ANTIMICROBIAL ACTIVITY OF *AVERRHOA BILIMBI* FRUIT EXTRACT ON SIMULATED ORAL BIOFILM

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ANTIMICROBIAL ACTIVITY OF *AVERRHOA BILIMBI* FRUIT EXTRACT ON SIMULATED ORAL BIOFILM

By

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DISSERTATION SUBMITTED IN FULFILLMENT
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ABSTRACT

This study was conducted to search for new active compounds from plant extracts that can be used as agents for plaque control. Streptococci dominate more than 600 bacterial species reported found in the dental plaque. At the initial phase of plaque or biofilm formation, the bacterial component colonising the tooth surface consisted mainly of the Gram-positive facultatives but as the biofilm becomes complex and matured, it shifts to consist of more Gram-negative anaerobes. While both *Streptococcus sanguinis* and *Streptococcus mitis* dominates the early phase of plaque formation, *Streptococcus mutans* tends to increase in population when the plaque has become more matured.

This study was carried out to investigate the susceptibility of dental plaque bacteria that includes *S. sanguinis, S. mitis* and *S. mutans* to the extract of five plants: *Averrhoa bilimbi, Brucea javanica, Euphorbia tirucalli, Nigella sativa* and *Vernonia amygdalina*. The screening test was carried out at four different amounts (5, 10, 15 and 20 mg) using the disc diffusion method while the minimum inhibitory concentration (MIC) was determined using the microdilution assay. 0.12% (w/v) of chlorhexidine gluconate (CHX) and sterile distilled water were used as the positive and negative controls, respectively. It was found that all three bacteria were susceptible to the aqueous extract of *A.bilimbi* and *V.amygdalina*. The MIC of *A.bilimbi* was 6.25 mg/mL for all three *Streptococcus* sp. while *V. amygdalina* exhibited weak antibacterial response. Based on this screening, only the extract of *A. bilimbi* was selected for futher tests. It was determined that *A. bilimbi* has a minimum bactericidal concentration of 12.5 mg/mL for *S. mutans* dan *S. mitis*, and 25.0 mg/mL for *S. sanguinis*. Three active compounds of *A. bilimbi* were identified as ascorbic acid, acetylvitisin B and chinic acid. *A. bilimbi* also demonstrated slight aggregative effect. At a minimal concentration of 6.25 mg/mL

(MIC), *A. bilimbi* extract demonstrated antiadhesion activity on the binding of bacteria to the acquired pellicle. Extract-treated pellicle was found to prevent bacteria adhesion and thus decreases bacteria population inside the biofilm by 64.5%. This extract also showed good antibacterial effect as it can decrease the hydrophobic properties of all three bacteria surfaces. Lost in hydrophobic properties is a negative effect to bacteria because it influences the bacteria adherence capacity during the early phase of biofilm formation. Exposure of biofilm-24hr to *A. bilimbi* for 30, 60 and 90 sec was found to be effective in reducing bacterial population in the biofilm by 24.1, 30.5 and 49.8%, respectively. At a minimal concentration of 6.25 mg/mL (MIC), *A. bilimbi* extract also exhibited antiadherence effect on the adhesion of bacteria to the acquired pellicle. Extract-treated pellicle showed antiadhesion activity by reducing the biofilm attachment to experimental pellicle by 64.5%. It also reduced the cell surface hydrophobicity of all streptococcus tested and make them less adherent. *A. bilimbi* also down regulated the expression of *gtfB* by almost 5-fold (RQ = 0.204), almost fully suppress *gtfC* (RQ = 0.008) and totally suppressed the expression of *vicR* gene (RQ = 0.000).

The significant antibacterial properties exhibited by *A.-bilimbi* extract suggested its potential use for plaque control.

ABSTRAK

Kajian ini dijalankan untuk mencari bahan aktif baru daripada ekstrak tumbuhan yang boleh digunakan sebagai agen bagi tujuan pengawalan plak gigi. Streptococci menguasai lebih daripada 600 spesies bakteria yang dilaporkan terdapat di dalam biofilem. Pada peringkat awal pembentukan plak atau biofilem, komponen bakteria yang mengkoloni permukaan gigi terdiri daripada Gram-positif fakultatif tetapi semakin biofilem menjadi kompleks dan matang, ianya berubah kepada lebih banyak Gram-negatif anarob. Sementara kedua-dua *Streptococcus sanguinis* dan *Streptococcus mitis* menguasai peringkat awal pembentukan plak, populasi *Streptococcus mutans* pula lebih cenderung untuk meningkat apabila plak menjadi lebih matang.

Kajian ini dijalankan untuk menyiasat perencatan bakteria plak gigi yang terdiri daripada *S. sanguinis, S. mitis* dan *S. mutans* terhadap ekstrak daripada lima tumbuhan: *Averrhoa bilimbi, Brucea javanica, Euphorbia tirucalli, Nigella sativa* dan *Vernonia amygdalina*. Ujian saringan dijalankan pada empat amaun berbeza (5, 10, 15 and 20 mg) menggunakan kaedah ujian resapan cakera manakala kepekatan perencatan minima (MIC) ditentukan menggunakan asai pencairan-mikro sampel. 0.12% (w/v) chlorhexidine gluconate (CHX) dan air suling yang disterilkan digunakan sebagai kontrol positif dan negatif. Hasilnya, perencatan positif kesemua tiga bakteria hanya dilihat terhadap ekstrak *A. bilimbi* dan *V.-amygdalina*. Nilai MIC bagi *A. bilimbi* ialah 6.25 mg/mL untuk ketiga-tiga *Streptococcus* sp. manakala *V. amygdalina* menunjukkan tindak balas antibakteria yang lemah. Berdasarkan hasil saringan ini, hanya ekstrak *A. bilimbi* digunakan untuk ujian selanjutnya. Kajian menunjukkan ekstrak *A. bilimbi* mempunyai nilai kepekatan minimum

bakterisidal (MBC) 12.5 mg/mL untuk *S. mutans* dan *S. mitis*, dan 25.0 mg/mL bagi *S. sanguinis*. Tiga bahan aktifnya adalah asid askorbik, asetilvitisin B dan asid khinik. *Averrhoa bilimbi* juga mempamer sedikit kesan aggregatif. Pendedahan biofilem-24jam kepada *A. bilimbi* untuk 30, 60 and 90 s didapati berjaya menurunkan populasi bakteria dalam biofilem tersebut kepada, 24.1, 30.5 and 49.8%. Pada kepekatan minima 6.25 mg/mL (MIC), ekstrak *A. bilimbi* turut menunjukkan kesan antipelekatan bakteria kepada pelikel perolehan. Pelikel yang dirawat dengan ektrak didapati menghalang pelekatan bakteria lalu menyebabkan pengurangan populasi bakteria dalam biofilem sebanyak 64.5%. Ekstrak ini juga menunjukkan kesan antibakteria yang baik kerana dapat mengurangkan sifat hidrofobik permukaan sel ketiga-tiga bakteria. Kehilangan sifat hidrofobik memberi kesan negatif kepada bakteria kerana ia mempengaruhi kapasiti pelekatan bakteria ketika peringkat awal pembentukan biofilem. *A.-bilimbi* juga telah menurunkan tahap ekspresi *gtfB* gen hampir 5 kali ganda (RQ = 0.204), hampir merencatkan sepenuhnya *gtfC* gen (RQ = 0.008) dan merencatkan sepenuhnya ekspresi gen *vicR* (RQ = 0.000).

Kesan ketara anti-bakteria yang ditunjukkan oleh ekstrak *A. bilimbi* menunjukkan ianya berpotensi digunakan untuk mengawal plak.

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LIST OF ABBREVIATIONS

Abbreviation	Description
BHI	Brain Heart Infusion
Cm	Centimetre
CFU/Ml	Colony forming units per millilitre
˚C	Degree Celsius
dH_2O	Distilled water
et al.	et alia (and others)
G	Gram
g/Ml	Gram per millilitre
g/L	Gram per litre
L	Litre
µg	Microgram
µg/mL	Microgram per millilitre
mL	Millilitre
Mg	Milligram
mg/mL	Milligram per millilitre
Mm	Milimetre
Min	Minute
Nm	Nanometer
OD	Optical density
PBS	Phosphate Buffered Saline
pp.	Pages
%	Percentage

LIST OF ABBREVIATIONS

Sec	Seconds
sp.	Species
qPCR	Quantitative Polymerase Chain Reaction

CHAPTER ONE

1.1 INTRODUCTION

Streptococci are Gram-positive bacteria that grow in chain or in pair and predominate in oral biofilm. Most of these cocci are non-pathogenic but a few like *Streptococcus sanguinis* and *Streptococcus gordonii* have been associated with infective endocarditis when they spread through the blood stream during dental treatment (Kreth *et al*., 2009; Hahn *et al*., 2005). Oral biofilm or dental plaque is formed through a sequence of steps that start from the initial deposition of salivary pellicle, followed by colonization of bacteria and finishes with the complex formation of a mature biofilm. At the initial colonization phase of plaque formation, over 80% of the microorganisms consist mainly of streptococci that include *S. sanguinis* and *S. mitis* (Rosan and Lamont, 2000). Immediately after cleaning, the tooth surfaces and gingivae of healthy individuals are colonized mainly by Gram-positive cocci, and subsequently followed by the Gram-positive rods and filaments, with very little of the Gram-negative cocci. However in the matured state, oral biofilm becomes dominated by the Gram-negative anaerobes (Xie, 2000).

When in the form of a biofilm the bacteria have an increased resistance towards antibiotics and chemotherapeutic agents. The bacteria are also less able to be phagocytised by the host's inflammatory cells (Costerton *et al*., 1999; Socransky and Haffajee, 2002). The pathogenicity of oral biofilm depends very much on the solubility of the extracellular matrix as well as the structure of the biofilm. In early biofilm, the structure of the matrix is thin, loose and easily penetrable by saliva. In matured biofilm however, the extracellular matrix becomes compact, thick and non-penetrable to saliva. This is when the biofilm becomes pathogenic as it allows for the accumulation of acids

and other bacterial by-products. Plaque bacteria also synthesize extracellular polysaccharides (EPS) from dietary sugar like sucrose (Bowen, 2002) that contribute to the architecture of the biofilm. Sucrose is used as a substrate for the synthesis of EPS (Bowen, 2002). The gelatinous and sticky nature of EPS enhances the aggregation of bacteria to the developing plaque that influence the biochemical and physical characteristic of the biofilm. Sucrose is also utilised to form intracellular polysaccharides (IPS) which when nutrients are limited, can be metabolized by bacteria to generate energy that ensure its viability (Zero *et al*., 1986).

Dental caries and periodontal diseases are two common plaque-associated oral diseases that are caused by the activities of bacteria. Caries is attributed to the acidic by-products of saccharolytic bacteria such as *S. mutans,* which accumulates in thick plaque and causes demineralization of tooth mineral (Shay, 2002; Fathilah *et al*., 2017). The symptoms of dental caries are toothache, sensitivity to sweet, hot or cold foods or drinks and pain when chewing. Periodontal diseases on the other hand are serious inflammatory disease of the gum and tissues like cementum, alveolar bone and gingivae that surround and support the teeth (Kim and Amar, 2006). This inflammation is triggered by nitrogen-based by-products from the metabolism of asaccharolytic bacteria that dominates the plaque formed below the gingival margin. Severe periodontitis can lead to tooth loss and can be found in 5-20% of adult population worldwide (Albandar, 2005; Haynes, 2006; Khalili, 2007). Among the symptoms are swollen and bleeding gum, sensitive teeth and loose teeth.

Conventional methods in plaque control include tooth brushing and flossing. However of late, rinsing using mouthrinses with added active compounds has become popular. Though effective, some active agents and the alcohol solvent used in its preparation has been associated with side effects like tooth staining, oral cancer, and (Addy, 1986; Smigel, 1991; Llewelyn, 1994) shift in the normal flora of the intestine and oral cavity (Sofowora *et al*., 2013). These side effects prompted researchers to find alternative agents with antimicrobial activity that can be added to oral rinses as antiplaque agent, with no or less side effects.

Plants have long been investigated for potential sources of active compounds to be used as components in traditional and folklore medicine. Active compounds reported present in plants include alkaloids, phenols, flavonoids, tannins and terpenoids (Cowan, 1999; Leon *et al*., 2001; Al-Zubaydi *et al*., 2009).

1.2 Objectives of Research:

To evaluate the antimicrobial activity of aqueous extracts of *Averrhoa bilimbi*, *Euphorbia tirucalli*, *Nigella sativa*, *Vernonia amygdalina* and *Brucea javanica* on the early oral biofilm consisting of *S. sanguinis, S. mitis* and *S. mutans*.

Specific objectives:

1. To screen the respective plant extracts for antimicrobial activities based on susceptibility test and, the MIC and MBC values towards *S. sanguinis, S. mitis* and *S. mutans*
2. To determine active compounds of the selected plant extracts with effective antimicrobial acivities using Liquid chromatography–mass spectrometry (LC-MS)
3. To determine antibacterial properties of the selected plant extract that included aggregative, killing, antiadherence and cell surface hydrophobicity activities on mixed-culture of *S. sanguinis, S. mitis* and *S. mutans.*
4. To determine effect of the selected plant extract on specific genes *gtfB, gtfC* and *vicR* of the oral streptococci (*S. sanguinis, S. mutans, S. mitis*)

CHAPTER TWO

LITERATURE REVIEW

2.0 Oral Ecosystem

The oral ecosystem is contributed by major components that include the host's structures, oral fluid and microorganisms. Within this ecosystem, dynamic interactions between these components result in an ecological balance that mutually benefits both host and the microbial inhabitants. Under a balanced condition, the oral cavity is protected from inflammatory and infectious pathologies like gingivitis, periodontitis, candidiasis and caries. In situations where this balance is destabilized because of factors like changes in salivary flow and inadequate oral care, an opportunistic condition is created which initiates a shift in the microbial components that favours plaque associated diseases and weakens the oral tissues (Lacoste-Ferré *et al*., 2013).

2.1 Oral Structures

The oral cavity has numerous sites that serve as suitable habitats for specific microorganisms. Structures such as the lips, hard and soft palates, attached gingiva, cheek, tongue, gingival sulcus and teeth provides surfaces for microbial adhesion. Soft tissue surfaces which undergo continuous desquamation harbour less microorganisms as compared to surfaces of the hard tooth tissue. Due to the non-shedding property of the hard tissue, microorganisms are able to adhere and accumulate forming a biofilm coating the tooth surface known as dental plaque. The anatomy of teeth greatly influences the sites of plaque accumulation. Pit and fissures on the occlusal area, as well as interproximal surface between adjacent teeth allows retention of food remnants and growth of microorganisms, leading to accumulation of dental plaque that is hard to reach for cleaning. Positions of teeth also influence the site of plaque accumulation with

the posterior teeth that are difficult to clean having more plaque compared to the anterior teeth (Straub *et al*., 1998).

2.2 Oral Fluids

Saliva and gingival crevicular fluid (GCF) are oral secretions containing host defence factors that help maintain the integrity of oral cavity by supplying nutrients, water, antimicrobial components and receptor sites for microbial adherence. Saliva moistens the supragingival environment, while GCF bathes the subgingival area (Taylor and Preshaw, 2016).

2.2.1 Saliva

Saliva is composed of 99% water and consists of organic and inorganics components. Different types of electrolytes like calcium, chloride and bicarbonate contributes the major inorganic constituents, while proteins that include immunoglobulins, enzymes, mucosal glycoproteins, some oligopeptides and polypeptides form the majority of the organic components. Glucose and nitrogenous products of microorganisms such as ammonia and urea are also present in saliva (Humphrey and Williamson, 2001; Taylor and Preshaw, 2016). Saliva performs various biochemical and mechanical functions such as protection, lubrication, buffer system and initial digestion of starch. The mean pH of saliva is between 6.25-7.25 (de Almeida *et al*., 2008). Saliva acts as a buffer system to neutralize acids and bases produce by oral bacteria (Taylor and Preshaw, 2016). Sialin, urea and ammonia are the salivary components that increase the biofilm pH. The most important buffer system in stimulated saliva is the carbonic acid-bicarbonate system while in unstimulated saliva is the phosphate buffer system (Tenovuo *et al*., 1994). Both buffer systems help maintain the integrity of the enamel structure. Saliva plays an important role in forming and maintaining a dynamic oral ecosystem. It helps to increase the pH and provides ions

such as phosphate, magnesium, calcium and fluoride that are involved in the remineralization of tooth enamel (Marcotte and Lavoie, 1998). The demineralization and remineralization process result in a net loss of mineral and this leads to tooth decay (Kidd and Fejerskov, 2004). Salivary proteins deposited from the saliva are important components of the acquired pellicle, source of food for oral microbes, possess antimicrobial activity and promote bacteria aggregation. Proteins that are involved in maintaining the oral ecosystems are lysozymes, peroxidase, histidine, agglutinins, lactoferrin, secretory immunoglobulin A, secretory immunoglobulin M (IgM) and G (IgG) (Liébana, 2002).

2.2.2 Gingival Crevicular Fluid (GCF)

Serum constituents reach the mouth via GCF (Cimasoni, 1983). In addition to IgG that is predominant in GCF, IgM, IgA, neutrophils, lymphocytes and monocytes are also present. At healthy sites, the flow of GCF is slow but faster rate has been observed during inflammatory responses such as those associated with periodontal problems. With the flow, GCF removes weakly-adherent microbial cells, but at the same time introduces additional constituents that act as nutrients for its anaerobic inhabitants. Though temperature inside the mouth remains relatively constant at 37˚C, the temperature in sub-gingival area has been known to increase in presence of inflammatory response (Marsh, 2000). Based on its properties, GCF can be used as a biochemical indicator to determine the progression of periodontitis (Armitage, 1996).

2.3 Oral Microflora

2.3.1 Normal Microorganism

Components of the normal flora in the mouth change with age. The mouth is sterile at birth, but several microbes are immediately acquired due to the rapid colonization of epithelial surfaces of the newborn mouth that are passively transferred from milk, water, general environment and from mother to the baby. Early colonizers of the oral surfaces consist mostly of the aerobic and facultative anaerobic species. Following tooth eruption within the first year of life, mutans streptococci and *S. sanguinis* colonize the hard tissue surfaces of the teeth. As the age increases the oral flora composition becomes more complex as the pioneer community modifies the environment and provide suitable conditions for colonization by other populations (Marsh, 2000). Novel nutrients developed by these pioneer species can be used by other microorganisms as part of a food chain, change the local redox potential (Eh), pH and expose or modify new receptors on surfaces for adherence (Marsh, 2000). Continuous accumulation of these microbes increases species diversity that leads to a stable climax environment harbouring the Gram-positives, Gram-negatives, facultative and obligate anaerobes, and archaea (Aas *et al*., 2005; Paster *et al*., 2006). In supragingival plaque, the microbial population constitutes mainly of the saccharolytic Gram-positive *S. mitis*, *S. mutans* and *S. sanguinis* while, the subgingival plaque is dominated by the Gram-negative anaerobes *Fusobacterium nucleatum*, *Prevotella intermedia* and *Tannerella forsythia* (He and Shi, 2009). The important role of normal flora is to keep the oral tissue surfaces in good condition by acting as a colonization barrier against adhesion by potentially pathogenic microbes (Dodds *et al*., 2005).

2.3.2 Ecological Balance and Pathogenic Flora

Within the oral environment, there is a balance between colonization and removal of its microbial flora. Maintenance of this ecological balance keeps the oral cavity in a healthy condition (Ruby and Goldner, 2007; Filoche *et al*., 2010). When oral hygiene is challenged by bad habits such as excessive intake of high carbohydrate-containing foods, saccharolytic bacteria such as *S. mutans* tends to flourish and increase the percentage of acid-tolerant bacteria. The acidic environment enhances demineralization of teeth.

Under such condition, the normal bacteria now change to become an opportunistic pathogen. In other words, an altered ecological balance of the oral environment may cause a shift in the predominance of the normal microbes.

2.4 Oral Biofilm

Biofilm is defined as a specific but highly variable structural entity that consists of a group of microorganisms and their product embedded in a matrix of polymers of host and bacterial origin (Flemming and Wingender, 2010). In addition to those that form on tooth surfaces and dental prostheses, biofilms also forms readily at various sites inside the human body such as on catheters, implants, external surfaces of the eye, contaminated prosthetic joints, endotracheal tubes, and valves of an artificial heart (Gurenlian, 2007).

2.4.1 Mechanism of Formation

Biofilm forms in a sequential manner beginning with adhesion of bacteria to the acquired pellicle. Acquired pellicle is a thin protein film that is formed by the selective adsorption of salivary proteins on clean tooth surface (Siqueira *et al*., 2007; Huang *et al*., 2011; Vukosavljevic *et al*., 2014). Colonization of bacteria can be irreversible if it involves adhesion to specific receptors present in the acquired pellicle. The adhesion maybe a reversible interaction if the binding is mediated by forces such as the van der Waals forces, Coulomb interactions, hydrophobic interactions or Lewis acid-base interactions (Hannig and Hannig, 2009). Within the first 4 to 8 hours after the tooth is thoroughly cleansed, 60-80% of the primary colonizers consist of oral streptococci (Diaz *et al*., 2006; Dige *et al*., 2009). Once anchored on the tooth surface, these colonizers synthesize extracellular polymeric matrix which hold the bacterial mass together and enhance stronger attachment of the bacteria (Kaplan, 2010). One of the main components in the matrix is extracellular polysaccharides (EPS). Its gelatinous structure can trap nutrients from the surrounding, influences iron exchange within biofilms and can block harmful agent outside of the biofilm (Huang *et al*., 2011). As the biofilm matures the number of bacteria adhering balance those that are dislodged from the biofilm and a climax community is achieved. The microbial-plaque communities communicate via physical interactions termed co-adhesion and co-aggregation. Co-adhesion is the process of attachment between planktonic bacterial cells and already adherent cells on the tooth surface. Co-aggregation occurs when a particular cell-to-cell reaction takes place between distinct bacterial cells and this interaction can be specific and site-specific. Recognition of receptors on the primary colonizers by complementary adhesins on the secondary colonizers makes this possible. Studies have shown *Fusobacterium nucleatum* acts as a bridging organism between the early colonizers such as streptococci, and the late colonizers such as the periodontal pathogens (Jakubovics

and Kolenbrander, 2010; Huang *et al*., 2011). A mature oral biofilm creates various microenvironments with different oxygen concentration, pH and nutrient availability.

2.4.2 Young and Matured Biofilms

A newly formed biofilm is thin and the matrix is loose due to the low volume of EPS forming an open structure biofilm that allows free movement of oral fluid to neutralise any production of acids (or bases) by the bacteria. As the biofilm matures more EPS are synthesized which decrease the porosity of the biofilm. Under such conditions, the cell numbers and the pattern of microbial colonization change from mono-layered chains at 6-hour (Dige *et al*., 2007) to mixed-species microcolonies at 8-hours (Palmer *et al*., 2003). Mature biofilms consist of highly heterogeneous matrix structure, fluid-filled pores and channels that are surrounded by matrix aggregates and colonized by scattered bacteria (Wood *et al*., 2000; Dige *et al*., 2007). Mature biofilms also contain voids that cannot be stained by different fluorochromes that probably indicate the presence of biological substances such as glycoproteins and exopolysaccharides (Auschill *et al*., 2001).

2.4.3 Plaque Metabolism

2.4.3.1 Glycolysis and Acid Production

The initiation and progression of tooth decay is strongly connected to consumption of sucrose (Moye *et al*., 2014). The metabolism of dietary carbohydrates started with sucrose splitted into fructose and glucose molecules by oral bacteria through glycolytic pathway then organic acids such as lactic, acetic and formic acids are produced (Kleinberg, 2002; Muñoz-Elías and Mc Kinney, 2006) that will decrease the pH value of dental plaque to below 5.5 (Marsh, 2006) and cause the demineralization of the teeth (Schafer and Adair, 2000; Caufield and Griffen, 2000; Dogan, 2013).

2.4.3.2 Synthesis of Extracellular Polysaccharides (EPS)

Sucrose is converted to water-insoluble glucans by glucosyltransferase enzymes (Gtf) to form extracellular polysaccharide (EPS) that assist bacterial biofilm formation and attachment to tooth surfaces and to one another (Marsh *et al*., 1985). It was reported by Yamashita *et al*., (1993) that inactivation of the Gtf enzymes helps to prevent caries in an animal model study. The *gtfB* and *gtfC* genes were reported to express Gtf enzyme activity (Aoki *et al*., 1986; Hanada and Kuramitsu, 1988)

2.5 The Genus *Streptococcus*

2.5.1 Cell Wall Structure and Function

The streptococci are Gram-positive bacteria with thick cell wall that may extend to 50 nm from the cell's membrane. It is mainly composed of peptidoglycan, with carbohydrates and protein that are covalently attached (Koch, 2000; Brown *et al*., 2005; Scott and Barnett, 2006; Vollmer *et al*., 2008; Weidenmaier and Peschel, 2008). The cell envelope of Gram-positive bacteria contains carbohydrate-based anionic polymers that act as scaffolds for enzymes required in cell wall metabolism and has been shown to play important role in extracellular interactions.

2.5.2 Oral Streptococci

S. sanguinis, S. mutans and *S. mitis* are the predominant streptococci of the oral cavity (Paik *et al*., 2005). These cocci are saccharolytic and metabolises carbohydrates for energy.

2.5.2.1 *Streptococcus sanguinis*

S. sanguinis is a facultative anaerobe and a member of the viridians group. Previously known as *S. sanguis,* this species is one of the most abundant streptococci and a common agent of infective endocarditis (Paik *et al*., 2005). *S. sanguinis* is a primary colonizer that starts colonizing infants after the emergence of teeth (Okahashi *et*

al., 2011). It binds directly to the pellicle-coated teeth assisted by the presence of numerous fimbriae on its cell surface (Nobbs *et al*., 2009; Okahashi *et al*., 2011). Higher levels of *S. sanguinis* and lower levels of mutans streptococci have been reported in the saliva of children (Caufield *et al*., 2000). Lower risk to caries is also attained when the *S. mutans* to *S. sanguinis* ratio is small (Loesche *et al*., 1975).

2.5.2.2 *Streptococcus mitis*

S. mitis is α-hemolytic and is also a member of the viridians streptococci group of bacteria (Matsui *et al*., 2013). In addition to the oral cavity, it is also a normal inhabitant of the gastrointestinal system, female genital system and oropharynx (Lu *et al*., 2003; Lyytikainen *et al*., 2004). *S. mitis* has been associated with bacteremia, septicaemia and infective endocarditis (Mitchell, 2011).

2.5.2.3 *Streptococcus mutans*

S. mutans is the initiator of dental caries (Li *et al*., 2013) and other than in the mouth, it also inhabits the intestine and pharynx (Loesche, 1986). This species has been implicated with pyogenic and other infections in heart, mouth, skin, joints, central nervous system and muscle. An important virulence determinant of *S. mutans* is its ability to produce acids in biofilms and its tolerance for acidic condition by maintaining its intracellular pH (Nishimura *et al*., 2012). Exposures of teeth to low pH may initiate the process of tooth decay as acids at a critical pH of 5.5 demineralizes tooth enamel (Busuioc *et al*., 2009; Forssten *et al*., 2010). Intra and extracellular polysaccharides that are produced by many of its strain determines the cariogenicity of dental plaque. This sticky EPS helps the adhesion of bacteria to the acquired pellicle by serving as binding sites (Schilling *et al*., 1989; Dedeoglu *et al*., 2015).

2.6 Biofilm Associated Diseases

Dental caries and periodontal diseases are two main diseases associated with oral biofilms. Biofilm formed above and below the gingiva and is respectively referred to as the supragingival and subgingival plaque. In the form of biofilms, bacteria are 1000 times more resistance to antibiotics and chemotherapeutic agents than in the planktonic cells (Gurenlian, 2007; Jakubovics and Kolenbrander, 2010). Biofilm bacteria are more resistance because of the up-regulation of antimicrobial system in biofilm cells, tough intercellular matrices and slow growing cells (Zhang and Mah, 2008).

2.6.1 Dental Caries

Dental caries is a common health problem that was first reported in Miller's chemoparasitic theory in 1890 (Touger-Decker and van Loveren, 2003). It affects about 60% to 90% of both adults and children (Marinho *et al*., 2013). In Malaysia, a survey carried out in 2005 by The Malaysian Oral Health showed that caries prevalence in 5-year-old children was 76.2%, in 12-year olds caries-free prevalence was 57.8% compared to 25.9% in 1991 while in 16-year-olds 30.2% compared to 9.9% in 1991. In the same year for periodontal health, 98.6% and 95.8% of primary school children and secondary school children were free from gingivitis (Oral Health Division, 2005; Oral Health Division, 2006). Although caries is a multifactorial disease (Selwitz *et al*., 2007), in general it is initiated when there is prolonged exposure of tooth surface to low pH environment created by bacteria in matured plaque. When a critical pH is achieved at about pH 5.5, the tooth structure starts to demineralise and caries is initiated. Around 40 bacterial species have been associated to caries (Kutsch, 2014; Blanc *et al*., 2014) but *Lactobacilli*, *S. sobrinus* and *S. mutans* were identified the main acid-tolerant bacteria involved in its formation (Touger-Decker and Loveren, 2003; Nurelhuda *et al*., 2010).

2.6.2 Periodontal Disease

Periodontal disease is a localized infection and inflammatory disease that is mainly associated with the anaerobic Gram-negative bacteria. This disease is initiated when oral hygiene is poor and plaque is allowed to extend into the gingival sulcus. This subgingival plaque harbours anaerobic Gram-negative microbes. Following microbial interaction with the body's defence system, lysis of these microbs leads to the release of various pathogenic determinants such as endotoxins, collagenase, hyaluronidase that result in inflammation of the periodontium that eventually leads to loosening of the tooth. In contrast to caries that is closely associated with acidic pH, destruction of the supporting tissues results in an increased pH within the periodontal pocket. Bacteria closely associated with this region of the oral cavity include *Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella nigrescens, Prevotella intermedia, Treponema sp*. (*Treponema denticola*), *S. intermedius, Peptostreptococcus micros, Bacteroides forsythus* and *Tannerella forsythensis* (Haffajee and Socransky, 1994; Arigbede *et al*., 2012). The growth of these species is supported by the increased protein concentration in the GCF that serves as substrates for energy generation by the anaerobic bacteria. It has been reported that about 5–20% of the adult population worldwide have severe periodontitis that if untreated, may lead to tooth loss (Albandar, 2005; Haynes, 2006). In Malaysia, adults subject affected with periodontal disease was 90.2% in 2000 compared to 92.8% in 1990.

2.7 Biofilm Control

There are several alternative approaches to biofilm control which includes the inhibition of bacterial colonization (Fathilah *et al*., 2003), interruption of growth and metabolism (Fathilah *et al*., 2009), modification of plaque biochemistry, alteration of plaque ecology and disruption of established plaque (Chandki *et al*., 2011). However, in

view of the defence role played by the normal microbes in the oral cavity, it is crucial that the presence of this microbial population is maintained. Thus, the aim of biofilm control with regards to the oral environment is not to totally remove but to maintain the population at its minimum (Fathilah, 2011) and this can be obtained via the use of chemical-based and plant-based antimicrobial agents.

2.7.1 Chemical-based Active Compounds

Chemical-based active compounds used in plaque control includes phenols (thymol and delmopinol), quarternary ammonium compounds (benzalkonium chloride and cetylpyridinium chloride), bisbiguanides (chlorhexidine and alexidine), bispyridines (octenidine), metallic salts (zinc, tin and copper), amino alchohols (octapenol and decapenol) and other surfactants (sodium lauryl sulphate) (Chandki *et al*., 2011). Generally these compounds work by inhibiting sugar transport, acid production, polysaccharide synthesis, bacterial enzymes and damanging the cell membrane (Marsh, 2003; Marsh, 2010; Jafer *et al*., 2016). Chlorhexidine that is known as the gold standard among antiplaque agents, kills bacteria by adsorbing onto the cell wall which then leads to cell leakage and release of bacteria and lead to intracellular components leakage. Therefore, at low concentration chlorhexidine causes leakage of small molecular weight substances like phosphorus and potassium thus, resulting in a bacteriostatic activity. At high concentration however, a bactericidal effect will occur resulting in cell lysis (Gomes *et al*., 2001).

2.7.2 Plant-based Agents

Many plants have secondary metabolites with antimicrobial properties to defend itself from continuous microbial assaults. Some examples of these compounds are quercetin, allicin, guaijaverin and sanguinarine. Quercetin is a plant pigment (flavonoid) found in many fruits, vegetables leaves and grains. Quercetin in raw red onion was

reported to exhibit excellent antibacterial actions against periodontal pathogens (Shu *et al*., 2011)**.** Allicin isolated from garlic is another active compound effective against Gram-positive species *Streptococcus mutans*, *S. sobrinus*, and *Actinomyces oris* (Bachrach *et al*., 2011). Guaijaverin from *Psidium guajava* and sanguinarine from *Sanguinaria canadensis* demonstrated anti-*Streptococcus mutans* activity (Prabu *et al*., 2006) and inhibition of bacterial adherence to newly formed pellicle (Godowski, 1989), respectively.

2.8 Plants with Antimicrobial Properties

Plants are natural sources of antimicrobial agents (Nalina and Rahim, 2007; Fathilah, 2011; Himratul *et al*., 2011). Compounds from plants are perceived to have fewer side effects compared to currently used antibiotics and chemical-based agents that have been found to be nephrotoxic, neurotoxic, hypertensive and/or cytotoxic. Some bacteria have developed resistance towards antibiotics, with a few that have been reported to cause bone marrow depression and severe damage of the liver (Chong and Pagano, 1997). *Averrhoa bilimbi, Brucea javanica, Euphorbia tirucalli, Nigella sativa and Vernonia amygdalina* are plants common in traditional medicine in treating microbial-associated illnesses

2.8.1 *Averrhoa bilimbi*

2.8.1.1 Botanical Information

A. bilimbi belongs to the family of *Oxalidaceae* and is native to India. It is known by the English as cucumber tree, tree sorrel or bilimbi. Locally in Malaysia it is called belimbing buloh, b'ling, belimbing asam or billing-billing (Morton, 1987; Lima *et al*., 2001). *A. bilimbi* can reach up to 15 m of height, long-lived with short trunk that divides into a number of upright branches. The leaves are alternate, imparipinnate,

clustered at the branch tips with 30-60 cm long, ovate, downy, with pointed tip and rounded base. It is medium-green on the upper surface and pale on the underside, at 2-10 cm long and 1.2-1.25 cm wide. Fruits are fairly cylindrical of 4-10 cm long, faintly 5-sided, capped by a thin, star shaped calyx at the stem-end, and tipped with five hair-like floral remnants at the apex. The fruits are crispy when unripe and the external green colour changes to yellowish-green when ripe. The outer skin is glossy, soft, very thin and tender while the flesh is green, juicy, jelly-like and extremely acidic. *A. bilimbi*'s flower is fragrant, small, purplish or yellowish-green marked with dark-purple and 5-petalled. (Mathew *et al*., 1993; Ashok *et al*., 2013).

2.8.1.2 Active Components and Health Benefits

The fruit of *A. bilimbi* is used to treat coughs, biliousness and beri-beri. The extracts of leaves and fruit have anti-microbial, cytotoxic activities, antifertility, anti-oxidant, antibacterial (Zakaria *et al*., 2007) and antidiabetic activities (Ashok *et al*., 2013). Malaysians take the leaves fresh or fermented as a treatment for venereal disease (Ashok *et al.*, 2013).The fruits of *A. bilimbi* contain saponins, triterpenoid and flavonoids. The chemical components of *A. bilimbi* include citric acid, amino acids, cyanidin-3-O-h-D-glucoside, potassium ion, vitamin A, phenolics and sugars. Saponins, alkoloids and flavonoids are also found present in extracts of the bark. The nutritional values for 100 g of edible portion are iron, thiamine, riboflavin, ascorbic acid, niacin, phosphorus, calcium, fiber, protein, vitamin A, vitamin B1 (thiamine), oxalic acid and carotene (Ashok *et al.*, 2013).

(i)

(ii)

Figure 2.1: (i) The *A. bilimbi* plant. (ii) Fruits of *A. bilimbi*

2.8.2 *Brucea javanica*

2.8.2.1 Botanical Information

B. javanica is a member of *Simaroubaceae* family and native to India, Northern Australia and Southeast Asia (Kamperdick *et al*., 1995; Liu *et al*., 2012). Locally it is known as melada pahit. In Indonesia, the fruit is known as buah Makassar while to the Chinese, the fruit is known as Ya-Tan-Tze. *B. javanica* shrubs stands up to 3 m tall with small fruits of about 0.5 cm long. The flowers are greenish white to greenish red or purple.

2.8.2.2 Active Components and Health Benefits

Aqueous extracts of *B. javanica* seeds has been reported to exhibit antifungal potential on oral candida (Nordin *et al*., 2013) and antiproliferative activity on oral cancer cells (Majid *et al.*, 2014). Among the chemical compounds isolated from *B. javanica* are terpenoids and lignans (Luyengi *et al*., 1996), quassinoid glycosides (Sakaki *et al*., 1986), alkaloids (Karin *et al*., 1990), alkaloid glycosides (Kitagawa *et al*., 1994) and quassinoids (Kim *et al*., 2003), anthraquinone, oleic acid, olein, linoleic acid (Wang *et al*., 2011), pregnane glucosides (Chen *et al*., 2011; Liu *et al*., 2011), sesquiterpenes (Chen *et al*., 2009) and tetracyclic triterpene quassinoids (Chen *et al*., 2011). The main active component in *B. javanica* is tetracyclic triterpene quassinoids (Chen *et al*., 2013).

(i)

(ii)

Figure 2.2: (i) *B. javanica* plant (Kamaruddin, 2007). (ii) Seeds of *B. javanica* (Cumming, 2014)

2.8.3 *Euphorbia tirucalli*

2.8.3.1 Botanical Information

E. tirucalli is a small tree of 7-12 m high within the family of *Euphorbiaceae.* Found in the tropical and sub-tropical regions of Asia (Khan and Malik, 1990), this plant is known as pencil-tree because of its pencil-like branches. The bark of *E. tirucalli* is cracked, rough and greenish brown that releases a milky sap when cut. The branch lets whorled that will modified into phylloclade (Baniakina and Eyme, 1997). *E. tirucalli* has high drought tolerance and salinity and can survive in a wide range of habitats (Janssens *et al*., 2009) even under unfavourable condition in which most crops plants cannot grow. It can live in areas that have low rainfall, high altitudes and poor saline soils but it cannot endure frost (Van Damme, 2001).

2.8.3.2 Active Components and Health Benefits

The bioactive components in *E. tirucalli* among others include diterpenes, steroids, alkaloids, flavonoids, tannins (Fauconneau *et al*., 1997). It exhibits activities such as antimicrobial, anticarcinogenic and antitumour, and has been used to treat whooping cough, leprosy, asthma, jaundice, enlargement of spleen, bladder stones, colic pains and tumours (Waczuk *et al*., 2015). The stem latex is used to treat tooth ache, warts, asthma, cough, ear ache, abdominal pain, leprosy, tumors, skin diseases, rheumatism and intestinal worms (Prasad *et al*., 2011).

Figure 2.3: *E. tirucalli* plant (Morad, 2011)

2.8.4 *Nigella sativa*

2.8.4.1 Botanical Information

N. sativa belongs to the *Ranunculaceae* family. Can grow up to 30 cm, the flower of this plant is pale blue and the fruit consists of follicles that contain the seeds (Shuid *et al*., 2012), known black cumin in English or in Arabic, *Habbatul Barakah* or *Habbah Sawda* which mean *Seeds of blessing*. In Bangladesh it is known as *Kalo jeera*, in old Latin as Panacea, in Chinese as *Hak Jung Chou* and in India as *Kalonji* (Aggarwal *et al*., 2008). *N. sativa* is being used in folk medicine in the East Asia, Arabian Gulf region, Egypt, Africa, Middle East, Europe and Greece (Tariq, 2008; Khan *et al*., 2011).

2.8.4.2 Active Components and Health Benefits

N. sativa contains alkaloids, proteins, saponin, and 0.4–2.5% essential oil (Ali and Blunden, 2003). The essential oil of *N. sativa* contains active components such as dithymoquinone, thymoquinone, thymol and thymohydroquinone (Ghosheh *et al*., 1999). Among these, thymoquinone has been reported the most abundant and contributes to 30–48% of the total compounds (Shuid *et al*., 2012).

N. sativa has been reported to exhibit, antifungal (Khan *et al*., 2003) and antimicrobial activities against a wide range of microorganisms (Sokmen *et al*., 1999). It has a wide range of healing properties and has been used among many to treat cough, asthma, chronic headache, fever and gastrointestinal problems. The crude seed extract of this plant was reported by Ab Rahman *et al*., (2014) to exhibit promising wound healing activities on cultured oral mucosal cells. It also has been used as a stimulant, emmenagogue, diuretic, carminative, anthelmintic and externally it is applied directly to nasal ulcers, abscesses, orchitis, swollen joints and eczema (Tariq, 2008; Nadkarni, 1976).

(i)

(ii)

Figure 2.4: (i) *N. sativa* plant (Lumiparta, 2012). (ii) Seeds of *N. sativa* (Nache, 2013)

2.8.5 *Vernonia amygdalina*

2.8.5.1 Botanical Information

V. amygdalina is a small, soft wooded shrub within the family of *Asteraceae*. It is commonly called bitter leaf because of its bitter taste (Khalili *et al*., 2014).

2.8.5.2 Active Components and Health Benefits

The leaves of *V. amygdalina* contain sesquiterpene lactones (Jisaka *et al*., 1992; Igile *et al*., 1995), steroid glucosides (Ohiagashi, 1994; Areghore *et al*., 1997), vitamin C and tannins, saponins (Igile *et al*., 1995), and flavonoids luteolin. Extracts of this plant have been used to treat emesis, loss of appetite-induced ambrosia, diabetes (Nwanjo, 2005; Igbakin and Oloyede, 2009), nausea, dysentery and other gastrointestinal tract problems, antimalarial, antihelmint and laxative herb (Igile *et al*., 1994; Khalili *et al*., 2014). In Nigeria, extracts of the plant are used to treat cough, used as tonic, in the control of tick, feverish condition, hypertension and constipation (Regassa, 2000; Kambizi and Afolayan, 2001; Amira and Okubadejo, 2007). It has been reported that the leaves of *V. amygdalina* exhibit antimicrobial activities on Gram positive and Gram negative bacteria (Okoh *et al*., 1995; Akinpelu, 1999).

(i)

(ii)

Figure 2.5: (i) *V. amygdalina* plant (Scamperdale, 2009). (ii) Leaves of *V. amygdalina* (Nelindah, 2014)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Refer Appendix 1.

3.1.1 Plant Samples

Table 3.1: List of plant selected for the screening of their antimicrobial properties

Species name	**Family name**	**Local name**	**Part used in extraction**	**Place of purchase**
Averrhoa bilimbi	Oxalidaceae	Belimbing buloh	Fruits	Rimba Ilmu Botanical Garden, University Malaya.
Brucea javanica	Simaroubaceae	Melada pahit	Seeds	Rimba Ilmu Botanical Garden, University Malaya.
Euphorbia tirucalli	Euphorbiaceae	Patah tulang	Branch	Local farm in the district of Setiu, Terengganu
Nigella sativa	Ranunculaceae	Habbatussauda	Seeds	Local agricultural market, Kuala Lumpur
Vernonia amygdalina	Compositae	Bismillah leaves	leaves	Rimba Ilmu Botanical Garden, University Malaya.

3.1.2 *Streptococcus* species

The bacterial strains were purchased from the American Type Culture Collection (ATCC)

Table 3.2: List of *Streptococcus* species

Streptococcus species	ATCC No.
S. mitis	ATCC® 49456™
S. sanguinis	ATCC® BAA-1455™
S. mutans	ATCC® 25175 ™

3.2 Research Design

This study aimed to screen the aqueous extracts of five plants namely, *A. bilimbi, E. tirucalli, N. sativa, V. amygdalina* and *B. javanica* for antibacterial activities against three main species of oral streptococci. Disc diffusion test which is an adoption of the Kirby-Bauer antibiotic test was carried out in the screening process (Bauer *et al*., 1966). Each species of streptococci was screened for their susceptibility towards the respective plant extracts. Based on the screening results, extract/s that produced positive response from all the three bacteria was selected for further analysis to determine their antimicrobial, antiadherence and antibiofilm potential. This is because all three bacteria are commonly associated in the formation of oral biofilm (Whiley and Beighton, 1998; Facklam, 2002). Molecular study was performed to identify gene/s that could be involved in these activities. The methodology of this study is summarised in Figure 3.1.

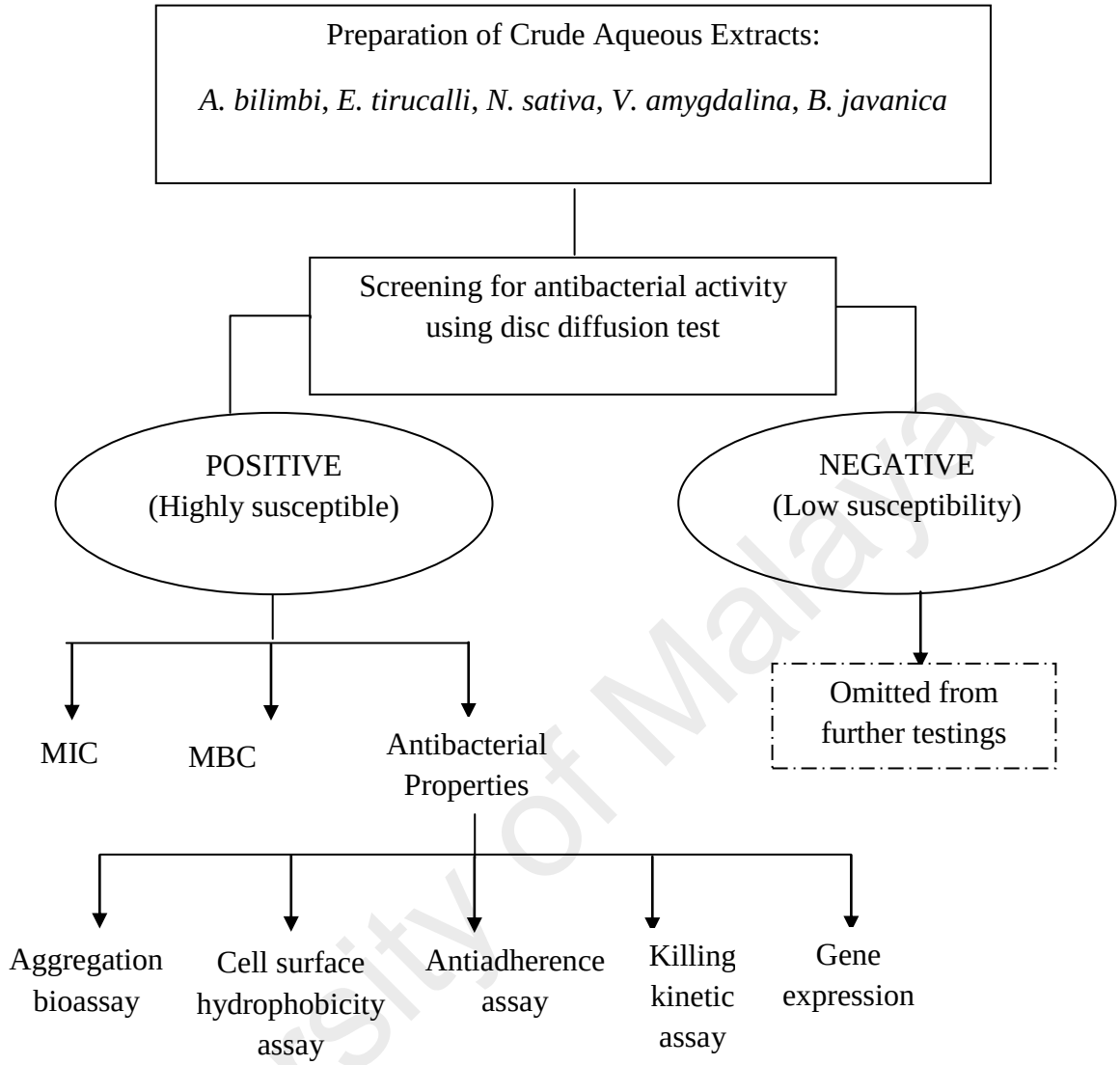


Figure 3.1: Outline of research

3.2.1 Collection of Plant Specimens

Five local plants with history in folklore medicinal practices were chosen as research specimens. The fresh materials which include the leaves, fruits and seeds were purchased or obtained from sources in Terengganu and Rimba Ilmu Botanical Garden, University Malaya.

3.2.2 Preparation of Plant Extracts

Decoctions of the plant materials were prepared using sterile distilled water as solvent.

3.2.2.1 *E. tirucalli* and *V. amygdalin* Extracts

Fresh soft branches of *E. tirucalli* and leaves of *V. amygdalin* were cleaned under running tap water, dried using tissue paper and weighed. The specimens were cut to small pieces and homogenised in distilled water at a ratio of sample to water of 1:10. The homogenate was left to boil until the volume was reduced to one-third of the original. The decoction was filtered through a filter paper to remove debris before it was further boiled to a final volume of 100 mL. The decoction was then concentrated by an overnight freeze drying. The dried concentrate was collected in sterile Falcon tubes, sealed and stored at 4 ˚C. When required, a stock solution of 200 mg/mL was aseptically prepared.

3.2.2.2 *A. bilimbi* Extract

Fresh fruits of *A. bilimbi* were washed, weighed and blend. 100g of the preparation was suspended in distilled water (1:10) and left soaked for 24 hours. The suspension was filtered to remove debris. The filtrate was concentrated to a final volume of 100 mL using a rotary vacuum evaporator and then freeze-dried overnight in a freeze

dryer. The dried concentrate was collected in sterile Falcon tubes, sealed and stored at 4 ˚C. When required, a stock solution of 200 mg/mL was aseptically prepared.

3.2.2.3 *B. javanica* and *N. sativa* Extracts

The seeds of these plants were dried and weighed. The specimens were homogenised in distilled water at a ratio of samples to water of 1:10. Similar procedure as in Section 3.2.2.1 was repeated.

3.3.3 Preparation of Culture Media

Brain Heart Infusion (BHI) agar and broth were used as growth media for the streptococci. The BHI powder was weighed as directed by the manufacturer, dissolved in distilled water and sterilised at 121 ˚C for 15 min in an autoclave. Once warm to the touch, the liquid agar media was poured into sterile Petri dishes, each in a volume of about 25-30 mL. The agar plates were left for 30 minutes under a laminar flow to solidify. Once cool both broth media and the agar plates were kept refrigerated at 4 ˚C for later use. Refer Appendix 2 for details in media preparations.

3.3.4 Revival and Preparation of Stock Culture

The freeze dried bacteria obtained from the American Type Culture Collection (ATCC) was rehydrated with 0.3 to 0.4 mL of BHI broth and mixed well. An aliquot was inoculated into 5 mL BHI broth in a tube and cultured on BHI agar, respectively. The broth and agar were incubated for 24 hours following which 500 µL of the bacterial suspension was aliquoted and mixed with 500 µL glycerol to be stored as stock cultures. The stock cultures were revived (activated) by transferring the stocks into tubes containing BHI broth. The tubes were incubated at 37 ^{o}C for 24 hours in an incubator. The bacteria suspension were then cultured on agar and incubated for 24 hours. The grown colonies were used in the preparation of *Streptococcus* suspension.

3.3.5 Preparation of Bacterial Suspension

Bacterial colonies of 20-24 hr were harvested and dispensed in 5 mL of sterile BHI broth. The turbidity of the suspension was adjusted and standardized spectrophotometrically to an optical density (OD_{550nm}) of 0.144 which is equivalent to $1x10^8$ cells/mL or to #0.5 McFarland standard (Ismail *et al*., 2006).

3.3.6 Antibacterial Screening Using Susceptibility Test

Sterile paper discs were impregnated with extracts of *E. tirucalli*, *V. amygdalina*, *A. bilimbi*, *B. javanica* and *N. sativa* to concentrations of 50, 100, 150, 200 mg/mL. The discs were placed on top of the agar plates which have been inoculated with the respective streptococci strains. The extracts from the discs diffuses through the agar, creating a concentration gradient around the discs. The antibacterial property of the extracts was judged based on the diameter of growth inhibition zone that formed around the discs.

3.3.6.1. Preparation of Extract Discs

Stock extracts of the respective plants was centrifuged for 10 min at 10,000 rpm to remove unwanted debris and then filter-sterilized through a 0.22 µm filter. The respective extracts were then impregnated onto sterile paper discs to give final amounts of 5, 10, 15 and 20 mg of extract. The discs were left to dry at room temperature. This procedure was adopted and modified from the Kirby-Bauer antibiotic test (Bauer and Kirby, 1959). Sterile distilled water and CHX-containing mouthrinse was similarly impregnated into paper discs to act as the negative and positive controls, respectively. Whatman AA discs of 6 mm diameter were used in the tests.

3.3.6.2. Susceptibility Test

BHI agar plates were aseptically inoculated with 100 mL of the respective *S. mitis*, *S. sanguinis* and *S. mutans* suspension prepared in Section 3.3.5. Paper discs with various concentrations of the respective extracts were placed on the agar as illustrated in Figure 3.2. The plates were then incubated at 37 °C for 18-24 hrs following which, the diameter of growth inhibition zone produced around the discs was measured. All experiments were performed in triplicate. Plant extracts that produced no growth inhibitory zones on all three streptococcal strains was/were screened out from further analysis.

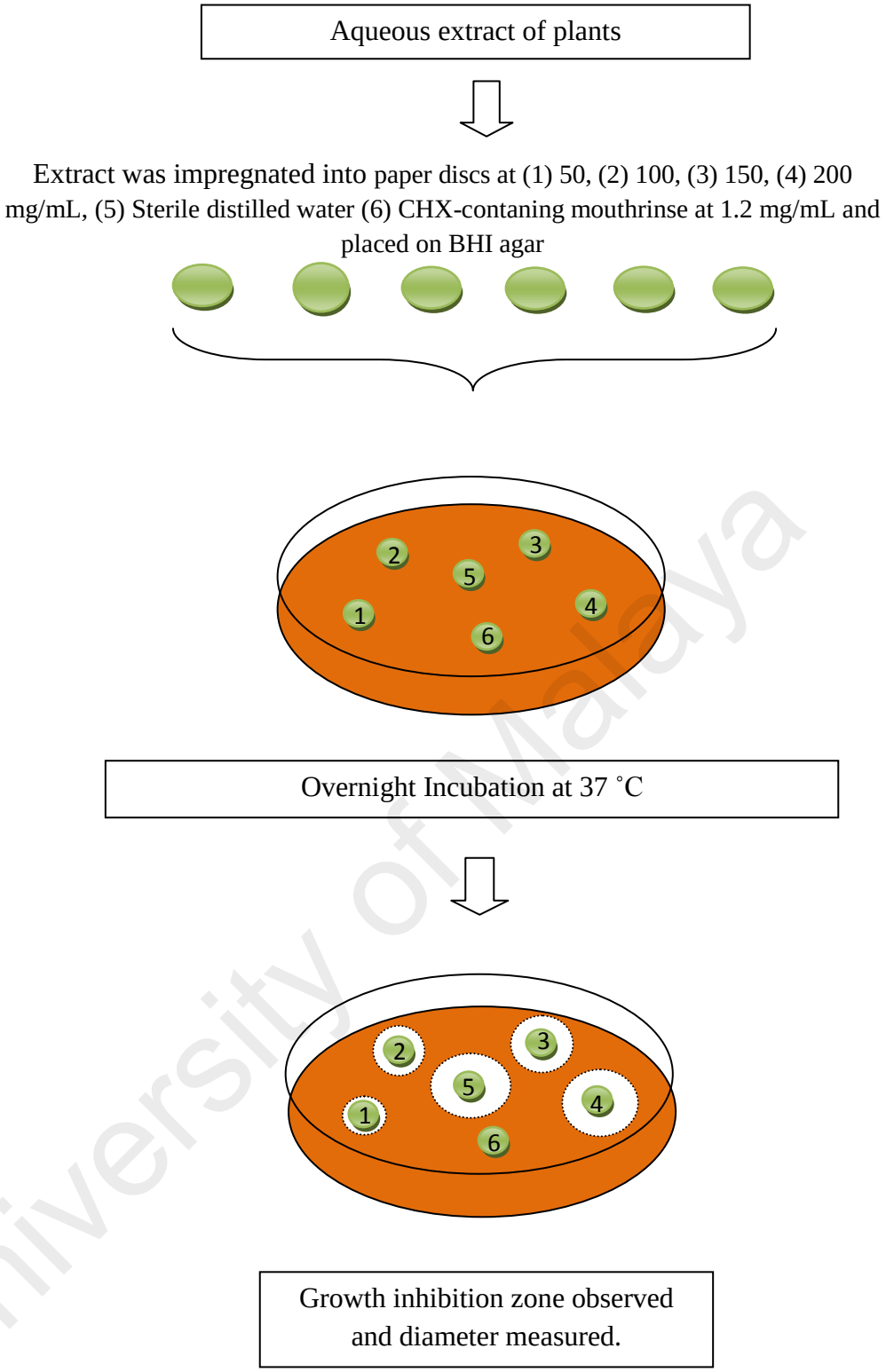


Figure 3.2: An illustration of the screening method using the disc diffusion susceptibility test. The inhibited growth zone produced by 1.2 mg/mL CHX-containing mouthrinse (disc 5) was used as a positive reference while that of the sterile distilled water (disc 6) was used as a negative response.

3.3.7 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentrations (MICs) of plant extract against the bacteria species was determined according to the microdilution method of the CLSI (formerly NCCLS) (M27-A) with some modifications. A 96-well microtiter plate was used in place of test tubes. 100µL of BHI broth was dispensed into the microtiter plate with wells labelled as Well 1 (W1) to Well 12 (W12). 100µL of extract solution (200 mg/mL) was added into W1 and two-fold serial dilution was performed from W1 to W9, giving final concentrations of extract in W1 through W9, 100, 50, 25, 12.5, 6.25, 3.13, 1.57, 0.79 and 0.4 mg/mL, respectively. W10 that has only the CHX-containing mouthrinse and BHI broth represented the positive control, while W11 with only the BHI broth represented as negative control. 100 µL of the respective bacterial suspension was then added into W1 to W12, except for W11. The microtitre plates were incubated overnight at 37 ˚C, following which an ELISA reader was used to read the absorbance. The well with concentration at which no bacterial growth was read, a subculture was made on fresh BHI agar plates, followed by incubation at 37 ^{o}C for 24 hours. Absence of any colony forming units in the plates would mean the concentration is the minimal bactericidal concentration (MBC) of the extract. This concentration was considered the lowest concentration at which microorganisms are totally inhibited from growth. The MIC and MBC values obtained were used to indicate the antibacterial strength of the extracts.

3.3.8 Compounds of *A. bilimbi* Extract

Liquid chromatography–mass spectrometry (LC-MS) method was used to determine the content of active compounds in the selected plant extract. This procedure however was not done in the laboratory but out sourced to GENEION BIO SDN. BHD (Petaling Jaya, Selangor). Pure extract of *A. bilimbi* was used in the determination.

Table 3.3: LC-MS detail description

	Detail Description
Brief method information	• Method: LCMS/MS = full scan with MS/MS data collection • Ionisation mode: Negative • Column: Zorbax C18 – 150mm X 4.6mm X 5µM • Buffer: A: water with 0.1% formic acid and 5mM ammonium formate B: Acetonitrile with 0.1% formic acid and 5mM ammonium formate • Rapid screening at 15 min run time • AB Sciex 3200Q Trap LCMS/MS with Perkin Elmer FX 15 uHPLC system • Gradient run program • 10% B to 90%B from 0.01 min to 8.0 min, hold for 3 min and back to 10% B in 0.1 min and re-equilibrated for 5 min

MS setting and conditions	• Voltage IS: -4500V • Source temperature: 500˚C • Desolvation gas: 40psi • Source gas: 40psi • Scan range: 100-1200m/z for full scan, 50-1200m/z for MS/MS scan • Declustering potential: 40V • Enhance potential: 10V • Collision energy: Spread of 35eV +/- 15 eV

3.3.9 Antibacterial Properties of Plant Extracts on *S. mitis*, *S. sanguinis* and *S. mutans*

Evaluation of the antibacterial properties of the selected plant extract included its aggregative, killing, antiadhesive and biofilm dislodging activities against *S. mitis*, *S. sanguinis* and *S. mutans*.

3.3.9.1 Determination of Aggregative Activity

The selected plant extract was prepared into a solution of 20 mg/mL using sterile distilled water as solvent. The suspension was vortexed for 2 min and filter-sterilised through 0.02 μm pore size. Specific volumes of bacteria suspension, extract and BHI broth were added into the wells as indicated in Table 3.4 (a) to give final concentrations in ascending order from W0 to W3. Following similar steps, CHX-containing mouthrinse was included in the experiment to represent as positive control [(Table 3.4 (b)].

For the assay, 150 μL BHI broth medium was aseptically dispensed into each well of a microtiter plate labelled as W0 to W5. The selected plant extracts within the range of 0-70 μL was added to each well. BHI broth medium was added to make a final volume of 200 μL in each well. The final concentrations of extract and CHX in wells W0 to W5 were 0 - 7 mg/mL and 0 – 0.3 mg/mL, respectively. The microtiter plate was placed in an incubator with shaker for 1 hour at 37 °C following which the microtiter plate was removed and examined under a binocular microscope. A positive result was noted with the appearance of precipitate or residue at the bottom of the well. The degree of aggregative activity was indicated by the number of (+) sign.

Table 3.4: Final concentrations of (a) selected plant extracts and (b) CHX-containing mouthrinse in wells W0-W5.

(a)

Well no.	Bacteria suspension (µL)	Stock xtract at 20 mg/mL (µL)	BHI broth (µL)	Total volume (µL)	Final concentration of plant extract (mg/mL)
W0	120	0	80	200	0.0
W1	120	50	30	200	5.0
W2	120	60	20	200	6.0
W3	120	70	10	200	7.0

(b)

Well no.	Bacteria suspension (µL)	CHX-containing mouthrinse (µL)	BHI broth (µL)	Total volume (µL)	Final concentration of CHX-containing mouthrinse (mg/mL)
W0	150	0	50	200	0.0
W1	150	10	40	200	0.06
W2	150	20	30	200	0.12
W3	150	30	20	200	0.18
W4	150	40	10	200	0.20
W5	150	50	0	200	0.30

3.3.9.2 Determination of Time-killing Activity

a. Experimental Design

Bacteria behave differently when in the form of biofilm compared to when they are planktonic. Existing in the biofilm form enables bacteria to develop some degree of colonization resistance (Socransky and Haffajee, 2002). This experiment aimed to evaluate the response time of the biofilm reacting to the plant extract. This period of the response time may suggest the effectiveness of the extract as an antibiofilm agent.

In this assay, 24-hr biofilms of mixed culture of S. *mitis, S. sanguinis* and *S. mutans* (ratio of 1:1:1) were developed. Following exposure to the extract over periodic duration time, reduction in bacterial counts of the biofilms were determined and analysed. An illustration of the methodology is given in Figure 3.3.

b. Collection of saliva and Preparation of 24-hr Biofilms on Pellicle-coated Glass Beads

Saliva was aseptically collected from a single volunteer to minimize any variation that may arise between individuals. The volunteer was requested to thoroughly gargle with tap water to remove any food debris in the mouth before he chewed on a sugar-free gum to stimulate saliva production. Whole saliva was collected into ice-chilled test tubes and then centrifuged at 17,000 × g for 30 min, at 4 ˚C. The supernatant was collected and filter sterilised through a disposable 0.2 µm pore size low protein-binding filter. Sterile glass beads were placed in a Petri dish and covered with whole saliva that was collected and prepared. The dish was placed in a shaking incubator at 37 ºC for 2 min to allow for uniform pellicle formation around the beads. Following this, the saliva was discarded and the beads washed with PBS. Once rid off excess saliva, 6 mL of bacterial suspension as prepared in Section 3.3.5, was added and the Petri dish was returned to

the shaking incubator to allow for uniform formation of 24-hr biofilm around the glass beads.

c. Killing Kinetic Assay

Following incubation the growth suspension was discarded. A 3 mL volume of fresh broth and 3 mL volume of the respective plant extract at its MIC was added. The Petri dish was returned for further incubation. At every following 30 sec up until 90 sec, nine beads were taken out and dispensed into three separate microfuge vials, each containing 1 mL of PBS. The vials were sonicated for 10 sec (50Hz) followed by 60 sec vortexing to dislodge viable adherent bacteria. Serial ten times dilution was then carried out on each vials down to the final concentration of 1×10^{-6} in tube T7. Tube T1 contained the undiluted bacterial concentration. 100 uL was then pipetted out and plated on three separate BHI agar plates. Finally a total of 21 agar plates were incubated over a period of 24 hr at 37 ^{o}C. Viable bacteria colonies was enumerated and plates from a dilution that gave a cfu count of 30-300 cells were selected for determination. The growth population cfu mL^{-1} was determined using the formula:

$$\text{Growth population} = \text{CFU count per plate} \times \text{dilution factor}$$

The result was represented by the mean of cfu count of three plates. Sterile distilled water and CHX-containing mouthrinse was used as the negative and positive controls, respectively.

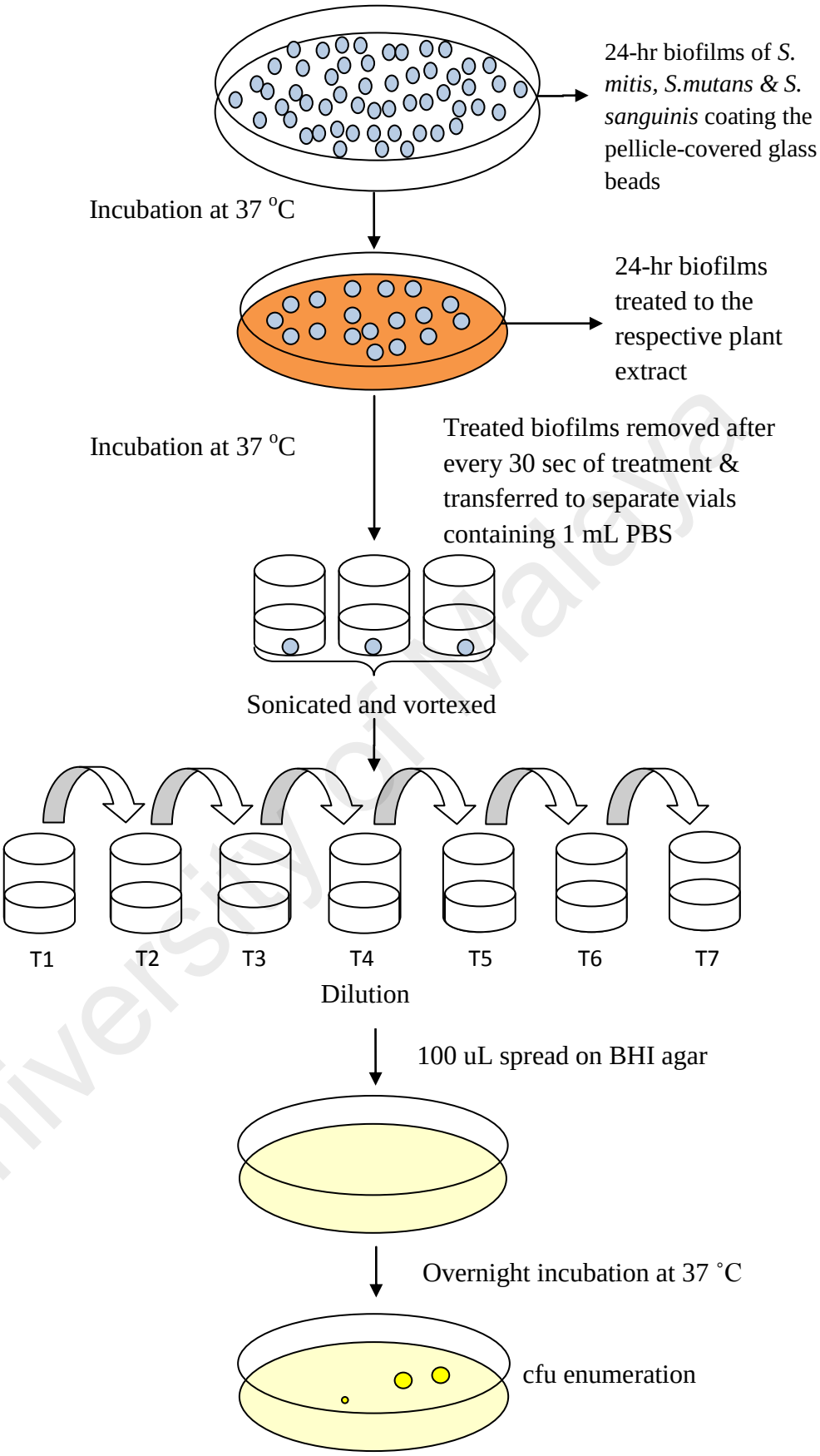


Figure 3.3: An illustration of the killing-time assay perfomed on 24-hr biofilms of *S. mitis*, *S. mutans* and *S. sanguinis*

3.3.9.3 Determination of Antiadherence Activity

a. Experimental Design

Bacteria colonises tooth surfaces by adhering to the acquired pellicle coating the teeth either primarily through specific interactions between receptors in the dental pellicle with specific molecules termed adhesins on the bacterial surface or via other secondary binding such as hydrophobic interaction. It is thus hypothesised that any interference with this interaction, would lead to an inhibition of the adhesion process that may lead to reduced bacterial adherence and result in minimal biofilm formation. The plant extract with such property would then possess an antiadherence activity.

In this experiment, the Nordini's Artificial Mouth (NAM) model (Rahim *et al*., 2008) which was designed to mimic the oral environment was used. Sterile glass beads coated with whole saliva was used as substratum for pellicle formation to mimic the acquired pellicle on tooth surface (Figure 3.4).

b. Collection of Saliva and Preparation of Pellicle-coated Glass Beads

Sterile glass beads were placed in the glass pipette (Figure 3.4) which represented a chamber mimicking the oral cavity. Sterile saliva prepared in Section 3.3.9.2 (b) was flowed-in for 2 min at a rate of 0.3 mL/min to allow for pellicle formation. After 2 min, sterile distilled water was run into the artificial mouth system to rinse excess saliva from the pellicle-coated glass beads.

c. Antiadherence Assay

The pellicle-coated glass beads were treated with the plant extract by allowing the flow of the respective extract into the system for 2 min. Following a wash with a 2 min flow of sterile distilled water, the extract-treated biofilm were exposed to the flow of bacterial suspension consisting of *S. mitis, S. mutans and S. sanguinis* (ratio of 1:1:1) in 1% of sucrose that was aseptically introduced into the bacterial reservoir (Figure 3.4). The suspension was pumped into the system at a rate of 0.3 mL/min to mimic the flow of saliva under the unstimulated condition. The system was allowed to run for 24 hr (overnight) at condition set at 37 ˚C that mimic condition in the oral cavity.

After 24 hrs the glass beads were removed and immersed in 1 mL phosphate-buffered saline (PBS) in microcentrifuge vials, following which, the vials were sonicated for 60 sec to dislodge any attached bacteria. The population of adherent bacteria was indicated by the optical absorbance (OD) measured at 550 nm using a spectrophotometer (Ismail *et al*., 2006). The antiadherence activity of extract was evaluated based on the reduction of adherent bacterial cells to the extract-treated biofilm on the glass beads when compared to the untreated biofilm. Similar steps were followed to test the adherence of bacteria to water-coated and CHX-coated glass beads, which represented as the negative and positive controls, respectively. Data gathered will be statistically analyzed using SPSS (version 13.0).

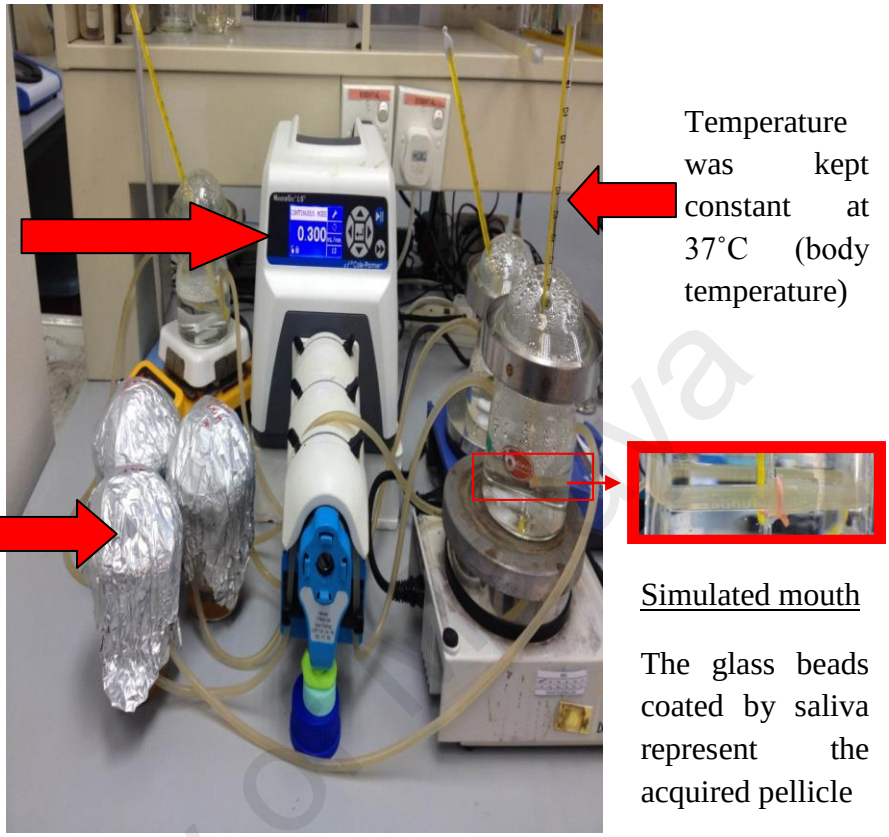


Figure 3.4: An illustration of the Nordini's Artificial Mouth (NAM) model adopted in this study (Rahim *et al*., 2008).

3.3.9.4 Determination of the Effect of *A. bilimbi* Extract on Cell Surface Hydrophobicity of Mixed and Individual Streptococci

a. Experimental Design

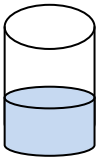
Cell surface hydrophobicity (CSH) was conducted according to the protocol of Fathilah *et al*., (2006) with some modifications. Effect of *A. bilimbi* extract on CSH of the streptococci was determined following treatment of the bacteria to *A. bilimbi* at sub-MIC concentration. The relative CSH was calculated as described in the equation:

$$\% \text{ change in } A_{550} = \frac{(At-Au)}{A_t} \times 100$$

Where, At is the initial absorbance in the absence of hexadecane and Au is the final absorbance in the presence of hexadecane. A summary of the study was shown in Figure 3.5.

b. Preparation of Standard Bacterial Cell Suspension

An overnight mixed and individual cultures of the streptococci was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded while the cells were washed with phosphate buffered saline and resuspended in fresh BHI broth. The turbidity of the suspension was adjusted and standardized spectrophotometrically to an optical density of 0.6 read at a wavelength of 550nm (OD_{550nm}).

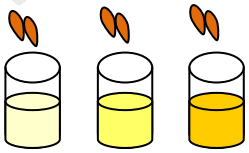


Bacterial suspension was prepared in sterile tube

2 mL bacterial suspension was dispensed into tube containing 2 mL *A. bilimbi* extract at 2, 10 & 20 mg/mL

1 mL aliquoted to measure OD (A_t)

200 µL of hexadecane was added



Vortex (1 min) & left to stand at room temperature for 15 min

OD was measured at 550 nm wavelength (A_u)

% change in A550 = $[(A_t\text{-}Au)/ A_t] \times 100$

Figure 3.5: Outline for the determination of streptococcal cell surface hydrophobicity

c. CSH Assay

2 mL of *S. mutans*, *S. sanguinis* and *S. mitis* culture suspensions were separately dispensed into sterile tube containing 2 mL *A. bilimbi* extract at different concentration to give final concentrations of 1, 5 and 10 mg/mL. 1 mL was pipetted out to measure the absorbance at a wavelength of 550 nm (A_t). 200 µL of hexadecane was added into each tube. The tubes were vigorously agitated for 1 min and left to stand at room temperature for 15 min. 1 mL of the lower aqueous phase was gently aliquoted out into a cuvette and absorbance was measured at OD_{550nm} (A_u). The percentage of bacteria adsorbed to hexadecane was calculated as described in section 3.3.9.4 (a). Similar procedure was performed on mixed-culture streptococci suspension.

3.3.10 Determination of Effect of *A. bilimbi* on Expression of *gtfB, gtfC* and *vicR* using Real Time PCR (qPCR)

a. Experimental Design

Specific genes *gtfB, gtfC* and *vicR* were identified through literature review to be involved in the adherence of bacteria to the pellicle-coated surface. Glucosyltransferases B and C (*gtfB* and *gtfC*) and the response regulator (*vicR*) genes are virulence factor in the pathogenicity of *Streptococcus* species. Gtf B synthesizes insoluble glucans while Gtf C produces soluble and insoluble glucans that can lead to the initiation of plaque formation and caries. Hence, this study was continued to investigate the expression pattern gene of interest after 24 h of growth in reaction to the *A.bilimbi* at MIC concentration (6.25 mg/ml).

Any changes to the expression of these genes upon treatment with the selected plant extract may indicate antimicrobial property of the extract. (Koo *et al*., 2002)

b. Preparation of 24-hr Biofilm and Bacterial Cells for Gene Extraction

24-hr biofilms on pellicle-coated glass beads consisting of all three streptococci in a ratio of 1:1:1 were developed based on description in Section 3.3.5. The biofilm was treated with the selected plant extract for 24 hr at the sub-MIC concentration. Following this, the beads were sonicated to dislodge the adherent cells and centrifuged at 10,000 rpm for 10 min to pellet the cells down. The pellet was washed with PBS prior to use.

c. RNA Extraction and cDNA Formation by Reverse Transcriptase

Total RNA from the biofilm was isolated using HiYield^{TM} Total RNA Mini Kit (Real Biotech Corp., Taiwan). The concentration and purity of extracted RNA was determined based on the optical absorbance reading at 260 and 280 nm (ND-1000 spectrophotometer, NanoDrop Technologies). The concentration to carry out RT-PCR was standardized to 40µg/µl. Equal amounts of RNA (2 µg in 20 µL reactions) were reverse-transcribed with specific gene primer using SuPrimeScript RT (GeNet Bio).

d. Quantitative PCR (qPCR) using the Listed Primer in Table 3.5

Sequences of *gtfB, gtfC* and *vicR* genes were obtained from previous study (Shemesh *et al*., 2007). *16S rRNA* gene was used as housekeeping gene. Real-time PCR was performed in PCR tubes using the ABI 7500 fast real-time PCR machine (Applied Biosystems, Rotkreuz, Switzerland) using Titan HotTaq EvaGreen® qPCR Mix (ROX) (Bioatlas). 2 µL of diluted cDNA samples and 18 µL of mastermix (containing the primers) were added to the tube. Real-time PCR reactions were performed started with initial denaturation at 95°C for 5 min, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60°C. Expression of genes was calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001)

Table 3.5: Oligonucleotide sequences of *gtfB*, *gtfC*, *vicR* and *16S rRNA*

Gene target	Primer Sequences (5´- 3´)	Tm(˚C)[b]	(bp)[c]
gtfB			
Forward	AGCAATGCAGCCAATCTACAAAT	54.2	23
Reverse	ACGAACTTTGCCGTTATTGTCA	53.9	22
gtfC			
Forward	CTCAACCAACCGCCACTGTT	57.4	20
Reverse	GGTTTAACGTCAAAATTAGCTGTATTAGC	56.8	29
vicR			
Forward	TGACACGATTACAGCCTTTGATG	56	23
Reverse	CGTCTAGTTCTGGTAACATTAAGTCCAATA	58.3	30
16S rRNA			
Forward	CCTACGGGAGGCAGCAGTAG	61.6	20
Reverse	CAACAGAGCTTTACGATCCGAAA	56	23

[a] Gene templates were selected based on Shemesh *et al*., 2007.

[b] Melting temperatures were examined with Oligo Analyzer 1.2.

[C] Oligo length (bp, base pair).

CHAPTER FOUR

RESULTS

4.1 Susceptibility of *S. sanguinis, S. mitis* and *S. mutans* to *A. bilimbi, B. javanica, E. tirucalli, N. sativa* and *V. amygdalina* Extracts

4.1.1 Sensitivity of Oral Streptococci to Plant Extracts

Based on the diameter of growth inhibitory zone formed around paper discs impregnated with extracts from the various plants, selective growth inhibitory effect was observed on *S. sanguinis, S. mitis and S. mutans* and at varying degree of inhibitory strength (Table 4.1- 4.5). The extract of *A. bilimbi* exhibited growth inhibitory activity on all three streptococci, with *S. mitis* being the most susceptible followed by *S. sanguinis* and *S. mutans* (Table 4.1). The growth of *S. sanguinis* was also found to be inhibited in descending order, by *N. sativa, E. tirucalli, V. amygdalina* and *B. Javanica* (Table 4.2- 4.5). *S. mitis* was resistant towards *B. javanica* and *E. tirucalli*, and weakly affected by *N. sativa* and *V. amygdalina.* Among the three streptococci, *S. mutans* was determined most resistant and was only affected by *A. bilimbi* and *V. amygdalina*. In general, the growth inhibitory effect of the plant extracts where observed, was in a dose dependent manner. CHX that was used as a positive control, exhibited zones of inhibition with diameter within the range of 20.5 - 25.3 mm. Data obatained was also presented in Figure 4.1- 4.5 (Appendix 3).

Table 4.1: Susceptibility of *S. sanguinis*, *S. mitis* and *S. mutans* to *A. bilimbi* measured by the diameter of inhibition zone produced at four concentrations. CHX-containing mouthrinse (0.12 % w/v) represented as positive control. The values were presented as diameter in mm ± standard deviation.

Bacteria species	Diameter of inhibition zone (mm)				
	Extract concentration				
	20mg	15mg	10mg	5mg	CHX
S. sanguinis	27.3 ± 1.0	24.0 ± 1.5	20.3 ± 0.5	15.3 ± 2.1	25.3 ± 2.2
S. mitis	34.5 ± 2.1	32.0 ± 2.8	28.0 ± 2.8	24.5 ± 0.7	24.0 ± 1.4
S. mutans	20.3 ± 0.5	19.0 ± 0.9	16.7 ± 0.5	9.7 ± 1.4	20.5 ± 2.1

Table 4.2: Susceptibility of *S. sanguinis*, *S. mitis* and *S. mutans* to *B. javanica* measured by the diameter of inhibition zone produced at four concentrations. CHX-containing mouthrinse (0.12 % w/v) represented as positive control. The values were presented as diameter in mm ± standard deviation.

Bacteria species	**Diameters of inhibition zone (mm)**				
	Extract concentration				
	20mg	15mg	10mg	5mg	CHX
S. sanguinis	13.7 ± 0.12	11.7 ± 0.15	11.5 ±0.07	10.5 ±0.07	25.3 ± 2.2
S. mitis	No inhibition zone				24.0 ± 1.4
S. mutans	No inhibition zone				20.5 ± 2.1

Table 4.3: Susceptibility of *S. sanguinis*, *S. mitis* and *S. mutans* to *E. tirucalli* measured by the diameter of inhibition zone produced at four concentrations. CHX-containing mouthrinse (0.12 % w/v) represented as positive control. The values were presented as diameter in mm ± standard deviation.

Bacteria species	**Diameters of inhibition zone (mm)**				
	Extract concentration				
	20mg	15mg	10mg	5mg	CHX
S. sanguinis	22.0 ± 3.5	19.0 ± 2.6	15.3 ± 1.5	10.3 ± 1.5	25.3 ± 2.2
S. mitis	No inhibition zone				24.0 ± 1.4
S. mutans	No inhibition zone				20.5 ± 2.1

Table 4.4: Susceptibility of *S. sanguinis*, *S. mitis* and *S. mutans* to *N. sativa measured* by the diameter of inhibition zone produced at four concentrations. CHX-containing mouthrinse (0.12 % w/v) represented as positive control. The values were presented as diameter in mm ± standard deviation.

Bacteria species	**Diameter of inhibition zone (mm)**				
	Extract concentration				
	20mg	15mg	10mg	5mg	CHX
S. sanguinis	22.0 ± 1.5	21.0 ± 2.3	19.0 ± 1.0	16.0 ± 2.1	25.3 ± 2.2
S. mitis	19.0 ± 1.0	17.0 ± 1.4	15.0 ± 1.4	12.0 ± 0.0	24.0 ± 1.4
S. mutans	No inhibition zone				20.5 ± 2.1

Table 4.5: Susceptibility of *S. sanguinis*, *S. mitis* and *S. mutans* to *V. amygdalina* measured by the diameter of inhibition zone produced at different concentrations. CHX-containing mouthrinse (0.12 % w/v) represented as positive control. The values were presented as diameter in mm ± standard deviation.

Bacteria species	**Diameters of inhibition zone (mm)**				
	Extract concentration				
	20mg	15mg	10mg	5mg	CHX
S. sanguinis	19.5 ± 0.7	16.5 ± 0.7	10.0 ± 0.5	No inhibition zone	25.3 ± 2.2
S. mitis	12.7 ± 4.0	9.3 ± 2.9	9.0 ± 0.0	No inhibition zone	24.0 ± 1.4
S. mutans	11.3 ± 2.5	11 ± 1.4	No inhibition zone		20.5 ± 2.1

4.1.2 MIC and MBC of Plants Extracts

The MIC of *A. bilimbi* on all three streptococci was determined at an equal concentration of 6.25 mg/mL. The MICs of the other four plants extracts was only determined for *S. sanguinis* and at values higher than 6.25 mg/mL (Table 4.6). MIC values were also determined for *A. amygdalina* on all the three streptococci. The values were however, much higher than that observed for *A. bilimbi*.

With regard to MBC, the values obtained for *A. bilimbi* were 12.5 mg/mL for both *S. mutans* and *S. mitis* and 2-fold higher at 25.0 mg/mL for *S. sanguinis*. The MBCs for *B. javanica, E. tirucalli, N. sativa* and *V. amygdalina* exceeded 50 mg/mL and thus, were determined as resistant in the test.

Table 4.6: Minimum inhibitory concentration (MIC) of plants extracts on *S. sanguinis, S. mitis* and *S. mutans*

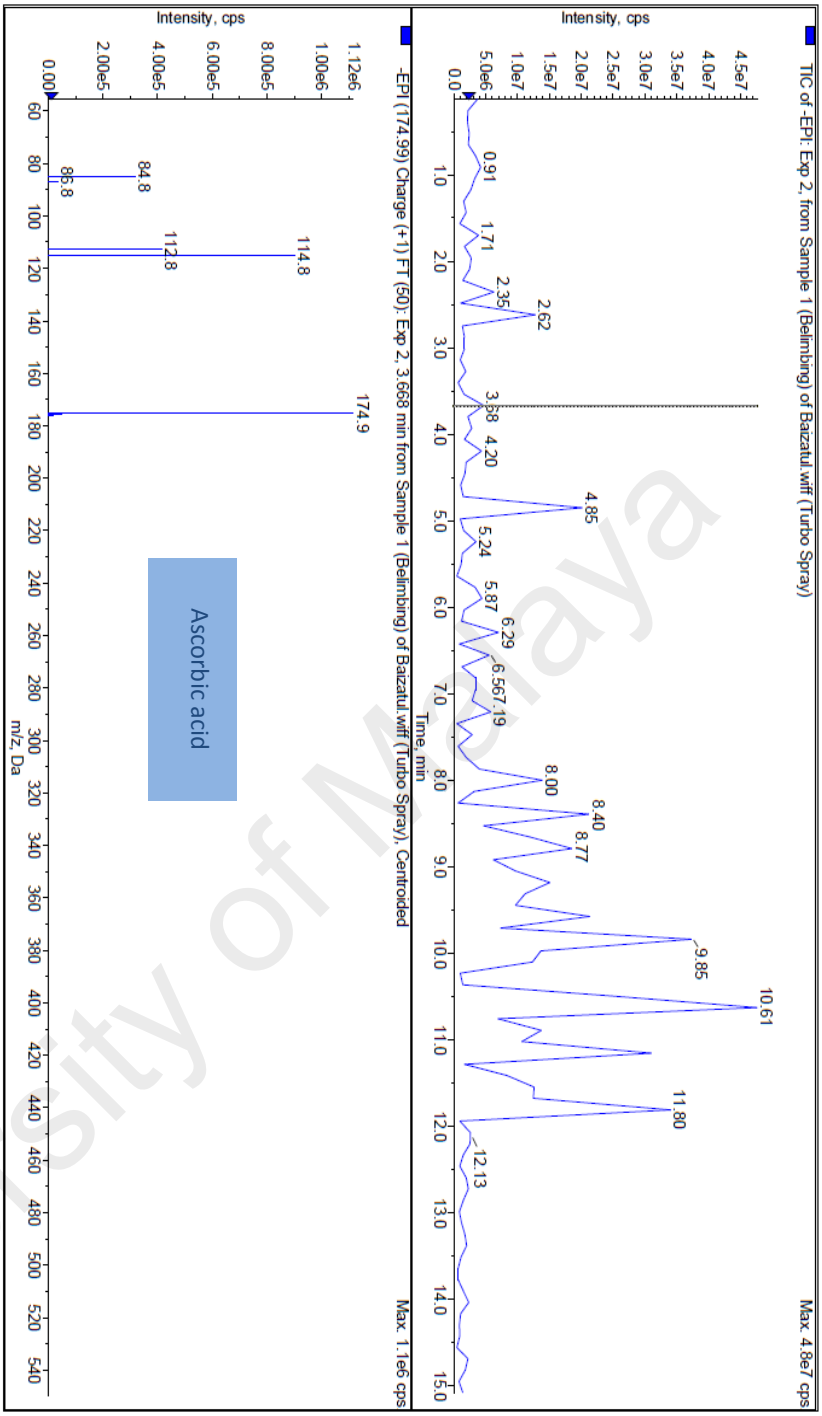
Bacteria species	**Concentration of extracts (mg/mL)**				
	A. bilimbi	*B. javanica*	*E. tirucalli*	*N. sativa*	*V. amygdalina*
S. sanguinis	6.25	50.00	12.50	25.00	12.50
S. mitis	6.25	Resistant	Resistant	50.00	50.00
S. mutans	6.25	Resistant	Resistant	Resistant	Resistant

Results from susceptibility test and MIC were used to screen out extracts that showed weak or negative antibacterial response to *S. sanguinis, S. mitis* and *S. mutans*. Data suggested only the extract of *A. bilimbi* showed antibacterial potential and was thus, selected for further testing to verify its antibacterial activity. The extracts of *B. javanica, E. tirucalli, N. sativa* and *V. amygdalina* were omitted from further testings.

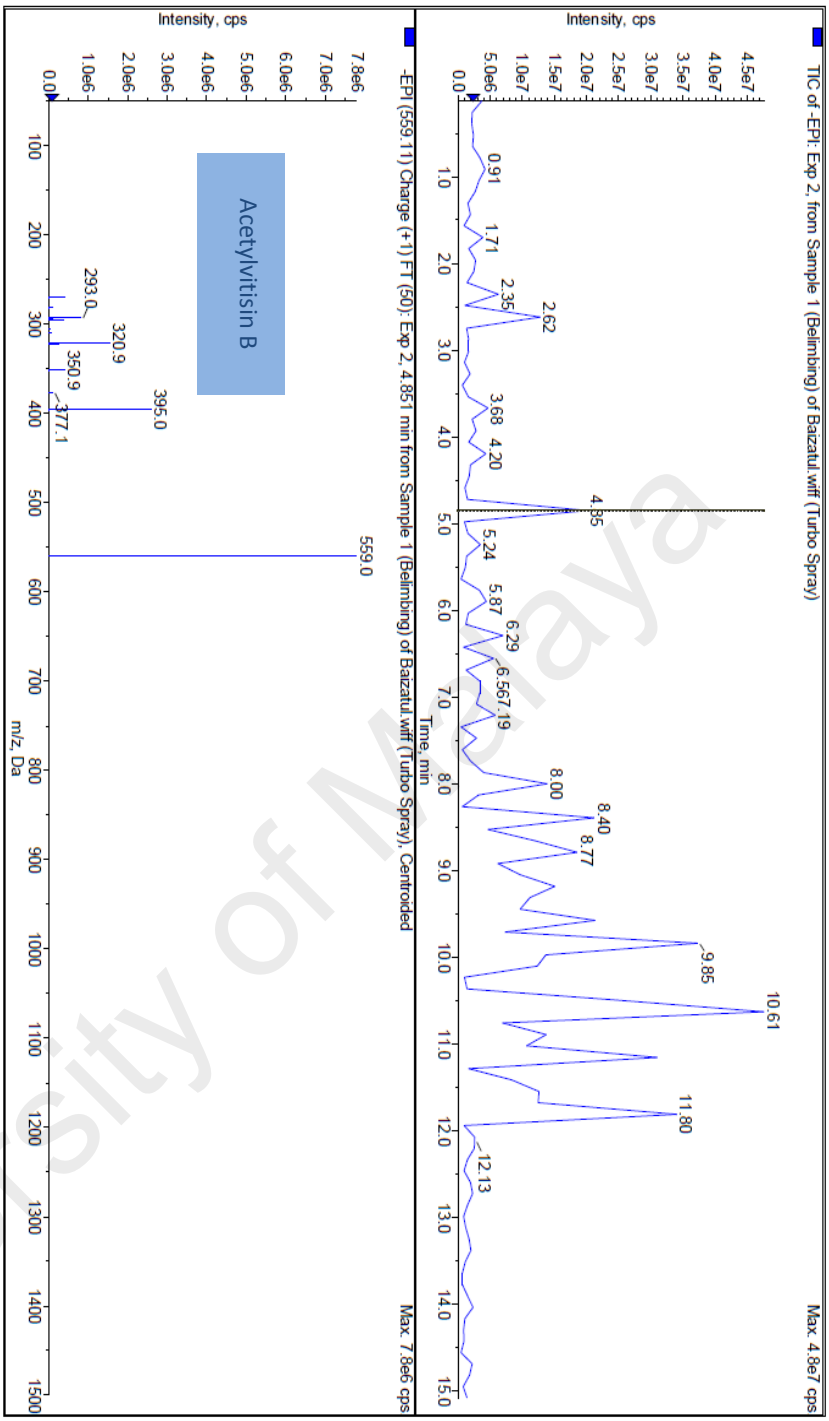
4.1.3 Compounds of *A. bilimbi* Extract

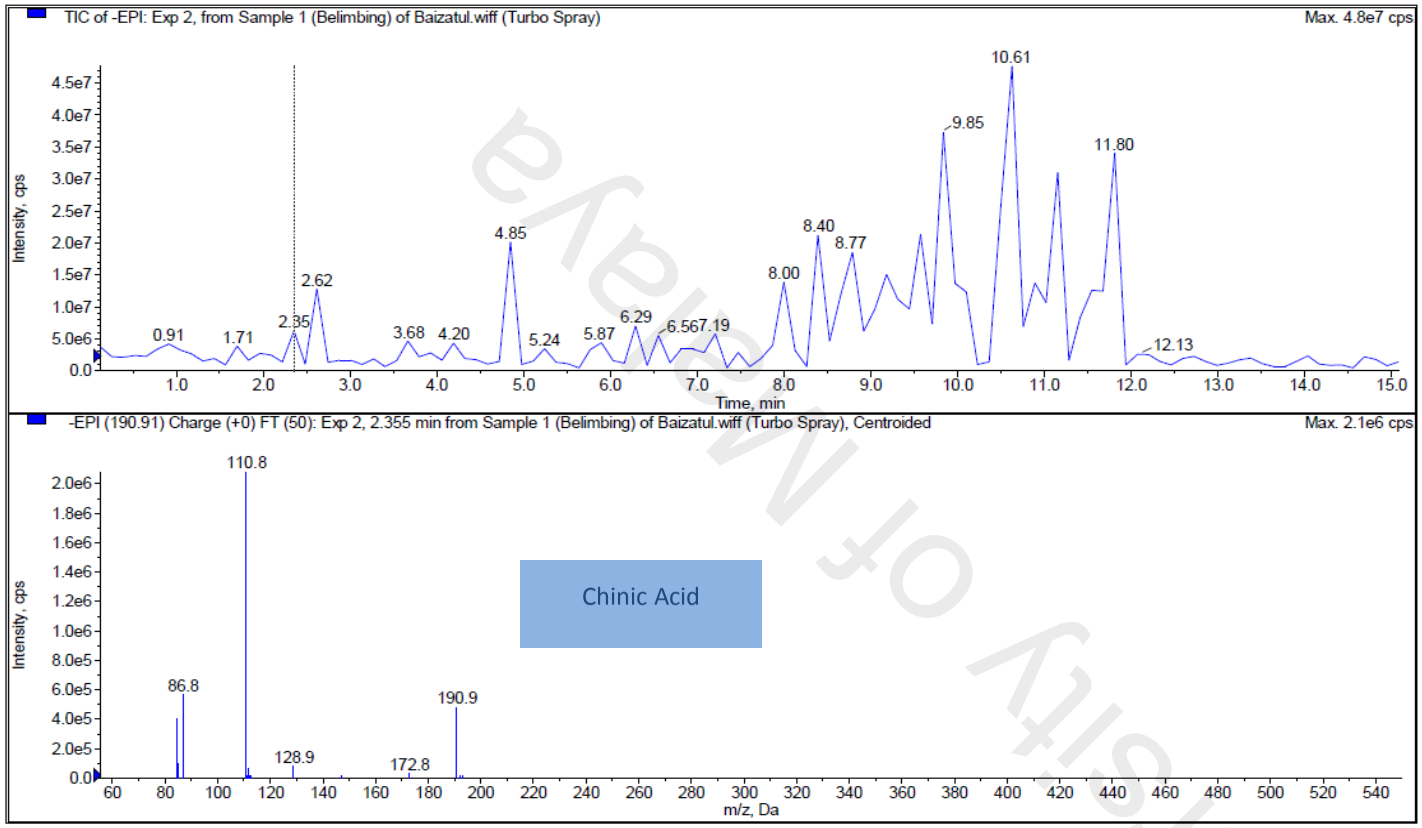
Three compounds were found present in *A. bilimbi* extract that included ascorbic acid, acetylvitisin B and chinic acid. Separation of ascorbic acid was obtained at LC-MS run at a retention time (R_t) of 3.68min. Acetylvitisin B and chinic acid were separated out later at R_t of 4.85 and 2.35min, respectively (Figure 4.6).

(i)



(ii)





(iii)

Figure 4.6: Profiles of compounds isolated from *A. bilimbi* obtained from LC-MS; (i) Ascorbic acid, (ii) Acetylvitisin B and (iii) Chinic acid.

4.1.4 Aggregative Activity of *A. bilimbi* Extract

Table 4.7 shows the aggregative effect of *A. bilimbi* extract on the streptococci was within the range of the MIC and ±1 mg/mL extract concentrations. *A. bilimbi* was found to cause bacterial aggregation at 5.0 mg/mL and this activity remains unchanged when the concentration was increased to 6.0 and 7.0 mg/mL. It appeared that the different in the volume of BHI used (Table 3.1) has no effect on the aggregation activity of *A. bilimbi*. No aggregative activity was exhibited by CHX-containing mouthrinse (0 to 0.3 mg/mL) on *S. mutans, S. mitis* and *S. sanguinis.*

Table 4.7: The aggregative effect of *A. bilimbi* extract on *S. mutans, S. mitis* and *S. sanguinis* observed at concentration range of 5.0 – 7.0 mg/mL. The (+) sign indicated the presence of aggregating reaction while (-) sign indicated absence of aggregative activity observed.

Bacteria species	**Concentration of extract (mg/mL)**			
	0.0	5.0	6.0	7.0
S. mutans	-	+	+	+
S. mitis	-	+	+	+
S. sanguinis	-	+	+	+

Table 4.8: No aggregative effect of chlorexidine on *S. mutans, S. mitis* and *S. sanguinis* observed at concentration of up to 0.3 mg/mL. The (-) sign indicated absence of aggregative activity.

Bacteria species	**Concentration of CHX (mg/mL)**			
	0.0	0.1	0.2	0.3
S. mutans	-	-	-	-
S. mitis	-	-	-	-
S. sanguinis	-	-	-	-

4.2 Antibacterial Properties of *A. bilimbi* on Mixed-culture of *S. mitis, S. sanguinis and S. mutans*

4.2.1 Killing Effect of *A. bilimbi* Extract on 24hr-biofilm of Mixed – culture of Streptococci

A. bilimbi displayed almost immediate killing of bacteria in the 24hr-biofilm, showing a significant drop in the population count (Figure 4.7). A 24.1% reduction of population count was seen in the first 30 sec of exposure to the extract. Extended exposure periods to 60 and 90 sec were observed to exert further reduction in bacterial count by 30.5 and 49.8%, respectively (Figure 4.8). The optimum killing activity of *A. bilimbi* was observed almost immediate within the first 30 sec of exposure at a calculated killing rate of 40.94 cell/min. Extension of exposure to *A. bilimbi* to 60 and 90 sec showed significant reduction in the killing activity of the extract as indicated by reduction of the killing rate to 37.5 and 27.1 cell/min, respectively. Comparative to *A. bilimbi*, CHX-containing mouthrinse exhibited a 93% killing effectiveness at 30 sec of exposure (Figure 4.8). No reduction of cell population was recorded under the untreated (control) condition.

Table 4.9: The killing effect of *A. bilimbi* on Mixed-culture of *S. mitis, S. sanguinis and S. mutans*.

	CFU at time		
Time (sec)	30	60	90
A. bilimbi	20.47	18.75	13.55
CHX-containing mouthrinse	1.80	1.62	1.62
Untreated	26.97	26.97	26.97

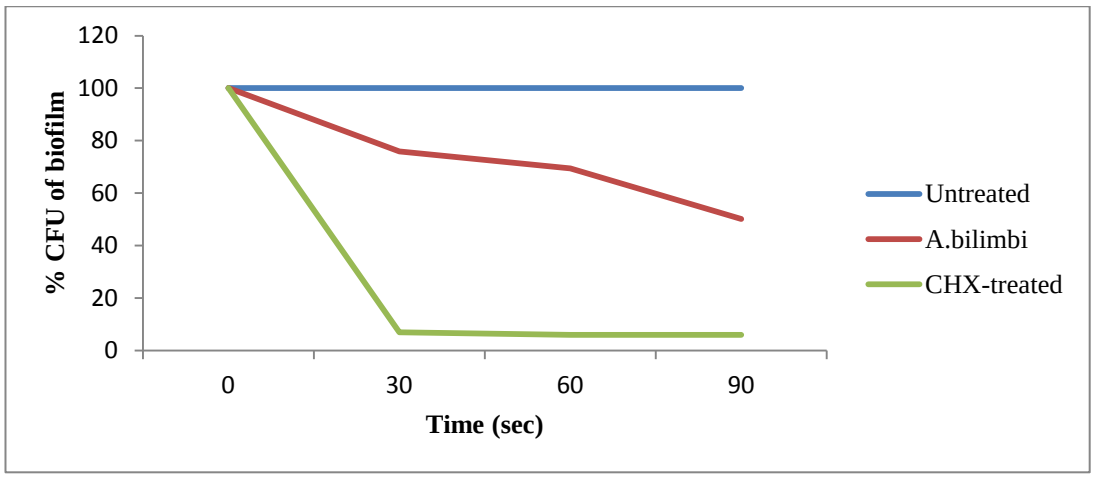


Figure 4.7: Killing effect of *A. bilimbi* comparative to CHX exhibited by reduction in the percentage of bacterial population

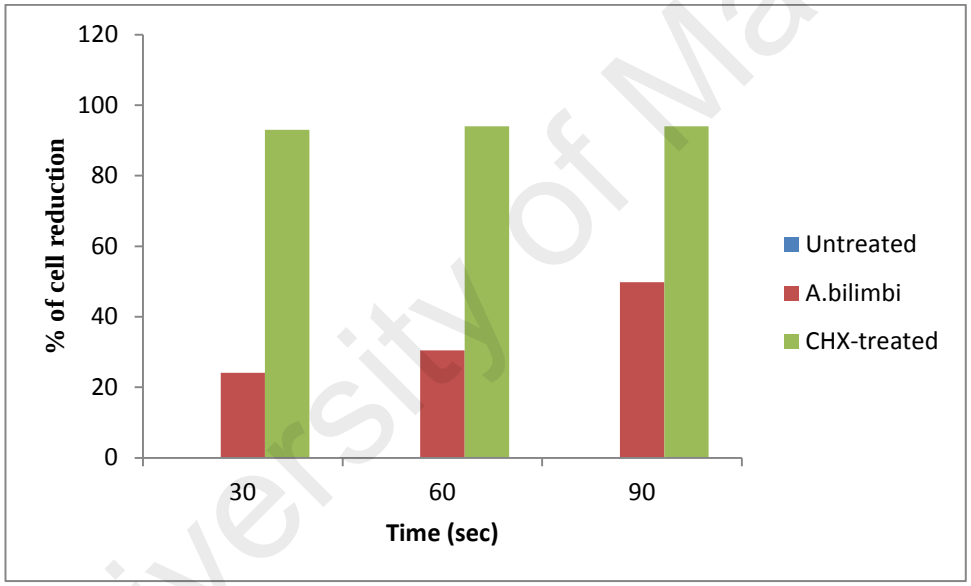


Figure 4.8: Killing effect of *A. bilimbi* as measured by percentage reduction of population count in 24hr mixed-culture biofilm formed on saliva-coated glass beads (sGB). The values plotted were compared to the negative (untreated) and positive (CHX-treated) controls. Values plotted were mean of triplicate tests.

4.2.2 Antiadherence Effect of *A. bilimbi* on the Adhesion of Mixed-Culture of Streptococci to Saliva-coated Glass Beads

When saliva was run through the NAM system, a layer of experimental pellicle formed on the surface of the glass beads. This layer of experimental pellicle is important as it has salivary components that provides binding receptors for the attachment of oral streptococci to the glass beads. Therefore, under the untreated (distilled water-coating) condition, the cells count of streptococci determined present in the biofilm would represent the optimum number of bacteria that is able to attached to the sGB. This cells count was taken to indicate a 100% of bacteria cells adherence. Following treatment of the sGB with *A. bilimbi* extract however, showed a reduction in bacterial adherence to 35.5% (Figure 4.10). This suggested an inhibition of adherence activity of the streptococci to sGB by 64.5% ($p<0.001$). It was determined that treatment of sGB with CHX-mouthrinse showed greater inhibition of adhesion by 96% ($p<0.001$).

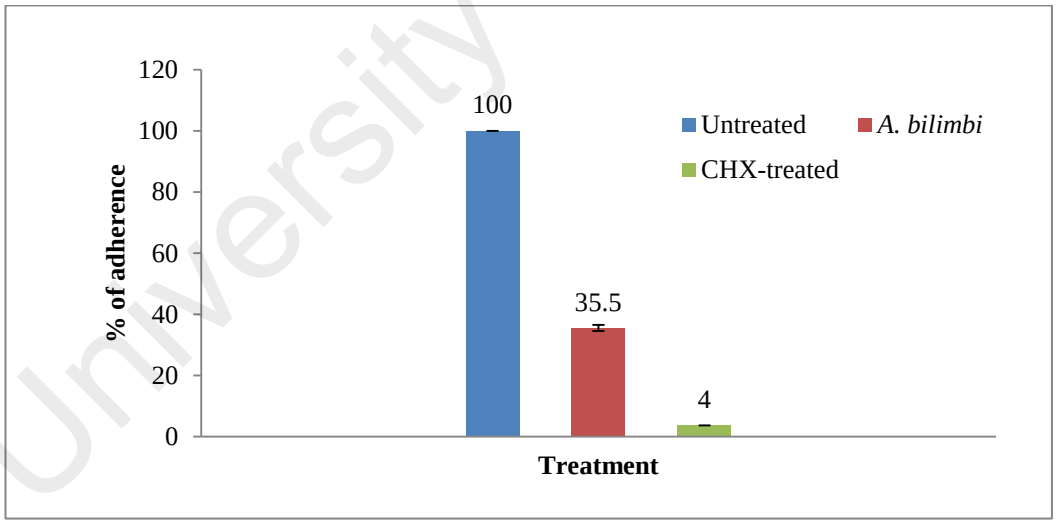


Figure 4.9: Percentage of adherent bacterial cells following treatment of sGB with *A. bilimbi* extract. The inhibitory effect was compared to the untreated (control) and CHX-treated sGB. Values plotted were mean of triplicate tests.

4.2.3 Effect of *A. bilimbi* Extract on Cell Surface Hydrophobicity of Mixed and Individual Culture of Streptococci

The effect of *A. bilimbi* on CSH was selective with greatest reduction seen on *S. sanguinis* and *S. mitis*, followed by *S. mutans*. Compared to 1 mg/mL, the CSH was drastically reduced at 5 mg/mL on *S. sanguinis, S. mitis, S. mutans* and mix species. At 5 mg/mL, *A. bilimbi* extract reduced the CSH by approximately 50% of *S. sanguinis* (52.35%), *S. mutans* (40.57%), *S. mitis* (47.92%) and mix species (48%). Higher reduction was seen at higher *A. bilimbi* concentration of 10 mg/mL indicated that the effect was concentration dependent (Figure 4.10).

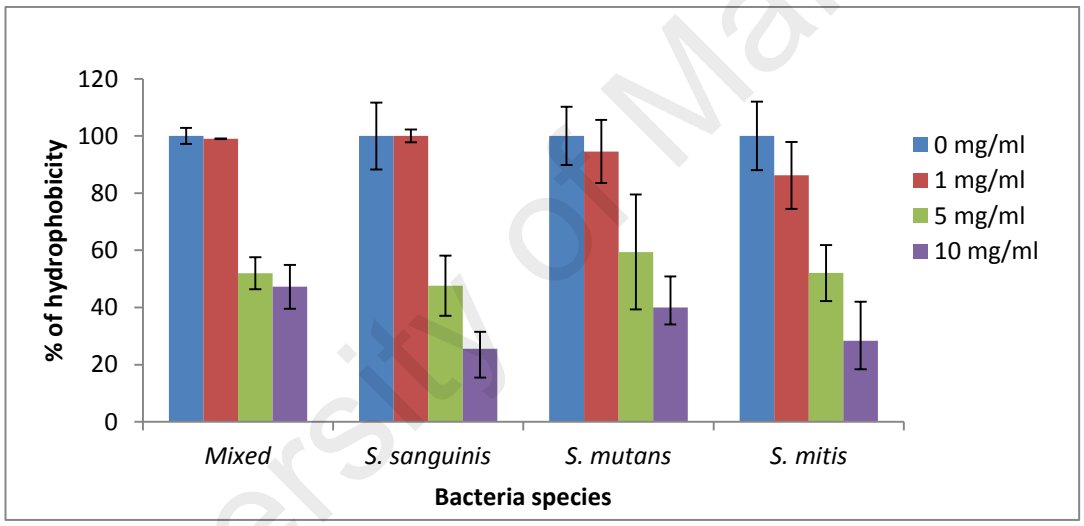


Figure 4.10: Effect of *A. bilimbi* extracts on cell surface hydrophobicity of streptococci when in mixed culture comparative to individual *S. sanguinis*, *S. mutans* and *S. mitis*. Values plotted were mean of triplicate tests performed thrice (n=9).

When in mixed population, *A. bilimbi* at 1 mg/mL did not show much effect and was almost similar to the untreated bacteria cells. Reduction in CSH on both mixed and individual cultures was however significantly observed at higher concentrations of 5 mg/mL and 10 mg/mL.

4.2.4 Effect of *A. bilimbi* Extract on the Expression of Glucosyltransferases (*gtfB* and *gtfC*) and Response Regulator (*vicR*) Genes

Based on data from real-time PCR, it was found that treatment of the oral streptococci with *A. bilimbi* extract had significantly affected the expression of *gtfB*, *gtfC* and *vicR* genes. Based on the RQ values, the extract was found to down regulate the expression of *gtfB* by almost 5-fold (RQ = 0.204) while for *gtfC*, the gene was almost fully suppressed (RQ = 0.008). *A. bilimbi* extract also totally suppressed the expression of *vicR* gene (RQ = 0.000) (Figure 4.11).

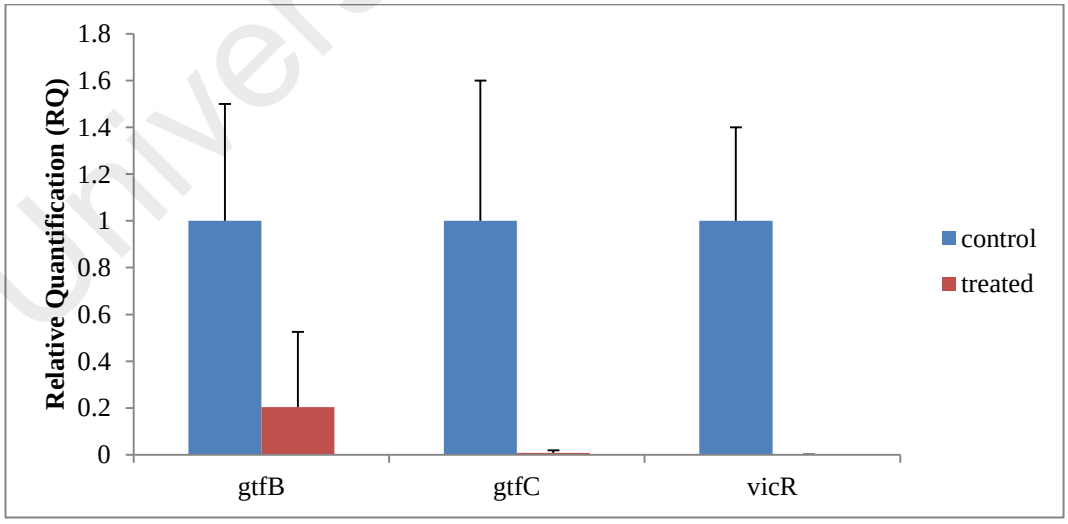


Figure 4.11: Effect of *A. bilimbi* treatment of 24hr-mixed culture biofilm of *S. mitis*, *S. sanguinis* and *S. mutans* on the expression of *gtfB*, *gtfC* and *vicR* genes

CHAPTER FIVE:

DISCUSSION

S. mutans, *S. mitis* and *S. sanguinis* are categorized as commensal and non-pathogenic bacteria of the oral cavity. Studies have shown that the status and role of these streptococci may change as determined by changes in the oral environment of the human host. *S. sanguinis* for example, despite being a normal oral bacteria has been known to cause infective endocarditis when it enters the blood stream as a result of dental procedures (Herzberg, 1996; Herzberg *et al*., 1997; Matta *et al*., 2009). *S. mutans* is another oral commensal which becomes an opportunistic pathogen strongly associated in caries development (Hamada and Slade, 1980; Russell, 2008). Being aciduric, this *Streptococcus* easily tolerates and survives in the acidic pH accumulated in oral biofilm formed on tooth surfaces, which eventually leads to demineralization of the enamel surface once a critical pH of 5.5 is reached or exceeded (Dawes, 2003). Unlike the *S. mutans* and *S. sanguinis*, *S. mitis* has remained clear of any association with oral disease. Based on the high percentage and common presence in dental plaque (Whiley and Beighton, 1998; Facklam, 2002), these three species were used in this study as representative microbes of the dental biofilm.

One method to reduce caries incidence would be to control the formation of dental biofilm and to maintain its biomass thin and healthy. Several approaches have been reported to meet this objective. Manipulation of the plaque ecology and inhibition of bacterial colonization by using active compounds from plant have shown promising outcomes that can be used in the development of health care products that contribute to general maintenance of good oral health (Palombo, 2011). In this study, aqueous extraction and preparation of plant extracts was used. This was to mimic the traditional

way of herbs processing by the locals and to ensure extracts obtained are safe for human use. It is also important to promote this green technology as it is perceived by the public as safer for human use and consumption (Institute of Medicine and National Research Council, 2005).

In this study five plants that include *A. bilimbi*, *B. javanica*, *E. tirucalli*, *N. sativa* and *V. amygdalina* were screened for their antibacterial effect on three *Streptococcus* species common in the oral cavity. Based on the Kirby-Bauer sensitivity test, all three streptococci were found to be susceptible to extracts of only two plants, *A. bilimbi* and *V. amygdalina*. The diameter of inhibition zones showed that the extracts for *A. bilimbi* and *V. amygdalina* exhibited varying degrees of antibacterial activity towards different *Streptococcus* species. *A. bilimbi* exhibited stronger antibacterial activity compared to *V. amygdalina* (Table 1 & Table 5). Based on a previous study, aqueous extract of *A. bilimbi* was reported effective in inhibiting the growth of *S. aureus*, *S. epidermis*, *B. cereus* and *K. rhizophila* (Zakaria *et al*., 2007) while, the aqueous extract of *V. amygdalina* was effective in inhibiting the growth of *S. aureus*, *E. coli*, *P. earuginosa*, *Klebsiella* spp., *C. albicans* and *S. mutans* (Anibijuwon *et al*., 2012; Ghamba *et al*., 2014; Kigigha and Onyema, 2015), although high concentrations were used in these studies. Unlike *S. mitis* and *S. mutans*, *S. sanguinis* was found susceptible to all five plant extracts, but at varying strength. The antibacterial effect of *B. javanica*, *E. tirucalli* and *N. sativa* extracts on the other hand, showed selectivity to only *S. sanguinis* or *S. sanguinis* and *S. mitis* (Table 4.1, 4.2 and 4.3).

However, compared to the sub-MIC of CHX-containing mouthrinse (18.75µg/mL) which was used as a positive control in the study, the sub-MIC of *A. bilimbi* was much higher (6.25mg/mL). This may be explained by the nature of both

active compounds. As a crude preparation, *A. bilimbi* extract may contain impurities that can influence its antibacterial properties towards the streptococci. CHX on the other hand is a pure chemical active agent.Throughout the study, *A. bilimbi* extract at the sub-MIC was used. At this concentration, a minimum bacteria population is allowed to remain viable and not totally killed. This is important as streptococci constitute a common commensal of the oral cavity. Any bactericidal agent that could kill these commensals would expose the mouth to potential pathogenic infection. In other words, indigenous bacteria such as streptococci influences pathogenic colonization of the oral surfaces (van der Waaij *et al*., 1971; Drenkard and Ausubel, 2002; Wardwell *et al*., 2011). It has been reported that antibiotic usage that totally kills the oral bacteria, disrupts the resident communities and increases colonization of pathogens that leads to the onset of oral diseases (Pepin *et al*., 2005; Adams *et al*., 2007).

Based on the sub-MIC and MBC results, the antibacterial activity of *A. bilimbi* towards *Streptococcus* species was determined to be stronger compared to the aqueous extracts of *B. javanica, E. tirucalli, N. sativa* and *V. amygdalina.* As for *V. amygdalina,* although it exhibited antibacterial activity towards all three streptococci, the effective concentrations were comparatively high (Table 4.6). Thus, it was decided that only the extract of *A. bilimbi* was suitable for subsequent analysis in this study.

The antibacterial effect of *A. bilimbi* fruits has long been reported and studied (Zakaria *et al*., 2007; Das *et al*., 2011). No single compound was associated with this property. It has been suggested that all active compounds present, together with acidity of the extract contributed synergistically to its antibacterial effect (Giannuzzi and Zaritzky, 1996). Ascorbic acid present in the extract, contributed to the low pH of *A. bilimbi,* as also reported earlier (Giannuzzi and Zaritzky, 1996). Other fruits that also

have antibacterial properties like pomegranate fruit (*Punica granatum*) (Vasconcelos *et al*., 2006; Reddy *et al*., 2007; Opara *et al*., 2009) also have low pH and high content of ascorbic acid. Previously it has been proposed that an acidic pH enhances the antibacterial activity of plant extracts (Molan, 1992). The US Food and Drug Administration (FDA, 2007) reported pH range for pomegranate in its natural state to be 2.93-3.20.

The acidity of *A. bilimbi* fruits is also due to present of other weak organic acids (Liepina *et al*., 2013). This low pH has been associated by Friedman and Jurgens, (2000) to the antimicrobial properties of its phenolic compounds. This dependence on pH, and the relationship between acidity of extracts and its antibacterial activity, was however claimed by Krisch *et al*., (2008) as poor. Further support to this claim was made by Liepina *et al*., (2013) who demonstrated that pH does not determine the antimicrobial activity of *A. bilimbi* extract. Based on this understanding, this study proceeded to investigate the antibacterial activity of *A. bilimbi* extract on three selected common streptococci of the oral cavity.

Based on LC-MS results, three active compounds were identified to be present in *A. bilimbi* extract although few others have been reported (Section 2.8.12). These included ascorbic acid, acetylvitisin B and chinic acid (Figure 4.6). Acetylvitisin B and chinic acid have not been extensively studied. Acetylvitisin B is an anthocyanin pigment identified as 3-acetylglucoside or acetylvitisin B (Bakker and Timberlake, 1997). It is grouped as flavonoids and information from previous study reported the presence of acetylvitisin B in grapes and red wine (Lorrain *et al*., 2013).

Chinic acid is also known as quinic acid according to the PubChem Open Chemistry Database (Kim *et al*., 2015). Previously used in bacterial differentiation (Butcher, 1926), its synonym quinic acid has been used in the synthesis of anti-influenza/anti-swine flu medication (Khatoon *et al*., 2015). Research done by Pero and Lund, (2009) showed that quinic acid was synthesized to nicotinamide and tryptophan via gastrointestinal tract microflora which were known as powerful antioxidants that enhance DNA repair. Quinic acid can be used to treat prostate cancer with lesser toxic effect (Inbathamizh and Padmini, 2013)

Ascorbic acid, also known as ascorbate or vitamin C, is a water-soluble ketolactone having two ionizable hydroxyl groups. The number and sites of hydroxyl groups have been associated with the toxicity of this compound towards microorganism. Toxicity was reported to increase when hydroxylation increased (Girón *et al*., 1988). The strong antimicrobial effect of ascorbic acid has been demonstrated (Tajkarimi and Ibrahim, 2011; Isela *et al*., 2013). A number of oral microbes including *S. mutans, S. aureus, P. gingivalis, C. albicans and E. faecalis* are affected by ascorbic acid (Isela *et al*., 2013). The antibacterial activity was however suggested to arise via the antioxidant property of ascorbic acid. Earlier study showed ascorbic acid as an effective antioxidant agent (Barrita and Sanchez, 2013). As an antioxidant, this acid alters bacterial cell's oxido-reduction after penetrating the cells. Kallio *et al*., (2012) showed that pure vitamin C was able to inhibit *S. aureus* at millimolar concentrations and suggested it may have affected the bacterium through its metabolism. He also suggested that the effectiveness of ascorbic acid was not solely attributed to its low pH that causes pH alteration of the culture medium because maximal inhibition was also showed when acidity of the compound was neutralized.

Other than its growth inhibitory effect on the streptococci, *A. bilimbi* was determined to induce aggregation between *S. mutans*, *S. mitis* and *S. sanguinis* (Table 4.7). A very low concentration (5 mg/mL) of the extract was enough to initiate aggregation between the three streptococci. Within the oral environment, bacterial aggregation is important as it helps in bacterial clearance. Some constituents of the oral fluid are known to promote similar bacterial clearance mechanism to clear microorganisms and prevent them from adhering to tooth and mucosal surfaces of the mouth (Courtney and Hasty, 1991). Even though the aggregative effect was not so pronounced, it gives additional credit to *A. bilimbi* extract as an antibacterial agent.

Attachment of bacteria to oral surfaces especially the teeth, involves specific and non-specific interactions between bacteria cell wall and the acquired pellicle on teeth (Gibbons and Etherden, 1983; Fathilah *et al.*, 2003). The former occurs when specific receptor-adhesin binding happened between bacterial extracellular appendages and receptors on the acquired pellicle on the saliva-coated tooth surface. In addition to this, the bacterial cell surface of streptococci is in general hydrophobic due to the presence of numerous hydrophobic cell wall proteins. This contributes to nonspecific interactions that in a way, stabilises the binding process both cell-to-pellicle and cell-to-cell (Bodet *et al*., 2008).

Previous reports have shown that among the three bacteria tested, *S. sanguinis* is the most hydrophobic. This property helps it to attach strongly to saliva–coated surfaces compared to other tested bacteria (Nesbitt *et al*., 1982; McNab *et al*., 1995; Jenkinson, 1997; Fathilah *et al*., 2006). Bacteria possess surface hydrophobicity due to the presence of lipoteichoic acids (LTA), hydrophobic proteins on the cell-wall and external appendages (Jenkinson, 1986; McNab and Jenkinson, 1992). The extract of *A. bilimbi*

was shown to reduce the CSH of *S. mutans, S. mitis* and *S. sanguinis* with the highest hydrophobicity reduction was observed on *S. sanguinis* (Figure 4.10). The hydrophobicity reduction of all bacteria tested increased with increased concentration of the extract suggested that the effect is concentration dependent. The acidity of ascorbic acid, which is one of the main compounds present in the extract, may have altered and modified the cell wall proteins, losing the hydrophobic nature of the proteins. Similar observations have been made in bacterial adherence studies involving active compounds (Jenkinson and Lamont, 1997; Nostro *et al*., 2004; Prabu *et al*., 2006; Fathilah *et al.*, 2006). This finding showed the potential of *A. bilimbi* in reducing nonspecific adhesion of oral streptococci to surfaces, which in general interferes with the attachment process of bacteria to tooth surface. Poor attachment support from nonspecific interactions may result in poor adhesion of the streptococci to the tooth surface as well as between cells. This has also been reported by Nesbitt *et al*., (1982) who observed significant reduction of *S. sanguinis* adherence in the presence of hydrophobic bond inhibitors.

An experimental biofilm to mimic dental plaque was used to simulate conditions in the mouth. The biofilms were created by allowing all three streptococci *S. mutans, S. mitis* and *S. sanguinis*, to form biofilm on saliva-coated glass beads. These glass beads were placed in the NAM artificial mouth model (Rahim *et al*., 2008). Glass beads were used in this study to represent tooth surfaces as was reported by Pratt-Terpstra *et al*., (1989) who demonstrated that the glass surfaces and enamel have similar properties once coated with saliva. Salivary glycoproteins adhered to the hard substratum and form the acquired pellicle that is required for bacterial colonisation. These proteins serve as binding receptors for the streptococci through adhesins on the bacterial cell surface with complementary receptors within the acquired pellicle (Marsh and Martin, 1999). This binding, forms the earliest adhesion in the colonisation process. In this study, this first

binding interaction could be interrupted by the extract of *A. bilimbi*. Reduction of bacterial adherence to the extract treated experimental pellicle was observed, and this suggests an interference with the receptors involved in bacterial adhesion had occurred. The extracts may also alter the cell wall properties (Fathilah *et al*., 2006) or the binding sites for bacterial attachment on the experimental pellicle when it was first treated with the extract. The bacterial cell wall could be the main target of the extract to attack the bacterial cell as most of the plant extracts contained polyanionic bioactive compounds. The polyanionic compounds present in the extract may interact with the cations in the bacterial cell wall and disturb the cell wall stability leading to cell destruction. Moreover, the bioactive compounds present in the extract may interact with the lipid layer or sialic acid in the bacterial cell wall that affect the lipid membrane in the cell wall causing the cells to lyse (Blondelle *et al*., 1996). The antibacterial substances present in the extract probably disturbed the production of peptidoglycan layer of the cell wall (Okemo *et al*., 2001; Mandal *et al*., 2007). This interruption may also include the various binding forces involved such as hydrogen bonds, van der Waals forces and electrostatic interactions (Hannig and Hannig, 2009). However, how *A. bilimbi* affected the individual species could not be determined as all three streptococci were in a suspension to mimic the multispecies bacteria present in oral cavity. Fathilah and Rahim, (2003) and Fathilah *et al*., (2006) have published several reports on responses of several common individual oral streptococci to treatments with *Piper betle* and *Psidium guajava* extracts. The extracts were reported to interrupt both the primary and secondary adhesions of *S. mitis*, *S. sanguinis* and *Actinomyces* sp. to an experimental pellicle-coated surface. In the former the extracts were suggested to work by altering the specific binding receptors for bacteria present in the pellicle. While in the later, the extracts work by diminishing the hydrophobicity of becteria cell surface, making them less able to form strong binding with the pellicle-coated surface.

In designing an oral health care rinse, the effectiveness of the product in reducing microbial population in the oral biofilm is one of the main aim. Comparing the killing effect to that of CHX-containing mouthrinse, the effectiveness of *A. bilimbi* was much lower than the commercial mouthrinse (Figure 4.7 and 4.8). The killing effect of an extract was suggested by several researchers as due to inhibition of macromolecular synthesis, enzyme dysfunction, loss of energy production and leakage of cellular contents that can lead to cell death (Tsuchiya and linuma, 2000; Zasloff, 2002).

Some oral streptococci have the ability to produce extracellular polysaccharides that in a way, increase the biomass of dental plaque. These chains of glucose residues, termed glucans, play a role in adhesion as their sticky nature enhances attachment of bacterial cells to the tooth surface. In oral biofilms sticky and gelatinous glucans are mainly synthesized by the *S. mutans* using sucrose as the substrate. Both glucosyltransferase B (GtfB) and glucosyltransferase C (GtfC) are required for extracellular polysaccharide formation in dental biofilm (Barrientos and Rodriguez, 2010). GtfC can bind to the pellicle while GtfB remains on the streptococcal surface but it can bind to other bacteria species on their surfaces (Kreth and Herzberg, 2015). Water insoluble glucans synthesized by GtfB and GtfC supply a source of fermentable substrates that increased the cariogenic potential of extracellular polysaccharides by allowing substantial access to nutrients (Forssten *et al*., 2010) and promote other oral bacteria adhesion by providing binding sites (Kim *et al*., 2012). Glucan-binding protein attach to glucans mediated bacteria aggregation, by that enhance dental plaque formation and causing the enamel surface to demineralize (Hamada and Slade, 1980; Mattos-Graner *et al*., 2001; Tamesada *et al*., 2004).

Differential expression of specific genes *gtfB*, *gtfC* and *vicR* genes of the streptococci was observed in this study following exposure of the bacteria to *A. bilimbi* extract. *VicR* gene was found to be completely suppressed when treated with *A. bilimbi* extract, which may indicate the killing effect was due to suppression of this gene. And because the product of *vicR* gene regulates the expression of *gtfB* and *gtfC* genes (Senadheera *et al*., 2005), it is thus reasonable to suggest that the suppression of *gtfB* and *gtfC* may have occurred as a result of the suppression of *vicR* gene (Figure 4.11). The *vicR* gene encode for response regulator protein. Previous study by Senadheera *et al*., (2005) reported *vicR* gene as important for bacteria survival and this was agreeable with this finding.

Conclusion

Out of five aqueous plant preparations screened, only the extract of *A. bilimbi* exhibited susceptibility towards the common plaque bacteria *S. mutans*, *S. mitis* and *S. sanguinis*. The extracts of *B. javanica, E. tirucalli, N. sativa* and *V. amygdalina* showed selective susceptibility towards one or two bacteria only. Hence, *A. bilimbi* was selected. Three main compound present in *A. bilimbi* extract were ascorbic acid, chinic acid and acetylvitisin B. *A. bilimbi* extract showed antibacterial activity by inducing aggregation and killing activities. It also exhibited antibiofilm activity by reducing the adhesion of streptococci to tooth surfaces, both through specific binding to pellicle-coated surfaces, and secondary binding through interference of bacterial cell surface hydrophobicity. The suppression of *gtfB*, *gtfC* and *vicR* by *A. bilimbi* may minimize the synthesis of insoluble glucans that reduces and controls biomass build up in oral biofilms, that indirectly reduces subsequent attachment of bacteria to the tooth surface. Even though the aggregative effect of *A. bilimbi* extract was not great, it gives additional credit to the extract as it may assist bacterial clearance from the oral cavity. The outcomes of this study thus suggest the potential of *A. bilimbi* extract to be promoted as an active component in antimicrobial oral health care products as it may minimize bacterial adhesion to tooth surface and maintain loose biofilm structure. The plant is also widely available and the fruit is rich in ascorbic acid, which may help in the maintenance of oral soft tissues.

REFERENCES

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology, 43*(11): 5721-5732.

Ab Rahman, M. R., Abdul Razak, F., & Mohd Bakri, M. (2014). Evaluation of wound closure activity of *Nigella sativa*, *Melastoma malabathricum*, *Pluchea indica*, and *Piper sarmentosum* extracts on scratched monolayer of human gingival fibroblasts. *Evidence-Based Complementary and Alternative Medicine, 2014*.

Adams D. A., Riggs M. M., Donskey C. J. (2007). Effect of fluoroquinolone treatment on growth of and toxin production by epidemic and nonepidemic *Clostridium difficile* strains in the cecal contents of mice. *Antimicrobial Agents and Chemotherapy, 51*: 2674–2678.

Addy, M. (1986). Chlorhexidine compared with other locally delivered antimicrobials. *Journal of Clinical Periodontology, 13*: 957–964.

Aggarwal, B. B., Kunnumakkara, A. B., Harikumar, K. B., Tharakan, S. T., Sung, B. & Anand, P. (2008). Potential of spice-derived phytochemicals for cancer prevention. *Planta Medica, 74*(13): 1560-9.

Akinpelu, D. A. (1999). Antimicrobial activity of *Vernonia amygdalina leaves*. *Fitoterapia, 70*(4): 432-434.

Albandar, J. M. (2005). Epidemiology and risk factors of periodontal diseases. *Dental Clinics of North America, 49*(3): 517–532.

Ali, B. H. & Blunden, G. (2003). Pharmacological and toxicological properties of *Nigella sativa*. *Phytotherapy Research, 17*(4): 299–305.

Al-Zubaydi, S. R., Al-Hmdany, M. A., & Raesan, S. J. (2009). Antibactrial effect of some medicinal plant extracts against some pathogenic bacteria strains. *Journal of Duhok University, 12*(1): 244-249.

Amira, C. A. & Okubadejo, N. U. (2007). Frequency of complementary and alternative medicine utilization in hypertensive patients attending an urban tertiary care centres in Nigeria. *BMC Complementary and Alternative Medicine, 7*: 30–48.

Anibijuwon, I. I., Oladejo, B. O., Adetitun, D. O., & Kolawole, O. M. (2012). Antimicrobial activities of *Vernonia amygdalina* against oral microbes. *Global journal of Pharmacology, 6*(3): 178-185.

Aoki, H., Shiroza, T., Hayakawa, M., Sato, S., & Kuramitsu, H. K. (1986). Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. *Infection and Immunity, 53*(3): 587-594.

Areghore, E. M., Makkar, H. P. S. & Becker, K. (1997). Chemical composition and tannins in leaves of some browse plants from Delta (Central Nigeria) eaten by ruminants. *Proceeding of the Society of Nutrition Physiolgy, 5*: 11-15.

Arigbede, A. O., Babatope, B. O. & Bamidele, M. K. (2012). Periodontitis and systemic diseases: A literature review. *Journal of Indian Society of Periodontology*, *16*(4): 487-91.
Armitage, G. C. (1996). Periodontal diseases: diagnosis. *Annals of Periodontology*, *1*(1): 37-215.

Ashok Kumar, K., Gousia, S. K., Anupama, M. & Naveena Lavanya Latha, J. (2013). A review on phytochemical constituents and biological assays of *Averrhoa bilimbi*. *International Journal of Pharmacy and Pharmaceutical Science Research*, *3*(4): 136-139.

Auschill, T. M., Arweiler, N. B., Netuschil, L., Brecx, M., Reich, E. & Sculean, A. (2001). Spatial distribution of vital and dead microorganisms in dental biofilms. *Archives of Oral Biology*, *46*: 471-476.

Bachrach, G., Jamil, A., Naor, R., Tal, G., Ludmer, Z. & Steinberg, D. (2011). Garlic allicin as a potential agent for controlling oral pathogens. *Journal of Medicinal Food*, *14*(11): 1338-43.

Baniakina, J. & Eyme, J. (1997). Studies on the morphological and anatomical structures in the Family Euphorbiaceae. *Revue de medicines et pharmacopees Africaines*, *12*: 27 – 48.

Bakker, J., & Timberlake, C. F. (1997). Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *Journal of Agricultural and Food Chemistry*, *45*(1): 35-43.

Barrientos, S., & Rodríguez, A. (2010). Production of glucosyltransferase B and glucans by *Streptococcus mutans* strains isolated from caries-free individuals. *Acta odontologica latinoamericana: AOL*, *24*(3): 258-264.

Barrita, J. L. S., & Sánchez, M. D. S. S. (2013). Antioxidant role of ascorbic acid and his protective effects on chronic diseases. *Morales-González JA. Oxidative Stress and Chronic Degenerative Diseases-A Role for Antioxidants. Rejeka Intech*, 449-84.

Bauer, A. W., & Kirby, W. M. M. (1959). Single disc antibiotic sensitivity testing of staphylococci. *A.M.A Archives of Internal Medicine Journals*, *104*: 208-216.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*(4): 493.

Blanc, V., Isabal, S., Sanchez, M. C., Llama-Palacios, A., Herrera, D. & Sanz, M. (2014). Characterization and application of a flow system for in vitro multispecies oral biofilm formation. *Journal of Periodontal Research*, *49*: 323-32.

Blondelle, S. E., Takahashi, E., Houghten, R. A., & Pérez-payá, E. (1996). Rapid identification of compounds with enhanced antimicrobial activity by using conformationally defined combinatorial libraries. *Biochemical journal*, *313*(1): 141-147.

Bodet, C., Grenier, D., Chandad, F., Ofek, I., Steinberg, D., & Weiss, E. I. (2008). Potential oral health benefits of cranberry. *Critical reviews in food science and nutrition*, *48*(7): 672-680.

Bowen, W. H. (2002). Do we need to be concerned about dental caries in the coming millennium? *Critical Reviews in Oral Biology & Medicine*, *13*(2): 126-131.

Brantner, A., Maleš, Ž., Pepeljnjak, S., & Antolić, A. (1996). Antimicrobial activity of *Paliurus spina-christi* Mill. (Christ's thorn). *Journal of Ethnopharmacology*, *52*(2): 119-122.

Brown, C. K., Gu, Z. Y., Matsuka, Y. V., Purushothaman, S. S., Winter, L. A., Cleary, P. P., Olmsted, S. B., Ohlendorf, D. H. & Earhart, C. A. (2005). Structure of the streptococcal cell wall C5a peptidase. Proceeding of the National Academy of Sciences of the United States of America, *102*(51): 18391-6.

Busuioc, M., Mackiewicz, K., Buttaro, B. A. & Piggot, P. J. (2009). Role of Intracellular Polysaccharide in Persistence of *Streptococcus mutans*. *Journal of Bacteriology*, *191*(23): 7315-22.

Butcher, B. H. (1926). Use of chinic acid in the differentiation of the colon-aerogenes groups. *Journal (American Water Works Association)*, *15*(2), 171-173.

Caufield, P. W., Dasanayake, A. P., Li, Y., Pan, Y., Hsu, J. & Hardin, J.M. (2000). Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity. *Infection and Immunity*, *68*(7): 4018-23.

Caufield, P. W. & Griffen, A. L. (2000). Dental caries. An infectious and transmissible disease. *Pediatric Clinics of North America*, *47*(5): 1001–19.

Chandki, R., Banthia, P. & Banthia, R. (2011). Biofilms: A microbial home. *Journal of Indian Society of Periodontology*, *15*(2): 111-4.

Chen, H., Bai, J. & Fang, Z. F. (2011). Indole alkaloids and quassinoids from the stems of *Brucea mollis*. *Journal of Natural Products*, *74*(11): 2438–2445.

Chen, M., Chen, R., Wang, S., Tan, W., Hu, Y., Peng, X. & Wang, Y. (2013). Chemical components, pharmacological properties,
and nanoparticulate delivery systems of *Brucea javanica*. *International Journal of Nanomedicine*, *8*: 85-92.

Chen, Q. J., Ouyang, M. A., Tan, Q. W., Zhang, Z. K., Wu, Z. J. & Lin, Q. Y. (2009). Constituents from the seeds of *Brucea javanica* with inhibitory activity of tobacco mosaic virus. *Journal of Asian Natural Products Research*, *11*(6): 539–547.

Chen, Y. Y., Pan, Q. D. & Li, D. P. (2011). New pregnane glycosides from *Brucea javanica* and their antifeedant activity. *Chemistry & Biodiversity*, *8*(3): 460–466.

Chong, K. T. & Pagano, P. J. (1997). In vitro combination of PNV-140690, a Human Immunodeficiency Virus type 1 protease inhibitor with Ritonavir against

Ritonavir-sensitive and Resistant Clinical Isolates. *Antimicrobial Agents and Chemotherapy*, *41*(ll): 2367-2377.

Cimasoni, G. (1983). Crevicular fluid updated. *Monographs in Oral Science*, *12*(III-VII): 1-152.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*, *284*(5418): 1318-1322.

Courtney, H. S., & Hasty, D. L. (1991). Aggregation of group A streptococci by human saliva and effect of saliva on streptococcal adherence to host cells. *Infection and Immunity*, *59*(5): 1661-1666.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12*(4): 564-582.

Cumming, R. (2014). *Brucea javanica*. Retrieved from https://www.flickr.com/photos/58828131@N07/14541824695/in/photolist-47hPTQ-47dK7a-RTb78L-RTao2G-oa1DRr-ckYaEo-ckYcE9-ckY9kq-jpCwjg-jpCsBg-bmW7i1-jpASfV-jpCEoT-bmW9Du-ckY8P7-ckYbwm-bmW65y-o7YgHA-ckXZ3w-jpEbUy-bmWqhU-bmW8zm-bzQUmt-ckYc8Q-bzQTcB-cgk1nJ-9zwsY1-ckXYxs-bzQRM2-bmW175-6pSfXo-jpD525-jpB3Wz-ckY3P7-6WhT99-cErejm-5EDGYY-ckY2Gh-S7JJxF-ckXXDo-bzR1cp-JVbe5o-giYu7v-nSwgyr-eSPjC3-9zwrPW-jpCzSD-giZUak-giZU5k-a4i7vc/

Das, S. C., Sultana, S., Roy, S., Hasan, S. S. (2011). Antibacterial and cytotoxic activities of methanolic extracts of leaf and fruit parts of the plant *Averrhoa bilimbi* (Oxalidaceae). *American Journal of Scientific and Industrial Research*, *2*(4): 531-536.

Dawes, C. (2003). What is the critical pH and why does a tooth dissolve in acid? *Journal-Canadian Dental Association*, *69*(11): 722-725.

De Almeida Pdel V, Grégio AM, Machado MA, de Lima AA, Azevedo LR. (2008). Saliva composition and functions: a comprehensive review. *Journal of Contemporary Dental Practice*, *9*(3): 72-80.

Dedeoglu, N., DeLuca, V., Isik, S., Yildirim, H., Kockar, F., Capasso, C. & Supuran, C. T. (2015). Sulfonamide inhibition study of the β-class carbonic anhydrase from the caries producing pathogen *Streptococcus mutans*. *Bioorganic & Medicinal Chemistry Letters*, *25*(11): 2291–2297.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmanan, A. & Wade, W. G. (2010). The human oral microbiome. *Journal of Bacteriology*, *192*(19): 5002-17.

Diaz, P. I., Chalmers, N. I. & Rickard, A. H. (2006). Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Applied and Environmental Microbiology*, *72*: 2837–2848.

Dige, I., Nilsson, H., Kilian, M. & Nyvad, B. (2007). *In situ* identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning

microscopy and fluorescence *in situ* hybridization. *European Journal of Oral Sciences*, *115*: 459-467.

Dige, I., Nyengaard, J. R., Kilian, M. & Nyvad, B. (2009). Application of stereological principles for quantification of bacteria in intact dental biofilms. *Oral Microbiology and Immunology*, *24*: 69–75.

Dodds, M. W. J., Johnson, D. A. & Yeh, C. K. (2005). Health benefits of saliva: a review. *Journal of Dentistry*, *33*(3): 223-233.

Dogan, O. (2013). Dental Caries: The Most Common Disease Worldwide and Preventive Strategies. *International Journal of Biology*, *5*(4): 55-61.

Drenkard E., Ausubel F. M. (2002). Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*, *416*: 740–743.

Duke, J. A. (2002). *Handbook of medicinal herbs*. CRC press.

Edgar, W. M. (1992). Saliva: its secretion, composition and functions. *British Dental Journal*, *172*(8): 305-312.

Facklam, R. (2002). What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clinical Microbiology Reviews*, *15*: 613–630.

Fathilah, A. R, Baharuddin, B. A, Akbar, E. F. M, Norizan, A. H, Ibrahim, N. F, Musa, M. Y. (2017).
Alternative sweeteners influence the biomass of oral biofilm. *Archives of Oral Biology*, *80*:180-184.

Fathilah, A. R. (2011). *Piper betle* L. and *Psidium guajava* L. in oral health maintenance. *Journal of Medicinal Plants Research*, *5*(2): 156-163.

Fathilah, A. R., Rahim, Z. H., Othman, Y., & Yusoff, M. (2009). Bacteriostatic effect of *Piper betle* and *Psidium guajava* extracts on dental plaque bacteria. *Pakistan Journal of Biological Sciences: PJBS*, *12*(6): 518-521.

Fathilah, A. R., & Rahim, Z. H. A. (2003). The anti-adherence effect of *Piper betle* and *Psidium guajava* extracts on the adhesion of early settlers in dental plaque to saliva-coated glass surfaces. *Journal of Oral Science*, *45*(4): 201-206.

Fathilah, A. R., Othman, R. Y., & Rahim, Z. H. A. (2006). The effect of *Piper betle* and *Psidium guajava* extracts on the cell-surface hydrophobicity of selected early settlers of dental plaque. *Journal of Oral Science*, *48*(2): 71-75.

Fauconneau, B., Waaffo-Tequo, F., Hugnet, F., Barries, I., Decandit, A. & Merillon, J. M. (1997). Comparative study of radical scavenger and antioxidant properties of phenolic compounds from *Vitas vinfera* cell culture using in vitro tests. *Life Sciences*, *16*: 2103-2110.

FDA. (2007). Approximate pH of foods and food products. Rockville, MD: US Food and Drug Administration.

Filoche, S., Wong, L., & Sissons, C. H. (2010). Oral biofilms: emerging concepts in microbial ecology. *Journal of Dental Research*, *89*(1): 8-18.

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, *8*(9): 623-633.

Forssten, S. D., Björklund, M., & Ouwehand, A. C. (2010). *Streptococcus mutans*, caries and simulation models. *Nutrients*, *2*(3): 290-298.

Friedman, M., & Jürgens, H. S. (2000). Effect of pH on the stability of plant phenolic compounds. *Journal of Agricultural and Food Chemistry*, *48*(6): 2101-2110.

Forssten, S. D., Björklund, M. & Ouwehand, A. C. (2010). *Streptococcus mutans*, caries and simulation models. *Nutrients*, *2*(3): 290–298.

Ghamba, P., Balla, H., Goje, L., Halidu, A., & Dauda, M. (2014). In vitro antimicrobial activities of *Vernonia amygdalina* on selected clinical isolates. *International Journal of Current Microbiology and Applied Sciences*, *3*: 1103-1113.

Ghosheh, O. A., Houdi, A. A. & Crooks P. A. (1999). High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa* L.). *Journal of Pharmaceutical and Biomedical Analysis*, *19*(5): 757–762.

Giannuzzi, L., & Zaritzky, N. E. (1996). Effect of Ascorbic Acid in Comparison to Citric and Lactic Acid on *Listeria monocytogenes* Inhibition at Refrigeration Temperatures. *LWT-Food Science and Technology*, *29*(3): 278-285.

Gibbons, R. J., & Etherden, I. (1983). Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles. *Infection and immunity*, *41*(3): 1190-1196.

Girón, L. M., Aguilar, G. A., Cáceres, A., & Arroyo, G. L. (1988). Anticandidal activity of plants used for the treatment of vaginitis in Guatemala and clinical trial of a *Solanum nigrescens* preparation. *Journal of Ethnopharmacology*, *22*(3): 307-313.

Godowski, K. C. (1989). Antimicrobial action of sanguinarine. *Journal of Clinical Dentistry*, *1*(4): 96-101.

Gomes, B. P. F. A., Ferraz, C. C. R., ME, V., Berber, V. B., Teixeira, F. B., & Souza-Filho, F. J. (2001). In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *International Endodontic Journal*, *34*(6): 424-428.

Gurenlian, J. R. (2007). The Role of Dental Plaque Biofilm in Oral Health. *Journal of Dental Hygiene*, *81*(5): 1-11.

Haffajee, A. D. & Socransky, S. S. (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000*, *5*: 78–111.

Hahn, C. L., Schenkein, H. A., & Tew, J. G. (2005). Endocarditis-associated oral streptococci promote rapid differentiation of monocytes into mature dendritic cells. *Infection and Immunity*, *73*(8): 5015-5021.

Hamada, S., and Slade, H.D. (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiological Reviews*, *44*: 331– 384.

Hanada, N. O. B. U. H. I. R. O., & Kuramitsu, H. K. (1988). Isolation and characterization of the *Streptococcus mutans gtf*C gene, coding for synthesis of both soluble and insoluble glucans. *Infection and Immunity*, *56*(8): 1999-2005.

Hannig, C., & Hannig, M. (2009). The oral cavity—a key system to understand substratum-dependent bioadhesion on solid surfaces in man. *Clinical Oral Investigations*, *13*(2): 123-139.

Haynes, D. R. (2006). Emerging and future therapies for the treatment of bone loss associated with chronic inflammation. *Inflammopharmacology*, *14*(5-6): 193– 197.

He, X. S. and Shi, W. Y. (2009). Oral microbiology: past, present and future. *International Journal of Oral Science*, *1*(2): 47.

Herzberg, M.C. (1996). Platelet-streptococcal interactions in endocarditis. *Critical Reviews in Oral Biology & Medicine*, *7*: 222–236.

Herzberg, M.C., Meyer, M.W., Kilic, A., and Tao, L. (1997). Host-pathogen interactions in bacterial endocarditis: streptococcal virulence in the host. *Advances in Dental Research*, *11*: 69–74.

Himratul-Aznita, W. H., Mohd-Al-Faisal, N., & Fathilah, A. R. (2011). Determination of the percentage inhibition of diameter growth (PIDG) of *Piper betle* crude aqueous extract against oral Candida species. *Journal of Medicinal Plants Research*, *5*(6): 878-884.

Huang, R., Li, M. & Gregory, R. L. (2011). Bacterial interaction in dental biofiilm. *Virulence*, *2*(5): 435-444.

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: normal composition, flow, and function. *The Journal of Prosthetic Dentistry*, *85*(2): 162-169.

Igbakin, A.P. & Oloyede, O.B. (2009). Comparative studies on the hypoglycaemic, hypoproteinaemic, hypocholesterolaemic and hypolipidaemic properties of ethanolic and normal saline extracts of the root of *Vernonia amygdalina* in diabetic rats. *Advances in Environmental Biology*, *3*: 33-38.

Igile, G. O., Oleszek, W., Jurzysta, M., Burda, S., Fafunso, M. A. & Adetunde, A. F. (1994). Flavonoids from *Vernonia amygdalina*. *Journal of Agricultural and Food Chemistry*, *42*(11): 2445-8.

Igile, G. O., Oleszek, W., Jurzysta, M., Aquino, R. P., De Tommasi, N. & Pizza, C. (1995). Vernoniosides D and E, two novel saponins from *Vernonia amygdalina*. *Journal of Natural Products*, *58*: 1438-43.

Inbathamizh L, Padmini E (2013). Quinic acid as a potent drug candidate for prostate cancer – a comparative pharmacokinetic approach. *Asian Journal of Pharmaceutical Clinical Research*, *6*(4): 106-112.

Institute of Medicine and National Research Council. (2005). *Dietary Supplements: A Framework for Evaluating Safety.* Washington, DC: The National Academies Press.

Isela, N. N. R., Sergio, N. C., José, M. S. J., Rene, H. D., & Claudio, C. R. (2013). Ascorbic acid on oral microbial growth and biofilm formation. *Journal of Pharmaceutical Innovation*, *2*(4).

Ismail, W. H., Razak, F. A., & AbdulRahim, Z. H. (2006). The role of sucrose in the development of oral biofilm in a simulated mouth system. *Online Journal of Biological Sciences*, *6*: 62-6.

Jafer, M., Patil, S., Hosmani, J., Bhandi, S. H., Chalisserry, E. P., & Anil, S. (2016). Chemical Plaque Control Strategies in the Prevention of Biofilm-associated Oral Diseases. *The Journal of Contemporary Dental Practice*, *17*(4): 337.

Jain, S. C., Sharma, R., Jain, R., & Sharma, R. A. (1996). Antimicrobial activity of *Calotropis procera*. *Fitoterapia*, *67*(3): 275-277.

Jakubovics, N. S. & Kolenbrander, P. E. (2010). The road to ruin: the formation of disease-associated oral biofilms. *Journal of Oral Diseases*, *16*(8): 729-39.

Janssens, M. J., Keutgen, N. & Pohlan, J. (2009). The role of bio-productivity on bio-energy yield. *Journal of Agriculture and Rural Development in the Tropics, 110*: 39–47.

Jenkinson, H. F. (1986). Cell-surface proteins of *Streptococcus sanguis* associated with cell hydrophobicity and coaggregation properties. *Microbiology*, *132*(6): 1575-1589.

Jenkinson, H. F., & Lamont, R. J. (1997). Streptococcal adhesion and colonization. *Critical Reviews in Oral Biology & Medicine*, *8*(2): 175-200.

Jisaka, M., Kawanaka, M., Sugiyama, H., Takegawa, K., Huffman, M. A., Ohigashi, H. & Koshimizu, K. (1992). Antischistosomal activities of sesquiterpene lactones and steroid glucosides from *Vernonia amygdalina*, possibly used by wild chimpanzees against parasite-related diseases. *Bioscience, Biotechnology & Biochemistry*, *56*(5): 845-6.

Kalemba, D., & Kunicka, A. (2003). Antibacterial and antifungal properties of essential oils. *Current medicinal chemistry*, *10*(10): 813-829.

Kallio, J., Jaakkola, M., Mäki, M., Kilpeläinen, P., & Virtanen, V. (2012). Vitamin C inhibits *Staphylococcus aureus* growth and enhances the inhibitory effect of quercetin on growth of *Escherichia coli* in vitro. *Planta medica*, *78*(17): 1824-1830.

Kamaruddin, M. S. (2007). *Brucea javanica*. Retrieved from https://www.flickr.com/photos/kmatsalleh/2040252186/in/photolist-47hPTQ-47dK7a-RTb78L-RTao2G-oa1DRr-ckYaEo-ckYcE9-ckY9kq-jpCwjg-jpCsBg-bmW7i1-jpASfV-jpCEoT-bmW9Du-ckY8P7-ckYbwm-bmW65y-o7YgHA-ckXZ3w-jpEbUy-bmWqhU-bmW8zm-bzQUmt-ckYc8Q-bzQTcB-cgk1nJ-9zwsY1-ckXYxs-bzQRM2-bmW175-6pSfXo-jpD525-jpB3Wz-ckY3P7-6WhT99-cErejm-5EDGYY-ckY2Gh-S7JJxF-ckXXDo-bzR1cp-JVbe5o-giYu7v-nSwgyr-eSPjC3-9zwrPW-jpCzSD-giZUak-giZU5k-a4i7vc/

Kambizi, L. & Afolayan, A. J. (2001). An ethnobotanical study of plants used for the treatment of sexually transmitted diseases (njovhera) in Guruve District, Zimbabwe. *Journal of Ethnopharmacology*, *77*: 5–9.

Kamperdick, C., Van Sung, T., Thuy, T. T., Van Tri, M. & Adam, G. (1995). (20R)-O-(3)-alpha-L-arabinopyranosyl-pregn-5-en-3beta, 20-diol from *Brucea javanica*. *Phytochemistry*, *38*(3): 699–701.

Kaplan, J. Á. (2010). Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of Dental Research*, *89*(3): 205-218.

Karin, S. L., Yang, M. F., Roberts, & Phillipson, J. D. (1990). Canthin-6-one alkaloids from cell suspension cultures of *Brucea javanica*. *Phytochemistry*, *29*(1): 141–143.

Khan, A. Q. & Malik, A. (1990). A New Macrocyclic Diterpene Ester from the Latex of *Euphorbia tirucalli*. *Journal of Natural Products*, *53*(3): 728–731.

Khan, M. A., Ashfaq, M. K., Zuberi, H. S., Mahmood, M. S. & Gilani, A. H. (2003). The in vivo antifungal activity of the aqueous extract from *Nigella sativa* seeds. *Phytotherapy Research*, *17*: 183–6.

Khan, M. A., Chen, H. C., Tania, M. & Zhang, D. Z. (2011). Anticancer activities of *Nigella sativa* (black cumin). *African Journal of Traditional, Complementary and Alternative Medicine*, *8*(5): 226-32.

Khalili, J. (2007). Periodontal disease: an overview for medical practitioners. *Likars' ka sprava/Ministerstvo okhorony zdorov'ia Ukrainy*, (3-4): 10-21.

Khalili, R. M. A., Norhayati A. H., Naim, R., Atif, A. B. & Khairil, M. (2014). Study on Antioxidant Capacity and Anticancer Activity of Bismillah Leaf (*Vernonia amygdalina*). *World Journal of Pharmaceutical Research*, *3*(6): 14-29.

Khatoon, N., Jain, P., & Choudhary, A. K. (2015). Phytochemical Studies on Seed and Leaf Extracts of *Solanum torvum Sw*. *Indo American Journal of Pharmaceutical Research*, *5*(5): 1649-1656.

Kidd, E. A. M. & Fejerskov, O. (2004). What Constitutes Dental Caries? Histopathology of Carious Enamel and Dentin Related to the Action of Cariogenic Biofilms. *Journal of Dental Research*, *83*(C): C35-C38.

Kigigha, L. T., & Onyema, E. (2015). Antibacterial activity of bitter leaf (*Vernonia amygdalina*) soup on *Staphylococcus aureus* and *Escherichia coli*). *Sky Journal of Microbiology Research, 3*(4): 041 – 045.
Kim, J. & Amar, S. (2006). Periodontal disease and systemic conditions: a bidirectional relationship. *Odontology*, *94*(1):10-21.

Kim, M. A., Lee, M. J., Jeong, H. K., Song, H. J., Jeon, H. J., Lee, K. Y., & Kim, J. G. (2012). A monoclonal antibody specific to glucosyltransferase B of *Streptococcus mutans* GS-5 and its glucosyltransferase inhibitory efficiency. *Hybridoma*, *31*(6): 430-435.

Kim, I. H., Suzuki, R., Hitotsuyanagi, Y. & Takeya, K. (2003). Three novel quassinoids, javanicolides A and B, and javanicoside A, from seeds of *Brucea javanica*. *Tetrahedron*, *59*(50): 9985–9989.

Kim, S., Thiessen, P. A., Bolton, E. E., Chen, J., Fu, G., Gindulyte, A., & Wang, J. (2015). PubChem substance and compound databases. *Nucleic Acids Research*, gkv951.

Kitagawa, I., Mahmud, T., Simanjuntak, P., Hori, K., Uji, T. & Shibuya, H. (1994). Indonesian medicinal plants. VIII. Chemical structures of three new triterpenoids, bruceajavanin A, dihydrobruceajavanin A, and bruceajavanin B, and a new alkaloidal glycoside, bruceacanthinoside, from the stems of *Brucea javanica* (Simaroubaceae). *Chemical and Pharmaceutical Bulletin*, *42*(7): 1416–1421.

Kleinberg, I. (2002). A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. *Critical Reviews in Oral Biology & Medicine*, *13*(2): 108-125.

Koch, A. L. (2000). The exoskeleton of bacterial cells (the sacculus): still a highly attractive target for antibacterial agents that will last for a long time. *Critical Reviews in Microbiology*, *26*: 1–35.

Koo, H., Rosalen, P. L., Cury, J. A., Park, Y. K., Bowen, W. H. (2002). Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrobial* Agents *Chemotherapy*, *46*(5):1302-9.

Kreth, J., Merritt, J., & Qi, F. (2009). Bacterial and host interactions of oral streptococci. *DNA and Cell Biology*, *28*: 397–403.

Kreth, J., & Herzberg, M. C. (2015). Molecular Principles of Adhesion and Biofilm Formation. In *The Root Canal Biofilm* (pp. 23-53). Springer Berlin Heidelberg.

Krisch, J., Galgóczy, L., Tölgyesi, M., Papp, T., & Vágvölgyi, C. (2008). Effect of fruit juices and pomace extracts on the growth of Gram-positive and Gram-negative bacteria. *Acta Biologica Szegediensis*, *52*(2): 267-270.

Kutsch, V. K. (2014). Dental caries: an updated medical model of risk assessment. *Journal of Prosthetic Dentistry, 111*: 280-5.

Lee, K. H., Hayashi, N., Okano, M., Nozaki, H. & Juichi, M. (1984). Antitumor agents, 65. Brusatol and cleomiscosin-A, antileukemic principles from *Brucea javanica*. *Journal of Natural Products*, *47*(3): 550-551.

Lacoste-Ferré, M. H., Hermabessiere, S., Jézéquel, F., & Rolland, Y. (2013). Oral ecosystem in elderly people. *Geriatrie et psychologie neuropsychiatrie du vieillissement*, *11*(2): 144-150.

León, J., Rojo, E., & Sánchez-Serrano, J. J. (2001). Wound signalling in plants. *Journal of Experimental Botany*, *52*(354): 1-9.

Li, M. Y., Huang, R. J., Zhou, X. D. & Gregory, R. L. (2013). Role of sortase in *Streptococcus mutans* under the effect of nicotine. *International Journal of Oral Science*, *5*(4): 206-11.

Liébana J, González MP, Liébana MJ, Parra L. (2002). Composición y ecología de la microbiota oral. En: LíébanaJ, ed. Microbilogía oral. 2ª ed. Madrid. MacGraw-Hill-Interamericana, p. 515-25

Liepiņa, I., Nikolajeva, V., & Jākobsone, I. (2013). Antimicrobial activity of extracts from fruits of *Aronia melanocarpa* and *Sorbus aucuparia*. *Environmental and Experimental Biology*, *11*: 195-199.

Lima, V. L. A. G., Mélo, E. A. & Lima, L. S. (2001). Physicochemical characteristics of bilimbi (*Averrhoa bilimbi* L.). *Revista Brasileira de Fruticultura*, *23*(2): 421-423.

Liu, J. H., Zhao, N., Zhang, G. J., Yu, S. S., Wu, L. J., Qu, J., Ma, S. G., Chen, X. G., Zhang, T. Q., Bai, J., Chen, H., Fang, Z. F., Zhao, F. & Tang, W. B. (2012). Bioactive quassinoids from the seeds of *Brucea javanica*. *Journal of Natural Products*, *75*(4): 683-8.

Liu, J. Q., Wang, C. F., Li, X. Y., Chen, J. C., Li, Y. & Qiu, M. H. (2011). One new pregnane glycoside from the seeds of cultivated *Brucea javanica*. *Archives of Pharmacal Research*, *34*(8): 1297–1300.

Llewelyn J. (1994). Oral squamous cell carcinoma. Mouthwashes may increase risk. *British Medical Journal*, *308*:1508.

Loesche, W. J., Rowan, J., Straffon, L. H. & Loos, P. J. (1975). Association of *Streptococcus mutans* with human dental decay. *Infection and Immunity*, *11*: 1252–1260.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiology and Molecular Biology Reviews*, *50*: 353-380.

Lorrain, B., Ky, I., Pechamat, L., & Teissedre, P. L. (2013). Evolution of analysis of polyhenols from grapes, wines, and extracts. *Molecules*, *18*(1): 1076-1100.

Lu, H. Z., Weng, X. H., Zhu, B., Li, H., Yin, Y. K. & Zhang, Y. X. (2003). Major outbreak of toxic shock-like syndrome caused by *Streptococcus mitis. Journal of Clinical Microbiology*, *41*(7): 3051–3055.

Lumiparta. (2012). *Nigella sativa*. Retrieved from https://www.flickr.com/photos/vukki/7538067268/in/photolist-9vyRAu-6rarjf-wGDyfT-86KLkY-cu7U6b-nJdvCK-D1NWJs-F9XWV7-tZvY9X-qWCNg5-cu7yM3-GDQ354-gkNhHQ-nQfKLE-9Sfdw8-ou8mp9-etKb6J-nrDpNt-9Sfdya-87JUrg-66B6Fh-6R58Pc-8p6DCv-9VdRsv-4wGqtg-exNdEV-tC7zXp-qreAAW-9vv4uw-cmhB6N-6oR7tJ-nM5H96-6oLX6t-H1efB-9vvPRR-6qEmSf-71oipw-6F1ggD-adBkcY-d3RB8w-6F5uG1-8oqZ79-HMf1py-uYc4nT-ZzaAo3-8ikA8V-6qAaDK-mWwfSM-6oLXRz-camtfy/

Luyengi, L., Suh, N., Fong, H. H. S., Pezzuto, J. M. & Kinghorn, A. D. (1996). A lignan and four terpenoids from *Brucea javanica* that induce differentiation with cultured HL-60 promyelocytic leukemia cells. *Phytochemistry*, *43*(2): 409–412.

Lyytikainen, O., Rautio, M., Carlson, P., Anttila, V. J., Vuento, R., Sarkkinen, H. (2004). Nosocomial bloodstream infections due to viridans streptococci in haematological and non-haematological patients: species distribution and antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*, *53*(4): 631–634.

Majid, M. Z., Mohamad Zaini, Z., & Abdul Razak, F. (2014). Apoptosis-inducing effect of three medicinal plants on oral cancer cells KB and ORL-48. *The Scientific World Journal*, *2014*.

Mandal, S., Mandal, M. D., & Pal, N. K. (2007). Antibacterial potential of *Azadirachta indica* seed and *Bacopa monniera* leaf extracts against multidrug resistant *Salmonella enterica* serovar Typhi isolates. *Archives of Medical Science*, *3*(1): 14.

Marcotte, H. and Lavoie, M. C. (1998). Oral microbial ecology and the role of salivary immunoglobulin A. *Microbiology and Molecular Biology Reviews*, *62*(1): 71-109.

Marinho, V. C., Worthington, H. V., Walsh, T. & Clarkson, J. E. (2013). Fluoride varnishes for preventing dental caries in children and adolescents. *Cochrane Database of Systematic Reviews*, *7*: 2279.

Marsh, P. D. (2000). Role of the Oral Microflora in Health. *Microbial Ecology in Health and Disease*, *12*(3): 130–137.

Marsh, P. D. (2003). Are dental diseases examples of ecological catastrophes? *Microbiology*, *149*(2): 279-294.

Marsh, P. D. (2006). Dental plaque as a biofilm and a microbial community–implications for health and disease. *BMC Oral Health*, *6*(1): S14.

Marsh, P. D. (2010). Controlling the oral biofilm with antimicrobials. *Journal of Dentistry*, *38*: S11-S15.

Marsh, P. and Martin. (1999). Oral Microbiology. 4th Ed. Great Britain: Wright

Marsh, P. D., McDermid, A. S., Keevil, C. W., & Ellwood, D. C. (1985). Environmental regulation of carbohydrate metabolism by *Streptococcus sanguis* NCTC 7865 grown in a chemostat. *Microbiology*, *131*(10): 2505-2514.

Matsui, N., Ito, M., Kuramae, H., Inukai, T., Sakai, A. & Okugawa, M. (2013). Infective endocarditis caused by multidrug-resistant *Streptococcus mitis* in a combined immunocompromised patient: an autopsy case report. *Journal of Infection and Chemotherapy*, *19*(2): 321-5.

Matta, M., Gousseff, M., Monsel, F., Poyart, C., Diebold, B., Podglajen, I., and Mainardi, J. L. (2009). First case of *Streptococcus oligofermentans* endocarditis based on *sodA* gene sequences determined after amplification directly from valvular samples. *Journal of Clinical Microbiology*, *47*: 855–856.

Mathew, L., George, S. T., Babylatha, A. K. & Geetha, C. K. (1993). Flowering and fruit development in bilimbi (*Averrhoa bilimbi* L.). *South Indian Horticulture, Kerala*, *41*(1): 41-42.

Mattos-Graner, R. O., Jin, S., King, W. F., Chen, T., Smith, D. J., & Duncan, M. J. (2001). Cloning of the *Streptococcus mutans* gene encoding glucan binding protein B and analysis of genetic diversity and protein production in clinical isolates. *Infection and immunity*, *69*(11): 6931-6941.

Merchant, A. T. (2011). Periodontitis and Dental Caries Occur Together. *Journal of Evidence Based Dental Practice*, *11*(3): 151-152.

McNab, R., & Jenkinson, H. F. (1992). Gene disruption identifies a 290 kDa cell-surface polypeptide conferring hydrophobicity and coaggregation properties in *Streptococcus gordonii*. *Molecular microbiology*, *6*(20): 2939-2949.

McNab, R., Holmes, A. R., & Jenkinson, H. F. (1995). Cell-surface polypeptides as determinants of hydrophobicity in *Streptococcus gordonii* and *Streptococcus sanguis*. *Colloids and Surfaces B: Biointerfaces*, *5*(3): 135-142.

Mitchell, J. (2011). *Streptococcus mitis*: walking the line between commensalism and pathogenesis. *Molecular Oral Microbiology*, *26*(2): 89–98.

Molan, P. C. (1992). The antibacterial activity of honey: 1. The nature of the antibacterial activity. *Bee World*, *73*(1): 5-28.

Morad, A. F. (2011). *Euphorbia tirucalli*. Retrieved from https://www.flickr.com/photos/adaduitokla/6073964956/in/photolist-afJEaJ-aDCMD5-odSHCR-QZ7MNA-4AHJpF-mRDhmd-N52ctC-qGv3uu-q1iNYj-qYUxa4-Uf1Knm-X2Ze2K-8Dw23M-gkd1J1-JWotQi-KHqHuC-SqyrTw-ugah7f-tAJq9G-Q8dNry-Q8dW7J-ehtBrZ-aNYBKM-7aQpC3-6K4H9A-52YkBS-6nYgqr-9LC2BW-HCzd4k-LgpAPJ-uxKHcP-61kXi-eagrWB-8BL185-qkttpt-8SVK3k-brdn86-bqKGyy-64tsi5-6m1Gz9-W2PD6-f3oUcb-byhwze-bE8rwA-jBdgL5-8KZ3F8-4vf3rt-gve1BX-7QuA5n-bT35Zv

Morton, J. (1987). Bilimbi. p. 128–129 In: Fruits of warm climates. Julia F. Morton, Miami, Florida.

Moye, Z. D., Zeng, L., & Burne, R. A. (2014). Fueling the caries process: carbohydrate metabolism and gene regulation by *Streptococcus mutans*. *Journal of Oral Microbiology*, *6*.

Muñoz-Elías, E. J., & McKinney, J. D. (2006). Carbon metabolism of intracellular bacteria. *Cellular Microbiology*, *8*(1): 10-22.

Muttalib K. A. (2009) Caries in 5-year-olds in Malaysia. World Congress on Preventive Dentistry.

Nache, M. N. (2013). *Nigella sativa*. Retrieved from https://www.flickr.com/photos/nache/10069396346/in/photolist-gkNhHQ-9Sfdw8-ou8mp9-etKb6J-nrDpNt-D1NWJs-9Sfdya-87JUrg-66B6Fh-6R58Pc-8p6DCv-9VdRsv-4wGqtg-exNdEV-tC7zXp-qreAAW-cmhB6N-9vv4uw-6oR7tJ-nM5H96-6oLX6t-H1efB-tZvY9X-9vvPRR-6qEmSf-71oipw-6F1ggD-adBkcY-d3RB8w-6F5uG1-HMf1py-8oqZ79-uYc4nT-8ikA8V-6qAaDK-6oLXRz-mWwfSM-qWCNg5-camtfy-pyxxDH-6nxcRj-YHYBHS-ctisUd-5EpkZo-Hd7Gnc-dV1oAP-aepho9-JEYihu-7fNTP1-7PUdjr

Nadkarni, A. K. (1976). Indian materia medica. 3rd ed. Mumbai: Popular Prakashan Pvt. Ltd., pp: 301–40.

Nalina, T. and Rahim, Z. H. A. (2007). The crude aqueous extract of *Piper betle* L. and its antibacterial effect towards *Streptococcus mutans*. *American Journal of Biotechnology Biochemistry*, *3*(1): 10-15.

Nelindah. (2014). *Vernonia amygdalina*. Retrieved from https://www.flickr.com/photos/51463027@N02/13886092344/in/photolist-Tuc3wY-na4Rtf-brUUbT-na3mvX-Rbz4vo-bDvedk-bBqcue-e3zB2o-k17gmG-7zYs3y-6LRKXd-nrrqAH-Rm9rrB-dyJ6CB-D93KKB-S1omXs-DfqtKw-7BbefP-na4Fdf-DR8QsN-7zYrAy-7BbeEZ-SpwmQW-7Bbe8T-a6jz2w

Nesbitt, W. E., Doyle, R. J., & Taylor, K. G. (1982). Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxylapatite. *Infection and Immunity*, *38*(2): 637-644.

Nishimura, J., Saito, T., Yoneyama, H., Lan Bai, L., Okumura, K. & Isogai, E. (2012). Biofilm formation by *Streptococcus mutans* and related bacteria. *Advances in Microbiology*, *2*(3): 208-215.

Nobbs, A. H., Lamont, R. J. & Jenkinson, H. F. (2009). Streptococcus adherence and colonization. *Microbiology and Molecular Biology Reviews*, *73*(3): 407-450.

Nordin, M. A. F., Harun, W. H. A. W. & Razak, F. A. (2013). Antifungal susceptibility and growth inhibitory response of oral Candida species to *Brucea javanica* Linn. extract. *BMC Complementary and Alternative Medicine*, *13*(1): 1.

Nostro, A., Cannatelli, M. A., Crisafi, G., Musolino, A. D., Procopio, F., & Alonzo, V. (2004). Modifications of hydrophobicity, in vitro adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Letters in Applied Microbiology*, *38*(5): 423-427.

Nurelhuda, N. M., Al-Haroni, M., Trovik, T. A., Bakken, V. (2010). Caries experience and quantification of *Streptococcus mutans* and *Streptococcus sobrinus* in saliva of Sudanese school children. *Caries Research*, *44*(4): 402–7.

Nwanjo, H. U. (2005). Efficacy of aqueous leaf extract of *Vernonia amygdalina* on plasma lipoprotein and oxidative status in diabetic rat models. *Nigerian Journal of Physiological Sciences*, *20*: 39-42.

Ohiagashi, M. (1994). Assessment of antibacterial activity of some traditional medicine plants. *Ethiopian Journal of Health Development*, *13*: 211-216

Okahashi, N., Nakata, M., Terao, Y., Isoda, R., Sakurai, A., Sumitomo, T., Yamaguchi, M., Kimura, R. K., Oiki, E., Kawabata, S. & Ooshima, T. (2011). Pili of oral *Streptococcus sanguinis* bind to salivary amylase and promote the biofilm formation. *Microbial Pathogenesis*, *50*(3-4): 148-54.

Okemo, P.O., Mwatha, W.E., Chhabra, S.C., Fabry, W. (2001). The kill kinetics of *Azadirachta indica* a. juss. (meliaceae) extracts on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans. African Journal of Science and Technology*, *2*: 113-118.

Okoh, I. A., Babalola, G. O., & Ilori, M. O. (1995). Effect of methanol extract of *Vernonia amygdalina* on malting and brewing properties of sorghum. *Technical quarterly (Master Brewers Association of the Americas) (USA)*.

Opara, L. U., Al-Ani, M. R., & Al-Shuaibi, Y. S. (2009). Physico-chemical properties, vitamin C content, and antimicrobial properties of pomegranate fruit (*Punica granatum* L.). *Food and Bioprocess Technology*, *2*(3): 315-321.

Oral Health Division, Ministry of Health Malaysia. National Oral Health Survey of Preschool Children 2005 (NOHPS 2005).

Oral Health Division, Ministry Of Health Malaysia. Oral Healthcare for School Children in Malaysia 2006.

Ou T‐I. Bactericide composition. US Patent Application Publication 2009/0253785 A1; 2009.

Paik, S., Senty, L., Das, S., Noe, J. C., Munro, C. L. & Kitten, T. (2005). Identification of virulence determinants for endocarditis in *Streptococcus sanguinis* by signature-tagged mutagenesis. *Infection and immunity*, *73*(9): 6064-74.

Palmer, R. J. Jr., Gordon, S. M., Cisar, J. O. & Kolenbrander, P. E. (2003). Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. *Journal of Bacteriology*, *185*: 3400-3409.

Palombo, E. A. (2011). Traditional Medicinal Plant Extracts and Natural Products with Activity against Oral Bacteria: Potential Application in the Prevention and Treatment of Oral Diseases. *Evidence-Based Complementary and Alternative Medicine*, *2011*:1–15.

Paster, B. J., Olsen, I., Aas, J. A., & Dewhirst, F. E. (2006). The breadth of bacterial diversity in the human periodontal pocket and other oral sites.*Periodontology 2000*, *42*(1): 80-87.

Pépin, J., Saheb, N., Coulombe, M. A., Alary, M. E., Corriveau, M. P., Authier, S., & Nguyen, M. (2005). Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*–associated diarrhea: a cohort study during an epidemic in Quebec. *Clinical Infectious Diseases*, *41*(9): 1254-1260.

Pero, R. W., & Lund, H. (2009). In Vivo Treatment of Humans with Quinic Acid Enhances DNA Repair and Reduces the Influence of lifestyle Factors on Risk to Disease. *International Journal of Biotechnology and Biochemistry*, *5*(3): 293-305.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, 29(9): e45-e45.

Prabu, G. R., Gnanamani, A. & Sadulla, S. (2006). Guaijaverin -- a plant flavonoid as potential antiplaque agent against *Streptococcus mutans*. *Journal of Applied Microbiology*, *101*(2): 487-95.

Prasad, S. H. K. R., Swapna, N. L. & Madan, Prasad. (2011). Efficacy of *Euphorbia Tirucalli* (L.) Towards Microbicidal Activity Against Human Pathogens. *International Journal of Pharmacy and Biological Sciences*, *2*(1): 229-235.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher H.J., 1989. The effects of pellicle formation on streptococcal adhesion to human enamel and artificial substrata with various surface free-energies. *Journal of Dental Research*, 68: 463-467.

Rahim, Z. H. A., Fathilah, A. R., Irwan, S., & Wan Nordini Hasnor, W. I. (2008). An artificial mouth system (NAM model) for oral biofilm research. *Research Journal of Microbiology*, *3*(6): 466-473.

Reddy, M. K., Gupta, S. K., Jacob, M. R., Khan, S. I., & Ferreira, D. (2007). Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta medica*, *53*(05): 461-467.

Regassa, A. (2000). The use of herbal preparation for tick control in Western Ethiopia. *Journal of the South African Veterinary Association*, *71*: 240–3.

Rosan, B. & Lamont, R. J. (2000). Dental plaque formation. *Microbes and Infection*, *2*(13): 1599-1607.

Ruby, J., & Goldner, M. (2007). Nature of symbiosis in oral disease. *Journal of dental research*, *86*(1): 8-11.

Russell, R. R. (2008). How has genomics altered our view of caries microbiology? *Caries Research*, *42*: 319–327.

Saad, B., Azaizeh, H., & Said, O. (2005). Tradition and perspectives of Arab herbal medicine: a review. *Evidence-Based Complementary and Alternative Medicine*, *2*(4): 475-479.

Sakaki, T., Yoshimura, S., Tsuyuki, T., Takahashi, T., Honda, T., & Yadanzioside P, (1986). A new antileukemic quassinoid glycoside from *Brucea*

javanica (L.) Merr with the 3-O-(beta-D-glucopyranosyl) bruceantin structure. *Chemical and Pharmaceutical Bulletin*, *34*(10): 4447–4450.

Scamperdale. (2009). *Vernonia amygdalina*. Retrieved from https://www.flickr.com/photos/36517976@N06/3789418000/in/photolist-na4Rtf-brUUbT-nrrqAH-na3mvX-Rm9rrB-D93KKB-S1omXs-DfqtKw-7BbefP-na4Fdf-DR8QsN-7zYrAy-7BbeEZ-7Bbe8T-a6jz2w-bBqcYT-b8dC4n-b8dxVg-7Bf3Yb-7zYqZ7-7Bf3GQ-b8dzS6-EhD7AG-k15wL2-7Bf44E-k17iYq-7zYsB7-7zUGF6-k15Aup-k15Abi-7BbdXx-7Bf3PG-7Bf48w-7zYskh-Rbz4i9-5XWcoy-Ewg8VX-c6KvZN-yNVoLP-HHxss6-sNYpCz-D9ppeU-RbwAoS-EbKK2Y-b8dDuM-8Vj7Eh-7Bf3MC-7zYsJw-6LRKX7-5XWcib

Schafer, T. E. & Adair, S. M. (2000). Prevention of dental disease. The role of the pediatrician. *Pediatric Clinics of North America*, *47*(5): 1021–42.

Schilling, K. M., Blitzer, M. H. & Bowen, W. H. (1989). Adherence of *Streptococcus mutans* to glucans formed in situ in salivary pellicle. *Journal of Dental Research, 68*: 1678–1680.

Scott, J. R. & Barnett, T. C. (2006). Surface proteins of Gram-positive bacteria and how they get there. *Annual Review of Microbiology, 60*: 397–423.

Selwitz, R. H., Ismail, A. I. & Pitts, N. B. (2007). Dental caries. *Lancet, 369*(9555): 51-9.

Senadheera, M. D., Guggenheim, B., Spatafora, G. A., Huang, Y. C. C., Choi, J., Hung, D. C. & Cvitkovitch, D. G. (2005). A *VicRK* signal transduction system in *Streptococcus mutans* affects *gtfBCD, gbpB,* and *ftf* expression, biofilm formation, and genetic competence development. *Journal of Bacteriology, 187*(12): 4064-4076.

Shay, K. (2002). Infectious complications of dental and periodontal diseases in the elderly population. *Clinical Infectious Diseases, 34*(9): 1215-1223.

Shemesh, M., Tam, A., & Steinberg, D. (2007). Expression of biofilm-associated genes of *Streptococcus mutans* in response to glucose and sucrose. *Journal of Medical Microbiology, 56*(11): 1528-1535.

Shu, Y., Liu, Y., Li, L., Feng, J., Lou, B., Zhou, X. & Wu, H. (2011). Antibacterial activity of quercetin on oral infectious pathogens. *African Journal of Microbiology Research, 5*(30): 5358–5361.

Shuid, A. N., Mohamed, N., Mohamed, I. N., Othman, F., Suhaimi, F., Mohd Ramli, E. S, Muhammad, N. & Soelaiman, I. N. (2012). *Nigella sativa*: A Potential Antiosteoporotic Agent. *Evidence-Based Complementary and Alternative Medicine, 2012*: 1-6.

Siqueira, W. L., Helmerhorst, E. J., Zhang, W., Salih, E., & Oppenheim, F. G. (2007). Acquired enamel pellicle and its potential role in oral diagnostics. *Annals of the New York Academy of Sciences, 1098*(1): 504-509.

Smigel, K. (1991). High alcohol mouthwashes are under scrutiny. *Journal of the National Cancer Institute, 83*:751.

Socransky, S. S. & Haffajee, A. D. (2002). Dental biofilms: difficult therapeutic targets. *Periodontology 2000, 28*: 12-55.

Sofowora, A., Ogunbodede, E., Onayade, A. (2013). The role and place of medicinal plants in the strategies for disease prevention. ***African Journal of Traditional, Complementary and Alternative medicines*****,** *10*(5): 210-29.

Sokmen, A., Jones, B. M. & Erturk, M. (1999). The in vitro antibacterial activity of Turkish medicinal plants. *Journal of Ethnopharmacology*, *67*: 79–86.

Straub, A. M., Salvi, G. E. & Lang, N. P. (1998). Supragingival plaque formation in the human dentition. In Lang N. P, Attstrom R., Loe H. *Proceeding of the European workshop on mechanical plaque control*. Chicago: Quintessence, 72-84.

Tajkarimi, M., & Ibrahim, S. A. (2011). Antimicrobial activity of ascorbic acid alone or in combination with lactic acid on *Escherichia coli* O157: H7 in laboratory medium and carrot juice. *Food Control*, *22*(6): 801-804.

Tamesada, M., Kawabata, S., Fujiwara, T., & Hamada, S. (2004). Synergistic effects of streptococcal glucosyltransferases on adhesive biofilm formation. *Journal of dental research*, *83*(11): 874-879.

Tariq, M. (2008). *Nigella Sativa* Seeds: Folklore Treatment in Modern Day Medicine. *Saudi Journal of Gastroenterology*, *14*(3): 105–106.

Taylor, J. J., & Preshaw, P. M. (2016). Gingival crevicular fluid and saliva. *Periodontology 2000*, *70*(1): 7-10.

Tenovuo, J., & Lagerlöf, F. (1994). Saliva. *Thylstrup A, Fejerskov O. Textbook of clinical cariology. 2nd ed. Copenhagen: Munksgaard*, 713-17.

Touger-Decker, R. & van Loveren, C. (2003). Sugars and dental caries. *American Journal of Clinical Nutrition*, *78*(4): 881-892.

Tsuchiya, H., & Iinuma, M. (2000). Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from *Sophora exigua*. *Phytomedicine*, *7*(2): 161-165.

Van Damme, P. L. J. (2001). *Euphorbia tirucalli* for high biomass production. In: Schlissel A, Pasternak D, editors. Combating desertification with plants, Kluwer Academic Pub., pp. 169–187.

Van der Waaij D., Berghuis-de Vries J. M., Lekkerkerk L.-v. (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *Journal of Hygiene*, *69*: 405–411.

Vasconcelos, L. C. D. S., Sampaio, F. C., Sampaio, M. C. C., Pereira, M. D. S. V., Higino, J. S., & Peixoto, M. H. P. (2006). Minimum inhibitory concentration of adherence of *Punica granatum* Linn (pomegranate) gel against *S. mutans*, *S. mitis* and *C. albicans*. *Brazilian Dental Journal*, *17*(3): 223-227.

Vollmer, W., Blanot, D., de Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*, *32*: 149–167.

Vukosavljevic, D., Custodio, W., Buzalaf, M. A., Hara, A. T., & Siqueira, W. L. (2014). Acquired pellicle as a modulator for dental erosion. *Archives of Oral Biology*, *59*(6): 631-638.

Waczuk, E. P., Kamdem, J. P., Abolaji, A. O., Meinerz, D. F., Caeran Bueno, D., Nascimento, T. K., Do Canto-Dorow, T. S., Boligon, A. A., Athayde, M. L., Da Rocha, J. B & Avila, D. S. (2015). *Euphorbia tirucalli* aqueous extract induces cytotoxicity, genotoxicity and changes in antioxidant gene expression in human leukocytes. *Toxicology Research*, *4*: 739-748.

Wang, H. W., Liu, Y. Q., Yan, Z. J., Wei, S. L. & Ye, J. Q. (2011). A gas chromatography-mass spectrometry analysis of the essential oils from *Brucea javanica* extracted with different methods. *Fine Chemicals*, *28*(7): 668–697.

Wardwell, L. H., Huttenhower, C., & Garrett, W. S. (2011). Current concepts of the intestinal microbiota and the pathogenesis of infection. *Current Infectious Disease Reports*, *13*(1): 28-34.

Weidenmaier, C. & Peschel, A. (2008). Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nature Reviews Microbiology*, *6*: 276–287.

Whiley, R. A. & Beighton, D. (1998). Current classification of the oral streptococci. *Oral Microbiology and Immunology*, *13*:195–216.

Wild, R. (1994). The complete book of natural and medicinal cures. *Emmaus, Pa: Rodale Press, Inc*, 50-56.

Wood, S. R., Kirkham, J., Marsh, P. D., Shore, R. C., Nattress, B. & Robinson, C. (2000). Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. *Journal of Dental Research*, *79*: 21-27.

Xie, H., Cook, G. S., Costerton, J. W., Bruce, G., Rose, T. M., & Lamont, R. J. (2000). Intergeneric communication in dental plaque biofilms. *Journal of Bacteriology*, *182*(24): 7067-7069.

Yamashita, Y., Bowen, W. H., Burne, R. A., & Kuramitsu, H. K. (1993). Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. *Infection and Immunity*, *61*(9): 3811-3817.

Zakaria, Z. A., Zaiton, H., Henie, E. F. P., Mat Jais, A.M. & Engku Zainuddin, E. N. H. (2007). In vitro Antibacterial Activity of *Averrhoa bilimbi* L. Leaves and Fruits Extracts. *International Journal Tropical Medicine*, *2*(3): 96-100.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature, 415*(6870): 389-395.

Zero, D. T., Van Houte, J., & Russo, J. (1986). Enamel demineralization by acid produced from endogenous substrate in oral streptococci. *Archives of Oral Biology, 31*(4): 229-234.

Zhang, L. & Mah, T. F. (2008). Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *Journal of Bacteriology, 190*: 4447–4452.