

**EVALUATION OF SYNERGISTIC ANTIPLAQUE  
ACTIVITY OF *SALVADORA PERSICA L.* AND  
*CAMELLIA SINENSIS VAR. ASSAMICA*: A  
COMPARATIVE CLINICAL STUDY**

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**THESIS SUBMITTED IN FULFILMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF  
PHILOSOPHY/ DENTISTRY**

**FACULTY OF DENTISTRY  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2017**

**UNIVERSITY OF MALAYA**  
**ORIGINAL LITERARY WORK DECLARATION**

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Name of Degree: DOCTOR OF PHILOSOPHY

Title of Project Thesis: EVALUATION OF SYNERGISTIC ANTIPLAQUE  
ACTIVITY OF *SALVADORA PERSICA L.* AND *CAMELLIA SINENSIS VAR.*  
*ASSAMICA*: A COMPARATIVE CLINICAL STUDY

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## ABSTRACT

Green tea, non-fermented leaves of *Camellia sinensis* var. *assamica*, is widely consumed as healthy beverage since thousands of years in Asian countries. Chewing sticks (miswak) of *Salvadora persica* L. are traditionally used as natural toothbrush to ensure oral health in developing countries. Both green tea and *Salvadora persica* L. extracts were reported to have antibacterial activity against many dental plaque bacteria. However, their combination has never been tested to have antibacterial and antiadherence effects against primary dental plaque colonizers, playing an initial role in the dental plaque development, which was investigated in this study. Thus, the aim of this study was to evaluate the synergistic antiplaque activity of the combination of green tea and *Salvadora persica* L. aqueous extracts both *in vitro* and *in vivo*.

In the *in vitro* part of the study, two-fold serial micro-dilution method was used to measure minimal inhibitory concentration (MIC) of aqueous extracts of green tea, *Salvadora persica* L. and their combinations. Adsorption to hexadecane was used to determine the cell surface hydrophobicity (CSH) of bacterial cells. Glass beads were used to mimic the hard tissue surfaces, and were coated with saliva to develop experimental pellicle for the adhesion of the primary colonizing bacteria. While in the *in vivo* part of the study, a double-blinded, randomized, 24 hours plaque re-growth crossover clinical trial was carried out involving 14 participants who rinsed with test (combination of green tea and *Salvadora persica* L. aqueous extracts), 0.12% chlorhexidine (CHX) and placebo mouth rinses for 24 hours. A week before the trial, all participants received scaling, polishing and oral hygiene instruction. On the trial day, the participants received polishing at base line and rinsed with 15 ml of randomly allocated mouth rinse twice a day without oral hygiene measures. After 24 hours, plaque index was scored and then the participants entered a six days washout period with usual

oral hygiene measures after which they repeated the same protocol for the following two mouth rinses.

*In vitro* results have revealed that green tea aqueous extracts exhibited better antiplaque effect than *Salvadora persica L.* aqueous extracts. Their combination, equivalent to 1/4 and 1/2 of MIC values of green tea and *Salvadora persica L.* aqueous extracts respectively, showed synergistic antiplaque properties with fractional inhibitory concentration (FIC) equal to 0.75. This combination was found to significantly reduce CSH ( $p < 0.05$ ) and lower the adherence ability ( $p < 0.003$ ) towards experimental pellicle. *In vivo*, significant differences were found between mouth rinses by means of plaque index. Test mouth rinse significantly reduced plaque accumulation when compared with placebo and CHX mouth rinses. CHX mouth rinse non-significantly reduced plaque accumulation when compared with placebo.

In conclusion, combination between green tea and *Salvadora persica L.* aqueous extracts exhibited synergistic antiplaque activity against primary dental plaque colonizers *in vitro*. Rinsing with 15 ml of this combination twice daily has a significant antiplaque effect better than CHX for a 24 hours period *in vivo*; therefore it could be utilized as a natural alternative mouth rinse to CHX.

## ABSTRAK

Teh hijau, daun *Camellia sinensis* var. *assamica* yang tidak dieram, telah digunakan secara meluas sebagai minuman kesihatan sejak ribuan tahun di kalangan negara-negara Asian. Kayu sugi (miswak) *Salvadora persica* L. telah digunakan secara tradisional sebagai berus gigi untuk menjamin kesihatan oral di negara-negara membangun. Kedua-dua ekstrak teh hijau dan *Salvadora persica* L. secara berasingan telah dilaporkan mempunyai aktiviti antibakteria terhadap bakteria plak gigi. Walaubagaimanapun, kombinasi kedua-dua ekstrak tidak pernah diuji untuk kesan antibakteria dan antilekatan terhadap koloni utama plak gigi, yang memainkan peranan awal di dalam pembentukan plak gigi, telah diuji di dalam kajian ini. Oleh itu, tujuan kajian ini adalah untuk menilai kesan sinergistik aktiviti antiplak oleh kombinasi ekstrak akuas teh hijau dan *Salvadora persica* L. secara *in vitro* dan *in vivo*.

Di dalam kajian *in vitro*, kaedah pencairan bersiri (gandaan dua) telah digunapakai untuk menyukat konsentrasi minima yang menghalang (MIC) ekstrak akuas teh hijau, *Salvadora persica* L. dan kombinasi keduanya. Penjerapan kepada hexadecane telah diguna untuk menentukan hidrofobisiti permukaan sel (CSH) untuk sel bakteria. Bebola kaca telah digunakan sebagai mimik permukaan tisu keras, dan telah disaluti dengan air liur bagi membentuk pelikel ekperimental sebagai lekatan untuk koloni bakteria utama. Sementara itu di dalam bahagian *in vivo*, kajian melibatkan teknik ‘double blinded’ secara rawak, dalam tempoh 24 jam pembentukan plak secara percubaan klinikal silang telah dijalankan ke atas 14 peserta yang menggunakan ubat kumuran ujian (kombinasi ekstrak akuas teh hijau dan *Salvadora persica* L.), 0.12% CHX dan plasebo selama 24 jam. Seminggu sebelum percubaan, semua peserta menerima penskaleran, pengilapan dan arahan oral higin. Pada hari percubaan, semua peserta menerima pengilapan dan diminta berkumur dengan 15 ml ubat kumuran secara rawak sebanyak 2 kali tanpa

sebarang penjagaan kebersihan mulut. Selepas 24 jam, indek plak telah diambil dan peserta memasuki tempoh 'wash out' selama 6 hari tanpa penjagaan kebersihan mulut dan protokol yang sama diulang untuk 2 ubat kumuran berikutnya.

Hasil *in vitro* menunjukkan ekstrak akuas teh hijau telah memberi lebih kesan antiplak daripada ekstrak *Salvadora persica* L. Kombinasi keduanya, iaitu 1/4 dan 1/2 nilai MIC ekstrak akuas teh hijau secara respektif, menunjukkan kesan sinergistik antiplak dengan 'fractional inhibitory concentration' (FIC) bernilai 0.75. Kombinasi ini telah menurunkan CSH secara signifikan ( $p < 0.05$ ) dan mengurangkan kebolehekatan ( $p < 0.003$ ) terhadap pelikel experimental. Secara *in vivo*, perbezaan yang signifikan telah diperolehi di antara ubat kumuran melalui pengukuran plak index. Ubat kumuran ujian telah mengurangkan plak terkumpul secara signifikan apabila dibandingkan dengan plasebo and CHX. Ubat kumuran CHX telah menurunkan plak terkumpul secara tidak signifikan bila dibandingkan dengan placebo.

Secara rumusan, kombinasi ekstrak akuas teh hijau dan *Salvadora persica* L. telah menunjukkan kesan sinergistik aktiviti antiplak terhadap bakteria utama plak gigi secara *in vitro*. Berkumur dengan 15 ml kombinasi ekstrak sebanyak 2 kali sehari dapat memberi kesan antiplak yang signifikan berbanding dengan CHX untuk tempoh 24 jam secara *in vivo*; oleh itu ianya boleh digunakan sebagai ubat kumuran semulajadi sebagai alternative kepada CHX.

## ACKNOWLEDGEMENTS

This research would not have been successful without the support and help from many parties.

Syukur Alhamdulillah with ALLAH's blessings this research could be completed. It is nonetheless a product of interaction and timeless hard work of my supervisors, and friends who helped me through all the way. For this reason, I express my deepest gratitude to all the contributors for their comments, criticisms, questions, support and encouragement. Regrettably, but inevitably, the following list of names is incomplete and I hope that those who are missing will forgive me, and accept my sincere appreciation of their influence on my work.

My deepest gratitude is to my supervisors, Associate Prof. Dr. Nor Adinar Baharuddin and Associate Prof. Dr. Wan Harun Himratul-Aznita who supervised me through the PhD research. I have been amazingly fortunate to have supervisors who gave me the freedom to explore on my own and at the same time the guidance to recover when my steps faltered. Their patience and support helped me overcome many difficult situations towards the completion of this thesis. I am deeply grateful to them for the long discussions that helped me sort out the details of my work.

Most importantly, none of this would have been possible without the love and patience of my lovely wife Enas, my kid Mohammed, my newborn Ameen, parents, and brothers to whom this thesis is dedicated to.

I am grateful to Dr. Nor Lidhe for her statistical consultation at different stages of my research. I am also indebted to the staff of the Balai Ungku Aziz Research Laboratory and Postgraduate Clinics, in Faculty of Dentistry / University of Malaya.

I am thankful to my friends Alfaisal, Pn Zaleha, Firdaus, Shahril Amin and Dr Mazin Saleh who helped me through the hurdles that came during my study and to many other friends who have helped me stay sane through these difficult years.

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## LIST OF SYMBOLS AND ABBREVIATIONS

$\mu$	: Micro
$\mu\text{M}$	: Micromole
$^{\circ}\text{C}$	: Degree centigrade
<i>A. actinomycetemcomitans</i>	: <i>Aggregatibacter actinomycetemcomitans</i>
ATCC	: American Type Culture Collection
BHI	: Brain Heart Infusion
BUARL	: Balai Ungku Aziz Research Laboratory
CG	: (–)-catechin gallate
CONSORT	: Consolidation Standards of Reporting Trials
CSH	: Cell surface hydrophobicity
DTC	: Dilute test combination
DTC1	: Dilute test combination 1
DTC2	: Dilute test combination 2
EC	: (–)-epicatechin
ECG	: (–)-epicatechin gallate
EGC	: (–)-epigallocatechin
EGCG	: (–)-epigallocatechin gallate
FIC	: Fractional inhibitory concentration
G	: Gram
GC	: (+)-gallocatechin
GCG	: (–)-gallocatechin gallate
Gt	: Green tea
Kg	: Kilogram
L	: Liter



Mg	: Milligram
MIC	: Minimum inhibition concentration
MIC <sub>gt</sub>	: Minimum inhibition concentration of green tea
MIC <sub>sp</sub>	: Minimum inhibition concentration of <i>Salvadora persica L.</i>
ml	: Milliliter
NAM	: Nordini's Artificial Mouth
OD	: Optical density
OHE	: Oral hygiene education
PBS	: Phosphate buffer saline
PCR	: Polemerase chain reaction
PI	: Plaque Index
SD	: Standard deviation
Sp	: <i>Salvadora persica L.</i>
SWS	: Stimulated whole saliva
TC	: Test combination
TC1	: Test combination 1
TC2	: Test combination 2
TC3	: Test combination 3

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## CHAPTER 1: GENERAL INTRODUCTION

Dental plaque is the soft biofilm deposit that develops on hard surfaces in the oral cavity, including tooth surfaces (Allison et al., 1995). It is composed of over 500 bacterial species which adhere to tooth surfaces, proliferate and interact with each other within the dental plaque leading to plaque maturation (Rosan et al., 2000). The dental plaque is classified into two categories namely: supragingival plaque and subgingival plaque. Supragingival plaque is located at and coronal to the dento-gingival junction. Subgingival plaque is located apical to the dento-gingival junction which is usually divided into tooth adherent zone, epithelial adherent zone and non-adherent zone in the tooth gingival crevice (Chetrus et al., 2013). Dental plaque may be clinically recognized on teeth as a white, grayish, or yellow mass with a globular appearance after one day with no oral hygiene measures (Manganiello et al., 1977). In the periodontal health, oral bacteria cause no damage to either the bacteria or the host periodontal tissues. Any disruption to this state of balance causes alterations in both the host periodontal tissues and biofilm bacteria and results ultimately in the destruction of the periodontium (Newman et al., 2012).

The preventive measure that aims to remove dental plaque and prevent it from recurring is termed as plaque control which can be accomplished either mechanically or chemically or both procedures are combined in some cases (Axelsson et al., 1981). Mechanical plaque control is the manual removal of supragingival plaque. Toothbrushes are the most adopted tool for teeth cleaning in mechanical plaque control (Perry et al., 2001), but they do not clean interproximally in most cases so their role in teeth cleaning is accomplished with at least one additional interproximal cleaning aids such as tooth picks and interdental brushes (Graves et al., 1989).

Mechanical plaque control is not properly performed by most individuals despite its essential and important role in the prevention of gingivitis and periodontitis (Teles et al., 2009). Most dentate adults who claim to brush their teeth on a regular basis have visible plaque (Morris et al., 2001). Brushing techniques are particularly limited in their access to interproximal plaque of molar and pre-molar areas, and the control of supragingival biofilm accumulation on these areas requires the use of other devices such as interproximal cleaning aids (Axelsson et al., 2002). It was reported that low percentage of individuals use daily interproximal cleaning aids (Bakdash, 1995; Crocombe et al., 2012). Mechanical plaque control procedures focus solely on the hard surfaces of the oral cavity which only represent 21 - 23% of the total area of the oral cavity (Kerr et al., 1991). In fact, gingivitis and periodontitis can develop from microbial plaque accumulated on soft oral tissue that serves as a source of bacteria to colonize tooth surface (Socransky et al., 2005). On the other hand, epidemiological studies have reported high prevalence of gingivitis among school children, adolescents, adults and older individuals (Ismail et al., 1990; Dhar et al., 2007). Thus, in an attempt to resolve the limitation of the mechanical plaque control and high prevalence of gingivitis, chemical plaque control may be used as an adjunctive measures to mechanical plaque control (Sugano, 2012) as the use of these requires minimal skill (Al-Bayaty et al., 2010).

The chemical plaque control is an adjunct to an established oral care measures rather than a substitute for tooth brushing and other interdental cleansing methods (Claffey, 2003). On the other hand, there are certain situations in which individuals are unable to carry out mechanical plaque control measures and thus chemical plaque control may be essential to remove their dental plaque. Such situations include individuals with postoperative oral and periodontal surgeries, mental and/or physical disability and facial trauma with or without inter-maxillary fixation (Oppermann et al.,

2010). Chemotherapeutic agents could reverse or inhibit gingivitis by reducing dental plaque to a threshold below of that causing periodontal disease or alteration to the dental plaque bacterial composition in such a way that health status would not turn into disease (Mhaske et al., 2012). Among these chemotherapeutic agents, mouth rinses, such as chlorhexidine (CHX), provide a convenient way to enhance plaque control by mechanical plaque control measures (Jones, 1997). CHX mouth rinse is the most effective antiseptic oral rinse for dental plaque inhibition and prevention of gingivitis (Ribeiro et al., 2007). It is a broad-spectrum antiseptic with pronounced antimicrobial effects on gram negative and gram positive bacteria as well as on fungi and some viruses (Rölla et al., 1975). CHX is a positively charged bisbiguanide that can absorb to different negatively charged intraoral sites such as salivary pellicle on tooth surfaces, and many components of the biofilm on the tooth surfaces. Two most common concentrations of CHX mouth rinses which are commercially available and extensively used are 0.2% and 0.12%. In oral use as a mouth rinse, CHX has been reported to have a number of side effects including: brown discoloration of the teeth, some restorative materials and mucosa, bitter taste, sometimes sloughing of oral mucosa (Helldén et al., 1981; Addy et al., 1995), unilateral or bilateral swelling of the parotid gland and enhanced supra-gingival calculus formation which restricts its general use (Kapoor et al., 2011). These side effects of CHX may encourage many researchers to find an alternative mouth rinse with less side effects and comparable antiplaque efficacy.

Chewing sticks were used by the ancient populations for cleaning teeth (Chaurasia et al., 2013). Among at least 182 plant species suitable for preparing these chewing sticks, the most common plant is *Salvadora persica* L. (Elvin-Lewis, 1982). *Salvadora persica* L. is a small tree with a crooked trunk whose stems and roots are spongy that can easily be crushed between the teeth and become soft when soaked in water (Almas et al., 2004).

*Salvadora persica* L. chewing sticks were recommended by the World Health Organization who encouraged researchers to carry out further research on their biological activities on oral health (Löe, 2000). *Salvadora persica* L. has a high antibacterial activity against oral bacteria (Sofrata et al., 2011a; Sofrata et al., 2011b; Chelli-Chentouf et al., 2012; Ahmad et al., 2013). This antimicrobial activity is attributed to biologically active compounds present in *Salvadora persica* L. (Akhtar et al., 1981; Tenovuo et al., 1981; Darout et al., 2000b). However, CHX remains the gold standard oral antimicrobial agent as compared with *Salvadora persica* L. mouth rinse which exhibits lower antimicrobial activities (Moeintaghavi et al., 2012). On the other hand, herbal medicines are safer with negligible side effects, and low mammalian toxicity. They are also user friendly (Deshpande et al., 2011).

Tea plant is a small bushy plant about 3 to 4 feet high. It is recognized as *Camellia sinensis* (L.) Kuntze (family: *Theaceae*) by botanists (Graham, 1992). It has two main varieties including *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica*. Generally, *Camellia sinensis* var. *sinensis* is mainly grown in Japan, China and Taiwan, whereas *Camellia sinensis* var. *assamica* is predominately grown in South and Southeast Asian countries including Malaysia and Australia (Chan et al., 2007). Leaves of *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica* are used to produce 3 main types of tea according to the fermentation degree of the plant's leaves during processing. These main types include non-fermented, semi-fermented, and fermented tea which are known as green tea, oolong tea, and black tea respectively (Bancirova, 2010).

Polyphenols are present in green tea and mainly include catechins. Major catechins are (–)-epicatechingallate (ECG), (–)-epicatechin (EC), (–)-epigallocatechin (EGC) and (–)-epigallocatechingallate (EGCG), and these are thought to be responsible

for the health benefits that have traditionally been attributed to green tea. It was reported that green tea polyphenols exhibit antibacterial activity against gram positive and gram negative bacteria (Otake et al., 1991; Makimura et al., 1993; Arakawa et al., 2004; Yoda et al., 2004; Shimamura et al., 2007; Forouzanfar, 2011). An earlier epidemiologic study showed daily intake of green tea is inversely associated with periodontal disease. It was suggested that daily intake of green tea at meals and breaks is an easy habit to maintain a healthy periodontium (Kushiyama et al., 2009).

This study was designed to investigate the synergistic antiplaque effect of a combination between green tea, non-fermented leaves of *Camellia sinensis* var. *assamica*, and *Salvadora persica* L. aqueous extracts and to formulate a mouth rinse of this combination which is hypothesized to exhibit a significant antiplaque efficacy over a period of 24 hours.

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Dental plaque

The oral cavity is moist and warm, and can support the growth of various microorganisms such as bacteria, viruses, fungi, archaea, mycoplasma, and protozoa. These microbes colonize tooth (non-shedding) and mucosal (shedding) surfaces in the oral cavity to form a 3-dimensional, structurally organized, multispecies soft deposit that is termed as biofilm. This results in accumulation of large number of matrix embedded with oral microorganisms that are adhered to each other or/and to oral surfaces, especially at stagnant and hard-to-reach sites, unless subjects practice effective plaque control measures (Wilson, 2005; Marsh et al., 2009).

The metabolic activity and distribution of resident oral microbes are influenced by a number of environmental factors. The mouth temperature is maintained at around 35 – 37 °C. This favours the growth of a wide range of oral microbes. During inflammation, temperature increases at subgingival sites, and this can result in alteration of bacterial gene expression that favours the growth and protease activity of some periodontal pathogens within the subgingival plaque (Marsh et al., 2011). Generally, oral bacteria are either facultative or obligate anaerobic. Their distribution is generally related to the redox potential which is the measure of the degree of oxidation–reduction at a site. In a healthy mouth, the gingival crevice has the lowest redox potential, and thus harbours more obligate anaerobes (Kenney et al., 1969). Within dental plaque, facultative anaerobic species can grow in aerobic sites by existing in close partnership with oxygen-consuming species. Moreover, many oral anaerobes release enzymes that scavenge the low levels of oxygen in the environment to enable them to survive.

The intraoral pH is another major determinant of bacterial metabolism and distribution. It is maintained at around neutrality by the buffering activity of saliva. This



condition is suitable for the growth of members of the resident oral microbiota. Changes in intraoral pH frequently occur, and these drive major shifts in the proportions of bacteria within dental plaque such as decreased intraoral pH after sugar consumption (Marsh et al., 2009) and increased pH within gingival crevices during gingival inflammation (Eggert et al., 1991). Gingival crevicular fluid and saliva also have a major effect on bacterial distribution by providing potential nutrients for oral microorganisms such as amino acids, proteins and glycoproteins.

The lifestyle of an individual can also affect the metabolism and distribution of oral bacteria such as frequency of intake of fermentable carbohydrates and smoking. Other factors affecting the composition of the oral microorganisms include age and female hormonal changes during pregnancy. Generally, the microbial composition of the dental plaque at any site remains stable over time once it is established, unless a main perturbation occurs in key environmental determinants. This perturbation will lead to a shift in the balance of the microbiota and thus increase the risk of disease (Lang et al., 2015).

Biofilm formed on teeth surfaces is referred to as dental plaque. It contains multiple species of oral microorganisms that engage in various metabolic, physical, and molecular interactions, and form a microbial community. This community lifestyle provides many benefits to the participating microorganisms. This can be seen, for example, in the metabolism of primary colonizers that alters the local environment which, in turn, facilitates the attachment and growth of later colonizing species. In the microbial community, there is an increased metabolic efficiency and diversity, and an enhanced tolerance to environmental stress, host defenses, and antimicrobial agents. Some microbial species within the microbial biofilm can produce neutralizing enzymes ( $\beta$ -lactamase, catalase, IgA protease, etc.) that protect inherently susceptible other

species organisms from inhibitors. Also, horizontal gene transfer is more efficient in multispecies biofilm. Microbial communities could also provide physical protection from phagocytosis to microbial cells deep within biofilm. Microorganisms that individually cannot cause disease are capable to do so when they are present in microbial community such as dental plaque (pathogenic synergism). Thus, the microbial community properties are more than the sum of its microbial species cells (Lang et al., 2015).

### **2.1.1 Structure of dental plaque**

Dental plaque is heterogeneous in structure. There is profound evidence about the presence of open fluid-filled channels running through the plaque mass. Various nutrients make contact with the attached microcolonies by diffusion from the water channels rather than from the intercellular matrix. The plaque forming bacteria present and proliferate within the plaque matrix through which the channels run. This matrix provides a specialized environment, which distinguishes oral bacteria that exist within the biofilm from the planktonic bacteria that are free-floating in saliva or gingival crevicular fluid. The dental plaque matrix acts as a barrier within the biofilm. Metabolites produced by bacteria during metabolism are engaged and concentrated within the biofilm, which enhances metabolic interactions among the different plaque bacteria. The intercellular plaque matrix is composed of inorganic and organic materials that derived from gingival crevicular fluid, saliva, and bacterial products. The inorganic elements of plaque are predominantly phosphorus and calcium, with traces of other minerals such as potassium, sodium, and fluoride. The source of inorganic elements of supragingival plaque is mainly saliva. When the mineral content increases within plaque, the mass starts to calcify to form dental calculus. The organic components of the dental plaque matrix include proteins, polysaccharides, glycoproteins, and lipid material. The lipid material is composed of debris from the membranes of host and

disrupted bacterial cells, and probably food debris. Glycoproteins coming from saliva are important constituents that initially coat clean tooth surfaces to form the acquired pellicle. They also become integrated into the developing dental plaque. Polysaccharides produced by oral bacteria are also one of the organic portions of the plaque matrix, which are playing a main role in maintaining the integrity of the dental plaque (Lang et al., 2015).

Dental plaque consists mainly of microorganisms. By using polymerase chain reaction (PCR) techniques for microbial identification, it has been found that more than 500 microbial species could be present as natural residents of dental plaque. A wet weight of 1 gram of plaque contains approximately  $10^{11}$  bacteria. It was reported that over  $10^9$  bacterial cells can be present in supragingival plaque on a single tooth surface. The bacterial counts can range from  $10^3$  in a healthy crevice to more than  $10^8$  in a deep periodontal pocket. Other non-bacterial microorganisms that are present in dental plaque include yeasts, archaea, protozoa, and viruses (Aas et al., 2005; Newman et al., 2012).

### **2.1.2 Classification of dental plaque**

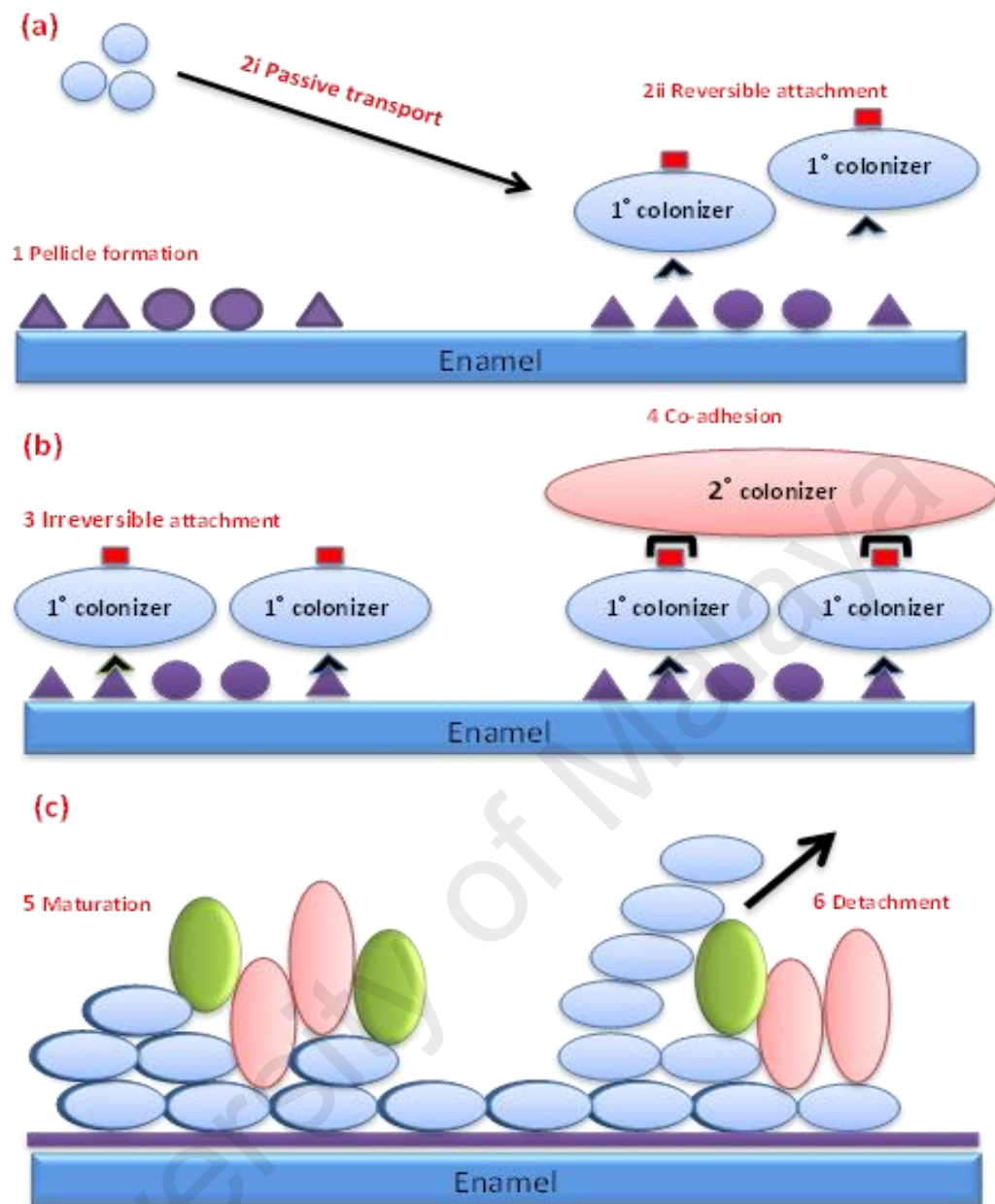
Dental plaque is generally classified according to its position on the tooth surface in relation to the gingival margin (Newman et al., 2012):

- 1- Supragingival plaque which is located at or coronal to the gingival margin; it is referred to as marginal plaque when plaque comes in direct contact with the gingival margin.
- 2- Subgingival plaque which is located apical to the gingival margin, between the tooth surface and the gingival pocket epithelium.

### **2.1.3 Formation of a dental Plaque Biofilm**

The process of plaque formation follows an ordered sequence of events. As shown in Figure 2.1 (Lang et al., 2015), the distinct phases in dental plaque formation involve:

- 1- Adsorption of the acquired pellicle (conditioning film) on teeth surfaces immediately after tooth cleaning.
- 2- Reversible adhesion of the primary plaque colonizers to the acquired pellicle covering tooth surfaces.
- 3- More irreversible attachment involving interactions between specific molecules on the primary plaque colonizers cell surface (i.e. adhesins) and complementary molecules (i.e. receptors) found in the acquired pellicle.
- 4- Co-adhesion, in which secondary plaque colonizers adhere to receptors on the already attached primary colonizing bacteria, result in increasing microbial diversity.
- 5- Proliferation of the attached cells, leading to an increase in the plaque biomass and synthesis of exo-polymers to form the dental plaque matrix (plaque maturation).
- 6- Detachment of some attached cells to help colonization elsewhere in the oral cavity.



**Figure 2.1: Dental plaque formation steps**

(a) Acquired pellicle forms on a clean tooth surface (1). Bacteria are passively transported to the tooth surfaces (2i), and may reversibly be held by weak forces of attraction (2ii). (b) Attachment of bacteria becomes irreversible through specific interactions between adhesins on the bacterial cell and complementary receptors in the pellicle (3) Secondary plaque colonizers attach to the already attached primary colonizers by co-adhesion (4). (c) Growth results in biofilm maturation (5). Bacterial cells detach to colonize elsewhere (6). Adapted and modified from Lang et al. (2015)

### **2.1.3.1 Formation of the acquired pellicle**

In the oral cavity, the hard and soft tissues surfaces are always coated with a thin layer of the acquired pellicle, which contains many protein molecules that act as receptors (adhesion sites) for bacteria. There are more than 180 different proteins, peptides, and glycoproteins, including mucins, keratins, proline-rich proteins, histidine-rich proteins, phosphoproteins (e.g. statherin) present in the acquired pellicle (Sanz et al., 2005; Siqueira et al., 2007; Siqueira et al., 2009). Salivary pellicle can be identified on tooth enamel surfaces within 1 minute after tooth brushing. After 2 hours, the acquired pellicle is principally in equilibrium between adsorption and detachment, although further maturation can be noticed for several hours. The transmission electron microscopy shows that the acquired pellicle is composed of two layers. One of these layers is a thin basal layer that is very difficult to detach even with harsh mechanical and chemical treatments. The second layer is a thicker globular layer, about 1  $\mu\text{m}$  or more, which is more easier to detach (Hannig, 1999; Hannig et al., 2005). According to these facts, one can conclude that tooth enamel is permanently coated with an acquired pellicle immediately after teeth eruption and/or cleaning. So, oral bacteria that adhere to tooth surfaces do not actually contact the enamel but they interact with the acquired pellicle covering the tooth enamel (Newman et al., 2012). The acquired pellicle is not simply a passive adhesion matrix. It contains many incorporated enzymes, and some of these may influence the metabolism and physiology of adhering bacterial cells such as peroxidases, lysozyme, and  $\alpha$ -amylase (Hannig et al., 2004; Hannig et al., 2008; Hannig et al., 2009b).

### **2.1.3.2 Initial adhesion of the primary plaque colonizers**

Tooth brushing eliminates most of the bacteria from the exposed tooth surfaces. Recolonization of oral bacteria begins immediately which can be detected within 3 minutes (Hannig et al., 2007). The initial steps of transport and interaction of bacteria

with the tooth surface are essentially non specific. Specific interactions occur between the complimentary adhesins of the primary colonizers and receptors on the salivary pellicle. These interactions allow bacteria to firmly adhere to the tooth surface. In fact, a relatively small proportion of oral bacteria have adhesins which interact with the host pellicle receptors, and these bacteria are generally the most dominant bacteria found in biofilm on tooth enamel shortly after tooth cleaning. During the first 4 to 8 hours after tooth brushing, streptococci constitute about 60% to 80% of oral bacteria present in the biofilm formed on tooth surfaces (Nyvad et al., 1987; Dige et al., 2009), in addition to *Haemophilus* sp. and *Neisseria* sp., *Actinomyces* sp. and *Veillonella* sp. (Aas et al., 2005; Diaz et al., 2006). These bacterial species are referred to as the primary colonizers of tooth surfaces. The primary colonizers offer new binding sites for the subsequent adhesion by other oral bacteria, i.e. secondary colonizers, once they attach to tooth surface. The primary colonizers, through their metabolic activity, modify the local microenvironment in ways that can enhance the ability of other bacteria to survive in the dental plaque biofilm. For example, by removing oxygen, the primary plaque colonizers offer conditions of low oxygen tension that allow the survival and growth of obligate anaerobes (Newman et al., 2012).

The initial steps of bacterial colonization on teeth surfaces are as follow:

- 1- Phase 1 (Transport to the surface): Involves the initial attraction of the primary plaque colonizer towards tooth surface. Several random contacts between the bacteria and tooth surface may occur, for example, through sedimentation of bacteria, through Brownian motion (an average displacement of 40  $\mu\text{m}/\text{hour}$ ), through active bacterial movement, or through liquid flow. However, the flow of saliva and mechanical contact between teeth and oral soft tissues are certainly the most important factors

that enhance the primary plaque colonizers to be in contact with teeth (Newman et al., 2012).

- 2- Phase 2 (Initial adhesion): This is the initial, non-specific, reversible adhesion of the primary plaque colonizers to the acquired pellicle covering tooth surfaces. This process starts when the primary colonizers are in close proximity to the tooth surface (separation distance about 50 nm). Short and long range forces, such as electrostatic repulsive forces and van der Waals attractive forces, operate at this distance. The bacterial cells behavior at this distance can be described by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloid stability (Hermansson, 1999). According to the theory, the total interaction energy (also known as the total Gibbs energy), is the sum of the electrostatic repulsion and the attractive forces. At the physiologic ionic strength of saliva, the van der Waal's forces give rise to a net attraction of primary colonizing bacteria at distances of tens of nm from the tooth surface. The primary plaque colonizing bacteria are reversibly bound when their distance to the tooth surface is approximately 10 nm as there will be a net energy of electrostatic repulsion preventing them from getting even closer to the tooth surface. Many bacterial cells possess structures, such as fimbriae, that protrude from the bacterial cell surface. These structures may carry hydrophobic molecules involving in cell-surface interactions. In addition, bacterial cell surfaces are not uniformly charged negative charge. There may be regions of the bacterial cell surface that are positively charged and thus the bacterial cell will be attracted to the negatively charged pellicle (Newman et al., 2012).
- 3- Phase 3 (Firm attachment): After initial weak adhesion, a firm anchorage between primary colonizers and tooth surface is established. On rough



surfaces, bacteria can better withstand shear forces so that a change from reversible to irreversible binding may occur more easily and frequently. The substratum free-energy surface is important, since the water film between the interacting surfaces has to be eliminated before the involvement of short-range forces. At this point, firmer binding is provided by more specific interactions between bacterial adhesins and receptors in the acquired pellicle on tooth surfaces. It was reported that 10 to 50 adhesin-receptor interactions are required to achieve essentially irreversible binding of a primary colonizing bacterial cell to the acquired pellicle (Newman et al., 2012). Numerous proteins found in the acquired pellicle can act as receptors for streptococci adhesins including acid proline-rich proteins,  $\alpha$ -amylase, statherin, and salivary agglutinin glycoprotein (Scannapieco, 1994).

#### **2.1.3.3 Co-adhesion with secondary plaque colonizers**

Once primary colonizers are firmly attached to tooth surface, they start to multiply. In addition, their metabolism modifies the local environment to be more suitable for secondary plaque colonizers, for example by making the local environment more anaerobic after consumption of oxygen and the production of reduced end-products of metabolism. As the biofilm develops further, adhesins on the cell surface of fastidious secondary plaque colonizers, such as obligate anaerobes, bind to receptors on already attached primary colonizing bacteria by a process termed co-adhesion or co-aggregation, and the composition of the developing plaque becomes more diverse. Co-adhesion ensures that bacteria co-locate with other microorganisms with complementary metabolic functions (Lang et al., 2015).

## **2.2 Dental plaque control**

Dental plaque control can be defined as the preventive procedures aiming at removing dental plaque from oral surfaces and preventing it from recurring (Weijden et al., 2015). Dental plaque adhered to tooth surfaces is classified into supragingival and subgingival dental plaque in relation to gingival margin. Supragingival plaque is exposed to natural self-cleansing mechanisms inside the oral cavity. For example, friction through mastication removes supragingival plaque, but this effect is limited to teeth occlusal surfaces and incisal edges. However, these natural self-cleansing mechanisms are found to have a negligible effect in optimal supragingival plaque control (Löe, 2000; Thomas, 2004).

Supragingival plaque control measures can alter the amount and composition of subgingival dental plaque (Dahlén et al., 1992). This fact was confirmed at a later date by Haffajee et al. (2001) who reported that optimal supragingival plaque control can modify subgingival microbiota composition and lower the levels of periodontal pathogens inside the periodontal pockets. Thus, optimum dental plaque control is important for the preservation of oral health, and prevention and control of gingivitis, periodontitis and dental caries. This can be achieved through two methods: either mechanical or chemical plaque control. Sometimes the two methods are combined to achieve optimal oral cleanliness (Axelsson et al., 2002).

### **2.2.1 Mechanical plaque control**

Mechanical plaque control is the regular removal of supragingival microbial deposit and the prevention of its accumulation on tooth surfaces, adjacent gingival surfaces and other hard surfaces found in the oral cavity (like crowns, bridges, etc.) by self or dental care professional and it is essential for periodontal health throughout life (Axelsson et al., 2002). Toothbrushes are the most adopted and accepted tool for

mechanical plaque control. They can remove dental plaque from facial, oral and occlusal tooth surfaces (Perry et al., 2001; Weijden et al., 2015). To date, although there are different methods for tooth brushing there is no method that clearly appears to be superior in terms of dental plaque removal efficiency. Nevertheless, the recommended method of tooth brushing must ensure removal of considerable amount of dental plaque without traumatizing gingival tissue (Frandsen, 1986).

Generally, it is advisable for patients to brush their teeth at least 2 times per day (Weijden et al., 1993; Gallagher et al., 2009). The use of dentifrice during tooth brushing provides fluoride which helps in controlling dental caries. Unfortunately, individuals who live stressful and busy life may fail to keep brushing their teeth twice daily (Thomas, 2004). Epidemiological and clinical studies have revealed that mechanical plaque control measures performed by most individuals are insufficient to control supragingival plaque accumulation (Sheiham et al., 2002; Crocombe et al., 2012).

Toothbrush does not reach the interdental surfaces during tooth brushing. Thus, supplementary interdental cleansing aids such as interdental brush or interdental wood sticks are necessary to ensure proper plaque removal from these surfaces (Lang et al., 1977; Hugoson et al., 1979; Weijden et al., 2015). With current understanding that periodontal diseases are more pronounced in the interproximal surfaces, particularly in molars and premolars, than other facial or oral surfaces (Löe, 1979), therefore, the use of interdental cleansing aids as a complementary to toothbrush is a fundamental principle for prevention of periodontal diseases (Warren et al., 1996; Crocombe et al., 2012).

### **2.2.2 Limitation of mechanical plaque control**

The efficacy of tooth brushing to remove dental plaque depends on individuals' dexterity. Several studies were conducted to measure the efficacy of tooth brushing for dental plaque control. The results of these studies showed that tooth brushing alone was found to be less effective in controlling dental plaque. It was previously reported that most individuals only remove 50% of dental plaque by tooth brushing (Jepsen, 1998). Another study reported that only 20% of 4170 dentates cleaned interproximally on daily basis. Only 55.5% of them brushed twice daily, and 27.6% of them had a plaque score of two or more (Crocombe et al., 2012). In the 1998 UK Adult Dental Health survey, Morris et al. (2001) observed dental plaque control performance and reported that the mean proportion of teeth with plaque deposit was 30% and 44% in the 25 – 34 and the 65 – above year age groups respectively, while about one-third of teeth in 72% of all dentate adults examined had visible dental plaque. Weijden et al. (1998) conducted a study to assess the effectiveness of a single 1-minute tooth brushing exercise. The participants involved in this study were asked to keep their routine tooth brushing methods. The results showed that approximately 39% of the plaque on their teeth had been removed.

In a meta-analysis study, Van der Weijden et al. (2005) assessed the effectiveness of tooth brushing using manual toothbrush with respect to the level of plaque and gingivitis in 9 controlled studies of at least 6 months duration. The results of this study showed poor self-performed mechanical plaque removal that needed to be improved. Also they suggested using chemical plaque control as adjunctive to tooth brushing. As a conclusion, most individuals do not properly brush their teeth and therefore they live with huge amount of plaque on their teeth, even though they brush on a regular basis.

In developed countries, tooth brushing with toothpaste is the most common and effective oral hygiene measures practiced by individuals (Frandsen, 1986; Jepsen, 1998). Interdental cleaning aids are secondary adjunct to tooth brushing to clean toothbrush inaccessible interdental spaces. This seems highly important in individuals who are susceptible to periodontal diseases (Kinane, 1998). Unfortunately, surveys conducted in many developed countries revealed that the percentage of individuals who claimed to use interproximal cleaning aids on a daily basis ranged from 11 - 51% (Bakdash, 1995). Most individuals do not properly clean their teeth by toothbrushes and interdental cleansing aids. Thus, high prevalence of gingivitis was reported even though in early age toothbrush users (Lavstedt et al., 1982; Addy, 1986). For example, the prevalence of gingivitis was 84.37% in 1,587 government school children of Udaipur district in the age group of 5 - 14 years children (Dhar et al., 2007). In another study, a survey was conducted during 1982 - 1984 on different age groups 5 - 17, 18 - 44 and 45 - 74 years old in 5,983 Mexican Americans, 1,192 Cuban Americans, and 2,226 Puerto Ricans. This survey revealed that the prevalence of gingivitis for Mexican Americans was 76.6%, 82.4% and 59.1%, for Cuban Americans was 65.1%, 81.2% and 72.3%, while for Puerto Rican was 90%, 90% and 65.1% in respect to age groups (Ismail et al., 1990). Two reasons for this, it is either or both from lacking dexterity with tooth cleaning measures, tooth brushing and interdental cleaning, or from failing to comply with the recommendation to clean teeth on regular basis (Frandsen, 1986). Also, it was found that even though individuals brush their teeth for 2 minutes duration, they fail to remove half of the dental plaque that has accumulated on their teeth (De la Rosa et al., 1979). This can happened due to the fact that no or little attention is given by individuals to certain tooth surfaces during tooth cleaning process (Rugg-Gunn et al., 1978; MacGregor et al., 1979).

On the other hand, mechanical plaque control procedures merely focus on the hard surfaces of the oral cavity which represent 21 - 23% of the total area of the oral cavity that provide an excellent surface for the establishment and growth of dental plaque (Kerr et al., 1991). The accumulation of dental plaque on soft oral tissue, such as tongue and oral mucosa, serves as a source of bacteria which initiate gingivitis and periodontitis (Socransky et al., 2005).

The limitations of mechanical tooth cleansing measures and the high prevalence of gingivitis, suggest that chemical plaque control could be useful as an adjunctive measure to mechanical plaque control, and require minimal cooperation and skill (Van der Weijden et al., 2005; Al-Bayaty et al., 2010; Sugano, 2012).

### **2.2.3 Chemical plaque control**

Chemical plaque control is the preventive measure that helps in prevention of dental plaque formation and re-accumulation on plaque free oral surfaces. For home self-care, these preparations can be delivered into the oral cavity through various vehicles, including mouth rinses, toothpastes, chewing gum and gels (Axelsson et al., 2002). Chemical plaque control measures can be used as adjunctive measures by subjects with difficulties in achieving proper plaque control using only mechanical measures (Teles et al., 2009).

#### **2.2.3.1 Chlorhexidine**

Many products have been produced to control dental plaque re-formation and gingivitis. CHX is the most effectively used antiseptic for that purpose. It is available in three forms including acetate, hydrochloride and digluconate salts. Among these forms, the latter has been used in manufacturing of most oral products. CHX was developed in England by Imperial Chemical Industries in 1940s. In 1954, it was first marketed as an antiseptic for skin wounds. Later, it was commonly used in medicine including

gynecology, obstetrics, urology and pre-surgical skin disinfection for both surgeon and patients. Its first use in dentistry was for pre-surgical disinfecting oral operating area and in endodontic treatment (Lang et al., 2015).

The role of CHX for prevention of plaque accumulation and gingivitis was initially studied by L  e et al. (1970). In this study, CHX mouthwash was prepared at a concentration of 0.2%. The participants were asked to rinse for 60 seconds with 10 ml of the mouth rinse twice daily in the absence of mechanical control measures. The results showed inhibition of dental plaque re-growth by approximately 60% and reduction in gingivitis severity by about 50 – 80%. Later, many studies were conducted and confirmed the role of CHX in reducing dental plaque accumulation and gingivitis. Strydonck et al. (2012) conducted a meta-analysis based on 30 publications. They aimed to evaluate CHX mouth rinse effect on dental plaque and gingivitis in the presence of mechanical oral hygiene. They reported that CHX mouth rinse significantly reduced plaque, approximately 33%, and gingivitis scores, approximately 26%, in gingivitis patients as compared to placebo or other control mouth rinses.

The two most common commercially available concentrations of CHX mouth rinses are 0.12% and 0.2%. Despite the difference, patient receives approximately an equal dosage of CHX: 15 ml of 0.12% CHX contains 18 mg, while 10 ml of 0.2% CHX contains 20 mg per volume. With respect to dental plaque inhibition, some studies reported no statistical differences between 0.12% and 0.2% CHX mouth rinses (Segreto et al., 1986; Smith et al., 1995; Kapoor et al., 2011). In a meta-analysis of 7 studies comparing the antiplaque effect 0.12% and 0.2% CHX mouth rinses, the latter was significantly better with small difference. However, this difference is probably negligible in terms of clinical relevance (Berchier et al., 2010).

***(a) Mechanism of action of chlorhexidine***

CHX digluconate is a cationic bis-biguanide. It has a molecular structure consisting of a hexamethylene bridge with terminal four chlorophenyl groups (Matthijs et al., 2002). The magnitude of the antibacterial effect of CHX depends on its concentration. At low concentration, CHX exhibits bacteriostatic effects. The positively charged CHX molecule is rapidly attracted to the negatively charged bacterial cell wall through specific and vigorous adsorption to phosphate containing compounds. This disturbs the integrity of the bacterial cell membrane. Thus, CHX becomes attracted towards the inner bacterial cell membrane and binds to its phospholipids. As a result, low molecular weight compounds will leak from the bacterial cells. This stage is reversible at low CHX concentration. At higher concentrations, CHX can exhibit a bactericidal effect by penetrating the bacterial cell wall and inducing precipitation of the bacterial cytoplasm. Thus, rinsing with CHX can provide an immediate reduction in the salivary bacterial counts. On clean tooth surface, CHX can bind to the acquired pellicle and enamel. This tooth surface-bound CHX can interfere with the adherence of oral bacteria to the tooth surface (Jones, 1997). One of the properties of CHX is substantivity which gives CHX the advantage over other compounds. This property is linked to CHX's ability to adsorb onto and bind to oral soft and hard tissues. By this, CHX mouth rinse is able to act for prolonged periods, up to 12 hours within oral cavity (Jones, 1997; Tomás et al., 2010).

***(b) Limitation of chlorhexidine mouth rinse***

CHX mouth rinse has beneficial effects in reducing dental plaque accumulation and gingivitis severity. Rinsing with CHX in addition to mechanical plaque measures result in approximately 33% reduction of plaque and 26% reduction of gingivitis. Unfortunately, rinsing with CHX has several observed side effects. Extrinsic tooth and tongue staining was the most commonly observed side effect. Besides, change of taste



sensation and increased calculus formation were frequently observed side effects. Other less frequent complaints were mucosal lesions, hypersensitivity, burning and anaesthetized sensations (Flötra et al., 1971; Strydonck et al., 2012).

Staining side effects of CHX could be explained by a local precipitation reaction occurring between tooth and tongue bound CHX with chromogens found within beverages and foodstuffs. This effect may be reduced by minimizing the intake of such beverages and foods during treatment with CHX mouth rinse, especially just after rinsing. For example, it would be preferable to reduce the intake of coffee and tea immediately after the morning rinse with CHX, as the orally bound CHX will be at the highest concentration. Likewise, rinsing with CHX last thing at night is to be recommended, as no foods or beverages will be consumed during sleeping (Jones, 1997).

The enhanced calculus formation side effect of CHX was reported to be due to the cationic property of CHX that amplify the precipitation of salivary proteins on tooth surface resulting in acquired pellicle thickness and/or amplified precipitation of inorganic salts in or on the pellicle layer (Addy et al., 2008).

Another common side effect of CHX mouth rinses is taste perturbations. Van Strydonck et al. (2005) concluded that the perturbation of taste perception after rinsing with 0.12% CHX is significantly lower compared to rinsing with 0.2% CHX mouth rinses. This conclusion was supported by Pizzo et al. (2006). On the other hand, earlier studies by Steenberghe et al. (2001) and Keijser et al. (2003) concluded that there was no significant difference in terms of taste perception, alteration of taste and duration of taste. Therefore, a certain conclusion with respect to taste perception could not be drawn. Sensitivity to CHX was also reported. It was found that the oral mucosa showed sensitivity to contact with CHX in some cases (Yusof, 1988).

In conclusion, despite of beneficial effects of CHX on oral health, unfortunately it has several side effects that may cause discomfort to its users. This may encourage researchers to find a substitute that has the beneficial effects of CHX with fewer side effects.

### **2.3 Medicinal traditional plants**

Natural medicinal plants are the source of several biologically active compounds, many of which have been used for the development of new chemicals for pharmaceuticals (Palombo, 2011). For thousands of years, medicinal plants have been used as traditional therapy for numerous human diseases around the world. In developing countries' rural areas, people continue to use medicinal plants as the primary source of medicine (Chitme et al., 2004). Approximately 80% of the people in these developing countries used traditional medicines for their health care (Kim, 2005). Medicinal plants compose the main component of traditional medicine. In other words, about 3300 million people regularly use medicinal plants (Farnsworth, 1994). In Latin America, the World Health Organization Regional Office for the Americas reported that 71% of Chileans and 40% of Colombians used traditional medicine for treatment. In the US, patients prefer to use complementary alternative medicine than western medicine. While in Japan, traditional medicines are prescribed for patients in clinics by up to 70% of allopathic doctors (Bussmann et al., 2010). In several Asian countries, traditional medicine is usually used even though western medicine is readily available. Complementary alternative medicine is becoming increasingly popular in several developed countries (WHO, 1998).

The secondary metabolites of the medicinal plants have an important role in conventional western medicine. They are obtained after the processing of fresh or dried plant material. Most researchers preferred to use dried plant material compared with

fresh plants for the following causes: (a) fewer extraction problems with dried plant material; (b) fresh plants need time for processing after collection because of their water content that affects their solubility during extraction; (c) the antimicrobial properties are stable in dried plant material; (d) most traditional healers use dried plant material or its aqueous extract (Eloff, 1998). Many constituents of plants are pharmacologically-active. It was reported that 119 secondary plant metabolites were used as drugs. This pharmacological activity of the constituents of the plants were discovered after following up on ethnomedical use of the plant (Farnsworth et al., 1991).

### **2.3.1 *Camellia sinensis* (L.) Kuntze**

Recently, much attention has been driven to natural plants rich in polyphenols. Many of these natural plants may provide food and beverages full with beneficial effects in human (Ferrazzano et al., 2011). Polyphenolic compounds isolated from these plants were found to exhibit many biological activities such as antioxidant, antiviral, antibacterial, anti inflammatory, anti-allergy, anti-cancer and immune-stimulant activities (Scalbert et al., 2005). Tea is one of the main sources of the natural polyphenolic compounds in the daily human diet (Ferrazzano et al., 2011).

Tea was known by ancient Chinese for thousands of years as long ago as 2700 BC. They added tea leaves, to be more palatable, into drinking water and boiled for hygienic reason. Nowadays with the exception of drinking water, tea is considered the most widely consumed beverage around the world. It is consumed by over 60% of the world population. For example, the United Kingdom population consumes an average of 644.1 tonnes of tea every day. The UK Tea Council revealed that the Britons drink 165 million cups of tea per day, an average that reaches 60.2 billion cups of tea per year. Tea production has reached approximately 4.52 million tonnes in 2010 by different world's countries. The Republic of China is the largest producer of tea producing as

much as 1,467,467 tonnes followed by India who produces about 991,180 tonnes (Bansal et al., 2013).

Tea is prepared from the leaves of the tea plant *Camellia sinensis* (L.) Kuntze. Different types of tea are found according to the degree of fermentation of the tea plant leaves. Tea is generally consumed as green, oolong, black, and Pu-erh tea. Around the world, consumers vary in their preferences to different tea types. People of Japan and China prefer green tea over other tea types. In contrast, people of western countries prefer black tea which dominates western markets. Pu-erh tea is exclusively consumed in Asia (Balentine et al., 1997).

The most important constituents of the tea are polyphenols, particularly monomeric flavanols, which are known as catechins. Many *in vivo* and *in vitro* studies have investigated the catechins' biological activities. Catechins were reported to have antioxidant activity by stabilizing or deactivating free radicals generated by metabolic pathways within the body tissue, thereby lowering free radical-mediated damage of cells and tissues in an organism (Von Staszewski et al., 2011). In addition, catechins were reported to have a role in inhibition of carcinogenesis (Otsuka et al., 1998), lowering of plasma cholesterol levels (Ikeda et al., 1992), protection from cardiovascular diseases (Mukamal et al., 2002), activation of leukocytes (Sakagami et al., 1992), enhanced loss of body fat (Klaus et al., 2005), protection from the effects of radiation (Uchida et al., 1992), improvement in type 2 diabetes (Shoji et al., 2006), protection from neurodegenerative diseases (Ramassamy, 2006), increase of bone density (Devine et al., 2007) and antimutagenic activity (Hayatsu et al., 1992). With the emergence of resistant microbial strains to current antibiotics, polyphenols derived from tea leaves were reported to help in the development of novel plant-derived antimicrobials (Friedman, 2007).

### 2.3.2 Types of *Camellia sinensis* (L.) Kuntze

*Camellia sinensis* (L.) Kuntze (family: *Theaceae*), the tea plant, was reported to grow in about 30 countries worldwide (Graham, 1992). There are two main varieties of *Camellia sinensis* (L.) Kuntze used to prepare tea. Chinese tea is prepared from *Camellia sinensis* var. *sinensis*, while Assam tea is prepared from *Camellia sinensis* var. *assamica*. Generally, *Camellia sinensis* var. *sinensis* is mainly grown in Japan, China and Taiwan, whereas *Camellia sinensis* var. *assamica* is predominately grown in South and Southeast Asian countries including Malaysia and Australia (Chan et al., 2007).

Tea is classified into 3 main types according to the fermentation degree during processing. These main types include non-fermented, semi-fermented and fermented tea. The non-fermented tea, known as green tea, is produced by drying and steaming the fresh plant leaves to inactivate polyphenol oxidase and prevent oxidation. Semi-fermented tea, known as oolong tea, is produced by subjecting the fresh plant leaves to partial fermentation before drying process. In contrast, the fermented tea, known as black and Pu-erh tea, is produced by subjecting the fresh plant leaves to full fermentation after harvesting and before drying and steaming stages (Bancirova, 2010).

### 2.3.3 Non-fermented *Camellia sinensis* (L.) Kuntze (Green Tea)

Green tea is a popular drink worldwide. It is obtained from fresh leaves of the shrub *Camellia sinensis* (L.) Kuntze. The fresh tea leaves are steamed and heat dried (Bancirova, 2010). There is an increased attention to green tea effect on human health due to biological activities of its components. As a result of several epidemiological studies, green tea consumption was found to be related with lower incidence of many systemic diseases including strokes, cardiovascular diseases, cancer and obesity (Hertog et al., 1993; Keli et al., 1996; Bell et al., 2002; McKay et al., 2002). These effects of

green tea were interpreted, in part, to its free radical scavenging and antioxidant activities of its polyphenols (Laughton et al., 1991; Scott et al., 1993). Moreover, green tea polyphenols, like catechins, were suggested to have a modulating effect on the physical structure of human cell membrane, thus interfering with several membrane dependent processes, like cell proliferation (Agarwal et al., 1992), mitochondrial functioning and apoptosis (Spencer et al., 2001), cell signaling and cell cycle (Chung et al., 1999), through interaction of catechins with cell membrane phospholipids. Polyphenolic (–)-epigallocatechin gallate has been shown to have an offensive effect on tumor cells, not their normal counterparts, in animal models by inducing apoptosis, cell cycle arrest and interfering with various cellular signal transduction pathways (Chen et al., 2004). As a conclusion, green tea provides a natural source for several beneficial health effects in human through its different biologically active constituents.

#### **2.3.3.1 Chemical composition of green tea**

The chemical composition of green tea is complex. Many factors can affect the tea chemical composition including climate, season, age of leaves at collection time and horticultural practices. Chemical composition of green tea includes free amino acids 1 – 5.5%, amino acid theanine 4%, carbohydrates, alkaloids (theophylline 0.02 – 0.04%, caffeine 3.5%, theobromine 0.15 – 0.2%), polyphenols, volatile compounds, several minerals and traces of elements. Among all these constituents, polyphenols particularly flavonoids are the most important due to their significant role in the tea biological activity (Graham, 1992; Cabrera et al., 2003).

The most common flavonoids in green tea are the flavan-3-ols (flavanols or flavans). The flavan-3-ols are sub-classified according to the degree of polymerization into monomers including catechins, dimers including theaflavins and oligomers including derived tannins (thearubigins). The other flavonoids including flavones

(luteolin and apigenin) and flavonols (myricetin, kaempferol, and quercetin) are found in green tea but in relatively lesser quantity (as shown in Table 2.1) (Peterson et al., 2005; Savić et al., 2014).

Polyphenolic catechin fractions comprise up to 30% of the dry weight of green tea leaves. Catechins, have a pronounced antioxidant and antimicrobial effects, comprise a group of free catechins including (+)-catechin, (–)-epicatechin (EC), (+)-gallocatechin (GC) and (–)-epigallocatechin (EGC), in addition to the galloyl catechins including (–)-catechin gallate (CG), (–)-epicatechin gallate (ECG), (–)-gallocatechin gallate (GCG) and (–)-epigallocatechin gallate (EGCG). Among these catechins, (EGCG) is present in higher amount in green tea comprising about 50% of catechin pool, while (EGC), (ECG) and (EC) account for 20%, 13%, 6% respectively (Hara, 2001).

**Table 2.1: Flavonoid content of green tea (Peterson et al., 2005)**

Sub-classes		Concentration (mg/100 g dry green tea)
<b>Flavan-3-ols</b>	(–)-Epicatechin	793 ± 411
	(–)-Epicatechin-3-gallate	1755 ± 1056
	(–)-Epigallocatechin	1712 ± 1466
	(–)-Epigallocatechin-3-gallate	8975 ± 5923
	(+)-Catechin	24 ± 17
<b>Flavones</b>	Apigenin	77 ± 73
	Luteolin	8 ± 9
<b>Flavonols</b>	Kaempferol	130 ± 34
	Myricetin	101 ± 43
	Quercetin	175 ± 48

### **2.3.3.2 Antimicrobial activity of green tea**

The antimicrobial activity of tea was first revealed at the beginning of the last century by a Major, McNaught, in the British Army Medical Corps. He investigated the bactericidal activity of brewed black tea against *Salmonella typhi* and *Brucella melitensis*. He concluded with a suggestion that adding black tea into water bottles of British Army troops can prevent diseases caused by these microbes. However, studies investigating the antimicrobial activity of green tea did not start until the late 1980s. Polyphenolic catechins were reported to be the major players responsible for the antibacterial activity of green tea. Among green tea catechins, EGC, EGCG and ECG are the most important and powerful antibacterial agents. Notwithstanding these antibacterial components of green tea, it was found that green tea had a weak antibacterial activity when used systemically as conventional antibacterial agents. However, it may be suitable to be used as topical agent for superficial bacterial infections (Stapleton et al., 2004; Taylor et al., 2005).

For that reason, green tea catechins are used topically for various preventive and antiseptic purposes. For example, it is effectively used as topical antibiotic mixture ointment for the treatment of impetigo, formulated for oral rinses and skin creams and it has been incorporated into vacuum cleaner filters and face masks in order to minimize air born microbial contamination (Hara, 2001; Taylor et al., 2005).

### **2.3.3.3 Epidemiological studies on the benefit of green tea on oral health**

Many epidemiological studies have been carried out to investigate the effect of green tea on oral health. These studies provided evidence on the beneficial effects of drinking green tea in terms of minimizing dental caries and improving periodontal health. Among these studies, Elvin-Lewis et al. (1986) examined 106 American children, and they reported significant reduction in plaque and caries scores among



children who drank tea, 1 - 3 cups/day, compared with those who did not drink tea. In another epidemiological study involving 800 children in Japan, the results showed lower incidence of pits and fissures caries in the teeth of children who daily drank one cup of tea than those who did not consume tea (Onisi, 1985).

In United Kingdom, a study was performed to survey the oral health of over 6000 children after drinking tea, sugared and carbonated drinks. It was found that lower caries incidence was detected among children who consumed tea (Jones et al., 1999). Kushiya et al. (2009) analysed the relationship between green tea intake, by means of green tea cups per day, and periodontal condition of 940 Japanese men aged between 49 to 59 years. After adjusting for other confounding variables, they found that drinking one green tea cup per day was significantly associated with a 0.23 mm reduction in the mean of probing depth, a 0.28 mm reduction in the mean of clinical attachment loss, and a 0.63% reduction in bleeding on probing. Thus, they concluded that there was a modest inverse relationship between green tea intake and periodontal disease.

By analyzing cross sectional data from the Ohsaki Cohort 2006 Study, Koyama et al. (2010) examined the association between green tea consumption and tooth loss in Japan population. They analyzed self-administered questionnaires of 25,078 participants (13,059 women and 12,019 men) aged between 40 to 64 years. The results of this study revealed that drinking  $\geq 1$  cup daily of green tea was significantly associated with decreased tooth loss which was sex independent.

In conclusion, the results of these epidemiological studies demonstrate sufficient evidence for a beneficial effect of green tea on oral health. This encourages researchers to extensively investigate the burden secret of its health beneficial effect on the oral hygiene.

#### 2.3.3.4 Oral antimicrobial activity of tea and its polyphenols

Many studies were carried out to investigate the antibacterial activity of green tea extract and its polyphenols against dental plaque bacteria. A group of acid-producing bacterial species of the genus *Streptococcus*, in particular *S. sobrinus* and *S. mutans*, found in human dental plaque were reported to have the major infective role in the development of dental caries. In the oral cavity, salivary amylase hydrolyses food starch to carbohydrates including oligo- and monosaccharides (e.g. glucose, maltose). Oral streptococci species secrete enzymes which are able to ferment carbohydrates and produce organic acids responsible for dental caries.

Ooshima (2005) evaluated the anticaries activity of 3 types of tea and their polyphenolic constituents by determining their ability to inhibit insoluble glucan synthesis. The author found that the concentration of purified oolong tea polyphenol to inhibit insoluble glucan synthesis by 50% was 2 µg/ml, 8 µg/ml with polyphenols purified from black tea leaves, 40 µg/ml with oolong tea extract, 250 µg/ml with a green tea extract. This inhibitory effect was found to be attributed to the ability of the tea polyphenols to inhibit glucosyltransferase activity of *S. mutans*. Moreover, the author found that tea polyphenols reduced the surface hydrophobicity of oral streptococci. These effects collectively might account for the anticaries activity of green tea polyphenols. The tea polyphenols, in particular EGCG, inhibitory effect on glucosyltransferase activity was further investigated by Xu et al. (2011). They found that EGCG was able to inhibit glucosyltransferase activity and prevent the growth of *S. mutans*. Furthermore, Xu et al. (2012) described a mechanism by which EGCG inhibit dental plaque accumulation. They hypothesized that EGCG was able to suppress *gtf* genes in *S. mutans* at the transcriptional level. Thus EGCG was able to disrupt the initial attachment of *S. mutans* and prevent the formation of mature biofilm.

Hirasawa et al. (2006) examined the effect of EGCG on the inhibition of acid production in dental plaque *S. mutans* among 15 volunteers rinsed with EGCG and placebo, i.e. water. It was found that the pH values of plaque samples after rinsing with EGCG were significantly higher than the pH values of plaque samples after rinsing with water. In another study, Hassani et al. (2008) evaluated the efficacy of black and green tea extracts against *S. mutans*, *S. mitis*, and *S. sanguinis* that are responsible for dental caries. They found that both extracts were able to inhibit the growth of oral streptococci. Black tea extracts at a concentration of 1 mg/ml inhibited biofilm formation. In contrast, a higher concentration of green tea extract was needed to inhibit biofilm formation. The Korean green tea polyphenols were evaluated for their antimicrobial effects and inhibition of biofilm formation properties against 12 oral microorganisms. The authors used scanning electron microscopy to analyze the morphological changes in the bacteria after treating with green tea polyphenols. The results of this study revealed various morphological changes, such as formation of cell aggregates, the presence of perforations, and leakage of cytoplasmic materials from cells treated with tea polyphenols, depending on the bacteria. Moreover, the authors found that tea polyphenols exhibited an inhibitory effect against adherent cells of *S. mutans* and *S. sanguinis* (Cho et al., 2010). The growth inhibitory effects of Iranian green and black tea extracts on *S. mutans* were also studied (Naderi et al., 2011). They found that the minimum inhibitory concentrations of Iranian green and black tea extracts were 150 and 50 mg/ml, respectively. Green tea extracts have an effect in reducing the salivary load of many oral microorganisms. It was found that rinsing with green tea extract mouth rinse resulted in significant reduction of colony numbers of salivary *S. mutans* and *Lactobacillus* (Tehrani et al., 2011).

*In vitro* studies revealed the antimicrobial activity of green tea against dental plaque bacteria and periodontal pathogens. It was found that green tea catechins prevent

the growth of *P. gingivalis*, *P. intermedia*, and *P. nigrescens* (Sakanaka et al., 1996; Hirasawa et al., 2002). In addition, these catechins were reported to inhibit *P. gingivalis* adherence onto human buccal epithelial cell by binding the hydroxyl- group in green tea catechins, mainly in EGCG, ECG and GCG, to the fimbria which is the adhesive factor of *P. gingivalis* (Sakanaka et al., 1996). They were also reported to have inhibited the production of its toxic end metabolites (Sakanaka et al., 2004).

In a pilot clinical study evaluating the local delivery of green tea catechins into pockets in periodontal patients, with respect to their periodontal status, green tea catechins exhibited a bactericidal effect against black pigmented, gram negative anaerobic rods, and periodontal status improved when combined with mechanical treatment (Hirasawa et al., 2002). Green tea catechins were found to prevent the attachment of oral streptococci to tooth enamel. It was reported that these catechins prevented the attachment of *S. mutans* to saliva coated hydroxyapatite discs. This effect was due to modification of bacterial phenotype through catechins-mediated denaturation of extracellular protein ligands such as fibrils and fimbria (Otake et al., 1991; Xiao et al., 2000). Also, it was found that green tea ECG and EGCG could adsorb to salivary pellicle proteins and prevented their further formation by inducing modification in their physical properties (Joiner et al., 2004). In an investigation on antibacterial activity of green tea extracts against cariogenic and periodontal pathogens, Araghizadeh et al. (2013) determined the minimum inhibition concentration of the green tea extracts as low as 3.28 mg/ml, 6.25 mg/ml, 12.5 mg/ml and 12.5 mg/ml for *S. mutans*, *Aggregatibacter actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* respectively using broth micro-dilution. From these data, they concluded that the green tea exhibited positive antibacterial activity, and suggested to utilize green tea extracts in the preparation of mouthwashes for the prevention of dental caries and periodontal diseases.

Among normal oral flora, primary dental plaque colonizers are present in saliva and initially adhere to tooth surface after tooth brushing. They gain attention because of their initial and important role in starting the development of dental plaque. Many *in vitro* studies investigated the antibacterial activity of green tea extracts against these plaque bacteria. Tsai et al. (2008) found that methanolic extract of green tea at a concentration of 4 mg/ml was able to inhibit the growth of *S. sanguinis*. In contrast, higher concentrations were needed to inhibit the growth of *S. mutans* and *S. sobrinus*. In an *in vivo* study, it was reported that rinsing with 2 mg/ml of tea polyphenols for 5 minutes exhibited bactericidal effect against *S. mutans*, *S. sanguis*, *S. sobrinus*, *S. mitis*, and *S. salivarius*. Moreover, the tea polyphenols were found to inhibit the adherence of *S. mutans* and *S. sanguis* to human tooth samples (Cho et al., 2010). Recently green tea aqueous extract was found to inhibit the growth of *S. sanguinis* (ATCC 10556) and *S. oralis* (ATCC 9811) with minimum inhibition concentrations of 512 µg/ml and 128 µg/ml respectively (Das et al., 2015).

The tea polyphenols, in particular catechins, were reported to exhibit antiviral activity. EGCG has the ability to inhibit influenza virus by binding to the biological molecules, enhancing its agglutination, and finally inhibiting its adsorption to the target host cells. Moreover, EGCG can directly bind to the receptors on the viral cell surfaces and disturbs the viral infectivity (Shimamura et al., 2007).

Green tea extracts and its polyphenols have antifungal activity particularly against *C. albicans*. Tea polyphenols were reported to inhibit biofilm formation and inactivate the proteasome of *C. albicans*. Cultures treated with 1 µM of EGCG showed a 75% reduction of viable candida cells during biofilm formation. EGCG was also found to reduce established candida biofilm (Evensen et al., 2009). In another study, the antifungal activity of tea polyphenols (theaflavins and catechins) against *Candida*

species was studied. The polyphenols exhibited antifungal activity against all tested *Candida* species with a minimum inhibition concentration of 6.25 mg/ml for *C. albicans*. The fungus *C. glabrata* was found to be the most sensitive species followed by *C. parapsilosis*, *C. albicans*, *C. krusei*, and *C. tropicalis* (Sitheeque et al., 2009).

In conclusion, green tea extract and its polyphenolic constituents exhibit an antimicrobial activity against various oral microorganisms providing a natural source that might be utilized in the development of oral hygiene products.

#### **2.3.3.5 Mechanism of antibacterial activity of green tea extracts**

Green tea extracts have been reported to exhibit antibacterial activity against various gram positive and gram negative oral bacteria (Ikigai et al., 1993; Araghizadeh et al., 2013). This antibacterial activity was reported to be due to the presence of its biologically active polyphenolic catechins in the green tea extracts. The most potent catechins are EGCG and ECG. Their high antibacterial activity was reported to be attributed to the presence of a hydroxyl group, i.e. a galloyl moiety (Hamilton-Miller, 1995).

The mechanism behind this antibacterial activity of green tea has not yet been clearly elucidated. A suggested hypothesis has been proposed for explaining the mechanism of antibacterial activity of the green tea catechins. When gram positive bacteria are exposed to catechins, EGCG can directly bind to peptidoglycan within the bacterial cell membrane and induce its precipitation. This will result in damage to the bacterial cell wall and interfere with cell wall biosynthesis. Such an effect of EGCG was reported on *Staphylococcus* (Juven et al., 1994; Shimamura et al., 2007). In another suggested hypothesis, EGCG may react with reactive oxygen species in the presence of superoxide dismutase, resulting in generation of hydrogen peroxide and contributes to the bactericidal activity of EGCG against gram negative bacteria (Arakawa et al., 2004).

By using atomic force microscopy (AFM), Cui et al. (2012) compared morphological changes in gram positive and gram negative bacteria induced by EGCG at a concentration lower than its minimum inhibitory concentration. The authors found that EGCG caused aggregates in the cell envelopes of *S. aureus* and *S. mutans*, i.e. gram positive bacteria. Whereas, EGCG caused microscale grooves in the cell envelopes of *Pseudomonas aeruginosa* and *E. coli*, i.e. gram negative bacteria. These results suggested that the morphological alterations of gram negative bacterial cell walls induced by EGCG depended on oxidative stress by H<sub>2</sub>O<sub>2</sub> which was confirmed by flow cytometry. In contrast, hydroxyl groups and conjugated double bonds present in catechins may be involved in binding to the gram positive bacterial cell membrane compounds. It was found that EC and EGCG have high affinity to bacterial cell membrane lipid bilayers. This affinity is attributed to their content of gallic acid esters (Hashimoto et al., 1999; Kamihira et al., 2008), and thus catechins galloyl and gallic moieties disturb lipid bilayer membrane and eventually result in cell death through loss of cell structure and function (Ikigai et al., 1993; Tsuchiya et al., 1996; Cox et al., 2001).

#### **2.3.3.6 *In vivo* studies (antiplatelet activity) of green tea**

Initially, *in vitro* studies focused on the antimicrobial activity of green tea extract and its polyphenols against oral microbes. These studies concluded that green tea has a variable antimicrobial activity against various oral microorganisms. Daily oral intake of green tea has a beneficial, positive effect on periodontal diseases for both prophylactic and therapeutic purposes (Deshpande et al., 2012). Considering this fact, subsequent *in vivo* studies have been carried out to investigate the beneficial effects of green tea extracts.

The antiplaque efficacy of green tea aqueous extract mouth rinse of a concentration of 5 mg/ml, i.e. 0.5%, was evaluated in a double-blinded, parallel, randomized, plaque re-growth clinical trial. In this study, 30 participants rinsing with green tea aqueous extracts twice daily showed an effective reduction in plaque re-growth over a period of 4 days. Although the positive control 0.2% CHX showed better antiplaque effect than green tea, the difference was not statistically significant (Das et al., 2015). This antiplaque activity of 0.5% green tea aqueous extract was demonstrated in another blinded, randomized, parallel clinical trial for two weeks, i.e. longer period of time (Hambire et al., 2015). This study was carried out on 60 healthy children who were divided into 3 groups and asked to rinse twice daily with either 0.5% green tea aqueous extract, 0.2% CHX gluconate or 0.05% sodium fluoride. No statistical differences were found between 0.5% green tea aqueous extract and 0.2% CHX in reducing dental plaque and gingival scores over two weeks period.

In another recent parallel, randomized controlled clinical trial, the antiplaque activity of a commercially available green tea mouth rinse (Colgate plax Fresh tea<sup>®</sup>) was compared with other commercially available Listerine and 0.2% CHX mouth rinses. In this study, 48 subjects had plaque, gingival and bleeding indices scored at 2 and 3 weeks after baseline. Again, the antiplaque effect of commercial green tea mouth rinse was comparable to commercially available Listerine and 0.2% CHX mouth rinses (Biswas et al., 2015). The effects of Iranian green tea mouth rinse, containing 1% tannin, on dental plaque accumulation and gingivitis were explored. In a double blinded, parallel, randomized clinical trial, 40 volunteers comprising of dental students were divided into two groups who rinsed with 15 ml of either green tea aqueous extract containing 1% tannin or 0.12% CHX twice a day. After 1 and 4 weeks, green tea provided an antiplaque effect and reduction in gingival index scores comparable to 0.12% CHX mouth rinse. This effect was accompanied by reduced tooth staining



compared with CHX. This study supports the idea of using biologically active plant extracts as natural chemicals alternative to synthetic drugs to gain safety and cost effect advantages (Radafshar et al., 2015). The antiplaque efficacy of 0.25% green tea catechins mouth rinse was studied in a crossover clinical trial among 30 participants. By rinsing twice a day for one week, 0.25% green tea catechins mouth rinse was found to be able to reduce plaque accumulation with a comparable effectiveness to 0.12% CHX mouth rinse (Kaur et al., 2014).

Overall results of the above mentioned *in vivo* clinical studies supports the effectiveness of green tea extract and its polyphenols mouth rinses as antiplaque agents. The advantages of this include increased safety, the use of natural materials rather than synthetic, and it is cost effective.

#### **2.3.3.7 Toxicity of green tea**

Green tea is one of the most consumed beverages in the world, particularly in the cultures of China and Japan. Due to this widespread consumption, its potential biological effects have been investigated both *in vitro* and *in vivo*. A considerable amount of research had reported many health beneficial effects of green tea. This was believed to be due to green tea antimicrobial (Araghizadeh et al., 2013), anti-cancer (Bode et al., 2009) and antioxidant properties (Higdon et al., 2003). Recently, products containing green tea extracts have been marketed commercially as dietary supplements for health benefits in some countries (Chengelis et al., 2008).

Bun et al. (2006) investigated the toxic effect of green tea extracts on liver function in female Wistar rats. The experiment was of 12 week duration and the rats were delivered aqueous green tea extracts (1400 mg/kg body weight /day) and alcoholic green tea extracts (2000 mg/kg body weight /day) by intra-gastric gavage. The results showed no signs of liver clinical toxicity or behavioral changes in the rats. Hsu et al.

(2011) evaluated the safety of aqueous green tea extract using an animal model (mice). In this study, three doses of green tea extracts were orally administered to both genders of mice. The doses were 625, 1250 and 2500 mg/kg body weight /day for 28 days. The results revealed that the highest extract concentration, i.e. 2500 mg/kg body weight / day, did not cause adverse effects regarding body weight, organs weight, serum biochemistry, hematology, urinalysis or histopathology in mice of either gender.

Wang et al. (2012b) evaluated the oral sub-chronic toxicity of aqueous green tea extract in 80 male and female Sprague Dawley rats. The green tea extracts were delivered orally by using gavage at doses 1250, 2500, and 5000 mg/kg body weight / day for 91 consecutive days. Adverse effects were monitored by measuring clinical observations including survival, serum biochemistry, hematology, urinalysis and histopathological examination. This study concluded that the dose at 2500 mg / kg / day of aqueous green tea extract was safe with no adverse effects for either gender. The beneficial health effects obtained with drinking green tea are mostly considered to be due to tea catechins constituents. Thus the safety of green tea catechins has been properly investigated through many studies. Chengelis et al. (2008) evaluated the potential adverse effects of green tea catechins in rats. Catechins dosage of 2000 mg/kg body weight /day was given orally by gastric intubation for 28 consecutive days. The clinical condition of the rats, motor activity, functional observational battery, clinical pathology, and organ weights were evaluated at the end of the study. No observed adverse effects were found and the catechins dosage of 2000 mg/kg body weight /day was reported to be systemically safe. The antiplaque and antigingivitis efficacy of green tea extract mouth rinse was evaluated through many in vivo clinical trials using various extract concentration. No study reported adverse toxic effects observed with the use of the green tea extract (Moghbel et al., 2011; Tehrani et al., 2011; Jenabian et al., 2012; Kaur et al., 2014; Hambire et al., 2015). In conclusion of these mentioned studies,

aqueous green tea extract with a dosage of 2500 mg/kg body weight /day is safe when administered orally with no systemic observed adverse effects.

#### 2.3.4 *Salvadora persica L.*

People in rural areas, mainly young, traditionally prefer to use natural chewing sticks such as that of *Salvadora persica L.* more than synthetic toothbrushes and tooth pastes. These chewing sticks give the benefits of keeping their teeth healthy with whiter pleasant appearance (Goyal et al., 2011). *Salvadora persica L.* chewing sticks has several synonyms in different Arabic dialects and countries include miswaak, misswak, miswaki, meswak, mswaki, sewak, siwak, and siwaki (Hattab, 1997). These chewing sticks are known by different names in other different cultures including koyoji in Japanese, qesam in Hebrew, qisa in Aramaic, and mastic in Latin (Bos, 1993). *Salvadora persica L.* chewing stick was used by the ancient Babylonians (7000 years ago) followed by the Greek, Romans, Jews, Egyptians and Islamic empires. Nowadays, it is being used in Africa, South America, Asia and the Middle East including Saudi Arabia and throughout the Islamic countries (Chaurasia et al., 2013). *Salvadora persica L.* (belong to *Salvadoraceae* family) is a 4 – 6 meter tall green tree with a short trunk, smooth green leaves and white bark. Its branches are long which are either semi-climbing or pendulous and often pubescent or glabrous. The leaves are sub-succulent with long coriaceous blades which are elliptic to orbicular, acute to round at apex and sub-cordate to cuneate at base. *Salvadora persica L.* has small greenish white flowers in terminal and lateral panicles. The stems and roots are spongy and can easily be crushed between the teeth and pieces of its roots are usually scented and become soft when soaked in water (Almas et al., 2004).

#### 2.3.4.1 Chemical composition of *Salvadora persica* L.

Various natural bioactive constituents have been identified in *Salvadora persica* L. extracts by many investigators. Many of these constituents are considered essential for good dental and oral hygiene. GC-MS analysis of the volatile oil extracted from *Salvadora persica* L. leaves identified eugenol, eucalyptol, benzyl nitrile, thymol, isothymol, isoterpinolene, and  $\beta$ -caryophyllene as important components (Alali et al., 2003). Salvadoricine, an indole alkaloid, was also extracted from the leaves of *Salvadora persica* L. (Malik et al., 1987). Eucalyptol (46%),  $\alpha$ -caryophellene (13.4%),  $\beta$ -pinene (6.3%), and 9-epi-(E)-caryophellene are the main essential oil constituents of *Salvadora persica* L. stem that have been detected (Alali et al., 2005).

By using capillary electrophoresis techniques, aqueous extracts of the root and stem of *Salvadora persica* L. have also been examined for some antimicrobial anionic constituents. It was found that *Salvadora persica* L. root and stem extracts contain thiocyanate, sulfate chloride and nitrate (Darout et al., 2000b). Four derivatives of benzylamides were isolated from the stems of *Salvadora persica* L. including butanediamide (Khalil, 2006). The phytochemical investigation revealed that it also contains linolic, oleic and stearic acids. Among the constituents identified are esters of aromatic acids and of fatty acids, and some terpenoids (Abdelrahman et al., 2003). Glycosides, the flavonoids rutin and quercetin were also detected in the stem of *Salvadora persica* L. (Abdel-Wahab et al., 1990; Ohtani et al., 1992). Arora et al. has identified alkaloids, glycosides, steroids, triterpenoids and flavonoids including kaempferol and quercetin in alcoholic extract of the stems of *Salvadora persica* L. (Arora et al., 2013).

The air dried root bark of *Salvadora persica* L. was found to contain 27.1% ash by physicochemical analysis. This ash consists of significant amounts of salts, mainly

chlorides. Considerable amount of alkloidal constituents (such as trimethyl amine), lesser amount of resin and coloring substances, and traces of saponins and tannins were also identified in *Salvadora persica L.* roots. Higher concentration of fluoride, sulfur, silica, vitamin C, lesser amount of sterols and flavonoids were also reported (Kirtikar et al., 1987; Bhandari, 1990). Salvadoarea and Benzylisothiocynate have also been isolated from the root of *Salvadora persica L.* sticks (Ray et al., 1975; Bader et al., 2002). Flavonoids including kaempferol, quercetin were identified in the roots of *Salvadora persica L.* sticks (Abdel-Wahab et al., 1990). Chewing sticks from *Salvadora persica L.* have been analyzed and it was found that these sticks contain fluoride, calcium, phosphorus, and silica. There was a considerable amount of silica in the ashes of *Salvadora persica L.* chewing sticks (Hattab, 1997).

#### **2.3.4.2 Epidemiological studies of *Salvadora persica L.***

In the first dental health survey conducted in Sudan, Emslie (1966) reported lower caries prevalence among people using chewing sticks than in those using the usual toothbrushes. The same finding was observed in people of Southern Ghana. It was found that the lower caries prevalence among chewing stick users was in spite of carbohydrate rich diet intake and absence of modern dental prophylactic measures (Elvin-Lewis et al., 1980). In a comprehensive epidemiological study involving several thousand school children in Zimbabwe, Sathananthan et al. (1996) reported that children who used chewing sticks for teeth cleaning had fewer caries lesions than those children who cleaned their teeth using conventional toothbrushes and paste.

In an epidemiological study, Baghdady et al. (1979) compared the caries prevalence between 724 Sudanese and 1617 Iraqi schoolchildren. They used the WHO DMFT index and reported that Sudanese schoolchildren exhibited lower caries prevalence due to using *Salvadora persica L.* chewing sticks for teeth cleaning in

addition to their diet type. The same results were recorded in Saudi Arabian children aged 13 to 15 years when compared with western countries children (Younes et al., 1982). Again, the main preventive factor reported was using *Salvadora persica* L. chewing sticks for teeth cleaning.

Using chewing sticks for teeth cleaning could be as effective as the conventional toothbrush if proper instructions on using chewing sticks were given to their users. In an epidemiological study, it was revealed that Tanzanian schoolchildren who were chewing sticks users showed significantly higher plaque at baseline than did their comparator toothbrush users. The researchers found that the schoolchildren when participating in a school program that emphasized effective tooth brushing were able to improve their oral hygiene regardless of whether they used chewing sticks or toothbrush (Palenstein Helderma et al., 1992).

Low periodontal treatment needs have been reported for Saudi Arabian adults who used *Salvadora persica* L. chewing sticks (Al-Khateeb et al., 1991; Guile, 1992). Eid et al. (1990) revealed that there was no significant difference in the gingival or bleeding indices between *Salvadora persica* L. chewing sticks users and toothbrushes users. In another study, Darout et al. (2000a) compared the periodontal status among adult Sudanese who either habitually used *Salvadora persica* L. chewing sticks or used toothbrushes. The Community Periodontal Index was used to score gingival bleeding, supragingival calculus, probing depth and loss of attachment of the index teeth. The investigators found that the periodontal status of the *Salvadora persica* L. chewing sticks users in the studied Sudanese population was better than that of conventional toothbrushes users. The efficacy of the chewing sticks used for teeth cleaning was comparable to or slightly better than that of the toothbrushes. It is believed that

*Salvadora persica* L. chewing sticks should be recommended for use in motivated individuals in the developing countries due to low cost and availability.

In survey conducted between November 2010 and April 2011, the prevalence and reasons for *Salvadora persica* L. chewing sticks use among 220 adult Muslims of Banyo in the Adamawa region of Cameroon were determined. It was found that 187 (85%) participants used chewing sticks for teeth cleaning and the majority was males. The using of chewing sticks increased with ageing. Chewing stick users believe the practice of using chewing sticks has a relationship with religion, and provide a positive effect in the mouth, compared with non-users. Chewing stick users were less likely to visit dentists and experienced less mouth malodor. They also reported less oral health problems than the non users. Most of the participants used chewing sticks alone while a few of them used chewing sticks with charcoal, salt and toothpaste (Agbor et al., 2013).

#### **2.3.4.3 Antimicrobial properties of *Salvadora persica* L.**

Decades ago, many *in vitro* and *in vivo* studies were carried out to search for a simple, effective and inexpensive method to prevent and control dental plaque re-formation in response to high prevalence of dental caries and periodontal diseases. Natural plants have many benefits for humans which are attributed to their phytochemical compounds such as *Salvadora persica* L. chewing sticks. Using these chewing sticks as natural dental brushes has been recommended by the World Health Organization (Löe, 2000). In a previous study, the antibacterial activity of *Salvadora persica* L. aqueous extract was analyzed via the streaked plate method, the ditch plate method and the tube dilution method to determine the minimal inhibition concentration of the extract against several oral microorganisms. The study found that *Salvadora persica* L. aqueous extract has high antibacterial activity against several oral bacteria, mainly *S. mitis*, *S. mutans* and *S. aureus*. In addition, the extract was effective against

anaerobic *Streptococcus*. The authors concluded that *Salvadora persica L.* chewing sticks have both mechanical and chemical role in controlling dental plaque formation by possessing antimicrobial activity against oral bacteria. For that reason, they encouraged the use of these chewing sticks on a daily basis as a natural toothbrush to gain all its benefits of effectiveness, inexpensiveness and availability (Al lafi et al., 1995).

Using *Salvadora persica L.* chewing sticks to clean teeth provides an antimicrobial effect against salivary oral microorganisms. In a previous study, the salivary levels of 25 oral bacterial species were investigated among 30 users of *Salvadora persica L.* chewing sticks and 26 users of toothbrushes. The checkerboard DNA-DNA hybridization method using whole genomic DNA probe was used. This study concluded that *Salvadora persica L.* had a selective antimicrobial effect against different oral bacteria with a significant effectiveness against *P. intermedia*, *L. acidophilus*, *E. corrodens*, *F. nucleatum*, *S. sputigena* and oral streptococci species including *S. mitis*, *S. sanguis*, *S. oralis* and *S. salivarius*. These oral streptococci species are the primary colonizing bacteria that primarily adhere to tooth surfaces and initiate the dental plaque development. The reduced bacterial salivary levels were found to be independent of gender, age and periodontal status (Darout et al., 2002).

The use of *Salvadora persica L.* chewing sticks also affects subgingival bacteria in the oral cavity. The subgingival microbiota composition in both *Salvadora persica L.* chewing sticks and toothbrushes users was investigated after standardization of the type, the size and the method of use for both *Salvadora persica L.* chewing sticks and toothbrushes. By using DNA probe and checkerboard methods, it was found that the subgingival plaque of *Salvadora persica L.* chewing sticks users contained statistically lower level of *A. actinomycetemcomitans* compared with toothbrushes users. This finding suggested a potent inhibitory effect of *Salvadora persica L.* chewing sticks, i.e.



by *Salvadora persica* L. constituents, against this periodontal pathogen (Al-Otaibi et al., 2004). In another study, fresh *Salvadora persica* L. root pieces were investigated to discover their antibacterial properties against *S. mutans*, *Lactobacillus acidophilus*, *P. gingivalis*, *A. actinomycetemcomitans* and *H. influenzae*. It was found that these root pieces had a high antibacterial effect represented by wide inhibition zone ranging between 10.9 cm to 14 cm on gram negative bacteria and 1.4 cm to 3.2 cm on gram positive bacteria. The results of this study provided another evidence on considering *Salvadora persica* L. root sticks as an appreciable oral hygiene tool through its chemical action, in addition to its mechanical action (Sofrata et al., 2008). The whole antimicrobial benefits of *Salvadora persica* L. can be achieved by using the bark and pulp of its roots, that was found to be more effective than using them separately (Almas et al., 1999).

In addition to the studies investigating the antimicrobial activities of the chewing sticks of *Salvadora persica* L., many studies were carried out to investigate their extracts. It was reported that *Salvadora persica* L. aqueous extract's glucosionlates are hydrolyzed to either isothiocyanate, sulphate and glucose at neutral pH (pH 7) or to nitrate, sulphate, glucose and sulphur at low pH (pH 3 - 4) by the action of tissue plant myrosinase. Isothiocyanate is decomposed into its alcoholic part and thiocyanate (Ahmed et al., 1972). *Salvadora persica* L. thiocyanate has antimicrobial effect by amplifying the antimicrobial action of salivary peroxidase- thiocyanate and hydrogen peroxidase system in the oral cavity. This can occur during oxidation of thiocyanate and generation of hypothiocyanate by salivary peroxidase with the presence of hydrogen peroxide from oral bacteria and leukocytes. The hypothiocyanate oxidases sulfhydryl groups in the bacterial cytoplasmic membrane. This will lead to bacterial cell death by loss of the ability to transport glucose and leakage of peptide, amino acids and potassium (Tenovuo et al., 1981).

Benzyl isothiocyanate is a dominant essential oil constituent found in *Salvadora persica* L. It was found that it had a strong bactericidal effect against gram negative bacteria including medically important pathogen *P. aeruginosa*, *S. enterica* and *H. influenza*. It also had rapid and robust killing effect against periodontal pathogens including *P. gingivalis* and *A. actinomycetemcomitans*. This high bactericidal effect of benzyl isothiocyanate of *Salvadora persica* L. was explained after using electron microscopy. Treating gram negative bacteria with *Salvadora persica* L. extracts has been shown to result in formation of protrusions in the bacterial cell membrane and thus deteriorate the cell membrane integrity. In contrast, benzyl isothiocyanate of *Salvadora persica* L. was found to have no or limited bactericidal effect on gram positive oral bacteria like *L. acidophilus* and *S. mutans* (Sofrata et al., 2011b).

Another study investigated the antibacterial efficacy of alcoholic and aqueous extracts of *Salvadora persica* L. against several oral bacteria including: *S. mutans*, *E. coli*, *S. aureus*, *L. acidophilus* and *P. aeruginosa*. The alcoholic and aqueous extracts were prepared by maceration. It was found that both alcoholic and aqueous extracts of *Salvadora persica* L. had antibacterial efficacy with more potency was shown in alcoholic extract rather than aqueous extract. This probably might be explained by possessing different phytochemical constituents after phytochemical screening of both extracts. The phytochemical screening of aqueous extract of *Salvadora persica* L. revealed slight presence of tannis, saponins and other reducing components while the phytochemical screening of alcoholic extract of *Salvadora persica* L. showed moderate presence of tannis, sterols, saponins and slight presence of flavonoids, basic alkaloids and other reducing components. It was also noticed that *Salvadora persica* L. extracts were more powerful against gram positive bacteria than gram negative bacteria. This finding may be due to simpler cell wall structure of gram positive bacteria than gram negative bacteria. By consideration to these findings, it was concluded that *Salvadora*

*persica L.* have antibacterial efficacy and can be considered as medicinal plant that may be utilized as oral therapeutic agent (Mohammed, 2013).

In another study, aqueous extract of *Salvadora persica L.* was found to have antimicrobial activity against *S. mutans*, *S. aureus*, *S. faecalis*, *P. aeruginosa*, *L. acidophilus*, *S. pyogenis* and *C. albicans*. This microbial inhibition of aqueous extract was found to be more efficient than alcohol extract, and the strongest observed effect was found against *S. faecalis* (Al-Bayati et al., 2008). *Salvadora persica L.* extract has also antibacterial activity against anaerobic bacteria. It was concluded that 50% aqueous extract of *Salvadora persica L.*, prepared by boiling dried *Salvadora persica L.* in distilled water, effectively inhibited the growth of black pigmented *P. gingivalis* and may be used as antibacterial agent for the prevention and curing of periodontal diseases (Mohammad, 2013). In another study, the antibacterial effect of 60% ethanol extracted *Salvadora persica L.* was investigated on supragingival dental plaque collected from 10 patients by using disk diffusion method. A remarkable antibacterial effect was obtained at a concentration of 10 mg/ml of alcohol extract of *Salvadora persica L.* However, its antibacterial effect was lower than 0.2% CHX (Al-Bayat et al., 2010). Furthermore, the antimicrobial activity of alcohol extract of *Salvadora persica L.* was investigated against different microbial species isolated from the oral cavity of children. It was found that methanol extract of *Salvadora persica L.* showed a varying antimicrobial activity. The highest antimicrobial activity was observed against *E. coli* followed by *S. aureus* and *S. mutans*. In addition to these bacterial species, methanol extract of *Salvadora persica L.* showed growth inhibition effect against several fungi including *Candida* and *Penicillium* species (Chelli-Chentouf et al., 2012). These antimicrobial activity may be due to constituents present in *Salvadora persica L.* including chloride, trimethylamine, salvadorine, thiocyanate, tannis, nitrate and sulphur (Vahabi et al., 2011). Darout et al

identified some antimicrobial anionic compounds of *Salvadora persica L.* aqueous extract including nitrites, sulphate, chloride and thiocyanate (Darout et al., 2000b).

Fluoride has antibacterial effect when applied topically in the oral cavity through inhibition of bacterial enzymes. This effect is minimal when fluoride is ingested systemically (Featherstone, 1999). As fluoride can be found in *Salvadora persica L.* extracts, so it is possible to use chewing sticks or extract mouth rinses of *Salvadora persica L.* as a source for topical delivery of fluoride in the oral cavity. This will provide the benefits of antibacterial properties by bacterial enzymes inhibition, in addition to inhibition of tooth surfaces demineralization and enhancement of re-mineralization (Goyal et al., 2011). In a study performed to investigate the antibacterial property of *Salvadora persica L.* extracts containing toothpaste on cariogenic *S. mutans*, it was found that this toothpaste had antibacterial activity against caries producing *S. mutans* (Talha et al., 2013). *Salvadora persica L.* extracts was found to have a neutralizing effect on dental plaque pH after consumption of a sucrose solution. This dental plaque pH raising effect of *Salvadora persica L.* extracts was found to last longer when compared to rinsing with water. These results were explained by the buffering capacity of *Salvadora persica L.* extracts through increasing salivation due to its relatively strong taste, and thus leading to dilute and wash out acids in oral cavity. Also its elevated dental plaque pH effect could be due to its antimicrobial effects against acid releasing bacteria (Edgar et al., 1986; Sofrata et al., 2007; Talha et al., 2013).

#### **2.3.4.4 Antiplaque activity of *Salvadora persica L.***

Chewing sticks, like *Salvadora persica L.*, have a significant role in controlling supragingival dental plaque and enhance good oral hygiene when they are properly used. They can be as effective as toothbrushes in removing supragingival dental plaque

through combined effect of salivation enhancement and mechanical cleansing action (Goyal et al., 2009).

In a single randomized controlled clinical trial, the antiplaque effect of *Salvadora persica* L. mouth rinse was investigated in comparison to CHX 0.2% mouth rinse. In this study, all participants were asked to rinse with their blindly assigned mouth rinse for 4 weeks. In the first two weeks, participants kept their own habitual tooth brushing technique in addition to rinsing with the assigned mouth rinse twice per day. Later on, their brushing technique was standardized by using bass tooth brushing technique for the following two weeks. The results of this study showed no statistical difference between *Salvadora persica* L. mouth rinse and 0.2% CHX mouth rinse as adjunctive method to tooth brushing (Rahmani et al., 2005).

In five days crossover clinical trial conducted on ten dental students, the effect of 60% ethanol extract of *Salvadora persica* L. was evaluated on supragingival dental plaque formation. As a result from this study, alcohol extract of *Salvadora persica* L. was found to have a preventing effect on supragingival dental plaque accumulation, but it was significantly less effective than 0.2% CHX mouth rinse (Al-Bayaty et al., 2010). A randomized clinical trial was conducted on 62 participants for an attempt to investigate the antiplaque effect of fresh *Salvadora persica* L. chewing sticks. All participants were randomly allocated into two groups of either fresh or inactivated *Salvadora persica* L. chewing sticks users for 3 weeks with refrainment of mechanical measures. Data collected from this study revealed significant antiplaque effect of fresh *Salvadora persica* L. chewing sticks. This effect was explained to be attributed to its chemical constituents in addition to its mechanical action (Sofrata et al., 2011a).

The count of several oral microbes in the oral cavity showed significant reduction after rinsing with 400 mg/ml methanol extract of *Salvadora persica* L. mouth

rinse for three and eight days testing periods. This finding highlighted the role of *Salvadora persica L.* extracts in growth inhibition of oral microbes by exerting either bacteriostatic or bactericidal effects. Hence, *Salvadora persica L.* was suggested to be used in controlling dental plaque either chemically by using its extracts to prepare mouthwashes or mechanically by using it as chewing stick, since it is easy to find and inexpensive (Chelli-Chentouf et al., 2012).

In another clinical trial, the antiadherence property of *Salvadora persica L.* twigs aqueous extracts in a mouth rinse was evaluated. It was found that it caused a significant 84% reduction in adherence of *S. mutans* to buccal epithelial cells (Hammad et al., 2005). Recently, a clinical study was carried out to compare 4 hourly brushing using *Salvadora persica L.* chewing sticks versus toothbrushes with 0.12% CHX. It was found that *Salvadora persica L.* chewing sticks was as effective as combined toothbrush and CHX in improving oral care and reducing dental plaque score (Hafez et al., 2015).

The profound beneficial effect of *Salvadora persica L.* on oral health encourages its involvement in the content of various commercial herbal mouth rinses. Many studies were performed to evaluate the antiplaque efficacy of these *Salvadora persica L.* extracts containing herbal mouth rinses. In a clinical study conducted on 28 healthy subjects, it was found that Persica™ mouth rinse, containing *Salvadora persica L.* extracts, significantly improved gingival health and reduced the salivary load of the carcinogenic *S. mutans*. In contrast, no appreciable effect of Persica™ mouth rinse in reducing plaque accumulation was found (Khalessi et al., 2004). Later on, a comparative clinical study was carried out to investigate the antibacterial properties of both Persica™ and 0.2% CHX mouth rinses on *S. mutans* among orthodontic patients. The results showed that both mouth rinses had significant antibacterial effects against *S. mutans*. Although Persica™ mouth rinse was not efficient as CHX mouth rinse, but it

significantly reduced *S. mutans* count in the oral cavity. Persica™ mouth rinse used in this study consisted of three herbs' extracts including milfoil, miswak (*Salvadora persica* L.) and spearmint. The observed antibacterial effect of Persica™ mouth rinse was explained to be attributed to *Salvadora persica* L. herb extracts. Another finding from this study was that the complains of unpleasant taste with burning sensation and tooth discoloration were much higher in patients used CHX mouth rinse than those used Persica™ mouth rinse (Salehi et al., 2006).

The antiplaque effect of poly herbal mouth rinse containing *Salvadora persica* L. extracts was studied in a randomized clinical study on 48 volunteers. The poly herbal mouth rinse was found to significantly reduce plaque regrowth, but it was less effective compared with nonalcoholic 0.2% CHX mouth rinse (Singh et al., 2013). In a later study, this finding was further supported after conducting a randomized controlled clinical trial among 72 volunteers (Bhat et al., 2014).

In a conclusion, *Salvadora persica* L. chewing sticks and its extracts have an antiplaque activity. This effect is attributed to its chemical constituents in addition to its mechanical action when used as toothbrush. Hence, *Salvadora persica* L. can be used to control dental plaque either chemically by using its extracts to prepare different oral hygiene products like mouth rinse, or mechanically by using it as natural toothbrush. This might be highly encouraged by its advantages of safety, availability and inexpensiveness.

#### **2.3.4.5 Antioxidant activity *Salvadora persica* L.**

Several oral bacterial species (especially streptococci) produce hydrogen peroxides which are oxygen radicals that have the ability to destroy a variety of molecules within oral cavity cells by oxidative stress. These oxygen radicals, with the activation of immune system, are important factors that play a role in periodontal

diseases progression (Ryan et al., 1995; Wei et al., 2004). Peroxidase, heme-containing enzymes, is an oxidoreductase that can reduce hydrogen peroxide by using it as electron acceptor. So, it plays a role in oxidative stress neutralization and enhance extracellular defense against oral pathogens (Vojinović et al., 2007). By using chromatography, three types of peroxidase (PO I, PO II and PO III) were separated from *Salvadora persica L.* roots. Among these peroxidases, PO II was more stable and withstood denaturation induced by heat, pH, metal chelators, metal ions and proteolytic activity (Mohamed et al., 2012). In a previous study, researchers tried to investigate the antioxidant capacity of *Salvadora persica L.* chewing sticks extracts which were extracted by using different solvents (methanol 80%, ethanol 80%, acetone 80% and distilled water). They found that *Salvadora persica L.* methanol extract had the highest amount of crude extracts. After GC-MS spectrometer analysis, it was found that methanol extract of *Salvadora persica L.* had furan derivatives containing hydroxyl group that may be responsible for its antioxidant capacity. Moreover, GC-MS analysis revealed several antioxidant enzymes in methanol extract of *Salvadora persica L.* including catalase, peroxidase and polyphenol oxidase. Hence, *Salvadora persica L.* chewing sticks have antioxidant effect that attributed to the synergy between its antioxidant compounds and enzymes (Mohamed et al., 2013).

#### **2.3.4.6 Toxicity of *Salvadora persica L.***

Recently, there is a widespread interest in the use of medicinal plants for the maintenance of oral health. This may be to compensate for the synthetic medications with all its undesirable side effects. One of these medicinal plants is *Salvadora persica L.*, whose roots and branches were widely used as a traditional oral hygiene tool in Middle East and African countries where they grow. There are many reports highlighted the beneficial effects of the extract of this plant for oral health. It has been reported that its extract are capable of reducing pathogenic microorganisms in the oral cavity and



preventing dental caries (Yarde et al., 1995; Darout et al., 2000a; Almas, 2002). The safety of *Salvadora persica* L. extract for oral use was the topic for many studies.

An earlier study reported that neither aqueous nor alcoholic extracts of *Salvadora persica* L. were toxic to mice at doses of up to 1200 mg/kg body weight (Ezmirly, 1979). Verma et al. (2012) investigated the acute oral toxicity of *Salvadora persica* L. aqueous extract at the doses of 300 and 5000 mg/kg body weight in Swiss albino mice. After oral administration and mice monitoring for one week, no adverse effect or mortality was observed. This result has supported the earlier study of acute oral toxicity of *Salvadora persica* L. aqueous extract report (Ahmad et al., 2011). Another study reported that 2 g/kg body weight of *Salvadora persica* L. extract was safe and well tolerated in albino mice model (Al-Bayat et al., 2010).

In an attempt to evaluate the safety profile of *Salvadora persica* L. root sticks' alcoholic extract, acute and sub-chronic toxicity assessments were carried out on mice. Regarding to acute toxicity, a dose of extract up to 5g/kg body weight was delivered intraperitoneally in mice, and monitored for behavioral changes including signs of toxicity, and mortality within 24 hours. During 7 days, the surviving mice were examined for signs of delayed toxicity. As for the sub-chronic toxicity, mice were administrated daily with the extract at a dose of 400 mg/kg body weight for 30 days. Finally, the biochemical and hematological parameters were evaluated in serum and blood samples including the weights of vital organs. The extract was safe and showed no mortality or signs of delayed toxicity in the acute toxicity test. Overall, *Salvadora persica* L. root sticks aqueous extract is safe concerning liver and kidney functions and hematological assessments (Ibrahim et al., 2012). In a conclusion of above studies, a dosage up to 5 g/kg body weight of *Salvadora persica* L. root sticks extract is systemically well tolerated with no signs of toxicity.

## 2.4 Extraction of plants

Plants are considered the source of different drugs groups such as anti-cancer, antimicrobial, antispasmodics etc. Worldwide, tribal people extensively use large number of plants for curing several diseases. They claim that these plants possess antibiotic properties from traditional point of view. In Ayurveda, plants cure various diseases. Therefore, many researchers emphasized on evaluating the relation between different plants constituents and relief of diseases based on traditional claims. In this aspect, the researchers pay attention to the extraction process of plants bioactive constituents as a challenging task (Tiwari et al., 2011).

Pharmaceutically, the term “extraction” is used for the process of separation of medicinally active constituents of plant tissues from the inert plants components by using particular solvents (menstruum). The yields obtained from plants by extraction are relatively complex mixtures of metabolites, in either liquid state or semisolid state or in dry powder form (after eliminating the solvent), and can be intended for external or oral use. These include preparations groups known as powdered extracts, semisolid extracts, fluid extracts, tinctures, infusions or decoctions. Galenicals are the popular name for these preparations as an indication to Galen (second century Greek physician) (Remington et al., 2006).

Maceration, infusion and percolation are the basic techniques used for the purpose of extraction of medicinal plants and mostly applied for obtaining galenical preparations by separating the therapeutically wanted plants constituents (obtaining) from the inert unwanted plants constituents (eliminating) after treatment with a selective solvent (menstruum). The principle mechanism is by dissolution of soluble plant constituents from solid plant mass using appropriate solvent, which is generally termed to as leaching. Many factors affect this process including the transport rate of solvent into the plant mass, the solubility rate of the soluble plant constituents by the solvent

and the transport rate of solution out of the insoluble plant mass. The quality and quantity of an extract depends on many basic parameters. Generally, these parameters include (Handa et al., 2008):

- 1- The plant part to be extracted: The effect of plant material on extract varies according to the difference in plant material nature, its origin, moisture content, degree of processing, and particle size.
- 2- The solvent selected for extraction: There are many types of solvents can be used for plant material extraction. The nature of such solvent, its concentration and its polarity affect the quantity as well as secondary metabolite composition of an extract.
- 3- The extraction methods: Variation in extraction methods includes the type of extraction process, duration of extraction process and temperature at which the process of extraction runs.

As a basic principle, the plants tissue should be grind into finer pieces in order to increase the surface area of the plants materials to be in contact with the selected solvent for extraction. This will result in an increased rate of the extraction process. For mixing plants materials to the solvent, 10:1 (selected solvent volume to plants materials weight) mixing ratio has been used as ideal (Das et al., 2010).

#### **2.4.1 Solvents used in extraction of plants**

There are many solvents used in the process of plants extraction. The mostly used solvents are as follow:

- 1- Water: It is considered as universal environment friendly solvent. It is inexpensive and used to extract plant products with antimicrobial activity. Researchers primarily use water extracts for initial screening of plants for

possible antimicrobial activity (Cowan, 1999). However, plant extracts extracted by using other organic solvents have been found to give better consistent antimicrobial activity comparing with water extracts (Das et al., 2010).

- 2- Acetone: It can dissolve many hydrophilic and lipophilic components from plants used. It is volatile, miscible with water and has a low toxicity. It is the solvent of choice when more phenolic compounds with antimicrobial activity are required to be extracted (Eloff, 1998). Previous studies reported that extraction of tannins and other phenolic constituents was better in aqueous acetone than in aqueous methanol (Eloff, 1998; Das et al., 2010).
- 3- Alcohol: Using alcohol as a solvent can produce plant extracts with higher antimicrobial activity as compared to water extracts. This is attributed to the fact that alcohol solvent is more efficient in plant cell wall and seeds degradation than water solvent. As a result, more constituent with antimicrobial properties can be obtained when using alcohol like polyphenols (Lapornik et al., 2005). Two types of alcohol are commonly used as solvent for plants extraction: ethanol and methanol. For ethanol, it was found that pure ethanol solvent can extract more bioactive constituents when diluted with water. A 70% aqueous ethanol is more powerful than pure ethanol as a solvent due to increased polarity and easier to penetrate plant cellular membrane to extract plants ingredients. Although methanol solvent has more polarity than ethanol which can extract more plant constituents, it is more cytotoxic than ethanol. This makes it unsuitable for extraction in certain kind of studies (Wang et al., 2010; Bimakr et al., 2011).

#### **2.4.2 Extraction methods**

There are many techniques for the purpose of extraction of medicinal plants constituents. The most commonly used techniques are the basic procedures including maceration, infusion and percolation which can be used for initial and bulk extraction (Handa et al., 2008).

##### **2.4.2.1 Maceration**

Maceration is one of the basic techniques for extraction. The general procedure of maceration includes soaking a suitable grinded plant material into a selected solvent (menstruum) in a closed vessel, and the mixture is allowed to stand for at least three days with occasional shaking at room temperature. After that, the liquid of the mixture is strained off leaving solid residue of the mixture (marc) which is then pressed to obtain as much occluded liquid as possible from the marc. Then, the pressed and strained liquid is mixed and filtered to clarify it from the coarse plant material. The general principle of maceration is generally referred to as leaching which is affected by various factors, including the transport rate of the solvent into the plant material, solubilization rate of plant soluble constituents into the solvent and the transport rate of the solution out of the plant insoluble material. Thus, minimizing the plant material size by grinding is essential to increase the surface area of contact between the plant material and the solvent, and decreasing the radial distances traversed between the plant solid materials resulting in better transport rate of the solvent into the plant material and solution out of insoluble material. Occasional shaking during maceration is important, it assists the diffusion process and ensures dispersal of the concentrated solution around the plant material surface and brings fresh solvent to the plant material surface for further extraction (Handa et al., 2008).

#### **2.4.2.2 Infusion**

Fresh infusions are prepared by macerating the plant material for a short period with boiling or cold water. These are dilute solutions of the readily soluble constituents of the plant material (Handa et al., 2008).

#### **2.4.2.3 Percolation**

This procedure is most frequently used to extract active constituents in the preparation of tinctures and fluid extracts. A percolator (cylindrical vessel with a conical bottom containing a discharge valve) is generally used. Plant material is soaked in a suitable solvent inside the percolator until equilibrium. Following that, the solvent extract is drained out through the discharge valve of the percolator. Fresh solvent is added at the top into the percolator and the solvent extract drips out through the bottom discharge valve after acquiring equilibrium. Washing plant material is repeated four to five times until the material gets exhausted (Handa et al., 2008).

### **CHAPTER 3: ANTIBACTERIAL EFFECT OF A COMBINATION OF *CAMELLIA SINENSIS* VAR. *ASSAMICA* AND *SALVADORA PERSICA* L. AGAINST PRIMARY COLONIZERS OF DENTAL PLAQUE**

#### **3.1 Introduction**

Dental plaque can be defined as the soft deposit that form a 3 dimensional, structurally organized, multispecies biofilm adhering to the non-shedding hard surfaces in the oral cavity, including tooth surfaces, and removable and fixed restorations (Allison et al., 1995). The formation of dental plaque is a sequential process that is initiated by adhesion of the primary plaque colonizers to salivary pellicle covering tooth surfaces (Rosan et al., 2000). It was reported that after tooth brushing, streptococci, *Actinomyces* sp. and *Veillonella* sp., as well as obligate aerobes such as *Haemophilus* sp. and *Neisseria* sp. are the predominant oral bacteria present in the dental plaque formed on tooth surfaces (Nyvad et al., 1987; Aas et al., 2005; Diaz et al., 2006; Dige et al., 2009). These primary colonizers start to multiply immediately after their firm attachment to tooth surface and their metabolism modifies the local environment. As the biofilm develops, the already attached primary colonizers provide complementary receptors for the adhesins on the surface of secondary plaque colonizers and mediate their attachment by a process termed as co-adhesion or co-aggregation, and the composition of the developing plaque becomes more diverse (Lang et al., 2015). Subsequently, the dental plaque formation goes through physiologic and physical interactions among secondary colonizers resulting in maturation (Rosan et al., 2000).

Accumulation of dental plaque on tooth surfaces may contribute to the development of gingivitis, dental caries and periodontal diseases. Therefore, a regular plaque control performed at the early stage of plaque development is an essential preventive measure that interferes with plaque development and maintains good oral hygiene (Axelsson et al., 1981).

Chemical plaque control are the preventive measures using preparations containing chemical agents that are delivered into the oral cavity through various vehicles, including mouth rinses, toothpastes, chewing gum and gels (Axelsson et al., 2002). Chemical plaque control measures can be used as adjunctive measures by subjects who experience difficulties in achieving proper plaque control using only mechanical measures (Teles et al., 2009). CHX mouth rinse is the most effective antiseptic used for that purpose. Unfortunately, several side effects are experienced with its use and this may encourage researchers to find an alternative mouth rinse that has the beneficial effects of CHX with fewer side effects.

Traditional natural medicinal plants are widely available and have been utilized as stable, safe and biologically active plant-derived galenicals as alternative to synthetic drugs (Farnsworth et al., 1985). One of these traditional plants, *Salvadora persica* L. chewing stick was recommended by the World Health Organization and further research on their biological activities on oral health was encouraged (Löe, 2000). The antibacterial effect of *Salvadora persica* L. extracts against dental plaque bacteria was well documented (Sofrata et al., 2011a; Sofrata et al., 2011b; Chelli-Chentouf et al., 2012). In concern to the primary plaque colonizers, earlier studies had shown effective antibacterial effect of *Salvadora persica* L. against primary colonizing bacteria in dental plaque development, particularly oral streptococci species including *S. mitis*, *S. sanguinis* (Al lafi et al., 1995; Darout et al., 2002) and *A. viscosus* (Darmani et al., 2006; Vahabi et al., 2011). Toothpastes containing *Salvadora persica* L. were found to exhibit an antibacterial effect against *S. sanguinis* and *A. viscosus* (Poureslami et al., 2007).

In addition to *Salvadora persica* L., green tea, produced from non-fermented leaves of *Camellia sinensis* var. *assamica*, is a popular drink which has beneficial effect



on periodontal tissues (Kushiyama et al., 2009), and has been proven to exhibit antibacterial effects against bacterial species of dental plaque (Shumi et al., 2014). Primary plaque colonizers are among the oral bacteria that are reported to be susceptible to the antibacterial activity of green tea extracts. Tsai et al. (2008) prepared green tea extract by mixing 10 g of dried leaves with 50 ml of methanol at room temperature for 3 hours. They found that this extract showed an antibacterial activity against *S. sanguinis* with minimum inhibition concentration values of 4 mg/ml. Subsequent study supported this antibacterial activity of alcoholic green tea extracts against *S. sanguinis*, but at a higher concentration, i.e. 0.08 g/ml (López Rodríguez, 2014). In another study, it was reported that alcoholic extract of green tea at a concentration of 3 mg/ml exhibited bactericidal effects against *S. mitis* and *S. sanguinis* (Hassani et al., 2008). The alcoholic extract of green tea was also reported to have antibacterial activity against *A. viscosus* (Sunitha et al., 2012). Green tea polyphenols were reported to have bactericidal effects against *S. mitis* and *S. sanguinis* at a concentration of 2 mg/ml (Cho et al., 2010).

In conclusion, both *Salvadora persica* L. and green tea extracts are reported to exhibit antibacterial activity against several dental plaque bacteria including primary colonizers. This study is the first trial that investigate the synergistic antibacterial effects of combined *Salvadora persica* L. and green tea aqueous extracts against primary plaque colonizers *in vitro*.

### **3.2 Null hypothesis**

A combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts exhibits no synergistic antibacterial activity against primary plaque colonizers.

### **3.3 Aim of the study**

The primary aim of this study was to evaluate the synergistic antibacterial activity of the combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts against primary plaque colonizers *in vitro*.

### **3.4 Objectives of the study**

#### **3.4.1 Antimicrobial Analysis**

- 1- To determine the susceptibility of the primary plaque colonizers to the aqueous extracts of *Camellia sinensis* var. *assamica*.
- 2- To determine the susceptibility of the primary plaque colonizers to the aqueous extracts of *Salvadora persica* L.
- 3- To determine the antibacterial activity of the aqueous extracts of *Camellia sinensis* var. *assamica* against primary plaque colonizers.
- 4- To determine the antibacterial activity of the aqueous extracts of *Salvadora persica* L. against primary plaque colonizers.

#### **3.4.2 Combination formulation**

- 1- To formulate a mixture of aqueous extracts comprising of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. that exhibits the best synergistic activity as an antibacterial agent.

### **3.5 Materials and methods**

#### **3.5.1 Plant material**

##### **3.5.1.1 *Salvadora persica* L.**

Commercial *Salvadora persica* L. root sticks (AL KHAIR, B. NO. AK. 108/140222 SP) were purchased from local markets in Kuala Lumpur in sealed wrappers. They are the roots of *Salvadora persica* L. plant (family: *Salvadoraceae*) which is growing in Pakistan (known as Peelu tree). These commercial *Salvadora persica* L. root sticks were manufactured in Pakistan and imported to Malaysia.

##### **3.5.1.2 *Camellia sinensis* var. *assamica***

Green tea powder in commercial ready-made tea packs (BOH, B. NO. 50722) were purchased from local markets in Kuala Lumpur. The commercial Green tea powder was manufactured in Malaysia from non-fermented leaves of the tea plant *Camellia sinensis* var. *assamica* (family: *Theaceae*) grown in Sri Lanka. The non-fermented leaves of the tea plant were produced by drying and steaming the fresh plant leaves to inactivate polyphenol oxidase and prevent oxidation.

#### **3.5.2 Extraction**

*Salvadora persica* L. and green tea aqueous extracts were prepared by maceration process as described by Seidel (2012). The process of extraction is clearly expressed in Figure 3.1.

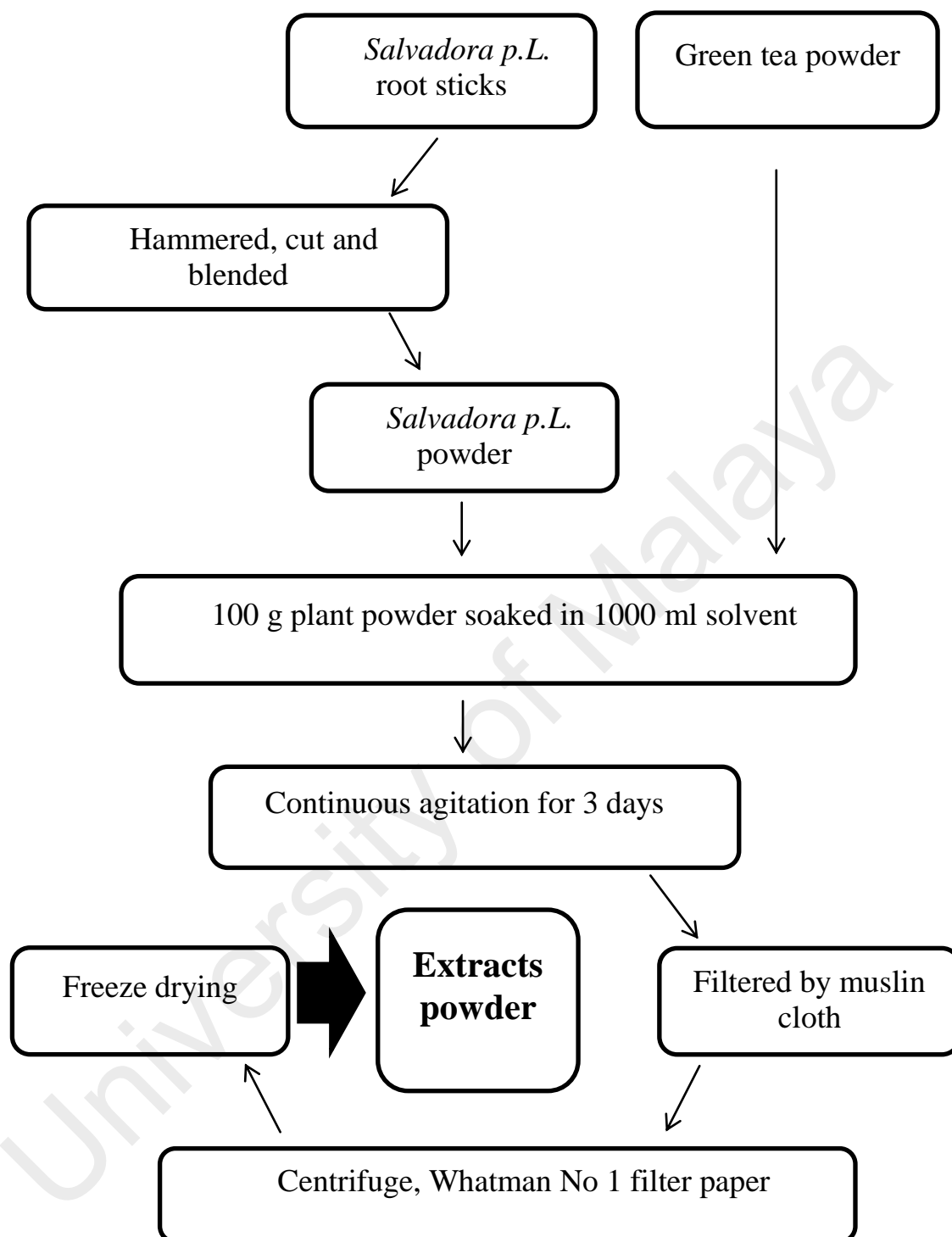
##### **3.5.2.1 Extraction of *Salvadora persica* L. aqueous extracts**

*Salvadora persica* L. root sticks were hammered with a hammer and cut into small pieces (about 1 cm) using a sharp pair of scissors, then the pieces were ground into fine pieces using a commercial grinder. Following that, *Salvadora persica* L. root sticks powder (100 g) was soaked into 1000 ml of non-ionized distilled water with a 1 g plant / 10 ml solvent mixing ratio. The mixture was left for 3 days at room temperature

with continuous shaking in an electrical shaker device (IKA<sup>®</sup> KS 4000 i control) to accelerate the extraction process. After that, the aqueous extracts were filtered using a muslin cloth to remove coarse plant material; the muslin cloth was twisted to release excess of aqueous extracts from the exhausted plant material. The aqueous extracts were centrifuged for 10 minutes at 10000 rpm and once again filtered using filter paper (Whatman No 1, diameter 150 cm). The filtered aqueous extracts were freeze dried using (EYELA FDV-1200) device (as shown in Appendix A) to obtain *Salvadora persica* L. aqueous extracts powder. The powdered extract was stored at room temperature, and the desired working concentrations (w/v) were prepared accordingly prior to the experiment.

#### **3.5.2.2 Extraction of green tea aqueous extracts**

The green tea powder (100 g) was soaked in 1000 ml of non-ionized distilled water with a 1 g plant / 10 ml solvent mixing ratio. The mixture was left for 3 days at room temperature with continuous shaking in an electrical shaker device (IKA<sup>®</sup> KS 4000 i control) to accelerate the extraction process. After that, the aqueous extracts were filtered using a muslin cloth to remove coarse plant material; the muslin cloth was twisted to release excess of aqueous extracts from the exhausted plant material. The aqueous extracts were centrifuged for 10 minutes at 10000 rpm and once again filtered using filter paper (Whatman No 1, diameter 150 cm). The filtered aqueous extracts were freeze dried using (EYELA FDV-1200) device (as shown Appendix A) to obtain green tea aqueous extracts powder. The powdered extract was stored at room temperature, and the desired working concentrations (w/v) were prepared accordingly prior to the experiment.



**Figure 3.1: Extraction process (Maceration)**

### **3.5.3 Bacteria**

#### **3.5.3.1 Selection of bacteria**

*Streptococcus sanguinis* (ATCC BAA-1455), *Streptococcus mitis* (ATCC 49456) and *Actinomyces viscosus* (ATCC 43146) were selected for this study since they are considered as primary bacterial colonizers in human dental biofilm (Li et al., 2004). These bacteria were obtained from the 20% glycerol stocks stored in -80 °C in the Balai Ungku Aziz Research Laboratory (BUARL), Faculty of Dentistry University of Malaya.

#### **3.5.3.2 Preparation of culture media for bacterial culturing**

The non-selective Brain Heart Infusion (BHI) agar culture media (CM1136, Oxoid Ltd, Hampshire, UK) was used for preparing working cultures for *S. mitis*, *S. sanguinis* and *A. viscosus*. This culture media was prepared by suspending 47 g of medium powder in 1000 ml distilled water then heating with frequent agitation by boiling for 1 minute to completely dissolve the powder. The solution was sterilized by autoclaving at 121 °C for 15 minutes. The sterile solution was allowed to cool to 70 °C - 80 °C. By using biological safety cabinet, the sterile solution was aseptically dispensed in petri dishes and then kept at 4 °C in refrigerator until be used.

BHI broth culture media (CM1135, Oxoid Ltd, Hampshire, UK) was used for preparing bacterial suspensions for *S. mitis*, *S. sanguinis* and *A. viscosus*. It was prepared by suspending 37 g of medium powder in 1000 ml distilled water then boiled with frequent agitation for 1 minute to completely dissolve the powder. The solution was sterilized by autoclaving at 121 °C for 15 minutes. The sterile solution was allowed to cool to room temperature and then kept at 4 °C in refrigerator until further used.

#### **3.5.3.3 Stock Cultures Preparation**

The respective 20% glycerol stocks of each bacterial species, kept frozen at -80 °C, were allowed to thaw at room temperature. Following that, 100 µl of each respective

thawed stock was used to inoculate BHI agar media plates and then incubated at 37 °C for 18-24 hours. For short term storage, these BHI agar cultures were stored at 4 °C for further use in the experiments as working culture. Throughout the study period, these bacteria cultures were maintained on agar media at 4 °C up to maximum of two weeks in which sub-culturing was carried out every two weeks to ensure cells viability. For long term storage, the strains were grown in BHI broth to late log phase and 20% glycerol were added to the bacterial suspension. All vials were kept in -80 °C for long term storage.

#### **3.5.3.4 Preparation of Standard Bacterial Cell Suspension**

The growth bacterial colonies of working stock culture were transferred respectively (picking only 3 - 5 colonies by inoculating loop) into 5 ml of BHI broth and incubated for 18 – 24 hours at 37 °C. After an overnight incubation, the turbidity of the respective bacterial suspension was adjusted at a wavelength of 550 nm using a spectrophotometer (Shimadzu UV-1700, Japan) to an absorbance of 0.144 which is equivalent to 10<sup>6</sup> cells/ml (Rahim et al., 2014). Throughout the experiments, three species biofilm mixture of *S. mitis*, *S. sanguinis* and *A. viscosus* was used with mixing ratio of 1:1:1 by volume.

#### **3.5.4 Antimicrobial evaluation of the extracts**

##### **3.5.4.1 The Kirby-Bauer Susceptibility Test (disk diffusion)**

The protocol of disk diffusion method used for antimicrobial susceptibility test in this study was as described by Bauer et al.(1966). The BHI agar plates were inoculated with 100 µl of each respective *S. mitis*, *S. sanguinis*, *A. viscosus* and their biofilm mixture suspensions. Sterile filter paper disks (6 mm in diameter) were impregnated with 50 µl of each respective aqueous extracts of *Salvadora persica* L. and green tea with a concentration of 1 g/ml, 0.75 g/ml, 0.5 g/ml and 0.25 g/ml as test.

Sterilized distilled water and nonalcoholic 0.12% CHX were used as the negative and positive controls respectively. All these disks which had been incorporated with respective extracts at different concentrations, sterilized distilled water and 0.12% CHX were left overnight to dry before placement on agar surfaces. All the agar plates were incubated at 37 °C for 24 hours. Following incubation, the average diameter of inhibition zone surrounding the disks containing the test solution was measured using a millimeter ruler. The test was repeated 3 times in triplicate to ensure reproducibility of results.

#### 3.5.4.2 Minimum inhibition concentration (MIC)

MIC of each respective aqueous extracts of *Salvadora persica* L. (MIC<sub>sp</sub>) and green tea (MIC<sub>gt</sub>) was determined by two-fold serial broth micro-dilution (Cavalieri et al., 2005). This test was repeated 3 times in triplicate to ensure reproducibility of the results. A 100 µl of BHI broth was dispensed into each eight wells of two rows, labelled W1 to W8, in 96-well microtiter trays (NUNC™ Brand products). For determining MIC<sub>sp</sub>, 100 µl of aqueous extract of *Salvadora persica* L. with a concentration of 125 mg/ml was added into W1 of each respective row and two-fold serial dilution was carried out from W1 through W6. Thus the final concentrations of aqueous extract of *Salvadora persica* L. in the wells were 62.5 mg/ml, 31.25 mg/ml, 15.63 mg/ml, 7.81 mg/ml, 3.9 mg/ml and 1.95 mg/ml respectively. W7, which was the negative control, contained nutrient broth only. In contrast, W8 contained 100 µl of the mixture of non-alcoholic 0.12% CHX with nutrient broth, and it acted as the positive controls. Except for the first row which was considered as a blank row, all wells of the second row were inoculated with 10 µl of *S. mitis*, *S. sanguinis*, *A. viscosus* mixture suspension and incubated overnight at 37 °C. After incubation, the inhibition of bacterial growth in the wells was determined by loss of turbidity observed via naked eye. MIC was representing the concentration of extract in the well that showed no growth of the



bacteria. The same procedure was also repeated for each single strain of the respective bacteria, including *S. mitis*, *S. sanguinis* and *A. viscosus*.

For determining MIC<sub>gt</sub>, 100 µl of aqueous extract of green tea with a concentration of 7.8 mg/ml was added into W1 of each respective row. Then, two-fold serial dilution was carried out from W1 through W6, thus the final concentrations of aqueous extract of green tea in the wells were 3.9 mg/ml, 1.95 mg/ml, 0.98 mg/ml, 0.49 mg/ml, 0.24 mg/ml and 0.12 mg/ml respectively, and the procedure was continued as for determining MIC<sub>sp</sub>.

#### **3.5.4.3 Minimum bactericidal concentration (MBC)**

After determination of MICs, 50 µl from tubes identified with the MICs including the next two concentrations that did show any turbidity of all the respective bacteria were pipetted out and sub-cultured onto BHI agar plates. The agar plates were incubated at 37 °C for 24 to 48 hours until visible growth of colonies was detected. The MBC value was represented as the concentration where no growth or fewer than three colonies were obtained that gave an approximately 99 to 99.5% killing activity (Cavalieri et al., 2005).

#### **3.5.5 Antimicrobial and synergistic evaluation of the extracts combinations**

##### **3.5.5.1 Formulation of green tea and *Salvadora persica* L. test combinations**

Three test combinations (TC) of *Salvadora persica* L. and green tea aqueous extracts were prepared depending on their MIC values as illustrated in Table 3.1. The first TC (TC1) was prepared by mixing concentrated four folds of MIC<sub>gt</sub> with four folds of MIC<sub>sp</sub> to achieve a green tea : *Salvadora persica* L. concentration ratio of (4 : 4). The second TC (TC2) was prepared by mixing four folds of MIC<sub>gt</sub> with two folds of MIC<sub>sp</sub> to achieve a green tea : *Salvadora persica* L. concentration ratio of (4 : 2). Finally, the

third TC (TC3) was prepared by mixing two folds of MIC<sub>gt</sub> with four folds of MIC<sub>sp</sub> to achieve a green tea : *Salvadora persica* L. concentration ratio of (2 : 4).

**Table 3.1: Ratio and concentration of green tea (Gt) and *Salvadora persica* L. (Sp) test combinations**

Test combination (TC)	MIC Concentration Values				Gt : Sp
	Gt		Sp		Concentration Ratio
	Concentration	mg	Concentration	mg	
TC1	4x MIC	3.92	4x MIC	62.52	4 : 4
TC2	4x MIC	3.92	2x MIC	31.26	4 : 2
TC3	2x MIC	1.96	4x MIC	62.52	2 : 4

### 3.5.5.2 Antimicrobial and synergistic evaluation of the green tea and *Salvadora persica* L. test combinations

The antimicrobial evaluation for each test combination, including TC1, TC2 and TC3, was determined by adding 100 µl of BHI broth into each six wells of two rows, labelled W1 to W6, in a 96-well microtiter plate (NUNC™ Brand products). A 100 µl of the respective test combination was added into W1 of each respective row and two-fold serial dilution was carried out from W1 through W4. The negative control was nutrient broth only contained in W5. In contrast, W6 contained 100 µl of the mixture of nonalcoholic 0.12% CHX with nutrient broth, and it acted as the positive control as shown in Table 3.2. Except for the first row which was considered as a blank row, all wells of the second row were inoculated with mixture suspension of *S. mitis*, *S.*

*sanguinis* and *A. viscosus*. The microtiter plate was then incubated overnight at 37 °C. Following incubation, the inhibition of bacterial growth in the wells was determined by recording optical density (OD) of the bacterial suspensions in the wells at a wavelength of 550 nm using spectrophotometer (μQuant, FC-BIOS).

**Table 3.2: Two-fold serial dilutions of green tea and *Salvadora persica* L. test combinations in wells (W) of microtiter plate**

Test combinations	green tea : <i>Salvadora persica</i> L. MIC ratio					
(TC)	W1	W2	W3	W4	W5	W6
TC1	2 : 2	1 : 1	1/2 : 1/2	1/4 : 1/4	BHI broth	0.12% CHX
TC2	2 : 1	1 : 1/2	1/2 : 1/4	1/4 : 1/8	BHI broth	0.12% CHX
TC3	1 : 2	1/2 : 1	1/4 : 1/2	1/8 : 1/4	BHI broth	0.12% CHX

From the test combinations, combination dilutions were selected for further investigations which had an OD value close to that of the positive control 0.12% CHX. This experiment was carried out in triplicate at 3 different times to ensure accuracy and reproducibility of the results. Then, fractional inhibitory concentration (FIC) index was calculated for each selected combination using the following equation:

$$\begin{aligned}
 FIC \text{ index} &= FIC_{Gt} + FIC_{Sp} \\
 &= (\text{concentration of green tea, in combination} / MIC_{Gt, \text{ in test alone}}) \\
 &+ (\text{concentration of } Salvadora \text{ persica L., in combination} / MIC_{Sp, \text{ in test alone}}).
 \end{aligned}$$

The extracts interaction in the selected combinations was described as synergistic ( $FIC \leq 0.5$ ), partial synergistic ( $0.5 < FIC < 1$ ), additive ( $FIC = 1$ ), indifferent ( $1 < FIC < 4$ ) or antagonistic ( $FIC \geq 4$ ) (Lorian, 2005).

### 3.6 Results

The aqueous extracts of *Salvadora persica* L. and green tea were prepared by using maceration method. 100 g of each plant powder was soaked in 1000 ml of solvent, and then the filtered aqueous extracts were freeze dried to obtain plants aqueous extracts powder. The yield aqueous extract powder after extracting 100 g green tea plant material was 21 g powder. While, the yields aqueous extract powder after extracting 100 g of *Salvadora persica* L. plant material was 13 g powder as shown in Table 3.3.

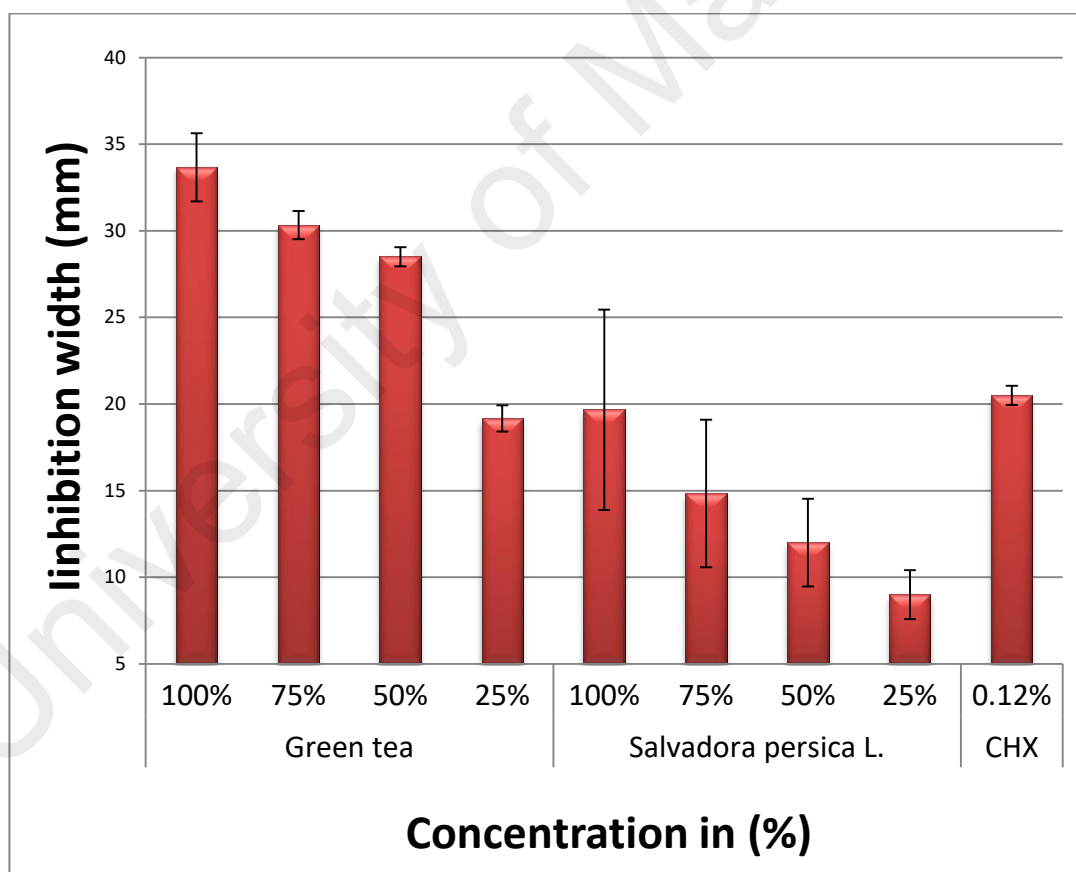
**Table 3.3: Yield aqueous extract powder after extracting 100 g of each green tea and *Salvadora persica* L. plant material.**

Plant material (100 g)	Yield aqueous extract powder
Green tea	21 g
<i>Salvadora persica</i> L.	13 g

The susceptibility of the primary dental plaque colonizers namely *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension to green tea and *Salvadora persica* L. aqueous extracts was determined by using disk diffusion method. In this method, BHI agar plate was used and inoculated with the primary dental plaque colonizers. The susceptibility of the primary colonizing bacteria to both extracts was represented by the width of growth inhibition zones formed around disks impregnated with different concentrations (i.e. 100%, 75%, 50% and 25%) of each aqueous extract of green tea and *Salvadora persica* L. respectively. The outcomes of the bacterial susceptibility analysis revealed that all primary dental plaque colonizers (*S. mitis*, *S. sanguinis* and *A. viscosus*) and their mixture suspension were found to be susceptible to green tea and *Salvadora persica* L. aqueous extracts with higher susceptibility observed to green tea aqueous

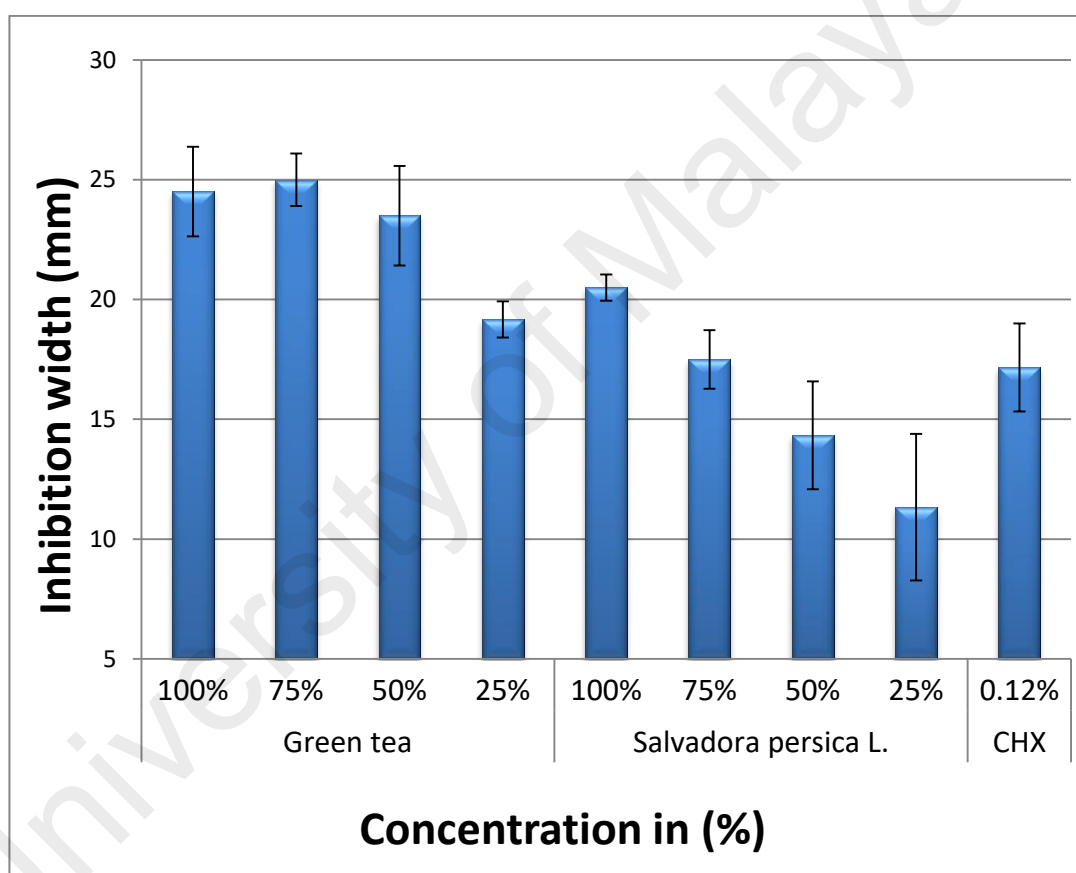
extract compared with *Salvadora persica L.* aqueous extract in a dose dependent manner.

In this study, *S. mitis* was found to be susceptible to both green tea and *Salvadora persica L.* aqueous extracts as shown in Figure 3.2. This susceptibility was in dose dependent manner where wider zones of inhibition were formed around 100% of both green tea and *Salvadora persica L.* aqueous extracts, while narrower zones were formed around 25%. Another finding that *S. mitis* was more susceptible to green tea aqueous extract than *Salvadora persica L.* aqueous extract at 100%, 75%, 50% and 25% concentrations respectively.



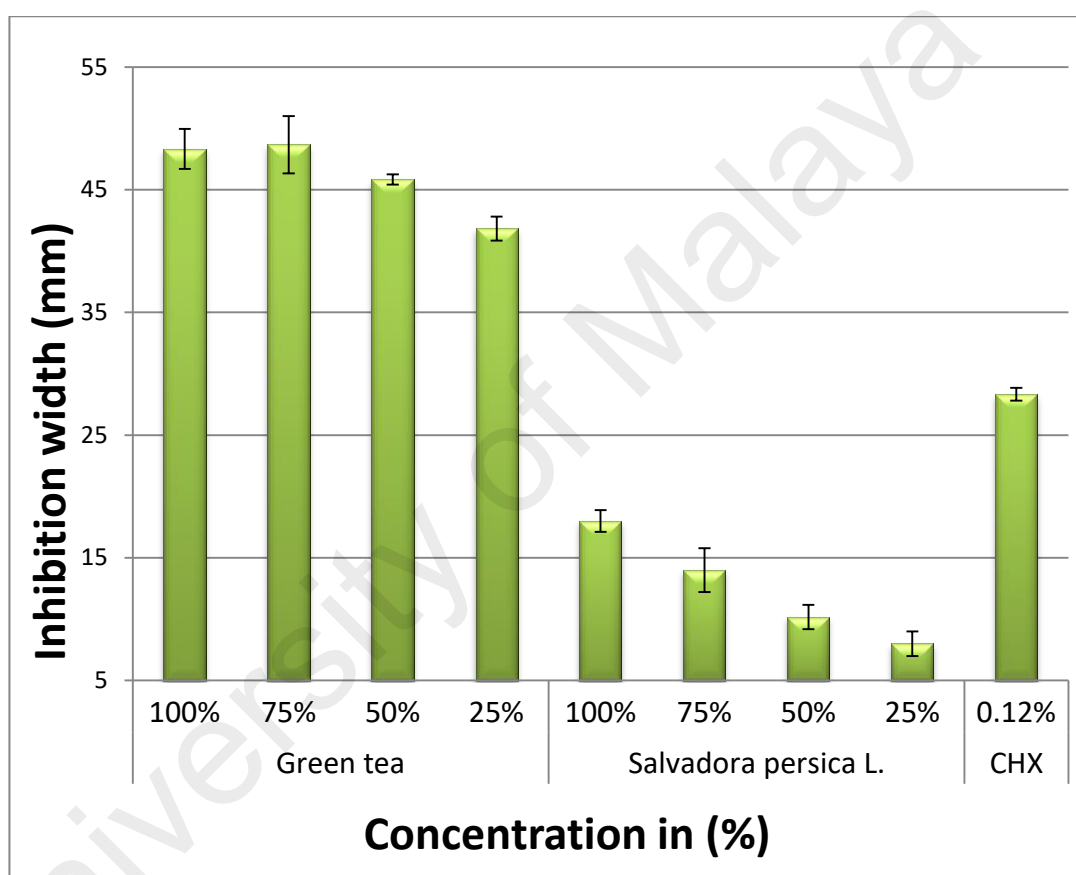
**Figure 3.2: Zone of inhibition of different concentration of green tea and *Salvadora persica L.* aqueous extracts against *S. mitis***

Figure 3.3 shows the susceptibility of *S. sanguinis* towards different concentrations of green tea and *Salvadora persica* L. aqueous extracts which is represented by bars of inhibition width. *S. sanguinis* was found to be more susceptible to green tea aqueous extract when compared with *Salvadora persica* L. aqueous extracts at all respective concentrations. In respect to each extract, *S. sanguinis* was found to have a dose dependent susceptibility to each particular extract where higher susceptibility was observed to higher extract concentrations.



**Figure 3.3: Zones of inhibition of different concentrations of green tea and *Salvadora persica* L. aqueous extracts against *S. sanguinis***

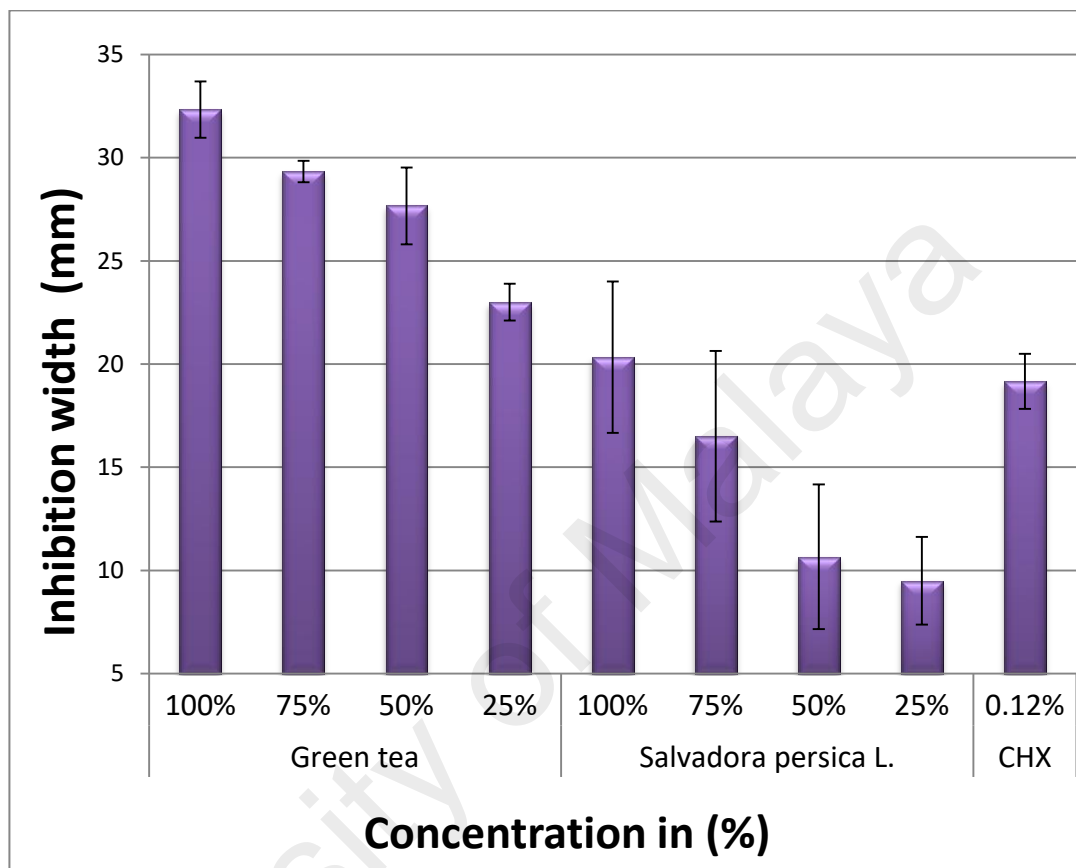
For *A. viscosus*, the outcomes of the disk diffusion test revealed high susceptibility of this primary colonizing bacterium towards green tea aqueous extracts compared with *Salvadora persica L.* aqueous extracts. The susceptibility was found to be dose dependent where wider zones of growth inhibition were observed around higher concentrations of both green tea and *Salvadora persica L.* aqueous extracts as shown in Figure 3.4.



**Figure 3.4: Zones of inhibition of different concentrations of green tea and *Salvadora persica L.* aqueous extracts against *A. viscosus***

Zones of inhibition of different concentrations of green tea and *Salvadora persica L.* aqueous extracts against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension are illustrated in Figure 3.5. The results of this study revealed that the primary colonizing bacteria mixture suspension was more susceptible to green tea

aqueous extracts compared with *Salvadora persica* L. aqueous extracts as shown in the figure. This susceptibility was found to be dose dependent in which higher susceptibility of the bacteria was observed to higher concentration in respect for each extract.

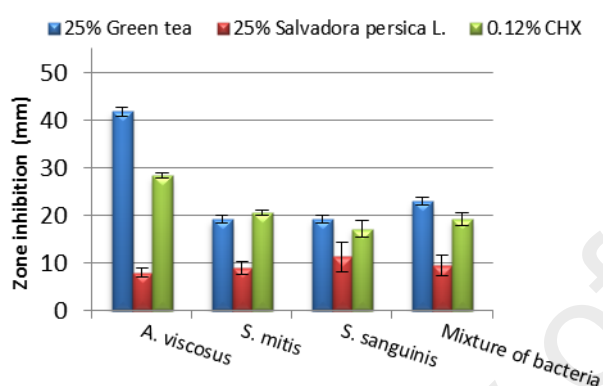


**Figure 3.5: Zones of inhibition of different concentrations of green tea and *Salvadora persica* L. aqueous extracts against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension**

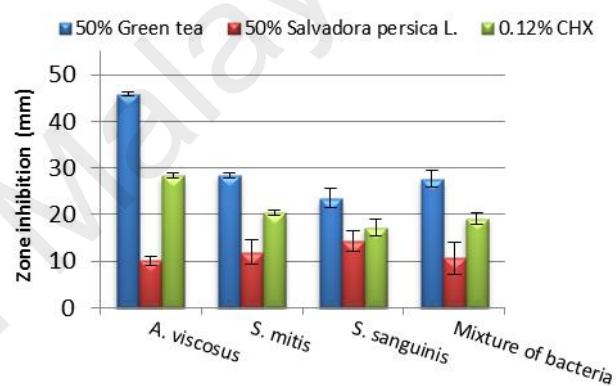
Figure 3.6 shows the comparison of the susceptibility of each respective bacterium (*S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension) to green tea aqueous extract with the susceptibility of these bacteria to *Salvadora persica* L. aqueous extract at the same concentration. *A. viscosus* was found to be more susceptible to green tea aqueous extract than *S. mitis*, *S. sanguinis* and the mixture suspension at each respective concentration of 25% (see Figure 3.6 A), 50% (see Figure 3.6 B), 75% (see Figure 3.6 C) and 100% (see Figure 3.6 D). There were no large differences between the



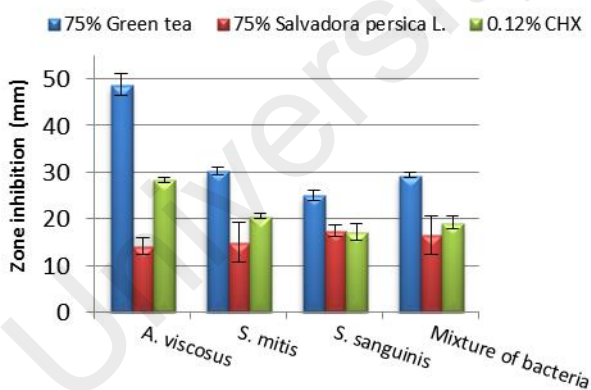
susceptibility of *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension to *Salvadora persica L.* aqueous extract at each respective concentration of 25% (see Figure 3.6 A), 50% (see Figure 3.6 B), 75% (see Figure 3.6 C) and 100% (see Figure 3.6 D). All the primary colonizing bacteria were more susceptible to green tea aqueous extract than *Salvadora persica L.* aqueous extract at each respective concentration of 25% (see Figure 3.6 A), 50% (see Figure 3.6 B), 75% (see Figure 3.6 C) and 100% (see Figure 3.6 D).



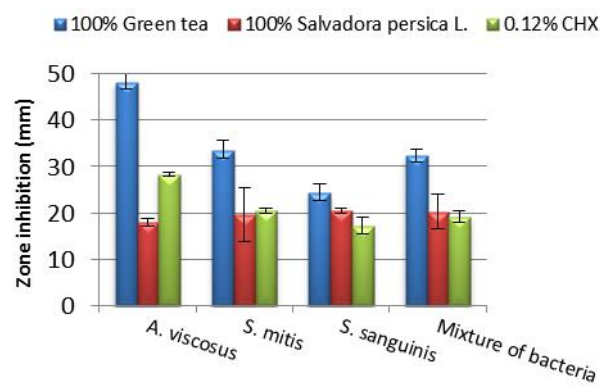
(A)



(B)



(C)



(D)

**Figure 3.6: Zones of inhibition of green tea and *Salvadora persica L.* aqueous extracts against *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension at concentration (A) 25%, (B) 50%, (C) 75% and (D) 100%**

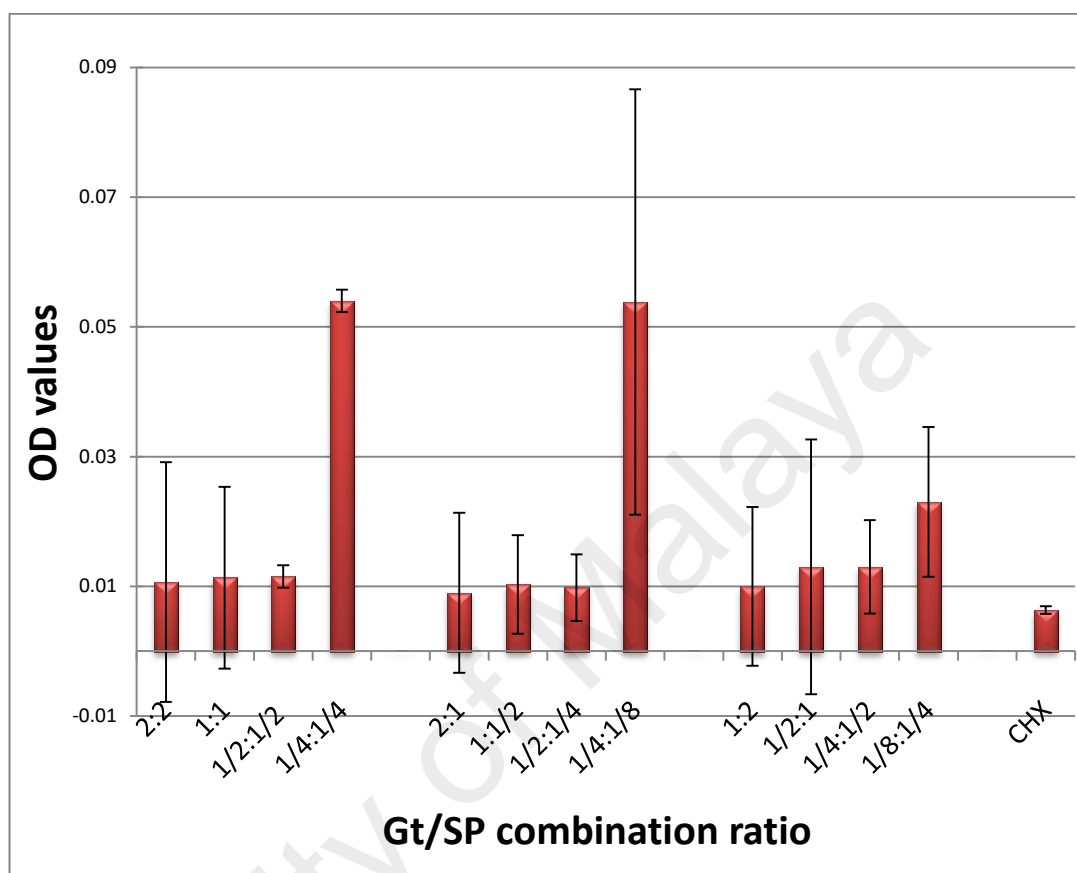
In this study, MIC values of green tea and *Salvadora persica L.* aqueous extracts against *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixtures were determined by observing loss of turbidity via naked eye of inoculated two-fold serial dilutions of each extract after incubation. In contrast, MBC values were determined by inhibiting the bacterial growth (three or less colonies) on BHI agar plates. According to MIC and MBC values as shown in Table 3.4, green tea aqueous extract exhibited higher antibacterial activity than *Salvadora persica L.* aqueous extract. It was found that the minimum concentrations of green tea aqueous extract required to inhibit the growth of *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 0.98 mg/ml, 0.98 mg/ml, 0.06 mg/ml and 0.98 mg/ml respectively. In contrast, the minimum concentrations of *Salvadora persica L.* aqueous extract required to inhibit the growth of *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 15.63 mg/ml, 15.63 mg/ml, 3.91 mg/ml and 15.63 mg/ml respectively. For bactericidal effects, it was found that the minimum concentrations of green tea aqueous extract required to provide bactericidal activity against *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 1.95 mg/ml, 1.95 mg/ml, 0.12 mg/ml and 1.95 mg/ml respectively. On the other hand, the minimum concentrations of *Salvadora persica L.* aqueous extract required to provide bactericidal activity against *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 31.25 mg/ml, 31.25 mg/ml, 15.63 mg/ml and 31.25 mg/ml respectively.

**Table 3.4: MIC and MBC values of green tea and *Salvadora persica* L. aqueous extracts against *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixtures.**

Bacteria	Green tea		<i>Salvadora persica</i> L.	
	MIC	MBC	MIC	MBC
	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)
<i>A. viscosus</i> (A.v)	0.06	0.12	3.91	15.63
<i>S. mitis</i> (S.m)	0.98	1.95	15.63	31.25
<i>S. sanguinis</i> (S.s)	0.98	1.95	15.63	31.25
MIX (A.v + S.m + S.s)	0.98	1.95	15.63	31.25

After determination of the MIC values of green tea aqueous extract and *Salvadora persica* L. aqueous extract against the *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension, these MIC values were used to formulate 3 test combinations of green tea and *Salvadora persica* L. aqueous extracts. For antibacterial analysis, two-fold serial dilution was carried out for the test combinations which were then inoculated with *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension and incubated overnight. Depending on the optical density (OD) values of the test combination dilutions, it was found that 3 dilutions have antibacterial activity close to that of 0.12% CHX as shown in Figure 3.7. These test combination dilutions were: a) 1/2:1/2 dilution which was equivalent to 1/2 MIC value of green tea aqueous extract to 1/2 MIC value of *Salvadora persica* L. aqueous extract; b) 1/2:1/4 dilution (DTC2) which was equivalent to 1/2 MIC value of green tea aqueous extract to 1/4 MIC value of *Salvadora persica* L. aqueous extract; c) 1/4:1/2 dilution (DTC1) which was equivalent to 1/4 MIC value of green tea aqueous extract to 1/2 MIC value of *Salvadora persica* L. aqueous extract (see Figure 3.7 and Table 3.5). Among these dilutions of test combination, DTC1 and DTC2 were selected to calculate their fractional inhibitory concentration (FIC) index as described in

Table 3.5. In both DTC1 and DTC2, green tea aqueous extract and *Salvadora persica* L. aqueous extract interaction exhibited partial synergism with a FIC index value of 0.75.



**Figure 3.7: The optical density (OD) values following exposure of bacterial suspension to various green tea / *Salvadora persica* L. (Gt/Sp) combinations.**

CHX is 0.12% chlorhexidine. Untreated OD was  $0.529 \pm 0.054$  (not shown)

**Table 3.5: Extracts concentration and interaction of dilute test combinations of green tea aqueous extract (Gt) and *Salvadora persica* L. aqueous extract (Sp) having antibacterial activity close to 0.12% CHX.**

Dilute test combination (DTC)	MIC Concentration Values					FIC	Extracts interaction
	Gt		Sp		Gt : Sp Concentration Ratio		
	Concentration	mg	Concentration	mg			
DTC1	1/4x MIC	0.25	1/2x MIC	7.82	1/4 : 1/2	0.75	Partial synergism
DTC2	1/2x MIC	0.49	1/4x MIC	3.91	1/2 : 1/4	0.75	Partial synergism

### 3.7 Discussion

*S. mitis*, *S. sanguinis* and *A. viscosus* are normal oral flora primarily which adhere to acquired pellicle covering tooth surfaces and form a bacterial initial layer for subsequent dental plaque buildup (Li et al., 2004). The salivary levels of *S. mitis* and *S. sanguinis* are significantly lower in *Salvadora persica* L. chewing stick users than toothbrush users (Darout et al., 2002). Moreover, the extracts of *Salvadora persica* L. were reported to have a positive antibacterial effect against *A. viscosus* (Darmani et al., 2006; Vahabi et al., 2011). Further, primary plaque colonizers were reported to be susceptible to the antibacterial activity of green tea extracts. Tsai et al. (2008) and López Rodríguez (2014) found that green tea alcoholic extract inhibited the growth of *S. sanguinis*. In another study, it was reported that alcoholic extract of green tea showed a bactericidal effect against *S. mitis* and *S. sanguinis* (Hassani et al., 2008). The alcoholic extract of green tea was also reported to have antibacterial activity against *A. viscosus* (Sunitha et al., 2012). Green tea polyphenols were reported to kill *S. mitis* and *S. sanguinis* at a concentration of 2000 mg/ml (Cho et al., 2010). So far, there is no data about the antibacterial effects of the combination of these popular *Salvadora persica* L. chewing sticks and green tea aqueous extracts against the primary dental plaque colonizers. Thus, it was aimed, in this study, to investigate the antibacterial effect of *Salvadora persica* L. and green tea aqueous extract combination on primary dental plaque colonizers, i.e. *S. mitis*, *S. sanguinis* and *A. viscosus*.

In this study, it was decided to use commercial dried plant material for preparing both *Salvadora persica* L. and green tea aqueous extracts rather than fresh plants. This decision was made to avoid time delay between plant material collection and processing. Eloff (1998) reported that it is difficult to work with fresh plant material because of the differences in water content that may affect solubility or subsequent separation by liquid to liquid extraction. On the other hand, the problems associated

with the extraction process for dried plant material were reported to be fewer than the problems experienced with the extraction process for fresh plant material. Moreover, most traditional healers used plants in the dried form or as aqueous extract because the secondary plant constituents have to be relatively stable particularly if it is to be used as an antimicrobial agent (Eloff, 1998). Also, commercial products of plant material can provide full information about the used plants including batch numbers, scientific plants' names, the used part of the plant, and the place where the plant was grown and collected. For all above reasons, it was opted to use green tea powder in commercial ready-made tea packs (BOH, B. NO. 50722) and commercial *Salvadora persica* L. root sticks (AL KHAIR, B. NO. AK. 108/140222 SP).

In this study, maceration was selected as a suitable method for green tea and *Salvadora persica* L. crude plants extraction. The principle mechanism of maceration is leaching out soluble plant constituents from the solid plant mass by soaking in an appropriate solvent at room temperature. Unlike other possible soxhlet and serial exhausting extraction methods, heat was avoided in maceration which may affect some of thermolabile constituents which might be present in the plants extracts (Seidel, 2012). For example, polyphenolic catechins in the green tea were reported to undergo degradation when green tea drinks are heated at a temperature of 98 °C for 15 minutes. This causes reduction in green tea catechins content by about 1 – 15%. Heating green tea drinks at 120 °C for 20 minutes causes a 23% degradation of catechins (Wang et al., 2000). Also green tea catechins were found to be stable in water at room temperature. High-performance liquid chromatography (HPLC) analysis revealed that about 10 – 15% green tea catechins would be lost if tea was prepared and brewed in boiling water (Chen et al., 2001). On the other hand, it was reported that boiling active *Salvadora persica* L. chewing sticks in water for 2 hours could deactivated its bioactive compounds, thus leading to lose their antibacterial activity (Sofrata et al., 2011a).

In the extraction process, the plant material was ground into finer pieces in order to increase the surface area of the plant material to be in contact with the solvent. This resulted in an increased rate of the extraction process. For mixing plants materials to the solvent, a 1 : 10 (plants materials weight to selected solvent volume) mixing ratio was used as this ratio has been reported as ideal (Das et al., 2010).

The selection of the solvent between water and alcohols (i.e. ethanol and methanol) for plants extraction was a challenge. The antibacterial effects of the aqueous extracts were initially assessed *in vitro*. In the third part of this study, a test mouth rinse of the aqueous extracts and evaluation of its antiplaque activity was carried out *in vivo*. Although alcoholic extracts exhibit better antimicrobial effect (Das et al., 2010), but it was reported that they have restrictions when used intraorally in certain patients for example pregnant and nursing women, alcoholics, diabetic, children and patients with xerostomia (Mankodi et al., 2005; Van Strydonck et al., 2005; Witt et al., 2005). In addition, it could cause unwanted effects such as composite filling surface softening (Penugonda et al., 1994). For that reason, distilled water was decided to be used for extracting plant constituents with antimicrobial activity since it is the safest and inexpensive universal solvent (Das et al., 2010).

In the antibacterial effect experiments, *S. sanguinis*, *S. mitis* and *A. viscosus* were selected for this study since they are considered as primary bacterial colonizers in human dental biofilm (Li et al., 2004). Throughout the experiments, three species biofilm mixture of *S. mitis*, *S. sanguinis* and *A. viscosus* was used with mixing ratio of 1:1:1 by volume. During the first 4 to 8 hours after tooth brushing, streptococci are the most predominant oral bacteria present in the biofilm formed on tooth surfaces (Nyvad et al., 1987; Dige et al., 2009). Other bacteria species are commonly present at this time include obligate aerobes such as *Haemophilus* sp. and *Neisseria* sp., as well as

facultative anaerobes including *Actinomyces* sp. and *Veillonella* sp. (Aas et al., 2005; Diaz et al., 2006). These proportions of bacteria continuously change with time until maturation of dental plaque. By reviewing the literature, there is no information about the ratio between planktonic *S. sanguinis*, *S. mitis* and *A. viscosus* in human saliva or within dental plaque. For that reason, it was opted to use the 3 species biofilm mixture of *S. mitis*, *S. sanguinis* and *A. viscosus* with mixing ratio of 1:1:1 by volume, i.e. equal proportions for each bacterium.

In this study, the disk diffusion experiment results revealed that all tested bacteria and their mixture were sensitive to both green tea and *Salvadora persica* L. aqueous extracts in a dose-dependent manner. Also, MIC values of both green tea and *Salvadora persica* L. aqueous extracts revealed good antibacterial activity against tested bacteria. This finding agrees with the earlier studies which showed effective antibacterial effect of *Salvadora persica* L. against primary colonizer bacteria in dental plaque development, particularly oral streptococci species including *S. mitis*, *S. sanguinis* (Al lafi et al., 1995; Darout et al., 2002; Das et al., 2015) and *A. viscosus* (Darmani et al., 2006; Vahabi et al., 2011). Cho et al. found that 2 mg/ml of tea catechins were toxic to *S. mitis* and *S. sanguinis* (Cho et al., 2010). Hassani *et al.* reported that the growth of *S. mitis* and *S. sanguinis* was inhibited by 3 mg/ml of green tea extract (Hassani et al., 2008). There is only one study (Sunitha et al., 2012) which investigated the antibacterial effect of aqueous tea extract against *A. viscosus* that concluded no effect of the extract against these bacteria. This finding disagrees with our results which revealed high sensitivity of *A. viscosus* to Gt extract. This disagreement may be attributed to the differences in the preparation of the aqueous extract of the plant.



The MIC values revealed a higher antibacterial activity observed in green tea extract than *Salvadora persica* L. extract (0.98 mg/ml and 15.63 mg/ml respectively). This may be attributed to the difference of the constituents found in green tea and *Salvadora persica* L. extracts. Green tea aqueous extract was reported to contain tannins, saponin, glycosides, terpenoids and flavonoids including polyphenolic catechins (Subhashini et al., 2010). Previous studies found that the antibacterial activity of green tea extract against primary bacterial colonizers of dental plaque has been linked to the green tea catechins and particularly EGCG, GCG, EGC, EC and ECG (Hassani et al., 2008; Cho et al., 2010). The most potent catechins reported to exhibit antibacterial activity are EGCG and ECG. Their high antibacterial activity was reported to be attributed to the presence of hydroxyl group (galloyl moiety) (Hamilton-Miller, 1995). The bacterial outer cell membrane, essentially composed of phospholipid bilayer and proteins, is the first site that interacts with antibacterial agents. Any damage to this membrane can result in cell death through either inhibiting of membrane associated enzymes, physical disruption or membrane proton motive force dissipation (Juven et al., 1994; Shimamura et al., 2007).

One possible mechanism suggested for green tea extract antibacterial activity is that EGCG has a damaging effect on the gram positive bacterial cell wall. Cui *et al.* (2012) reported in their study using the atomic force microscopy, that EGCG can damage the cell wall of *Staphylococcus aureus* by binding to peptidoglycan through its hydroxyl group resulting in peptidoglycan cross-linking bridges break down and degradation (Shimamura et al., 2007). Ikigai *et al.* (1993) reported the ability of EGCG to induce damage to the gram positive bacterial cell membrane and leakage of micro-molecules. Moreover, it was reported that major green tea extracts polyphenols may disturb bacterial cell morphology and cytoplasmic membrane through altering the cell osmotic pressure and leading to cell constituents leakage and death (Sivaroooban et al.,

2008). On the other hand, the antibacterial activity of *Salvadora persica L.* may be attributed to its phytochemical constituents including chloride, trimethylamine, salvadorine, thiocyanate, tannis, nitrate and sulphur, in addition to the anionic compounds of aqueous extract including nitrites, sulphate, chloride and thiocyanate which were found to be responsible for the antibacterial effect (Akhtar et al., 1981; Darout et al., 2000b; Vahabi et al., 2011).

Fluoride also can be found in *Salvadora persica L.* Goyal et al. (2011) reported that it might have antibacterial properties demonstrated by bacterial glycolytic enzymes inhibition and possible interaction with intracellular polysaccharides. Glucosionlates can be found in *Salvadora persica L.* aqueous extracts. These constituents are hydrolyzed to isothiocyanate, sulphate and glucose at neutral pH or to nitrate, sulphate, glucose and sulphur at low pH by the action of tissue plant myrosinase. Isothiocyanate is decomposed into its alcoholic part and thiocyanate (Ahmed et al., 1972). *Salvadora persica L.* thiocyanate has antimicrobial effect by amplifying the antimicrobial action of the antibacterial peroxidase-thiocyanate and hydrogen peroxidase system. This occurs during oxidation of thiocyanate and generation of hypothiocyanate by peroxidase with the presence of hydrogen peroxide from oral bacteria. The hypothiocyanate oxidases sulfhydryl groups in bacterial cytoplasmic membrane leading to loss of the ability to transport glucose and leakage of peptide, amino acids and potassium and thus inhibiting bacterial glycolysis and death (Tenovuo et al., 1981; Thomas et al., 1994). Hypothiocyanate was reported to show antibacterial effect against *S. sanguinis* at a level beyond its normal salivary level (Welk et al., 2009). Nitrate also can be found in *Salvadora persica L.* aqueous extracts. It has been reported to have antimicrobial activity against *E.coli*, *S. faecalis*, *P. aeruginosa* and *S. aureus* by interfering with active transport of proline, oxidative phosphorylation and oxygen uptake (Yarbrough et al., 1980). In addition, two flavonoids (quercetin and kaempferol) were identified in

green tea extract (Savić et al., 2014) and *Salvadora persica* L. extract (Arora et al., 2013). Guan and his colleagues (Guan et al., 2012) reported that both flavonoids, isolated from *Nidus Vespa* (honeycomb), actively inhibit the growth of *S. sanguinis* and *A. viscosus*. This activity might be due to their ability to complex extracellular proteins, and then bind to bacterial cell wall (Cowan, 1999). Thus, quercetin and kaempferol in green tea and *Salvadora persica* L. may have this antibacterial activity against *S. mitis*, *S. sanguinis* and *A. viscosus*.

The aim of this study was to investigate the synergistic antibacterial effect of the combination of green tea and *Salvadora persica* L. extracts against primary plaque colonizers. In addition to the fact that *S. mitis*, *S. sanguinis* and *A. viscosus* are normal oral flora playing important role in the initiation of dental plaque development. Thus the combination concentration should inhibit the growth rather than completely killing these bacteria in an attempt to reduce their load that inversely affect dental plaque development. In this study, positive control CHX optical density (OD) value was used as a reference for the selection of such combination concentration. The DTC1 and DTC2 were selected as these are the lowest concentrations that have absorbance values close to that of the positive control CHX and they showed partial synergism (FIC=0.75) as shown in Table 3.5. Our findings revealed that the primary colonizer bacteria were sensitive to the combinations. Thus, these green tea and *Salvadora persica* L. combinations (DTC1 and DTC2) possess the advantage of being an antibacterial agent at a concentration lower than their MICs values.

Two hypothesized explanations are proposed for the synergistic activity observed in these green tea and *Salvadora persica* L. combinations, i.e. DTC1 and DTC2. First, the observed synergy activity might be attributed to a competitive inhibition at the site of action of some constituents for example quercetin and

kaempferol. These constituents were found in both green tea (Savić et al., 2014) and *Salvadora persica* L. (Arora et al., 2013) extracts and were reported to have antibacterial activity against primary dental plaque colonizers (Guan et al., 2012). The second hypothesized explanation is that phytochemical compatibility between the combined extracts might also attribute to the observed synergy for example catechins in green tea, fluoride and thiocyanate in *Salvadora persica* L, which have different antibacterial mechanisms. In conclusion, DTC1 and DTC2 combinations showed better antibacterial effect at lower concentration of both green tea and *Salvadora persica* L. extracts, and simultaneously minimizing possible side effects. Furthermore, it is also cost effective for the purpose of commercialization of product.

### 3.8 Conclusions

- 1- The primary plaque colonizers including *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were susceptible to both green tea, non-fermented leaves of *Camellia sinensis* var. *assamica*, and *Salvadora persica* L. aqueous extracts in a dose-dependent manner.
- 2- The aqueous extract of green tea showed antibacterial activity against the primary plaque colonizers. The minimum concentration of green tea aqueous extract that inhibits the growth of each respective *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 0.98, 0.98, 0.06 and 0.98 mg/ml respectively. While, the minimum concentration of green tea aqueous extract that shows bactericidal activity on each respective *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 1.95, 1.95, 0.12 and 1.95 mg/ml respectively.
- 3- The aqueous extract of *Salvadora persica* L. showed antibacterial activity against the primary plaque colonizers. The minimum concentration of *Salvadora persica* L. aqueous extract that inhibits the growth of each respective *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 15.63, 15.63, 3.91 and 15.63 mg/ml respectively. While, the minimum concentration of *Salvadora persica* L. extract that shows bactericidal activity on each respective *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension were 31.25, 31.25, 15.63 and 31.25 mg/ml respectively.
- 4- Two combinations of green tea and *Salvadora persica* L. aqueous extracts exhibited synergistic antibacterial activity against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension, i.e. primary plaque colonizers. The first is the combination of 0.25 mg/ml green tea aqueous extract and 7.82 mg/ml *Salvadora persica* L. aqueous extract (DTC1), equivalent to 1/4 and 1/2 of MIC values of

green tea and *Salvadora persica* L. aqueous extracts respectively. While the second is the combination of 0.49 mg/ml green tea aqueous extract and 3.91 mg/ml *Salvadora persica* L. aqueous extract (DTC2), equivalent to 1/2 and 1/4 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively. Both combinations showed partial synergism with a FIC equal to 0.75. Thus, these combinations might be used as a useful active agent for the development of oral health products, such as tooth paste or mouthwash, to help in chemical dental plaque control.

**CHAPTER 4: ANTIADHERENCE EFFECT OF A COMBINATION OF  
*CAMELLIA SINENSIS* VAR. *ASSAMICA* AND *SALVADORA PERSICA* L.  
AGAINST PRIMARY COLONIZERS OF DENTAL PLAQUE**

**4.1 Introduction**

Various microorganisms such as bacteria, viruses, fungi, archaea, mycoplasma, and protozoa can grow in the moist and warm oral cavity niche. These microbes colonize dental and mucosal surfaces in the oral cavity to form multispecies soft deposit that is termed as dental plaque or biofilm. This result in the buildup of large numbers of matrix embedded oral microorganisms that are adhered to each other or/and to oral surfaces, especially at stagnant and hard-to-reach sites, and this may lead to the development of periodontal diseases and dental caries (Wilson, 2005; Marsh et al., 2009).

Dental plaque formation is a complex sequential process. It starts by adsorption of the acquired pellicle on teeth surfaces immediately after tooth cleaning. This acquired pellicle acts as a conditioning film covering tooth surfaces that provides a substrate for the reversible adhesion of the primary plaque colonizers. With time, this reversible adhesion of the primary colonizers becomes more irreversible attachment involving interactions between specific molecules on the primary plaque colonizers cell surface (i.e. adhesins) and complementary molecules (i.e. receptors) found in the acquired pellicle. After firm attachment of the primary plaque colonizers, they offer receptors for the attachment of the secondary plaque colonizers by a process called co-adhesion, leading to an increase in the microbial diversity of the dental plaque. Following that, proliferation of the attached cells occurs, and this results in increasing the plaque biomass and synthesis of exo-polymers to form the dental plaque matrix, i.e. plaque maturation (Lang et al., 2015).

By tooth brushing, most of the oral bacteria are eliminated from the exposed tooth surfaces. Recolonization of oral bacteria, more specifically primary plaque colonizers, begins immediately after tooth brushing. It was reported that oral bacteria can be detected within 3 minutes on tooth surfaces (Hannig et al., 2007). These primary plaque bacterial colonizers are initially attached to the tooth surfaces, modify the local microenvironment through their metabolic activity and provide new binding sites for the adhesion of the secondary plaque colonizers (Newman et al., 2012).

The attachment of primary plaque colonizers onto tooth surfaces is a complex process. Initially, the planktonic primary plaque colonizers in the saliva transport to the tooth surfaces through saliva flow or mechanical contact between teeth and oral soft tissues. As the primary plaque colonizers become about 50 nm in close approximate to tooth surface, they reversibly attach to the tooth surface through non-specific, long-range forces such as electrostatic repulsive forces, van der Waals attractive forces and hydrophobic forces. Many of primary plaque colonizers possess structures, such as fimbriae, that protrude from the bacterial cell surface and carry hydrophobic molecules involving in cell-surface hydrophobic interactions. This results in eliminating the water film between the interacting bacterial surfaces and tooth surfaces. Thus, it provides substratum free-energy surfaces which are important for the involvement of irreversible short-range forces. At this point, firmer binding between primary plaque colonizers and tooth surfaces is provided by more specific interactions between bacterial adhesins and receptors in the acquired pellicle on tooth surfaces (Newman et al., 2012).

Among the primary plaque colonizers, *S. mitis*, *S. sanguinis* and *A. viscosus* adhere to salivary pellicle on tooth surfaces and mediate the subsequent adhesion of the secondary plaque colonizers, thus playing an initial role in the dental plaque formation (Li et al., 2004; Bathla, 2011). In the oral cavity, *Actinomyces viscosus* is a prominent



bacterium in human dental plaque. This filamentous microorganism preferentially colonizes the tooth surfaces, and it appears to actually require the presence of teeth in order to colonize the mouth, since it is usually not present in the oral cavities of pre-dentate infants (Ellen, 1976). In dentate adults, *A. viscosus* has highly been associated with gingivitis (Syed et al., 1978) and root surface decay (Ellen et al., 1985). *A. viscosus* possesses two types of fimbriae, which can be distinguished functionally and antigenically. Type 2 fimbriae are associated with a N-acetylgalactosamine- and galactose-specific lectin that mediates attachment to receptors on certain bacteria and mammalian cells (Cisar et al., 1984; Sandberg et al., 1986). In contrast, type 1 fimbriae mediate attachment of *A. viscosus* cells to salivary pellicle formed on tooth surfaces (Wheeler et al., 1980; Cisar et al., 1988). The attachment of *A. viscosus* cells to apatite surfaces is prominently promoted by adsorbed salivary proline-rich proteins and the protein statherin on experimental pellicle which serve as receptors for type 1 fimbriae of *A. viscosus* (Gibbons et al., 1988b). Adsorption of *A. viscosus* bacteria to experimental pellicle prepared from pure proline-rich proteins is not inhibited by lactose. This suggests that type 1 rather than type 2 fimbriae are responsible for the attachment of *A. viscosus* cells to experimental pellicle. Furthermore, adsorbed salivary proline-rich proteins molecules do not induce the attachment of *A. naeslundii* cells which possess only type 2 fimbriae (Gibbons et al., 1988a). Concerning to *S. sanguinis*, this oral bacterium was reported to possess pili. Each pilus is simply composed of three tandem pilus subunits (PilA, B, and C) and a single sortase C. The pili of *S. sanguinis* have an ability to bind to extracellular proteins such as fibronectin, and may contribute to the adherence of *S. sanguinis* to host tissues. Also, they have been reported to bind salivary  $\alpha$ -amylase, including adsorbed  $\alpha$ -amylase on acquired pellicle, through its PilC subunits. Thus, these pili participate in specific-adhesion of *S. sanguinis* to tooth surfaces (Okahashi et al., 2010; Okahashi et al., 2011). Moreover, *S. sanguinis* possess

several cell surface proteins, such as Ssp5, that were reported to bind to salivary proteins such as salivary agglutinin (Demuth et al., 1990). Further, *S. mitis* strains have been reported to have abundant surface fibrils after examination by transmission electron microscopy (Cowan et al., 1992). Moreover, Zähler et al. (2011) identified PI-2 pili in *S. mitis*. These pili can be associated with a broad range of *S. mitis* traits which involve in early adhesion to tooth surfaces in dental plaque development. PI-2 pili have previously been identified in *S. pneumoniae* which are composed of the pilus backbone protein PitB (Bagnoli et al., 2008). In contrast, in oral *S. mitis*, PI-2 pili are composed of PitB and PitA proteins. PitA is suggested to be a putative adhesin that contributes for the bacterial adhesion to tooth surfaces (Zähler et al., 2011). Also, *S. mitis* was reported to possess proteins on its bacterial cell surface that bind salivary sialic acid-containing oligosaccharides. Thus, these proteins may serve as adhesins that participate for the bacterial specific-adhesion to tooth surface (Murray et al., 1986).

In the previous chapter, two combinations of green tea and *Salvadora persica* L. aqueous extracts were found to exhibit synergistic antibacterial activity against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension. The first combination was composed of 0.25 mg/ml green tea aqueous extract and 7.82 mg/ml *Salvadora persica* L. aqueous extract (DTC1), equivalent to 1/4 and 1/2 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively. While the second combination was composed of 0.49 mg/ml green tea aqueous extract and 3.91 mg/ml *Salvadora persica* L. aqueous extract (DTC2), equivalent to 1/2 and 1/4 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively. Both combinations showed partial synergism with FIC index equal to 0.75. This study was designed to investigate the antiadherence activity of these two combinations of green tea and *Salvadora persica* L. aqueous extracts, i.e. DTC1 and DTC2, on *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension by using artificial mouth model. Among these combinations, the

combination which had better antiadherence activity was further investigated for its effect on bacterial cell surface hydrophobicity (CSH) which is considered as one of the important initial non-specific forces for oral bacteria to adhere to the tooth surface (Weiss et al., 1982).

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#### **4.2 Null hypothesis**

A combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts exhibits no synergistic antiadherence activity against primary plaque colonizers.

#### **4.3 Aim of the study**

The primary aim of this study was to evaluate the synergistic antiadherence activity of the combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts against primary plaque colonizers *in vitro*.

#### **4.4 Objectives of the study**

- 1- To determine the antiadherence effect of the combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts on primary plaque colonizers biofilm by means of the Nordini's Artificial Mouth (NAM) model (Rahim et al., 2008).
- 2- To determine the effect of the combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts on cell surface hydrophobicity (CSH) of the primary plaque colonizers.

## **4.5 Materials and methods**

### **4.5.1 Bacteria**

The same bacterial species were selected for this study as detailed in Chapter 3.

#### **4.5.1.1 Preparation of culture media for bacterial culturing**

Culture media was prepared as previously detailed in Chapter 3.

#### **4.5.1.2 Stock Cultures Preparation**

Stock cultures were prepared as previously detailed in Chapter 3.

#### **4.5.1.3 Preparation of Standard Bacterial Cell Suspension**

For the antiadherence experiment, the growth bacterial colonies of working stock culture were transferred respectively by inoculating loop into of BHI broth and incubated for 18 – 24 hours at 37 °C. After an overnight incubation, the turbidity of the respective bacterial suspension was adjusted at a wavelength of 550nm using a spectrophotometer (Shimadzu UV-1700, Japan) to an absorbance of 0.144 which is equivalent to  $10^6$  cells/ml (Rahim et al., 2014). Throughout the experiment, three species suspension mixture of *S. mitis*, *S. sanguinis* and *A. viscosus* was used with mixing ratio of 1:1:1 by volume.

For the Cell Surface Hydrophobicity (CSH) experiment, a  $1 \times 10^8$  cells/ml of bacterial suspension was used. The respective bacterial species were inoculated in fresh BHI broth and incubated at 37 °C for 18 - 24 hours where the log phase was attained. Following this, the cells were harvested by centrifugation at 8000 g for 10 minutes. The cell pellets were washed twice with phosphate buffer saline (PBS) and re-suspended in the same solution with cell density adjusted to an absorbance of 0.600 at 600 nm wavelength which is equivalent to  $1 \times 10^8$  cells/ml using a spectrophotometer (Shimadzu UV-1700, Japan) (Razak et al., 2006). Throughout the experiment, three species

suspension mixture of *S. mitis*, *S. sanguinis* and *A. viscosus* was used with mixing ratio of 1:1:1 by volume in addition to each respective bacterial suspension.

#### **4.5.2 Plant extract combinations**

Two combinations of green tea and *Salvadora persica* L. aqueous extracts were prepared. The first combination was composed of 0.25 mg/ml green tea aqueous extract and 7.82 mg/ml *Salvadora persica* L. aqueous extract (DTC1), equivalent to 1/4 and 1/2 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively. The second combination was composed of 0.49 mg/ml green tea aqueous extract and 3.91 mg/ml *Salvadora persica* L. aqueous extract (DTC2), equivalent to 1/2 and 1/4 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively.

#### **4.5.3 Antiadherence evaluation of the extracts combinations**

Antiadherence effects of green tea and *Salvadora persica* L. aqueous extracts and two of their combinations that showed partial synergism were determined by using Nordini's Artificial Mouth (NAM) model as described earlier (Rahim et al., 2008). All tests of antiadherence evaluation of the extracts were conducted in triplicate to ensure accuracy and reproducibility of the results.

##### **4.5.3.1 Saliva collection**

Stimulated whole saliva (SWS) was collected from a single donor at 9 am to 11 am and prepared following the protocol described by De Jong and Van der Hoeven (1987). At the beginning, the donor rinsed his mouth with tap water to remove any possible remnants of food. Next, approximately 40 ml of SWS was collected into ice-chilled tubes by expectoration after chewing sugar-free paraffin wax. Then, 1,4-Dithio-D,L-threitol (DTT) was added to the collected SWS samples with a concentration of 2.5mM. DTT was used to help in minimizing the aggregation of proteins in the SWS samples. After adding the DTT, the SWS was stirred slowly for 10 minutes and

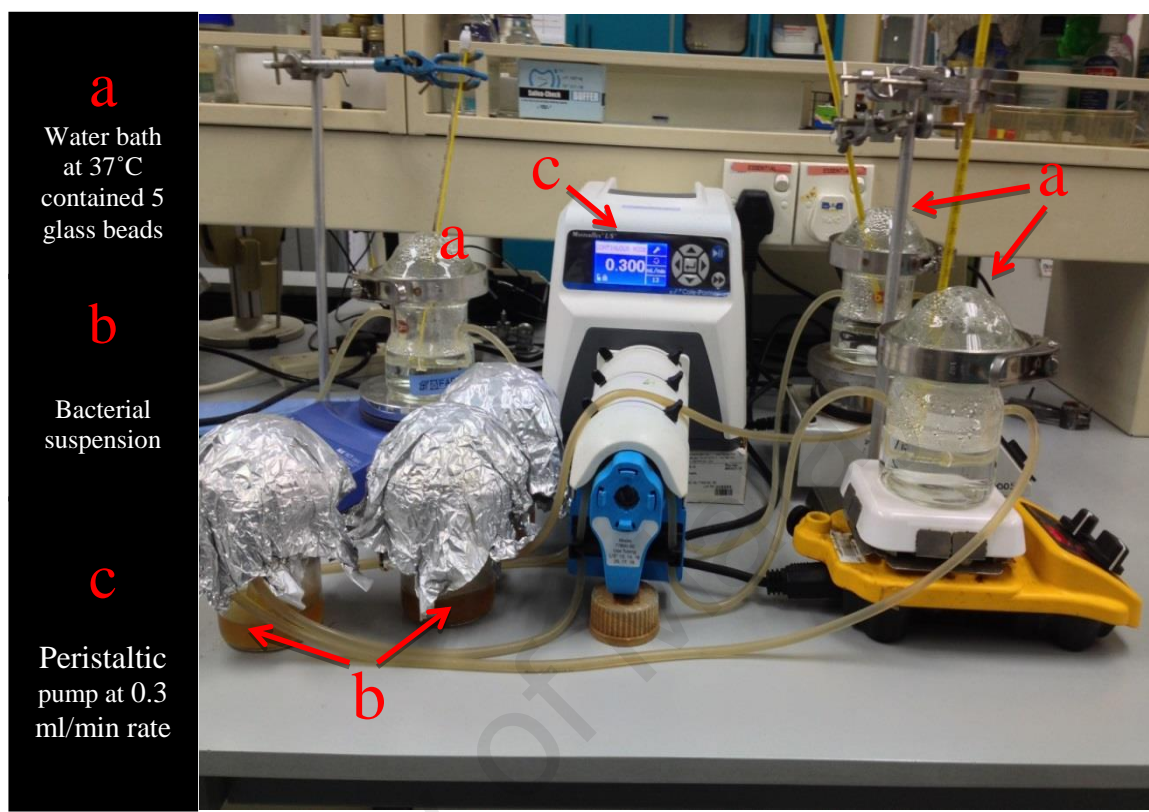
centrifuged at 800 g for 30 minutes. After centrifugation, the supernatant was obtained and then sterilized by filtration using a disposable 0.2 µm (Supor® Membrane) low protein-binding filter (Acrodisc® Syringe Filters, Pall Corp, USA). The sterile SWS was then stored in refrigerator at -20 °C. Prior to the experiment, the frozen SWS were thawed and centrifuged to eliminate any possible precipitants.

#### **4.5.3.2 Preparation of acquired pellicle and antiadherence evaluation procedure**

In this study, sterile glass beads were used to serve as a tooth surface. Five beads were placed in a glass chamber within a water bath at 37 °C to mimic human body temperature (Figure 4.1). They were used to develop acquired pellicle by running sterile saliva into the NAM model at a constant rate of 0.3 ml/min for 2 minutes followed by 2 minutes running of sterile distilled water to rinse off excess saliva. For the negative control, *S. sanguinis*, *S. mitis* and *A. viscosus* mixture suspension was pumped into the NAM model at a rate of 0.3 ml/min for 2 hours. While for the test, 15 ml of the test combination was run into the NAM model for 30 seconds, followed by running sterile distilled water for 30 seconds to wash off the excess of test combination. Then, bacterial suspension was pumped into the NAM model for 2 hours at the same flow rate, i.e. 0.3 ml/min. The count of adhered bacterial cells to glass beads was determined by transferring each glass bead to a sterile micro-centrifuge tube containing 1 ml of PBS. The tubes were sonicated for few seconds; vortex for a 1 minute to dislodge attached cells, and then serially diluted using PBS to a final dilution of 1:10 (6<sup>th</sup> dilution). A 100 µl of bacterial suspension from each tube was pipetted out and inoculated on three BHI agar plates. Following incubation at 37 °C for 24 hours, the plates with dilution that gave a CFU count of between 30 - 300 cells were selected for enumeration by using the following formula (Chess, 2009):

$$Total\ CFU/ml = No.\ of\ formed\ colonies / (Dilution\ factor \times Volume\ used)$$

This procedure was repeated using 15 ml of 0.12% CHX in place of test combination to serve as a positive control.



**Figure 4.1: NAM model for antiadherence evaluation of extracts combinations**

#### **4.5.4 Determination of cell surface hydrophobicity (CSH)**

This experiment was carried out using *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension respectively three times in triplicate to ensure accuracy reproducibility of the results.

##### **4.5.4.1 CSH of bacterial species**

CSH of the bacterial cells was determined following the protocol of Klotz et al. (1985), by using the adsorption of bacterial cells to hexadecane, acting as hydrophobic tooth surface, to measure the hydrophobic interaction of bacterial cells. A volume of 2 ml of bacterial suspension was added to a sterile glass tube containing 2 ml of PBS to



get a final volume of 4 ml. By using PBS as a blank, the absorbance ( $A_a$ ) was read at OD550 nm which represented the absorbance of bacterial cells in the absence of hexadecane. A volume of 200  $\mu$ l of hexadecane, acting as the hydrophobic surface, was added to the tube which was then agitated vigorously for 1 minute. The tube was left on the bench to stand at room temperature for 20 minutes to allow hexadecane to separate from the lower aqueous phase within the tube. Following this, the lower aqueous phase was gently pipetted out, and the absorbance ( $A_b$ ) was read at OD550 nm representing the absorbance of bacterial cells in the presence of hexadecane. The relative CSH was presented as percentage of adsorption of the bacterial cell to hexadecane, and was measured by using the following equation:

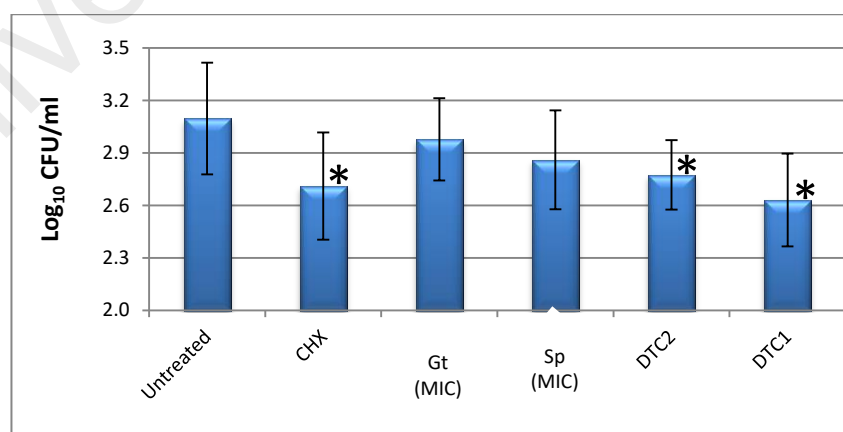
$$CSH = [(A_a - A_b) / A_a] \times 100$$

#### **4.5.4.2 CSH of bacterial species treated with test combination of green tea and *Salvadora persica* L.**

A volume of 2 ml of green tea and *Salvadora persica* L. stock extracts mixture was dispensed into a sterile glass tube containing 2ml of bacterial suspension to give a final desired concentration of green tea and *Salvadora persica* L. extracts test combination in a final volume of 4 ml within the glass tube. The optical density ( $A_a$ ) was read and represented the absorbance in the absence of hexadecane. Following then, 200  $\mu$ l of hexadecane was added and the optical density was again recorded and considered as ( $A_b$ ). Thus, the effect of the test combination on CSH of bacterial cells was represented as reduction in CSH following treatment with the test combination.

## 4.6 Results

The  $\log_{10}$  CFU/ml of *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension adhered to the experimental pellicle on glass beads treated with 0.12% CHX (CHX), green tea, i.e. Gt (MIC), and *Salvadora persica* L., i.e. Sp (MIC), aqueous extracts at their MIC values, DTC2 and DTC1 in the NAM model is shown in Figure 4.2. As appears in the figure, it was found that CHX, DTC2 and DTC1 significantly reduced the amount of bacterial mixture adhered to experimental pellicle when compared with untreated glass beads. The higher antiadherence effect was observed with DTC1 followed by CHX and DTC2 respectively. CHX, DTC1 and DTC2 were found to reduce the amount of bacterial mixture adhered to the experimental pellicle when compared with green tea and *Salvadora persica* L. aqueous extracts at a concentration of their MIC values. Another finding was that *Salvadora persica* L. aqueous extract at its MIC value exhibited better antiadherence effect, reduced bacterial adherence to the experimental pellicle, when compared with the effects exhibited by green tea aqueous extract at its MIC value with no statistical difference.



**Figure 4.2:  $\log_{10}$  CFU/ml of mixture of bacteria adhered to experimental pellicles treated with different solutions.**

Gt (MIC) and Sp (MIC) are green tea and *Salvadora persica* L. aqueous extracts at their MIC values respectively. \* Statistical analysis of Mann Whitney test showed DTC1, DTC2 and 0.12% CHX to be significant ( $p < 0.003$ ) when compared with untreated.

Table 4.1 summarizes the antiplaque effect of DTC1 and DTC2 against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension compared with 0.12% CHX (positive control) and untreated (negative control). Both DTC1 and DTC2 were found to have antibacterial effect, OD values of 0.013 and 0.010 respectively, close to that of 0.12% CHX which had an OD value of 0.006 (data obtained from chapter 3). All of DTC1, DTC2 and 0.12% CHX were found to inhibit the growth of bacteria when compared with untreated which had an OD value of 0.529. The antiadherence efficacy was expressed by the  $\log_{10}$  CFU/ml of *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension adhered to experimental pellicle. DTC1 was found to exhibit the best antiadherence efficacy followed by 0.12% CHX and DTC2 respectively. DTC1, DTC2 and 0.12% CHX were found to significantly reduce the amount of bacteria adhering to the experimental pellicles compared with untreated glass beads.

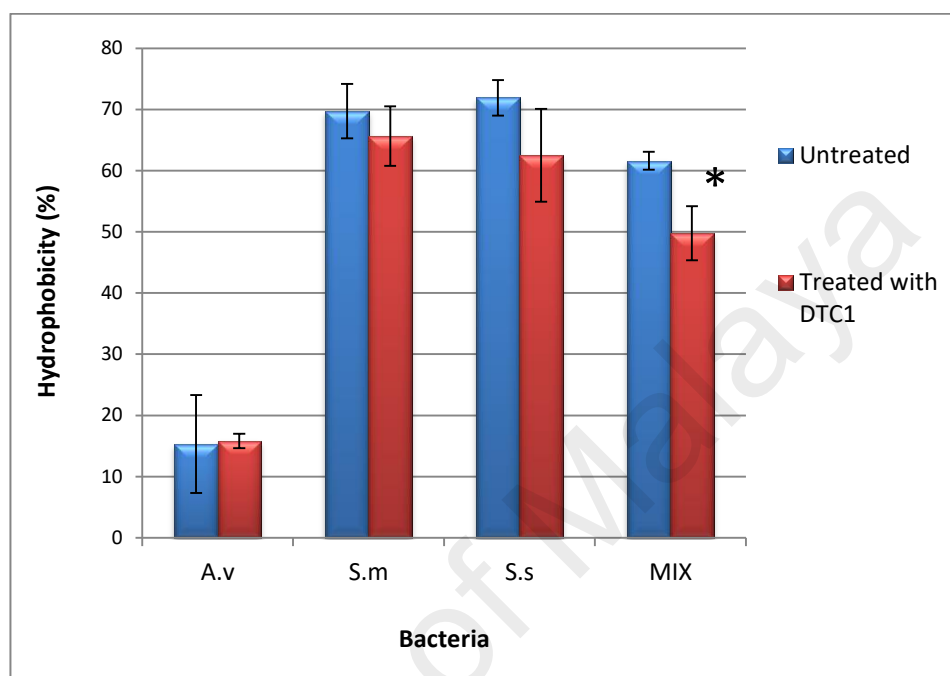
**Table 4.1: Antiplaque effect of different test combinations against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension**

Solutions	Antiadherence [Microbial population of plaque ( $\log_{10}$ CFU/ml)]	Antibacterial (OD values)
<b>DTC1</b>	*2.631	0.013
<b>DTC2</b>	*2.775	0.010
<b>0.12% CHX</b>	*2.711	0.006
<b>Untreated</b>	3.097	0.529

\* Statistical analysis of Mann Whitney test showed DTC1, DTC2 and 0.12% chlorhexidine (0.12% CHX ) to be significant ( $p < 0.003$ ) when compared with untreated.

Adsorption to hexadecane was used to determine the CSH of the primary colonizing bacteria with and without treating the bacteria with DTC1. As shown in Figure 4.3, DTC1 was found to have no effect on the CSH of *A. viscosus*, while it reduced the CSH of *S. mitis* and *S. sanguinis* without statistical significant difference. In

contrast, DTC1 was found to significantly ( $p < 0.05$ ) reduce the CSH of the *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension.



**Figure 4.3: CSH of treated and untreated bacteria with DTC1.**

*A. viscosus* (A.v), *S. mitis* (S.m), *S. sanguinis* (S.s) and their mixture suspension (MIX), \* Statistical analysis of Mann Whitney test showed DTC1 to be significant ( $p < 0.003$ ) when compared with untreated.

#### 4.7 Discussion

Among the normal oral flora, *S. mitis*, *S. sanguinis* and *A. viscosus* are the pioneer bacteria that adhere to acquired pellicle covering tooth surfaces and form the initial bacterial layer on tooth surfaces. These primary plaque colonizers provide new binding sites for the subsequent secondary plaque colonizers that contribute to dental plaque buildup. Thus, adherence of primary colonizer bacteria to the acquired pellicle formed immediately on tooth surfaces after tooth brushing can be considered as a crucial step in the dental plaque formation which embodies specific and non-specific adhesion (Rosan et al., 2000).

The specific adhesion involves lectin-like interaction between specific receptors, often sugars or oligosaccharides, found in the acquired pellicle covering oral surfaces and the surface proteins, usually adhesins such as polymeric fimbriae (or pili), of primary colonizer bacteria allowing for long range adherence (Busscher et al., 1992; Nobbs et al., 2009). *S. mitis*, *S. sanguinis* and *A. viscosus* have been reported to possess numerous surface adhesins that selectively mediate attachment of these bacteria to specific receptors on the salivary pellicle (Gibbons et al., 1988b; Cowan et al., 1992; Vadillo-Rodríguez et al., 2004; Okahashi et al., 2011).

In the previous chapter, it was found that two combinations of green tea and *Salvadora persica* L. aqueous extracts exhibited synergistic antibacterial activity against *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension. The first combination was composed of 0.25 mg/ml green tea aqueous extract and 7.82 mg/ml *Salvadora persica* L. aqueous extract (DTC1), equivalent to 1/4 and 1/2 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively. While the second combination was composed of 0.49 mg/ml green tea aqueous extract and 3.91 mg/ml *Salvadora persica* L. aqueous extract (DTC2), equivalent to 1/2 and 1/4 of MIC values of green tea and

*Salvadora persica L.* aqueous extracts respectively. Both combinations showed partial synergism with FIC index equal to 0.75. After best reviewing of the literature, no previous studies have been carried out to investigate the antiadherence activity of green tea and *Salvadora persica L.* aqueous extracts or their combination against *S. mitis*, *S. sanguinis* and *A. viscosus*. Thus, this is the first study that evaluates the antiadherence activity of both extracts and their combinations, i.e. DTC1 and DTC2, which exhibited antibacterial activity against these primary plaque colonizers.

Nordini's Artificial Mouth (NAM) model was used to determine the antiadherence effects of green tea and *Salvadora persica L.* aqueous extracts and two of their combinations. In this model, glass beads served as tooth surfaces. They were covered by experimental pellicles which were formed by the flow of saliva in the model. It was decided that the plant extracts and their combinations should flow over the coated glass beads for 30 seconds. This is because the 0.12% CHX mouth rinse was used as the positive control in this experiment. On a clean tooth surface, CHX can bind to the acquired pellicle and enamel. This tooth surface-bound CHX can interfere with the adherence of oral bacteria to the tooth surface (Jones, 1997). The direction of use of the CHX mouth rinse is rinsing with 15 ml for 30 seconds. Thus in this experiment, it was opted to flow 15 ml of the test solutions for 30 seconds over the experimental pellicle-covered glass beads. After that, *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension was pumped over the glass beads for duration of 2 hours. This duration was decided to evaluate the immediate effects of the test plant solutions on the adherence of the primary colonizers to the experimental pellicle.

In this experiment, the results revealed that *Salvadora persica L.* aqueous extract at a concentration equivalent to its MIC value showed better but non-significant antiadherence activity when compared with green tea aqueous extract at a concentration

equivalent to its MIC value. However the antiadherence activity of both extracts was statistically non-significant when compared to untreated glass beads, i.e. negative control, as shown in Figure 4.2. This difference in the antiadherence activity may be attributed to the different constituents found in both green tea and *Salvadora persica L.* aqueous extracts. One explanation for this antiadherence activity is that the constituents of the *Salvadora persica L.* and green tea aqueous extracts may have altered some receptors on the acquired pellicle, thereby disturbing the recognition of adhesins on the bacterial cells, hence diverting particular interactions between the two surfaces. For example, salivary  $\alpha$ -amylase is a constituent of the acquired dental pellicle (Örstavik et al., 1974). It may play an important role in the formation of dental plaque by serving as a receptor for primary plaque colonizers adhesion to tooth surfaces and providing nutrients through hydrolysis of starches to the growing plaque colonizers (Scannapieco et al., 1993; Okahashi et al., 2011). It was reported that a significantly lower level of dental plaque  $\alpha$ -amylase was found among *Salvadora persica L.* chewing sticks users compared with regular brush users. Moreover, dental plaque  $\alpha$ -amylase activity was reduced after sugar intake among *Salvadora persica L.* chewing sticks users (Khalil et al., 2013). This finding may be attributed to the polyphenolic tannins of *Salvadora persica L.* which was reported to inhibit salivary  $\alpha$ -amylase (Kandra et al., 2004). Moreover, dietary tannins were reported to bind both salivary histatin and proline-rich protein (Yan et al., 1995; Lu et al., 1998). Thus, tannins in *Salvadora persica L.* may bind salivary  $\alpha$ -amylase, histatin and proline-rich protein present in experimental pellicle and therefore modified the pellicle surface. Moreover, fluoride was one of the identified constituents of *Salvadora persica L.* chewing sticks extract (Hattab, 1997). Recently, it was reported that rinsing with a fluoride- based mouth rinse results in a slight fluoride accumulation at the surface of the pellicle-coated enamel. This might have occurred either by incorporation of the fluoride in the pellicle through binding to

proteins or by interaction with the hydroxyapatite of the enamel surface (Weber et al., 2015). Thus, constituents of *Salvadora persica L.* extract may modify the experiment pellicle through binding to salivary proteins adsorbed on the glass beads. On the other hand, green tea was found to have an antiadherence efficacy against oral streptococci. However, its effect was the lowest when compared with Cistus tea, red wine and grape juice (Hannig et al., 2009a). In an earlier study, it was reported that a concentration of 1 to 4 mg/ml tea polyphenols can inhibit the preliminary adherence of *S. mutans* and *A. viscosus* to saliva coated hydroxyapatites effectively (Xiao et al., 2000). These findings are not surprising when one knows that tea polyphenol components including theaflavin, ECG and EGCG are capable to adsorb onto acquired pellicle, cause dramatic changes within the pellicles, and subsequently modify its structure (Joiner et al., 2004).

The antiadherence activity of two combinations of green tea and *Salvadora persica L.* aqueous extracts, which are DTC1 and DTC2, were investigated in this experiment. The highest significant effect was observed in the DTC1 followed by 0.12% CHX and the DTC2 respectively ( $p < 0.003$ ) as shown in Figure 4.2 and Table 4.1. One explanation is that the constituents of the green tea and *Salvadora persica L.* aqueous extracts may have synergistically modified some receptors on the experimental pellicle, thereby disturbing the recognition of adhesins on the bacterial cells, hence diverting particular interactions between the two surfaces. In this context it is noteworthy that the combinations of green tea and *Salvadora persica L.* aqueous extracts yielded the best results, which were superior to those of single extracts. It may be hypothesized that no single components but the biologically compatible interactions of all components of green tea and *Salvadora persica L.* aqueous extracts are responsible for the beneficial modification of the pellicle.



As mentioned above, the combination DTC1 exhibited the best antiadherence activity; therefore it was further investigated whether it had an effect on bacterial CSH which is considered as one of the important initial non-specific forces for oral bacteria to adhere to the tooth surface (Weiss et al., 1982). The hydrophobic force is fundamentally attributed to the tendency of polar water molecules to exclude other non-polar molecules, resulting in segregation of these polar and non-polar substances. Thus, any deviation occurred to this affinity may affect the adherence mechanism of the primary plaque colonizers (Chandler, 2005). In the CSH experiment, hexadecane was used to mimic hydrophobic tooth surfaces and it was found that all the tested primary plaque colonizers were hydrophobic. *S. sanguinis* showed the highest CSH followed by *S. mitis* and *A. viscosus*, and this is in agreement with an earlier study (Razak et al., 2006). High CSH observed for *S. sanguinis* might be attributed to the presence of a hydrophobic cell wall polypeptide of a molecular mass 16kDa (Jenkinson, 1986; Jenkinson et al., 1997). Other factors for example cell wall lipoteichoic acids, hydrophobic proteins and external appendages may confer the overall CSH of these bacteria. The latter have many hydrophobic domains of non-polar amino acids which participate in hydrophobicity interactions with acquired pellicle hydrophobic proteins at a greater distance from the negatively charged tooth surface (Fives-Taylor et al., 1985; Jenkinson, 1986).

In this CSH experiment, the combination DTC1 exhibited no effect on CSH of *A. viscosus*, while it non-significantly reduced the CSH of *S. mitis* and *S. sanguinis* respectively. In contrast, this combination has significantly reduced the CSH of the *S. mitis*, *S. sanguinis* and *A. viscosus* mixture suspension. These findings may be due to the fact that the behavior of a single bacterium differs when it is mixed with other bacteria in a suspension. It is noteworthy to conclude that this finding of DTC1-based significant reduction in CSH of the primary plaque colonizers, i.e. *S. mitis*, *S. sanguinis*

and *A. viscosus* mixture suspension, may explain the DTC1 high antiadherence activity observed in the NAM model test.

This profound reduction in bacterial CSH might be attributed to some constituents found in the combination DTC1 which might have an effect on the bacterial cell membrane. Quercetin and kaempferol were reported to be found in both green tea and *Salvadora persica L.* extracts. These polyphenols have been reported to complex extra-cellular proteins, and then bind to bacterial cell wall (Cowan, 1999). Green tea catechins has been reported to target bacterial cell membrane and inducing irreversible changes (Ikigai et al., 1993). It was reported that green tea polyphenolic EGCG can directly bind to peptidoglycan of bacterial cell membrane of gram positive bacteria and induce its precipitation. This results in damaging the bacterial cell wall and interference with cell wall biosynthesis (Shimamura et al., 2007).

By using atomic force microscopy (AFM), Cui et al. (2012) reported that morphological changes in gram positive bacteria were induced by polyphenolic EGCG at a concentration lower than its minimum inhibitory concentration. The authors found that EGCG caused aggregates in the cell envelopes of gram positive bacteria. Moreover, hydroxyl groups and conjugated double bonds present in the active components of the green tea catechins may involve in binding to the bacterial cell membrane compounds. It was found that EC and EGCG have high affinity to bacterial cell membrane lipid bilayers. This affinity is attributed to their content of gallic acid esters (Hashimoto et al., 1999; Kamihira et al., 2008), and thus catechins galloyl and gallic moieties disturb lipid bilayer membrane and eventually result in cell death through loss of cell structure and function (Ikigai et al., 1993; Tsuchiya et al., 1996; Cox et al., 2001). Furthermore, major green tea extract polyphenols may disturb bacterial cell morphology and cytoplasmic membrane through altering the cell osmotic pressure and leading to cell constituents

leakage and death (Sivarooban et al., 2008). On the other hand, Sofrata et al. (2011b) revealed, by using electron microscopy, the ability of benzyl isothiocyanate constituent of *Salvadora persica L.* to induce membrane protrusion in bacterial cell membrane of *A. actinomycetemcomitans*. Thiocyanate can be found in the *Salvadora persica L.* aqueous extract (Darout et al., 2000b) which can be oxidized to hypothiocyanate by peroxidase with the presence of hydrogen peroxide from oral bacteria. The hypothiocyanate oxidases sulfhydryl groups in bacterial cytoplasmic membrane leading to death by loss of the ability to transport glucose and leakage of peptide, amino acids and potassium (Tenovuo et al., 1981). Thus, these components of green tea and *Salvadora persica L.* extracts in the combination DTC1 may alter the surface characteristic of the bacterial cells and therefore reduce their adsorption to hexadecane.

#### 4.8 Conclusions

- 1- Green tea and *Salvadora persica* L. aqueous extracts at a concentration equivalent to their MIC values exhibited non-significant antiadherence activity against primary plaque colonizers.
- 2- *Salvadora persica* L. aqueous extract at a concentration equivalent to its MIC value exhibited better, but non-significant, antiadherence activity against primary plaque colonizers than green tea aqueous extract at a concentration equivalent to its MIC value.
- 3- Two combinations of green tea and *Salvadora persica* L. aqueous extracts exhibited a significant synergistic antiadherence activity against primary plaque colonizers. The first combination was composed of 0.25 mg/ml green tea aqueous extract and 7.82 mg/ml *Salvadora persica* L. aqueous extract (DTC1), equivalent to 1/4 and 1/2 of MIC values of green tea and *Salvadora persica* L. aqueous extracts respectively. While the second combination was composed of 0.49 mg/ml green tea aqueous extract and 3.91 mg/ml *Salvadora persica* L. aqueous extract (DTC2), equivalent to 1/2 and 1/4 of MIC values of green tea and *Salvadora persica* L. aqueous extracts, respectively.
- 4- The combination DTC1 exhibited the best significant antiadherence activity followed by CHX and DTC2 respectively.
- 5- The combination DTC1 could be used as a useful active agent for the development of oral health products, such as tooth paste or mouthwash, to help in chemical dental plaque control.

## **CHAPTER 5: ANTIPLAQUE EFFICACY OF A FORMULATED COMBINATION OF *CAMELLIA SINENSIS* VAR. *ASSAMICA* AND *SALVADORA PERSICA* L.: A RANDOMIZED 24 HOURS PLAQUE RE- GROWTH CLINICAL TRIAL**

### **5.1 Introduction**

Dental plaque is the soft deposit forming biofilm on the tooth surfaces in the oral cavity (Allison et al., 1995). It can be clinically recognized on tooth surfaces with or without disclosing agents in less than 24 hours (L  e et al., 1965; Teles et al., 2012). Dental plaque is the main cause of dental caries, gingivitis and periodontitis. Therefore, an effective dental plaque control initiated at the early stage of plaque development is an essential procedure to maintain good oral hygiene (Axelsson et al., 1981).

Plaque control is either mechanical or chemical procedures or sometimes by both procedures (Axelsson et al., 1981). Mechanical plaque control is the regular removal of supragingival dental plaque from hard surfaces found in the oral cavity including tooth surfaces and other hard surfaces (such as crowns, bridges, etc.) by self or dental care professional (Axelsson et al., 2002). The most adopted and accepted tool for teeth cleaning in mechanical plaque control is the toothbrush. It can remove the supragingival dental plaque from facial, oral and occlusal tooth surfaces (Perry et al., 2001). There are many techniques available for tooth brushing. However, there is no superior brushing technique over other techniques in plaque removal. Many factors might determine the recommended brushing technique for each individual including morphology of the dentition and the patient's own manual dexterity (Weijden et al., 2015). The ideal technique is the one that allows for complete dental plaque removal in the least possible time, without inducing any trauma to tissues (Hansen et al., 1971). However, individuals do not appear to remove plaque by tooth brushing as might be expected despite their apparent efforts (Weijden et al., 2015). De la Rosa et al. (1979)

evaluated plaque removal with daily tooth brushing over a 28 days period following dental prophylaxis. They found that approximately 60% of the dental plaque remained after self-performed tooth brushing. In the 1998 UK Adult Dental Health Survey, Morris et al. (2001) reported that the percentage of teeth appeared with plaque pellicle were 30% and 44% in the 25 to 34 years and 65 years to older age groups respectively.

How often teeth should be brushed and how much plaque on teeth should be removed for the prevention of dental disease are not yet known. Thus, there is no true consensus on the optimal frequency of tooth brushing (Weijden et al., 2015). In a longitudinal study, Kressin et al. (2003) evaluated the effect of oral hygiene measures on tooth retention. They found that consistent tooth brushing including at least once daily practice resulted in a 49% reduction in the risk of tooth loss when compared with lack of consistent oral hygiene measures. The minimum frequency of tooth brushing needed to prevent the development of gingivitis is once every day or every second day (Lang et al., 1973). Bosman et al. (1977) evaluated the effect of tooth brushing frequency in resolving induced experimental gingivitis. They found that in participants who properly brushed their teeth once per day or every second day, the gingiva resolved within 7 to 10 days. However, proper tooth brushing combined with interdental cleaning once every 24 hours is adequate to prevent the onset of interdental caries as well as gingivitis (Kelner et al., 1974; Weijden et al., 2015). From a practical standpoint, tooth brushing at least twice daily is generally recommended as the patients can remove dental plaque and apply fluoride through using of dentifrices to prevent caries. The American Dental Association recommends brushing teeth twice per day with fluoride toothpaste, in addition to daily interdental cleaning by interdental cleansing aids (Weijden et al., 2015).

Individuals usually believe that they spend more time on brushing their teeth than they actually do (Saxer et al., 1998). Brushing time for any given toothbrush is consistently correlated with the efficiency of plaque removal. Many clinical studies in literature indicate the importance of prolong duration of brushing on enhancing the plaque removal (Hawkins et al., 1986; Preber et al., 1990; Van der Weijden et al., 1996; McCracken et al., 2003; McCracken et al., 2005; Gallagher et al., 2009). Weijden et al. (1993) evaluated the effect of 5 different brushing times (30, 60, 120, 180, and 360 seconds) on plaque removal. The authors found that an optimum in plaque removing efficacy was achieved with tooth brushing at 2 minutes duration. This finding was further confirmed by Slot et al. (2012) in a systematic review. In this systematic review, the authors evaluated the effect of a single brushing exercise in relation to the brushing time. They found that the estimated weighted mean efficacy as represented in Quigley-Hein plaque index scores reduction was 41% after 2 minutes and 27% after 1 minute. Thus, brushing for 2 minutes or longer must be encouraged regardless the type of the dental brush used.

The toothbrush cannot reach the tooth interproximal surfaces during tooth brushing. Thus, these surfaces cannot be cleaned by toothbrush alone and need more supplementary interdental cleansing tools such as interdental brush or wood sticks to ensure proper plaque removal. In susceptible patients, gingivitis and periodontitis are usually more pronounced in the interproximal surfaces, particularly in molars and premolars, than other facial or oral surfaces (Löe, 1979). Therefore, the use of interdental cleansing tools complementary to toothbrushing is fundamental for prevention of periodontal diseases and dental caries (Lang et al., 1977; Hugoson et al., 1979).

Unfortunately, there are many limitations for mechanical plaque control measures. First, an effective plaque removal by tooth brushing and interdental cleaning aids needs high level of dexterity from the individual. Several studies were conducted to evaluate the efficacy of tooth brushing for dental plaque control. It was reported that most individuals do not properly brush their teeth and they (Jepsen, 1998). In the UK Adult Dental Health survey, Morris et al. (2001) found that the mean proportion of teeth with plaque deposit was 30% and 44% in the 25 to 34; and the 65 and above years age groups respectively, while about 1/3 of teeth in 72% of all dentate adults examined had visible dental plaque. In a meta-analysis study, Van der Weijden et al. (2005) assessed the effectiveness of tooth brushing using manual toothbrush with respect to the level of plaque in 9 controlled studies of at least 6 months duration. The results of this study revealed high plaque presence despite self-performed mechanical plaque removal. Interdental cleaning aids are adjuncts to tooth brushing, and are used to clean interdental spaces that are inaccessible to toothbrushing. This seems highly important in individuals who are considered susceptible to periodontal diseases.

Development of gingivitis and periodontitis is usually localized in the interproximal surfaces, particularly in molars and premolars, than other facial or oral surfaces (Löe, 1979; Kinane, 1998). It was reported that 11- 51% of individuals in many developed countries use interproximal cleaning aids on a daily basis (Bakdash, 1995). As a conclusion, most individuals do not properly brush their teeth and/or use interdental cleansing aids; therefore they live with huge amount of plaque on their teeth, even though they brush on a regular basis.

Moreover, many epidemiological studies revealed a high prevalence of gingivitis even though in early age (Lavstedt et al., 1982; Addy, 1986; Ismail et al., 1990; Dhar et al., 2007). The reasons for this are either lack of dexterity in tooth cleaning measures,



tooth brushing and interdental cleaning, and/or failure to comply with the recommendation to clean teeth on regular basis (Frandsen, 1986). Also, it was found that even though individuals brush their teeth for 2 minutes duration, they fail to remove half of dental plaque accumulated on their teeth (De la Rosa et al., 1979). Moreover, high prevalence of dental caries was also reported (Bagramian et al., 2009). This can happened due to the fact that no or little attention is given by individuals to certain tooth surfaces during tooth cleaning process (Rugg-Gunn et al., 1978; MacGregor et al., 1979). On the other hand, mechanical plaque control procedures merely remove plaque from the hard oral surfaces (Kerr et al., 1991). Microbial mass accumulated on soft tissue of the oral cavity, such as tongue and oral mucosa, can serve as a source of bacteria to colonize tooth surface and cause gingivitis and periodontitis (Socransky et al., 2005). These limitations of mechanical tooth cleansing measures and the high prevalence of gingivitis and dental caries suggest the use of chemical plaque control as an adjunctive measures to mechanical plaque control (Van der Weijden et al., 2005; Sugano, 2012) which require minimal cooperation and skill in their use (Al-Bayaty et al., 2010).

Among chemical plaque measures, CHX is the gold standard mouthwash used for chemical plaque control (Strydonck et al., 2012). Unfortunately, CHX has some undesirable side effects such as extrinsic staining on teeth and restorations, unpleasant taste, sloughing of oral mucosa and calculus formation enhancement (Helldén et al., 1981; Addy et al., 1995; Kapoor et al., 2011). In an attempt to solve CHX extrinsic staining side effect, CHX mouthwashes containing anti-discolouration agents were reported to have no consistent beneficial effects on plaque and gingivitis (Solís et al., 2011; Li et al., 2014). This encourages researchers to find alternatives for CHX mouth rinse such as herbal formulations. Many researchers have reported antiplaque efficacy of several herbal formulations when used as mouth rinses (Mehta et al., 2013; Gupta et

al., 2015; Pedrazzi et al., 2015; Prasad et al., 2015). *Salvadora persica L.* is one of the herbs that has been reported to exhibit an antiplaque efficacy after rinsing with its extracts (Al-Bayaty et al., 2010). The same observation was reported for green tea aqueous extract (Kaur et al., 2014; Hambire et al., 2015).

Primary plaque colonizers play a crucial initial role in the development of dental plaque (Rosan et al., 2000; Nobbs et al., 2011). In chapters 3 and 4 on *in vitro* investigations, it was found that a combination of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica L.* aqueous extracts (DTC1) exhibited significant synergistic antibacterial and antiadherence effects against primary plaque colonizers biofilm, i.e. *S. mitis*, *S. sanguinis* and *A. viscosus*. In addition, a 24 hours plaque re-growth protocol has been used successfully in earlier clinical studies to evaluate the antiplaque efficacy of various oral formulations (Claydon et al., 1995; Claydon et al., 1999; Claydon et al., 2002; Yévenes et al., 2009). Hence, this clinical study was carried out to evaluate the antiplaque efficacy of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica L.* aqueous extracts combination mouth rinse as compared with a placebo and CHX mouth rinses, negative and positive controls respectively, over a 24 hours period.

## **5.2 Null hypothesis**

There is no difference in antiplaque efficacy of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts mouth rinse and distilled water over a period of 24 hours *in vivo*.

## **5.3 Aim of the study**

The primary aim of this study was to determine the efficacy of aqueous extracts of *Camellia sinensis* var. *assamica* in combination with *Salvadora persica* L., as an *in vivo* antiplaque mouthwash.

## **5.4 Objectives of the study**

- 1- To compare the antiplaque activity of a combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts mouth rinse with a placebo mouth rinse following a 24 hours plaque re-growth clinical trial by means of modified Quigley-Hein Plaque Index (Turesky 1970).
- 2- To compare the antiplaque activity of a combination of *Camellia sinensis* var. *assamica* and *Salvadora persica* L. aqueous extracts mouth rinse with a 0.12% CHX mouth rinse following a 24 hours plaque re-growth clinical trial by means of modified Quigley-Hein Plaque Index (Turesky 1970).

## **5.5 Materials and methods**

### **5.5.1 Study design**

This study was a randomized, double blinded, 24 hours plaque re-growth cross-over clinical trial. It was carried out at the Periodontology Clinic at the Faculty of Dentistry, University of Malaya between February and June 2015. This study was approved by the Medical Ethical Committee, Faculty of Dentistry, University of Malaya (DF RD1501/0010 (P)). This study followed the Consolidation Standards of Reporting Trials (CONSORT) Statement and was registered at [clinicaltrials.gov](http://clinicaltrials.gov), number NCT02624336.

### **5.5.2 Sample population**

The sample population was postgraduate students of the Faculty of Dentistry University of Malaya who wished to volunteer in this study. The volunteers who met the inclusion and exclusion criteria were invited to participate in this study.

The inclusion criteria included those who:

- 1- were between 25 to 40 years old;
- 2- were in good general health;
- 3- had more than 20 teeth.

The exclusion criteria included those who

- 1- had active cavitated caries and/or periodontal disease;
- 2- were currently undergoing orthodontic treatment;
- 3- had a history of antibiotics within the past 4 months;
- 4- required prophylactic antibiotic coverage;
- 5- required systemic and/or topical non-steroidal anti-inflammatory drugs for the past 4 months;

- 6- were pregnant or intended to and lactating mother;
- 7- had a known intolerance or allergy to mouthwashes;
- 8- had heart valve replacement and/or any systemic disease.

Written informed consent was obtained from all participants prior to the beginning of the trial.

### **5.5.3 Sample size of participants**

The sample size was calculated by conducting a pilot study with five participants. The calculation was based on mean PI and standard deviation of each group of the three tested mouth rinses. By using G\*Power software (version 3.1.9.2), it was found that sample size of 14 pairs was enough to reject the null hypothesis between green tea and *Salvadora persica* L. extracts combination (test) and distilled water (negative control) mouth rinses at probability power of 0.95 and 0.05 type I error probability.

### **5.5.4 Clinical measurement**

The plaque quantity was recorded using modified Quigley-Hein PI (Turesky et al., 1970) with the aid of a plaque disclosing agent (erythrosine tablets). In this index, a score of 0 to 5 was assigned to each labial / buccal and lingual / palatal surface of all teeth except the third molars and any filled tooth surface. The scoring criteria were as follows:

- 0 = No plaque,
- 1 = Separate flecks of plaque at the cervical margin of the tooth,
- 2 = A thin continuous band of plaque (up to one mm) at the cervical margin of the tooth,

- 3 = A band of plaque wider than one mm but covering less than one-third of the crown of the tooth,
- 4 = Plaque covering at least one-third but less than two-thirds of the crown of the tooth,
- 5 = Plaque covering two-thirds or more of the crown of the tooth.

All measurements were recorded by a single calibrated examiner. The distance from the gingival margin to the edge of the disclosed area was measured to the nearest 0.5 mm using a calibrated periodontal probe and the scores were recorded in a PI record form for each participant. The mean of PI for each participant was calculated by collecting the scores over the total number of surfaces examined.

#### **5.5.5 Examiner calibration**

The PI scores for all participants were recorded by a single examiner. Calibration of the examiner, in particular scoring system, were carried out with the assistance of an experienced examiner as described by Hefti et al. (2012). One week before the clinical calibration session, the protocol, the case report form (as shown in Appendix B) and the criteria of modified Quigley-Hein PI (Turesky, 1970) were reviewed and discussed by the examiner with the experienced examiner.

The standardization and calibration exercise was carried out on 4 participants with 1 hour time interval between the assessments. Intra- and interexaminer calibrations were carried out using the same protocol. The discrepancies in the scores were discussed between the examiners and the dissimilarity in recording the scores were resolved.

At the end of the calibration session, data were analyzed and then intra- and interexaminer agreements were assessed according to the six-level nomenclature of agreement by Landis et al. (1977) as follow:

- Poor agreement:  $< 0.00$ ,
- Slight agreement:  $0.00-0.20$ ,
- Fair agreement:  $0.21-0.40$ ,
- Moderate agreement:  $0.41-0.60$ ,
- Substantial agreement:  $0.61-0.80$ ,
- Almost perfect agreement:  $0.81-1.00$ .

Data analysis for intra and interexaminer agreements is shown in Table 5.1. This enhanced the reliability of the study in which the data were able to be measured in a reproducible manner which in turn improved the discriminative power of the study (Hefti et al., 2012).

**Table 5.1: Intra- and interexaminer agreement for the study examiner for modified Quigley-Hein PI mean**

Test	Intra examiner	Inter examiner
Kappa value	0.827	0.888
Agreement	Almost perfect	Almost perfect

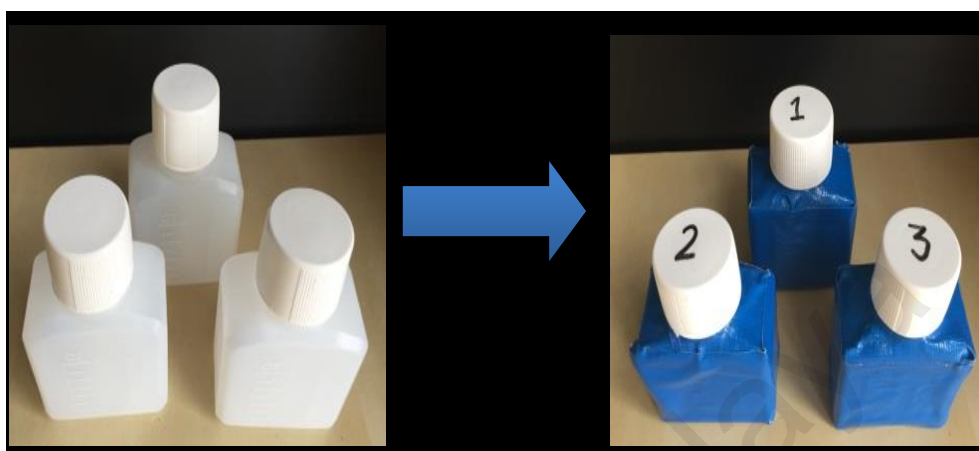
### 5.5.6 Interventions

The test formulation, 0.12% CHX mouth rinse (positive control) and distilled water (negative control) were used in this clinical study. A full description of the mouth rinses is shown in Table 5.2. The mouth rinses were aliquoted in identical opaque bottles as shown in Figure 5.1. These bottles were randomly given sequential number codes (1, 2 and 3) by a laboratory technician not involved in this study. All participants had an equal probability of assignment to the interventions sequence which was randomly selected by using a computer random number generator (Microsoft Excel 2010). The examiner received a number coded interventions sequence list to assign the blinded intervention. In order to achieve allocation concealment, this trial was double blinded in which examiner, nursing staff and participants were unable to identify the corresponding intervention. Decoding was done at the end of the study.

**Table 5.2: Description of interventions**

Interventions	Positive control	Test formulation	Negative control
<b>Ingredients &amp; concentration</b>	0.12% Chlorhexidine gluconate (w/v) (active ingredient)	Combination of <i>Camellia sinensis</i> var. <i>assamica</i> (0.25mg) + <i>Salvadora persica</i> L. (7.82mg) extracts/1ml distilled water	Distilled water
<b>Dosage/ Regimen</b>	15ml twice daily /rinse for 30sec / refrain from eating or drinking for 30min	15ml twice daily /rinse for 30sec / refrain from eating or drinking for 30min	15ml twice daily /rinse for 30sec / refrain from eating or drinking for 30min
<b>Duration</b>	24 hours	24 hours	24 hours
<b>Color</b>	Light blue	Light yellow	Colorless
<b>Preparation</b>	Used commercial oral rinse (Oradex™)	Prepared in Balai Ungku Aziz Research Laboratory (BUARL), Faculty of Dentistry University of Malaya	Prepared in Balai Ungku Aziz Research Laboratory (BUARL), Faculty of Dentistry University of Malaya





**Figure 5.1: Sequentially number-coded identical opaque bottles used in the clinical study**

### **5.5.7 Preparatory period**

After selection of the participants, the aim and flow of the clinical study were explained for them in a preparatory clinical session 7 days prior clinical trial period. At that time, all participants were given oral hygiene education, and received professional tooth cleaning by the examiner.

#### **5.5.7.1 Oral hygiene education OHE**

In this study, all participants were given OHE on the role of dental plaque as an essential causative agent for the development of dental caries and periodontal diseases as well as the importance of controlling its accumulation on tooth surfaces. Instructions and demonstration on how to perform tooth brushing, using the modified Bass technique for 2 minutes and how to use dental floss were carried out by the examiner for all participants. The protocol for mouth rinsing was explained to all participants. Participants were informed to rinse with undiluted 15 ml of the allocated mouth rinse

for 30 seconds, followed by refraining from eating and drinking for the next 30 minutes. All participants were supplied with similar start-up pack containing toothpaste (Colgate®), dental floss (Colgate®) and a soft toothbrush (Colgate®) as illustrated in Figure 5.2. The participants used this oral hygiene set during the preparatory period before the clinical trial period (7 days) and each wash out period (6 days).



**Figure 5.2: Oral hygiene set (tooth paste, tooth brush and interdental floss)**

#### **5.5.7.2 Professional tooth cleaning**

After OHE had been given to all volunteered participants, they received scaling and polishing by the examiner using ultrasonic scaler (SATELEC P5 Newtron XS, UK) and Gracey curettes (Hu-Friedy, Chicago, IL, USA).

#### **5.5.8 Data collection**

After the preparatory period, each participant passed through three phases of the clinical trial with intervening wash out periods. A different mouthrinse intervention was allocated for each phase. PI scores were recorded after each clinical phase.

#### **5.5.8.1 Clinical trial period**

In this period, the participants attended the dental clinic twice, at baseline (0 hour) and after 24 hours. PI scores were recorded after rinsing with each mouth rinse intervention.

##### ***(a) Baseline period***

At the start of each period, the participants' teeth were disclosed with a disclosing agent (erythrosine tablets) by asking the participants to chew the disclosing tablet for 1 minute and rinse their mouth with water 3 times, and then the participants' teeth were polished to have plaque free teeth surfaces at baseline. After that, the participants were asked to rinse with undiluted 15 ml of the allocated mouth rinse solution for 30 seconds under supervision and instructed to refrain from eating and drinking for 30 minutes after rinsing. After the first rinsing, the participants were asked to repeat the second rinsing at home after 12 hours and to refrain from mechanical oral hygiene measures or using chewing gum for 24 hours at which time the teeth were again disclosed and PI was recorded.

##### ***(b) At 24 hours period***

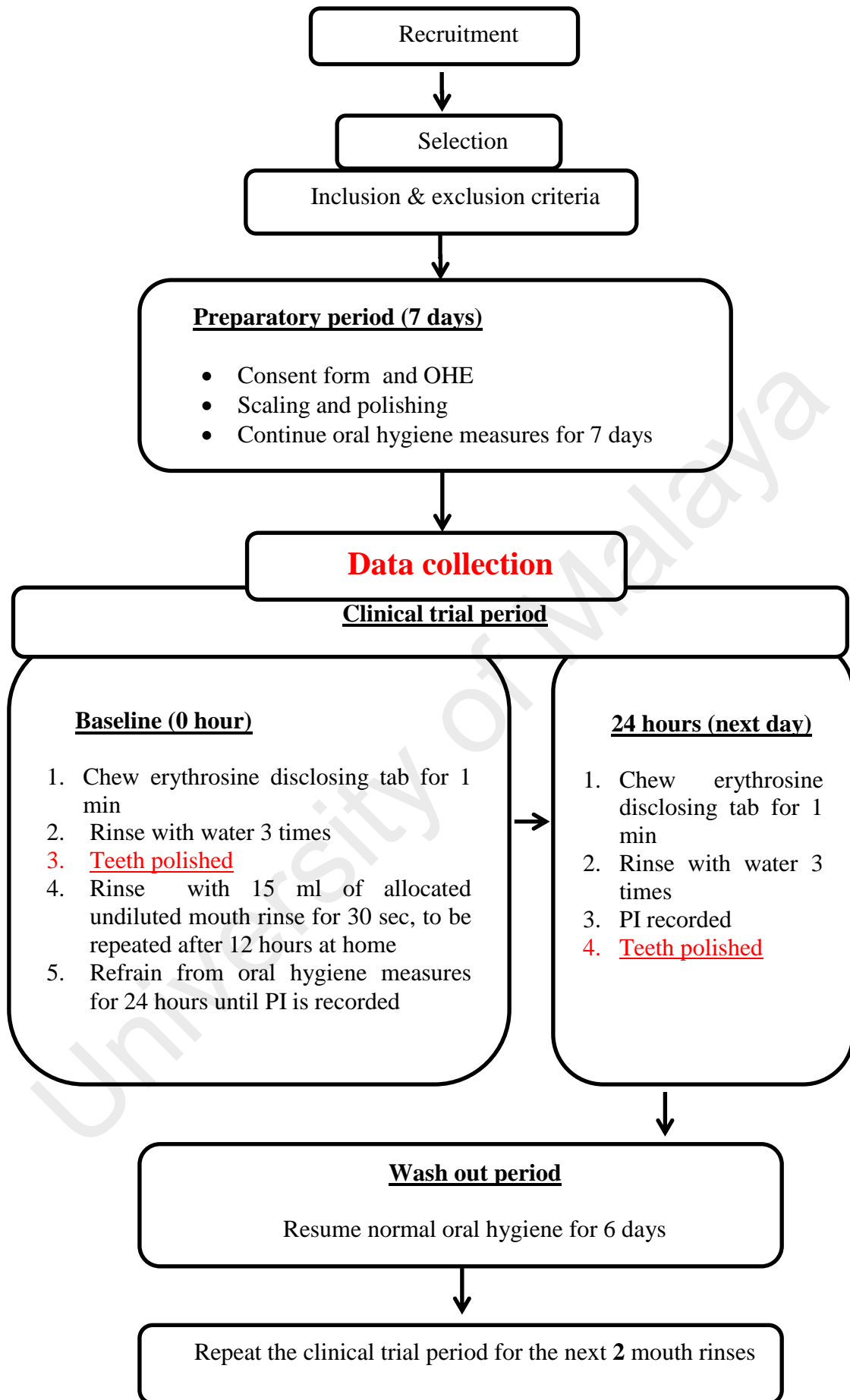
The following day, the participants were asked to chew a disclosing tablet for 1 minute and rinse their mouth with water 3 times, then modified Quigley-Hein PI was recorded (as shown in Figure 5.3), and finally participant's teeth were polished. The use of systemic antibiotics or other antibacterial medications was reported and resulted in participant exclusion from the study.



**Figure 5.3: Dental plaque after 24 hours with no oral hygiene measures**

#### **5.5.8.2 Wash out period**

After recording PI and teeth polishing, the participants entered a 6 days wash out period to eliminate the tested solution effect on the enamel and gingiva, which may potentially be absorbed when using the mouth rinse solutions. During this period, the participants were asked to resume oral hygiene measures, i.e. tooth brushing and flossing. After the wash out period, the same protocol was repeated for the next two mouth rinse interventions as shown in Figure 5.4. On the last day of the study, each participant received professional polishing of all his / her teeth.



**Figure 5.4: Flow of the clinical trial**

### **5.5.9 Safety**

Oral examinations of oral surfaces including the buccal, labial and sublingual mucosa, gingiva, tongue, mucobuccal fold, hard and soft palate, uvula, oropharynx, teeth and dental restorations were carried out for all participants at the time of PI scoring after rinsing twice daily for 24 hours following each intervention. Adverse events were recorded except intervention-unrelated abnormalities, such as traumatic ulcers, food burns and lip bites.

### **5.5.10 Statistics**

The data of this study were analyzed using Statistical Package of Social Science (SPSS) version 16.0 for windows. For mouth rinse interventions comparison, PI scores were described in term of mean and standard deviation. Kruskal-Wallis H test ( $p < 0.05$ ) was used to detect any difference among interventions. Then, Mann-Whitney U test ( $p < 0.0167$ ) was used to test the difference between each pair of interventions. The intra- and interexaminer agreements were assessed by using kappa test. G\*Power software (version 3.1.9.2) was used to determine sample size, intervention effect size and power of the study.

## 5.6 Results

### 5.6.1 Sociodemographic

The CONSORT 2010 flow diagram of this study is illustrated in Figure 5.5. In total, 16 participants were assessed for eligibility for the clinical study in February 2015. However, only 14 subjects who fulfilled the study criteria began the trial and completed both preparatory and clinical trial periods. No participants dropped out of the study.

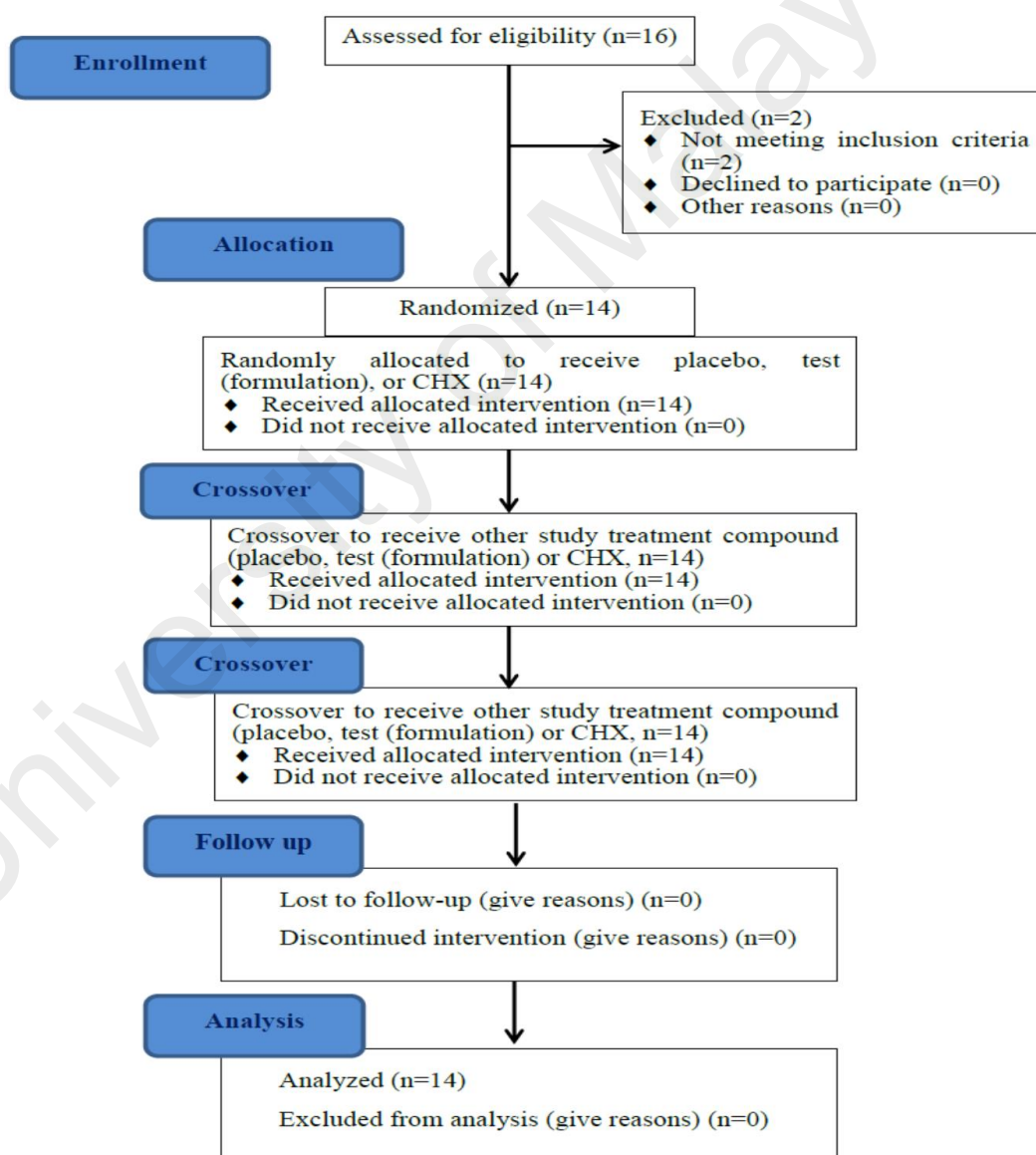


Figure 5.5: Consort 2010 flow diagram summarized design and conduct of the study.

The demographic data of those participants were summarized in Table 5.3. The mean age for the participants was  $30.79 \pm 5.22$  years. The majority of the participants were male (13, 93%). About 36% of the participants were from Malay ethnicity.

**Table 5.3: Demographic data of study participants**

Total		
N=14		
	Mean $\pm$ SD	Range
<b>Age (years)</b>	$30.79 \pm 5.22$	25 – 39
N (%)		
<b>Gender</b>		
Male	13 (93)	
Female	1 (7)	
<b>Ethnicity</b>		
Malay	5 (36)	
Others	9 (64)	

#### **5.6.2 Means and SD of PI of different interventions at 24 hours**

Table 5.4 summarises the mean PI for the different interventions after 24 hours. The test group had the lowest mean compared to CHX and placebo groups. However, the mean PI showed a significant difference between different groups at 24 hours after tooth polishing ( $p < 0.05$ ).



**Table 5.4: Mean and SD of PI of the different interventions at 24 hours**

Intervention	N	Mean	SD	Kruskal-Wallis H test
Test (formulation)	14	0.931	±0.372	p< 0.05
CHX	14	1.317	±0.344	
Placebo	14	1.440	±0.498	

The data were further subjected to Mann-Whitney U test statistical analysis to test the difference between each couple of interventions. The achieved effect size and achieved power for the different intervention groups were also determined. Table 5.5 summarizes the comparison of mean PI of different interventions groups with achieved effect size and power. The mean PI of the test group was significantly lower than mean PI of placebo ( $p<0.0167$ ) with an effect size of 1.158 and achieved power of 0.897 at  $\alpha$  error probability 0.05. Interestingly, the mean PI of the test group was also significantly lower than that of the CHX group ( $p<0.0167$ ) with an effect size of 1.077 and power of 0.856. There was no significant difference between mean PI of CHX and placebo groups.

**Table 5.5: Comparison of mean PI of different interventions groups with achieved effect size and power**

Interventions groups comparison	Mann-Whitney U test	Achieved effect size	Achieved power at $\alpha$ error probability 0.05
Test (formulation) versus placebo	$p<0.0167$	1.158	0.897
Test (formulation) versus CHX	$p<0.0167$	1.077	0.856
CHX versus placebo	$p>0.0167$	0.287	0.178

## 5.7 Discussion

In chapters 3 and 4, it was found that a combination of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica* L. aqueous extracts exhibited significant synergistic antibacterial and antiadherence effects against primary plaque colonizers. To confirm these preliminary *in vitro* results and to evaluate its impact on plaque formation, this clinical study was carried out over a 24 hours period.

### 5.7.1 Sample size and pilot study

There was no published data on the anti-plaque activity of the test mouthrinse that could be used to determine sample size. Therefore, the sample size was determined by conducting a pilot study prior to the clinical study. In addition to sample size determination, there are other advantages of conducting pilot study in advance such as refining protocols and testing assessments (Houser, 2007). For that reason, a pilot study was performed to estimate the sample size and fine-tune the study protocol.

The pilot study showed that sample size of 14 participants was enough to detect the difference between test mouth rinse and placebo in reducing plaque. The interesting finding was that this sample size was also enough to detect the difference between test mouth rinse and 0.12% CHX. This might be due to high effectiveness (as shown in Table 5.5) of the test mouth rinse that was underestimated by the pilot study.

All participants completed the study with no dropouts. This might be due to short term design of the study. Each participant completed the study within 21 days including 7 days for preparatory period, 3 days for 3 clinical trial periods and 12 days for 2 washout periods. Moreover, the participants were postgraduate students of the Faculty of Dentistry University of Malaya. Thus, they were routinely available in the Faculty of Dentistry where the study was carried out and there were no difficulties in contacting them.

### **5.7.2 Randomization, blinding and allocation concealment**

This study was a randomized controlled trial. This type of study offers the highest level of evidence for medical interventions effectiveness (Sackett et al., 2000). Moreover, a well-designed and conducted randomized controlled trial can balance confounders and minimize bias. Hence, it can produce the most reliable estimate of the intervention effects (Montenegro et al., 2002). In this study, randomization was achieved by giving the interventions sequential number codes (1, 2 and 3) and generating a sequence list of number coded interventions, by using a computer random number generator (Microsoft Excel 2010), to randomly assign the blinded intervention to the participants. Thus, all participants had an equal probability of assignment to the interventions sequence.

This study was double blinded in which the examiner, nursing staff and participants were unable to identify the allocated intervention. Achieving study blinding was very important since inadequate blinding can exaggerate the size of interventions' effects (Colditz et al., 1989; Montenegro et al., 2002). Moreover, allocation concealment was achieved by giving the interventions' identical opaque bottles sequential number codes (1, 2 and 3) and generating a number coded interventions sequence list to blindly allocate the interventions to the participants. Achieving such allocation concealment was very important since inadequate allocation concealment can greatly exaggerate the size of the interventions' effects (Juni et al., 2001; Montenegro et al., 2002).

In conclusion, randomization, blinding, and concealment of interventions allocation were achieved in this study. These element are able to enhance the quality of this study (Montenegro et al., 2002).

### 5.7.3 Study design and methodology

In designing this clinical study, two possible study designs were considered, crossover or conventional parallel. In parallel design, the participants must be divided into three groups and each group rinse with only one intervention mouth rinse. While in crossover design, all participants rinse with the three interventions mouth rinses separated by washout periods. In this study, the variations among the participants were considered. Individuals differ in the rate of plaque formation. They are either slow or heavy plaque formers (Newman et al., 2012). Many factors may explain this variation including the clinical wettability of the tooth surfaces, the relative salivary flow conditions and the saliva-induced aggregation of oral bacteria (Simonsson, 1988). Other factors that may explain the individuals' variation in the rate of plaque formation are diet, chewing fibrous food, the presence of copper amalgam, smoking, tongue and palate brushing, the chemical composition of the pellicle, antimicrobial factors present in the saliva, and the retention depth of the dentogingival area (Newman et al., 2012). In an earlier study, de novo plaque formation was investigated among slow and heavy plaque formers. After day 1, the slow plaque formers showed less plaque with less complex supragingival structure (Zee et al., 1997). Thus, it was opted for a crossover design in preference to conventional parallel design in which each participant would serve as his/her own control to improve the sensitivity for detection of relative changes in plaque accumulation. By using crossover design, many advantages were obtained including avoidance of confounding variables, increasing the power of the statistical test conducted to confirm the existence of the intervention effect, and minimizing the sample size of participants (Wellek et al., 2012).

A protocol with no mechanical hygiene measures for 24 hours was used because the deposition of dental plaque at the gingival margin occurs on all teeth surfaces which can be clinically recognized with or without disclosing agents in less than 24 hours (L  e

et al., 1965). Also, the 24 hours plaque reformation can be safely measured as previously done (Claydon et al., 1995; Claydon et al., 1999; Claydon et al., 2002; Yévenes et al., 2009). Furthermore, the test mouth rinse was earlier found to exhibit a significant antiadherence activity (as shown in chapter 4). Thus, it was decided to use 24 hours plaque re-growth protocol to confirm this *in vitro* result and to evaluate its impact on plaque formation. Unfortunately, the effect of the test mouth rinse on gingival tissue cannot be evaluated as it requires a longer-term protocol. For that reason, it was decided to only use plaque index in this clinical study.

In this study, a washout period was needed to rule out any carryover effects of the interventions mouth rinses (Wellek et al., 2012). Therefore, a washout period of 6 days was used in this study. This wash period was believed to be enough to rule out any carryover effects of interventions as previously done in studies using 24 hours plaque re-growth protocol (Claydon et al., 1999; Claydon et al., 2002).

The modified Quigley-Hein Plaque Index (Turesky et al., 1970) was used to record plaque quantity. It is a sensitive index for plaque recording as it has discriminatory power for plaque at the gingival third of tooth surface. Moreover, all teeth can be scored by this index with the aid of plaque disclosing agents. Disclosed plaque is clinically easier to detect and therefore scoring plaque is more objective. This index is recommended for clinical studies testing therapeutic agents (Darby, 2013).

#### **5.7.4 Results**

In this clinical study, a combination of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica* L. aqueous extracts mouth rinse (test) was able to significantly reduce plaque accumulation as compared to placebo after 24 hours re-growth. Interestingly, this formulated mouthwash was also able to significantly reduce plaque quantity as compared to 0.12% CHX mouthwash. In the *in vitro* antibacterial

investigation as mentioned in chapter 3, this same combination was found to exhibit antibacterial activity comparable to 0.12% CHX against *S. mitis*, *S. sanguinis*, *A. viscosus* mixture suspensions, i.e. primary plaque colonizers. It also significantly reduced the adherence of those bacteria to saliva covered glass beads which acted as tooth surfaces. When compared the antiadherence effect of the combination with 0.12% CHX, fewer primary colonizers adhered but it was statistically non-significant as mentioned in chapter 4. Therefore, this formulated mouth rinse might exhibit these observed *in vitro* antibacterial and antiadherence activity and thus significantly reduced the accumulated plaque after 24 hours re-growth *in vivo*.

The initial adherence of primary plaque colonizers to oral surfaces is important as those bacteria provide new receptors for subsequent adhesion of secondary bacterial colonizers, and thus promoting dental plaque development (Nobbs et al., 2011). The finding suggested that the significant antiplaque effect of the test mouth rinse was attributed to fewer primary plaque colonizers' receptors available on tooth surfaces for subsequent secondary colonizers adhesion, therefore resulting in retarded dental plaque development.

This proposed explanation is further supported by earlier reported findings of lower salivary levels of primary plaque colonizers among *Salvadora persica* L. chewing sticks users than toothbrush users; an effect attributed to chemical constituents of *Salvadora persica* L. (Darout et al., 2002). Furthermore, polyphenolic tannins were reported to inhibit salivary  $\alpha$ -amylase and bind both salivary histatin and proline-rich protein (Yan et al., 1995; Lu et al., 1998; Kandra et al., 2004). Recently, it was reported that rinsing with a fluoride-based mouth rinse resulted in a slight fluoride accumulation at the surface of the pellicle-coated enamel (Weber et al., 2015). Both tannins and fluoride were identified in *Salvadora persica* L. aqueous extracts (Hattab, 1997;

Mohammed, 2013) and they may bind salivary proteins of the acquired pellicle covering tooth surfaces, modify the acquired pellicle surface and therefore interfere with the adherence of the primary plaque colonizers to tooth surfaces.

On the other hand, gargling with green tea polyphenols, catechins, was reported to reduce intraoral load of primary plaque colonizers including *S. sanguinis*, *S. salivarius*, *S. mitis* and *S. sobrinus* (Cho et al., 2010). Also, green tea was found to have an antiadherence activity against oral streptococci (Hannig et al., 2009a). In a previous study, it was reported that a concentration of 1 to 4 mg/ml tea polyphenols could inhibit the preliminary adherence of *A. viscosus* and *S. mutans* to saliva coated hydroxyapatites effectively (Xiao et al., 2000). These findings might be explained by the capability of tea polyphenols to adsorb onto acquired pellicle, subsequently modify its structure, and therefore interfere with the bacterial adherence to tooth surface (Joiner et al., 2004).

Some constituents found in green tea and *Salvadora persica L.* extracts were reported to exhibit beneficial activities intraorally. These constituents may be biologically compatible and synergistically exert beneficial efficacy when the participants rinsed with the formulated test mouth rinse. The green tea polyphenolic EGCG was found to inhibit insoluble glucan synthesis in the oral cavity through inhibiting glucosyltransferase activity of *S. mutans* (Ooshima, 2005; Xu et al., 2011). In another study, Xu et al. (2012) described a mechanism by which EGCG inhibit dental plaque accumulation. They hypothesized that EGCG was able to suppress *gtf* genes in *S. mutans* at the transcriptional level. Thus EGCG was able to disrupt the initial attachment of *S. mutans* and prevent the formation of mature biofilms.

In another study used scanning electron microscopy, green tea polyphenols was found to induce various morphological changes in oral bacterial cells, such as formation of cell aggregates, the presence of perforations, and leakage of cytoplasmic materials

from bacterial cells. Moreover, the authors found that tea polyphenols were effective against adherent cells of *S. mutans* and *S. sanguinis*, suggesting the potential use in the prevention and treatment of dental caries (Cho et al., 2010). Green tea catechins were also found to prevent the attachment of oral streptococci to tooth surface. It was reported that these catechins prevented the attachment of *S. mutans* to saliva coated hydroxyapatite discs. This effect was due to modification of bacterial cells through catechins mediated denaturation of extracellular protein ligands such as fibrils and fimbria (Otake et al., 1991; Xiao et al., 2000). Thiocyanate, a component of *Salvadora persica* L. extract, was found to amplify the antimicrobial action of salivary peroxidase-thiocyanate and hydrogen peroxidase system in the oral cavity. This can occur during oxidation of thiocyanate and generation of hypothiocyanate by salivary peroxidase with the presence of hydrogen peroxide from oral bacteria and leukocytes. The hypothiocyanate oxidases sulfhydryl groups in bacterial cytoplasmic membrane leading to death by loss of the ability to transport glucose and leakage of peptide, amino acids and potassium (Tenovuo et al., 1981).

Fluoride is also found in *Salvadora persica* L. extracts, so a mouth rinse of *Salvadora persica* L. extract can provide a source for topical delivery of fluoride in the oral cavity. Fluoride has antibacterial properties by bacterial enzymes inhibition, in addition to inhibition of tooth surfaces demineralization and enhancement of remineralization (Goyal et al., 2011). Moreover, *Salvadora persica* L. extracts was reported to neutralize dental plaque pH for a longer time when compared to rinsing with water. This effect is attributed to the buffering capacity of *Salvadora persica* L. extracts through increasing salivation due to its relatively strong taste, and thus leading to wash out acids in oral cavity (Edgar et al., 1986; Sofrata et al., 2007; Talha et al., 2013).



Earlier relevant clinical studies have investigated the antiplaque efficacy of green tea and *Salvadora persica* L. extracts separately. It was reported that neither commercial *Salvadora persica* L. extract mouth rinse (Persica™) nor its comparator placebo reduced dental plaque accumulation (Khalessi et al., 2004). In contrast, Al-Bayaty et al. (2010) found that rinsing with 10 ml of *Salvadora persica* L. extract at 100 mg/ml 3 times a day significantly reduced dental plaque scores but did not reach the better effect of 0.2% CHX. Recent clinical trials reported that rinsing with green tea extracts at 50 and 250 mg/ml significantly reduced dental plaque accumulation and the antiplaque effect of both concentrations was comparable to 0.12% CHX (Kaur et al., 2014; Hambire et al., 2015). In conclusion, both *Salvadora persica* L. and green tea extracts have antiplaque effects which are in accordance to the current results but at much higher concentrations. The novelty of this study relies on investigating the antiplaque effect of the combination between green tea and *Salvadora persica* L. aqueous extracts at lower concentrations than previously studied. The significant antiplaque activity of this combination observed in this study might be attributed to the synergistic antibacterial and antiadherence activity of this combination as described in chapters 3 and 4. Thus, this combination has the advantage of both cost effective and safety.

In this clinical study, rinsing with the test formulated mouth rinse has significantly reduced dental plaque scores when compared to 0.12% CHX with an effect size of 1.077. The comparison was powerful (achieved power = 0.856). This result was not surprising as the earlier *in vitro* results had revealed better synergistic antiadherence effect of the green tea and *Salvadora persica* L. extracts combination against primary plaque colonizers than 0.12% CHX, green tea extract and *Salvadora persica* L. extract alone. Less primary plaque colonizers biofilm adhered to glass beads treated with the combination when compared to 0.12% CHX after 2 hours (as shown in chapter 4).

Although the difference was not significant, but this might have an effect on the dental plaque quantity formed after 24 hours by providing less binding sites for the secondary plaque colonizers *in vivo*.

#### **5.7.5 Generalizability of the findings**

In this clinical study, the test formulated mouth rinse significantly reduced supragingival plaque in healthy gingiva participants. Since succession of supragingival plaque reformation is similar for both healthy and periodontitis subjects (Teles et al., 2012), this clinical finding suggests that the antiplaque effect could also be seen in periodontitis subjects.

#### **5.7.6 Safety**

In this study, rinsing with the test formulated mouth rinse was safe with no adverse effects detected in all participants. This finding agrees with earlier preclinical studies investigated the toxicity of green tea and *Salvadora persica L.* extracts. According to these studies, green tea aqueous extract up to 2500 mg/kg body weight /day (Bun et al., 2006; Isbrucker et al., 2006; Chengelis et al., 2008; Hsu et al., 2011; Wang et al., 2012a) and *Salvadora persica L.* root stick extract up to 5000 mg/kg body weight /day (Ezmirly, 1979; Al-Bayat et al., 2010; Ahmad et al., 2011; Ibrahim et al., 2012; Verma et al., 2012) are safe when administered orally with no systemic observed adverse effects. In this study, the concentrations of green tea and *Salvadora persica L.* extracts used in the test mouth rinse were 0.25 mg/ml and 7.82 mg/ml respectively. The dosage was 15 ml of the test mouth rinse twice daily. This value is much lower than the earlier reported maximum safe concentration of green tea and *Salvadora persica L.* extracts. Moreover, the green tea and *Salvadora persica L.* aqueous extracts were topically, not systemically, given as a mouth rinse for only 30 seconds. Furthermore, there are many previous clinical trials that investigated the *Salvadora persica L.* and

green tea extracts mouth rinses separately (Al-Bayaty et al., 2010; Bhat et al., 2011; Moghbel et al., 2011; Tehrani et al., 2011; Chelli-Chentouf et al., 2012; Jenabian et al., 2012; Kaur et al., 2014; Hambire et al., 2015) with no any toxicity reports, but this clinical trial was the first to investigate the combination between them.

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## 5.8 Conclusion

- 1- This study demonstrated that rinsing with 15 ml of 0.25 mg/ml green tea, non-fermented leaves of *Camellia sinensis* var. *assamica*, and 7.82 mg/ml *Salvadora persica* L. aqueous extracts combination twice daily can significantly reduce dental plaque accumulation after 24 hours re-growth.
- 2- This study demonstrated that rinsing with 15 ml of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica* L. aqueous extracts combination twice daily can significantly reduce dental plaque accumulation after 24 hours re-growth when compared with 0.12% CHX.
- 3- These results confirm the earlier reported antibacterial and antiadherence effects of this combination (as shown in chapters 3 and 4) which may explain its significant antiplaque effect.
- 4- Rinsing with 15 ml of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica* L. aqueous extracts combination twice daily may provide a natural alternative mouth rinse to 0.12% CHX for controlling dental plaque up to 24 hours period.
- 5- Rinsing with 15 ml of 0.25 mg/ml green tea and 7.82 mg/ml *Salvadora persica* L. aqueous extracts combination twice daily can safely reduce significant amount of dental plaque after 24 hours re-growth with no adverse effects.

## CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

### 6.1 Conclusions

This study initially evaluated the antibacterial activity of each respective green tea, non-fermented leaves of *Camellia sinensis* var. *assamica*, and *Salvadora persica* L. aqueous extracts against primary plaque colonizers, i.e. *S. mitis*, *S. sanguinis*, *A. viscosus* and their mixture suspension. According to the results, it was concluded that a combination of green tea and *Salvadora persica* L. aqueous extracts has synergistic antibacterial activity

After evaluating the antiadherence activity of the combination of green tea and *Salvadora persica* L. aqueous extracts against primary plaque colonizers *in vitro* by using NAM model and CSH test, it was concluded that this combination has synergistic antiadherence activity.

By using green tea and *Salvadora persica* L. aqueous extracts combination, i.e. DTC1, as a mouth rinse after conducting 24 hours plaque re-growth crossover clinical trial, it was concluded that this combination can exhibit significant antiplaque activity.

### 6.2 Recommendations

In the *in vitro* part of this study, green tea and *Salvadora persica* L. aqueous extracts combination, i.e. DTC1, was found to exhibit significant antibacterial and antiadherence activity against primary plaque colonizers. These findings may encourage other researchers to evaluate the effects of this combination on other oral microorganisms including:

- oral bacteria which are the significant contributors to dental caries such as *Streptococcus mutans* and *Lactobacillus* species.

- periodontal pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*.
- oral fungi which are significant contributors to oral candidiasis such as *Candida albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata*.

In the clinical part of this study, the green tea and *Salvadora persica* L. aqueous extracts combination, i.e. DTC1, was found to exhibit a significant antiplaque effect when used as a mouth rinse for a period of 24 hours. Longer-term clinical studies are highly encouraged to confirm the results of the clinical part of this study for longer-term, and to evaluate the effect of this combination on gingival tissue.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### Publications

- 1- Abdulbaqi Hayder Raad, Himratul-Aznita Wan Harun, Baharuddin Nor Adinar. Anti-plaque Effect of a Synergistic Combination of Green Tea and *Salvadora persica* L. against Primary Colonizers of Dental Plaque. Archives of Oral Biology. 70 (2016) 117–124 <http://dx.doi.org/10.1016/j.archoralbio.2016.06.011>. (Appendix G)
- 2- Abdulbaqi, Hayder Raad, Wan Harun Himratul-Aznita, and Nor Adinar Baharuddin. "Evaluation of *Salvadora persica* L. and green tea anti-plaque effect: a randomized controlled crossover clinical trial." BMC Complementary and Alternative Medicine 16.1 (2016): 493. DOI 10.1186/s12906-016-1487-0. (Appendix H)

### Papers Presented

- 1- Participation (poster and oral presentation) in 29th Annual Scientific Meeting of IADR-SEA Division. (Appendix E)