INHIBITION OF ORAL BIOFILMS BY ESSENTIAL OILS EXTRACTED FROM ASIAN HERBS

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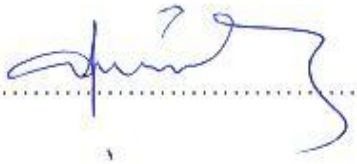
Assoc. Prof. Dr. Yeşim Ekinci ..... 
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To my beloved family, who offered me
unconditional love and support throughout my life.

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ABSTRACT

INHIBITION OF ORAL BIOFILMS BY ESSENTIAL OILS EXTRACTED FROM ASIAN HERBS

The vast majority of bacteria causing infection, including oral diseases, exist within complex bacterial communities, known as biofilms. Although planktonic cells can easily be eliminated with antimicrobial agents, the biofilm cells can survive and cause recontamination. Therefore, discovery of novel compounds against biofilms is critical. The aim of this study was investigating novel biofilm inhibitors obtained from natural, non-toxic traditional Asian plants. Essential oils (EOs) were extracted from traditional Asian herbs, namely seaweed Wakame, Hojicha, Genmaicha and Sencha teas and Shiitake edible mushroom by hydrodistillation for 3h using Clevenger apparatus. Antimicrobial activity of EOs were tested on four periodontal pathogens; *S.mutans*, *A.actinomycetemcomitans*, *F.nucleatum*, and *P.gingivalis*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each EO against planktonic cells were determined. Moreover, the inhibitory and disruptive effects of oils on biofilm formation and developed biofilms were analyzed using microdilution method and crystal violet staining. Chlorhexidine digluconate was used as positive control (0.09375 - 12 mg/l). Inhibitory and destructive effects of EOs on biofilms formed on coverslips were confirmed by SEM. All EOs were found to be antibacterial with highest zone of inhibition with Sencha, Genmaicha and Shiitake on *A.actinomycetemcomitans* (17.3, 18.8 and 18.8 mm in diameter, respectively). Moreover, Wakame and Sencha EOs had strong inhibitory effect on *S.mutans* biofilm formation at their sub-MIC values. EOs were also significantly disrupt pre-formed biofilms of *A.actinomycetemcomitans* and *F.nucleatum*. In conclusion, EOs of Asian herbs may be considered as alternative therapeutic strategy against biofilm associated oral diseases due to their high anti-biofilm effects.

ÖZET

ORAL BİYOFİLMLERİN ASYA KITASINA ÖZGÜ BİTKİLERDEN ELDE EDİLEN UÇUCU YAĞLAR KULLANILARAK ENGELLENMESİ

Ağız hastalıkları gibi enfeksiyonel hastalıklara sebep olan bakterilerin büyük bir kısmı biyofilm olarak bilinen kompleks bakteri komiteleri halinde bulunmaktadırlar. Planktonik bakteriler antimikrobiyel maddelerle kolayca ortadan kaldırılabilirken, biyofilm hücreleri bu maddelerden etkilenmezler ve kontaminasyonun tekrar oluşmasına sebep olurlar. Bu yüzden biyofilm oluşumunu engelleyebilecek yeni maddelerin keşfi büyük önem taşımaktadır. Bu çalışmanın amacı Asya kıtasına özgü bitkilerde bulunan doğal, toksik olmayan yeni biofilm inhibitörlerinin araştırılıp elde edilmesidir. Çalışmada Wakame adlı bir deniz yosunu, Hojicha, Genmaicha ve Sencha yeşil çayları ve yenebilen bir mantar türü olan Shiitake kullanılmıştır. Bu bitkilerden 3 saat boyunca hidrodistilasyon methodu ile Clevenger aparatı kullanılarak uçucu yağlar (EO) elde edilmiştir. Yağların antimikrobiyel etkileri dört periodontal patojen (*S.mutans, A.actinomycetemcomitans, F.nucleatum ve P.gingivalis*) üzerinde test edilmiştir. EOların ayrıca planktonik hücrelere karşı etkili oldukları minimum inhibisyon (MIC) ve bakterisidal konsantrasyonları (MBC) belirlenmiştir. Bunun yanı sıra, mikrodilüsyon yöntemi ve kristal viyole boyaması kullanılarak EOların biyofilm oluşumunu engelleyici ve/ya olgunlaşmış biyofilmleri yıkıcı etkilerinin olup olmadığı test edilmiştir. Klorheksidin glukonat solüsyonları pozitif kontrol olarak kullanılmıştır (0.09375-12 mg/l). EOların lamel üzerinde oluşturulan biyofilmi engelleyici ve yokedici etkileri SEM kullanılarak da doğrulanmıştır. Çalışmada kullanılan bütün EOlar antimikrobiyel etki göstermiş olup en büyük inhibisyon alanı *A.actinomycetemcomitans* bakterisine karşı Sencha, Genmaicha ve Shiitake EOlarında tespit edilmiştir. Ayrıca, Wakame ve Sencha EOları MIC değerlerinin altında dahi *S.mutans* biyofilm oluşumunu engellemişlerdir. EOlar *A.actinomycetemcomitans* ve *F.nucleatum* bakterilerin oluşturmuş olduğu biyofilmleri de etkili bir şekilde bozmuşlardır. Sonuç olarak bu çalışmadan elde edilen bulgular Asya kıtasına özgü bitkilerden elde edilen

EOların biyofilm bağlantılı oral hastalıklara karşı alternatif bir tedavi yöntemi olarak kullanılabileceğini göstermiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

AL-2	Autoinducer-2
ATCC	American Type Culture Collection
BHB	Brain Heart Broth
cfu/ml	Colony forming units per mililiter
CHX	Chlorhexidine
CO_2	Carbon dioxide
CSP	Competence Signaling Peptides
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EPS	Extracellular polymeric substance
EO	Essential oil
FAME	Fatty acid methyl ester
GC-MS	Gas Chromatography equipped with Mass Spectrometry
GSg	Genmaicha used in this study
GSh	Hojicha used in this study
GSko	Kombu used in this study
GSku	Kukicha used in this study
GSn	Nori used in this study
GSs	Sencha used in this study
GSsh	Shiitake used in this study
GSw	Wakame used in this study
HMDS	Hexamethyldisilazane
IL-1	Interleukin-1
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
OD	Optical Density
PCR	Polymerase Chain Reaction
PBS	Phosphate buffered saline

rDNA	Ribosomal DNA
SEM	Scanning Electron Microscopy
TBE	Tris/Borate/EDTA buffer
TCM	Traditional Chinese Medicine
TNF-α	Tumor necrosis factor- alpha

1. INTRODUCTION

Biofilm can be defined as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription"[1]. The formation of biofilms is an important survival strategy for bacterial cells. Once established, biofilm infections are rarely destroyed by host defense mechanisms and other external factors such as antibiotics, detergents, dehydration, UV exposure. Moreover, *in vitro* biofilm studies have shown that biofilms are resistant to antibiotics at hundreds or even a thousand times higher concentrations than that found for planktonic bacteria. This leads to failure of therapies at low doses or use of long term high doses of antibiotics for successful treatment, which may, however, cause emergence of resistant strains against these antibiotics in the population [2].

Dental plaque is also a biofilm community that accumulates through sequential and ordered colonization of multiple oral bacteria [3] and today it has been known that almost all oral diseases such as dental caries, gingivitis and periodontitis are caused by oral biofilms. The high prevalence of these diseases in the population and the treatment costs make scientists to find some treatment methods against oral bacteria. The current therapies are mechanical scaling and root planing, use of antibodies and use of chemotherapeutic agents (chlorhexidine, povidone-iodine, fluoride, sodium chlorite). However, all these methods can not completely remove all oral biofilms and they have considerable side effects including risk of antibiotic resistance, immunotoxicity, carcinogenicity, genotoxicity, reprotoxicity, risk of development of hyperthyroidism, staining of teeth, disturbances in taste sensing and increased calculus accumulation, etc [4-7]. Therefore, investigating novel anti-biofilm compounds that can efficiently inhibit oral biofilm formation and/or eradicate mature dental plaque without causing significant health problems is one of the hot topics in microbiology and dentistry.

In nature, eukaryotes live closely associated with virulent prokaryotes. This has forced mammals to evolve different defense systems. Plants and fungi, however, do not possess active immune systems; instead they have to rely on physical and chemical defenses. For these reasons, it might be expected that plants and fungi have evolved to produce chemical compounds to inhibit bacterial biofilms. Therefore, a great attention is developed for investigation of novel anti-biofilm compounds extracted from plants.

Essential oils are volatile aromatic constituents of the plants and secreted as secondary metabolites by all organs of the plant (flowers, twigs, stems, seeds, fruits, buds, leaves, etc). In nature they have an important role in protection of plants against bacteria, virus, and fungi infections [8]. Moreover, the antimicrobial, antiviral and antibiofilm activities of essential oils extracted by steam distillation, hydrodistillation, expression and many other methods from several plants such as thyme, euganol, rosemary have been shown in studies [9-13].

Traditional medicine refers to the health practices, approaches, knowledge and beliefs incorporating plant based medicines and it has been used for maintenance of health and for prevention and treatment of diseases in Asian countries for long time and currently in Europe and America. Therapeutic effects of several plants cultivated in Asia (seaweeds, kelps, green tea) have been found to be effective against several diseases like diabetes, cholesterol and cancer [14-17]. Although solvent extracts of these herbs have been studied widely against microorganisms, there is very little or no information on antibacterial and anti-biofilm activities of essential oils extracted from these traditional Asian plants. Investigation of essential oils extracted from five Asian plant (green teas, edible seaweed and mushroom) for their potential antibacterial and antibiofilm effects against oral pathogens and biofilms, and finding novel natural and harmless biofilm inhibitors for the treatment of oral diseases were aimed in this thesis.

2. THEORETICAL BACKGROUND

2.1. DENTISTRY

2.1.1. Oral Cavity

The oral cavity (mouth) is the entrance of digestive system and it contains a complex system of tissues and organs necessary for intake, processing and passing of food to the rest of the gastrointestinal tract [18]. In addition to this primary role of mouth, in humans it plays an important role in communication.

The oral cavity consists of cheeks, the hard and soft palates, the tongue and teeth, as shown in Figure 2.1.

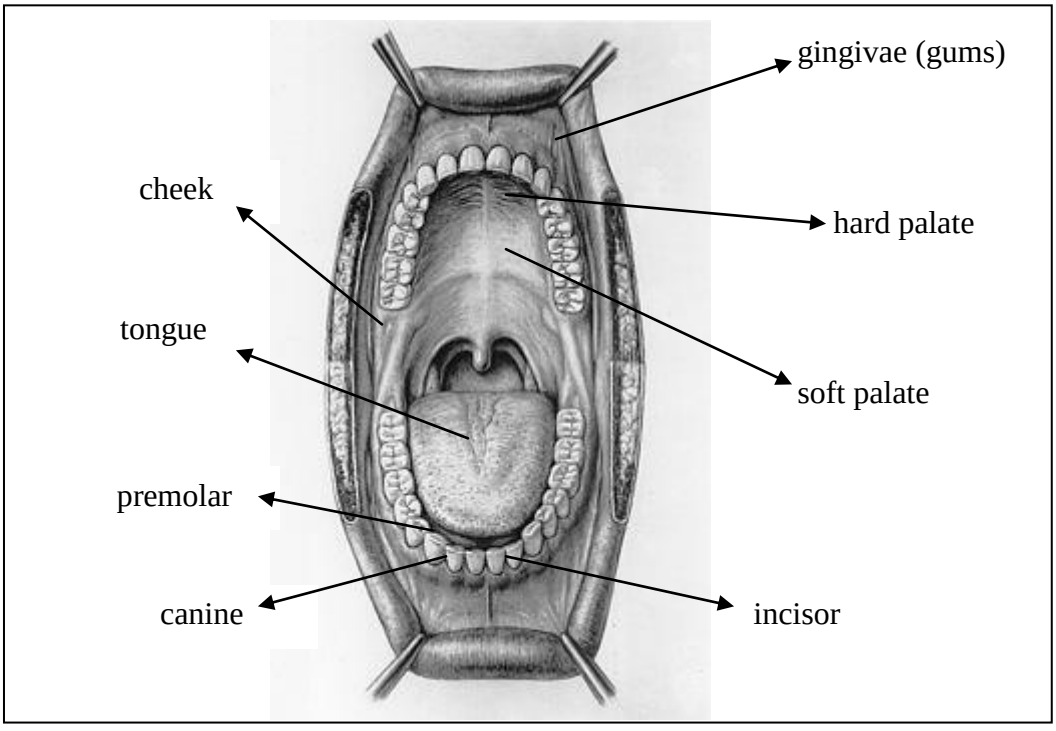

Figure 2.1. Anatomical features of the oral cavity

The cheeks comprise the lateral walls of the oral cavity while hard and soft palate comprise the roof of the mouth [18]. The tongue, on the other hand forms the floor of the mouth.

The teeth are highly mineralized appendages of the mouth and they are necessary for tearing, scraping and chewing the ingested food [19]. This mechanical digestion increases the surface area of the food which improves the activity of the digestive enzymes found in the saliva. It is also known that teeth help speech with the lips and tongue [20]. Humans usually have 20 primary teeth, known also as “baby” or “milk” teeth, and 32 permanent teeth. There are four classes of teeth, namely incisors, canines, premolars and molars [21].

Teeth protrude into the oral cavity within alveolar bone whose upper regions are covered with gingivae (gum) tissue [18].

2.1.2. Anatomy of Tooth

All mammalian teeth have the same general features consisting of mainly two tissues; hard and soft tissue. Hard tissue contains an extremely hard material called "enamel", a layer of bony material, "dentine" and bone-like structure called "cementum", which are represented in Figure 2.2. Dental pulp which forms the soft tissue is divided into crown and root [22, 23].

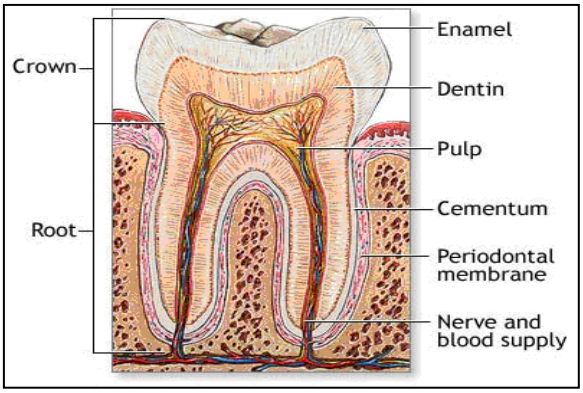

Figure 2.2. Main parts of a tooth

2.1.2.1 Enamel

Enamel is the mineralized part covering the crown of the tooth in mammals and it is considered as the hardest tissue of the body due to its high inorganic content (96-97 per cent) an low organic matter (1 per cent, mainly soluble and insoluble proteins and peptides) and water (2-3 per cent) content [18, 24]. Enamel is not a living tissue due to absence of blood supply and nerves [24]. The thickness of the enamel varies along the surface of the crown (approximately 2μm thick on the crown of the tooth) and it is often thickest at the biting edges (i.e. cusps) [25]. The hardness of enamel comes from two main minerals that it contains; namely hydroxyapatite and fluorapatite and the latter makes enamel more resistant to acids present in the diet [18]. Unlike dentin and bone, enamel does not contain collagen. Instead, two unique proteins, namely, amelogenins (90 per cent) and enamelins (10 per cent) are present in enamel [26].

2.1.2.2. Dentine

Dentine forms the bulk of the mineralized portion of the tooth and it is produced by odontoblasts which continuously deposit on dentine. Dentine is covered by enamel in the region of the tooth crown and cementum over the root surface [18, 27]. The composition of dentine is 70 per cent inorganic material (hydroxyapatite), 18-20 per cent organic material (mainly collagen) and 10-12 per cent water [18, 24]. Calcium salts, fluoride, copper, zinc and iron are the other inorganic minerals present in dentine. However, this composition changes as the tooth ages because of continuous mineralization [24].

2.1.2.3. Cementum

Cementum is a bone-like structure covering roots of the tooth and binding it to the periodontal ligament [28, 29]. Because of this function of cementum, it is also classified as a part of the periodontium although it is one of the parts of tooth [24]. It is composed of 55-60 per cent inorganic material and remainder being inorganic matter and water [18, 28]. Moreover, cementum may provide an important role when root is fractured or reabsorbed.. In these cases, cementum may replace the lost root tissue and provide repairing of the root [28].

2.1.2.4. Dental pulp

Dental pulp is the space present at the center portion of the crown and root, and it is covered by dentin. The part of the pulp in the crown is called the pulp chamber while that in root is called the root canal(s) [24, 28]. Pulp is made up of living connective tissue, odontoblasts, tiny blood vessels and nerves. Because of this composition, pulp is mainly responsible for dentin production (by its odontoblasts), for keeping organic materials of dentin stable by supplying nutrients and water. Moreover, nerves of pulp provide sensation of physical effects to dentin or pulp such as extreme temperature, pressure or trauma [30].

2.1.3. Anatomy of Periodontium

The periodontium represents all the tissues whose main function is to support the tooth and attach it to the jaw [31]. It consists of four tissues, as shown in Figure 2.3, namely, cementum (discussed above), periodontal ligament, alveolar bone and gingiva.

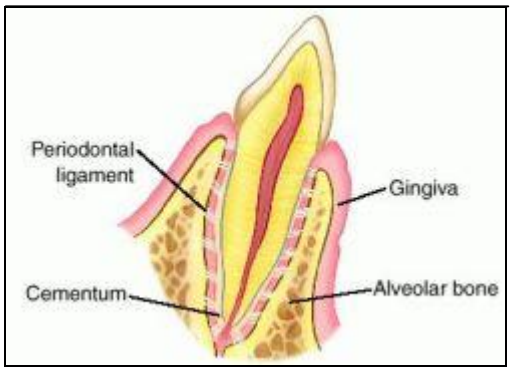

Figure 2.3. Anatomy of the periodontium

2.1.3.1. Periodontal ligament

Periodontal ligament is the term used for fibrous connective tissue that suspend the tooth in the socket and support the gingiva [27]. Osteoblasts that form bone, cementoblasts that form cementum and fibroblasts that form fibrous tissue are present in periodontal ligament. Therefore, this structure of periodontium has formative functions. Moreover, the presence of nerves and blood vessels in the ligament gives sensory and nutritive functions to this tissue [28].

2.1.3.2. Alveolar bone

Alveolar bone is a specialized bony tissue that supports the tooth in the jaw. This structure is made of compact bone which forms and protect the root socket and provides attachment for the fibers of the periodontal ligament [27, 28, 32]. Periodontal disease, which is the focus of this study to find a treatment of it, firstly causes loss of alveolar bone in the socket and makes a crater-like depression which extends over time and results in the widening of the tooth socket [33]. The reasons and process of periodontal disease is discussed in detail in the "Oral Diseases" part.

2.1.3.3. Gingiva

The gingiva is one portion of the oral mucosa and it is the most peripheral component of the periodontium [34]. As Figure 2.4 shows below, it starts at the mucogingival junction, covers the alveolar bone and ends at the cervix of each tooth, where it forms epithelial attachment.

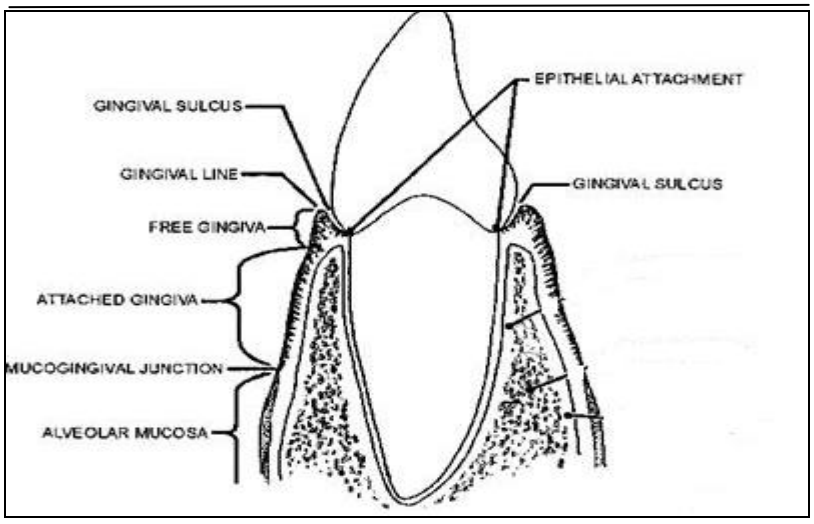

Figure 2.4. Structures of gingiva

The gingiva is highly vascular and receives blood supply. It helps in the support of the teeth, and protects the alveolar bone and periodontal ligament from bacterial invasion [35].

Figure 2.5 shows the healthy gingiva that can be characterized clinically in terms of its color, size, consistency and surface texture [21, 34].

- Color of the healthy gingiva is pale pink. However, it can be lighter or darker according to the person's complexion.
- Normal size of healthy gingiva depends on cellular and intercellular elements and blood supply to the tissue. As it will be mentioned below, changes in size of the gingiva (i.e. swollen and inflamed gingiva) show gingival disease.
- Firmness of the gingiva is an indicator of oral health.
- The healthy gingiva is stippled and has an orange peel texture. The reduction or loss of this property occurs in the case of gingival disease.

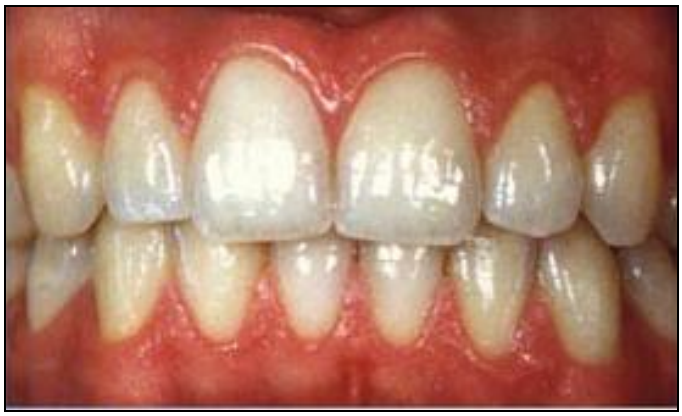

Figure 2.5. Healthy gingiva

Gingiva is composed of different parts [21, 35]:

- Marginal (free or unattached) gingiva: it is the terminal edge of the gingiva surrounding the tooth. It is not bound directly to the tooth or bone and it forms the soft tissue wall of the gingival sulcus (Figure 2.4). In a healthy mouth, marginal gingiva has approximately 1-3 mm wide. The marginal gingiva is composed of gingival margin (narrow band of gingiva which shows symptoms of gingivitis firstly), gingival sulcus (area between the marginal gingiva and the tooth) and epithelial attachment (the part that connects the gingiva to the tooth surface).

- Attached gingiva: The portion of gingiva present between marginal (free) gingiva and mucogingival junction (Figure 2.4). It is tightly bound to the underlying bone of alveolar process.

  To understand the health of the gingiva, the width of attached gingiva is important.
- Interdental gingiva: it is the portion of gingiva filing the interproximal space below the contact areas of two adjacent teeth and it is represented in Figure 2.6.

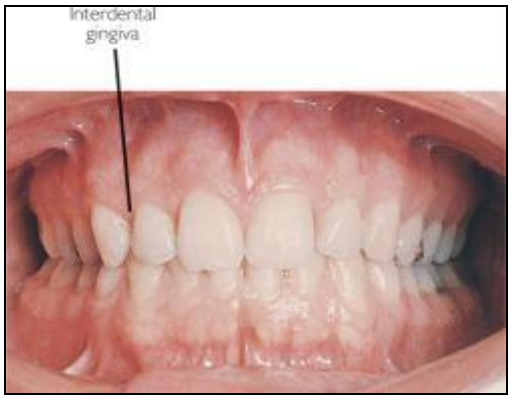

Figure 2.6. Gingiva tissue showing interdental gingiva

2.2. ORAL MICROBIOTA

External surfaces of the human body provide a home for many microorganisms and most of these commensal microbes are in harmony with the host. In fact, these microorganisms play a role in the development of the immune system of the host. Oral microbiota, however, are the only part of the human body whose commensal microbes cause diseases. Dental caries and periodontal diseases, for instance, occur due to the interactions between the bacterial community of the mouth, environment and defense system of the host [36]. Therefore, researchers have attempted to isolate, characterize and identify the oral microbiota species since Anton Leeuwenhoek observed "animalcules" in the human dental plaque over 300 years ago.

Oral cavity has some features that make it a unique microbial habitat. Teeth, for example, provide hard non-shedding surfaces on which large masses of microorganisms can accumulate (dental plaque). Moreover, highly papillated structure of tongue supports

growth of high number of microorganisms. Saliva, which continuously bath the oral cavity has a significant effect on microbiota; for instance, the pH of saliva (6.75-7.25) favors the growth of many bacteria. On the other hand, gingival crevicular fluid (GCF), a plasma derived fluid that flows through the junctional epithelium, carries nutrients to the microbes in the gingival tissue and plays a role in regulation of the microflora in the gingival tissue by carrying host immune components to it [37]. With the development of culture studies (anaerobic methods and culture-independent molecular methods) more than 700 species living in the oral cavity have been identified [38]. Initially, scientists focused on identifying microbes causing oral diseases (caries, gingivitis, periodontitis, etc) and they found some "key" microorganisms (e.g. *Porphyromonas gingivalis* in periodontitis), to be the main cause of the disease. However, then they realized that without knowing the normal oral community, finding the disease specific pathogens could not be possible.

The oral microbiota is complex and all four types of microorganisms (viruses, fungi, protozoa and bacteria) colonize many oral sites including the tongue, oral mucous membranes, subgingival and supragingival tissues and teeth [36, 37].

2.2.1. Viruses

Herpes simplex virus, cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) are the viruses detected most frequently in the mouth. Although their prevalence has increased in the case of oral diseases such as periodontitis and periodontal abscesses, they have also been detected in healthy subjects [39, 40]. Possible reasons of transmission of these viruses are breastfeeding and sexual contact [41]. These viruses can stay dormant in the mouth unless stress, cold, other virus infection or disease weakens the immune system of the host. Then they become active and take place in oral diseases with oral pathogenic bacteria.

It was shown that presence of two herpes viruses in the periodontal pockets increased the occurrence of subgingival *P. gingivalis, B. forsythus, P. intermedia, P. nigrescens, Treponema denticola* and *A. actinomycetemcomitans* [42, 43]. Slots and Contreras [44] hypothesized a new model for periodontitis in which herpes virus in periodontal tissue causes suppression of periodontal immune defense and therefore pathogens associated to

periodontitis can increase in number which result in release of pro-inflammatory cytokines and chemokines, initiation of cytotoxic or immunopathological events, and finally breakdown of periodontal tissues.

2.2.2. Fungi

Yeasts are commensal microorganisms in the oral cavity and they may not cause disease unless there are predisposing host factors, such as other disease [45]. The most prevalent yeast genus isolated from the oral cavity is Candida (*Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida guilliermondii*). Other than *Candida* species, *Rhodotorula* spp., *Saccharomyces cerevisiae*, and *Cryptococcus* spp. were also detected in the mouth at low concentrations [46].

Among *Candida* species, *Candida albicans* is the most common yeast found in the mouth. It colonizes several surfaces in the oral cavity such as tongue, epithelial cells of the cheek, and tooth surfaces.

Overgrowth or infection of the oral cavity by *Candida* species causes oral candidiasis, the most common human fungal infection especially in children and elder people. However, as mentioned before, Candida species do not cause the disease generally unless the immune system has been weaken. For instance, carriage rates of the oral Candida species have been reported as 20 -75 per cent without any symptoms [47].

2.2.3. Protozoa

Protozoa are not commonly detected in the oral cavity. Only in the absence of oral hygiene, protozoa such as *Entamoeba gingivalis* (Figure 2.7a) and *Trichomonas tenax* (Figure 2.7b) appear, significantly [48]. These protozoa species are harmless and they can be found between the gingival pockets and near the base of the teeth associated with poor oral hygiene [37].

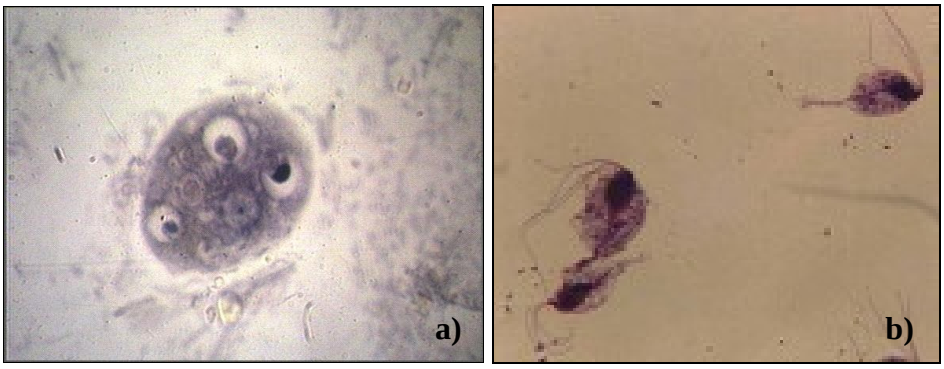

Figure 2.7. Light microscope image of a. *Entamoeba gingivalis* stained with iron hematoxylin stain b. *Trichomonas tenax* stained with Giemsa dye

2.2.4. Bacteria

The most prevalent microorganisms of the mouth are bacteria and they are found in huge numbers in the mouth; for instance 100 million bacteria are present in per milliliter of saliva, while 1 billion bacterial cells living in per milligram of dental plaque [36]. All types of bacteria are present in the oral cavity, shown in Table 2.1; Gram positive, Gram negative, obligate aerobes, facultative anaerobes, obligate anaerobes and there are all morphological shaped (cocci, bacilli, filaments, fusiform bacilli, spirochaetes) bacteria.

With the development of molecular studies (16S rRNA, checker board DNA-DNA hybridization, real time PCR), bacterial species that do not grow on artificial media have been identified and their frequency in the healthy and diseased sites of the oral cavity can be determined [49].

By these culture methods and culture-independent molecular methods approximately 36 phyla/divisions within *Bacteria* domain were detected in the mouth [36] and eight phyla that are found frequently in the mouth are listed as *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Protebacteria*, *Spirochaetes*, '*Synergistes*', and TM7 and shown in Figure 2.8. Among these huge diversity, 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 have role in dental plaque formation and in plaque associated oral diseases (dental caries, gingivitis and periodontitis).

Table 2.1. Bacterial genera found in the oral cavity

	Gram positive	**Gram negative**
Cocci	Abiotrophia Enterococcus Peptostreptococcus Streptococcus Staphylococcus Stomatococcus	Moraxella Neisseria Veillonella
Rods	Actinomyces Bifidobacterium Corynebacterium Eubacterium Lactobacillus Propionibacterium Pseudoramibacter Rothia	Actinobacillus Campylobacter Cantonella Capnocytophaga Centipeda Desulfobacter Eikenella Fusobacterium Haemophilus Leptotrichia Porphyromonas Prevotella Selenomonas Treponema Wolinella

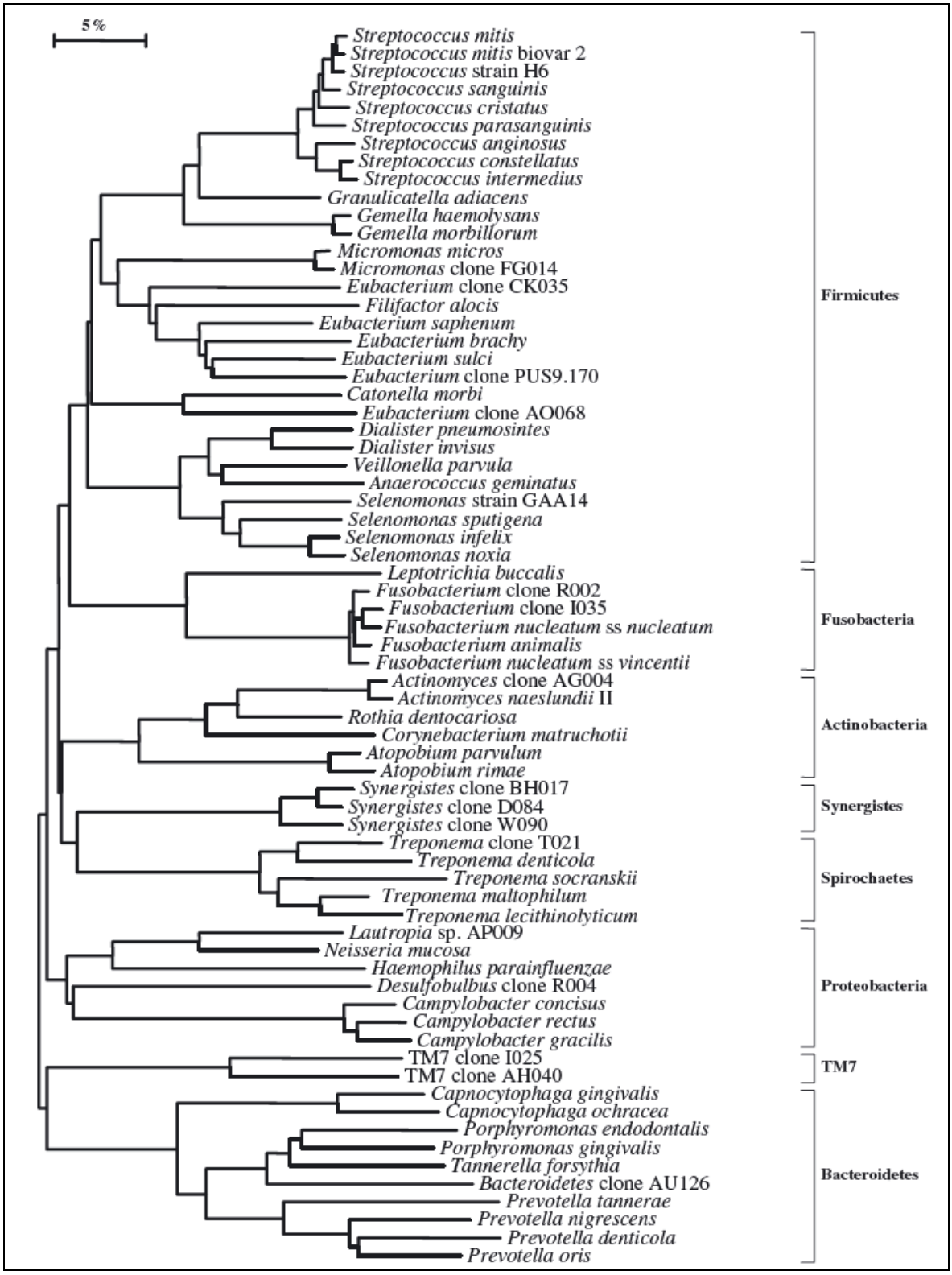

Figure 2.8. Phylogenetic tree of predominant cultivable and non-yet cultivable species in the human mouth [38]

2.3. ORAL DISEASES and TREATMENT METHODS

The mouth is an essential part of the human body and oral health is directly related to the health of the rest of the body. Many researches showed that poor oral health affects the general health; for instance, periodontal diseases were found to contribute to cardiovascular diseases [50]. There are several oral diseases, however, dental caries and periodontal diseases (gingivitis and periodontitis) have been detected worldwide and studied in this thesis.

2.3.1. Dental Caries

Dental caries, shown in Figure 2.9 is an infectious microbiological teeth disease developed by localized dissolution and destruction of the tooth hard tissues [51]. Tooth decay is the second most common cause of tooth loss and it is a worldwide public health problem [51, 52]. Not only children and adolescents, adults are also susceptible to dental caries; but adults may develop caries on the root surface instead of on the crown.

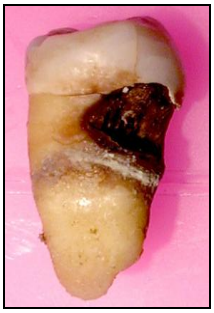

Figure 2.9. Tooth with a late stage dental caries

It has been reported that in 1997 more than 98 percent of people worldwide suffered from dental caries [53]. However, the prevalence of tooth decay has decreased gradually due to the development of effective preventative agents.

2.3.1.1. Etiology of the dental caries

Composition, morphological characteristics and position of the teeth affect the susceptibility to dental caries. For example, teeth having less fluorine, zinc and iron, and teeth with deep, narrow occlusal fissures are more likely to develop dental caries [51]. Moreover, more viscous saliva and reduced saliva levels can increase the susceptibility to tooth decay while saliva with neutral/alkaline pH can neutralize the acids produced by the bacteria and so prevent dental caries [54]. The composition of the diet is also effective on dental caries formation, i.e., diet rich in carbohydrates, especially sucrose, induces while diet containing vitamins A, B,D, K, calcium, and fluorine inhibits tooth decay [55].

2.3.1.2. Formation of dental caries

Dental caries is a multifactorial disease that depends on host factors (teeth and saliva), dental plaque microorganisms and diet. Dental plaque, a thin microbial biofilm, is formed on the surface of the tooth when oral hygiene is not done sufficiently. Microorganisms present in the dental plaque, such as *S. mutans* and *S. sobrinus*, ferment carbohydrates, especially sucrose, in the diet and produce acids which result in a decrease in pH to 5.0-5.5. At this pH, most of the bacteria present in the plaque cannot grow. However, this low pH also causes demineralization of the inorganic substances (hydroxyapatite) of the tooth. As hydroxyapatite dissolves, it acts as a buffer and allows the growth of acid tolerant streptococci and lactobacilli in the plaque. The proteolytic enzymes of these bacteria then start to disintegrate the organic components of the tooth which is assumed to be the beginning of progression of tooth decay. If teeth are not cleaned and dental plaque is not removed, the remineralizing potential of the saliva can not reverse the demineralization of the tooth and so a white spot will occur on the tooth surface which then progresses to dental caries [51, 56].

2.3.2. Gingivitis

Gingivitis is defined as the inflammation of the gum tissue (gingiva) caused by accumulation of dental plaque (biofilm) on the teeth at a location adjacent to the gingiva. Microorganisms associated to dental biofilm and their virulence factors induce the host immune response in the gum tissue and as a result swelling, reddening and bleeding of

gingival tissue shown in Figure 2.10 occur [57]. Gingivitis being the mildest form of periodontal disease affects 50 per cent of all adults in the world [56].

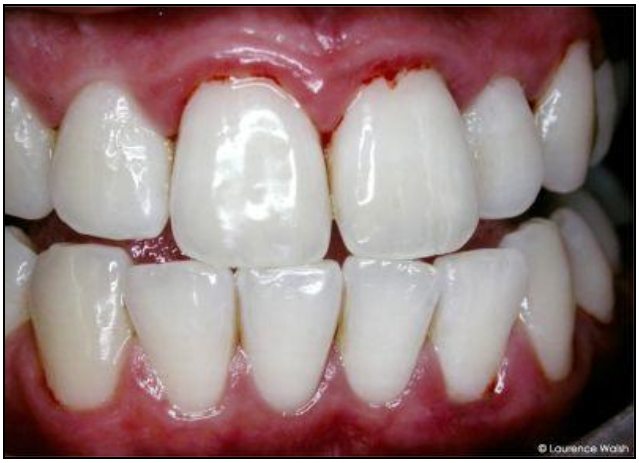

Figure 2.10. Red, swollen, bleeding gingiva of a patient with gingivitis

2.3.2.1. Etiology of gingivitis

Formation and accumulation of dental biofilms on the tooth surface near the gum tissue is the main factor causing gingivitis. As mentioned before in dental caries, accumulation of the plaque is due to poor oral hygiene. However, gingivitis can be easily reversed in the early stages if dental plaque is removed by adequate oral hygiene [58]. Theilade *et al.* [59] showed the effect of oral hygiene on periodontal health by finding that the absence of tooth brushing for 28 days increased biofilm amount so that gingivitis occurred in all periodontally healthy subjects within 2-3 weeks. Moreover, removal of dental plaque and re-tooth brushing eliminated the symptoms of gingivitis and resulted in healthy gingiva in the same subjects just in one week.

In addition to dental plaque, there are some indirect factors including hormonal disturbances (such as at puberty, pregnancy, menopause), tobacco use, drugs, malnutrition and some systemic diseases may lead to gingivitis [51].

2.3.2.2. Development of gingivitis

There is no specific bacteria causing gingivitis, as mentioned before, it is a consequence of accumulation of dental plaque at the gingival margin. At the initial stages

of supragingival plaque, Gram positive aerobic bacteria such as *Streptococcus*, *Lactobacillus* and *Actinomyces* species are primarily present. As biofilm develops further, the microbiota of the supragingival plaque is shifted to more anaerobic, Gram negative bacteria including *Campylobacter*, *Fusobacterium*, *Aggregatibacter*, *Prevotella* and *Porphyromonas* species that increase in number over time [60]. This shift begins the gingivitis.

Four stages of gingivitis leading to acute gingivitis and further to chronic gingivitis is represented in Figure 2.11 [61]:

- Initial lesion of gingivitis occurs between 2-4 days after biofilm formation has started. This stage has initiated by the shift of microbiota of biofilm from streptococci and *Actinomyces* species to Gram negative anaerobes. At this stage although inflammation of gingival tissue begins, there is no clinical sign of inflammation. The LPS structure and virulence factors of these Gram negative anaerobes activate the macrophages which start to produce cytokines such as IL-1 and TNF-α. IL-1 then activates mast cells which produce histamine, a vasoactive substance that can induce vascular permeability and vasodilatation. Leukocytes, especially neutrophils are now able to move from the blood vessels to connective tissue of the gingiva as a result of vasodilatation. These leukocytes kill some of the bacteria in the biofilm by releasing degradative enzymes. However, these enzymes also lead to destruction of collagen and connective tissue of surrounding vessels.

- Early lesion of gingivitis (4-7 days) is the stage at which spirochetes dominate the biofilm matrix and the processes of the initial lesion continue to develop with worse results. For instance increased blood flow and accumulation of leukocytes from blood to gingival tissue cause swelling (edema) and redness of the gingiva. Moreover another type of leukocyte, namely lymphocytes starts to accumulate in gingival tissue. Dissolving of collagen may reach up to 60-70 per cent.

- Established lesion (14-21 days) is the stage when acute gingivitis starts to shift to chronic gingivitis. B cells interact with macrophages in the gingival tissue and

become plasma cells which produce antibodies. At that stage, plasma cell do not cause bone loss. However, loss of collagen still continues.

After that stage, many lesions develop into advanced lesion, while some of them continue to remain as established lesions for long times.

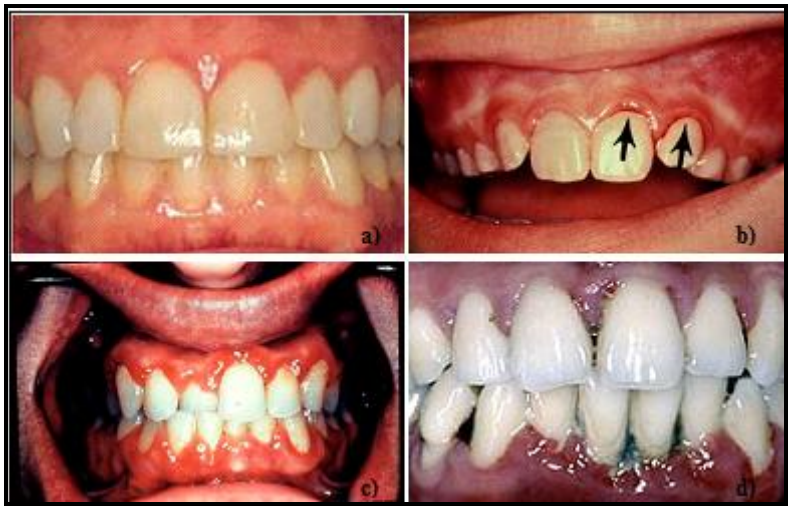

Figure 2.11. Four stages of gingivitis lesions a. initial lesion, b. early lesion c. established lesion, d. advanced lesion (periodontitis)

- Advanced lesion is the stage that severe gingivitis proceeds into periodontitis, an oral disease characterized by periodontal pocket depth > 4 mm and bone loss which eventually will lead to tooth loss.

2.3.3. Periodontitis

Periodontitis is the chronic inflammation of periodontal membrane which results in irreversible loss of connective tissue attachment and alveolar bone and it is represented in Figure 2.12 [62]. The most severe forms of periodontitis cause significant destruction of periodontal ligament and osseous tissue and ultimately loss of teeth. Periodontitis is always preceded by gingivitis although gingivitis does not always progress to periodontitis [63]. Therefore, early symptoms of periodontitis (red, swollen and easily bleeding gums) are similar to that of gingivitis. The reasons why the inflammation of gingiva expands to cause

the breakdown of collagen tissue and bone resorption in some patients are not clear. It has been suggested that there are underlying genetic mechanisms or risk factors such as, smoking, diabetes, stress, other systemic diseases etc that provoke this progression to periodontitis in certain people and not in others [64].

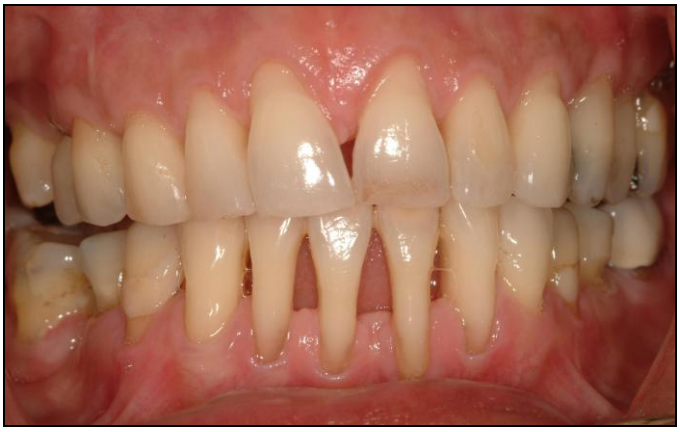

Figure 2.12. Patient with advanced periodontitis showing severe bone and gum loss

It has been reported that 30 per cent of adult population have periodontitis with a periodontal pocket depth ≥ 4 mm and 5-15 per cent of these patients have advanced periodontitis (pocket depth ≥ 6mm) [56].

2.3.3.1. Etiology of periodontitis

Similar to gingivitis, periodontitis is also consequence of a complex interactions between pathogenic bacteria reside in biofilm, host responses and other systemic and environmental risk factors. These risk factors are summarized in in Figure 2.13.

Among these factors, however, presence of periodontal pathogens colonized in three dimensional structure of biofilm matrix is the primary etiologic factor. When biofilm is started to form on tooth surface close to gum tissue due to the poor oral hygiene, it firstly colonizes supragingivally then subgingivally. As biofilm develops, the environment becomes favorable for growth of Gram negative anaerobic pathogens such as *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum* and *T. forsythensis* whose number increases in the subgingival biofilm with time. The virulence factors of these bacteria

induce host response which may cause collagen tissue breakdown. As the connection between tooth and gum tissue widens, red complex periodontal pathogens (*P. gingivalis, T. denticola* and *T. forsythensis*) can colonize deeper pockets and cause more collagen tissue breakdown and bone resorption together with immune responses [57].

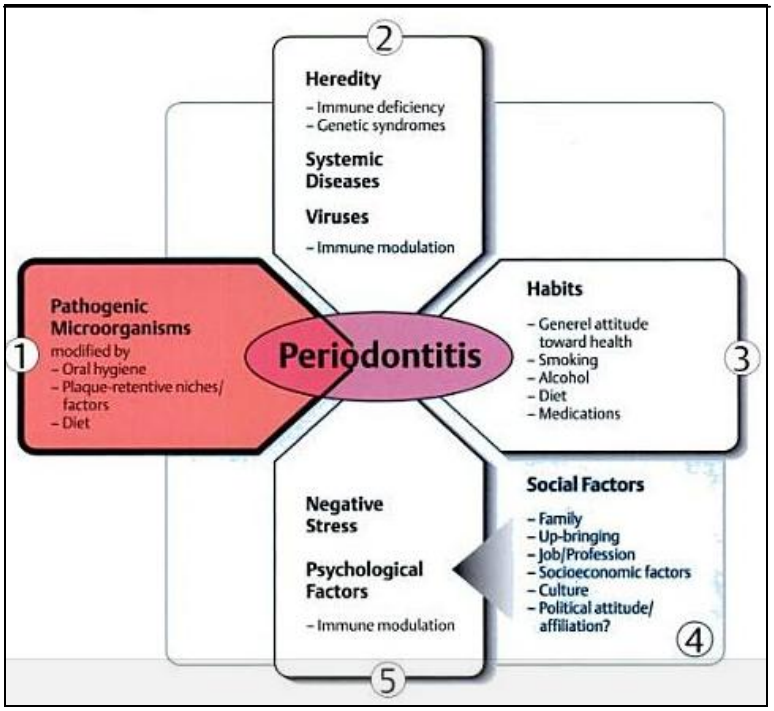

Figure 2.13. Factors causing the development of periodontitis [34]

The host inflammatory response to periodontal pathogens is the second important etiological factor underlying periodontitis. As mentioned before some bacterial products (LPS, proteases, toxic products) released from the dental plaque stimulate the immune cells such as neutrophils and macrophages, which produce pro-inflammatory cytokines like interleukin -1β, tumor necrosis factor-α , interleukin-6. These cytokines and neutrophils, however, can stimulate tissue destruction and deleterious alveolar bone resorption in the host.

Since gingivitis progresses into periodontitis in some patients while disease state is arrested at gingivitis in other patients, it was suggested that genetic differences between the patients may be a risk factor for periodontitis. Many results mentioned in the previous study have proven this hypothesis. Polymorphisms in genes of IL-1 family [65], Fc

receptor and human leukocyte antigens [66] were detected when healthy and periodontitis DNAs were compared.

The association between periodontitis and other factors such as diabetes mellitus [67] and tobacco use [68] were also shown in several researches.

2.3.3.2. Development of periodontitis

The stages in the progression from gingivitis to periodontitis were mentioned in detail above. Therefore, here a summary of this stages leading to periodontitis is given.

The initiation and progression of periodontitis is thought to be due to the colonization of periopathogenic bacteria in mature subgingival biofilm in which bacteria can be protected with diffusion barriers and migrate to adjacent periodontal sites and to periodontal tissues. If the development of mass of biofilm is not stopped by some mechanical and chemical methods, numbers of "orange complex" (*Prevotella* spp. and *Fusobacterium* spp.) and "red complex" bacteria (*T. forsythia,P. gingivalis* and *T. denticola*) increase in the biofilm and inflammatory responses leading to destructive periodontitis have been stimulated [69].

As described before, the lipopolysaccharide (LPS) layer of Gram negative bacteria stimulates the complex host response (Figure 2.14). Virulence factors induce production of cytokines (IL-1, TNF-α and prostaglandins) from host immune cells. Metalloproteinases (MMPs) are also activated by the immune cells. While TNF-α inhibits collagen formation by reducing the fibroblast activity, MMPs increase the collagen breakdown of gingival tissue [70]. Moreover, TNF-α also increases osteoclast activity, which removes bone tissue by releasing mineralized matrix and breaking of organic substances, and so bone resorption occurs [71]. It was also shown that some extracellular enzymes of periopathogens such as proteases, collagenase, and hyaluronidase and chondroitin sulfatase could cause direct damage to connective tissue and bone [72].

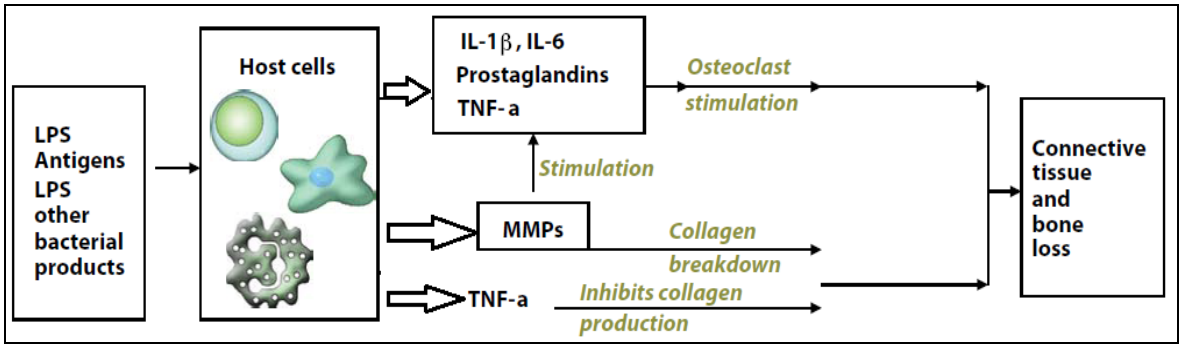

Figure 2.14. Pathogenesis of periodontitis

It can be concluded that periodontal bacteria are required for infective periodontal disease, while individual responses determines disease progression.

2.3.4. Prevention and treatment methods of oral diseases

Inhibition of plaque formation and disruption of existing plaque to clear the tooth surfaces and periodontal pocket from pathogenic microorganisms are promising ways for prevention and treatment of oral diseases [51]. Moreover, control of plaque formation on the tooth surfaces and on supragingival area is important to prevent recolonization of the same areas by oral pathogens [73]. There are several mechanical and chemical methods available against oral diseases.

2.3.4.1. Mechanical methods

The main and the most important mechanical method for prevention of oral diseases is tooth brushing. It has been shown in many studies that adequate daily oral hygiene is sometimes enough to prevent oral diseases [58]. Daily removal of pre-formed biofilms by toothbrushing can easily prevent colonization of pathogenic bacteria and further biofilm development which has started to form after 2 days of last tooth brushing on tooth and supragingival tissues [61]. However, tooth brushes should be renewed every 3 months since they are also colonized by mixed community of biofilms after several months of use and so they are reservoirs for opportunistic pathogens [74].

In the absence of oral hygiene, dental plaque formation and accumulation by pathogenic oral microorganisms including Gram negative anaerobes is the destiny. Continuous plaque formation results in hardened plaque called calculus or tartar which can not be removed by simple tooth brushing [75]. Moreover, as described above, plaque containing Gram negative anaerobes might cause gingivitis and periodontitis which result in formation of periodontal pockets deeper than 3mm that will provide colonization deeper parts of subgingival tissue. Since tooth brushing can not clean of periodontal pocket, mechanical scaling and root planing, shown in Figure 2.15 and sometimes surgery are used to remove dental plaque.

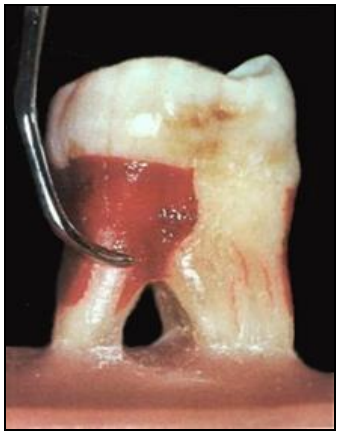

Figure 2.15. Scaling and root planing procedure

Scaling, an important step of periodontal therapy, is the removal of plaque, calculus, microbial flora, stains from the surfaces the tooth by a simple scaler [76].

Root planing, an advanced specific periodontal therapy, includes removal of the infected roughened cementum and surface dentin and microorganism to clean the root surfaces. This process smoothens the root surfaces to prevent adherence of calculus onto it [76].

Decrease in inflammation, probing depth and bleeding of gums after scaling and root planing therapy and following adequate oral hygiene have been showed in many studies [77-79]. However, since in some cases plaque and calculus can not be removed completely,

especially when pocket depths are large, growth of remaining bacteria and recolonization of the infected sites have also been observed [80].

An alternative method which is still in experimental stage is use of photodynamic therapy to kill periodontopathogenic bacteria and to disrupt their biofilm matrix [81]. This system, briefly, includes the destructive action of solar light on microorganisms which are absorbed by specific dyes. However, since it is not efficient to kill plaque residing bacteria and since it has the potential of phototoxic and photoallergic side effects, this current photodynamic therapy may not be a promising treatment method.

2.3.4.2. Chemical methods

According to the microbial etiology of the oral diseases, scientists and dentists have used both local and systemic chemotherapeutic agents in combination with mechanical scalping and root planing methods to remove dental plaque effectively. None of the current chemical agents are effective against subgingival plaque when applied alone, their action sites are limited to supragingival plaque. Therefore, they are preferred to be applied in periodontal pockets after disturbing subgingival plaque by mechanical methods in periodontitis treatment.

- Antibiotics are natural or synthetic organic compounds which able to inhibit or kill specific microorganisms even at low doses. The microbial etiology of dental diseases has favored the use of antibiotics to treat these diseases during more than twenty years. Penicillin, metronidazole, doxycycline, tetracycline, clindamycin and amoxicillin are some of the examples of antibiotics that are commonly used in dentistry [5].

  Systemic delivery and local delivery of the antibiotics are the methods for taking the drug into the body. In systemic delivery, antibiotics enter the periodontal tissues and periodontal pocket via serum so they can reach to the oral sites where mechanical cleaning methods can not reach [5]. However, these systemic antibiotics have potential to increase resistance of periodontal bacteria. For instance, amoxicillin resistance was observed in some periodontal pathogens including *Fusobacterium* and *Prevotella* species [6]. In another study, increased resistance of periodontal

pathogens to three commercial antibiotics were shown as that before the administration of the drugs, the 6 per cent of bacteria exhibited resistance to doxycyclin, 0.5 per cent to amoxicillin and 50 per cent to metronidazole. After two weeks, this numbers increased to 35, 25 and 80 percentages, respectively [82]. Moreover, since biofilms have some mechanisms (it will be discussed below) to be protected from drugs, much higher concentrations of drugs are necessary to kill biofilms than that required to kill planktonic bacteria. Therefore, the risk of development of antibiotic resistant strains increases due to excessive use of antibiotics. Uptake of antibiotics by systemic way has also some adverse effects such as gastrointestinal disturbances, headache, dizziness, hypersensitivity, etc [81].

In local delivery, on the other hand, chemotherapeutic agent is placed directly into the infection site (e.g. periodontal pocket) where the content of the drug is released in a controlled manner [83]. This kind of delivery system may eliminate the drawbacks of systemic antibiotics such as development of antibiotic resistance and gastrointestinal disturbances. However, which kind of polymer is better for delivery of drugs is still a question mark. Natural polymers are biodegradable, but they have limited half-life, their composition is very complex and they may cause immunogenicity. More durable polymers, on the other hand, are non-biodegradable so they have to be removed from the body after complete dug release since they may cause inflammation and irritation at the delivery site [83]. Moreover, using these local delivery systems needs high costs.

- Fluoride is a chemical found in the nature and also be synthesized chemically. Fluoride is also present naturally in low concentrations in drinking water, sea water and foods. The anti-cariogenic effect of fluoride was shown in several studies [84, 85]. Therefore, tap water is fluoridated in USA and UK, toothpastes and mouthwashes containing fluoride are sold and used worldwide, fluoride varnishes having longer duration of action are used, consuming of fluoride containing foods such as fish and tea is recommended as strategies against dental caries [51].

However, there are also many criticisms against use of fluoride for human health. The opponents of fluoride advocate that fluoride is a toxic chemical and it should not be used in mouthwashes/toothpaste and drinking water must not be fluoridated in USA. There is even an association called Fluoride Action Network working for stopping fluoridation [86]. The level of fluoride that is taken daily into the body is very important. It has been estimated that an adult ingests 0.042 mg/kg of fluoride while a child ingests 0.127 mg/kg fluoride daily from drinking water, tooth brushing and diet [4]. Moreover, the same study listed the side effects of intake of increased levels of fluoride as dental and skeletal fluorosis, immunotoxicity, carcinogenicity, genotoxicity, reprotoxicity, renal toxicity and gastrointestinal tract toxicity. By the use of fluoride in mouthwashes and toothpastes, by drinking of fluorinated water and also tea prepared from this water and by consuming fluoride containing foods, reaching the critical level of daily intake of fluoride should not be hard.

- Sodium hypochlorite (NaOCl), the household bleach, has been used as disinfectant for more than 100 years and it was incorporated into oral medicine when Hecker was used it to treat periodontal disease in 1913 [5]. It has many advantages as antimicrobial agent; it has wide spectrum antimicrobial and rapid bactericidal activities, at low concentrations it is not toxic to human cells or cause any color change on tooth [5]. The antibacterial activity of NaOCl was shown to be due to the irreversible oxidation of sulfhydryl groups of essential enzymes of bacteria, disruption of metabolic functions of bacteria and deleterious effects on DNA [87].

  While 0.05 to 0.1 per cent sodium hypochlorite may be used for oral irrigation, 150-600 folds concentrated sodium hypochlorite was shown to be effective on biofilms [88]. Since, sodium hypochlorite causes irritation of mucous membranes at such high concentrations [5], individual use of this disinfectant is not an efficient and possible treatment against dental plaque related oral diseases.

- Povidone-iodine consists of a povidone (polyvinylpyrrolidone) polymer that entraps iodine and releases it in small amounts at a defined rate. Since elemental iodine has been shown to be effective against several microorganisms, and since povidone-

idonide does not irritate mucosa, discolorize teeth and tongue and cause emergence of resistant microorganisms, commercial moutrinses containing povidone-iodine has been sold in the market. It was showed that 10 per cent povidone-iodine could disrupt *in vitro* biofilms [89]. However, commercially available 1 per cent povidone iodine mouth washes did not have significant plaque inhibitory activity [90]. In some cases, commercial povidone-iodine mouth wash (Betadine) was applied after disrupting the plaque with mechanical scaling and root planing to increase its penetration into the biofilms [91]. The most important side effect of this agent, on the other hand, is the risk of development of hyperthyroidism due to the excessive incorporation of iodine in the thyroid gland [5].

- Chlorhexidine is a diphenyl compound which is widely used as antiseptic agent in several hand washing and oral products. It is highly effective against Gram positive and Gram negative (e.g. periodontal pathogens) bacteria and also yeasts due to its cationic nature [87]. It was found that chlorhexidine was bactericidal at high concentrations while bacteriostatic at low concentrations [92]. The mechanism of chlorhexidine by which it affects the bacterial cell have been widely studied; damage to LPS layer and cytoplasmic membrane and so the leakage of the cell constituents cause death of bacteria[87].

  The significant effect of chlorhexidine on plaque and gingivitis were detected and this effect was found to be related to the reduction of pellicle formation and attachment of bacteria on tooth surface [93]. Therefore, chlorhexidine has been used as active ingredient of two medicaments that are accepted by American Dental Association (ADA).

  A commercial 0.12% chlorhexidine gluconate mouthrinse was tested on gingivitis patients during six month [7]. It was observed that chlorhexidine group had significantly less gingivitis (31% reduction), gingival bleeding (39% reduction), and plaque (49% reduction) compared to the placebo group. However, the side effects of chlorhexidine including staining of teeth, disturbances in taste sensing and increased calculus accumulation, were also detected in that study [7].

- Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a bisphenol antimicrobial agent with a broad range of activity. Many contemporary consumer and personal health-care products (deodorants, toothpastes, mouth washes), household items (plastics and textiles) include triclosan [94].

  Triclosan have bacteriostatic activity at concentrations ranging between 0.025 and 100μg/mL, and bactericidal activity at higher concentrations [95]. At bactericidal concentrations triclosan kills the bacteria nonspecifically; i.e., it disrupts bacterial cell wall functions. However, at sub-lethal concentrations triclosan affects some specific targets [94] such as it blocks fatty acid synthesis by specifically inhibiting the NADH-dependent enoyl-acyl carrier protein reductase FabI [95].

  However, it was detected that some Gram negative bacteria such as *E. coli* and *Salmonella* and some dermal, intestinal, and environmental microorganisms could develop resistance mechanisms against triclosan [94]. Other side effects of triclosan include development of allergies in children that higher exposed to triclosan and toxicity of triclosan to aquatic bacteria at levels found in the environment.

- Listerine is a mouthrinse composed 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 firstly formulated as formulated as a surgical antiseptic in 1879. Today, it is used also for oral care since Food and Drug Administration Advisory Panel has recommended Listerine as a safety and effective antiplaque and antigingivitis therapeutic solution.

  The mechanism of action of Listerine was reported as bacterial cell wall destruction, bacterial enzymatic inhibition, and extraction of bacterial lipopolysaccharides [96]. Listerine could disrupt the pre-formed biofilm since it can penetrate into the biofilm and kill biofilm associated bacteria [97, 98]. Moreover, in contrast to chlorhexidine and triclosan, Listerine has very little disadvantages such as unpleasant taste with a burning sensation on the mucosa and little if any staining on tooth. Furthermore, long

term studies of Listerine have showed that it did not induce any resistant strains in plaque and not encourage undesirable oral pathogens [97].

As Table 2.2 presented, Listerine is as much as effective as chlorhexidine and much more effective than triclosan on plaque and gingivitis reduction [99]. Considering the essential oil composition and strong effectiveness of Listerine on plaque and gingivitis development, screening of new plants to find essential oils having efficient anti-biofilm activity and to formulate a new mouth rinse was the main idea of this thesis.

Table 2.2. Results of some published studies showing six-month plaque/gingivitis mouthrinse clinical researches

Active Ingredient	**Market product**	**Per cent of plaque reduction**	**Percent of gingivitis reduction**
0.12 per cent Chlorhexidine	Peridex	50.3-60.9	30.5-42.5
Fixed combination of essential oils (0.064 per cent thymol, 0.092 per cent eucalyptol, 0.06 per cent methyl salicylate, 0.042 per cent menthol)	Listerine	13.8-56.1	22.1-35.9
0.03 per cent Triclosan	Plax	24.0-29.1	16.9-23.0

2.4. ORAL BIOFILMS

There are several different definitions of biofilm; one of which was given by Costeron *et al* [100] "complex communities of microorganisms attached to a surface or interface enclosed in a exopolysaccaharide matrix of microbial and host origin to produce a spatially organized three-dimensional structure". Biofilms have been found to be the reason of almost 80 per cent of microbial infections including urinary tract infections, middle ear infections, catheter infections and dental diseases [101]. Actually today it is known that almost all dental diseases are caused by dental biofilms [3].

Dental plaque is a biofilm community that accumulates through sequential and ordered colonization of multiple oral bacteria [3]. This bacterial community is composed of bacterial microcolonies, an extracellular slime matrix, fluid channels and complex communication systems [102].

Microcolonies are the clusters of sessile thousands of bacteria that form independent micro-niche which is significantly different from surrounding conditions. The extracellular slime matrix is a polymeric substance surrounding the biofilm to protect microcolonies from external factors (host defenses, antibiotics, etc). Through this matrix, fluid channels that provide nutrient and oxygen to internal microcolonies while removing wastes from biofilm are present. Biofilms have a complex communication systems including metabolic communication, genetic exchange, production of inhibitory factors (e.g. bacteriocins) and quorum-sensing , all of which determine the metabolism and composition of the biofilm residents [3, 102].

In 1998, Socransky *et al* [103] grouped bacterial species in dental plaque into six bacterial complexes which are represented in Figure 2.16. As described below, the situation of biofilm (healthy vs diseased) depends on the presence and number of these complexes in the plaque. In previous studies, presence of orange and red complex bacteria has been associated to the periodontitis [104].

2.4.1. Oral biofilm formation

As Figure 2.17 shows formation of oral biofilm is a complex, sequential colonization of various oral bacteria on tooth surface and onto each other. After tooth surface is completely cleaned from bacteria by professional methods, tooth pellicle, a thin film made from saliva proteins, is covered the tooth surface in a very short time. Oral bacteria of blue and yellow complexes are known as "early colonizers" and they can adhere to tooth surface by binding to the proteins of pellicle. For instance, Streptococcus *gordonii*, an early colonizer of dental biofilm, can bind to the proline-rich proteins of the pellicle [3]. In a previous study, it was shown that 50-70 per cent of bacteria colonized on tooth surface just

4-8 hours after tooth cleaning were Streptococcus species (yellow complex), while 15 per cent were Actinomyces (blue complex) and Campylobacter (green complex) species [104].

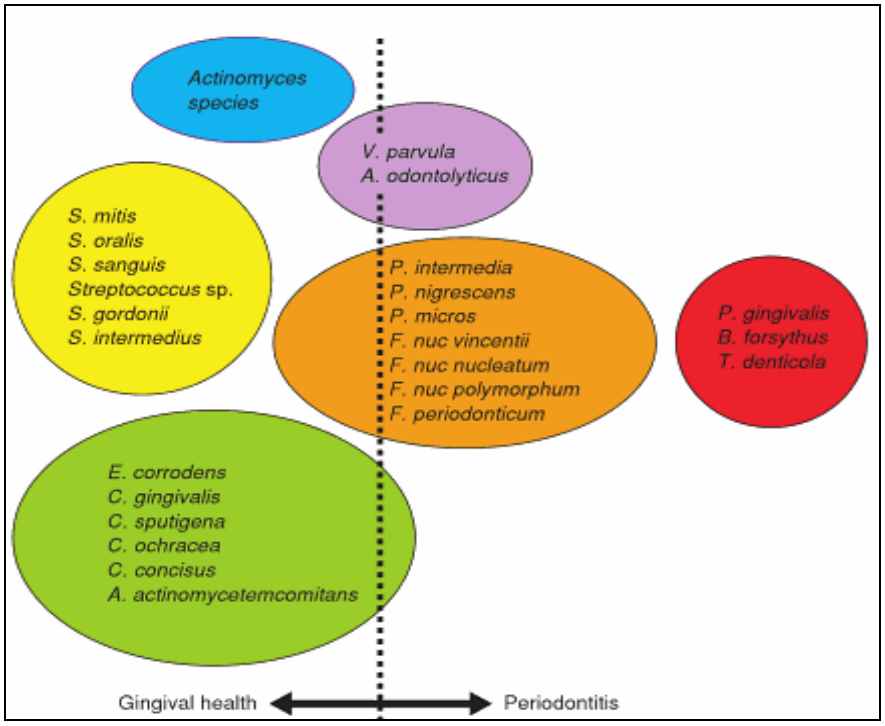

Figure 2.16. Bacterial complexes present in dental plaque [105]

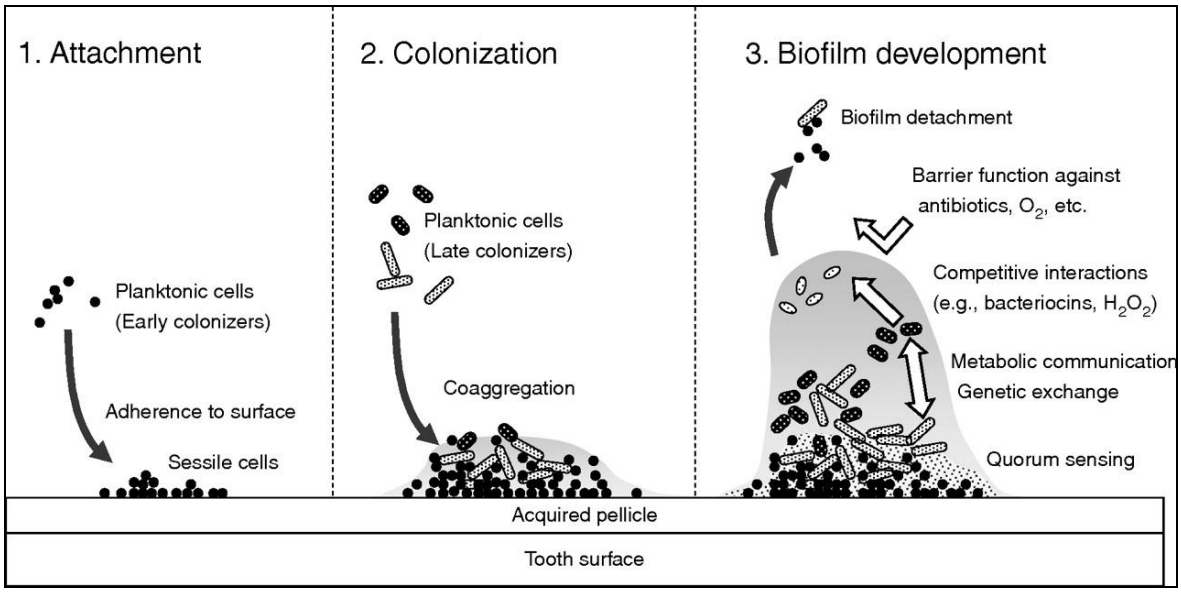

Figure 2.17. Schematic representation of dental biofilm formation on tooth surface [3]

After early colonizers attach to the tooth pellicle, they provide the colonization of other planktonic bacteria that can not directly bind to the tooth surface. These planktonic bacteria bind to the surface of early colonizers through some receptors and these cell-to-cell interactions between bacteria are known as co-aggregation. The bacteria that bind to the early colonizers are called secondary colonizers and they provide attachment surface for other bacteria in development of biofilm. *Actinomyces naeslundii*, for instance, has two fimbriae by one of which it attaches to surface receptors of early colonizers such as *Streptococcus*, and by other fimbriae it binds to the other planktonic bacteria that can not bind to early colonizers [106]. However, there are also some late colonizers that can directly bind to early colonizer bacteria; such as *P. gingivalis*, a member of red complex that is associated to periodontitis, has long fimbriae by which it can bind to *S. oralis* surface. Most of the late colonizers, on the other hand, need a bridge bacterium to bind to the biofilm matrix. *Fusobacterium nucleatum* provides bridging streptocooci and obligate anaerobes [3] by specific surface interactions. Moreover, *F. nucleatum* supports the growth of anaerobic bacteria (e.g. *P. gingivalis*) in an oxygenated environment by reducing the oxygen concentration due to its metabolism. Binding of late colonizers forms the biofilm matrix, shown in Figure 2.18, but biofilm continues to mature as bacterial density increases in the biofilm and also some bacteria can deattach from biofilm during maturation to disperse other oral sites.

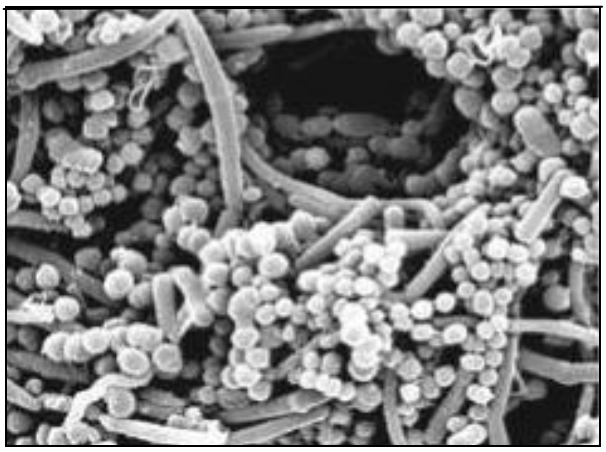

Figure 2.18. Scanning electron micrograph of mature dental plaque [104]

In addition to cell-to-cell interactions as represented in Figure 2.19, metabolic interactions in Figure 2.20 and quorum sensing are also important for biofilm formation and development. Metabolic communication occurs through use of a product of metabolism of a bacteria species as nutrient by another different species. For instance, *Veillonella* species produce Vitamin K_2, which is required for the growth of *Prevotella* and *Porphyromonas* species [3].

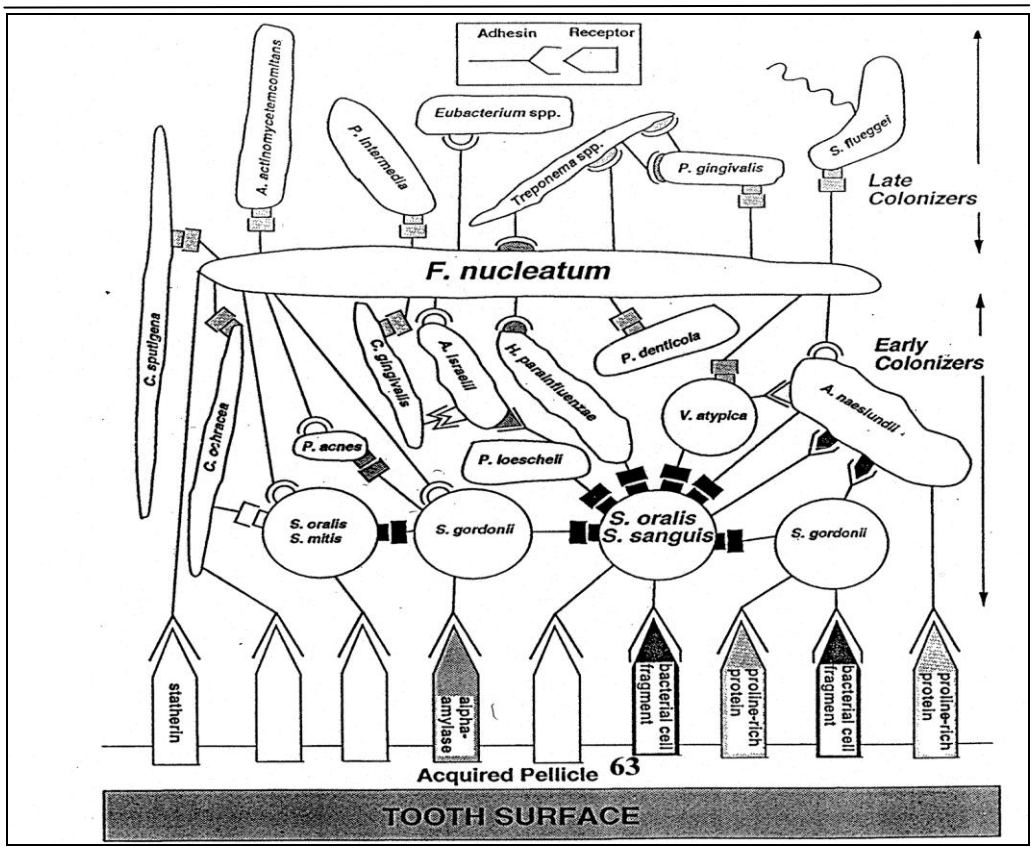

Figure 2.19. Cell-to-cell interactions in mature oral biofilm [107]

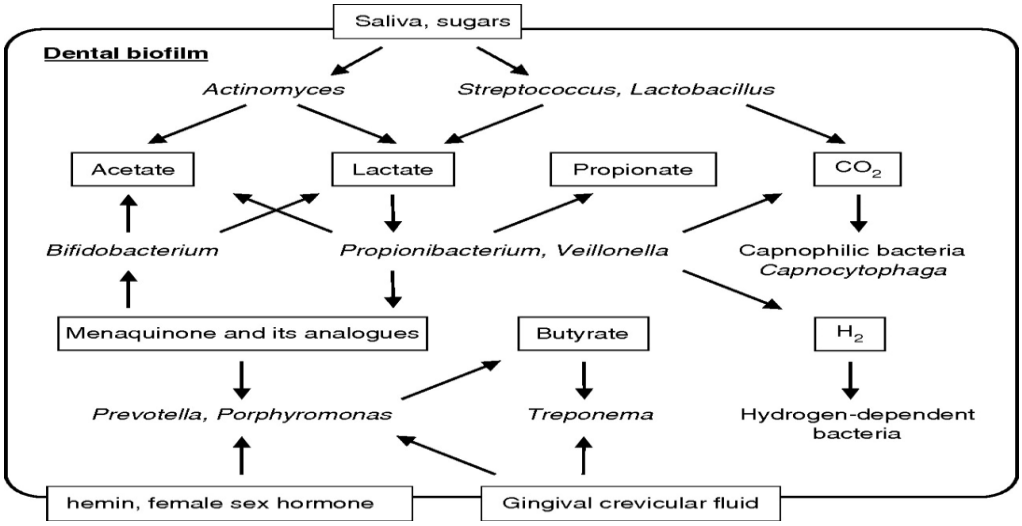

Figure 2.20. Schematic view of metabolic interactions among oral bacteria [3]

Quorum sensing, gene regulation in response to cell density, is another mechanism that affects biofilm formation, as well as virulence factors and acid tolerance [3]. Gram positive oral bacteria (*S. mutans*, *S. gordonii* and *S. intermedius*) produce competence signaling peptides (CSP) and autoinducer -2 (AL-2) molecules while Gram negative ones (*P. gingivalis*, *A. actinomycetemcomitans*) produce AL-2 whose functions in biofilm formation were shown in several studies [108, 109, 110].

2.4.2. Biofilm of Healthy Oral Cavity

Healthy gingivae contains a very simple supragingival plaque composition with 1–20 layers of bacteria dominated by Gram positive cocci (*Streptococcus* species), Gram positive rods and filaments (*Actinomyces* species) and very few Gram negative cocci (*Veillonella parvula*; *Neisseria* species). According to "ecological plaque hypothesis" suggested by Marsh [111], the presence of some protective bacteria on the tooth surface may protect the tooth surfaces from binding of pathogens. These protective bacteria belong to yellow complex that were shown to be associated with pockets < 3 mm [103]. *Streptococcus sanguinis*, for instance, is one of the protective bacteria and its effect on prevention of tooth decay was shown in previous studies [112]. If this bacteria is early colonized on tooth surface, it decreases the colonization of decay forming *S. mutans* bacteria on the tooth surface [112]. Moreover, antagonistic effect of *S. sanguinis* was also

detected against periodontal pathogens such as *T. forsythia* and *A.actinomycetemcomitans* [113].

2.4.3. Biofilm of Diseased Oral cavity

2.4.3.1. Dental Caries

Cariogenic bacteria (*S. mutans* and *S. sobrinus*) may be found naturally in dental plaque, but since pH on the tooth surface is neutral with a diet low in carbohydrates and since these bacteria grows slowly at this pH, their numbers remain low in the total plaque community. However, the number of these acid tolerant bacteria increases when pH is decreased due to diet containing high fermentable carbohydrates and enamel demineralization occurs [114].

Although *S. mutans* and *S. sobrinus* were found more frequent in saliva than dental plaque, they need to be incorporated into dental plaque to cause caries.

2.4.3.2. Periodontal Diseases

As Figure 2.19 shows the biofilm causing periodontal diseases is very complex and requires sequential colonization of bacteria; from primary colonizers to late colonizers. For instance, development of gingivitis and periodontitis occurs when an organized dental plaque composed of several cell layers (100-300) is formed. These diseases associated plaques contain less numbers of Gram positive cocci, rods and filaments as early colonizers, but much more late colonizer Gram negative anaerobic bacteria such as *F. nucleatum, Prevotella intermedia, Prevotella nigrescens* (orange complex), *A. actinomycetemcomitans* (green complex), *P. gingivalis, T. denticola* (i.e. Spirochetes) and *T. forsythia* (red complex). As the following Figure 2.21 demonstrates red complex species *T. denticola* (Spirochetes) and *P. gingivalis* were more frequently detected in gingivitis and periodontitis than in health [115].

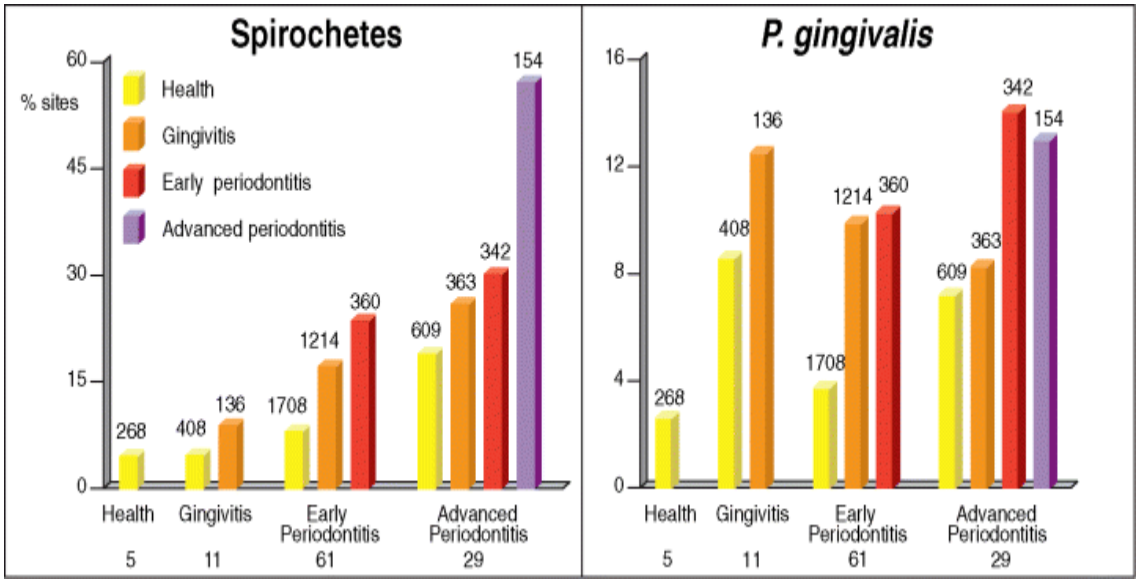

Figure 2.21. Prevalence of Spirochetes and *P. gingivalis* in health and periodontal diseases [115]

2.4.4. Why do bacteria form biofilms?

Once bacteria is cooperated into biofilm, they exhibited different behavioral patterns which provide them mainly two advantages [116, 117]:

a) When bacteria exist within biofilm matrix, they expose to increased rates of genetic exchange among other biofilm residents. This may result in evolution of new pathogens due to transfer of genes responsible for virulence factors, antibiotic resistance, etc.

b) Biofilm-associated bacteria are more resistant to external factors (antibiotics, detergents, UV exposure, dehydration, salinity, phagocytosis) than their planktonic counterparts do. The resistance of biofilm bacteria may be explained by three mechanisms; firstly, the extracellular polymeric substance (EPS) layer act as a barrier and it can neutralize reactive or charged antimicrobial agents or bind them and prevent their actions. Moreover, these agents may be diluted to sublethal concentrations as passing through the EPS layer, so they can not kill all bacteria in the biofilm. Secondly, the limited nutrients in the biofilm causes slow growing or stationary mode of bacteria which provides resistance to antibiotics (e.g. β-lactams)

that are effective only against rapidly dividing bacteria. It has been shown that the concentration of antibiotic to kill biofilm associated bacteria is more than 250 times that required to kill planktonic cells [118]. Lastly, the resistance of biofilm bacteria against antimicrobial agents may due to the presence of a subpopulation of resistant phenotypes in the biofilm. For instance presence of small numbers of bacteria having β-lactamase enzymes to degrade β-lactams can increases the resistance of the biofilm [3].

2.5. TRADITIONAL ASIAN HERBAL PLANTS

By combining knowledge, skills, theories, beliefs, and experiences specific to different cultures, traditional medicine has been used for maintenance of health and for prevention and treatment of diseases.

Several herbs endemic to the Asian countries, especially to China and Japan, have been widely used in medicine in Asia, Europe and America. In China, plants have been used to enhance flavor and preserve foods and to heal the sickness for thousands of years. This therapeutic method has then spread to all over the world and increased in popularity which result in a new term called TCM indicating "Traditional Chinese Medicine".

Five edible plants endemic to and cultivated in Asian countries studied in this project are described as below.

2.5.1. Wakame (Undaria pinnatifida)

Undaria pinnatifida, shown in Figure 2.22 and Figure 2.23, a brown algae endemic to Korea, Japan and China, has extended its geographical distribution to the shores of Europe and to New Zealand [119]. It has a very thin blade, flat stipe which forms a midrib running through the middle of the blade, haptera and sporophylls. At maturity, the edges of the stipe widen and form wing-like reproductive structures called sporophylls. The sporophyte generally grows to 1.5-2 m, within a year and shows heteromorphic alternation of generation.

Figure 2.22. *Undaria pinnatifida* brown algae

U. pinnatifida was one of the first seaweeds in the world to be harvested and cultivated, primarily in Korea, China and Japan where it is a commercially important food, *U. pinnatifida* cultivation and marketing In Korea in 2005 was recorded about 286,611 metric tons (wet) and 44,000,000 US $ [120].

Figure 2.23. Dried wakame (Shandong, China) used in this study

The nutritional value of *U. pinnatifida* seaweed was studied in several previous studies [121-123] and it was shown that this seaweed has high ash content, high protein level and amino acid composition which make it an interesting potential source of food proteins. Moreover, this brown algae is a healthy low-fat food because of its low lipid content and high levels of polyunsaturated fatty acids (17.9–52.3 per cent of total fatty acid methyl esters). *Undaria* is also rich in many minerals including macrominerals (17875 mg/100g; Na, K, Ca, Mg) and trace elements (10.17 mg/100 g; Fe, Zn, Mn, Cu).

Furthermore, in the same study it was shown that lower Na/K ratio of *Undaria* (0.81) might be correlated to the anti-hypertensive effect of it since the intake of diets with a high Na/K ratio have been related to the incidence of hypertension.

In addition to its economical and nutritional importance, wakame seaweed has many therapeutic benefits. For instance, a major component of aqueous extract of *Undaria pinnatifida*, galactofucan, was shown to have antiviral activity on herpes simplex virus [124]. Moreover, antihypertensive activity of four dipeptides derived from the hot water extract of wakame on hypertensive rats by oral administration was proven in a previous study of *Suetsuna et al*. [14]. Fucoxanthin obtained from algae could induce apoptosis of human leukemic cancer cells [15] and reduce white adipose tissue weight of obese rats [125].

2.5.2. Japanese teas (*Camellia sinensis*)

Camellia sinensis, an evergreen tree or shrub of the Theaceae family that grows 0.6-1.5 m under cultivation, is native to China, Japan, South and Southeast Asia [126] but today it is also cultivated in other countries that have a tropical environment.

As Figure 2.24 shows the leaves of *C. sinensis* are short stalked, light green, alternate, 5-30 cm in length and about 4 cm in wide while the flowers of *C. sinensis* are yellow-white, 2.5-4 cm in diameter, solitary or in clusters of two or four [126].

Figure 2.24. *Camellia sinensis* a. leaves b. flowers

The first cultivation and enjoyment of green tea recorded in China at 2700 BC and in Japan about 100 BC [126]. Today tea is one of the most popular beverages around the world and green tea accounts for 20 per cent of tea consumption all over the world [127].

Although all Japanese teas come from the evergreen shrub of *Camellia sinensis*, they have different names such as Sencha, Genmaicha, Hojicha, Bancha, Kukicha, etc. due to the different processing methods of tea which result in different tastes, colors and physiological effects [128]. Among several types of Japanese teas, Sencha, Hojicha and Genmaicha were studied in this project.

2.5.2.1 Sencha

Sencha (Figure 2.25), non-fermented tea, is the most popular Japanese green tea with its pleasant, fresh taste which is almost grassy. Sencha differs from other Asian teas in terms of its production process; firstly, Sencha is steamed for between 15–20 seconds to prevent oxidization of the leaves. Then, the leaves are rolled, shaped, and dried. Finally, after drying, the leaves are fried to aid in their preservation and to add flavor [129].

Over 75 per cent of all tea produced gardens in Japan are for Sencha cultivation and Sencha makes up to 90 per cent of the consumption of non-fermented tea in Japan [129].

Figure 2.25. Dried leaves of Sencha (Hibiki-an, Japan) used in this study

Nutritional composition of green tea [130] is made of proteins (15–20% dry weight), aminoacids (1–4% dry weight) such as glutamic acid, tryptophan, glycine etc., carbohydrates (5–7% dry weight) such as cellulose, pectins, glucose, fructose and sucrose; lipids (linoleic and α-linolenic acids); sterols (stigmasterol); vitamins (B, C, E); caffeine minerals and trace elements (5% dry weight).

Use of green tea in medicinal applications in Asia dated back to 4,700 years ago. Several studies have shown that green tea Sencha is rich in polyphenols and catechin (tea tannins) which exhibited antimutagenic activity [131], suppressive effect on liver injury [132], anti-cancer activity [16], antioxidant activity [133] and, antibacterial [134] activity. Moreover, Sencha contains high levels of antioxidant minerals such as Cr (238.6 ng/g), Mn (1069.7 μg/g), Se (92.9 ng/g) and Zn (24.6μg/g) [130]. Significant effect of intake of green tea on reduction of caries formation was observed in *S. mutans* infected rats fed with a diet containing green tea polyphenols[135]. Moreover, a recent study[136] showed that green tea catechins could reduce periodontal breakdown of gingival tissue induced by *P. gingivalis.*

2.5.2.2. Hojicha

Hojicha, shown in Figure 2.26, is a green tea made from large unrolled leaves of tea plant or from Bancha, Sencha or Kukicha. The production process of Hojicha is different than other teas in terms of that it is roasted in a porcelain pot over charcoal [137]. This process replaces the vegetative tones of standard green tea with a toasty, slightly caramel-like flavour and changes the color of tea to a light or reddish brown appearance [138].

The production process of Hojicha decreases the caffeine and catechins content of the tea . Less caffeine provides softer drinking, while less catechin, however, lowers the antioxidant activity of the tea. Therefore, Hojicha would not be a good choice for someone drinking green tea for health benefits [138].

Figure 2.26. Dried leaves of Hojicha (Hibiki-an, Japan) used in this study

Nutritional values of Hojicha leaves (100 g) were reported [139] as follows: protein (18.2 g), lipid (4.8 g), carbohydrate (39.2 g glucose and 18 g fiber), inorganic compounds (2690 mg), vitamins (A, B1, B2, niacin, C), caffeine (1.9 per cent) and catechin (9.5 per cent). A previous study [140] has shown that the roasting process of Hojicha might increase the antimutagenicity by an antimutagen developed during roasting. Decrease in the catechin content of Hojicha was also proven in the same study. Another study, on the other hand, displayed anti-tumor activity of Hojicha against urinary bladder tumors which was less than unfermented green tea due to lower catechin content of Hojicha [139].

2.5.2.3. Genmaicha

Genmaicha, shown in Figure 2.27, is a special Japanese tea which is prepared by blending leaves of green tea with fire-roasted rice [138], which makes the taste of tea as mild, slightly toasty and grainy. This tea sometimes is called as "popcorn" tea because a few grains of rice can pop during roasting and can be seen in the final product.

Figure 2.27. Dried leaves of Genmaicha (Hibiki-an, Japan) used in this study

In terms of nutritional value of Genmaicha, only catechin content of the tea was studied before [141]. It was shown that Genmaicha contained 88 mg total catechin in per gram of tea leaf, which was much lower than that of Sencha and higher than that of Hojicha. This result also shows the reduction activity of roasting on catechin levels of the tea, because Genmaicha is made from unroasted green tea.

2.5.3. Shiitake (Lentinula edodes)

Shiitake, edible mushroom growing usually on oak logs, is endemic to East Asia and it is shown in Figure 2.28 and Figure 2.29. Cultivation of the Shiitake mushroom began in China almost a thousand years ago and then it was introduced into Japan. Today, it also is popular in Europe and in the USA. Worldwide production of Shiitake is now approximately 200.000 tones/year [142].

Figure 2.28. Shiitake mushroom

Nutritional composition of Shiitake is as follows [143, 144]: protein with 2-2.6 per cent of fresh and 25.9 per cent of dry mushroom, amino acids (14 per cent of dry mushroom), minerals such as Ca, Mg, Zn, K, P) and vitamins (B2, C, ergosterol).

Figure 2.29. Dried Shiitake mushroom (Shirakiku, Japan) used in this study

The shiitake mushroom has been used as folk medicine in China and Japan for centuries. Even today it is used for treatment coughs and heart disease in Okinawa since it contains an amino acid that helps reduce cholesterol levels [144]. In addition to these therapeutic effects, flavor components of essential oil of Shiitake was shown to have inhibitory effect on platelet aggregation [145]. Moreover, a polysaccharide, called lentinan, isolated from Shiitake had macrophage boosting activity [144] which result in increased immunity. The Shiitake mushroom had also exhibited anticarcinogenic activity most probably due to the immune booster effect of it [146]. In another study conducted by Hearst *et al* [147] aqueous extract of Shiitake could kill most of the bacterial and half of the fungal species tested. Furthermore, rats feeding with Shiitake fibers had significantly lower total cholesterol and LDL levels compared to rats fed with cellulose powder [17].

2.6. ESSENTIAL OILS

Essential oils are the volatile, natural, organic constituents of aromatic plants and they contribute to both flavor and odor of the plant [8,148]. Essential oils are synthesized

as secondary metabolites by all organs of the plant (flowers, twigs, stems, seeds, fruits, buds, leaves, etc) and stored in secretory cells, epidermic cells or in glandular trichomes [8]. In nature they have an important role in protection of plants against bacteria, virus, fungi infections and also against insects and herbivores by reducing their appetite for the plant [8]. Essential oils may also have a role in the dispersion of plant seeds and pollens by attracting desired insects [8].

2.6.1. Historical and Current Uses of Essential oils

The term *'essential oil'* is thought to be derived from an effective component of a drug *Quinta essentia* which was named by a Swiss reformer of medicine, Paracelsus von Hohenheim, in the 16th century [149]. However, essential oils had been known and used in the East countries much earlier than 16th century. Distillation method for extracting essential oils was first used 20000 years ago by Egypt, India, Persia and was improved by Arabs in 9th century [149]. By the 13th century, essential oils had been extracted from plants by pharmacies and so their use in medicine had started [150]. Use of essential oils in Europe, however, was not widespread until the 16th century but after that century distillation and use of different essential oils such as rosemary, turpentine, lavender, tea tree oil, etc in medicine were started to be documented. Antibacterial activity of vaporous essential oils were shown by De la Croix in 1881 [151]. However, by 19th and 20th century, interest for the use of essential oils against diseases has decreased gradually while their use for flavor and aroma have become more popular [149].

In addition to pharmaceutical and therapeutical applications of essential oils, they have been used in food preservation, perfumes, cosmetics, aromatherapy, phototherapy, spices and nutrition for many years [152]. Today, approximately 3000 essential oils are known and 300 of them are commercially available in European Union as in food (as flavourings), in perfumes (fragrances and aftershaves), in aromatherapy and in pharmaceuticals (due to their functional properties) [153]. Moreover, essential oils and their components are being used in dental root canal sealers, antiseptics and feed supplements for cows and piglets [154,155] due to their antibacterial property. They are also used as insect repellents [156].

2.6.2. Extraction of Essential Oils

There are many extraction methods used for extraction of essential oils from different parts of plants. These methods significantly affect the yield, chemical constituents and composition of essential oils which may in turn affect the biological activity of the extracted oil [157].

2.6.2.1. Steam Distillation

Steam distillation is the most common method for extraction of essential oils. This method uses the extraction ability of the steam (and sometimes pressure) to extract the aromatic components from the plant material.

As Figure 2.30 represents, in steam distillation, plant material from which essential oils are extracted is put in a round flask which is placed on another flask containing boiling water. As steam passes through the plant material, the essential oil molecules are lifted and then they vaporize. Then this essential oil saturated vapor passes through the cooling coil (condenser) where it condenses. Both precipitated water and essential oil are collected in a funnel with bulb shape, but since essential oil is more lighter than water, it moves to the top of the water. Then, by opening the valve, essential oil can be drained off and so separated from water [158,159].

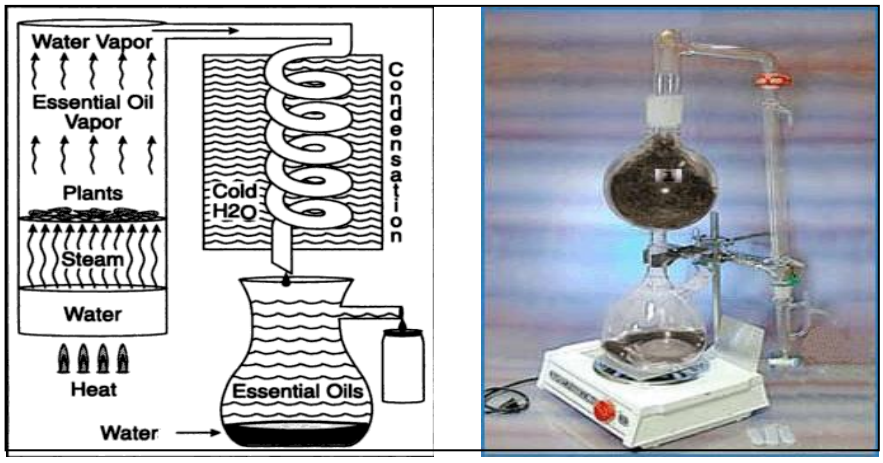

Figure 2.30. Steam distillation of essential oils

2.6.2.2. Hydrodistillation

Hydrodistillation is a traditional method for essential oil extraction from plants. It is very similar to the steam distillation. The only difference is that plant material is put in the flask containing water and both plant and water are carried out to boiling. The vapor mixture (water + oil) produced in the flask is then passes through the condenser and so water and oil are condensed and collected in a bulb funnel. Due to the density differences of oil and water, oil floats to the top and drained off easily [160].

Clevenger type apparatus, represented in Figure 2.31, is the most commonly used apparatus for hydrodistillation of essential oils. It was also used in our study as shown in Figure 2.31b.

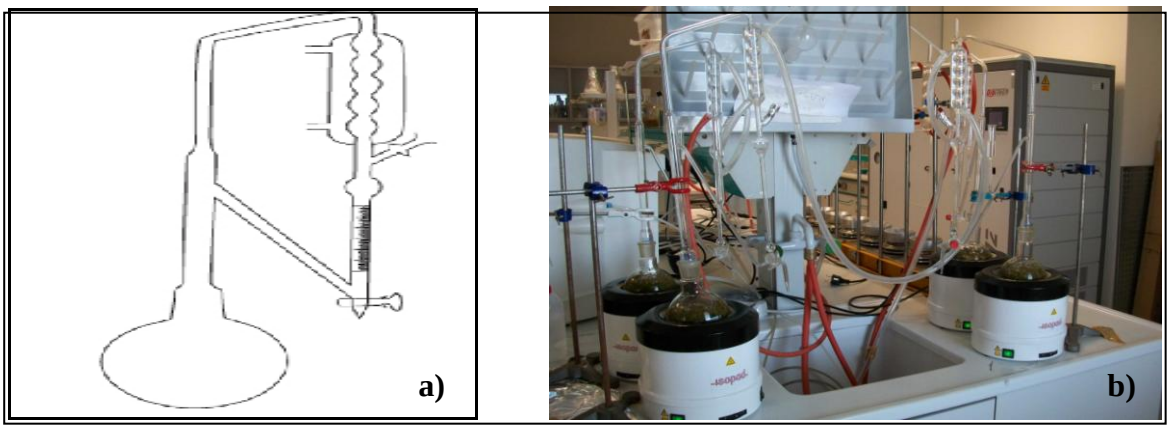

Figure 2.31. Hydrodistillation a. Schematic view of Clevenger type apparatus b. Essential oil extraction system composed of four Clevenger apparatus and heaters was used in this study

2.6.2.3. Cold Pressing (Expression)

This method of essential oil extraction is used primarily for citrus fruits (such as lemon, orange, grapefruit etc) due to their high oil content. Briefly, essential oils found on the skin of the citrus plant are extracted through pressing the citrus peel [159, 161]. Cold pressing has some advantages and disadvantages. For instance, this technique is appropriate for isolation of heat labile oxygen-containing species found in the fruit [157].

However, the isolated essential oils are not pure, they tend to oxidize easily and their storage life is short compared to essential oils extracted by other methods [159].

2.6.2.4. Solvent Extraction

Solvent extraction method is more appropriate for essential oil extraction from some flowers, for instance jasmine and violet, which are destroyed under steam distillation. Briefly, flowers are firstly exposed to hydrocarbon solvents such as petroleum ether and hexane to which penetrate the flowers and dissolve necessary plant materials including the aromatic molecules, waxy matter and pigment. Then, this solvent is separated from the mixture by evaporation at low temperatures under vacuum. After this step either a resin or a concentrated concrete remain at the bottom of the flask. To separate the essential oils from this concrete, alcohol is added and then removed by a final distillation process [158,159]. The major drawback of this method is that extracted essential oil is not pure since residual solvents might remain [162].

2.6.2.5. Supercritical Carbon dioxide Extraction

Since the initial use of the supercritical fluid extraction method for essential oils in the 1980s, popularity of this method increases day by day. Development of this method yields supercritical carbon dioxide (CO2) extraction, a solvent-free method provides little degradation of essential oils during extraction when compared to other extraction techniques [163] and since it does not leave any residue, the extracted essential oil are totally pure [157]. Moreover, this technique enables extraction of essential oils having different compositions from the same plant by changing the temperature and pressure of the extracting fluid CO_2 [157]. The flow diagram illustrating the supercritical fluid extraction process is shown in Figure 2.32.

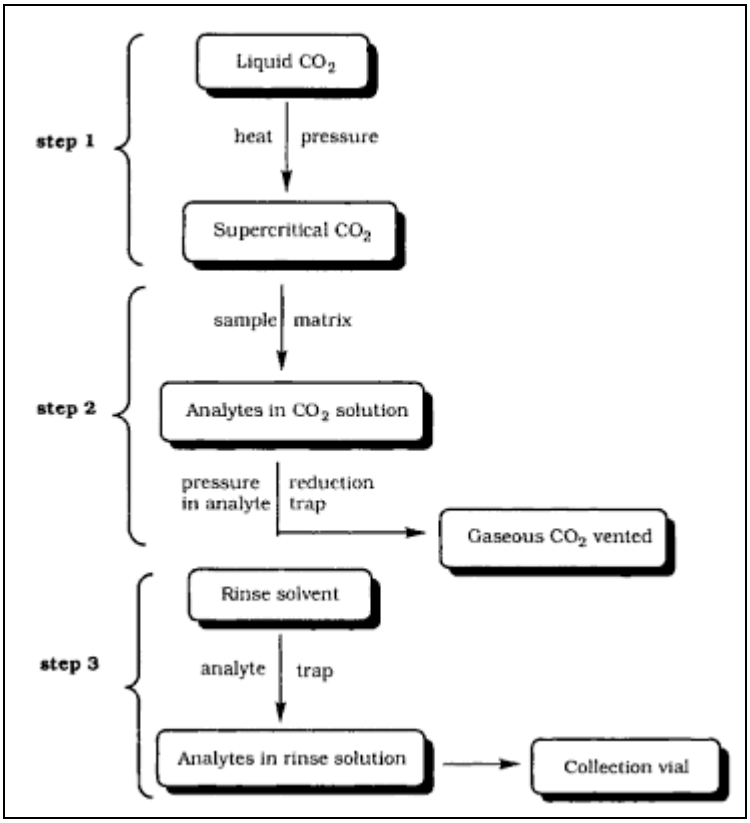

Figure 2.32. Flow diagram of supercritical fluid extraction process

Briefly, pure CO_2 gas is converted into liquid CO_2 under high pressure which has the ability to extract essential oils from the plant material that is exposed to liquid CO_2. Then, this liquid is drained off and depressurized which resulted in removal of CO_2 gas and left of essential oils. Then, essential oils are rinsed with a solvent and collected in a vial [159].

2.6.2.6. Microwave-assisted solvent extraction(MASE)

Microwave-assisted extraction is a modified form of solvent extraction if hydrocarbon solvents used or of hydrodistillation if water is used as solvent. This method was patented in 1990 as Microwave Assisted Process (MAP) and then used for extraction of several kinds of compounds such as essential oils, colors, biophenols and other primary and secondary compounds from plants in very short times of extraction [163]. Briefly, plant material is immersed in a solvent (hexane or water) and heated and irradiated by microwave energy. Plant cells (such as glands storing essential oil) are exposed to high temperature and localized high pressures due to microwave heating which cause increased pressure on the cell walls and result in rupturing of the plant cell and leaking of active

constitutes (such as essential oil) to the surrounding solvent (hexane or water) [164]. After extraction, essential oil is separated and obtained by liquid-liquid distillation [165].

2.6.2.7. Solvent free microwave extraction(SFME)

Solvent free microwave extraction method is a recent patented method, in 2004, specifically essential oil can be extracted without using a hydrocarbon solvent or water under microwave heating [165] distillation at atmospheric pressure. Schematic presentation of SFME is shown in Figure 2.33. Briefly, plant material is placed in a microwave reactor in the absence of solvent or water. The microscopic traces of water in gland cells are heated (internal heating) and evaporated which generates pressure on the cell walls of the gland cells. Finally, cells are ruptured and the essential oils are released, vaporized and then condensed by the cooling system outside the microwave reactor [166].

This technique has many advantages such as use very short extraction time, less solvent consumption and high yields of essential oils. Moreover, oxygen containing constituents of essential oil are extracted without decomposition by thermal and hydrolytic reactions [165]. A previous study [167] showed that essential oil containing more oxygenated compounds due to SFME was more effective against microorganisms than essential oil extracted by hydrodistillation.

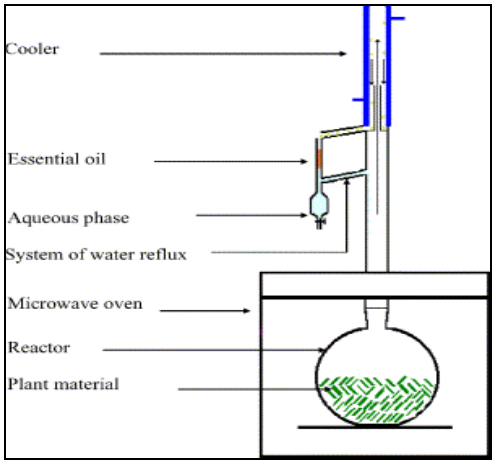

Figure 2.33. Schematic presentation of SFME system [166]

2.6.3. Composition of Essential Oils

Essential oils are chemically very complex mixtures containing 20-60 components in different concentrations [148]. Most of the oils are composed of one to three major components at fairly high concentrations (20-70 per cent) which are responsible for characteristic odor and taste of the plant and biological activity of the essential oil [8]. Essential oils also contain many minor constituents present in trace amounts. The chemical composition of essential oil is characteristic for each plant species and it differs in subspecies or even in harvesting seasons and in geographical sources [148, 168].

Analysis of chemical constituents of essential oils is performed by a gas chromatography (GC) equipped with mass spectrometry (MS) which provides both qualitative and quantitative analysis. GC-MS analysis provides a data in which GC retention times and MS results are shown and identification of the components of essential oil in question is carried out by comparison of this GC-MS data with that of reference standards [148].Essential oils are made of four major components namely terpenes, terpenoids, aromatic constituents and aliphatic constituents all having low molecular weight [8].

2.6.3.1. Terpenes

Terpenes, compounds composed of carbon and hydrogen atoms only, made from the 5-carbon isoprene unit, whose chemical formulation is given in Figure 2.34. All terpenes are the different combinations of several isoprene units.

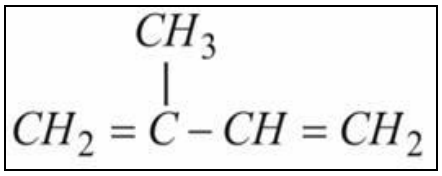

Figure 2.34. Isoprene unit

Essential oils mainly contain two forms of terpenes, namely monoterpenes (C_{10}) and sesquiterpenes (C_{15}). Monoterpenes, shown in Figure 2.35 are composed of two isoprene

units, so they contain 10 carbons. They are the most common major constituent of the essential oils, i.e., they constitute 90 per cent of the oil [8]. They are classified according to their functional groups as shown in Table 2.3.

Table 2.3. Monoterpenes and sesquiterpenes according to their functional groups

	Terpene	
Functional group	**Monoterpenes**	**Sesquiterpenes**
Carbure	acyclic: myrcene, ocimene monocyclic: terpinenes, p-cimene, phellandrenes bicyclic: pinenes, camphene, sabinene	azulene, b-bisabolene, cadinenes, b-caryophyllene, logifolene, curcumenes, elemenes, farnesenes, zingiberene
Alcohol	acyclic: geraniol, linalol, citronellol, lavandulol, nerol monocyclic: menthol, a-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, b-vetinone, turmerones
Ester	acyclic: linalyl acetate or propionate, citronellyl acetate monocyclic: menthyl or a-terpinyl acetate bicyclic: isobornyl acetate	
Ether	1,8-cineole, menthofurane	
Peroxyde	ascaridole	
Phenol	thymol, carvacrol	

Sesquiterpenes, shown in Figure 2.35 are the compound made from assembly of three isoprene units; they contain 15 carbon molecules. Like monoterpenes, sesquiterpenes are also named according to the their functional group, as represented in Table 2.3. Some plants containing sesquiterpenes can be exemplified as lavandin, lavender, orange, lemeon, lemongraa, peppermint, thyme, rosemary and so on [8].

2.6.3.2. Terpenoids

Terpenoids (Figure 2.35) are the terpenes containing oxygen molecule [8]. The antiviral actitvty of terpenoids were shown in several studies [169-171].

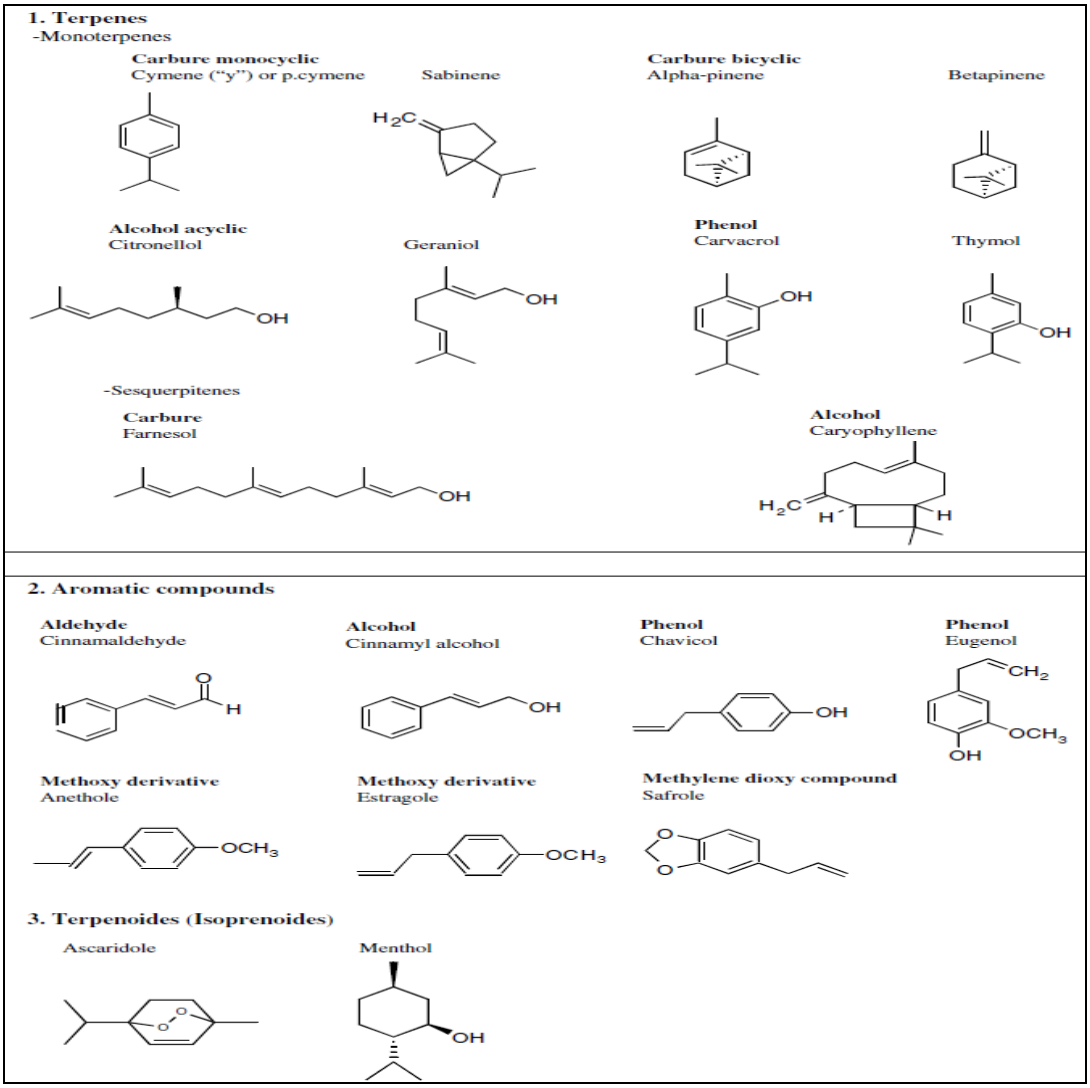

Figure 2.35. Chemical structures of some components of essential oils [8]

2.6.3.3. Aromatic constituents

Aromatic constituents of essential oils, shown in Figure 2.35, are derived from phenylpropane and they are present in plants at lower amounts than terpenes [8]. 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, as shown in Table 2.4.

Table 2.4. Aromatic constituents according to functional groups

Functional group	
Alcohol	Cinnamic alcohol
Aldehyde	cinnamaldehyde
Phenol	chavicol, eugenol
Methoxy derivatives	anethole, elemicine, estragole, methyleugenol
Methylene dioxy compound	apiole, myristicine, safrole

2.6.3.4. Aliphatic constituents

Low molecular weight aliphatic constituents of essential oils are extracted from plants by steam distillation. Examples of these compounds are hydrocarbons, acids (C_3-C_{10}), alcohols, aldehydes, acyclic esters or lactones [148].

Roasted (like the tea samples studied in this project), grilled or torrefied plants have characteristic nitrogenous or sulphured compounds as secondary metabolites [8, 148].

2.6.4. Essential oils from Asian edible seaweed (Wakame), green teas (Sencha, Hojicha, Genmaicha) and mushroom (Shiitake)

Although volatile compounds of several edible herbs cultivated in Asian countries have been widely studied, there are very few studies analyzing essential oil compounds of Wakame, Sencha, Hojicha, Genmaicha and Shiitake.

2.6.4.1. Essential oils of Wakame (Undaria pinnatifida)

The only available study that determined the essential oil components of Wakame (*Undaria pinnatifida*) was that of Kajiwara *et al*., [172] showing that only two compounds , β-ionone and a sesquiterpene alcohol, called epicubenol, were detected in *U. pinnatifida*. Moreover, it was shown that this sesquiterpene alcohol makes approximately 90 % of the volatile compounds of this seaweed. However, a more recent study [173] determined a total of 127 volatile compounds including 4 organic acids, 34 aldehydes, 19 alcohols, 34 ketones, 8 esters, 12 hydrocarbons, 5 sulfur-containing compounds, and 11 more of different nature in *U. pinnatifida*. Since full article is not available online, the detailed composition of essential oil of the seaweed could not be found.

On the other hand, a study [174] detected volatile compounds of another Japanese edible kelp, called Kombu, as 1-iodooctane, nonanal, (E)-2-nonenal, (E,Z)-2,6-nonadienal, 1-octen-3-ol, (E)-2-nonen-1-ol, (E,Z)-2,6-nonadien-1-ol, diacetyl and β-ionone. .

2.6.4.2. Essential oils of Asian green teas

Sencha and its volatile components have been studied more than Hojicha and Genmaicha. No analysis of volatile components of Genmaicha even could be found in the literature. Kumazawa and Masuda [175] showed in their study that steam distillate of green tea Sencha had 36 volatiles and they named 23 of them. The major components of odor fraction were identified as: 4-methoxy-2-methyl-2-butanethiol, (*Z*)-1,5-octadien-3-one, 4-mercapto-4-methyl-2-pentanone, (*E*,*E*)-2,4-decadienal, *β*-damascone, *β*-damascenone, (*Z*)-methyl jasmonate and indole.

In another study [176], major volatile compounds of green tea and roasted tea that showed antioxidant activity were analyzed and it was observed that green tea contained six major components detected in GC-MS at high concentrations; 3,7-dimethyl-1,6-octadien-3-ol (8.04 mg/kg), 3,7-dimethyl-1,6-octadien-3-ol (6.1 mg/kg), 3-hexen-1-ol (6.01 mg/kg), benzyl alcohol (4.67 mg/kg), 1-ethylcyclohexene (4.44 mg/kg), methyl salicylate (3.76 mg/kg). On the other hand, it was observed that major constituents of volatile extracts from roasted green tea were pyrazines (0.17-7.67 mg/kg) which are heterocyclic compounds formed in heat treated foods and beverages like roasted green tea. Moreover, it was known

that the pleasant toasted flavor of roasted green teas is due to the presence of alkylpyrazines [176].

Similar results of presence of pyrazines at higher amounts in volatile fraction of tea were observed in Hojicha which is also prepared by roasting of the green tea. Yamanishi *et al* [177] identified 66 volatile compounds, of which 21 were pyrazines. They also found that furans and pyrroles were presented at higher concentrations than in Sencha.

2.6.4.3. Essential oils of Shiitake (Lentinus edodes)

Data for analysis of volatile components of Shiitake (*Lentinus edodes*) were limited since most of the studies were in Japanese language. In two previous studies, volatile compounds present in essential oil of Shiitake extracted either by solid phase microextraction [178] or solvent extraction [147] were identified as shown in Table 2.5.

Table 2.5. Volatile components of dried Shiitake mushroom identified in previous studies

Chung-May and Ziyang [178]	**Shimada *et al* [147]**
Dimethyl disulfide, l-Octen-3-ol, 3-Octanone, Dimethyl trisulfide, l ,2 ,4-Trithiolane, 1 - (Methylthio) dimethyl disulfide, l ,2,4,5 - Tetrathiane, l ,2,4,6-Tetrathiepane, Lenthionine, Hexathiepane	1,2,4-Trithiolane, 1,2,4,5-Tetrathiane, 1,2,3,5-Tetrathiane, 1,2,4,5,7-Pentathiocane, Lenthionine, Hexathiepane

2.7. BIOLOGICAL ACTIVITIES OF ESENTIAL OILS AND THEIR CONSTITUENTS

It is known that biological actions of essential oils depend on the chemical composition of the essential oil [148]. Both major components (alcoholic, phenolic, terpenic or ketonic compounds) and minor components present in the oil have role in these biological activities.

2.7.1. Antimicrobial Activity

Antibacterial activity of essential oils can be determined by different methods including disc diffusion, agar diffusion, agar dilution, broth dilution, time-kill analysis, survivor curves and scanning electron microscopy [168].

Several studies have shown that essential oils have diverse antibacterial and antifungal activities. For instance, rosemary, oregano, lemongrass, sage, clove and thyme essential oils exhibited antibacterial activity against many food borne pathogens including *E. coli*, *B. cereus*, *S. aureus*, *S. typhimurium* and *L. monocytogenes* [11, 179, 180, 181]. Moreover, antibacterial activity of some essential oil components such as carvacrol, geraniaol, thymol and euganol were also detected against foodborne pathogens [182, 183] .

Essential oils and their components were also effective against several oral bacteria as shown in Table 2.6.

There is no available study testing antimicrobial activity of volatile oil or its constituents of Wakame on any microorganisms. However, effectiveness of essential oils of other algae species against bacteria were shown; in a study conducted by Karabay *et al* [185] volatile oil of a red algae was found to be effective against *S. faecalis*, *S. aureus*, *E. coli and S. typhimurium* while volatile oil of red algae, *Porphyra tenera* [186], effective against both *Bacillus pyocyaneus* and *Staphylococcus aureus*.

Two previous studies [187, 188] shown that essential oils of Sencha had antimicrobial activity on tested microorganism, but since these papers are not available online, which microorganisms were tested could not be known. Furthermore, antibacterial effect of constituents of green tea essential oil were mentioned in a study of Kubo *et al* [189]. Briefly, growth inhibitory effects of β-ionone (on *B. subtilis* and *P. acnes*), α-terpineol (on *B. subtilis, P. acnes, S. aureus and S.mutans*), δ-cadinene (on *P. acnes*), indole (on *B. subtilis*), 1-octanol (on *S.mutans* and *P. acnes*) and cis-jasmine (on on *B. subtilis* and *P. acnes*) were determined.

Table 2.6. Essential oils tested in vitro against oral bacteria

Essential oil/its component	**Species of bacteria**	**References**
Peppermint	*P. intermedia, P. nigrescens, P. gingivalis, A.actinomycetemcomitans, F. nucleatum, Treponema denticola*	[9]
Sage	*P. nigrescens A.actinomycetemcomitans, P. gingivalis, F. nucleatum, Treponema denticola*	[9]
Australian tree oil	*A.actinomycetemcomitans, P. gingivalis, F. nucleatum*	[9]
Manuka	*S. sobrinus, S. mutans, P. gingivalis, A.actinomycetemcomitans, F. nucleatum*	[10]
Tea tree	*S. sobrinus, S. mutans, P. gingivalis, A.actinomycetemcomitans, F. nucleatum, S. bovis, S. gordonii, S. intermedius, S. oralis, Actinomyces spp, Lactobacillus, P. endodontalis, Veillonella spp.*	[10,12]
Eucalyptus	*S. sobrinus, S. mutans, P. gingivalis, A.actinomycetemcomitans, F. nucleatum*	[10]
Lavandula	*S. sobrinus, S. mutans, P. gingivalis, A.actinomycetemcomitans, F. nucleatum*	[10]
Romarinus	*S. sobrinus, S. mutans, P. gingivalis, A.actinomycetemcomitans, F. nucleatum*	[10]
Satureja hotensis L.	*A.actinomycetemcomitans, P. gingivalis, F. nucleatum, P. nigrescens, P. intermedia,*	[184]
Euganol	*P. nigrescens, A.actinomycetemcomitans, P. gingivalis*	[9]
Thymol	*P. nigrescens, A.actinomycetemcomitans, P. gingivalis*	[9]

No study could be found in the literature showing antimicrobial effect of essential oil extracted from Shiitake mushroom. However, lenthionine, one of the major components of Shiitake essential oil, was shown as antimicrobial against several tested microorganisms including *B. subtilis*, *S. aureus*, *E. coli*, *P. vulgaris*, *C. albicans* and *S. ceraevisiae* [190].

2.7.2. Antibiofilm Activity

Different methods are available to determine the effect of essential oils on biofilms such as crystal violet staining, XTT reduction assay and scanning electron microscopy, which are summarized in Table 2.7. Moreover, some researchers have examined the susceptibility of the bacteria incorporated into biofilm against essential oils by scraping the biofilm and inoculating the bacteria extracted from biofilm on agar medium [193].

Table 2.7. Test methods used to measure the antibiofilm activity of essential oils and their constituents

Purpose	Test method	Reference
Screening of inhibitory effect of essential oils on biofilm formation	***Crystal violet staining assay*** (by measuring turbidity, color intensity and absorbance)	[191, 192]
Screening of disruptive effect of essential oils on mature biofilms	***Crystal violet staining assay*** (by measuring turbidity, color intensity and absorbance)	[192]
Determination of effect of essential oils on metabolic activity of bacteria incorporated into biofilm matrix	***XTT reduction assay*** (by measuring color intensity and absorbance)	[191, 192]
Determination of susceptibility of bacteria in mature biofilm against essential oils	***Viable count***	[193]
Observation of physical effects of essential oils on biofilms	***Scanning electron microscopy***	[191, 193]

For an agent to be a biofilm inhibitor, it needs to be effective on biofilm formation at doses lower than its MIC [194]. As Marsh [194] discussed, the biofilm formation inhibitory effect of an agent at higher concentrations than its MIC might due to bactericidal or bacteriostatic effects of the agent. On the other hand, lower concentrations than MIC might inhibit biofilm formation by different modes of action against the production of virulence factors resulting in reduced adherence, membrane disruption, etc, as shown in Table 2.8.

Table 2.8. Modes of action of an antimicrobial agent at supra- and sub-MIC values against biofilm formation

High Concentration (> MIC)	**Low Concentration (< MIC)**
Bactericidal	Reduced adherence
Bacteriostatic	Membrane disruption
Reduced plaque accumulation	Perturbation of ion gradients
	Inhibition of sugar metabolism (transport, glycolysis, glucan formation)
	Inhibition of proteases
	Inhibition of amino acid metabolism
	Reduction of bacterial growth rate

Several essential oils and their constituents were shown to have antibiofilm activity. Tea tree oil, for instance, could both inhibit biofilm formation of *P. aeruginosa* and *C. albicans* at concentrations lower than its MIC and could destroy pre-formed biofilms of *P. aeruginosa* and *S. epidermis* [192]. However, tea tree oil was effective on biofilm formation of *S. epidermis* only at MIC and supra-MIC doses which was due to the reduction of growth in the presence of essential oil.

In another study conducted by Nostro *et al* [13], oregano essential oil and its two major components namely carvacrol and thymol exhibited significant biofilm formation inhibitory activity against *S. epidermis* and *S. aureus* bacteria at their 0.5X MICs. It was also shown that oregano essential oil was less effective on biofilms than individual carvacrol and thymol components which do not work in a synergic way [13].

Efficiency of essential oils were also studied on oral biofilms. Essential oils extracted from *Satureja hortensis L.* plant were tested on biofilm formation of different oral pathogens *including P. gingivalis, A. actinomycetemcomitans, F. nucleatum, T. forsythia, P. intermedia* and *P. nigrescens* [184]. Although this essential oil showed strong antibacterial activity against tested oral bacteria, it was found that biofilm inhibitory activity of oil at its sub-MIC concentrations was limited.

2.7.3. Other biological activities

In addition to the antibacterial and antibiofilm activities, essential oils and their components have several biological activities such as, antioxidant, antimutagenic, anti-inflammatory, insecticidal, etc.

Essential oils extracted from *Melaleuca armillaris* species were exhibited strong antiviral activity (up to 99 per cent) [195]. Similar results were obtained by essential oil of *Salvia fructicosa* and its major component, thujone, against herpes simplex virus-1 [196] (80-95 per cent virucidal activity at low doses of oil). Moreover, other components of *S. fructicosa* such as borneol, bornyl acetate, and isoborneol exhibited considerable antiviral activity.

Antioxidant activity of essential oils was presented in many previous studies. For instance, *M. armillaris* volatile oil was found to be possible free radical suppressor [195]. Moreover, oregano essential oil, rich in thymol and carvacrol was shown to inhibit hydroperoxide formation [197] and had a considerable antioxidant activity [198].

Essential oils extracted from some *Alpinia* species have found that they are moderate anti-inflammatory compounds [148]. Moreover, linalool and linalyl acetate constituents of essential oils have a significant role in anti-inflammatory activity of some essential oils having these two components [199]. *Eucalyptus citriodora* , a plant used in folk medicine in Brazil, is used as anti-inflammatory in some respiratory diseases. In a study [200], anti-inflammatory action of this plant was found to be due to its essential oil composition.

2.8. MECHANISM OF ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF ESSENTIAL OILS

The mechanisms by which essential oils and their constituents cause cidal effects have been widely studied and it has been understood that their antimicrobial effect cannot be due to a specific mechanism, there should be many targets in the bacteria since essential oils are made from several complex constituents [168].

Antimicrobial action of essential oils and their components have been generally thought against membrane structure and function [201]. Figure 2.36 shows the possible targets of essential oils on bacterial cell.

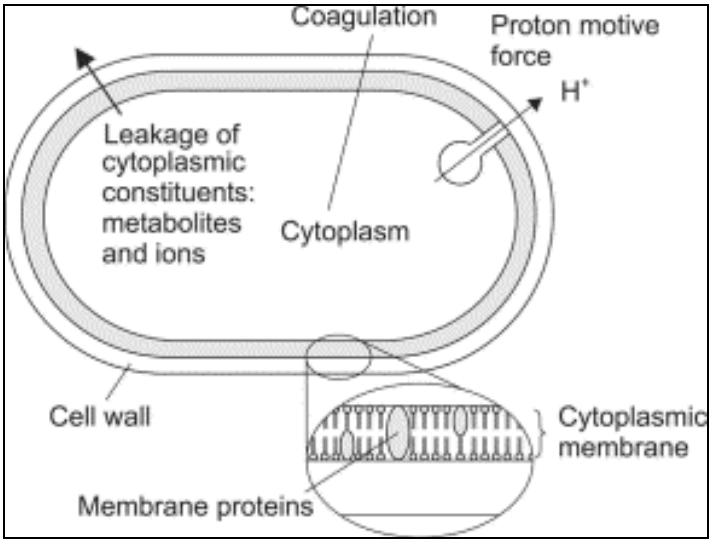

Figure 2.36. Targets or essential oil in the bacteria cell [168]

The lipophilic structure of essential oils is one of the most important characteristic of them by which they can pass through the cell wall and cytoplasmic membrane, disrupt the structures (polysaccharides, fatty acids and phospholipids) present on cell wall and membrane, and so permealize them [202]. Then, this permeabilization of cell wall and membrane might result in loss of ions, and so reduction of membrane potential, collapse of proton pump and finally depletion of ATP and death of the cell [8]. Another possible result of damage of cell wall and membrane by essential oils is leakage of cytoplasmic constituents, metabolites and ions [202]. Bacteria may not die if it can tolerate to a certain

amount of leakage of cell constituents. However, extensive leakage of critical cellular molecules leads to cell death [168]. Essential oils may also cause coagulation of the cytoplasm [203] and damage lipids and proteins of the cell membrane [204].

Mechanism of antimicrobial activity of several essential oils was proven in many studies. For instance, essential oils containing high percentage of phenolic compounds such as carvacrol, euganol, and thymol cause dissolving of cell wall and phospholipid membrane of *B. cereus* which leads to lysis of the bacterial cell [168]. Moreover, oregano essential oil exhibited cidal effects on *S. aureus* and *P. aeruginosa* by causing leakage of the cell components [205]. Another study conducted by Trombetta *et al* [201] showed that the antimicrobial effect of (+)menthol, thymol, and linalyl acetate may be a result of disturbance of the lipid fraction of bacterial plasma membranes which leads to alterations of membrane permeability and in leakage of intracellular materials.

The cytotoxic effects of essential oils were shown to depend on the state of cell growth, i.e., dividing cells are more sensitive to essential oils since oils can penetrate more at the budding sites [8].

Although the mechanisms for antimicrobial activity of essential oils have been studied extensively, how essential oils inhibit or disrupt biofilms is not still clear. Some possible explanations of anti-biofilm activity of essential oils are that essential oils may prevent bacteria from aggregating with Gram-positive pioneer species, they may slow down the rate of bacterial division, and they may extract endotoxins from Gram-negative pathogens [206, 207]. All these mechanisms lead to decrease in bacterial load, slowing plaque maturation, and decrease in plaque mass and pathogenicity [206].

Farnesol, an acyclic sesquiterpene alcohol, is one of the major constituents of essential oils extracted from lemongrass, rose, musk and balsam. Farnesol is the most studied essential oil component that inhibits biofilm formation significantly. Therefore, the mechanisms how farnesol could affect formation of biofilms were examined in many studies. For instance, farnesol was shown to inhibit synthesis of polysaccharides important in biofilm matrix formation by *S. mutans* [208, 209]. Moreover, it was reported that

farnesol could prevent the formation of the fibrin matrix of *S. aureus* [208, 210]. In another study [211], the mechanisms of antibiofilm activity of farnesol against S. mutans biofilms were indicated as disruption of the proton-permeability of the *S. mutans* membrane, and inhibition of acid production and glucan synthesis by bacteria that exist in biofilms matrix.

In addition to farnesol, cinnamaldehyde has been shown to inhibit *E. coli* biofilms by its ability to inhibit both Al-1 and Al-2 receptor systems [212].

3. MATERIALS

3.1. BACTERIAL STRAINS

Five pathogenic microorganisms known to play a role in dental implant failure and some oral diseases such as dental caries, periodontitis and endodontic infections were studied in this project. Strict anaerobes, *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 25586, and facultative anaerobe *Aggregatibacter actinomycetemcomitans* FDC Y4, were obtained as freeze dried cultures from Dr. Philip Bird, Queensland University, Australia. *Streptococcus mutans* was regrown from -80°C culture collection of Yeditepe University. Another strain of *Porphyromonas gingivalis* was isolated from failed implant samples and identified by 16S rDNA analysis.

3.2. GROWTH MEDIA

Brain Heart Broth (Merck, Germany), Fastidious Anaerobe Broth (LabM, UK), Fastidious Anaerobe agar (LabM, UK) and Sheep Blood agar plates (Salubris, Massachusetts, USA) were used for the cultivation of oral bacteria throughout the study and prepared according to manufacturer's instructions.

3.3. CHEMICALS, BUFFERS AND SOLUTIONS

3.3.1 Solutions used in DNA isolation

3.3.1.1. 0.05 M Sodium Hydroxide (NaOH) solution

5 M stock solution of sodium hydroxide (NaOH) was diluted with distilled water until the final concentration was 0.05 M in 10 ml. This NaOH solution was filter sterilized and stored at 4 °C until used.

3.3.1.2. Tris-HCl (pH 7) solution

1.21 g of Tris base was measured and suspended in 10 ml of distilled water. The pH of the solution was adjusted to 7 with concentrated hydrochloric acid (HCl). The solution was filter sterilized and stored at 4 °C until being used.

3.3.2. Buffers and solutions used in PCR

Taq polymerase (5 units/µl), 10 X Taq polymerase buffer, 25 mM $MgCl_2$ solution (Fermentas, Canada), 40mM dNTP mix (Promega, USA) and primers [27F (5' AGAGTTTGATCATGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3')] were purchased and stored at -20 °C until being used.

3.3.3. Buffers and solutions used in agarose gel electrophoresis

3.3.3.1 Tris-Boric acid-EDTA (TBE) buffers

One liter of 5X stock solution of TBE buffer was prepared as follows: 7.3 g of EDTA (Sigma, Germany) was measured and put into 50 ml of distilled water. By adding NaOH, the pH of the solution was adjusted to 8 at which EDTA could only be dissolved completely. Then, 54.0 g of Tris base (Sigma, Germany) and 27.5 g of boric acid (Applichem, Germany) was measured and put in 1000 ml flask. Onto them, completely dissolved EDTA solution was added and the volume of TBE buffer was completed to one liter with distilled water. 1X TBE buffer was used for preparation of agarose solution by diluting 5X stock TBE solution five-fold with distilled water. 0.5X TBE buffer was used in gel tank by making 10-fold dilution of 5X stock TBE solution with distilled water.

3.3.3.2. 1 per cent Agarose solution

To visualize PCR products, agarose gel electrophoresis was applied for which 1 per cent of agarose solution was prepared by dissolving 0.7 g of agarose powder (Invitrogen, Carlsbad,CA) in 70 ml of 1X TBE buffer by heating.

3.3.3.3. Ethidium bromide

Ethidium bromide solution used in agarose gel electrophoresis for visualization of DNA bands under UV light was purchased from Merck (Germany).

3.3.3.4. Loading dye (buffer)

6X loading dye (buffer) (Takara, Japan) was used for preparation of DNA samples for loading on agarose gel. For gel electrophoresis, 6X loading dye was diluted to 1X by mixing 1 µl of dye with 5 µl of DNA sample.

3.3.3.5. DNA marker (ladder)

Commercially available Wide Range DNA ladder (Takara, Japan) containing 16 DNA fragments with different sizes ranging from 10000 bp to 50 bp (Figure 3.1) was used in agarose gel electrophoresis.

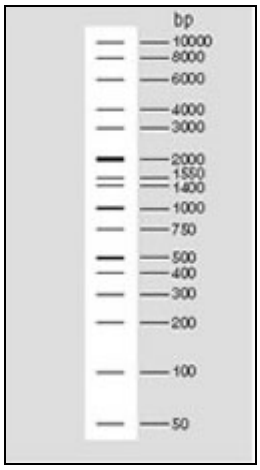

Figure 3.1 Binding patterns of Wide Range DNA ladder (Takara, Japan).

3.3.4. Chemicals for crude lipid extraction from plants

Methanol (Sigma, Germany) was used as extraction solvent for extraction of crude lipids from Asian herbs

3.3.5. Chemicals for fatty acid methyl ester (FAME) preparation

Reagent 1 (150 ml of HPLC grade methanol + 150 ml distilled water + 45 gr of sodium hydroxide pellets), Reagent 2 (325 ml of 6 N Hydrochloric acid + 275 ml of HPLC grade methanol), Reagent 3 (200 ml of HPLC grade methyl tert-butyl ether + 200 ml of HPLC grade hexane) and Reagent 4 (10.8 g of sodium hydroxide + 900 ml of distilled water) were prepared according to the manufacturer's manual and used during FAME preparation

3.3.6. Chemicals for essential oil extraction

Xylene (Sigma, Germany) and anhydrous sodium sulphate powder (Sigma, Germany) were used during essential oil extraction.

3.3.7. Chemicals and solutions for anti-microbial and anti-biofilm tests

3.3.7.1. 1X Phosphate buffered saline (PBS) solution (pH 7.4)

1X PBS solution at pH 7.4 was prepared as follows: 8.18 g of NaCl (Merck, Germany), 0.186 g of KCl (AppliChem, 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 distilled water. After pH of the solution was adjusted to 7.4 and final volume was completed to one liter, the solution was autoclaved.

3.3.7.2. Mc. Farland No.1 Standard solution

To prepare the standard solution, firstly stock solutions of one per cent barium chloride ($BaCl_2$) and one per cent hydrochloric acid (H_2SO_4) were prepared as follows: 10 ml of one per cent $BaCl_2$ stock solution was prepared by dissolving 244 mg of $BaCl_2$ powder (AppliChem, Germany) in 10 ml of distilled water. 100 ml of one per cent H2SO4 stock solution was prepared by taking 1.05 ml of H2SO4 (95-97 per cent) (Sigma, Germany) and adding it onto 99 ml of distilled water.

Mc. Farland No.1 standard solution was then prepared by mixing 0.5 ml of one per cent barium chloride ($BaCl_2$) and 99.5 ml of one per cent sulfuric acid (H_2SO_4). The absorbance of this mixture was then measured at 600 nm. (OD_{600nm} = 0.233)

3.3.7.3. Brain Heart Broth with 0.1 per cent Tween 80

Tween 80 is a nonionic emulsifier which facilitated mixing of immiscible broth medium and essential oil in this study. Brain Heart broth was prepared and sterilized as described above. 50 µl of broth was taken and discarded from 50 ml of BHB medium. 50 µl of Tween 80 (Sigma, Germany) was added in BHB to make the final concentration of Tween 80 0.1 per cent in 50 ml of BHB.

3.3.7.4. Brain Heart Broth with 1 per cent sucrose

Stock solution of 10 per cent sucrose solution was prepared by weighing 20 g of sucrose (BioBasic, Canada) and dissolving it in 200 ml of distilled water.

BHB medium with 1 per cent sucrose was prepared as follows; 50 ml of 10 per cent sucrose solution was added onto 450 ml of BHB while passing the sucrose through 0.25 µm filters.

3.3.7.5. Brain Heart Broth with 0.1 per cent Tween 80 and 1 per cent sucrose

Brain Heart broth with 1 per cent sucrose was prepared as described above. 50 ml of this medium was taken into a new sterile falcon tube and 50 µl of it was replaced with 50 µl of Tween 80. This resulted in BHB medium with 0.1 per cent Tween 80 and 1 per cent sucrose.

3.3.7.6. Methanol

Methanol is a fixative agent used to fix the adhered biofilms on wells of the microplate for quantification of biofilms with crystal violet stain.

3.3.7.7. 0.1 per cent crystal violet stain

1 per cent of stock solution of crystal violet was prepared by dissolving 1 gram of crystal violet powder (Sigma, Germany) in 100 ml of distilled water.

0.1 per cent of crystal violet used in the biofilm quantification protocol was prepared by diluting 5 ml of 1 per cent crystal violet solution in 45 ml of distilled water. Both stock and working solutions of dye were kept away from light and stored at room temperature.

In this study, crystal violet solution was used to stain bacteria attached to the biofilms formed on the walls of the microplate wells. Therefore, color density of dye gave the amount of biofilm formation in the absence/presence of essential oils.

3.3.7.8. Ethanol (95 per cent)

Ethanol (95 per cent) was added into the crystal violet stained wells to leach and solubilize the remaining dye from biofilms after washing the wells with water. 95 per cent of ethanol (50 ml) was prepared by mixing 47.74 ml of absolute ethanol (99.5 per cent) (Tekkim, Turkey) with 2.26 ml of distilled water.

3.3.8. Chemicals and solutions for scanning electron microscopy (SEM)

3.3.8.1 0.15 M Sodium cacodylate buffer

0.15M of sodium cacodylate buffer was prepared by dissolving 4.815 g of sodium cacodylate trihydrate powder (Sigma, Germany) in 150 ml of distilled water. After adjusting the pH of the solution to 7.2, cacodylate buffer was sterilized by autoclaving at 121 °C for 15 minutes. 0.15 M sodium cacodylate buffer was used for preparation of 2.5 per cent of glutaraldehyde solution and for washing the coverslips after glutaraldehyde fixation.

3.3.8.2. Glutaraldehyde (2.5 per cent) solution in sodium cacodylate buffer

Glutaraldehyde, a fixative agent, was used to prepare the biofilms on coverslips for electron microscopy. Stock solution of glutaraldehyde solution (25 per cent) was purchased from Sigma (Germany) and stored at - 20 °C until being used. To prepare 2.5 percent glutaraldehyde solution, 2.5 ml of stock solution was suspended in 22.5 ml of sterile cacodylate buffer. This working solution of glutaraldehyde was filter-sterilized, protected from light and stored at - 20 °C.

3.3.8.3. Ethanol series

Ascending concentrations of ethanol solutions are used for dehydration of the samples prepared for electron microscopy. 30 per cent, 50 per cent, 70 per cent, 80 per cent, 90 per cent, 95 per cent and 100 per cent of ethanol solutions, 50 ml of each, were prepared as follows:

- 30 per cent ethanol : 15 ml of absolute ethanol + 35 ml of sterile distilled water
- 50 per cent ethanol : 25 ml of absolute ethanol + 25 ml of sterile distilled water
- 70 percent ethanol : 35 ml of absolute ethanol + 15 ml of sterile distilled water
- 80 per cent ethanol : 40 ml of absolute ethanol + 10 ml of sterile distilled water
- 90 per cent ethanol : 45 ml of absolute ethanol + 5 ml of sterile distilled water
- 95 per cent ethanol : 47.5 ml of absolute ethanol + 2.5 ml of sterile distilled water
- 100 per cent ethanol : 50 ml of absolute ethanol

3.3.8.4. Hexamethyldisilazane (HMDS)

Commercially available hexamethyldisilazane (Sigma, Germany) was purchased and adequate amounts of 100 per cent of chemical were used for final drying of biofilm samples on coverslips.

3.4. KITS

Purelink PCR Purification kit (Invitrogen, Carlsbad,CA) was used for purification of PCR products, i.e., to remove any remaining primers, dNTPs, enzymes and salts from PCR products. Purification of PCR products were done according to manufacturer's instructions.

3.5. PLANT SAMPLES

Traditional Asian dried herbs namely seaweed Wakame (Shandong, China), and a kind of dried forest mushroom called Shiitake (Shirakiku, Japan) were purchased from local market. Houjicha, Sencha and Genmaicha tea leaves (Hibiki-an, Japan) were obtained from a collaborative Japanese company. All plant samples were stored at room temperature until being used.

3.6. LABORATORY EQUIPMENTS

Some of the laboratory equipments used during this study were anaerobic workstation, benchtop centrifuge, biological safety cabinet, spectrophotometer (microplate reader), autoclave, scanning electron microscope, vortex, turbidimeter, chemical hood, -20 °C and -80 °C refrigerators, nanodrop, automatic thermocyclers, gel electrophoresis, gel visualization systems, heat block, micropipettes, microwave, Soxhlet and Clevenger apparatuses.

3.7. EXTERNAL SERVICES

Sequencing of 16S rDNA samples were carried out in RefGen Company (Ankara, Turkey). Analysis of biofilm samples under scanning electron microscope was carried out in Gazi University.

4. METHODS

4.1. Growth and Identification of Bacteria

Essential oils were tested against oral anaerobes known to play a role in dental implant failure and other oral diseases such as dental caries, periodontitis and endodontic infections. *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 25586 and *Aggregatibacter actinomycetemcomitans* FDC Y4 were obtained as freeze dried cultures from Dr. Philip Bird, Queensland University, Australia. A facultative anaerobe, *Streptococcus mutans* was provided from -80°C culture collection of Yeditepe University. Another strain of *Porphyromonas gingivalis* was isolated from failed implant sample.

Both freeze dried bacteria, and -80 °C stock bacteria were cultivated in either Brain Heart Broth (BHB) (Merck, Germany) or Fastidious Anaerobe Broth (FAB) (LabM, UK) under aseptic conditions. Bacteria were then incubated at 37 °C for 48-72 hrs in an anaerobic workstation (Don Whitley Scientific, UK) containing 10 per cent CO_2, 10 per cent H_2, and 80 per cent N_2 gas mixture. Further viability and purity of the bacteria were checked by inoculating them on sheep blood agar (Salubris, Massachusetts, USA) or Fastidious Anaerobe Agar (LabM, UK) with 5 per cent defibrinated sheep blood. The identity of bacteria of clinical isolate was determined and the identity of ATCC cultures were confirmed by 16S rDNA sequencing analysis.

4.1.1. Maintenance of viability and purity of bacteria

During the subculturing processes, it was determined that commercially ready- sheep blood agar plates provided the best optimum growth of all test anaerobes. Therefore, all anaerobes were subcultured on Sheep blood agar plates using the streaking method under aseptic conditions. Bacteria were incubated in an anaerobic cabinet containing 10 per cent CO_2, 10 per cent H_2, and 80 per cent N_2 gas mixture.

4.1.2. Identification of Anaerobes

4.1.2.1. 16S rDNA sequencing

16S rDNA analysis was applied to identify anaerobes with molecular methods.

- DNA isolation from bacteria: The protocol for bacterial DNA extraction described by Woo *et al* [213] was applied with some modifications. Bacteria were inoculated on sheep blood agar plates were incubated under anaerobic conditions for 18-24 h, and then half a loop full of fresh bacterial culture was suspended in 100 µl of sterile distilled water. Following vortexing and addition of 80 µL of filter sterilized 0.05 M NaOH, tubes were incubated at 60 °C for 45 min in a heating block. Then, 8 µL of Tris-HCl (pH 7) was added and ,the mixture was diluted 100 times with sterile distilled water. The concentration (ng/µL) and purity of the isolated DNA was measured with nanodrop (Implen, Germany).

- PCR amplification of 16S rDNA region of bacteria: 50 µL PCR reaction was prepared for each DNA sample. All the following reagents were added into the PCR tubes while tubes were on ice. Master mix was prepared as follows: 29.7 µl distilled water, 5.0 µl $MgCl_2$ (25mM), 5.0 µl Taq polymerase buffer, 2 µl forward (10µM) and reverse (10 µM) primers, 1.0 µl dNTP (10 mM), 5 µl template DNA (1-50 ng/µl), 0,3 µl Taq polymerase (5units/µl). PCR amplification was carried out with an automated thermocycler (Figure 4.1) under the following conditions for 27f/1492r primer set: 94 °C for 5 minutes as an initial denaturation step, 94 °C for 30 seconds, 55 °C for 45 seconds, 72 °C for 2 minutes as 32 cycles and a final elongation step at 72 °C for 5 minutes.

- Gel electrophoresis: One percent agarose gel was prepared by adding 0.7 g of agarose powder in 70 ml of 1 X TBE (Tris-Boric acid-EDTA) buffer. After dissolving the agarose completely by heating and adding 2.4 µL of ethidium bromide (final concentration 0.5 µg/ml), the agarose solution was poured into a gel tray and the comb was placed. When the gel was solidified at room temperature for 20-30

min, the comb was removed and the gel tray was placed in gel tank which was filled with 0.5 X TBE buffer.

5 µl of each DNA sample was mixed with 1 µl of 6X loading dye and then samples were loaded to the wells of the agarose gel. After loading of 3 µl of DNA ladder to the first well, gel electrophoresis was run at 100 V for 30 minutes. DNA bands of PCR products on the gel were visualized and photographed under UV light with UV-transilluminator (Bio-Rad, US).

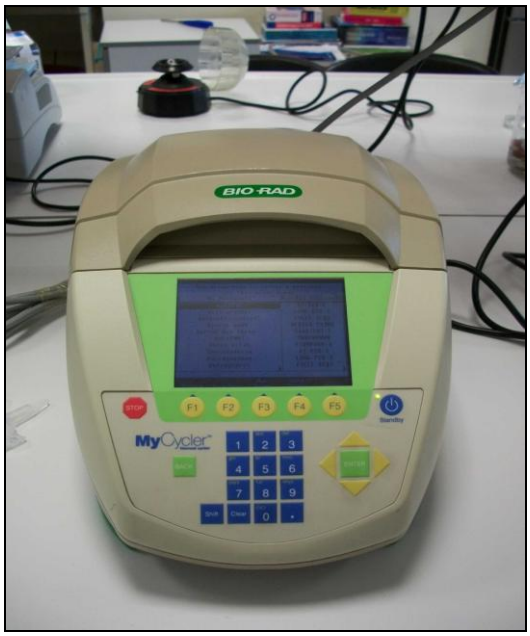

Figure 4.1. Automated thermocycler for amplification of DNA samples

- PCR purification: A commercial kit, named Purelink PCR Purification kit (Invitrogen, Carlsbad, CA) was used to remove any remaining primers, dNTPs, enzymes and salts from PCR products. Purification of PCR products were carried out according to the manufacturer's instructions: Before starting, buffers were prepared according to the manufacturer's instructions. Four volumes of PureLink Binding buffer (~200 µl) was added onto one volume of PCR (~50 µl) product and mixed well. This mixture was then transferred into a spin column with a collection tube supplied with the kit. After centrifugation of the column at room temperature at 10000g for 1 min, the flow through in the collection tube was discarded. Then, 650

µl of Wash Buffer was added into the column and after centrifugation at room temperature at 10000g for 1 min, the flow through was discarded again. The column was centrifuged at maximum speed at room temperature for 2-3 mins to remove any remaining residuals on the column. After centrifugation, the collection tube was discarded together with the flow through and spin column was placed in a clean elution tube supplied with the kit. When 50 µl of elution buffer was added to the center of the column, the column was incubated at room temperature for 1-2 min and after last centrifugation at maximum speed for 2 min, the tubes contained the purified PCR product were stored at -20 °C until being used.

- 16S rDNA sequencing: Approximately 45 µL of purified PCR products with at least 50 ng/ µl concentrations were sent for DNA sequencing (Ref-Gen,Ankara). The sequence results were then analyzed with BLAST DDBJ (DNA Data Bank of Japan) to determine the identity of test bacteria.

4.2. Crude Lipid Extracts of Asian Herbs

As a preliminary study, Asian herbs, namely, Wakame and Nori edible seaweeds, Kombu edible kelp and Houjicha and Kukicha green teas were purchased from the market. Crude lipid extracts of plants were obtained by methanol by Soxhlet apparatus. Total lipids were then converted to fatty acid methyl esters (FAME) and FAME composition of each plant were identified by gas chromatography (GC).

4.2.1. Extraction of crude lipids from Asian herbs by Soxhlet apparatus

All plant samples were ground separately, weighed and 100 g of them transferred in separate cone-shaped filter papers which were then placed in Soxhlet apparatus. Crude lipids of each plant were extracted by 500 ml of methanol in a round bottom flask.
When the color of extract in the column became colorless, extraction was stopped. To remove the solvent of the extract (methanol) the flask was connected to a rotary evaporator (Heidolph, Germany) and methanol was evaporated under reduced pressure at 40 °C until

20 ml of solvent remained. Extracts were aliquoted into two; one for FAME preparation and other for antimicrobial activity test.

4.2.2. Preparation of Fatty acid methyl esters (FAMEs)

For FAME analysis, solvent of methanolic extract was evaporated completely at room temperature. The total fatty acids were saponified, methylated and extracted from 20 mg of dry crude lipid extract by a standard protocol described by MIDI (MIDI, Newark, Del). Briefly, following addition of 1.0 ml of Reagent 1, tubes were vortexed and incubated at 100 °C for 5 min. After incubation, tubes were re-vortexed and put at 25 min more incubation at 100 °C. Tubes were cooled, 2.0 ml of Reagent 2 added and incubation at 80 °C for 10 min was carried out. After cooling the tubes, 1.25 ml of Reagent 3 was added and tubes were placed in a laboratory rotator to mix the tubes gently at 12 rpm for 10 min and to form two phases. The lower phase was discarded carefully and 3.0 ml of Reagent 4 was added to the remaining upper phase. Tubes were again placed in a laboratory rotator and mixed for 5 min. When two distinct phases were formed, the upper phase was collected and transferred into a clean GC vial.

4.2.3. Determination of fatty acid composition

The analyses of FAME samples were conducted via gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector and auto sampler. FAMEs were separated with a fused-silica capillary column (25m by 0.2mm) with cross-linked 5% phenyl methyl silicone. The operating parameters for the study were set and controlled automatically by computer program. Cellular fatty acids having nine to thirty carbon length of each plant species were identified by comparing the commercial databases (Eukary) with the MIS software package. The results were expressed as chromatog with peak retention times. Names and percent of each peak in total FAME were also specified by the software.

4.2.4. Anti-microbial assay using disc diffusion method

The agar disc diffusion method was employed for the determination of antimicrobial activity of crude lipid extract and FAME of Asian herbs. Briefly, overnight culture of bacteria were collected from agar surface and suspended in 1X phosphate buffered saline solution (pH 7.4) and vortexed to obtain a homogenous suspension. The turbidity of the bacterial suspension was adjusted to turbidity of McFarland No.1 standard solution by using turbidimeter (BIOLOG,Hayward CA) to have $3x10^8$ cfu/ml bacteria in the suspension. 100 µl of inoculum was taken and spread on Sheep Blood agar (Salubris, USA) with a sterile cotton swab and bacteria were allowed to dry on agar surface for 5 min. Meanwhile, blank filter paper discs (6 mm in diameter) were individually impregnated with 15 µl of crude lipid extract in methanol and FAME. Discs were placed on the inoculated plates which were then incubated under anaerobic conditions for 24 hr at 37 °C. After incubation, presence of inhibition zones around the paper discs were examined and diameters of inhibition zones were measured in millimeters.

4.3. Essential oils of Asian Herbs

The ineffectiveness of crude lipid extracts and FAMEs of studied Asian herbs against oral pathogens directed the project to use of essential oil extracts of Asian herbs. Two plants having beneficial fatty acids at high percentages from the previous study and three new Asian herbs (green teas Genmaicha and Sencha and an edible mushroom Shiitake) were studied.

4.3.1. Extraction of essential oil of Asian herbs by hydrodistillation:

20 g of finely ground dried leaves were put in 500 ml round bottomed flask placed on a suitable heating device. Then, 250 ml of distilled water was added in the flask and a Clevenger type apparatus was connected to the flask as shown in figure (Figure 4.2)

Distilled water was added to the apparatus from the filling funnel "N" until the water levels became equal at "B" and "H" points which provided pressure balance during

hydrodistillation. Then, 0.5 ml of xylene was added to the apparatus from tube "K" to entrap the extracted oil. When xylene is added, it flows on the water surface and during distillation, the little amount of extracted essential oil will be collected in xylene. The "K" end from where xylene was added was closed tightly with a piece of aluminum foil and distillation carried out for 3 h. After 3 h, essential oil was collected from the column into glass vials. Small amount of anhydrous sodium sulphate was added to the essential oil to absorb residual water that the essential oil contained. After separation of essential oil from dried and aggregated sodium sulphate, oil was put into a clean glass vial and then stored at 4 °C until required.

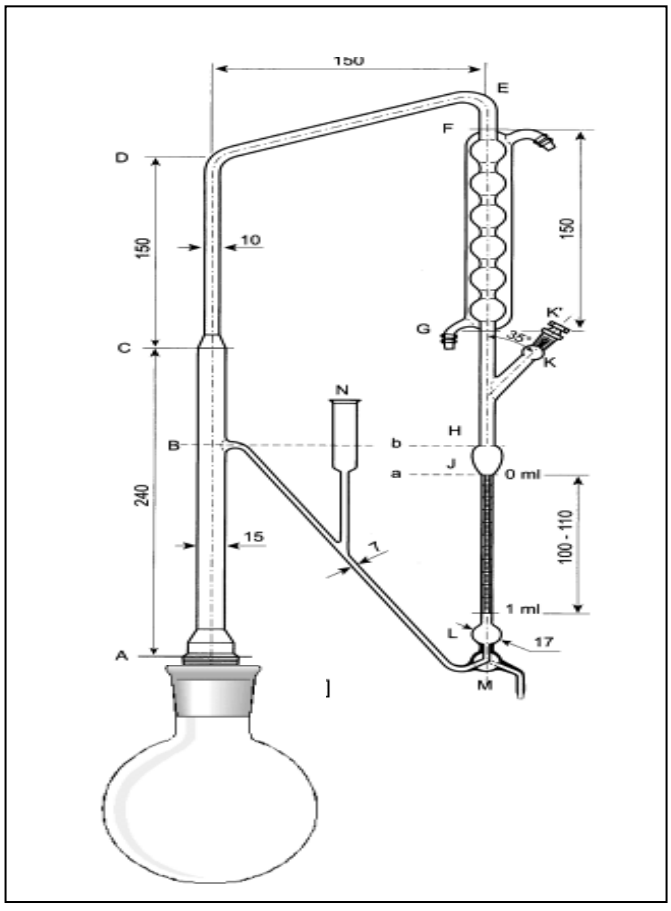

Figure 4.2. Clevenger type apparatus for essential oil extraction from plants. Dimensions in millimeters [124]

4.3.2. Screening of anti-microbial effect of essential oils

The agar disc diffusion method was employed for the determination of antimicrobial activity of the essential oils of Asian herbs. Briefly, tested bacteria were inoculated in Brain Heart Broth (BHB) medium and incubated overnight at 37 °C under anaerobic conditions. After incubation, bacteria were centrifuged at 4000 rpm for 5 min and the supernatant was discarded and pellet was dissolved in 2 ml of 1X phosphate buffered saline solution (pH 7.4) and vortexed to obtain a homogenous suspension. The turbidity of the bacterial suspension was adjusted to turbidity of McFarland No.1 standard solution by using a turbidimetry (BIOLOG, Hayward CA). 100 µl of inoculum (10^8 cfu/ml) was taken and spread on sheep blood agar (Salubris,USA) with a sterile cotton swab and bacteria were allowed to dry on agar surface for 5 min. Meanwhile, blank filter paper discs (6 mm in diameter) were individually impregnated with 15 µl of the undiluted oil aliquots and then discs were placed on the inoculated plates in an orientation shown below (Figure 4.3). The plates were incubated anaerobically for 24 h at 37 °C. After incubation, presence of inhibition zones around the paper discs were examined and diameters of inhibition zones were measured in millimeters.

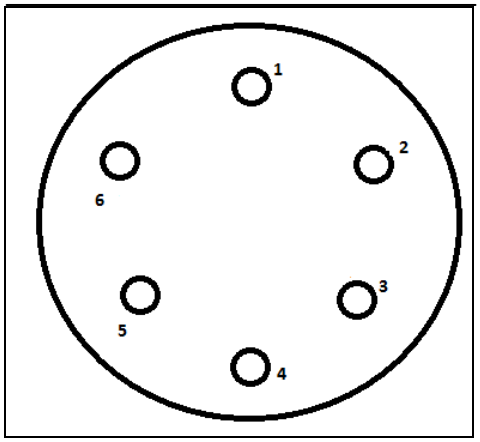

Figure 4.3. The orientation of filter paper discs containing tested essential oils for antimicrobial assay. 1= Wakame, 2= Houjicha, 3= Sencha, 4= Genmaicha, 5= Shiitake, 6= positive control (chlorhexidine gluconate).

4.3.3. Determination of Minimum inhibitory concentrations (MICs) and Minimum Bactericidal concentrations (MBCs) of essential oils

Bacteria (*S. mutans, A. actinomycetemcomitans, F. nucleatum* and isolated strain of *P. gingivalis*) were further tested to determine the concentrations of essential oils at which they exhibited bacteriostatic and bactericidal effects using a broth microdilution technique. ATCC strain of *P.gingivalis* was excluded from study because it could not be grown in BHB medium. Serial two fold dilutions of essential oils (1/4 to 1/512 (v/v)) were prepared in wells of sterile polystyrene flat bottom 96-well microplates with lids (TPP, Switzerland) as shown in the Figure 4.4. Briefly, all selected wells were loaded with 100 µl of BHB medium containing 1 per cent sucrose and 0.1 per cent Tween 80 which was shown to increase the dissolving capacity of the oil in the medium without killing the bacteria. Then, 1000 µl of 1:2 diluted essential oil were put in wells indicated as "E.O 1/4" in the Figure 4.4. Serial two fold dilutions were carried out from these wells until the wells labeled as "E.O 1/512". Excess broth (100 µl) was discarded from wells in which the last dilution was prepared. Then 100 µl of inoculum was added to each well, except blanks.

The inoculum was prepared as follows: 16-18 hours broth culture was collected from broth by centrifugation at 4000 rpm for 5 minutes. The pellet was then resuspended in 2 ml of 1X PBS solution (pH 7.4) and the turbidity of the bacterial suspension was adjusted to the turbidity of Mc Farland No.1 reference solution. Bacteria ($3x10^8$ cfu/ml) were then diluted 10^3 times with BHB containing 1 per cent sucrose to adjust final concentration of bacteria approximately to $3x10^5$ cfu/ml and wells, except blanks, were loaded with this bacterial suspension.

Three wells of negative control (containing inoculum but no essential oil) and three wells of blank of negative control (containing 200 µL of medium) were included on each microplate. A microplate for positive control, chlorhexidine, was also prepared as described above at concentrations ranging from 12 mg/l to 0.09375 mg/l to determine MIC and MBC of chlorhexidine on tested pathogens. The contents of the wells were mixed and the microplates were incubated at 37°C under anaerobic conditions for 24-36 hours.

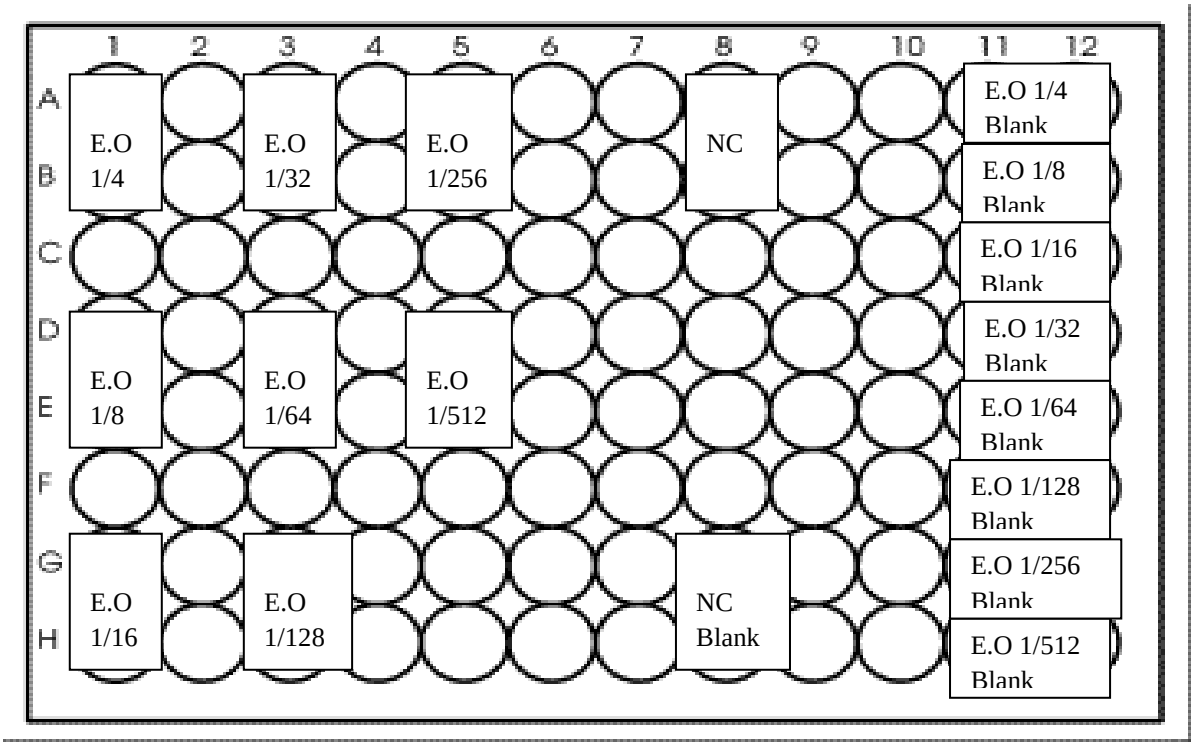

Figure 4.4. Microplate orientation of wells loaded with different concentrations of essential oils

After incubation, the contents of wells were mixed again at moderate speed for 30 seconds with microplate shaker device and the absorbance of wells were measured at 595 nm by microplate reader (ThermoLab Systems, Germany).

Bacterial growth in a well containing diluted essential oil was determined as

Average of $O.D_{595nm}$ *of essential oil - Average of* $O.D_{595nm}$ *of blank* $\geq$ 0,05 (growth)
Average of $O.D_{595nm}$ *of essential oil - Average of* $O.D_{595nm}$ *of blank* $\leq$ 0,05 (no growth)

MIC was defined as the lowest concentration of essential oil at which no growth ($O.D_{595nm} \leq 0.05$) was detected.

To find MBC, 5 µl of inoculum was taken from each well and inoculated as drop on sheep blood agar surface. After incubating the plates at 37°C under anaerobic conditions

for 24-36 h, the growth of bacteria on agar was observed. The lowest concentration at which no bacterial growth was observed was defined as MBC.

4.3.4. Biofilm Inhibition and Biofilm Disruption Assays

4.3.4.1. Biofilm inhibition assay

The effect of essential oils on biofilm formation by oral pathogens was examined using the microdilution method. Same flat bottom 96-well microplate prepared for MIC test was used for biofilm inhibition assay. After taking 5 µl of inoculums from the wells of MIC microplate for MBC test, all remaining medium was decanted and planktonic cells were removed by washing the wells three times with sterile 1X PBS solution (pH 7.4). The biofilms formed on the wells of the microplate were then fixed with 200 µl of methanol for 15 min and after incubation methanol was discarded and microplate was allowed to air dry at room temperature. Formed biofilms were then stained with 150 µl of 0.1 per cent (w/v) Crystal Violet (Sigma, Germany) solution for 15 min and then wells were rinsed thoroughly with water until the control wells became colorless. To quantify biofilm formation 200 µl of 95% ethanol was added into each well and plates were rocked for 30 minutes at room temperature. Absorbance values at 595 nm (OD_{595}) were determined for each well using microplate reader.

The percentage of biofilm formation in the absence/presence of essential oils at different concentrations was calculated by using the following equation:

$$(OD_{595} \text{ of the test well} / OD_{595} \text{ of non-treated control well}) \times 100 \qquad (4.1)$$

4.3.4.2. Biofilm Disruption Assay

To examine the effects of essential oils on the one day developed biofilms, biofilm disruption assay was done. Briefly, wells of polystyrene flat bottom 96-well microtitre plate were loaded with 100 µl of BHB containing one per cent sucrose and 100 µl of inoculum, except blanks. Blank wells were filled only with 200 µl of medium. Two wells of negative control (containing inoculum but no essential oil) were included on each microplate. A microplate for positive control, chlorhexidine, at concentrations ranging

from 12 mg/l to 0.09375 mg/l to determine disruptive effect of chlorhexidine on pre-formed biofilms was also included. The inoculum was prepared in the same way as described above. Final concentration of bacterial cells in each well was adjusted to approximately 1.5 x 10^5 cfu/ml and microplates were then incubated at 37 °C for 24 h under anaerobic conditions. After incubation, the cell growth was examined by measuring the absorbance at 595 nm with microplate reader. Then, the culture supernatant (medium) from each well was discarded and planktonic cells were removed by washing the wells three times with sterile 1X PBS solution at a pH 7.4.

In a separate clean, sterile 96 well microplate, serial two fold dilutions of essential oils (1/2 - 1/256) were prepared in BHB containing one per cent sucrose and 0.1 per cent Tween 80, as described above in the MIC experiment. Then, these diluted oils were transferred to the 1 day biofilm microplate and the microplate was further incubated at 37 °C for 24 h under anaerobic conditions.. After incubation, biofilms were fixed, stained and quantified as described above in the protocol of biofilm inhibition assay.

The percentage of biofilm amount was calculated by using the following equation:

$$(OD_{595} \text{ of the test well} / OD_{595} \text{ of non-treated control well}) \times 100 \qquad (4.2)$$

4.5.4. Scanning Electron Microscopy (SEM)

Examination of biofilm formation and effect of essential oils on formation of new biofilms and disruption of existing biofilms were done by scanning electron microscopy (SEM). The most effective essential oils on corresponding bacterial biofilm were chosen for SEM analysis.

4.3.5.1. Effect of essential oil on biofilm formation

Sterile coverslips were placed in each well of a polystyrene 6-well tissue culture plate (TPP, Switzerland). 2X MIC, 1X MIC and 0.5X MIC dilutions of essential oil were prepared in 3600 µl of BHB containing one per cent sucrose and 0.1 per cent Tween 80. Then 400 µl of bacterial suspension having a final concentration of 10^6 cfu/ml in 4 ml of

medium was added into each well. This inoculum was prepared as follows: The turbidity of 16-18 hours grown culture was adjusted to that of McFarland No.1 reference solution as described above and it was diluted 10 times with BHB medium. When 400 µl of this bacterial suspension was added into 3600 µl of medium containing diluted essential oil, final concentration of bacteria in the well became 10^6 cfu/ml.

Biofilm formation on coverslips in the absence (negative control well) or presence (test well) of essential oil was allowed by incubating the plate at 37 °C for 24 hours under anaerobic conditions.

4.3.5.2. Effect of essential oil on pre-formed biofilms

Sterile coverslips were placed in each well of a polystyrene 6-well tissue culture plate. 3600 µl of BHB containing one per cent sucrose and 0.1 per cent Tween 80 was added into each well. The final concentration of bacterial suspension, prepared as described above, was adjusted to 10^6 cfu/ml in each well by adding 400 µl of inoculum (10^7 cfu/ml) on 3600 µl of medium. Biofilms were formed on coverslips by incubating the tissue culture plate at 37 °C for 24 h under anaerobic conditions.

After incubation, the culture medium was removed carefully from each well and coverslips were washed once with sterile 1X PBS solution (pH 7.4). 2X MIC, 1X MIC and 0.5X MIC dilutions of essential oil prepared in 4 ml of BHB containing one per cent sucrose and 0.1 per cent Tween 80 were added onto coverslips in corresponding wells. The tissue culture plate was then incubated for a further 24 h at 37 °C under anaerobic conditions.

4.3.5.3. Preparing the biofilms on coverslips for SEM analysis

All coverslips on which biofilms were allowed to form in the absence/presence of essential oils for both biofilm formation and biofilm disruption assays were washed once with 1X PBS solution (pH 7.4) after culture medium in the wells was discarded. 2.5 per cent of glutaraldehyde solution prepared in 0.15 M cacodylate buffer was added in the wells to fix the biofilms on coverslips which were then incubated for overnight at room temperature.

After incubation, glutaraldehyde was removed from the wells and coverslips were rinsed three times, 10 min each, with 0.15 M cacodylate buffer. Samples were dehydrated by exposing the coverslips to ascending ethanol series (30 per cent → 50 per cent → 70 per cent → 80 per cent → 90 per cent → 95 per cent → 100 per cent) for 10 min each. Final drying step of coverslips was done by three rinses (10 min each) of coverslips with hexamethyldisilazane (≥ 99 per cent, Sigma, Germany). In final rinse, hexamethyldisilazane was not discarded but was allowed to evaporate slowly at room temperature.

When hexamethyldisilazane was completely evaporated, coverslips were coated with gold for 165 s and biofilm formation on coverslips in the absence/presence of essential oils was examined with scanning electron microscopy (Jeol JSM 6060LV, Japan) done at Gazi University under the supervision of Prof.Dr. Zekiye Suludere. Images were taken at different magnifications ranging from 500X-20000X at 10 or 15 kv.

5. RESULTS and DISCUSSION

5.1. RESULTS

In this study *in vitro* effects of methanol extracts, FAMEs and essential oils extracted from Asian herbs on oral anaerobic pathogens were evaluated. The effects of essential oils on formation and disruption of biofilms formed by these microorganisms were also tested.

5.1.1. Chemical Composition of Fatty Acid Methyl Esters (FAME)

The fatty acid composition of the crude lipid extracts of plants was determined by gas chromatography (GC) as fatty acid methyl esters (FAMEs). Fatty acid composition and content of Asian herbs, showing percentage of each fatty acid among total FAME of the corresponding plant, were summarized in Table 5.1. Common fatty acids were detected in herbs, such as saturated myristic, palmitic and stearic acids and unsaturated oleic and linoleic acids. Palmitic and linoleic fatty acids were present in all five plant samples. Moreover, palmitic acid was predominant with approximately 18 per cent in Kombu edible kelp. This fatty acid was also found to be at high concentrations in Nori (31 per cent), Kukicha (29 per cent) and Hojicha (27 per cent). Green teas Kukicha and Hojicha also contained high amounts of linoleic acid (19 per cent and 14 per cent, respectively). On the other hand, these green teas were shown to be rich in fraction of linolenic acids and oleic acids (18:3 (n-3)/18:1 (n-8)) with 48 per cent and 45 per cent, respectively.

α-parinaric acid, a conjugated polyunsaturated fatty acid was the predominant fatty acid in Wakame (21 per cent) and it was not detected in any other studied plants. While fatty acids, myristic acid, palmitoleic acid, arachidonic acid and eicosapentaenoic acid (EPA) were present only in edible marine plants, they were not detected in green tea samples. Moreover, EPA was shown to be the fatty acid with the highest percentage (approximately 39 per cent) in Nori seaweed.

Table 5.1. Fatty Acid profiles of studied Asian Herbs

Fatty acid	Common name	Per cent in WAKAME	Per cent in KOMBU	Per cent in NORI	Per cent in KUKICHA	Per cent in HOJICHA
14:0	Myristic acid	4.98	11.65	3.37		
16:1 w7c	Palmitoleic acid	0.97	6.91	2.13		
16:0	Palmitic acid	17.22	17.91	30.83	29.42	27.07
16:1 cis 7 DMA (w 9)		1.75				
C14 Dicarboxilic		5.83				
18:1 w9t Alcohol	Oleyl alcohol	1.72				
16:0 3OH	3-hydroxyhexadecanoic acid	0.73		1.95		
18:3 w6c	Calendic acid	1.48	7.20			
18:4 w3c	α- parinaric acid	21.27				
18:2 w6c	Linoleic acid	6.30	8.68	2.99	19.41	13.97
18:3 w3c / 18:1 w8c	Linoleic, oleic acids	16.60			48.31	45.22
18:0	Stearic acid	1.48		4.50		3.80
20:4 w6c	Arachidonic acid	10.68	14.62	2.69		
20:5 w3c	Eicosapentaenoic acid	8.98	9.67	38.92		
Fucosterol		---	---			
18:1 w9c	Oleic acid		16.90	12.17		
24:0	Lignoceric acid			0.44		
Coprostane				---		
Cholesterol-palmitat				---		---
Unknown 25.052					2.86	7.59
C9 Dicarboxylic acid						2.34

Oleic acid, a monounsaturated omega-9 fatty acid that makes up 59-75 per cent of pecan oil, 36-67 per cent of peanut oil and 15-20 per cent of grape seed oil, was detected in Kombu and Nori plants with 17 per cent and 12 per cent, respectively. The saturated fatty acid, lignoceric acid, was present at trace levels in Nori (0.44 per cent).

5.1.2. Extraction of essential oil of Asian herbs by hydrodistillation

The concentrations of essential oils obtained by hydrodistillation of dried plants; Wakame (GS_w), Sencha (GS_s), Hojicha (GS_h), Genmaicha (GS_g) and Shiitake (GS_{sh}), were found as 260 mg/l, 185 mg/l, 180 mg/l, 280 mg/l and 125 mg/l, respectively.

5.1.3. Antimicrobial Activity

5.1.3.1. Disc diffusion assay

In the first part of the study, antimicrobial activity of crude lipid methanol extracts and fatty acid methyl esters of Asian herbs; Wakame (GS_w), Kombu (GS_{ko}), Nori (GS_n), Kukicha (GS_{ku}) and Hojicha (GS_h), was evaluated against oral anaerobes by the disc diffusion method. It was observed that methanol extracts and FAMEs of studied plants did not have antimicrobial activity (data not shown).

In the second part of the study, antimicrobial activity of essential oils of Asian herbs, Wakame (260 mg/l), Sencha (185 mg/l), Hojicha (180 mg/l), Genmaicha (280 mg/l) and Shiitake (125 mg/l), against oral anaerobes were examined by the disc diffusion method. The diameters of growth inhibition zones measured in millimeters are presented in Table 5.2. Results in Figure 5.1 showed that *Aggregatibacter actinomycetemcomitans* was the most sensitive microorganism to essential oils with the largest inhibition zones (18.8 mm) for Genmaicha and Shiitake oils.

Figure 5.1. Antimicrobial test of essential oils on *A. actinomycetemcomitans* on Sheep blood agar by disc diffusion assay (1: Wakame, 2: Sencha, 3: Hojicha, 4: Genmaicha, 5: Shiitake, P.C.: positive control, chlorhexidine gluconate (1200mg/l))

Both ATCC and isolated strains of *Porphyromonas gingivalis* exhibited significant susceptibility, with >10 mm zone of inhibition for all essential oils, as shown in Figure 5.2. With the exception of Shiitake oil (inhibition zone diameter 12.5 mm), the effect of all other essential oils on *Fusobacterium nucleatum* was seen to be weak (<10 mm inhibition diameter), as shown in Figure 5.3.

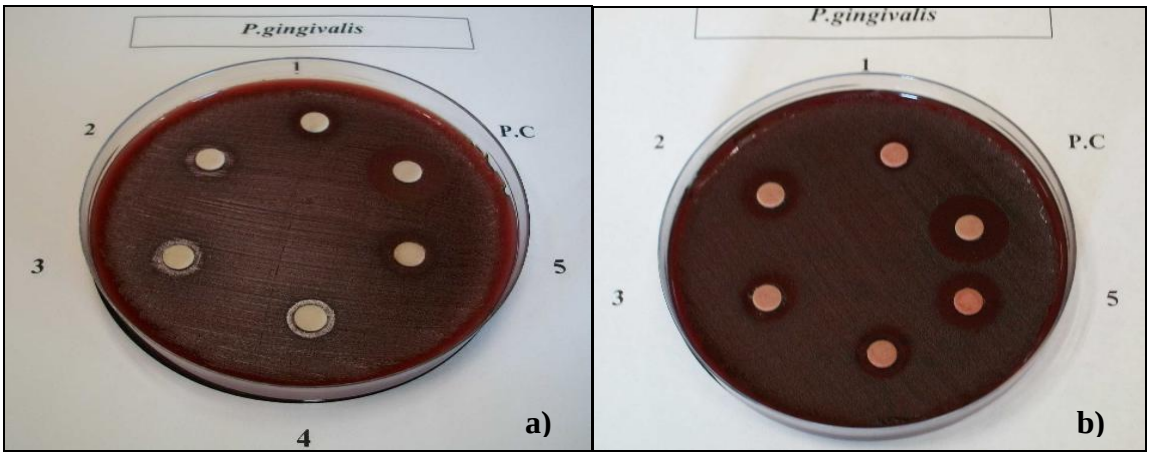

Figure 5.2. Disc diffusion test results of essential oils and positive control against a. *Porphyromonas gingivalis* ATTC strain and b. *Porphyromonas gingivalis* isolated strain on Sheep blood agar (1: Wakame, 2: Sencha, 3: Hojicha, 4: Genmaicha, 5: Shiitake, P.C.: positive control, chlorhexidine gluconate (1200mg/l))

Table 5.2. Diameter of inhibition zone (mm) of essential oils extracted from Asian herbs and positive control (chlorhexidine gluconate) for oral pathogens[a]

	Wakame	**Sencha**	**Houjicha**	**Genmaicha**	**Shiitake**	**Positive control (1200mg/L chlorhexidine gluconate)**
S. mutans	3,3 ± 5,8 mm (BS)	-[b]	-[b]	6,8 ± 5,9 mm (BS)[c]	10,4 ± 1 mm (BS)[c]	15,8 ± 3,4 mm
A. actinomycetemcomitans	16,4 ± 1,7 mm	17,3 ± 2,9 mm	16,3 ± 1,8 mm	18,8 ± 5,6 mm*	18,8 ± 2,5 mm*	19,2 ± 2,8 mm
F. nucleatum	5,9 ± 1,8 mm	6,7 ± 2,3 mm	6,3 ± 2,0 mm	6,7 ± 2,2 mm	12,5 ± 0,2 mm*	25,3 ± 0,1 mm
P. gingivalis (ATCC strain)	11,1 ± 1,6 mm	12,4 ± 1 mm	12,2 ± 0,2 mm	12,4 ± 0,9 mm	13,6 ± 1,2 mm*	21,5 ± 3,2 mm
P. gingivalis(isolated strain)	10,6 ± 0,5 mm	11,8 ± 1,4 mm	11,7 ± 1,1 mm	12 ± 0,7 mm	13,3 ± 1 mm*	21,2 ± 4 mm

[a] Each value is mean ± standard deviation. Each experiment was carried out three or four times

[b] "-" shows no inhibitory effect of tested agent on corresponding bacteria.

[c] "BS" shows the biostatic effect of the agent on corresponding bacteria

* The most effective essential oil on corresponding bacteria

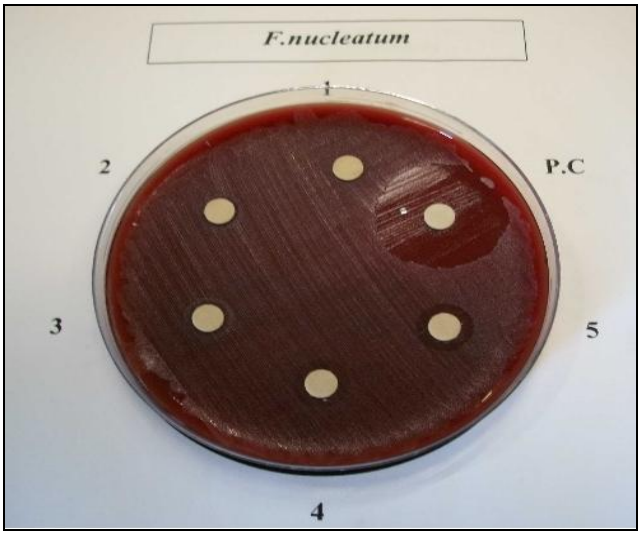

Figure 5. 3. Antimicrobial test result showing inhibition zones of essential oils towards *F. nucleatum* on Sheep blood agar *(*1: Wakame, 2: Sencha, 3: Hojicha, 4: Genmaicha, 5: Shiitake, P.C.: positive control, chlorhexidine gluconate (1200mg/l))

On the other hand, it was found that essential oils of Asian herbs in this study were not able to kill *S. mutans*. The zones seen in the Figure 5.4 around the discs were not inhibition zones, since growth was observed but at a lesser extent on agar plate was examined under stereomicroscope. This kind of growth slowing effect of an agent is known as the biostatic effect.

As shown in Table 5.2, antibacterial activity of the essential oils was similar to that of the positive control, chlorhexidine gluconate (CHX) for *A. actinomycetemcomitans*. For other tested bacteria, the inhibition diameters of the positive control (CHX) were higher than that of essential oils.

Figure 5.4. Disc diffusion result of *Streptococcus mutans* tested against essential oils and positive control (1: Wakame, 2: Sencha, 3: Hojicha, 4: Genmaicha, 5: Shiitake, P.C.: positive control, chlorhexidine gluconate (1200mg/l)). The growth inhibition zone was formed for only positive control. Zones around discs containing essential oils showing biostatic effect (slight growth of bacteria was observed around discs under stereomicroscope).

5.1.3.2. MIC and MBCs of essential oils of Asian herbs

When antimicrobial activity of essential oils on oral pathogens were determined by disc diffusion test, the minimum concentrations of the essential oils and positive control required to inhibit (MIC) and to kill (MBC) planktonic oral bacteria were analyzed by microdilution methods and results were summarized in Table 5.3.

Results in Table 5.3 showed all five essential oils could inhibit the growth of all microorganisms tested at concentration lower than the undiluted oil. When the MICs of essential oils on each bacteria were compared it was observed that Shiitake oil was the most effective essential oil against *S.mutans* (MIC: 1.9 mg/l) and *P. gingivalis* (0.5 mg/l). Moreover, while *A. actinomycetemcomitans* was most sensitive to Genmaicha (1.1 mg/l), Hojicha exhibited the lowest MIC (1.4 mg/l) against *F. nucleatum*.

Similar to disc diffusion results, MIC test results also showed that essential oils were most effective against *A. actinomycetemcomitans* and *P. gingivalis* among tested oral

pathogens since the MIC values for these two bacteria were much lower than that of other bacteria.

Mix of tested bacteria was also studied for MIC, MBC and biofilm tests to determine whether the sensitivity of bacteria changed when they were incorporated as a mixed culture. Although it could not be said MIC results of mix culture were lower or higher than that of individual bacteria, it was concluded that the efficiency of essential oils did not decrease in a mixed culture.

When MIC results of positive control, chlorhexidine gluconate, were examined, it was observed that *S. mutans* was the most sensitive bacteria (MIC 1.5 mg/l), followed by *A. actinomycetemcomitans* (MIC 3 mg/l) and then *F. nucleatum* (MIC 0.5 mg/l), *P. gingivalis* (MIC 0.5 mg/l) and the mixture of bacteria (MIC 0.5 mg/l).

Table 5.3. Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of Essential Oils and positive control for tested oral pathogens

Bacteria	**Essential oil**	**MIC % (w/v)**	**MBC % (w/v)**
Streptococcus mutans	Wakame	25 (65 mg/l)	25 (65 mg/l)
	Sencha	12.5 (23.1 mg/l)	12.5 (23.1 mg/l)
	Houjicha	1.6 (2.8 mg/l)	25 (45 mg/l) ↑
	Genmaicha	6.2 (17.5 mg/l)	12.5 (35 mg/l) ↑
	Shiitake	1.6 (1.9 mg/l)*	12.5 (15.6 mg/l) ↑
	Chlorhexidine gluconate	0.125 (1.5 mg/l)	0.25 (3 mg/l) ↑
Aggregatibacter actinomycetemcomitans	Wakame	3.1(8.1 mg/l)	25 (65 mg/l) ↑
	Sencha	3.1 (5.8 mg/l)	12.5 (23.1 mg/l) ↑
	Houjicha	0.8 (1.4 mg/l)	25 (45 mg/l) ↑
	Genmaicha	0.4 (1.1 mg/l)*	25 (70 mg/l)↑
	Shiitake	3.1 (3.9 mg/l)	25 (31.2 mg/l) ↑
	Chlorhexidine gluconate	0.25 (3 mg/l)	0.5 (6 mg/l) ↑

Table 5.3. Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of Essential Oils and positive control for tested oral pathogens (Continue)

Bacteria	Essential oil	MIC % (w/v)	MBC % (w/v)
Fusobacterium nucleatum	Wakame	6.2 (16.2 mg/l)	50 (130 mg/l) ↑
	Sencha	12.5 (23.1 mg/l)	25 (46.2 mg/l)↑
	Houjicha	0.8 (1.4 mg/l)*	50 (90 mg/l) ↑
	Genmaicha	25 (70 mg/l)	25 (70 mg/l)
	Shiitake	25 (31.2 mg/l)	25 (31.2 mg/l)
	Chlorhexidine gluconate	0.5 (6 mg/l)	0.5 (6 mg/l)
Porphyromonas gingivalis	Wakame	0.4 (1.0 mg/l)	1.6 (4.1 mg/l) ↑
	Sencha	1.6 (2.9 mg/l)	1.6 (2.9 mg/l)
	Houjicha	0.8 (1.4 mg/l)	3.1 (5.6 mg/l) ↑
	Genmaicha	0.4 (1.1 mg/l)	25 (70 mg/l) ↑
	Shiitake	0.4 (0.5 mg/l)*	6.2 (7.8 mg/l) ↑
	Chlorhexidine gluconate	0.5 (6 mg/l)	0.5 (6 mg/l)
Mix bacteria	Wakame	3.1 (8.1 mg/l)	12.5(32.5 mg/l) ↑
	Sencha	1.6 (2.9 mg/l)	12.5 (23.1 mg/l) ↑
	Houjicha	1.6 (2.8 mg/l)	12.5 (22.5 mg/l) ↑
	Genmaicha	1.6 (4.4 mg/l)	12.5 (35 mg/l) ↑
	Shiitake	1.6 (1.9 mg/l)*	12.5 (15.6 mg/l) ↑
	Chlorhexidine gluconate	0.5 (6 mg/l)	0.5 (6 mg/l)

* Essential oils with the lowest MIC on corresponding bacteria

"↑" shows the essential oil having a MBC value higher than its MIC value. This indicates that the concentrations of extracted oil between MIC and MBC values had inhibitory effect on growth of bacteria, rather than killing the bacteria.

Minimum Bactericidal Concentrations (MBCs) of essential oils were determined to understand whether the MIC concentrations were also showing the cidal concentrations of oils. As Table 5.3 showed, most of the essential oils required a higher concentration than the MIC in order to kill the bacteria. Exceptions were generally for essential oils having high MIC values. For instance, Wakame (25 per cent) and Sencha (12.5 per cent) for *S.mutans* and Genmaicha (25 per cent) and Shiitake (25 per cent) for *F.nucleatum*, had same MIC and MBC values against corresponding bacteria. Chlorhexidine gluconate had

generally equal values for MIC and MBC for tested bacteria, except for *S. mutans* and *A. actinomycetemcomitans* whose MBCs were higher than MICs.

5.1.4 Quantitative Biofilm Assays

5.1.4.1. Inhibition of biofilm formation by essential oils

The effect of different concentrations of essential oils (ranging from 25 per cent to 0.2 per cent) on biofilm-forming ability of oral pathogens was tested on polystyrene flat-bottomed microtitre plates and the results were shown in the following tables and graphs. As mentioned before, an agent is assumed to be a specific biofilm inhibitor if it is effective on biofilm formation at doses lower than its MIC [194] since at these doses biofilm formation might be inhibited by a different mode of action rather than killing the bacteria. Such a biofilm inhibitor agent might affect the production of virulence factors resulting in reduced adherence, membrane disruption, etc.

Table 5.4 and Figure 5.5 showed the percentage of biofilm formation by *S. mutans* in the presence of essential oils at different concentrations. Although all essential oils extracted from Asian herbs seemed to be effective (biofilm formation was less than 60 per cent of untreated control) on *S. mutans* biofilms at high concentrations, only Wakame (MIC 25 per cent, 65 mg/l) and Sencha (MIC 12.5 per cent, 23.1 mg/l) showed biofilm inhibitory activity at their sub-MIC values (0.5X MIC). Other extracted oils, on the other hand, were effective on biofilm formation only at their supra-MIC or MBC values. The effect of essential oils on biofilm formation of *A. actinomycetemcomitans* was shown in Table 5.5 and Figure 5.6. Results demonstrated that neither sub-MIC nor supra-MIC concentrations of extracted oils could inhibit the biofilm formation significantly (Biofilm formation percentages were > 60 per cent of untreated control). Inability of oils to inhibit biofilm formation by *A. actinomycetemcomitans* at sub-MIC concentrations might be explained by ineffectiveness of extracted oils on virulence factors required to form biofilms. Interestingly, low concentrations of Sencha (at 0.4 and 0.2 per cent) seemed to increase biofilm formation.

Table 5.6 and Figure 5.7 showed the percentage of biofilm formation by *F. nucleatum* in the presence of essential oils at different concentrations. The results were similar that obtained for *A. actinomycetemcomitans*, i.e, both sub- and supra-MIC values of all essential oils were incapable of inhibiting biofilm formation of *F. nucleatum*. When MIC and MBC results were also considered, it can be observed that minimum concentrations required to inhibit growth or to kill bacteria were high, except MIC of Hojicha. Furthermore, it was observed that the percentage of biofilm formation by *F. nucleatum* was significantly more than 100 per cent at high concentrations of Genmaicha and Shiitake while almost all concentrations of Wakame oil seemed to enhance the biofilm formation of that bacteria.

Table 5.4. Effects of essential oils on formation of biofilms by *S. mutans*

		Biofilm Formation (per cent of untreated control)[a]							
	Essential oil	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**	**0.2%**
Streptococcus mutans	**Wakame**	10.4±8.0*	17.7±6.4[b]	94.6±13.9	101.8±4.7	101.1±0.7	101.0±6.6	103.5±4.3	103.5±6.8
	Sencha	21.6±5.2*	29.0±19.6	25.3±19.4 [b]	92.8±16.9	99.8±4.5	107.0±1.0	106.1±3.5	100.2±10.1
	Houjicha	45.5±9.3*	50.6±19.9	93.8±7.2	100.1±0.8	99.8±0.7	90.0±14.7	92.7±15.5	91.2±14.0
	Genmaicha	7.0±3.3*	10.8±7.3	95.3±10.1	94.3±6.1	96.3±5.0	97.8±6.9	97.5±8.3	101.1±1.3
	Shiitake	6.1±3.7	5.5±1.5*	11.9±7.0	94.1±7.6	100.0±4.3	99.4±3.0	99.4±1.2	101.5±2.8

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm inhibition activity.

[b] Significant reduced biofilm formation at sub-MIC values of essential oil was shown in red color

*The most inhibitory concentration of the essential oil in biofilm formation made by corresponding bacteria.

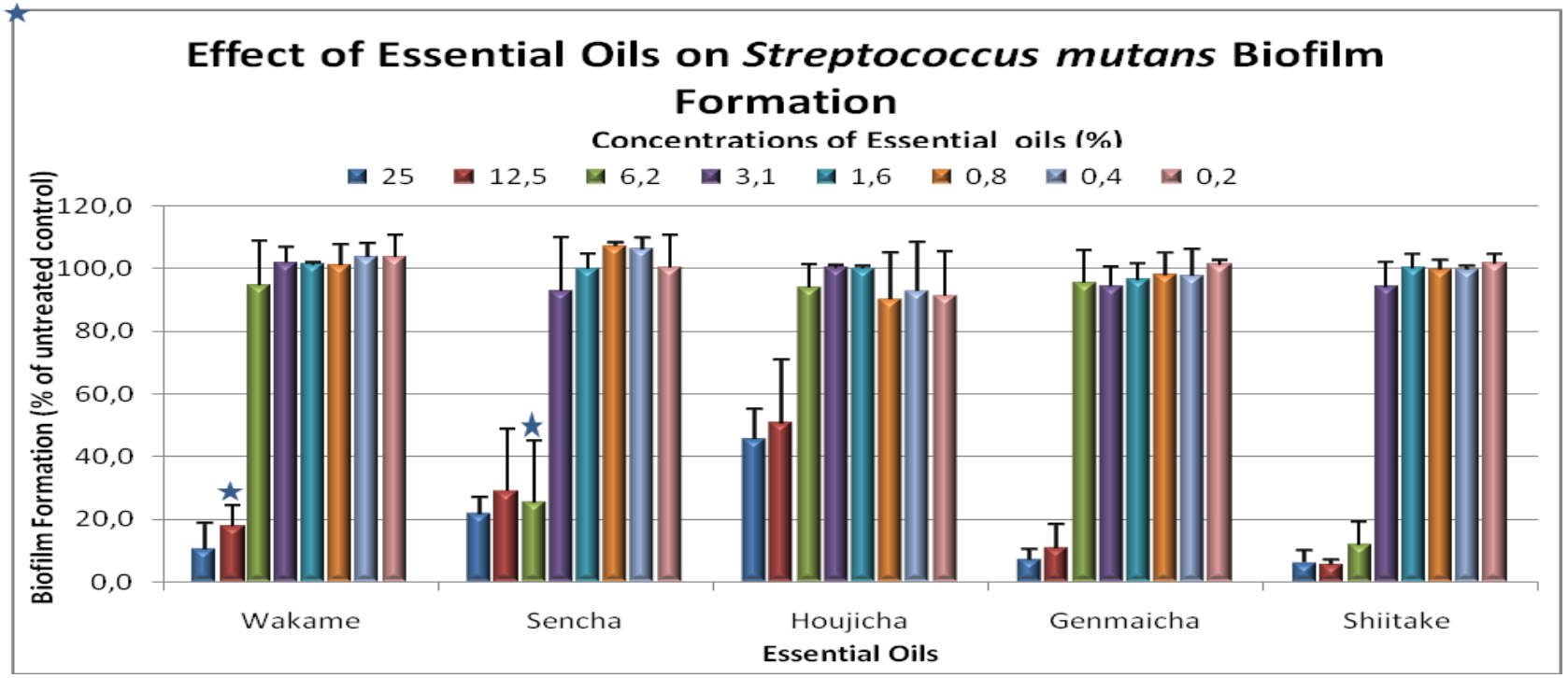

Figure 5. 5. Effects of essential oils on formation of biofilms by *S. mutans*.

"★" indicates the significant reduced biofilm formation at sub-MIC value of essential oil

Table 5.5. Effects of essential oils on formation of biofilms by *A. actinomycetemcomitans*

		Biofilm Formation (% of untreated control) [a]							
	Essential oil	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**	**0.2%**
Aggregatibacter actinomycetemcomitans	**Wakame**	92.0±10.2	86.3±18.3	77.9±7.9	80.7±10.1	74.9±15.0	72.3±12.0*	74.5±6.7	76.3±18.2
	Sencha	118.5±5.0	86.5±17.7	79.5±2.2*	86.3±6.0	80.7±14.4	100.1±4.7	115.2±21.5	139.8±15.0
	Houjicha	119.9±25.7	101.7±10.6	97.8±14.1	82.2±17.4*	83.6±16.3	82.5±8.1	89.6±13.7	103.2±24.2
	Genmaicha	97.5±22.5	79.0±14.7	64.8±12.1*	65.7±8.3	65.6±14.0	70.4±14.1	74.0±15.6	85.0±10.3
	Shiitake	90.1±14.5	92.1±14.8	87.2±17.1	82.6±19.1	78.4±13.5*	82.9±13.7	78.9±11.9	83.6±7.6

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm inhibition activity.

*The most inhibitory concentration of the essential oil in biofilm formation made by corresponding bacteria.

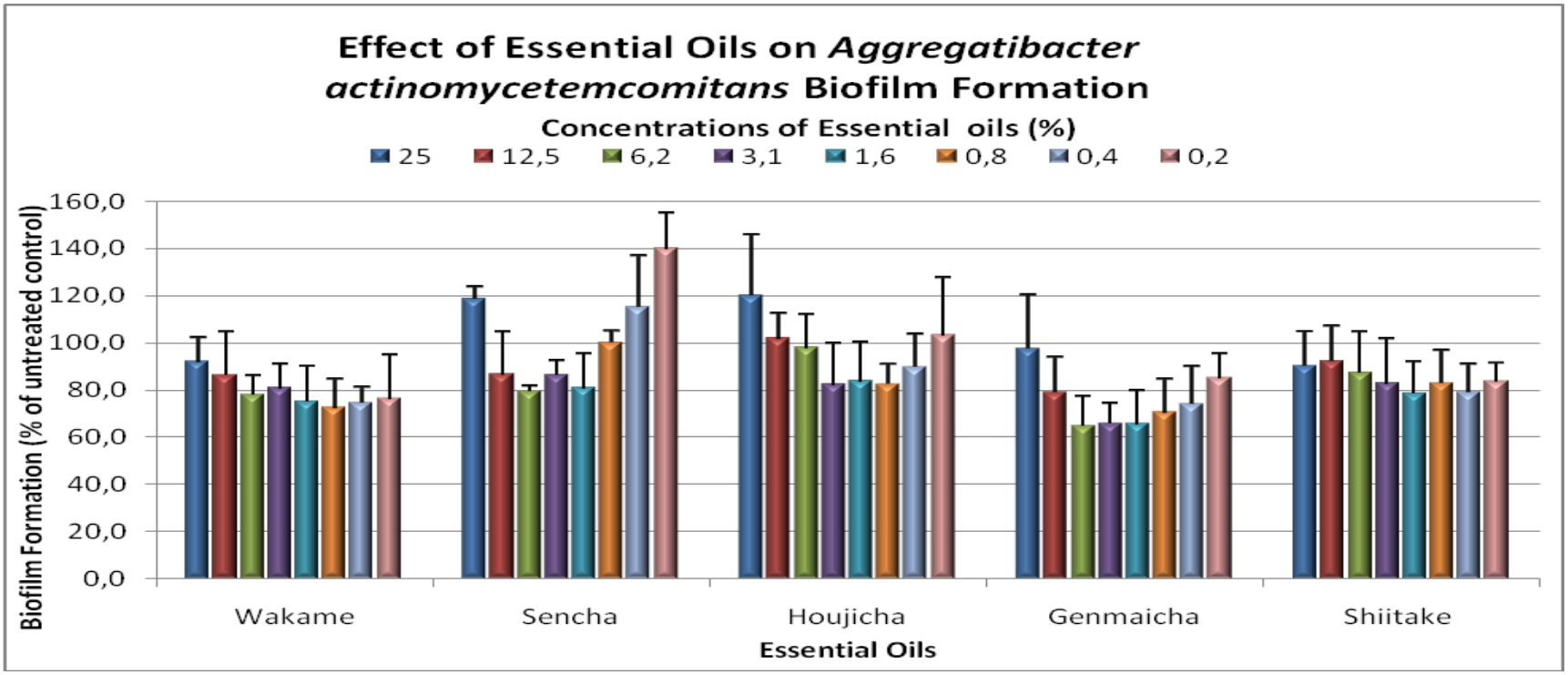

Figure 5.6. Effects of essential oils on formation of biofilms by *A. actinomycetemcomitans*.

Table 5.6. Effects of essential oils on formation of biofilms by *Fusobacterium nucleatum*

		Biofilm Formation (% of untreated control) [a]							
	Essential oil	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**	**0.2%**
Fusobacterium nucleatum	**Wakame**	129.4±12.5	137.3±9.2	110.6±6.2	119.1±13.7	122.3±29.4	99.5±2.8	105.2±10.3	98.1±4.6*
	Sencha	91.1±25.6	86.0±27.8*	90.6±4.6	99.5±6.5	99.0±1.0	102.8±1.7	104.5±0.1	105.5±6.1
	Houjicha	109.1±21.9	107.3±17.0	99.4±37.1	103.3±5.8	114.3±13.5	107.4±2.2	71.4±20.4*	106.0±8.6
	Genmaicha	116.6±3.7	103.5±13.8	107.9±6.4	99.7±8.4	91.1±12.5*	94.5±5.7	106.8±12.0	91.2±7.8
	Shiitake	123.2±19.5	114.9±9.3	106.4±9.0	105.0±14.9	91.5±5.4	90.8±3.8	94.9±8.6	85.6±4.0*

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm inhibition activity.

*The most inhibitory concentration of the essential oil in biofilm formation made by corresponding bacteria.

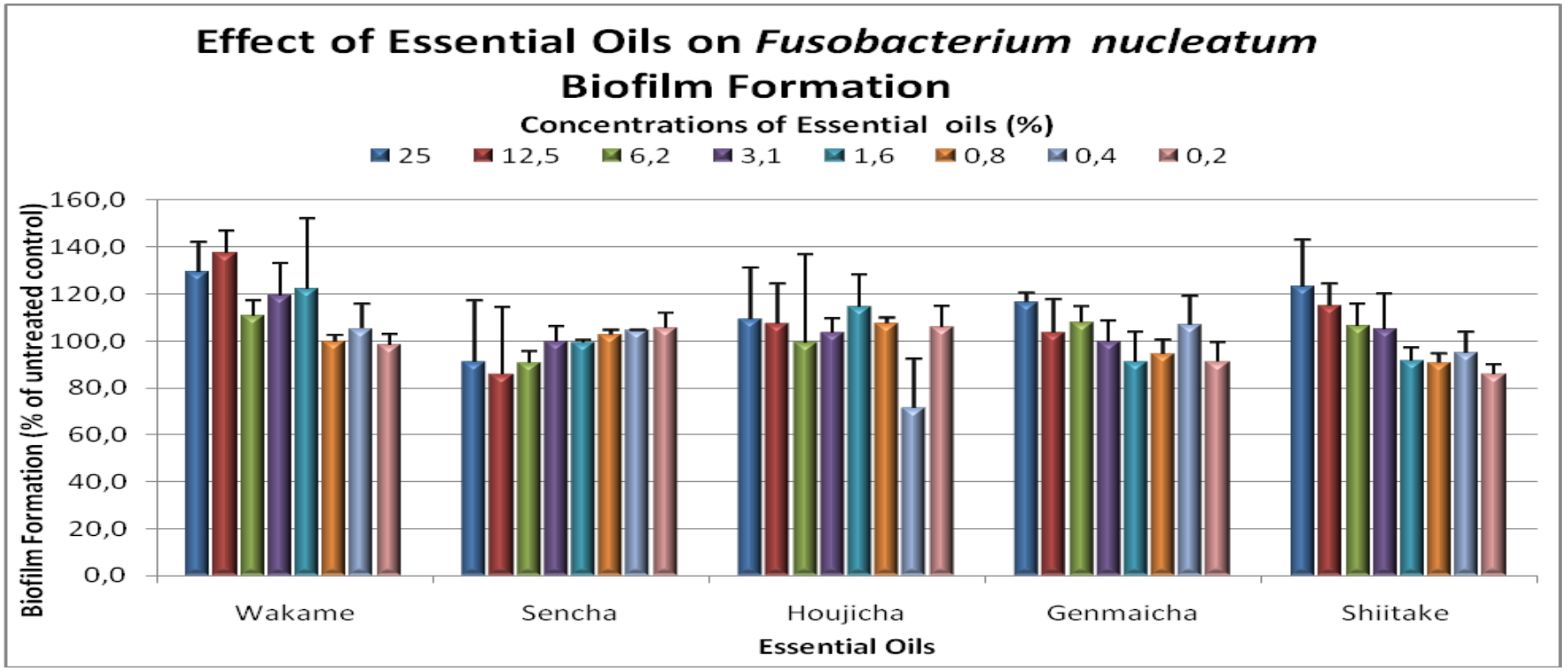

Figure 5.7. Effects of essential oils on formation of biofilms by *F. nucleatum*

Table 5.7. Effects of essential oils on formation of biofilms by *Porphyromonas gingivalis*

		Biofilm Formation (% of untreated control) [a]							
	Essential oil	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**	**0.2%**
Porphyromonas gingivalis	**Wakame**	104.8±18.5	84.7±10.9	97.9±13.3	92.2±14.6	81.6±14.1*	85.0±12.3	92.4±5.2	95.5±3.5
	Sencha	91.3±12.5	73.2±11.3	63.9±4.4	61.1±6.0*	62.0±4.7	61.7±4.8	70.9±7.7	80.4±13.5
	Houjicha	111.1±17.6	98.0±8.9	80.8±4.9	70.0±8.8	70.8±14.6	62.6±14.0*	68.5±10.9	66.5±1.5
	Genmaicha	119.0±21.7	89.7±11.4	102.4±12.6	85.3±4.2	80.2±7.3*	82.0±2.6	91.5±12.0	94.5±7.8
	Shiitake	87.5±15.4	78.8±13.9	61.2±5.3	67.6±11.3	64.8±10.8	55.9±6.1*	68.4±12.2	102.5±15.4

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm inhibition activity.

*The most inhibitory concentration of the essential oil in biofilm formation made by corresponding bacteria.

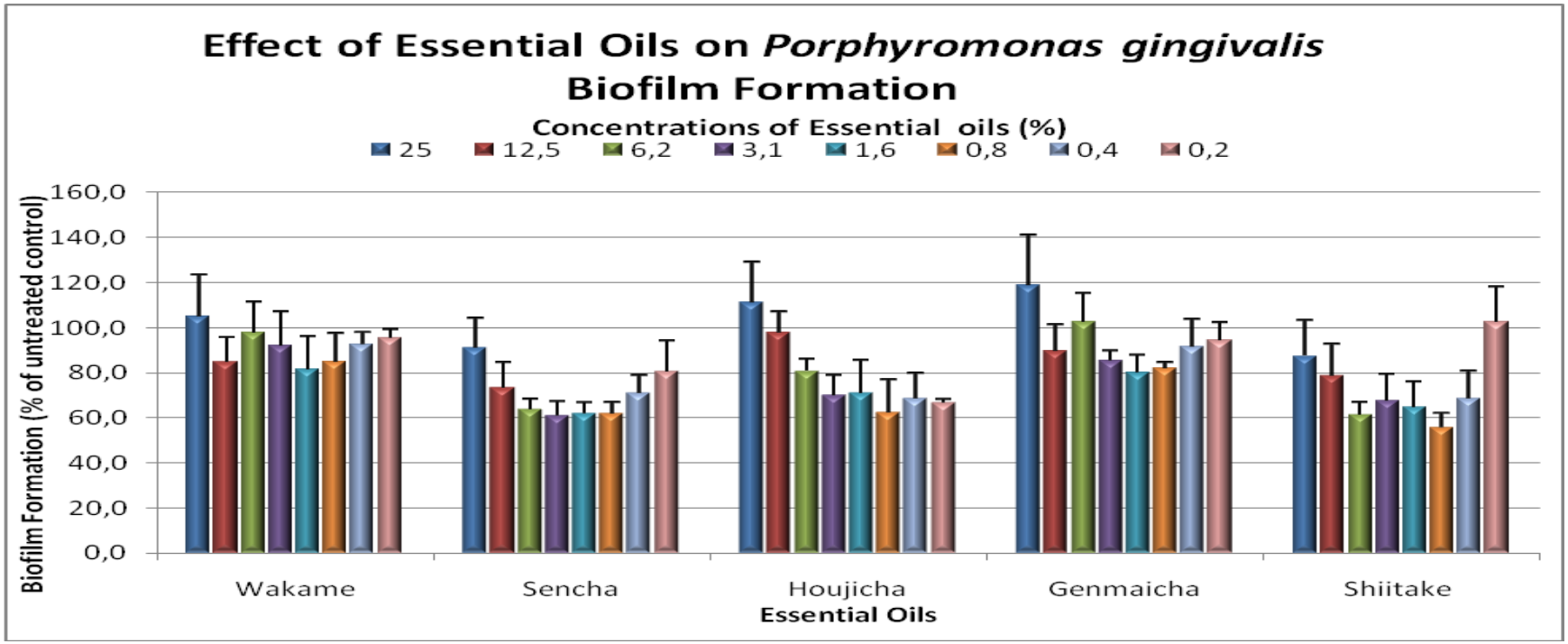

Figure 5.8. Effects of essential oils on formation of biofilms by *P. gingivalis*

The effect of essential oils on biofilm formation of *P. gingivalis* was also examined and the results shown in Table 5.7 and Figure 5.8. No significant biofilm inhibition could be observed at sub-MIC concentrations of extracted oils (biofilm formation > 60 per cent of untreated control). However, Sencha (MIC: 1.6 per cent, 2.9 mg/l) and Hojicha (MIC: 0.8 per cent, 1.4 mg/l) oils exhibited slight biofilm formation inhibitory effect (percentage of biofilm formation was around 70 per cent) at their sub-MIC values. When supra-MIC concentrations of essential oils were examined, it was seen that Shiitake oil at its 2X MIC could inhibit biofilm formation of *P. gingivalis* (amount of biofilm formation was 55.9 per cent), significantly which might occur due to the bacteriostatic effect of essential oil. Moreover, supra-MIC concentrations of Shiitake and Sencha oils seemed to have slight effect of biofilm formation.

The effects of essential oils at different concentrations on formation of multi-species biofilms were also studied and results shown in Table 5.8 and Figure 5.9 indicate that extracted oils could inhibit the formation of multi-species biofilms significantly only at high concentrations (4X-16X MICs of oils). Moreover, this biofilm inhibitory effect was observed at MBC and 2X MBC values of essential oils, therefore, it might be said that biofilm inhibition was most probably due to bactericidal activity of essential oils on bacteria incorporated in multi-species biofilm.

Inhibitory effects of chlorhexidine gluconate, positive control of this study, on biofilm formation of single species and multi-species biofilms were also examined. As shown in Table 5.9 and Figure 5.10, chlorhexidine had a strong biofilm inhibition activity on *S. mutans* and multi-species biofilms, followed by *F. nucleatum* biofilms. Moreover, chlorhexidine caused slight reduction in biofilm formation of *P. gingivalis* while it had no significant effect on formation of biofilms by *A. actinomycetemcomitans*. However, when these results were analyzed in detail, it can be understood that inhibitory effect of chlorhexidine against *S. mutans* and *P. gingivalis* biofilm formation was due to the bactericidal and/or bacteriostatic activity of chemical agent (since inhibitory effect was seen at concentrations higher than MICs and MBCs of the oils.

Table 5.8. Effects of essential oils on formation of multi-species biofilms

Bacteria	Essential oil	Biofilm Formation (% of untreated control) [a]							
		25%	12.5%	6.2%	3.1%	1.6%	0.8%	0.4%	0.2%
Mix bacteria	Wakame	6.6±2.2*	11.0±5.8	88.9±7.6	92.2±8.7	97.9±2.4	99.5±2.9	95.3±5.1	99.6±0.8
	Sencha	6.0±1.3*	15.8±6.6	97.3±2.0	94.5±8.5	101.0±2.4	99.2±1.3	100.6±0.7	101.2±1.6
	Houjicha	7.4±5.9*	10.0±6.0	85.0±8.4	96.6±4.6	98.4±2.6	99.1±3.1	101.5±1.7	103.0±4.2
	Genmaicha	5.8±2.2	4.8±0.7*	89.2±5.6	90.1±10.0	98.6±0.9	100.0±2.3	96.2±6.3	100.1±2.9
	Shiitake	7.0±2.3	4.7±0.5*	64.2±9.1	95.9±7.5	96.7±6.0	98.0±1.4	94.0±7.7	96.5±4.6

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm inhibition activity.

*The most inhibitory concentration of the essential oil in biofilm formation made by corresponding bacteria.

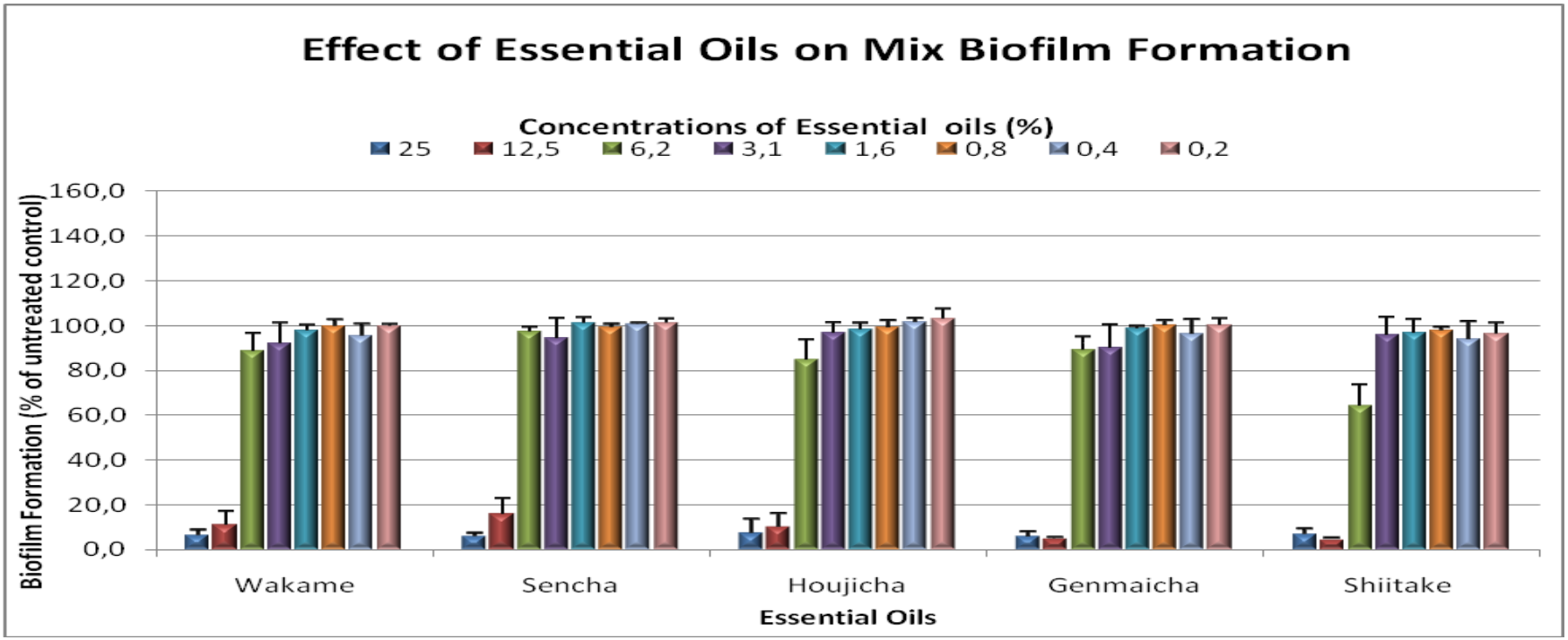

Figure 5.9. Effects of essential oils on formation of multi-species biofilms

Table 5.9. Effects of chlorhexidine gluconate on formation of biofilms

Bacteria	**Biofilm Formation (% of untreated control) [a]**							
	Positive control (Chlorhexidine gluconate)							
	12 mg/l	**6 mg/l**	**3 mg/l**	**1.5 mg/l**	**0.75 mg/l**	**0.375 mg/l**	**0.1875 mg/l**	**0.09375 mg/l**
Streptococcus mutans	3.8±0.4	3.6±0.3*	3.6±0.3*	3.8±0.4	78.7±25.7	98.3±1.9	99.8±1.1	100.6±1.0
Actinobacillus actinomycetemcomitans	67.4±14.5*	82.7±18.7	131.8±14.1	99.9±26.9	109.0±12.8	112.1±12.4	140.4±17.3	112.7±20.2
Fusobacterium nucleatum	5.1±0.04	6.1±1.3	4.9±0.3[b]*	102.7±0.3	101.7±0.02	100.4±2.9	100.4±2.9	107.8±4.3
Porphyromonas gingivalis	52.3±2.9*	61.9±7.4	87.2±1.3	90.9±10.2	106.3±13.3	113.5±5.6	104.2±13.9	106.4±17.7
Mix bacteria	5.1±1.6	4.1±0.3*	8.5±3.6[b]	9.0±7.2[b]	96.2±7.4	100.2±11.4	98.5±2.4	98.4±8.3

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm inhibition activity.

[b] Significant reduced biofilm formation at sub-MIC values of positive control was shown in red color

*The most inhibitory concentration of the positive control in biofilm formation made by corresponding bacteria

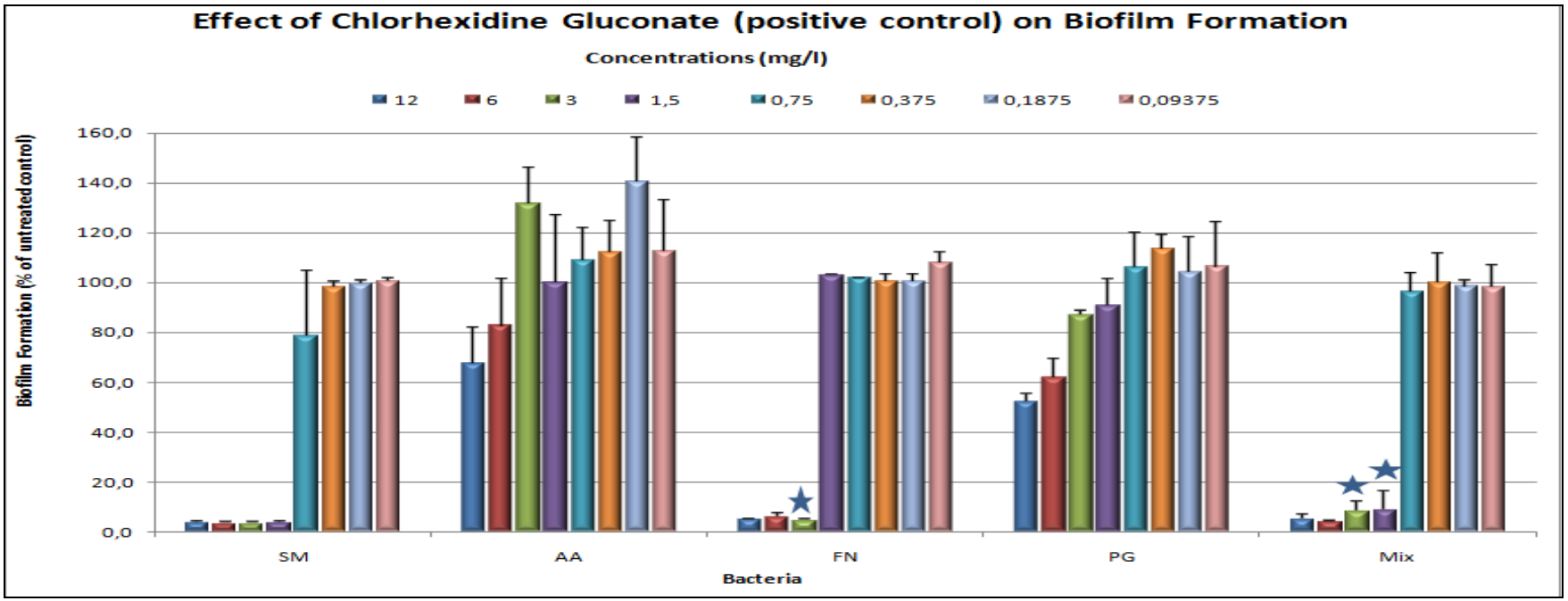

Figure 5.10. Effects of chlorhexidine on formation of biofilms by oral bacteria.

"★" indicates the significant reduced biofilm formation at sub-MIC value of agent.

On the other hand, chlorhexidine reduced biofilm formation of *F. nucleatum* and mix bacteria significantly (> 90 per cent) at its sub-MIC values which may show that chlorhexidine has an inhibitory activity on virulence factors required to form single species *F. nucleatum* biofilms and multi-species biofilms.

5.1.4.2. Disruption of pre-formed biofilms by essential oils

Tables 5.10.-5.14 and Figures 5.11.-5.15 show the disruptive effect of different concentrations of essential oils (ranging from 50 per cent to 0.4 per cent) on mature biofilms of oral pathogens.

It is known that once established, biofilms have greater resistance to external agents such as antibiotics, detergents or biocides than their planktonic cells [191] and therefore disruption of pre-formed biofilms tend to have higher MIC values than killing of planktonic cells in suspension [214]. By considering this fact, disruptive effects at MIC and 2X MIC values of essential oils were also assumed significant.

Results of effect of essential oils on pre-formed biofilms of *S. mutans* were displayed in Table 5.10 and Figure 5.11. It was observed that essential oils extracted from five Asian herbs did not show disruption effect on mature biofilms of *S. mutans* even at high-folds of MIC values.

Significant disruptive effects of essential oils tested on established biofilms of *A. actinomycetemcomitans* were shown in Table 5.11 and Figure 5.12. With the exception of Genmaicha, all extracted oils could destruct pre-formed biofilms of *A. actinomycetemcomitans* at their sub-MIC values; 0.5X MICs of Wakame, Sencha and Hojicha reduced the biofilm amount significantly (> 67 per cent). Shiitake, on the other hand, was significantly effective even at its 0.25X MIC concentration.

Genmaicha, on the other hand, disrupted biofilms of *A. actinomycetemcomitans* at folds of its MIC (2X-128X MIC). Moreover, where Wakame, Sencha and Shiitake essential oils were effective at high concentrations (50 and 25 per cent), Hojicha oil did not

exhibited this property and more interestingly this volatile oil at 50 per cent seemed to increase biofilm formation.

Table 5.12 and Figure 5.13 displayed the biofilm disruptive activity of essential oils on mature *F. nucleatum* biofilms. Wakame essential oil caused biofilm enhancement in pre-formed biofilm as it did in early-biofilm formation of *F. nucleatum* (Table 5.6). Possible reasons of induction of biofilm formation in the presence of essential oil are mentioned in the discussion part.

Sencha and Genmaicha, on the other hand, eradicated mature biofilms of *F. nucleatum* at their sub-MIC concentrations significantly by more than 77 per cent. Moreover, Sencha was also effective at its MIC and supra-MIC values, while Genmaicha was at 2X MIC. Unlike Sencha and Genmaicha, Hojicha could disrupt biofilms of *F. nucleatum* only at its 2X and 4X MIC values by approximately 50 per cent.

P. gingivalis biofilms exhibited more resistance to essential oils compared to the biofilms formed by other tested individual bacteria and results were shown in Table 5.13 and Figure 5.14. None of the essential oils could disrupt mature *P. gingivalis* biofilms at their sub-MIC concentrations. Significant results were obtained only at folds of MICs of Sencha (2X-32X MIC), Hojicha (64X MIC), Genmaicha (32X MIC) and Shiitake (4X-16X MIC). Wakame essential oil, on the other hand, had slight disruption activity on pre-formed biofilms at its supra-MIC concentrations.

Table 5.10. Effects of essential oils on pre-formed biofilms of *S. mutans*

		Biofilm Formation (% of untreated control)[a]							
	Essential oil	**50%**	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**
Streptococcus mutans	**Wakame**	101.9±0.4	100.6±1.8	100.4±2.2	101.4±0.3	100.3±2.1	94.8±9.5*	101.3±1.3	100.7±0.9
	Sencha	100.3±0.7	99.9±2.2*	104.5±2.5	100.5±1.4	100.1±2.0	99.9±0.6*	100.9±1.4	100.5±0.9
	Houjicha	107.8±6.5	107.7±7.6	105.9±7.2	107.7±5.6	105.8±6.9	105.4±6.7*	106.7±7.4	108.3±6.0
	Genmaicha	100.4±1.8	101.9±1.2	100.3±1.7	101.0±1.8	100.4±2.0	101.3±2.3	102.1±1.9	99.9±1.3*
	Shiitake	103.8±2.9	101.5±1.2	100.3±3.0*	100.4±3.0	100.6±2.0	100.4±3.1	104.0±5.2	100.4±1.1

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm disruption activity.

* The most effective concentration of the essential oil in disruption of pre-formed biofilm formed by the corresponding bacteria.

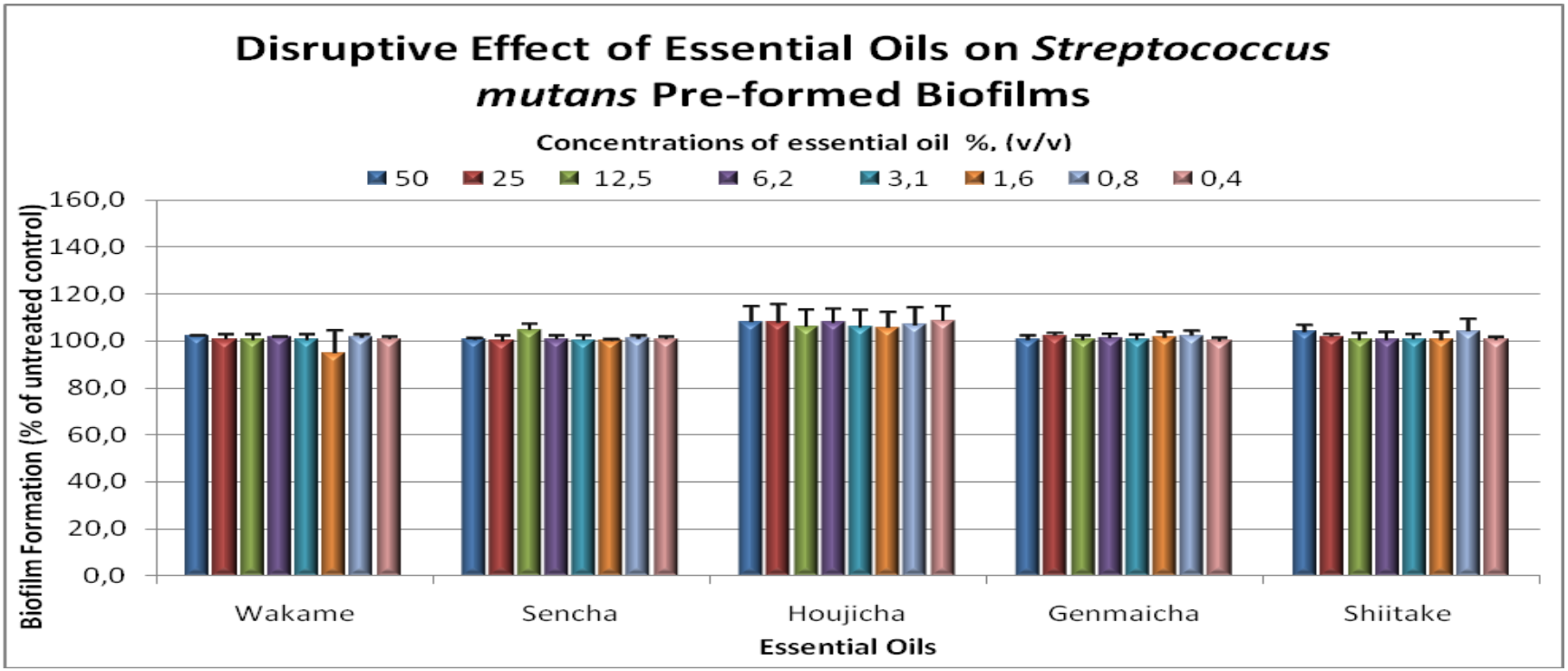

Figure 5.11. Effects of essential oils on pre-formed biofilms of *S. mutans*

Table 5.11. Effects of essential oils on pre-formed biofilms of *A. actinomycetemcomitans*

		Biofilm Formation (% of untreated control) [a]							
	Essential oil	**50%**	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**
Aggregatibacter actinomycetemcomitans	**Wakame**	52.3±2.5	59.7±6.2	44.0±14.8	32.5±0.7 [b] *	36.5±5.1 [b]	51.5±15.2 [c]	104.7±2.4	108.0±3.6
	Sencha	37.6±9.6	49.4±25.4	44.1±9.0	34.6±1.1 [b] *	36.2±11.4 [b]	39.9±15.2 [c]	62.0±18.3	99.0±15.5
	Houjicha	127.1±14.5	83.5±13.1	40.4±1.0	29.4±5.3	35.9±21.1	47.9±19.7 [b]	22.0±9.2 [b]*	32.8±16.5 [c]
	Genmaicha	34.6±6.2	22.0±11.9	27.5±13.2	11.9±4.9*	11.9±1.2*	13.0±0.9	15.9±4.0 [b]	92.5±15.0
	Shiitake	43.5±3.1	19.4±4.5*	20.1±1.3	43.3±9.4 [b]	39.7±14.0 [b]	48.3±24.8 [c]	46.3±4.4 [c]	69.4±16.4

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm disruption activity.

[b] Significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil.

[c] Significant reduced biofilm amount at sub-MIC values of essential oil was shown in red color

*The highest disruptive concentration of the essential oil on mature biofilm formed by corresponding bacteria

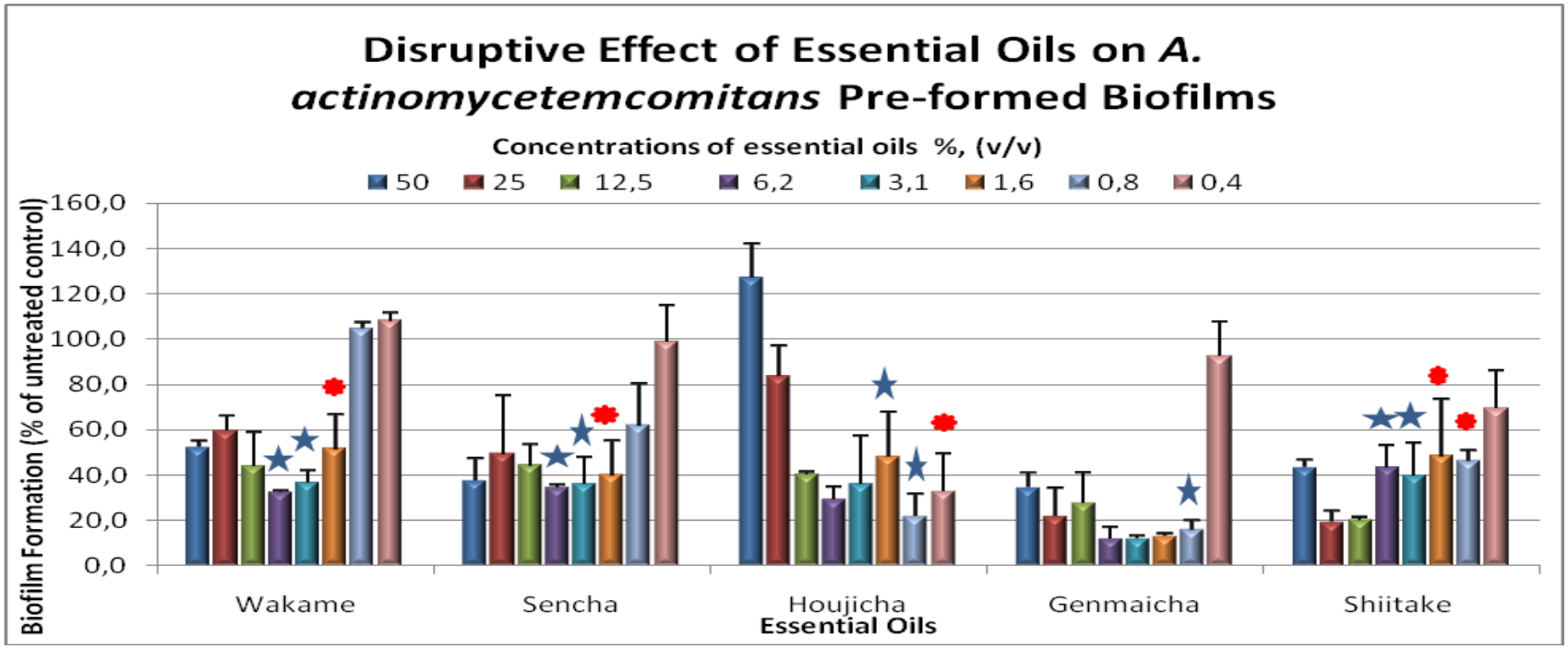

Figure 5.12. Effects of essential oils on pre-formed biofilms of *A. actinomycetemcomitans*.

"★" indicates the significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil

"✿" indicates the significant reduced biofilm amount at sub-MIC values of essential oil

Table 5.12. Effects of essential oils on pre-formed biofilms of *F. nucleatum*

Bacteria	Essential oil	Biofilm Formation (% of untreated control) [a]							
		50%	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**
Fusobacterium nucleatum	**Wakame**	120.2±29.0*	133.1±32.5	129.9±19.8	124.0±35.1	136.6±31.0	164.4±13.0	122.7±25.3	137.0±31.5
	Sencha	20.2±0.9	35.4±17.9 [b]	45.4±13.5[b]	12.6±0.2[c]*	23.6±7.8 [c]	28.2±3.7 [c]	29.2±10.7 [c]	80.1±10.6
	Houjicha	87.0±18.4	81.6±21.3	77.6±18.4	79.3±21.4	58.6±3.0	49.5±10.3 [b]*	120.2±4.0	101.1±21.3
	Genmaicha	50.9±11.1 [b]	57.2±17.4 [b]	26.2±3.3 [c]	24.6±3.5 [c] *	28.4±6.0 [c]	40.2±13.2 [c]	30.2±0.9 [c]	90.0±12.7
	Shiitake	54.9±13.7 [b] *	73.8±22.2	131.0±19.8	61.3±26.9	86.1±30.7	134.8±18.4	79.2±11.3	92.6±14.0

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm disruption activity.

[b] Significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil.

[c] Significant reduced biofilm amount at sub-MIC values of essential oil was shown in red color

* The highest disruptive concentration of the essential oil on mature biofilm formed by corresponding bacteria

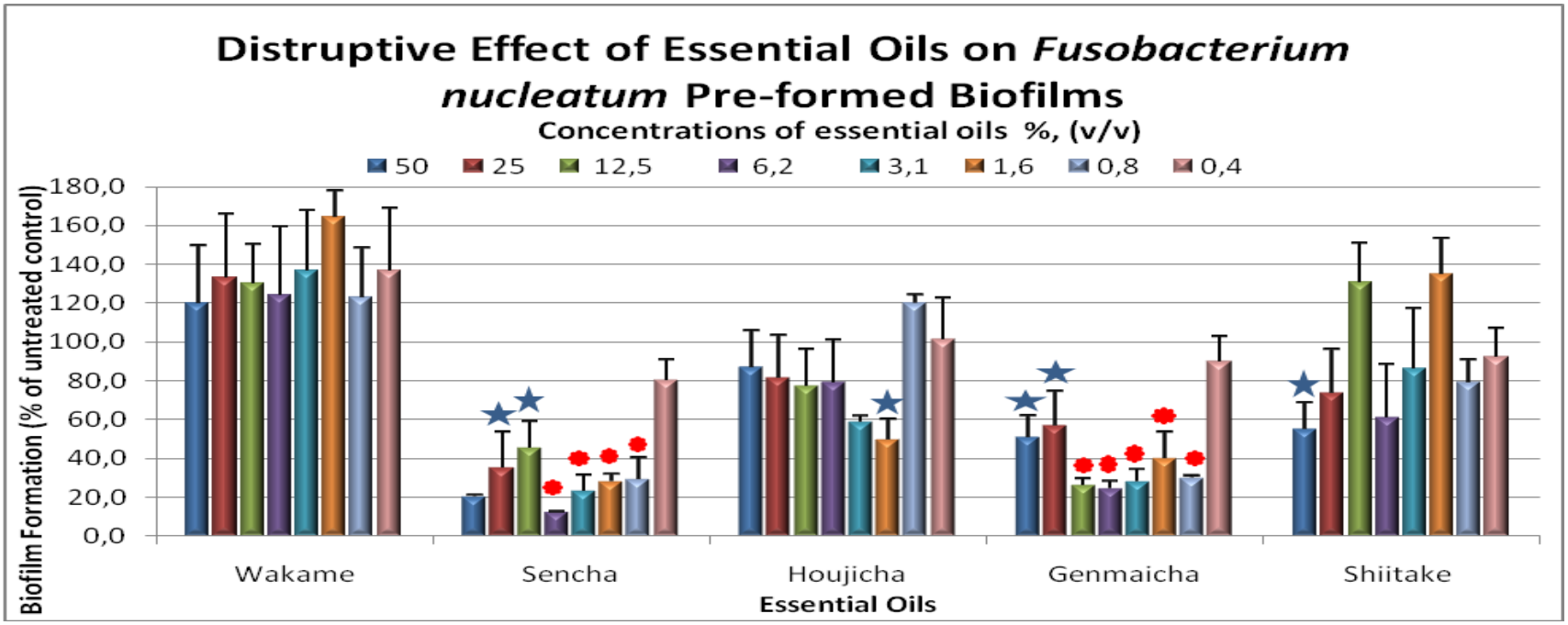

Figure 5.13. Effects of essential oils on pre-formed biofilms of *F. nucleatum*.

"★" indicates the significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil

"✿" indicates the significant reduced biofilm amount at sub-MIC values of essential oil

Table 5.13. Effects of essential oils on pre-formed biofilms of *P. gingivalis*

Bacteria	Essential oil	Biofilm Formation (% of untreated control) [a]							
		50%	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**
Porphyromonas gingivalis	**Wakame**	75.6±14.9	81.3±17.7	75.1±19.2	64.8±18.9*	68.3±15.1	86.3±12.6	74.1±13.7	102.5±30.8
	Sencha	46.3±6.4	15.1±4.1	14.0±4.1*	14.4±2.3	27.5±13.9[b]	70.2±28.4	78.6±15.8	110.5±20.6
	Houjicha	25.6±7.8*	91.9±4.8	91.3±16.3	89.1±1.8	69.2±7.0	75.2±5.8	75.5±9.7	104.9±28.6
	Genmaicha	101.9±25.7	76.0±17.4	50.3±22.6*	64.8±23.8	80.1±7.3	75.1±11.8	97.2±15.7	93.9±8.0
	Shiitake	116.8±14.1	70.9±9.9	60.2±15.9	50.7±4.2*	54.0±7.7	59.5±10.4	73.4±27.7	84.6±19.1

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm disruption activity.

[b] Significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil.

* The highest disruptive concentration of the essential oil on mature biofilm formed by corresponding bacteria

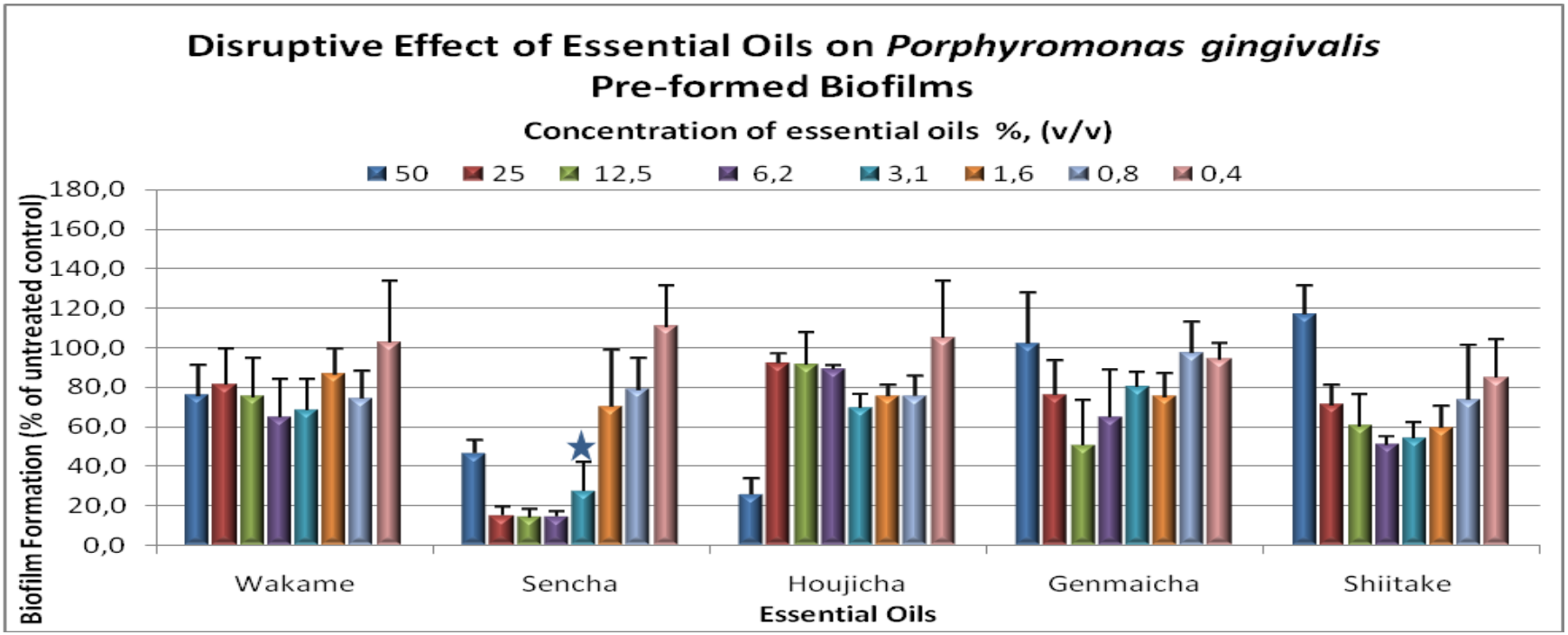

Figure 5.14. Effects of essential oils pre-formed biofilms of *P. gingivalis*.

"★" indicates the significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil

The disruptive activity of essential oils and positive control (chlorhexidine gluconate) on pre-formed multi-species biofilms were shown in the Table 5.14 and Figure 5.15 and Table 5.15 and Figure 5.16, respectively. It was observed that multi-species biofilms were more resistant to extracted oils and chlorhexidine gluconate then single-species biofilms were; *i.e.*, all oils studied did not cause eradication of mature multi-species biofilms even at their supra-MIC values. On the other hand, while chlorheixidine could disrupt mature biofilms of *A. actinomycetemcomitans* and *P. gingivalis* at sub-MIC, 2X and4X MIC values, mature biofilms of *F. nucleatum* and *S. mutans* exhibited resistance to all test concentrations of chlorhexidine gluconate.

Table 5.14. Effects of essential oils on multi-species pre-formed biofilms

Bacteria	Essential oil	Biofilm Formation (% of untreated control) [a]							
		50%	**25%**	**12.5%**	**6.2%**	**3.1%**	**1.6%**	**0.8%**	**0.4%**
Mix bacteria	**Wakame**	101.6±0.6	101.8±2.3	99.0±1.8*	100.9±2.4	100.1±1.3	100.8±4.6	100.9±0.9	100.1±1.2
	Sencha	101.7±1.4	102.3±1.7	100.7±2.6	99.3±0.6	99.5±0.8	99.1±2.3*	99.1±1.7*	100.6±1.5
	Houjicha	100.8±1.6	100.1±1.7	99.8±1.9	100.3±0.8	99.9±1.3	99.1±2.7*	99.8±1.1	100.3±0.5
	Genmaicha	101.3±2.5	101.1±1.4	100.4±2.6	99.8±2.2	100.7±1.2	100.7±1.7	97.4±0.5*	99.7±2.0
	Shiitake	102.6±2.5	100.6±1.6	100.9±1.0	99.1±3.4	99.8±2.5	99.7±0.7	97.2±1.0*	98.8±0.4

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm disruption activity.

* The highest disruptive concentration of the essential oil on mature biofilm formed by corresponding bacteria

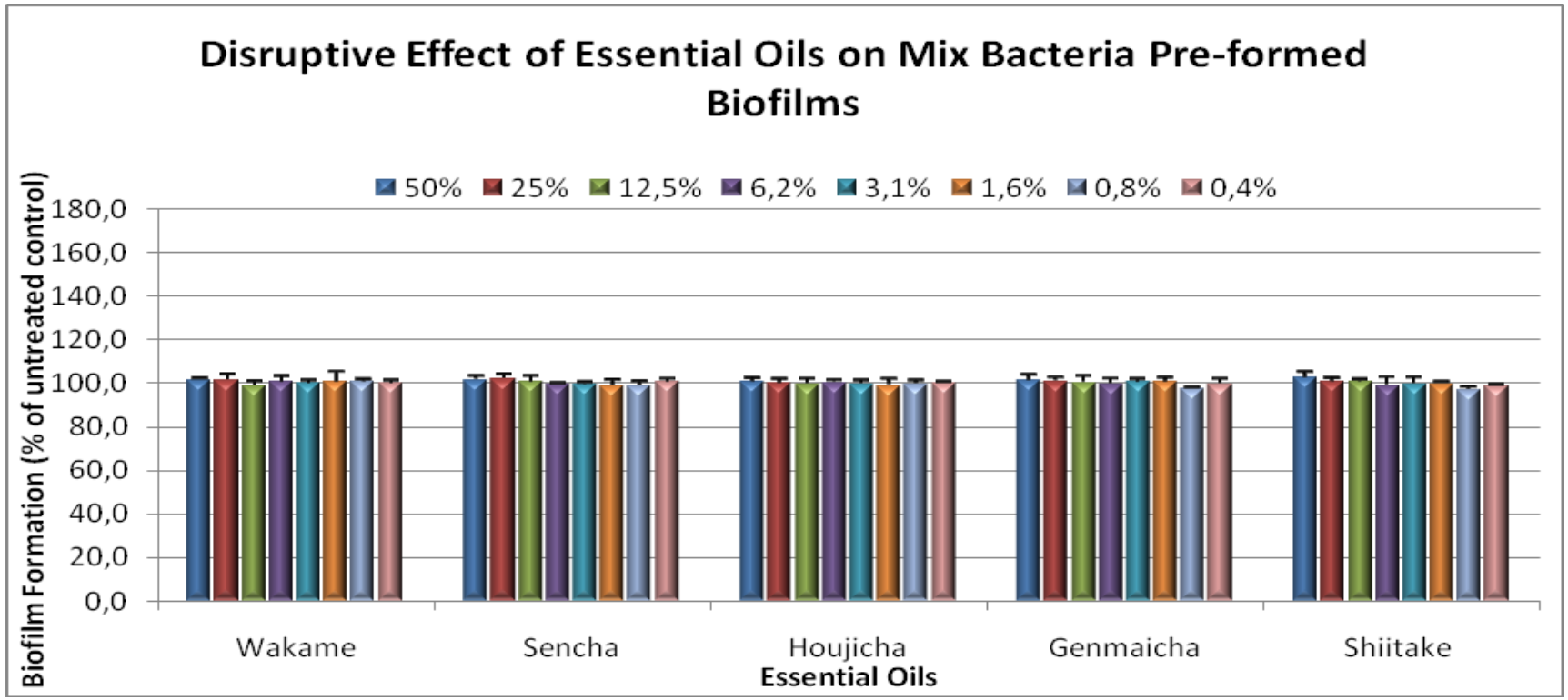

Figure 5.15. Effects of essential oils on multi-species pre-formed biofilms.

Table 5.15. Effects of chlorhexidine gluconate on pre-formed biofilms

Bacteria	**Biofilm Formation (% of untreated control) [a]**							
	Positive control (Chlorhexidine gluconate)							
	12 mg/l	**6 mg/l**	**3 mg/l**	**1.5 mg/l**	**0.75 mg/l**	**0.375 mg/l**	**0.1875 mg/l**	**0.09375 mg/l**
Streptococcus mutans	100.8±1.1	102.2±0.9	100.5±2.2*	100.9±1.0	102.7±2.1	101.1±2.4	101.7±1.1	102.5±1.3
Actinobacillus actinomycetemcomitans	31.4±20.8	33.9±2.3 [b]	30.7±4.7[b]*	44.6±9.4[c]	54.7±15.8 [c]	56.9±27.9 [c]	68.3±26.1	68.8±21.5
Fusobacterium nucleatum	83.6±10.9	75.2±20.1*	85.1±15.5	82.5±14.6	86.5±16.9	79.9±9.6	94.2±11.6	80.7±8.0
Porphyromonas gingivalis	25.8±8.4 [b]	79.4±13.9	79.0±0.4	23.6±4.6 [c] *	55.6±6.1 [c]	81.1±9.6	83.2±18.5	76.2±4.5
Mix bacteria	97.8±2.2*	99.0±2.5	99.2±1.6	98.7±1.4	99.3±1.9	100.3±2.8	99.8±1.7	99.6±0.9

[a] Biofilm formation values were calculated as (OD_{595} treated well) / (mean OD_{595} control well) x 100. Values are expressed as means ± standard deviations. Biofilm formation $\leq$ 60 per cent of untreated control indicates significant biofilm disruption activity.

[b] Significant reduced biofilm amount at 2X and/or 1X MIC values of essential oil.

[c] Significant reduced biofilm amount at sub-MIC values of essential oil was shown in red color

*The most disruptive concentration of the positive control on mature biofilm formed by corresponding bacteria

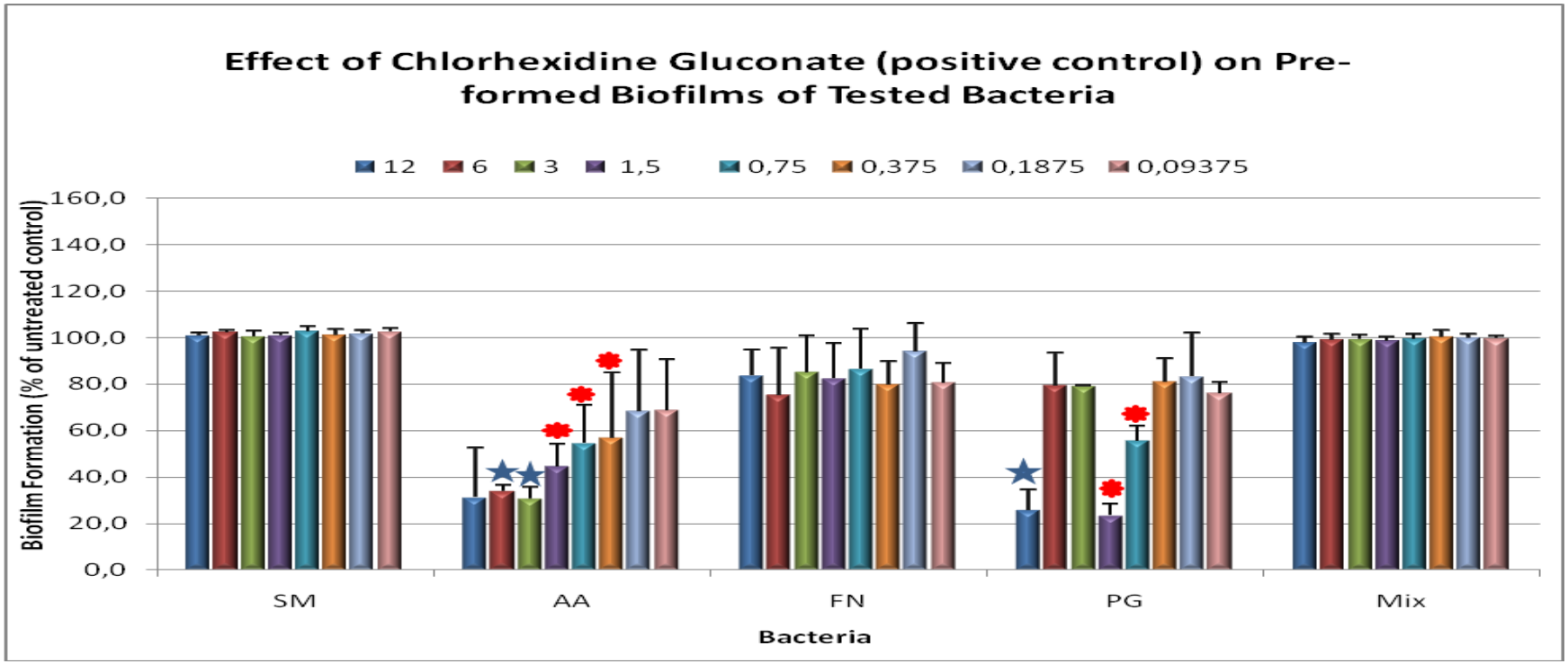

Figure 5.16. Effects of chlorhexidine on pre-formed biofilms by oral bacteria.

"✚" indicates the significant reduced biofilm amount at 2X and/or 1X MIC values of chlorhexidine gluconate

"✿" indicates the significant reduced biofilm amount at sub-MIC values of chlorhexidine gluconate

5.1.5. Scanning Electron Microscopy

5.1.5.1. Inhibitory effect of Sencha essential oil on S. mutans biofilm formation

To observe the effects of essential oils on biofilm formation, scanning electron microscopy (SEM) analysis was carried out. For this experiment, *Streptococcus mutans* and Sencha essential oil were chosen because during quantitative biofilm inhibition assays this oil exhibited biofilm inhibition activity against *S. mutans* at its 2X MIC, 1X MIC and 0.5X MIC concentrations. Negative control without essential oil was also included to see the normal biofilm growth.

As Figure 5.17 showed *S. mutans* could form multi-layers of biofilms when no essential oil was added. When biofilms were examined at high magnification, the fimbriae which provide the attachment of bacteria to the surface was observed, as shown in Figure 5.17c. However, when essential oil at sub-inhibitory (0.5X MIC) concentration was added, the multi-layer of biofilm structure of *S. mutans* could not be formed, i.e., bacteria only formed a mono-layeri as in Figure 5.18. Furthermore, essential oil did not cause any morphological change or cidal effect, as expected, since a sub-MIC concentration was used. As Figure 5.19 and Figure 5.20 show, no aggregation of bacteria even in a monolayer was observed at 1X and 2X MIC concentrations of the Sencha oil. This results may be a result of bactericidal effects of oil which could be seen in Figure 5.19b and Figure 5.20a. Change in the morphology and hemolysis which are the indicators of bacteria death were observed in these figures. Moreover, embedding of bacteria into the medium that contained high concentrations of essential oil was detected, as shown in Figure 5.19a during SEM analysis.

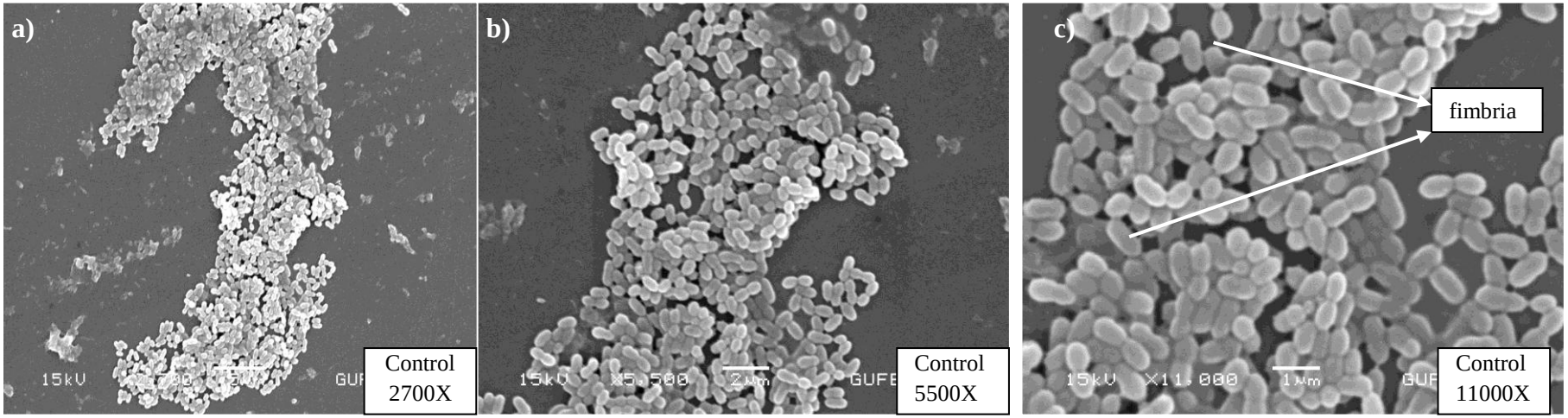

Figure 5. 17. Scanning electron microscopy images of *S. mutans* biofilms without essential oils a. at 2700X b. at 5500 X c. at 11000 X

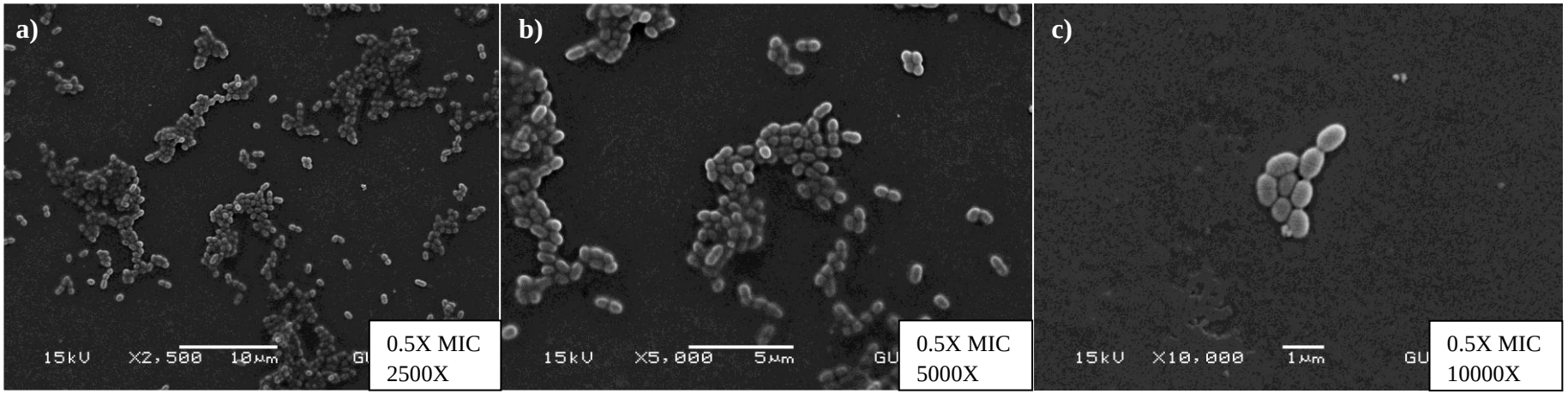

Figure 5. 18. Scanning electron microscopy images of *S. mutans* biofilms in the presence of 0.5X MIC Sencha essential oil a. at 2500X b. at 5000 X c. at 10000 X

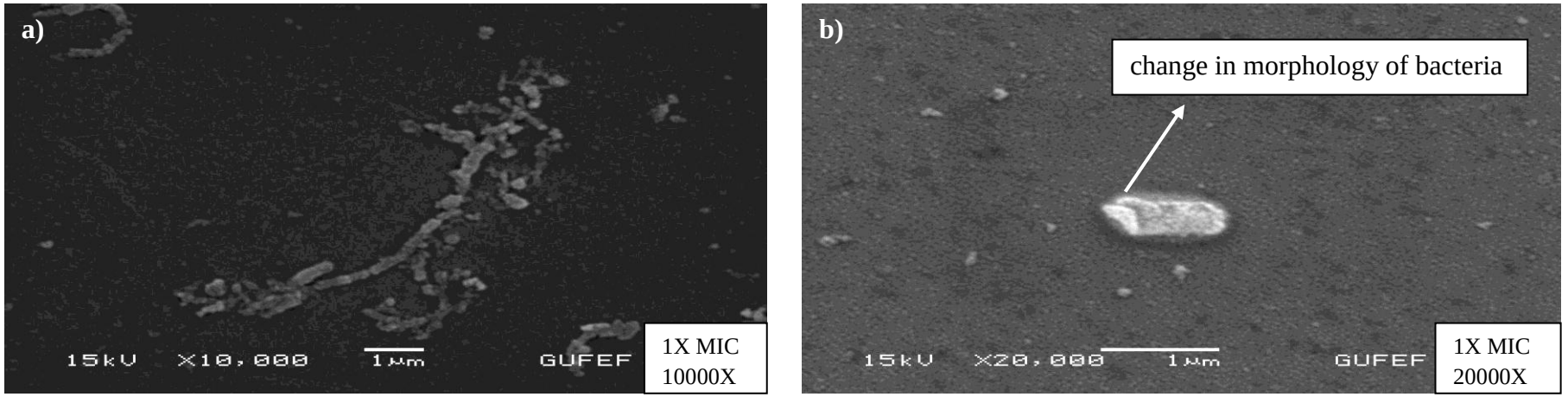

Figure 5.19. Scanning electron microscopy images of *S. mutans* biofilms in the presence of 1X MIC Sencha essential oil a. at 10000X b. at 20000 X

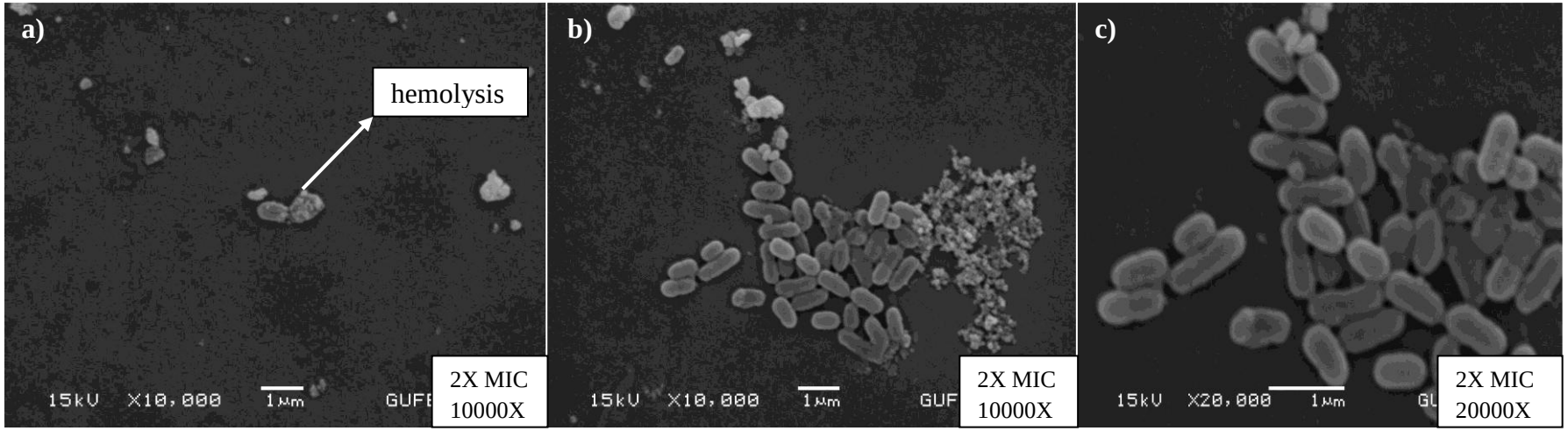

Figure 5.20. Scanning electron microscopy images of *S. mutans* biofilms in the presence of 2X MIC Sencha essential oil a. at 10000X b. at 10000 X c. at 20000 X

5.1.5.2. Disruptive effect of Hojicha essential oil on A. actinomycetemcomitans pre-formed biofilms

To observe the effects of essential oils on mature biofilms, scanning electron microscopy analysis was done. For this experiment, *A. actinomycetemcomitans* and Hojicha essential oil were chosen because during quantitative biofilm disruption assays this oil exhibited strong biofilm destructive activity against pre-formed biofilms of *A. actinomycetemcomitans* at its 2X MIC, 1X MIC and 0.5X MIC concentrations. Negative control without essential oil was also included to see the normal biofilm growth. The three dimensional matrix of *A. actinomycetemcomitans* biofilm was observed under SEM at different magnifications, as shown in Figure 5.21 without essential oil. Holes which can be seen in Figure 5.21b-c were most probably the fluid channels to deliver water and nutrients to the cells at the inner portion of the biofilm [215]. When 0.5X MIC of Hojicha was applied to the pre-formed biofilms, significant reduction in biofilm matrix was clearly observed in Figure 5.22.

As it can bee seen clearly in Figure 5.23, treatment of mature biofilms with 1X MIC values of essential oil caused significant disruption of pre-formed biofilms. Moreover, when magnified, an interestingly, oil caused asymmetrical cell division of bacteria (white arrow in Figure 5.23c). 2X MIC essential oil, on the other hand, disrupt mature biofilms significantly, as shown in Figure 5.24, compared to the control and the reason of this reduction might be the cidal activity of extracted oil, which can be seen in Figure 5.24c. However, when SEM results of 1X and 2X MICs were compared, more biofilm was observed in 2X MIC treated sample. This may be explained by viscous nature of high concentrated essential oil which may cause embedding of bacteria in the oil and holding them together.

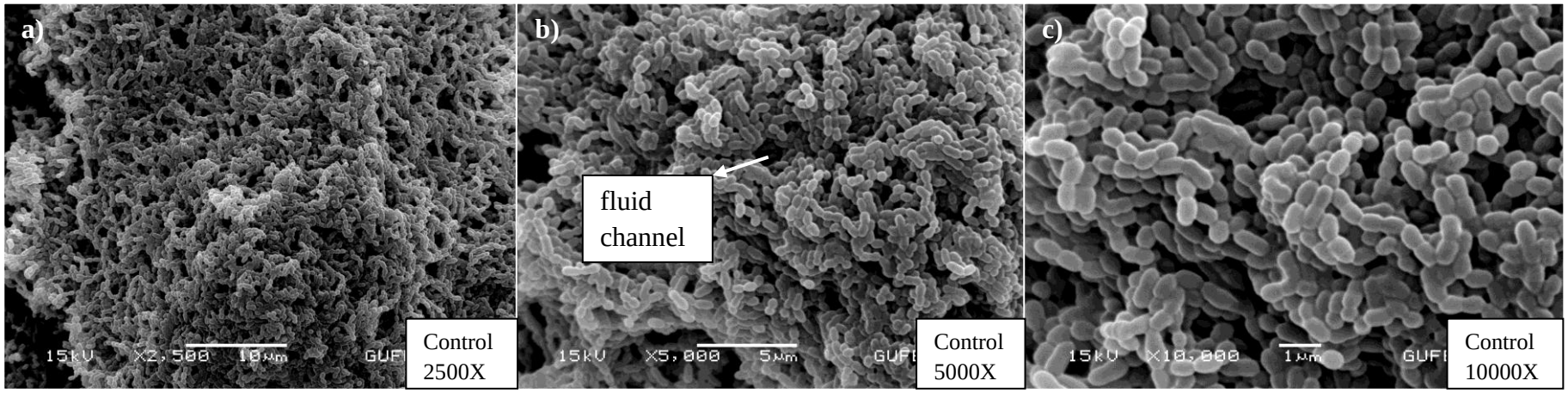

Figure 5.21. Scanning electron microscopy images of *A. actinomycetemcomitans* biofilms without essential oils

a. at 2500X b. at 5000 X c. at 10000 X

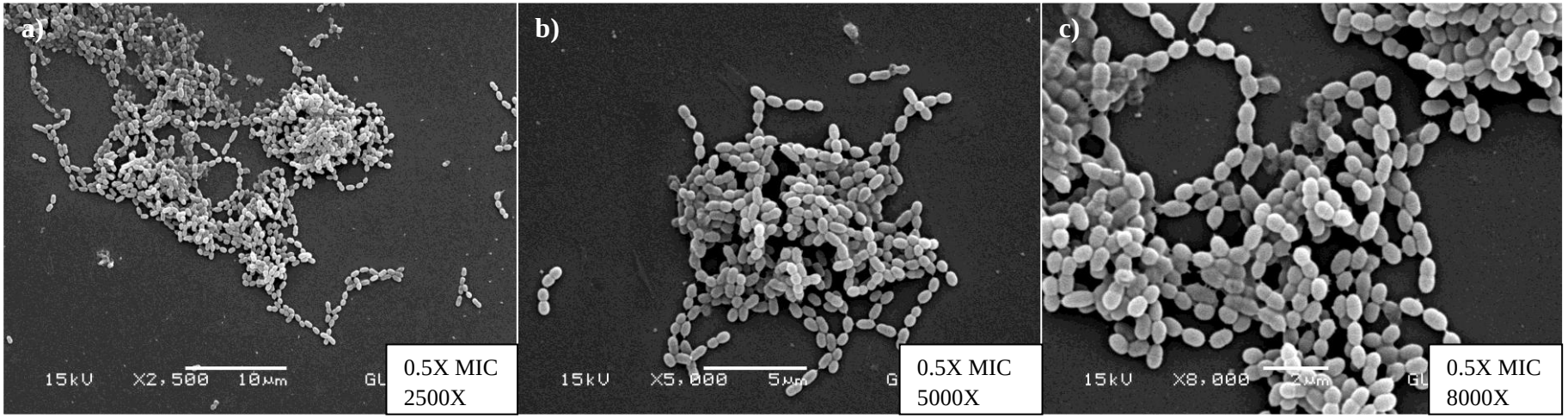

Figure 5.22. Scanning electron microscopy images of *A. actinomycetemcomitans* biofilms in the presence of 0.5X MIC Hojicha essential oil

a. at 2500X b. at 5000 X c. at 8000 X

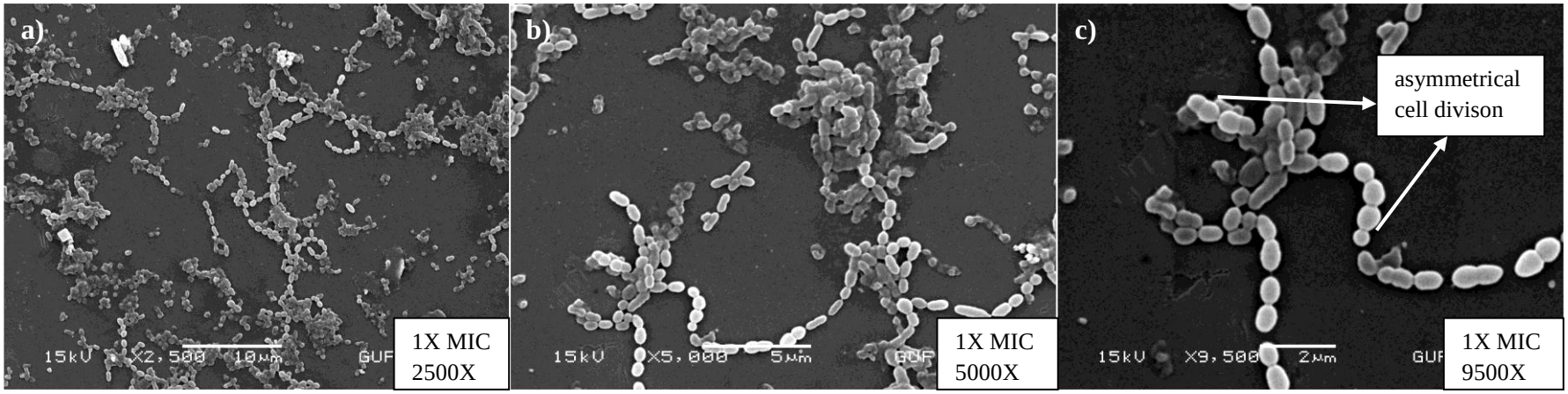

Figure 5.23. Scanning electron microscopy images of *A. actinomycetemcomitans* biofilms in the presence of 1X MIC Hojicha essential oil a. at 2500X b. at 5000 X c. at 9500 X

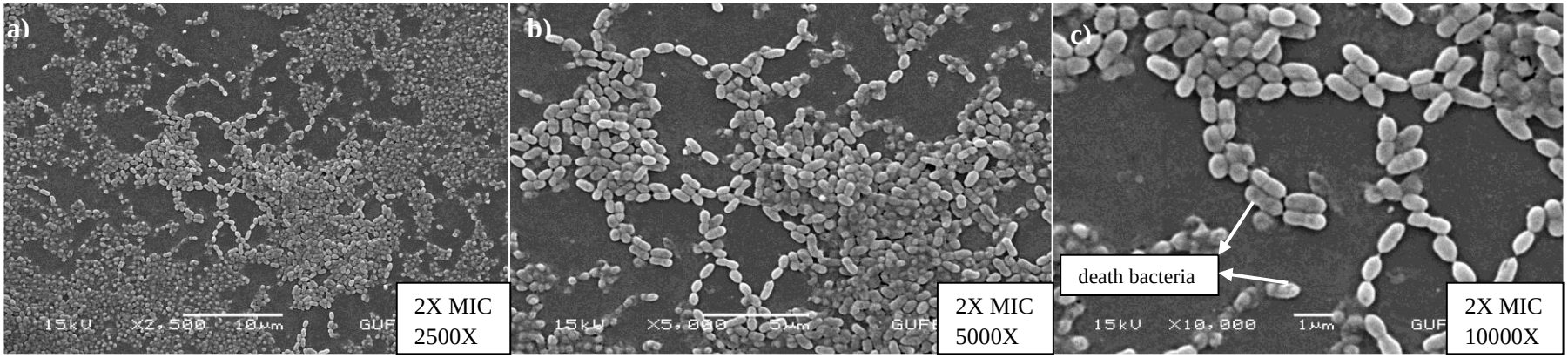

Figure 5.24. Scanning electron microscopy images of *A. actinomycetemcomitans* biofilms in the presence of 2X MIC Hojicha essential oil a. at 2500X b. at 5000 X c. at 10000 X

5.2. DISCUSSION

5.2.1. Chemical Composition of Fatty Acid Methyl Esters (FAME)

The therapeutic importance of each fatty acid detected in the studied plants was searched in the scientific literature and it was observed that these Asian herbs were rich in beneficial fatty acids. Therapeutic effects of some fatty acids include reduction of inflammation (eicosapentaenoic, arachidonic, linoleic and calendic acids), cytotoxicity to cancer cells (linoleic, calendic and α- parinaric acids), acne reduction (linoleic acid) and wound healing (calendic acid). Moreover, antibacterial and antibiofilm properties of individual fatty acids such as palmitic, stearic, oleic and linoleic acids [216, 217] have been detected in some previous researches. These properties gave the idea for them to be used against pathogen anaerobes that play a role in oral diseases. However, the ineffectiveness of crude lipid extracts and total fatty acid methyl esters of these plants against tested microorganisms directed the study to use essential oils extracted from three new herbs (Sencha and Genmaicha green teas and edible mushroom Shiitake) and two plants (Wakame and Hojicha) selected from previous FAME study.

5.2.2. Antimicrobial Activity

5.2.2.1. Disc diffusion assay

In the first part of the study, antimicrobial activity of crude lipid methanol extracts and fatty acid methyl esters of Asian herbs; Wakame (GS_w), Kombu (GS_{ko}), Nori (GS_n), Kukicha (GS_{ku}) and Hojicha (GS_h), against oral anaerobes was evaluated by the disc diffusion method and it was observed that neither crude lipid extracts nor FAMEs of studied plants exhibited antimicrobial activity. Antimicrobial studies of free fatty acids isolated from Asian herbs [218], however, showed antimicrobial activity. In two previous studies, free fatty acids [219] and α-parinaric acid (18:4 w3c) [220] isolated from Wakame were shown to possess antimicrobial activity on *B. subtilis* and *S. mutans*, respectively. The α-parinaric acid was also seen to be the predominant fatty acid in GS_w used in this study, However, this study found FAMEs of GS_w to have no antimicrobial activity against any of the test bacteria in disc diffusion assay. The different results might be due to the

lower concentrations of fatty acid in the total FAMEs in this research or due to the use of different extraction protocols of lipids.

Although crude lipid extracts of GS_{ko} did not exhibit any antimicrobial activity on oral bacteria, another study showed that at high concentrations of methanolic extracts of Kombu (*L. digitata*) of Irish origin had antimicrobial effects on food pathogens such as *L. monocytogenes, S. abony*, *E. faecalis* and *P. aeruginosa* [221].The ineffectiveness of extracts of GS_{ko} against test bacteria in this study might be explained by the use of anaerobes whose metabolic activities are different than aerobic bacteria.

No previous study could be found on antimicrobial effects of crude lipid extracts or fatty acid methyl esters of Nori, Kukicha and Hojicha. However, in a previous study conducted by Bancirova [222] the antimicrobial activity of infusion, rather than lipid extract, of green tea Hojicha on *Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa* were observed.

In the second part of the study, antimicrobial activity of essential oils of Asian herbs, Wakame (GS_w), Sencha (GS_s), Hojicha (GS_h), Genmaicha (GS_g) and Shiitake (GS_{sh}), against oral anaerobes were examined by the disc diffusion method.

While essential oils did not show a cidal effect on *S. mutans* by disc diffusion test, they were able to inhibit and kill the bacteria in broth at concentrations lower than that was loaded on the discs (Table 5.3). In many studies in the literature, the correlation of disc diffusion and MIC results were discussed and some studies showed that these tests do not always correlate 100 per cent [223, 224]. The unrelated results of disc diffusion and MIC tests observed in this study might occur due to the viscous nature of essential oils which might prevent complete diffusion of oil from the disc.

Although there is no study that tests volatile oils extracted from Wakame on any microorganisms including anaerobes, essential oils of algae species were studied against bacteria such as *Streptococcus faecalis, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Enterobacter cloacae, Escherichia coli and Salmonella typhimurium.* [185, 186]. In the study conducted by Karabay *et al* [185]

the volatile oil of a red algae was shown to be effective against *S.f aecalis, S. aureus, E. coli and S. typhimurium* while infective against *B. subtilis, S. epidermidis, P. aeruginosa* and *E. cloacae*. Another study [186] showed that the volatile oil from *Porphyra tenera* had an inhibitory effect on both *B. pyocyaneus* and *S. aureus*, but not on *B. subtilis* and *E. coli*.

Two previous studies [187, 188] showed similar results as in this study when antimicrobial activity of Sencha essential oil was observed. On the other hand, no study could be found in the literature showing antimicrobial effect of essential oil extracted from Genmaicha and Hojicha green teas and Shiitake mushroom. However, some previous studies presented the antimicrobial activity of solvent extracts of Shiitake, rather than essential oils, on several bacteria such as *M. luteus* and *B. cereus* [225] and oral pathogens *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *Porphyromonas* spp [226].

5.2.2.2. MIC and MBCs of essential oils of Asian herbs

The results of MIC indicated that all five essential oils could inhibit the growth of all microorganisms tested. However, as mentioned before, the results of disc diffusion and MIC results of essential oils on *S. mutans* did not correlate. For example, while *S. mutans* seemed to be resistant to Hojicha essential oil according to disc diffusion results (Table 5.2), the minimum inhibitory concentration of the same oil was very low (1.6 per cent, 2.8 mg/l), as shown in Table 5.3. This confusing result might occur due to inefficient dissolving capacity of the oil from blank discs. Moreover, essential oils having bacteriostatic effect on *S. mutans* according to disc diffusion test showed bactericidal activity in MBC results on *S. mutans* at concentrations lower than that was loaded in the disc. This might show that essential oils were more effective on bacteria when bacteria are in liquid culture than on agar surface. Similar results were obtained in previous studies [227, 228]. When Burt and her colleagues [227] showed that the essential oils carvacrol and thymol were less effective in the gelatin gel than in broth, Mahboubi and Farzin [228] noted that dissolution of *A. sieberi* essential oil in aqueous media was different from the agar layer.

The statement"when zone of growth inhibition decreases, MIC increases" presents the correct correlation between inhibition zone diameter and MIC value [229]. However,

Hojicha results for disc diffusion and MIC against *A. actinomycetemcomitans* and *F. nucleatum* did not obey this rule. Results in Tables 5.2 and 5.3showed that for *A. actinomycetemcomitans* and *F. nucleatum* MIC values of Hojicha essential oil (0.8 per cent, 1.4 mg/l for both oils) were lower than MICs of Sencha [3.1 per cent (5.8 mg/l) and 12.5 per cent (23.1 mg/l), respectively] and Shiitake [3.1 per cent (3.9 mg/l) and 25 per cent (31.2 mg/l), respectively] whose inhibition zones were higher than that of Hojicha. This might due to experimental error or due the lower dissolution capacity of oil from the disc as mentioned above.

Similar errors were also seen in MIC results of essential oil extracted from Wakame against *P. gingivalis*. Although Wakame displayed the smallest diameter of inhibition zone (11.1 mm), MIC value of this oil also had the lowest concentration (0.4 per cent, 1.0 mg/l). Results of disc diffusion and MIC tests of other oils for *P. gingivalis* correlated 100 per cent and essential oils displayed strong bactericidal effect against test bacteria (MIC values ranged between from 0.4 to 1.6 per cent).

The MIC results of positive control on tested pathogens were similar as found in previous studies. For instance, Wei *et al* [230] and Greenberg *et al* [231] showed that MIC of chlorhexidine gluconate against *S. mutans* were 1.3 mg/l and 2 mg/l, respectively which were not significantly different than our finding (1.5 mg/l). Moreover, two studies [231, 232] displayed MICs of chlorhexidine against *P. gingivalis* (8 mg/l), *F.nucleatum* (8 mg/l) and *A. actinomycetemcomitans* (4 mg/l) which were similar to findings of this study; 6 mg/l, 6 mg/l and 3 mg/l, respectively. Furthermore, the whole MIC results of chlorhexidine gluconate obtained in this study were in correlation with the general statement that Gram-positive bacteria are more susceptible to chlorhexidine than Gram-negative bacteria [233]. Here, it was shown that *S. mutans*, the only Gram-positive bacteria of this study, was affected the most by chlorhexidine gluconate.

5.2.3. Quantitative Biofilm Assays

5.2.3.1. Inhibition of biofilm formation by essential oils

The inhibitory effects on biofilm formation of essential oils extracted from Asian herbs were examined by microdilution and crystal violet staining methods.

Wakame and Sencha on *S.mutans*, exhibited biofilm inhibitory activity at their sub-MIC values (0.5X MIC) which indicated that these essential oils might have an effect on virulence factors that are required for biofilm formation by *S. mutans* and therefore, these two essential oils might be potential therapeutics for biofilm control during oral diseases, such as dental caries. Other extracted oils, however, were effective on biofilm formation of *S. mutans* only at their supra-MIC or MBC values measuring that their biofilm inhibitory effect was due to either bacteriostatic or bactericidal activity of the oils.

Biofilm formation by *A. actinomycetemcomitans,* on the other hand, was not inhibited by essential oils from both at their sub-MIC and supra-MIC concentrations. Inability of essential oils to inhibit biofilm formation by *A. actinomycetemcomitans* at sub-MIC concentrations might be explained by ineffectiveness of extracted oils on virulence factors required to form biofilms. Interestingly, low concentrations of Sencha (at 0.4 and 0.2 per cent) seemed to increase biofilm formation. Similar results were obtained in a previous study testing inhibitory effects of antibiotics on biofilm formation of *A. actinomycetemcomitans* [219], briefly, low concentration of erythromycin and ampicillin (0.1 mg/L) were shown to increase biofilm formation significantly compared with the controls.

Non- inhibitory effects of oils at supra-MIC concentrations on biofilm formation of *A. actinomycetemcomitans* might be explained in two ways: First explanation was briefly as follows: During experiments, it was observed that essential oils at high concentrations (50, 25 and 12.5 percentages) remained on the walls of the wells even after washing steps and they absorbed crystal violet dye, so dye could not be removed during washing steps. This excess crystal violet on those wells might increase the absorbance at 595 nm which resulted in more biofilm formation percentage than expected, even more than 100 per cent was sometimes observed. Moreover, planktonic bacteria may embed in these viscous essential oils at high concentrations and may not be removed during washings. This might also explain why percentage of biofilm formation was sometimes more than 100 per cent or why more biofilm formation percentage was obtained at higher concentrations than lower concentrations of the essential oils. This effect of viscous essential oils was also detected in the wells containing high concentrations of Genmicha and Shiitake oils on *F. nucleatum.* (Percentage of biofilm formation > 110 per cent). A second explanation could

be the transient inhibitory effect of essential oils on biofilm formation. Cells might attach to the surface of the wells and although essential oil may reduce the growth or cause detachment in the biofilm, since essential oils are volatile compounds, the effect of oil may decrease over time and biofilm might be reformed.

Essential oils were found to be ineffective against formation of biofilms by *F. nucleatum.* MIC and MBC results also showed that killing/ inhibition of planktonic cells of *F. nucleatum* require high concentrations oils. This partial resistance of planktonic and full resistance of biofilm mode of *F. nucleatum* against essential oils might be explained by presence of some specific protein, acriflavin resistance protein, which can protect bacterium from hydrophobic inhibitors [234], such as essential oils.

It was observed that the pattern of inhibition of mixed bacteria biofilms were similar to that of *S. mutans biofilms* by essential oils, i.e., multi-species biofilms were sensitive only to high folds of MICs of oils. Since *S. mutans* is the first colonizing bacteria in oral biofilm formation, if its attachment and/or biofilm formation was inhibited by essential oils, other bacteria (secondary and late colonizers) could not attach on *S. mutans* and therefore further biofilm development might be prevented. Similar results were displayed in a study of Arslan *et al.* [235] in which lactoferrin was showed to inhibit the initial attachment of *S. gordonii*, an initial colonizer, but not that of *F. nucleatum* and *P. gingivalis*. However, attachment of multi-species biofilm composed of two oral bacteria, *S. gordonii* and *F. nucleatum* or *S. gordonii* and *P. gingivalis*) was significantly reduced in the presence of lactoferrin.

When biofilm formation inhibitory effects of essential oils on each tested bacteria were compared, it can be noted that despite *F. nucleatum* and *S. mutans* had similar MIC values for Sencha (12.5 per cent, 23.1 mg/l), biofilm formation by *F. nucleatum* was not affected from Sencha significantly while biofilm formation of *S. mutans* was reduced by > 61 per cent in the presence of Sencha oil. Biofilm formation of *S. mutans* at high MIC of Wakame and Sencha was inhibited to a greater extent than that of *A. actinomycetemcomitans* and *P. gingivalis* with lower MIC values for the same oils.

Results of previous studies showing the effect of chlorhexidine gluconate on *S. mutans* [236], *F. nucleatum* and *P. gingivalis* [184] biofilm formation supported our findings; chlorhexidine gluconate at 0.1 per cent concentration reduced biofilm formation of first two bacteria while it did not affect biofilm formation by the later bacteria [184].

Sensitivity of *A. actinomycetemcomitans* biofilm formation against chlorhexidine was shown in a study by Haase *et al*. [237] in which chlorhexidine at 9.6 mg/l and 0.6mg/l concentrations effectively inhibit biofilm formation of two strains of *A. actinomycetemcomitans*. Conflicting results in study of Hasee *et al*. and in this thesis might be explained by use of different strains of bacteria. Chlorhexidine gluconate was shown to inhibit biofilms composed of multi-species in a previous study [238] in which higher concentrations of chlorhexidine gluconate (16-40 mg/l) was necessary to inhibit mixed culture biofilms by 90 per cent. However, in our study we showed that even 1.5 mg/ml concentration of chlorhexidine was enough to reduce biofilm formation of multi- bacteria by the same amount. Yanti *et al.* used a mixture of *S. mutans, S. sanguis* and *A. viscous* cells to form multi species biofilms, while *S. mutans*, *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis* were used in this study. The different composition of multi-species biofilms might explain the different susceptibility of biofilms to chlorhexidine.

5.2.3.2. Disruption of pre-formed biofilms by essential oils

External agents such as antibiotics, detergents or biocides can disrupt pre-formed biofilms at higher MIC values than killing of planktonic cells in suspension [191, 214]. Therefore, in addition to sub-MIC values, disruptive effects at MIC and 2X MIC values of essential oils were also assumed significant.

Essential oils extracted from Asian herbs did not show disruptive effect on mature biofilms of *S. mutans* even at high-folds of MIC values. It is generally known that, Gram negative bacteria are more resistant to antimicrobial compounds due to the presence and features of outer membrane, namely, extracellular lipopolysaccharide (LPS) layer [239]. However, this extracellular layer may also increase the susceptibility of Gram negative bacteria to some agents, such as small terpenoid and phenolic compounds found in essential oils extracted from herbs, which cause disintegration and release of the LPS layer [168, 240].

All extracted oil, except Genmaicha, could destruct pre-formed biofilms of *A. actinomycetemcomitans* at their sub-MIC values, which may not the bactericidal or bacteriostatic effect of oils rather some pathways or processes induced by these essential oils may cause extracellular matrix or detachment of cells from biofilms.

Hojicha volatile oil at 50 per cent concentration seemed to increased biofilm of *A. actinomycetemcomitans*. The enhanced biofilm formation in the presence of essential oil may indicate the stress response and so the survival mechanism of the bacteria. It is also possible that essential oil may increase the uptake of nutrients into the biofilm matrix by increasing the permeability of biofilm matrix [192].

Significant disruptive effects of essential oils on mature *P. gingivalis* were detected at folds of MICs which indicated that cidal effects of essential oils were the most probable reasons for disruption of established biofilms of *P. gingivalis*.

When all the results showing destruction of pre-formed single species biofilms in the presence of essential oils were considered, it can be said that essential oils were highly effective against mature biofilms and most of the time they can disrupt these biofilms at sub-MIC concentrations. As mentioned above, established biofilms usually exhibit resistance to disinfectants and MIC concentrations required to destroy pre-formed biofilm are many times higher than those required for planktonic cells. In the literature, some possible mechanisms of resistance of mature biofilms to drugs were cited as [241]:

1. limited penetration of drugs inside the exopolymer matrix
2. a decreased growth rate or nutrient limitations
3. expression of resistance genes, such as genes encoding efflux pumps

Since our findings showed the significant activity of essential oils on mature biofilms, it may be said that essential oils were able to overcome resistance mechanisms and so they showed strong disruption activity on biofilms even at 0.5X of the MIC of planktonic cells. Another explanation of this disruptive activity of essential oils on mature biofilms may be explained by the hydrophobic nature of extracted oils which enables diffusion of oils

through the polysaccharide matrix of the biofilm and then oils destabilize biofilm due to their strong intrinsic antimicrobial properties [242].

The inability of essential oils to destruct the pre-formed multi-species biofilms was observed even at their supra-MIC values. This shows that biofilms become more resistant to essential oils formed by multiple microorganisms [243]. The penetration capability of oils may be limited through multi-species biofilms. Moreover, the insufficiency of oils to disrupt biofilms of first colonizer, i.e., *S. mutans*, might be the reason of development of biofilms in the presence of essential oils. Furthermore, since we did not examine the composition of multi-species biofilms under scanning electron microscope, the biofilms that we assumed as mix biofilm may just contain *S. mutans* bacterium since essential oils may kill most of the other bacteria in the biofilm.

In this study, similar results were found to previous studies which tested chlorhexidine gluconate on mature biofilms of single or multi-species. For instance, a large effect and small effect of chlorhexidine on developed biofilms of *A. actinomycetemcomitans* [244] and *F. nucleatum* [245], respectively were detected,. Moreover, the effect of chlorhexidine on artificial mature biofilms was reported as minor and superficial since it could only disrupt 6-hours old biofilms while it was ineffective against 24 and 48 hours-old mature multi-species biofilms [246]. In another study [247], it was also shown that 0.12 per cent chlorhexidine gluconate mouthrinse had little antiplaque and antigingivitis effect on mature biofilms.

Comparison of effects of essential oils on early biofilm inhibition and preformed biofilm disruption for each bacteria showed that essential oils had significant effect either on formation of early biofilms or on eradication of pre-formed biofilms. For instance, while essential oils could not inhibit biofilm formation of *A. actinomycetemcomitans* and *F. nucleatum* at MIC or sub-MIC values, they could disrupt mature biofilms of these two bacteria even at their sub-MIC concentrations. This might be explained by the effect of oils on a component or a protein that is found only in mature biofilms and absent in early-formed biofilms. On the other hand, early biofilm formation of *S. mutans* was inhibited by sub-MIC concentrations of Wakame and Sencha oils, but none of the other four extracted oils could disrupt pre-formed biofilms of mutans streptococci. This result may show that

these essential oils may affect some virulence factors that play a role in early biofilm formation; i.e., once the biofilm was established, they became ineffective.

6. CONCLUSION

In the present study, essential oils were extracted from five traditional Asian plant, namely Wakame, Sencha, Hojicha, Genmaicha and Shiitake, and their activities against four oral pathogens and biofilms formed by these species were examined.

All tested essential oils exhibited antibacterial activity against all tested bacteria species, except against *S. mutans* whose growth was not inhibited, but slowed down by the action of essential oils. Moreover, biofilm formation by *S. mutans* and mix bacteria were inhibited significantly by all five essential oils. However, only Wakame and Sencha could inhibit *S. mutans* biofilm formation at their sub-MC concentrations which showed that anti-biofilm activity of these two oils were not because of bacteriostatic or bactericidal activity, but they may affect *S. mutans* virulence factors that are required to form biofilms. Therefore, these Wakame and Sencha essential oils may be used as an ingredient of a toothpaste or mouthwash for prevention of dental caries.

On the other hand, essential oils had strong disruptive effects on pre-formed biofilms of *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis* even at their sub-MIC values which were also confirmed by scanning electron microscopy. However, none of the essential oil could disrupt the mature multi-species biofilm. Therefore, they may not suitable for the treatment of periodontal diseases. Actually, to suggest the use of essential oils as preventative and/or therapeutic agents against sub- and supragingival plaque in the mouth, it is necessary to test them on artificial plaques obtained from gingivitis or periodontitis patients which will give more realistic result.

In conclusion, antimicrobial and anti-biofilm activities of Wakame, Sencha, Hojicha, Genmaicha and Shiitake essential oils, which have not been studied before, against oral pathogens and biofilms were detected in this thesis.

7. FUTURE STUDIES

The mechanism of oral antibiofilm activity of each essential oil is planning to be found by several extra tests. The viability of bacterial species in biofilm matrix after exposure to the essential oil will be analyzed to compare the minimum inhibitory concentrations of planktonic and biofilm associated bacteria. Moreover, XTT assay, a measure of metabolic activity of bacteria, is planning to be applied to understand how essential oils affect biofilm metabolism. Fatty acid composition of membrane of the bacterial cells in biofilm before and after essential oil treatment should be analyzed by FAME analysis and gas chromatography to determine the membrane dissolving and fatty acid disruption effects which were assumed to be possible biofilm inhibition mechanisms of essential oils.

Chemical composition of essential oils will also be analyzed to detect the constituents which may be the reason of the antibacterial and antibiofilm activities of each essential oil.

Moreover, cytotoxicity tests should be done to prove that essential oils do not have toxic effects on healthy human cells before these substances can reliably be used in commercial applications.

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