liposome suspensions were filtered through a filter medium with a 200 nm and 400 nm pore size, one would expect the size of the resultant liposomes to be smaller than or equal to this value. In our study, the size of unloaded liposomes was observed lower than 200nm (146.9 ± 2.8 and 147.8 ± 2.1 nm) according to DLS.

Liposomes are extruded through polycarbonate filters at temperatures higher than their transition temperatures, meaning as they pass through the 200 and 400 nm pore, they are in a fluid state, are easy to deform and can therefore sometimes, regain their initial size after extrusion. The transition temperatures for PL85G, was room temperature so for the extrusion process 30 $^{\circ}$C was used. Another phenomenon which could explain the increase in liposome size, post extrusion, is secondary particle growth. With certain mixtures of lipids, liposome sizes reach a minimum, followed by a re-increase in size with further extrusion cycles. Fusion, which is a gradual increase in size of bigger particles at the expense of smaller ones by means of molecular diffusion, could be the reason behind secondary particle growth [184].

Moreover, the results showed that the mean particle size of unloaded liposomes were smaller than loaded liposomes. This is expected as it is known that empty vesicles are always smaller than loaded ones. This is a result of higher cohesion and packing among the apolar chains in vesicular membrane [185].

Furthermore, zeta potential values of liposomes were evaluated. The zeta potential is a function of the overall charge of a particle and changes in size can affect aggregation or fusion [186]. Zeta potential measurements of liposomes provide direct information on the structure of the lipid head group. The significance of zeta potential is that its value can be related to the stability of colloidal dispersion. Zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in a disperse system. For particles that are small enough, a high zeta potential will confer stability; dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will flocculate. Therefore, disperse systems with high zeta potentials (negative or positive) are electrically stabilized while those with low zeta potentials tend to coagulate [184, 186]. The optimum value should be around 25 mV. It can be concluded that the studied formulations were relatively stable.

From the zeta potential measurements in Table 6.2, the zeta potentials for UL1 and UL2 formulations were positive 34.8 ± 2.2 mV and 46.5 ± 1.8 mV, respectively. Following loading with EO, the zeta potential of vesicles were -36.6 ± 0.7 mV for L1 and -29.9 ± 1.7 for L2 loaded liposomes. Furthermore, when all the zeta potential values were statistically

evaluated, it was determined that UL1 and UL2 have significantly different zeta potential values ($p<0.05$) and following loading with EO liposomes, the zeta potential values of L1 and L2 were significantly different ($p<0.05$). After incorporation loaded liposomes became negatively charged, due to the lipid structure of the oil.

It has been reported that low PDI values (≤0.3), indicate a homogenous vesicle population. Larger PDI values (>0.3), indicate a high heterogeneity [167]. When the PDI values were statistically evaluated for all liposomal formulations, results showed that PDI values of the UL1 and UL2 unloaded liposomes were not significantly different ($p>0.05$). However, L1 and L2 loaded liposomes showed significantly different PDI values ($p< 0.05$). This is due to the heterogeneous particle size distribution PDI value of L1 (0.41 ± 0.02) which is larger than 0.3 and the homogenous particle size distribution PDI value (0.16 ± 0.07) of L2 (smaller than 0.3). Although, the L2 formulation was seen to be more homogenous than the L1 formulation, this was observed due to the aggregation of the vesicles, leading to low PDI values for L2.

7.3.2. Morphological characterization of liposomes (PLM and AFM)

Liposomes were further examined by PLM and AFM in order to evaluate their morphological structures i.e. bilayer structures. According to the images formulations L1 and L2 were MLV type vesicles.

The microscopic examination of liposomes confirmed that L2 had a larger size thatn L1 supporting particle size data (L2= 2171 ± 151.0, L1= 412.5 ± 13.93). These findings confirm aggregation has occurred in L2, and leads us to conclude that L1 formulation has better characterization properties than the L2 formulation. Furthermore, microscopic examination revealed L1 vesicles to have an intact shape and a homogenous distribution (Figure 6.3 and 6.4). Therefore, according to overall results of the characterization studies L1 was observed as the more suitable liposome to be used in this study.

7.4. ANTIMICROBIAL ACTIVITY TESTING

Studies on the antimicrobial mechanism of EOs have shown that they generally affect bacterial cell membrane functions such as; electron transport, nutrient uptake, protein and nucleic acid synthesis and enzyme activity. This results in a change to the membrane permeability, thus causing release of intracellular components and eventually cell death [116, 151]. Several studies have shown the antimicrobial activity of plant EOs against different cariogenic and periodontopathic bacteria [187-189]. The EO of *Shitake* mushroom, demonstrated significant antimicrobial activity on several periodontal pathogens [190]. Moreover, EOs from traditional Asian herbs, namely *Wakame, Houjicha, Genmaicha* and *Sencha* teas were tested on four oral pathogens involved in periodontitis. Results displayed all EOs to have antibacterial properties [191].

A comparative antimicrobial study on the EO from different *Satureja* species, showed *S. hortensis L.* EO to have the strongest antimicrobial effect against bacteria, yeast and fungi [144]. This effect has been attributed to the high contents of monoterpenes and oxygenated compounds, such as γ-terpinene, p-cymene, thymol and carvacrol [169, 172, 173]. Similar to our findings, Gursoy and Cakmakcı found that free EO which was extracted by hydrodistillation method for 3h from *S.hortensis* from the southern west part of Turkey also had antimicrobial activity against *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum*. Diktas and Sahin also reported that the EO of *S. hortensis* had a very strong fungicidal effect on *A. flavus* [154]. Furthermore, *S. hortensis* L. EO has previously shown inhibitory effects on two phytopathogenic fungi *Alternaria mali* and *Botrytis cinerea* [149].

Investigations on *S.hortensis* L. EO have also reported conflicting results. *S.hortensis* L. EO from Serbia, displayed an antimicrobial activity against *B. subtilis*, whereas a strain from Yusufeli in Turkey did not exhibit any antimicrobial effect against same bacteria [136]. As with our present findings, it has been found that the EO of *S.hortensis* successfully inhibited phytopathogenic fungi and some food spoilage fungi including *C. albicans* [187]. However, in contradiction, another study found no antimicrobial affect against *C. albicans* [192]. These conflicting results could be due to many factors including; strain type of plant, bacterial strain studied, geographical location of plant, extraction process, experimental parameters, part of plant used and other environmental factors.

The use of liposomes as a drug delivery system is fast becoming the method of choice based on the advantageous properties that differ according to its formulation. Some studies have shown that encapsulation of certain substances are able to increase bioavailability and thus enhance its bioactive properties [169, 170]. While others, have shown a decrease in cytotoxicity due to the slow releasing nature [161, 165].

To date there are no reports on the antimicrobial activity of liposomal incorporation of EO from *S.hortensis L*, or on liposomes against oral microorganisms. However, several studies relating to liposomal incorporation of other plant extracts and their antimicrobial effects have been carried out [77, 169, 170]. Following the liposomal incorporation of *Artemisia arborescens* EO an increase in antiviral activity against *Herpes simplex* viruses was observed [77]. Antiviral assay results also showed that *A. arborescens* incorporated EO increased its *in vitro* antiherpetic effect.

The major components of *Origanum dictamnus* EO have previously been successfully encapsulated into phosphotidyl choline-based liposomes. Results showed antimicrobial properties against *S.aureus, S.epidermis, P.aeruginosa, E.cloacae, K. pneumoniae, E.coli, S.mutans, S.viridans, C.albicans, C.tropicalis, C.glabrata* and *L.monocytognes* [169]. Although this study did not compare the antimicrobial effect of free EO with loaded liposomes due to the difference in concentrations, a slow releasing property of the EO over a period of 120 h was observed (appendix p. 107). Kinetic studies and time kill assays will need to be performed in order to confirm this.

The antimicrobial efficiency of antimicrobial compounds may differ, according to the Gram type of the test microorganism [193]. Gram-positive organisms are said to be more sensitive than Gram negative ones. This is due to the presence of an outer membrane, surrounding the cell wall of Gram negative bacteria, making it less susceptible to the antibacterial substances. This outer membrane may restrict the diffusion of hydrophobic compounds through its lipopolysaccaride covering. On the other hand, Gram positive bacteria are always in direct contact with the hydrophobic constituents thus enabling binding to the phospholipids bilayer of the bacterial cell membrane, thereby increasing ion permeability [194].

Liposomal formulations have been studied for their use in overcoming reduction in cellular resistance by overcoming cellular permeability [195, 196]

Results from the present study have shown no discrimination in antimicrobial activity based on Gram type. L1 and L2 liposomes exhibited varying degrees of antimicrobial activity against all tested microorganisms. These results cannot be correlated with the Gram stain of the bacteria, i.e. cell membrane, or with its respiratory functions (aerobic or anaerobic). Findings may suggest a possible therapeutic role for these liposomes against both bacterial groups.

The weak inhibition activity observed for both L1 and L2 on the mixed culture is to be expected since, bacteria grow in communities known as biofilms and therefore cell to cell communication (quorum sensing) and possible surface alterations allow the bacterium to reduce its interactions with the antimicrobial compound, thus making it resistant [197]. Further studies are required in order to understand the resistance mechanism of these microorganisms against antimicrobial compounds, which may contribute to the present results.

8. CONCLUSION

S. hortensis EO can successfully be incorporated into liposome formulations L1 (P85G (7:1:2) and L2 (P85G (10:1:4) loaded liposomes by the Bangham method. Furthermore, following 120h incubation antimicrobial activity was observed against all test microorganisms.

To the best of our knowledge, this is the first study showing the successful incorporation of *S.hortensis* EO into two different liposome formulations suitable for antimicrobial use in the oral cavity. Such data suggest that incorporation of EO in liposomes could be useful in the management of oral infections induced by oral microorganisms.

Therefore, further detailed studies are necessary in order to understand the mechanisms, kinetics and time release properties of liposomes. and develop effective formulations.

REFERENCES

1. Petersen, P. E., D. Bourgeois., and H.Ogawa., "The Global Burden of oral diseases and risk to oral health", *Bulletin of The World Health Organization*, Vol. 83, pp. 661-669, 2005.

2. Plambo, E.A., " Traditional Medicinal Plant Extracts and Natural Products with Activity Against Oral Bacteria: Potential Application in the Prevention and Treatment of oral diseases", *Evidence-Based Complementary and Alternative Medicine*, pp. 15 pages, 2009.

3. Petersen, P.E., " The burden of oral disease: challenges to improving oral health in the 21st century", *Bulletin of the World Health Organization*, Vol. 81, pp. 3, 2005.

4. Petersen, P.E., " The World Oral Health Report 2003: continuous improvement of oral health in the 21st century—the approach of the WHO Global Oral Health Programme", *Community Dentistry and Oral Epidemiology*, Vol. 31, pp. 3-24, 2003.

5. Kathleen, P.T., "*Foundations in Microbiology*", McGraww Hill, New York, 2005.

6. Marsh, P.D., " Are dental diseases examples of ecological catastrophes?", *Microbiology-Sgm*, Vol.140, pp. 279-294, 2003.

7. Marsh, P.D., " Dental plaque as a microbial biofilm", *Caries Res.*, Vol. 38, pp. 204-211, 2004.

8. Hojo, K., S. Nagaoka, T. Ohshim, N. Maeda," Bacterial Interactions in Dental Biofilm Development", *Journal of Dental Research*, Vol.88, pp. 982-988, 2009.

9. Holt, J., N. Krieg, P. Sneath, J. Staley, and S. Williams, "*Manual of Determinative Bacteriology*", 9th ed, Williams & Wilkins: Baltimore, MA, USA, 1994.

10. Davey, M. E., G. A. O'Toole, "Microbial Biofilms: From Ecology to Molecular Genetics", *Microbiology Molecular Biology Review*, Vol. 64, pp.847-867, 2000.

11. Ximenez-Fyvie, L. A., A. D.Haffajee, L. Martin, A. Tanner, and P. Macuch, "Identification of Oral *Actinomyces* species using DNA Probes", *Oral Microbial. Immunolgy*, Vol. 14, pp. 257-265,1999.

12. Marsh, P., "Dental Plaque as a Biofilm and a Microbial Community Implications for Health and Diseases", *BMC Oral Health*, 2006.

13. Kreth, J., J. Merritt, F, Qi, "Bacterial and Host Interactions of Oral *Streptococci*". *DNA And Cell Biology*. Vol.28, pp. 397-40, 2009.

14. Lenon. M.A and P.E. Petersen," Dental Caries in Developed and Developing Countries", *Caries Res*. Vol. 24, pp. 24-43, 1990.

15. Loesche, W., "Dental caries and periodontitis: contrasting two infections that have medical implication", *Infectious Disease Clinics of North America*, Vol.21, pp.471-502, 2007.

16. Toda, Y., I. Moro, T. Koga, H. Asakawa, and S. Hamada, "Ultra Structure of Extracellular Polysaccharides Produced by Serotype , *Streptococcus mutans"*. *Journal Dental. Res.* Vol.66, pp.1364-1369, 1987.

17. Trahan, L., M. Bareil, L. Gauthier, C. Vadebocoeur, "Transport and Phosphorylation of Xylitol by a Fructose Phosphotransferase System in *Streptococcus mutans", Caries Res.*, Vol.18, pp.53-63, 1985.

18. Capelli, D.P., and C. Mobley, "Prevention in Clinical Oral Health Care", *Elsevier Health Science*, 2008.

19. Teng, Y.T, , G.W. Taylor, F. Scannapieco, D.F. Kinane, M. Curtis, and J.D. Berck, "Periodontal health and systemic disorders", *J. Can. Dent. Assoc.* Vol.68, pp.188-192, 2002.

20. Vyas, S.P., "'Controlled and Targeted Drug Delivery Strategies Towards Intra Periodontal Pocket Diseases", *J. Clin. Pharm. Ther.*, Vol. 25, pp. 21-42, 2005.

21. Steele, D.G., and C.A., Bramblett, "*The Anatomy and Biology of the Human Skeleton"*, Texas A&M University Press, 1994.

22. Aas, J.A., B. J. Paster, L. N. Stokes, I. Olsen, and F.E. Dewhirst, "Defining the Normal Bacteria Flora of the Oral Cavity", *Journal Clin. Microbiol*, Vol.43, pp. 5721-5732, 2005.

23. Jian, B., A. S. Kolansky, Z. W. Baloach, and P. K. Gupta, "*Entamoeba gingivalis* Pulmonary Abscess-Diagnosed by Fine Needle Aspiration", *Cyto Journal*, Vol. 5, No. 1, pp. 12, 2008.

24. Lindsay, D., and A. Von Holy, "Bacterial Biofilms Within the Clinical Setting: What Healthcare Professionals Should Know", *J. Hosp. Infect*, Vol. 64, pp. 313-325, 2006.

25. Henderson, B., M. Curtis, and R. Seymour, "*Periodontal medicine and systems biology"*, Blackwell Pub, 2009

26. Narhi, T. O., A. Ainamo, and J. H. Meurman, "Salivary yeasts, saliva, and oral mucosa in the elderly", *Journal of dental research*, Vol. 72, No. 6, pp. 1009, 1993

27. Sutherland, I. W., "The biofilm matrix-an immobilised but dynamic environment", *Trends Microbiol*. Vol.9, pp. 222-227, 2001.

28. Lin, J., S.Qui, K. Lewis, and A. M. Klibanov, "Bactericidal properties of flat surfaces and nanoparticles derivatized with alkylated polyethylenimines", *Biotechnol. Prog.* Vol.18, pp.1082-1086, 2002.

29. Tatarciuc, M., I. C. Zamfirache, M. E. Crăciun, S. Dunca, Ş. Surdu, C. M. Roşu, and M. Ştefan, *"Study Regarding The Adherence Of Oral Microbiota On Composite Plating Materials*", TOM IX, 2008

30. Walling, D. M., A. L. Brown, W. Etienne, W. A. Keitel, and P. D. Ling, "Multiple Epstein-Barr virus infections in healthy individuals", *Journal of virology*, Vol. 77, No. 11, pp. 6546, 2003.

31. Ikuta, K., Y. Satoh, Y. Hoshikawa, and T. Sairenji, "Detection of Epstein-Barr virus in salivas and throat washings in healthy adults and children", *Microbes and infection*, Vol. 2, No. 2, pp. 115–120, 2000.

32. Imbronito, A. V., S. R. Grande, N. M. Freitas, O. Okuda, R. F. Lotufo, and F. D. Nunes, "Detection of Epstein-Barr virus and human cytomegalovirus in blood and oral samples: comparison of three sampling methods", *Journal of Oral Science*, Vol. 50, No. 1, pp. 25–31, 2008.

33. Fejerskov, O., E.Kidd, " *Dental caries: The disease and its clinical management* ", 2nd edn. Oxford: Blackwell Munksgaard; pp. 265-77, 2008.

34. Baehni, P.C., and Y. Takeuchi., "Anti-plaque agents in the prevention of biofilm-associated oral diseases". *Oral Diseases*, Vol. 32, pp. 7-15, 2005.

35. Maleskey, G., "*Nature's medicines: from asthma to weight gain, from colds to high cholesterol: the most powerful all-natural cures"*, Rodale Books, 1999.

36. Tebbs, S. E., and T. S. J. Elliott, "A novel antimicrobial central venous catheter impregnated with benzalkonium chloride, *"Journal Antimicrob. Chemother*, Vol. 13, pp. 595-606, 2001.

37. Ricardo, P.T., "Antimicrobial agents used in the control of periodontal biofilms: effective adjuncts to mechanical plaque control," *Periodontitics*, Sao Paola, Brazil, 2008.

38. Morris, A. J., J. Steele, and D. A. White, "The total cleanliness and periodontal health of UK adults in 1998", *Br Dent.J*ournal,Vol.191,pp. 186-92, 2001.

39. Talwar, R., "*Your Guide to Teeth Care"*, New Dawn Press, 2005.

40. Brothers, J. and others, "*Essentials of clinical periodontology and periodontics*", Jaypee Brothers Publishers, 2008.

41. Cobb, C.M., "Non-surgical pocket therapy: mechanical.", *Annals of periodontology/the American Academy of Periodontology*, Vol. 1, No. 1, pp. 443, 1996.

42. Caffesse, R. G., P. L. Sweeney, and B. A. Smith, "Scaling and root planing with and without periodontal flap surgery", *Journal of Clinical Periodontology*, Vol. 13, No. 3, pp. 205–210, 1986.

43. Cugini, M. A., A. D. Haffajee, C. Smith, R. L. Kent Jr, and S. S. Socransky, "The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results", *Journal of Clinical Periodontology*, Vol. 27, No. 1, pp. 30–36, 2000.

44. Meisel, P. and T. Kocher, "Photodynamic therapy for periodontal diseases: state of the art", *Journal of Photochemistry and Photobiology B: Biology*, Vol. 79, No. 2, pp. 159–170, 2005.

45. Socransky, S.S., and Haffajee. A.D., "Periodontal microbial ecology". *Periodontol. 2000*, Vol. 38, pp.135-87, 2005

46. Siqueira, J.F., I. N. Jr, Rôças, S.S. Paiva, T. Guimarães-Pinto, K.M. Magalhães, and K.C. Lima, "Bacteriologic investigation of the effects of sodium hypochlorite and chlorhexidine during the endodontic treatment of teeth with apical periodontitis", *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, Vol. 104, No.1, pp 122-130, 2007.

47. Gosau1, M., S. Hahnel, F. Schwarz, T. Gerlach, T. E. Reichert, R. Bürgers, "Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm", *Clinical Oral Implants Research*, Vol.21, No.8, pp 866-872, 2001.

48. Adams, D. and M. Addy, "Mouthrinse" *ADR*, Vol.8, No.291, 1994:

49. Ouhayoun J.P.,"Penetrating the plaque biofilm: impact of essential oil mouthwash", *Journal of Clinical Periodontology* , Vol.30 ,pp 10-12, 2003.

50. Yazdankhah, S. P., A. A. Scheie, E. A. Høiby, B.T. Lunestad, E. Heir, T. Ø. Fotland, K. Naterstad, and H. Kruse, " Triclosan and Antimicrobial Resistance in Bacteria: An Overview", *Microbial Drug Resistance*, Vol 12, No. 2, pp 83-90, 2006.

51. Andres, C. J., J. C. Shaeffer, and A. S. Windeler, "Comparison of antibacterial properties of stannous fluoride and sodium fluoride mouthwashes", *Journal of dental research*, Vol. 53, No. 2, pp. 457, 1974

52. Banoczy, J., B. Ritlop, G. Solymosi, A. Gombik, and A. Adatia, "Anticariogenic effect of fluoridated milk and water in rats.", *Acta physiologica Hungarica*, Vol. 76, No. 4, pp. 341, 1990.

53. Shah, N. "*Oral and Dental Diseases: Causes, prevention and treatment strategies. In Burden of Disease in India*", National Commission on Macroeconomics and Health, Ministry of Health & Family Welfare & Ministry of Finance, Government of India, 2005.

54. Fluride Action Network, *http://www.fluoridealert.org/*

55. Harrison, P. T., "Fluoride in water: A UK perspective", *Journal of fluorine chemistry*, Vol. 126, No. 11-12, pp. 1448–1456, 2005

56. Slots J., "Selection of antimicrobial agents in periodontal therapy", *J Periodont Res,*Vol. 37, pp.389-398, 2002

57. Musk, D. J. Jr., and P. J. Hergenrother, "Chemical Countermeasures for the Control of Bacterial Biofilms: Effective Compounds and Promising Targets", *Current Medicinal Chemistry,*Vol. 13, pp 2163-2177, 2006.

58. Spratt, D. A., J. Pratteni, M. Wilson, and K. Gulabivala,"An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates", *International Endodontic Journal*, Vol. 34, pp. 300-307, 2001.

59. Eley, B. M., "Antibacterial agents in the control of supragingival plaque, a review", *British Dental Journal*, Vol. 186, pp 286-296, 1999.

60. Ferres, M., A.D. Haffajee, K. Allard, S. Som, J.M. Goodson and S.S. Socransky, "Antibiotic resistance of subgingival species during and after antibiotic therapy", *J. Clin. Periodontol.* Vol.29, pp. 724–735, 2002.

61. Fosse T., I. Madinier, L. Hannoun, C. Giraud Morin, C. Hitzig, Y. Charbit and S. Ourang, "High prevalence of cfxA β-lactamase in aminopenicillin-resistant *Prevotella* strains isolated from periodontal pockets", *Oral Microbiol. Immunol*,.Vol. 17, pp. 85–88, 2002

62. Tichy, J. and Novak, J. “Extraction, assay, and analysis of antimicrobials from plants with activity against dental pathogens (*Streptococcus sp*.),” *Journal of Alternative and Complementary Medicine*, vol. 4, no. 1, pp. 39–45, 1998.

63. Burt, S. A., "Essential oils: Their antibacterial properties and potential applications in foods-a review", *International Journal of Food Microbiology*, Vol.94, pp. 223–253, 2004.

64. Meskin, M.S., W.R.Bidlack., A.J.Davies, and S.T.Omaye, "Phytochemicals in Nutrition and health", *CRC,Boca Raton*, 2002.

65. Evans, R. J., and G. S., Reyhout, "Alternatives to synthetic antioxidants, in charalambous,G.(ed)", *Food science and human nutrition*, *Elsevier*, *Amsterdam*, pp.27-42, 1992.

66. Madsen, H.L., and G. Bertelsen, "Spices as Antioxidants", *Trends Food Scie. Techn*. Vol.6, pp.271-277, 1995.

67. Venskutonis, P.R., "Food additives: the dilemmaof synthetic or natural", *Acta Alimentaria* Vol.33(1), pp.1-5, 2004.

68. Cowan, M. M., "Plant products as antimicrobial agents", *Clinical Microbiology Reviews*, Vol. 12, No. 4, pp. 564–582, 1999.

69. Kalemba, D., and A. Kunicka, "Antibacterial and antifungal properties ofessentialoils,"*CurrentMedicinal Chem*istry, Vol.10, No. 10, pp. 813–829, 2003.

70. Chitme, H. R., R. Chandra, and S. Kaushik, "Studies on antidiarrheal activity of calotropis gigantea R. Br. in experimental animals," *Journal of Pharmacy & Pharmaceutical Sciences*, Vol.7, pp. 70–75, 2003.

71. Kim, H.-S., "Do not put too much value on conventional medicines" *Journal of Ethnopharmacology*, Vol. 100, no. 1-2,pp. 37–39, 2005.

72. Shu., Y.Z, "Recent natural products based drug development: a pharmaceutical industry perspective". *Journal of Natural Products* Vol.61, pp.1053–1071, 1998.

73. http://www.naturalpedia.com/P/Plants-United_States.html

74. Kalemba, D., and A. Kunicka, "Antibacterial and antifungal properties of essential oils," *Current Medicinal Chemistry*, Vol.10, no.10, pp.813–829, 2003.

Smith-Palmer, A., J. Stewart, and L. Fyfe, "Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens", *Letters in Applied Microbiology*, Vol. 26, No. 2, pp. 118–122, 1998.

75. Kim, J., M. R. Marshall, and C. Wei, "Antibacterial activity of some essential oil components against five foodborne pathogens", *Journal of Agricultural and Food Chemistry*, Vol. 43, No. 11, pp. 2839–2845, 1995.

76. Sinico, C., De Logu, A., Lai, F., Valenti, D., Manconi, M., Loy, Giuseppe., Bonsignore, L., Fadda, A.M., "Liposomal incorporation of *Artemisia arborescens L*. essential oil and in vitro antiviral activity," *European Journal of Pharmaceutics and Biopharmaceutics*, Vol. 59, pp. 161–168, 2005.

77. Gumus.T.,"Determination of the changes of antifungal properties of *Satureja hortensis*, *Thymus vulgar*is and *Thymbraspicata* exposed to gamma irradiation"., *Radiation Physics and Chemistry*, Vol.79 , pp.109–114, 2010.

78. Vivek, K., H.K.Baek, and C. Kang, "Control of *Salmonella* in foods by using essential oils: a review", *Food Research International*, 2011.

79. Hajhashemi, V., et al, "Anti-inflammatory and analgesic properties of the leaf extracts and essential oil of *Lavandula angustifola*," *Mill. J.Ethnopharmacol*, Vol.89, pp.67-71, 2003.

80. Miller, L. and B. Miller, "*Ayurveda & aromatherapy: the Earth Essential guide to ancient wisdom and modern healing"*, Lotus Pr, 1995.

81. Boutekedjiret, C., F. Bentahar, R. Belabbes, and J. M. Bessiere, "Extraction of rosemary essential oil by steam distillation and hydrodistillation", *Flavour and Fragrance Journal*, Vol. 18, No. 6, pp. 481–484, 2003

82. Rahman, A., "Studies in Natural Products Chemistry", *Elsevier,*Vol. 21, No. 2, 2000.

83. Anna.C., Manzan. C. M., Fabio S. Toniolo , Eliane Bredow , and Nanci P. Povh, "Extraction of essential oil and pigments from *Curcuma longa L*. by steam distillation and extraction with volatile solvents", *J. Agric. Food Chem.*, Vol. 51 (23), pp 6802–6807, 2007.

84. Ericksen, M., "*Healing with Aromatherapy"*, Keats Pub, 2000.

85. Damjanovic, B., Z. Lepojevic, V. Zivkovic, and A. Tolic, "Extraction of fennel (Foeniculum vulgare Mill.) seeds with supercritical CO2: Comparison with hydrodistillation", *Food Chemistry*, Vol. 92, No. 1, pp. 143–149, 2005.

86. Linskens, H.F, J.F. Jackson, and M.S., "Allen, *Plant volatile analysis"*, Springer, 1997.

87. Mandal, V., Y. Mohan, and S. Hemalatha, "Microwave assisted extraction-an innovative and promising extraction tool for medicinal plant research", *Pharmacog Rev*, Vol. 1, pp. 7–18, 2007.

88. Sawamura, M., "*Citrus Essential Oils"*, Flavor and Fragrance, Wiley, 2010

89. Lucchesi, M.E., F. Chemat and J. Smadja, "Solvent-free microwave extraction of essential oil from aromatic herbs: comparison with conventional hydro-distillation", *Journal of Chromatography A*, Vol.1043, No.2, pp 323-327, 2004.

90. Okoh, O.O., A.P. Sadimenko and A.J. Afolayan, "Comparative evaluation of the antibacterial activities of the essential oils of *Rosmarinus officinalis* L. obtained by hydrodistillation and solvent free microwave extraction methods", *Food Chemistry*, Vol. 120, No.1, pp 308-312, 2010.

91. Bouhdid, S., Skali, S. N., Idaomar, M., Zhiri, A., Baudoux, D., Amensour, and M., Abrini, J."Antibacterial and antioxidant activities of *Origanum compactum* essential oil ", *African Journal of Biotechnology*, Vol. 7 (10), pp. 1563-1570, 16 May, 2008

92. Perry, N.S., and et. al, "Salvia for dementia threapy: review of pharmacological activity and pilot tolerability clinical trial, "*Pharmacol. Biochem. Behav.* Vol.75, pp. 651-659, 2003.

93. Silva, J., "Analgesic and anti-inflammatory effects of essential oils of eucalyptus," *J. Ethanopharmacol*. Vol. 89, pp. 277-283, 2003.

94. Carson, C.F., B.J. Mee., T.V. Riley.,"Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphyloccus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy," *Antimicrobial Agents and Chemotherapy*, Vol. 46, pp. 1914-1920, 2002.

95. Suresh P, Ingle VK, Vijayalakshmi." Antibacterial activity of eugenol in comparison with other antibiotics". *Journal Food Sci. Technol.*Vol. 9, pp.256–257, 1992.

96. Ceylan, E., and D. Y .C. Fung, "Antimicrobial activity of species," *Journal of Rapid Methods and Automation in Microbiology*, Vol. 12, pp.15-20, 2004.

97. Bakkali, F., S.Averbeck, D. Averbeck, and M. Idaomar, "Biological effects of essential oils'-review", *Food and Chemical Toxicology* Vol.46, pp.446-475, 2008.

98. Goni, P., P.Lopez, C.Sanchez, and R. Gomez-lus, "Antimicrobial activity of vapour phase of a combination of cinnamon and clove essential oils", *Food Chemistry*, Vol.116, pp. 982-989, 2009.

99. Syamsir, D. R., "Essential oils and biological activities of three selected wild *Alpinia* species", *Industrial Crops and Products*, Vol. 40, , pp. 254-260, 2009.

100. Busatta, C., Vidal, R. S, A.S., Popiolski, A.J., Mossi, and R.L.," Application of *Origanum majorana L*. essential oil as an antimicrobial agent in sausage", *Food Microbiol*. Vol. 25, pp. 207-211, 2008.

101. Edris, A.," Pharmaceutical and Therapeutic potentials of essential oils and their individual volatile constituents: a review", *Phytotherapy Research*, Vol. 21,pp: 308-323, 2007.

102. Tajkarimi, M.M., Ibrahim, S.A., Cliver, D.O.,"Antimicrobial herb and spice compounds in food", *Food Control*, Vol.21, pp: 1199-1218, 2010.

103. .Cushnie, T.P., Lamb, A.J.,"Antimicrobial activity of flavonoids", *International Journal of Antimicrobial Agents*, Vol.26, pp: 343-356, 2005.

104. Chen, X., Y. Zhang, Y. Zu, Y. Fu, and W. Wang, "Composition and Biological activities of the essential oil from *Schisandra chinensis* obtained by solvent-free microwave extraction", *Food Science and Technology*, Vol.44, pp. 2047-2052, 2011.

105. Santos, F.S., and M.G.Novales, "Essential oils from aromatic herbs as antimicrobial agents", *Current opinion in Biotechnology*"Vol.23, pp.1-6, 2011.

106. Nychas, G.J.E.," Natural antimicrobials from plants. in G.W.Gould, New methods of food preservation," *London, Blackie Academic and Professional*, pp. 58-89, 1995.

107. Shelef, L.A.," Antimicrobial effects of spices," *Journal of Food Safety*, Vol.6, pp. 29-44, 1983.

108. Sikkema, J., J. A. De Bont, and B. Poolman, "Interactions of cyclic hydrocarbons with biological membranes.", *Journal of Biological Chemistry*, Vol. 269, No. 11, pp. 8022, 1994.

109. Cox, S.D., C.M.Mann, J.L.Markham, and J.E.Gustafson, "The mode of antimicrobial action of essential oil of *Melaleuca alternifola*", Journal of Applied Microbiology, Vol. 88, pp. 170-175, 2000.

110. Denyer, S.P., and W.B.Hugo, "Biocide-induced damage to the bacterial cytoplasmic membrane". *International Biodeterioration and Biodegradation*, Vol. 36,pp. 227-245, 1995.

111. Fei, Lv, H. Liang, Q. Yuan and C.Li "In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms ", *Food Research International*, .Vol.44, pp. 3057-3064, 2011.

112. Denyer, S.P., and W.B. Hugo, "Mechanisms of action of chemical biocides". *The society for applied bacteriology*. Technical series no. 27 (pp 171-188), Oxford, Oxford Blackwell scientific publication.

113. Frag, R.S., Z. Y. Hewedi, and G. S. A El-Baroty, "Antimicrobial activity of some Egyptian spice essential oils", *Journals of food protection*, Vol.52(9), pp. 665-667, 1989.

114. Mohagheghzadeh A, M. Shams-Ardakani, and A. Ghannadi, "Volatile constitutes of callus and flower-bearing tops of *Zataria multiflora Boiss* (Lamiaceae)". *Flavour Fragrance J*ournal, Vol.15, pp. 373-6, 2000.

115. Ultee, A., R.A. Slump, G.Steging, and E. J. Smid, "Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice", *Journal of food Protection*, Vol.63 (5), pp. 620-624, 2000.

116. Knobloch, K., A. Pauli, and B.Iberl, *"Antibacterial and antifungal properties of essential oil components*" Journal of essential oil research,Vol.1, pp. 119-128, 1989.

117. Juven. B., J. Kanner, F.Schved, and Weisslowicz, "Factors that interact with the antibacterial action of thyme essential oil and its active constituents", *Journal of Applied Bacteriology*,Vol.76, pp. 626-631, 1994.

118. Trombetta, D., F. Castelli, M. G. Sarpietro, V. Venuti, M. Cristani, C. Daniele, A. Saija, G. Mazzanti, and G. Bisignano, "Mechanisms of antibacterial action of three

monoterpenes", *Antimicrobial agents and chemotherapy*, Vol. 49, No. 6, pp. 2474, 2005.

119. Sedifkon, F., Z. Jamzad and M. Mirza, "Chemical variation in the essential oil of *Satureja sahendica* from Iran," *Food Chemistry*, Vol.88, pp. 325-2-328, 2004.

120. Ultee, A., M. H. J. Bennik, and R. Moezelaar, "The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus''*, *Applied and Environmental Microbiology*, Vol. 68, No. 4, pp. 1561, 2002.

121. Dormans. H.J.D, and S.G. Deans, "Antimicrobial agents from plants : antibacterial activity of plant volatile oils", *Journal of Applied Microbiology*, Vol. 88, pp. 308-316, 2000.

122. Delaquis, P.J., K.Stanich, B.Girard and Mazza,"Antimicrobial activity of individual and mixed fractions of *dill, cilantro, coriander,* and *eucalyptus* essential oils". *International Journal of food Microbio*. Vol. 74, pp. 101-109, 2002.

123. Carson, C., and T.V.Riley. "Safety efficacy and provenance of the tea tree oil", *Contact Dermatitis*, Vol.45, pp. 65-67, 2001.

124. Skandamis, P.N. and G. J. E. Nychas. "Effect of *Oregano* essential oil on microbiological and physic-chemical attributes of minced meat stored in air and modified atmospheres", *Journal of Applied Microbiology*, Vol. 91, pp.1011-1022, 2001.

125. Gill, A. O., P.Delaquis, P.Russo and R.A.Holley, "Evaluation of antilisterical of cilantro oil on vacuum packed ham", *International Journal of Food Microbial*, Vol.73, pp.83-92, 2002.

126. Lambert, R.J., and P.N. Skandamis, "A study of the minimum inhibitory concentration and mode of *Oregano* essential oil, thymol and carvacrol," *Journal of Applied Microbiology*, Vol.91, pp. 453-462, 2001.

127. Lopez, P., C. Sanchez, and R. Batlle, "Vapor-Phase Activities of Cinnamon, Thyme, and *Oregano* Essential Oils and Key Constituents against Foodborne Microorganisms," *Journal of Agricultural and Food Chemistry*, Vol. 55, pp. 4348-4356, 2007.

128. Mondello, F., and F. Bernardis., "In vivo activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* (tea tree) oil against azole-susceptible and resistant human pathogenic *Candida* species," *BMC Infect Diseases*,Vol. 6, pp. 158, 2006.

129. Delaquis, P.J., K. Stanich, B. Girard and Mazza., "Antimicrobial activity of individual and mixed fractions of *dill, Cilantro, Coriander,* and *Eucalyptus* essential oils," *International Journal of food Microbio.* Vol. 74, pp.101-109, 2002.

130. http://www.fungoceva.it/erbe_ceb/satureja_hortensis.htm.

131. K.H.C. Baser, "Essential oils from aromatic plants which are used as herbal tea in Turkey, In; Flavours, Fragnance and Essential Oils", *Proceeding of the 13th international congress of flavours*, Fragrances and Essential oils, 1995.

132. Baser. K., and G. Tumen, et al, "Composition and antibacterial acitivity of the essential oils from *Saturaje wiedemanniana*", *Journal Essential oil Res*,Vol. 56, pp. 731-738, 2001.

133. Baser.K., and G. Tümen, "The essential oil of *Saturaje cilicica P.H.Davis*", *Journal Essential oil Res*. Vol.5, pp. 547-548, 2002.

134. Tümen, G., and N.Kırıme, "The essential oils of *Saturaje L* occurring in Turkey". *In :Proceeding of the 27 th international symposium on essential oils*, Allured Publishing Corporation, Vienna pp.250-254, 1997.

135. Güllüce, M., M. Sökmen., and D. Daferena., "In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and

callus cultures of *S.hortensis L," Journal of Agriculture And Food Chemistry,* Vol. 51, pp. 3958-3965, 2003.

136. Vagionas.K., K. Graiko, O. Ngassapa, D. Runyoro, and I. Chinou, "Composition and antimicrobial activity of the essential oils of three *Satureja* species growing tanzania", *Food Chemistry*, Vol.103, pp.319-324, 2007.

137. Krstev.T.M., D. Radnovic, D. Kıtıc, Z. S. Radıc, and B. Zlatkovıc,,"Antimicrobial activity of *Satureja hortensis L*. Essential oil against pathogenic microbial strains", *Arch.Biol.Sci.Belgrade*, Vol. 62, pp. 159-166, 2010.

138. Krstev, B.T., and D.Radnovic, "Antimicrobial activity of *Saturaje hortensis L*.essential oil against pathogenic microbial strains", *Arch.Biol.Sci.*, Vol. 62(1), pp. 159-166, 2010.

139. Smallfield, B.M., J. W. Van Klink, and N. B .Perry, "*Coriander* spice oil: effects of fruit crushing and distillation time on yield and composition", *Journal of Agric. Food.Chem.*, Vol. 49, pp. 118-123, 2001.

140. Özkalp. B., M. M. Özcan, "Antibacterial activity of several concentration of sater (*Satureja hortensis* L.) essential oil on spoliage and pathogenic food-related microorganisms", *World Applied Sciences Jou*rnal, Vol. 6, pp. 509-514, 2009.

141. Razzaghi-Abyaneh.M., M. Shams-Ghahfarokhi, T. Yoshinari, M.B. Rezaee, K. Jaimand, H. Nagasawa, and S. Sakuda, "Inhibitory effects of *Satureja hortensis L*. essential oil on growth and aflatoxin production by *Aspergillus parasiticus*", *International Journal of Food Microobiology*, Vol. 123, pp. 228-233, 2008.

142. Sahin, F., I. Kahraman, M. Gulluce, "Evaluation of Antimicrobial Activities of *Satureja hortensis L*", *Journal of Ethnoparmacology,* Vol. 87, pp. 61-65, 2003.

143. Gursoy, U.K., M. Gursoy, O. V. Gursoy, L. Cakmakcı, E. Konenen, and V. J. Uitto, "Anti-biofilm properties of *Satureja hortensis L.* essential oil against periodontal pathogens ", *Anaerobe*, Vol. 15, pp. 164–167, 2009.

144. Skocibusic.M., and N. Bezic, "Phytochemical analysis and in vitro antimicrobial activity of two *Satureja* species Essential oils", *Phytotherapy Resear*ch, Vol. 18, pp. 967-970, 2004

145. Sefidkon, F., K.Abbasi., Z.Jamzas., and S.Ahmadi., "The effect of distillation methods and stage of plant growth on the essential oil content and Composition of *Satureja rechingeri* Jamzad", *Food Chemi*stry, Vol.100, pp. 1054-1058, 2007.

146. Sefidkon, K., Z. Jamzad, "Chemical composition of the essential oil of three iranian *Satureja species* (*S.mutica, S.macrantha and S.intermedia*)", *Food Chemistr*y, Vol. 91, pp.1-4, 2005

147. Azaz. D., F. Demiric, F. Satıl, M. Kurkcuoglu, K.H.C. Baser, "Antimicrobial activity of some *Satureja* essential oils" *Z.Naturforsch,Vol*. 57c, pp. 817-821, 2002.

148. Azaz. D., Demiric.F., Satıl.F., Kurkcuoglu.M., Base.K.H.C.,"In vitro anrimicrobial activity and chemical composition of some *Satureja* essential oils", *Flavour and Fragrance Journal*, Vol. 20, pp. 587-591, 2005.

149. Tozlu.E., A. Cakır, S. Kordali, G. Tozlu, H. Ozer, and T. A. Akcın, "Chemical compositions and insecticidal effects of essential oils isolated from *Achillea gypsicola, Satureja hortensis, Origanum acutidens* and *Hypericum scabrum* against broadbean weevil", *Scientia Horticulturae,*Vol. 130, pp. 9-17, 2011.

150. Esquivel. M.M., M. A. Ribeiro, M. G. Bernardo-Gil, "Supercritical extraction of savory oil:study of antioxidant activity and extraction characterization", *Journal of Supercritical,* Vol. 4, pp. 129-138, 1999.

151. Khajeh. M.,"Optimization of process variables for essential oil components from *Satureja hortensis* by supercritical fluid extraction using Box-Behnken experimental design", *Journal of Super*critical, Vol. 55, pp. 944-948, 2011.

152. Öztürk. M., "Anticholinesterase and antioxidant activities of Savory (*Satureja thymbra L.*) with identified major terpenes of the essential oils", *Food Chemistry*, Vol. 134, pp.48-54, 2012.

153. Dikbas, N., R. Kotan, F. Dadasoglu, and F. Sahin, "Control of *Aspergillus flavus* with essential oil and methanol extract of *Satureja hortensis* ", *International Journal of Food Microbiology*, Vol. 124, pp. 179-182, 2008.

154. Sefidkon, F., K. Abbasi, and G. B. Khaniki ,"Influence ofdrying and extraction methods on yield and chemical composition of the essential oil of *Satureja hortensis*", *Food Chemistry*, Vol. 99,pp. 19–23, 2006.

155. Bangham, A.D., M.M. Standish., and J.C. Watkins, "Liposomes" *Journal Mol. Biol*.,Vol.13, pp. 34, 1965.

156. Lasic, D.D., "Liposomes, From Physics to Applications," *Elsevier, Amsterdam/ London, New York*, Tokyo, 1993.

157. Keller, B.C., "Liposomes in Nutrition," *Trends in Food Science and Technology*, Vol. 12, pp. 25-31, 2001.

158. Lasic, D.D., "Liposomes in Gene Delivery," *CRC Boca Raton.*, 1997.

159. Hirokazu, F., and H. Yasushi., "Improvement of transduction efficiency of recombinant adenovirus vector conjugated with cationic liposome for human oral squamous cell carcinoma cell lines," *Oral Oncology*, Vol. 39, pp. 601-609, 2003.

160. Donsi, F., M. Annunziata., and M. Sessa., "Nano encapsulation of essential oils to enhance their antimicrobial activity in foods," *Food Science and Technology*, Vol. 44 pp. 1908-1914, 2011

161. Helfrich, W., "Size Distributions of Vesicles: The Role of Effective Rigidity of Membranes", *Journal of Physc*.Vol. 47, 1984.

162. Sharma, A., and U. Sharma, "Liposomes in drug delivery:progress and limitations", *International Journal of Pharmaceutics*, Vol.154, pp. 123-140, 1997.

163. Steinberg, D., and M. Friedman, "Dental drug-delivery devices; local and sustained release applications," *Crt. Rev. Ther. Drug Carrier Syst*., Vol. 16, pp. 425-459, 1999.

164. Beaulac. C, S. Sachetelli, and J. Lagace, "In-vitro bactrical efficacy of Sub-MIC concentration of liposome encapsulated antibiotic against Gram- negative and Gram positive bacteria", *Journal of Antimicrobial Chemothreapy*, Vol. 41, pp. 35-41, 1998.

165. Zasadzinsk,J.A., B. Wong, N. Forbes, G. Braun, G. Wu, "Novel methods of enhanced retention in and rapid, targeted release from liposomes", Vol. 16, pp. 203-214, 2011.

166. Fleischer, S., G, Rouser, B.Fleischer, B. Cash, and Kritcheusky, "Phospholipids," *Journal of Lipid Res*.Vol.8,pp.170-180, 1967.

167. Johnson. T., and C. Case, "Chemical Methods of control", *Laboratory experiments in Microbiology*, 4th Edition,Redwood City,CA, September 11, 2006.

168. Liolious. L.L, O. Gortzi, S. Lalas, J. Tsaknis, and I. Chinou, "Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus L*. and in vitro antimicrobial activity", *Food Chemistry*, Vol. 112, pp .77-83, 2009.

169. Ortan, A., G. H. Campeanu, C. Dinu, and L. Popescu, "Studies concerning the entrapment of *Anethum graveolens* essential oil in liposomes", *Roumanian Biotechnology Letters*,Vol.14, pp.4411-4417, 2008.

170. Espina, L., M. Somolinos, and S. Loran, "Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobail activity acting alone or in combined process", *Food Control*, Vol. 22, pp. 896-902, 2011.

171. Ghannadi, A., "Composition of the essential oil of *Satureja hortensis L*. seeds from Iran", *Journal of Essential Oil Research*, Vol. 14, pp. 35-39, 2002.

172. Arora, D.S., and I. Kaur, "Antimicrobial activity of species", *Journal of Antimicrobial Agents*, Vol. 12, pp. 257-262, 1999.

173. Joulain, D., W. A. Ko¨ nig, and D. H. Hochmuth, "Terpenoids and related constituents of essential oils", *Hamburg, Germany: Library of MassFinder*, 2001.

174. McLafferty, F. W., D. B. Stauffer, "The Wiley/NBS registry of mass spectral data", *New York: J. Wiley and Sons*,1989.

175. Adams, R. P., "Identification of essential oil components by gas chromatography/mass spectroscopy", *Allured Publishing Corp*., Carol Stream, USA, 1995.

176. Mirjana, S., and N. Bezic, "'Phytochemical analysis and in viro antimicrobial activity of two *Satureja sepecies* essential oils," *Phytothreapy Research*, Vol.18, pp. 967-970, 2004.

177. Tumen, G., K. H. C. Baser, B. Demirci, and N. Ermin, "The essential oils of *Satureja coerulea* Janka and *Thymus aznavourii* Velen", *Flavour and Fragrance Journal*, Vol. 13, pp. 65-67, 1998.

178. Cosentino, S., C. I. G. Tuberoso, B. Pisano, M. Satta, V. Mascial, E. Arzedi, and F. Plamas, " In-vitro antimicrobial activity and chemical composition of *Sardinian Thymus* essential oils," *Letters in Applied Microbiology*, Vol. 29, pp. 130-135, 1999.

179. Szoka, F. Jr., and D. Papahadjopoulos, "Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes)", Annual *Review of Biophysics and Bioengineering*, Vol. 9, pp. 467-508, 1980.

180. Kumar. B., P. Praveen, B. Kaur, B. Mittu and N. Garg, "Antimicrobial and Spermicidal Activity Of Native And Recombinant Pediocin CP2: A Comparative Evaluation", *Clinical Microbiology*, Vol.3, No. 3, 2012

181. Nguyen, S., M. Hiorth, M. Rykke, and G. Smistad, "The potential of liposomes as dental drug delivery systmes", *Eur. Journal Pharm. Biopharm*., Vol. 77, pp. 75-83, 2011.

182. Campanha. M. T. N., E. M. Mamizuki, and A. M. Carmona, "Interaction between cationic liposomes and bacteria: the physical- chemistry of the bactericidal action" *Journal of Lipid Research*, Vol. 40, 1999.

183. King, D., A. Staab, A. J. Kaplita, D. Yurgaitis, R. Firestone, and S. Lasch, "BR96 conjugates of highly potent anthracyclines", *Bioorganic & Medicinal Chemistry Letters*,Volume 13, pp. 2119–2122, 2003.

184. Calvagno, M.G , C, Christian, P. Donatella, C. Donato, C. Iannone, C. Francesco, D. Patrizia, and F. Massimo, "Effects of Lipid Composition and Preparation Conditions on Physical-Chemical Properties, Technological Parameters and In Vitro Biological Activity of Gemcitabine-Loaded Liposomes", *Current Drug Delivery*, Vol.4, pp. 89-101, 2007.

185. Shoji. Y., and H. Nakashima, "Nutracutics and delivery systems", *Journal of Drug Targeting*, Vol. 12, pp. 385-391, 2004.

186. Shapiro, S., A. Meier, and B. Guggenheim, "The antimicrobial activity of essential oils and essential oil components towards oral bacteria", *Oral Microbiology Immunology*, Vol. 9, pp. 202–20864, 1994.

187. Chami, N., S. Bennis, F. Chami, A. Aboussekhra, and A. Remmal," Study of anticandidal activity of carvacrol and eugenol in vitro and in vivo", *Oral Microbiology Immunology*, Vol. 20, pp. 106–111, 2005.

188. Hammer, K. A., L. Dry, M. Johnson, E. M. Michalak, C. F. Carson, and T. V. Riley,"Susceptibility of oral bacteria to Melaleuca alternifolia (tea tree) oil in vitro", *Oral Microbiology Immunology*, Vol. 18, pp. 389–392, 2003.

189. Solmaz, G., P. S. Bird, Y. Ekinci, M. Korachi, "Growth Inhibition Properties of Shiitake Extract on Pathogenic Oral Anaerobes", *IADR*, Marh 16-19, 2011, no. 2237, San diego, California.

190. Solmaz, G., P. S. Bird, Y. Ekinci, M. Korachi," Biofilm Inhibition Properties of Natural Asian Herbs ", *IADR*, Macrh 16-19, 2011, no. 3026, San Diego, California.

191. National Committee for Clinical Laboratory standards, Method for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Approved standard, 6th ed., *A.Wayne, Pennsylvania USA: NCCLS document M7-A6*, ısbn 1-56238-486, 2003.

192. Rongen, H.A.H., A. Bult, W. P. Van Bennekom, "Liposomes and Immunoassays", *Journal of Immunological Methods*, Vol. 204, pp. 105-103, 1997.

193. Oke, F., B. Aslim, S. Ozturk, and S. Altundag, " Essential Oil composition, antimicrobial and antioxidant activities of *Satureja cuneifola*", *Food Chemistry*, Vol. 112, pp. 874-879, 2009.

194. Mugabe..C., R. T. Liggins, D. Guan, I. Manisali, and H. M. Burt," Development and in vitro characterization of paclitaxel and docetaxel loaded into hydrophobically

derivatized hyperbranched polyglycerols", *Pharmaceutical Nanotechnology*,Vol. 404, Issues 1-2, pp.238-249, 2011.

195. Colas, J.C., W. Shi,V. S. N. Malleswara Rao, A. Omri, M. R. Mozafari, H. Singh, "Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting", *Micron 38* , pp.841–847, 2007.

196. Hasegawa, T.,."Cenomanian–Turonian carbon isotope events recorded in terrestrial organic matter from northern Japan", *Palaeogeogr. Palaeoclimatol*. Palaeoecol. Vol.130, pp.251– 273, 1997.

APPENDIX A: GC-MS ANALYSIS RESULTS

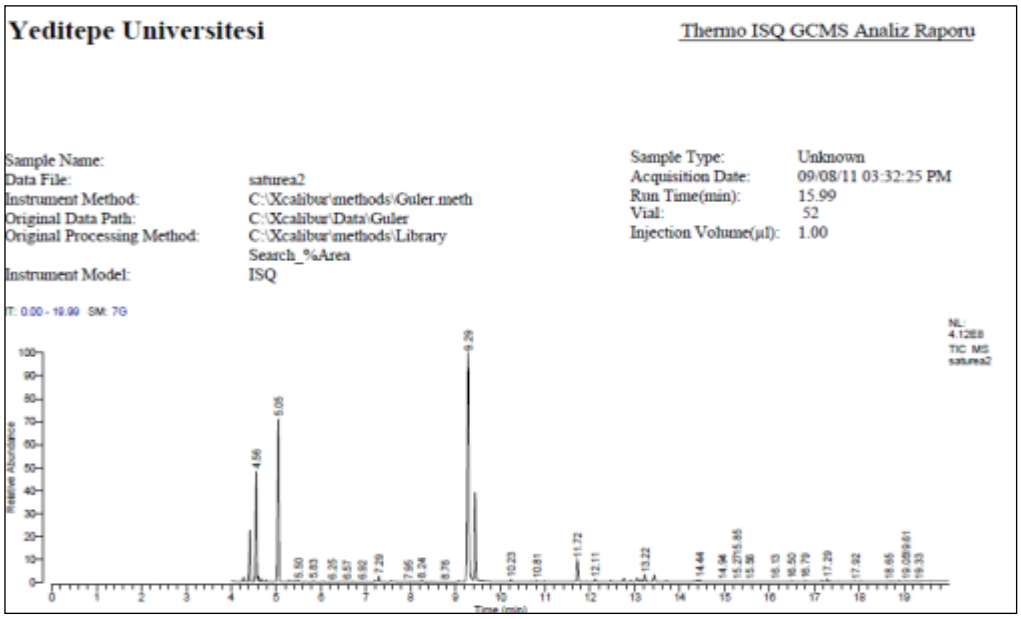

Figure A.1. Chromotogram Graphic Of *Satureja hortensis L*. Essential Oil

Table A.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS)

RT	Compound Name	SI	Cas #	Library	Area	Area %
4.27	l-Phellandrene	936	99-83-2	WILEY7	14094544	0.49
4.27	l-Phellandrene	923	99-83-2	WILEY7	14094544	0.49
4.27	à PHELLANDRENE	920	1529-99-3	WILEY7	14094544	0.49
4.41	à-Terpinene	937	99-86-5	WILEY7	141543335	4.91
4.41	à TERPINENE	936	99-86-5	WILEY7	141543335	4.91
4.41	à-Humulene	932	6753-98-6	WILEY7	141543335	4.91
4.56	Benzene, 1-methyl-4-(1-methylethyl)- (CAS)	942	99-87-6	WILEY7	351987521	12.22
4.56	Benzene, 1-methyl-4-(1-methylethyl)- (CAS)	936	99-87-6	WILEY7	351987521	12.22
4.56	Benzene, 1-methyl-4-(1-methylethyl)- (CAS)	926	99-87-6	WILEY7	351987521	12.22
4.67	Sabinene	921	3387-41-5	WILEY7	7961643	0.28
4.67	Sabinene	896	3387-41-5	WILEY7	7961643	0.28
4.67	á-Phellandrene	894	555-10-2	WILEY7	7961643	0.28
4.77	1,3,6-Octatriene, 3,7-dimethyl-, (E)- (CAS)	918	3779-61-1	WILEY7	4678366	0.16
4.77	cis-Ocimene	914	6874-10-8	WILEY7	4678366	0.16

TableA.1. Percentages of the Chemical Composition of the *S.hortensis L*. EO (GC-MS) (continued)

RT	Compound Name	SI	Cas #	Library	Area	Area %
4.77	1,3,6-Octatriene, 3,7-dimethyl-, (E)- (CAS)	918	3779-61-1	WILEY7	4678366	0.16
4.77	cis-Ocimene	914	6874-10-8	WILEY7	4678366	0.16
4.77	1,3,6-Octatriene, 3,7-dimethyl-, (E)- (CAS)	906	3779-61-1	WILEY7	4678366	0.16
5.05	ç-Terpinene	931	99-85-4	WILEY7	573406192	19.90
5.05	ç-Terpinene	924	99-85-4	WILEY7	573406192	19.90
5.05	ç-Terpinene	922	99-85-4	WILEY7	573406192	19.90
5.29	CIS-SABINENE HYDRATE	905	15826-82-1	WILEY7	3019532	0.10
5.29	TRANS-SABINENE HYDRATE	890	17699-16-0	WILEY7	3019532	0.10
5.29	TRANS-SABINENE HYDRATE	872	546-79-2	WILEY7	3019532	0.10
5.36	1-PHENYL-4-CYANO-1-OCTANONE	921	58422-89-2	WILEY7	1778938	0.06
5.36	Ethanone, 1-phenyl- (CAS)	918	98-86-2	WILEY7	1778938	0.06
5.36	7,7-dimethyl-tetracyclo[4.1.0.0(2,4).0(3,3)]hepta	917	124345-62-6	WILEY7	1778938	0.06
5.43	Benzaldehyde, 4-methyl- (CAS)	917	104-87-0	WILEY7	3900358	0.14
5.43	Tolualdehyde [methylbenzaldehyde]	911		NA	WILEY7	3900358
5.43	Benzaldehyde, 3-methyl- (CAS)	905	620-23-5	WILEY7	3900358	0.14
5.50	à-TERPINOLENE	896	586-62-9	WILEY7	4414349	0.15
5.50	à-Terpinene	896	99-86-5	WILEY7	4414349	0.15
5.50	à-TERPINOLENE	895	586-62-9	WILEY7	4414349	0.15
5.68	Benzene,	638	77611-58-6	WILEY7	1101402	0.04
5.68	3-PHENYL-1-PROPANOL-1,2-D2	636	19718-72-0	WILEY7	1101402	0.04
5.68	Benzaldehyde, 4-methyl- (CAS)	616	104-87-0	WILEY7	1101402	0.04
5.83	CIS-SABINENE HYDRATE	894	15826-82-1	WILEY7	2640984	0.09
5.83	TRANS-SABINENE HYDRATE	885	17699-16-0	WILEY7	2640984	0.09
5.83	trans Sabinene hydrate	878	17699-16-0	WILEY7	2640984	0.09
5.43	Benzaldehyde, 3-methyl- (CAS)	905	620-23-5	WILEY7	3900358	0.14
5.43	Benzaldehyde, 3-methyl- (CAS)	905	620-23-5	WILEY7	3900358	0.14
5.50	à-TERPINOLENE	896	586-62-9	WILEY7	4414349	0.15
5.50	à-TERPINOLENE	895	586-62-9	WILEY7	4414349	0.15
5.68	Benzene,	638	77611-58-6	WILEY7	1101402	0.04
5.68	3-PHENYL-1-PROPANOL-1,2-D2	636	19718-72-0	WILEY7	1101402	0.04
5.68	Benzaldehyde, 4-methyl- (CAS)	616	104-87-0	WILEY7	1101402	0.04
5.83	CIS-SABINENE HYDRATE	894	15826-82-1	WILEY7	2640984	0.09
5.83	TRANS-SABINENE HYDRATE	885	17699-16-0	WILEY7	2640984	0.09
5.83	trans Sabinene hydrate	878	17699-16-0	WILEY7	2640984	0.09
6.05	5-ETHYL-2-METHYL-PYRIDIN-4-YLAMINE	737	5350-64-1	WILEY7	1079104	0.04

Table A.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS) (continued)

RT	Compound Name	SI	Cas #	Library	Area	Area %
6.05	TRANS-2-CAREN-4-OL	723	6617-35-2	WILEY7	1079104	0.04
6.05	(1,3-DIMETHYL-2-METHYLENE-CYCLOPE	712		NA	WILEY7	1079104
6.25	CIS-SABINENE HYDRATE	776	15826-82-1	WILEY7	969837	0.03
6.25	1-TERPINEOL	770	586-82-3	WILEY7	969837	0.03
6.25	TRANS-SABINENE HYDRATE	766	17699-16-0	WILEY7	969837	0.03
7.20	1-BORNEOL	874	464-45-9	WILEY7	3593541	0.12
7.20	endo-Borneol	859	507-70-0	WILEY7	3593541	0.12
7.20	1-BORNEOL	852	464-45-9	WILEY7	3593541	0.12
7.29	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	938	562-74-3	WILEY7	18634227	0.65
7.29	(CAS)					
7.29	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	938	562-74-3	WILEY7	18634227	0.65
7.56	3-Cyclohexene-1-methanol, à,à,4-trimethyl-,	878	10482-56-1	WILEY7	3346572	0.12
7.56	(S)- (CAS)					
7.56	á FENCHYL ALCOHOL	876	470-08-6	WILEY7	3346572	0.12
8.24	CARVACROL METHYL ETHER	906	6379-73-3	WILEY7	4648681	0.16
8.24	THYMYL METHYL ETHER	888	1076-56-8	WILEY7	4648681	0.16
8.24	2-Isopropyl-1-methoxy-4-methylbenzene	881	31574-44-4	WILEY7	4648681	0.16
9.08	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	866	499-75-2	WILEY7	3305182	0.11
9.08	Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	861	89-83-8	WILEY7	3305182	0.11
9.08	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	859	499-75-2	WILEY7	3305182	0.11
9.28	Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	924	89-83-8	WILEY7	1200230288	41.65
9.28	Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	921	89-83-8	WILEY7	1200230288	41.65
9.28	Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	918	89-83-8	WILEY7	1200230288	41.65
9.44	Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	920	89-83-8	WILEY7	296527363	10.29
9.44	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	918	499-75-2	WILEY7	296527363	10.29
9.44	Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	903	89-83-8	WILEY7	296527363	10.29
10.23	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	879	499-75-2	WILEY7	6740285	0.23
10.23	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	878	499-75-2	WILEY7	6740285	0.23
10.23	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	878	499-75-2	WILEY7	6740285	0.23
10.68	à-Copaene	831	3856-25-5	WILEY7	1406115	0.05
10.68	à-Ylangene	807	14912-44-8	WILEY7	1406115	0.05
10.68	à-Cubebene	804	17699-14-8	WILEY7	1406115	0.05
10.81	à-Copaene	888	3856-25-5	WILEY7	2975957	0.10
10.81	à-Copaene	864	3856-25-5	WILEY7	2975957	0.10
10.81	à-Copaene	862	3856-25-5	WILEY7	2975957	0.10

Table A.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS) (Continued)

RT	Compound Name	SI	Cas #	Library	Area	Area %
11.72	trans-Caryophyllene	953	87-44-5	WILEY7	84750505	2.94
11.72	trans-Caryophyllene	940	87-44-5	WILEY7	84750505	2.94
11.72	trans-Caryophyllene	923	87-44-5	WILEY7	84750505	2.94
11.92	GERMACRENE-D	794	23986-74-5	WILEY7	2410892	0.08
11.92	GERMACRENE-D	792	23986-74-5	WILEY7	2410892	0.08
11.92	á-Cubebene	778	13744-15-5	WILEY7	2410892	0.08
12.11	AROMADENDRENE	909	489-39-4	WILEY7	9254544	0.32
12.11	Aromadendrene	900	489-39-4	WILEY7	9254544	0.32
12.11	Alloaromadendrene	899	25246-27-9	WILEY7	9254544	0.32
12.46	à-Humulene	860	6753-98-6	WILEY7	3857765	0.13
12.46	à-Humulene	852	6753-98-6	WILEY7	3857765	0.13
12.46	à-Humulene	849	6753-98-6	WILEY7	3857765	0.13
12.75	ç-Cadinene	896	39029-41-9	WILEY7	11992948	0.42
12.75	à-Amorphene	895	23515-88-0	WILEY7	11992948	0.42
12.75	Naphthalene,	892	30021-74-0	WILEY7	11992948	0.42
12.83	à-Muurolene	813	31983-22-9	WILEY7	1090512	0.04
12.83	à-Amorphene	773	23515-88-0	WILEY7	1090512	0.04
12.83	à-Muurolene	771	31983-22-9	WILEY7	1090512	0.04
12.90	GERMACRENE-D	883	23986-74-5	WILEY7	4381163	0.15
12.90	GERMACRENE-D	873	23986-74-5	WILEY7	4381163	0.15
12.90	Germacrene D	848	23986-74-5	WILEY7	4381163	0.15
13.05	Ledene	885	21747-46-6	WILEY7	16340283	0.57
13.05	LEDENE	879	21747-46-6	WILEY7	16340283	0.57
13.05	1H-Cycloprop[e]azulene,	879	49826-80-4	WILEY7	16340283	0.57
13.15	à-Muurolene	884	31983-22-9	WILEY7	8621361	0.30
13.15	Valencene	856	4630-07-3	WILEY7	8621361	0.30
13.15	ç-Gurjunene	851	22567-17-5	WILEY7	8621361	0.30
13.22	á-Bisabolene	888	495-61-4	WILEY7	22144824	0.77
13.22	1H-Benzocycloheptene,	888	3853-83-6	WILEY7	22144824	0.77
13.22	2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-m					
13.43	ë-Cadinene	922	483-76-1	WILEY7	27216108	0.94
13.43	ë-Cadinene	892	483-76-1	WILEY7	27216108	0.94
13.43	ë-Cadinene	891	483-76-1	WILEY7	27216108	0.94
13.54	BENZOL,	653		NA	WILEY7	1044066
13.54	1-(1-FORMYLETHYL)-4-(1-BUTEN-3-YL)-					

Table A.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS) (Continued)

RT	Compound Name	SI	Cas #	Library	Area	Area %
13.70	à-Copaene	830	3856-25-5	WILEY7	3483850	0.12
13.70	à-Muurolene	822	31983-22-9	WILEY7	3483850	0.12
13.70	Zingiberene	820	495-60-3	WILEY7	3483850	0.12
14.27	10,13-Octadecadiynoic acid, methyl ester (CAS)	680	18202-24-9	WILEY7	977191	0.03
14.27	ISOAROMADENDRENEPOXID	662		NA	WILEY7	977191
14.27	METHYL ESTER OF	656		NA	WILEY7	977191
14.37	(+) spathulenol	871	77171-55-2	WILEY7	4345416	0.15
14.37	(-)-Spathulenol (CAS)	862	77171-55-2	WILEY7	4345416	0.15
14.37	SPATHULENOL	833	6750-60-3	WILEY7	4345416	0.15
14.44	(-)-Caryophyllene oxide	918	1139-30-6	WILEY7	5575333	0.19
14.44	(-)-Caryophyllene oxide	891	1139-30-6	WILEY7	5575333	0.19
14.44	(-)-Caryophyllene oxide	890	1139-30-6	WILEY7	5575333	0.19
15.02	isospathulenol	684		NA	WILEY7	864027
15.02	(+) spathulenol	670	77171-55-2	WILEY7	864027	0.03
15.02	(-)-Spathulenol (CAS)	665	77171-55-2	WILEY7	864027	0.03
15.11	ë-Cadinol	651	36564-42-8	WILEY7	1014990	0.04
15.11	à-Muurolene	644	31983-22-9	WILEY7	1014990	0.04
15.11	à-Amorphene	639	23515-88-0	WILEY7	1014990	0.04
15.27	Junipene	717	475-20-7	WILEY7	944771	0.03
15.27	EPIGLOBULOL	699	552-02-3	WILEY7	944771	0.03
15.27	Junipene	694	475-20-7	WILEY7	944771	0.03
16.50	6,10,11,11-TETRAMETHYL-TRICYCLO[5.3.0	834	489-39-4	WILEY7	1777976	0.06
16.50	.1(2,3)]UNDEC-1(7)ENE					
16.50	ë-Cadinene	833	483-76-1	WILEY7	1777976	0.06
16.79	4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalen	694	13943-77-6	WILEY7	1638605	0.06
16.79	e					
16.79	4-(2,2,6-TRIMETHYL-BICYCLO[4.1.0]HEPT	693	77143-20-5	WILEY7	1638605	0.06
17.17	4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalen	717	13943-77-6	WILEY7	3233723	0.11
17.17	e					
17.17	Silane, trimethylphenyl- (CAS)	714	768-32-1	WILEY7	3233723	0.11
17.29	4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalen	739	13943-77-6	WILEY7	5588444	0.19
17.29	e					
17.29	Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	711	499-75-2	WILEY7	5588444	0.19
17.37	2,6,10,14,18,22-Tetracosahexaene,	682	7683-64-9	WILEY7	1343495	0.05
17.37	2,6,10,15,19,23-hexamethyl- (CAS)					

Table A.1. Percentages of the Chemical Composition of the *S.hortensis* L. EO (GC-MS) (Continued)

RT	Compound Name	SI	Cas #	Library	Area	Area %
17.92	4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalen	682	13943-77-6	WILEY7	958126	0.03
17.92	e					
17.92	2H-Inden-2-one,	672	54725-16-5	WILEY7	958126	0.03

A.1. THE CALCULATION OF ESSENTIAL OIL OF *S. hortensis L.* CONCENTRATION

Table A.2. Explanation of Equation for Calculation of Essential Oil Density

m_3 (mg)	Mass of 10 ml distilled water
m_1 (mg)	Mass 9 ml distilled water
m_2 (mg)	oil + distilled water= 26.409 (mass of mixture)
m_3 (mg)	26.4985 mg = Mass of 10 ml distilled water)
d= m_l (mg)	25.4970 mg = Mass of 9 ml distilled water
d	m_2-m_1/ m_3-m_1
d	26.4095-25.4970/26.4985-25.4970
d	0.912/ 1.0015
d	0.911

APPENDIX B: PREPARATION AND CHARACTERIZATION OF LIPOSOMES AND STATISTICAL ANALYSIS RESULTS

Table B.1. Liposome Formulations, Their Compositions, Measurements For Particle Size

Code	**Composition**		**Average Diameter (nm)**	**Ortalama**	**Standart Deviations**
UL1	P 85 G:CHOL:SA	1.replicate	144,00	146,9	2,8
		2.replicate	147,1		
		3.replicate	149,6		
UL2	P85G:CHOL:SA	1.replicate	150,2	147,8	2,113
		2.replicate	146,4		
		3.replicate	146,7		
L1	P85 G:CHOL:SA+E.O	1.replicate	384,3	412,5	33,27
		2.replicate	449,2		
		3.replicate	404		
L2	P85G:CHOL:SA+E.O	1.replicate	1997	2171	151
		2.replicate	2255		
		3.replicate	2262		

Table B.2. Liposome Formulations, Their Compositions, Measurements For Particle Zeta Potential

Code	**Composition**		**Average Diameter (nm)**	**Ortalama**	**Standart Deviations**
UL1	P 85 G:CHOL:SA	1.replicate	37,4	34,,8	2,2
		2.replicate	33,6		
		3.replicate	33,5		
UL2	P85G:CHOL:SA	1.replicate	44,4	46,5	1,8
		2.replicate	47,7		
		3.replicate	47,3		
L1	P85 G:CHOL:SA+E.O	1.replicate	-35,9	-36,7	0,709
		2.replicate	-36,8		
		3.replicate	-37,3		
L2	P85G:CHOL:SA+E.O	1.replicate	-31,7	-29,9	1,66
		2.replicate	-29,7		
		3.replicate	-28,4		

Table B.3. Liposome Formulations, Their Compositions, Measurements For Particle PDI Values

Code	Composition		Average Diameter (nm)	Ortalama	Standart Deviations
UL1	P 85 G:CHOL:SA	1.replicate	0.387	0.396	0.013
		2.replicate	0.389		
		3.replicate	0.411		
UL2	P85G:CHOL:SA	1.replicate	0.316	0.304	0.012
		2.replicate	0.305		
		3.replicate	0.292		
L1	P85 G:CHOL:SA+E.O	1.replicate	0.387	0.41	0.02
		2.replicate	0.423		
		3.replicate	0.42		
L2	P85G:CHOL:SA+E.O	1.replicate	0.144	0.164	0.07
		2.replicate	0.104		
		3.replicate	0.245		

Table B.4. Liposome Formulations, Their Compositions, Measurements For Particle Size, Zeta Potential And PDI Values

Code	Composition	Molar Ratio	Average diameter (nm)	Zeta potential (mV)	PDI	Observations
UL1	PL 85 G:CHOL:SA	7;1;2	146.9 ± 2.8	34.8 ± 2.2	0.396 ± 0.01	Milky dispersions
UL2	PL 85G:CHOL:SA	10;1;4	147.8 ± 2.11	46. 5 ± 1.8	0.304 ± 0.012	Milky dispersion
L1	PL85 G:CHOL:SA+ EOs	7;1;2	412.5 ± 33.27	36.7 ± 0.7	0.41 ± 0.02	Milky dispersion
L2	PL 85G:CHOL:SA+ EOs	10;1;4	2171 ± 151.0	29.9 ± 1.66	0.164 ±0.07	Milky dispersion

B.1. STATISTICAL EVALUATION OF LIPOSOME CHARACTERIZATION

Table B.5. Mean particle size of Loaded Liposomes L1 L2 And Unloaded Liposomes UL1 And UL2

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 3 8570456 2856819 477,61 0,000
Error 8 47852 5981
Total 11 8618308

S = 77,34 R-Sq = 99,44% R-Sq(adj) = 99,24%

Individual 95% CIs For Mean Based on
Pooled StDev

Level N Mean StDev ---------+---------+---------+---------+
L1 3 412,5 33,3 (-*-)
L2 3 2171,3 151,0 (-*-)
UL1 3 146,9 2,8 (*-)
UL2 3 147,8 2,1 (*-)
---------+---------+---------+---------+
600 1200 1800 2400

Pooled StDev = 77,3

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0,05
Critical value = 2,42

Intervals for level mean minus largest of other level means

Level Lower Center Upper --------+---------+---------+---------+-
L1 -1911,4 -1758,8 0,0 (*----------)
L2 0,0 1758,8 1911,4 (----------*)
UL1 -2177,0 -2024,4 0,0 (*----------)
UL2 -2176,2 -2023,6 0,0 (*----------)
--------+---------+---------+---------+-
-1200 0 1200 2400

Table B.5. Mean particle size of Loaded Lıposomes L1 L2 And Unloaded Lıposomes UL1 And UL2 (Continue)

Grouping Information Using Tukey Method

C1	N	Mean	Grouping
L2	3	2171,3	A
L1	3	412,5	B
UL2	3	147,8	C
UL1	3	146,9	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 98,74%

C1 = L1 subtracted from:

C1	Lower	Center	Upper	---------+---------+---------+---------+
L2	1556,6	1758,8	1961,1	(-*)
UL1	-467,9	-265,6	-63,3	(-*)
UL2	-467,0	-264,7	-62,5	(-*)
				---------+---------+---------+---------+
				-1200 0 1200 240

C1 = L2 subtracted from:

C1	Lower	Center	Upper	---------+---------+---------+---------+
UL1	-2226,7	-2024,4	-1822,2	(-*-)
UL2	-2225,8	-2023,6	-1821,3	(-*-)
				---------+---------+---------+---------+
				-1200 0 1200 2400

C1 = UL1 subtracted from:

C1	Lower	Center	Upper	---------+---------+---------+---------+
UL2	-201,4	0,9	203,1	(-*-)
				---------+---------+---------+---------+
				-1200 0 1200 2400

Table B.6. Zeta Potential Of Loaded Liposomes L1 And L2 And Unloaded Ul1 And Ul2 Statistical Results

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 3 16676,82 5558,94 1941,42 0,000
Error 8 22,91 2,86
Total 11 16699,72

S = 1,692 R-Sq = 99,86% R-Sq(adj) = 99,81%

Individual 95% CIs For Mean Based on
Pooled StDev

```
Level  N    Mean  StDev  ------+---------+---------+---------+---
L1     3  -36,667  0,709  (*)
L2     3  -29,933  1,662    (*)
UL1    3   34,833  2,223                      (*)
UL2    3   46,467  1,801                         (*
                  ------+---------+---------+---------+---
                     -25        0       25       50
```

Pooled StDev = 1,692

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0,05
Critical value = 2,42

Intervals for level mean minus largest of other level means

```
Level   Lower   Center   Upper  ---------+---------+---------+---------+
L1     -86,472  -83,133   0,000  (*--------------------------)
L2     -79,739  -76,400   0,000   (-*-----------------------)
UL1    -14,972  -11,633   0,000                    (*---)
UL2      0,000   11,633  14,972                       (---*)
                        ---------+---------+---------+---------+
                              -60       -30        0       30
```

Grouping Information Using Tukey Method

C1 N Mean Grouping
UL2 3 46,467 A
UL1 3 34,833 B

Table B.6. Zeta Potential Of Loaded Liposomes L1 And L2 And Unloaded UL1 And UL2 Statistical Results (Continued)

L2 3 -29,933 C
L1 3 -36,667 D

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 98,74%

C1 = L1 subtracted from:

```
C1   Lower  Center  Upper  ------+---------+---------+---------+---
L2    2,308  6,733  11,159              (*)
UL1  67,074  71,500  75,926                        (*)
UL2  78,708  83,133  87,559                         (*)
                    ------+---------+---------+---------+---
                       -50        0       50      100
```

C1 = L2 subtracted from:

```
C1   Lower  Center  Upper  ------+---------+---------+---------+---
UL1  60,341  64,767  69,192                       (*)
UL2  71,974  76,400  80,826                        (*)
                    ------+---------+---------+---------+---
                       -50        0       50      100
```

C1 = UL1 subtracted from:

```
C1   Lower  Center  Upper  ------+---------+---------+---------+---
UL2  7,208  11,633  16,059               (*)
                    ------+---------+---------+---------+---
                       -50        0       50      100
```

Table B.7. Pdi Of Loaded Liposomes L1 And L2 And Unloade Liposomes UL1 And UL2 Statistical Data

One-Way Anova: C2 Versus C1

Source DF SS MS F P
C1 3 0,11488 0,03829 25,53 0,000
Error 8 0,01200 0,00150
Total 11 0,12689

S = 0,03873 R-Sq = 90,54% R-Sq(adj) = 86,99%

Individual 95% CIs For Mean Based on
Pooled StDev

Level	N	Mean	StDev	---------+---------+---------+---------+
L1	3	0,41000	0,01997	(----*----)
L2	3	0,16433	0,07267	(----*-----)
UL1	3	0,39567	0,01332	(-----*----)
UL2	3	0,30433	0,01201	(----*-----)

---------+---------+---------+---------+
0,20 0,30 0,40 0,50

Pooled StDev = 0,03873

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0,05
Critical value = 2,42

Intervals for level mean minus largest of other level means

Level	Lower	Center	Upper	-------+---------+---------+---------+--
L1	-0,06209	0,01433	0,09076	(-----*------)
L2	-0,32209	-0,24567	0,00000	(------*-------------------)
UL1	-0,09076	-0,01433	0,06209	(------*-----)
UL2	-0,18209	-0,10567	0,00000	(-----*--------)

-------+---------+---------+---------+--
-0,24 -0,12 0,00 0,12

Grouping Information Using Tukey Method

C1	N	Mean	Grouping
L1	3	0,41000	A

Table B.7. Pdi Of Loaded Liposomes L1 And L2 And Unloade Liposomes UL1 And UL2 Statistical Data (Continued)

UL1 3 0,39567 A B
UL2 3 0,30433 B
L2 3 0,16433 C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 98,74%

C1 = L1 subtracted from:

```
C1    Lower   Center   Upper  -------+---------+---------+---------+--
L2   -0,34697 -0,24567 -0,14436  (----*----)
UL1  -0,11564 -0,01433  0,08697           (----*----)
UL2  -0,20697 -0,10567 -0,00436       (----*----)
                       -------+---------+---------+---------+--
                          -0,20      0,00      0,20      0,40
```

C1 = L2 subtracted from:

```
C1    Lower   Center   Upper  -------+---------+---------+---------+--
UL1  0,13003  0,23133  0,33264                    (----*----)
UL2  0,03870  0,14000  0,24130                (----*----)
                       -------+---------+---------+---------+--
                          -0,20      0,00      0,20      0,40
```

C1 = UL1 subtracted from:

```
C1    Lower   Center   Upper  -------+---------+---------+---------+--
UL2  -0,19264 -0,09133  0,00997       (----*----)
                       -------+---------+---------+---------+--
                          -0,20      0,00      0,20      0,40
```

B.2. ATOMIC FORCE MICROSCOPY RESULTS

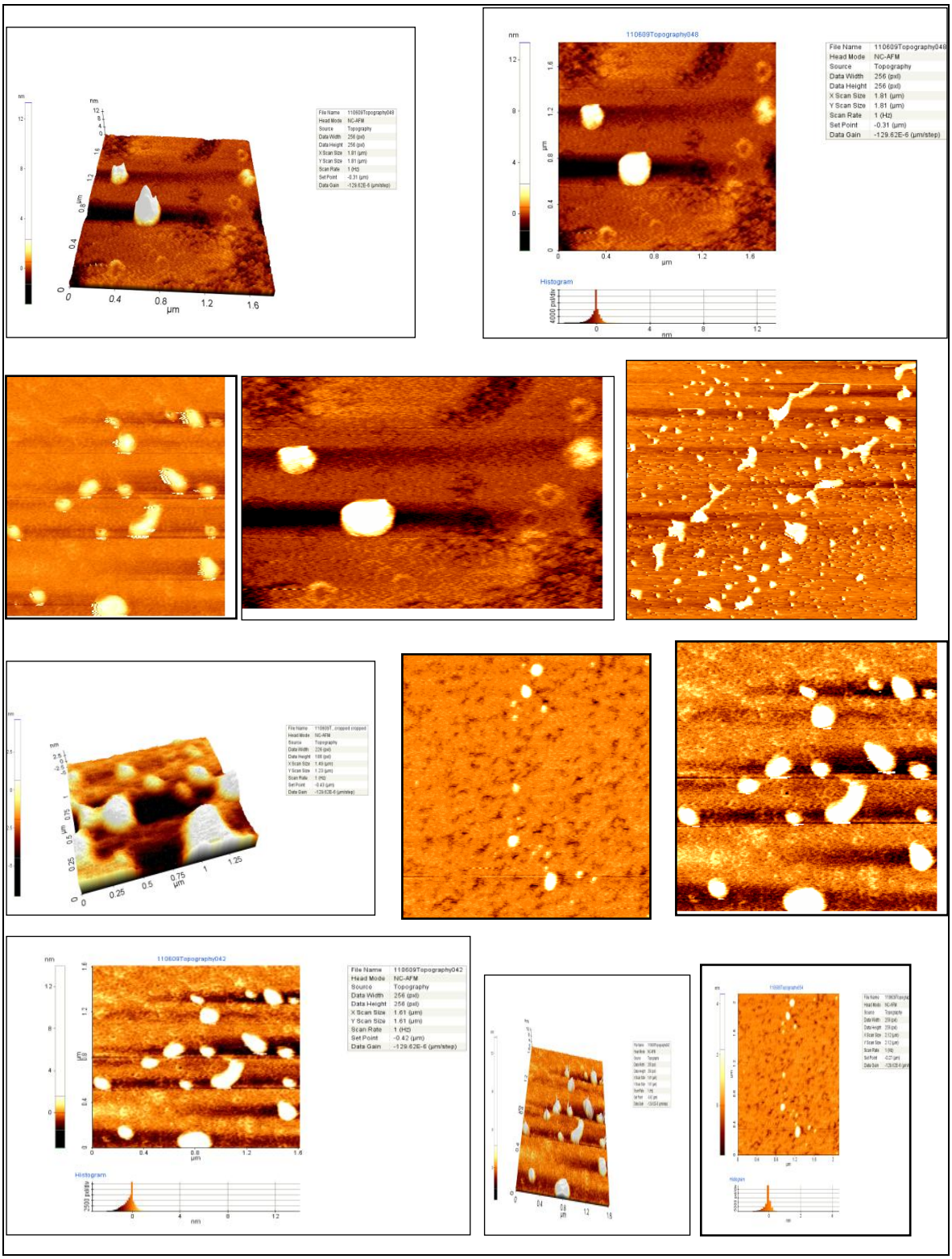

Figure B.1 3D Images of Loaded liposomes L1 (Topography of vesicles)

B.3. POLARIZE LIGHT MICROSCOPY (A)

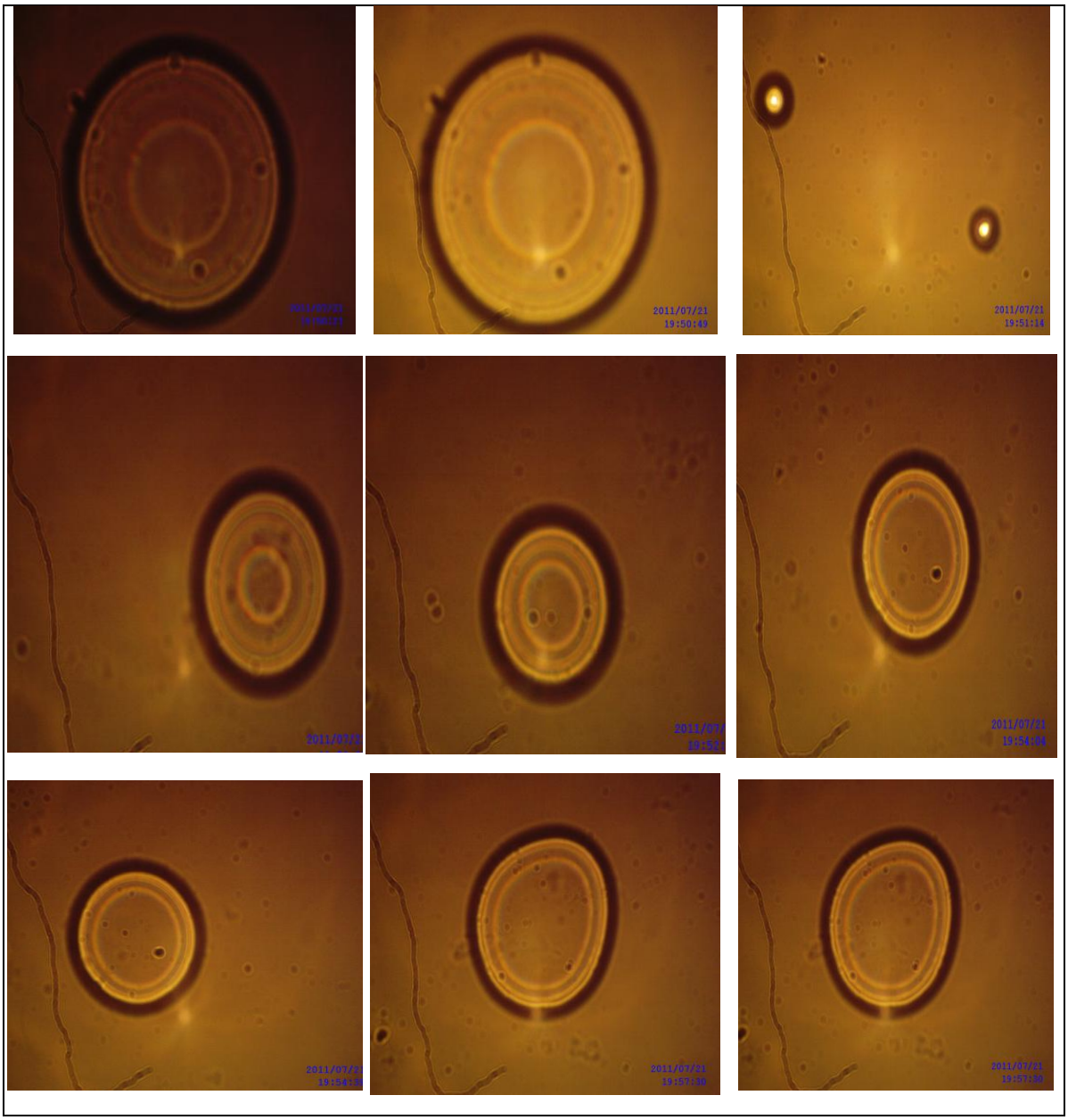

Figure B.2 Polarized Light Microscopy images of UL1 at 100X

B.4. POLARIZE LIGHT MICROSCOPY (B)

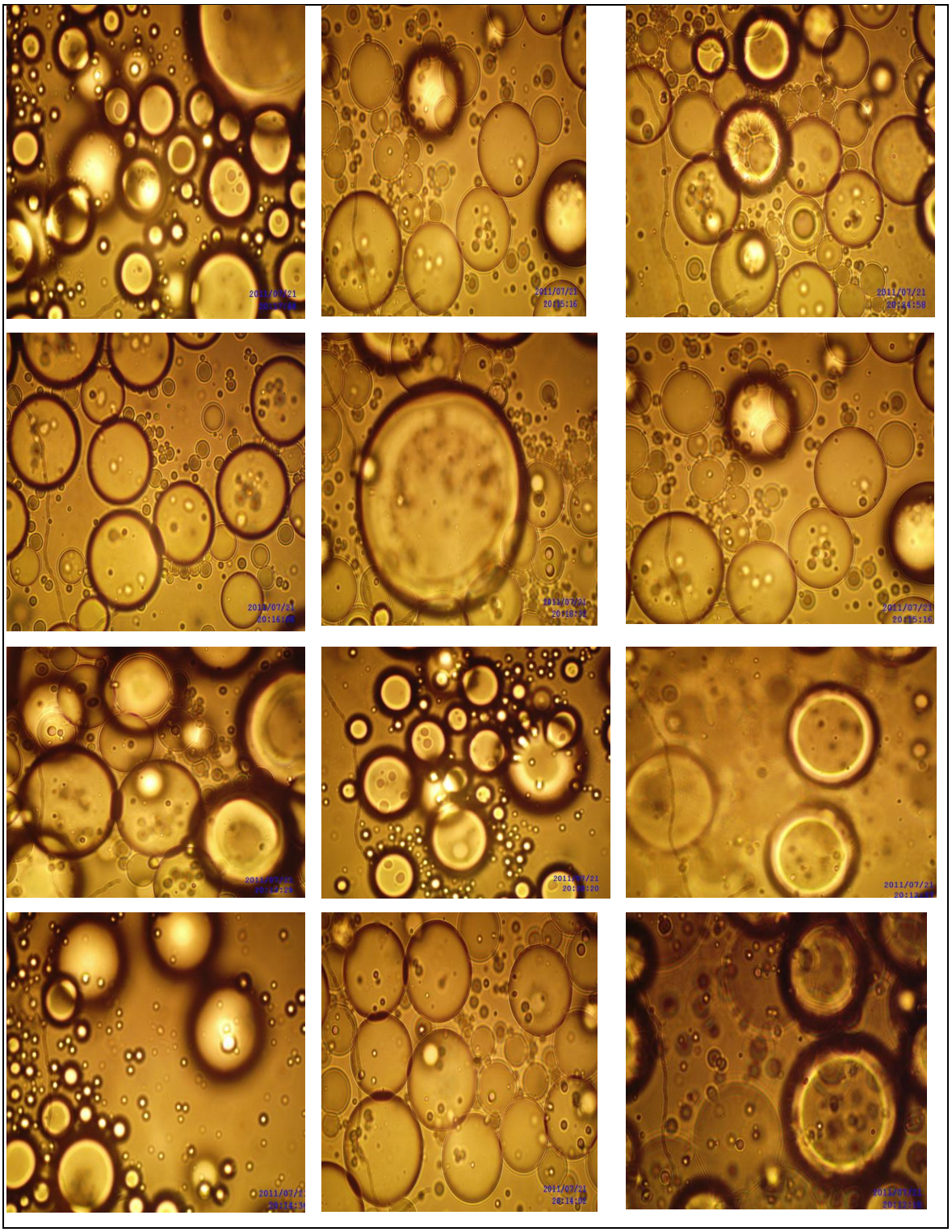

Figure B.3. Polarized Light Microscopy images of
L1 at 100X

APPENDIX C: ANTIMICROBIAL ACTIVITY TESTING AND THEIR STATISTICAL EVALUATION RESULTS

Table C.1. Antimicrobial Activity of L1 and L2 against Oral Microorganisms following 120 h Incubation.

	1st repeat				2nd repeat			3rd repeat		
	Days	**E.O**	**L1**	**L2**	**E.O**	**L1**	**L2**	**E.O**	**L1**	**L2**
S.mutans	**24**	24,0	16	6	23,0	16	12	23,0	16	12
	48	30	16	7	30	16	13	30	16	13
	72	35	16	10	39	16	14	39	16	14
	96	39	16	15	39	16	15	39	16	15
	120	39	19	16	39	19	16	39	19	16
F.nucleatum	**24**	21,0	9	0	15,0	9	0	22,0	9	0
	48	24	12	18	25	12	19	24	14	18
	72	24	14	20	23	12	19	24	14	20
	96	24	15	20	23	15	19	24	15	20
	120	25	16	21	25	15	21	25	15	21
P.gingivalis	**24**	26,0	12	12	32,0	12	12	30,0	12	13
	48	29	12	13	32	12	12	32	13	18
	72	34	13	14	34	13	14	34	13	18
	96	35	13	15	35	13	15	35	14	18
	120	36	23	20	36	23	20	36	24	20
P.intermedia	**24**	24,0	0	0	20,0	0	0	29,0	0	2
	48	26	12	13	26	12	13	32	12	14
	72	26	13	14	26	13	14	34	13	14
	96	29	16	14	29	16	14	29	16	14
	120	29	16	14	29	16	14	29	16	14
A.actinomycetemcomitans	**24**	24,0	0	13	21	0	15	19,0	0	15
	48	33	8	13	21	8	16	20	8	15
	72	36	17	13	32,0	17	16	32,0	14	15
	96	37	17	16	32	17	16	32	14	18
	120	56	17	16	56	17	16	56	17	18
C.albicans	**24**	44,0	0	7	46,0	0	7,5	40,0	0	7
	48	46	8	16	46	7,5	17	41	8	17
	72	47	10	22	46	9	24	42	10	26
	96	48	29	27	48	29	27	46	29	27
	120	48	30	30	48	30	30	48	30	30

Table C.1. Antimicrobial activity of L1 and L2 against Oral Microorganisms following 120 h Incubation (continued).

		1st repeat			2nd repeat			3rd repeat		
Mix	**24**	15,0	12	11	15,0	12	11	15,0	12	11
	48	16	12	11	16	12	11	16	12	11
	72	17	12	11	18	12	11	17	12	11
	96	17	13	12	18	13	11	17	13	11
	120	18	13	12	18	13	12	18	13	12
	24	15,0	12	11	15,0	12	11	15,0	12	11

Table C 2. Antimicrobial Activity of L1 and L2 against Oral Microorganisms following 120 h Incubation (mean and standart deviation)

	EO		**L1**		**L2**	
	mean	**standart deviation**	**mean**	**standart deviation**	**mean**	**standart deviation**
S.mutans	23.3	0.577350269	16	0	10	3.46410162
	30.0	0	16	0	11	3.46410162
	37.7	2.309401077	16	0	12.66666667	2.30940108
	39.0	0	16	0	15	0
	39.0	0	19	0	16	0
F.nucleatum	19.3	3.785938897	9	0	0	0
	24.3	0.577350269	12.66666667	1.154700538	18.33333333	0.57735027
	23.7	0.577350269	13.33333333	1.154700538	19.66666667	0.57735027
	23.7	0.577350269	15	0	19.66666667	0.57735027
	25.0	0	15.33333333	0.577350269	21	0
P.gingivalis	29.3	3.055050463	12	0	12.33333333	0.57735027
	31.0	1.732050808	12.33333333	0.577350269	14.33333333	3.21455025
	34.0	0	13	0	15.33333333	2.30940108
	35.0	0	13.33333333	0.577350269	16	1.73205081
	36.0	0	23.33333333	0.577350269	20	0
P.intermedia	24.3	4.509249753	0	0	0.666666667	1.15470054
	28.0	3.464101615	12	0	13.33333333	0.57735027
	28.7	4.618802154	13	0	14	0
	29.0	0	16	0	14	0
	29.0	0	16	0	14	0
A.a	21.3	2.516611478	0	0	14.33333333	1.15470054
	24.7	7.234178138	8	0	14.66666667	1.52752523
	33.3	2.309401077	16	1.732050808	14.66666667	1.52752523
	33.7	2.886751346	16	1.732050808	16.66666667	1.15470054
	56.0	0	17	0	16.66666667	1.15470054

Table C.2. Antimicrobial Activity of L1 and L2 against Oral Microorganisms following 120 h Incubation (mean and standart deviation) (continued)

	EO		L1		L2	
C.albicans	43.3	3.055050463	0	0	7.166666667	0.28867513
	44.3	2.886751346	7.833333333	0.288675135	16.66666667	0.57735027
	45.0	2.645751311	9.666666667	0.577350269	24	2
	47.3	1.154700538	29	0	27	0
	48.0	0	30	0	30	0
Mix	15.0	0	12	0	11	0
	16.0	0	12	0	11	0
	17.3	0.577350269	12	0	11	0
	17.3	0.577350269	13	0	11.33333333	0.57735027
	18.0	0	13	0	12	0

C.1. STATISTIC RESULTS OF ANTIMICROBIAL TESTS

Table C.3. Statistical Evaluation of Antimicrobial Activity of Loaded Liposomes L1 And L2 on Oral Microorganisms

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 6 606,4762 101,0794 1061,33 0,000
Error 14 1,3333 0,0952
Total 20 607,8095

S = 0,3086 R-Sq = 99,78% R-Sq(adj) = 99,69%

Individual 95% CIs For Mean Based on
Pooled StDev

Level N Mean StDev -----+---------+---------+---------+----
a.a 3 17,000 0,000 (*)
c.alb 3 30,000 0,000 (*)
f.nucleatum 3 15,333 0,577 (*
mix 3 13,000 0,000 (*)
p.g 3 23,333 0,577 (*
p.i 3 16,000 0,000 (*)
s.mutans 3 19,000 0,000 (*)

Table C.3. Statistical Evaluation of Antimicrobial Activity of Loaded Liposomes L1 And L2 on Oral Microorganisms (Continued)

```
                    -----+---------+---------+---------+----
                        15,0      20,0      25,0      30,0

Pooled StDev = 0,309
Grouping Information Using Tukey Method

C1           N    Mean  Grouping
c.alb        3  30,000  A
p.g          3  23,333    B
s.mutans     3  19,000      C
a.a          3  17,000       D
p.i          3  16,000         E
f.nucleatum  3  15,333         E
mix          3  13,000           F

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 99,58%

C1 = a.a subtracted from:

C1           Lower  Center   Upper  --------+---------+---------+---------+-
c.alb        12,139  13,000  13,861                          (*)
f.nucleatum  -2,527  -1,667  -0,806              (*)
mix          -4,861  -4,000  -3,139             (*)
p.g           5,473   6,333   7,194                    (*)
p.i          -1,861  -1,000  -0,139              (*)
s.mutans      1,139   2,000   2,861                   (*)
                        --------+---------+---------+---------+-
                            -10        0       10       20
C1 = c.alb subtracted from:
C1           Lower   Center    Upper
f.nucleatum  -15,527  -14,667  -13,806
mix          -17,861  -17,000  -16,139
p.g           -7,527   -6,667   -5,806
p.i          -14,861  -14,000  -13,139
s.mutans     -11,861  -11,000  -10,139
C1           --------+---------+---------+---------+
```

Table C.3. Statistical Evaluation of antimicrobial activity of Loaded Liposomes L1 And L2 on Oral Microorganisms (Continued)

```
nucleatum    (*)
mix          (*)
p.g                (*)
p.i           (*)
s.mutans          (*)
          --------+---------+---------+---------+-
               -10        0       10       20


C1 = f.nucleatum subtracted from:

C1        Lower  Center   Upper --------+---------+---------+---------+-
mix       -3,194  -2,333  -1,473               (*)
p.g        7,139   8,000   8,861                     (*)
p.i       -0,194   0,667   1,527               (*)
s.mutans   2,806   3,667   4,527                     (*)
                          --------+---------+---------+---------+-
                               -10        0       10       20


C1 = mix subtracted from:

C1        Lower  Center   Upper --------+---------+---------+---------+-
p.g       9,473  10,333  11,194                       (*)
p.i       2,139   3,000   3,861                (*)
s.mutans  5,139   6,000   6,861                      (*)
                          --------+---------+---------+---------+-
                               -10        0       10       20
C1 = p.g subtracted from:
C1        Lower  Center   Upper --------+---------+---------+---------+-
p.i       -8,194  -7,333  -6,473           (*)
s.mutans  -5,194  -4,333  -3,473              (*)
                          --------+---------+---------+---------+-
                               -10        0       10       20
C1 = p.i subtracted from:
C1        Lower  Center  Upper --------+---------+---------+---------+-
s.mutans  2,139   3,000  3,861                  (*)
                          --------+---------+---------+---------+-
                               -10        0       10       20
```

Table C.4. Statistical Evaluation of Antimicrobial Activity of Loaded Liposomes L2 on Oral Microorganisms

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 6 638,571 106,429 558,75 0,000
Error 14 2,667 0,190
Total 20 641,238

S = 0,4364 R-Sq = 99,58% R-Sq(adj) = 99,41%

Individual 95% CIs For Mean Based on
Pooled StDev

Level N Mean StDev -------+---------+---------+---------+--
a.a 3 16,667 1,155 (*)
c.alb 3 30,000 0,000 (*)
f.nucleatum 3 21,000 0,000 (*)
mix 3 12,000 0,000 (*)
p.g 3 20,000 0,000 (*)
p.i 3 14,000 0,000 (*)
s.mutans 3 16,000 0,000 (*)
-------+---------+---------+---------+--
15,0 20,0 25,0 30,0

Pooled StDev = 0,436

Grouping Information Using Tukey Method

C1 N Mean Grouping
c.alb 3 30,000 A
f.nucleatum 3 21,000 B
p.g 3 20,000 B
a.a 3 16,667 C
s.mutans 3 16,000 C
p.i 3 14,000 D
mix 3 12,000 E

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 99,58%

C1 = a.a subtracted from:

C1 Lower Center Upper ---------+---------+---------+---------+
c.alb 12,116 13,333 14,550 (*-)
f.nucleatum 3,116 4,333 5,550 (*-)
mix -5,884 -4,667 -3,450 (*-)
p.g 2,116 3,333 4,550 (*-)
p.i -3,884 -2,667 -1,450 (*-)
s.mutans -1,884 -0,667 0,550 (*-)
---------+---------+---------+---------+

Table C.4. Statistical Evaluation of Antimicrobial Activity of Loaded Liposomes L2 on oral microorganisms (Continued)

```
                                -10        0       10       20
C1 = c.alb subtracted from:
C1            Lower   Center    Upper
f.nucleatum  -10,217   -9,000   -7,783
mix          -19,217  -18,000  -16,783
p.g          -11,217  -10,000   -8,783
p.i          -17,217  -16,000  -14,783
s.mutans     -15,217  -14,000  -12,783
C1          ---------+---------+---------+---------+
f.nucleatum           (*)
mix          (*)
p.g                (*)
p.i          (*)
s.mutans         (*)
           ---------+---------+---------+---------+
                 -10        0       10       20
C1 = f.nucleatum subtracted from:

C1         Lower  Center   Upper  ----------+---------+---------+---------+
mix       -10,217  -9,000  -7,783          (*)
p.g        -2,217  -1,000   0,217                (*)
p.i        -8,217  -7,000  -5,783           (*)
s.mutans   -6,217  -5,000  -3,783             (*)
                           ---------+---------+---------+---------+
                                -10        0       10       20
C1 = mix subtracted from:
C1        Lower  Center  Upper  ---------+---------+---------+---------+
p.g       6,783   8,000  9,217                      (*)
p.i       0,783   2,000  3,217                (*)
s.mutans  2,783   4,000  5,217                   (*)
                         ---------+---------+---------+---------+
                              -10        0       10       20

C1 = p.g subtracted from:

C1        Lower  Center   Upper  ---------+---------+---------+---------+
p.i       -7,217  -6,000  -4,783            (*)
s.mutans  -5,217  -4,000  -2,783              (*)
                          ---------+---------+---------+---------+
                              -10        0       10       20
C1 = p.i subtracted from:
C1        Lower  Center  Upper  ---------+---------+---------+---------+
s.mutans  0,783   2,000  3,217                   (*)
                         ---------+---------+---------+---------+
                              -10        0       10       20
```

Table C.5. Statistical Evaluation of Antimicrobial Effect of *S.mutans* on L1,L2 and EO

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 2 938,0000 469,0000 * *
Error 6 0,0000 0,0000
Total 8 938,0000
S = 0 R-Sq = 100,00% R-Sq(adj) = 100,00%

Individual 95% CIs For Mean Based on
Pooled StDev
Level N Mean StDev ---+---------+---------+---------+------
EO 3 39,0000 0,0000 *
L1 3 19,0000 0,0000 *
L2 3 16,0000 0,0000 *
---+---------+---------+---------+------
18,0 24,0 30,0 36,0
Pooled StDev = 0,0000
Grouping Information Using Tukey Method

C1 N Mean Grouping
EO 3 39,0000 A
L1 3 19,0000 B
L2 3 16,0000 C
Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 97,80%
C1 = EO subtracted from:

C1 Lower Center Upper ---+---------+---------+---------+------
L1 -20,0000 -20,0000 -20,0000 *
L2 -23,0000 -23,0000 -23,0000 *
---+---------+---------+---------+------
-21,0 -14,0 -7,0 0,0
C1 = L1 subtracted from:

C1 Lower Center Upper ---+---------+---------+---------+------
L2 -3,0000 -3,0000 -3,0000 *
---+---------+---------+---------+------
-21,0 -14,0 -7,0 0,0

Table C6. Statistical Evaluation of Antimicrobial Effect of *F.nucelatum* on L1,L2 and EO Oral Microorganisms

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 2 141,556 70,778 637,00 0,000
Error 6 0,667 0,111
Total 8 142,222

S = 0,3333 R-Sq = 99,53% R-Sq(adj) = 99,38%

Individual 95% CIs For Mean Based on Pooled StDev
Level N Mean StDev +---------+---------+---------+---------
EO 3 25,000 0,000 (*-)
L1 3 15,333 0,577 (*-)
L2 3 21,000 0,000 (-*-)
+---------+---------+---------+---------
15,0 18,0 21,0 24,0

Pooled StDev = 0,333
Grouping Information Using Tukey Method

C1 N Mean Grouping
EO 3 25,000 A
L2 3 21,000 B
L1 3 15,333 C
Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 97,80%
C1 = EO subtracted from:
C1 Lower Center Upper -+---------+---------+---------+--------
L1 -10,502 -9,667 -8,831 (-*)
L2 -4,835 -4,000 -3,165 (-*-)
-+---------+---------+---------+--------
-10,0 -5,0 0,0 5,0
C1 = L1 subtracted from:
C1 Lower Center Upper -+---------+---------+---------+--------
L2 4,831 5,667 6,502 (*-)
-+---------+---------+---------+--------
-10,0 -5,0 0,0 5,0

Table C.7. Statistical Evaluation of Antimicrobial Effect of *P.gingivalis* L1, L2 and EO

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 2 427,556 213,778 1924,00 0,000
Error 6 0,667 0,111
Total 8 428,222

S = 0,3333 R-Sq = 99,84% R-Sq(adj) = 99,79%

Individual 95% CIs For Mean Based on Pooled StDev

Level N Mean StDev -+---------+---------+---------+--------
EO 3 36,000 0,000 (*)
L1 3 23,333 0,577 (*)
L2 3 20,000 0,000 (*)
-+---------+---------+---------+--------
20,0 25,0 30,0 35,0

Pooled StDev = 0,333
Grouping Information Using Tukey Method

C1 N Mean Grouping
EO 3 36,000 A
L1 3 23,333 B
L2 3 20,000 C

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 97,80%
C1 = EO subtracted from:
C1 Lower Center Upper --------+---------+---------+---------+-
L1 -13,502 -12,667 -11,831 (-*)
L2 -16,835 -16,000 -15,165 (*-)
--------+---------+---------+---------+-
-12,0 -6,0 0,0 6,0
C1 = L1 subtracted from:

C1 Lower Center Upper --------+---------+---------+---------+-
L2 -4,169 -3,333 -2,498 (*-)
--------+---------+---------+---------+-
-12,0 -6,0 0,0 6,0

Table C.8. Statistical Evaluation of Antimicrobial Effect of *P.intermedia* L1, L2 and EO

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 2 398,0000 199,0000 * *
Error 6 0,0000 0,0000
Total 8 398,0000

S = 0 R-Sq = 100,00% R-Sq(adj) = 100,00%

Individual 95% CIs For Mean Based on
Pooled StDev
Level N Mean StDev -----+---------+---------+---------+----
EO 3 29,0000 0,0000 *
L1 3 16,0000 0,0000 *
L2 3 14,0000 0,0000 *
-----+---------+---------+---------+----
16,0 20,0 24,0 28,0
Pooled StDev = 0,0000
Grouping Information Using Tukey Method
C1 N Mean Grouping
EO 3 29,0000 A
L1 3 16,0000 B
L2 3 14,0000 C
Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 97,80%
C1 = EO subtracted from:

C1 Lower Center Upper +---------+---------+---------+---------
L1 -13,0000 -13,0000 -13,0000 *
L2 -15,0000 -15,0000 -15,0000 *
+---------+---------+---------+---------
-15,0 -10,0 -5,0 0,0

C1 = L1 subtracted from:

C1 Lower Center Upper +---------+---------+---------+---------
L2 -2,0000 -2,0000 -2,0000 *
+---------+---------+---------+---------
-15,0 -10,0 -5,0 0,0

Table C.9. Statistical Evaluation of Antimicrobial effect of *A.actinomycetemcomitans* L1, L2 and EO

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 2 3122,000 1561,000 * *
Error 6 0,000 0,000
Total 8 3122,000

S = 0 R-Sq = 100,00% R-Sq(adj) = 100,00%

Individual 95% CIs For Mean Based on
Pooled StDev

Level N Mean StDev ----+---------+---------+---------+-----
EO 3 56,0000 0,0000 *
L1 3 17,0000 0,0000 *
L2 3 16,0000 0,0000 *
----+---------+---------+---------+-----
20 30 40 50

Pooled StDev = 0,0000
Grouping Information Using Tukey Method

C1 N Mean Grouping
EO 3 56,0000 A
L1 3 17,0000 B
L2 3 16,0000 C
Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 97,80%
C1 = EO subtracted from:

C1 Lower Center Upper ---+---------+---------+---------+------
L1 -39,0000 -39,0000 -39,0000 *
L2 -40,0000 -40,0000 -40,0000 *
---+---------+---------+---------+------
-36 -24 -12 0
C1 = L1 subtracted from:
C1 Lower Center Upper ---+---------+---------+---------+------
L2 -1,0000 -1,0000 -1,0000 *

Table C.10. Statistical Evaluation of Antimicrobial Effect of *C.albicans* L1, L2 and EO

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 2 648,0000 324,0000 * *
Error 6 0,0000 0,0000
Total 8 648,0000

S = 0 R-Sq = 100,00% R-Sq(adj) = 100,00%

Individual 95% CIs For Mean Based on Pooled StDev
Level N Mean StDev +---------+---------+---------+---------
EO 3 48,0000 0,0000 *
L1 3 30,0000 0,0000 *
L2 3 30,0000 0,0000 *
+---------+---------+---------+---------
30,0 35,0 40,0 45,0
Pooled StDev = 0,0000
Grouping Information Using Tukey Method

C1 N Mean Grouping
EO 3 48,0000 A
L2 3 30,0000 B
L1 3 30,0000 C
Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 97,80%
C1 = EO subtracted from:
C1 Lower Center Upper ------+---------+---------+---------+---
L1 -18,0000 -18,0000 -18,0000 *
L2 -18,0000 -18,0000 -18,0000 *
------+---------+---------+---------+---
-15,0 -10,0 -5,0 0,0
C1 = L1 subtracted from:
C1 Lower Center Upper ------+---------+---------+---------+---
L2 0,0000 0,0000 0,0000 *
------+---------+---------+---------+---
-15,0 -10,0 -5,0 0,0

Table C.11. Statistical Evaluation of Antimicrobial effect of *mix microorganisms* n L1, L2 and EO

* **One-way ANOVA: C2 versus C1**

Source DF SS MS F P
C1 2 62,00000 31,00000 * *
Error 6 0,00000 0,00000
Total 8 62,00000

S = 0 R-Sq = 100,00% R-Sq(adj) = 100,00%

Individual 95% CIs For Mean Based on Pooled StDev
Level N Mean StDev +---------+---------+---------+---------
EO 3 18,0000 0,0000 *
L1 3 13,0000 0,0000 *
L2 3 12,0000 0,0000 *
+---------+---------+---------+---------
12,0 13,5 15,0 16,5

Pooled StDev = 0,0000
Grouping Information Using Tukey Method
C1 N Mean Grouping
EO 3 18,00000 A
L1 3 13,00000 B
L2 3 12,00000 C

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 97,80%
C1 = EO subtracted from:

C1 Lower Center Upper +---------+---------+---------+---------
L1 -5,00000 -5,00000 -5,00000 *
L2 -6,00000 -6,00000 -6,00000 *
+---------+---------+---------+---------
-6,0 -4,0 -2,0 0,0
C1 = L1 subtracted from:

C1 Lower Center Upper +---------+---------+---------+---------
L2 -1,00000 -1,00000 -1,00000 *
+---------+---------+---------+---------
-6,0 -4,0 -2,0 0,0

Table C.11. Statistical Evaluation of Antimicrobial Effect of *mix microorganisms* n L1, L2 and EO (Continued)

*

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 6 3140,571 523,429 * *
Error 14 0,000 0,000
Total 20 3140,571

S = 0 R-Sq = 100,00% R-Sq(adj) = 100,00%

Individual 95% CIs For Mean Based on
Pooled StDev

Level	N	Mean	StDev	--+---------+---------+---------+-------
a.a	3	56,0000	0,0000	*
c.alb	3	48,0000	0,0000	*
f.nucleatum	3	25,0000	0,0000	*
mix	3	18,0000	0,0000	*
p.g	3	36,0000	0,0000	*
p.i	3	29,0000	0,0000	*
s.mutans	3	39,0000	0,0000	*

--+---------+---------+---------+-------
20 30 40 50

Pooled StDev = 0,0000

Grouping Information Using Tukey Method

C1	N	Mean	Grouping
a.a	3	56,0000	A
c.alb	3	48,0000	B
s.mutans	3	39,0000	C
p.g	3	36,0000	D
p.i	3	29,0000	E
f.nucleatum	3	25,0000	F
mix	3	18,0000	G

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 99,58%

C1 = a.a subtracted from

Table C.11. Statistical Evaluation of Antimicrobial Effect of *mix microorganisms* n L1, L2 and EO (Continued)

```
*

C1             Lower    Center     Upper
c.alb        -8,0000   -8,0000   -8,0000
f.nucleatum  -31,0000  -31,0000  -31,0000
mix          -38,0000  -38,0000  -38,0000
p.g          -20,0000  -20,0000  -20,0000
p.i          -27,0000  -27,0000  -27,0000
s.mutans     -17,0000  -17,0000  -17,0000

C1          ---------+---------+---------+---------+
c.alb                    *
f.nucleatum      *
mix          *
p.g                 *
p.i              *
s.mutans                 *
          ---------+---------+---------+---------+
                -20        0       20       40


C1 = c.alb subtracted from:

C1             Lower    Center     Upper
f.nucleatum  -23,0000  -23,0000  -23,0000
mix          -30,0000  -30,0000  -30,0000
p.g          -12,0000  -12,0000  -12,0000
p.i          -19,0000  -19,0000  -19,0000
s.mutans      -9,0000   -9,0000   -9,0000
C1          ---------+---------+---------+---------+
f.nucleatum          *
mix              *
p.g                   *
p.i                *
s.mutans                  *
          ---------+---------+---------+---------+
                -20        0       20       40


C1 = f.nucleatum subtracted from:

C1         Lower   Center    Upper  ---------+---------+---------+---------+
mix       -7,0000  -7,0000  -7,0000                *
p.g       11,0000  11,0000  11,0000                        *
p.i        4,0000   4,0000   4,0000                   *
```

Table C.11. Statistical Evaluation of Antimicrobial Effect of *mix microorganisms* n L1, L2 and EO (Continued)

```
s.mutans  14,0000  14,0000  14,0000                    *
                          ---------+---------+---------+---------+
                               -20        0       20       40


C1 = mix subtracted from:

C1          Lower   Center    Upper  ---------+---------+---------+---------+
p.g       18,0000  18,0000  18,0000                       *
p.i       11,0000  11,0000  11,0000                   *
s.mutans  21,0000  21,0000  21,0000                         *
                          ---------+---------+---------+---------+
                               -20        0       20       40
C1 = p.g subtracted from:

C1          Lower   Center    Upper  ---------+---------+---------+---------+
p.i       -7,0000  -7,0000  -7,0000             *
s.mutans   3,0000   3,0000   3,0000                *
                          ---------+---------+---------+---------+
                               -20        0       20       40
C1 = p.i subtracted from:
C1          Lower   Center    Upper  ---------+---------+---------+---------+
s.mutans  10,0000  10,0000  10,0000                   *
                          ---------+---------+---------+---------+
                               -20        0       20       40
One-way ANOVA: C2 versus C1

Source  DF       SS      MS  F  P
C1       6  3140,571  523,429  *  *
Error   14     0,000    0,000
Total   20  3140,571
S = 0   R-Sq = 100,00%   R-Sq(adj) = 100,00%
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level       N     Mean   StDev  --+---------+---------+---------+-------
a.a         3  56,0000  0,0000                                  *
c.alb       3  48,0000  0,0000                              *
f.nucleatum 3  25,0000  0,0000        *
mix         3  18,0000  0,0000  *
p.g         3  36,0000  0,0000                  *
```

Table C.12. Statistical Evaluation of Antimicrobial Effect of *S. mutans* L1,L2 and EO (L1-L2- EO- Control -Different Times) (120hrs)

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--+---------+---------+---------+-------
24	3	23.333	0.577	(*-)
48	3	30.000	0.000	(-*-)
72	3	37.667	2.309	(-*)
96	3	39.000	0.000	(-*-)
120	3	39.000	0.000	(-*-)
240	3	13.000	0.000	(-*-)
241	3	16.000	0.000	(-*-)
242	3	10.000	3.464	(-*-)
480	3	13.000	0.000	(-*-)
481	3	16.000	0.000	(-*-)
482	3	11.000	3.464	(-*-)
720	3	14.000	0.000	(-*-)
721	3	16.000	0.000	(-*-)
722	3	12.667	2.309	(-*)
960	3	15.000	0.000	(-*-)
961	3	16.000	0.000	(-*-)
962	3	15.000	0.000	(-*-)
1200	3	16.000	0.000	(-*-)
1201	3	19.000	0.000	(-*-)
1202	3	16.000	0.000	(-*-)

--+---------+---------+---------+-------
10 20 30 40

Pooled StDev = 1.323

Grouping Information Using Tukey Method

	C1	N	Mean	Grouping
EO-	120	3	39.000	A
EO-	96	3	39.000	A
EO-	72	3	37.667	A
EO-	48	3	30.000	B
EO-	24	3	23.333	C
L1-	120	3	19.000	D
L2-	120	3	16.000	D E
C -	120	3	16.000	D E
L1-	96	3	16.000	D E
L1-	72	3	16.000	D E
L1-	48	3	16.000	D E

Table C.12. Statistical Evaluation of Antimicrobial Effect of *S. mutans* L1,L2 and EO (L1-L2- EO- Control -Different Times) (120hrs) (Continued)

L1- 24	3	16.000	D E
L2- 96	3	15.000	D E F
c - 96	3	15.000	D E F
c - 72	3	14.000	E F G
c - 48	3	13.000	E F G
c - 24	3	13.000	E F G
L2- 72	3	12.667	E F G
L2- 48	3	11.000	F G
L2- 24	3	10.000	G

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 99.95%

One-way ANOVA: C2 versus C1

Source	DF	SS	MS	F	P
C1	19	1761.93	92.73	40.03	0.000
Error	40	92.67	2.32		
Total	59	1854.60			

S = 1.522 R-Sq = 95.00% R-Sq(adj) = 92.63%

Table C.13. Statistical Evaluation of Antimicrobial Effect of *F. nucleatum* L1, L2 and EO (L1- L2- EO- Control -Different Times) (120hrs)

```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level  N    Mean  StDev  --------+---------+---------+---------+-
  24   3  19.333  3.786                    (--*-)
  48   3  24.333  0.577                        (--*-)
  72   3  23.667  0.577                       (--*-)
  96   3  23.667  0.577                       (--*-)
 120   3  25.000  0.000                         (--*-)
 240   3  13.000  0.000            (--*-)
 241   3   9.000  0.000        (--*-)
 242   3   3.000  5.196  (-*--)
 480   3  13.000  0.000            (--*-)
 481   3  12.667  1.155            (-*--)
 482   3  18.333  0.577                 (-*--)
 720   3  14.000  0.000             (--*--)
 721   3  13.333  1.155             (-*--)
 722   3  19.667  0.577                   (-*--)
 960   3  15.000  0.000              (-*--)
 961   3  15.000  0.000              (-*--)
 962   3  19.667  0.577                   (-*--)
1200   3  16.000  0.000               (--*-)
1201   3  15.333  0.577              (--*-)
1202   3  21.000  0.000                    (--*--)
                   --------+---------+---------+---------+-
                       7.0     14.0     21.0     28.0

Pooled StDev = 1.522


Grouping Information Using Tukey Method
C1       N     Mean      Grouping
 EO 120  3     25.000  A
 EO 48   3     24.333  A B
 EO 96   3     23.667  A B C
 EO 72   3     23.667  A B C
 L2 120  3     21.000  A B C D
 L2 96   3     19.667    B C D E
 L2 72   3     19.667    B C D E
 EO 24   3     19.333      C D E
 L2 48   3     18.333        D E F
 C  120  3     16.000          E F G
 L1 12   3     15.333          E F G
```

Table C.13. Statistical Evaluation of Antimicrobial Effect of *F. nucleatum* L1,L2 and EO (L1- L2- EO- Control -dDifferent Times) (120hrs) (Continued)

L1	96	3	15.000 E F G
C	96	3	15.000 E F G
C	72	3	14.000 F G
L1	72	3	13.333 G H
C	48	3	13.000 G H
C	24	3	13.000 G H
L1	48	3	12.667 G H
L1	24	3	9.000 H
L2	24	3	3.000 I

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 99.95%

Table C.14. Statistical Evaluation of Antimicrobial Effect of *P. gingivalis* L1, L2 and EO (L1- L2- EO- Control -Different Times) (120hrs)

One-way ANOVA: C2 versus C1

Source	DF	SS	MS	F	P
C1	19	4496.86	236.68	131.49	0.000
Error	40	72.00	1.80		
Total	59	4568.86			

S = 1.342 R-Sq = 98.42% R-Sq(adj) = 97.68%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-----+---------+---------+---------+----
EO	24	3	29.333	3.055 (-*-)
EO	48	3	31.667	2.517 (-*-)
EO	72	3	34.333	0.577 (-*-)

Table C.14. Statistical Evaluation of Antimicrobial Effect of *P. gingivalis* L1, L2 and EO (L1- L2- EO- Control -Different Times) (120hrs) (Continued)

```
EO   96   3  35.000  0.000                          (-*-)
 EO   120   3  36.000  0.000                           (-*--)
 C    24  3  23.700  0.000              (-*-)
 L1   24   3  12.000  0.000  (-*-)
 L2   24   3  12.333  0.577  (--*-)
 C    48   3  28.300  0.000                   (-*--)
 L1   48   3  12.333  0.577  (--*-)
 L2   48  3  14.333  3.215    (-*--)
 C   72   3  29.300  0.000                    (-*-)
 L1  72   3  13.000  0.000   (--*-)
 L2  72   3  15.333  2.309      (-*-)
 C   96  3  30.700  0.000                     (-*-)
 L1  96   3  13.333  0.577    (-*-)
 L2  96  3  16.000  1.732       (-*-)
C   120   3  32.000  0.000                        (--*-)
L1  120   3  23.333  0.577              (-*--)
L2  120   3  20.000  0.000           (--*-)
                  -----+---------+---------+---------+----
                    14.0      21.0      28.0      35.0

Pooled StDev = 1.34
Grouping Information Using Tukey Method

       C1       N  Mean   Grouping
 C      120      3  36.000    A
 EO     96       3  35.000    A B
 EO     72       3  34.333    A B C
 C      120      3  32.000    A B C D
 EO     48       3  31.667      B C D
 C      96       3  30.700        C D
 EO     24       3  29.333           D
 C      72       3  29.300           D
 C      48      3  28.300           D
 C      24      3  23.700             E
L1      120     3  23.333             E
L2      120     3  20.000             E F
L2      96      3  16.000               F G
L2      72      3  15.333                 G
L2      48      3  14.333                 G
L1      96      3  13.333                 G
L1    72   3  13.000           G
L1    48   3  12.333           G
```

Table C.14. Statistical Evaluation of Antimicrobial Effect of *P. gingivalis* L1, L2 and EO (L1- L2- EO- Control -Different Times) (120hrs) (Continued)

L2 24 3 12.333 G
L1 21 3 12.000 G
Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1

Individual confidence level = 99.95%

Table C.15. Statistical Evaluation of Antimicrobial Effect of *P. intermedia* L1,L2 and EO (L1- L2- EO- Control -Different Times) (120hrs)

One-way ANOVA: C2 versus C1

Source	DF	SS	MS	F	P
C1	19	6533.22	343.85	120.65	0.000
Error	40	114.00	2.85		
Total	59	6647.22			

S = 1.688 R-Sq = 98.28% R-Sq(adj) = 97.47%

Table C.15. Statistical Evaluation of Antimicrobial Effect of *P. intermedia* L1,L2 and EO (L1- L2- EO- Control -Different Times) (120hrs) (Continued)

```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
 EO  24   3  24.333  4.509                  (*-)
 EO  48   3  28.000  3.464                    (*-)
 EO  72   3  28.667  4.619                    (-*-)
 EO  96   3  29.000  0.000                    (*-)
 EO  120  3  29.000  0.000                      (*-)
 C   24   3  25.000  0.000                  (-*)
 L1  24   3   0.000  0.000  (-*-)
 L2  24   3   0.000  0.000  (-*-)
 C   48   3  28.300  0.000                    (-*)
 L1  48   3  12.000  0.000          (-*-)
 L2  48   3  12.667  0.577          (-*)
 C   72   3  32.600  0.000                     (*-)
 L1  72   3  13.000  0.000          (-*)
 L2  72   3  13.667  0.577           (*-)
 C   96   3  35.000  0.000                      (*-)
 L1  96   3  16.000  0.000            (*-)
 L2  96   3  14.667  1.155           (*-)
C    120  3  37.000  0.000                       (-*)
L1   120  3  16.000  0.000            (*-)
L2   120  3  14.667  1.155           (*-)
                  --+---------+---------+---------+-------
                    0       12       24       36

Pooled StDev = 1.688
Grouping Information Using Tukey Method
C1   N    Mean  Grouping
1200  3  37.000  A
 960  3  35.000  A
 720  3  32.600  A B
 120  3  29.000    B C
  96  3  29.000    B C
  72  3  28.667    B C
 480  3  28.300    B C
  48  3  28.000    B C
 240  3  25.000      C
  24  3  24.333      C
1201  3  16.000        D
 961  3  16.000        D
1202  3  14.667        D
 962  3  14.667        D
```

Table C.15. Statistical Evaluation of Antimicrobial Effect of *P. intermedia* L1,L2 and EO (L1- L2- EO- Control -Different times) (120hrs) (Continued)

```
722  3  13.667       D
 721  3  13.000       D
 482  3  12.667       D
 481  3  12.000       D
 242  3   0.000        E
 241  3   0.000        E

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of C1
Individual confidence level = 99.95%
```

Table C.16. Statistical Evaluation of Antimicrobial Effect of *A.a* L1,L2 and EO (L1- L2- EO- Control -Different times) (120hrs)

One-way ANOVA: C2 versus C1

```
Source  DF      SS     MS     F     P
C1      19  7614.08  400.74  92.48  0.000
Error   40   173.33    4.33
Total   59  7787.41

S = 2.082   R-Sq = 97.77%   R-Sq(adj) = 96.72%


Individual 95% CIs For Mean Based on
Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
EO  24   3  21.333  2.517              (*-)
EO  48   3  24.667  7.234               (*-)
EO  72   3  33.333  2.309                   (-*)
EO  96   3  35.000  2.646                    (-*)
EO  120  3  56.000  0.000                              (-*-)
C   240  3  12.300  0.000         (-*)
L1  24   3   0.000  0.000  (-*-)
L2  24   3  14.333  1.155          (-*)
C   48   3  16.700  0.000           (*-)
L1  48   3   8.000  0.000       (-*-)
L2  48   3  14.667  1.528           (*-)
```

Table C.16. Statistical Evaluation of Antimicrobial Effect of *A.a* L1,L2 and EO (L1- L2- EO- Control -Different Times) (120hrs) (Continued)

```
C   72   3  16.300  0.000          (*-)
L1  72   3  16.000  1.732          (-*-)
L2  72   3  15.667  2.517          (-*)
C   96   3  15.700  0.000          (-*)
L1  96   3  17.000  0.000           (-*)
L2  96   3  16.667  1.155           (*-)
C    120  3  26.000  0.000               (*-)
L1   120  3  17.000  0.000           (-*)
L2   120  3  16.667  1.155           (*-)
--+---------+---------+---------+-------
0       16       32       48
Pooled StDev = 2.082
Grouping Information Using Tukey Method

C1    N   Mean  Grouping
EO   120  3  56.000  A
EO   96   3  35.000    B
EO   72   3  33.333    B
C    120  3  26.000     C
EO   48   3  24.667     C
EO   24   3  21.333     C D
L1  120   3  17.000       D E
L1  96    3  17.000       D E
C   48    3  16.700       D E
L2  120   3  16.667       D E
L2  96    3  16.667       D E
C   72    3  16.300       D E
L1  72    3  16.000       D E
C   96    3  15.700       D E
L2  72    3  15.667       D E
L2  48    3  14.667         E
L2  24    3  14.333         E F
C   24    3  12.300         E F
L1  48    3   8.000          F
L1  241   3   0.000            G
Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
                                    All Pairwise Comparisons among Levels of C1
                                        Individual confidence level = 99.95%
```

Table C.17. Statistical Evaluation of Antimicrobial Effect of *C. albicans* L1,L2 and EO (L1- L2- EO- Control -Different Times) (120hrs)

One-way ANOVA: C2 versus C1

Source DF SS MS F P
C1 19 12224.09 643.37 417.32 0.000
Error 40 61.67 1.54
Total 59 12285.76

S = 1.242 R-Sq = 99.50% R-Sq(adj) = 99.26%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-+---------+---------+---------+--------
24	3	43.333	3.055	(*)
48	3	44.333	2.887	(*)
72	3	45.000	2.646	(*)
96	3	47.333	1.155	(*)
120	3	48.000	0.000	(*)
240	3	14.300	0.000	(*
241	3	0.000	0.000	(*)
242	3	7.167	0.289	(*)
480	3	15.300	0.000	(*)
481	3	7.833	0.289	(*)
482	3	16.667	0.577	(*)
720	3	17.000	0.000	(*)
721	3	9.667	0.577	(*)
722	3	24.000	2.000	(*)
960	3	19.000	0.000	(*)
961	3	29.000	0.000	(*)
962	3	27.000	0.000	(*)
1200	3	21.000	0.000	(*)
1201	3	30.000	0.000	(*)
1202	3	30.000	0.000	(*)

-+---------+---------+---------+--------
0 15 30 45

Table C.17. Statistical Evaluation of Antimicrobial Effect of *C. albicans* L1, L2 and EO (L1- L2- EO- control -different times) (120hrs) (Continued)

Grouping Information Using Tukey Method

C1	N	Mean	Grouping
EO	120	3	48.000	A
EO	96	3	47.333	A
EO	72	3	45.000	A B
EO	48	3	44.333	A B
EO	24	3	43.333	B
L2	120	3	30.000	C
L1	120	3	30.000	C
L1	96	3	29.000	C
L2	96	3	27.000	C D
L2	72	3	24.000	D E
C	12	3	21.000	E F
C	96	3	19.000	F G
C	72	3	17.000	G H
L2	48	3	16.667	G H
C	48	3	15.300	G H
C	240	3	14.300	H
L1	72	3	9.667	I
L1	48	3	7.833	I
L2	24	3	7.167	I
L1	24	3	0.000	J

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

All Pairwise Comparisons among Levels of C1

Individual confidence level = 99.95%

Table C.18. Statistical Evaluation of antimicrobial effect of *Mix of microorganisms* L1,L2 and EO (L1, L2, EO- Control -Different Times) (120hrs)

Pooled StDev = 0.224

Grouping Information Using Tukey Method

C1	N	Mean	Grouping
C	120	25.000	A
C	96	24.000	B
EO	120	18.000	C
EO	96	17.333	C
EO	72	17.333	CC
72	3	16.000	D
EO	48	16.000	D
C	48	15.000	E
EO	24	15.000	E
C	24	14.000	F
L1	120	13.000	G
L1	96	13.000	G
L2	120	12.000	H
L1	72	12.000	H
L1	48	12.000	H
L1	24	12.000	H
L2	96	11.333	H I
L2	72	11.000	I
L2	48	11.000	I
L2	24	11.000	I

Means that do not share a letter are significantly different.