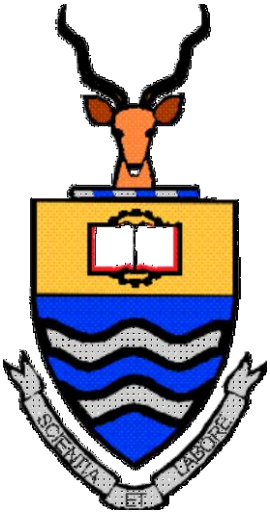
Effect of *Punica granatum* L. (pomegranate) on the oral pathogens and the identification of metabolites responsible for beneficial effects

Zandiswa Modjadji Gulube



A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Doctor of Philosophy in Medicine

Johannesburg, 2019

DECLARATION

I, Zandiswa Modjadji Gulube, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in Medicine to University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……..Signature of candidate

……day of ……February……..2019

DEDICATION

Roselina Nonzwanga Salman

Poster presentation:

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ABSTRACT

Introduction

Dental caries and periodontal diseases are infections caused by accumulation and multiplication of bacteria in the oral cavity. Dental caries is caused by demineralisation of the tooth due to the acids produced by *Streptococcus mutans* from fermentable carbohydrates. Pathogenic characteristics of *S. mutans* include biofilm formation, production of extracellular polysaccharides, acidogenicity and aciduricity. Periodontal diseases which are caused by many anaerobic oral bacteria are diseases of the tooth supporting tissues. Among these anaerobes, *Porphyromonas gingivalis (Pg)* has been identified as a keystone pathogen which also produces cysteine proteases, also called gingipains that cause tissue destruction and inactivate the host immune system. Both these oral infections can be prevented by controlling bacterial biofilm also called plaque with antimicrobial oral hygiene products. Medicinal plants have shown antimicrobial activity against these oral bacteria. Various parts of the *Punica granatum* L. fruit, commonly known as pomegranate are known to have antimicrobial effect against many bacteria. Its use in the oral cavity to control plaque is also highlighted. However, the effect of the peel extracts on the virulence factors of cariogenic bacteria and the periodontal pathogen, and the responsible beneficial compound has not been studied. The aim of this study was to evaluate the effect of pomegranate fruit peel on the oral pathogens and identify the metabolites responsible for the beneficial effects.

Methods

Pomegranate peel and seed extracts were prepared using methanol, DCM:methanol, hexane, water and acetone. Minimum bactericidal concentrations (MBC) were obtained against *S. mutans* and lactobacilli using double dilution technique. Based on MBC, three different subinhibitory concentrations of the methanol peel extract were evaluated for their effect on biofilm formation, acid and extracellular polysaccharides production in *S. mutans*. The MBC

against *P. gingivalis, Fusobacterium, Capnocytophaga* sp. and *P. intermedia* were also determined. The DCM: methanol crude peel extract was fractionated using solvent-solvent extraction and chromatography. The MBC's of fractions and subfractions against *S. mutans* were determined. Bioactive subfractions with the best activity were examined for the inhibition effect on biofilm formation and acid production. The results were analysed using Kruskal-Wallis and Wilcoxon Rank Sum Tests. The effect of the crude extract and subfractions on the proteinase production in *P. gingivalis* was also studied. Purification and structural elucidation were performed by means of preparative HPLC, high resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR) and Fourier transform infra-red spectroscopy (FTIR). In addition, the cytotoxicity of the crude plant extract and the bioactive subfractions on HEK 293 embryonic kidney cell was also established.

Results

The MBCs for the crude peel and seed extract against *S. mutans* and Lactobacilli ranged between 6.25 and 25 mg/ml whereas for the periodontal pathogens *P. gingivalis, P. intermedia, Capnocytophaga* and *Fusobacterium* they ranged between 0.025 – 3.125 mg/ml. MBC values for the seed extracts were much higher than the peel extracts. After 6 and 24 h, at subinhibitory concentrations, the methanol crude peel extract significantly reduced biofilm-formation by 91 % and 65 % respectively ($p < 0.01$). It also significantly inhibited acid production in *S. mutans* ($p < 0.01$). The crude peel extract did not inhibit the production of soluble extracellular polysaccharides (EPS) in either the biofilm or the planktonic growth. However, it significantly reduced the insoluble EPS in the biofilm and the plantktonic ($p < 0.01$) form of *S. mutans*. Chemical analysis resulted in 4 fractions and 10 subfractions. The subfractions F 3.2 and F 4.1 displayed the best MBC on *S. mutans* of 6.25 and 12.5 mg/ml respectively. Subfractions F 3.2 and F 4.1 significantly reduced the biofilm formation ($p <0.01$) and acid production compared to the controls. In some tests the reduction in the acid production was due to the antibacterial

effect of the subfractions. The crude peel extract, subfractions F 3.2 and F 4.1 reduced proteolytic activity in *P. gingivalis* where Arg- gingipain was inhibited up to 54 % and Lys-gingipain up to 93 %. The UHPLC-MS showed presence of hydrolysable tannins, major bioactive components in the peel. Structural elucidation by NMR identified 2 compounds to be present, β-sitosterol in the subfration F 3.2 while galloyl-HHDP-hexoside was found in both subfractions. At 1.6 mg/ml concentration crude peel extract, subfrations F 3.2 and, F 4.1 showed cell viability of 62, 24 and 58 % respectively.

Conclusions

Antibacterial activity of the peel extracts of *P. granatum* proved to be better than the seed extracts therefore the peel extract was further explored. Subfractions, which were identified as β-sitosterol and galloyl-HHDP-hexoside showed anti-biofilm and anti-acidogenic activity which were present in the crude extract. However, the purified compounds did not show improved activity. The crude extract and the purified compounds also inhibited the growth of periodontal pathogens and proteolytic activity in *P. gingivalis*. At high concentrations the crude extract and the compounds will kill the cariogenic bacteria and periodontal pathogens. At low concentrations they will reduce the virulence of these two major oral pathogens. The crude extract and identified compounds proved to be safe. This suggests that *P. granatum* peel has the potential to control and prevent dental caries and periodontal diseases.

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LIST OF ABBREVIATIONS AND ACRONYMS

A. naeslundii	*Actinomyces naeslundii*
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Arg	Arginine
C	Carbon
CDC	Centre for disease control and prevention
CFU	Colony forming unit
CO_2	Carbon dioxide
DCM	Dichloromethane
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EPS	Extracellular polysaccharides
FTIR	Fourier-transform infrared spectroscopy
GTF	Glucosyltransferase
H	Hydrogen
HPLC	High performance liquid chromatography
IL-6-174	Interleukin-6
Lys-	Lysine gingipain

LPXTG	Leucine-Proline-any-Threonine-Glycine
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentrations
mRNA	Messenger ribonucleic acid
MS	Mitis salivarius agar
N	number
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
PBS	Phosphate buffered saline
PEP	Phosphoenolpyruvate
PG	Phosphoglyceric acid
PTS	Phosphotransferase system
Pg	*Porphyromonas gingivalis*
RPM	Revolutions per minute
S. mutans (SM)	*Streptococcus mutans*
TLC	Thin-layer chromatography
UHPLC-HRMS	Ultra high performance liquid chromatography-high resolution mass spectrometry

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Oral diseases continue to be the major health problem worldwide. Dental expenditure in the United States alone in 2003 was $74.3 billion with forty four percent paid out of pocket by patients and seven percent paid by state government sources (CDC 2011). Besides economics, the experience of pain, problem eating, chewing, smiling and communication due to oral diseases has a major impact on people's lives. The most prevalent oral diseases are dental caries, periodontal disease, oral abscesses and oral candidiasis. Most of these diseases are exacerbated by accumulation of bacterial plaque on tooth surface. The causative agents for such infections have been identified as bacteria or yeast. Several approaches have been emphasized for the prevention and treatment of these infections. In dental caries mouthwash and fluoride treatment are used in the initial stage while dental fillings and sealants are used for the advanced stage of demineralization. Periodontal diseases has been treated with scale and polish, root planning and surgery together with antimicrobial mouth rinses and antibiotics. However some of these mouth rinses, frequent use of antibiotics and drugs have side effects.

Healing power found in plants is an ancient idea with about 80% of the people in developing countries using traditional medicines for their health care. These traditional plants have also been used for treatment of oral infections. Pomegranate (*Punica gratunum* L.) is one of the traditional plants that have been widely researched and is known for its biological activities such as antibacterial, antiviral, antidiarrheal, antifungal, antitumor and anti-ulcer effect. Pomegranate derived extracts have been researched widely against dental plaque formation and oral diseases. Pomegranate has shown some significant reduction in clinical signs of chronic

periodontitis. Its crude hydroalcoholic extract has shown some antibacterial activity against dental plaque. However, the majority of these studies were clinical trials and they used visible inspection to evaluate the effect of extracts. Therefore, this study scientifically evaluated the antimicrobial effect of several pomegranate crude extracts obtained using six different solvents *in vitro*. It identified the most effective solvent in the preparation of crude extract. Three sub-inhibitory concentrations of the identified crude extract were studied for the anti-virulence properties on the cariogenic and periodontal bacteria. In addition, the identified extract was purified further using column chromatography and the obtained fractions/subfractions were screened for antimicrobial effect. Identification of biochemical constituents present in the bioactive subfractions were performed using mass spectrometry, nuclear magnetic resounance and fourier-transform infrared spectroscopy.

1. Literature review

1.1 Oral microbiome

The human oral cavity is a home to more than seven hundred bacterial species which reside in harmony with the host. Most of these bacteria are commensals and opportunistic pathogens. Different sites like teeth, buccal mucosa, dorsum of tongue, tooth surfaces (subgingingival and supragingival), crevicular epithelium and gingival crevice in the oral cavity can harbour these bacterial species. Some of these bacteria are listed in Table 1.1. Homeostasis is a characteristic of oral microbiota in healthy individual whereby these microorganisms are capable of cohabiting in the different sites of the mouth, depending on the pH, availability of nutrients and mucous surface. The oral microbiota function as part of the host defense by acting as a barrier e.g. by competing for essential nutrients and creation of unfavorable conditions to exogenous organisms that may be pathogenic to the host.

Most of these microbes are harmless, but under certain conditions some can cause oral infections (Sakamoto *et al.,* 2005). Microbial homeostasis can break down, and major shifts in the composition of the microflora can occur. The microbiota will then shift from being healthy towards a pathogenic state through interactions between bacterial activity and the environmental factors (Marsh 1994). The shift of microorganisms may be due to various factors such as bacterial overgrowth, change in lifestyle, diet and use of antimicrobials (Dagli *et al.,* 2016). Poor oral hygiene leads to biofilm build up that is more diverse and become pathogenic which leads to oral diseases like dental caries, gingivitis, periodontitis etc. The pathogenic state requires good oral hygiene practices like brushing with toothpastes or oral rinse (Wade 2013). These oral hygiene practices reduce the number of bacterial load in the oral cavity and keep homeostasis.

Table 1.1 Human oral cavity microbiota

Site	Bacteria	Gram reaction	Morphology
Tongue and buccal mucosa	*Streptococcus salivarius*	Positive	Cocci
	Veillonella	Negative	Cocci
	Diphtheroids	Positive	Bacilli
	Stomacoccus mucilagenosus	Positive	Cocci
Teeth (sub- and supragingival plaque)	*Streptococcus* species	Positive	Cocci
	S. mutans	Positive	Cocci
	Lactobacilli	Positive	Rods
	S. sanguinis	Positive	Cocci
Gingival crevice	*Streptococcus* species	Positive	Cocci
	Actinomyces	Positive	Rods
	Fusobacterium	Negative	Rod
	Treponema	Negative	Spiral
	Veillonella	Negative	Cocci
	Prevotella	Negative	Bacilli
	Porphyromonas and	Negative	Rods
	Stomacoccus mucilagenosus	Negative	Cocci

1.1.1 Factors influencing oral microbial flora

Several factors have been identified to influence the oral microbial flora such as host diet, host habits, age and the quality of saliva. Although the salivary glycoproteins are the major source of nutrient for these oral bacteria, dietary compounds do transiently provide nutrients and increase the growth of certain bacteria. For example, oral cavities of individuals with high sucrose intake would contain high numbers of *Streptococcus mutans* and lactobacilli. Similarly, smokers carry high number of *Fusobacterium* species in their oral cavities and females on oral contraceptives carry high number of yeast. Babies and edentulous adults would mostly carry *Streptococcus salivarius*, lactobacilli, *Candida* and *Staphylococcus* and no anaerobic bacteria. Interestingly, there is no difference found among the salivary oral microbiota of individuals from different countries worldwide (Nasidze *et al.*, 2009). Moreover, the worldwide similarity in oral microbiota is not influenced by what people eat and the environment but the principal contributing factor is the species carrier (Wade 2013).

Research has shown that the salivary bacterial profile is more diverse depending on the intraoral location (Paster *et al.*, 2006), especially with those individuals with established oral diseases such as dental caries and periodontitis when compared with orally healthy people (Belstrom *et al.*, 2016). Moreover, human commensal found in the oral cavity is responsible for dental caries and periodontal diseases in comparison to the microbiota found somewhere else in the body.

1.2 Dental Caries

Dental caries, or tooth decay is a disease of the mineralized tooth due to the acid production by oral bacteria from fermentable carbohydrate (Figure 1.1). It can lead to the loss of tooth structure, inadequate tooth function, pain, and infection. Dental caries is an ancient disease that has affected the human race through its history. It is the most prevalent multifactorial disease in the world whose onset and progression is influenced by many diverse factors such as bacteria, diet, environment and socioeconomics.

Dental caries results from an ecological imbalance in the physiological equilibrium between tooth minerals and oral microbial biofilms (Fejerskov 2004). Bacteria live on teeth in micro-colonies that are encapsulated in an organic matrix of polysaccharides, proteins, and DNA secreted by the cells. Teeth offer non-shedding surfaces for microbial colonisation and large numbers of bacteria and their by-products accumulate in a biofilm on tooth surfaces in healthy and diseased sites (Scheie and Petersen 2004).

Figure 1.1 Carious lesions on different sites of the teeth (Adapted from dentalcare.com)

In individuals who frequently ingest high levels of carbohydrates, there is high frequency of acid production (Hans *et al.*, 2016). This acid causes local pH values to fall below a critical

value of pH 5.5 resulting in the erosion of the enamel, cementum and/or dentin. If the diffusion of calcium, phosphate, and carbonate out of the tooth is allowed to continue, cavitation (dental caries) will eventually take place and this process is termed demineralisation (Featherstone 2004).

Demineralisation can be reversed in its early stages through uptake of calcium, phosphate and fluoride. Fluoride acts as a catalyst for the diffusion of calcium and phosphate into the tooth, which remineralises the crystalline structures in the lesion. Furthermore, the constant acid production, poor buffering capacity of saliva and frequent low pH in the mouth results apart from enamel decalcification in changing the composition of the oral microbiota to one that favours aciduric species (Takahashi and Nyvad 2011).

The aciduric species, particularly *S. mutans* and lactobacilli, continue to multiply while producing acid. The active state of these microbes under aciduric condition, thus exacerbates the damage to the dental hard tissues. While dental plaque continues to be formed by primary colonisers on the tooth surface, *S. mutans* and lactobacilli are among oral bacteria that co-aggregate joining the developing biofilm via co-aggregation interactions (Wade 2013). *S. mutans* is also responsible for the production of extracellular polysaccharides (EPS) which further enriches the biofilm matrix. The EPS serve as building blocks for the biofilm, cause ease of adherence of bacteria as well as food storage for *S. mutans* which cannot be accessed by any other microbes.

Therefore, biofilm formation, acidification of the milieu and the maintenance of the low pH environment at the tooth biofilm interface are major controlling virulence factors that modulate dental caries pathogenesis. Although the nature of the disease and the methods of treatment

have advanced tremendously during the past century, the disease is still not controlled worldwide (Shu *et al.,* 2000).

1.2.1 *Streptococcus mutans*

Streptococcus mutans, is a gram positive bacteria that plays a major role in the development of dental caries. These bacteria generally appear as pairs or chains, spherical to ovoid in shape, nutritionally fastidious and with fermentative metabolism - Figure 1.2 (Abachi *et al.,* 2016). They are classified based on the cell-surface rhamose-glucose polysaccharides into four different serological groups (c, e, f, and k). Serotype c constitutes 70-80 % of strains isolated from the oral cavity, with the rest being 20 % or less for serotype e and serotype k respectively (Nakano *et al.,* 2010). However, non-serotype c strains have been isolated in higher rates from the heart valves and atheromatous plaques with serotype k, 12 % more than in the oral cavity (Nakano *et al.,* 2010).

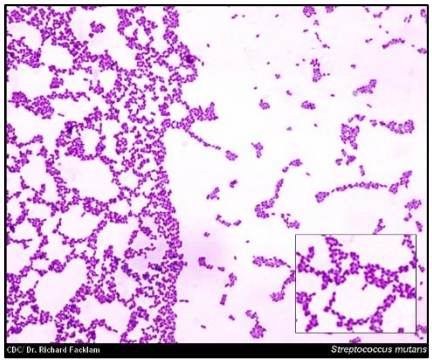


Figure 1.2 *S. mutans* gram positive cocci in chains (Adapted from Smith collection)

Mitis salivarius agar (MS) was one of the first media to be developed as a selective medium for culturing the streptococci species in laboratory (Carlsson 1967). Later it was used to identify *S. mutans* based on the unique appearance of the colonies grown on MS. Conversely, high levels of other streptococci and enterococci interferes the growth of *S. mutans* on MS, and resulted in false negatives and underestimations. Therefore, addition of bacitracin and tellurite

proved to be successful in selection of *S. mutans*. They are cultured by incubation at 37^0 C for 48 hours under 10 % CO_2 (Figure 1.3).

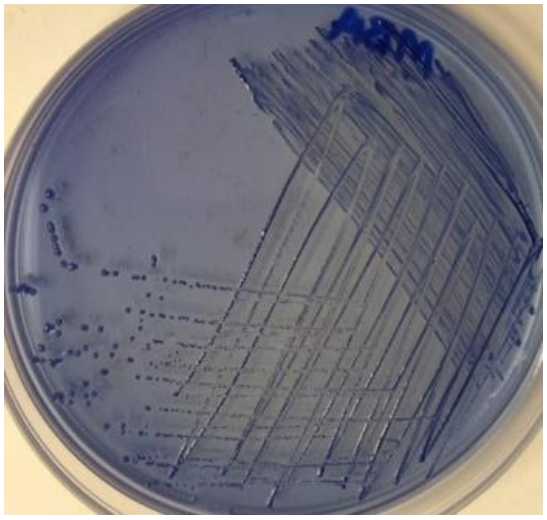


Figure 1.3 *S. mutans* colonies growing on a mutans bacitracin agar plate

S. mutans produces both lactic acids and extracellular polysaccharides through the process of metabolic activity of fermentable carbohydrates such as sucrose. They are acidogenic as well as aciduric with the ability to survive, grow and maintain their metabolism under acidic conditions (Marsh 2009). Their ability to tolerate acid is partly due to a membrane-bound protein called F-ATPase, which extrudes protons out of the cells, preventing a decrease in the intracellular pH and consequent damage to acid sensitive enzymes, DNA and proteins (Quivey *et al.*, 2001). In *S. mutans*, sucrose can be internalized by the sugar:phosphotransferase system (PTS) via ABC transporters which are members of the ATP-binding cassette (Webb *et al.*, 2008). Also, three major GTFs are encoded by *S. mutans*: GTFB and GTFC produce predominantly α 1,3-linked water-insoluble glucans, and GTFD produces an α 1,6-rich, water-soluble glucan (Bowen and Koo 2011).

This organism has also been isolated from the blood of several patients with bacteremia and infective endocarditis (Nakano *et al.*, 2010). Infective endocarditis (IE) affecting the cardiovascular system is a typical infectious disease caused by disseminating microorganisms into the bloodstream. Blood-borne staphylococci from contaminated medical devices or streptococci from gingival wounds during dental surgery are the most common endocarditis-inducing pathogens that can easily gain access into the bloodstream and colonize the injured heart valves to induce thrombus formation (Moreillon and Que 2004).

1.2.2 Pathogenesis

Although dental plaque contains microbial mix flora, *S. mutans* is the most important bacteria that is implicated in the initiation and progression of dental caries. In addition, lactobacilli are also implicated in the progression of this disease. Both of these pathogens are acidogenic (produce acid) and aciduric (withstand acid and continue metabolisms at low pH) bacteria that metabolize carbohydrates into acids, resulting in an acidic condition that causes dissolution of enamel in the adjacent teeth. Extracellular polysacharides formed in situ facilitate local accumulation of *S. mutans* (via membrane-associated glucan-binding proteins) while embedding them in a diffusion-limiting polymeric matrix (Takahashi and Nyvad 2011). *S. mutans* is the principal etiological agent of human caries and is one of the primary colonizers to the tooth surface. Pathogenic characteristics of *S. mutans* include biofilm formation, production of extracellular polysaccharides, acidogenicity and aciduricity (Loesche 1986).

While little is known about adhesive properties of lactobacilli (Menezes *et al.*, 2006). A strong correlation has been established between the salivary *Lactobacillus* count and dental caries (Badet and Thebaud, 2008). In some cases, lactobacilli could also play a beneficial role by

inhibiting the growth, adherence and production of extracellular polysaccharides (Chung *et al.*, 2004) by *S. mutans*.

1.2.3 Biofilm Formation

Biofilm that is firmly attached to the tooth surface is the prime biological factor associated with dental caries. Tooth pellicle is a thin film that covers the tooth soon after cleansing and it originates from salivary proteins. Oral bacteria such as viridans streptococci which are early colonizers can now colonize the pellicle on the tooth surface (Pereira *et al.*, 2006). *S. mutans* species are the first to adhere selectively to and colonize saliva coated teeth. The streptococcal cell wall component mediate adherence to various salivary receptors. The interaction between the binding proteins such as the bacterial alpha amylase and saliva amylase facilitate attachment of these bacteria to the tooth surface. In addition, *S. mutans* also expresses several surface adhesins that can bind to salivary pellicles formed on the teeth (Mitchell 2003), whereas sucrose-dependent adherence is mediated by glucan binding proteins and water-insoluble glucans produced from sucrose through glucosyltransferase (GTF) enzymes produced by this bacteria (Koga *et al.*, 1986).

Subsequent attachment of other bacteria involves a variety of specific co-aggregation reactions that involves many oral bacteria considered secondary colonizers. Lactobacillus is not involved in the initiation of biofilm formation but as caries lesion progresses it has been found to be one of the habitants. Previously, lactobacillus has demonstrated a poor ability to form mono-culture biofilm but in the presence of *A. naeslundii* which are also found in the oral cavity, it predominated in substantial amount of biofilm (Shu *et al.*, 2000).

Indeed, the biofilm will continue to form on the tooth surfaces which promotes accumulation of microcolonies. A polymeric matrix will be formed and it protects entrenched bacteria from disruption by the antibacterial agents and it offers 3D scaffold for interconnected progression (Xiao *et al.,* 2012). A study by Vinogradov *et al*., 2004 has shown similar viscoelastic properties in the biofilm to those of organic polymers (Vinogradov *et al.,* 2004). Moreover, the existence of EPS like glucans augment the power for *S. mutans* attachment on the apatitic surfaces which leads to glucan-rich matrix and cell–glucan adhesions in the critical structural integrity of the biofilm 3D architecture (Cross *et al.,* 2007).

1.2.4 Acid Production

The biofilm thickens and matures as the nutrients from saliva and carbohydrates derived from food are intermittently supplied. This plaque maturation results in anaerobic condition which allows acid production. These acidic and anaerobic conditions results in colonization by aciduric and oxygen labile bacteria such as mutans streptococci (Takahashi 2005). *S. mutans* have been identified as the one of the bacteria that is involved in acid production. They are able to ferment carbohydrates particularly sucrose from the diet through the Embed-Meyerhof-Parnas pathway to form lactic acid, formic, acetic, succinate and other organic acid. These acids thus begin the dissociation of the tooth enamel consequently leading to localized decalcification and breakdown of calcified dental tissue (Hardie 1992). This is an ongoing process because carbohydrates are frequently (2-5 times a day) consumed by humans. Each time sugars are eaten, the plaque pH drops within 5-10 minutes and it would remain at the critical pH of 5.5 depending on the oral hygiene, diet and the physiology of an individual. This phenomenon creates a curve called Stephan curve, a graph of pH verses time after sugar consumption (Figure 1.4). Therefore, an increase in the frequency of carbohydrate intake would increase the susceptibility to dental caries.

Even though there is little information regarding attachment and biofilm formation of lactobacillus, they have been identified as cariogenic. Lactobacilli are able to produce lactic, acetic and other acids and grow and survive in acidic environment. They are able to ferment carbohydrates in two metabolic processes that are homolactic fermentation and heterolactic fermentation (Badet and Thebaud 2008, Caufield *et al.,* 2007). Whatever method used by lactobacillus, it results in acidification of the environment.

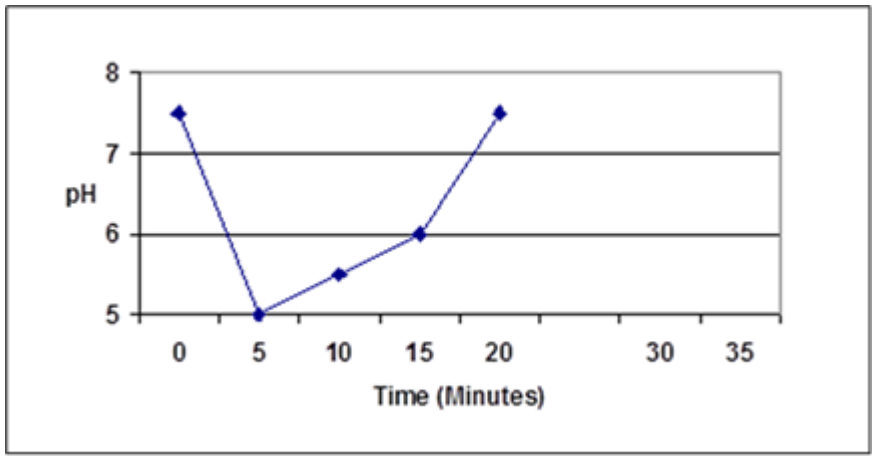


Figure 1.4 Stephan curve, buffering capacity of plaque

1.2.5 Extracellular polysaccharides production (EPS)

The EPS matrix is formed mainly by the *S. mutans* in the dental biofilms. They are synthesized by GTFs from *S. mutans* which the majority are glucans that are responsible for binding of bacteria on the tooth surface to institute pathogenicity of biofilm and also offer nutrients. Research has found EPS production to affect progression of dental caries tremendously. The three types of GTFs from *S. mutans* are products of the GTF B, GTF C, and GTF D genes. These GTFs synthesize soluble and insoluble glucans in the presence of sucrose (Leme *et al.,* 2006, Loesche 1986). The EPS formed in situ facilitate local accumulation of *S. mutans* (via membrane-associated glucan-binding proteins) while embedding them in a diffusion-limiting polymeric matrix (Bowen and Koo 2011). They attaches to the tooth surface through the salivary pellicle as well as other bacterial surfaces and produce organic acids (Hannig *et al.,*

2008). The capacity of *S. mutans* to use these polysaccharides as short-term storage compounds withdraws an additional ecological benefit while increasing the extent of acidification within the biofilm (Burne *et al.,* 1996). Thus, *S. mutans* thrives in the complex oral microbiome and effectively modulates the transition from nonpathogenic to cariogenic biofilms.

1.2.6 Control and prevention of dental caries

Prevention and control of dental caries can be achieved by taking into account all the factors that influence dental caries. These factors are teeth and its environment comprising of saliva, bacteria in the form of dental plaque, and the quantity and frequency of dietary carbohydrate intake.

1.2.7 Saliva

Saliva can play a major role in maintaining a healthy mouth as well as prevention of caries through several mechanisms. It participates in the formation of acquired enamel pellicle through covering all surfaces of the tooth. Saliva consists of a wide range of structurally and functionally complex host molecules (proteins and glycoproteins). These act as primary nutrients for the resident oral microbiota, which grow as multispecies biofilms on oral surfaces (Marsh *et al.,* 2016). Saliva contains inorganic ions such as bicarbonate and phosphate which neutralizes the acid that results in demineralization of the enamel and act in remineralization. It can also act by flushing/cleansing away food debris and microorganisms found in the oral cavity. It also contains cystatins which are cysteine proteinase inhibitors that are also antimicrobial and immunomodulatory (Marsh *et al.,* 2016). Moreover, this antimicrobial properties such as immunoglobulins, lactoperoxidases and lactoferin inhibit the growth of bacteria (Llena-Puy 2006). Therefore individuals with low salivary flow and xerostomia are

highly susceptible to dental caries. These individuals are often given regime (stimulation by chewing or chemicals) to improve saliva flow or given artificial saliva.

1.2.8 Dietary carbohydrates

Among all the dietary carbohydrates, sucrose is the most cariogenic sugars. Transiently present sucrose is rapidly taken up and metabolized by *S. mutans* and converted into strong acid such as lactic acid which causes demineralization of enamel. In addition, sucrose is converted into soluble and insoluble extracellular polysaccharides (EPS) as well as intracellular polysaccharide called glycogen and these compounds serve as nutrient for this bacteria when incoming nutrient is not available. EPS also serves as an adherence mechanism and non-penetrable moiety. Therefore reduction in the quantity and frequency of sucrose intake is important in the prevention and control of dental caries. This can be achieved by education and awareness. The incidence of dental caries has declined in developed countries but it has increased in the developing countries due to the improvement in the life style and the change in diet. WHO has therefore considered an oral health and particularly the control of dental caries a priority project (Petersen and Kwan 2009).

1.2.9 Dental plaque

Control of plaque can be achieved by mechanical oral hygiene procedures as well the addition of antimicrobial/antiplaque agents to the dental care products. Fluoride, triclosan and chlorhexidine are the most commonly used agents for the control of dental caries. These antimicrobial agents can work by reducing the accumulation of new plaque or removing the existing plaque. Fluoride has been available for use to the general population for the past many years in the prevention of dental caries. Fluoride concentrated in plaque and saliva inhibits the demineralization of sound enamel and enhances the remineralization. It inhibits bacterial

growth, acid production and polysaccharide production (Takahashi and Washio 2011). Triclosan has been shown to have some effect against bacteria that adhere to the teeth surface (Koga *et al.*, 1986).

Chlorhexidine, formulations remain undisputed as the gold standard among anti-plaque mouthrinses, but local side effects have tended to restrict their use to the short to medium term (Sheen and Addy 2003). It has an immediate bactericidal action and a prolonged bacteriostatic action due to adsorption onto the pellicle-coated enamel surface. The most significant adverse local side effect of chlorhexidine reported by McCoy *et al*., (2008) were taste change, stains on the teeth, tongue and/or dentures, sore mouth, sore tongue and/or throat and tongue-tip irritation as well as wheezing and shortness of breath (McCoy *et al.*, 2008).

Another oral hygiene utility is available in India, Pakistan, Arabian and some African countries. It is a traditionally used teeth-cleaning stick generally known as Miswak or Siwak (Dahiya *et al.*, 2012). Miswak is from a tree known as *Arak (Salvadora persica)* or the Toothbrush tree and is a stick with a size of a pen. It is chewed and the mechanical control results from the friction between the plant fibers and tooth surface. In some areas where they do not have this plant, they use other indigenous teeth-cleaning trees like *Citrus sinensis*, *Citrus aurantifolia*, and *Azadirachta indica*. Antibacterial effects of Miswak against dental caries and periodontal pathogens have been established (Sofrata *et al.*, 2008). It provides both mechanical and chemical plaque controlling effect. Controlling of each of the major virulence factors offers a selective therapeutic target for prevention.

1.3 Periodontal diseases

Periodontal disease is a condition of the periodontium caused by bacteria that multiply and build up on the teeth and gums leading to irritation, inflammation and bleeding (Figure 1.5). It is divided into two categories. The mildest form of periodontal disease is called the gingivitis (Pihlstrom *et al.,* 2005). Gingivitis is highly prevalent and readily reversible by simple, effective oral hygiene. Gingivitis does not affect the underlying supporting structures of the teeth. Inflammation that extends deep into the tissues and causes loss of supporting connective tissue and alveolar bone is called the periodontitis. It is initiated when oral pathogens gain access to the gingival tissue and stimulate a host immune and inflammatory response. Periodontitis results in softening of tissue pockets or deepened crevices between the gingival and tooth root. Severe periodontitis can result in tooth loosening, occasional pain and discomfort, impaired mastication, and eventual tooth loss (Pihlstrom *et al.,* 2005). The bacteria responsible for this disease are commensal anaerobic bacteria and they have been isolated from healthy people (Armitage 2010). The periodontal pathogen and their byproducts translocate via bacteremia after they easily penetrate the inflamed, ulcerated gingival tissue and enter the bloodstream. They can be dispersed systemically and elicit host responses in distal tissues and organs. Researchers have found periodontal pathogen in atherosclerotic plaque (Haraszthy *et al.,* 2000) and in the arterial walls as well (Deshpande *et al.,* 1998).

Figure 1.5 Periodontitis - inflammation of gums (taken from periobasics.com)

1.3.1 Pathogenesis

Periodontitis is a multifactorial disease that is caused by pathogenic microflora and their byproducts, genetic factors, presence of environmental factors and an excessive host response (D'Aiuto *et al.*, 2004, Kornman 2008, Salvi *et al.*, 2008). Response to infection varies enormously between individuals, and genetic factors may play a role in individual variations (Wang 2005). The genetic factors are mostly associated with gene polymorphisms that play a role in the immune response, tissue destructive process, or metabolic mechanism. In some situations, genetic polymorphisms could cause a change in the protein or its expressions, possibly resulting in alterations in innate and adaptive immunity, thus resulting in being the determinant in disease progression.

Generally, single nucleotide polymorphism on intron might affect splicing, and/or transcription, thereby changing quantity and/or quality of mRNA and protein expression (Suzuki *et al.*, 2004). Soncransky *et al*., (2000) has shown a particular genotype rendering host defense mechanism more selective against specific periodontal pathogens in chronic

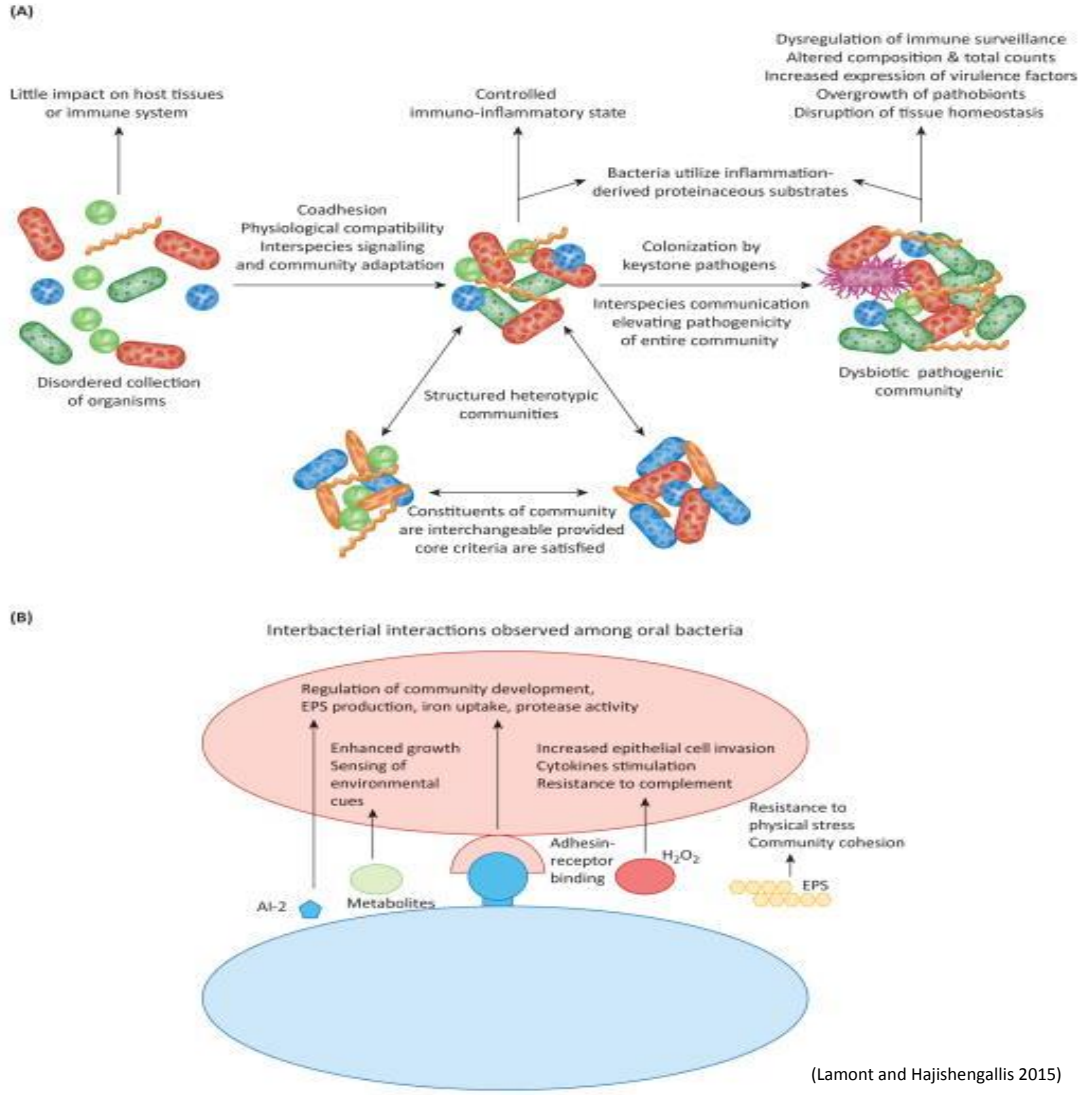
periodontitis patients (Socransky *et al.,* 2000). Furthermore, Nibali *et al*., (2007) suggested that IL-6-174 polymorphism may be important in determining susceptibility to colonisation with periodontal-pathogen (Nibali *et al.,* 2007).

Periodontitis progression and severity can be aggravated by the immune response to infections notably neutrophils, macrophages, lymphocytes and fibroblasts (Gonzales 2015). The effective suppression of infection by the pathogen relies on the response of the innate and adaptive immune systems. Specific attack to the intruder pathogen promotes adaptive immune system. This response can last for days to weeks in order to achieve the best effectiveness and will entail immunity reminiscence (Wilensky *et al.,* 2015). However, instant defence against invading pathogens without any immunologic memory will provide an innate immune sytem (Medzhitov and Janeway 2000). Functional defects in the host immune response or deficient numbers of different cell subsets have profound effects on the host's susceptibility to periodontitis (Hart *et al.,* 1994). In addition, co-infection with a virus, such as herpes simplex virus, Epstein–Barr virus or cytomegalovirus, promotes the release of pro-inflammatory cytokines, contributing to periodontal tissue breakdown (Slots 2015). Other risk factors are smoking, diabetes mellitus, poor oral hygiene, immunosuppression, hormonal changes and poor nutrition.

1.3.2 Periodontal pathogens

Bacterial plaque is the main causative agent among many other risk factors related to periodontal disease. Periodontitis is well recognized as an infectious disease, and several pathogens have been identified on the basis of their association with this disease. Certain clusters of bacterial species are associated with this infection such as the *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia (Tf), Treponema denticola (Td), Prevotella intermedia*

(*Pi*), *Aggregatibacter actinomycetemcomitans* (*Aa*) and Capnocytophaga (*Capno*) are considered the major periodontal pathogens (Torrungruang *et al.,* 2015). However, current developments in this topic states consistently that periodontitis is initiated by a polymicrobial synergy and dysbiosis termed-PSD (Figure 1.6) other than by selected 'periodontal pathogens' as traditionally thought (Lamont and Hajishengallis 2015). The keystone pathogens elevate the virulence of the entire microbial community through interactive communication with the accessory pathogens. *P. gingivalis* can have a community-wide pathogenic influence on the microbiota in animals, at least in part via host modulation (Hajishengallis and Lamont 2012, Hajishengallis *et al.,* 2011). Moreover, the introduction of *P. gingivalis* into a healthy multispecies biofilm alters the pattern of microbial community gene expression (Frias-Lopez and Duran-Pinedo 2012).



(Lamont and Hajishengallis 2015)

Figure 1.6: 'Model overview. Community formation driven by co-adhesion and physiological compatibility initially leads to a balanced interaction between host immunity and the metabolism of the inflammophilic microbiota. The identities of individual species are less important than the presence of the appropriate complement of genes. As established in mouse models, colonization by keystone pathogens such as *P. gingivalis* enhances community virulence through interactive communication with accessory pathogens such as *S. gordonii* and disruption of immune surveillance. The dysbiotic community increases in number, pathobionts (green) overgrow and become more active, and tissue destruction ensues. B. Summary of synergistic interbacterial interactions that have been documented among oral bacteria'(Lamont and Hajishengallis 2015)

1.3.3 *Porphyromonas gingivalis (Pg)*

Porphyromonas gingivalis, an anaerobic gram-negative bacterium, is associated with chronic form of the disease and the development and progression of periodontitis (Carranza and Newman 1996). It is the most aggressive pathogen and has been extensively researched. It exist practically in the subgingival crevice. Three distinct microenvironments coexist in this region for *Pg*. The multiplex embedded community will be found on the root surface; the fluid phase of the gingival crevicular; and the crevice in the epithelial cells of the gingival lining (Hajishengallis and Lamont 2014). Moreover, each region provides discrete opportunities and challenges but *Pg* can still adjust among these regions. And, its adjustment occurs on a universal scale and it regulates about 30 % of the expressed proteome according to it's the community, or planktonic or epithelial cell conditions (Hendrickson *et al.,* 2009, Kuboniwa *et al.,* 2009).

Porphyromonas gingivalis internalization to host cells requires bacterial virulence factors, such as the Fim A fimbrae (Hajishengallis and Lamont 2012), Ser B serine phosphatase and several proteases (Tribble and Lamont 2010). Once internalized, the bacteria are able to survive and replicate in the intracellular milieu, without activation of an acute immune response (Hajishengallis and Lamont 2012). *Porphyromonas gingivalis* also produces a broad spectrum of virulence factors including outer membrane vesicles, adhesins, lipopolysaccharides, hemolysin and proteinases (Cutler *et al.,* 1995, Force and Nahata 1995). Arg-gingipain and Lys-gingipain cysteine proteinases are the main endopeptidases produced by *Pg* and are believed to contribute to host colonization, evasion of host defense mechanism and destruction of periodontal tissues (Grenier and Tanabe 2010). They are responsible for approximately eighty five percent proteolytic and hundred percent trypsin-like activities of *Pg* (Hajishengallis and Lamont 2012). Arg-gingipain are encoded by two genes Rgp A and Rgp B while Lys-

gingipain is encoded by Kgp. Gingipains are highly active extracellular and surface proteinases and are of particular importance because they can destroy various components of periodontal tissue, including extracellular matrix proteins, cytokines, complement proteins, antibodies, and proteinase inhibitors (Curtis *et al.,* 1999, Kadowaki *et al.,* 2000). Rgp A and Kgp mediate adherence of *Pg* to the epithelial cells as well as gingival crevice while facilitating colonisation of the bacterial biofilm (Guo *et al.,* 2010). Recently, it has been shown that *Pg* infection which exerts pro-inflammatory effects in chronic periodontitis upregulates IL-33 expression in human gingival/oral epithelial cells via endogenous gingipain-dependent mechanisms (Tada *et al.,* 2016).

Porphyromonas gingivalis coaggregates with other bacteria such as *Treponema denticola, Fusobacterium nucleatum,* and the formation of these multi strain complex communities contribute to the pathogenesis of periodontitis (Lamont and Hajishengallis 2015). Other bacteria include *A. actinomycetemcomitans, Campylobacter rectus, Eikenella corrodens and Eubacterium* species. These bacteria are predominantly and frequently detected in periodontal pockets and are capable of colonizing and invading periodontal tissues. Both the host and bacteria in periodontal biofilm release proteolytic enzymes that damage tissues (Carranza and Newman 1996). Investigations on the mechanisms involved in coaggregation of these periodontal pathogens with *Pg* is an ongoing process. Elucidating these mechanisms may help to better understand the formation and development of periodontitis-associated dental plaque and, thereby, the pathogenesis of periodontal infection (Zhu and Lee 2016).

1.3.4 Control and prevention of periodontal diseases

Periodontal treatment aims to cure inflamed tissue, reduce the number of pathogenic bacteria and eliminate the diseased pockets. In controlling the disease mechanical therapy, plaque

controlling antimicrobial compounds and systemic administration of antibiotics are currently used. Conventional therapy includes scaling, clearing of inflamed tissue and root planning (Kumar *et al.*, 2009, Odds 1994). Bacterial virulence factors can also be targeted. Antimicrobial agents include povidone-iodine and chlorhexidine containing mouthrinses can be used (Slots 2002). They all have advantages and disadvantages. For example, povidone-iodine has a potential to induce hyperthyroidism and staining. Chlorhexidine causes numbness, irritation to oral mucosa, loss of taste buds and staining of teeth. Medicinal plants have also been studied for their beneficial effects in the prevention and treatment of periodontal diseases.

1.4 Medicinal plants

The use of medicinal plants dates back 4000-years ago in the Sumerian clay tablet that recorded plant remedies for various illnesses. Plants are used extensively for herbal treatment. The majority of people living in countries like Asia, Latin America and Africa use medication prepared from plant sources. These plants are accessible in the form of powder, pastes, decoction, infusion and pills and are available traditionally for the treatment of numerous human illnesses (Khan *et al.*, 2017). They have been used for treatment of diseases such as leprosy, influenza, bronchitis, wound healing, asthma, tuberculosis, jaundice, muscular cramps, high blood pressure, diabetes, viral hepatitis, dysentery, diarrhea, gynaecological disorders and cardio vascular disorders. Numerous *in vitro* studies have investigated the activity of traditionally used plant against diseases. Several plants such as *Punica granatum* L. (pomegranate), *Dodonaea viscosa* var. *angustifolia, Camelia sinensis* (tea plant), *Curcuma xanthorrhiza* (Javanese ginger), *Hyptis divaricata*, *Aloe vera*, *Glycyrrhiza uralensis* (Chinese liquorice), *Hypericum perforatum* (St. John's Wort) and *Origanum vulgare* (oregano) have been studied extensively and has shown effect against diseases including caries (Dziedzic *et al.*, 2013, Jain *et al.*, 2016, Khan *et al.*, 2017, Kim *et al.*, 2008, Miller *et al.*, 2015, Patel *et al.*,

2013, Suntar *et al.,* 2016, Villinski *et al.,* 2014, Yoshinaga *et al.*, 2014**).** Moreover, all these studies exhibited different modes of action some being anti-biofilm and others having anti-adherence properties.

Medicinal plants can contain molecules with a potential activity against periodontal pathogens (de-Oliveira *et al.,* 2016). They have been fundamental for thousands of years to provide bioactive molecules used to treat different types of human infirmities, such as inflammation, pain, and tumours. *Glycyrrhiza uralensis* (Chinese liquorice), *Vaccinium macrocarpon* (cranberry), *Mangifera indica* (mango), *Eucalyptus globulus (*eucalyptus leaves*), Myristica fragrans (*nutmeg*), Vitis vinifera (*grapevine*)* and *Salvadora persica (*miswak*)* have been found to be effective against oral diseases (Bairy *et al.,* 2002, Fani and Kohanteb 2012, Munoz-Gonzalez *et al.,* 2014, Nagata *et al.,* 2006, Shafiei *et al.,* 2012, Sofrata *et al.,* 2007, Villinski *et al.,* 2014, Yamanaka *et al.,* 2007). These medicinal plants have shown greater antibacterial efficacy on the cariogenic bacteria as well as periodontal diseases despite the heterogeneous methodological aspect.

Also, in South Africa medicinal plants have been used with a rich traditional practice by healers for a wide range of diseases and symptoms (Saeed *et al.,* 2016). They have tested about twenty six South African medicinal plants that are used for all sorts of ailments ranging from cancer, leprosy, influenza, bronchitis, wound healing, asthma, tuberculosis, jaundice, muscular cramps, high blood pressure, diabetes, viral hepatitis, dysentery, diarrhea, gynaecological disorders and cardio vascular disorders (Saeed *et al*., 2016). They looked at the cytotoxicity of these plants towards sensitive and multidrug-resistant cancer cells. In their study, they found these plants to be cytoxic towards sensitive and multi drug resistant leukemia cells

Figure 1.7 A selection of medicinal plants that traditional healer sell in South Africa.

Photo by Janelle Cabuco

Different parts of the plants produces diverse extensive variety of bioactive molecules that can be used as healing agents (Cragg and Newman 2001). The active ingredients in these medicinal plants are chemical compounds known as secondary metabolites (Escaich, 2008). The remedies for medicinal plants have advanced tremendously from herbalism into production of the first pure synthetic drug based on natural products (Figure 1.7 and Table 1.2).

Table 1.2 Traditional uses of South African medicinal plants Adapted from (Saeed *et al.,* 2016).

Table 1
Traditional uses of South African medicinal plants against cancer.

Family	Plant species	Part collected	Traditional uses	References
Acanthaceae	*Hypoestes aristata* Soland. ex Roem and Schult. var. *aristata*	Leaves	According to healers of the Xhosa tribe, the plant is also used to treat complicated and major diseases like **cancer**, arthritis, tuberculosis, bone fractures.	(Bhat, 2014)
Anacardiacaea	*Pistacia vera* L.	(Resin)	The plant is used for the treatment of eczema, throat infection, renal stone, and asthma. In addition, some *Pistacia* species have shown various biological activities including anti atherogenic, antioxidant, antifungal and anti-inflammatory. The oleo gum resin extract of *Pistacia vera* has shown **antiangiogenic and cytotoxic** effects.	(Bozorgi et al., 2013; Mirian et al., 2015)
	Cotinus coggygria (Scop)	Leaves	The *Cotinus coggygria* possesses diverse biological activities, including anti-oxidant, anti-inflammatory, and **anticancer** effects. Also it is used in the treatment of acute icteric infectious hepatitis.	(Kim et al., 2012; Matic et al., 2011; Savikin et al., 2009; Shen et al., 1991; Wang et al., 2015; Westenburg et al., 2000)
	Schinus molle L.	Leaves and resin	Traditionally *S. molle* is used as antibacterial, topical antiseptic, digestive, for respiratory and urinary infections, analgesic, and central depressant, purgative diuretic. Recently the **antioxidant and anticancer activities** have been reported.	(Bendaoud et al., 2010)
Apocynaceae	*Acokanthera oppositifolia (Lam.) Codd,*	Leaves	Traditionally used as highly effective arrow poison in South-Africa, which indicates his **cytotoxic** properties.	(Steyn, 1934; Van Wyk et al., 2002)
Asteraceae	*Artemisia afra* Jacq. ex Willd.	Leaves	It is widely used in South African traditional medicine to treat many different medical conditions, particularly respiratory and inflammatory. It has been reported that *A. afra* has **anticancer** potential.	(Fouche et al., 2008; Spies et al., 2011).
Celastraceae	*Maytenus bachmannii* (Loes.) Marais.	Leaves	*Maytenus* species are most frequently used in African traditional medicine to treat infectious and inflammatory diseases. In some part of Africa (Kenya) is used traditionally as **anticancer**.	(da Silva et al., 2011; Kupchan and Smith, 1977; Shirota et al., 1996)
Combretaceae	*Combretum kraussi* Hochst.	Leaves	*C. kraussi* has been used traditionally as antibacterial and antiinflammatory. It has been reported that the combretastatins which isolated from the seeds of *C. kraussi* have shown cytotoxic activity and influenced the tubulin polymerization.	(Eloff et al., 2005; Peuzzdni et al., 1993)
Fabaceae	*Castanospermum australe* A. Cunn & C. Fraser ex Hook	Leaves	The alkaloid, castanospermine, inside *C. australe* have shown, anti-parasitic, anti-HIV and **anticancer** properties	(Duke, 1989; Rhinehart et al., 1990)
Ginkgoaceae	*Ginkgo biloba* L.	(Root bark, leaves, fruits)	*G. biloba* extract has been used as herbal dietary supplements in several European countries and USA because of **chemo-preventive** properties. It may also have effects on angiogenesis, DNA damage, cell proliferation and death, and free radicals scavenging.	(Biggs et al., 2010; Chan et al., 2007; DeFeudis et al., 2003; Ye et al., 2007)
Lamiaceae	*Leonotis leonurus* (L.) R. Br.	Aerial parts, except *P. barbatus* (roots)	Tea made from the whole plant is used against **cancer**, arthritis, piles, bladder and kidney disorder, obesity, and rheumatism.	(Thring and Weitz, 2006)
	Orthosiphone serratus Schltr		Several *Orthosiphon* species traditionally used for expectorant, rheumatism indicated antioxidant activity and **anticancer** activity.	(Mukesh et al., 2015; Stampoulis et al., 1999; Yam et al., 2009)
	Plectranthus barbatus Andrews		used for a wide range of complaints, *e.g.* stomach ache and as purgative drug, against hypertension, congestive heart failure, eczema, colic, respiratory disorders, painful urination, insomnia, convulsions, and **cancer prevention**.	(Abdel-Mogib et al., 2002) (Mwitari et al., 2013)
	Plectranthus ciliates E. Mey. ex Benth.		Traditionally is used to treat diseases of digestive tract, respiratory system, nervous system, inflammation, infections, pain and **different types of cancers**	(Lukhoba et al., 2006)
	Salvia apiana Jeps.		It is recommended by herbalists to treat **prostate hyperplasia**.	(http://oneearthherbs.squarespace.com/diseases/benign-prostatic-hyperplasia-bph.html).
	Tetradenia riparia (Hochst.) Codd,		Traditionally it is used in cases of cough, dropsy, diarrhea, fever, headaches, malaria, antimicrobial and toothache. The terpenoids inside the plant have been shown to possess **cytotoxic and antioxidant** activities.	(Campbell et al., 1997; Gazim et al., 2013)
Lauraceae	*Laurus nobilis* L.,	Leaves	Used among other applications against skin diseases and **cancer** in the Middle East.	(Said et al., 2002)
Magnoliaceae	*Magnolia x soulangeana*	Leaves	Some of magnolia species are rich of lignans, therefore, are widely used in Chinese and Japanese traditional medicines for their **chemo-preventive** role and **cytotoxic** activity.	(Arora et al., 2012; Saeed et al., 2014; Singh et al., 2015)
Meliaceae	*Trichilia emetic* Sond.	Leaves	*T. emetic* is native to sub-Saharan Africa, it's possess hypotensive, antioxidant, antibacterial, anti-inflammatory and **cytotoxic** properties	(Konate et al., 2014; Traore et al., 2007)
Moraceae	*Broussonetia papyrifera* L. (Vent.)	Leaves	The plant is used traditionally as astringent, diuretic, vulnerary, tonic, ophthalmic, diaphoretic and laxative properties. The cytotoxicity activities against **breast and cervical cancer cells** have been reported recently.	(KUMAR et al., 2014; Pang et al., 2014)
Myoporaceae	*Bontia daphnoides* L.,	Leaves	*B. daphnoides* has been used traditionally for treating nephritis, hypertension,	(Ayensu, 1981; Williams et al., 2007)

1.4.1 Identification of chemical constituents from medicinal plants

Plants are the source of different classes of phytochemicals. These are secondary metabolites that are produced by plants and they do not have any known direct function in basic metabolism, and are often differentially distributed among limited taxonomic groups within the plant kingdom. They are phenolics such as flavonoids, tannins, lignin, salicylic acid. They belong to an aromatic group with one or more hydroxyl substituents (Genwali *et al.,* 2013). Benzoic acid and caffeic acid derivatives are the constituents of phenolic acids. The most common polyphenols in our diet are flavonoids and phenolic acids and are distributed widely in fruits, vegetables, cereals and beverages (Genwali *et al.,* 2013). The other groups comprise terpenoids which include aromatic oils, resins, waxes, steroids, rubber and carotenoids. They are the most widespread group of natural products. Inversely, some authors use the term "terpenes" more broadly, to include the terpenoids. Another major group are alkaloids often toxic, e.g., strychnine, nicotine, caffeine, cocaine, capsaicin (Zhang *et al.,* 2017). Many plants such as fruits and vegetables have demonstrated the most antioxidant activity, a beneficial health effect confined in numerous phytochemicals (Scalbert *et al.,* 2005). Moreover, vitamin C, E and carotenoids, have revealed strong antioxidant capacity (Rice-Evans *et al.,* 1995).

General methods of using different organic solvents are available for extraction of secondary constituents from plants. This is followed by chromatography, an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. Chromatography is based on the principle where a mixture is applied onto the solid stationary phase and molecules separated from each other while moving with the aid of a mobile phase (Coskun 2016). The factors effective in this separation process include molecular characteristics related to adsorption, partition, and affinity or differences among their molecular weights (Porath 1997). Various chromatography

methods have been developed and are used widely in the separation and purification of compounds. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography (Harwood and Moody 1989).

Purification of biomolecules can be achieved with the use of column chromatography. Firstly the stationary phase is packed and then the sample to be separated applied. The sample runs through a stationary phase by means of the mobile phase while separating based on several factors such as size and resulting bands are collected at the bottom of the column. The most widely used material for the stationary phase are silica, cellulose, polyamide and sephadex. Separation of compounds by the stationary phase is based on a number of principles such as ion-exchange. It uses electrostatic interactions among charged protein groups, and the matrix. The matrix will have a different ion charge to that of the molecule to be separated, and the attraction to each other will be achieved (Coskun 2016). The positively charged will adsorb the negatively charged molecules and vice versa.

Another method uses a gel-filtration chromatography which separates macromolecules on the basis of size. Small molecules will pass through the column faster than bigger molecules. Affinity chromatography, the molecule of interest will bind specifically to the material of the stationary phase. Then the bound material will later be released from the matrix through changing pH or addition of a salt solution (Firer 2001). Paper chromatography is another form whereby plant compounds can be separated or viewed. It comprises of highly water saturated layer of cellulose. Thick filter paper forms the base support settled with a stationary phase. This paper chromatography can be developed in a tank with a mobile phase (Firer 2001).

Thin-layer chromatography is a "solid-liquid adsorption" method. A solid adsorbent coated on glass plates forms the stationary phase (Coskun 2016). The adsorptive solid can be alumina, silica gel or cellulose. Mobile phase will migrate through the stationary phase by means of capillary action. Polarity of the material, the solid phase and the solvent being used will determine the speed of migration (Sherman *et al.,* 1991). Specific chemical substances can be used to enhance visibility that will enable material identification on the chromatograph. Room light as well as ultra violet light or fluorescence light can be used to enhance visibility (Coskun 2016). Column chromatography is commonly adopted in separation and purification but these traditional methods are time consuming, have low efficiency and are complicated in operation. Mixed components make it imposible to perform structural, and functional analysis, and purification of many molecules.

Subsequent purification using preparative high performance liquid chromatography (HPLC) is applied to enable structural, and functional analysis. Vital constituents of a HPLC machine are solvent, appropriate column, high- pressure pump, detector, and recorder. Perfect results in the separation, and identification of active molecules are generated by this technique. Atmospheric pressure is used move the mobile phase through columns, and with a set flow rate. Computerized system controls and runs the separation and material is accrued (Lochmüller *et al.,* 1996).

Other methods known as Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are available for functional studies and identification of any bioactive compounds (Ingle *et al.,* 2017). FTIR is used to identify the functional groups present and structure determination of the sample. Total

reflectance plates that are highly attenuated record spectra in relation to the percentage diffusion. It uses the reference given in Varian FTIR instrument manual to assign the peaks attained at specific wavenumbers by bonding and functional group (Hazra *et al.,* 2007). Mass spectrometry is another prominent technique in the identification, quantification and elucidation of the structure and chemical properties of molecules. It enables determination of the molecular weight of molecules (Ingle *et al.,* 2017).

Physical, chemical and biological properties of matter can be achieved through the use of a Nuclear Magnetic Resonance Spectroscopy. The 2D NMR enables structural elucidation of the complicated structure while the 1D could be used for routine diagnostic. (Ingle *et al.,* 2017). Determination of molecular structure of solids can be achieved with the use of solid state NMR spectroscopy. The C and H-NMR is used to identify the types of carbon and protons respectively that are present in the compound (Cos *et al.,* 2006).

1.4.2 Pomegranate

Pomegranate (*Punica granatum* L.) an ancient fruit, affectionately known as the 'jewel of winter,' is the predominant member of two species comprising the Punicaceae family (Figure 1.8). It is native to the Himalayas in northern India to Iran but has been cultivated and naturalized since ancient times over the entire Mediterranean region. Now it is cultivated almost everywhere. It is also found in India and more arid regions of Southeast Asia, the East Indies, and tropical Africa (Jurenka 2008). *Punica granatum* L. has been used extensively as traditional medicine in many countries. The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, and protection from ultraviolet (UV)

radiation. Other potential applications include infant brain ischemia, Alzheimer's disease, male infertility, arthritis, and obesity (Jurenka 2008)

Figure 1.8 Pomegranate tree and fruit grown from Pomona farm, Cape Town

Over the past decade, significant progress has been made in establishing the pharmacological mechanisms of pomegranate and the individual constituents responsible for them. Pomegranate fruit is divided into three parts: seeds, juice and peels. Extracts of all parts of the fruit appear to have therapeutic properties (Lansky and Newman 2007). Its juice, peel and oil have been reported to have anticancer and cardiovascular disease preventing properties (Kim *et al.,* 2002) and some studies report the bark, roots, and leaves of the tree have medicinal benefit as well. Fruit of pomegranate have been found to possess an antimicrobial activity against both gram positive and gram negative bacteria (Mathabe *et al.,* 2006). Different types of phytochemicals have been identified in these various parts of the fruit and the ongoing research shows the most therapeutically beneficial pomegranate constituents as ellagic acid, ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones listed in Table 1.3 (Lansky and Newman 2007). Pomegranate peel is a waste from juice processing and several studies have confirmed that pomegranate peel is a rich source of

bioactive compounds including ellagitannins, catechin, rutin and epicatechin among others (Fawole *et al.,* 2015, Glazer *et al.,* 2012).

Table 1.3 Pomegranate peel constituents and its bioactivity

1.4.2.1 Pomegranate and oral pathogens

Compound name	Bioactivity	Reference
Tannins		
2-o-galloylpunicalin		(Tanaka *et al.,* 1986)
2,3-(S)-HHDP-D-glucose		(Tanaka *et al*.,1986; Liu *et al*.,2007)
6-O-galloyl-2-3-(S)-HHDP-D-glucose		(Tanaka *et al*.,1986)
Punicacortein A	Anti-HIV	(Tanaka *et al*.,1986)
Punicacortein B	Anti-HIV	(Tanaka *et al*.,1986)
Punicacortein C	Anti-HIV	(Tanaka *et al*.,1986)
Punicacortein D	Anti-HIV	(Tanaka *et al*.,1986)
Punicalagin	Antioxidant, anti-hypertensive	(Anand *et al.,* 2004, Gil *et al.,* 2000, Liu and Li 2007)
	Antifungal	(Endo *et al.,* 2010)
	Antiproliferative	(Fischer *et al.,* 2011)
	Anticarcinogenic	(Ismail *et al.,* 2012)
Ellagitannins	Anti-atherosclerosis	(Khateeb *et al.,* 2010)
Punicalin	Antioxidant, Anti-viral Antifungal	(Wang *et al.,* 2013)) (Gil *et al*., 2000; Liu *et al*., 2007; Tanaka *et al*.,1986)
Punigluconin	Antioxidant	Tanaka *et al*.,1986
Granatin B, Strictinin A	Anti-inflammatory	(Lee *et al.,* 2008)
Major flavonoids		
Naringenin-4'-methylether-7-O-α-L-arabinofuranosyl(1→6)- β-D-glucopyranoside		(Srivastava *et al.,* 2001)
Quercetin-3,4-dimethylether-7-O α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside	Antioxidant	(Chauhan and Chauhan 2001)
Major Alkaloids		
Peelletierine		(Neuhofer *et al.,* 1993, Vidal *et al.,* 2003)
N-methylpelletierene		Neuhofer *et al*., 1993
Pseudopelletierene		Neuhofer *et al*., 1993
N-acetyl-sedridine		Neuhofer 1990

Some research has been done on the antiplaque activity of various parts of pomegranate. In these studies different results have been reported which show an antimicrobial effect of pomegranate. Recently, Dabholkar *et al*., (2016) evaluated antimicrobial effect of pomegranate-containing mouthwash on oral biofilm-forming bacteria *S. mutans* (Dabholkar *et al.*, 2016) and showed some positive results using disk diffusion method. The zone of inhibition for the *S. mutans* was visible until the concentration of 5 µg/ml. Another study investigated the effect of hydroalcoholic extract of *Punica granatum* L. on several oral pathogens such as *S. sanguinis, S. mutans, S. salivarius, S. sobrinus,* and *E. faecalis*. They discovered a significant antibacterial effect on all these common oral bacterial pathogens with maximum effect on *S. mutans* with MBC and MIC of 3.9 mg/ml, which is the main microorganism responsible for dental plaque and caries (Hajifattahi *et al.,* 2016). Menezes *et al*. (2006) also studied whole fruit and showed antiplaque activity by measuring total plaque bacteria (Menezes *et al*., 2006). On the other hand Vasconcelos *et al*. (2006) studied peel extract for their anti-adherence activity which is the primary cause of this infection (Vasconcelos *et al*., 2006). Unfortunately, in this study bacterial adherence was determined visually which is not a generally accepted technique.

Likewise, pomegranate mouth rinse has been evaluated for its activity against periodontal pathogens. In one study, they compared the effectiveness of the mouthrinse with pomegranate to reduce the bleeding index in patients with bleeding gingival condition. The pomegranate mouthrinse showed similar antimicrobial and anti-inflammatory properties to that of 0.12% chlorhexidine. This suggests that pomegranate mouthrinse can be used to replace chlorhexidine side effects in controlling bleeding gums in periodontal disease (Batista *et al.,* 2014). In their study they have not shown the part of the fruit that was used to prepare the mouthrinse. Bhadbhade *et al.* (2011) showed pomegranate fruit juice to have antimicrobial activity against

periodontal pathogens, *A. actinomycetemcomitans*, *P. gingivalis, P. intermedia* at a very high concentration (16.25-62.5 mg/ml). Such high concentrations are difficult to maintain in the oral cavity due to the constant saliva flow. They also showed visible reduction in plaque with no scientific or qualitative measurements to support their observations in the study.

In addition, studies have been conducted to determine the chemical constituents responsible for the beneficial effects. Given its potential to produce a significant therapeutic effect, pomegranate can be useful as drug or supplement in the treatment or management of oral diseases. However, further studies are required. Pomegranate peel is characterized by a substantial amount of phenolic compounds, hydrolysable tannins and flavanoids. These compounds account for the antimicrobial, antioxidative, antimutagenic and anti-inflammatory activities activity associated with the fruit (Wasila *et al.,* 2013). Moreover, the phytochemical concentration of the peel is high enough to be effective without further enrichment (Endo *et al.,* 2010). The spectral analysis identified punicalagin from the peel with a strong antifungal activity of 3.9 mg/ml against *Candida albicans*. A study by Fischer *et al.*, (2011) had shown that the free radical scavenging and antioxidant potential of the phenolic compounds might be responsible for the beneficial effect, especially the hydrolysable tannins of the fruit peel (Fischer *et al.,* 2011). The gallic acid and ellagic acid esters were the main constituents of the tannin fraction. The massive structural diversity is due to the big number of likely combinations of monomers (Fischer *et al.,* 2011). Gallotannins and ellagitannins, gallagylesters and punicalagin are subdivision structures of hydrolysable tannins (Haslam 2007). Dehydro-ellagitannins and transformed dehydro-ellagitannins are further subdivision group (Okuda *et al.,* 2000).

One study has been done to fractionate *P. granatum* peel and identify compounds that are responsible for the activity against *A. baumannii, E. coli, P. aeruginosa and S. aureus* that are

resistant to multiple drugs. In that study, bioactive compounds were identified with high pressure liquid chromatography (Khan *et al.,* 2017). The HPLC fractions demonstrated an antimicrobial activity against the MDR pathogens. The bioactive compounds in these fractions were identified as valoneic acid dilactone from aqueous F1 and F4, hexoside from ethanol F4 and coumaric acid from hexane F2 (Khan *et al.,* 2017). Additional, isolated compound punicalagin, exhibited antibacterial as well as antifungal properties against dermatophytes species, gram negative and gram positive bacteria. In this study, the bioactive compound was fractionated using liquid-liquid separation with ethyl acetate:butanol and purified further in a sephadex LH-20 as well as a lobar column (Foss *et al.,* 2014). The NMR was used for structural elucidation of the bioactive compound. Both crude extract and the fractionated punicalagin showed identical antifungal activity against *T. rubrum*. Pomegranate and its punicalagin demonstrated a considerable antifungal activity indicative of being used for anti-dermatophyte therapeutic agent (Foss *et al.,* 2014). Conversely, there are no studies available on the effect of pomegranate isolated compounds against dental caries and periodontal diseases.

In the past most studies have focused on the antimicrobial effect of either medicinal plant extracts or plant derived chemical constituents. This approach is appropriate because most of the time in the treatment of infections, complete elimination of pathogens is required. A novel approach of therapy has been suggested by some researchers, where the virulence of bacteria can be targeted instead of an antimicrobial property (Escaich 2008). The therapeutic compound would not kill the pathogens but simply render them avirulent. In a sense, this would be ideal where the causative organism is part of the normal flora such as *S. mutans,* a cariogenic and *P. gingivalis*, a periodontal pathogen in the oral cavity. The virulence factors of *S. mutans* are biofilm formation, production of acid and polysaccharides. The major virulence factor of *P.*

gingivalis is production of proteinases. Therefore in the prevention and control of these infections, the therapeutic compound can target these virulence factors.

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CHAPTER 2

STUDY DESIGN

2.1 Introduction

The human oral cavity can harbor up to 700 different types of commensal bacteria. A fraction of them can cause opportunistic infections such as dental caries to the hard tissue, periodontal diseases to the gingival tissue and oral candidiasis to the oral mucosa. Dental caries is caused by acidogenic and aciduric bacteria such as *S. mutans* and Lactobacilli. Periodontal diseases are caused by proteolytic bacteria such as *P. gingivalis, P. intermedia, F. nucleatum* and *Capnocytophaga* species (Samaranayake 1996). Oral candidiasis is caused by a unicellular fungi *Candida* species, with the most common being *Candida albicans*. These bacteria reside in the form of biofilm also called dental plaque. *S. mutans* initiates the adherence to form dental plaque, produce extra- and intracellular polysaccharides which serve as nutrient source and adherence moiety. As the condition becomes conducive, other oral bacteria become incorporated into the biofilm (Nisengard and Newman 1994). In addition, *S. mutans* produce acids that cause demineralization of enamel. *P. gingivalis* which is a keystone pathogen in the development of periodontal diseases and it also becomes incorporated into this biofilm and produce proteolytic enzymes, which cause gingival tissue destruction. In the prevention and treatment of these infections, use of antibacterial compounds is important. Furthermore, chemical agents that can target these virulence factors would be advantageous and they can provide additional benefit.

These preventative and treatment regimes are usually is expensive and not easily accessible, especially in developing countries; therefore medicinal plants are extensively used (Rosas-Pinon *et al.,* 2012). In dentistry, medicinal plants have attracted attention due to their antimicrobial effects against oral bacteria. For example propolis, *Mikania* species, *Nidus*

vespae have shown antiplaque properties (Duarte *et al.*, 2003, Xiao *et al.*, 2006, Yatsuda *et al.*, 2005). *Punica granatum* (pomegranate) has been used traditionally for treatment of infections throughout the world including South Africa (Howell and D'Souza 2013). The peel of the pomegranate fruit has antimicrobial properties against *Candida albicans* and gastro-intestinal pathogens (Prashanth *et al.*, 2001). At high concentrations, the fruit juice and whole fruit reduces oral plaque and periodontal pathogens (Bhadbhade *et al.*, 2011, Menezes *et al.*, 2006). However no study has investigated the effect *P. granatum* fruit peel on the virulence properties of *S. mutans*. Therefore, this study investigated the effect of *P. granatum* fruit peel on the virulence properties of the cariogenic bacteria *S. mutans* and periodontal pathogen *P. gingivalis*. In addition, compounds present in the bioactive subfractions were identified with reference to literature.

2.2 Aim and objectives

2.2.1 Aim

The aim of this study was to investigate the antimicrobial activity of pomegranate crude peel, seed extracts and purified fractions of the peel against oral pathogens responsible for dental caries and periodontal diseases. In addition, effect on the virulence of these bacteria was studied.

2.2.2 Objective

a. To investigate antimicrobial effect of methanol, dichloromethane:methanol, acetone, hexane and water crude extracts of pomegranate peel and seeds against *S. mutans*, lactobacillus, *P. gingivalis, P. intermedia, F. nucleatum,* and *Capnocytophaga.*
b. To determine the effect of the subinhibitory concentration of the crude peel extract on the biofilm formation by *S. mutans*.

c. To determine the effect of subinhibitory concentration of peel crude extract on the acid and extracellular polysaccharide production by *S. mutans*.
d. Fractionation of crude peel extract using column chromatography, and determination of antibacterial, anti-biofilm and anti-acidogenic activity of fractions.
e. To study the effect of subinhibitory concentration of crude peel extract and subfractions on the proteinase production by *P. gingivalis*.
f. To perform structural elucidation on beneficial subfractions to classify its chemicals composition.
g. To determine the cytotoxicity of crude peel extract, subfractions.

2.3 Study design and layout of the thesis

Figure 2.1 describes the study design and layout of the thesis chapter by chapter.

a. To investigate antimicrobial effect of methanol, dichloromethane:methanol, acetone, hexane and water crude extracts of pomegranate peel and seeds against *S. mutans*, lactobacillus, *P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *Capnocytophaga*

The pomegranate fruit was purchased, peel/seed separated and dried, and pulverized. Extracts were prepared using various solvents, dried and the yields were determined. Extracts were reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml. MBC values were obtained using microdilution technique. Based on the MBC values of methanol extract which proved to be the best, three subinhibitory concentrations 6.25, 3.125 and 1.56 mg/ml were selected for further studies for its effect on virulence properties.

Results of this section are described in Chapter 3.

b. To determine the effect of the subinhibitory concentration of crude peel extracts on *S. mutans* biofilm formation

The effect of the three subinhibitory concentrations on biofilm formation was examined using a well described method. Biofilm of *S. mutans* was grown in the presence of three subinhibitory concentrations of methanol crude extract. Water was used as a control. Biofilm formation was quantified using serial dilution technique at 6, 24 and 30 hours. This experiment was performed in triplicates using five strains of *S. mutans*. Bacterial counts of control and extracts were then compared using Student t-test.

Results of this section are described in Chapter 4.

c.* To determine the effect of subinhibitory concentration of crude peel extracts on the acid and extracellular polysaccharide production by *S. mutans

Streptococcus mutans culture were grown in the presence of three subinhibitory concentrations, (1.56, 3.125 and 6.25 mg/ml) of methanol peel extract for 16 h. The pH of the media was measured at 0, 10, 12, 14 and 16 h. Water was used as a control. *S. mutans* count was also determined at 0, 10, 12 and 14 h using a microdilution technique. This experiment was performed in triplicates using five strains of *S. mutans*. The test results were compared to the control results using Kruskal-Wallis test.

Results of this section are described in Chapter 5

The effect of pomegranate peel on the extracellular polysaccharides production was investigated using a well described technique. *S. mutans* (planktonic and biofilm) was allowed to grow in the presence and absence of peel extract in an environment conducive for the production of soluble (SEPS) and insoluble (IEPS) extracellular polysaccharides. Production

of soluble and insoluble polysaccharides extracted from the biofilm and planktonic cultures were measured. These experiments were repeated three times using five strains of *S. mutans*. The results on production of SEPS as well IEPS were compared to the control using Wilcoxon Rank Sum Test.

Results of this section are described in Chapter 6.

d. Fractionation of peel crude extract using liquid and column chromatography & the determination of MBC, Biofilm assay, Acid assay

Crude extract purification was done using liquid-liquid purification and column chromatography. Firstly, the dried crude extract was dissolved in the combination of methanol and water solvents on a 1:9 ratio. Sequential separation of polar and nonpolar compounds was performed using hexane and ethyl acetate. All extracts were spotted on a thin layer chromatography. The extracts with multiple bands were further purified using column chromatography. The bands that were obtained from the column were collected separately and tested for their antimicrobial activity. Two bands subfractions F 3.2 and F 4.1 with best bioactivity were screened for their ability to inhibit biofilm formation as well as acid production by *S. mutans*.

Results of this section are described in Chapter 7.

e. To study the effect of subinhibitory concentration of crude peel extracts and the subfractions F 3.2 and F 4.1 on the proteinase production by *P. gingivalis*

The effect of subinhibitory concentrations of peel extract, subfractions F 3.2 and F 4.1 on the production of Arg-gingipain and Lys-gingipain production was evaluated using a chromogenic test. *P. gingivalis* cells were exposed to the subinhibitory concentrations of peel extracts. A

colometric assay was performed to determine Arg-gingipain or Lys-gingipain. Relative enzymatic activity was calculated. The test results were compared to the controls. This experiment was repeated five times using a strain of *P. gingivalis*.

Results of this section are described in Chapter 8.

f. To perform structural elucidation on subfractions F 3.2 and F 4.1 to identify chemical composition of the beneficial effects.

Subfractions F 3.2 and F 4.1 were further purified using flash chromatography. Preparative HPLC, Mass spectrometry, NMR and FTIR were used for structural elucidation and identification of chemical composition of the subfractions.

Results of this section are described in Chapter 9.

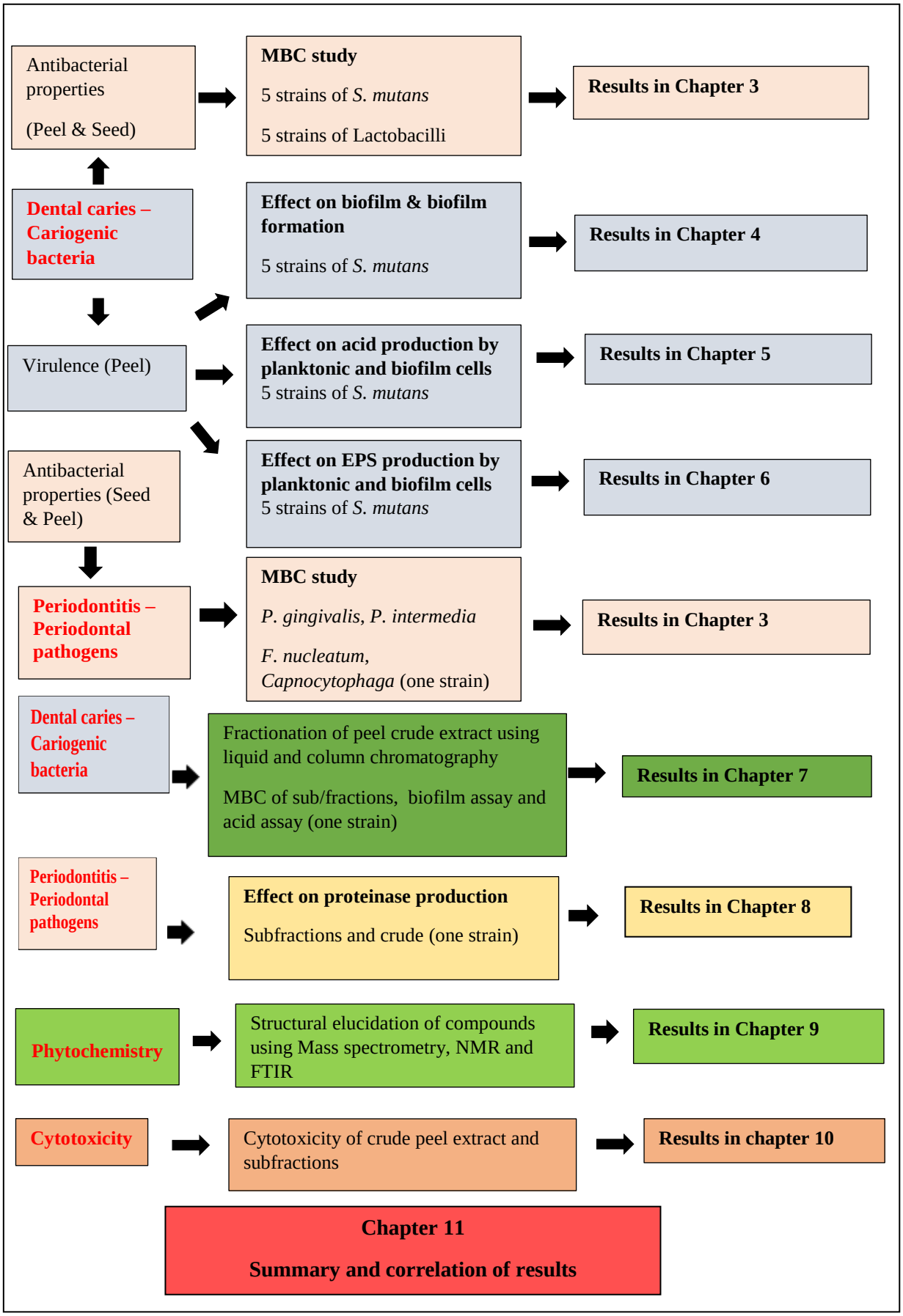
g. To determine the cytotoxicity of peel extract and most active compound identified

Cytotoxicity tests of crude peel extract, subfractions F 3.2 and F 4.1 were carried out using a HEK 293 kidney cell line and the MTT assay. The human cell line was exposed to different concentrations of test extract and compound. Cell viability was then measured and compared with controls.

Results of this section are described in Chapter 10.

h. A summary of results described in Chapter 3 to 10 are discussed in Chapter 11.

Figure 2.1 Study design and layout of the thesis



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CHAPTER 3

The antimicrobial effect of pomegranate peel and seed extracts against causative agents of dental caries and periodontal diseases

3.1 Introduction

The antimicrobial activity of pomegranate extracts as well as its constituents is well documented (Abdollahzadeh *et al.,* 2011, Cowan 1999, Naz *et al.,* 2007, Reddy *et al.,* 2007). The entire *Punica granatum* L fruit components abundant with tannins show relatively strong astringent effect (Shaygannia *et al.,* 2016). The pomegranate peel consists of about fifty percent of the total fruit weight and therefore accounts for the major active compounds of the fruit (Opara *et al.,* 2009). *P. granatum* peel extracts that are rich in ellagitannins exert remarkable antimicrobial activity against gram positive, negative pathogens and other microbes (Batista *et al.,* 2014, Pagliarulo *et al.,* 2016). Singh *et al*., 2002, postulated in their study that the antimicrobial effect of pomegranate may be attributed to its high antioxidant activity which may be directly correlated to the phenolic content in the various peel extracts (Singh *et al.,* 2002).

Few *in vitro* studies have been performed on pomegranate peel and oral pathogens. Abdollahzadeh *et al*., (2011) reported that pomegranate methanolic peel extract has an antimicrobial activity against *S. mutans* and lactobacilli at the concentrations of 8 and 12 mg/ml respectively (Abdollahzadeh *et al.,* 2011). In another study, Rosas-Pinon *et al*., 2012, used pomegranate fruit pericarp from the Altiplane in Mexico to prepare aqueous and ethanolic extraction (Rosas-Pinon *et al.,* 2012). They reported that the aqueous extract had better antibacterial property against *S. mutans* (MIC value of 12.5 µg/ml) and *P. gingivalis* with ethanol extract (MIC of 125 µg/ml).

Pomegranate seed oil contains 65-80 % conjugated linolenic acids (CLnAs) mostly punicic acid (Abbasi *et al.,* 2008). Punicic acid has anti-inflammatory properties since it can inhibit synthesis of tumor necrosis factor (TNF-α) and neutrophils myeloprexidase activity (Boussetta *et al.,* 2009). In addition, pomegranate seed oil has a high amount of tocopherols which exert strong natural antioxidant activity (Elfalleh *et al.,* 2011). No research has been done on the antimicrobial effect of pomegranate seed against oral pathogens.

Interestingly, pomegranate cultivars were reported from several locations all over the world, including Europe (Spain, France, Italy, Greece, and Cyprus), Asia (Turkey, Turkmenistan, Kirgizstan, Azerbaijan, Iran, India, China, Russia, Israel), and North Africa (Morocco, Tunisia, Egypt). Also, the botanical differences between wild pomegranates and cultivated pomegranates are not obvious (Holland *et al.,* 2009). In South Africa, there are more than ten cultivars that are being cultivated and Fawole *et al*., 2012 have shown variation in the antioxidant and anti-tyrosinase activities of the peel from methanolic extracts of the seven cultivars (Fawole *et al.,* 2012). They have also reported on the antibacterial effect of these cultivars against other pathogens such as *E. coli* and *K. pneumonia*. There is no doubt that more research has to be done on the antibacterial effect of the pomegranate peel and seed against oral pathogens especially on the South African cultivars.

The purpose of this chapter was to determine minimum bactericidal concentration (MBC) values of the peel and seed of the locally produced South African cultivar against oral pathogens and select the best solvent that would give a best yield for the subsequent studies. The selection of the MBC in the peel study together with the best performing solvent would

further allow for the identification of the three sub-inhibitory concentrations which could be used in the subsequent experiments.

Aim: This study investigated the antibacterial (minimal bactericidal concentrations) effect of methanol, acetone, hexane, water and dichloromethane/methanol extracts of the seed and peel against *S. mutans, lactobacillus* (cariogenic bacteria) and *P. intermedia, P. gingivalis, F. nucleatum,* and *Capnocytophaga* (periodontal pathogens).

3.2 Methods and Materials

3.2.1 Plant material

Pomegranate fruits were purchased from Zanddrift Farm in Cape Town. The pomegranate plant, fruit and peel were obtained from this farm and deposited in the University of Witwatersrand herbarium library with a voucher number specimen J36617. The fruit peel was separated, washed and air dried. The material was thereafter pulverised in an electric grinder (Figure 3.1). Pomegranate seeds were obtained from the fruits, air dried and pulverised. Methanol, acetone, dichloromethane:methanol, water and hexane were used for extraction. Crude extracts were obtained by mixing 10 g of peel or seed powder separately with one hundred milliliter of the relevant solvent. The solution was then shaken at 120 rpm at room temperature overnight. After filtration, the solvent extracts were evaporated under reduced pressure (Buchi rotary evaporator), below 40^0 C and the plant residue re-extracted twice. The solvent extracts were then fully dried under cold air current and stored at 4^0 C until required (Eloff 1999). The yield of a dried extract was obtained. All the dried extracts were then reconstituted to obtain 50 mg/ml concentrations using dimethyl sulfoxide (DMSO).

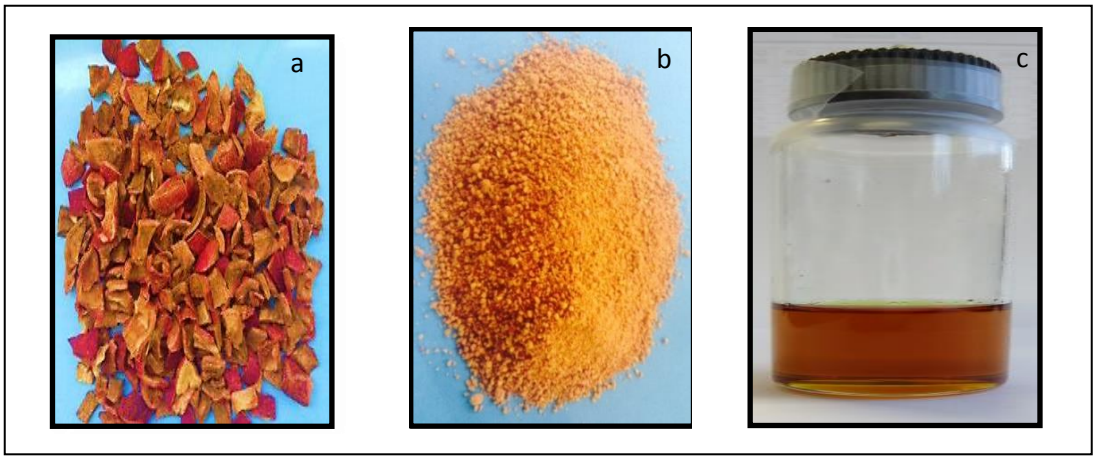

Figure 3.1 Dried peel (a), peel powder (b) and peel crude extract (c) of *P. granatum* fruit

3.2.2 Bacterial cultures

Cultures of *S. mutans*, lactobacilli, *P. intermedia, P. gingivalis, F. nucleatum,* and *Capnocytophaga* species were obtained from Oral Microbiology laboratory, Wits, Johannesburg. These cultures were collected with an ethical permission from University of Witwatersrand ethics committee (Ref: W-CPB-180919-01) and kept at -70^0 C. *S. mutans* and lactobacilli were isolated from saliva samples of patients attending Charlotte Maxeke Academic hospital Johannesburg at the Wits Dental Clinic. These were patients with active carious lesions. Saliva samples collected from these patients were plated on mutans bacitracin agar (MBA) and rogosa agar (RA) (Conda Lab, SA). Cultures were identified using gram reaction and biochemical reactions in the API 20 Strep standardized system for the *S. mutans* (BioMerieux, SA). They were verified using Microscan (Walkaway) S140 (Siemens US) as an additional identification method which uses a series of biochemical reactions.

S. mutans was cultured on blood agar (Oxoid Ltd, UK) for continual revival after the identification procedure was completed. These cultures were grown under CO_2 for forty eight hours at 37^o C. Bacterial suspensions were prepared in tryptone broth to obtain an optical density of 0.2 at 405nm which contained approximately 10^7 colony forming units per mililiter.

One hundred microliter of this suspension (inoculum) was used in the various test concentrations of plant extract.

Cultures of *P. intermedia* ATCC 25611 and *F. nucleatum* ATCC 25586 were obtained from Oral Microbiology laboratory. The cultures of *P. gingivalis* and *Capnocytophaga* species were obtained from patients attending Oral Medicine and periodontology clinic, Wits oral Health Centre, Johannesburg. Samples of periodontal pocket debris were collected by a clinician using paper points. Paper points were then placed in serum and transported into the laboratory for processing within an hour of collection. Samples were plated on blood agar plates supplemented with 20 µl of 5 mg/ml hemin and 1 mg/ml menadione and incubated at 37^{o} C for 7 days under anaerobic conditions. The anaerobic environment was created using the anaero-pack (MGC, Davies diagnostics). Identification of cultures was done with gram reaction and API 20A strip containing biochemical reaction. These cultures were used throughout the study. Five clinical strains of *S. mutans*, and one strain of each of *P. intermedia, F. nucleatum, P. gingivalis* and *Capnocytophaga* species (periodontal pathogens) were included in the study.

3.2.3 Antibacterial activity (MBC)

For *S. mutans* and lactobacilli, a two-fold dilution of crude peel extract (100 µl) was prepared using tryptone broth in a microtitre plate (Eloff 1998). The test concentrations were 25 mg/ml to 0.39 mg/ml Figure 3.2. Fresh inocula, with optical density of 0.2 (405 nm) containing approximately 10^5 - 10^6 cfu/ml were prepared and added to the wells. The plates were incubated at 37^{o} C under CO_2 for 48 hours. Chlorhexidine gluconate was used as a positive control and water as a negative control. Effect of 10 % DMSO on the bacterial growth was determined. After incubation, each well was subcultured on blood agar and incubated at 37^{o} C under CO_2 for 48 hours.

Similarly antibacterial activity against *P. gingivalis, Capnocytophaga* sp., *P. intermedia* and *F. nucleatum* was determined. The plant extracts were diluted in tryptone soy broth containing 5 mg/ml hemin and 1 mg/ml menadione for *P. gingivalis* and *P intermedia.* While, *Fusobacterium* and *Capnocytophaga* were diluted in plain tryptone soy broth. The microtitre plates were incubated anaerobically for 7 days at 37^{o} C. After incubation each dilution was cultured on blood agar and the lowest concentration of the extracts that showed no growth was recorded as MBC value.

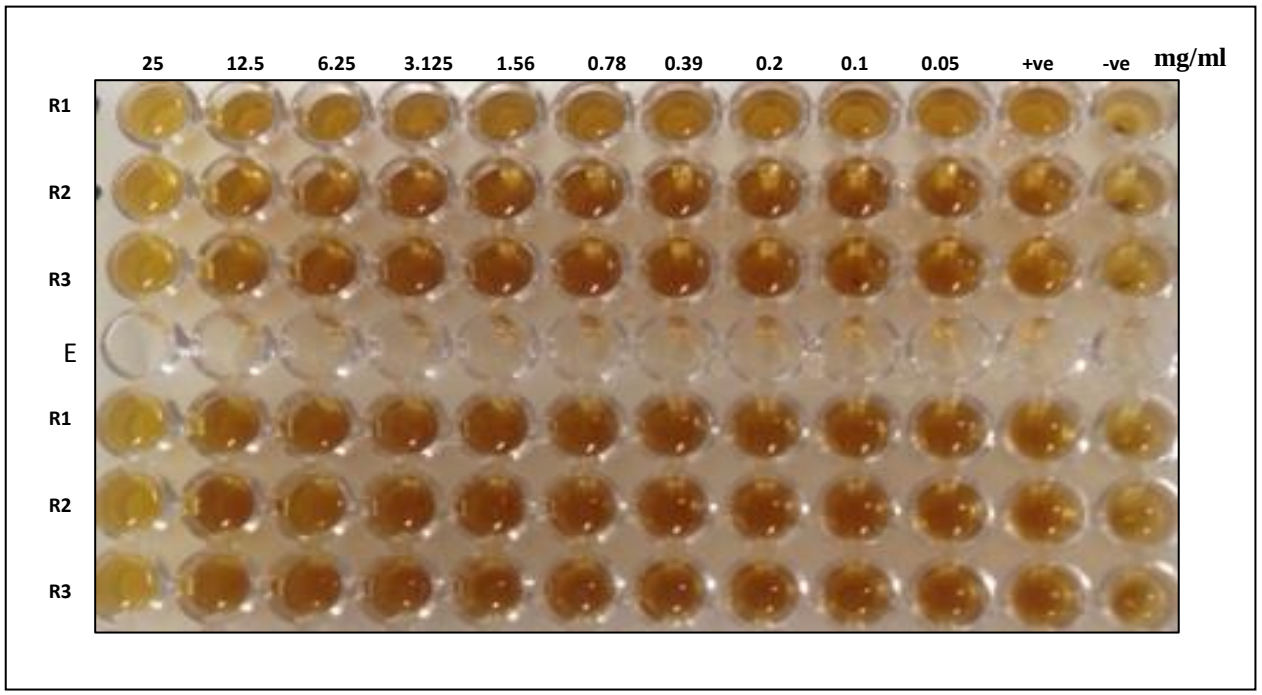

Figure 3.2 Microtitre plate containing various concentrations (mg/ml) of *P. granatum* peel extract (horizontal) and inoculum. Repeated experiments (three times for each test organism): R1, R2 and R3 and E row is empty. About 0.1 % chlorhexidine (+ve) positive control and inoculum (–ve) negative were used. The solvent that produced the best MBC for *S. mutans* were selected for the subsequent studies. Three sub-inhibitory concentrations of the crude extract were chosen for the subsequent studies such as acid, biofilm and EPS production studies.

3.3 Results

3.3.1 Pomegranate crude extract yield

The pomegranate dried peel and seeds were extracted with methanol, dichloromethane/methanol, hexane, water and acetone. The pomegranate peel DCM/Methanol extract produced the highest extract yield of 0.48 g/gram of dry powder followed by methanol at 0.47 g/gram of dry powder and water at 0.46 g/gram of dry powder (Table 3.1). The yields of seed extracts are given in Table 3.2. The water and methanol seed extracts produced a good yield (0.3 g/gram of powder) compared to the other solvents.

Table 3.1 Yield of pomegranate peel extracts

Repeats	**Yield per gram of pomegranate peel powder**				
	Methanol	**DCM/Methanol**	**Hexane**	**Water**	**Acetone**
1	0.441	0.510	0.080	0.532	0.290
2	0.330	0.720	0.007	0.330	0.290
3	0.507	0.550	0.001	0.263	0.139
4	0.440	0.260	0.005	0.530	0.124
5	0.540	0.370	0.007	0.530	0.094
6	0.570	0.008	0.008	0.590	0.373
Mean yield	**0.471**	**0.481**	**0.018**	**0.463**	**0.218**
% yield	**47.1**	**48.1**	**1.8**	**46.3**	**21.8**

Table 3.2 Yield of pomegranate seed extracts

Repeats	**Yield per gram of pomegranate seed powder**				
	Methanol	**DCM/Methanol**	**Hexane**	**Water**	**Acetone**
1	0.050	0.200	0.004	0.296	0.150
2	0.277	0.040	0.004	0.023	0.310
3	0.268	0.014	0.004	0.118	0.172
4	0.550	0.077	0.005	0.650	0.179
5	0.450	0.320	0.010	0.600	0.060
Mean yield	**0.319**	**0.130**	**0.005**	**0.337**	**0.174**
% yield	**31.9**	**13.0**	**0.5**	**33.7**	**17.4**

3.3.2 Antibacterial effect of the pomegranate peel extract against *S. mutans* and lactobacilli (MBC values)

The results of the antibacterial effect of pomegranate peel extract are shown in Table 3.3. For *S. mutans*, the MBC values of crude peel and seed extracts ranged from 6.25 to 25 mg/ml. For lactobacilli, the MBC values of crude peel and seed extracts ranged from 12.5 to 25 mg/ml. DCM/methanol extract of peel and acetone extract of seeds showed the best antibacterial activity against *S. mutans* (Table 3.3). Water extract of peel and DCM/methanol extract of seeds showed the best antibacterial activity against lactobacilli (Table 3.4).

Consequently, methanol was selected as a solvent mixture for the extractions for the subsequent antivirulence studies. For the biofilm, acid and extracellular polysaccharide assays, 3 subinhibitory concentrations of 1.56, 3.125 and 6.25 mg/ml were chosen. Dimethyl sulfoxide had no effect on bacterial growth of *S. mutans* and lactobacilli. The positive control 0.1 % chlorhexidine gluconate killed the test organism. The minimal inhibitory concentrations (MIC) could not be obtained because the peel extract precipitated with the culture medium.

Table 3.3 Minimum bactericidal concentrations of pomegranate peel and seed extracts against *S. mutans*.

Clinical strains	Repeats	**Pomegranate peel extract MBC (mg/ml)**					**Pomegranate peel extract MBC (mg/ml)**				
		Methanol	DCM/ Methanol	Hexane	Water	Acetone	Methanol	DCM/ Methanol	Hexane	Water	Acetone
SM 1	1	12.50	6.25	25.00	12.50	12.50	6.25	12.50	12.50	12.50	6.25
	2	6.25	12.50	12.50	12.50	12.50	12.50	12.50	25.00	12.50	12.50
	3	12.50	6.25	12.50	12.50	6.250	6.25	12.50	25.00	12.50	25.00
SM 6	1	12.50	6.25	25.00	12.50	12.50	12.50	12.50	12.50	12.50	12.50
	2	12.50	12.50	25.00	25.00	12.50	25.00	12.50	12.50	12.50	12.50
	3	12.50	25.00	12.50	12.50	6.25	12.50	12.50	12.50	12.50	25.00
SM 7	1	12.50	6.25	25.00	12.50	12.50	12.50	12.50	12.50	12.50	6.25
	2	6.25	12.50	25.00	25.00	12.50	12.50	12.50	12.50	12.50	6.25
	3	6.25	25.00	12.50	12.50	6.25	12.50	12.50	12.50	12.50	25.00
SM 12	1	6.25	6.25	12.50	12.50	12.50	25.00	12.50	12.50	12.50	6.25
	2	12.50	12.50	25.00	12.50	12.50	12.50	12.50	12.50	12.50	12.50
	3	12.50	25.00	12.50	12.50	6.25	6.25	12.50	12.50	12.50	25.00
SM 13	1	25.00	6.25	12.50	12.50	25.00	25.00	12.50	12.50	12.50	12.50
	2	25.00	6.25	25.00	12.50	25.00	25.00	12.50	12.50	12.50	25.00
	3	25.00	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	25.00
Median		**12.50**	**6.25**	**25.00**	**12.50**	**12.50**	**12.50**	**12.50**	**12.50**	**12.50**	**12.50**
± SD		**6.63**	**7.27**	**6.45**	**4.40**	**5.79**	**6.95**	**0.00**	**4.40**	**0.00**	**8.14**

Table 3.4 Minimum bactericidal concentrations of pomegranate peel and seed extracts against Lactobacilli.

Clinical strains	Repeats	**Pomegranate peel extract MBC (mg/ml)**					**Pomegranate seed extract MBC (mg/ml)**				
		Methanol	DCM/ Methanol	Hexane	Water	Acetone	Methanol	DCM/ Methanol	Hexane	Water	Acetone
L1	1	25.00	25.00	25.00	12.50	25.00	25.00	6.25	12.50	12.50	12.50
	2	25.00	12.50	25.00	12.50	25.00	12.50	12.50	12.50	12.50	6.25
	3	12.50	25.00	25.00	12.50	25.00	12.50	6.25	12.50	12.50	25.00
L2	1	12.50	25.00	25.00	12.50	25.00	25.00	12.50	12.50	12.50	25.00
	2	25.00	12.50	25.00	12.50	25.00	25.00	12.50	12.50	12.50	12.50
	3	12.50	25.00	25.00	12.50	25.00	12.50	12.50	12.50	12.50	12.50
L3	1	25.00	25.00	25.00	12.50	25.00	25.00	6.30	12.50	12.50	25.00
	2	6.25	6.25	25.00	12.50	12.50	25.00	12.50	12.50	12.50	25.00
	3	12.50	25.00	25.00	12.50	25.00	12.50	12.50	12.50	25.00	12.50
L4	1	25.00	25.00	12.50	6.25	25.00	25.00	12.50	12.50	12.50	25.00
	2	25.00	12.50	6.25	12.50	25.00	25.00	12.50	12.50	12.50	25.00
	3	6.25	25.00	6.25	6.25	25.00	12.50	12.50	6.25	25.00	12.50
L5	1	25.00	25.00	12.50	12.50	25.00	25.00	25.00	25.00	25.00	25.00
	2	25.00	12.50	12.50	12.50	12.50	25.00	25.00	25.00	25.00	25.00
	3	12.50	25.00	12.50	12.50	25.00	12.50	12.50	12.50	25.00	25.00
Median		**25.00**	**25.00**	**25.00**	**12.50**	**25.00**	**25.00**	**12.50**	**25.00**	**12.50**	**25.00**
± SD		**7.6**	**6.9**	**7.6**	**2.2**	**4.4**	**6.3**	**5.5**	**4.8**	**6.1**	**7**

Table 3.5 Summary results of Minimum bactericidal concentrations of pomegranate peel and seed extracts against *S. mutans* and Lactobacilli

Solvent	***S. mutans*** **Median MBC (mg/ml) n=15**		**Lactobacilli** **Median MBC (mg/ml) n=15**	
	Peel extract	**Seed extract**	**Peel extract**	**Seed extract**
Methanol	**12.50**	12.50	**25.00**	25.00
DCM/Methanol	**6.25**	12.50	**25.00**	12.50
Hexane	**25.00**	12.50	**25.00**	25.00
Water	**12.50**	12.50	**12.50**	12.50
Acetone	**12.50**	12.50	**25.00**	25.00

3.3.3 Antibacterial effect of the pomegranate peel and seed extract against *Capnocytophaga* (Cs), *F. nucleatum* (*Fn*), *P. gingivalis* (*Pg*) and *P. intermedia* (*Pi*) the periodontal pathogens.

The MBC values of peel and seed extracts against periodontal pathogens are shown in Table 3.6 and 3.7. For *P. gingivalis* the peel extract MBC ranges from 0.025 to 1.56 mg/ml for all solvents. Methanol extract was the most effective whereas hexane extract had the least effect. For *P. intermedia* the MBC values of peel extract ranged from 0.195 to 3.125 mg/ml. Methanol was the most effective solvent. For *F. nucleatum* the MBC values of peel extract ranged from 0.78 mg/ml to 6.25 mg/ml and acetone proved to be the most effective solvent at the lowest concentration. For *Capnocytophaga* species MBC values of the peel extract ranged from 0.39 mg/ml to 3.125 mg/ml. Acetone proved to be the best solvent because it showed the lowest MBC (Table 3.6).

For *P. gingivalis* the MBC of the seed extract ranges from 0.39 to 1.56 mg/ml. Acetone extract was most effective whereas hexane was the least effective solvent. For *P. intermedia* the MBC values ranged from 0.39 to 3.125 mg/ml. Methanol and acetone were the most effective solvents. For *F. nucleatum* the MBC values ranged from 3.125 mg/ml to 25 mg/ml. Methanol proved to be the most effective solvent. For *Capnocytophaga* species the MBC values ranged from 0.78 mg/ml to 6.25 mg/ml. Acetone and hexane proved to be the best solvents (Table 3.6).

Peel extracts showed better antibacterial activities against periodontal pathogens compared to the seed extract (Table 3.7). *P. gingivalis* was the most sensitive periodontal pathogen to pomegranate extracts (Table 3.7).

Table 3.6 Antibacterial effect of the pomegranate peel and seed extract against Capnocytophaga, *F. nucleatum, P. gingivalis* and *P. intermedia* (periodontal pathogens)

Extract	**Solvent**	**Repeat**	**MBC (mg/ml)**			
			Pg	Pi	Fn	Cs
Peel	Methanol	1	0.025	0.195	6.250	1.560
		2	0.025	0.195	6.250	1.560
		3	0.025	0.195	6.250	1.560
	Median		**0.025**	**0.195**	**6.250**	**1.560**
	DCM/Methanol	1	0.780	0.390	1.560	0.78
		2	0.780	0.390	3.125	0.780
		3	0.780	0.390	1.560	0.098
	Median		**0.780**	**0.390**	**1.560**	**0.780**
	Hexane	1	1.560	3.125	0.780	3.125
		2	1.560	3.125	0.780	3.125
		3	1.560	3.125	0.780	3.125
	Median		**1.560**	**3.125**	**0.780**	**3.125**
	Water	1	0.780	3.125	1.560	3.125
		2	0.780	3.125	1.560	3.125
		3	0.780	3.125	1.560	3.125
	Median		**0.780**	**3.125**	**1.560**	**3.125**
	Acetone	1	0.195	0.780	0.780	0.390
		2	0.195	0.780	0.780	0.390
		3	0.195	0.780	0.780	0.390
	Median		**0.195**	**0.780**	**0.780**	**0.390**
Seed	Methanol	1	1.560	0.390	6.250	1.560
		2	1.560	0.390	6.250	1.560
		3	1.560	0.390	6.250	1.560
	Median		**1.560**	**0.390**	**6.250**	**1.560**
	DCM/Methanol	1	0.780	3.125	25.000	6.250
		2	0.780	3.125	25.000	6.250
		3	0.780	3.125	25.000	6.250
	Median		**0.780**	**3.125**	**25.000**	**6.250**
	Hexane	1	0.780	1.560	6.250	0.780
		2	0.780	1.560	3.125	0.780
		3	0.780	1.560	3.125	0.780
	Median		**0.780**	**1.560**	**3.125**	**0.780**
	Water	1	1.560	0.780	25.000	6.250
		2	1.560	0.780	25.000	3.125
		3	1.560	0.780	25.000	3.125
	Median		**1.560**	**0.780**	**25.000**	**3.125**
	Acetone	1	0.390	0.390	6.250	0.780
		2	0.390	0.390	6.250	0.780
		3	0.390	0.390	6.250	0.780
	Median		**0.390**	**0.390**	**6.250**	**0.780**

Table 3.7 Summary of MBC results of peel and seed extracts against *P. intermedia, P. gingivalis, F. nucleatum,* and Capnocytophaga

Extract	**Solvent**	**Median MBC (mg/ml)**			
		Pg	**Pi**	**Fn**	**Cs**
Peel	Methanol	0.025	0.195	6.250	1.560
	DCM/Methanol	0.780	0.390	1.560	0.780
	Hexane	1.560	3.125	0.780	3.125
	Water	0.780	3.125	1.560	3.125
	Acetone	0.195	0.780	0.780	0.390
Seed	Methanol	1.560	0.390	25.000	6.250
	DCM/Methanol	0.780	3.125	25.000	6.250
	Hexane	0.780	1.560	3.125	0.780
	Water	1.560	0.780	25.000	3.125
	Acetone	0.390	0.390	6.250	0.780

3.4 Discussion

Dichloromethane:methanol extraction, a combination of a slight nonpolar (DCM) and a polar (methanol) solvent is the best method to extract a wide range of plant compounds. Generally, water has been used in preparation of home remedies due to its ease of availability. The challenge in using water is that it cannot extract nonpolar bioactive compounds. The type of solvent used in the extraction procedure determines the success of isolating compounds from plant material (Masoko and Makgapeetja 2015). Polar solvents such as methanol, ethanol, acetone, chloroform and ethyl acetate are popularly used for industrial scale extraction of phenolic compounds. And these solvents have greater capability for extraction of antioxidants compared to non-polar solvents (Ismail *et al.,* 2012). Different solvents produce different phenolic content and associated antioxidant activity especially the non-water solvents in the phenolic extraction of the peel (Negi and Jayaprakasha 2003, Zahin *et al.,* 2010).

A study by Li *et al*., (2016), demonstrated a significant variation in total phenolic content and the four major polyphenols from different fruit parts of pomegranate from China. The main polyphenols were gallic acid, ellagic acid, punicalagin and punicalin (Li *et al.,* 2016). The peel had the utmost content of the punicalagin A & B which attributes major contribution to the antioxidant activity of pomegranate. Seeds contained the highest quantity of oil. In a study, the lipid content from the Iran variety was higher than western pomegranate cultivars (Fadavi *et al.,* 2006). The leading fatty acid of the seed oils were linolenic acid followed by linoleic acid, oleic acid, stearic acid and palmitic acid. To a reduced extent, the saturated myristic acid and behenic acid were also found in some cultivars (Fadavi *et al.,* 2006). Again, Elfalleh *et al*., studied the phenolic and tocopherols content of fruits, peels and seed oils of the Tunasian pomegranates. The antioxidant influence correlated with phenolic content especially in the peel extract (Elfalleh *et al.,* 2011). High correlations were also found between peel hydroxybenzoic acids and antioxidant capacities. They identified tocopherols (vitamin E) to be the contributor in major part to the antioxidant activity of seed oil. They speculated that the bioactive compounds from the peel might be a potential resource for the development of antioxidant function in dietary food (Elfalleh *et al.,* 2011).

Pomegranate has been used widely throughout the world for treatment of infections including oral infections (Al-Zoreky 2009). The antimicrobial effect of pomegranate against gram positive and gram negative bacterial oral pathogens has been demonstrated (Abdollahzadeh *et al.,* 2011, Mehta *et al.,* 2014, Rosas-Pinon *et al.,* 2012). The pomegranate peel consists of about 50 % of the total fruit weight and it may therefore accounts for the major active compounds of the fruit (Opara *et al.,* 2009). In a clinical trial, Menezes *et al*. (2006) has shown the use of *P. granatum* for a month,can reduce plaque by 84 % (Menezes *et al.,* 2006). The present study demonstrated that *P. granatum* peel and seed extracts have antibacterial activity against gram

positive plaque bacteria (*S. mutans* and lactobacilli). The MBC concentrations for the cariogenic bacteria were high (6.25 mg/ml). These results are in agreement with Abdollahzadeh (2010) and de Oliveira *et al*., (2013) who showed anti-*S. mutans* and lactobacilli activity at the concentration of 8 and 12 mg/ml respectively (Abdollahzadeh *et al.,* 2011, Oliveira *et al.,* 2013). High concentrations are known to inhibit the growth of *S. mutans* (Dabholkar *et al.,* 2016, Dastjerdi *et al.,* 2014, Mehta *et al.,* 2014). The only report that has shown contrasting results is by Rosas-Pinon *et al*., (2012). They reported MBC values of 0.125 mg/ml against *S. mutans*. Nevertheless, *P. granatum* has shown to reduce the plaque bacteria in the actual oral plaque and therefore it was further exploited for its effect on the plaque formation (biofilm study), plaque metabolism (acid production) and plaque composition (EPS production).

In clinical trials, *P. granatum* has also been shown to have beneficial effects in periodontal diseases. It has been shown to improve the attachment level, pocket depth and bleeding (Batista *et al.,* 2014, Bhadbhade *et al.,* 2011, Sastravaha *et al.,* 2005). In a present study, *P. granatum* also showed antibacterial effect against gram negative periodontal pathogens (*P. gingivalis, P. intermedia, F. nucleatum and Capnocytophaga* species*).* The best MBC for *P. gingivalis* and *P. intermedia* were 0.025 mg/ml and 0.195 mg/ml respectively. *Capnocytophaga* was completely eliminated at 0.390 mg/ml. MBC value of *P. gingivalis* is similar to the values found by Rosas-Pinon *et al*, (2012) which were 0.250 mg/ml. However, high concentrations were reported by Bhadbhade *et al*., (2011) with the lowest concentration of 16.125 mg/ml, followed by 31.25 mg/ml and the highest of 62.50 mg/ml for *P. intermedia*, *P. gingivalis* and *A. actinomicetamcomitance* respectively. Although present study has shown that *P. granatum* has anti-periodontal pathogen activity, only the effect of *P. granatum* on the proteinase production by *P. gingivalis* will be studied. *P. gingivalis* is the primary pathogen and the

proteinase facilitates the pathogenesis of this infection. If *P. granatum* shows any reduction in the proteinase production, it would be an additional benefit in the oral cavity.

3.5 Conclusions

Solvents DCM/methanol produced the best yield from the *P. granatum* peel. At high concentrations (6.25 to 12.5 mg/ml) it completely killed cariogenic bacteria *S. mutans* and lactobacilli. Methanol extract performed best for the periodontal pathogens compared to all the solvents. At low concentrations (< 0.2 mg/ml) it killed periodontal pathogens especially *P. gingivalis* and *P. intermedia.* This study demonstrated that *P. granatum* have potential to be used to prevent dental caries and periodontal diseases. Further studies can be undertaken to study its effect on the virulence properties of these bacteria. These results also encourage further investigations to extract and identify the active chemical compounds responsible for the antibacterial effect observed.

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CHAPTER 4

The effect of *P. granatum* peel extract on the biofilm formation by *S. mutans*

4.1 Introduction

Dental biofilms develop after the initial microbial attachment of oral bacteria to the tooth surface which is preceded by formation and stabilization of highly structured microcolonies. These microcolonies grows larger due to the multiplication of bacteria, grow vertically connecting with one another via the extracellular polymers. (Ansari *et al.,* 2017). Biofilm plays a leading role in the development and progression of most common infectious diseases of the tooth *e.g* dental caries. Biofilm consists of oral bacteria, glucan (extracellular polysaccharides), salivary compounds and debris (Kim and Lee 2016). Moreover, throughout this matrix there's water channels transporting nutrients and intercellular signalling molecules (Donlan and Costerton 2002). The stability of the biofilm matrix network is strengthened by the presence of molecular polymers, and the water channels (Ansari *et al.,* 2017). It also regulates permeability by preventing and reducing diffusion of antibiotics, slows movement of nutrients through the matrix as well as the blocking predatory cells of the immune system (Costerton *et al.,* 1999).

S. mutans is a pioneer species and the first to adhere to the tooth surface. Therefore, it is a key contributor to biofilm formation and the main causative agent of dental caries. The tooth surface is the natural habitat of the *S. mutans* and this organism cannot be found in the edentulous human beings. *S. mutans* will strive better compared with other oral streptococci in an environment rich in sucrose, low pH, and low saliva flow and these conditions allow this bacteria to form biofilm. The biofilm formed in such conditions is called cariogenic biofilm and this induces caries lesions on the tooth surface (Kim and Lee 2016). *S. mutans* is responsible for the production of glucosyltransferases (GTF's) that synthesize extracellular

polysaccharide (EPS). Extracellular polysacharides, most importantly the water-insoluble glucans, plays a major role in the formation of biofilm, its stability and structural integrity through attachment of bacteria to tooth surfaces and protecting bacteria against noxious stimuli and other environmental attacks (Chen *et al.*, 2016). Also, present in the *S. mutans* biofilms are the adhesin proteins and extracellular DNA (Liao *et al.*, 2014). The bacterial attachment, stability of the matrix by EPS from *S. mutans* is the most critical step in the pathogenicity in a rat caries model (Yamashita *et al.*, 1993).

At the moment, dental plaque is removed mainly by nonspecific mechanical removal such as tooth brushing, the use of floss, application of oral rinses with antimicrobial activity such as chlorhexidine, fluoride, triclosan and essential oils. Other potential anti-cariogenic methods include sugar substitutes, probiotic strains, and arginine an insoluble calcium compound (Ren *et al.*, 2016). However, selectivity of these treatments have not been proven on *S. mutans*. Effective antimicrobials that selectively elimininate *S. mutans* which are targeted to the bacterial growth factors with minimal effect on the other oral microbes must be designed. This should be done to prevent caries and oral diseases that develop as a results of plaque accumulation without affecting the ecological balance between pathogens and commensal bacteria (Ren *et al.*, 2016). In addition, an alternative approach that will disarm bacteria of its pathogenicity such as targeting bacterial virulence can be developed for new antimicrobials (Burne *et al.*, 1996, Hannig *et al.*, 2008, Llena-Puy 2006). The overall strategy is to inhibit specific mechanisms that promote infection. A reduced presures of drug resistance and mutations should be provided by the stripping off these microorgansms of their virulence properties.Therefore, interfering with *S. mutans* matrix architecture by inhibiting bacterial adhesion or disrupting the intercellular communication as well as preventing the development of biofilm may be a better target for the prevention of dental caries.

Aim: The aim of this chapter was to examine the effect of the crude peel extract of *P. granatum* on biofilm formation by *S. mutans*.

4.2 Material and Methods

4.2.1 *S. mutans* cultures and inocula

Isolation and identification of *S. mutans* strains is described in Chapter 3.2.2. Strains stored at -70^0 C were retrieved and cultured. Five *S. mutans* (SM1, SM6, SM7, SM12 and SM13) strains were cultured on blood agar plates (Oxoid Ltd, UK) for these experiments. These cultures were grown under CO_2 for 48 hours at 37^o C. The *S. mutans* inocula were prepared in tryptone broth media.

4.2.2 Biofilm assay

The effect of crude peel extract on the biofilm formation by *S. mutans* was studied using glass slide technique (Naidoo *et al.*, 2012). Briefly, three glass slides were held side by side in an upright (90^o C) position with an autoclave tape in four separate sterile beakers containing 50 ml of tryptone broth. In each beaker, a sub-inhibitory concentrations of methanol extract (1.56, 3.125 and 6.25 mg/ml) was added. Sterile distilled water was added into the fourth beaker instead of plant extract and it was considered as a control. All the beakers were inoculated with a 500 µl of inoculum. This was then incubated for 6 and 24 hours at 37^o C under CO_2. At each incubation period, one slide was removed and rinsed with sterile distilled water. Then, the attached biofilm cells were scrapped off (Figure 4.1) using another clean sterile glass slide and the biofilms were suspended in phosphate buffered saline (PBS). The suspension was then vortexed and quantified using a serial dilution technique. A positive control containing 0.2 % chlorhexidine was included. This experiment was repeated three times.

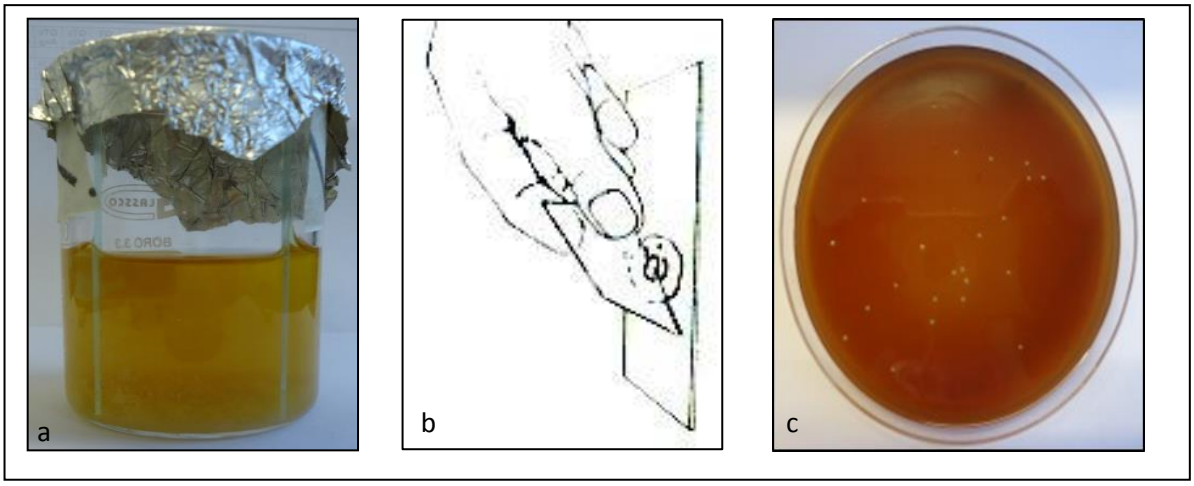

Figure 4.1 Biofilm assay (a) Beaker containing glass slides, *S. mutans* culture and plant extract (b) Removal of the biofilm, (c) *S. mutans* colonies on blood agar

4.2.3 Statistical analysis

Descriptive analysis was done for all the variables by computing means and standard deviation. The percentage reduction in the biofilm formation was calculated after 6 and 24 hours at all test concentrations. The formula used was $\left(\frac{x-y}{x}\right) x\ 100$ where x is the control while y is the test biofilm count. To determine the effect of different concentrations of the crude extract on biofilm formation by *S. mutans,* bacterial counts in the test biofilms were compared to the control counts using the Wilcoxon Rank Sum Test. Statistical significance was inferred at p value of < 0.05.

4.3 Results

The *S. mutans* counts in the biofilms grown in the presence of 1.56, 3.125 and 6.25 mg/ml crude extract and the controls are shown in Table 4.1, 4.2 and 4.3 respectively. In addition, the percentage reductions in the *S. mutans* counts of the biofilms are also shown in these tables. The summary of the results for all three concentration as well as their percentage reductions are shown in Table 4.4. After six hours, 1.56, 3.125, 6.25 mg/ml of crude *P. granatum* extract significantly reduced biofilm formation by 94.76 %, 96.6 % and 90.97 % respectively. The reduction was statistically significant with p value of < 0.01 (Tables 4.1 - 4.3, Figure 4.2 and 4.3). The concentration of 3.125 mg/ml was the most effective in decreasing the number of viable cells in *S. mutans* biofilms. At twenty four hours, exposure to 1.56, 3.125, 6.25 mg/ml of crude extract, the biofilm formation of *S. mutans* was also reduced by 65.4 %, 95.5 % and 87.6 % respectively (Tables 4.1 - 4.3, Figures 4.2 and 4.3). The reduction was significant with p value of < 0.01. Likewise, the concentration of 3.125 mg/ml had the highest percentage reduction thus most effective in decreasing the viable cells in the biofilm.

Table 4.1 The effect of 1.56 mg/ml crude extract of *P. granatum* peel on biofilm formation by *S. mutans*

Strains	Repeats	***S. mutans* counts in biofilm (cfu/ml)**					
		0 hrs		**6 hrs**		**24 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**
SM1	1	3.0×10^6	1.5×10^7	2.6×10^5	1.5×10^4	4.3×10^6	9.4×10^5
	2	3.1×10^6	7.1×10^6	3.4×10^5	6.0×10^3	1.3×10^6	1.5×10^6
	3	3.8×10^6	5.2×10^6	2×10^5	3.9×10^4	1.6×10^6	2.2×10^6
Mean		$\mathbf{3.2 \times 10^6}$	$\mathbf{9.2 \times 10^6}$	$\mathbf{2.6 \times 10^5}$	$\mathbf{1.9 \times 10^4}$	$\mathbf{2.4 \times 10^6}$	$\mathbf{1.5 \times 10^6}$
±SD		$\mathbf{4.7 \times 10^6}$	$\mathbf{5.3 \times 10^6}$	$\mathbf{7.2 \times 10^4}$	$\mathbf{1.9 \times 10^4}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{6.3 \times 10^5}$
SM6	1	4.5×10^6	9.0×10^6	4.7×10^6	3.0×10^4	8.0×10^7	1.5×10^6
	2	1.0×10^7	2.2×10^7	2.6×10^6	4.5×10^5	1.3×10^7	3.5×10^5
	3	5.4×10^6	2.5×10^7	1.7×10^7	2.8×10^5	1.5×10^7	3.0×10^5
Mean		$\mathbf{6.2 \times 10^6}$	$\mathbf{1.9 \times 10^7}$	$\mathbf{8.1 \times 10^6}$	$\mathbf{2.5 \times 10^5}$	$\mathbf{3.5 \times 10^7}$	$\mathbf{7.1 \times 10^5}$
±SD		$\mathbf{3.8 \times 10^6}$	$\mathbf{8.5 \times 10^6}$	$\mathbf{7.9 \times 10^6}$	$\mathbf{2.1 \times 10^5}$	$\mathbf{3.8 \times 10^7}$	$\mathbf{6.6 \times 10^5}$
SM7	1	2.2×10^6	1.2×10^7	1.3×10^6	4.9×10^5	3.1×10^6	2.8×10^7
	2	1.5×10^6	2.5×10^7	1.1×10^6	2.5×10^4	5.3×10^7	7.4×10^5
	3	1.8×10^6	1.3×10^7	4.0×10^5	4.0×10^4	1.5×10^6	8.5×10^5
Mean		$\mathbf{1.8 \times 10^6}$	$\mathbf{1.7 \times 10^7}$	$\mathbf{9.4 \times 10^5}$	$\mathbf{1.8 \times 10^5}$	$\mathbf{1.9 \times 10^7}$	$\mathbf{9.9 \times 10^6}$
±SD		$\mathbf{3.8 \times 10^5}$	$\mathbf{7.1 \times 10^6}$	$\mathbf{4.8 \times 10^5}$	$\mathbf{2.6 \times 10^5}$	$\mathbf{2.9 \times 10^7}$	$\mathbf{1.6 \times 10^7}$
SM12	1	4.4×10^6	2.6×10^7	3.4×10^6	5.0×10^4	7.9×10^5	7.8×10^5
	2	3.4×10^6	1.7×10^7	7.3×10^5	9.2×10^4	5.5×10^5	6.0×10^5
	3	1.9×10^6	6.5×10^6	2.5×10^5	3.3×10^4	3.6×10^6	1.2×10^6
Mean		$\mathbf{3.2 \times 10^6}$	$\mathbf{1.6 \times 10^7}$	$\mathbf{1.4 \times 10^6}$	$\mathbf{5.8 \times 10^4}$	$\mathbf{1.6 \times 10^6}$	$\mathbf{8.5 \times 10^5}$
±SD		$\mathbf{1.2 \times 10^6}$	$\mathbf{9.6 \times 10^6}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{3.0 \times 10^4}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{2.9 \times 10^5}$
SM13	1	2.5×10^6	4.6×10^6	4.7×10^4	2.7×10^4	1.0×10^7	6.0×10^6
	2	1.7×10^6	4.6×10^6	7.5×10^4	1.5×10^4	3.2×10^6	5.7×10^6
	3	1.3×10^4	1.1×10^7	3.8×10^6	3.2×10^5	6.2×10^6	1.6×10^7
Mean		$\mathbf{1.8 \times 10^6}$	$\mathbf{6.8 \times 10^6}$	$\mathbf{1.3 \times 10^6}$	$\mathbf{1.2 \times 10^5}$	$\mathbf{6.6 \times 10^6}$	$\mathbf{9.5 \times 10^6}$
±SD		$\mathbf{6.1 \times 10^5}$	$\mathbf{3.9 \times 10^6}$	$\mathbf{2.2 \times 10^6}$	$\mathbf{1.7 \times 10^5}$	$\mathbf{3.7 \times 10^6}$	$\mathbf{6.4 \times 10^6}$
Combined mean		$\mathbf{3.3 \times 10^6}$	$\mathbf{1.4 \times 10^7}$	$\mathbf{2.4 \times 10^6}$	$\mathbf{1.2 \times 10^5}$	$\mathbf{1.3 \times 10^7}$	$\mathbf{4.5 \times 10^7}$
Combined ±SD		$\mathbf{2.0 \times 10^6}$	$\mathbf{7.7 \times 10^6}$	$\mathbf{4.4 \times 10^6}$	$\mathbf{1.7 \times 10^5}$	$\mathbf{2.7 \times 10^7}$	$\mathbf{7.9 \times 10^6}$
% reduction $\left({}^{(Control - test)}/_{Control}\right)$ X 100				**94.76**		**65.4**	

Table 4.2 The effect of 3.125 mg/ml crude extract of *P. granatum* peel on biofilm formation by *S. mutans*

Strains	Repeats	***S. mutans* counts in biofilm (cfu/ml)**					
		0 hrs		**6 hrs**		**24 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**
SM1	1	3.0×10^6	1.5×10^7	2.6×10^5	4.0×10^4	4.3×10^6	3.2×10^3
	2	3.1×10^6	1.1×10^7	3.4×10^5	2.5×10^4	1.3×10^6	2.0×10^6
	3	3.8×10^6	7.0×10^6	2.0×10^5	2.4×10^4	1.6×10^6	1.4×10^6
Mean		$\mathbf{3.2 \times 10^6}$	$\mathbf{1.1 \times 10^7}$	$\mathbf{2.7 \times 10^5}$	$\mathbf{2.9 \times 10^4}$	$\mathbf{2.4 \times 10^6}$	$\mathbf{2.2 \times 10^6}$
±SD		$\mathbf{4.7 \times 10^5}$	$\mathbf{3.9 \times 10^6}$	$\mathbf{7.2 \times 10^4}$	$\mathbf{8.9 \times 10^3}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{9.4 \times 10^5}$
SM6	1	4.5×10^6	7.4×10^6	4.7×10^6	1.9×10^5	8.0×10^7	7.8×10^5
	2	1.0×10^7	2.1×10^7	2.6×10^6	1.7×10^5	1.3×10^7	6.5×10^5
	3	5.4×10^6	2.0×10^7	1.7×10^7	5.8×10^4	1.5×10^7	8.7×10^5
Mean		$\mathbf{6.7 \times 10^6}$	$\mathbf{1.6 \times 10^7}$	$\mathbf{8.1 \times 10^6}$	$\mathbf{1.4 \times 10^4}$	$\mathbf{3.6 \times 10^7}$	$\mathbf{7.6 \times 10^5}$
±SD		$\mathbf{3.3 \times 10^6}$	$\mathbf{7.6 \times 10^6}$	$\mathbf{7.9 \times 10^6}$	$\mathbf{7.2 \times 10^4}$	$\mathbf{3.8 \times 10^7}$	$\mathbf{1.1 \times 10^5}$
SM7	1	2.2×10^6	1.0×10^7	1.3×10^6	1.0×10^5	3.1×10^6	3.8×10^6
	2	1.5×10^6	1.4×10^7	1.1×10^6	5.0×10^3	5.3×10^7	5.5×10^4
	3	1.8×10^6	1.0×10^7	4.0×10^5	5.0×10^4	1.5×10^6	2.9×10^5
Mean		$\mathbf{1.8 \times 10^6}$	$\mathbf{1.1 \times 10^7}$	$\mathbf{9.4 \times 10^5}$	$\mathbf{3.5 \times 10^4}$	$\mathbf{1.7 \times 10^8}$	$\mathbf{1.3 \times 10^6}$
±SD		$\mathbf{3.8 \times 10^5}$	$\mathbf{2.0 \times 10^6}$	$\mathbf{4.8 \times 10^5}$	$\mathbf{5.6 \times 10^4}$	$\mathbf{3.0 \times 10^8}$	$\mathbf{2.1 \times 10^6}$
SM12	1	4.4×10^6	2.1×10^7	3.4×10^6	3.3×10^5	7.9×10^5	6.0×10^5
	2	3.4×10^6	2.0×10^7	7.3×10^5	1.8×10^5	5.5×10^5	1.2×10^6
	3	1.9×10^6	3.5×10^6	2.5×10^5	2.9×10^4	3.6×10^6	1.7×10^6
Mean		$\mathbf{3.2 \times 10^6}$	$\mathbf{1.4 \times 10^7}$	$\mathbf{1.4 \times 10^6}$	$\mathbf{1.7 \times 10^5}$	$\mathbf{1.6 \times 10^6}$	$\mathbf{1.5 \times 10^6}$
±SD		$\mathbf{1.2 \times 10^6}$	$\mathbf{9.9 \times 10^6}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{1.5 \times 10^5}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{5.6 \times 10^5}$
	1	2.5×10^6	5.1×10^6	4.7×10^4	4.9×10^4	1.0×10^7	1.8×10^6
	2	1.7×10^6	4.1×10^6	7.5×10^4	8.2×10^3	3.2×10^6	$8.1\ 10^6$
	3	1.3×10^4	1.0×10^7	3.8×10^6	2.5×10^4	6.2×10^6	3.7×10^6
Mean		$\mathbf{1.8 \times 10^6}$	$\mathbf{6.4 \times 10^6}$	$\mathbf{1.3 \times 10^6}$	$\mathbf{2.7 \times 10^4}$	$\mathbf{6.6 \times 10^6}$	$\mathbf{4.5 \times 10^6}$
±SD		$\mathbf{6.1 \times 10^5}$	$\mathbf{3.2 \times 10^6}$	$\mathbf{2.2 \times 10^6}$	$\mathbf{2.0 \times 10^4}$	$\mathbf{3.7 \times 10^6}$	$\mathbf{3.2 \times 10^6}$
Combined mean		$\mathbf{3.4 \times 10^6}$	$\mathbf{1.2 \times 10^7}$	$\mathbf{2.4 \times 10^6}$	$\mathbf{8.6 \times 10^4}$	$\mathbf{4.5 \times 10^7}$	$\mathbf{2.0 \times 10^6}$
Combined ±SD		$\mathbf{2.3 \times 10^6}$	$\mathbf{6.2 \times 10^6}$	$\mathbf{4.4 \times 10^6}$	$\mathbf{9.2 \times 10^4}$	$\mathbf{1.4 \times 10^8}$	$\mathbf{2.1 \times 10^6}$
% reduction $\left({}^{(Control - test)}/_{Control}\right)$ X 100				**96.6**		**95.5**	

Table 4.3 Effect of 6.25mg/ml crude extract of *P. granatum* peel on biofilm formation by *S. mutans*

***S. mutans* counts in biofilm (cfu/ml)**							
Strains	Repeats	**0 hrs**		**6 hrs**		**24 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**
SM1	1	1.4×10^6	1.6×10^6	6.5×10^3	3.0×10^3	1.9×10^6	1.5×10^4
	2	1.4×10^6	1.5×10^6	6.5×10^3	4.5×10^3	1.9×10^6	1.0×10^4
	3	2.7×10^6	4.3×10^6	3.1×10^5	1.8×10^4	2.8×10^6	2.9×10^5
Mean		**1.9×10^6**	**2.5×10^6**	**1.1×10^5**	**8.3×10^3**	**2.2×10^6**	**1.0×10^5**
±SD		**7.3×10^5**	**1.6×10^6**	**1.7×10^5**	**8.0×10^3**	**5.3×10^5**	**1.6×10^5**
SM6	1	6.5×10^5	2.4×10^6	9.7×10^5	3.2×10^3	1.5×10^7	5.6×10^5
	2	6.5×10^5	1.9×10^6	9.7×10^5	4.0×10^3	1.5×10^7	2.6×10^5
	3	4.3×10^6	1.8×10^7	3.1×10^6	1.7×10^5	2.8×10^7	1.2×10^7
Mean		**1.9×10^6**	**7.4×10^6**	**1.7×10^6**	**5.9×10^4**	**1.9×10^7**	**4.3×10^6**
±SD		**2.1×10^6**	**9.1×10^6**	**1.2×10^6**	**9.6×10^4**	**7.5×10^6**	**6.7×10^6**
SM7	1	8.0×10^5	4.2×10^6	1.1×10^5	1.5×10^4	2.6×10^8	7.1×10^6
	2	8.0×10^5	4.4×10^6	1.1×10^5	2.3×10^4	2.6×10^8	6.3×10^6
	3	5.5×10^6	1.1×10^7	$4.1\ 10^6$	2.6×10^5	3.4×10^7	1.3×10^7
Mean		**2.4×10^6**	**6.4×10^6**	**1.4×10^6**	**9.9×10^4**	**1.8×10^8**	**8.6×10^6**
±SD		**2.7×10^6**	**3.7×10^6**	**2.3×10^6**	**1.4×10^5**	**1.3×10^8**	**3.4×10^6**
SM12	1	2.0×10^6	8.1×10^6	1.5×10^5	5.0×10^5	5.1×10^6	1.8×10^6
	2	2.0×10^6	8.0×10^6	1.5×10^5	1.7×10^5	5.1×10^6	1.9×10^6
	3	5.2×10^6	1.4×10^7	2.9×10^6	6.9×10^4	1.5×10^6	1.1×10^7
Mean		**3.1×10^6**	**9.8×10^6**	**1.1×10^6**	**2.5×10^5**	**3.9×10^6**	**5.0×10^6**
±SD		**1.9×10^6**	**3.1×10^6**	**1.6×10^6**	**2.3×10^5**	**2.1×10^6**	**5.5×10^6**
SM13	1	1.0×10^6	8.6×10^6	1.5×10^5	4.3×10^4	1.7×10^6	1.0×10^7
	2	1.0×10^6	8.7×10^6	1.5×10^5	2.5×10^4	1.7×10^6	7.0×10^6
	3	8.5×10^5	6.5×10^6	5.6×10^6	3.8×10^5	1.6×10^7	7.7×10^6
Mean		**9.5×10^5**	**7.9×10^6**	**2.0×10^6**	**1.5×10^5**	**6.3×10^6**	**8.3×10^6**
±SD		**8.7×10^4**	**1.2×10^6**	**3.1×10^6**	**2.0×10^5**	**7.9×10^6**	**1.8×10^6**
Combined mean		**2.0×10^6**	**6.8×10^6**	**1.3×10^6**	**1.1×10^5**	**4.3×10^7**	**5.3×10^6**
Combined ±SD		**1.7×10^6**	**4.7×10^6**	**1.8×10^6**	**1.6×10^5**	**8.7×10^7**	**4.8×10^6**
% reduction $\left({}^{(Control - test)}/_{Control}\right)$ X 100				**90.97**		**87.6**	

Table 4.4 Summary results of the crude extract of *P. granatum* peel on biofilm formation by *S. mutans*

Time (hrs)		***S. mutans* counts in biofilm (cfu/ml)**					
		1.56 mg/ml		**3.125 mg/ml**		**6.25 mg/ml**	
		Control	**Plant**	**Control**	**Plant**	**Control**	**Plant**
6	**Mean**	2.4×10^6	1.3×10^5	2.4×10^6	8.6×10^4	1.3×10^6	1.1×10^5
	±SD	4.4×10^6	1.7×10^5	4.4×10^6	9.2×10^4	1.8×10^6	1.6×10^5
	% reduction	**94.76**		**96.6**		**90.97**	
24	**Mean**	1.3×10^7	4.5×10^6	4.5×10^7	2.0×10^6	4.3×10^7	5.3×10^6
	±SD	2.3×10^7	7.9×10^6	1.4×10^8	2.1×10^6	8.7×10^7	4.8×10^6
	% reduction	**65.4**		**95.5**		**87.6**	

Percentage Reduction (%) $= \left({}^{(Control - test)}/_{Control}\right) \text{X } 100$

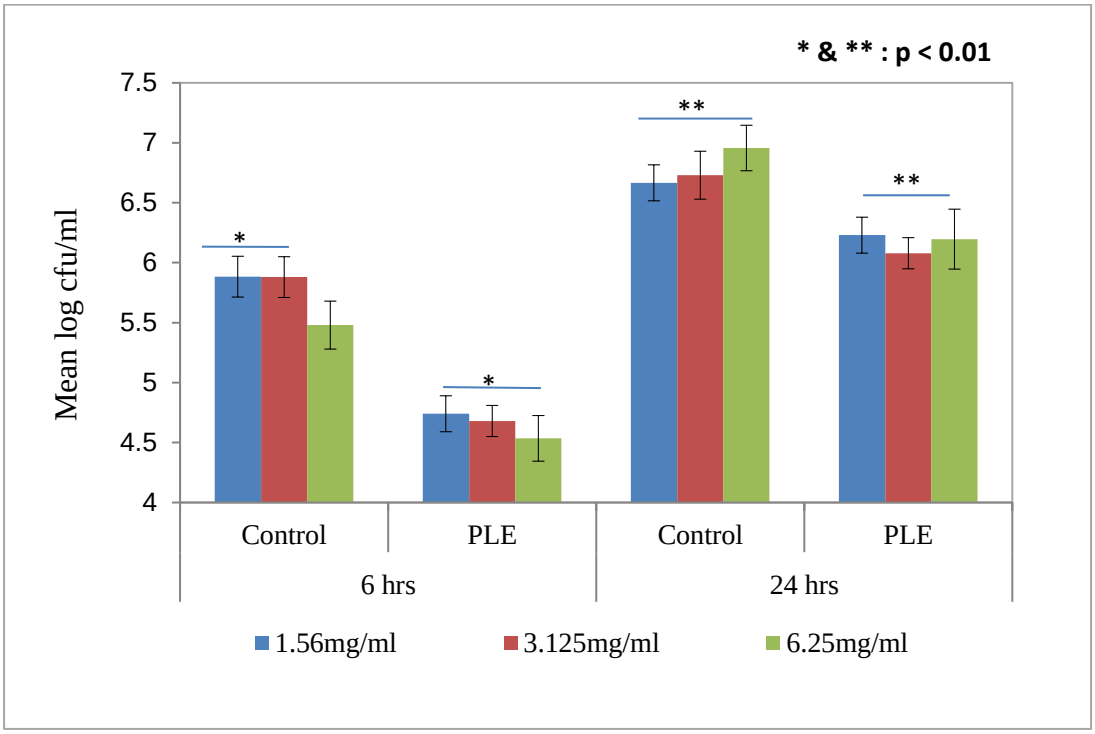

Figure 4.2 Effect of *P. granatum* on the biofilm formation by *S. mutans* (n = 15). Bars represent overall comparisons. *: At 6 h, comparison of controls to 3 sub-inhibitory concentrations ($p < 0.01$), **: At 24 h, comparison of controls to 3 sub-inhibitory concentrations ($p < 0.01$)

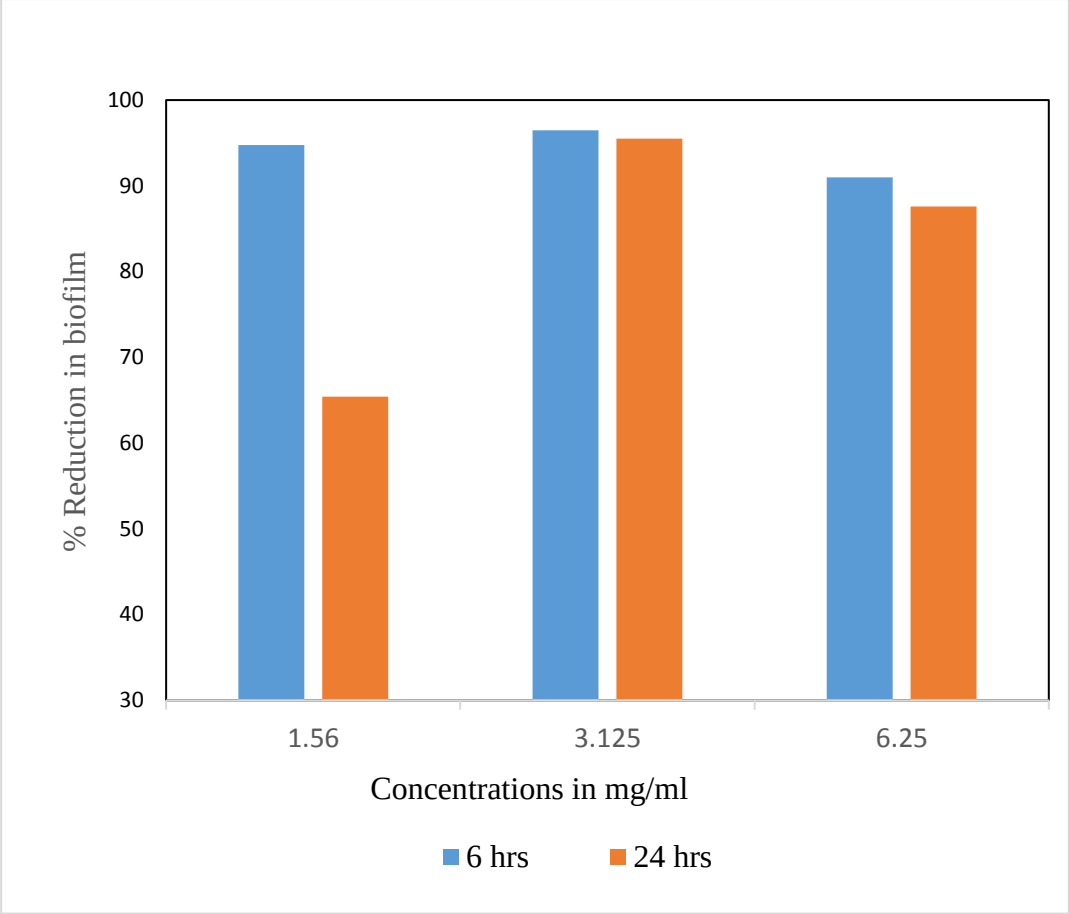

Figure 4.3 Percentage reduction in *S. mutans* biofilm formation by *P. granatum* peel

4.4 Discussion

Generally fully formed biofilms are difficult to remove and therefore, it is better to prevent the biofilm formation in which the adherence of bacteria to the surfaces can be targeted. In this study, after 6 and 24 hours *P. granatum* significantly decreased the number of viable cells in *S. mutans* biofilm when compared to that of negative control with a p value of < 0.01. It inhibited the biofilm formation by *S. mutans* at subinhibitory concentrations which suggests that the plant extract can provide a long term protection. The six hours of exposure at 1.56 mg/ml concentration reduced the biofilm formation of *S. mutans* by 94.8% which is beneficial because it may be used in the morning and/or evening in the form of mouth-rinse or a dentifrice. The inhibitory effect will last for six hours thereafter a thin layer of plaque may develop before

it is time for the second rinse. This thin layer of plaque will not support a complex microbiota and allow anaerobic metabolism and lactic acid production by the pioneer species *S. mutans*. Even thou there is a constant flow of saliva in the oral cavity, *S. mutans* will not be able to form biofilm in the presence of the plant extract. However, after 24 hours there was a reduction in the effectiveness of protection compared to the six hours observation. The biofilm formation was reduced by 65 % which was still significant and beneficial because of its extended protection time. The 3.125 mg/ml concentration performed better than 1.56mg/ml at both six and twenty four hours with percentage reduction of 96.6 % and 95.51 % respectively. While the 6.25 mg/ml concentration was the lowest performing among the three sub-inhibitory concentrations at 6 hours exposure with a percentage reduction of 91%. This was then followed by 87.6% after 24 hours. The diminishing antimicrobial effect of the extract cannot be explained. Accumulation of bacterial byproducts may have neutralized the plant extracts or the acidic pH due to the metabolism of bacteria may have had some effect.

Subinhibitory concentration of *Dodonaea viscosa var. angustifolia* plant crude extract had shown similar results of 95 and 97 % reduction in biofilm formation by *S. mutans* at 6 and 24 hours respectively (Naidoo *et al.,* 2012). Likewise, reduced biofilm formation by *S. mutans* after exposure to *Rhus coriaria L*. extract and methyl gallate on the polystyrene surface has been previously reported (Kacergius *et al.,* 2017). In their study, there was reduction in biofilm roughness and thickness with only 1 mg/ml of the major component methyl gallate when using optical profilometry assay (Kacergius *et al.,* 2017).

The effect of the plant extract might be attributed to interference with the production of the major EPS in the biofilm matrix, preventing strong attachment to the surface and connection between microcolonies in the biofilm (Koo *et al.,* 2010). It may have affected the production

of glucosyltransferases especially GTF B and C required for maximum biofilm formation through the production of insoluble EPS by *S. mutans*. Vahid-Dastjerdi *et al.*,(2016) reported down-regulation of glycosyl transferase genes in *S. mutans* by the presence of *P. granatum L.* flower and *Rhus coriaria L*. fruit water extracts (Vahid-Dastjerdi *et al.,* 2016). In an *in vivo* study done by Chen *et al*., (2016) reduction in EPS production resulted in reduced caries in rats to same extent as usage of fluoride (Chen *et al.,* 2016). Hattori *et al.,* 1990 have shown that catechin, epicatechin and their galloyl esters isolated from green and black tea inhibits glucosyltransferase production in *S. mutans* (Hattori *et al.*, 1990). Furthermore, gallotannins are known to inhibit water insoluble glucan synthesis (Wu-Yuan *et al.*, 1988). Another animal study has shown that polymeric polyphenols present in Oolong tea (leaves of *Camellia sinensis*) not only inhibits glucosyltransferase production in *S. mutans* but it also inhibits sucrose-dependent cell adherence of these bacteria thus preventing plaque accumulation and development of dental caries (Ooshima *et al.*, 1993).

P. granatum extract may have interfered with eDNA and exopolysacharides by altering the net charge at the surface of the cells which have also been shown to dictate stability of the exopolymer containing biofilm (Otto 2014). In an *in vitro* study using atomic force microscopy (AFM), Das *et al*., (2011) showed that eDNA enhanced *S. mutans* adherence to a substratum, with a more significant impact on a hydrophobic surface than on a hydrophilic one (Das *et al.,* 2011). In an adherence study by Liao *et al*., (2014), they discovered that the addition of DNase I to growth medium significantly reduced biofilm formation (Liao *et al.,* 2014). The sortase enzyme SrtA, which covalently links LPXTG (Leu-Pro-any-Thr-Gly) motif-containing proteins to the cell wall, is critical for biofilm formation in *S. mutans* (Ansari *et al.,* 2017). Several plant derived natural products, especially flavonoids have been found to inhibit *S. mutans* SrtA activity. Branched chains of flavone can also decrease SrtA activity, such as kaempferol-3-

rutinoside (Luo *et al.,* 2017, Wallock-Richards *et al.,* 2015). Similarly, Kaempferol-3-rutinoside has also been identified in *P. granatum* and could be responsible for altering the activity of this peptidoglycan-binding enzyme.

Vasconcelos *et al*, 2006 reported that *P. granatum* peel reduce the adherence of *S. mutans* to glass, however their results were obtained only by visual inspection (Vasconcelos *et al.,* 2006). Present study scientifically analysed the effect and showed further promising results. *S. mutans* contains cell surface protein adhesin P1 (also called antigen I/II, PAc, or SpaP) which are responsible for sucrose-independent bacterial adherence to the tooth surface via interactions with agglutinin and other glycoproteins in the salivary pellicles (Liao *et al.,* 2014). P1 attaches to the salivary agglutinin glycoprotein (SAG gp340) complex and facilitate the binding of this bacteria. Oli *et al*., (2012) has shown that P1, is an amyloid forming protein and that amyloids are present in human dental plaque and in *S. mutans* biofilms (Oli *et al.,* 2012). Crude extract of *P. granatum* may have affected these adhesion proteins.

Destruction of mature biofilms is a challenging task for antimicrobials, as biofilms provide protection for microorganisms against these compounds (Ren *et al.,* 2016), (Takahashi and Nyvad 2011). This study investigated the effect of pomegranate on *S. mutans* and discovered a reduction in the production of a biofilm formation. According to Marsh *et al*., (1994) oral care products should attempt to control the oral microbiota at levels compatible with health, without killing all bacteria and losing the key benefits delivered by these resident microbes. Even though *P. granatum* antimicrobial effect might be reduced after 6 hours, plant extract will still lower the bulkiness of the biofilm. The use of *P. granatum* as a mouth rinse or incorporated into a toothpaste or chewing gum will reduce individual's chances of susceptibility to plaque related dental diseases such as caries and gingivitis.

Ideally, the effect of *P. granatum* on *S. mutans* should be studied in the presence of mix flora and saliva, however most studies are performed on monoculture including this study. Hydroxyapatite discs can be used instead of glass surface which was not possible due to the lack of availability. However, in future studies discs cut from animal teeth will be used in the presence of mix flora and saliva as these plays a major role in the development of plaque.

4.5 Conclusion

A crude extract of *P. granatum* reduce biofilm formation by *S. mutans* in 6 hours at all test concentrations 1.56, 3.125 and 6.25 mg/ml by 94.76 %, 96.6 % and 90.97 % respectively. This suggests that *P. granatum* will provide a 6 hour protection against plaque formation and maintain good oral hygiene if used as a mouth-rinse twice a day. The inhibitory effect was reduced after 24 hrs compared to the 6 hours of exposure. However, if this plant extract is used once a day there will still be extended protection for up to a period of 24 hours. Again, further investigation needs to be performed to identify the active compound responsible for the anti-biofilm activity of *P. granatum* extract and to attain the mode of action of this active compound.

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CHAPTER 5

The effect of peel of *P. granatum* on the acid production by *S. mutans*

5.1 Introduction

Acid production is another major virulence factor responsible for dental caries. Acids are produced through the fermentation of sugars that are consumed in the diet. They cause demineralization of enamel by releasing calcium and phosphate ions (Figure 5.1A). A sugar-rich diet results in acid production that will induce an ecological shift in the plaque microflora to increase aciduric species, especially mutans streptococci and lactobacilli (Figure 5.1B). While many sugars that we consume can be utilized by plaque bacteria, sucrose is the most cariogenic carbohydrate and exposure to it influences the development of caries in multiple ways. Other than acid production, it serves as substrate for extracellular enzymes (GTF) of plaque bacteria which synthesize sucrose-derived polymers such as soluble and insoluble extracellular polysacharides. Therefore, metabolism of sucrose 'the arch-criminal in dental caries' is the common factor in dental plaque (Li *et al.,* 2016).

A.

$$\text{Sucrose} \rightarrow \text{acid}$$
$$\downarrow$$
$$HACa_{10}(PO_4)_6OH_2 \rightarrow Ca^{++} + H_2PO_4^- +$$

B.

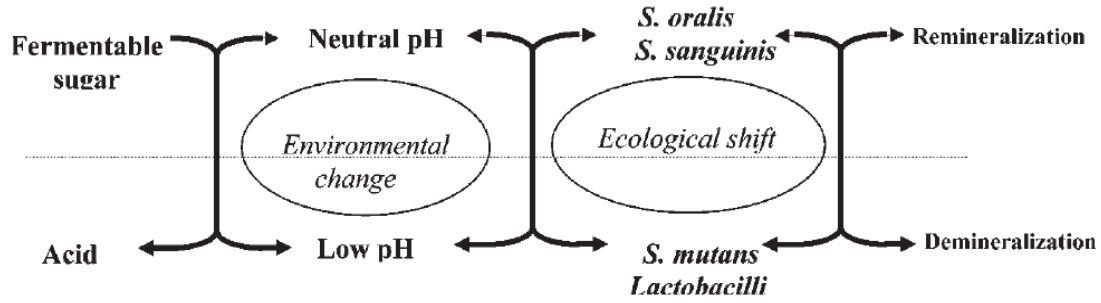

Figure 5.1 Schematic illustration of a cariogenic biofilm formation in the presence of fermentable sugars [source (Marsh 1994)]

S. mutans, a cariogenic bacteria within dental plaque produces acids by metabolizing sucrose. Frequent production and accumulation of these acids will lead to dental caries (Kawada-Matsuo *et al.,* 2017, Ma *et al.,* 2013). In a glycolytic pathway, lactate, formate, acetate, and ethanol are produced as fermentation products in a process of carbohydrate metabolism (Ajdic *et al.,* 2002) through the Embed-Meyerhof-Parnas pathway (Kawada-Matsuo *et al.,* 2017). Moreover, this process will produce ATP in the cytoplasm. (Kawada-Matsuo *et al.,* 2017). Lactate is the major product if the dietary glucose is abundant and the the precise distribution of fermentation products will depend on growth conditions (Dashper and Reynolds 1996).

S. mutans produces, and causes acid accumulation purely through the lactic acid production. Availability of strong acids in the milieu reduces plaque pH to a critical level that leads to demineralisation of enamel below pH 5.5 resulting in the initiation of caries process (Radcliffe *et al.,* 2002). The levels of mutans streptococci in the mouth responds rapidly to changes in the amount of sucrose in the diet (Wennerholm *et al.,* 1995). Direct uptake of sucrose encountered by streptococci is estimated at 90 %. The *S. mutans* crucial aspect is its virulence and the capabilities to initiate caries through acid production that would be suicidal without its aciduric ability (Hamilton and Buckley 1991). Moreover, the oral commensals will continue to experience fluctuations that is rapid and dynamic which is influenced mostly by the intake of carbohydrate resulting in a drop of pH from 7 to the acidic levels of 3 in less than 20 minutes (Hamilton 1990). *S. mutans* has evolved a repertoire of mechanisms to withstand the continuous cycles of acid shock i.e constitutive and acid-induced mechanism (Burne 1998), also known as the acid tolerance response or aciduricity (Svensater *et al.,* 1997).

Even though there is little information regarding lactobacillus, they have been identified as cariogenic bacteria. Lactobacillus is able to produce various acids including lactic acid, and survive this environment. It is able to ferment carbohydrates in two metabolic processes that are homolactic fermentation and heterolactic fermentation (Badet and Thebaud 2008, Caufield *et al.*, 2007). Whatever method used by lactobacillus, it results in acidification of the environment in the oral cavity generally. Nevertheless, *S. mutans* counts are much more higher (in millions) than the lactobacilli counts (around thousands) and *S. mutans* is a pioneer species in the development of dental biofilm.

Aim: The aim of this study was to investigate the effect of the peel of the *P. granatum* on the acid production by planktonic *S. mutans*.

5.2 Material and methods

5.2.1 Plant material

The pomegranate plant material was provided and fruit peel prepared as described in chapter 3 section 3.2.1.

5.2.2 *S. mutans* cultures

Five *S. mutans* strains were obtained as described in chapter 3 section 3.2.2.

5.2.3 Effect on acid production by planktonic cells of *S. mutans*

The effect of *P. granatum* crude peel extract on acid production by *S. mutans* was studied (Nalina and Rahim 2007). Cultures of *S. mutans* were grown on blood agar for forty eight hours under CO_2 at 37^o C. Fresh inoculum was prepared with distilled water to an OD_{600} of 0.5 containing approximately 10^5 - 10^6 cfu/ml. One hundred microliters of the inoculum was

transferred into 30 ml of 5 % sucrose tryptone broth containing the three sub-bactericidal concentrations of plant extract (0 as control, 1.56, 3.125 and 6.25 mg/ml) in a sterile sputum jar and incubated for 16 hours at 37^{o} C under CO_2. The pH of the media was measured at 0 and after 10, 12, 14 and 16 hours. A positive control of 0.2 % chlorhexidine and a negative control of water were included. The effect of DMSO was also determined. *S. mutans* counts was also measured at 0, 10, 12 and 14 hours using serial dilution technique. The experiments were performed in triplicates using the five strains of *S. mutans*.

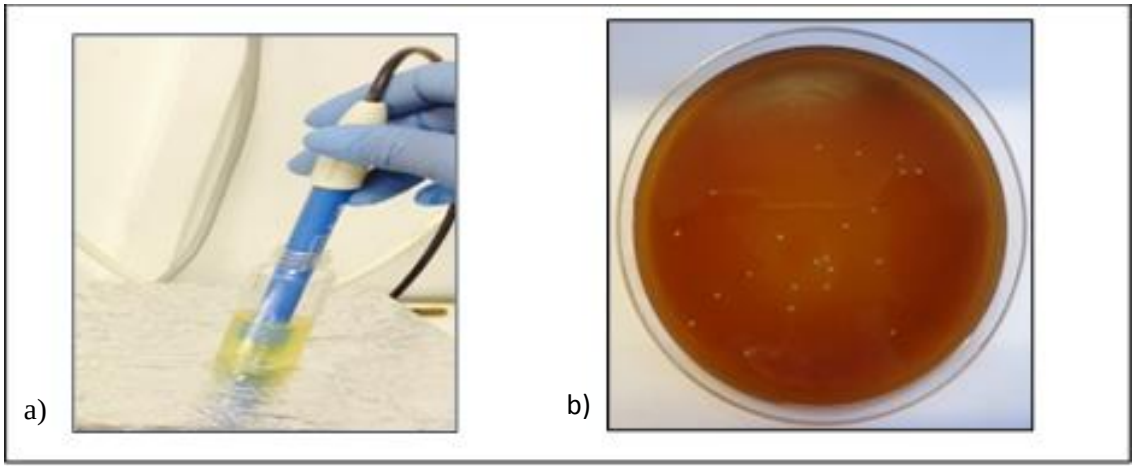

Figure 5.2 Acid assay: a) pH measurement b) *S. mutans* colonies on blood agar

5.2.4 Statistical analysis

The measured pH readings in the presence of the subinhibitory concentrations of plant extracts were compared with the control readings using the Kruskal Wallis test. Similarly, the bacterial counts were also compared.

5.3 Results

DMSO had no effect on the pH readings as well as bacterial counts. This means it had no effect on the growth and the acid production by *S. mutans*. Chlorhexidine killed *S. mutans* within 10 hours and therefore the bacteria could not produce acid.

The results of the pH readings in the presence of 1.56, 3.125 and 6.25 mg/ml crude extract are shown in table 5.1, 5.2 and 5.3 respectively. These results are summarized in table 5.4. The results of the *S. mutans* counts in the presence of 1.56, 3.125 and 6.25 mg/ml crude extract are shown in table 5.5, 5.6 and 5.7 respectively. These results are summarized in table 5.8.

The combined (all the test strains and all the repeats-15 readings) mean pH readings showed that at 0 hours the pH was 7 for the control as well as the test experiments. At 10, 12, 14 and 16 hours, the test pH was always higher than the control (Table 5.4). This suggest that the acid production was affected by the various concentrations of the test compound.

Bacterial counts were performed to eliminate the effect of bacterial growth on the acid production. The combined (all the test strains and all the repeats-15 readings) mean bacterial counts showed that the starting counts at 0 hours were similar for the control as well as the test experiments. At 10, 12, 14 and 16 hours, the test counts were not very different from the control counts (Table 5.8). This suggests that the bacterial counts were not affected by the various concentrations of the test extracts.

To verify and validate these observations, statistical analysis was performed. The mean pH and bacterial counts were plotted on graph (Figure 5.3). The pH readings and the bacterial counts of the controls were compared to the test compound (all concentrations were pooled together). The results showed that the bacterial counts of the test compounds were not significantly different from the control counts ($p = 0.23$) however, the pH readings were significantly higher in the tests compared to the control ($p < 0.01$). This suggests that the acid production by *S. mutans* was affected by the plant extract. The significant reduction in acid production was not due to the bacterial counts because the bacterial counts were not significantly reduced. It also showed that within 10 hours, the control pH reached a critical pH of 5.5 at which the enamel demineralization can occur whereas the presence of plant extract did not allow the drop in pH (Figure 5.3).

Table 5.1 The effect of 1.56 mg/ml of *P. granatum* crude extract on acid production by *S. mutans*

Acid production by *S. mutans* in the presence of 1.56 mg/ml extract (pH)											
Strains	**Repeats**	**0 hrs**		**10 hrs**		**12 hrs**		**14 hrs**		**16 hrs**	
		control	**plant**	**Control**	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**
SM 1	1	7	7	5.9	6.0	4.9	5.5	4.6	4.9	4.4	4.6
	2	7	7	6.1	6.4	5.1	5.9	4.6	5.2	4.4	4.7
	3	7	7	7.0	6.9	7.0	6.4	7.0	5.7	6.4	5.0
Mean		**7**	**7**	**6.3**	**6.5**	**5.7**	**5.9**	**5.4**	**5.2**	**5.1**	**4.8**
±SD		**0**	**0**	**0.6**	**0.4**	**1.1**	**0.5**	**1.4**	**0.4**	**1.2**	**0.2**
SM6	1	7	7	4.9	5.9	4.6	5.2	4.5	4.8	4.4	4.6
	2	7	7	4.8	5.3	4.4	4.8	4.3	4.6	4.3	4.4
	3	7	7	6.9	7.1	6.2	6.9	5.2	6.9	4.7	6.8
Mean		**7**	**7**	**5.5**	**6.1**	**5.1**	**5.6**	**4.7**	**5.4**	**4.5**	**5.3**
±SD		**0**	**0**	**1.2**	**0.9**	**0.9**	**1.1**	**0.5**	**1.3**	**0.2**	**1.4**
SM7	1	7	7	5.8	6.4	5.0	6.3	4.6	5.7	4.5	5.1
	2	7	7	5.0	6.0	4.5	5.6	4.4	5.0	4.3	4.7
	3	7	7	6.0	6.4	5.1	6.0	4.7	5.2	4.4	4.7
Mean		**7**	**7**	**5.6**	**6.3**	**4.9**	**5.9**	**4.6**	**5.3**	**4.4**	**4.8**
±SD		**0**	**0**	**0.5**	**0.2**	**0.3**	**0.4**	**0.1**	**0.4**	**0.1**	**0.2**
SM12	1	7	7	4.7	6.0	4.5	5.2	4.3	4.8	4.2	4.5
	2	7	7	4.4	4.7	4.2	4.5	4.2	4.5	4.7	5.0
	3	7	7	5.0	5.2	4.8	5.0	4.7	4.8	4.6	4.8
Mean		**7**	**7**	**4.7**	**5.3**	**4.5**	**4.9**	**4.4**	**4.7**	**4.5**	**4.8**
±SD		**0**	**0**	**0.3**	**0.6**	**0.3**	**0.4**	**0.2**	**0.2**	**0.3**	**0.2**
SM13	1	7	7	6.4	6.4	5.6	6.1	4.7	5.1	4.6	4.8
	2	7	7	4.7	5.1	4.5	4.8	4.4	4.6	4.3	4.5
	3	7	7	4.9	5.3	4.6	4.9	4.4	4.7	4.3	4.4
Mean		**7**	**7**	**4.7**	**5.0**	**4.3**	**4.7**	**4.0**	**4.3**	**3.9**	**4.6**
±SD		**0**	**0**	**2.0**	**0.7**	**1.8**	**0.7**	**0.2**	**0.3**	**0.1**	**0.2**
Combined mean		**7**	**7**	**5.60**	**6.04**	**5.10**	**5.61**	**4.76**	**5.14**	**4.58**	**4.86**
Combined ±SD		**0**	**0**	**0.88**	**0.67**	**0.81**	**0.71**	**0.70**	**0.64**	**0.54**	**0.59**

Table 5.2 The effect of 3.125 mg/ml of *P. granatum* crude extract on acid production by *S. mutans*

Acid production by *S. mutans* in the presence of 3.125 mg/ml extract (pH)											
Strains	**Repeats**	**0 hrs**		**10 hrs**		**12 hrs**		**14 hrs**		**16 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**
SM 1	1	7	7	5.9	6.1	4.9	5.6	4.6	5.1	4.4	4.8
	2	7	7	6.1	6.5	5.1	6.1	4.6	5.4	4.4	4.9
	3	7	7	7.0	7.1	7.0	7.0	7.0	7.0	6.4	6.7
Mean		**7**	**7**	**6.3**	**6.3**	**6.5**	**5.7**	**6.2**	**5.4**	**5.8**	**5.1**
±SD		**0**	**0**	**0.6**	**0.6**	**0.5**	**1.1**	**0.7**	**1.4**	**1.0**	**1.2**
SM6	1	7	7	4.9	6.0	4.6	5.3	4.5	4.9	4.4	4.7
	2	7	7	4.8	5.6	4.4	5.0	4.3	4.7	4.3	4.5
	3	7	7	6.9	6.8	6.2	6.1	5.2	5.4	4.7	4.9
Mean		**7**	**7**	**5.5**	**5.5**	**6.1**	**5.1**	**5.5**	**4.7**	**5.0**	**4.5**
±SD		**0**	**0**	**1.2**	**1.2**	**0.6**	**0.9**	**0.6**	**0.5**	**0.4**	**0.2**
SM7	1	7	7	5.8	6.4	5.0	6.2	4.6	5.8	4.5	5.2
	2	7	7	5.0	5.9	4.5	5.4	4.4	4.9	4.3	4.7
	3	7	7	6.0	6.4	5.1	6.1	4.7	5.4	4.4	4.9
Mean		**7**	**7**	**5.6**	**5.6**	**6.2**	**4.9**	**5.9**	**4.6**	**5.4**	**4.4**
±SD		**0**	**0**	**0.5**	**0.5**	**0.3**	**0.3**	**0.5**	**0.1**	**0.5**	**0.1**
SM12	1	7	7	4.7	6.2	4.5	5.5	4.3	4.9	4.2	4.6
	2	7	7	4.4	4.8	4.2	4.6	4.2	4.5	4.7	5.3
	3	7	7	5.0	5.4	4.8	5.1	4.7	4.9	4.6	4.8
Mean		**7**	**7**	**4.7**	**4.7**	**5.4**	**4.5**	**5.1**	**4.4**	**4.7**	**4.5**
±SD		**0**	**0**	**0.3**	**0.3**	**0.7**	**0.3**	**0.5**	**0.2**	**0.2**	**0.3**
SM13	1	7	7	6.4	6.4	5.6	6.2	4.7	5.2	4.6	4.9
	2	7	7	4.7	5.3	4.5	4.9	4.4	4.7	4.3	4.5
	3	7	7	4.9	5.2	4.6	4.8	4.4	4.5	4.3	4.4
Mean		**7**	**7**	**5.3**	**5.3**	**5.6**	**4.9**	**5.3**	**4.5**	**4.8**	**4.4**
±SD		**0**	**0**	**0.9**	**0.9**	**0.7**	**0.6**	**0.8**	**0.2**	**0.4**	**0.1**
Combined mean		**7**	**7**	**5.60**	**6.06**	**5.10**	**5.67**	**4.76**	**5.23**	**4.58**	**4.95**
Combined ±SD		**0**	**0**	**0.88**	**0.63**	**0.81**	**0.69**	**0.70**	**0.66**	**0.54**	**0.54**

Table 5.3 The effect of 6.25 mg/ml *P. granatum* crude extract on acid production by *S mutans*

Acid production by *S. mutans* in the presence of 6.25 mg/ml (pH)											
Strains	**Repeats**	**0 hrs**		**10 hrs**		**12 hrs**		**14 hrs**		**16 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**
SM 1	1	7	7	5.9	6.1	4.9	5.8	4.6	5.4	4.4	5.0
	2	7	7	6.1	6.5	5.1	6.3	4.6	5.9	4.4	5.3
	3	7	7	5.5	6.4	4.9	5.6	4.6	5.1	4.6	5.0
Mean		**7**	**7**	**5.8**	**5.8**	**6.3**	**5.0**	**5.9**	**4.6**	**5.4**	**4.5**
±SD		**0**	**0**	**0.3**	**0.3**	**0.2**	**0.1**	**0.4**	**0.0**	**0.4**	**0.1**
SM6	1	7	7	4.9	6.1	4.6	5.7	4.5	4.8	4.4	4.8
	2	7	7	4.8	5.5	4.4	4.9	4.3	4.7	4.3	4.6
	3	7	7	5.4	6.2	4.9	5.4	4.7	5.1	4.6	4.9
Mean		**7**	**7**	**5.0**	**5.0**	**5.9**	**4.7**	**5.4**	**4.5**	**4.9**	**4.4**
±SD		**0**	**0**	**1.9**	**1.9**	**0.4**	**0.2**	**0.4**	**0.2**	**0.2**	**0.2**
SM7	1	7	7	5.8	6.4	5.0	6.3	4.6	6.0	4.5	5.3
	2	7	7	5.0	6.0	4.5	5.4	4.4	5.0	4.3	4.7
	3	7	7	6.0	6.4	5.1	6.3	4.7	5.8	4.4	5.2
Mean		**7**	**7**	**5.6**	**5.6**	**6.2**	**4.9**	**6.0**	**4.6**	**5.6**	**4.4**
±SD		**0**	**0**	**0.5**	**0.5**	**0.2**	**0.3**	**0.5**	**0.1**	**0.6**	**0.1**
SM12	1	7	7	4.7	6.2	4.5	6.2	4.3	5.3	4.2	4.8
	2	7	7	5.0	5.3	4.8	5.1	4.7	4.8	4.6	4.8
	3	7	7	5.0	5.3	4.8	5.1	4.7	4.9	4.6	4.8
Mean		**7**	**7**	**4.9**	**4.9**	**5.6**	**4.7**	**5.5**	**4.6**	**5.0**	**4.5**
±SD		**0**	**0**	**0.2**	**0.2**	**0.6**	**0.1**	**0.6**	**0.2**	**0.2**	**0.2**
SM13	1	7	7	6.4	6.3	5.6	5.5	4.7	4.8	4.6	4.7
	2	7	7	4.7	5.4	4.5	5.0	4.4	4.8	4.3	4.6
	3	7	7	5.5	6.2	4.9	5.4	4.8	5.1	4.6	4.9
Mean		**7**	**7**	**5.5**	**5.5**	**6.0**	**5.0**	**5.3**	**4.6**	**4.9**	**4.5**
±SD		**0**	**0**	**0.9**	**0.9**	**0.5**	**0.6**	**0.3**	**0.2**	**0.2**	**0.2**
Combined mean		**7**	**7**	**5.4**	**6.0**	**4.8**	**5.6**	**4.6**	**5.2**	**4.5**	**4.9**
Combined ±SD		**0**	**0**	**0.6**	**0.4**	**0.3**	**0.5**	**0.2**	**0.4**	**0.1**	**0.2**

Table 5.4 Summarised results of acid production (pH) by *S. mutans* in the presence of *P. granatum* crude extract

Time (hrs)	Acid production by *S. mutans* (pH)					
	control	1.56 mg/ml	control	3.125 mg/ml	control	6.25 mg/ml
10	5.60	6.04	5.60	6.06	5.38	6.02
12	5.10	5.61	5.10	5.67	4.83	5.60
14	4.76	5.14	4.76	5.23	4.57	5.17
16	4.58	4.86	4.58	4.95	4.45	4.89

Table 5.5 The effect of 1.56 mg/ml of *P. granatum* crude extract on the *S. mutans* counts

Effect of 1.56 mg/ml *P. granatum* crude extract on the *S. mutans* counts (cfu/ml)									
Strains	**Repeats**	**0 hrs**		**10 hrs**		**12 hrs**		**14 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**
SM1	1	6.6×10^6	6.5×10^6	1.5×10^7	9.3×10^6	1.6×10^8	7.9×10^7	6.5×10^7	1.3×10^8
	2	4.4×10^6	5.2×10^6	1.5×10^7	3.2×10^7	4.0×10^7	3.0×10^7	3.9×10^7	7.1×10^7
	3	6.9×10^5	5.0×10^5	6.2×10^5	1.0×10^7	2.0×10^5	2.0×10^6	6.0×10^5	8.0×10^5
Mean		**3.9×10^6**	**4.0×10^6**	**1.1×10^7**	**1.8×10^8**	**6.9×10^7**	**3.7×10^7**	**3.5×10^8**	**6.9×10^8**
±SD		**3.0×10^6**	**2.8×10^6**	**8.7×10^6**	**4.1×10^6**	**8.6×10^7**	**2.2×10^7**	**3.3×10^7**	**5.0×10^7**
SM6	1	2.8×10^6	5.0×10^6	9.0×10^6	1.6×10^7	1.3×10^8	1.0×10^8	6.9×10^7	6.0×10^7
	2	7.4×10^6	1.1×10^7	2.3×10^7	2.4×10^7	5.2×10^7	1.8×10^7	1.0×10^8	1.9×10^8
	3	4.9×10^5	3.8×10^6	4.1×10^6	1.6×10^6	8.0×10^6	1.7×10^7	2.7×10^7	2.7×10^6
Mean		**3.5×10^6**	**6.5×10^6**	**1.2×10^7**	**1.4×10^7**	**6.6×10^7**	**4.2×10^7**	**6.6×10^7**	**8.3×10^7**
±SD		**3.5×10^6**	**2.2×10^6**	**9.8×10^6**	**1.1×10^7**	**5.9×10^7**	**2.9×10^7**	**3.1×10^7**	**2.7×10^7**
SM7	1	2.1×10^6	2.0×10^6	2.7×10^7	8.8×10^6	5.6×10^7	3.7×10^7	6.4×10^7	1.3×10^8
	2	3.7×10^6	4.5×10^6	2.8×10^7	1.9×10^7	2.0×10^7	5.9×10^7	2.6×10^7	6.7×10^7
	3	5.5×10^6	5.5×10^6	4.5×10^7	1.1×10^7	4.5×10^8	2.8×10^7	5.4×10^8	5.7×10^7
Mean		**3.8×10^6**	**4.0×10^6**	**3.4×10^7**	**1.3×10^7**	**1.8×10^8**	**4.1×10^7**	**2.1×10^8**	**8.4×10^7**
±SD		**1.7×10^6**	**2.2×10^6**	**9.7×10^6**	**4.3×10^6**	**2.4×10^8**	**3.3×10^7**	**2.9×10^8**	**5.2×10^7**
SM12	1	4.2×10^6	4.7×10^6	8.9×10^7	3.2×10^7	5.5×10^7	4.9×10^7	6.3×10^7	5.1×10^7
	2	1.5×10^7	1.7×10^7	3.3×10^8	4.0×10^6	1.9×10^7	3.3×10^7	7.9×10^6	4.2×10^7
	3	2.7×10^7	3.2×10^7	3.1×10^8	1.4×10^8	7.6×10^8	2.5×10^8	7.2×10^8	1.2×10^8
Mean		**1.6×10^7**	**1.8×10^8**	**2.5×10^8**	**1.1×10^8**	**2.8×10^8**	**1.1×10^8**	**2.6×10^8**	**2.0×10^8**
±SD		**1.1×10^7**	**8.6×10^6**	**1.4×10^8**	**9.1×10^7**	**4.2×10^8**	**1.8×10^8**	**4.0×10^8**	**1.8×10^8**
SM13	1	3.7×10^6	2.9×10^6	5.8×10^6	8.4×10^6	2.7×10^7	4.9×10^7	1.7×10^7	1.1×10^8
	2	4.9×10^6	1.3×10^7	1.7×10^7	2.8×10^7	4.6×10^8	3.1×10^7	5.9×10^6	3.4×10^7
	3	1.7×10^7	5.0×10^6	2.5×10^8	1.6×10^7	8.2×10^7	5.4×10^7	7.1×10^7	1.0×10^8
Mean		**8.4×10^6**	**7.2×10^6**	**9.3×10^7**	**1.7×10^7**	**1.9×10^8**	**4.5×10^7**	**3.1×10^7**	**8.4×10^7**
±SD		**7.1×10^6**	**1.5×10^7**	**1.4×10^8**	**1.3×10^7**	**2.3×10^8**	**2.5×10^7**	**3.5×10^7**	**2.8×10^7**
Combined mean		**7.1×10^6**	**7.9×10^6**	**7.9×10^7**	**3.4×10^7**	**1.6×10^8**	**5.5×10^7**	**1.2×10^8**	**1.0×10^8**
Combined ±SD		**7.2×10^6**	**9.0×10^6**	**1.2×10^8**	**4.2×10^7**	**2.2×10^8**	**8.3×10^7**	**2.1×10^8**	**8.0×10^7**

Table 5.6 The effect of 3.125 mg/ml *P. granatum* crude extract on the *S. mutans* counts

Effect of 3.125 mg/ml *P. granatum* crude extract on the *S. mutans* counts (cfu/ml)									
Strains	**Repeats**	**0 hrs**		**10 hrs**		**12 hrs**		**14 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**
SM1	1	6.6×10^6	4.0×10^6	1.5×10^7	2.9×10^6	1.6×10^8	4.2×10^7	6.5×10^7	4.9×10^7
	2	4.4×10^6	6.3×10^6	1.5×10^7	9.9×10^6	4.0×10^7	3.4×10^7	3.9×10^7	1.0×10^8
	3	6.9×10^5	7.2×10^5	6.2×10^5	2.9 x 10	2.0×10^5	2.0×10^5	6.0×10^5	4.7×10^6
Mean		$\mathbf{3.9 \times 10^6}$	$\mathbf{3.7 \times 10^7}$	$\mathbf{1.0 \times 10^7}$	$\mathbf{5.2 \times 10^7}$	$\mathbf{6.9 \times 10^7}$	$\mathbf{2.6 \times 10^7}$	$\mathbf{3.5 \times 10^7}$	$\mathbf{5.3 \times 10^7}$
±SD		$\mathbf{3.0 \times 10^6}$	$\mathbf{2.8 \times 10^6}$	$\mathbf{8.7 \times 10^6}$	$\mathbf{4.1 \times 10^6}$	$\mathbf{8.7 \times 10^7}$	$\mathbf{2.2 \times 10^7}$	$\mathbf{3.3 \times 10^7}$	$\mathbf{5.0 \times 10^7}$
SM6	1	2.8×10^6	4.5×10^6	9.0×10^6	6.1×10^6	1.3×10^8	9.6×10^7	6.9×10^7	4.7×10^7
	2	7.4×10^6	7.0×10^6	2.3×10^7	2.6×10^7	5.2×10^7	3.9×10^7	1.0 x 10	5.4×10^7
	3	4.9×10^5	2.8×10^6	4.1×10^6	7.1×10^6	8.0×10^6	7.6×10^6	2.7×10^7	4.0×10^6
Mean		$\mathbf{3.5 \times 10^6}$	$\mathbf{4.7 \times 10^6}$	$\mathbf{1.2 \times 10^7}$	$\mathbf{1.3 \times 10^7}$	$\mathbf{6.6 \times 10^7}$	$\mathbf{4.8 \times 10^7}$	$\mathbf{6.6 \times 10^7}$	$\mathbf{4.7 \times 10^7}$
±SD		$\mathbf{3.5 \times 10^6}$	$\mathbf{2.0 \times 10^6}$	$\mathbf{9.8 \times 10^6}$	$\mathbf{1.1 \times 10^7}$	$\mathbf{5.9 \times 10^7}$	$\mathbf{2.9 \times 10^7}$	$\mathbf{3.1 \times 10^7}$	$\mathbf{2.7 \times 10^7}$
SM7	1	2.1×10^6	2.3×10^6	2.7×10^7	2.7×10^6	5.6×10^7	1.3×10^7	6.4×10^7	1.8×10^7
	2	3.7×10^6	4.6×10^6	2.8×10^7	1.0×10^7	2.0×10^7	7.5×10^7	2.6×10^7	5.6×10^7
	3	5.5×10^6	6.8×10^6	4.5×10^7	9.2×10^6	4.5×10^8	2.5×10^7	5.4×10^8	5.7×10^7
Mean		$\mathbf{3.8 \times 10^6}$	$\mathbf{4.6 \times 10^6}$	$\mathbf{3.4 \times 10^7}$	$\mathbf{7.6 \times 10^6}$	$\mathbf{1.8 \times 10^8}$	$\mathbf{3.8 \times 10^7}$	$\mathbf{2.1 \times 10^8}$	$\mathbf{7.2 \times 10^8}$
±SD		$\mathbf{1.7 \times 10^6}$	$\mathbf{2.2 \times 10^6}$	$\mathbf{9.7 \times 10^6}$	$\mathbf{4.3 \times +0^6}$	$\mathbf{2.4 \times 10^8}$	$\mathbf{3.3 \times 10^7}$	$\mathbf{2.9 \times 10^8}$	$\mathbf{5.2 \times 10^7}$
SM12	1	4.2×10^6	7.2×10^6	8.9×10^7	1.2×10^7	5.5×10^7	2.7×10^7	6.3×10^7	2.8×10^7
	2	1.5×10^7	1.8×10^7	3.3×10^8	1.5×10^7	1.9×10^7	1.9×10^7	7.9×10^6	1.0×10^7
	3	2.7×10^7	2.4×10^7	3.2×10^8	1.7×10^8	7.6×10^8	1.8×10^8	4.0×10^8	1.8×10^8
Mean		$\mathbf{1.5 \times 10^7}$	$\mathbf{1.6 \times 10^7}$	$\mathbf{2.5 \times 10^8}$	$\mathbf{6.6 \times 10^7}$	$\mathbf{2.8 \times 10^8}$	$\mathbf{1.3 \times 10^8}$	$\mathbf{2.6 \times 10^8}$	$\mathbf{1.2 \times 10^8}$
±SD		$\mathbf{1.1 \times 10^7}$	$\mathbf{8.6 \times 10^6}$	$\mathbf{1.4 \times 10^8}$	$\mathbf{9.1 \times 10^7}$	$\mathbf{4.2 \times 10^8}$	$\mathbf{1.8 \times 10^8}$	$\mathbf{4.0 \times 10^8}$	$\mathbf{1.8 \times 10^8}$
SM13	1	3.7×10^6	3.2×10^6	5.8×10^6	4.9×10^6	2.7×10^7	7.6×10^7	1.7×10^7	3.4×10^7
	2	4.9×10^6	1.1×10^7	1.7×10^7	1.1×10^7	4.6×10^8	2.6×10^7	5.9×10^6	2.7×10^7
	3	1.7×10^7	3.3×10^7	2.5×10^8	3.0×10^7	8.2×10^7	4.9×10^7	7.1×10^7	7.6×10^7
Mean		$\mathbf{6.6 \times 10^6}$	$\mathbf{7.9 \times 10^6}$	$\mathbf{7.2 \times 10^7}$	$\mathbf{2.3 \times 10^7}$	$\mathbf{1.6 \times 10^8}$	$\mathbf{6.0 \times 10^7}$	$\mathbf{1.4 \times 10^8}$	$\mathbf{7.0 \times 10^7}$
±SD		$\mathbf{7.1 \times 10^6}$	$\mathbf{1.5 \times 10^7}$	$\mathbf{1.4 \times 10^8}$	$\mathbf{1.3 \times 10^7}$	$\mathbf{2.3 \times 10^8}$	$\mathbf{2.5 \times 10^7}$	$\mathbf{3.5 \times 10^7}$	$\mathbf{2.8 \times 10^7}$
Combined mean		7.1×10^6	9.0×10^6	7.9×10^6	2.1×10^7	1.6×10^8	5.8×10^7	1.2×10^8	6.8×10^7
Combined ±SD		7.2×10^6	9.1×10^6	1.2×10^8	4.2×10^7	2.2×10^8	8.3×10^7	2.1×10^8	8.0×10^7

Table 5.7 The effect of 6.25 mg/ml of *P. granatum* crude extract on the *S. mutans* counts

The effect of 6.25 mg/ml *P. granatum* crude peel on the *S. mutans* counts (cfu/ml)

Strains	**Repeats**	**0 hrs**		**10 hrs**		**12 hrs**		**14 hrs**	
		control	**plant**	**control**	**plant**	**control**	**plant**	**control**	**plant**
SM1	1	6.6×10^6	5.3×10^6	1.5×10^7	2.5×10^6	1.6×10^8	2.8×10^6	6.5×10^7	3.1×10^7
	2	4.4×10^6	3.9×10^6	1.5×10^7	8.6×10^6	4.0×10^7	3.5×10^7	3.9×10^7	9.5×10^7
	3	1.3×10^7	1.1×10^7	2.8×10^7	2.0×10^7	4.3×10^7	1.3×10^8	8.4×10^7	3.3×10^8
Mean		$\mathbf{8.2 \times 10^6}$	$\mathbf{6.8 \times 10^6}$	$\mathbf{2.0 \times 10^7}$	$\mathbf{1.0 \times 10^7}$	$\mathbf{8.3 \times 10^7}$	$\mathbf{6.4 \times 10^7}$	$\mathbf{6.3 \times 10^7}$	$\mathbf{1.5 \times 10^7}$
±SD		$\mathbf{4.7 \times 10^6}$	$\mathbf{3.7 \times 10^6}$	$\mathbf{7.1 \times 10^6}$	$\mathbf{8.9 \times 10^6}$	$\mathbf{7.2 \times 10^7}$	$\mathbf{5.7 \times 10^7}$	$\mathbf{2.3 \times 10^7}$	$\mathbf{1.6 \times 10^8}$
SM6	1	2.8×10^6	4.7×10^6	9.0×10^6	1.0×10^7	1.3×10^8	3.0×10^7	6.9×10^7	4.0×10^7
	2	7.4×10^6	6.7×10^6	2.3×10^7	1.2×10^7	5.2×10^7	3.0×10^7	1.0×10^8	4.6×10^7
	3	1.0×10^7	1.4×10^7	1.1×10^8	8.0×10^7	9.4×10^7	3.2×10^8	4.1×10^7	2.8×10^8
Mean		$\mathbf{7.1 \times 10^6}$	$\mathbf{8.6 \times 10^7}$	$\mathbf{4.7 \times 10^7}$	$\mathbf{3.4 \times 10^7}$	$\mathbf{9.5 \times 10^7}$	$\mathbf{1.3 \times 10^8}$	$\mathbf{7.1 \times 10^7}$	$\mathbf{1.2 \times 10^8}$
±SD		$\mathbf{4.1 \times 10^6}$	$\mathbf{5.2 \times 10^6}$	$\mathbf{5.5 \times 10^7}$	$\mathbf{4.0 \times 10^7}$	$\mathbf{4.3 \times 10^7}$	$\mathbf{1.7 \times 10^8}$	$\mathbf{3.1 \times 10^7}$	$\mathbf{1.4 \times 10^8}$
SM7	1	2.1×10^6	2.7×10^6	2.7×10^7	5.1×10^6	5.6×10^7	1.5×10^7	6.4×10^7	5.4×10^7
	2	3.7×10^6	5.7×10^6	2.8×10^7	1.4×10^7	2.0×10^7	3.5×10^7	2.6×10^7	5.6×10^7
	3	5.5×10^6	5.4×10^6	4.5×10^7	1.5×10^7	4.5×10^8	4.5×10^7	5.4×10^8	2.1×10^8
Mean		$\mathbf{3.8 \times 10^5}$	$\mathbf{4.6 \times 10^6}$	$\mathbf{3.4 \times 10^7}$	$\mathbf{1.1 \times 10^7}$	$\mathbf{1.8 \times 10^8}$	$\mathbf{3.2 \times 10^7}$	$\mathbf{2.1 \times 10^8}$	$\mathbf{1.1 \times 10^9}$
±SD		$\mathbf{1.7 \times 10^6}$	$\mathbf{1.6 \times 10^6}$	$\mathbf{9.7 \times 10^6}$	$\mathbf{5.4 \times 10^6}$	$\mathbf{2.4 \times 10^8}$	$\mathbf{1.5 \times 10^7}$	$\mathbf{2.9 \times 10^8}$	$\mathbf{9.0 \times 10^7}$
SM12	1	4.2×10^6	4.0×10^6	8.9×10^7	1.3×10^7	5.5×10^7	2.2×10^7	6.3×10^7	2.8×10^7
	2	2.7×10^7	3.5×10^7	3.1×10^8	1.7×10^8	7.6×10^8	1.8×10^8	7.2×10^8	8.4×10^8
	3	2.7×10^7	3.3×10^7	3.1×10^8	2.2×10^8	7.6×10^8	2.3×10^8	7.2×10^8	5.4×10^8
Mean		$\mathbf{1.9 \times 10^6}$	$\mathbf{2.4 \times 10^7}$	$\mathbf{2.4 \times 10^7}$	$\mathbf{1.4 \times 10^7}$	$\mathbf{5.2 \times 10^6}$	$\mathbf{1.4 \times 10^7}$	$\mathbf{5.0 \times 10^8}$	$\mathbf{4.8 \times 10^8}$
±SD		$\mathbf{1.3 \times 10^7}$	$\mathbf{1.8 \times 10^7}$	$\mathbf{1.3 \times 10^8}$	$\mathbf{1.1 \times 10^8}$	$\mathbf{4.1 \times 10^8}$	$\mathbf{1.1 \times 10^8}$	$\mathbf{3.8 \times 10^8}$	$\mathbf{4.1 \times 10^8}$
SM13	1	3.7×10^6	3.7×10^6	5.8×10^6	1.1×10^7	2.7×10^7	5.6×10^7	1.7×10^7	4.3×10^7
	2	4.9×10^6	9.4×10^6	1.7×10^7	9.3×10^6	4.6×10^8	2.6×10^7	5.9×10^6	1.6×10^7
	3	8.5×10^6	7.0×10^6	2.9×10^7	2.4×10^6	2.6×10^7	2.0×10^8	1.9×10^8	3.3×10^8
Mean		$\mathbf{5.7 \times 10^6}$	$\mathbf{6.6 \times 10^6}$	$\mathbf{1.7 \times 10^7}$	$\mathbf{7.6 \times 10^7}$	$\mathbf{1.7 \times 10^8}$	$\mathbf{9.5 \times 10^7}$	$\mathbf{7.0 \times 10^7}$	$\mathbf{1.3 \times 10^8}$
±SD		$\mathbf{2.5 \times 10^6}$	$\mathbf{2.9 \times 10^6}$	$\mathbf{1.2 \times 10^7}$	$\mathbf{4.6 \times 10^6}$	$\mathbf{2.5 \times 10^8}$	$\mathbf{9.6 \times 10^7}$	$\mathbf{1.0 \times 10^8}$	$\mathbf{1.7 \times 10^8}$
Combined mean		$\mathbf{8.8 \times 10^6}$	$\mathbf{1.0 \times 10^7}$	$\mathbf{7.2 \times 10^8}$	$\mathbf{4.0 \times 10^8}$	$\mathbf{2.1 \times 10^8}$	$\mathbf{9.2 \times 10^6}$	$\mathbf{1.8 \times 10^8}$	$\mathbf{2.0 \times 10^8}$
Combined ±SD		$\mathbf{8.0 \times 10^6}$	$\mathbf{1.0 \times 10^7}$	$\mathbf{1.0 \times 10^8}$	$\mathbf{6.7 \times 10^7}$	$\mathbf{2.6 \times 10^8}$	$\mathbf{9.6 \times 10^7}$	$\mathbf{2.5 \times 10^8}$	$\mathbf{2.4 \times 10^8}$

Table 5.8 Summarised results of bacterial counts (cfu/ml) by *S. mutans* in the presence of *P. granatum* crude extract

Time (hrs)		Summary results of the *P. granatum* subbactericidal concentrations on *S. mutans* counts (cfu/ml)				
		Control	**1.56 mg/ml**	**3.125 mg/ml**	**Control**	**6.25 mg/ml**
0	Mean	**7.1×10^6**	**7.9×10^6**	**9.0×10^6**	**8.8×10^6**	**1.0×10^7**
	±SD	7.2×10^6	7.9×10^6	9.1×10^6	8.0×10^6	1.0×10^7
10	Mean	**7.9×10^7**	**3.4×10^7**	**2.1×10^7**	**7.2×10^8**	**4.0×10^8**
	±SD	1.2×10^8	4.6×10^7	4.2×10^7	1.0×10^8	6.7×10^7
12	Mean	**1.6×10^8**	**5.5×10^7**	**5.8×10^7**	**2.1×10^8**	**9.2×10^6**
	±SD	2.2×10^8	6.6×10^8	8.3×10^7	2.6×10^8	9.6×10^7
14	Mean	**1.2×10^8**	**1.0×10^8**	**6.8×10^7**	**1.8×10^8**	**2.0×10^8**
	±SD	2.1×10^8	1.0×10^8	8.0×10^7	2.5×10^8	2.4×10^8

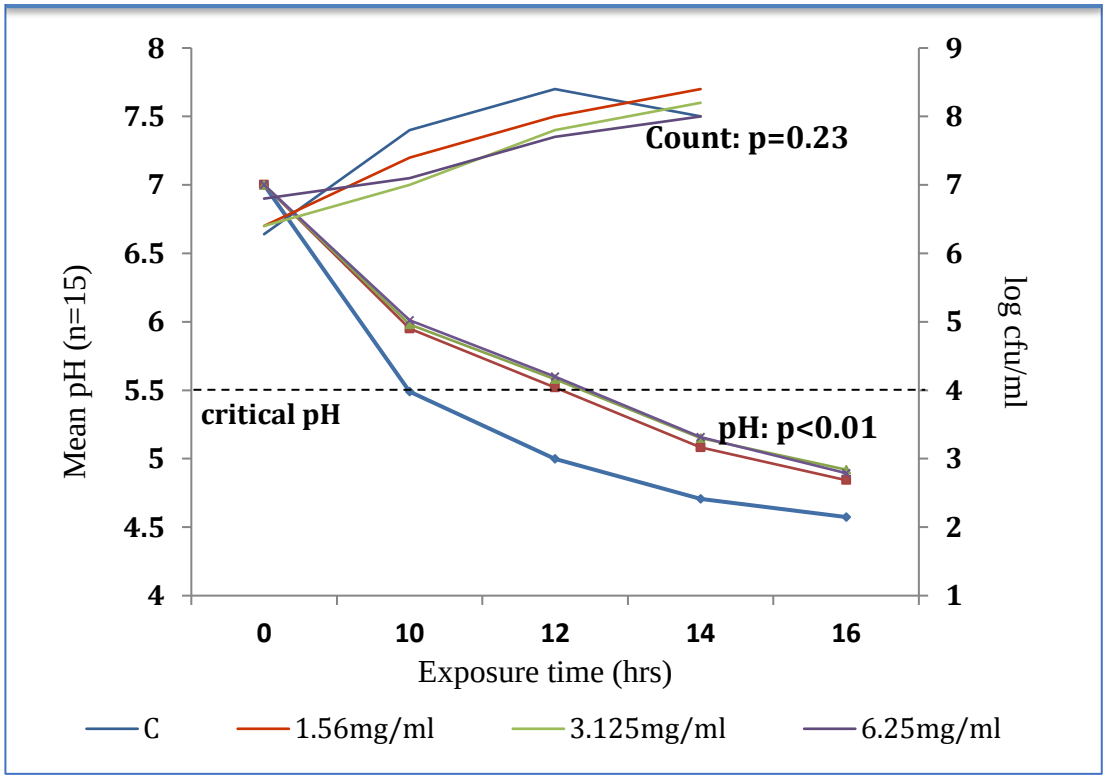

Figure 5.3 The effect of *P. granatum* crude on acid production by planktonic *S. mutans* (n=15)

5.4 Discussion

Dental care requires more investigation to be done for the development of new therapeutic agents in which the production of acids from fermentable carbohydrates can be interrupted (Xiao *et al.,* 2006). *Punica granatum* significantly inhibited acid production and it was independent from bacterial counts. With the control experiment, critical pH of 5.5 (cariogenic) when causes loss of enamel from the teeth was attained after ten hours of incubation which was delayed with plant extract by two hours. This suggests that the reduced acid production was due to the inhibitory effect of *P. granatum.*

Carbohydrate metabolism by *S. mutans* results in the formation of acids as end products that contribute to the demineralization of enamel, leading to dental caries (Cvitkovitch *et al.,* 1995). Bacteria can take up carbohydrates by specific sugar incorporation systems. The phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) is the principal sugar transport system used in oral streptococci for glucose and a variety of sugars, including mannose, fructose, sucrose, lactose, and maltose (Cvitkovitch *et al.,* 1995). In the PTS, after the sugar has been incorporated into the cytoplasm, the sugar molecule is processed and finally metabolized.

In the second last step of the glycolytic pathway, phosphoglyceric acid (PG) is converted to phosphoenol-pyruvate by the enzyme enolase with subsequent production of lactic acid. Fluoride, an inorganic chemical is known to interfere with the enzymes in the glycolysis pathway. It reacts with H^+ in the plaque/saliva to form HF ions that enables fluoride to penetrate the cell wall and bacterial membranes (Jenkins 1999). Once inside the cytoplasm it dissociates into H^+ and F molecules. The F will then inhibit the enolases thereby reducing the

formation of PEP which in turn will reduce the uptake of glucose by PEP-PTS thus reducing lactic acid production. Likewise, reduction of PEP production will interfere with the transfer of the phosphate group of PEP to ADP forming ATP. On another route of glucose uptake *i.e* the proton motive force, fluoride is also involved in the inhibition of ATPases responsible for this mechanism (Jenkins 1999).

Similarly, in the presence of *P. granatum* the reduction in the acid production by *S. mutans* may have been a direct effect on the glycolytic pathway. Some medicinal plants and its compounds, for example *Zingiber officinale* (ginger), Propolis, *Baccharis dracunculifolia*, Cranberry, Quercitrin and Deoxynojirimycin are known to inhibit acid production by *S. mutans* (Duarte *et al.,* 2006, Hasan *et al.,* 2015, Hasan *et al.,* 2014, Leitao *et al.,* 2004). *Zingiber officinale* crude extract and its methanolic fraction have been found to inhibit the glycolytic pH drop resulting in an increase in the cytoplasmic fluid leading to impairment of several enzymes in the cytoplasm (Hasan *et al.,* 2015). The rate of glycolytic pH drop reflects the acidogenic properties while the final pH values of suspension reflects acid tolerance (Gregoire *et al.,* 2007). Also, Quercitrin and Deoxynojirimycin a group of flavonols have been found to reduce glycolytic pH drop suggesting the impairment of acid production capability by *S. mutans* cells. Moreover, Gregoire *et al*., (2007) has shown reduced acid production by *S. mutans* in the presence of cranberry flavonols which was due to the disruption on the membrane-associated F-ATPase activity (Gregoire *et al.,* 2007). The cranberry flavonols also reduced acid production in the same study.

The ellagitannins, punicalagin, ellagic acid and gallic acid, which are found in *P. granatum*, have the ability to precipitate membrane proteins and they inhibit metabolic enzymes causing

cell lysis (Ismail *et al.*, 2012). Therefore, either these compounds or inhibition of enzymes in the glycolytic pathway may be responsible for the effect observed in this study. However, further research such as the glycolytic pH drop assay and F-ATPase activity needs to be done in order to elucidate the mechanism of action of the *P. granatum*. If *P. granatum* were to be incorporated in oral hygiene products there will be no acid production for 6 hours and no dissolution of ions at the critical pH value 5.5. The long lasting protection by the plant will therefore reduce caries development.

5.5 Conclusion

At subinhibitory concentrations of 1.56, 3.125 and 6.25mg/ml concentrations *P. granatum* peel extract significantly reduced the acid production by the *S. mutans*. The extracts did not have any effect on the bacterial counts. Therefore, the inhibition effect observed in the acid production is an indication of its therapeutic properties against virulence traits of *S. mutans*. Hence, it can be a promising prophylactic therapeutic agent for dental caries. However, further research needs to be done to investigate the mechanism of action by this eel extract.

5.6 References

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CHAPTER 6

The effect of *P. granatum* on soluble and insoluble extracellular polysaccharides production (EPS) by *S. mutans*

6.1 Introduction

Extracellular polysaccharides (EPS), are key contributor in the formation of dental plaque. They are synthesized by *S. mutans* that produces glucosyltransferases (GTFs). GTFs are responsible for the production of both water soluble and insoluble sticky glucan from sucrose (Kawada-Matsuo *et al.,* 2017). *S. mutans* initially attaches to the salivary pellicle covering the tooth surface via the cell surface adhesion antigen I/II. This biofilm becomes mature through glucosyltranferases synthesis of different forms of extracellular high molecular weight branched glucans. Oral streptococci and mutans will aggregate through the scaffolding provided by glucose polymers while interacting with bacterial cell-associated glucan-binding proteins (Smith 2002). Therefore, they provide binding sites that promote accumulation of microorganisms on the tooth surface (Figure 6.1). They provide nutrients between meals and further establish water insoluble pathogenic biofilm formation. Research has found EPS production to affect progression of dental caries tremendously.

S. mutans produces at least three GTFs, which are products of the GTF B, GTF C, and GTF D genes. GTF B synthesizes mostly insoluble glucans containing elevated amounts of α-1,3-linked glucose, GTF C synthesizes a mixture of insoluble and soluble glucans (rich in α-1,6-linked glucose), and GTF D synthesizes predominantly soluble glucans (Kawada-Matsuo *et al.,* 2017, Loesche 1986, Paes-Leme *et al.,* 2006, Ren *et al.,* 2016a). These glucosyltransferases has the most significant virulence factor in causing dental caries and they could be potential targets for inhibitory agents (Devulapalle and Mooser 2000). In addition, *S. mutans* produce fructosyltransferase, an active extracellular enzyme acting on sucrose to synthesize fructan

polymers and is encoded by *sac B*. They cleave sucrose to release free glucose (which is available for uptake into the cell) while linking the fructose parts of the molecule together to yield an inulin-like fructan (Colby and Russell 1997). Fructans are considered to function primarily as extracellular storage compounds, and can also act as binding sites for bacteria accumulation (Shemesh *et al.,* 2007). *S. mutans* is able to utilize these polysaccharides as short-term storage mechanism which provides an additional ecological benefit while increasing the extent of acidification within the biofilm (Burne *et al.,* 1996).

In addition to GTFs, another two cell-associated antigens (Ags) are correlated directly with the ability of *S. mutans* to adhere to EPS and accumulate in the tooth surface. These are antigen I/II (AgI/II) as well as the glucan-binding proteins (GbpA, GbpB, GbpC, GbpD) (Smith 2002). The AgI/II is a surface adhesin that facilitates interrelation with salivary, serum, extracellular matrix elements, host cells and other microbes in streptococci (Abachi *et al.,* 2016). The proline rich central portion of Ag I/II contains the adhesion epitome that plays a role in promoting the interaction between the cell and the EPS. The Gbps increase the binding of *S. mutans* to each other and to glucans deposited on tooth surfaces, contributing to the sucrose-dependent adherence to teeth (Smith 2002). They consist of a GTF domain responsible for the glucan synthesis. Glucan binding proteins contribute to the development of optimal and stable biofilm and they also provide protection to bacteria.

Moreover, insoluble glucan production has been considered an important virulence factor of *S. mutans*. *S. mutans* will thrive in the complex oral microbiome and effectively modulates the transition from nonpathogenic to a cariogenic biofilms. The inactivation of GTF genes in *S. mutans* leads to diminished virulence in rodent models, proving the importance of these genes in pathogenicity (Yamashita *et al.,* 1993). A defect in the GbpA influenced the expression of

genes controlling biofilm formation such as *GTF B, GTF C* and *GTF D* indicating its importance as a protein for firm and stable biofilm formation (Matsumi *et al.,* 2015).

Aim: The aim of this study was to evaluate the effect of *P. granatum* on the production of soluble and insoluble extracellular polysaccharides by *S. mutans*.

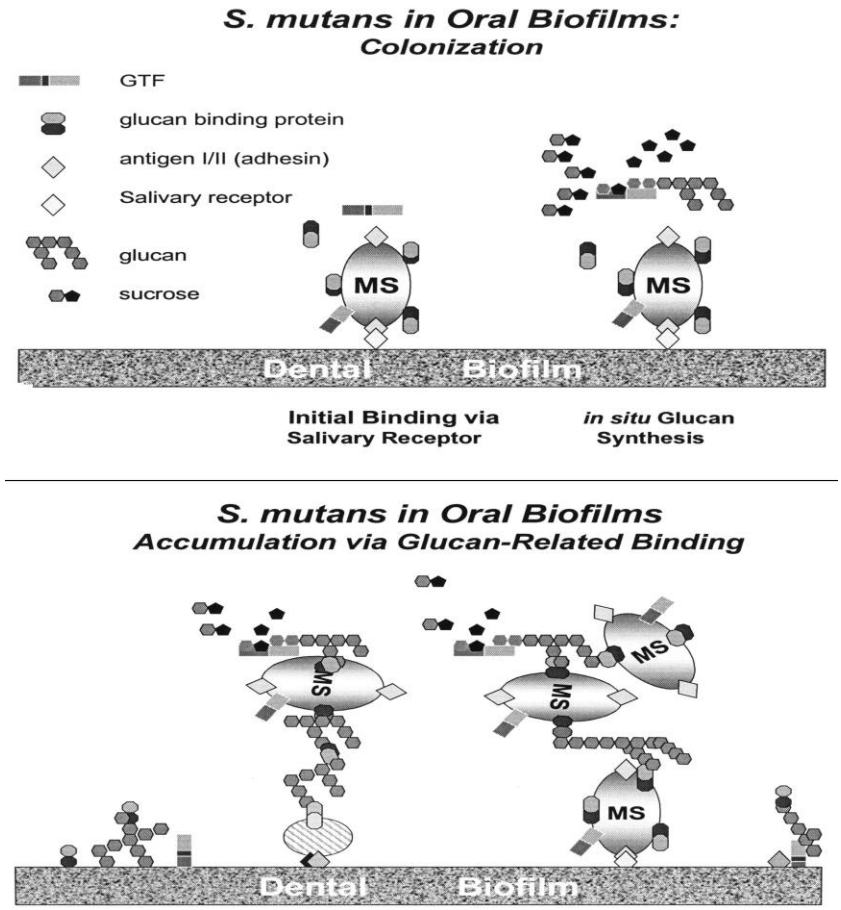

Figure 6.1 Models of mutans streptococcal (MS) colonization and accumulation in dental biofilms [Source : Smith, 2002]

6.2 Materials and methods

6.2.1 Plant material and preparation of extract

The pomegranate plant material was collected, fruit peel and extracts were prepared as described in chapter 3 section 3.2.1.

6.2.2 *S. mutans* cultures

Five *S. mutans* strains were obtained as described in chapter 3 section 3.2.2.

6.2.3 Biofilm EPS production

The effect of crude peel extract on the biofilm formation by *S. mutans* was studied (Naidoo *et al.,* 2012). This technique examined the effect of plant crude extract on EPS production by *S. mutans* biofilm. Three glass slides were held side by side in an upright (90^{o} C) position with an autoclave tape in four separate sterile beakers. Each beaker contained 50 ml of 5 % sucrose tryptone broth. Five hundred microliters of *S. mutans* was inoculated into each beaker. Three sub-inhibitory concentrations 1.56, 3.125 and 6.25 mg/ml of methanol crude extract were added separately. This was then incubated for 48 hours at 37^{o} C under CO_2. One slide was taken out, rinsed separately with sterile distilled water and the biofilm gently scraped-off in 5ml phosphate buffered saline (PBS) to harvest adherent cells and the production of EPS. Water was used as a control in the tryptone broth (0 concentration).

6.2.4 Planktonic EPS production

The effect of crude extract on planktonic growth of *S. mutans* and their production of EPS was examined using technique described by (Koo *et al.,* 2003). Cultures of *S. mutans* were grown on blood agar for 48 hours under CO_2 at 37^{o} C. Fresh inoculum was prepared with distilled water to an OD_{600} of ≈ 0.5 containing approximately 10^5 - 10^6 cfu/ml. Fifty milliliters of 5 %

sucrose tryptone broth containing the three sub-inhibitory concentrations of plant extract was inoculated with 500 µl of *S. mutans* suspension. The test concentrations were 1.56, 3.125 and 6.25 mg/ml. This was incubated for 48 hours at 37^{o} C under CO_2. Water was used as a control in the tryptone broth (0 concentration).

6.2.5 Soluble extracellular polysaccharide extraction

The extracellular polysaccharides were extracted and measured using a technique described by Aires *et al*., (2016) and Dubois *et al*., (1956) respectively. Five millilitres of the culture from either biofilm or planktonic suspension was centrifuged at 10,000 g for 5 min. Supernatant was collected and placed in a clean centrifuge tube. The pellet was resuspended in sterile distilled water and then centrifuged (repeated three times). All the supernatant from the same experiment were pooled together. Three volumes of ice cold absolute ethanol was added and placed at -20^{o} C for 30 min to precipitate polysaccharides. The mixture containing SEPS was then centrifuged, pellet was washed with 70 % alcohol and resuspended in 5 ml 1M NaOH. The total SEPS content in the samples were measured using phenol sulphuric assay in section 6.2.5.2.

6.2.5.1 Insoluble extracellular polysaccharide production (IEPS)

The mixture in section 6.2.5 was vortexed for 30 sec, agitated for 15 min. The sample was centrifuged for 5 min at 10000 g and the supernatant was transferred into a clean beaker. The remaining pellet was washed with 5 ml of 1M NaOH, centrifuged, and the supernatant was collected. This step was repeated three times. All supernatants were pooled together and three times volume of cold ethanol was added. The polysaccharides were allowed to precipitate at -20^{o} C for 30 min. The mixture was centrifuged and the pellet washed with 70 % ethanol. The

precipitate was then resuspended in 5 ml of NaOH. The total IEPS content in the samples were measured.

6.2.5.2 Phenol sulphuric assay

The total IEPS/SEPS content in the samples were measured separately using phenol sulphuric method (Dubois *et al.,* 1956). Two milliliters of 5 % aqueous solution of phenol and 5 ml of concentrated sulphuric acid was added rapidly to each mixture and was left to stand at room temperature in the dark for 10 min. Thereafter, it was vortexed for thirty seconds, placed in water bath at 25^{o} C for 20 min and the absorbance was read at 490 nm. These experiments were repeated 3 times for 5 strains of *S. mutans*. The percentage reduction was also calculated for each sub-inhibitory concentration.

6.2.6 Statistical analysis

The effect of the three sub-inhibitory concentrations 1.56 mg/ml, 3.125 mg/ml and 6.25 mg/ml of the crude extract on the soluble/insoluble extracellular polysaccharides production by *S. mutans* was determined by comparing OD readings of test to the controls using the Wilcoxon Rank Sum Test.

6.3 Results

The results of IEPS and SEPS production by the planktonic cells of *S. mutans* are shown in table 6.1 and the graph in Figure 6.2. Whereas, the results of IEPS and SEPS production by the biofilm form of *S. mutans* are shown in table 6.2 and the graph in Figure 6.3. The % reduction summary of all these results are depicted in table 6.3 and its graph in Figure 6.4.

The test concentrations of plant extract had no significant effect on the SEPS production in the planktonic growth of *S. mutans* with the p value > 0.05 at all test concentrations. The percentage reduction in this test ranged between 12.8 to 19.7 %. However, at high concentrations of 3.125 and 6.25 mg/ml the plant extract significantly reduced the IEPS production by *S. mutans* with a p value < 0.01. The % reduction at 1.56, 3.125 and 6.25 mg/ml was 18.9, 49.8 and 67.7 % respectively as shown in Figure 6.2 and 6.4. The reduction effect observed shows concentration dependence.

The test concentrations of the plant extract had no significant effect on the SEPS production in the biofilm growth of *S. mutans* with the p value > 0.05. In all the test concentrations *i.e* 1.56, 3.125 and 6.25 mg/ml the percentage reduction for SEPS production ranged between -14.3 to 21 %. However, at test concentrations of 1.56, 3.125 and 6.25 mg/ml the plant extract significantly (p value < 0.01) reduced the IEPS production in the biofilm growth by 54.05, 64.13 and 76.83% respectively as shown in Figure 6.3 and 6.4. Observations in table 6.3 and Figure 6.4 shows that the reduction effect was concentration dependent.

Table 6.1 The effect of *P. granatum* on the production of SEPS and IEPS by planktonic cells of *S. mutans*

Optical density at 490 nm												
		1.56 mg/ml				3.125 mg/ml				6.25 mg/ml			
Strain	Repeats	**SEPS**		**IEPS**		**SEPS**		**IEPS**		**SEPS**		**IEPS**	
		control	plant	control	plant	control	plant	control	plant	control	plant	control	plant
SM1	1	2.37	1.08	1.62	1.07	3.96	6.09	3.88	2.45	3.79	1.39	1.64	1.44
	2	1.36	1.97	2.45	1.36	4.77	4.62	3.22	1.87	1.48	1.26	1.48	0.47
	3	3.79	1.47	1.64	1.12	2.61	1.25	1.79	0.73	2.64	1.68	2.14	0.46
SM6	1	1.96	0.87	2.39	1.44	10.64	8.81	2.21	1.73	1.11	0.69	1.38	0.56
	2	3.89	3.83	1.68	1.99	0.85	1.37	3.93	0.53	1.10	4.50	2.09	0.65
	3	1.69	1.25	1.84	2.06	6.37	5.91	2.90	2.20	1.85	3.23	2.14	0.58
SM7	1	0.33	1.03	0.39	1.60	8.84	6.16	2.31	0.96	7.92	4.79	4.14	2.33
	2	4.70	3.04	0.96	1.70	9.31	5.03	2.25	0.57	3.47	1.67	1.382	0.37
	3	1.99	0.79	1.49	1.47	17.93	5.96	1.73	1.07	1.93	1.26	2.448	0.37
SM12	1	2.42	2.97	2.12	1.08	6.07	4.63	3.14	2.02	6.89	6.01	0.82	0.72
	2	4.35	3.47	1.68	0.84	1.93	1.05	1.57	0.47	2.07	1.20	1.40	0.31
	3	1.35	1.97	2.17	1.16	1.93	0.81	1.57	0.48	2.23	0.89	1.60	0.10
SM13	1	1.43	1.11	1.97	1.04	8.51	11.96	2.62	0.97	6.80	6.09	0.72	0.33
	2	2.80	2.08	1.36	1.15	1.80	10.42	2.78	1.94	1.75	2.23	2.47	0.33
	3	1.24	2.99	0.77	1.00	1.44	1.76	1.07	0.66	2.12	1.04	2.01	0.06
Mean		**2.38**	**1.99**	**1.64**	**1.33**	**5.8**	**5.06**	**2.47**	**1.24**	**3.14**	**2.52**	**1.86**	**0.60**
±SD		**1.28**	**1.02**	**0.58**	**0.36**	**4.66**	**3.46**	**0.85**	**0.71**	**2.24**	**1.90**	**0.82**	**0.57**
% reduction formulae		**16.4**		**18.9**		**12.4**		**49.8**		**19.7**		**67.7**	

Percentage Reduction (%) $= \left({}^{(Control - test)}/_{Control}\right)$ X 100

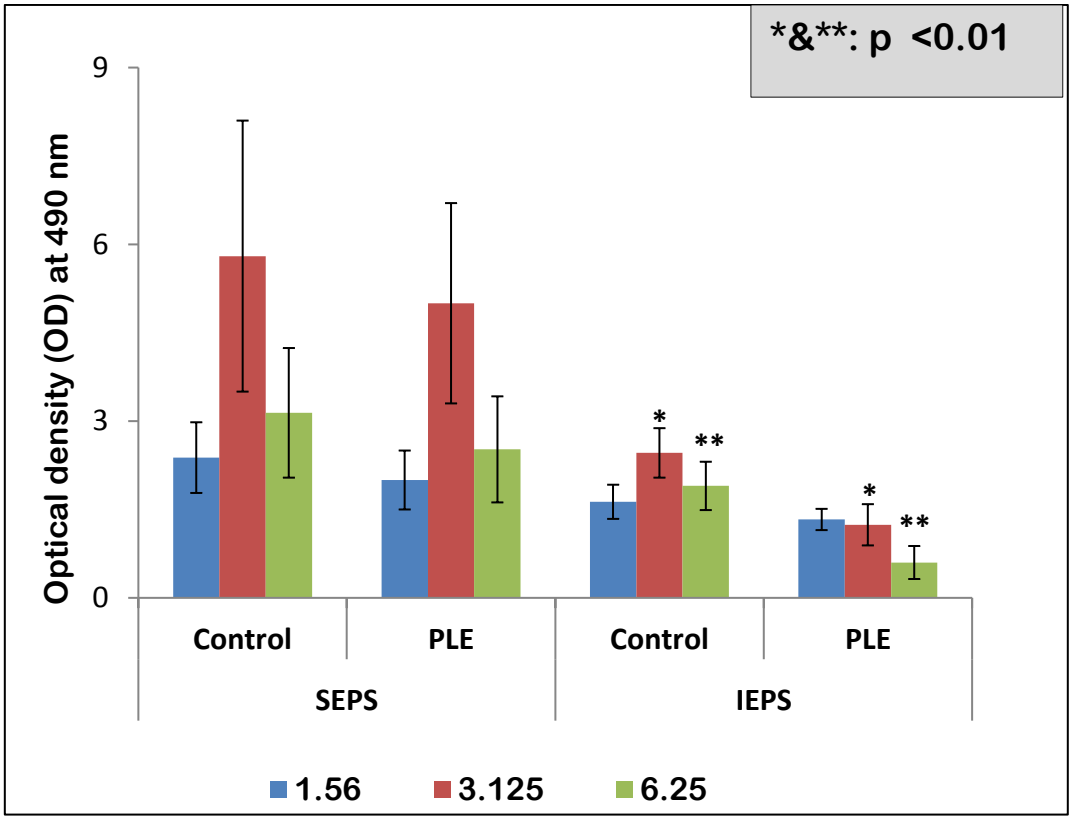

Figure 6.2 The effect *P. granatum* on soluble and insoluble extracellular polysaccharide production by planktonic *S. mutans* (n =15)

Table 6.2 The effect of *P. granatum* on the production of SEPS and IEPS by biofilm cells of *S. mutans*

Optical density at 490 nm												
		1.56 mg/ml				3.125 mg/ml				6.25 mg/ml			
		SEPS		**IEPS**		**SEPS**		**IEPS**		**SEPS**		**IEPS**	
Strain	Repeats	control	plant	control	plant	control	plant	control	plant	control	plant	control	plant
SM1	1	0.12	0.07	0.64	0.27	0.51	0.64	1.71	0.59	0.15	0.20	0.72	0.15
	2	0.06	0.06	0.24	0.12	0.21	0.53	0.85	0.46	0.08	0.06	1.48	0.46
	3	0.15	0.18	0.72	0.13	0.32	0.29	0.73	0.12	0.16	0.06	0.89	0.09
SM6	1	0.18	0.17	0.37	0.22	0.45	0.18	1.48	0.44	0.10	0.09	0.17	0.11
	2	0.12	0.09	0.20	0.40	0.36	0.18	0.75	1.27	0.08	0.05	0.26	0.14
	3	0.15	0.21	0.20	0.13	0.31	0.25	1.14	0.14	0.11	0.16	0.53	0.08
SM7	1	0.09	0.17	0.58	0.24	0.40	0.29	1.36	0.23	0.48	0.48	1.38	0.51
	2	0.05	0.08	0.26	0.11	0.27	0.26	1.29	0.21	0.15	0.05	1.14	0.15
	3	0.16	0.08	0.25	0.15	0.37	0.25	0.58	0.10	0.17	0.07	0.14	0.10
SM12	1	0.13	0.09	0.45	0.16	0.28	0.20	1.82	0.30	0.64	0.43	1.37	0.53
	2	0.17	0.34	0.67	0.30	0.05	0.08	0.12	0.142	0.10	0.03	1.05	0.03
	3	0.153	0.07	0.43	0.09	0.05	0.05	0.12	0.14	0.31	0.07	1.25	0.08
SM13	1	0.12	0.12	0.06	0.04	0.46	0.35	1.34	0.21	0.31	0.34	0.78	0.27
	2	0.20	0.10	0.15	0.16	0.13	1.23	0.32	0.43	0.05	0.06	0.58	0.04
	3	0.05	0.05	0.18	0.06	0.03	0.07	0.23	0.13	0.10	0.10	0.64	0.20
Mean		**0.13**	**0.13**	**0.37**	**0.17**	**0.28**	**0.32**	**0.92**	**0.33**	**0.19**	**0.15**	**0.82**	**0.19**
±SD		**0.05**	**0.08**	**0.20**	**0.10**	**0.16**	**0.30**	**0.57**	**0.30**	**0.17**	**0.15**	**0.45**	**0.17**
% reduction		**0**		**54.1**		**-14.3**		**64.1**		**21.1**		**76.8**	

Percentage Reduction (%) = $\left({}^{(Control - test)}/_{Control}\right)$ X 100

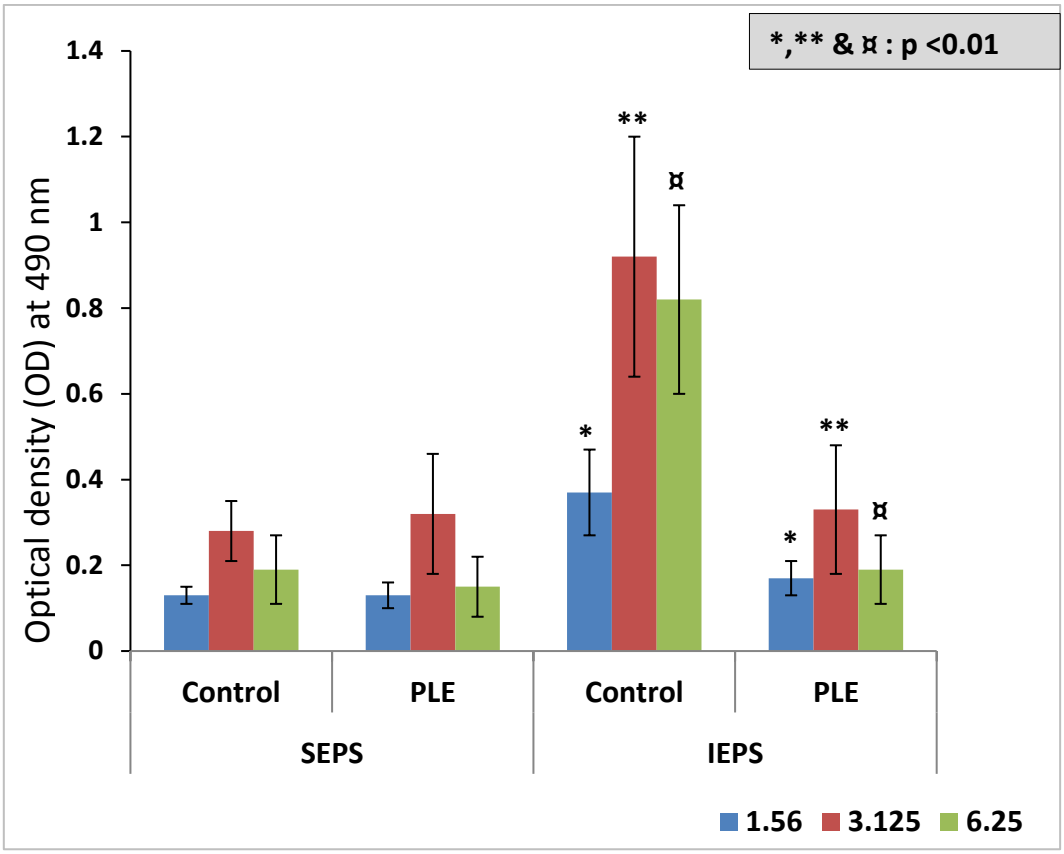

Figure 6.3 Effect of *P. granatum* on soluble SEPS and IEPS production by *S. mutans* biofilm (n = 15)

Table 6.3 Summary results (% reduction) of effect of *P. granatum* on soluble SEPS and IEPS production by *S. mutans* biofilm and planktonic cells

% reduction				
Concentrations (mg.ml)	Planktonic		Biofilm	
	SEPS	**IEPS**	**SEPS**	**IEPS**
1.56	**16.4**	**18.9**	**0.0**	**54.1**
3.125	**12.8**	**49.8**	**-14.3**	**64.1**
6.25	**19.7**	**67.7**	**21.1**	**76.8**

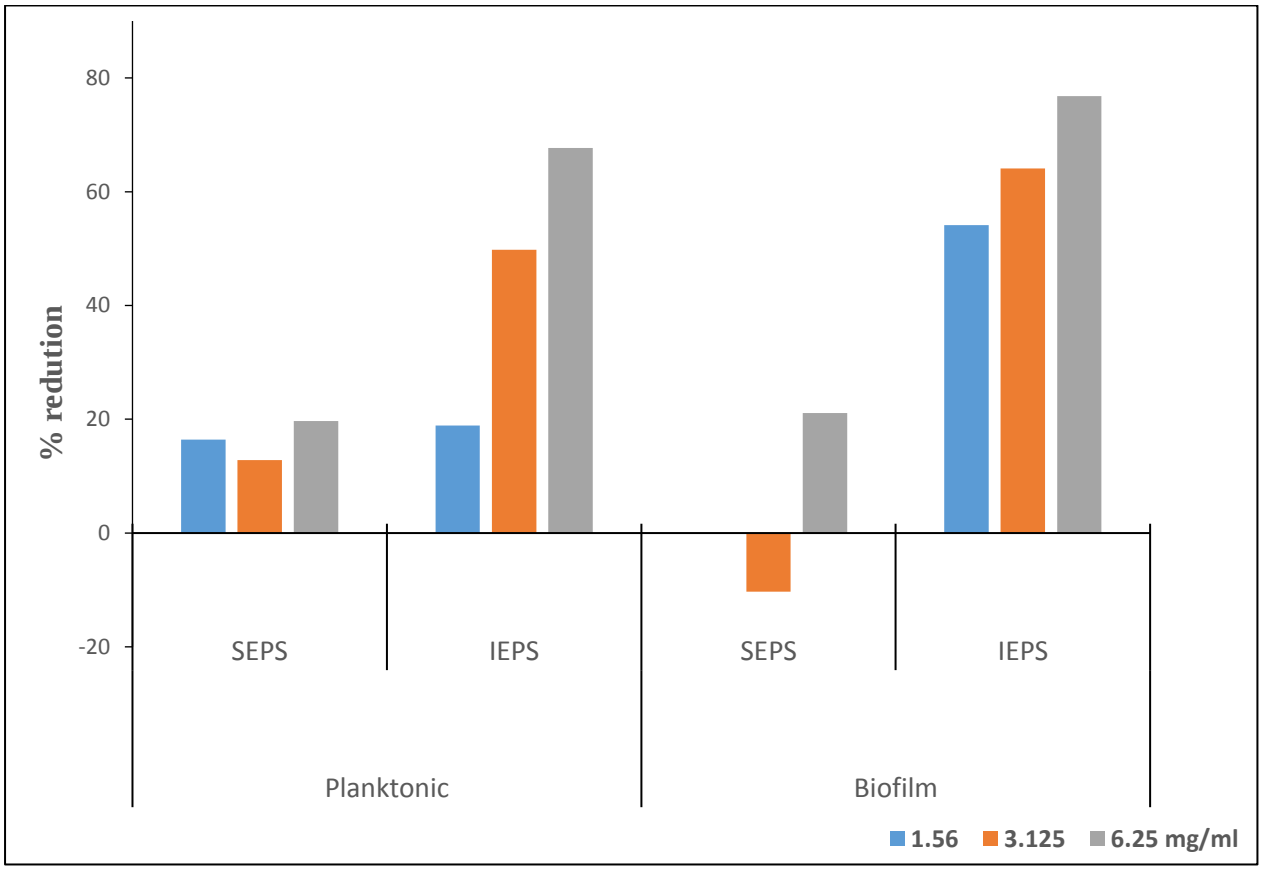

Figure 6.4 The % reduction of *P. granatum* on soluble SEPS and IEPS production by *S. mutans* (n = 15)

6.4 Discussion

Extracellular polysaccharides play a major role in the development of biofilm and its disruption is important for the control of dental caries. The objectives of this chapter was to determine the effect of pomegranate crude methanol extract on the production of IEPS and SEPS by *S. mutans*. This study showed that *P. granatum* crude extract reduced IEPS production by *S. mutans* in both biofilm and in planktonic cells in a concentration dependent manner (Figure 6.4). These IEPS are synthesized by GTF B and GTF C and bind avidly to the pellicle formed on the tooth surface. The reduction of IEPS production have major implications because EPS, particularly the IEPS, they increase the bulkness of plaque, provide nutrients to the cariogenic bacteria between meals and facilitate adherence of bacteria (Marsh and Martin. 2001). Even though *P. granatum* does not prevent biofilm production after six hours as shown in chapter 4 section 4.5, it will reduce the EPS production especially the insoluble glucans. The ability of *S. mutans* to facilitate cell adherence and biofilm development through the insoluble glucan production is an important virulence factor of *S. mutans*, largely due to its ability to facilitate cell adherence and biofilm formation (Guo *et al.*, 2015).

On the contrary, *P. granatum* did not have any significant effect on the production of SEPS by *S. mutans* in both planktonic and biofilm growth by *S. mutans*. Soluble EPS are synthesized by GTF C and GTF D genes of the *S. mutans*. Even though the mechanism of action or their contribution in the pathogenicity is not known, they have been shown to play role in the enhancement of caries development (Decker *et al.*, 2011). They provide nutrients when conditions are limited by serving as a source of fermentable carbohydrate for plaque bacteria (Jenkinson and Lamont 1997) and thus resulting in continual attack of the tooth surface. Research indicates that there is a positive cooperation of activity between GTF B and GTF D and it suggest that GTF D responsible for SEPS acts as an intrinsic primer for insoluble glucan

synthesis by GTF B enzymes (Venkitaraman *et al.*, 1995). The production of SEPS may aggravate the pathogenicity of *S. mutans*. SEPS is a soluble molecule that can be quickly degraded and solubilised by saliva. Therefore, the insignificant effect of the *P. granatum* on SEPS will not be dentrimental as they will be easily degraded and do not last long on the tooth surface.

An inhibition approach to target these GTFs has been explored for many years. A novel compound (2-(4-methoxyphenyl)-*N*-(3-{[2-4-methoxyphenyl) ethyl] imino} -1,4-dihydro-2 quinoxalinylide-ne) ethanamine that inhibit glucosyltransferases of *S. mutans* at 10 µg/ml with the capacity to inhibit pathogenicity of biofilm formation has been identified (Ren *et al.*, 2016). An *in vitro* assay of that study showed that the compound was capable of inhibiting EPS synthesis and biofilm formation in *S. mutans* by selectively antagonizing GTFs instead of by killing the bacteria directly. Moreover, the *in vivo* anti-caries efficacy of the same compound was evaluated in a rat model. They found that the compound significantly reduced the incidence and severity of smooth and sulcal-surface caries with a concomitant reduction in the percentage of *S. mutans* in the animals' dental plaque (Ren *et al.*, 2016). In another study, Long Zhang Gargle, made from Chinese herbs, had also shown EPS inhibition at a relatively low 2 % concentration (Yang *et al.*, 2016). *P. granatum* may have affected the production of glucosyltransferases especially GTF B and C required for maximum biofilm formation through the production of IEPS by *S. mutans*. Vahid-Dastjerdi *et al.*, 2016 have reported a down-regulation of glycosyl transferase genes in *S. mutans* by the presence of *P. granatum* flower and *Rhus coriaria L*. fruit water extracts (Vahid-Dastjerdi *et al.*, 2016). In another *in vivo* study, reduction of EPS production resulted in reduced caries in rats to same extent as usage of fluoride (Chen *et al.*, 2016). Furthermore, the hydroethanolic fraction and essential oils from the leaves of *Baccharis dracunculifolia* showed a change in the IEPS chemical composition

after treatment (Aires *et al.,* 2016). In this study, they found that the plant extract reduced IEPS production in the test experiment compared to the control. Plant derived compounds such as catechin-based polyphenols from the leaves of *Camellia sinensis*, flavovoids from *Apis melliifera propolis* and others have been shown to disrupt the enzymatic activity of GTFs (Ren *et al.,* 2016a). Nagasawa *et al*., (2017) showed that the presence of raffinose induced fructan synthesis by fructosyltransferase and aggregated extracellular DNA (eDNA, which is probably genomic DNA released from dead cells) into the biofilm (Nagasawa *et al.,* 2017). They attributed the inhibition effect to the reduced hydrophobicity of the cell surface which is mediated by the GTF B in the presence of sucrose. This was confirmed by deletion of GTF B that resulted in reduced hydrophobicity which is probably the mechanism in the GTFs inhibition (Nagasawa *et al.,* 2017).

Sucrose, the most cariogenic carbohydrate is a fermentable disaccharide and serves as a substrate for IEPS synthesis (Kreth *et al.,* 2008). Previously, it has been shown that sucrose can reduce the concentration of calcium, inorganic phosphorus, and fluoride in the dental biofilms. Loss of these ions plays a major role in the demineralisation of enamel and dentin of the oral cavity (Tenuta *et al.,* 2006). Its availability in the environment plays a role in the survival of *S. mutans* and development of cariogenic biofilm. There are three main functions in which synthesis of EPS from sucrose contributes to the development of dental caries. They provide sites of attachment on dental surfaces for microbial colonization, nutrients and water-insoluble matrix for biofilm formation (Ren *et al.,* 2016a). This study has shown that *P. granatum* reduced the development of biofilm as shown in chapter 4 as well as IEPS production by *S. mutans*. *P. granatum* might have disrupted the biofilm structure. It might have affected the GTF B activity as shown in the reduced IEPS production by blocking the ligand: enzyme activity. This results in reduced hydrophobic cell surface which in turn affects cell-cell or cell-

matrix interaction as well as production of IEPS. The incorporation of *P. granatum* in toothpaste or mouth rinses at sub-inhibitory concentrations will reduce the production of cariogenic biofilm especially IEPS. Whether *P. granatum* blocked the GTF B activity or other mechanism utilised, needs to be investigated further at a molecular level. The use of mix flora instead of monoculture in the presence of saliva needs further exploration for both biofilm and planktonic cell growth. Consequently, *in vivo* studies as well needs to be explored in order to examine the plant effect further.

6.5 Conclusion

P. granatum inhibited IEPS production by *S. mutans* in the biofilm form significantly (p value < 0.01). The percentage reduction at 1.56, 3.125 and 6.25 mg/ml plant extract was 54.05, 64.13 and 76.83 % respectively. The EPS reduction was concentration dependent. This will have major implications because it will reduce microbial colonization, adherent glucan production, and the interference with the stable biofilm formation. The planktonic growth IEPS were only reduced at higher concentrations of 3.125 and 6.25 mg/ml with a $p < 0.01$ compared to control. However, if *P. granatum* is used as a mouthrinse it will reduce IEPS in both planktonic and biofilm formation which results in less plaque formation between oral hygiene practice.

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CHAPTER 7

Fractionation of crude extract of *P. granatum* and, the effect of subfractions on the biofilm formation and acid production in *S. mutans*

7.1 Introduction

The effect of *Punica granatum* crude extract on biofilm and acid production by *S. mutans* has been described in chapters 4 and 5. It was shown that at the subinhibitory concentrations the crude extract reduced both acid production and biofilm formation. Significant reduction in acid production was observed with a $p = < 0.01$ in all test concentrations in the presence of the crude plant extract compared to the control. This effect was found to be concentration dependant. Generally, the healing power from plants has been attributed to its components especially the different classes of phytochemicals. Plants such as *Dodonaea viscosa* var. *angustifolia, Curcuma xanthorrhiza, Zingiber officinale, Withania somnifera*, and *Cimicifuga foetida* as well as *Punica gratunum L*. have been studied for their activity on *S. mutans*. Phytochemicals derived from these plants include flavonoids, alkaloids, carotenoids, tannins, antioxidants, anthocyaninis and phenolic compounds. For example, a flavone has been isolated and identified from *Dodonaea viscosa* var. *angustifolia* which has shown anti-*S. mutans*, anti-biofilm at 0.05 mg/ml and anti-acidogenic activity at 0.2 mg/ml (Ngabaza *et al.*, 2018*)* which was originally shown in the crude extract (Naidoo *et al.*, 2012).

Pomegranate peel comprises of different classes of phytochemicals that have been identified such as derivatives of gallotannins, ellagitannins, lipids, organic acids, flavonoids, lignans, triterpenoids, phytosterols, fatty acids, lipids, organic acids and phenolic acids (Wu and Tian 2017). Polyphenols, flavonoids, proanthocyanidins, polysaccharides, and proteins are the main components (Peng *et al.,* 2018). Punicalagin (0.234 %) containing pomegranate pericarp

extract has been shown to have an anti-*S. mutans* activity (Millo *et al.,* 2017). However, in this study punicalagin was not purified and screened for activity against *S. mutans*. In another study, HPLC fractionation was used to separate and identify bioactive compounds in the pomegranate peel extracts. Valoneic acid dilactone (aqueous), hexoside (methanol) and coumaric acid (hexane) were identified as bioactive compounds against multidrug resistant bacterial pathogens *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Khan *et al.,* 2017). The bioactivities of compounds from pomegranate peel have been explored in other fields but very little is known with regards to the antimicrobial activities against oral pathogens. Nothing is known about the bioactivity of the chemical compounds of pomegranate peel on *S. mutans and* its cariogenic activity. Moreover, no study is available on any of the South African cultivars against this microbe. Knowledge of phytochemicals also helps assess the structural factors that contribute to the plant activity.

Techniques for fractionation and purification of bioactive compounds from plants have undergone constant development. Finding an appropriate method that would screen for bioactivity like antioxidants, antibacterial together with simplicity, speed and specificity is the main goal in phytochemical analysis (Mulinacci *et al.,* 2004). Thin-layer chromatography (TLC) and column chromatography (CC) methods continue to be used widely owing to their suitability, economy as well as accessibility in different stationary phases. Cellulose, alumina, silica and polyamide are widely used and exhibit the most significance in purification of phytochemicals (Altemimi *et al.,* 2017). Plant materials are complex and that creates a difficult separation method. Hence, the use of multiple mobile phases with accumulative polarity is useful for extremely important separations, as well as using TLC to analyse the fractions of compounds from column chromatography (Altemimi *et al.,* 2017).

Research studies are available that have used complicated multi-step column chromatography successfully to purify punicalin a bioactive compound that is also found in pomegranate (Abdulla *et al.,* 2017, Aguilar-Zarate *et al.,* 2017, Martino *et al.,* 2004). These methods are labour-intensive and exhibit low yields and purity. Also, the purpose is not to only purify the punicalin but any class of the phytochemicals found in plants that exhibit beneficial effects. In addition, these plant compounds need to be produced in reasonable quantities in order to do more research.

Aim: The aim of this study was to fractionate the *P. granatum* crude extract and to study the effect of its bioactive subfraction/s on biofilm formation and acid production in *S. mutans*.

7.2 Materials and methods

7.2.1 Separation

7.2.1.1 Plant material and extract preparation

Punica granatum peel dried and pulverized (743 g) was macerated overnight with vigorous shaking in a mixture of dichloromethane (DCM): methanol (MeOH) solvents in a 1:1 ratio to a total volume of 5 liters. This step was repeated three times. The supernatant was filtered, evaporated on a rotary evaporator and complete dryness was achieved under a cold air stream to yield a crude extract.

7.2.1.2 Solvent extraction

The dried crude extract from section 7.2.1.1 was then suspended in a mixture of MeOH: water (H_2O) in a 9:1 ratio. The mixture was extracted with equal volume of hexane in a 500 ml

separation funnel with vigorous shaking for ten minutes. This step was repeated three times, the hexane layer pooled together in a beaker and dried overnight under cold air. The MeOH: H_2O mixture was re-extracted further with 500 ml ethyl acetate. The ethyl acetate layer was collected separately in a beaker and dried overnight under cold air. This was repeated three times. The remaining methanol residue was also dried under cold air.

7.2.1.3 Thin layer chromatography (TLC)

The silica gel GF 254 as well as reverse phase plates, 20 x 20 cm 1 mm thick were used. The dried extracts collected as described in section 7.2.1.2 of the hexane, ethyl acetate, and methanol were developed separately on a TLC plate using increasing polarity solvents. The developed plates were dried and the spots and bands developed with a vanillin spray reagent after which they were analysed under UV irradiation at 254 nm and 366 nm (Endo *et al.*, 2010).

7.2.1.4 Silica column chromatography

Further purification of the hexane extract was first done using with silica column. About 60 g silica column was suspended in 90 ml of a mixture of 9:1 hexane: ethyl acetate solvent mixture while swirling. The slurry was then poured onto a column while allowing the solvent to drain to prevent overflowing. The column was tapped gently to encourage bubbles to rise and allow silica to settle properly. The sample was loaded and then eluted with increasing polarity of solvent mixture working from the nonpolar hexane: ethyl acetate *i.e* 9:1, 8:2 $\rightarrow$6:4 ratios.

7.2.1.5 Sephadex column chromatography

The sephadex LH 20 column was prepared by dispersing 30g in 100 ml methanol. It was swirled and allowed to swell for 3 hours and then poured into the column. The hexane extract was loaded onto the sephadex column and ran with increasing concentrations of methanol, ethyl acetate and hexane. The second option had the hexane extracts reconstituted in water:ethyl acetate and eluted with alkaline water as well as gradual increase of the water pH to 12. Thirdly, a sephadex slurry was prepared in water at the pH 12 while the sample was reconstituted in water and eluted with pH 12 as well. The fractions collected were then neutralised.

7.2.1.6 Cellulose column chromatography separation 1

Separation of fractions from crude extract was attempted using cellulose (cellulose MN 100, Germany: native fibrous cellulose, standard grade with the average degree of polymerization 620-680, fiber length of 85 %, 20-100 µm ~6500 $cm_{2/g}$, residue on ignition < 10 000 ppm, < 20 Fe, < 5 ppm Cu, <7 ppm P, DCM extract <0.20 %). Briefly, 60 g of cellulose sorbents were dissolved in 200 ml sterile distilled water and agitated gently before pouring into a column. The hexane portion of the liquid-liquid extract was then loaded onto the column. The extract was then first eluted with about 6 ml of distilled water followed by aqueous solution (pH 12) in which two bands were collected. This was then followed by elution with 1:1 of water: tetrahydrofuran (THF) mixture where two more bands were collected. The fractions collected were then neutralised. The collected sample was then evaporated under dry cold air.

7.2.1.7 Cellulose column chromatography separation 2

To obtain sub-fractions F 3.2 and F 4.1, the bands collected as described above were further separated on a fresh cellulose column as described in section 7.2.1.3. The column was first flushed with 2 ml of 1:1 H_20: THF and the sample loaded thereafter. Optimal separation of bands was achieved by a gradual increase (in solvent polarity) from 1:1 → 7:3 → 9:1→1:0 of H_20: THF.

7.2.2 Antimicrobial effect of subfractions

7.2.2.1 *S. mutans* cultures

Five *S. mutans* strains were obtained as described in chapter 3 section 3.2.2.

7.2.2.2 Minimum bactericidal concentration

Minimum bactericidal concentrations for the cellulose extracted fractions and subfractions against *S. mutans* was obtained according to chapter 3 section 3.2.2 and section 3.2.3.

7.2.2.3 Biofilm assay

The effect of the subfractions F 3.2 and F 4.1 from the cellulose column 1 & 2 on biofilm formation by *S. mutans* was carried out as described in chapter 4 section 4.2.2.

7.2.2.4 Acid assay

The effect of the subfractions F 3.2 and F 4.1 from the cellulose column 1 & 2 assay on acid production by *S. mutans* was studied as described in chapter 5 section 5.2.3.

7.2.25 Statistical analysis

The percentage reduction in the biofilm formation was calculated after 6 and 24 hours at all test concentrations. The formula used was: (x-y)/x X 100 where x is the control while y is the test biofilm count. To determine the effect of different concentrations of the subfractions on biofilm formation by *S. mutans,* bacterial counts in the test biofilms were compared to the

control counts using the Wilcoxon Rank Sum Test. For the effect of subfractions on the acid production, the measured pH readings in the presence of the subinhibitory concentrations of plant extracts were compared with the control readings using Anova. The bacterial counts in the acid assay test were also compared to the control. Statistical significance was inferred at p value of < 0.05.

7.3 Results

7.3.1 Separation

7.3.1.1 Plant material and extract preparation

The plant crude extract from the DCM:MeOH was used because it is the most active extract against *S. mutans*. The plant crude extraction yielded about 282 g (150 + 89 + 43 g) after filtration and evaporation of the solvent *in vacuo*. It awarded a total of 38 % yield .

7.3.1.2 Solvent extraction (Hexane: Ethyl acetate)

Thin layer chromatography was performed for the analysis of fractions and the results are depicted in Figure 7.1. The hexane :ethyl acetate solvent 70: 30 mixture mixture was used as mobile phase. Lane 1 and 2 are the hexane samples from the liquid-liquid extracts showing 9 bands. Lane 3 is the ethyl acetate extract. The ethyl acetate had 3 bands. Lane 4 is the methanol:water with only 1 band.

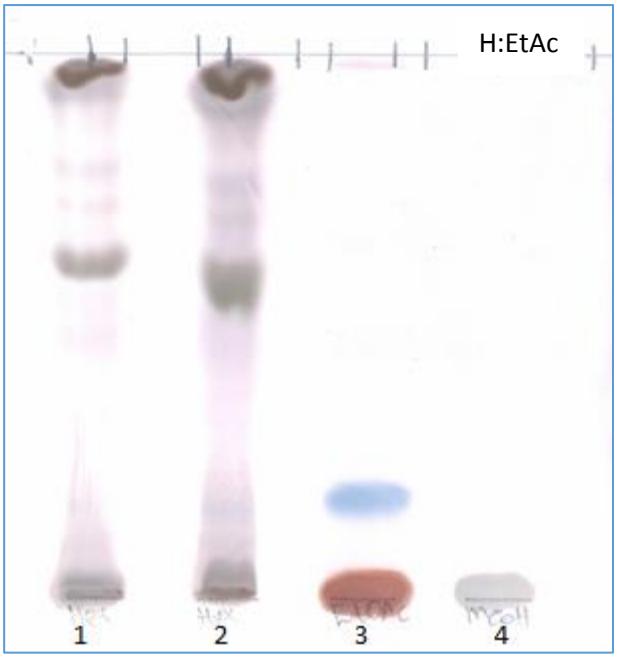

Figure 7.1 TLC of fractions (n-hexane, ethyl acetate and methanol fractions)

7.3.1.3 Column chromatography

The TLC results of n-hexane extract purified on a silica column are shown in Figure 7.2. It depicts the fractions collected from the silica column using 8:2 hexane: ethyl acetate as mobile phase. The bands could not be separated by the column and the different fractions collected gave similar pattern on the TLC plate figure 7.2 lane 1-4 having some similar compounds and lane 5 separate.

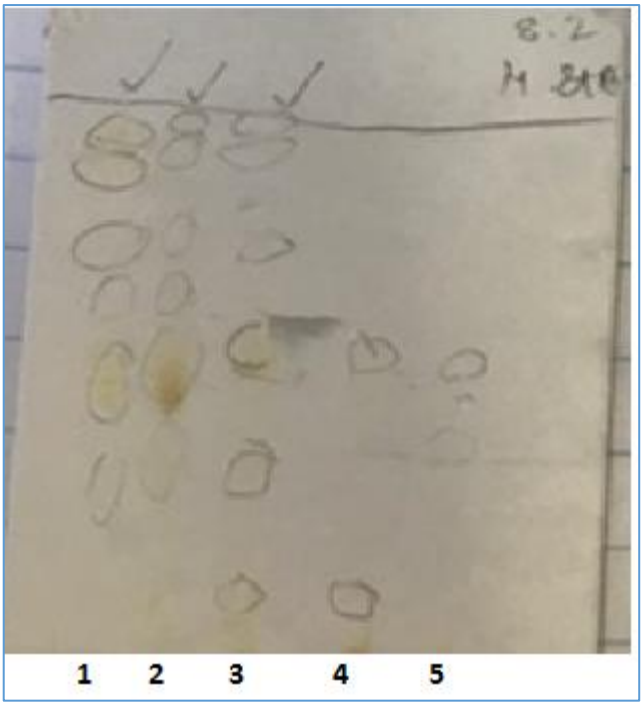

Figure 7.2 TLC of n-hexane extract purified on silica column fractions extracts using UV light

The problems encountered in the silica column required alternative methods. The sephadex LH 20 was then used to purify the n-hexane extract further. The hexane extract was awarded priority as the primary partitioning solvent for further purification due to indecisive inhibition activity of the liquid liquid extracts in the bioautography screening. The results of sephadex purification are shown in Figure 7.3. The column separation gave two bands that could not separate when spotted on a TLC plates they did not move either (results not shown).

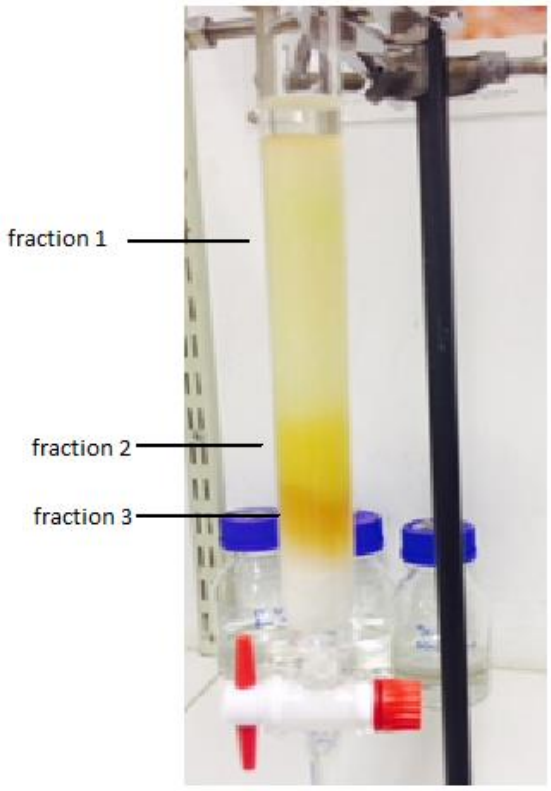

Figure 7.3 Purification of n-hexane extract on sephadex LH 20 column

The results of the n-hexane extract ran on a cellulose column 1 are shown in Figure 7.4. The figure depicts 3 fractions that separated initially. The fourth fraction would only move as the 3rd fraction was half way through the column as the gradual increase in solvent polarity was applied.

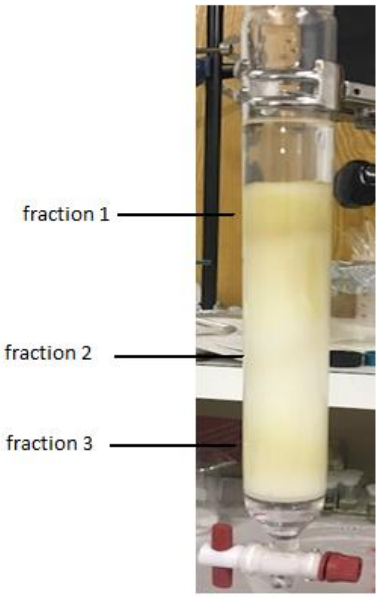

Figure 7.4 Three fractions of n-hexane extract on cellulose column

7.3.2 Antimicrobial effect

7.3.2.1 Minimum bactericidal concentrations

The minimum bactericidal concentrations of Fraction 1, Fraction 2, Fraction 3 and Fraction 4 obtained from the n-hexane extract separated on cellulose chromatography column 1 are shown in Table 7.1. All the fractions showed the same MBC of 12.5 mg/ml against *S. mutans* strain SM1. The 4 fractions obtained were separated further in a fresh cellulose chromatography column 2. Each fraction showed subsequent sub-fractions and their MBC's are shown in Table 7.1. The results show that all sub-fractions yielded an MBC that ranged between 6.25 and 25 mg/ml. Sub-fractions 3.2 (F 3.2) was the most active with an MBC of 6.25 mg/ml. This was followed by sub-fraction 4.1 (F 4.1) which yielded an MBC of 12.5 mg/ml. Further studies were then performed only on the 2 sub-fractions F 3.2 and F 4.1.

Table 7.1 The MBC of the fractions and sub-fractions of *P. granatum* against *S. mutans* SM1

strain

Strain	Repeats	Fractions MBC (mg/ml)				MBC of Sub-fractions (mg/ml)									
		F 1	F 2	F 3	F 4	F 1		F 2		F 3			F 4		
						F 1.1	F 1.2	F 2.1	F 2.2	F 3.1	F 3.2	F 3.3	F 4.1	F 4.2	F 4.3
SM1	1	12.5	12.5	12.5	12.5	25	25	25	25	25	6.25	12.5	12.5	25	25
	2	12.5	12.5	12.5	12.5	25	25	25	25	25	6.25	12.5	12.5	25	25
	3	12.5	12.5	12.5	12.5	25	25	25	25	25	6.25	12.5	12.5	25	25
Median		12.5	12.5	12.5	12.5	<25	<25	25	25	<25	6.25	12.5	12.5	25	25

F: fraction

7.3.2.2 Biofilm

For the subfraction F 3.2, two subinhibitory concentrations of 1.56, 3.125 and the MBC of 6.25 mg/ml were used in the biofilm study. Whereas, for the subfraction F 4.1, subinhibitory concentrations of 3.125 and 6.25 as well as the MBC of 12.5 mg/ml were selected.

The results of *S. mutans* counts in the biofilm grown in the presence of F 3.2 and the controls are shown in Table 7.2 and the plotted graph in Figure 7.5. In addition, the percentage reduction of the *S. mutans* counts on the biofilm is shown in Table 7.3 and Figure 7.6. After six hours, there was significant reduction in biofilm formation when compared to control at 6.25 mg/ml. *P. granatum* subfraction F 3.2 reduced biofilm formation by 58 % at this concentration. This was the only concentration with statistically significant difference among all tested concentrations with a p value of < 0.01 (Tables 7.2 and Figure 7.5). At concentrations of 3.125 and 1.56 mg/ml, the percentage reduction was less than 14 %. The observed effect after 6 hours was concentration dependent. After 24 hours of incubation, the purified fraction F 3.2 did not have any effect on the biofilm formation by *S. mutans*. Instead, it enhanced the growth of bacterial cells.

Table 7.2 The effect of subfraction F 3.2 of *P. granatum* on biofilm formation by *S. mutans*

			S. mutans counts in biofilm (cfu/ml)							
		0 hours	**6 hrs – F 3.2 in mg/ml**				**24 hrs – F 3.2 in mg/ml**			
Strain	**Repeats**	**Inoculum**	**Control**	**1.56**	**3.125**	**6.25**	**Control**	**1.56**	**3.125**	**6.25**
SM 6	1	2.3×10^6	2.2×10^5	3.7×10^5	5.20×10^5	4.6×10^5	3.9×10^5	1.9×10^5	4.6×10^5	6.6×10^5
	2		2.3×10^5	1.1×10^6	6.60×10^5	5.2×10^5	4.1×10^5	3.8×10^5	4.4×10^5	5.6×10^5
	3		1.8×10^5	9.6×10^5	8.80×10^5	8.8×10^5	4.0×10^5	4.6×10^5	4.0×10^5	1.8×10^5
Mean		2.3×10^6	$\mathbf{2.1 \times 10^5}$	$\mathbf{8.0 \times 10^5}$	$\mathbf{6.9 \times 10^5}$	$\mathbf{6.2 \times 10^5}$	$\mathbf{4.0 \times 10^5}$	$\mathbf{3.4 \times 10^5}$	$\mathbf{4.3 \times 10^5}$	$\mathbf{4.7 \times 10^5}$
±SD			$\mathbf{2.7 \times 10^4}$	$\mathbf{3.8 \times 10^5}$	$\mathbf{1.8 \times 10^5}$	$\mathbf{2.3 \times 10^5}$	$\mathbf{1.0 \times 10^4}$	$\mathbf{1.4 \times 10^5}$	$\mathbf{3.1 \times 10^4}$	$\mathbf{2.5 \times 10^5}$
SM 7	1	$\mathbf{2.2 \times 10^6}$	1.0×10^6	1.2×10^6	8.2×10^5	5.0×10^5	7.7×10^5	1.9×10^6	1.7×10^6	6.0×10^5
	2		1.7×10^6	3.4×10^6	3.5×10^5	4.3×10^5	3.8×10^6	1.7×10^6	5.8×10^5	6.9×10^5
	3		1.8×10^6	2.9×10^6	1.9×10^6	8.9×10^5	7.0×10^5	1.4×10^6	6.1×10^5	1.2×10^6
Mean		$\mathbf{2.2 \times 10^6}$	$\mathbf{1.5 \times 10^6}$	$\mathbf{2.5 \times 10^6}$	$\mathbf{1.0 \times 10^6}$	$\mathbf{6.1 \times 10^5}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{9.6 \times 10^5}$	$\mathbf{8.2 \times 10^5}$
±SD			$\mathbf{4.2 \times 10^5}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{8.1 \times 10^5}$	$\mathbf{2.5 \times 10^5}$	$\mathbf{1.8 \times 10^6}$	$\mathbf{2.5 \times 10^5}$	$\mathbf{6.3 \times 10^5}$	$\mathbf{3.0 \times 10^5}$
SM 12	1	$\mathbf{7.0 \times 10^5}$	1.3×10^6	1.1×10^6	1.2×10^6	4.8×10^5	2.9×10^5	4.9×10^6	1.6×10^6	8.0×10^3
	2		1.2×10^6	1.3×10^6	1.2×10^6	4.4×10^5	3.2×10^5	1.9×10^6	1.4×10^6	9.0×10^3
	3		1.2×10^6	1.1×10^6	1.1×10^6	2.7×10^5	1.7×10^5	2.8×10^6	2.7×10^6	2.0×10^6
Mean		$\mathbf{7.0 \times 10^5}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{4.0 \times 10^5}$	$\mathbf{2.6 \times 10^5}$	$\mathbf{3.2 \times 10^6}$	$\mathbf{1.9 \times 10^6}$	$\mathbf{6.9 \times 10^5}$
±SD			$\mathbf{1.3 \times 10^4}$	$\mathbf{9.3 \times 10^4}$	$\mathbf{2.5 \times 10^4}$	$\mathbf{9.1 \times 10^4}$	$\mathbf{6.5 \times 10^4}$	$\mathbf{1.3 \times 10^6}$	$\mathbf{5.7 \times 10^5}$	$\mathbf{9.6 \times 10^5}$
SM 13	1	$\mathbf{2.9 \times 10^6}$	2.9×10^6	1.9×10^5	7.5×10^5	3.0×10^5	1.1×10^4	1.7×10^6	7.2×10^5	2.0×10^6
	2		1.8×10^6	1.5×10^6	1.0×10^6	8.0×10^5	2.7×10^6	1.2×10^5	1.1×10^6	5.0×10^6
	3		1.4×10^6	1.4×10^6	2.4×10^6	3.1×10^5	2.9×10^6	5.0×10^5	2.1×10^5	1.7×10^6
Mean		$\mathbf{2.9 \times 10^6}$	$\mathbf{2.1 \times 10^6}$	$\mathbf{1.0 \times 10^6}$	$\mathbf{1.4 \times 10^6}$	$\mathbf{4.7 \times 10^5}$	$\mathbf{1.5 \times 10^6}$	$\mathbf{7.7 \times 10^5}$	$\mathbf{6.6 \times 10^5}$	$\mathbf{2.9 \times 10^6}$
±SD			$\mathbf{7.7 \times 10^5}$	$\mathbf{7.3 \times 10^5}$	$\mathbf{9.1 \times 10^5}$	$\mathbf{2.9 \times 10^5}$	$\mathbf{2.0 \times 10^6}$	$\mathbf{8.1 \times 10^5}$	$\mathbf{4.3 \times 10^5}$	$\mathbf{1.8 \times 10^6}$
Combined Mean		$\mathbf{2.0 \times 10^6}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{1.4 \times 10^6}$	$\mathbf{1.1 \times 10^6}$	$\mathbf{5.23 \times 10^5}$	$\mathbf{9.2 \times 10^5}$	$\mathbf{1.5 \times 10^6}$	$\mathbf{9.9 \times 10^5}$	$\mathbf{1.2 \times 10^6}$
Combined ±SD		$\mathbf{9.3 \times 10^5}$	$\mathbf{7.9 \times 10^5}$	$\mathbf{9.3 \times 10^5}$	$\mathbf{5.9 \times 10^5}$	$\mathbf{2.2 \times 10^5}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{1.4 \times 10^6}$	$\mathbf{7.3 \times 10^5}$	$\mathbf{1.4 \times 10^6}$
% reduction				**-11.1**	**14.1**	**57.9**		**-62.4**	**-7.2**	**-31.7**

Percentage Reduction (%) = $\left({}^{(Control - test)}/_{Control}\right)$ X 100

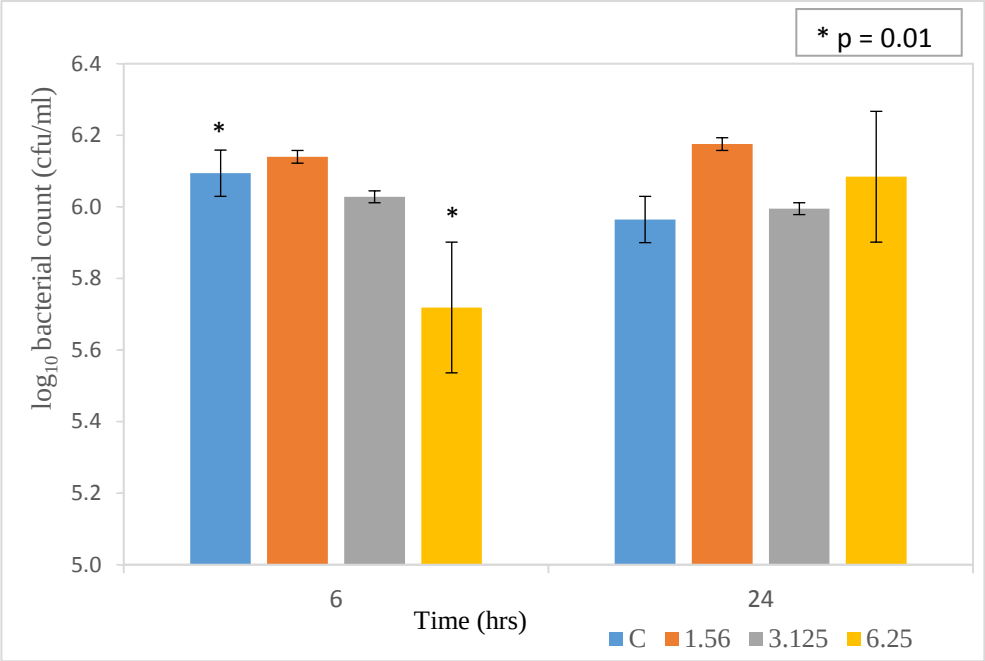

Figure 7.5 The effect of subfraction F 3.2 of *P. granatum* on the *S. mutans* biofilm formation

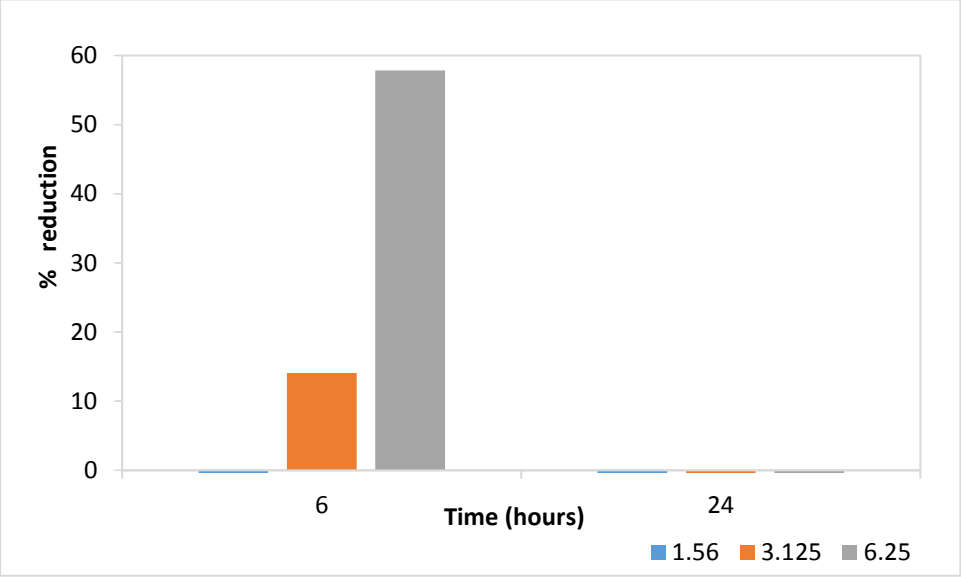

Figure 7.6 The percentage reduction in *S. mutans* biofilm formation by *P. granatum* subfraction F 3.2

The results of *S. mutans* counts in biofilm grown in the presence of F 4.1 are shown in Table 7.3, graphs in Figure 7.7 and the percentage reduction in Figure 7.8. A significant effect was observed at concentrations of 6.25 and 12.5 mg/ml with a p value of 0.003 after 6 hours of incubation. The subfraction F 4.1 inhibited *S. mutans* biofilm formation by 96 % at 12.5 mg/ml concentration after 6 hours. This was the most effective concentration in decreasing the viable cells in the biofilm. At 6.25 mg/ml percentage reduction was 61 % while 3.125 mg/ml showed a non-significant activity at 1.7 % reduction. The reduction effect observed was concentration dependent. After 24 hours of exposure, there was significant increase in biofilm formation at 3.125 mg/ml when compared to control. At the concentration of 6.25 mg/ml and 12.5 mg/ml, the concentration dependent effect observed in 6 hours was lost after 24 hours.

Table 7.3 The effect of subfraction F 4.1 of *P. granatum* on biofilm formation by *S. mutans*

***S. mutans* counts in biofilm (cfu/ml)**										
Strain	**Repeats**	**0 hrs**	**6 hrs – F 4.1 in mg/ml**				**24 hrs – F 4.1 in mg/ml**			
		Inoculum	**control**	**3.125**	**6.25**	**12.5**	**control**	**3.125**	**6.25**	**12.5**
SM 6	**1**	**1.3×10^6**	6.0×10^5	5.4×10^5	4.7×10^5	9.0×10^4	2.1×10^5	1.0×10^6	1.0×10^5	7.8×10^5
	2		6.4×10^5	8.0×10^5	3.0×10^5	4.4×10^4	1.4×10^5	1.6×10^5	1.2×10^5	6.3×10^5
	3		7.3×10^5	7.1×10^5	3.9×10^5	4.4×10^4	1.6×10^5	5.4×10^5	1.6×10^5	7.1×10^5
Mean		**1.3×10^6**	**6.6×10^5**	**6.8×10^5**	**3.9×10^5**	**5.9×10^4**	**1.7×10^5**	**5.7×10^5**	**1.3×10^5**	**7.1×10^5**
±SD			**6.7×10^4**	**1.3×10^5**	**8.5×10^4**	**2.7×10^4**	**3.5×10^4**	**4.2×10^5**	**3.0×10^4**	**7.5×10^4**
SM 7	**1**	**2.2×10^5**	1.3×10^6	2.6×10^6	6.8×10^5	2.0×10^5	3.2×10^6	1.2×10^6	7.0×10^6	5.2×10^6
	2		2.8×10^6	2.9×10^6	4.7×10^5	1.0×10^5	3.4×10^6	1.8×10^6	7.2×10^6	6.8×10^6
	3		1.6×10^6	3.1×10^6	5.3×10^5	1.0×10^5	3.3×10^6	1.6×10^6	5.9×10^6	4.6×10^6
Mean		**2.2×10^5**	**1.9×10^6**	**2.8×10^6**	**5.6×10^5**	**1.3×10^5**	**3.3×10^6**	**1.5×10^6**	**6.7×10^6**	**5.5×10^6**
±SD			**8.0×10^5**	**2.5×10^5**	**1.1×10^5**	**5.7×10^4**	**1.1×10^5**	**2.9×10^5**	**7.0×10^5**	**1.2×10^6**
SM 12	**1**	**1.1×10^6**	2.6×10^6	2.2×10^6	1.1×10^6	8.0×10^3	9.0×10^5	1.4×10^5	4.0×10^5	3.3×10^3
	2		3.1×10^6	2.4×10^6	1.5×10^6	5.2×10^4	4.6×10^5	3.1×10^5	8.6×10^5	1.0×10^4
	3		3.3×10^6	2.1×10^6	1.4×10^6	6.0×10^3	5.8×10^5	2.5×10^5	6.7×10^5	5.2×10^3
Mean		**1.1×10^6**	**3.0×10^6**	**2.2×10^6**	**1.4×10^6**	**2.2×10^4**	**6.5×10^5**	**2.3×10^5**	**6.4×10^5**	**6.2×10^3**
±SD			**3.5×10^5**	**1.5×10^5**	**2.1×10^5**	**2.6×10^4**	**2.3×10^5**	**8.6×10^4**	**2.3×10^5**	**3.5×10^3**
SM 13	**1**	**4.3×10^4**	2.1×10^6	1.1×10^6	4.3×10^5	3.9×10^4	8.8×10^6	2.0×10^6	1.4×10^3	1.6×10^5
	2		1.7×10^6	1.4×10^6	4.8×10^5	5.7×10^4	2.7×10^6	2.2×10^6	2.2×10^6	1.0×10^5
	3		1.8×10^6	1.9×10^6	8.4×10^5	8.1×10^4	3.5×10^6	2.4×10^6	2.7×10^4	9.0×10^4
Mean			**1.9×10^6**	**1.5×10^6**	**5.8×10^5**	**5.9×10^4**	**5.0×10^6**	**2.2×10^6**	**7.3×10^5**	**1.2×10^5**
±SD		**4.3×10^4**	**2.3×10^5**	**4.1×10^5**	**2.2×10^5**	**2.1×10^4**	**3.3×10^6**	**2.0×10^5**	**1.2×10^6**	**3.8×10^4**
Combined mean			**1.9×10^6**	**1.6×10^6**	**7.4×10^5**	**4.7×10^4**	**2.5×10^6**	**9.8×10^5**	**1.2×10^6**	**6.0×10^5**
Combined ±SD			**1.0×10^6**	**9.0×10^5**	**5.1×10^5**	**3.6×10^4**	**2.6×10^6**	**9.7×10^5**	**1.8×10^6**	**1.6×10^6**
% reduction				**1.7**	**60.1**	**96.3**		**50.7**	**10.3**	**30.5**

Percentage Reduction (%) = $\left({}^{(Control - test)}/_{Control}\right)$ X 100

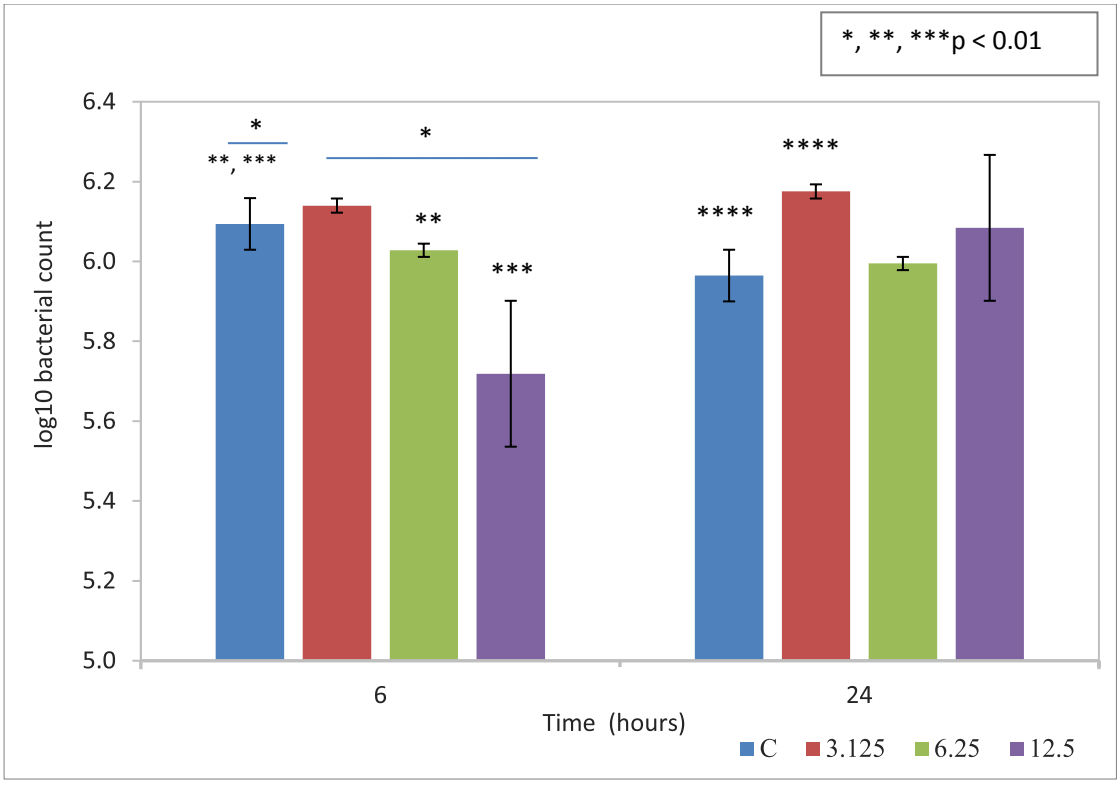

Figure 7.7 The effect of *P. granatum* subfraction F 4.1 on the *S. mutans* biofilm formation

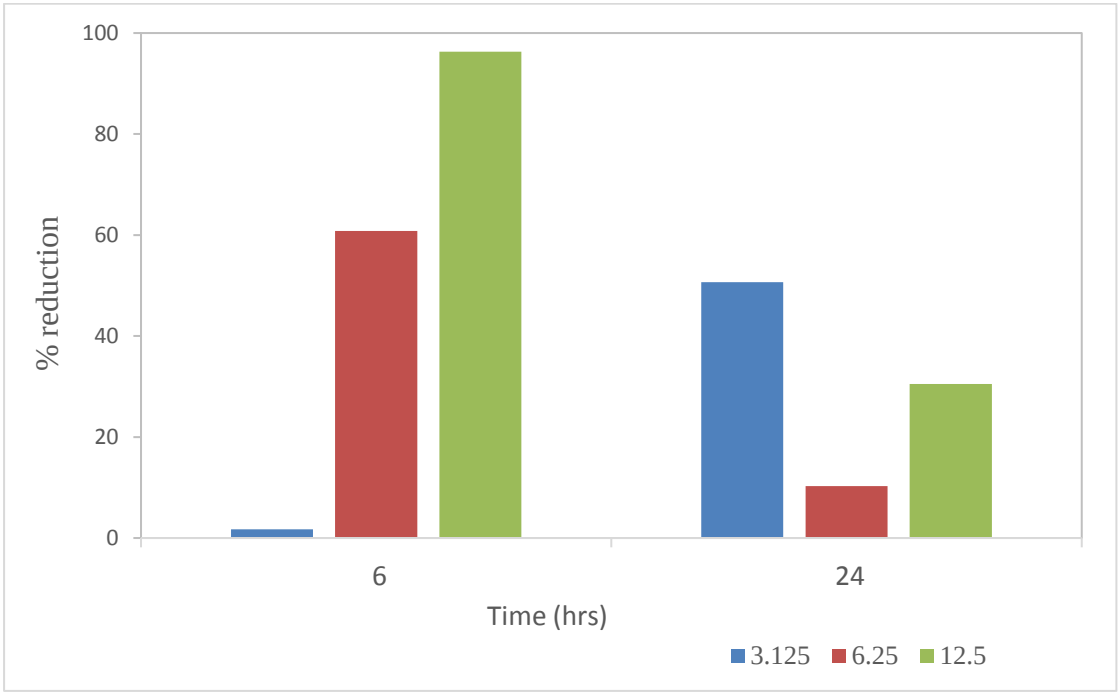

Figure 7.8 Percentage reduction in *S. mutans* biofilm formation by subfraction F 4.1

7.3.2.3 Acid assay

The results of the pH readings in the presence of 1.56, 3.125 and 6.25 mg/ml of purified subfraction F 3.2 are shown in Table 7.4 and Figure 7.9. At time 10, 12, 14 and 16 hours, the test pH was always higher than the control (Table 7.4, Figure 7.9). The observed effect was statistically significant with a p value < 0.01 and this effect was concentration dependent. This implies that the acid production was reduced in the presence of the purified fraction in a concentration dependent manner. After 10 hours, the pH drop was higher in the control compared to the test concentrations. At 16 hours, the control reached a pH of 5.5. This is the most critical time which causes demineralization of enamel. The pH with all three test concentrations did not reach a critical pH of 5.5 even after 16 hours.

Table 7.4 The effect of subfraction F 3.2 (mg/ml) on acid production by *S. mutans*

Acid production by *S. mutans* in the presence of subfraction F 3.2 (pH)																	
Strains		**0 hrs**	**10 hrs**				**12 hrs**				**14 hrs**				**16 hrs**			
	Repeats	**C**	**C**	**1.56**	**3.125**	**6.25**	**C**	**1.56**	**3.125**	**6.25**	**C**	**1.56**	**3.125**	**6.25**	**C**	**1.56**	**3.125**	**6.25**
SM 1	1	7.0	6.7	7.0	7.0	7.7	6.4	7.0	7.1	7.2	5.8	6.8	7.2	7.8	5.8	6.6	7.1	8.0
	2	7.0	6.5	6.9	7.0	7.7	6.3	6.8	7.2	7.5	5.6	6.7	7.2	7.8	5.6	6.7	7.0	8.1
	3	7.0	6.5	7.0	7.0	7.8	6.3	6.8	7.2	7.6	5.6	6.7	7.1	7.7	5.6	6.7	6.9	7.9
Mean		**7.0**	**6.5**	**6.9**	**7.0**	**7.7**	**6.3**	**6.9**	**7.2**	**7.4**	**5.7**	**6.7**	**7.1**	**7.8**	**5.7**	**6.7**	**7.0**	**8.0**
±SD		**0.0**	**0.1**	**0.0**	**0.0**	**0.1**	**0.1**	**0.1**	**0.0**	**0.2**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.0**	**0.1**	**0.1**
SM 6	1	7.0	6.8	7.3	7.8	8.2	6.0	7.2	7.8	8.1	5.7	6.4	7.4	8.0	5.7	6.4	6.9	7.7
	2	7.0	6.7	7.5	7.7	8.2	5.8	7.2	7.6	8.0	5.6	6.7	7.2	8.1	5.7	6.3	6.7	7.8
	3	7.0	6.7	7.4	7.7	8.2	5.8	7.1	7.5	8.1	5.6	6.5	7.1	8.1	5.6	6.3	6.7	7.9
Mean		**7.0**	**6.7**	**7.4**	**7.7**	**8.2**	**5.9**	**7.2**	**7.6**	**8.1**	**5.6**	**6.5**	**7.3**	**8.1**	**5.7**	**6.3**	**6.8**	**7.8**
±SD		**0.0**	**0.0**	**0.1**	**0.1**	**0.0**	**0.1**	**0.1**	**0.1**	**0.0**	**0.1**	**0.2**	**0.2**	**0.0**	**0.0**	**0.1**	**0.1**	**0.1**
SM 7	1	7.0	5.6	6.5	7.0	8.1	5.5	6.2	6.7	7.9	5.5	6.2	6.7	7.8	5.5	6.1	6.7	7.6
	2	7.0	5.6	6.6	7.2	8.0	5.4	6.2	6.7	8.0	5.4	6.2	6.7	8.9	5.4	6.2	6.8	7.9
	3	7.0	5.6	6.6	7.1	8.0	5.4	6.3	6.6	7.9	5.4	6.2	6.7	7.9	5.4	6.3	6.8	7.9
Mean		**7.0**	**5.6**	**6.6**	**7.1**	**8.0**	**5.4**	**6.2**	**6.7**	**7.9**	**5.4**	**6.2**	**6.7**	**8.2**	**5.4**	**6.2**	**6.8**	**7.8**
±SD		**0.0**	**0.0**	**0.1**	**0.1**	**0.1**	**0.1**	**0.0**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.6**	**0.0**	**0.1**	**0.1**	**0.2**
Combined mean		**7.0**	**6.3**	**7.0**	**7.3**	**8.0**	**5.9**	**6.8**	**7.2**	**7.8**	**5.6**	**6.5**	**7.0**	**8.0**	**5.6**	**6.4**	**6.8**	**7.9**
combined ±SD		**0.0**	**0.5**	**0.4**	**0.3**	**0.2**	**0.4**	**0.4**	**0.0**	**0.3**	**0.1**	**0.3**	**0.3**	**0.4**	**0.1**	**0.2**	**0.1**	**0.2**

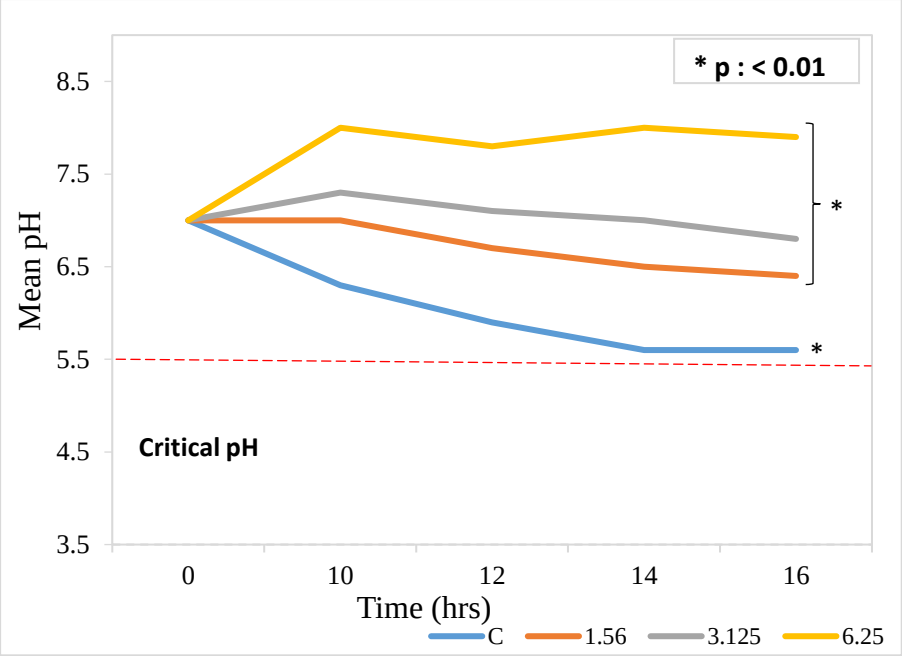

Figure 7.9 The effect of subfraction F 3.2 on acid production by *S. mutans*

The results of the *S. mutans* counts in the presence of 1.56, 3.125 and 6.25 mg/ml concentrations of subfraction F 3.2 are shown in Table 7.5 and Figure 7.10. Bacterial counts were also performed to eliminate the effect of subfraction on bacterial growth. At 10 and 14 hours, the test counts were statistically different compared to the control counts in all test concentrations with a p value of 0.01. The observed effect was concentration dependent. Moreover, the percentage reduction was 73.1 %, 99 % and 99.8 % in 1.56 mg/ml, 3.125 mg/ml and 6.25 mg/ml respectively after 10 hours (Table 8.5). After 14 hours the *S. mutans* count was reduced by 84.1 %, 85.7 % and 100 % at 1.56, 3.125 and 6.25 mg/ml respectively. This suggests that the effect of subfraction F 3.2 on the acid production might have been due to the growth inhibition because the bacterial counts were also reduced.

Table 7.5 The effect of subfraction F3.2 on the *S. mutans* counts in the acid assay

The effect of *P. granatum* F 3.2 extract on the *S. mutans* counts (cfu/ml)										
Strains	**Repeats**	**0 hrs**	**10 hrs**				**14 hrs**			
		Control	**Control**	**1.56**	**3.125**	**6.25**	**Control**	**1.56**	**3.125**	**6.25**
SM 1	**1**	1.5×10^7	3.2×10^7	1.8×10^6	5.2×10^5	4.0×10^4	3.2×10^8	3.7×10^6	3.5×10^6	2.0×10^4
	2	1.3×10^7	5.6×10^7	2.0×10^6	2.6×10^5	3.0×10^4	3.5×10^8	5.4×10^6	1.6×10^6	1.0×10^4
	3	1.3×10^7	2.1×10^7	1.3×10^6	2.6×10^5	6.0×10^4	3.9×10^8	2.8×10^6	1.9×10^6	1.0×10^4
Mean		$\mathbf{1.4 \times 10^7}$	$\mathbf{3.6 \times 10^7}$	$\mathbf{1.7 \times 10^6}$	$\mathbf{3.5 \times 10^5}$	$\mathbf{4.3 \times 10^4}$	$\mathbf{3.5 \times 10^8}$	$\mathbf{3.9 \times 10^6}$	$\mathbf{2.3 \times 10^6}$	$\mathbf{1.3 \times 10^4}$
±SD		$\mathbf{1.3 \times 10^6}$	$\mathbf{1.8 \times 10^7}$	$\mathbf{3.3 \times 10^5}$	$\mathbf{1.5 \times 10^5}$	$\mathbf{1.5 \times 10^4}$	$\mathbf{3.7 \times 10^7}$	$\mathbf{1.4 \times 10^6}$	$\mathbf{1.0 \times 10^6}$	$\mathbf{5.8 \times 10^3}$
SM 6	**1**	1.4×10^7	3.7×10^7	1.2×10^6	5.3×10^5	1.2×10^5	3.7×10^8	3.5×10^7	2.2×10^6	1.2×10^5
	2	1.3×10^7	3.5×10^7	1.1×10^6	2.6×10^5	3.2×10^5	2.9×10^8	2.9×10^6	2.0×10^6	2.6×10^5
	3	1.2×10^7	3.3×10^7	1.2×10^6	4.1×10^5	1.8×10^5	3.3×10^8	4.6×10^6	2.3×10^6	1.9×10^5
Mean		$\mathbf{1.3 \times 10^7}$	$\mathbf{3.5 \times 10^7}$	$\mathbf{1.2 \times 10^6}$	$\mathbf{4.0 \times 10^5}$	$\mathbf{2.1 \times 10^5}$	$\mathbf{3.3 \times 10^8}$	$\mathbf{1.4 \times 10^7}$	$\mathbf{2.2 \times 10^6}$	$\mathbf{1.9 \times 10^5}$
±SD		$\mathbf{1.2 \times 10^6}$	$\mathbf{2.1 \times 10^6}$	$\mathbf{7.5 \times 10^4}$	$\mathbf{1.4 \times 10^5}$	$\mathbf{1.0 \times 10^5}$	$\mathbf{3.8 \times 10^7}$	$\mathbf{1.8 \times 10^7}$	$\mathbf{1.3 \times 10^5}$	$\mathbf{7.0 \times 10^4}$
SM 7	**1**	2.4×10^7	1.9×10^8	1.8×10^7	2.3×10^6	5.0×10^5	1.4×10^8	3.3×10^7	2.3×10^7	4.0×10^4
	2	2.0×10^7	2.1×10^8	1.5×10^7	1.5×10^6	4.5×10^5	1.9×10^8	2.5×10^7	2.0×10^7	7.0×10^4
	3	2.7×10^7	2.0×10^8	2.1×10^7	1.9×10^6	3.8×10^5	1.7×10^8	2.1×10^7	2.8×10^7	9.0×10^4
Mean		$\mathbf{2.4 \times 10^7}$	$\mathbf{2.0 \times 10^8}$	$\mathbf{1.8 \times 10^7}$	$\mathbf{1.9 \times 10^6}$	$\mathbf{4.4 \times 10^5}$	$\mathbf{1.7 \times 10^8}$	$\mathbf{2.6 \times 10^7}$	$\mathbf{2.4 \times 10^7}$	$\mathbf{6.7 \times 10^4}$
±SD		$\mathbf{3.1 \times 10^6}$	$\mathbf{1.1 \times 10^7}$	$\mathbf{2.7 \times 10^6}$	$\mathbf{4.0 \times 10^5}$	$\mathbf{6.0 \times 10^4}$	$\mathbf{2.3 \times 10^7}$	$\mathbf{6.1 \times 10^6}$	$\mathbf{3.7 \times 10^6}$	$\mathbf{2.5 \times 10^4}$
Combined Mean		$\mathbf{1.7 \times 10^7}$	$\mathbf{8.9 \times 10^7}$	$\mathbf{6.9 \times 10^6}$	$\mathbf{8.8 \times 10^5}$	$\mathbf{2.3 \times 10^5}$	$\mathbf{2.8 \times 10^8}$	$\mathbf{1.5 \times 10^7}$	$\mathbf{9.4 \times 10^6}$	$\mathbf{9.0 \times 10^4}$
Combined ±SD		$\mathbf{5.4 \times 10^6}$	$\mathbf{8.1 \times 10^7}$	$\mathbf{8.3 \times 10^6}$	$\mathbf{8.0 \times 10^5}$	$\mathbf{1.8 \times 10^5}$	$\mathbf{9.3 \times 10^7}$	$\mathbf{1.4 \times 10^7}$	$\mathbf{1.1 \times 10^7}$	$\mathbf{8.7 \times 10^4}$
% reduction				**92.3**	**99.0**	**99.7**		**94.8**	**96.7**	**100**

Percentage Reduction (%) $= \left({}^{(Control - test)}/_{Control}\right)$ X 100

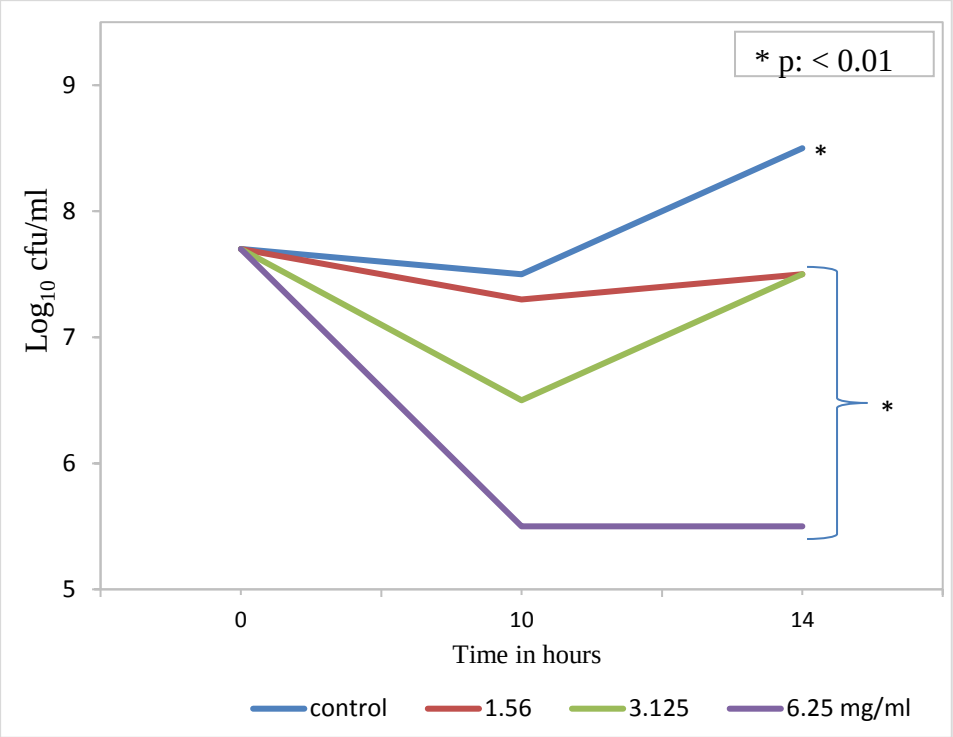

Figure 7.10 The effect of subfraction F3.2 on *S. mutans* counts in the acid assay

The results of the pH readings of the subfraction F 4.1 are shown in Table 7.6 and Figure 7.11. The plant extract significantly reduced the acid production with a p value of < 0.01 in all test concentrations compared to the control. The mean pH in the test experiment was always higher than the control at 10, 12, 14 and 16 hours. This effect was also concentration dependent. This subfraction was found to be alkaline and therefore the initial pH of the media containing test compound were higher than the control cultures. The control reached the critical pH of 5.5 at 14 hours whereas none of the test concentrations reached this pH even after 16 hours.

Table 7.6 The effect of *P. granatum* subfraction F 4.1 on acid production by *S. mutans*

Acid production by *S. mutans* in the presence of subfraction F 4.1 (pH)																
Strains	**Rep**	**10 hrs**				**12 hrs**				**14 hrs**				**16 hrs**			
		C	**3.125**	**6.25**	**12.5**	**C**	**3.125**	**6.25**	**12.5**	**C**	**3.125**	**6.25**	**12.5**	**C**	**3.125**	**6.25**	**12.5**
SM 1	1	5.8	6.4	7.2	8.2	5.7	6.3	6.9	8.2	5.6	6.3	6.8	8.2	5.7	6.4	6.9	8.4
	2	5.7	6.6	7.1	8.3	5.6	6.3	6.8	8.3	5.4	6.3	6.8	8.2	5.6	6.4	6.9	8.4
	3	5.7	6.6	7.1	8.2	5.6	6.4	6.9	8.2	5.6	6.4	6.8	8.1	5.6	6.5	6.8	8.4
Mean		**5.8**	**6.5**	**7.1**	**8.2**	**5.7**	**6.3**	**6.9**	**8.2**	**5.5**	**6.3**	**6.8**	**8.2**	**5.6**	**6.4**	**6.9**	**8.4**
±SD		**0.1**	**0.1**	**0.1**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.1**	**0.0**	**0.1**	**0.0**
SM 6	1	6.7	7.3	7.7	8.3	5.7	6.7	7.1	8.2	5.6	6.5	6.9	8.4	5.6	6.5	6.9	8.1
	2	6.8	7.3	7.6	8.4	5.6	6.5	7.1	8.3	5.5	6.4	6.8	8.4	5.5	6.5	6.8	8.1
	3	6.8	7.2	7.7	8.4	5.6	6.5	7.3	8.2	5.4	6.4	7.3	8.3	5.4	6.4	6.8	8.3
Mean		**6.8**	**7.3**	**7.7**	**8.4**	**5.7**	**6.6**	**7.2**	**8.2**	**5.5**	**6.4**	**7.0**	**8.4**	**5.5**	**6.4**	**6.8**	**8.2**
±SD		**0.0**	**0.0**	**0.1**	**0.0**	**0.1**	**0.1**	**0.1**	**0.0**	**0.1**	**0.0**	**0.3**	**0.1**	**0.1**	**0.0**	**0.1**	**0.1**
SM 7	1	6.5	6.9	7.7	8.6	5.9	6.3	7.1	8.4	6.0	6.6	6.9	8.4	6.2	6.6	6.8	8.7
	2	6.4	6.9	7.8	8.5	5.8	6.3	7.1	8.3	5.6	6.6	6.8	8.5	6.1	6.7	6.8	8.5
	3	6.4	6.8	7.7	8.6	5.7	6.3	7.0	8.3	5.8	6.6	6.8	8.4	6.0	6.7	6.7	8.5
Mean		**6.4**	**6.9**	**7.7**	**8.6**	**5.8**	**6.3**	**7.1**	**8.4**	**5.8**	**6.6**	**6.8**	**8.4**	**6.1**	**6.7**	**6.8**	**8.6**
±SD		**0.0**	**0.1**	**0.1**	**0.0**	**0.1**	**0.0**	**0.0**	**0.1**	**0.2**	**0.0**	**0.0**	**0.0**	**0.1**	**0.1**	**0.0**	**0.1**
Combined Mean		6.3	6.5	7.4	8.8	5.7	6.1	7.0	8.7	5.6	6.1	6.8	8.8	5.8	6.2	6.8	8.8
Combined ±SD		**0.0**	**0.5**	**0.3**	**0.3**	**0.1**	**0.1**	**0.1**	**0.2**	**0.1**	**0.2**	**0.1**	**0.2**	**0.1**	**0.3**	**0.1**	**0.1**

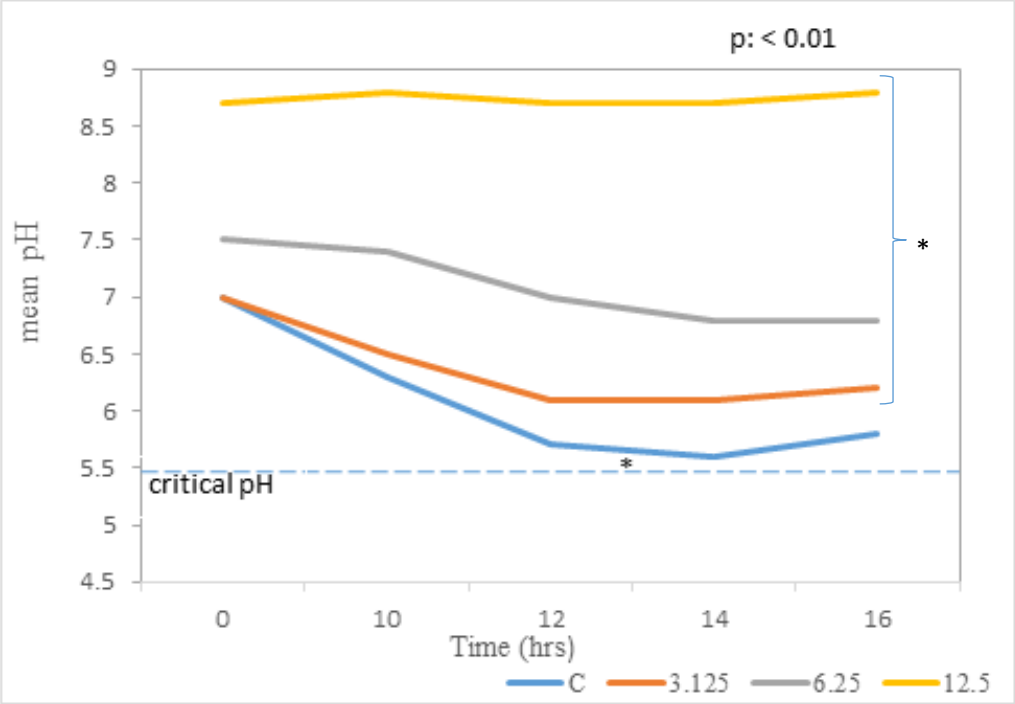

Figure 7.11 The effect of subfraction F 4.1 on acid production by *S. mutans*

The results of the effect of subfraction F 4.1 on the bacterial count in the acid assay are depicted in Table 7.7 and Figure 7.12. The mean values of the bacterial counts for the control were higher than all test concentrations at 10 hours. A non-significant effect was observed at 3.125 mg/ml after 10 hours. After 10 hours, at 3.125, 6.25 and 12.5 mg/ml the percentage reduction in the bacterial counts was 90.4 %, 93.4 and 99.9 % respectively. At 3.125 mg/ml the lowest concentration tested, the inhibition effect that was observed initially disappeared after 14 hours. The *S. mutans* counts were reduced by 93.3 % and 99.9 % at 6.25 and 12.5 mg/ml respectively. Therefore, the slight change in pH that was observed was due to the antibacterial effect of this fraction.

Table 7.7 The effect of *P. granatum* fraction F4.1 on the *S. mutans* counts in the acid assay

Effect subfraction F 4.1 (mg/ml) on the *S. mutans* counts (cfu/ml)										
Strains	**Repeats**	**0 hrs**	**10 hrs**				**14 hrs**			
		control	**control**	**3.125**	**6.25**	**12.5**	**control**	**3.125**	**6.25**	**12.5**
SM 1	1	1.7×10^7	5.5×10^8	1.0×10^7	4.8×10^7	3.3×10^5	8.7×10^7	1.5×10^7	1.6×10^7	6.3×10^4
	2	1.8×10^7	9.2×10^8	1.1×10^7	4.8×10^7	4.1×10^5	8.3×10^7	1.5×10^7	1.8×10^7	5.8×10^4
	3	1.6×10^7	8.5×10^8	9.7×10^6	5.1×10^7	5.1×10^5	8.8×10^7	2.2×10^7	1.6×10^7	6.5×10^4
Mean		**1.7×10^7**	**7.7×10^8**	**1.0×10^7**	**4.9×10^7**	**4.2×10^5**	**8.6×10^7**	**1.7×10^7**	**1.7×10^7**	**6.2×10^4**
±SD		**1.2×10^6**	**2.0×10^8**	**5.1×10^5**	**1.9×10^6**	**9.0×10^4**	**2.7×10^6**	**4.0×10^6**	**1.3×10^6**	**3.6×10^3**
SM 6	1	1.5×10^7	7.6×10^7	1.7×10^7	5.8×10^6	4.8×10^5	3.0×10^8	3.0×10^8	4.3×10^6	9.0×10^4
	2	1.3×10^7	3.1×10^7	1.2×10^7	4.2×10^6	2.7×10^5	3.2×10^8	2.6×10^8	6.4×10^6	1.1×10^5
	3	2.1×10^7	3.7×10^7	3.4×10^7	3.6×10^6	3.9×10^5	2.9×10^8	3.1×10^8	4.4×10^6	5.0×10^4
Mean		**1.6×10^7**	**4.8×10^7**	**2.1×10^7**	**4.5×10^6**	**3.8×10^5**	**3.0×10^8**	**2.9×10^8**	**5.0×10^6**	**8.3×10^4**
±SD		**3.9×10^6**	**2.4×10^7**	**1.2×10^7**	**1.1×10^6**	**1.1×10^5**	**1.9×10^7**	**2.6×10^7**	**1.2×10^6**	**3.1×10^4**
SM 7	1	6.1×10^7	2.6×10^7	4.1×10^7	2.2×10^6	4.4×10^5	3.2×10^8	6.0×10^8	2.2×10^7	1.7×10^5
	2	4.1×10^7	1.5×10^7	5.3×10^7	1.6×10^6	3.5×10^5	2.6×10^8	4.6×10^8	3.2×10^7	2.6×10^5
	3	8.9×10^7	4.8×10^7	5.7×10^7	2.8×10^6	5.7×10^5	3.2×10^8	4.3×10^8	1.9×10^7	3.3×10^5
Mean		**6.4×10^7**	**3.0×10^7**	**5.0×10^7**	**2.2×10^6**	**4.5×10^5**	**3.0×10^8**	**5.0×10^8**	**2.4×10^7**	**2.5×10^5**
±SD		**2.4×10^7**	**1.7×10^7**	**8.3×10^6**	**6.0×10^5**	**1.1×10^5**	**3.3×10^7**	**9.4×10^7**	**6.8×10^6**	**8.0×10^4**
Combined mean		3.2×10^7	2.8×10^8	2.7×10^7	1.9×10^7	4.2×10^5	2.3×10^8	2.7×10^8	1.5×10^7	1.3×10^5
Combined ±SD		2.7×10^7	3.8×10^8	1.9×10^7	2.3×10^7	9.4×10^4	1.1×10^8	2.1×10^8	9.1×10^6	1.0×10^5
% reduction				**90.4**	**93.4**	**99.9**		**-16.2**	**93.3**	**99.9**

Percentage Reduction (%) $= \left({}^{(Control - test)}/_{Control}\right) \times 100$

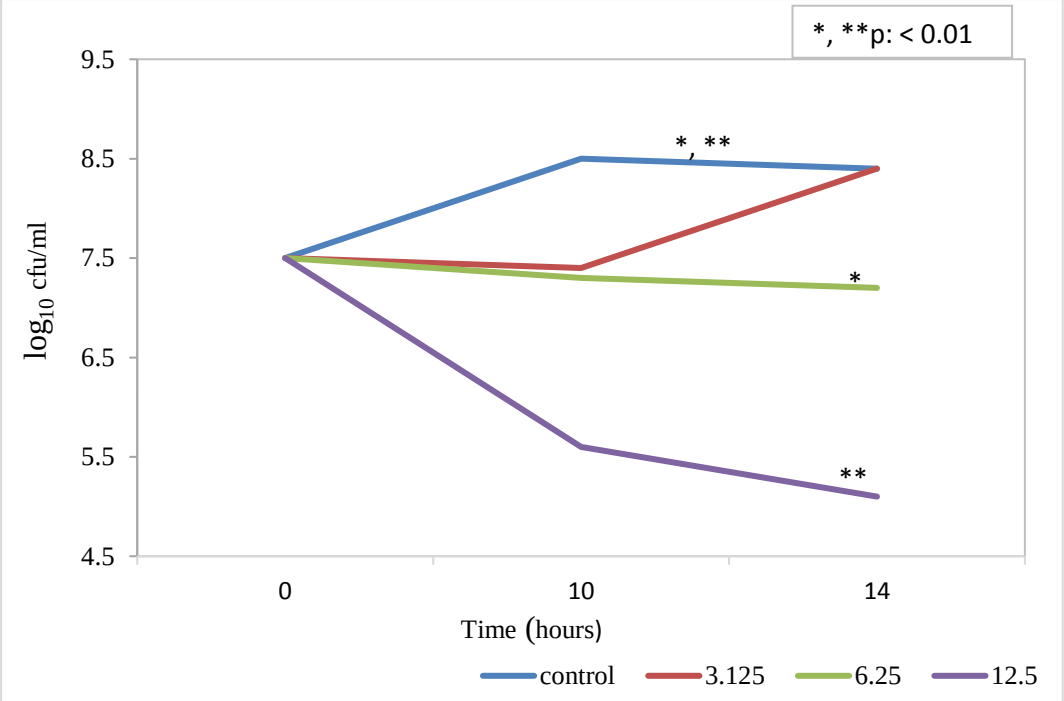

Figure 7.12 The effect of subfraction F 4.1 on *S. mutans* counts in the acid assay

7.4 Discussion

Diverse bioactive compounds containing beneficial phytochemicals are produced by plants. A widespread technique that employs multiple solvents with best polarity and the detection system is required for profiling of these bioactive compounds. These compounds are intended to be as wide-ranging as possible, therefore the initial extraction of compounds from a solid sample is important (Maria *et al.*, 2018). The composition of pomegranate crude peel extract was first investigated by studying the solvent with the most antibacterial activity. The *P. granautum* peel crude extract produced 6.25 mg/ml MBC for the DCM: Methanol which proved to be the most active solvent. Sequential and direct extraction was then used to target secondary bioactive metabolites in the *P. granatum* plant crude extract by using different proportions of aqueous solvent mixtures. There was no distinct inhibition activity with the autobiography screening observed when using sequential solvent extracts. Therefore the hexane extract gained the upperhand priority as the primary solvent used compared to ethyl

acetate and methanol. In theory, repeated extractions with hexane would have efficiently removed the non-polar compounds while methanol would remain with polar compounds. However, the non-polar hexane had the most metabolites. Contrary to this, a sequential extraction study using hexane, ethyl acetate and 50 % methanol showed more response to the polar molecules (Maria *et al.,* 2018). The high performance liquid chromatography-ultraviolet (HPLC-UV) and gas chromatography – flame ionization detector (GC-FID) analysis of these extracts had rough distribution of 10 % for hexane, 10 % for ethyl acetate, and 80 % for 50 % methanol of total signals for polar molecules. Moreover, the antimicrobial effect of these extracts was not evaluated.

In a study done previously, bioactive compounds of *P. granatum* were extracted using aqueous, methanol, ethanol, acetone, ether and chloroform solvents. However, in that study the water soluble alkaline fractions contained the highest number of phenolic acids and they were most effective against Gram positive bacteria (Prajna *et al.,* 2013). *Larrea tridentata* plant had shown confirmation of antibacterial activity by its fractions which were more effective in inhibiting the growth of gram positive bacteria when compared with gram negative bacteria especially the ethyl acetate fraction (Martins *et al.,* 2013). Nevertheless, Martins *et al*., 2013 reported no inhibiting effect for the hexane extract against the gram positive which is contradictory to the findings of this study.

Prevention or delay of biofilm formation is the major intervention in progression of dental caries. At a concentration of 6.25 mg/ml *P. granatum* crude extract significantly inhibited the biofilm formation in 6 hours by 90 %. The purified fractions F 3.2 (6.25 mg/ml) and F 4.1 (6.25 mg/ml) reduced biofilm formation by 58 % and 61 % respectively. Both purified fractions will

still not support the bacterial biofilm build up, establishment of anaerobic pathogens and acid production by *S. mutans*. Even though this effect disappears after 24 hours, the six hours protection will not allow an extended demineralization and biofilm build up in the enamel. The methanolic extract of *Opuntia ficus-indica* plant against nosocomial microorganisms showed an anti-biofilm activity and when its phytochemical analyses was evaluated it revealed the presence of flavonoids, tannins, and coumarines (Sanchez *et al.,* 2016). Possibly, these might be responsible for the anti-biofilm and anti-acid production effect observed in this study.

The production of acids by the pathogenic *S. mutans* is one of the virulence factors of this bacteria. Both F 3.2 and F 4.1 significantly reduced acid production at all test concentrations. Equally so, the bacterial count was also significantly reduced in all the test concentrations for subfraction F 3.2. This suggests then that the decrease in pH was due to the effect on antibacterial activity of subfraction F 3.2 not the inhibition of the acid production. The subfraction F 4.1 did not have any effect on the bacterial count at the subinhibitory concentrations of 3.125 mg/ml. This implies that the acid production effect was due to the inhibitory effect by subfraction F 4.1.

Development of the cutting edge analytic techniques has enabled the acquisition of a number of metabolites present in the *P. grantum* peel. Among these metabolites are the hydrolysable tannins such as ellagic acids, caffeic acid, luteolin and punicacic which play a major role in limiting the proliferation, metastasis and invasiveness of human cancer cells. Also, synergistic interactions might contribute towards the effectiveness of these compounds. Purified compounds of the purified F 3.2 or F 4.1 may contain compounds with the beneficial properties. The different cultivars of pomegranate are known to contain disparities in phytochemicals

composition (Qiu *et al.*, 2013). Exploration of phytochemicals from pomegranate peel with its diverse interactions as well as investigations of bioactivities, hold a big promise for therapeutic properties of this plant (Wu and Tian 2017).

7.5 Conclusion

This study isolated and purified the *P. granatum* bioactive compounds. Two fractions from the DCM: methanol crude extract being F 3.2 and F 4.1 performed better with MBC of 6.25 and 12.5 mg/ml respectively. At subinhibitory concentrations F 3.2 and F 4.1 inhibited the biofilm formation in *S. mutans.* Up to 58 % and 96 % reduction in the biofilm formation was shown by F 3.2 and F 4.1 respectively. In addition, these subfractions reduced the acid production in *S. mutans* excluding subfraction F 4.1 at 3.125 mg/ml. However, this effect was dependant on the antimicrobial property for the subfraction. The identity and quantity of compounds extracted from *P. granatum* DCM: methanol crude extract were strongly influenced by the solvents and the method of analysis. General conclusions on polarity of these fractions are difficult since sequential solvent extraction was used. However, the efficiencies of these fractions were confirmed but the different chromatography technique results are strongly convoluted with nonpolar fraction. In order to solve this complexity, further exploration towards identification of the beneficial compounds in the purified fractions F 3.2 and F 4.1 was carried out and is covered in chapter 9.

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CHAPTER 8

The effect of *P. granatum* crude peel extract, subfractions F 3.2 and F 4.1 on the *Porphyromonas gingivalis (Pg)* protease activity

8.1 Introduction

Periodontal disease, an infection in the subgingival area of the gums and teeth is caused by bacterial build up to form biofilm. It is divided into gingivitis and the aggressive form called periodontitis which causes destruction to the supporting structure of the tooth. Gingivitis is highly prevalent and readily reversible by simple and effective oral hygiene techniques. However, periodontal diseases extends deep and causes loss of supporting connective tissue and alveolar bone (Oliveira *et al.,* 2017). It is initiated when oral periodontal pathogens gain access to the gingival tissue and stimulate a host immune and inflammatory response. Some of these periodontal pathogens are *P. gingivalis, P. intermedia, Fusobacterium nucleatum, T. forsythia* and T*reponema denticola*.

Porphyromonas gingivalis, is considered as the major periodontal pathogen and is the keystone pathogen which elevates the virulence of the entire microbial community through co-operating communication with other pathogens (Hajishengallis and Lamont 2016) . In the whole mixed microbiota within the plaque biofilm, it persists and initiates infection (Wang *et al.,* 2018). Due to its strict anaerobic nature and fastidious nutritional requirement it exist in the subgingival crevice almost exclusively. Three distinct microenvironments exists for *Pg* in this region. The first region being the complex sessile community that resides at the root surface; crevicular fluid of the gingiva and the epithelial cells in and on the gingival line. Therefore, movement between the different regions provides diverse challenges and opportunities for the *Pg* (Hajishengallis and Lamont 2014).

Tissue destruction and crippling the immune system is caused by *P. gingivalis* through expression of a variety of virulence factors (Bostanci and Belibasakis 2012). Lipopolysaccharide fimbriae, hemagglutinin and proteases are virulence factors associated with this organism (Yamanaka *et al.,* 2007). Three cysteine proteases that reside within the fimbriae, bind and cleave a wide range of host proteins are responsible for the most potent adhesins and virulence factors of *Pg* namely gingipains (Oliveira *et al.,* 2017). Moreover, when antibodies were used to target these gingipains (Miyachi *et al.,* 2007, Yokoyama *et al.,* 2007) reduction in colonization and infectivity of *P. gingivalis* was observed in animal infection models. Gingipains function by degrading several human proteins as well as cytokines, and integrin. They are associated with all the stages of the pathogenecity in periodontal diseases starting from bacterial attachment and establishment to acquisition of nutrients as well as deactivation of host defenses (Nemoto and Ohara-Nemoto 2016). Gingipains are divided into two groups, the arginine-specific cysteine protease and the lysine-specific cysteine protease based on the respective substrate specificity (Oliveira *et al.,* 2017). The arginine-gingipains are encoded by a rgp A and B genes whereas the lysine-gingipain is encoded by the kgp gene.

Periodontal treatment should be able to reduce the bacterial load and pathogenicity through disarming the *Pg* of its virulence factors. Currently, periodontal therapy generally depends on surgical and non-surgical procedures that is aimed at reducing bacterial load from the infected sites. Conservative therapy includes clearing of inflamed tissue and scaling (Kumar *et al.,* 2009, Odds 1994). The therapy are usually supported by use of antibiotics that fail due to low concentration that reaches subgingival crevice fluids, organized biofilm that prevents effective penetration and the possible wash out effect in an anaerobic environment with low bacterial multiplication rates (Heitz-Mayfield *et al.,* 2002). Moreover, mechanical therapy including topical antimicrobial compounds are currently used. Topical antimicrobial agents includes

povidone-iodine and chlorhexidine containing mouthrinses (Slots 2002). They all have advantages and disadvantages. For example, povidone-iodine has a potential to induce hyperthyroidism and staining. Chlorhexidine causes numbness, irritation to oral mucosa, loss of taste buds and staining of teeth.

Also, a widespread use of synthetic chemicals and drugs has resulted in the emergence of uncommon infections and antimicrobial resistance. Medicinal plants appear to be suitable alternatives as they are available and accessible at affordable prices (Abdollahzadeh *et al.,* 2011). In a 4 day plaque re-growth study, pomegranate mouth-rinse prepared from a proprietary complex standardized to 30 % punicalagins reduced plaque formation in vivo (Bhadbhade *et al.,* 2011). In chapter 3 of this study, *P. grantum* crude peel extract showed antibacterial effect against periodontal pathogens *Aggregatibacter actinomyetemcomitans (Aa), Pg, Prevotella intermedia (Pi)* and *Fusobacterium* strains. The constant flow of saliva is the major challenge in the treatment of oral infections. The conservation of an effective antibacterial concentration for a longer period of time is the major challenge in the oral cavity. Therefore, targeting bacterial virulence factors at subinhibitory concentrations, a less explored approach can be a solution.

Aim: The aim of the study described in this chapter was to examine the effect of the subinhibitory concentrations of *P. granatum* crude peel extract, purified fractions F 3.2 and F 4.1 on the protease activity in *P. gingivalis*.

8.2 Materials and methods

8.2.1 *P. gingivalis* cultures

P. gingivalis strains were obtained as described in chapter 3 section 3.2.2.

8.2.2 Effect on protease activity by *P. gingivalis*

The inhibitory activity of the pomegranate peel extract against Arg-gingipain and Lys-gingipain production was evaluated using a well described technique (Yamanaka *et al.,* 2007). *P. gingivalis* was grown in 20 ml tryptic soy broth (Becton Dickinson Co., Sparks, MD, USA) supplemented with 20 µl of 5 mg/ml hemin and 20 µl of 1 mg/ml menadione anaerobically at 37^{o} C for 7 days. The cells were harvested by centrifugation. Media was decanted and the pellet rinsed three times with distilled water. The pellet was then resuspended in 50 mM phosphate-buffered saline (pH 7.4) and adjusted to an optical density of 2.0 at 660 nm. Benzoyl-arginine-*p*-nitroanilide and N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt in 0.1 M Tris-HCl (pH 8.0) containing 1 mM dithiothreitol was used as a substrate for Arg-gingipain and Lys-gingipain, respectively.

Subinhibitory concentrations were determined from the MBC values obtained in chapter 3 section 3.3.3. Arg-gingipain and Lys-gingipain substrates were dispensed into the wells of a microtitre plate separately. Various sub-inhibitory concentrations of plant extract and purified fractions were added to the wells with substrate. Bacterial cell suspension was added and incubated aerobically at 37^{o} C for 20 minutes. Controls were set up with substrate and bacterial culture only, as well as substrate and proteinase K. The effect of plant extract on DMSO and substrates was also tested. Adsorption at a wavelength of 405 nm was measured. Percentage reduction in the enzyme production was calculated as follows:

$[(A_{405}\ Control - A_{405}\ Plant - A_{405}\ DMSO) \div (A_{405}\ Control)\}\ X\ 100]$. This experiment was repeated 3 times using a strain of *P. gingivalis*.

8.3 Results

The effect of *P. granatum* crude extract together with subfractions F 3.2 and F 4.1 on the Arg-gingipain activity were analysed. The optical density is given in Table 8.1 and the percentage reduction of experiment compared to control is depicted in Figure 8.1. The concentration tested ranged between 0.34, 0.17, 0.09, 0.04 and 0.02 mg/ml. Proteinase K had an optical density of 0.05 which implies a 100 % reduction.

The results showed that *P. granatum* crude extract reduced the proteolytic activity by 57 % at 0.02 mg/ml followed by 50 % at 0.09 mg/ml. The reduction activity was not concentration dependant with the highest activity observed at 0.02 mg/ml, the lowest concentration tested (Table 8.1 and Figure 8.1).

The subfraction F 3.2 inhibited protease activity by 45 % at 0.02 mg/ml concentration followed by 41 % at 0.04 mg/ml. The reduction observed at 0.09 – 0.34 mg/ml was less than 40 %. The percentage reduction was concentration dependent.

The highest percentage reduction observed for the subfraction F 4.1 was 54 % at 0.09 mg/ml. The reduction was not concentration dependent for this subfraction.

Table 8.1 The effect of *P. granatum* crude, F 3.2 and F 4.1 extracts on ARG- protease activity of *P. gingivalis*

OD of ARG - Protease activity in the presence of crude, F 3.2 and F 4.1 concentrations (mg/ml)										
	No extract	**0.34**		**0.17**		**0.09**		**0.04**		**0.02**	
	Control	**DMSO**	**Test**	**DMSO**	**Test**	**DMSO**	**Test**	**DMSO**	**Test**	**DMSO**	**Test**
Crude extract	**1.39**	**0.795**	**1.62**	**0.72**	**1.51**	**0.62**	**1.31**	**0.63**	**1.41**	**0.66**	**1.30**
	1.38	**0.716**	**1.61**	**0.66**	**1.43**	**0.59**	**1.30**	**0.56**	**1.11**	**0.57**	**1.29**
	1.32	**0.698**	**1.46**	**0.60**	**1.43**	**0.68**	**1.32**	**0.075**	**1.26**	**0.68**	**1.10**
Mean	**1.36**	**0.74**	**1.56**	**0.66**	**1.46**	**0.63**	**1.31**	**0.42**	**1.26**	**0.64**	**1.23**
±SD	**0.97**	**0.30**	**0.89**	**2.19**	**0.89**	**2.55**	**0.91**	**4.05**	**0.89**	**5.05**	**0.86**
% reduction		**39.71**		**41.18**		**50**		**38.24**		**56.62**	
F3.2	**2.59**	**0.89**	**2.54**	**0.90**	**2.68**	**0.85**	**2.67**	**0.92**	**2.43**	**0.93**	**2.33**
	2.79	**0.87**	**2.96**	**0.89**	**2.55**	**0.92**	**2.43**	**0.87**	**2.41**	**0.91**	**2.31**
	2.69	**0.91**	**2.52**	**0.86**	**2.46**	**0.89**	**2.52**	**0.85**	**2.53**	**0.90**	**2.59**
Mean	**2.69**	**0.89**	**2.67**	**0.88**	**2.56**	**0.89**	**2.54**	**0.88**	**2.46**	**0.92**	**2.41**
±SD	**0.10**	**0.02**	**0.25**	**0.02**	**0.11**	**0.04**	**0.12**	**0.04**	**0.07**	**0.01**	**0.15**
% reduction		**33.82**		**37.54**		**38.66**		**41.26**		**44.61**	
F4.1	**1.04**	**0.45**	**0.96**	**0.44**	**0.98**	**0.42**	**0.98**	**0.42**	**1.00**	**0.50**	**0.99**
	1.03	**0.43**	**1.01**	**0.43**	**1.04**	**0.48**	**0.87**	**0.41**	**0.97**	**0.42**	**0.97**
	1.07	**0.51**	**0.96**	**0.45**	**0.99**	**0.45**	**0.93**	**0.46**	**0.95**	**0.43**	**0.96**
Mean	**1.05**	**0.46**	**0.98**	**0.44**	**1.00**	**0.45**	**0.93**	**0.43**	**0.97**	**0.45**	**0.97**
±SD	**0.02**	**0.04**	**0.03**	**0.01**	**0.03**	**0.03**	**0.06**	**0.03**	**0.03**	**0.04**	**0.01**
% reduction		**50.48**		**46.66**		**54.29**		**48.57**		**50.48**	

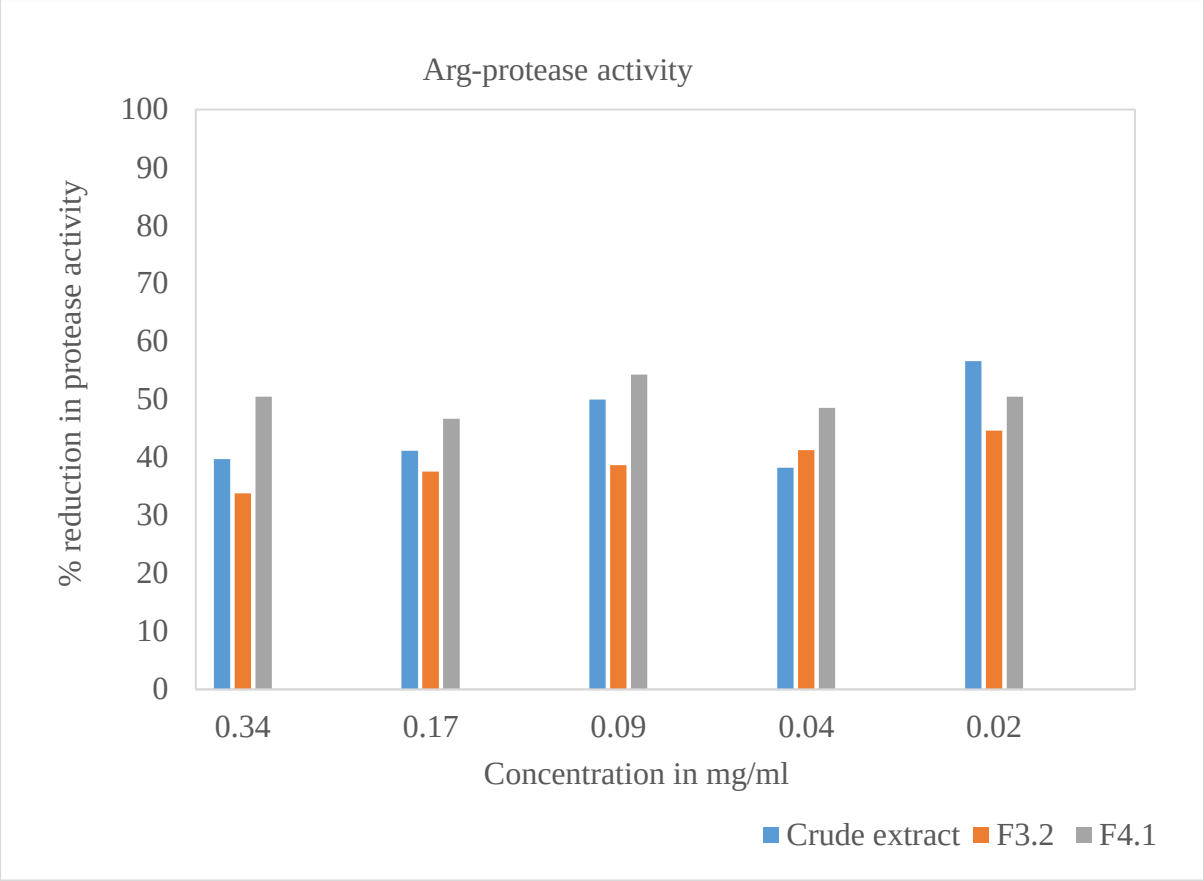

Figure 8.1 The effect of the crude extract, and subfractions F 3.2 and F 4.1 of *P. granatum* on the Arg-gingipain protease activity in *P. gingivalis*

The effect of *P. granatum* crude extract, subfractions F 3.2 and F 4.1 on the Lys-gingipain activity was analysed and the optical density is given in Table 8.2. The percentage reduction of experiment compared to control is depicted in a graph in Figure 8.2. The concentration tested ranged between 0.34, 0.17, 0.09, 0.04 and 0.02 mg/ml. The most proteolytic activity was 83 % at 0.02 mg/ml *P. granatum* crude extract. The least proteolytic activity (47 %) was observed at 0.34 mg/ml. The percentage reduction was concentration dependent with the lowest concentration exhibiting the highest proteolytic activity.

The highest inhibition of the proteolytic activity of the subfraction F 3.2 was 74 % at 0.02 mg/ml and the lowest was 53 % at 0.34 mg/ml. The effect was not concentration dependent. The subfraction F 4.1 had its highest activity at the concentration of 0.09 mg/ml with the observed reduction of 93 % and the lowest reduction was 70 % at 0.34 mg/ml. Again, the effect was not concentration dependent. The highest reduction in Lys-gingipain activity was 93 % by F 4.1 followed by 83 % by crude extract and 73 % by F 3.2 subfraction.

Table 8.2 The effect of *P. granatum* crude, F 3.2 and F 4.1 extracts on LYS- protease activity of *P. gingivalis*

OD of LYS - Protease activity in the presence of crude, F 3.2 and F 4.1 concentrations (mg/ml)										
	No extract	0.34		0.17		0.09		0.04		0.02	
	Control	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Crude extract	0.74	0.64	1.03	0.627	0.81	0.57	0.681	0.52	0.60	0.49	0.55
	0.71	0.65	1.05	0.56	0.84	0.49	0.969	0.49	0.69	0.51	0.69
	0.69	0.69	1.05	0.63	0.93	0.60	0.698	0.48	0.71	0.51	0.63
Mean	0.72	0.66	1.04	0.60	0.86	0.55	0.78	0.50	0.67	0.50	0.62
±SD	0.02	0.03	0.01	0.04	0.06	0.06	0.16	0.02	0.06	0.01	0.07
% reduction		47.22		63.89		68.05		76.39		83.33	
F3.2	0.74	0.394	0.71	0.48	0.76	0.42	0.71	0.38	0.73	0.37	0.55
	0.71	0.400	0.75	0.38	0.71	0.35	0.70	0.36	0.66	0.51	0.72
	0.69	0.400	0.76	0.46	0.72	0.46	0.75	0.35	0.70	0.49	0.69
Mean	0.72	0.40	0.74	0.44	0.73	0.41	0.72	0.36	0.69	0.46	0.65
±SD	0.02	0.00	0.03	0.06	0.03	0.06	0.03	0.01	0.03	0.08	0.09
% reduction		52.78		59.72		56.94		54.17		73.61	
F4.1	0.46	0.46	0.54	0.40	0.518	0.46	0.681	0.52	0.60	0.49	0.55
	0.42	0.36	0.55	0.44	0.521	0.45	0.969	0.49	0.69	0.51	0.69
	0.44	0.41	0.52	0.39	0.476	0.43	0.698	0.48	0.71	0.51	0.63
Mean	0.44	0.41	0.54	0.41	0.51	0.45	0.78	0.50	0.67	0.50	0.62
±SD	0.02	0.05	0.01	0.03	0.03	0.02	0.16	0.02	0.06	0.01	0.07
% reduction		70.45		77.27		93.18		84.09		88.63	

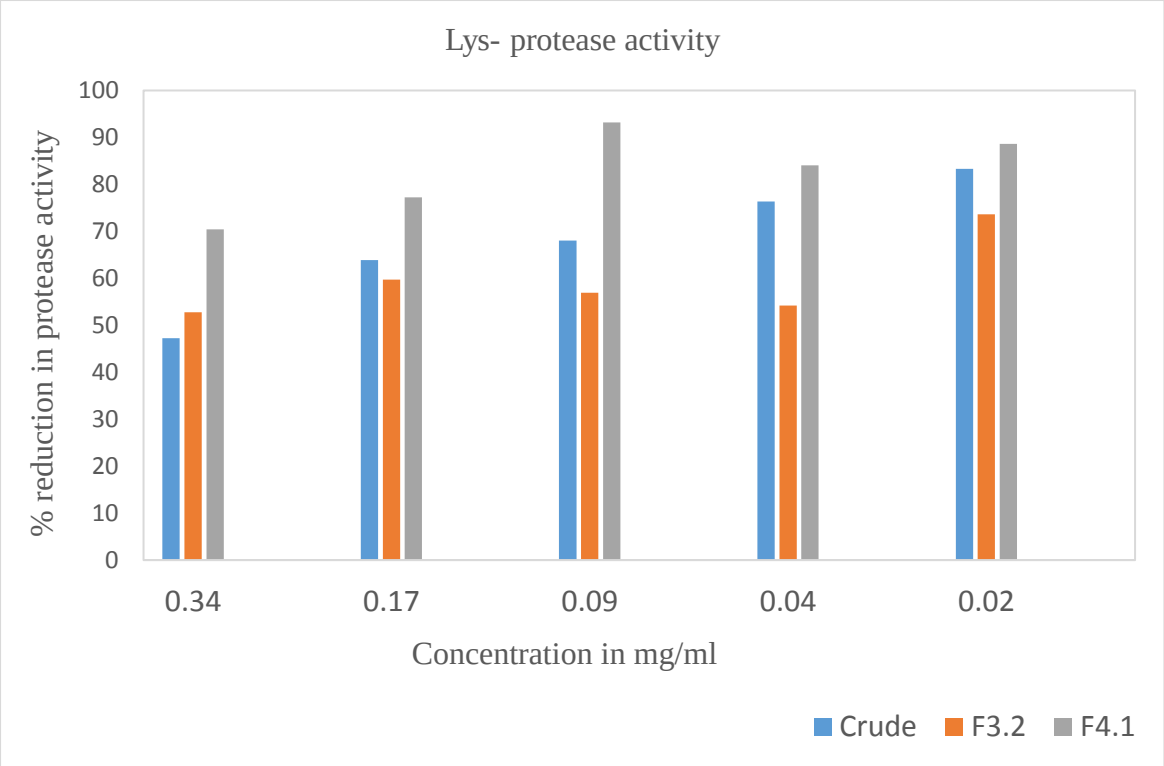

Figure 8.2 The effect of the crude extract, and subfractions F 3.2 and F 4.1 of *P. granatum* on the Lys-gingipain protease activity in *P. gingivalis*

8.4 Discussion

This study investigated the influence of crude extract, subfractions F 3.2 and F 4.1 of *P. granatum* on the major proteolytic enzymes of *P. gingivalis*, the Arg- and Lys-specific gingipain activities. The crude extract as well as the two subfractions showed higher proteolytic activity against Lys-specific cysteine protease compared to the Arg-gingipain. The highest percentage reduction observed by the Lys-gingipain for the crude extract, F 3.2 and F 4.1 was 83, 73 and 93 % respectively. While the crude extract showed the highest reduction in the proteolytic activity by the Arg-specific gingipain followed by F 4.1 and F 3.2 at 56 %, 54 % and 44 % respectively. The worst performing subfraction F 4.1 in the antimicrobial activity

chapter 7 section 7.3.2.1 was the most active in the anti-proteolytic activity of both gingipains. Nevertheless, these results showed that the crude extract, subfractions F3.2 and F 4.1 efficiently inhibited Arg-gingipain and Lys-gingipain activities in *P. gingivalis* at the lowest tested concentration of 0.02 mg/ml. Similar results have been reported by Patel *et al*, 2013 when they studied the effect of *D. viscosa* var. *angustifolia* (DVA) on the Arg-specific gingipain (Patel *et al.,* 2013). The DVA plant reduced the production of Arg-gingipain by 24 % and Lys-gingipain by 53 % at 0.02 mg/ml.

The initial key step in the development of periodontal diseases is adherence of bacteria and establishment of infections through plaque development. *Porphyromonas gingivalis* has been implicated in the damage of the soft tissue to cause infections. Its various adhesins would permit explicit adherence to the epithelial cells, erythrocytes and extracellular matrix (Lamont and Jenkinson 1998). But, the gingipains within the fimbriae are the most powerful adhesins and the pathogenic factor (Oliveira *et al.,* 2017). They are able to degrade collagen, fibronectin, immunoglobulin G and TNFα causing destruction of the host tissue (Oliveira *et al.,* 2017). Even though *P. granatum* will kill *P. gingivalis* at high concentrations, at subinhibitory concentrations it will reduce the proteolytic activity of the gingipains and reduce the most powerful virulence factor of the *Pg*.

Other plants such as *Rumex acetosa L.* and *Limonium brasiliense* have also shown effect against the gingipains from *P. gingivalis* (Oliveira *et al.,* 2017, Schmuch *et al.,* 2015). Plant extracts were prepared from rhizomes of the *L. brasiliense* with acetone: water to study its potential to inhibit the proteolytic activity of gingipains and the antiadhesive effects against *P. gingivalis* to human KB cells. In this study, *L. brasiliense* inhibited Arg-gingipain in a concentration-dependent manner. This plant reduced the proteolytic activity of the Arg-specific

Rgp gingipain by 75 % at 20 μg/ml. Whereas, Lys-gingipain activity was only reduced by 25 % (Oliveira *et al.,* 2017). Similarly, Schmuch *et al*., (2015) studied the effect of *Rumex acetosa L.* (RA1) extract on the adhesion of *P. gingivalis* to host cells (Schmuch *et al.,* 2015). The RA1 strongly interacted with the Arg-gingipain activity, while the corresponding Lys-gingipain was less affected at high concentrations. Further investigations showed that the RA1 interacts with surface proteins and changes functionality and reactivity (Schmuch *et al.,* 2015). In contrast, the present study showed a strong interference > 73 % with Lys-gingipain compared with Arg- gingipain in the crude extract as well as both subfractions.

Blocking of the bacterial proteases by natural products and the synthetic protease inhibitors diminishes bacterial adhesion and reduce proteolytic activity (Curtis *et al.,* 2002, Lohr *et al.,* 2011). These findings indicate that *P. granatum* crude extract, and the purified subfractions F3.2 and F 4.1 are a potent gingipain inhibitors partitularly Arg and Lys-specific gingipain. At 0.78 mg/ml *P. granatum* crude extract inhibits the growth of *P. gingivalis (*chapter 3 section 3.3.3 and chapter 8 section 8.3.2.1). Due to the constant flow of saliva and availability of gingival crevicular fluid (GCF) in patients suffering from periodontal diseases this concentrated plant extract can become diluted rapidly. At subinhibitory concentrations it inhibits the activity of Arg-gingipain and Lys-gingipain, which results in long lasting protection against *P. gingivalis* by disarming the surviving bacteria. Periodontal diseases are caused by different groups of bacteria, however *P. gingivalis* is a keystone pathogen in this microbiological community. It instigates this community to produce virulence factors and if targeted it will not be able to enhance its pathogenicity. Furthermore, research needs to be done to establish the mechanism of action of this plant.

8.5 Conclusions

At subinhibitory concentrations, the crude extract, subfractions F 3.2 and F 4.1 reduce Arg and Lys-gingipain proteolytic activity. The reduction in proteolytic activity was better in the Lys-gingipain compared to the Arg-gingipain. The increased Lys-gingipain reduction was 93 % with F 4.1, 83 % for crude extract and 73 % for F 3.2. Therefore, *P. granatum* will reduce the pathogenicity of the *P. gingivalis* in periodontal diseases. It has the potential to be developed into a therapeutic agent in the prevention and treatment of periodontal disease.

8.6 References

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CHAPTER 9

Identification and determination of pomegranate phytochemical present in the bioactive F 3.2 and F 4.1 purified fractions.

9.1 Introduction

Pomegranate fruit has drawn tremendous interest throughout the world due to its therapeutic properties, activity and phytochemical composition. Pomegranate is abundant and has a widespread variability of phytochemicals with different structures in plant tissues such as fruit, seed, leaves and peel as shown in Table 1. The efforts to examine the plant compounds have focussed more on the bioactive polyphenols particularly anthocyanins (ATs) and hydrolysable tannins (HTs). The peel of the pomegranate fruit is abundant in hydrolysable tannins, mainly ellagitannins (Wu and Tian 2017). About 85 % of the total tannins extracted from peel are the punicalagin isomers (Seeram *et al.,* 2005). Knowing the phytochemicals present in pomegranate assists in the determination of the structure of bioactive compounds as well as whether interactions in the complex mixtures are additive, antagonistic or synergistic (Wu and Tian 2017).

Punicalagins contain the richest source of antioxidants and the main ingredient present in pomegranate peel as evaluated by ferric reducing antioxidant power assay (Thangavelu *et al.,* 2017). The punicalagin and gallic acid of pomegranate have been found to show antibacterial activity against oral pathogens. These specific components act on the bacterial cell membrane and increase the bacterial cell wall breakage, prevent adhesion of bacteria to the tooth surface, precipitate the protein, and inhibit several bacterial enzymes (Abdollahzadeh *et al.,* 2011). Freshly prepared pomegranate juice inhibits plaque-forming bacteria by 84 % in comparison to golden standard 79 % chlorhexidine mouth rinse (Kote *et al.,* 2011).

In other studies, preparative high performance liquid chromatography has been employed to obtain pure punicalin from the pomegranate husk (Lu *et al.,* 2007, Wang *et al.,* 2013, Zhou *et al.,* 2011). The only disadvantages about these methods are their labour-intensive nature, and the low yields and purity of obtained compounds. Also, the operating process is time consuming and requires expensive equipment (Aguilar-Zarate *et al.,* 2017). Therefore, this limits characterisation of all the compounds in this plant. Quantification and identification of pomegranate phenol compounds from peel tissues could not be possible without the use of high-performance liquid chromatography diode array detector coupled with electrospray ionisation tandem mass spectrometry (Ambigaipalan *et al.,* 2016, Fischer *et al.,* 2011). Furthermore, is the elucidation of precise chemical structures by means of nuclear magnetic resonance (Nawwar *et al.,* 1994). Laboratories have designed their own strategies for the concentration and purification of bioactive compounds from plants. The polyphenols which are extracted from the pomegranate have many good properties such as antibacterial, antioxidant, anti-inflammatory, anti-proliferative, and DNA repair activities and thus can be used as a good alternative for systemic drugs. Identification of bioactive compounds from traditional plants is important in combating oral diseases. The purified compounds might be stronger and safer to use compared to the original crude plant extract.

The aim of this study was to characterise the constituents of the bioactive F 3.2 and F 4.1 subfractions.

Table 9.1 Different classes of phytochemicals identified from pomegranate. Adopted from (Wu and Tian 2017)

Classes	Phytochemicals
Ellagitannins, gallotannins and derivatives	Brevifolin, Brevifolin carboxylic acid, Brevifolin carboxylic acid 10-monopotassium sulphate, Castalagin, Casuariin, Casuarinin, Corilagin, Isocorilagin, Hippomanin A, Gemin D, Diellagic acid rhamnosyl(1→4) glucopyranoside, 1,2-Di-O-galloyl-4,6-O-(S)-hexahydroxydiphenoyl _β-D-glucopyranoside, Ellagic acid, 3,3'-Di-O-methylellagic acid, 3,3',4'-Tri-O-methylellagic acid, 3-O-Methylellagic acid, 4,4'-Di-O-methylellagic acid, 3'-O-Methyl-3,4-methylenedioxy-ellagic acid, Eschweilenol C (Ellagic acid 4-O-α-L-rhamnopyranoside), Ethyl brevifolincarboxylate, Eucalbanin B, Eucarpanin T1, Pomegraniin A, Pomegraniin B, Gallagic acid, Gallic acid 3-O-β-D-(6'-O-galloyl)-glucopyranoside, 6-O-Galloyl-2,3-(S)-hexahydroxydiphenoyl-D-glucose, 5-Galloylpunicacortein D, 2-O-Galloylpunicalin (2-O-Galloyl-4,6-(S,S)-gallagyl-D-glucose), Granatin A, Granatin B, 2,3-(S)-Hexahydroxydiphenoyl-D-glucose, Lagerstannin B, Lagerstannin C, 3-O-Methylellagic acid 4-O-_-L-rhamnopyranoside, 3,40-O-Dimethylellagic acid 4-O-α-L-rhamnopyranoside, Oenothein B, Pedunculagin I, Pedunculagin II, 1,2,3,4,6-Penta-O-galloyl-β-D-glucose, 3,4,8,9,10-Pentahydroxydibenzo [b,d] pyran-6-one (Urolithin M-5), Phyllanthusiin E, Pomegranatate, Punicacortein A, Punicacortein B, Punicacortein C, Punicacortein D, Punicafolin, Punicalagin A, Punicalagin B, Punicalin, Punicatannin A, Punicatannin B, Punigluconin, Strictinin [1-O-Galloyl-4,6-(S)-hexahydroxydiphenoyl-D-glucose], Tellimagrandin I, Tercatain [1,4-Di-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-_-glucopyranose], Terminalin (Gallagyl dilactone), 1,2,4,6-Tetra-O-galloyl-_-D-glucose, 1,2,3-Tri-O-galloyl-_-glucopyranose, 1,2,4-Tri-O-galloyl-_-glucopyranose, 1,2,6-Tri-O-galloyl-_-glucopyranose, 1,3,4-Tri-O-galloyl-β-glucopyranose, 1,4,6-Tri-O-galloyl-β-glucopyranose, 3,4,6-Tri-O-galloyl-β-glucopyranose, Valoneic acid dilactone
Flavonoids	Hovetrichoside C, Phloretin, Phlorizin, Eriodictyol-7-O-α-L-arabinofuranosyl (1-6)-β-D-glucoside, Granatumflavanyl xyloside, Naringin (Naringenin-7-O-rhamnoglucoside), Naringenin-4'methyl ether 7-O-α-L-arabinofuranosyl(1-6)-_-D-glucoside, Pinocembrin, Punicaflavanol, Apigenin, Apigenin 4'-O-β-glucopyranoside, Luteolin, Luteolin 3'-O-β-glucopyranoside, Luteolin 4'-O-β-glucopyranoside, Cynaroside (Luteolin 7-O-glycoside), Luteolin 3'-O-β_-xylopyranoside, Tricetin, Daidzein, Genistein, Amurensin (Noricaritin 7-_-D-glucopyranoside), Kaempferol, Astragalin (Kaempferol 3-O-glucoside), Kaempferol-3-O-rhamnoglucoside, Myricetin, Phellatin, Quercetin, Hirsutrin (Quercetin-3-O-glucoside), Quercimeritrin (Quercetin-7-O-glucoside), Quercetin 3-O-rhamnoside, Rutin (Quercetin-3-O-rutinoside), Quercetin-3,40-dimethyl ether 7-O-_-L-arabinofuranosyl(1-6)-_-D-glucoside, Cyanidin, Chrysanthemin (Cyanidin-3-O-glucoside), Cyanin (Cyanidin-3,5-di-O-glucoside), Antirrhinin (Cyanidin-3-O-rutinoside), Catechin-cyanidin-3-hexoside, Delphinidin, Myrtillin (Delphinidin-3-O-glucoside), Delphinidin-3,5-di-O-glucoside, Pelargonidin, Callistephin (Pelargonidin-3-O-glucoside), Pelargonin (Pelargonidin-3,5-di-O-glucoside), Catechin, Epicatechin, Epicatechin gallate, Epigallocatechin-3-O-gallate, Gallocatechin-(4→8)-catechin, Gallocatechin-(4→8)-gallocatechin, Catechin-(4→8)-gallocatechin, Procyanidin A2, Procyanidin B1, Procyanidin B2, Procyanidin B3
Lignans	Conidendrin, Isohydroxymatairesinol, Isolariciresinol, Matairesinol, Medioresinol, Phylligenin, Pinoresinol, Secoisolariciresinol, Syringaresinol, Pomegralignan, Punicatannin C
Triterpenoids and phytosterols	Asiatic acid, Betulinic acid (Betulic acid), Friedooleanan-3-one (Friedelin), Maslinic acid, Oleanolic acid, Punicanolic acid, Ursolic acid, Campesterol, Cholesterol, Daucosterol, _-Sitosterol, _-Sitosterol laurate, _-Sitosterol myristate, Stigmasterol
Fatty acids and lipids	Caproic acid (Hexanoic acid), Caprylic acid (Octanoic acid), Capric acid (Decanoic acid), Lauric acid (Dodecanoic acid), Myristic acid (Tetradecanoic acid), Myristoleic acid (9-cis-Tetradecanoic acid), Palmitic acid (Hexadecanoic acid), Palmitoleic acid (Hexadec-9-enoic acid), Punicic acid (9Z, 11E, 13Z-octadecatrienoic acid), Linoleic acid (cis, cis-9,12-Octadecadienoic acid), _-Linolenic acid (All-cis-9,12,15-octadecatrienoic acid), -Linolenic acid (All-cis-6,9,12-octadecatrienoic acid), Oleic acid (9Z-octadecenoic acid), Stearic acid (Octadecanoic acid), _-Eleostearic acid (9Z, 11E, 13E-octadecatrienoic acid), _-Eleostearic acid (9E, 11E, 13E-octadecatrienoic acid), Catalpic acid (9E, 11E, 13Z-octadecatrienoic acid), Arachidic acid (Eicosanoic acid), Gadoleic acid (9Z-icosenoic acid), Behenic acid (Docosanoic acid), Nervonic acid (cis-15-Tetracosenoic acid), 1-O-9E,11Z,13E-Octadecatrienoyl glycerol, 1-O-Isopentyl-3-O-octadec-2-enoyl glycerol, Tri-O-punicylglycerol, Di-O-punicyl-O-octadeca-8Z, 11Z, 13E-trienylglycerol, N-palmitoyl cerebroside
Organic acids and phenolic acids	Ascorbic acid, Citric acid, Fumaric acid, L-Malic acid, Oxalic acid, Quinic acid, Succinic acid, Tartaric acid, Caffeic acid, Chlorogenic acid, Cinnamic acid, o-Coumaric acid, p-Coumaric acid, cis-p-Coumaric acid, Coutaric acid, 7,8-Dihydroxy-3-carboxymethylcoumarin-5-carboxylic acid, Ferulic acid, Gallic acid, Methyl gallate, Neochlorogenic acid (5-O-Caffeoylquinic acid), Protocatechuic acid, Vanillic acid, Coniferyl 9-O-[*b*-D-apiofuranosyl(1!6)]-O-*b*-D-glucopyranoside, Sinapyl 9-O-[*b*-D-apiofuranosyl(1!6)]-O-*b*-D-glucopyranoside
Other compounds	Catechol, Coumestrol, Icariside D1, Phenylethylrutinoside, Syringaldehyde

9.2 Materials and Methods

9.2.1 Plant material and extract preparation

Punica granatum peel crude extract was obtained as described in **chapter 7 section 7.2.1.1.**

9.2.2 Solvent extraction

The hexane, ethyl acetate and methanol liquid-liquid separation performed as described in **chapter 7 section 7.2.1.2.**

9.2.3 Cellulose column chromatography separation 1 and 2

To obtain sub-fractions F 3.2 and F 4.1, purification was performed as described in chapter 7 section section **7.2.1.6 and 7.2.1.7.**

9.2.4 Flash chromatography analysis

The bioactive subfractions F 3.2 and F 4.1 were analyzed on an Agela Flash chromatography machine using AQ C18 column with 20-35 µm, 100À from Agela technologies. This machine is housed in the Oral microbiology laboratory. The F 3.2 analysis was carried out under the following conditions. The flow rate of 13 ml/min, detection at 200 nm, monitoring from 220 nm, solvent A 0.002 % ammonium water solution and solvent B as methanol. The elution steps were as follows:

Table 9.2 The flash chromatography machine steps for the analysis of active sub-fraction F 3.2

Stages	Start : End	Duration in minutes
	MeOH : 0.002 % NH_4	
Equilibrium	100.0 → 65.0	3.0
1	65.0 → 65.0	5.0
2	60.0 → 60.0	4.0
3	55.0 → 55.0	15.0
4	50.0 → 50.0	10.0
5	40.0 → 20.0	5.0
6	10.0 → 0.0	2.0
7	0.0 → 100.0	10.0
8	100.0 → 50.0	5.0
9	50.0 → 50.0	40.0

The F 4.1 analysis was carried out under the following conditions. The flow rate of 10 ml/min, detection at 200 nm, monitoring from 220 nm, solvent A 0.002 % ammonium water solution and solvent B as methanol. The elution steps were as follows:

Table 9.3 The flash chromatography machine steps for the analysis of active sub-fraction F 4.1

Stages	Start : End MeOH : 0.002 % NH_4	Duration in minutes
Equilibrate	100.0 → 65.0	2.0
1	65.0 → 65.0	5.0
2	60.0 → 60.0	4.0
3	55.0 → 55.0	15.0
4	50.0 → 50.0	10.0
5	40.0 → 20.0	5.0
6	10.0 → 0.0	2.0
7	0.0 → 100.0	10.0

9.2.5 Preparative high performance liquid chromatography

Dried extracts of F 3.2 and F 4.1 were processed for fractionation using HPLC analysis with Agilent 1200 series HPLC system (Waldbronn, Germany), that uses a quaternary solvent for delivery together with a diode array detection. Chromatographic separation was performed on an XBridge Shield RP C18 (5 µm, 4.6 mm × 250 mm) column with binary mobile phase system consisting of solvent A acetonitrile and solvent B water with a gradient that is linear (5 % - 95 % A) to (95 % : 5 % B) in 30 minutes with the 1.2 ml/min flow rate. The temperature of the column was kept at 35°C and the wavelength of the DAD was set at an acquisition range 215–300 nm. The injection volume was 600 µl.

9.2.6 UHPLC-HRMS Analysis

Mass spectrometry was performed using a Bruker Compact Q-TOF (time of flight) high resolution mass spectrometer in the Chemistry department, University of Witwatersrand according to Abdulla *et al.,* (2017). A 10µL of the sample was injected into the ultra-high-pressure liquid chromatography (UHPLC) and run through a loop at 50 % Solvent A consisting of 0.1 % formic acid in H_2O (v/v) and 50 % solvent B consisting of 0.1 % formic acid in Acetonitrile (v/v). The samples were diluted to a concentration of approximately 10 ppm. The mass spectrum analysis was processed using the Bruker Daltonics data analysis and sample identified with UHPLC- high resolution mass spectrometry (HRMS) Analysis. The TOF mass ranged from m/z 100 to 1500, and m/z 50 to 1500 was the mass of product ion scan range. The collision energy (CE) was set from 30 to 70 eV to generate more structural information. Calibration of the mass analyser was done using formic acid (2 ng/µl) with a syringe pump at a flow rate of 10 µl/min. Data acquisition and processing were performed by Analyst QS 2.0 software.

9.2.7 Nuclear Magnetic Resonance Spectroscopy

Further identification of the isolated compounds was carried out by NMR. NMR spectra were acquired on a Bruker 500 MHz 400DPX spectrometer with an Advance console and a dual proton–carbon, variable-temperature, inverse-detection probe. The ^{1}H, ^{13}C, HSQC or HMBC, COSY, DEPT 135 and NOESY spectra were obtained at 500 MHz. The structural elucidation was performed on APG 2 (414 m/z) and APG 6 (634 m/z). About 8.2 mg of the sample was dissolved in deuterated solvents of chloroform-d_3. The chemical shifts were reported in relation to the resonance peak of TMS (tetramethylsilane) chemical shift.

9.2.8 Fourier-transform infrared spectroscopy (FTIR)

The IR analysis of compounds APG 2 and APG 6 was done. The analysis used a Perkin Elmer FT-IR 100 spectrometer. The samples were dissolved in DMSO and one drop was placed on the surface plate for analysis. The samples were ran in the infrared region between 650 nm and 4000 nm.

9.3 Results

9.3 Subfractions F 3.2 and F 4.1 separation

The DCM:MeOH plant crude extract was dissolved in water:methanol mixture and subjected to hexane then ethyl acetate partitioning separately. Preliminary bioautography of these extracts were performed and the lack of definitive movement as well as inhibition was observed. Therefore, these results awarded the primary partitioning solvent hexane priority for further purification, screening and structural elucidation.

9.3.1 Flash chromatography analysis

9.3.1.1 Subfraction F 3.2

The cellulose column purified sub-fractions F 3.2 was ran on an Agela flash chromatography machine for further improvement and quantification. The results are shown in Figure 9.1(a). The fraction was collected under the dual collection and monitoring wavelength from 220. Distinct peaks were observed at 5 minutes in Peak 1 (P1), 6 minutes Peak 2 (P2) and 55 minutes Peak 3 (P3) minutes. Peak 1 was further purified and the results are shown Figure 9.1(b).

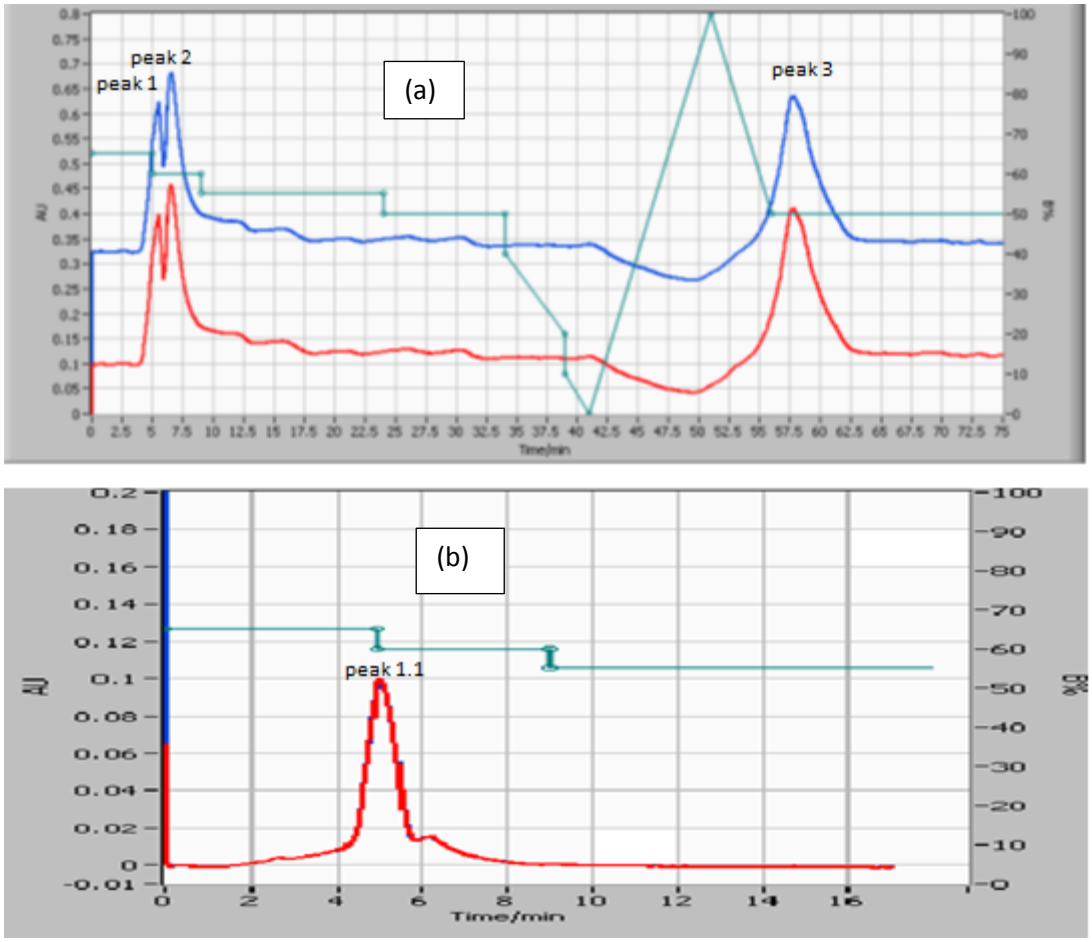

Figure 9.1 (a) Flash chromatograph purification of subfraction F 3.2 affording 3 peaks 1, 2 and 3 (b) Further resolution of peak 1 from (a).

9.3.1.2 Subfraction F 4.1

The flash chromatography results for the subfraction F 4.1 are shown in Figure 9.2.(a). The fraction was collected under the dual wavelengths of collection and monitoring from 220 nm. Distinct peaks were observed at 5 minutes in Peak 1 (P1), and a broad peak at between 8 to 18 minutes Peak 2 (P2) minutes. Peak 2 was further rerun on the flash chromatography and the results are shown in Figure 9.2.(b) (peak 2.1, 2.2 and 2.3). These spectra show that the broad peak 2 constituted of 3 different compounds. Further purification was not possible due to the machine breaking down and insufficient quantities for the further characterisation.

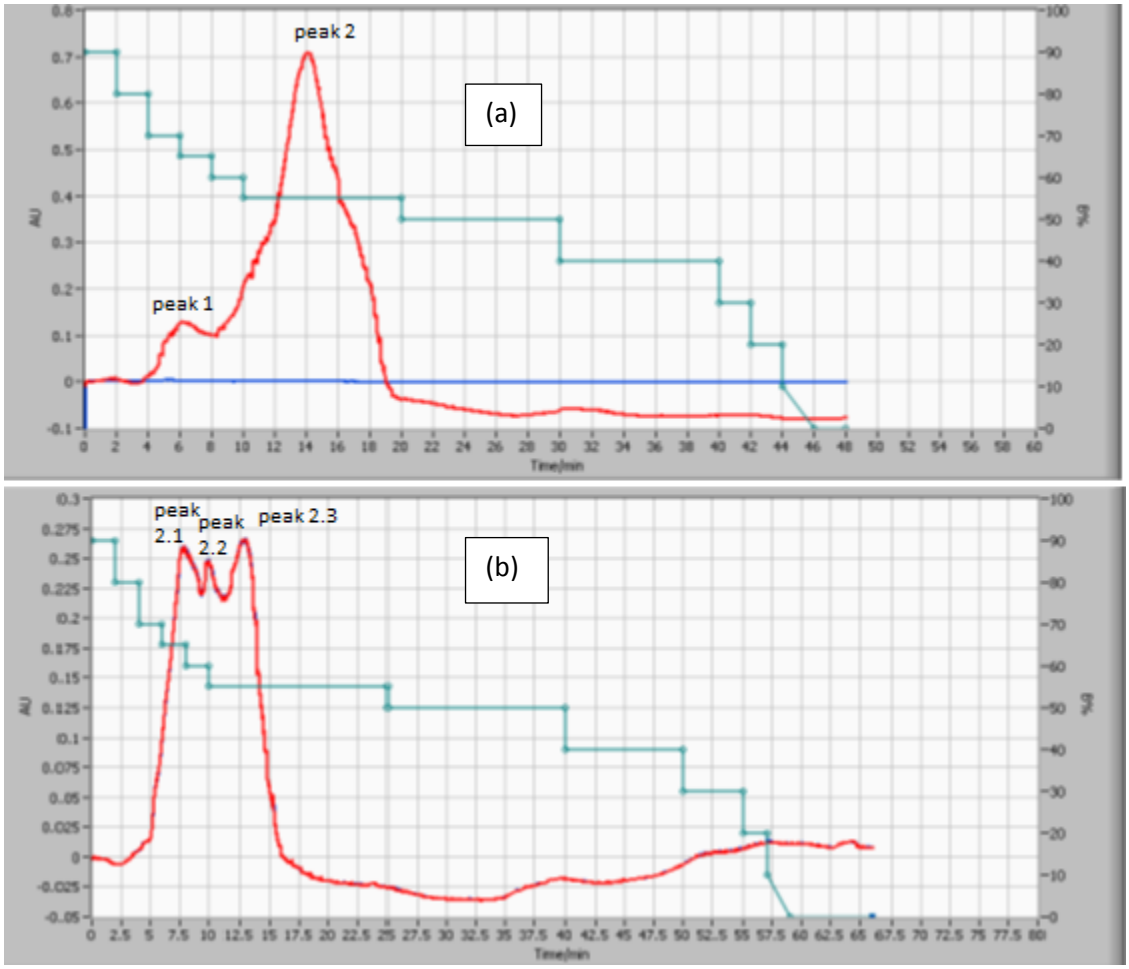

Figure 9.2 (a) Flash chromatograph purification of subfraction F 4.1 affording 3 peaks 1, 2 and 3 (b) Further resolution of peak 2 from (a).

9.3.2 Preparative HPLC

9.3.2.1 Subfraction F 3.2 prep HPLC

The HPLC analysis for F 3.2 gave nine peaks and the results shown in Figure 9.3 were recorded at 215 nm with different retention times. The polar compounds eluted first while the non-polar compounds were retained and eluted later. The retention time for subfraction F 3.2 compounds were A1 (3.33), A2 (3.59), A3 (8.08), and A4 (9.61). The first strong peak was collected in vial 1 starting at 3.17 minutes and finishing at 3.8344 minutes.

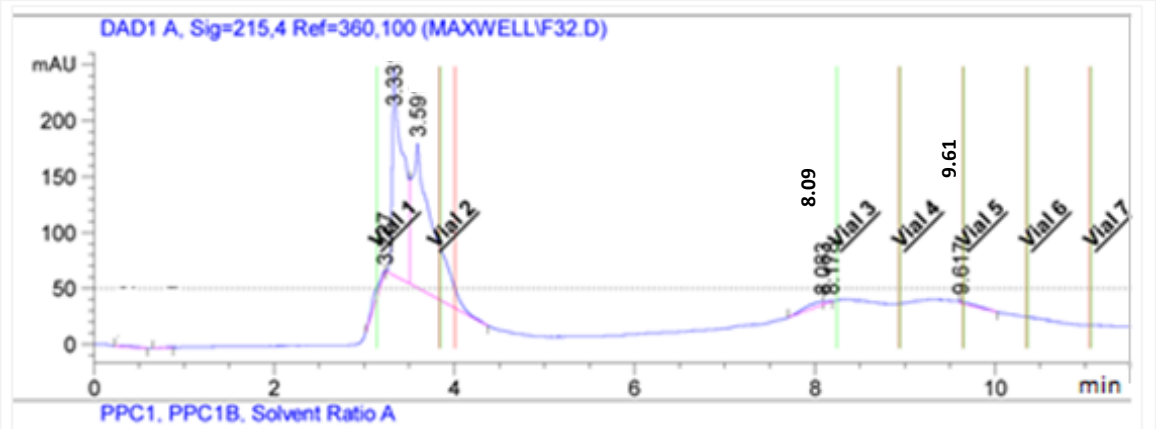

Figure 9.3 Preparative HPLC separation of subfraction F 3.2 at 215 nm

9.3.2.2 Subfraction F 4.1 prep HPLC

The spectrum obtained for preparative HPLC analysis for F 4.1 showed seven peaks and the results are shown in Figure 9.4. The identified compounds showed a typical visible absorption maximum between 215 to 360 nm. The elution order of the detected compounds was in agreement with the polarity. The retention time for compound **1** (1.941 minutes), **2** (2.255 minutes), **3** (4.149 minutes), **4** (3.815 minutes), **5** (5.119 -5.885 minutes), **6** (20.131 minutes) and **7** (21.886 minutes). These compounds were subjected to UHPLC-HRMS for further purification and quantification.

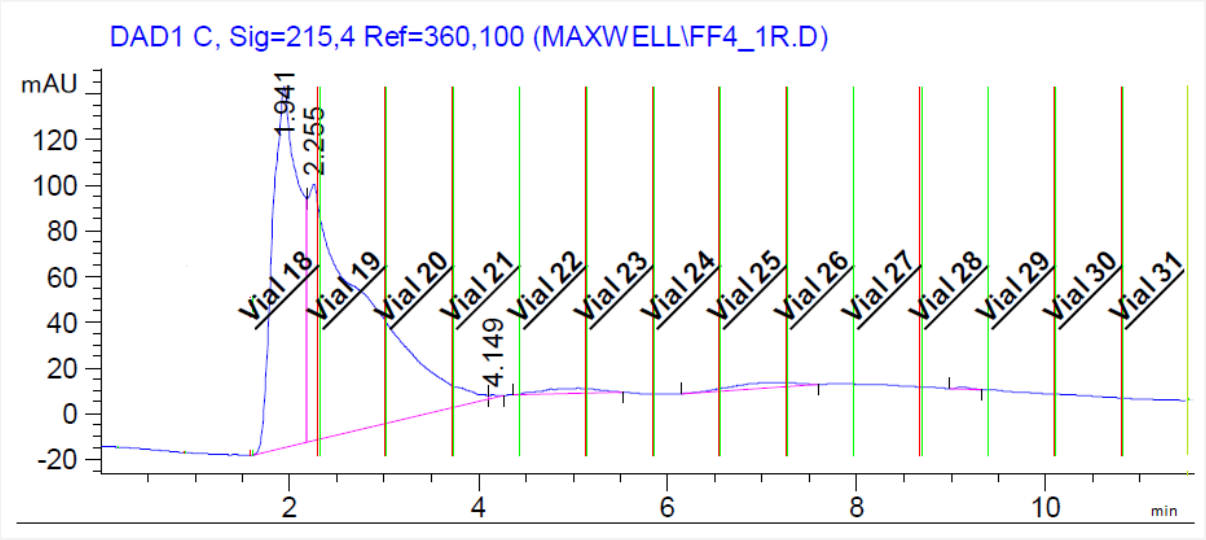

Figure 9.4 Preparative HPLC separation of subfraction F 4.1 at 360 nm

9.3.3 UHPLC-HRMS

9.3.3.1 Subfraction F 3.2

The HPLC subfraction peaks of F 3.2 were ran on the UHPLC-HRMS to reveal the typical mass spectrometric behaviour of electrospray ionisation (ESI). The UHPLC-HRMS spectra results are shown in Figure 9.5 (a) to (c) and the rest are in the appendices. The subfraction constituents observed are identified as different peaks as shown in Table 9.4. The main peak in this spectrum was detected at 430 m/z in Figure 9.5 (a) and (b). The compound at 431 m/z was identified according to literature as Ellagic acid-pentoside (433 m/z) with a molecular ion that shows deprotonation $[M - H]^{-2}$ previously isolated from the pomegranate outer skin (Ambigaipalan *et al.,* 2016). Moreover, the MS^2 fragmentation of a galloyl-HHDP gluconic acid releasing HHDP gluconic acid prompted the 497 m/z. The peak at 498 m/z $[M –H]^-$ was then attributed to galloyl-HHDP-gluconic acid (Ambigaipalan *et al.,* 2016). The compound at 566 m/z was identified as procyanidin dimer B1 (562 m/z) with protonation in molecular ion $[M + H]^{+4}$ (Ambigaipalan *et al.,* 2016). The compound at 634 was identified as Galloyl-HHDP-glucoside with protonation in molecular ion $[M + H]^+$ and the compound at 702 m/z was assigned as Di (HHDP) galloyl glucose pentose with molecular ion of $[M + H]^{+5}$ (Aguilar-Zarate *et al.,* 2017). While compounds at 770 with molecular ion $[M + H]^{+5}$ and 838 m/z $[M+H_2O+H]^{+3}$ were assigned as gallagyl-hexoside (punicalin) and procyanidin trimer A respectively according to literature (Abdulla *et al.,* 2017, Ambigaipalan *et al.,* 2016). Compound 18 isolated by Abdulla *et al*., 2017 generated ions at m/z 951 (Abdulla *et al.,* 2017). This compound produced fragment ions at m/z 907 indicating the natural loss of CO_2 and was identified as HHDP valoneoyl-glucoside. Therefore, the peak 907 was assigned HHDP valoneoyl-glucoside with a $[M - H]^-$ (Abdulla *et al.,* 2017).

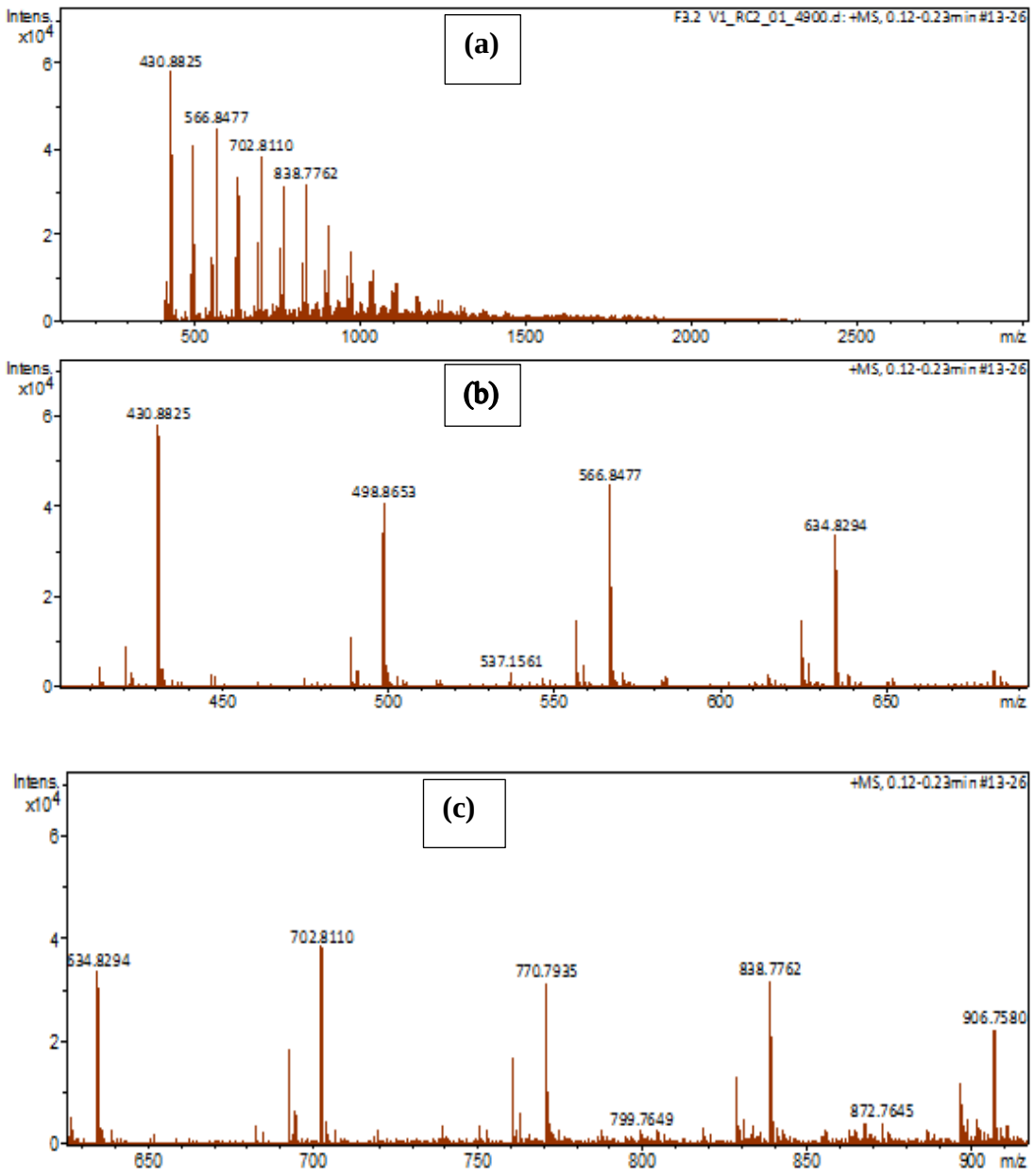

Figure 9. 5 (a) The UHPLC-HRMS spectra of subfraction F 3.2, (b), (c) enlarged range of (a)

Table 9.4 Identification of compounds in subfraction F 3.2 by UHPLC-HRMS

No.	m/z (study)	Intensity	Lit. m/z prediction	Identification	References
1.	431 $[M - H]^{-2}$	6.0×10^4	433	Ellagic acid-pentoside	(Ambigaipalan *et al.*, 2016)
2.	498 $[M + H]^{+}$	4.5×10^4	497	Galloyl-HHDP-gluconic (lagerstannin C)	(Ambigaipalan *et al.*, 2016) $C_{27}H_{22}O_{19}$
3.	566 $[M + H]^{+4}$	4.8×10^4	562	procyanidin dimer B1	(Ambigaipalan *et al.*, 2016) $C_{20}H_{17}O_{14}$
4.	635 $[M + H]^{+2}$	3.8×10^4	633	Galloyl-HHDP-hexoside	(Aguilar-Zarate *et al.*, 2017)
5.	703 $[M - H]^{-4}$	4.0×10^4	707	Di(HNDP)Galloyl Glucose Pentose	(Aguilar-Zarate *et al.*, 2017)
6.	771 $[M + H]^{+6}$	3.8×10^4	765	bis-HHDP glucoside (pedunculagin I)	(Abdulla *et al.*, 2017) $C_{34}H_{21}O_{22}$
7.	907 [M]	3.5 x 10	907	HHDP-valoneoyl glucoside	(Abdulla *et al.*, 2017)

Lit. = literature

9.3.3.2 Subfraction F 4.1

The UHPLC-HRMS spectra results for subfraction F 4.1 are shown in Figure 9.6 and the rest are shown in the Appendices 4.2. The proposed constituents are shown as major or product ion peaks in Table 9.5. The major peak for subfraction F 4.1 was observed at 663 m/z. An unknown compound with m/z 663 has been reported in the extractable ellagitannins fraction, and had fragments that are characteristic of the ellagitannins class (Perez-Ramirez *et al.,* 2018). Another compound that has been reported in literature at 649 m/z is a galloyl-HHDP-gluconic acid and lagerstannin C. The loss of a CO by the compound at 663 m/z [M –H-CO] identifies this compound as galloyl-HHDP-gluconic acid and lagerstannin C (Ambigaipalan *et al.,* 2016). The first peak at 413 m/z depicts presence of β-Sitosterol (414) with a molecular ion $[M-H]^{-1}$ according literature (Mohammad *et al.,* 2016). A peak was observed at 1000 m/z. According to Fischer *et al*., 2011, a peak produced at 967 belonged to derivative of lagerstannin B. and these can be hydroxylated at several positions in the structure. The rest of the peaks detected were identical to those found in subfraction F 3.2.

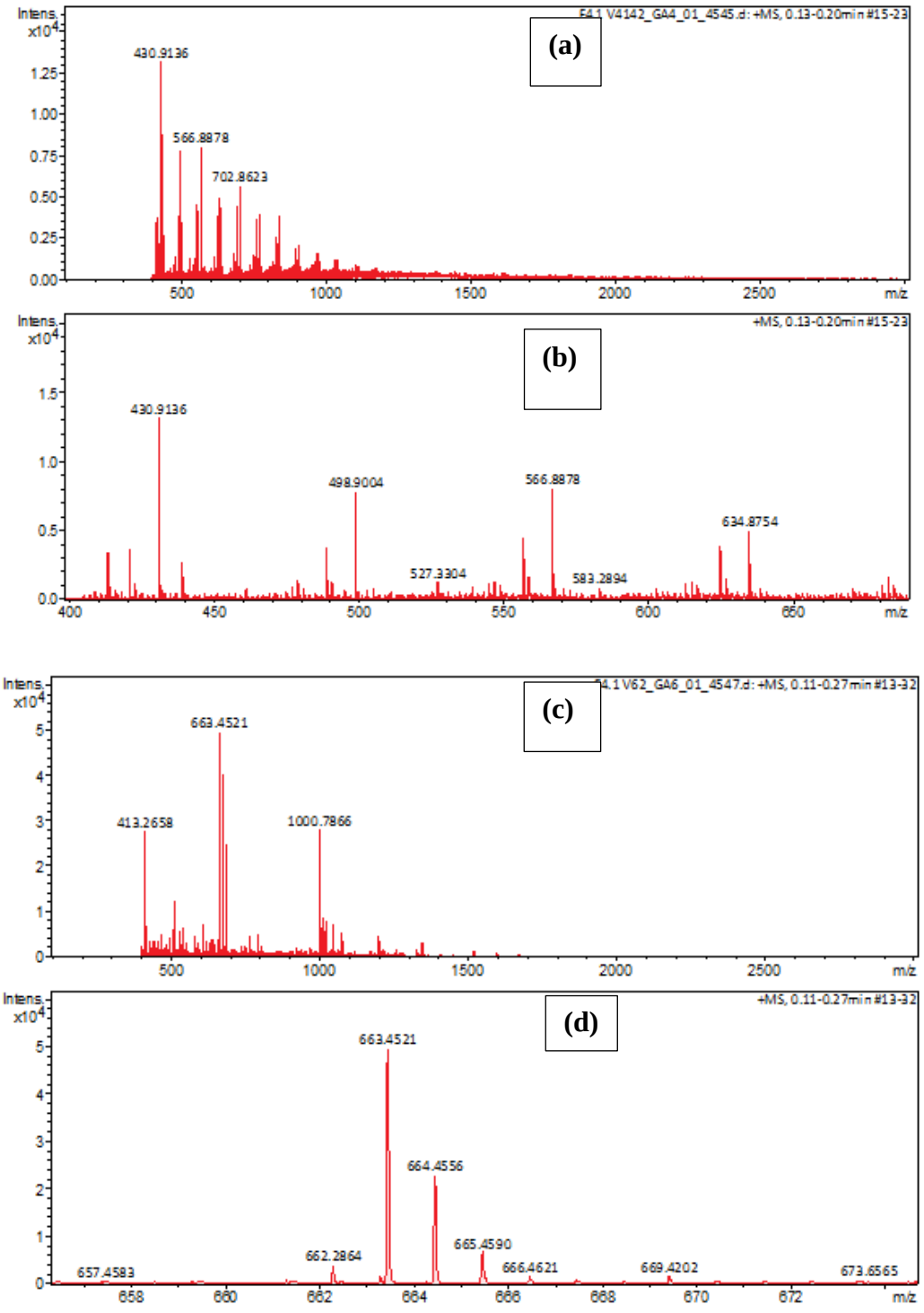

Figure 9.6 (a) UHPLC-HRMS spectra of subfraction F 4.1 (b), (c) enlarged range of (a) and (d) enlargement of peak 663.45 m/z

Table 9.5 Identification of compounds in subfraction F 4.1 by UHPLC-HRMS

No.	m/z(study)	Intensity	Lit. m/z/ prediction	identification	formulae
1.	413 $[M - H]^-$	3 X 10^4	414	β-Sitosterol	(Mohammad *et al.*, 2016) $C_{29}H_{50}O$
2.	430 $[M - H]^{-3}$	1.45 x 10^4	433	Pelargonidin-3-O-glucoside	(Ambigaipalan *et al.*, 2016) $C_{30}H_{26}O_{12}$
3.	499 $[M + H]^{+2}$	0.9 x 10^4	497	Galloyl-HHDP-gluconic (lagerstannin C)	(Ambigaipalan *et al.*, 2016) $C_{27}H_{22}O_{19}$
4.	566 $[M + H]^{+4}$	0.9 x 10^4	562	Procyanidin dimer B1	(Ambigaipalan *et al.*, 2016) $C_{20}H_{17}O_{14}$
5.	635 $[M + H]^{+2}$	0.6 X 10^4	633	Galloyl-HHDP-hexoside	(Aguilar-Zarate *et al.*, 2017)
6.	664 $[M +H]^+$ 664 [M – CO] [M – CO- H]	5 X 10^4	663 649	unknown galloyl-HHDP-glucoside (lagerstannin C)	(Perez-Ramirez *et al.*, 2018) (Wafa *et al.*, 2017) $C_{27}H_{21}O_{18}$

9.3.4 NMR spectroscopy

The NMR ^{1}H and ^{13}C shift of compound with APG 2 structural characterization results are shown in Figure 9.7. The ^{1}H and ^{13}C NMR data and from literature are presented in Table 9.6. Chemical shift analysis exhibited two signals in the upper value region. The highest signal resonated at δ 5.36 (t, 1H, 10.9, J = 4.06 Hz, Ar-H). It was followed by a signal slightly up field in the region of 3.53 (tt, 1H, J =11.1 Hz). The ^{1}H-NMR spectra of this compound also revealed a signal corresponding to the proton connected to C-3 hydroxyl group. The ^{13}C NMR chemical shift analysis in deuterated chloroform ($CDCl_3$) yielded 29 carbon signals as shown in Table 9.6. The spectra suggests that the analysed compound consists of a tetracyclic cyclopenta ring and a long flexible side chain at the C-17 carbon atom typical of a phytosterol. The four rings (A, B, C and D, from left to right) have trans ring junctures. The indirect dipole-dipole (J) coupling constants (reported in Hz) and resonance multiplicities were observed to be solvent independent. These results were confirmed by DEPT 135 and HMBC as shown in Figure 9.8 and results for the NOESY, HMBC, HSQC and COSY spectra are shown in the appendices. The structural elucidation shows the existence of a hydroxyl group on position 3 of the A ring. The APG 2 compound was identified as β-sitosterol as shown in Figure 9.9.

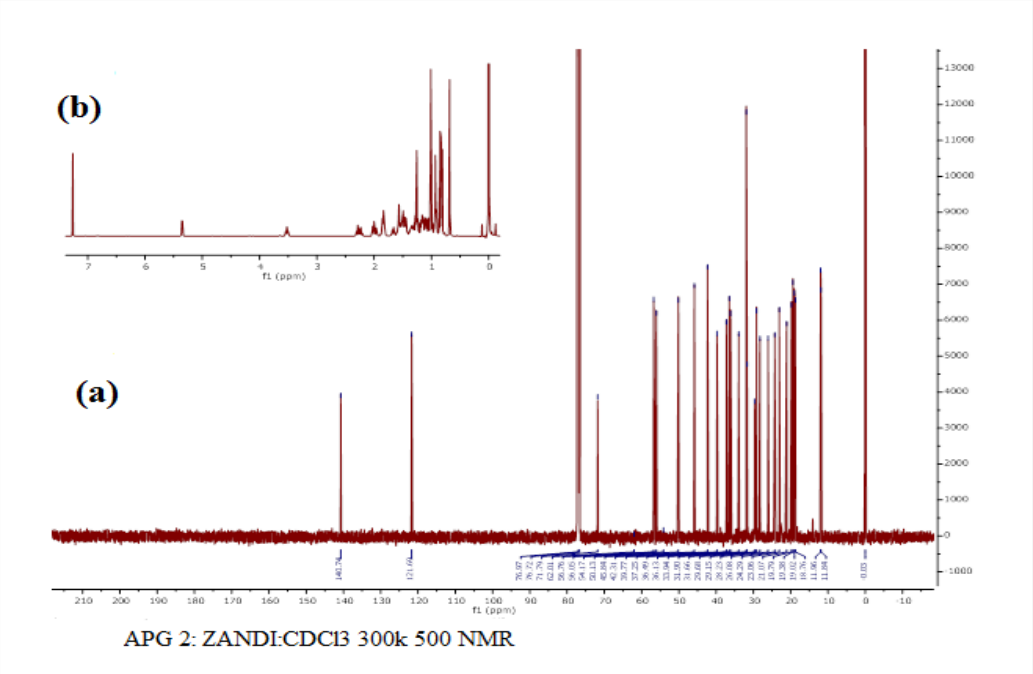

Figure 9.7 The NMR spectra of compound APG 2 (a) ^{13}C and (b) insert ^{1}H

Table 9.6 The ^{1}H NMR of compound APG 2

	APG 2			
	Experimental		**Literature**	
C Position	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
1	37.2		37.5	
2	31.7		31.9	
3	71.6	3.53 (tt, 1H, J =11.1 Hz, Cyclic-Alkyl (OH)	72	3.53 (tdd,1H, J=4.5,4.2,3.8 Hz)
4	41.8		42.5	
5	140.8	5.36 (t, 1H, 10.9, 4.06 Hz, Ar-H)	140.9	5.36 (t,1H, J=6.4 Hz)
6	121.8		121.9	
7	32.0		32.1	
8	31.8		32.1	
9	50.6		50.3	
10	37.7		36.7	0.93 (d, 3H, J=6.5 Hz)
11	21.1		21.3	
12	39.8		39.9	
13	42.7		42.6	
14	56.5		56.9	
15	26.3		26.3	
16	25.9		28.5	
17	56.2		56.3	
18	36.1		36.3	
19	19.3		19.2	0.93 (d, 3H, J= 6.5 Hz)
20	33.9		34.2	
21	26.3	0.92 (d, 3H, J= 6.5Hz Alkyl)	26.3	0.83 (d,3H, J= 6.4 Hz)
22	46.1		46.1	0.81 (d,3H, J= 6.4 Hz)
23	23.2		23.3	0.68 (s, 3H)
24	12.0	1.01 (s, 4H, Alkyl)	12.2	1.01 (s,3H)
25	30.4		29.4	
26	21.0		20.1	
27	19.4		19.6	
28	21.0		19.0	
29	12.0		12.0	

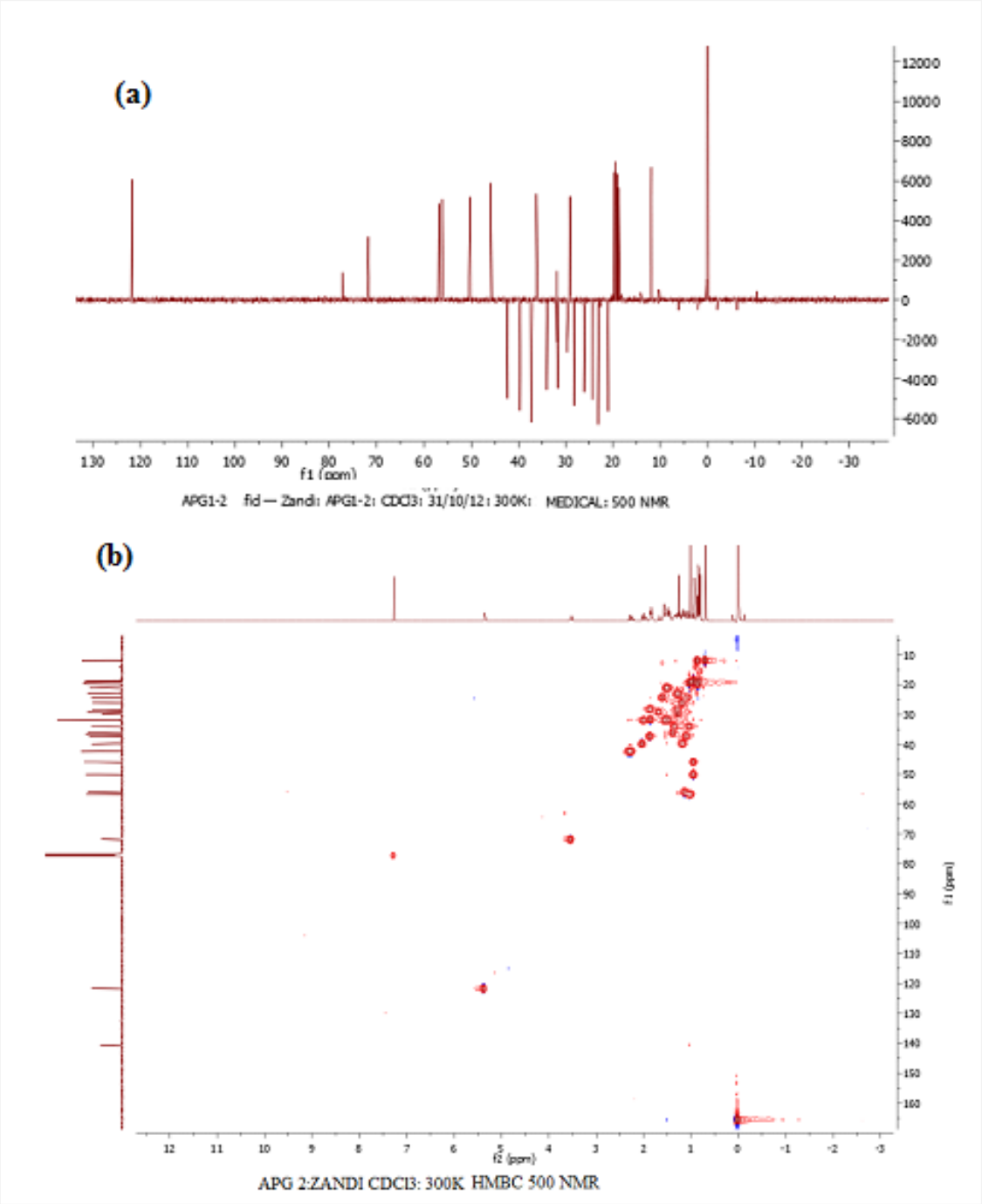

Figure 9.8 The NMR spectra of compound APG 2 using (a) DEPT 135 and (b) HMBC

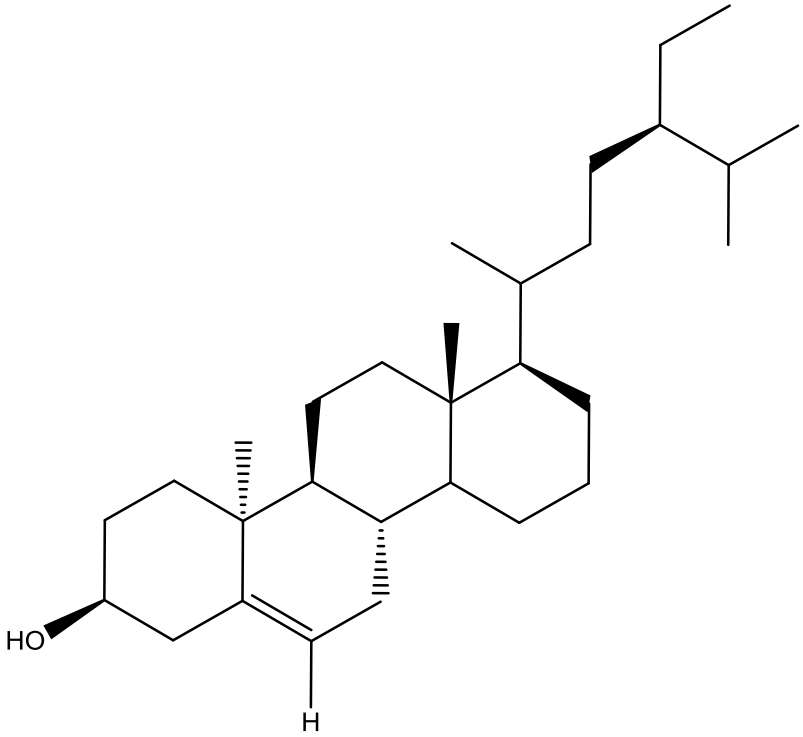

Figure 9.9 The APG 2 identified compound: β-sitosterol

The NMR ^{1}H and ^{13}C shifts of compound APG 6 structural characterization results are shown in Figure 9.10. The proton analysis results are shown in Table 9.7. The ^{1}H-NMR spectra of this compound revealed a signal corresponding to the proton connected to the three galloyl groups at δ_c 3.44 (s, 0H), 2.03, 1.64, 1.39, 3.24, 3.74, 7.26 (s, 1H) and a hexoside at 5.36 (t, J = 5.9 Hz, 2H), 3.37 (d, J= 11.6 Hz), 3.37 (d, J= 11.6 Hz), (OH), 4.38 (d, OH, J= 7.7 Hz). The ^{13}C NMR elucidation in $CDCl_3$ yielded δ_c three ester carbonyl carbons with one at 165.9 and two at 168.6. A hexoside with δ_c 95.1, 72.6, 71.9, 68.6, 73.9 and 64.0. The structural results were confirmed by DEPT 135 and HMBC as shown in Figure 9.11, the NOESY, COSY, HSQC spectra are shown in appendices. The characterized compound has a proposed structure as shown in Figure 9.12. These results show the existence of the galloyl, phenolic and hexoside groups. The galloyl-HHDP-hexoside has been identified at 634 m/z (Ambigaipalan *et al.,* 2016)

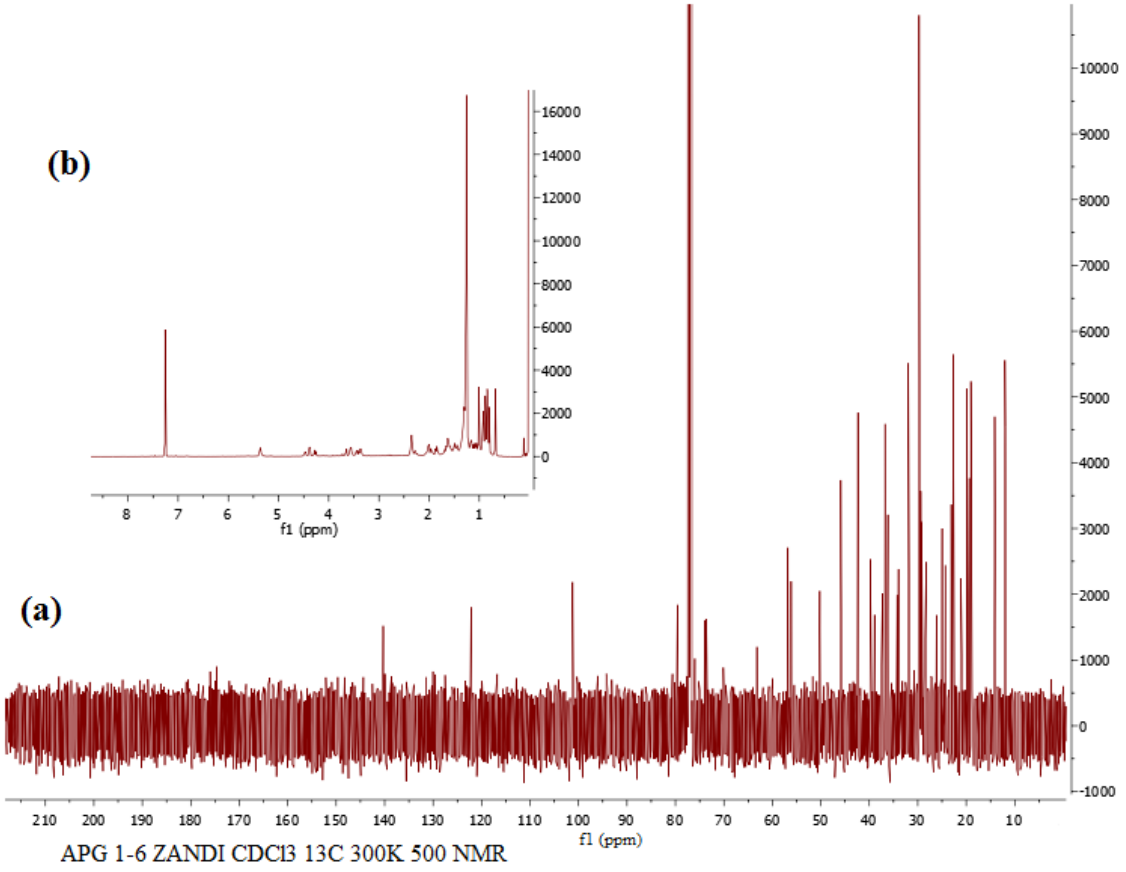

Figure 9.10 The NMR structural results of APG 6 compound (a) ^{13}C and insert (b) ^{1}H

Table 9.7 The ^{1}H NMR of APG 6

APG 6				
	Experiment		**Literature**	
C position	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
Galloyl				
1	121.2	3.44 (s, 0H),	120.9, 120.8	
2	109.6	2.03	110.4, 110.1	7.21 (1H, s), 7.09 (1H, s)
3	146.1	1.64, 1.39	146.1, 146.0	
4	140.3	3.24	139.3, 139.1	
5	146.1	3.74	146.1, 146.0	
6	109.6	7.26 (s, 1H)	110.4, 110.2	7.21 (1H, s), 7.09 (1H, s)
7	165.9		166.1, 165.3	
Glc				
1	95.1		95.4	5.94 (1H, d, J =8.1 Hz)
2	72.6	3.37 (d, J= 11.6 Hz)	75.5	5.53 (1H, t, J =9.6 Hz)
3	71.9	3.37 (d, J= 11.6 Hz)	72.2	3.94 (1H, t, J =9.6 Hz)
4	68.6		69.9	5.27 (1H, t, J =9.6 Hz)
5	73.9	(OH)	76.5	3.94(1H, t, J =12.0 Hz)
6	64.0	4.38 (d, OH, J=7.7 Hz)	61.7	3.70 (1H, dd, J =4.8 Hz)

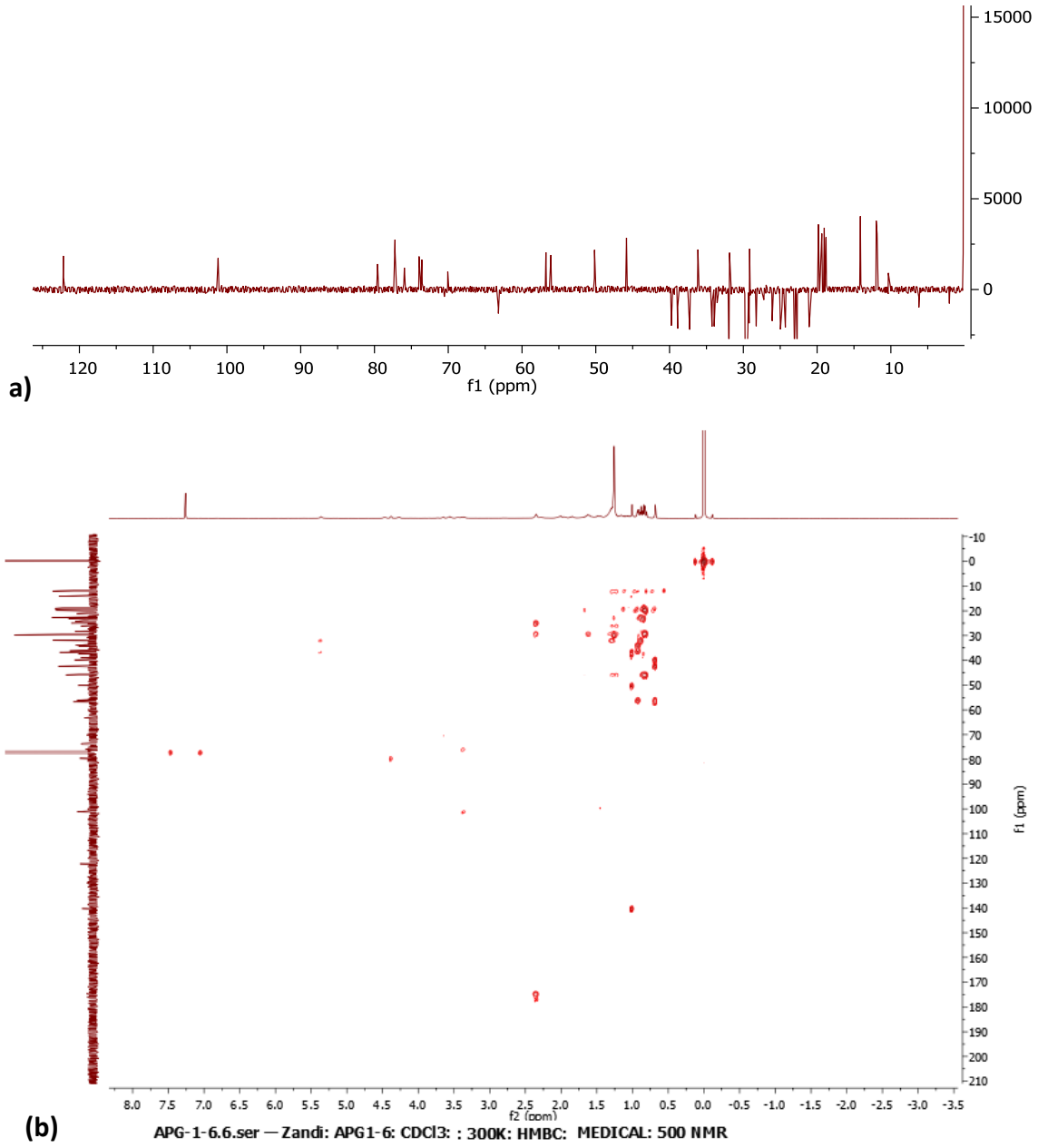

Figure 9.11 The NMR spectra of APG 6 compound (a) DEPT 135 (b) HMBC

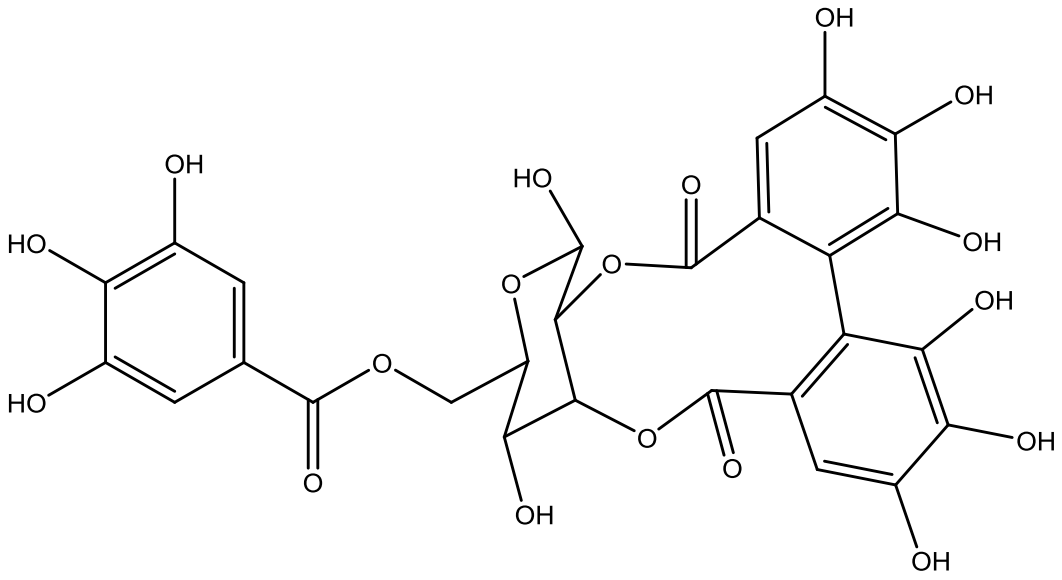

Figure 9.12 The proposed (yet inconclusive) APG 6 compound: galloyl-HHDP-hexoside

9.3.4 Infrared spectroscopy

The FTIR spectra results for compound APG 2 are shown in Figure 9.13. The functional group analysis is shown in Table 9.8. The IR analysis showed absorption bands of hydroxyl moieties at 3330, CH_3 at 2920, CH_2 at 2850 cm^{-1}. The IR analysis showed the presence of hydroxyl moieties, alkene functionalities, and aliphatic groups. The broad band between 3000 and 3500 cm^{-1} is due to the hydroxyl groups.

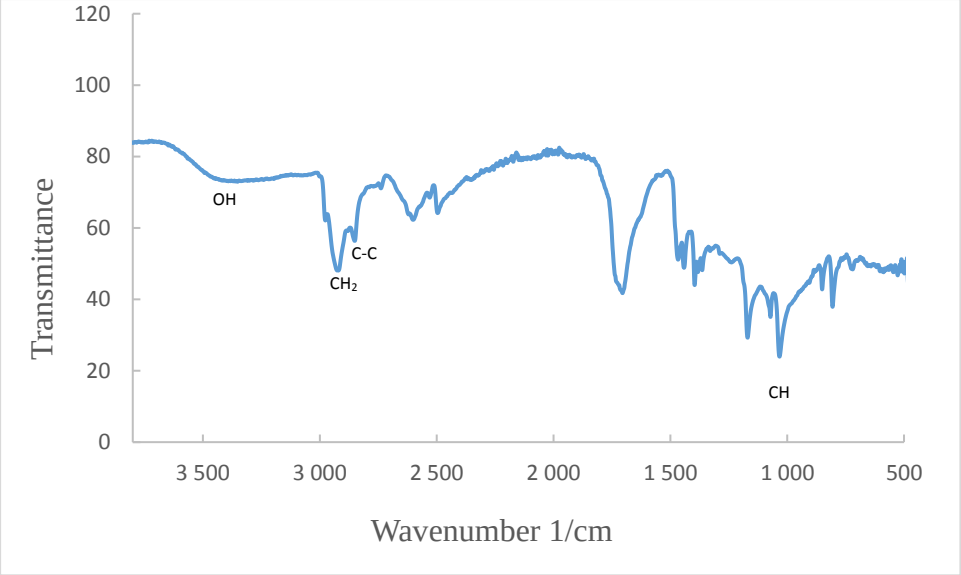

Figure 9.13 The FTIR spectrum of compound APG 2

Table 9.8 FTIR peak values of compound APG 2

APG 2 peak numbers (cm^{-1})	Functional group	Group frequency (cm^{-1})	Reference
3330	O-H stretch	3200-3600	(Mohammad *et al.*, 2016)
2920	C-H_3 stretch	2850- 2970	
2850	C-H_2 stretch		
1566	C-O	1,500–1,600	(Baker *et al.*, 2014)
1450	CH_2 bending Cyclic methylene	1,400 - 1600	
900	C-H Alkenes	1.000 - 1150	
864	C-H Alkenes	675-995	
765	C-H Alkenes bending	675-995	(Mohammad *et al.*, 2016)

The FTIR spectra results for compound APG 6 are shown in Figure 9.14. The functional group analysis is shown in Table 9.9.

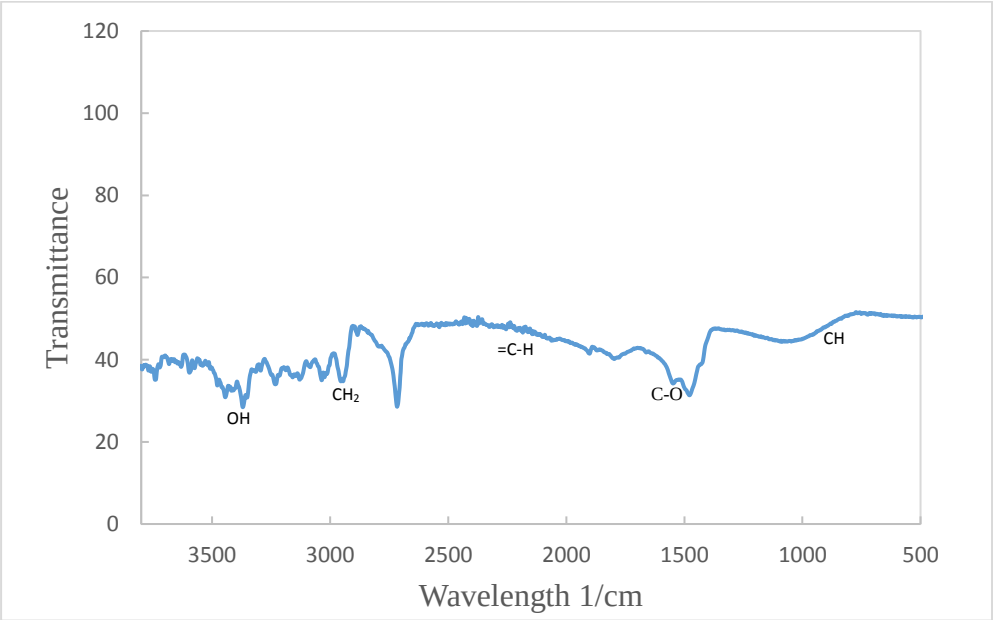

Figure 9.14 The FTIR spectrum of compound APG 6

Table 9.9 The FTIR peak values of compound APG 6

APG 6 peak numbers (cm^{-1})	Functional group	Group frequency (cm^{-1})	Reference
3330	O-H stretch	3200-3600	(Mohammad *et al.*, 2016)
2920	C-H_2 stretch	2850- 2970	
2850	C-C stretch		
1700	C=O stretch Saturated ester Aryl ketone stretch	1,600–1,700 1680-1700	(Baker *et al.*, 2014)
1566	C-O C-O-C C=C stretch	1,500–1,600 1400-1600	
1450	CH_2	1,400 – 1600	
900	Aryl ring	1.000 - 1150	

9.4 Discussion

A large body of literature has linked pomegranate polyphenols, particularly anthocyanins and hydrolyzable tannins to the health-promoting activities of pomegranate fruit extracts (Wu and Tian 2017). Plant materials are particularly challenging because they contain thousands of compounds with a wide range of chemistry, large dynamic ranges, and complex matrices (Wernisch and Pennathur 2016). Subfractions F 3.2 and F 4.1 needed characterisation of their constituents to classify the active compounds. Efficient purification and isolation of analytes in subfractions F 3.2 and F 4.1 was required to achieve these goals. Agela flash chromatography was used as the first step in the purification of these subfractions. Broad peaks were observed and further resolution of these peaks led to the discovery of new peaks which imply presence of multiple constituents. Low quantities of purified peaks obtained and the persistent damage with long period of maintenance were the main challenges that arose with the flash chromatography machine. It is for this reason that we could not proceed with the purification using this machine.

The subfractions F 3.2 and F 4.1 were then subjected to purification using the preparative HPLC. The peaks obtained from the preparative HPLC were characterised further using HPLC-DAD-ESI-HRMS. Thus, the use of the novel bi-analytic method to enhance resolution and decrease analysis time, coupled with high resolution mass spectrometry would improve sensitivity and specificity of obtaining the compounds. The preparative HPLC of subfraction F 3.2 gave 9 peaks with its vial 1 displaying 8 compounds on the HRMS spectra. While, vial 1 of subfraction F 4.1 gave 6 compounds on the HRMS spectra. This proves the extent of complexity of pomegranate plant metabolites. These are the common challenges that emerge not only from the complexity and large dynamic range of metabolites in plant extracts but also from the chemical diversity of the metabolome (Wishart 2011).

The HRMS characterised constituents were then ascribed according to the previously identified pomegranate peel compounds that are in literature. Detailed characterisation revealed that the presence of hydrolysable glucosides, tannins and an anthocyanide amongst other compounds as observed from the MS analysis. The compounds whose main peaks appear at 633 m/z, 414 m/z and 1000 m/z correspond to galloyl-HHDP-hexoside, β-sitosterol and lagerstannin B derivative respectively (Aguilar-Zarate *et al.*, 2017, Ambigaipalan *et al.*, 2016, Fischer *et al.*, 2011, Mohammad *et al.*, 2016) and some of these belong to hydrolysable tannins group of the pomegranate peel (Ambigaipalan *et al.*, 2016). The HRMS spectrum shows that the majority of peaks present in vial 1 of both subfractions F 3.2 and F 4.1 were indistinguishable. The peaks from subfraction F 3.2 at 702, 770, 838 and 906 m/z were the only distinct major peaks for subfraction F 3.2. While the peaks at 413, 663, 664, 665 and 1000 m/z were distinct for F 4.1. These peaks were then attributed according to literature. This demonstrates that the pigment profile of pomegranate peel may be much more complex.

Isolation and characterisation of the phenolic compounds in pomegranate plant extract have been explored extensively in the recent years. A study was conducted on the composition of *Punica granatum* L. peel extract fromTunisia. Their study revealed two main groups of polyphenolic compounds the anthocyanins and ellagitannins (Wafa *et al.*, 2017). It was in agreement with observations from Spain, Turkey and Iran that showed phenolic anthocyanins and ellagitannins as the main chemicals in the pomegranate peels (Gomez-Caravaca *et al.*, 2013). No study has been conducted on the bioactivity of anthocyanins and ellagitannins content of the South African cultivars against oral pathogens. Ellagitannins and anthocyanins were present among other compounds found in the subfractions of F 3.2 and F 4.1. They played a major role in the antibacterial activity of *Salmonella* strains (Wafa *et al.*, 2017) and are the foremost determined compounds (Gomez-Caravaca *et al.*, 2013). Likewise, they may have

been responsible for the antimicrobial, anti-biofilm and anti-acid production effect observed in this study. A β-sitosterol compound has been identified from methanolic peel extract of pomegranate with a MS fragment ion of 414 m/z (Mohammad *et al.,* 2016). Wafa *et al*., (2017) have identified an anthocyanin pelargonidin-3-glycoside at 433 m/z and a delphinidin-3, 5-diglycoside at 627 m/z. This study has also shown peaks at 413, 435, 499 and 634 m/z using UHPLC-HRMS.

Gomez-Caravaca *et al*., (2013) used different mixtures of water acidified with diverse concentrations of acetic and formic acid as mobile phase A for the determination of anthocyanins. The concentrations of acid they used varied from 1 to 5 % (Gomez-Caravaca *et al.,* 2013). In this study, the UHPLC-HRMS used the formic, water and acetronitrile as mobile phase. However, in another study the alkaline fractions that were water soluble were highly effective against gram positive microbes and had the uppermost number of phenolic acids (Prajna *et al.,* 2013). The subfractions F 3.2 and F 4.1 were purified in a cellulose column using aqueous solution (pH 12). Its UHPLC-HRMS shows the presence of the anthocyanins among the identified compounds. Also, the HPLC fractions from *Punica granatum* peel extracts showed antibacterial activity on the clinical isolates with multidrug resistance from *E. coli, A. baumannii* and *P. aeruginosa* (Khan *et al.,* 2017). In that study, the ethanol extract was used and monogalloyl-hexoside and hexahydroxydiphenoyl-hexoside were identified as bioactive compounds.

The ^{1}H and ^{13}C NMR analysis was carried out on the 2 compounds isolated from *P. granatum* and structures were confirmed by HMBC, COSY, DEPT, HSQC and NOESY techniques. The two compounds that were identified are shown in Figure 10.10 and 10.13. The compound APG 2 (β-sitosterol) a main dietary phytosterol found in plants correlates to the product ion peak at

413.26 m/z. This compound was also identified by Mohammad *et al*., (2016) from the pomegranate peel. The compound APG 6 was proposed to be protonated galloyl-HHDP-hexoside. The FTIR analysis confirmed the presence OH in the β-sitosterol. While carbonyl, OH and COC vibrations for the proposed galloyl-HHDP-hexoside were observed in agreement with NMR results.

In traditional herbal medicine, the principle of synergy among the plant compounds has been has long been explored. Mixtures of natural products have been used for many years for treatment of infections and to challenge drug tolerance or resistance (Wu and Tian 2017). There are studies available that suggest synergistic connections among pomegranate chemicals, which need to be explored further. Cooperative interactions have been discovered in limiting the proliferation, metastasis, and invasiveness of prostate cancer cells *in vitro* from phytochemicals in pomegranate fruit peel extracts, fermented pomegranate juice, and pomegranate seed oil (Lansky *et al.*, 2005). A subsequent investigation with pure compounds like EA, caffeic acid, luteolin, and punicic acid, also demonstrated synergy in suppressing the invasion of prostate cancer cells. The polyphenolic extracts of pomegranate fruit also synergistically interacted with the antibacterial drug ciprofloxacin, though various bacterial strains responded differently to the phytochemical-drug synergy, and the underlying mechanism of such synergy remains unknown (Dey *et al.*,)

9.5 Conclusion

The study reveals that the pomegranate peel composition is complex. One vial from the preparative HPLC produced multiple compounds with both subfractions F 3.2 and F 4.1. The two compounds identified were galloyl-HHDP-hexoside (633 m/z) and β-sitosterol (414 m/z), and they belong to the hydrolyzable tannins and phytosterols respectively. They have been

identified in the active subfractions of the pomegranate peel extract. They are known for their therapeutic activity against bacteria, tumors, cancer cells, inflammation and viruses.

9.6 Challenges

Obtaining purified samples in appreciable quantities was a major challenge. Labour intensity, time consumption and purification difficulties are also some of the main challenges facing characterisation of active compounds in traditional plants. The constant break down of the Agela flash chromatograph machine without a local technician available for the repairs was another challenge.

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CHAPTER 10

The cytotoxicity of crude extract, and subfractions F 3.1 and F 4.1 of *P. granatum* against the human embryonic kidney cells

10.1 Introduction

Pomegranate has been widely consumed by people in many different cultures throughout the world without any incident of being unsafe for over 4000 years. It has been traditionally used for treatment of diseases, and ailments. Furthermore, reports about its therapeutic qualities have echoed from generation to generation. It has anticancer, antiviral, anti-inflammatory, antiproliferation and antioxidant properties. It is now being recognized as a potential chemo-preventive and anticancer agent because of its toxicity to cancer cells (Tang *et al.,* 2017).

An *in vivo* study has looked into the toxicity of the methanol peel extract in mice and the lack of any adverse effects supported its use for certain oral infection therapy following oral administration (Bassiri-Jahromi *et al.,* 2015). In another study, researchers showed that the pomegranate peel extract acted either as an antioxidant to inhibit reactive oxygen species production or its scavenger in the presence of particulate matter (pm) inside human acute monocytic leukemia cell line. Particulate matter, a complex mixture of solids and liquids in the air is toxic to human health by causing induction of oxidative stress and activation of nuclear factor kappa B (NF-κB) pathway, leading to inflammation (Park *et al.,* 2016). Moreover, in an *in vitro* study , water extract from pomegranate peel induced apoptosis through DNA fragmentation in a dose dependent manner (Settheetham and Ishida 1995). The observed effect was after 24 and 48 hour incubation in a dose dependent manner.

The health benefits of pomegranate have been attributed to its wide range of phytochemicals. The most represented phenolic compound punicalagin, (2, 3 – hexahydroxydiphenoyl – gallagyl – D - glucose; Figure 10.1) is the major ellagitannin with the most bioactive component. It is known for its therapeutic properties also found in the crude peel extract – anticancer, antiproliferation etc (Adaramoye *et al.,* 2017, Russo *et al.,* 2018, Tang *et al.*, 2017). Punicalagin inhibited viability in human prostate cancer cells in a concentration dependent manner (Adaramoye *et al.,* 2017).

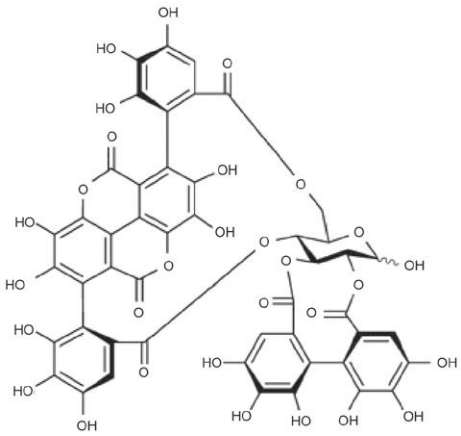

Figure 10.1 Chemical structure of punicalagin [Adapted from (Tang *et al.,* 2017)

Safety of traditional and herbal medicine must always be researched even though its treatment for various diseases has been established (Bassiri-Jahromi *et al.,* 2015). Marginal toxicity is important or even better no toxicity in the screening of plants products for the successful development of plant derived therapeutic medicine. Considering the worldwide use of pomegranate in dietary supplements, it is important to determine if any toxicological effects can occur from treatment. Toxicity, safety and tolerability of pomegranate is still controversial and has not been studied intensively.

Aim: The aim of this study is to evaluate the cytotoxic effect of *P. granatum* DCM:MeOH crude extract, F3.1 and F 4.1 purified fractions on human embryonic HEK 293 cells.

10.2 Materials and methods

10.2.1 Cytotoxicity

The effect of the crude extract from pomegranate peel, subfractions F 3.2 and F 4.1 on human cells was examined by using cytotoxicity assay. Human cells HEK 293 embryonic kidney cells were obtained from the School of Molecular Biological Sciences, University of the Witwatersrand Johannesburg, South Africa and were stored at -80° C in the tissue culture laboratory.

10.2.2 Cell lines harvest

The HEK 293 cells were regularly well-maintained in the supplemented Dulbeco's modified eagle's medium (88 %) with fetal calf serum (10 %), L-glutamine (1 %) and penicillin/streptomycin (1%). To subculture/cultivate and eliminate dead cells, old cells medium was removed and replaced with a PBS prewarmed to 37°C. Phosphate buffered saline was discarded and the cells were detached with 2 ml of Trypsin EDTA. The flasks were incubated at 37° C for 2 minutes. Subsequent to incubation, a slight knock on the sides of the flasks were given to support cell dissociation while preventing clumps of cells. About 2 ml supplemented medium was added to neutralize trypsin. Two milliliters of resulting suspension was removed for stock purposes and new T75 flasks used. The cytotoxic assay was performed on T25 flask with 1 ml resulting suspension. All flasks received fresh medium accordingly. The cells were cultured at 37° C in humidified 5 % CO_2 incubator.

10.2.3 Measurement of cell viability

Confluent HEK 293 cells in medium were stained in a 0.4 % trypan blue of a 1:1 ratio. A twenty microliter volume of this mixture was then placed on a hemocytometer and spread with a cover slip. Numbers of total viable cells were counted in each quadrant using a light microscope at 1000x magnification. Values are expressed as a percent of control culture (100 %) and the number of viable cells needed to get the desired concentration of 111000 cells per ml was then calculated using the following equation:

$$No.of\ cells = \frac{\#\ of\ cells\ in\ 4\ grids}{4} \times 2\ \times 10000$$

In order to fill the applicable wells of a microtiter plate the cell number was then multiplied by the amount of cell suspension.

10.2.4 Cytotoxicity assay/Cell proliferation assay

The crude peel extract as well as purified fractions F 3.2 and F 4.1 were tested for *in vitro* cytotoxicity using HEK 293 kidney cell line with 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay technique (Mosmann 1983). This technique relies on mitochondrial enzyme succinate dehydrogenase obtained from the viable cells to reduce MTT and produce purple insoluble product called formazan. Briefly, 100 µl of medium with appropriate calculated number of HEK 293 cells were dispensed into each well of the 96 well plate. After 24 hours of incubation, cell growth on the microtiter plate was viewed at x1000 under the microscope. The cells had grown and attached to the surface. The culture medium was replaced with fresh medium. About hundred microliter plant extract containing different concentrations ranging from 25 - 0.01 mg/ml of DCM:MeOH crude extract, subfraction 3.2 and 4.1 were added to their corresponding wells.

Any interference during absorbance reading of the values for test wells was eliminated by addition of blanks. Blank test control wells were prepared with medium only control solution. Positive control was a prepared with a 2 % Triton-X. Dimethyl sulphoxide control was also prepared as vehicle control used to dissolve the crude extract. Cell solution was also included for cell culture controls. Sample test wells contained 100 µl of cell suspension into applicable wells. The microtitre plates were then incubated for 24 hours at 37° C under 5 % carbon dioxide in a humidified incubator.

After 24 hours of incubation, the media was removed from all the test samples and controls and replaced with 100 µl of fresh medium and incubated briefly. Medium was removed and 100 µl of the MTT reagent was added. Further incubation of plates for 3 hours at 37° C and 5 % carbon dioxide was carried out. The MTT reagent was removed and substituted with 100 % DMSO to dissolve crystals of formazan. Plates were then read with a spectrophotometer at 570 nm after 30 minutes of incubation. The Figures 10.2 and 10.3 depicts the visual inspection of the experiment before the absorbance reading. The 50 % cytotoxicity concentration (CC_{50}) was determined according to Bustillo *et al*., (2009). It is defined as the quantity of plant extract generating 50 % of cell viability, compared to control (Bustillo *et al.,* 2009). The values of the percentage of cell viability were plotted against various concentrations of *P. granatum* plant crude extract and subfractions F 3.2 and F 4.1.

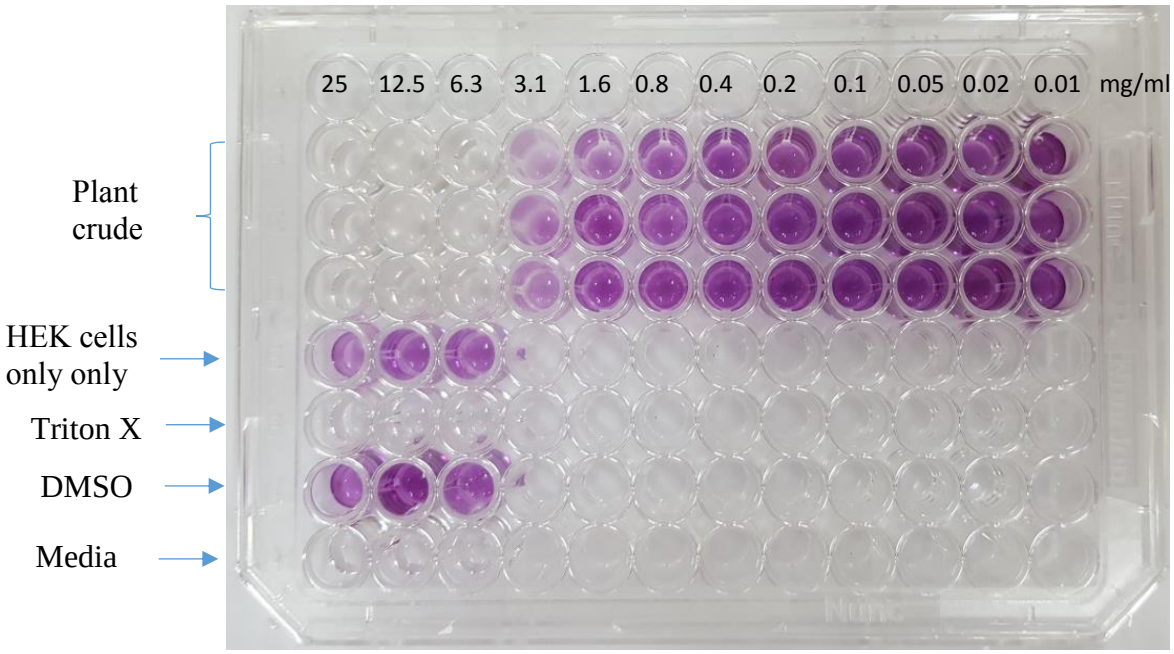

Figure 10.2 The effect of *P. granatum* crude extract on the viability of human HEK cells

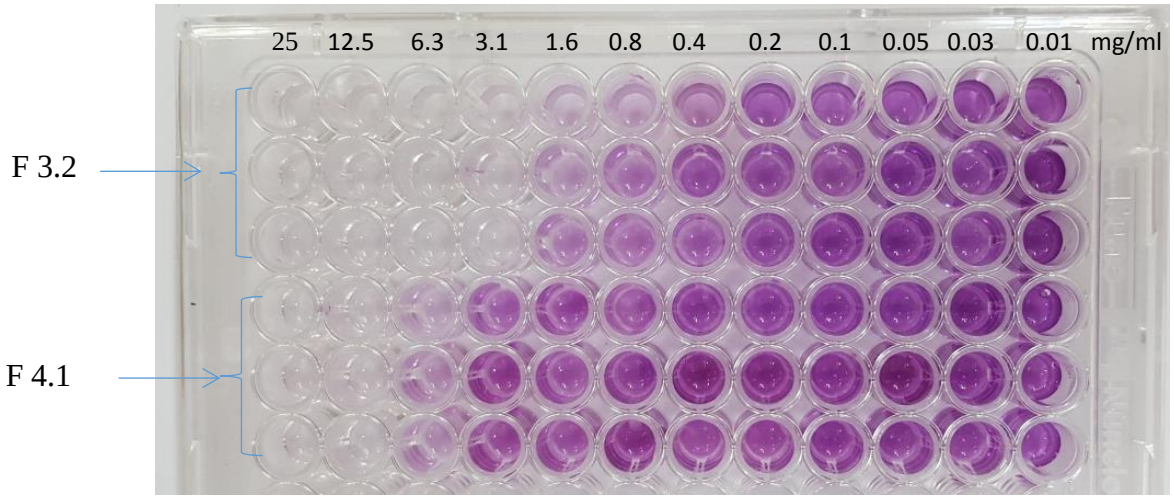

Figure 10.3 The effect of *P. granatum* purified fractions F 3.2 and F 4.1 on the viability of human HEK cell

10.3 Results

10.3.1 Cytotoxicity of crude extract

Cytotoxicity results of the crude extract of *P. granatum* are shown in Table 10.1, graph in Figure 10.4, summary in Table 10.4 and Figure 10.7. The table depicts the absorbance readings in the wells of microtiter plate whereas the figure 10.4 shows calculated cell viabilities at test concentrations. The cell viability was concentration dependent. Only 5 % of the cells were viable in the test concentrations ranging from 25 to 3.1 mg/ml. The CC_{50} is ~2.4 mg/ml. At concentrations of 0.8 and 1.6 mg/ml the cell viability was higher than CC_{50} compared to the control. While at concentrations of 0.4 - 0.01 mg/ml the cell viability was higher than the control.

Table 10. 1 The effect of *P. granatum* crude fractions on the viability of HEK 293 cells

	OD_{570} of controls				OD_{570} in the presence of various concentrations of crude extract (mg/ml)											
	blank	**triton X**	**DMSO**	**untreated**	**25**	**12.5**	**6.3**	**3.1**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**0.05**	**0.02**	**0.01**
crude	0.038	0.018	0.193	0.182	0.010	0.003	0.008	0.006	0.149	0.149	0.234	0.246	0.304	0.276	0.294	0.297
	0.035	0.039	0.232	0.216	0.010	0.009	0.003	0.008	0.105	0.135	0.258	0.254	0.251	0.284	0.3	0.323
	0.036	0.046	0.383	0.265	0.010	0.003	0.004	0.003	0.16	0.097	0.215	0.249	0.253	0.266	0.287	0.309
Mean	0.036	0.030	0.270	0.221	0.010	0.005	0.005	0.006	0.138	0.127	0.236	0.250	0.269	0.275	0.294	0.310
±SD	0.002	0.015	0.100	0.042	0.000	0.003	0.003	0.003	0.029	0.027	0.022	0.004	0.030	0.009	0.007	0.013
% Red.	**-**	**84.5**	**-**	**-**	**95.5**	**97.7**	**97.7**	**97.4**	**37.6**	**42.5**	**-6.6**	**-13.0**	**-21.9**	**-24.6**	**-32.9**	**-40.1**
CV	**-**	**-22.7**	**100**	**100**	**4.5**	**2.3**	**2.3**	**2.6**	**62.4**	**57.5**	**106.6**	**113.0**	**121.9**	**124.6**	**132.9**	**140.1**

Percentage Reduction (%) = $\left({}^{(Control-test)}/_{Control}\right)$ X 100

Red. = reduction, CV= cell viability

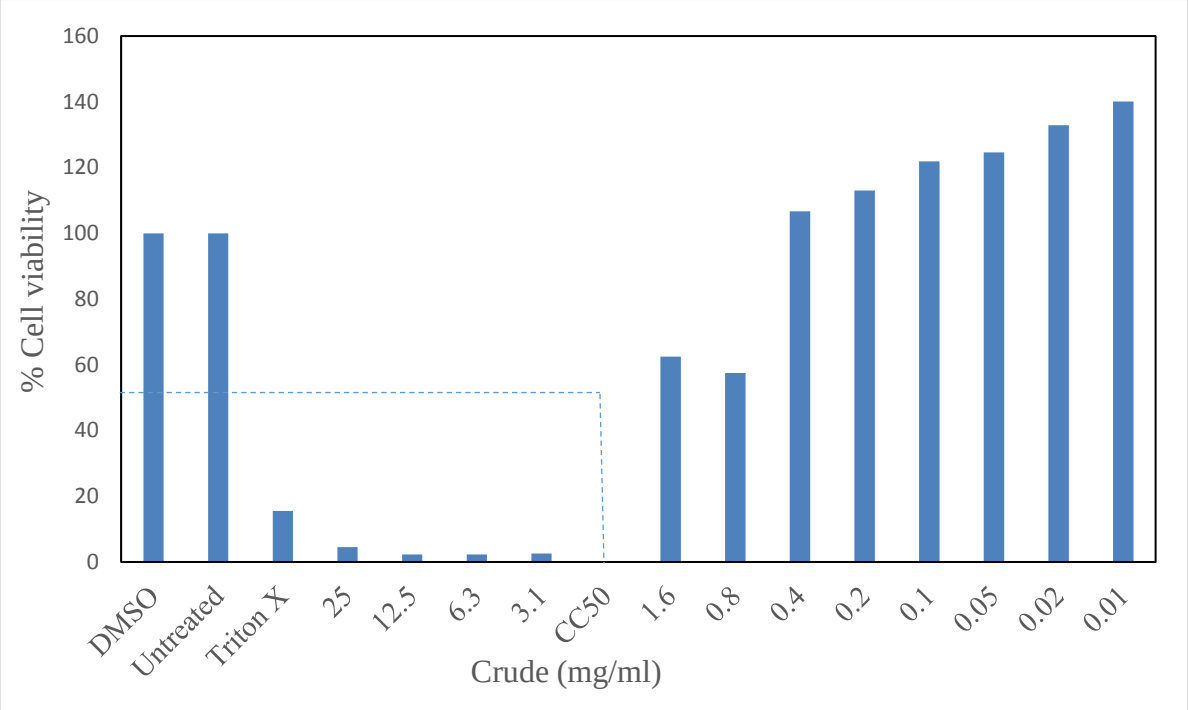

Figure 10.4 The effect of *P. granatum* crude extract on the viability of HEK 293 cells

10.3.2 Cytotoxicity of subfraction F 3.2

Cytotoxicity results of purified subfraction F 3.2 are shown in Table 10.2, Figures 10.5 and in the summarized Table 10.4 and Figure 10.7. The cell viability was concentration dependent. Only up to 24 % of cells were viable in the test concentrations, ranged between 25 to 1.6 mg/ml. The CC_{50} is ~1.2 mg/ml. At the concentration of 0.8 mg/ml the cell viability was at 69 % which is higher than the CC_{50}. Low concentrations ranging from 0.4 to 0.01 mg/ml exhibited higher cell viability when compared to the control.

Table 10. 2 The effect of *P. granatum* purified F 3.2 fractions on the viability of HEK 293 cells

OD_{570} of controls					OD_{570} in the presence of various concentrations of crude extract (mg/ml)											
	blank	Triton x	DMSO	Untreated	25	12.5	6.3	3.1	1.6	0.8	0.4	0.2	0.1	0.05	0.02	0.01
F 3.2	**0.038**	0.018	**0.193**	**0.182**	**0.004**	**0.014**	**0.027**	**0.068**	**0.060**	**0.182**	**0.238**	**0.269**	**0.319**	**0.337**	**0.356**	**0.351**
	0.035	**0.039**	**0.232**	**0.216**	**0.008**	**0.012**	**0.035**	**0.044**	**0.055**	**0.148**	**0.26**	**0.278**	**0.274**	**0.322**	**0.292**	**0.363**
	0.036	**0.046**	**0.383**	**0.265**	**0.040**	**0.004**	**0.012**	**0.022**	**0.042**	**0.129**	**0.209**	**0.278**	**0.286**	**0.311**	**0.317**	**0.344**
Mean	**0.036**	**0.034**	**0.269**	**0.221**	**0.017**	**0.010**	**0.025**	**0.045**	**0.052**	**0.153**	**0.236**	**0.275**	**0.293**	**0.323**	**0.322**	**0.353**
±SD	**0.002**	**0.015**	**0.100**	**0.042**	**0.020**	**0.005**	**0.012**	**0.023**	**0.009**	**0.027**	**0.026**	**0.005**	**0.023**	**0.013**	**0.032**	**0.010**
% Red.	**-**	**84.5**	**-**	**-**	**92.2**	**95.5**	**88.8**	**79.8**	**76.3**	**30.8**	**-6.6**	**-24.4**	**-32.6**	**-46.3**	**-45.6**	**-59.6**
CV	**-**	**15.5**	**100**	**100**	**7.7**	**4.5**	**11.2**	**20.2**	**23.7**	**69.2**	**106.6**	**124.4**	**132.6**	**146.3**	**145.6**	**159.6**

Percentage Reduction (%) = $\left({}^{(Control - test)}/_{Control}\right)$ X 100

Cell viability (CV) = $\left(\frac{Test}{Control}\right) X\ 100$

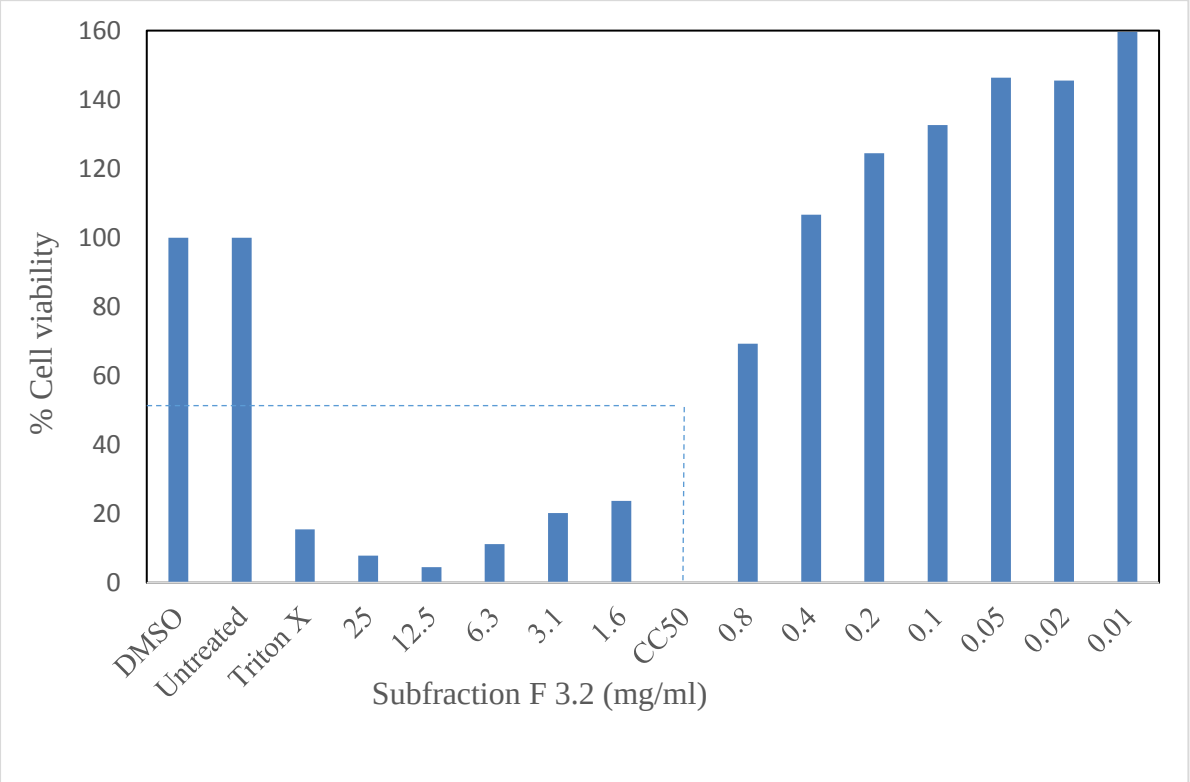

Figure 10.5 The effect of *P. granatum* purified F 3.2 on the viability of HEK 293 cells

10.3.3 Cytotoxicity of subfraction F 4.1

Cytotoxicity results of purified subfraction F 4.1 are shown in Table 10.3, Figures 10.6 and in the summarized Table 10.4 and Figure 10.7. The cell viability was not concentration dependent. Only up to 12 % of cells were viable in the test concentrations, ranged between 25 to 6.3 mg/ml. At the concentration of 1.6 to 0.2 mg/ml the cell viability was higher than at CC_{50}. At concentrations of 0.1 to 0.01 mg/ml cell viability was higher when compared to the control.

Table 10. 3 The effect of *P. granatum* purified F 4.1fractions on the viability of HEK 293 cells

OD_{570} of controls					OD_{570} in the presence of various concentrations of crude extract (mg/ml)											
	blank	Triton X	DMSO	Untreated	25	12.5	6.3	3.1	1.6	0.8	0.4	0.2	0.1	0.05	0.02	0.01
F 4.1	**0.038**	**0.018**	**0.193**	**0.182**	**0.063**	**0.045**	**0.055**	**0.123**	**0.111**	**0.174**	**0.222**	**0.241**	**0.297**	**0.312**	**0.286**	**0.326**
	0.035	**0.039**	**0.232**	**0.216**	**0.028**	**0.009**	**0.005**	**0.068**	**0.152**	**0.207**	**0.113**	**0.137**	**0.217**	**0.222**	**0.285**	**0.258**
	0.036	**0.046**	**0.383**	**0.265**	**0.021**	**0.144**	**0.021**	**0.116**	**0.118**	**0.185**	**0.183**	**0.208**	**0.212**	**0.257**	**0.283**	**0.301**
Mean	**0.04**	**0.034**	**0.269**	**0.22**	**0.037**	**0.066**	**0.027**	**0.102**	**0.127**	**0.189**	**0.173**	**0.195**	**0.242**	**0.264**	**0.285**	**0.295**
±SD	**0.002**	**0.015**	**0.100**	**0.042**	**0.023**	**0.070**	**0.026**	**0.030**	**0.022**	**0.017**	**0.055**	**0.053**	**0.048**	**0.045**	**0.002**	**0.034**
% Red	**-**	**84.5**	**-**	**-**	**83.0**	**70.0**	**87.7**	**53.5**	**42.3**	**14.2**	**21.5**	**11.2**	**-10.0**	**-19.8**	**-29.4**	**-34.1**
CV	**-**	**13.6**	**100**	**100**	**17.0**	**30.0**	**12.3**	**46.5**	**57.7**	**85.8**	**78.5**	**88.8**	**110.0**	**119.8**	**129.4**	**134.1**

Percentage Reduction (%) $= \left({}^{(Control - test)}/_{Control}\right) * 100$

Cell viability (CV) =) = $\left(\frac{Test}{Control}\right) X\ 100$

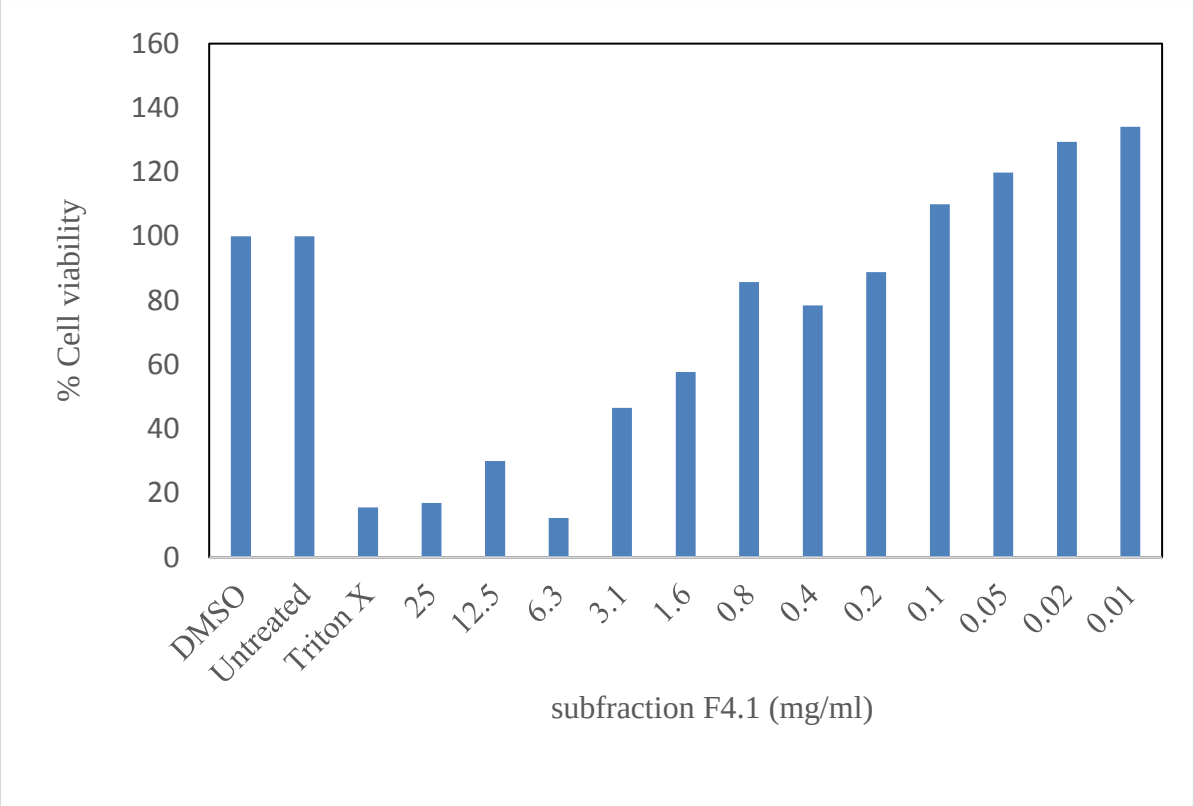

Figure 10.6 The effect of *P. granatum* purified F 4.1 on the viability of HEK 293 cells

Table 10.4 Summary table with cell viability for crude extract and subfractions F 3.2 and F 4.1

Cell viability %												
	25	**12.5**	**6.3**	**3.1**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**0.05**	**0.02**	**0.01**
Crude	**4.5**	**2.3**	**2.3**	**2.6**	**62.4**	**57.5**	**106.6**	**113.0**	**121.9**	**124.6**	**132.9**	**140.1**
F 3.2	**7.7**	**4.5**	**11.2**	**20.2**	**23.7**	**69.2**	**106.6**	**124.4**	**132.6**	**146.3**	**145.6**	**159.6**
F 4.1	**17.0**	**30.0**	**12.3**	**46.5**	**57.7**	**85.8**	**78.5**	**88.8**	**110.0**	**119.8**	**129.4**	**134.1**

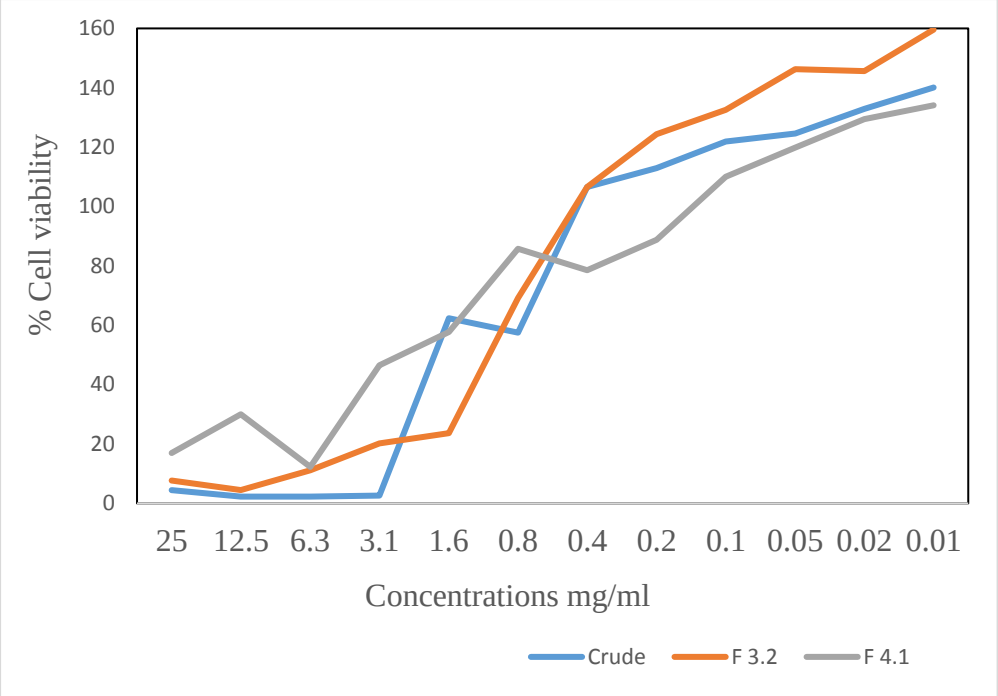

Figure 10.7 Summary graph of cell viability of crude extract, and subfractions F 3.2 and F 4.1

10.4 Discussion

Pomegranate is an ancient fruit with an eminent medical history due to its recognized beneficial properties and is thus considered generally safe. Although the fruit is safe to eat, the peel used in this study is abundant in phytochemicals and might not be safe to use. Cytotoxicity assay is among the popular *in vitro* assays used to assess the possibility of a compound to cause hostile effects (Oliveira *et al.*, 2017). The highest concentration of the crude extract that is safe to use was 1.6 mg/ml with 62 % cell viability, while highest toxicity was 97 % at 3.1 mg/ml. The MBC against *S. mutans* is 6.25 mg/ml, chapter 3 section 3.3.2. However, a concentration of 6.25 mg/ml will still be acceptable for dental caries therapeutic purposes due to the constant flow of saliva which will dilute the plant extract instantly in the oral cavity. This will also limit the exposure time to this concentration. At the concentrations of 0.78 mg/ml the crude plant extract will kill the *P. gingivalis*, a principal instigator in the periodontal infections. Admittedly, therapeutic chemicals used in the oral cavity are mouth rinse and toothpastes

based. Therefore, if the plant extract is used for gargling or tooth paste little will be absorbed into the body. The higher cell viability at low concentration of 0.4-0.01 mg/ml compared to control showed that the plant acted as cell division stimulant or exhibited enhanced cell proliferation instead of inhibition. On the other hand, at the concentration of 6.25 mg/ml, *P. granatum* will reduce biofilm formation by 95 % and inhibit acid production by *S. mutans*.

The results for the F 3.2 purified fraction showed the cell viability of 69 % at 0.8 mg/ml and sensitivity of 76 % at 1.56 mg/ml and. The F 3.2 fraction reduced biofilm formation by 58 % after 6 hours of exposure to 6.25 mg/ml concentration and it had little or no effect at lower concentration. It reduced acid production when compared to the control after 10 hours of exposure at the MBC of 1.56 mg/ml. Therefore, if F 3.2 is used as a mouthrinse or toothpaste at 6.25 mg/ml it will disrupt the biofilm, and as it gets diluted due to the constant flow of saliva it will reduce acid production. The cell viability of 11 % at 6.25 mg/ml concentration was observed after 24 hours which is acceptable. The contact time as a mouth rinse will be shorter and this concentration will not be maintained for extensive periods of time. There is also shedding of the oral mucosa in the oral cavity and hence it would prove safe to use.

Purified subfraction F 4.1 showed 12 % cell viability at 6.25 mg/ml concentration. At low concentrations it acted as cell stimulant and influenced cell viability in the range of 0.1 mg/ml to 0.001 mg/ml. It reduced biofilm formation by 61 % at 6.25 mg/ml. This concentration will be safe to use as it gets diluted by saliva.

Punica granatum methanolic peel extract showed no toxicity when administered through oral cavity under 7.5 mg/kg with repeated doses in mice (Bassiri-Jahromi *et al.*, 2015). And this

concentration is higher than the 6.25 mg/ml concentration evidenced in this study. In another study, cytotoxicity of *P. granatum* whole fruit including peel was done. They discovered that 0.1 mg/embryo was not toxic with a mortality rate of 3.1 % compared with 5 % of control. The toxic effects occurred at higher doses of 0.2 mg/embryo with 18 % mortality (Vidal *et al.,* 2003). Repeated administering the fruit extract revealed no toxicity or behavioural changes in rats at the dose range of 0.4–7 mg/kg through the nasopharyngeal route. No local irritation or change in the nasal mucosa was observed (Vidal *et al.,* 2003). The crude plant extract of this study shows toxicity at 3.1 mg/ml, and the subfractions F 3.2 and F 4.1 at 1.6 mg/ml and 6.3 mg/ml respectively *in vitro*. Subsequent to Vidal *et al*., 2003 study findings that administered upto 7 mg/kg without any adverse effect, higher concentrations of crude extract and the subfractions in this study would not show any toxicity or local irritation when administered orally. Punicalagin had low toxicity on cells *in vitro* at 10, 50 and 100 mM concentrations incubated for 24 hours. Intrinsic biochemical changes were the main mediators in the growth inhibition but not the destruction of membranes of cells (Adaramoye *et al.,* 2017).

Chlorhexidine (CHLX), a gold standard against which the efficacy of alternative oral therapeutic agents are measured. It is a broad-spectrum antiseptic and disinfectant widely used in dental settings for treatment and prevention of oral diseases. It may produce some undesirable side effects such as discolouration of dental enamel, pigmentation of anterior restorations, irritation of oral mucosa, and taste alteration (Escamilla-Garcia *et al.,* 2017). It can also cause cellular damage with chromatin condensation, pyknosis and nuclear disorganisation along with mitochondrial disintegration. At 0.12 % the CHLX exhibited toxicity by disorganizing the cytoplasma into the culture media (Escamilla-Garcia *et al.,* 2017). Most studies have shown that CHLX tends to damage different cell lines such as osteoblastic, endothelial, and fibroblastic cells (Giannelli *et al.,* 2008). Another study demonstrated CHLX's

toxic effect on stem cells from human exfoliated deciduous teeth at similar therapeutic concentrations over different periods of time (Tu *et al.,* 2015). The MIC and toxicity in KB cells of CHLX against *P. gingivalis* were compared. The antimicrobial agent CHLX was more toxic in eukaryotic cells than to prokaryotic cells (Oliveira *et al.*, 2017). Moreover, this golden standard agent is still used to date in clinical settings even though its concentration is toxic to eukaryotic cells.

In a study by Park *et al*., (2016), pomegranate peel plant extract showed safety by attenuating the cytotoxicity induction of the particulate matter and further purification of the crude identified the punicalagins as responsible for this protection effect (Park *et al.,* 2016). Therefore, the plant crude extract and its subfractions in this study might work in a similar manner by protecting the eukaryotic cells while very sensitive to bacteria. The 6.25 mg/ml concentration in this study is still safe to use as an oral mouth rinse or toothpaste because of the constant flow of saliva which would confer a dilution effect. These results suggest that the crude extract of *P. granatum* and its subfractions could form the basis of phyto-therapeutic preparations.

10.5 Conclusion

Lack of toxicity of crude peel extract of *Punica granatum,* F 3.2 and F 4.1 at low concentrations demonstrated enriched cell proliferation against HEK 293 cells. At 1.6 mg/ml only 62 %, 24 % and 58 % cells were viable *in vitro* for crude extract, F 3.2 and F 4.1 respectively. Toxicity was dose dependent for the crude extract and F 3.2 but not F 4.1. However, higher concentrations *in vivo* are required for sensitivity compared to *in vitro* studies. Therefore, this will have major implications because at higher concentrations it will reduce microbial colonization, adherent glucan production, and the interference with the stable biofilm

formation. Above all, if the crude extract and its subfractions were to be used as mouth rinse and tooth paste, little would be absorbed. More and detailed *in vivo* clinical studies are needed in order to confirm efficacy of these products and to study the mode of action.

10.6 References

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CHAPTER 11

Summary and correlation of results

11.1 Study summary

Oral infections continue to affect majority of people throughout the world including South Africa.The global oral health has not improved despite intensive focus on understanding the aetiology of the two major infections, dental caries and periodontal diseases. They are the most prevalent and costly diseases. Accumulation of causative microorganisms and their metabolic byproducts lead to the development of these infections. Oral hygiene products containing antimicrobial compounds can prevent and control these infections. Plant derived chemicals are the major source of therapeutic antimicrobial compounds. Pomegranate has been identified as a source of antimicrobial compounds. It is a medicinal fruit traditionally used for many infections including oral infections. Seven pomegranate fruit cultivars (Arakta, Bhagwa, Ganesh, Herskawitz, Molla de Elche, Ruby, and Wonderful) are commercially grown in South Africa. The pomegranate peel has been found to be effective in alleviating some oral conditions. The antibacterial action, its anti-inflammatory, and antiseptic activities are attributable to the large amounts of tannins in the fruit skin (Batista *et al.,* 2014). Knowledge of phytochemicals present in this plant and their therapeutic properties as well as their additive, antagonistic or synergistic interactions are important (Wu and Tian 2017).

This study investigated the antibacterial effect of pomegranate peel against oral pathogens responsible for dental caries and periodontal diseases. Secondly, the effect of subinhibitory concentrations on the virulence factors of *S. mutans* and *P. gingivalis* were also studied. The pomegranate peel extract, was fractionated, the beneficial properties were established and the chemical constituents were identified. Lastly, the cytotoxic effect of the crude extract and the

2 subfrations on embryonic cells was evaluated. A series of objectives were drawn and the summary of results is depicted in Figure 11.1.

a) **To investigate antimicrobial effect of methanol, dichloromethane:methanol, acetone, hexane and water crude extracts of pomegranate peel and seeds against *S. mutans*, lactobacillus, *P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *Capnocytophaga*.**

The present study demonstrated that *P. granatum* Wonderful cultivar peel and seed extracts grown in the Zanddrift farm in Cape Town, South Africa has an antibacterial activity against gram positive plaque bacteria, *S. mutans*, lactobacilli as well as anaerobic periodontal pathogens, *Prevotella intermedia, Porphyromonas gingivalis, Fusobacterium nucleatum*, and Capnocytophaga species. The MBC against the cariogenic bacteria were high (chapter 3) however, the MBC values for the periodontal pathogens were good. *Porphyromonas gingivalis* was the most sensitive with the lowest MBC of 0.025 mg/ml. These findings imply that pomegranate would be more effective against periodontal pathogens compared to cariogenic bacteria which is not a miraculous result. But, these pathogens are oral human commensals that reside in harmony under homeostasis. Oral hygiene products are used not to kill all the microorganisms but to control and prevent over growth of normal flora. Primarily, cariogenic bacteria need to attach and form biofilm, and produce metabolic byproducts including acids in order to cause dental caries. These high concentrations (MBC) are difficult to maintain in the oral cavity due to the constant salivary flow. However, studies have shown that *P. granatum* can be used to control oral biofilm and hence caries (Bhadbhade *et al.,* 2011). The lack of strong antibacterial effect directed investigation towards exploring the effect of the subinhibitory concentrations on the virulence properties of these oral pathogens.

Among the seven South African cultivars tested, the peel exhibited strong antibacterial, antioxidant and tyrosinase-inhibition activities (Fawole *et al.,* 2012) which was not the case in our study. The pomegranate Wonderful fruit peel was one of the cultivars they investigated and it is for this reason that they suggested instead of the peel being wasted, it could be explored for further therapeutic properties. In this study, the seed extracts did not have a good antimicrobial effect when compared to the peel against cariogenic and periodontal pathogens. The plant extract yield was more in the peel when compared to that obtained from the seed. Therefore, the effect of *P. granatum* peel extract on virulence properties of cariogenic bacteria and periodontal pathogens was studied.

b) To determine the effect of the subinhibitory concentration of crude plant extract of the peel on *S. mutans* biofilm formation.

Tooth adherent *S. mutans* form a biofilm and community which is considered a virulence factor in the pathogenesis of dental caries. Even though the MBC against *S. mutans* was 12.5 mg/ml, there was a complete biofilm formation inhibition in all subinhibitory concentrations including the lowest concentration of 1.56 mg/ml after 6 hours. Accumulation of biofilm is due to the attachment of the microbial community on the surface and establishment of pathogenic milleu on the tooth surface. The biofilm build up leads to the development and progression of infection of the tooth. *S. mutans* as a pioneer species of this community will not be able to adhere to tooth surface in the presence of the pomegranate peel crude extract from a minimum level of 1.56 mg/ml and above. Adherence is achieved through the binding surface proteins present on the cell surface of *S. mutans*. Therefore, this plant extract may have affected the *S. mutans* binding surface proteins or the extract may have created a layer on the surface blocking the adherence sites on the acquired pellicle. Nevertheless, the inhibition of biofilm formation will provide 6 hours protection to the tooth surface which will reduce plaque and maintain good

oral hygiene. The protective effect observed initially disappeared after 24 hours. However, if this plant is used as the mouthrinse twice a day it will provide protection for up to 24 hours. While the pomegranate peel extract will have an antibacterial effect at higher concentration of 12.5 mg/ml and it will reduce the formation of biofilm at reduced concentrations.

c) To determine the effect of subinhibitory concentration of peel crude extract on the acid and extracellular polysaccharide production by *S. mutans*.

Acid production in *S. mutans* is one of the most important virulence factors that contributes towards the pathogenesis of dental caries. Significant reduction in acid production was observed at all subinhibitory concentrations. *S. mutans* metabolises carbohydrates from the diet and produces acids and extracellular polysaccharides. Acid production induces ecological shift to aciduric community and results in the release of phosphate and calcium ions from the tooth enamel (demineralisation). To eliminate the growth dependent effect on the acid production, bacterial counts were performed together with the pH measurements. The crude extract did not have any effect on the bacterial count at all tested subinhibitory concentrations. Therefore, the inhibition in the acid production observed in this study was an indication of the extract interfering with the glycolytic pathway (Embed-Meyerhof-Parnas pathway) and inhibiting the production of lactic acid. Therefore, inhibition of acid production is the therapeutic property of the plant against virulence traits of *S. mutans*. These results suggest that the extracts will not only inhibit the biofilm formation but within the biofilm there will be no acid production which creates release of phosphate and calcium ions from the enamel.

Carbohydrates such as sucrose serve as substrate for the extracellular enzymes (GTF) of plaque bacteria which synthesize sucrose-derived polymers such as soluble and insoluble extracellular

polysacharides. These EPS are produced by bacteria for extra food storage and because they are sticky, they serve for their adherence. The pomegranate plant extract significantly reduced production of insoluble EPS at all test concentrations in the biofilm form. This will have major implications because it will reduce microbial colonization, adherence through glucan production, and interfere with the stability of the biofilm. The IEPS in the planktonic growth were only reduced at higher concentrations of 3.125 and 6.25 mg/ml compared to control. However, if *P. granatum* is used as a mouthrinse it will reduce IEPS in both planktonic and biofilm formation which results in less plaque formation between oral hygiene practise. No significant effect on the soluble EPS production in the biofilm as well as in the planktonic growth was noted. Nevertheless, soluble EPS are less of the problem because of the fast clearance in the oral cavity.

d) Fractionation of crude extract of *P. granatum* and, the effect of subfractions on the biofilm formation and acid production in *S. mutans*

The pomegranate peel is the major waste product derived from the pomegranate juice industry and is a potential source of ellagitannins such as punicalagin, punicalin, ellagic acid, ellagic acid-hexosides and hexahydroxydiphenoyl (HHDP)-hexosides (Aguilar-Zarate *et al.,* 2017). The plant crude extraction was carried out using the combination of methanol and dichloromethane (1:1 ratio). This solvent combination exhibited the highest inhibition against *S. mutans.* In addition, it also produced a 1 % more extract yield compared to the methanol. Therefore, these extracts were further fractionated. A series of purification steps using liquid-liquid separation coupled with column chromatography and ultra-high performance liquid chromatography were performed. Sequential liquid-liquid extraction with hexane, ethyl acetate, methanol - water yielded more bands in the hexane extract compared to ethyl acetate and methanol:water extracts. The hexane extract was purified further using the column

chromatography and it yielded 4 fractions and 10 subfractions. All these fractions and subfractions were screened for the antimicrobial effect against *S. mutans*. The subfractions F 3.2 and F 4.1 exhibited the highest antimicrobial activity. These 2 subfractions were used for further investigations.

e) To determine the effect of subinhibitory concentration of subfractions F 3.2 and F 4.1 on the biofilm formation and acid production by *S. mutans*

The observed MBC for the subfractions F 3.2 and F 4.1 were 6.25 and 12.5 mg/ml respectively. The MBC's from subfraction F 3.2 was similar to the DCM and methanol crude extract. This implies that the effect of the purified subfractions did not improve from the crude extract. In essence, this means the bioactive compounds were still present in the purified fractions. Foss *et al* ., (2014) had also shown similar findings with both crude extract and fractionated punicalagin exhibiting identical antifungal activity against *T. rubrum* (Foss *et al*., 2014) Moreover, the effect of bioactive compounds probably was not influenced by a synergistic effect while in the crude extract and there was no improvement in the activity due to the purification. Nevertheless MBC's of the subfractions were used to determine subinhibitory concentrations. These were then used to study the retained beneficial effects such as inhibition of biofilm formation and acid production in *S. mutans*.

The subfraction F 3.2 significantly reduced biofilm formation after 6 hours at the MBC concentrations of 6.25 mg/ml. At subinhibitory concentrations, the observed inhibition effect was 14 % and less. Significant effect on biofilm formation was also observed at the MBC of 12.5 mg/ml and the subinhibitory concentration of 6.25 mg/ml with the subfraction F 4.1 after 6 hours of incubation. The crude extract significantly reduced biofilm formation by 95 % at the minimal subinhibitory concentration tested of 1.56 mg/ml after 6 hours. Meaning, the crude extract had strongest inhibitory effect at the lowest subinhibitory concentrations tested and that

effect was reduced with the subfrations. The subfraction F 3.2 and F 4.1 will still protect the teeth from plaque formation and still maintain a good oral hygiene. At high concentrations these subfractions will have an antibacterial effect and as they become diluted with saliva, they will prevent the *S. mutans* attachment to the tooth surface for up to 6 hours providing protection.

Significant reduction in acid production was observed at the MBC and subinhibitory concentrations with subfraction F 3.2. When the bacterial counts in the acid assay were investigated, significant reduction was observed in all tested concentrations. The subfraction F 4.1 had significant reduction in acid production at all test concentrations. The bacterial count was also reduced significantly. This then implies that there was significant inhibition in acid production together with antibacterial effect. Indeed, the subfraction F 3.2 and F 4.1 will protect the enamel from demineralisation due to the acids produced through fermentation of sugars by *S. mutans*.

f) To study the effect of subinhibitory concentration of peel crude extract on the proteinase production by *P. gingivalis*.

Porphyromonas gingivalis (*Pg*), is considered as the major periodontal pathogen and is the keystone pathogen which elevates the virulence of the entire microbial community. It expresses a variety of virulence factors to cripple the immune system and causes tissue destruction (Bostanci and Belibasakis 2012). Its pathogenicity has been attributed to a number of virulence factors, including proteases, fimbriae, lipopolysaccharide and hemagglutinin (Yamanaka *et al.*, 2007). The most potent adhesins and virulence factors of *Pg* located within the fimbriae are the gingipains. The crude extract and the subfractions inhibited the growth of *Pg*. At subinhibitory concentrations crude extract reduced Arg and Lys-gingipain proteolytic activity. The reduction

in proteolytic activity was better in the Lys-gingipain compared to the Arg-gingipain. The increased Lys-gingipain reduction was 83 % by crude extract. Therefore, *P. granatum* crude extract as well as the subfractions will reduce the pathogenicity of the *P. gingivalis* in periodontal diseases. It has potential to be developed into therapeutic agent in the prevention and treatment of periodontal disease.

The pomegranate crude extract has a potential to be used as a therapeutic agent against dental caries and periodontal pathogens. At high concentrations it will have an antimicrobial effect against dental caries and periodontal pathogens. Due to constant flow of saliva, high concentrations will become diluted and at these lower concentrations, the pomegranate crude extract and subfractions will not allow attachment of *S. mutans* to the tooth surface. It will prevent production of acid from metabolism of carbohydrates. It will prevent production of IEPS which play a major role in the plaque formation and providing nutrients to plaque bacteria. It will prevent the growth of *P. gingivalis* and its proteolytic activity. *P. granatum* can positively influence all the pathogenic factors required for development of dental caries and periodontal diseases.

g) To perform phytochemical analysis on peel crude extract to isolate and identify major chemicals and identify the chemical/s responsible for the beneficial effects.

The two subfractions were subjected to structural elucidation using UHPLC-HRMS. From the mass spectroscopy, the majority of compounds present in the subfractions belonged to the hydrolyzable tannins (ellagitannins and gallotannins) and the anthocyanins group which are known as the major bioactive compounds in the pomegranate peel. The importance of ellagitannins resides in their biological properties such as anti-inflammatory, anti-carcinogenic, antioxidant, antimicrobial, antitumor and chemotherapy. Two compounds present were

subjected to structural characterization by NMR. These compound were APG 2 and APG 6. They were identified as β-sitosterol and galloyl-HHDP-hexoside for APG 2 (414 m/z) and APG 6 (635 m/z) respectively. The galloyl-HHDP-hexoside was present in both subfractions when accessed using HRMS, while the β-sitosterol was only identified in F 4.1.

Galloyl-HHDP-hexoside, a hydrolysed derivative of gallotannins is known to be present in pomegranate juice and husk (Aguilar-Zarate *et al.,* 2017, Lantzouraki *et al.,* 2015, Mena *et al.,* 2012). However, the antimicrobial and anticariogenic property of this compound is not known which was shown here. β-sitosterol is known to be present in pomegranate juice and seeds. It is known to cause uterine contraction and, attenuation of macrophage foam cell formation and atherogenesis (Promprom *et al.,* 2010, Rosenblat *et al.,* 2013). The compound β-sitosterol has been previously identified in the active n-hexane *Picea abies* bark extract using GCMS and that extract was found to be highly effective compared to the ethanol extract (Burcova *et al.,* 2018). This extract was tested against *Escherichia coli* (CCM 3988), *Bacillus cereus, Pseudomonas aeruginosa Bacillus subtilis, Staphylococcus aureus* and *Listeria monocytogenes*. In another study, β-sitosterol from the mince of *Schimpera arabica* leaves and stems methanol crude followed by sequential liquid-liquid n-hexane solvent extraction demonstrated a significant antioxidant as well as antibacterial effect (Hidayathulla *et al.,* 2018). They found β-sitosterol to be the major constituent in the hexane fraction. It demonstrated the most antibacterial effect together with the ethyl acetate extract on *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli*. The β-sitosterol also alleviates apoptosis and has anti-inflammatory activity in Alzheimer's disease (Lee *et al.,* 2018). Moreover, a liver with an induced injury showed improvement in its function, relieving the pathological changes of liver tissue, decreasing the level of pro-inflammatory cytokines,

and thus balancing the pro- and anti-inflammatory cytokines (Yang *et al.*, 2018). Nevertheless, the anticariogenic and antiperiodontal activity of β-sitosterol is not known.

When the efficacy of the two isolated compounds is compared, galloyl-HHDP-hexoside (F 3.2) seems to be better against cariogenic properties whereas the mixture of galloyl-HHDP-hexoside and β-sitosterol (F 4.1) showed better activity against periodontal pathogens (Table 11.1). β-sitosterol present in F 4.1 may have improved the activity against periodontal pathogen. The cytotoxicity results were also better with F 4.1 compared to the F 3.2. However, this viability was tested after 24 hours and in the oral cavity these compounds do not last for long due to the constant salivary flow. In addition, anticariogenic and antiperiodontal compound containing products are proposed to be used topically rather than systemically. Therefore these compounds will be safe to use in the oral cavity to prevent and control these two infections.

Table 11.1 Comparison of subfractions isolated from *P. granatum* peel

Test	**F 3.2 (galloyl-HHDP-hexoside)**	**F 4.1 (galloyl-HHDP-hexoside + β-sitosterol)**	**Interpretation (better subfraction)**
Anticariogenic			
MBC	6.25 mg/ml	12.5 mg/ml	F 3.2
Biofilm inhibition	At 6.25 mg/ml	At 12.5, 6.25 mg/ml	F 3.2
Acid inhibition	At all conc.	At all conc.	Both the same
Anti-periodontal			
Arg-protease reduction	Low %	High %	F 4.1
Lys-protease reduction	Low %	High %	F 4.1
Cytotoxicity (1.6 mg/ml)	24 % cells viable	58 % cells viable	F 4.1

The galloyl-HHDP-hexoside also has an anti-inflammatory, antitumor and apoptotic properties. Research has found the HHDP-galloyl glucose in green tea as one of the compounds responsible for the α glucosidase inhibition activity (Guo *et al.,* 2018). A polyphenolic compound penta-O-galloyl-β-d-glucose has been found to induce cytotoxicity and decrease proliferation of cancer cancer cells without affecting normal colon fibroblast cells (Kawk *et al.,* 2018). They also discovered that this compound induced p53 expression.

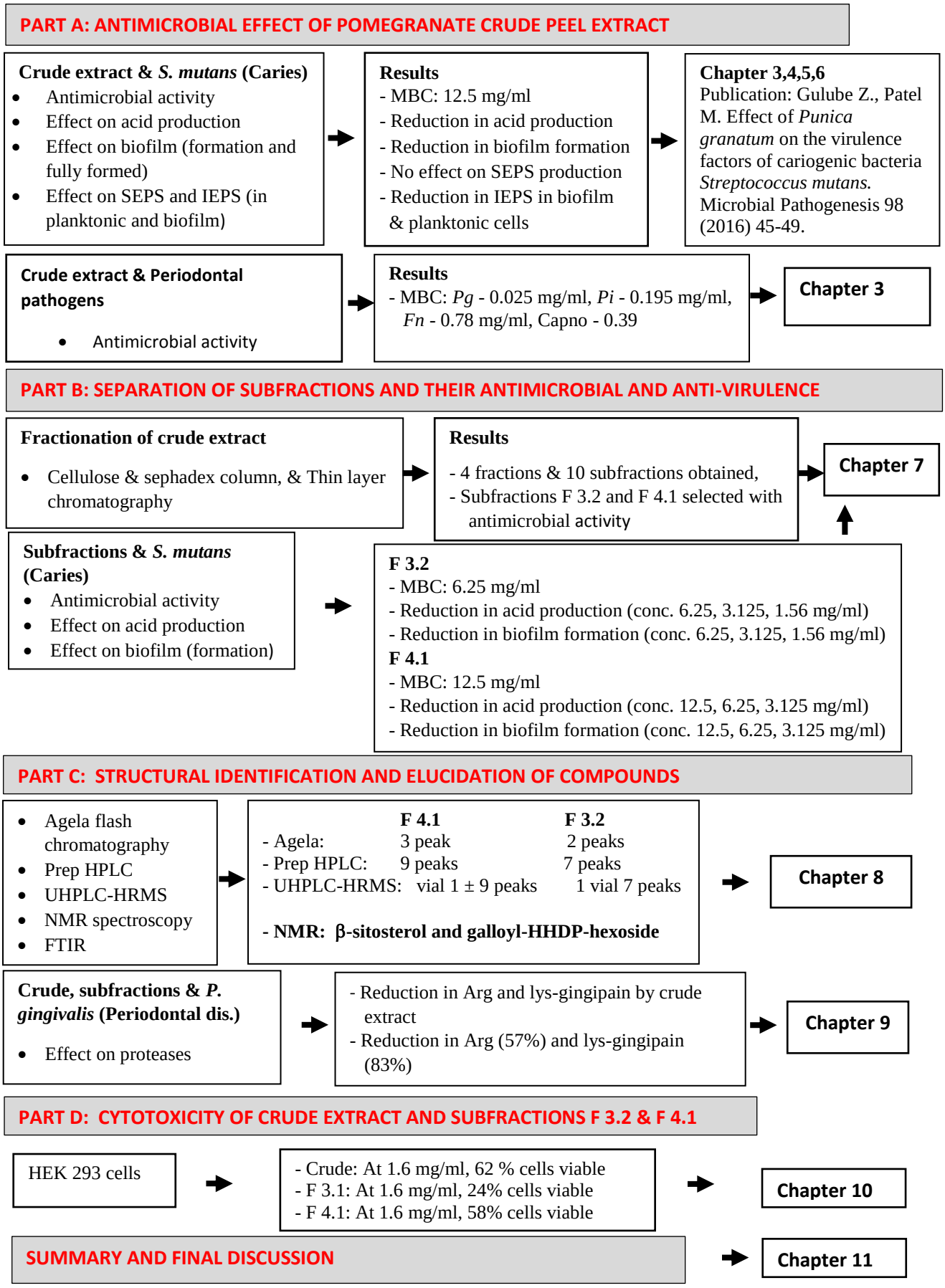
h) To determine the cytotoxicity of peel crude extract and subfractions F 3.2 and F 4.1.

Lack of toxicity of crude peel extract of *Punica granatum,* F 3.2 and F 4.1 at subinhibitory concentrations demonstrated enhanced cell proliferation against HEK 293 cells at low concentrations. Pomegranate plant crude extract and its purified subfractions acted as cell stimulant and influenced cell viability when compared to control in the range of 0.1 to 0.001 mg/ml. At the lowest beneficial concentration (1.56 mg/ml) for the subfractions, the cell viability was more 24 – 50 %. Nevertheless these compounds are exposed briefly to the oral mucosa whereas the test exposures were 24 hours. Therefore, the crude extract and the subfractions will be safe to use.

This plant and its subfractions will have major implications in dental caries and periodontal disease. At higher concentrations it will reduce microbial colonization, adherent glucan production, and the interference with the stability biofilm formation which is the primary requirements for the development of both dental caries and periodontal diseases. Admittedly, therapeutic chemicals used in the oral cavity are mouth rinse and toothpastes based. The crude extract and its subfractions if used as mouth rinse and tooth paste, will be absorbed minimally by the mucosa. More and detailed *in vivo* clinical studies are needed in order to confirm efficacy of these products and to study the mode of action. Simultaneously, at the concentration of 6.25

mg/ml, *P. granatum* will reduce biofilm formation and inhibit acid production by *S. mutans*. The constant shedding and less absorption in the oral mucosa makes the MBC of the pomegranate plant safe to use as brief topical application such as in the form of mouth rinse or toothpaste.

Figure 11.1 Study summary



11.2 Future research

- Miscellaneous phytochemicals with bioactivity have been identified in the pomegranate peel as well as inconsistencies regarding their presence/absence in different cultivars have been reported (Abachi *et al.,* 2016, Wu and Tian 2017). Therefore, attempts to obtain all the compounds from preparative HPLC and the UHPLC-HRMS peaks in good quantities for structural elucidation can be pursued. This will enable better understanding on the composition of the South African cultivars and its bioactivity.
- There was no improvement on the antimicrobial activity by the hexane purified subfractions when compared to the crude extract. Previously, a β-sitosterol isolated from hexane extract of a spruce bark had revealed a synergistic and bacteriostatic effect (Burcova *et al.,* 2018). Therefore, any synergistic, additive or antagonist influence among the bioactive compounds from *P. granatum* can be investigated.
- Ethyl acetate sequential extract purification and identification of the active metabolites.
- *Streptococcus mutans* strive and form organised microcolonies and became the major species in the mature biofilm with multiple species. Therefore, ideally mixflora should be used in this type of studies. However, in literature there are many antimicrobial studies that have used monoculture of *S. mutans* (Koo *et al.,* 2006, Ngabaza *et al.,* 2018, Patel *et al.,* 2013) because it is the pioneer species. Mix flora and saliva can be used to perform this type of studies which may provide more information.
- This study was an in vitro study. It the oral cavity this compounds may behave differently and therefore in vivo studies can be performed. Pomegranate rinse consisting of 30 % punicalagins demonstrated antimicrobial effect against periodontal pathogens and had no adverse effect in vivo (Bhadbhade *et al.,* 2011). Therefore similar studies can be done to determine the effect of the compounds from the locally produced cultivars.
- Use of buccal mucosa and epithelial cells in the cytoxicity study.

11.3 Conclusion

At high concentrations crude extracts of *P. granatum* inhibited the growth of cariogenic bacteria and at sub-inhibitory concentrations it inhibited the biofilm formation and, acid and extracellular polysaccharides production in *S. mutans*. Fractionation of the crude extract produced 2 bioactive subfractions F 3.2 and F 4.1. Both subfractions inhibited the *S. mutans* growth at levels equivalent to the crude extract. In addition, both subfractions retained the original activities of crude extract such as inhibition of biofilm formation and acid production which was shown in the crude extract. The crude extract, subfractions F 3.2 and F 4.1 inhibited growth of *P. gingivalis* and reduced its protease activity. The crude extract and subfractions F 4.1 showed more than C_{50} viability of HEK 293 cells at 1.6 mg/ml concentration while for subfraction F 3.2 this was observed at 0.8 mg/ml. Subfraction F3.2 was identified as galloyl-HHDP-hexoside whereas F4.1 contained β- sitosterol and a galloyl-HHDP-hexoside. These compounds present in the South African pomegranate cultivar – Wonderful showed anticariogenic and anti-periodontal pathogen activity. Therefore, *P. granatum* peel extract and extract derived chemical compounds can be used in the oral hygiene formulation to prevent dental caries and periodontal diseases. The findings provide scientific basis to promote value-adding of pomegranate fruit peel for pharmaceutical purposes. Further studies on the analysis and identification of other compounds present in the subfractions, investigation of their antimicrobial activity and search for ingredients for bioactivity needs to be carried out.

11.4 Limitations

- Ideally the effect of *P. granatum* on *S. mutans* should be studied in the presence of mix flora and saliva, however most studies are performed on monoculture including this study.
- Hydroxyapatite discs can be used instead of glass surface which was not possible due to the lack of availability. However, in future studies discs cut from animal teeth will be used.
- Accumulation of good quantities of purified samples was a challenge due to constant break-up of machines.
- Combination of compounds in subfractions might improve the bioactivity observed since the subfractions did not perform any better compared to the crude in this study.

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APPENDIX 1

Media composition and preparations

Api 20 Strep

API 20 Strep strip

API GP Medium: 2 ml L-cystine

0.5 g Tryptone (bovine/porcine origin)

20 g Sodium chloride

5 g Sodium sulfite

0.5 g Phenol red 0.17 g

Demineralized water to make 1000 ml pH: 7.4 - 7.6

Bacitracin:

0.01 g bacitracin

100 ml distilled water

Filter sterilize

Nα-Benzoyl-L-Arginine 4-nitroalilide hydrochloride (BAPNA)

0.02 g BAPNA

100 ml distilled water

N-(p-Tosyl) – Gly-Pro-Lys 4-nitroanilide acetate salt

0.03 g N - (p - Tosyl) – Gly – Pro - Lys 4 - nitroanilide acetate salt

100 ml distilled water

Blood agar plates

42.5 g Colombia base agar

1000 ml water

Dissolve by stirring and heating

Autoclave at 121^0C, 150 kPa for 10 minutes

Allow media to cool to 50^0 C and add 5 % sterile defibrinated blood

Pour plates, allow to set and refrigerate

Dithiothreitol (DTT)

0.154 g DTT

1000 ml distilled water

Human cell culture medium

88 % Dulbeco's modified eagle's medium

10 % Fetal calf serum

1 % L-glutamine

1 % Streptomycin

Mutans bacitracin agar (MBA)

90g MSA (mitis salivarius agar)

85g Sucrose

1000 ml water

Dissolve by boiling

Autoclave at 121^0 C for 15 minutes, Cool to 55^0 C

Add 0.25 ml Potasium tellurite in 250 ml media

2.5 ml Bacitracin Swirl & pour plates

Rogosa agar

75 g Rogosa agar

1000 ml sterile distilled water

Heat to dissolve

Cool and add 10 ml of 10 % tween 80

Add 1.32 ml acetic acid

Heat to 90^0 C for 2 minutes

Do not autoclave

Allow to cool and pour plates

5 % Sucrose broth

10g tryptone

5 g protease peptone no.3

5 g protease peptone

1 g glucose

50 g sucrose

4 g diphotassium phosphate

1000 ml distilled water

Autoclave at 150 kPa, 121^0 C for 10 minutes

Tryptone broth

12.5 g tryptone powder

7.5 g yeast extract

5 g sucrose

500 ml distilled water

Adjust media to pH 7.0

Autoclave at 150 kPa, 121^0 C for 10 minutes

Tryptone soya broth

30 g Tryptone soya broth

500 ml distilled water

Autoclave at 150 kPa, 121^0 C for 10 minutes

1x Phosphate buffered saline

8 g sodium chloride

0.24 g potassium hydrogen phosphate

0.2 g potassium chloride

1.44 g sodium hydrogen phosphate

800 ml distilled water

Autoclave at 150 kPa, 121^0 C for 10 minutes

Potassium tellurite

1g Potassium tellurite

100ml distilled water, filter sterilize

Use 1ml per litre

Proteinase K

0.01 g proteinase K

100 ml distilled water

APPENDIX 2

Statistical analysis

2.1 *Punica granatum* crude extract biofilm formation assay

anova lncount time treat

```
                   Number of obs =      90     R-squared     =  0.3854
                   Root MSE      = 1.53475     Adj R-squared =  0.3639
      Source |  Partial SS    df       MS          F     Prob > F
-----------+----------------------------------------------------
       Model |  127.000718     3  42.3335727      17.97     0.0000
             |
        time |  104.629074     2  52.3145368      22.21     0.0000
       treat |  22.3716445     1  22.3716445      9.50     0.0028
             |
    Residual |  202.568335    86  2.35544576
-----------+----------------------------------------------------
       Total |  329.569053    89  3.70302307
```

oneway lncount time, bonferroni

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	104.629074	2	52.3145368	20.23	**0.0000**
Within groups	224.93998	87	2.58551701		
Total	329.569053	89	3.70302307		

Bartlett's test for equal variances: chi2(2) = 6.2089 Prob>chi2 = 0.045

Comparison of lncount by time

(Bonferroni)

Row Mean-| Col Mean	6	24
24	2.61579	
	0.000	
30	1.62361	-.992177
	0.001	0.057

2.2 *Punica granatum* crude extract acid production

```
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Zandi.log
  log type:  text
 opened on:   6 Mar 2014, 13:01:44

. use "Zandi.dta"

.
.
. label define testlab 0 "Control" 1 "Test"

. label values test testlab

.
.
. *? Difference between C and T in pH combined all concentrations
. sum ph, detail

                            pH
-------------------------------------------------------------
      Percentiles      Smallest
 1%           4.2           4.2
 5%          4.43           4.2
10%          4.53           4.2       Obs                 360
25%         4.825           4.2       Sum of Wgt.         360

50%         5.575                     Mean           5.737889
                        Largest       Std. Dev.      .9816892
75%             7           7.04
90%             7           7.04      Variance       .9637136
95%             7           7.05      Skewness        .107272
99%          7.04           7.05      Kurtosis        1.44823

. ranksum ph, by(test)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs    rank sum    expected
-------------+---------------------------------
     Control |      180       28098       32490
        Test |      180       36882       32490
-------------+---------------------------------
    combined |      360       64980       64980

unadjusted variance   974700.00
adjustment for ties   -15283.33
                      ----------
adjusted variance     959416.67

Ho: ph(test==Control) = ph(test==Test)
            z =  -4.484
    Prob > |z| =   0.0000

.
. *DIFFERENCE HIGHLY SIGNIFICANT, WE LOOK AT INDIVIDUAL CONCENTRATIONS
.
. bysort concmgml:ranksum ph, by(test)

-------------------------------------------------------------------------------
-> concmgml = 1.56
```

```
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs    rank sum    expected
-------------+---------------------------------
     Control |       60        3252        3630
        Test |       60        4008        3630
-------------+---------------------------------
    combined |      120        7260        7260

unadjusted variance    36300.00
adjustment for ties     -568.11
                       ---------
adjusted variance      35731.89

Ho: ph(test==Control) = ph(test==Test)
             z =  -2.000
    Prob > |z| =   0.0455

-------------------------------------------------------------------------------
-> concmgml = 3.125

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs    rank sum    expected
-------------+---------------------------------
     Control |       60      3173.5        3630
        Test |       60      4086.5        3630
-------------+---------------------------------
    combined |      120        7260        7260

unadjusted variance    36300.00
adjustment for ties     -568.74
                       ---------
adjusted variance      35731.26

Ho: ph(test==Control) = ph(test==Test)
             z =  -2.415
    Prob > |z| =   0.0157

-------------------------------------------------------------------------------
-> concmgml = 6.25

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs    rank sum    expected
-------------+---------------------------------
     Control |       60      2962.5        3630
        Test |       60      4297.5        3630
-------------+---------------------------------
    combined |      120        7260        7260

unadjusted variance    36300.00
adjustment for ties     -571.01
                       ---------
adjusted variance      35728.99

Ho: ph(test==Control) = ph(test==Test)
             z =  -3.531
    Prob > |z| =   0.0004

.
. *ALL ARE SIGNIFICANT BUT THERE IS A TREND IN THE P VALUES, IT GETS LOWER WITH
```

HIGHER CONCENTRATIONS

```
. *? Difference between C and T in counts combind all concentrations
.
. sum countscfuml, detail

                        counts (cfu/ml)
-------------------------------------------------------------
      Percentiles      Smallest
 1%       490000         200000
 5%      2095000         200000
10%      3675000         200000       Obs                 360
25%      6975000         490000       Sum of Wgt.         360

50%     2.61e+07                      Mean           7.90e+07
                        Largest       Std. Dev.      1.49e+08
75%     6.49e+07       7.59e+08
90%     2.25e+08       7.59e+08       Variance       2.21e+16
95%     4.54e+08       7.59e+08       Skewness       3.108487
99%     7.59e+08       8.40e+08       Kurtosis       12.82857

.
. ranksum countscfuml, by(test)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs     rank sum    expected
-------------+---------------------------------
     Control |      180        33663       32490
        Test |      180        31317       32490
-------------+---------------------------------
    combined |      360        64980       64980

unadjusted variance   974700.00
adjustment for ties      -32.34
                     ----------
adjusted variance     974667.66

Ho: counts~l(test==Control) = counts~l(test==Test)
             z =   1.188
    Prob > |z| =   0.2348

.
. *COUNT NOT SIGNIFICANT
.
. bysort concmgml:ranksum countscfuml, by(test)

-------------------------------------------------------------------------------
-> concmgml = 1.56

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs     rank sum    expected
-------------+---------------------------------
     Control |       60       3645.5        3630
        Test |       60       3614.5        3630
-------------+---------------------------------
    combined |      120         7260        7260

unadjusted variance    36300.00
adjustment for ties       -0.38
```

```
                           ---------
adjusted variance      36299.62

Ho: counts~l(test==Control) = counts~l(test==Test)
             z =   0.081
    Prob > |z| =   0.9352

-------------------------------------------------------------------------------
-> concmgml = 3.125

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs    rank sum    expected
-------------+---------------------------------
     Control |       60        3812        3630
        Test |       60        3448        3630
-------------+---------------------------------
    combined |      120        7260        7260

unadjusted variance    36300.00
adjustment for ties       -0.38
                       ---------
adjusted variance      36299.62

Ho: counts~l(test==Control) = counts~l(test==Test)
             z =   0.955
    Prob > |z| =   0.3394

-------------------------------------------------------------------------------
-> concmgml = 6.25

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

        test |      obs    rank sum    expected
-------------+---------------------------------
     Control |       60      3804.5        3630
        Test |       60      3455.5        3630
-------------+---------------------------------
    combined |      120        7260        7260

unadjusted variance    36300.00
adjustment for ties       -1.01
                       ---------
adjusted variance      36298.99

Ho: counts~l(test==Control) = counts~l(test==Test)
             z =   0.916
    Prob > |z| =   0.3597

.
. *NO SIGNIFICANT DIFFERENCE IN COUNT AT VARIOUS CONCENTRATIONS
.
.
.
.
.

. set more on

. log close
      name:  <unnamed>
```

2.3 *Punica granatum* crude extract extracellular polysaccharides production assay

log: C:\Users\zandi\Documents\Analysis\analysis.log
opened on: 6 May 2014, 16:11:50.
. use "C:\Users\zandi\Desktop**biofilm seps 1 56mg.**dta", clear
. ranksum control, by (group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

group	obs	rank sum	expected
0	15	244.5	232.5
1	15	220.5	232.5

Prob > |z| = **0.6182**

. use "C:\Users\zandi\Desktop**plank seps 6 25mg**.dta", clear
. ranksum control, by(group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

group	obs	rank sum	expected
0	15	264.5	232.5
1	15	200.5	232.5

Prob > |z| = **0.1843**

. use "C:\Users\zandi\Desktop**plank seps 3 125mg**.dta", clear
. ranksum control, by(group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

group	obs	rank sum	expected
0	15	246	232.5
1	15	219	232.5

Prob > |z| = **0.5755**

. use "C:\Users\zandi\Desktop**plank seps 1 56mg**.dta", clear
. ranksum control, by(group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

group	obs	rank sum	expected
0	15	255	232.5
1	15	210	232.5

Prob > |z| = **0.3506**

. use "C:\Users\zandi\Desktop**biofilm ieps 6 25mg**.dta", clear
. ranksum control, by(treatment)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

treatment	obs	rank sum	expected
0	15	327	232.5
1	15	138	232.5

Prob > |z| = **0.0001**

. use "C:\Users\zandi\Desktop\biofilm **3 125mg ieps.**dta", clear

```
. ranksum  control, by( treatment)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test
  treatment |      obs    rank sum    expected
------------+---------------------------------
          0 |       15      300.5       232.5
          1 |       15      164.5       232.5
------------+---------------------------------
   Prob > |z| =   0.0048

. use "C:\Users\zandi\Desktop\biofilm ieps 1 56mg 2.dta", clear
. ranksum  control, by( group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test
      group |      obs    rank sum    expected
------------+---------------------------------
          0 |       15         305       232.5
          1 |       15         160       232.5
------------+---------------------------------

   Prob > |z| =   0.0026

-------------------------------------------------------------

. use "C:\Users\zandi\Desktop\plank ieps 6 25mg.dta", clear
. ranksum  control, by( group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test
      group |      obs    rank sum    expected
------------+---------------------------------
          0 |       15         328       232.5
          1 |       15         137       232.5
------------+---------------------------------
   Prob > |z| =   0.0001

. use "C:\Users\zandi\Desktop\3 125 IEPS PLANK.dta", clear
. ranksum  control, by( group)
Two-sample Wilcoxon rank-sum (Mann-Whitney) test
      group |      obs    rank sum    expected
------------+---------------------------------
          0 |       15       312.5       232.5
          1 |       15       152.5       232.5
------------+---------------------------------
   Prob > |z| =   0.0009

. log close
      name:  <unnamed>
       log:  C:\Users\zandi\Documents\Analysis\analysis.log
  log type:  text
 closed on:   6 May 2014, 16:11:50
-------------------------------------------------------------------------------------------------------------------
```

2.4 Subfractions F 3.2 biofilm formation

```
Welcome to Minitab, press F1 for help.
Retrieving project from file: 'C:\Users\00300399\Documents\My
Documents1\Study 2012\2018 SUBFRACTION STATS\Minitab BIOFIM F3.2 mpj.mpj'
```

Results for: Worksheet 4

Wilcoxon Signed Rank Test: diff

F 3.2 BIOFILM 1.56 mg/ml 6 HOURS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
diff  12     11       43.0  0.398     202500
```

Results for: Worksheet 5

Wilcoxon Signed Rank Test: DIFF

F 3.2 BIOFILM 1.56 mg/ml 24 HOURS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
DIFF  12     12       47.0  0.556     442500
```

Results for: Worksheet 6

Wilcoxon Signed Rank Test: diff

F 3.2 BIOFILM 3.125 mg/ml 6 HOURS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
diff  12     12       35.0  0.784     -70000
```

Results for: Worksheet 7

Wilcoxon Signed Rank Test: DIFF

F 3.2 BIOFILM 3.125 mg/ml 24 HOURS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
DIFF  12     11       34.0  0.965      32500
```

Results for: Worksheet 8

Wilcoxon Signed Rank Test: DIFF

F 3.2 BIOFILM 6.25 mg/ml 6 HOURS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
DIFF  12     12        7.0  0.013    -787500
```

Wilcoxon Signed Rank Test: diff

F 3.2 BIOFILM 6.25 mg/ml 24 HOURS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
diff  12     12       42.0  0.845      81750
```

2.5 Subfractions F 4.1 biofilm formation

Results for: Worksheet 4

Wilcoxon Signed Rank Test: DIFFERENCE

F 4.1 6HRS 3.125 mg/ml BIOFILM mtw

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
DIFFERENCE  12     12       35.0  0.784     -50000
```

Results for: Worksheet 5

Wilcoxon Signed Rank Test: DIFFERENCE

F 4.1 24HRS 3.125mg/ml BIOFILM mtw

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
DIFFERENCE  12     12       12.0  0.038    -825000
```

Wilcoxon Signed Rank Test: difference

F 4.1 BIOFILM 6 HOURS 6.25 MG/ML

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
difference  12     12        0.0  0.003   -1087500
```

Results for: Worksheet 2

Wilcoxon Signed Rank Test: DIFFERENCE

F 4.1 BIOFILM 24 HOURS 6.25 MG/ML

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
DIFFERENCE  12     12       38.0  0.969     -11000
```

Results for: Worksheet 3

Wilcoxon Signed Rank Test: difference

F 4.1 BIOFILM 6 HOURS 12.5 MG/ML

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
difference  12     12        0.0  0.003   -1767000
```

Results for: Worksheet 4

Wilcoxon Signed Rank Test: DIFFERENCE

F 4.1 BIOFILM 24 HOURS 12.5 MG/ML

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
DIFFERENCE  12     12       34.0  0.724    -168350
```

2.6 Subfractions F 3.2 acid production assay

```
Welcome to Minitab, press F1 for help.
```

Wilcoxon Signed Rank Test: DIFF

ACID PRODUCTION F 3.2 ALL CONCENTRATION 10HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
          N for   Wilcoxon         Estimated
       N   Test  Statistic      P     Median
DIFF  27     27      378.0  0.000      1.037
```

Results for: Worksheet 2

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 ALL CONCENTRATION 12 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
D	27	27	378.0	**0.000**	1.308

Results for: Worksheet 3

Wilcoxon Signed Rank Test: diff

ACID PRODUCTION F 3.2 ALL CONCENTRATION 14 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
diff	27	27	378.0	**0.000**	1.550

Results for: Worksheet 4

Wilcoxon Signed Rank Test: d

ACID PRODUCTION F 3.2 ALL CONCENTRATION 16 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
d	27	27	378.0	**0.000**	1.465

Results for: Worksheet 5

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 1.56MG/ML 10 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
D	9	9	45.0	**0.009**	0.7000

Results for: Worksheet 6

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 1.56MG/ML 12 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
D	9	9	45.0	**0.009**	0.8800

Results for: Worksheet 7

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 1.56MG/ML 14 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
d	9	9	45.0	**0.009**	0.8950

Results for: Worksheet 8

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 1.56MG/ML 16 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFF	9	9	45.0	**0.009**	0.8050

Results for: Worksheet 9

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 3.125 MG/ML 10 HRS

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
D	9	9	45.0	**0.009**	0.9950

Results for: Worksheet 10

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 3.125 MG/ML 12 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon         Estimated
      N   Test  Statistic      P     Median
DIFF  9      9       45.0  0.009      1.250
```

Results for: Worksheet 11

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 3.125 MG/ML 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009      1.455
```

Results for: Worksheet 12

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 3.125 MG/ML 16 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
d  9      9       45.0  0.009      1.270
```

ACID PRODUCTION F 3.2 6.25 MG/ML 10 HRS

Wilcoxon Signed Rank Test: D

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009      1.735
```

Results for: Worksheet 14

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 6.25 MG/ML 12 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon         Estimated
      N   Test  Statistic      P     Median
DIFF  9      9       45.0  0.009      1.915
```

Results for: Worksheet 15

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 6.25 MG/ML 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009      2.335
```

Results for: Worksheet 16

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 3.2 6.25 MG/ML 16 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
              N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
DIFFERENCE  9      9       45.0  0.009      2.300
```

2.7 Subfractions F 3.2 acid bacterial count

Wilcoxon Signed Rank Test: DIFFERENCE

F 3.2 ACID COUNT 10HRS ALL CONCENTRATION

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFFERENCE	27	27	0.0	**0.000**	-101702500

Results for: Worksheet 2

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 14 HRS ALL CONCENTRATION

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFF	27	27	0.0	**0.000**	-268333750

Results for: Worksheet 3

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 10 HRS 1.56 MG/ML

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFF	9	9	0.0	**0.009**	-97540000

Results for: Worksheet 4

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 14 HRS 1.56 MG/ML

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFFERENCE	9	9	0.0	**0.009**	-268125000

Results for: Worksheet 5

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 10 HRS 3.125 MG/ML

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFF	9	9	0.0	**0.009**	-107090000

Results for: Worksheet 6

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 14 HRS 3.125 MG/ML

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
DIFF	9	9	0.0	**0.009**	-266225000

Results for: Worksheet 7

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 10 HRS 6.25 MG/ML

Test of median = 0.000000 versus median ≠ 0.000000

	N	N for Test	Wilcoxon Statistic	P	Estimated Median
D	9	9	0.0	**0.009**	-108230000

Results for: Worksheet 8

Wilcoxon Signed Rank Test: D

F 3.2 ACID COUNT 10 HRS 6.25 MG/ML

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon          Estimated
      N   Test  Statistic      P      Median
DIFF  9      9        0.0  0.009  -279950000
```

2.8 Subfractions f 4.1 acid production assay

Wilcoxon Signed Rank Test: difference

ACID PRODUCTION F 4.1 ALL CONC. 10 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
               N for   Wilcoxon          Estimated
            N   Test  Statistic      P     Median
difference  27     27      378.0  0.000      1.267
```

Results for: Worksheet 2

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 4.1 ALL CONC. 12 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
       N for   Wilcoxon          Estimated
    N   Test  Statistic      P     Median
D  27     27      378.0  0.000      1.547
```

Results for: Worksheet 3

Wilcoxon Signed Rank Test: d

ACID PRODUCTION F 4.1 ALL CONC. 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
       N for   Wilcoxon          Estimated
    N   Test  Statistic      P     Median
d  27     27      378.0  0.000      1.690
```

Results for: Worksheet 4

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 4.1 ALL CONC. 16 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
       N for   Wilcoxon          Estimated
    N   Test  Statistic      P     Median
D  27     27      378.0  0.000      1.560
```

Results for: Worksheet 5

Wilcoxon Signed Rank Test: d

ACID PRODUCTION F 4.1 3.125mg/ml 10 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon          Estimated
   N   Test  Statistic      P     Median
d  9      9       45.0  0.009     0.5350
```

Results for: Worksheet 6

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 4.1 3.125mg/ml 12 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon          Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009     0.7100
```

Results for: Worksheet 7

Wilcoxon Signed Rank Test: d

ACID PRODUCTION F 4.1 3.125mg/ml 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
d  9      9       45.0  0.009     0.8400
```

Results for: Worksheet 8

Wilcoxon Signed Rank Test: D

ACID PRODUCTION F 4.1 3.125mg/ml 16 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009     0.7850
```

Wilcoxon Signed Rank Test: d

Acid production F 4.1 6.25 mg/ml 10 hrs

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
d  9      9       45.0  0.009      1.155
```

Results for: Worksheet 2

Wilcoxon Signed Rank Test: D

Acid production F 4.1 6.25 mg/ml 12 hrs

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009      1.305
```

Results for: Worksheet 3

Wilcoxon Signed Rank Test: DIFF

Acid production F 4.1 6.25 mg/ml 14 hrs

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon         Estimated
      N   Test  Statistic      P     Median
DIFF  9      9       45.0  0.009      1.255
```

Results for: Worksheet 4

Wilcoxon Signed Rank Test: diff

Acid production F 4.1 6.25 mg/ml 16 hrs

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon         Estimated
      N   Test  Statistic      P     Median
diff  9      9       45.0  0.009      1.055
```

Wilcoxon Signed Rank Test: DIFF

F 4.1 ACID PRODUCTION 12.5 MG/ML 10 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon         Estimated
      N   Test  Statistic      P     Median
DIFF  9      9       45.0  0.009      2.070
```

Results for: Worksheet 2

Wilcoxon Signed Rank Test: D

F 4.1 ACID PRODUCTION 12.5 MG/ML 12 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       45.0  0.009      2.560
```

Results for: Worksheet 3

Wilcoxon Signed Rank Test: diff

F 4.1 ACID PRODUCTION 12.5 MG/ML 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon         Estimated
      N   Test  Statistic      P     Median
diff  9      9       45.0  0.009      2.730
```

Results for: Worksheet 4

Wilcoxon Signed Rank Test: DIFFERENCE

F 4.1 ACID PRODUCTION 12.5 MG/ML 16 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
              N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
DIFFERENCE  9      9       45.0  0.009      2.615
```

2.9 Subfractions F 4.1 acid bacterial count

Results for: Worksheet 2

Wilcoxon Signed Rank Test: D

ACID COUNT F 4.1 ALL CONCENTRATIONS 14 HRS

Test of median = 0.000000 versus median ≠ 0.000000

```
       N for   Wilcoxon          Estimated
    N   Test  Statistic      P      Median
D  27     27       46.0  0.001  -161890000
```

Wilcoxon Signed Rank Test: DIFF

ACID COUNT F 4.1 3.125 MG/ML 10 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon          Estimated
      N   Test  Statistic      P      Median
DIFF  9      9       10.0  0.155  -262300000
```

Results for: Worksheet 4

Wilcoxon Signed Rank Test: D

ACID COUNT F 4.1 3.125MG/ML 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon         Estimated
   N   Test  Statistic      P     Median
D  9      9       26.0  0.722   19500000
```

Results for: Worksheet 5

Wilcoxon Signed Rank Test: diff

ACID COUNT F 4.1 6.25 MG/ML 10 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
         N for   Wilcoxon          Estimated
      N   Test  Statistic      P      Median
diff  9      9        0.0  0.009  -263050000
```

Results for: Worksheet 6

Wilcoxon Signed Rank Test: d

ACID COUNT F 4.1 6.25 MG/ML 14 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon          Estimated
   N   Test  Statistic      P      Median
d  9      9        0.0  0.009  -193250000
```

Results for: Worksheet 7

Wilcoxon Signed Rank Test: D

ACID COUNT F 4.1 12.5 MG/ML 10 HRS

```
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon          Estimated
   N   Test  Statistic      P      Median
D  9      9        0.0  0.009  -287615000
```

Results for: Worksheet 8

Wilcoxon Signed Rank Test: d

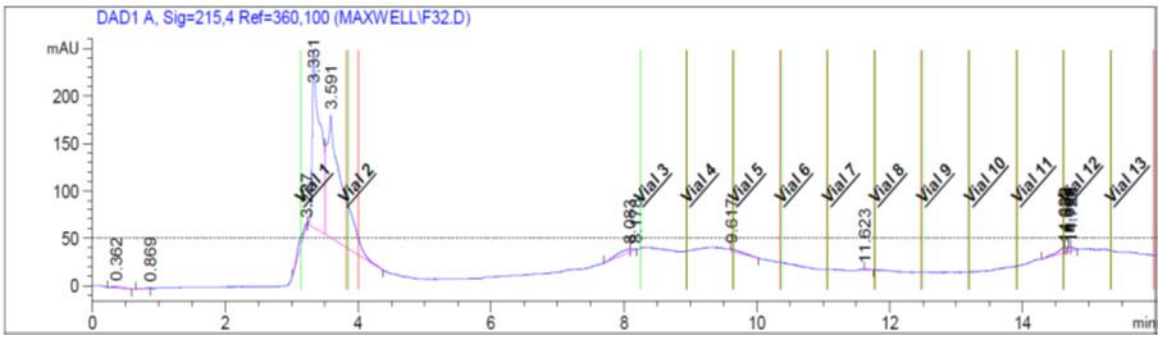
```
ACID COUNT F 4.1 12.5 MG/ML 14 HRS
Test of median = 0.000000 versus median ≠ 0.000000
      N for   Wilcoxon          Estimated
   N   Test  Statistic      P      Median
d  9      9        0.0  0.009  -204802500
```

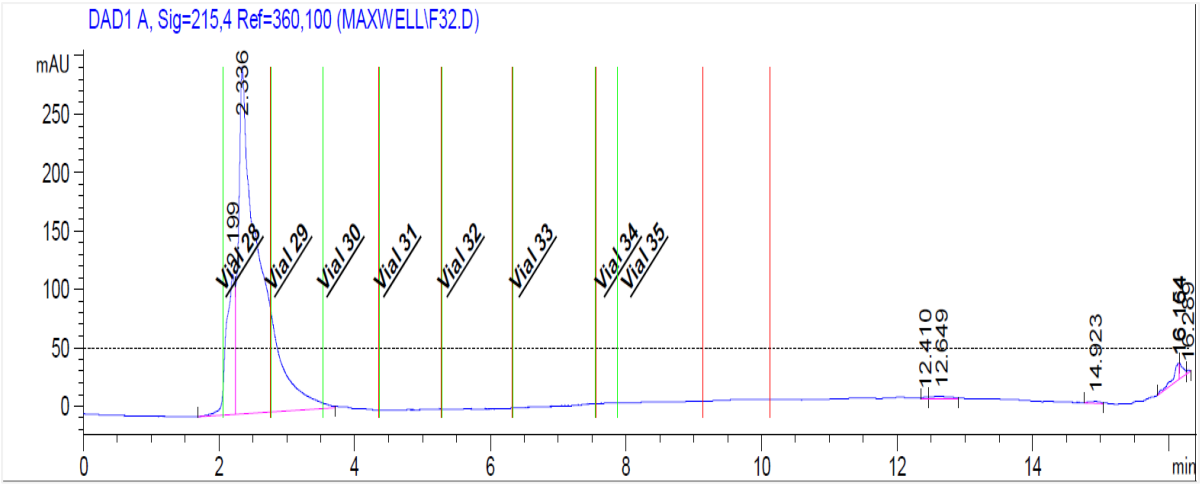
APPENDIX 3

Preparative HPLC

3.1 Preparative HPLC of subfraction F 3.2

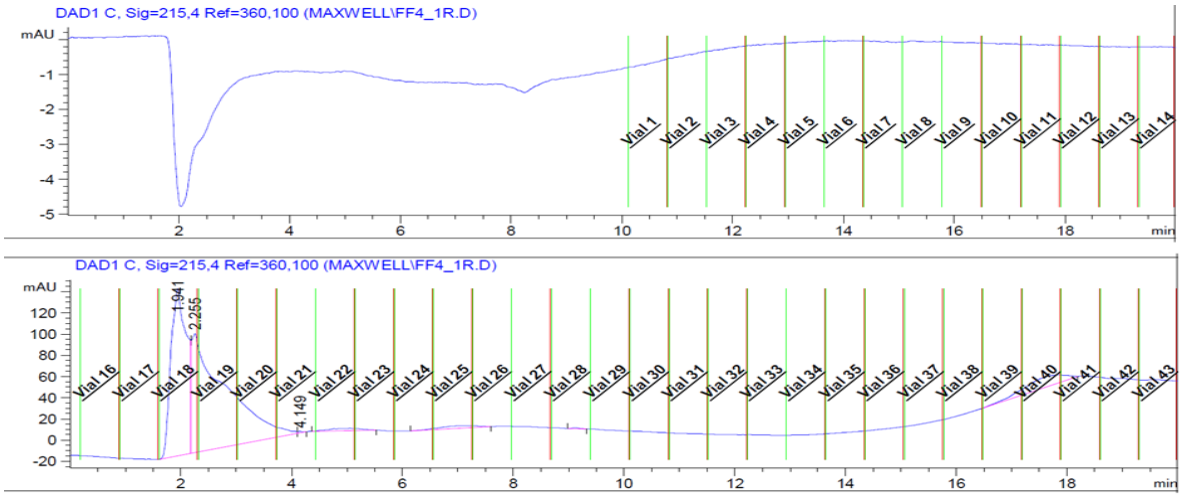
```
Data File C:\CHEM32\1\DATA\MAXWELL\F32.D
Sample Name: F32

    ======================================================================
    Acq. Operator   : SYSTEM
    Sample Operator : SYSTEM
    Acq. Instrument : University of Witwatersrand     Location : Vial 1
    Injection Date  : 8/15/2017 15:22:59
                                                  Inj Volume : 500.000 µl
    Method          : C:\CHEM32\1\METHODS\MAXWELL 5-95 MM 16 MIN.M
    Last changed    : 8/15/2017 15:21:14 by SYSTEM
                      (modified after loading)
    Sample Info     : F32
```





3.2 Preparative HPLC of subfraction F 4.1

```
==========================================================================
Acq. Operator    : SYSTEM
Sample Operator  : SYSTEM
Acq. Instrument  : University of Witwatersrand     Location : Vial 1
Injection Date   : 5/6/2017 14:15:42
                                                  Inj Volume : 500.000 µl
Acq. Method      : C:\CHEM32\1\METHODS\MAXWELL 50-95%.M
Last changed     : 5/6/2017 14:35:59 by SYSTEM
                   (modified after loading)
Analysis Method  : C:\CHEM32\1\METHODS\PIOUS\INSTRUMENT TEST.M
Last changed     : 5/1/2018 18:45:30 by SYSTEM
Sample Info      : Test run
```



APPENDIX 4

UHPLC-HRMS Analysis

1 Jan Smuts Avenue,
Braamfontein, 2001
Private Bag X3, WITS,
2050

Humphrey Raikes Building
Jorissen Street, Braamfontein
Johannesburg
South Africa

Client:	**Quote No:**	QLRGC000017-0
Contact Person: Maxwell Thatyana	**Sample Identification:** UHPLC-HRMS Analysis	
Address: Humphrey Raikes Building Jorissen Street, Braamfontein Johannesburg South Africa	**Date Received:** 2017	
Tel:	**WITS Contact:** E-mail: maya.makatini@wits.ac.za : A0048861@wits.ac.za Tel: +27 (0) 11 717 6708 Tel: +27 (0) 11 717 6761	
Email: maxwell.thatyana1@students.wits.ac.za	**Project No:**	
Title: Loop injection and UHPLC-HRMS Analysis		

Mass Spectrometry (HRMS) analysis
The analysis of (sample X) was performed on a Bruker Compact Q-TOF high resolution Compact mass spectrophotometer. A 10µL of the sample was injected into the UHPLC and run through a loop at 50% Solvent A consisting of 0.1 % formic acid in H2O (v/v) and 50% solvent B consisting of 0.1 % formic acid in Acetonitrile (v/v).
Sample Preparation: The samples were diluted to a concentration of approx. 10 ppm.
Data analysis : The mass spectrum analysis was processed using the Bruker Daltonics data analysis.
Sample Identification: UHPLC-HRMS Analysis.

Date:	Analysis done by	Approved by
29 September 2017	Thapelo Mbhele	Dr Maya Makatini

4.1 Subfractions F 3.2 UHPLC-HRMS Analysis

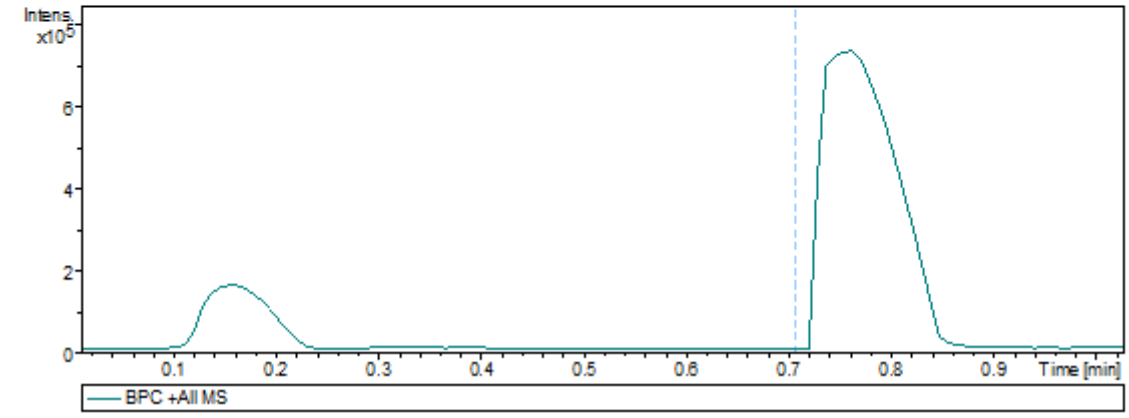
Display Report

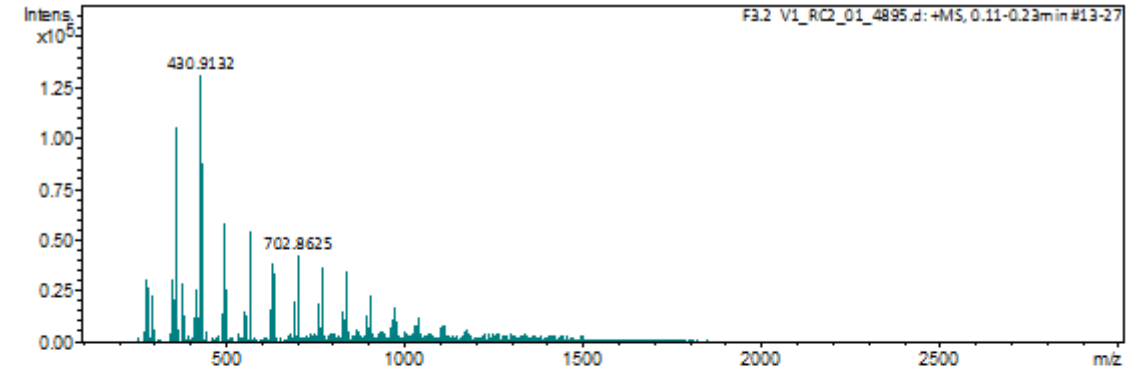
Analysis Info

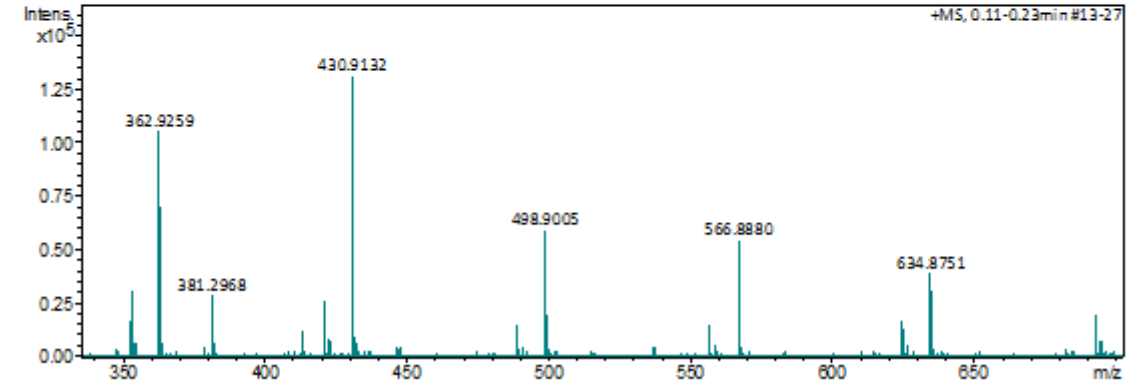
AnalysisName	Z:\Maxwell\28-09-2017\F3.2V1_RC2_01_4895.d	AcquisitionDate	2017/09/2804:28:32PM
Method	Loop MiDMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F3.2 V1	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F3.2 V1_RC2_01_4895.d

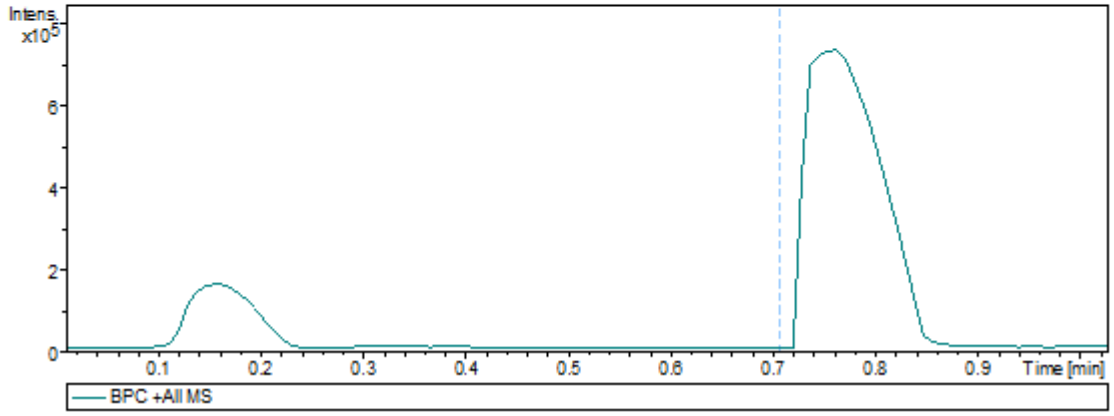
Display Report

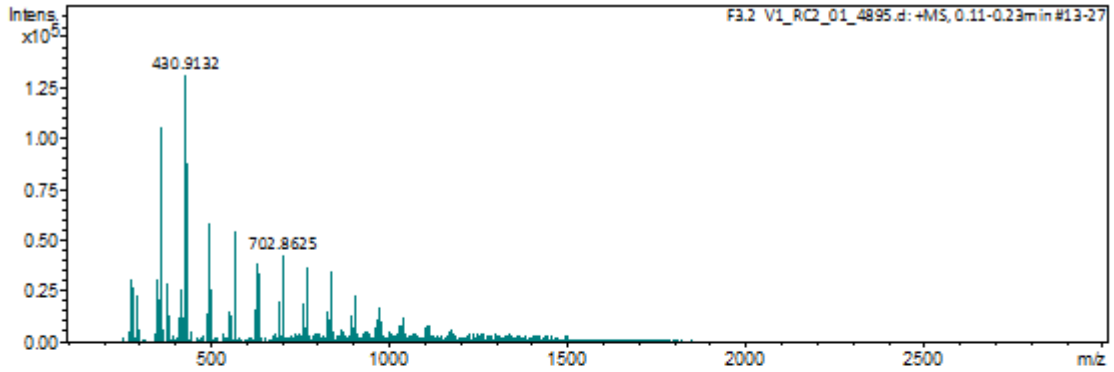
Analysis Info

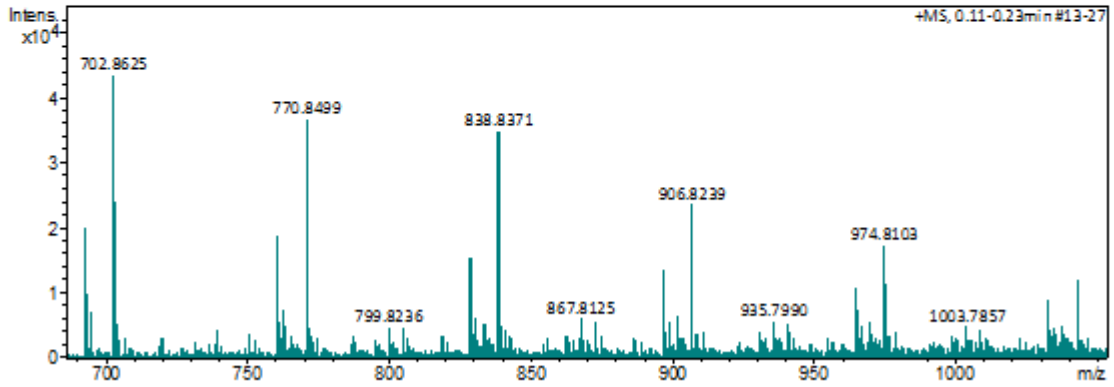
AnalysisName	Z:\Maxwell\28-09-2017\F3.2V1_RC2_01_4895.d	AcquisitionDate	2017/09/2804:28:32PM
Method	Loop MiDMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F3.2 V1	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F3.2 V1_RC2_01_4895.d

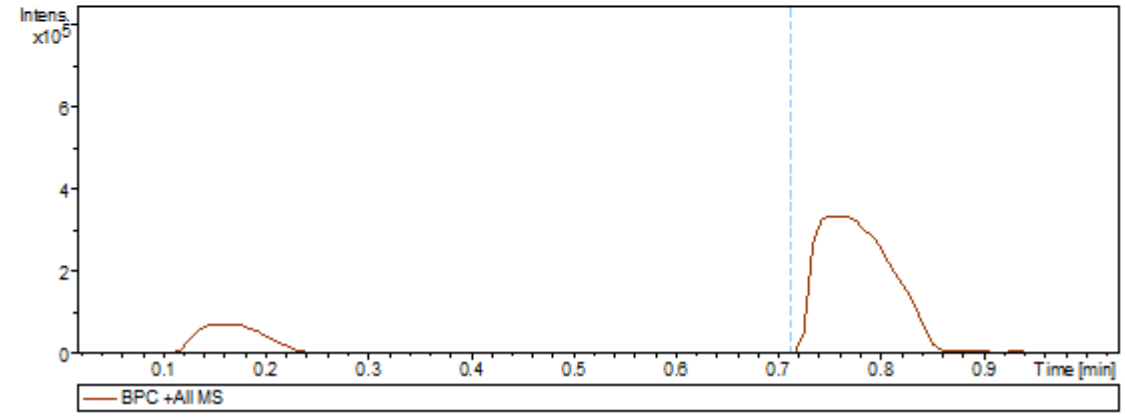
Display Report

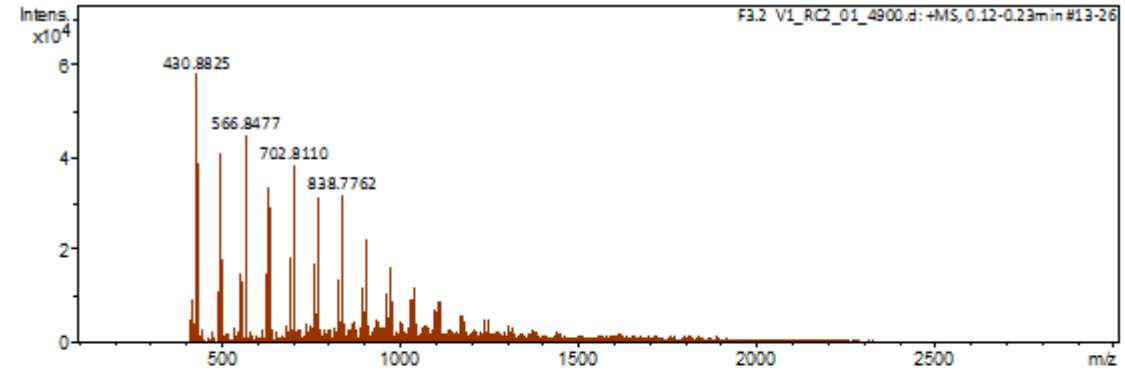
Analysis Info

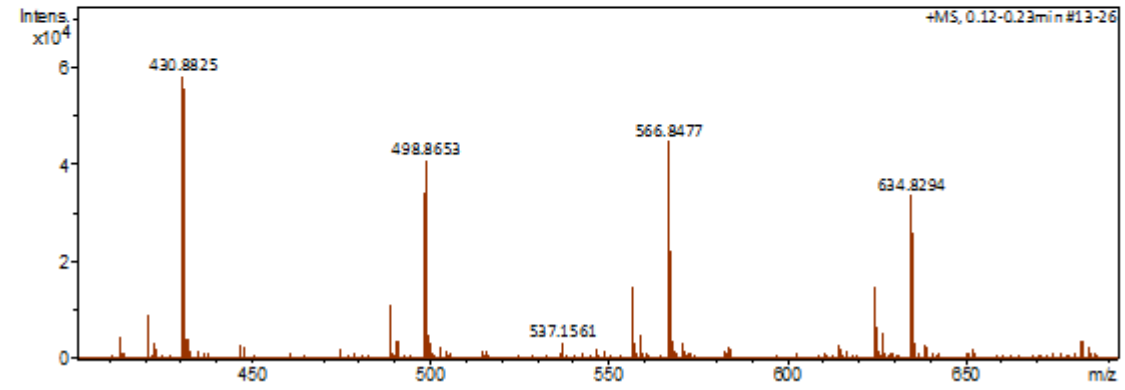
AnalysisName	Z:\Maxwell\28-09-2017\F3.2V1_RC2_01_4900.d	AcquisitionDate	2017/09/2804:40:48PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F3.2 V1	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F3.2 V1_RC2_01_4900.d

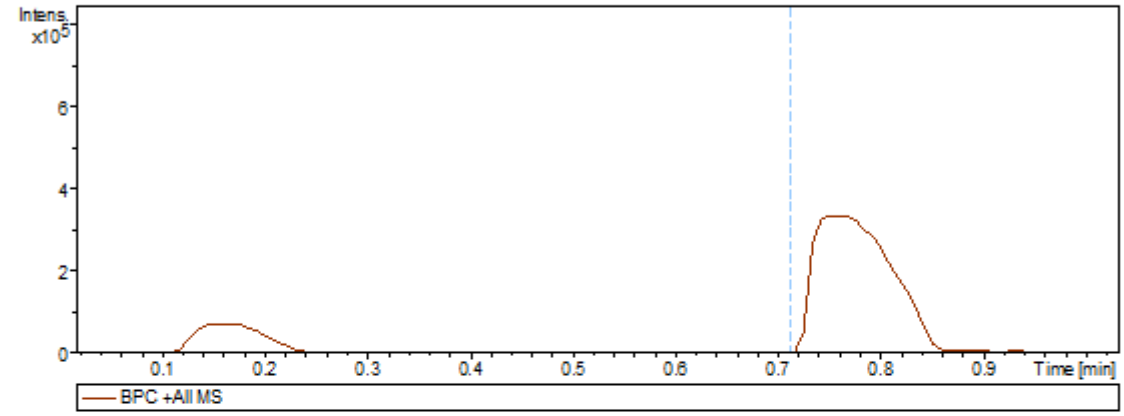
Display Report

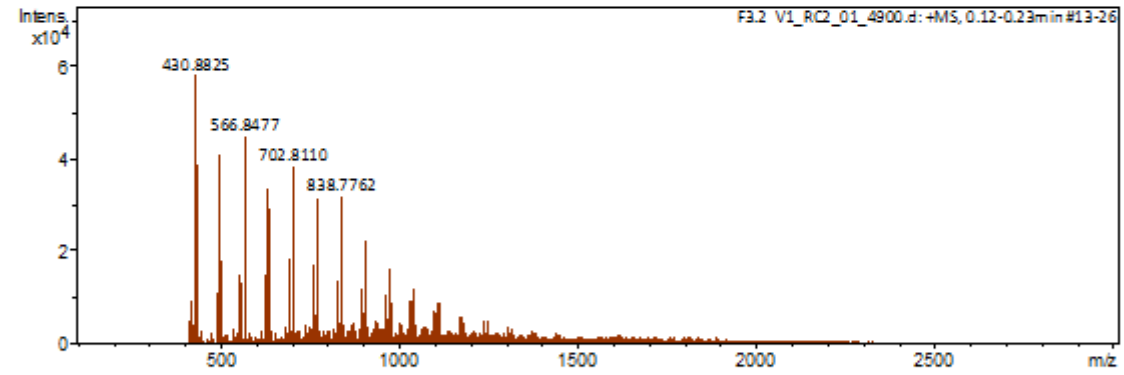
Analysis Info

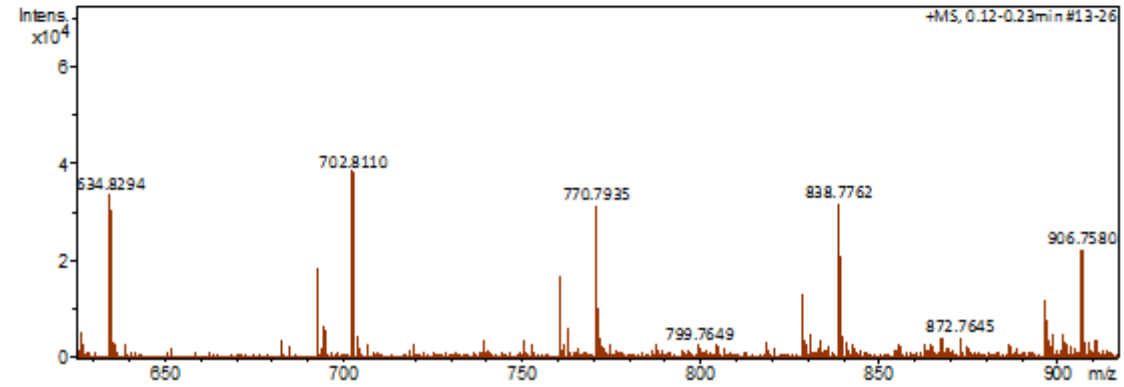
AnalysisName	Z:\Maxwell\28-09-2017\F3.2\V1_RC2_01_4900.d	AcquisitionDate	2017/09/2804:40:48PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F3.2 V1	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar		
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C		
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min		
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste		
		Set Corona	0 nA	Set APCI Heater	0 °C		







F3.2 V1_RC2_01_4900.d

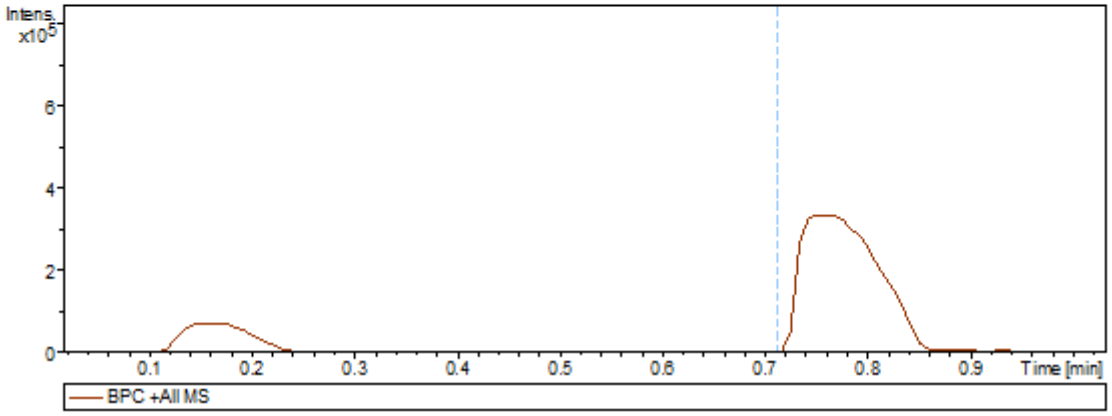
Display Report

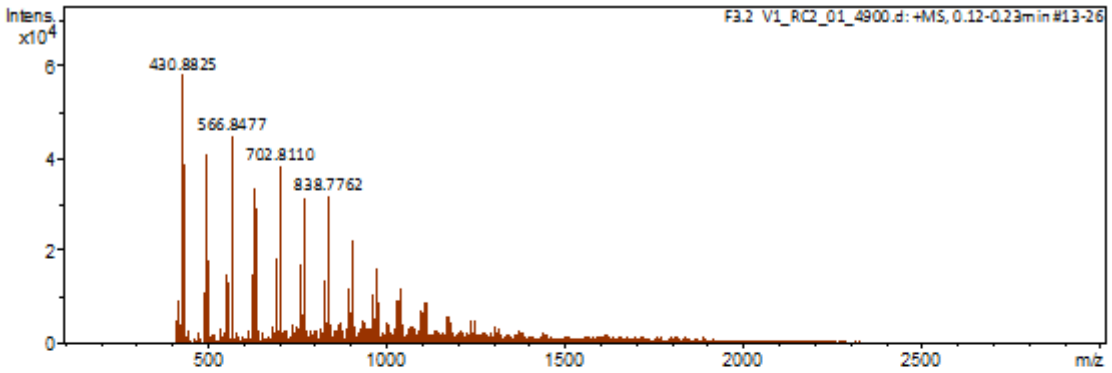
Analysis Info

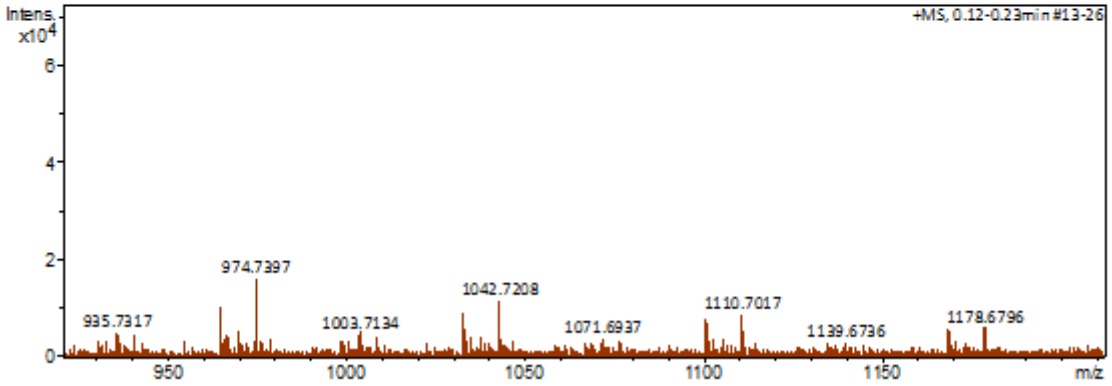
AnalysisName	Z:\Maxwell\28-09-2017\F3.2V1_RC2_01_4900.d	AcquisitionDate	2017/09/2804:40:48PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F3.2 V1	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F3.2 V1_RC2_01_4900.d

4.2 Subfractions F 4.1 UHPLC-HRMS

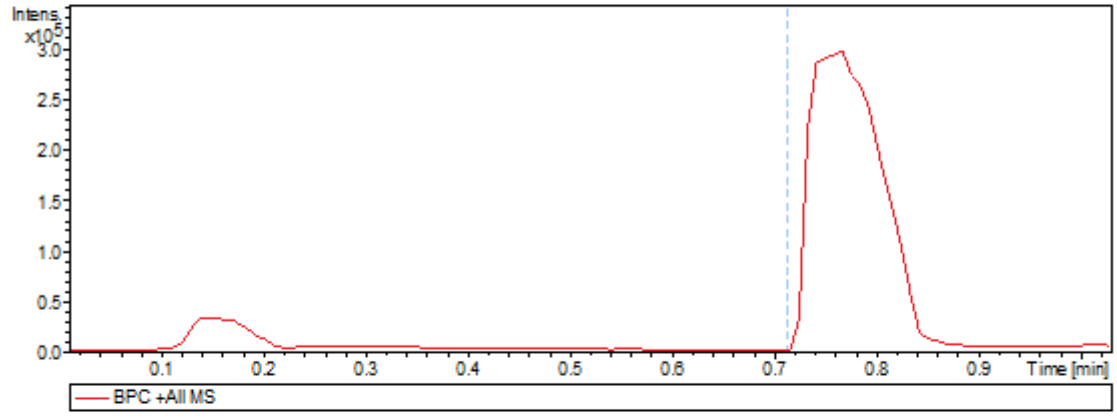
Display Report

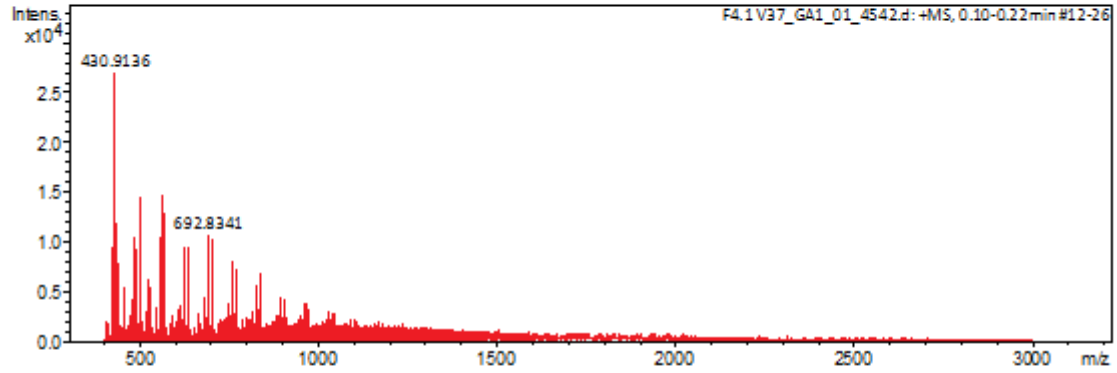
Analysis Info

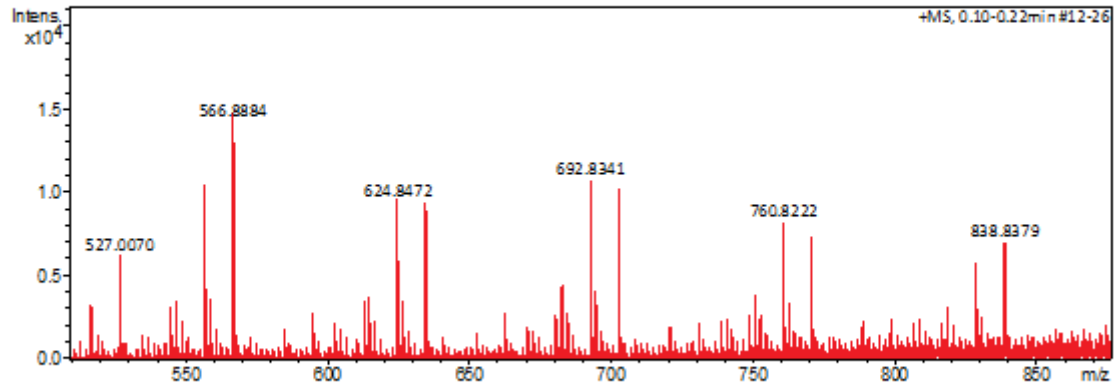
AnalysisName	Z:\Maxwell\08-09-2017\F4\V37_GA1_01_4542.d	AcquisitionDate	2017/09/0806:41:00PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V37	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V37_GA1_01_4542.d

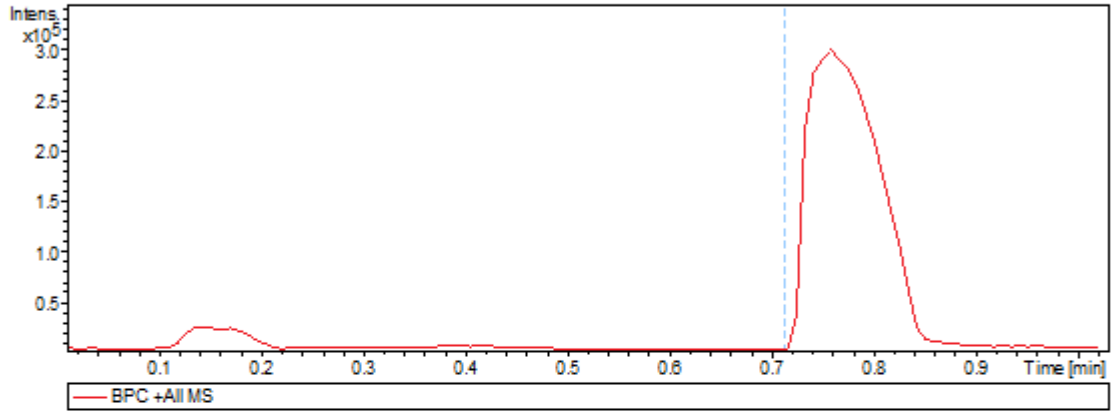
Display Report

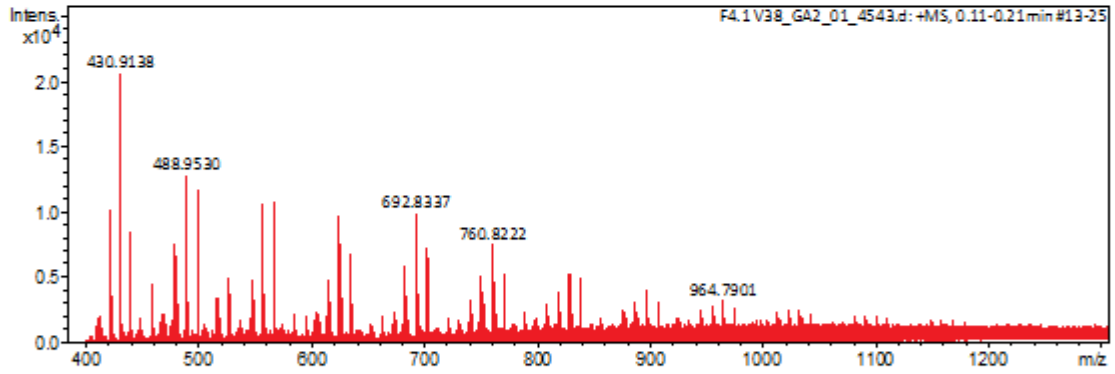
Analysis Info

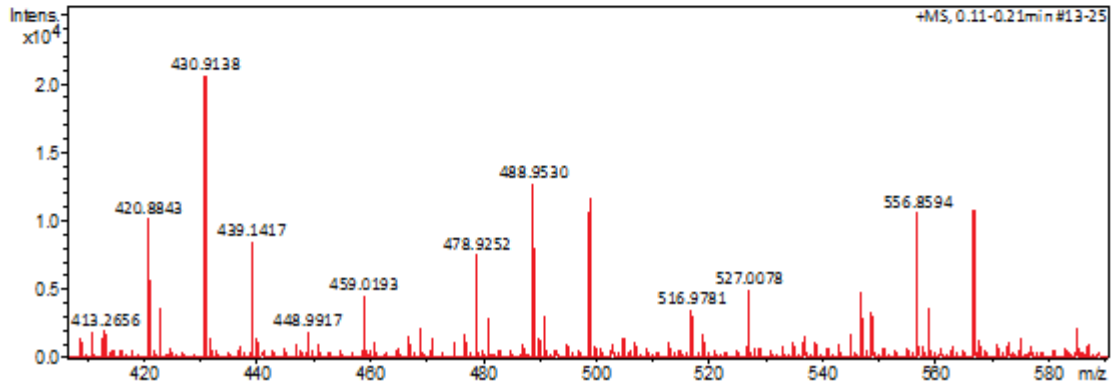
AnalysisName	Z:\Maxwell\08-09-2017\F4\V38_GA2_01_4543.d	AcquisitionDate	2017/09/0806:43:27PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V38	Instrument	compact 8255754.20116
Comment			

AcquisitionParameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V38_GA2_01_4543.d

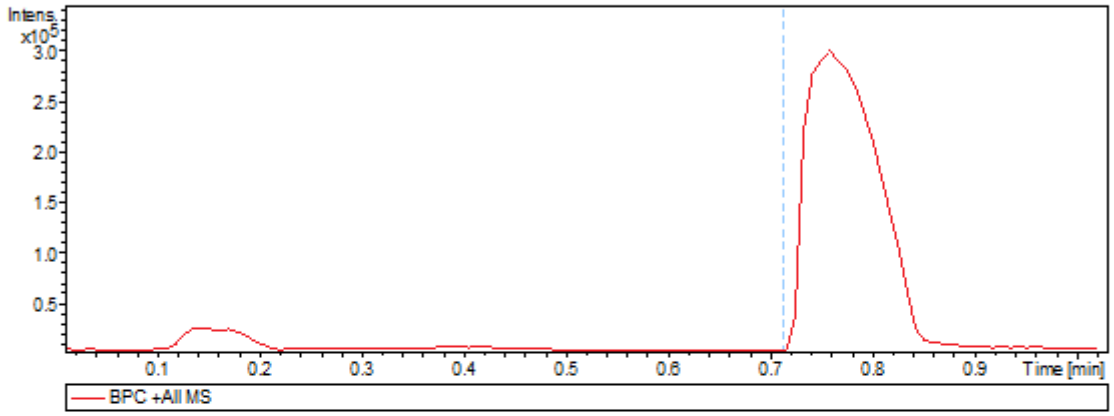
Display Report

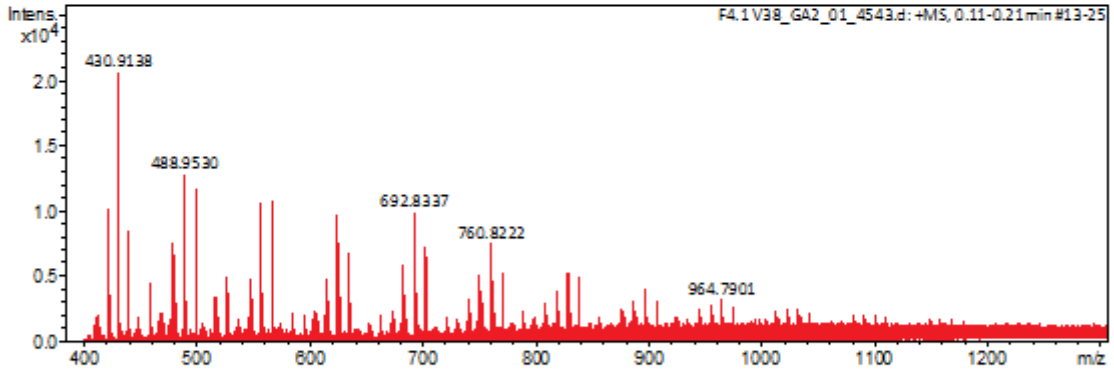
Analysis Info

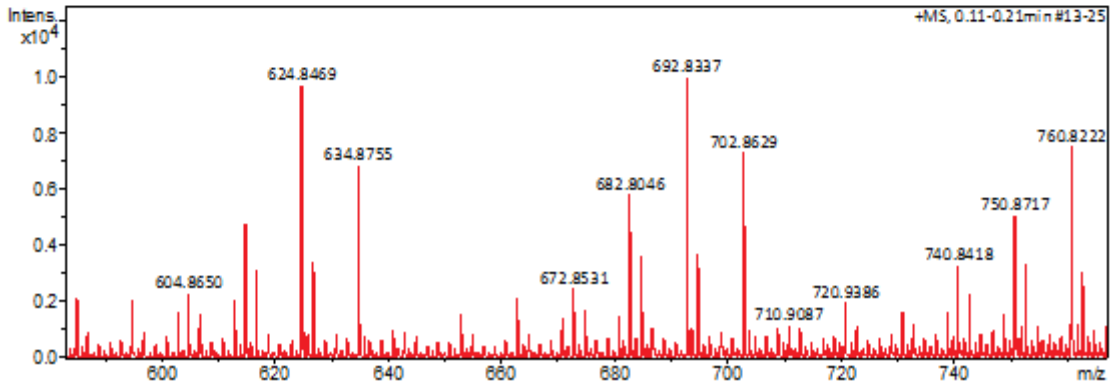
AnalysisName	Z:\Maxwell\08-09-2017\F4\V38_GA2_01_4543.d	AcquisitionDate	2017/09/0806:43:27PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V38	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V38_GA2_01_4543.d

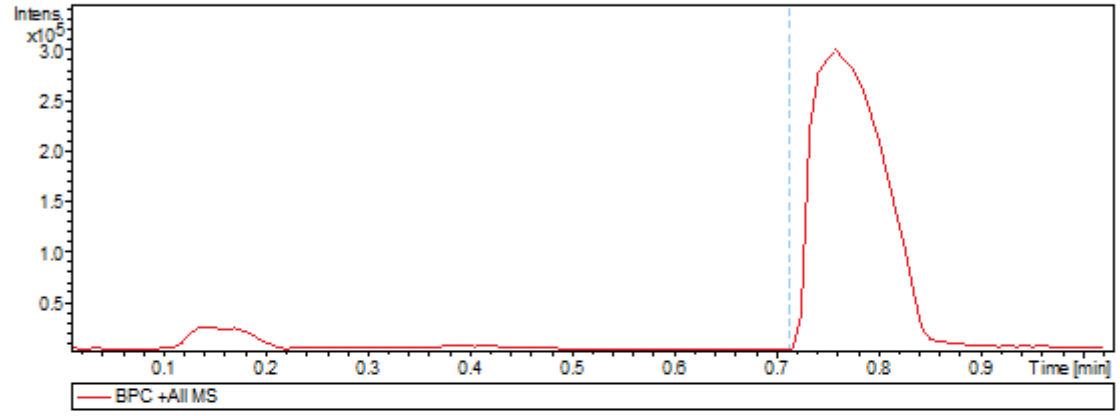
Display Report

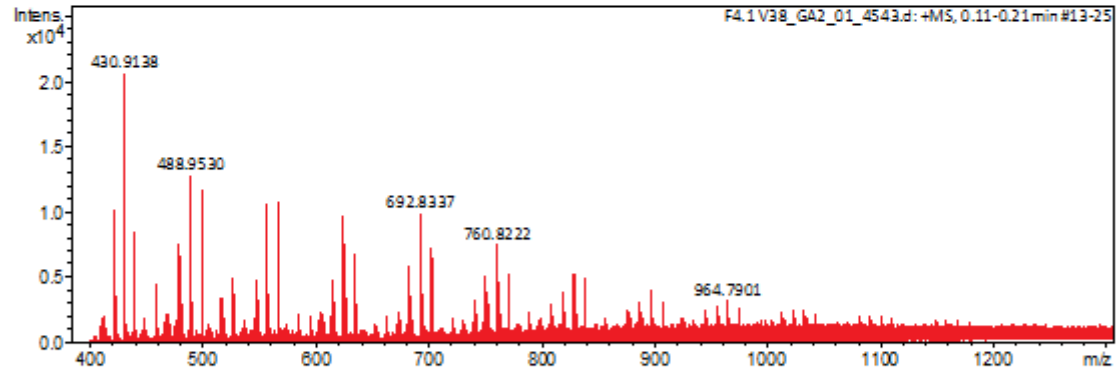
Analysis Info

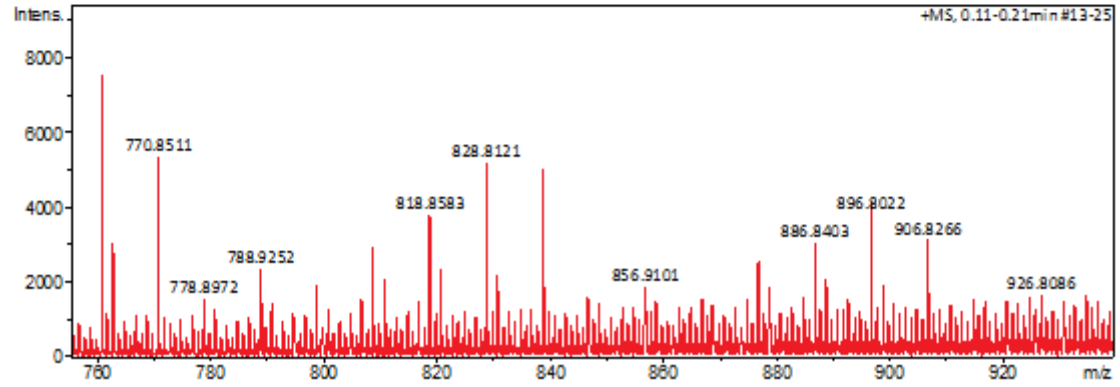
AnalysisName	Z:\Maxwell\08-09-2017\F4\V38_GA2_01_4543.d	AcquisitionDate	2017/09/0806:43:27PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V38	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V38_GA2_01_4543.d

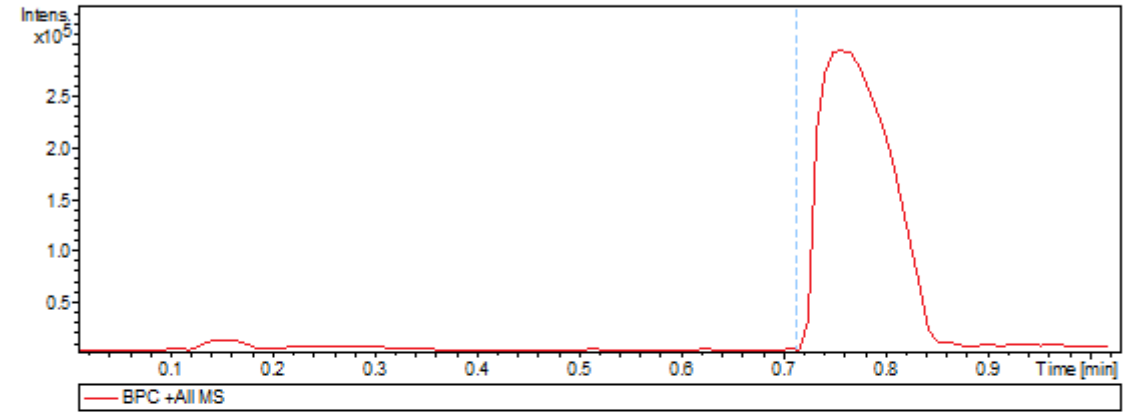
Display Report

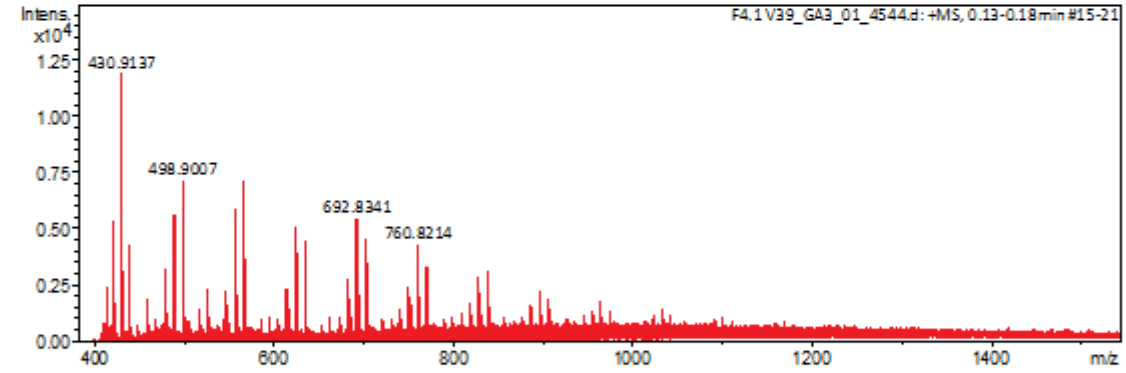
Analysis Info

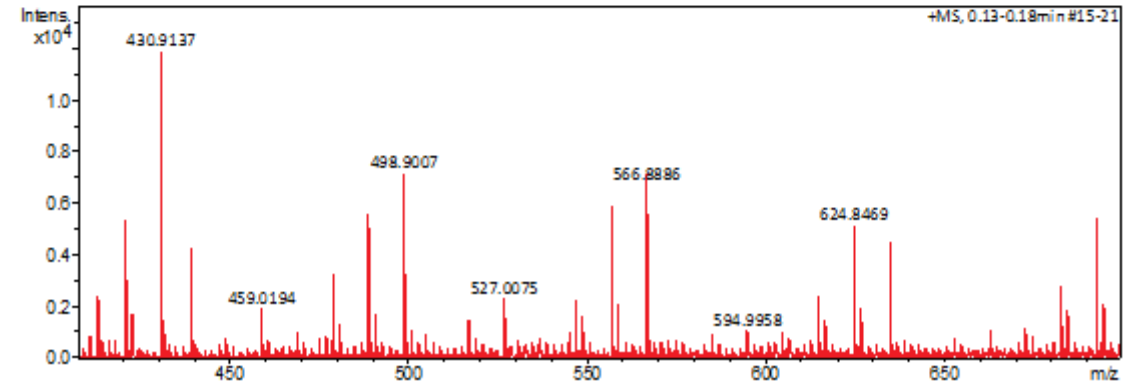
AnalysisName	Z:\Maxwell\08-09-2017\F4\V39_GA3_01_4544.d	AcquisitionDate	2017/09/0806:45:56PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V39	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V39_GA3_01_4544.d

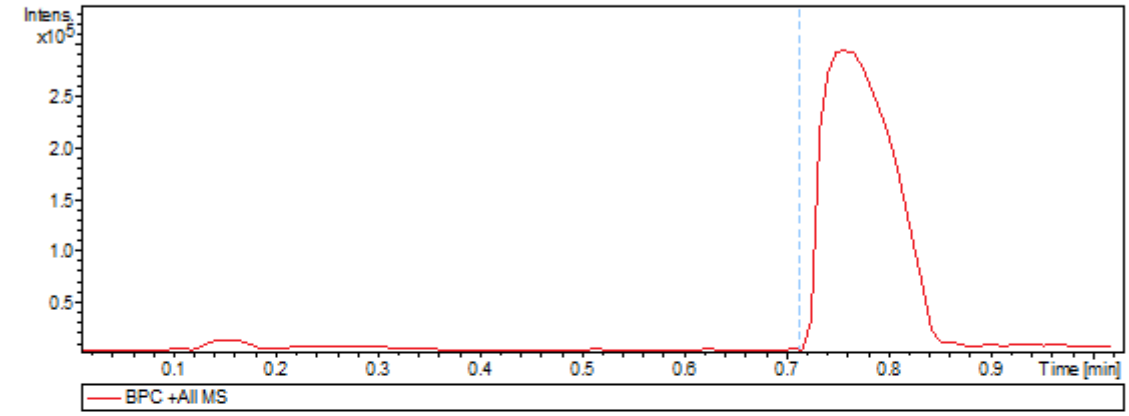
Display Report

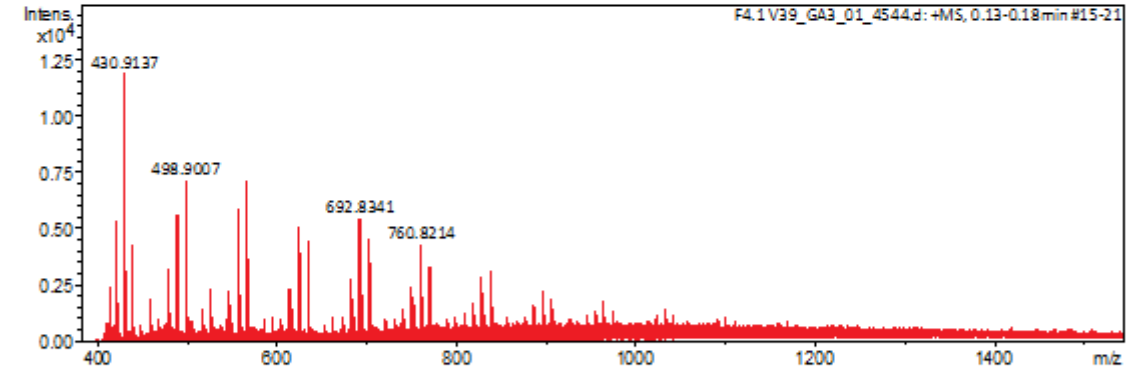
Analysis Info

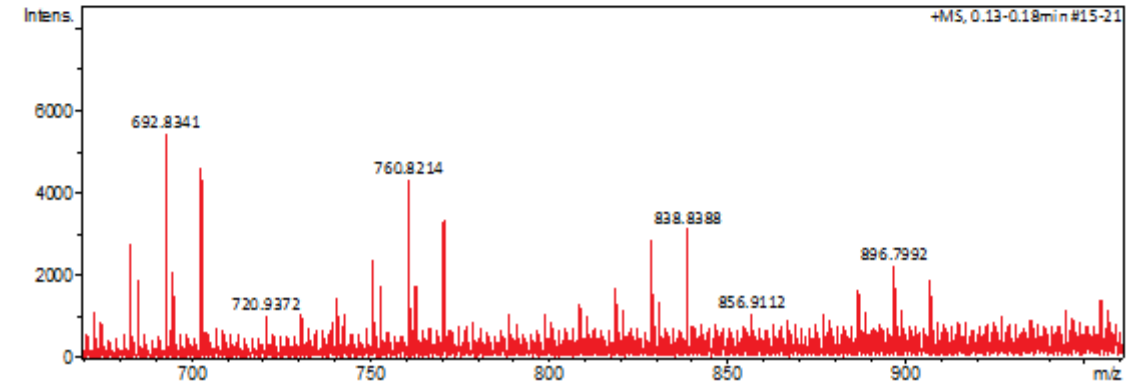
AnalysisName	Z:\Maxwell\08-09-2017\F4\V39_GA3_01_4544.d	AcquisitionDate	2017/09/0806:45:56PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V39	Instrument	compact 8255754.20116
Comment			

AcquisitionParameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V39_GA3_01_4544.d

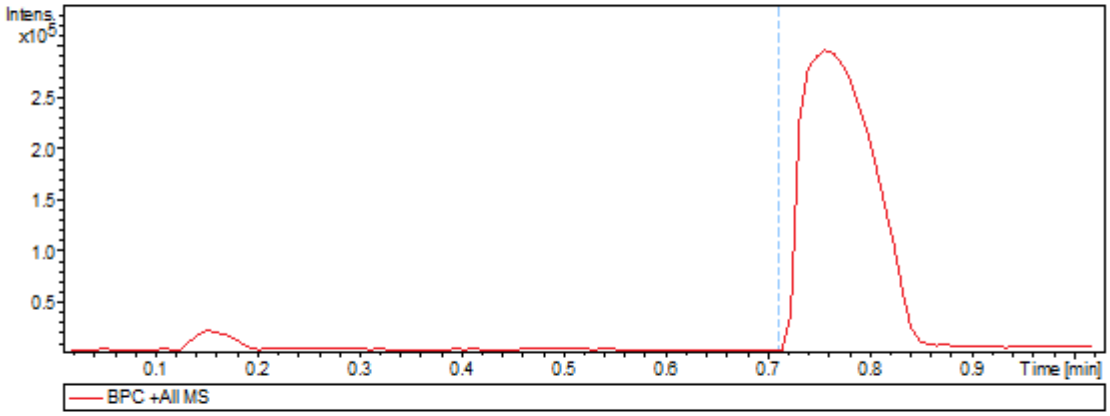
Display Report

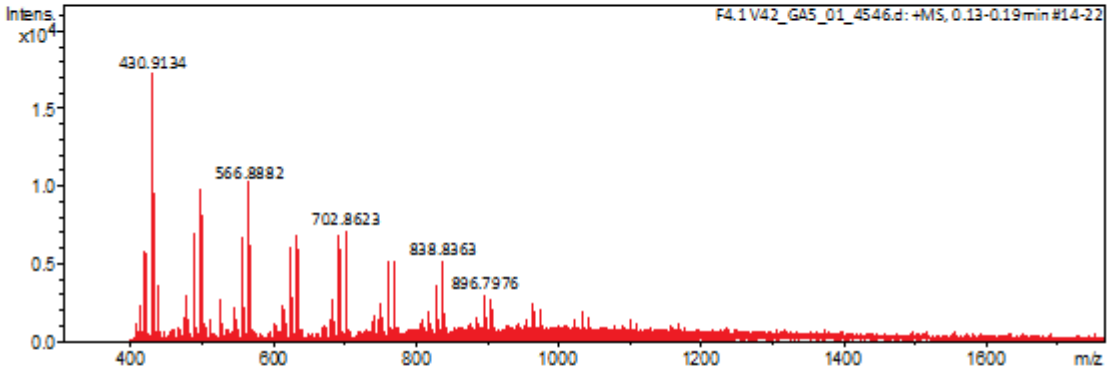
Analysis Info

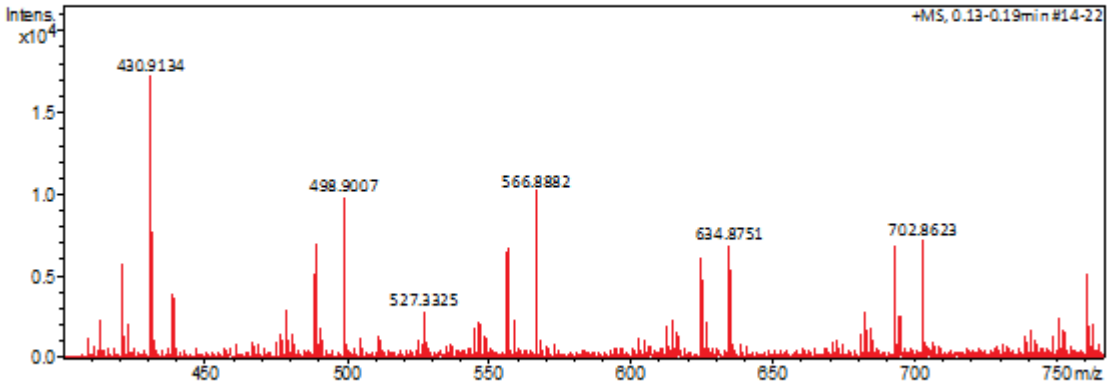
AnalysisName	Z:\Maxwell\08-09-2017\F4\V42_GA5_01_4546.d	AcquisitionDate	2017/09/08 6:50:49 PM
Method	Loop HIGH MS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V42	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V42_GA5_01_4546.d

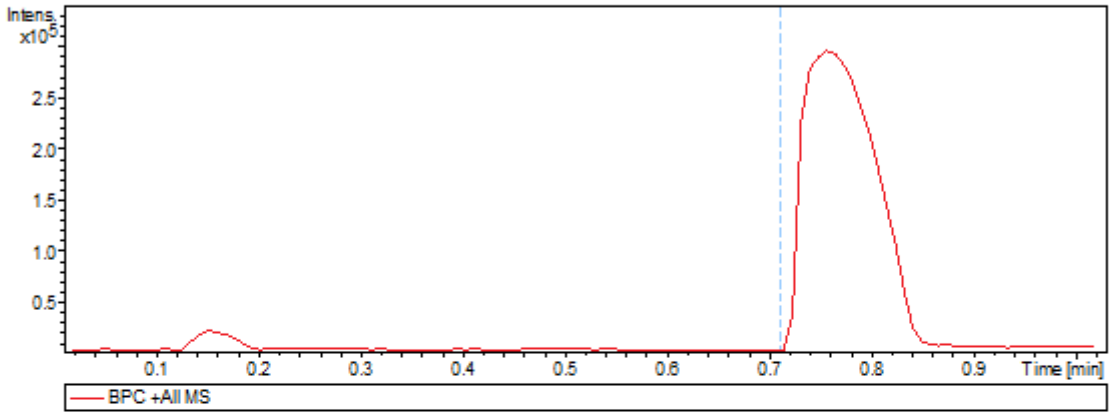
Display Report

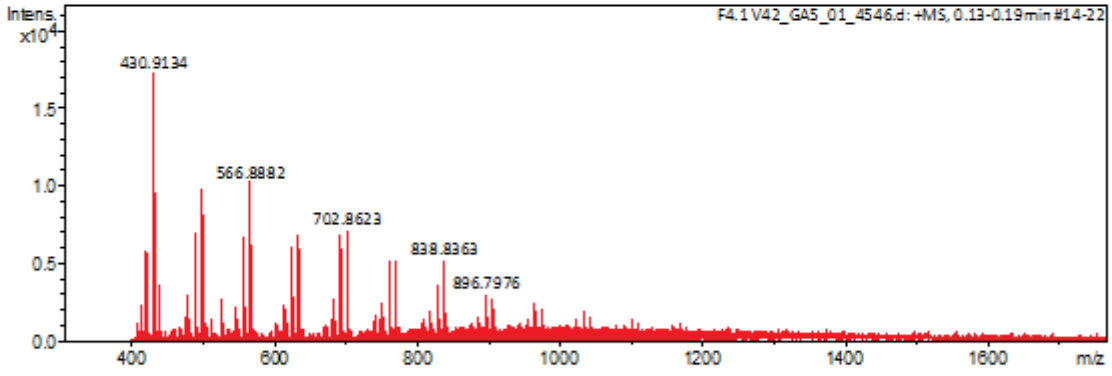
Analysis Info

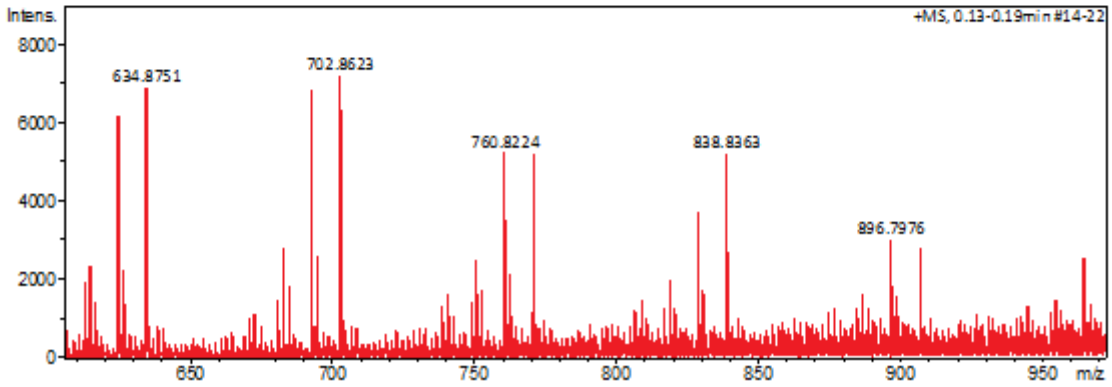
AnalysisName	Z:\Maxwell\08-09-2017\F4\V42_GA5_01_4546.d	AcquisitionDate	2017/09/0806:50:49PM
Method	Loop HIGH MS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V42	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V42_GA5_01_4546.d

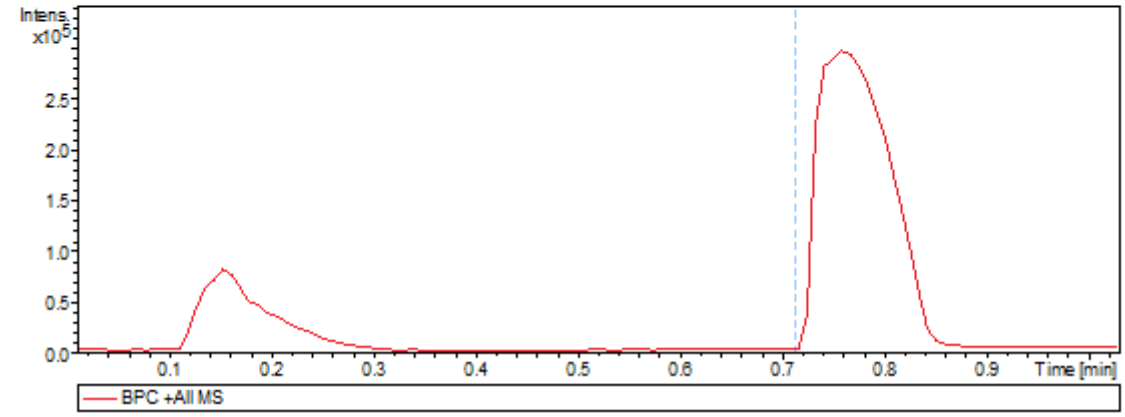
Display Report

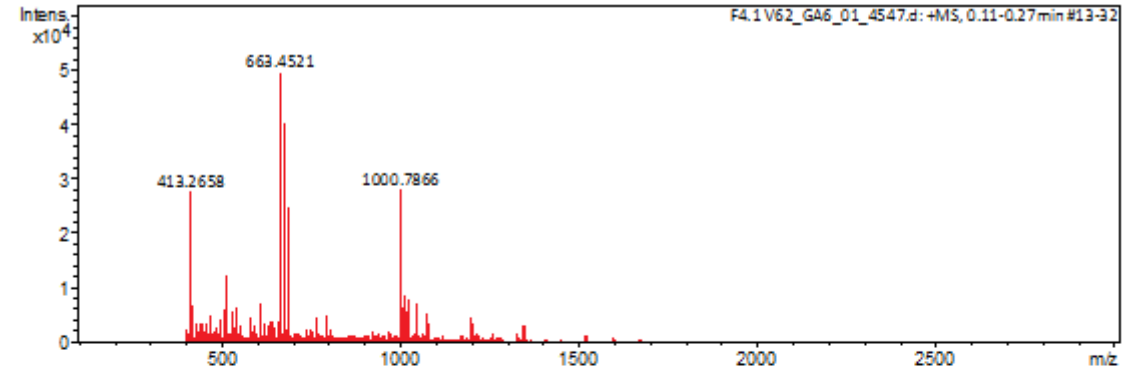
Analysis Info

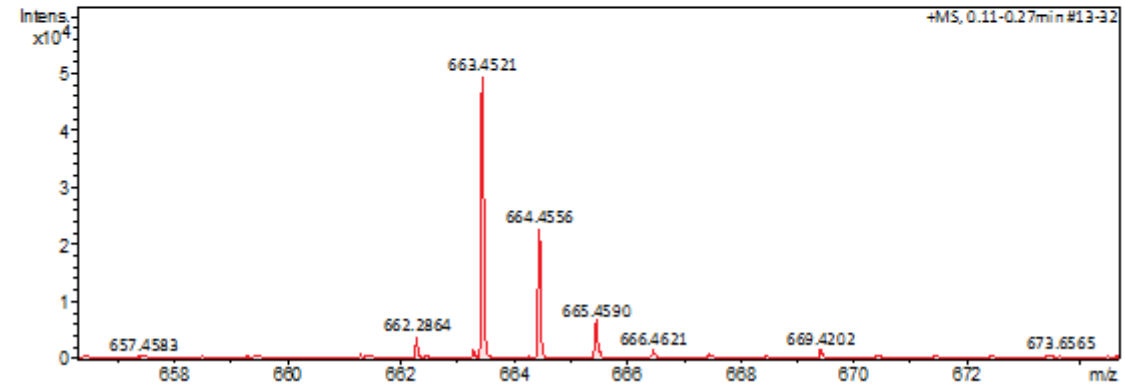
AnalysisName	Z:\Maxwell\08-09-2017\F4\V62_GA6_01_4547.d	AcquisitionDate	2017/09/0806:53:15PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1 V62	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V62_GA6_01_4547.d

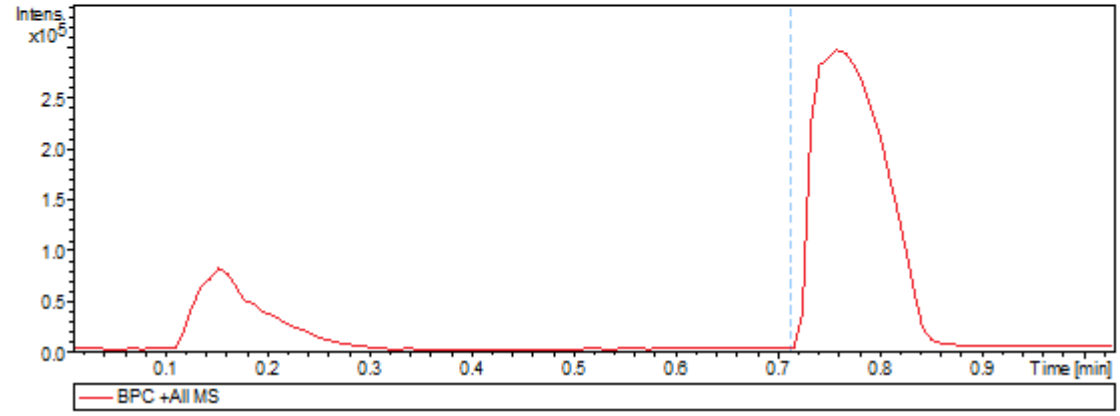
Display Report

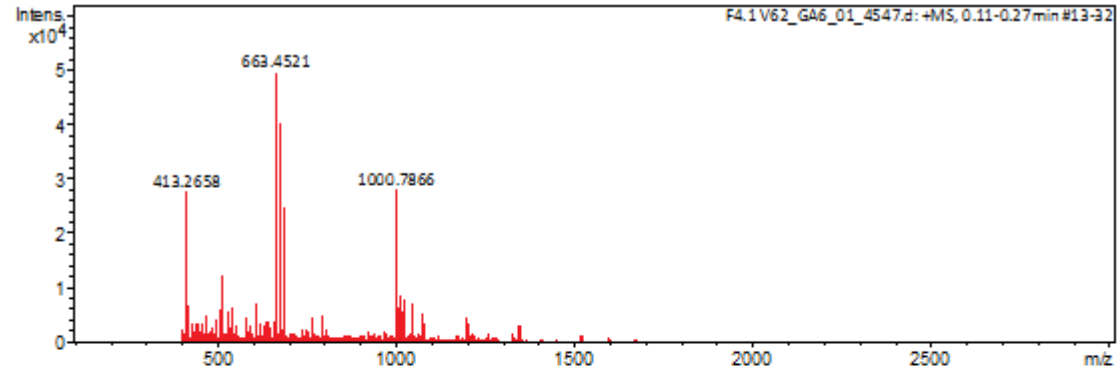
Analysis Info

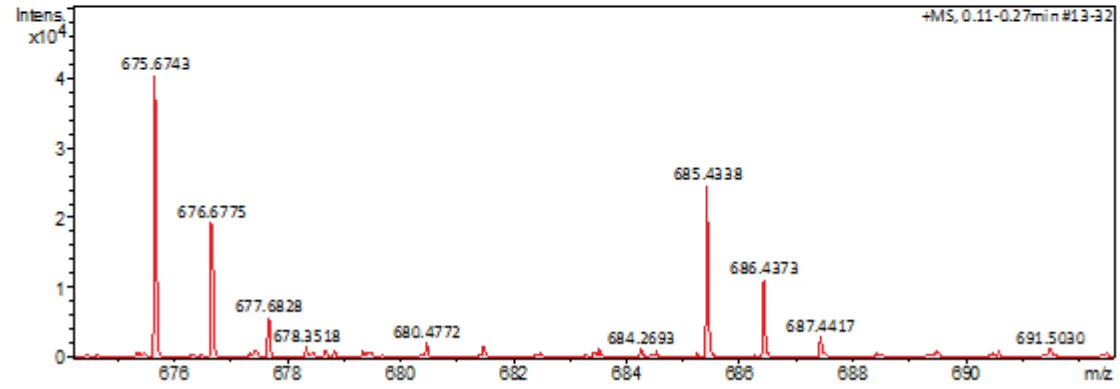
AnalysisName	Z:\Maxwell\08-09-2017\F4\V62_GA6_01_4547.d	AcquisitionDate	2017/09/0806:53:15PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V62	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar		
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C		
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min		
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste		
		Set Corona	0 nA	Set APCI Heater	0 °C		







F4.1 V62_GA6_01_4547.d

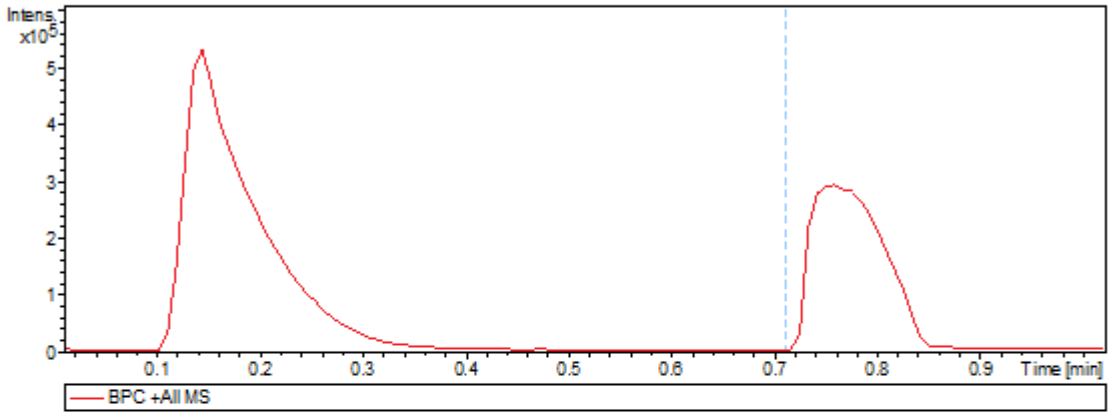
Display Report

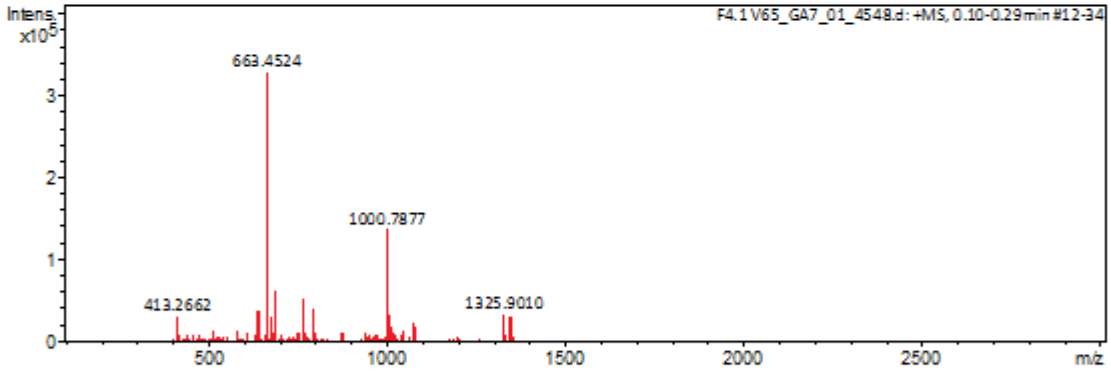
Analysis Info

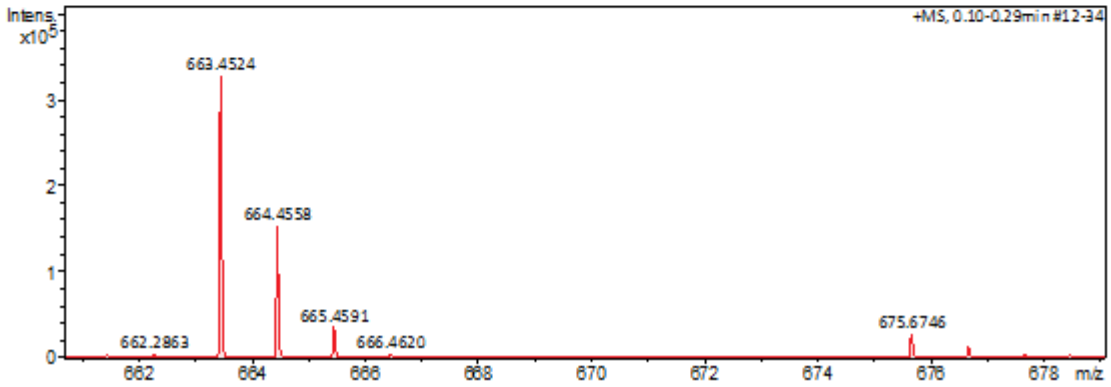
AnalysisName	Z:\Maxwell\08-09-2017\F4\V65_GA7_01_4548.d	AcquisitionDate	2017/09/0806:55:44PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V65	Instrument	compact 8255754.20116
Comment			

AcquisitionParameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V65_GA7_01_4548.d

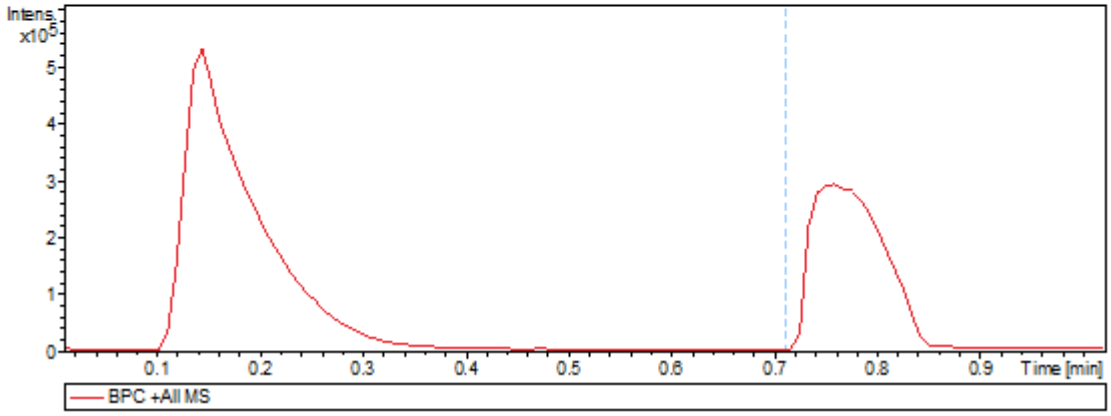
Display Report

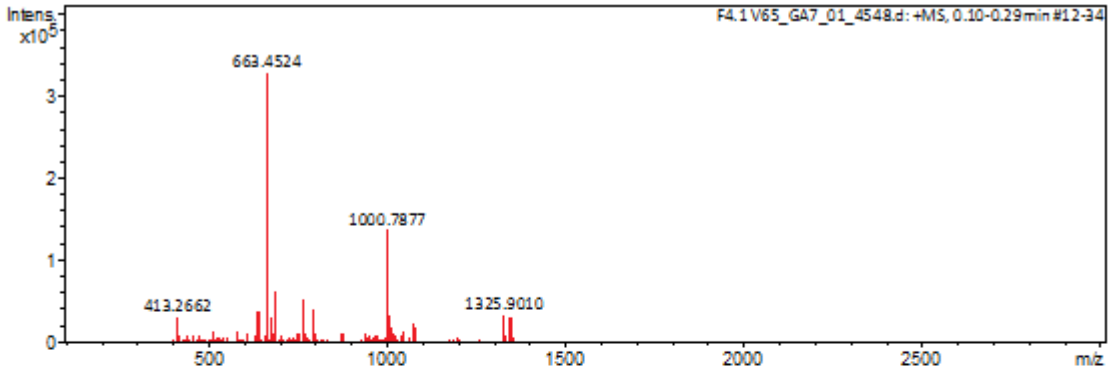
Analysis Info

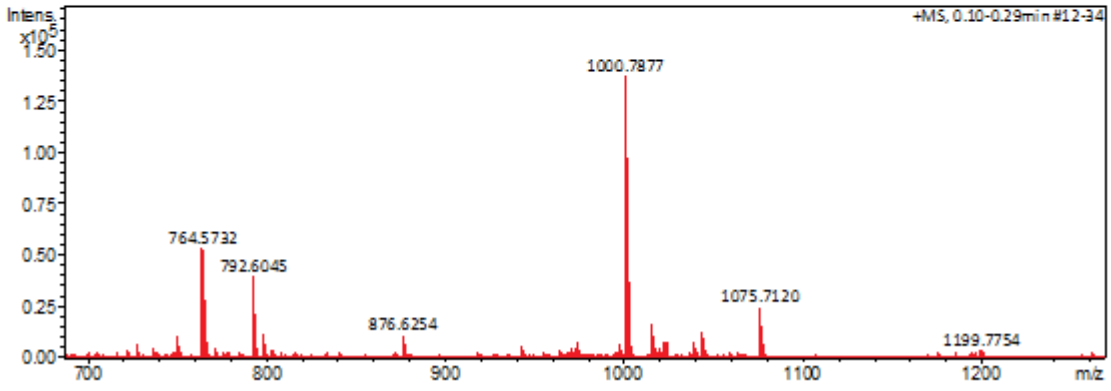
AnalysisName	Z:\Maxwell\08-09-2017\F4\V65_GA7_01_4548.d	AcquisitionDate	2017/09/0806:55:44PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V65	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V65_GA7_01_4548.d

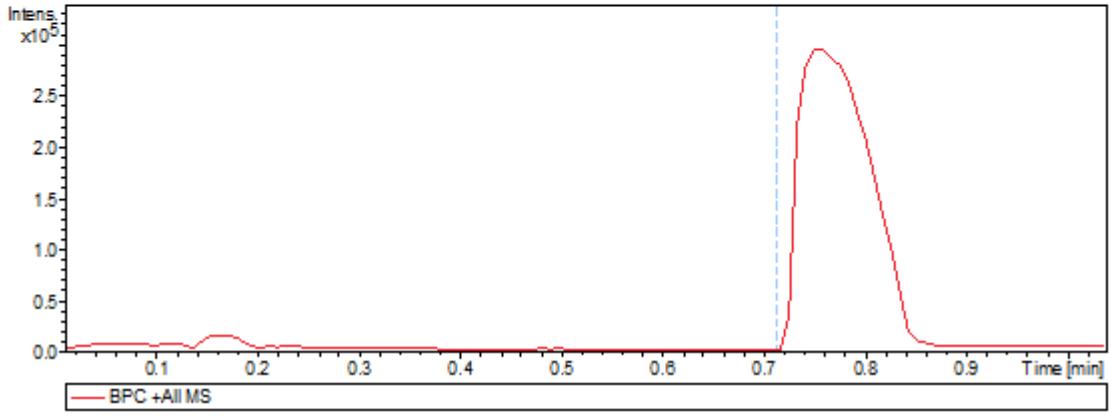
Display Report

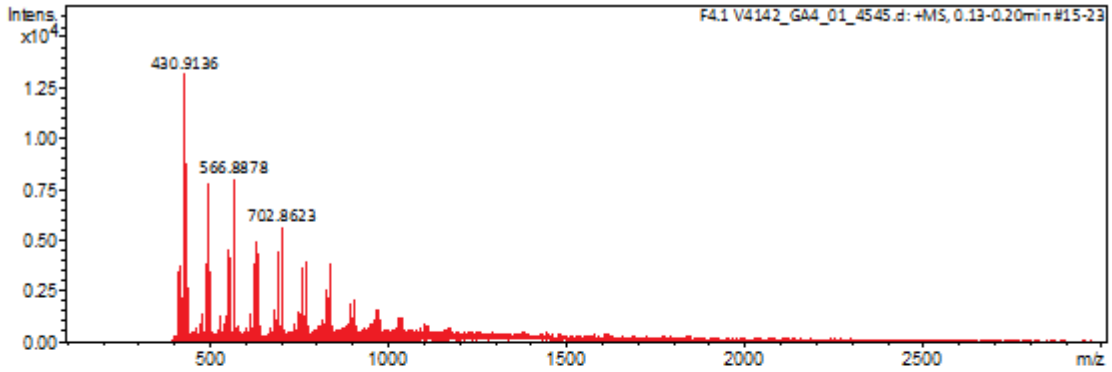
Analysis Info

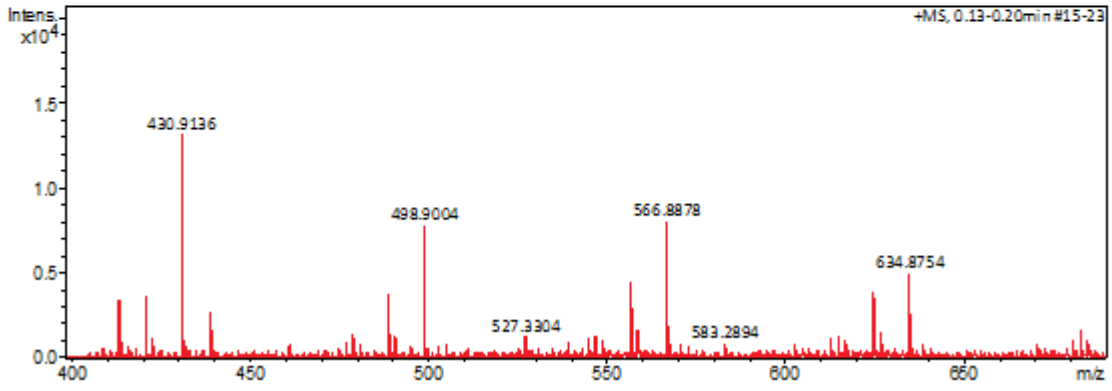
AnalysisName	Z:\Maxwell\08-09-2017\F4V4142_GA4_01_4545.d	AcquisitionDate	2017/09/0806:48:21PM
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V4142	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







F4.1 V4142_GA4_01_4545.d

Display Report

Analysis Info

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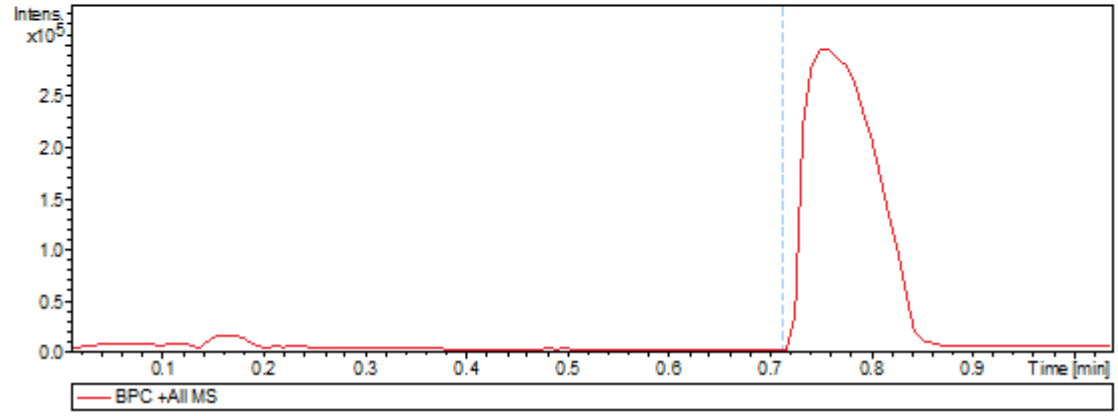
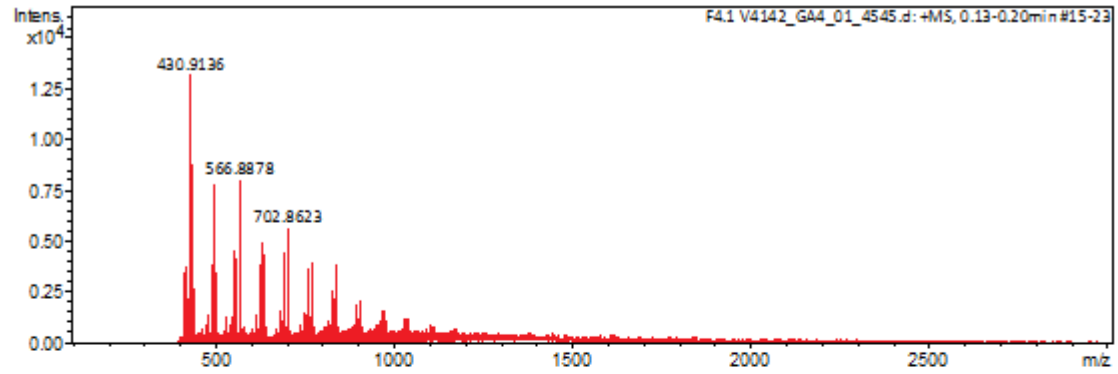
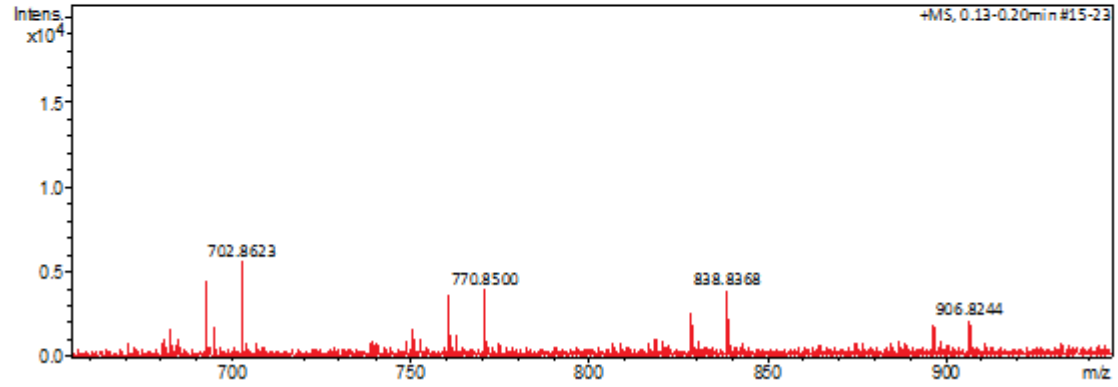
Method	Loop HIGHMS 100-1300 pos.m	Operator	RefilweMoepya
SampleName	F4.1V4142	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	3000 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C







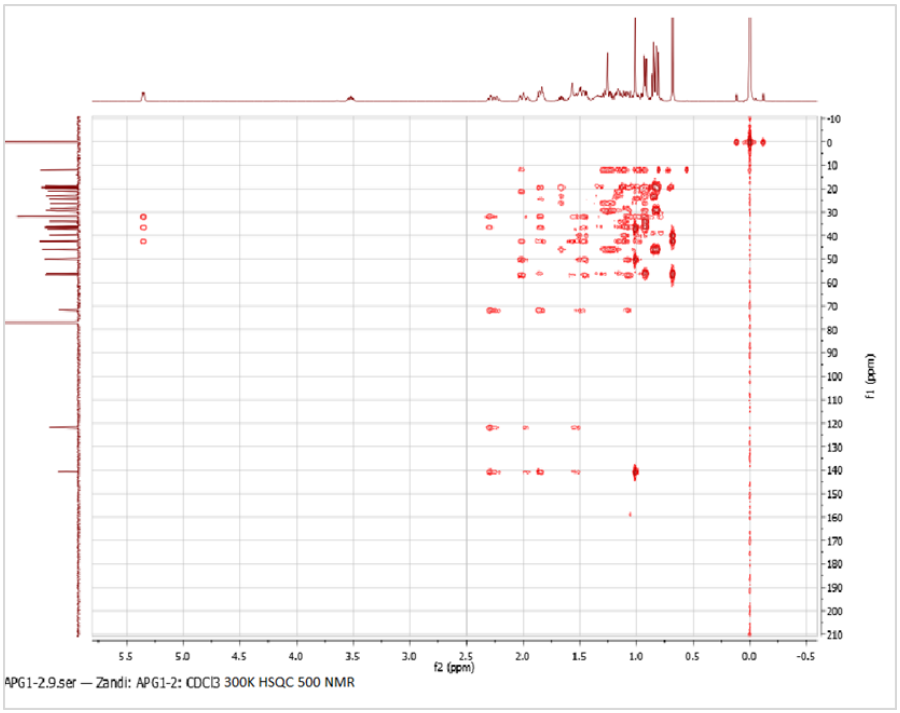
F4.1 V4142_GA4_01_4545.d

APPENDIX 5

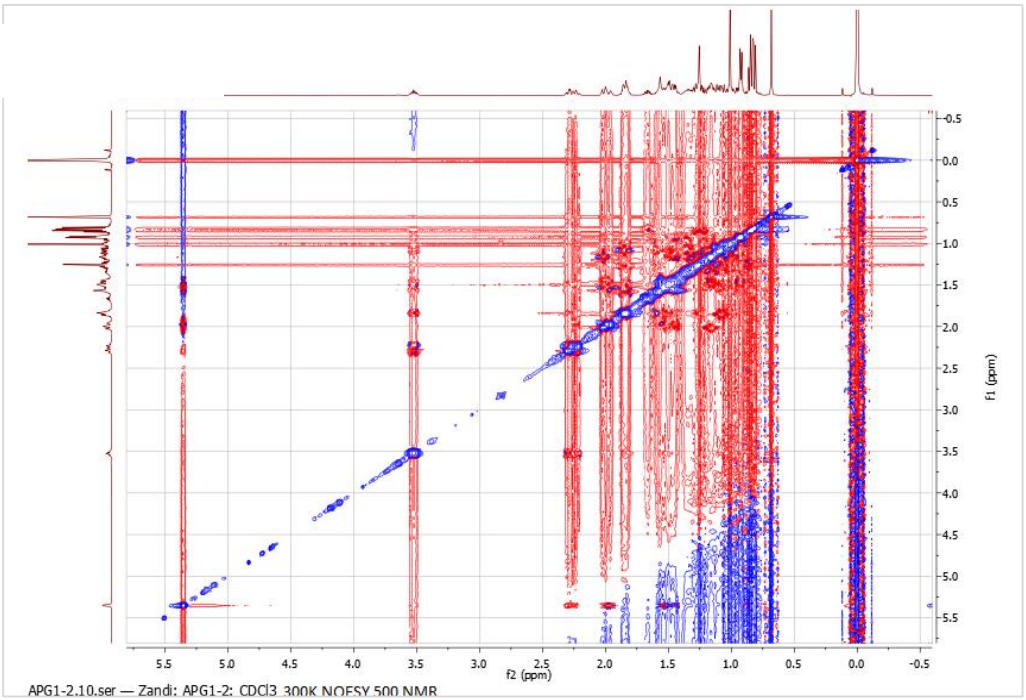
NMR SPECTRA

5.1 APG 2

HSQC 500 NMR in CDl3

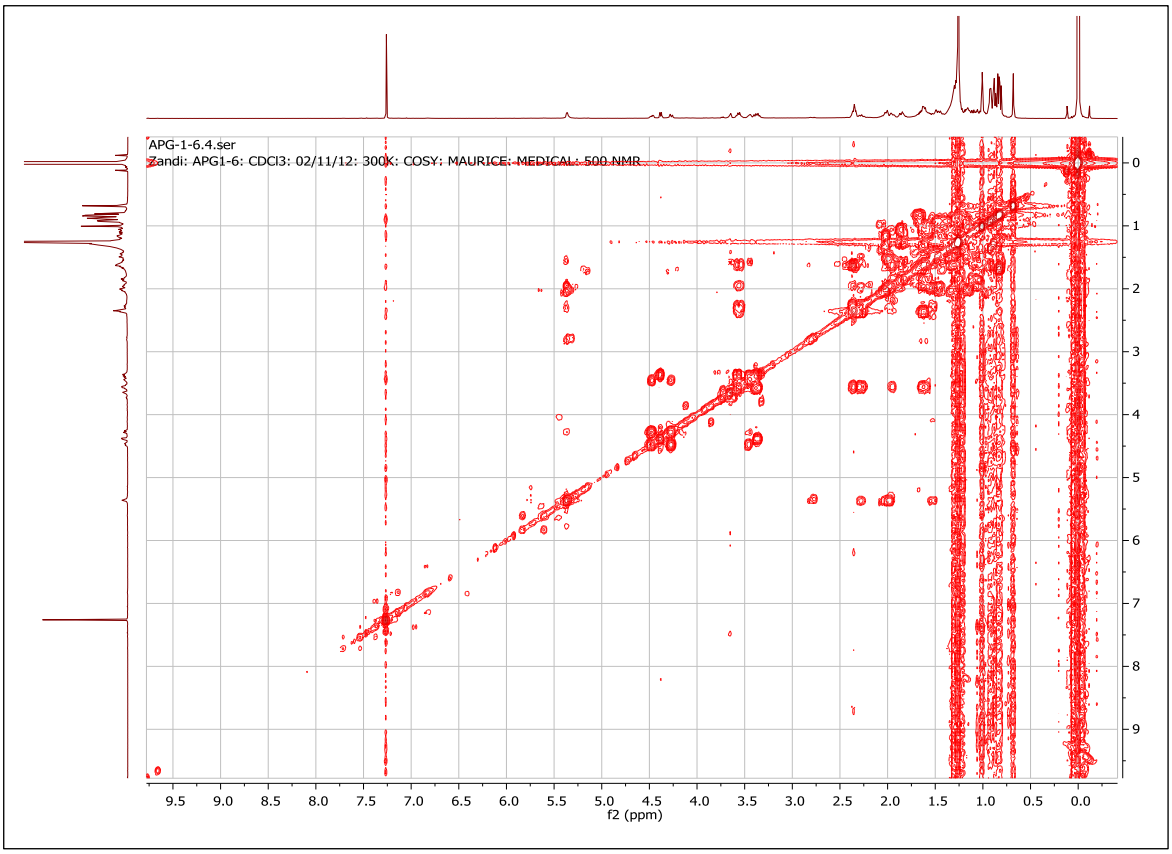


NOESY 500 NMR in CDl3

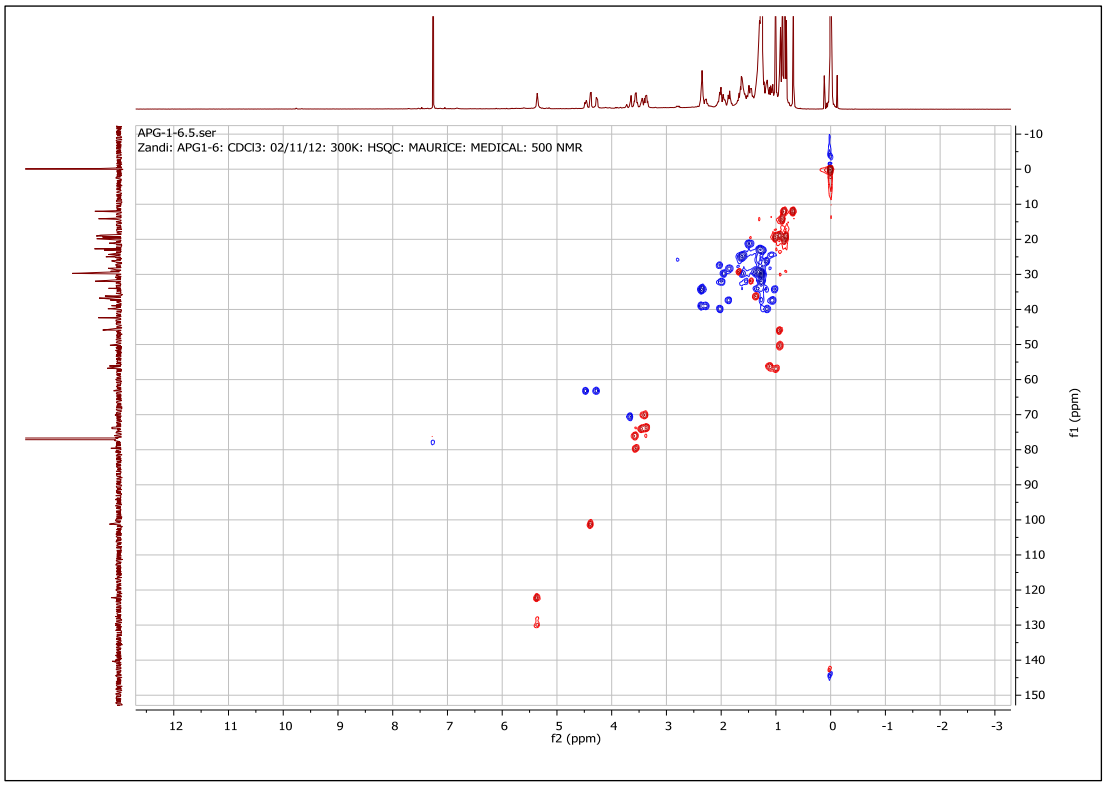


5.2 APG 6

COSY 500 NMR in CDl3



HSQC 500 NMR in CDl3


APG-1-6.5.ser
Zandi: APG1-6: CDCl3: 02/11/12: 300K: HSQC: MAURICE: MEDICAL: 500 NMR
f2 (ppm)
f1 (ppm)

APPENDIX 6

TURN IT IN REPORT

Ms

by Zandiswa Modjadji Gulube

Submission date: 06-Sep-2018 02:37PM (UTC+0200)
Submission ID: 997689063
File name: Zandi_Salman_Gulube.docx (10.54M)
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Ms

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APPENDIX 7

Ethics Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

19/09/2018

Ref: W-CBP-180919-01

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Ms Z Gulube
Student No. (if appropriate): 99 0914 9J
Staff No. (if appropriate): 00300399

Supervisor: Professor M Patel

School: Pathology
Department: Clinical Microbiology & Infectious Diseases
NHLS

Project title: *Effect of Punica gratunum Linn (pomegranite) on the oral pathogens and the identification of metabolites responsible for the beneficial effects*

Reason: Laboratory study.
No human participants will be involved in the study.

Professor CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu and Rhulani Mkansi.

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za.
Website:http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/