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# The effect of Dodonaea viscosa var. angustifolia on oral pathogens

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# The effect of *Dodonaea viscosa* var. *angustifolia* on oral pathogens

Roxanne Naidoo

**Degree of Master of Science in Medicine by research only**

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg 2012.

# DECLARATION

I, Roxanne Leandi Naidoo, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

I declare that this thesis has the approval of The Committee for Research on Human Subjects (Medical). Ethical clearance (M10205).

..... (Signature of candidate)

……. day of ……... 2012

# DEDICATION

To my Family,

Who were always there

# PUBLICATIONS AND PRESENTATIONS

1. Part of the work described in this thesis has been submitted under the title: Antibacterial characteristics of *Dodonaea viscosa* var. *angustifolia* extract on *Streptococcus mutans* biofilm. *Archives of Oral Biology*, 2011, submitted.

2. Poster presentation: *Dodonaea viscosa* var. *angustifolia* an indigenous South African medicinal plant and cariogenic bacteria. Postgraduate Cross Faculty Symposium (Best poster – 2nd place). University of the Witwatersrand, Johannesburg, South Africa, 2010.

3. Poster presentation: *Dodonaea viscosa* var. *angustifolia* an indigenous South African medicinal plant and cariogenic bacteria. XLIII Scientific Meeting of the South African Division – IADR, Pretoria, South Africa, 2010.

4. Poster presentation: *Dodonaea viscosa* var. *angustifolia* an indigenous South African medicinal plant and cariogenic bacteria. Faculty Research Day, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, 2010.

# ABSTRACT


**Introduction**

Dental caries and periodontal diseases are the most common bacterial oral infections in humans. Prevention and treatment of these infections are oral hygiene measures including antimicrobial compounds which can be chemical agents or natural products. *Dodonaea viscosa* var. *angustifolia* is an indigenous South African plant that has antibacterial, antiviral and anticandida properties. This study investigated the antibacterial and antivirulence effect of *D. viscosa* var. *angustifolia* on cariogenic bacteria and the causative organisms of periodontal diseases.

**Materials and methods**

Plant leaves were extracted in acetone, methanol and ethanol. The minimum bactericidal concentration of extracts were determined for cariogenic bacteria including *Streptococcus mutans* NCTC 10919, 4 clinical isolates of *S. mutans* and *Lactobacillus* spp. , *Lactobacillus casei* and periodontal pathogens including *Porphyromonas gingivalis, Prevotella intermedia, Capnocytophaga* spp. *and Fusobacterium nucleatum*. The effect of the subinhibitory concentration of crude ethanol plant extract on the biofilm (dental plaque), extracellular polysaccharide production (EPS) and acid production by *S. mutans* were investigated. The effect of the crude ethanol plant extract on protease activities such as Arg-gingipain and Lys-gingipain of *P. gingivalis* were also studied.

**Results**

Cariogenic bacteria were eliminated at concentrations of 1.56 - 12.5 mg/ml and periodontal pathogens at concentrations of 0.02 - 0.09 mg/ml of crude plant extract. The ethanol extract was the most effective against *S. mutans* and was used for all subsequent experiments. The

crude ethanol plant extract significantly inhibited the adherence of cells in preformed biofilms when exposure time was 24 hours ($p < 0.01$) rather than limited to 1 minute. However, it significantly reduced the formation of biofilms at 6, 24 and 30 hours. No effect of the crude ethanol plant extract on EPS production was observed. However it reduced the production of acid by planktonic cells but it had no effect on the production of acid in biofilms. The activity of Arg-gingipain and Lys-gingipain was significantly reduced by approximately 28 % and 50 % respectively.

**Conclusion**

*Dodonaea viscosa* var. *angustifolia* at high concentrations eliminates cariogenic bacteria. At low concentrations it will continue to kill periodontal pathogens and at subinhibitory concentrations it will render *P. gingivalis* avirulent. At subinhibitory concentrations the plant extract will not allow biofilm formation and acid production by *S. mutans* which are the major virulent factors in the development of dental caries. This study has shown that *D. viscosa* var. *angustifolia* has a potential to be developed into a preventative and therapeutic agent for commonly occurring oral diseases, dental caries and periodontal diseases. However, further research is required to establish active chemical constituents, cytotoxicity and *in vivo* efficacy of *D. viscosa* var. *angustifolia.*

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# TABLE OF CONTENTS

**Page**

**DECLARATION**..... **ii**

**DEDICATION**..... **iii**

**PUBLICATIONS AND PRESENTATIONS**..... **iv**

**ABSTRACT**..... **v**

**ACKNOWLEDGEMENTS**..... **vii**

**TABLE OF CONTENTS**..... **ix**

**LIST OF FIGURES**..... **xiv**

**LIST OF TABLES**..... **xvii**

**LIST OF ABBREVIATIONS AND ACRONYMS**..... **xix**

**CHAPTER 1: INTRODUCTION**

**1.1 Literature Review**..... **1**

**1.1.1 Oral pathogens**..... **1**

**1.1.2 Dental caries**..... **2**

**1.1.2.1 Microorganisms of dental caries**..... **4**

**1.1.2.1.1** *Streptococcus mutans*..... **4**

**1.1.2.1.2** Habitat, morphology and growth requirements..... **4**

**1.1.2.1.3** Pathogenicity of *Streptococcus mutans*..... **5**

**1.1.2.2 *Lactobacillus* species**..... **9**

**1.1.2.2.1** Habitat, morphology and growth requirements..... **9**

**1.1.2.2.2** Pathogenicity of *Lactobacilli*..... **10**

**1.1.2.3 Pathogenesis of dental caries**..... **10**

**1.1.2.4 Control and prevention**..... **11**

# TABLE OF CONTENTS

**Page**

**1.1.2.5 Natural prevention of dental plaque**……**12**

**1.1.2.6 Chemicals used in prevention and control**……...**13**

**1.1.2.6.1** Chlorhexidine gluconate……..……**13**

**1.1.2.6.2** Fluoride…….……**13**

**1.1.2.6.3** Iodine……**14**

**1.1.2.6.4** Triclosan……....**15**

**1.1.2.6.5** Essential oils……**15**

**1.1.3 Periodontal diseases**……...**15**

**1.1.3.1 Microorganisms of periodontal diseases**……..**18**

**1.1.3.1.1** *Porphyromonas gingivalis*……..……..**18**

**1.1.3.1.2** *Prevotella intermedia*……**20**

**1.1.3.1.3** *Fusobacterium nucleatum*……**22**

**1.1.3.1.4** *Capnocytophaga* species……..…….……**23**

**1.1.3.2 Pathogenic characteristics**……**25**

**1.1.3.3 Control and prevention**……..**26**

**1.1.3.3.1** Mechanical control……....**26**

**1.1.3.3.2** Chemical control……..….**27**

**1.1.4 Medicinal plants**……**28**

**1.1.5 *Dodonaea viscosa* var. *angustifolia***……**30**

**1.2 Aim**……...**33**

**1.3 Specific objectives**……...**33**

**CHAPTER 2: MATERIALS AND METHODS**

**2.1 Plant material**……**34**

**2.2 Plant extract**……**34**

# TABLE OF CONTENTS


**Page**

**2.3 Bacterial cultures**……**35**

**2.4 Antibacterial activity assay**……...**38**

**2.5 Biofilm assay**……...**40**

**2.5.1 Effect of crude ethanol plant extract on *Streptococcus mutans* biofilm (preformed)**…….....**40**

**2.5.2 Effect of crude ethanol plant extract on biofilm formation by *Streptococcus mutans***…….....**42**

**2.6 Effect of crude ethanol plant extract on the production of extracellular polysaccharides**……...**44**

**2.7 Acid production**……..**46**

**2.7.1 Effect on the acid production by biofilms of *Streptococcus mutans*** ……..**46**

**2.7.2 Effect on the acid production by planktonic cells of *Streptococcus mutans***……**47**

**2.8 Effect on protease activity by *Porphyromonas gingivalis***……....**47**

**2.9 Statistical analysis**……..**50**

**CHAPTER 3: RESULTS**

**3.1 *Dodonaea viscosa* var. *angustifolia* crude extract yield**……..…..**52**

**3.2 Identification of *Streptococcus mutans* using the API 20 Strep standardized system**……..…**54**

**3.3 Antibacterial activity assay**……..…..……**55**

**3.4 *Streptococcus mutans* biofilm assay**…….……**60**

**3.4.1 Effect of crude ethanol plant extract on preformed biofilm of *Streptococcus mutans***…….....**60**

# TABLE OF CONTENTS

**Page**

**3.4.2 Effect of crude ethanol plant extract on *Streptococcus mutans* biofilm formation**……**63**

**3.5 Extracellular polysaccharide analysis**……..**65**

**3.6 Acid production**……..**70**

**3.6.1 Effect of crude ethanol plant extract on the acid production by *Streptococcus mutans* in biofilm**……...……...……..**70**

**3.6.2 Effect of crude ethanol plant extract on the acid production by planktonic cells of *Streptococcus mutans***…….....……**72**

**3.7 Effect of the crude ethanol plant extract on the activity of proteases of *Porphyromonas gingivalis***……..**76**

**CHAPTER 4: DISCUSSION**……**82**

**4.1 Minimum bactericidal concentration assay**…….....**83**

**4.2 Inhibitory effects on *Streptococcus mutans* biofilm**……..……...**86**

**4.3 Effect on extracellular polysaccharide production in planktonic and biofilm forms**……....**91**

**4.4 Acid production in biofilms and planktonic cells**…….....**93**

**4.5 Inhibition of proteolytic activity of *Porphyromonas gingivalis***……...**97**

**CHAPTER 5: CONCLUSION, FUTURE RESEARCH AND LIMITATIONS TO THE STUDY**…….....**104**

**CHAPTER 6: REFERENCES**……**109**

**CHAPTER 7: APPENDICES**……**129**

# TABLE OF CONTENTS

**Page**

**Appendix 1 Composition and preparation of media**……..……**129**

**Appendix 2 Results of *Porphyromonas gingivalis* protease activity assay**……..…….....**133**

**Table 2.1 The protease activity (OD) of Arg-gingipain exposed to subinhibitory concentrations of *Dodonaea viscosa* var. *angustifolia* where controls are those for each concentration of crude plant extract using dimethyl sulphoxide (Figure 2.7B)**…….……**133**

**Table 2.2 The protease activity (OD) of Lys-gingipain exposed to subinhibitory concentrations of *Dodonaea viscosa* var. *angustifolia* where controls are those for each concentration of crude plant extract using dimethyl sulphoxide (Figure 2.7B)**…….....**134**

**Appendix 3 Ethical clearance**…….……**135**

# LIST OF FIGURES

**Figure 1.1** Root surface caries lesions with heavy microbial deposits particularly in the upper left canine (Fejerskov and Kidd, 2008)…….....3

**Figure 1.2** Spherical or oval, white colonies of *Streptococcus mutans* cultured on blood agar for 48 hours at 37 °C under $CO_2$…….....…....6

**Figure 1.3** The metabolic fate of dietary carbohydrates in the mouth (Marsh and Martin, 1999)……..7

**Figure 1.4** Round, white colonies of *Lactobacillus casei* cultured on Rogosa agar for 48 hours at 37 °C under $CO_2$……..…….....9

**Figure 1.5** Redness and swelling of gingiva and plaque build-up on teeth surfaces (Wen. 1985)…….....17

**Figure 1.6** Severe periodontitis: Swelling of gingiva due to the plaque formation above and below gingival margin (Wen, 1985)……...……...18

**Figure 1.7** The black pigmented colonies of *Porphyromonas gingivalis* cultured on blood agar for 7 days at 37 °C under anaerobic condition..…….....20

**Figure 1.8** Black pigmented colonies of *Prevotella intermedia* cultured on blood agar for 7 days at 37 °C under anaerobic condition……..…21

**Figure 1.9** Dull, white granular colonies with irregular rhizoid edges of *Fusobacterium nucleatum* cultured on blood agar for 7 days at 37 °C under anaerobic condition……...23

**Figure 1.10** White spreading colonies of *Capnocytophaga* species cultured on blood agar for 7 days at 37 °C under $CO_2$…..….....24

**Figure 1.11** The indigenous plant *Dodonaea viscosa* var. *angustifolia* may be found in these regions (green on map) in South Africa (Van Wyk *et al*, 2002)….....31

**Figure 1.12** The dioecious or monoecious plant *Dodonaea viscosa* var. *angustifolia*…...32

**Figure 1.13** The long, narrow leaves and flowers of *Dodonaea viscosa* var. *angustifolia*..32

**Figure 2.1** Microtitre plate showing the growth of *Prevotella intermedia* after an incubation period of 7 days at 37 °C under anaerobic condition with various concentrations of crude plant extract……..…….....…..39

**Figure 2.2** Chemical structure of Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride used as a substrate for Arg-gingipain obtained from Sigma-Aldrich, SA…...48

**Figure 2.3** Chemical structure of N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt used as a substrate for Lys-gingipain obtained from Sigma-Aldrich, SA…….49

**Figure 2.4** (A) Wells showing tests and positive controls, (B) wells showing dimethyl sulphoxide controls for each concentration of crude ethanol plant extract, (C) the formula used to calculate percentage enzymatic activity……..….51

**Figure 3.1** The 20 biochemical reactions characteristic of species that gave 99.9 % *Streptococcus mutans* match on an API 20 strep strip…….....…….…54

**Figure 3.2** The results sheet showing the numerical profile of species that gave 99.9 % *Streptococcus mutans* match on an API 20 strep strip……..…...55

**Figure 3.3** Bacterial counts (log CFU/ml) in *Streptococcus mutans* biofilm after 6 hours (two 1 minute exposures) and 24 hour exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract. * = p: < 0.01..….62

**Figure 3.4** Growth of *Streptococcus mutans* biofilm in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract (log CFU/ml) in

biofilm after 6, 24 and 30 hour exposures to plant extract. *, **, *** = p: < 0.01.....65

**Figure 3.5** Extracellular polysaccharides produced by *Streptococcus mutans* in biofilm after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract……..….67

**Figure 3.6** Extracellular polysaccharides produced by *Streptococcus mutans* planktonic cells in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract…….…69

**Figure 3.7** Mean pH values of *Streptococcus mutans* in biofilm after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract……72

**Figure 3.8** Mean pH values and the bacterial counts (log CFU/ml) of *Streptococcus mutans* in planktonic form after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract…….....75

**Figure 3.9** The protease activity of Arg-gingipain after exposure to different subinhibitory concentrations of crude ethanol plant extract. *, **, #, $, ~ = p: < 0.01……...……...……78

**Figure 3.10** The protease activity of Lys-gingipain after exposure to different subinhibitory concentrations of crude ethanol plant extract. *, **, #, $, ~ = p: < 0.01……..80

**Figure 3.11** The protease activity of Arg-gingipain and Lys-gingipain produced by *Porphyromonas gingivalis* exposed to various subinhibitory concentrations of crude ethanol plant extract. *, **, #, $, ~ = p: < 0.01…..81

**Figure 4.1** Effect of *Dodonaea viscosa* var. *angustifolia* on the cariogenic bacteria and periodontal pathogens: A summary of the findings……..….103

# LIST OF TABLES

**Table 2.3.1** Reading table for API 20 Strep standardized system (BioMerieux, SA).……37

**Table 3.1.1** The dry weight of acetone extracts of *Dodonaea viscosa* var. *angustifolia*….52

**Table 3.1.2** The dry weight of methanol extracts of *Dodonaea viscosa* var. *angustifolia*..52

**Table 3.1.3** The dry weight of ethanol extracts of *Dodonaea viscosa* var. *angustifolia*.....53

**Table 3.3.1** Minimum bactericidal concentrations of crude plant extracts of *Dodonaea viscosa* var. *angustifolia* against *Streptococcus mutans*.……..……56

**Table 3.3.2** Minimum bactericidal concentrations of crude plant extracts of *Dodonaea viscosa* var. *angustifolia* against *Lactobacillus* species…….....……..57

**Table 3.3.3** Minimum bactericidal concentrations of crude plant extracts of *Dodonaea viscosa* var. *angustifolia* against periodontal pathogens……..……58

**Table 3.3.4** Minimum bactericidal concentrations of dimethyl sulphoxide against periodontal pathogens……..….....59

**Table 3.3.5** Summary of median minimum bactericidal concentrations of acetone, ethanol and methanol extracts of *Dodonaea viscosa* var. *angustifolia* against cariogenic bacteria and periodontal pathogens……..…..60

**Table 3.4.1.1** Effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on preformed biofilm of *Streptococcus mutans*……...61

**Table 3.4.2.1** Effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on biofilm formation of *Streptococcus mutans*……64

**Table 3.5.1** Extracellular polysaccharide production by *Streptococcus mutans* biofilm after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract…….….66

**Table 3.5.2** Extracellular polysaccharide production by planktonic cells of *Streptococcus mutans* in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract…….….68

**Table 3.6.1.1** The effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on acid production by biofilms of *Streptococcus mutans*…..**.....**71

**Table 3.6.2.1** The effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on the acid production by planktonic cells of *Streptococcus mutans*……..73

**Table 3.6.2.2** The bacterial counts (CFU/ml) of *Streptococcus mutans* planktonic cells in the presence and absence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract……...74

**Table 3.7.1** Arg-gingipain activity (%) by *Porphyromonas gingivalis* exposed to subinhibitory concentrations of crude ethanol plant extract…….…...77

**Table 3.7.2** Lys-gingipain activity (%) by *Porphyromonas gingivalis* exposed to subinhibitory concentrations of crude ethanol plant extract…….....79

# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AgI/II:** | Antigen I/II |
| **API:** | Analytical Profile Index |
| **ATP:** | Adenosine triphosphate |
| **α:** | Alpha |
| **b:** | bar |
| **ß-galactosidase:** | Beta-galactosidase |
| **BHI:** | brain heart infusion media |
| **CFU/ml:** | colony forming units per millilitre |
| ***C. albicans:*** | *Candida albicans* |
| **CHX:** | chlorhexidine gluconate |
| **$CO_2$:** | carbon dioxide |
| **Da:** | Daltons |
| **DMSO:** | dimethyl sulphoxide |
| **DNA:** | deoxyribonucleic acid |
| **DTT:** | dithiothreitol |
| ***D. viscosa* var. *angustifolia*:** | *Dodonaea viscosa* var. *angustifolia* |

| | |
|---|---|
| **EPS:** | Extracellular polysaccharides |
| **F-ATPase:** | ATP synthase |
| ***F. nucleatum*:** | *Fusobacterium nucleatum* |
| **FTF:** | fructosyltransferase |
| **g:** | gram |
| **g/ml:** | gram per millilitre |
| **GC-MS:** | Gas chromatography-mass spectrometry |
| **GTFs:** | glucosyltransferases |
| **HIV:** | Human Immunodeficiency Virus |
| **HPLC:** | high performance liquid chromatography |
| **hrs:** | hours |
| **IgG:** | Immunoglobulin G |
| **IgAI:** | Immunoglobulin AI |
| **IL-1:** | Interleukin 1 |
| **IL-8:** | Interleukin 8 |
| **kDa:** | kilo Daltons |
| **MBC:** | minimum bactericidal concentration |
| **MC:** | Carlsson medium with sulphonamide |

| | |
|---|---|
| **mg:** | milligrams |
| **mg/ml:** | milligrams per millilitre |
| **MIC:** | minimum inhibitory concentration |
| **min:** | minutes |
| **ml:** | millilitre |
| **mM:** | millimolar |
| **MSA:** | Mitis salivarius agar |
| **MSB:** | Mitis salivarius bacitracin agar |
| **NCTC:** | National Collection of Type Cultures |
| **nm:** | nanometres |
| **OD:** | optical density |
| **PBS:** | phosphate buffered saline |
| **PCR:** | polymerase chain reaction |
| ***P. gingivalis*:** | *Porphyromonas gingivalis* |
| ***P. intermedia*:** | *Prevotella intermedia* |
| **PRPs:** | proline-rich proteins |
| **rpm:** | revolutions per minute |
| **SAG:** | salivary agglutinin glycoprotein |

| | |
|---|---|
| ***S. mutans*:** | *Streptococcus mutans* |
| **spp:** | species |
| ***S. sobrinus:*** | *Streptococcus sobrinus* |
| **Tris-HCL:** | hydrochloric acid (buffer) |
| **WHO:** | World Health Organization |
| **XTZ:** | xanthorrizol |
| **µg/ml:** | micrograms per millilitre |
| **µl:** | microlitres |
| **µm:** | micrometres |

# CHAPTER 1: INTRODUCTION

Most individuals suffer at some time in their life from localized episodes of disease in the mouth caused by the imbalance in the composition of their natural oral flora (Susumu and Hiroshi, 2005). These diseases include dental caries, periodontal diseases and oral candidiasis. The preventative measures commonly used are chemical agents which have antimicrobial properties (Nisengard and Newman, 1994). The use of medicinal plant extracts and natural products as alternative antimicrobial agents in the treatment of oral diseases has been reported (Tapsoba and Deschamps, 2005). This study investigated the antimicrobial properties of an indigenous South African medicinal plant that can potentially be used in the control and prevention of dental caries and periodontal diseases.

## 1.1 Literature Review

### 1.1.1 Oral Pathogens

The oral cavity is a complex ecosystem comprised of many surfaces coated with a wide variety of natural microflora that exists in harmony with the host. Over 750 species of bacteria colonize different surfaces of the oral cavity due to the binding of specific adhesins that are present on the bacterial surface to complementary specific receptors on a specific oral surface resulting in variation of microbial colonies (Jenkinson and Lamont, 2005; Aas *et al*, 2005). However, streptococci and gram-positive rods comprise the majority of the total viable count (Hamada and Slade, 1980). An imbalance between the host and the opportunistic microflora may occur due to dietary constituents, systemic illnesses, poor saliva flow and oral hygiene resulting in the alteration of microbial

communities which leads to the development of disease (Susumu and Hiroshi, 2005). The two most common diseases are dental caries which results in the demineralization of the tooth and periodontal diseases that affect the tooth supporting tissues. Dental caries is most common in children and periodontal diseases are common in adults (Lemos and Burne, 2008; Nisengard and Newman, 1994). The gram-positive cocci and bacilli (dental caries) and gram-negative rods (periodontal diseases) are present in high proportions in areas of the mouth in which these diseases are prevalent (Hamada and Slade, 1980).

### 1.1.2 Dental caries

Dental caries is one of the most common childhood diseases, although people may be susceptible to the disease throughout their lifetime (Selwitz *et al,* 2007). It is a chronic disease that causes the demineralization of the hard dental tissues due to acids (Figure 1.1, page 3) and initiates within the bacterial biofilm or dental plaque on the surface of the tooth (Koo *et al,* 2000). There are many types of caries affecting various tooth surfaces within the mouth. These surfaces include, buccal and lingual smooth surfaces (smooth surface caries), approximal sites (approximal surface caries) and fissures on occlusal surfaces are the most caries prone sites (fissure caries). Rampant caries usually develops in immunocompromised individuals such as xerostomic patients with a reduced salivary flow due to radiation treatment, individuals who suffer from Sjogrens syndrome or could be due to certain medications (Marsh and Martin, 1999).



**Figure 1.1:** Root surface caries lesions with heavy microbial deposits particularly in the upper left canine (Fejerskov and Kidd, 2008).

Generally the formation of dental plaque on the tooth surface is natural and beneficial to the host as it prevents colonization of exogenous species (Marsh, 1994). It is essentially a microbial biofilm formed by organisms tightly bound to each other and to the solid substratum and enclosed in an exopolysaccharide matrix produced by oral streptococci on the tooth surface (Koo *et al*, 2002; Sbordone and Bortolaia, 2003). These biofilms can be considered a community of over 500 bacterial species varying from their planktonic state by displaying different physiological properties, susceptibility to antimicrobial agents, immunological responses and its interaction with host tissues (Sbordone and Bortolaia, 2003). The colonization of bacteria and the formation of the biofilm follow a regular pattern which involves the adhesion of initial colonizers usually coccal bacteria such as *Streptococcus mutans* to the enamel salivary pellicle followed by interbacterial adhesion

(secondary colonization). Adhesins and molecular interactions contribute to plaque development and integrity (Rosan and Lamont, 2000).

Dental caries is a multifactorial disease affected by oral bacteria, salivary flow and composition, exposure to fluoride and consumption of dietary sugars (Selwitz *et al*, 2007). Oral plaque bacteria interact with dietary constituents particularly sucrose resulting in the production of organic acids such as lactic, acetic, formic and propionic acid which leads to the slow decomposition of teeth (Nisengard and Newman, 1994; Featherstone, 2004). Sucrose is the major fermentable carbohydrate which also acts as a substrate for the synthesis of polysaccharides in dental biofilms. Starch can also be seen as an important source of fermentable carbohydrates but is considered non-cariogenic although combinations of starch and sucrose have the potential to be more cariogenic than either carbohydrate alone (Duarte *et al*, 2008).

#### 1.1.2.1 Microorganisms of dental caries

##### 1.1.2.1.1 *Streptococcus mutans*

##### 1.1.2.1.2 Habitat, morphology and growth requirements

The main causative agents of dental caries are the gram-positive bacteria, *Streptococcus mutans. Streptococcus mutans* belongs to a group of cariogenic species called Mutans streptococci (Yoo *et al*, 2005). They are a homogenous group of non-motile, catalase-negative, gram-positive bacteria (Hamada and Slade, 1980). The natural habitat of *S. mutans* is the human mouth and it is the main causative agent in the development and

establishment of cariogenic biofilms (Song *et al,* 2007). They are spherical bacteria varying in diameter between 0.5 and 2 µm (Nisengard and Newman, 1994).

The most widely used agar for the selective isolation of *S. mutans* is mitis salivarius agar (MSA). *Streptococcus mutans* produces a characteristic colonial morphology on MSA which differentiates it from other oral streptococci (Emilson and Bratthall, 1976). This agar can be modified to be more selective by the addition of bacitracin (MSB agar), sulphonamide (MC agar), polymyxin and supplemented sucrose (Hamada and Slade, 1980). A non-selective medium such as blood agar (Figure 1.2, page 6) is often used to quantitatively relate *S. mutans* to the total number of recoverable bacteria (Emilson and Bratthall, 1976).

#### 1.1.2.1.3 Pathogenicity of *Streptococcus mutans*

*Streptococcus mutans* is implicated in the initiation of dental caries. There are three major virulence factors that play a role in pathogenesis, these include its ability to adhere and form biofilms on the tooth surface (plaque formation), to produce organic acids through fermentation of carbohydrates (acidogenic) whilst withstanding environmental stresses including acidic conditions (aciduric) and to produce extra- and intracellular polysaccharides (Lemos and Burne, 2008). The adherence of *S. mutans* to the surface of the tooth is necessary for colonization and infection to occur. Adhesion results due to specific stereo-chemical interactions between bacterial adhesins present on the microbial cell surface and receptors in the saliva-derived conditioning film, the acquired enamel pellicle (Marsh and Martin, 1999; Shimotoyodome *et al,* 2007).

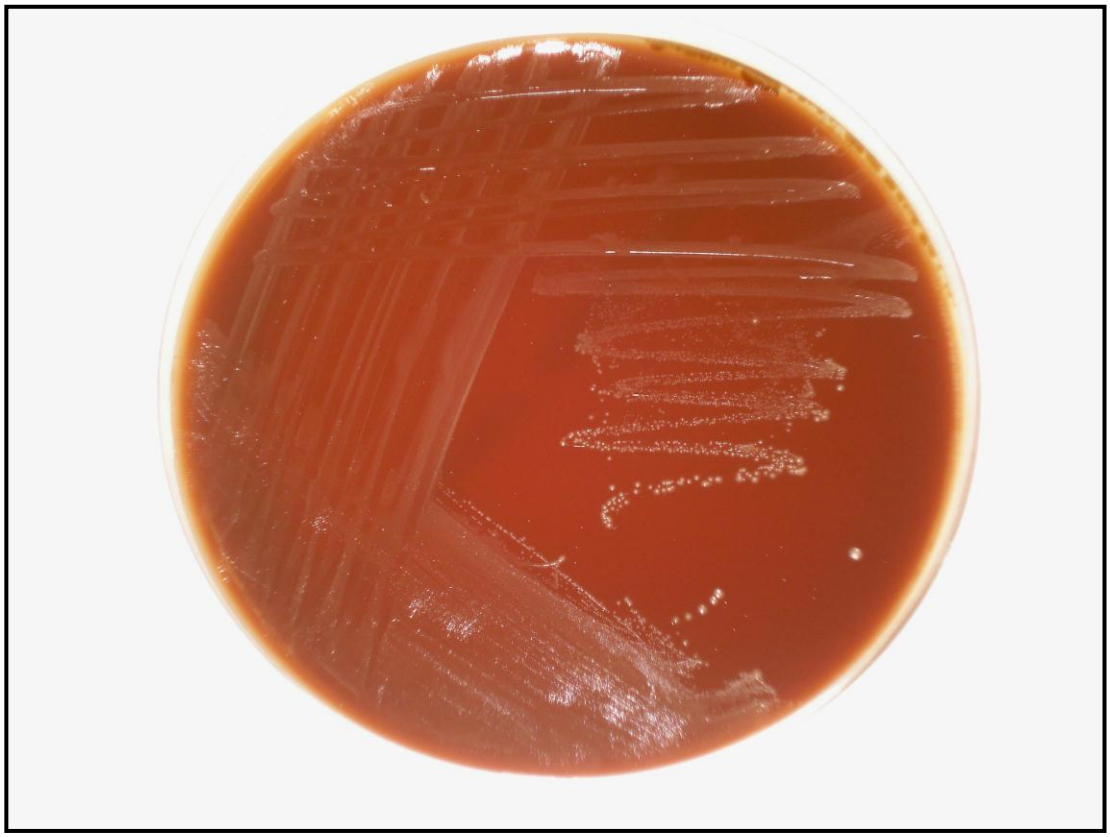


**Figure 1.2:** Spherical or oval, white colonies of *Streptococcus mutans* cultured on blood agar for 48 hours at 37 °C under $CO_2$.

The acquired pellicle is formed by the adsorption of heterogeneous salivary proteins to dental enamel hydroxyapatite promoting the adhesion of *S. mutans* through specific and nonspecific mechanisms (Shimotoyodome *et al*, 2007). *Streptococcus mutans* readily adheres to hydroxyapatite treated with proline-rich proteins (PRPs). Particular organisms will therefore bind to different regions of the acidic PRPs. The mutans streptococci usually bind to the glucan receptor present on the tooth surface (acquired pellicle) with a glucan binding protein (Marsh and Martin, 1999). There are however a number of proteins that function as adhesins.

*Streptococcus mutans* also synthesizes extracellular polysaccharides (Figure 1.3) from carbohydrates especially dietary sucrose. Polysaccharides are condensation polymers of monosaccharides which are joined together by glycosidic bonds (Smelcerovic *et al*, 2008). The polysaccharides produced may be soluble which are more labile and are often metabolized by other bacteria or insoluble which contributes to the structural integrity of the plaque and facilitates with the attachment of bacteria within the plaque. Sucrose is more readily metabolized because it acts as a substrate as the glucose and fructose bond moieties have sufficient energy on cleavage to support polysaccharide synthesis (Marsh and Martin, 2009).

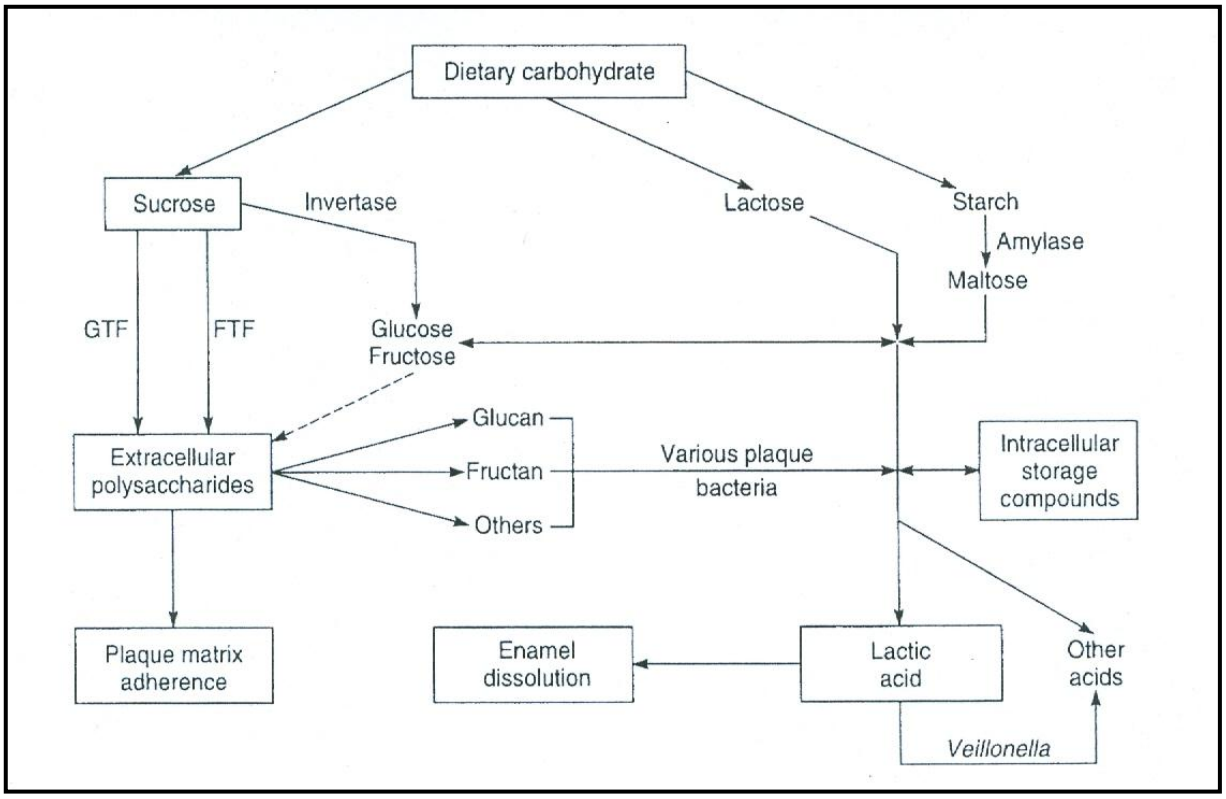



**Figure 1.3:** The metabolic fate of dietary carbohydrates in the mouth (Marsh and Martin, 1999).

Polysaccharides are synthesized by glucosyltransferases (GTFs). *Streptococcus mutans* produces three GTFs, GTF B which synthesizes α 1,3-linked insoluble glucans, GTF C synthesizes α 1,6-linked soluble and insoluble glucans and GTF D which is responsible for the synthesis of the soluble glucans. However, the essential virulence factors associated with dental caries are GTF B and C (Koo *et al,* 2003). The mixed-linkage (α 1,3, α 1,6) water-insoluble glucans are collectively referred to as mutans. A high content of α 1,3-linkages are mostly associated with water insolubility and α 1,6-linked side chains contributes to the adhesive properties of the biopolymers (Wiater *et al,* 2004). The size and structures of mutans produced is dependant upon the type of GTF and mutan properties depend on the relative activity and interactions of different GTF enzymes (Colby and Russell, 1997). Mutans contribute to the stabilization of the plaque biofilm by mediating the attachment of bacteria to the tooth surface and to each other (Wiater *et al,* 2004). They are also resistant to enzymes present in plaque. Water-insoluble glucans comprise approximately 1.5 % of the total dry material of dental plaque.

*Streptococcus mutans* also produces fructosyltransferase (FTF), which acts as a catalyst for the synthesis of fructan from fructose derived from sucrose (Ebisu *et al,* 1975). Fructans are usually metabolized during periods of nutrient deprivation. One of the main types of fructans are levans which are naturally occurring homopolymers of fructose. Its main chain is composed of repeating five-member fructofuranosyl rings which are connected by β-2,6 links and branched at C-1. Bacterial levans are large ranging from 2 - 100 million Da and are synthesized by a single inducible exoenzyme, levansucrase which utilizes sucrose as a substrate (Smelcerovic *et al,* 2008).

### 1.1.2.2 *Lactobacillus* species

#### 1.1.2.2.1 Habitat, morphology and growth requirements

*Lactobacilli* are saprophytes that comprise a group of more than 80 species that inhabit ecological habitats such as plants, animals, dairy products, the oral cavity (less than 1%), gastrointestinal tract and female genital tract (Caufield *et al*, 2007; Samaranayake, 2002). They are cylindrical cells (large or small rods), rounded, square or tapered at the ends and are approximately 0.2 to 1 µm in diameter and 10 µm in length. The majority of the *Lactobacilli* grow readily under anaerobic conditions, however they are capable of growing under a low oxygen tension with 5 – 10 % carbon dioxide (Nisengard and Newman, 1994). They grow at an acidic pH (6.0) under microaerophilic conditions. Growth is favored by a media enriched with glucose or blood (Samaranayake, 2002). The most commonly used agar is Rogosa agar (Figure 1.4).

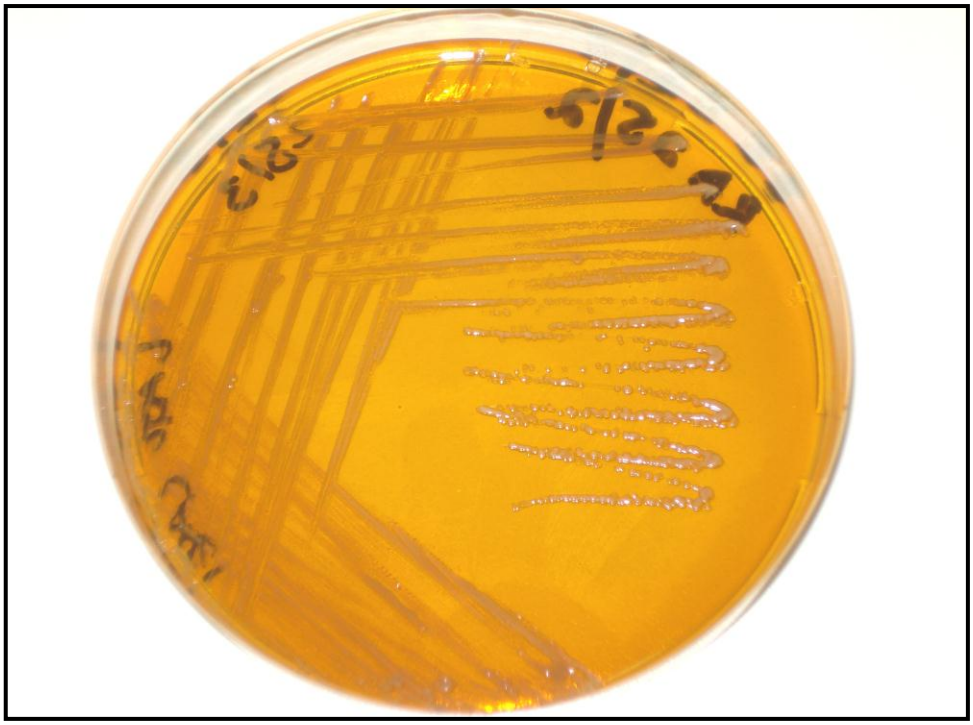


**Figure 1.4:** Round, white colonies of *Lactobacillus casei* cultured on Rogosa agar for 48 hours at 37 °C under $CO_2$.

#### 1.1.2.2.2 Pathogenicity of *Lactobacilli*

*Lactobacilli* are the major etiological agents of dental caries and are implicated in the progression of caries (Caufield *et al,* 2007). The most common species present in the oral cavity are *L. planetarium, L. salivarius, L, oris* and *L. casei* (Nisengard and Newman, 1994). They have been isolated in high numbers in both superficial and deep carious lesions where the pH tends to be acidic (Byun *et al,* 2004). *Lactobacilli* are usually the secondary invaders as they are only associated with caries after the lesion is formed and predominate in advanced lesions in adults (Caufield *et al,* 2007).

They may be classified into three groups namely, homofermenters which produce lactic acid from glucose fermentation (*L. casei*), heterofermenters which produce acetate, ethanol, carbon dioxide as well as lactic acid (*L. fermentum*) and facultative heterofermenters which primarily produce lactic acid but also have inducible enzymes capable of producing other fermentation products (Samaranayake, 2002). They are prolific lactic acid producers because they ferment carbohydrates to form acids and have a high tolerance for acidic environments (Samaranayake, 2002). Some *Lactobacilli* are capable of producing extra and intracellular polysaccharides.

### 1.1.2.3 Pathogenesis of dental caries

The mechanism of dental caries is similar for all types of caries. The causative bacteria, *S. mutans* have three properties that distinguish them from other bacteria, namely: (1) the rapid transportation of sugars if in competition with other plaque bacteria, (2) the ability to rapidly produce acid from these sugars, (3) acidogenic and aciduric properties which

allow them to maintain these activities under extreme environmental conditions and influence the local pH values in the mouth causing it to drop, resulting in demineralization of the teeth (Marsh and Martin, 1999). Demineralization occurs due to the loss of hydroxyapatite crystals when acid diffuses into the enamel, dentin or cementum of the teeth (Featherstone, 2004).

The causative bacteria colonize and live on the non-shedding surfaces of the teeth in microcolonies encapsulated in an organic matrix of polysaccharides, salivary proteins and DNA secreted by cells (Selwitz *et al,* 2007). This allows them to be protected from host defenses and offers resistance to antimicrobial agents. Cavitation my eventually result if there is continuous diffusion of carbonate, calcium and phosphate out of the teeth leading to demineralization. This is usually caused by acidogenic and aciduric bacteria. Demineralization of dentin results in the exposure of the collagen fibrils. Bacterially derived enzymes particularly proteolytic enzymes such as proteases usually produced by gram-negative anaerobic bacteria then breakdown the collagen present in the dentin (Clarkson *et al,* 1986; Kawasaki and Featherstone 1997). Therefore, the pathology of dental caries is mainly associated with bacterial metabolism. This causes the destruction of biomolecules due to catabolism of nutrients present in saliva, the host's diet, reactive oxygen species and other factors (Lemos and Burne, 2008).

#### 1.1.2.4 Control and Prevention

Control and prevention of bacteria, particularly *S. mutans* and *Lactobacilli* which are found in biofilm (dental plaque) is important. Prevention and control of dental plaque will

in turn control dental caries. This can be achieved by controlling plaque formation or removing fully formed plaque.

#### 1.1.2.5 Natural prevention of dental plaque

The mouth retains moisture and lubrication due to the presence of saliva which flows over all internal surfaces of the oral cavity (Marsh and Martin, 1999). Saliva is produced by and enters the mouth via the submandibular and sublingual glands and the major paired parotid and minor glands. Saliva has four important functions in the maintenance of oral health: (1) lubrication and mechanical washing action which are powerful mechanisms causing bacteria to be washed and swallowed thus killed my gastric acid, (2) buffering action (bicarbonate system) and pH which prevents colonization of pathogenic microorganisms by stabilizing pH to about 6.7 keeping the mouth alkaline, (3) maintenance and tooth integrity, (4) antibacterial activity due to the presence of immunoglobulins that may act as specific agglutinins which interact with surface receptors present on bacteria (Humphrey and Williamson, 2001; Nisengard and Newman, 1994). Saliva contains proteins and glycoproteins such as lactoperoxidases which alters the metabolism of plaque bacteria and lactoferrin and lysozymes that inhibit the growth and colonization of exogenous bacteria. These proteins and glycoproteins are also a source of nutrients to resident microflora and adsorb to the tooth surface forming the acquired pellicle to facilitate the attachment of microorganisms which are later cleared from the mouth by swallowing (Marsh and Martin, 1999).

Dental caries may also be controlled by good oral hygiene practices such as the mechanical removal of plaque by brushing teeth regularly. A reduction in the amount and the frequency of sugar intake may reduce the chances of developing caries, therefore a healthy diet should be maintained. Another approach is the application of fissure sealants containing antimicrobial compounds which acts as a barrier over caries prone areas such as occlusal pits and fissures (Marsh and Martin, 1999).

#### 1.1.2.6 Chemicals used in prevention and control

##### 1.1.2.6.1 Chlorhexidine gluconate

Chemical agents have also shown to be potentially effective against dental caries (Nisengard and Newman, 1994). Chlorhexidine gluconate (CHX) is an antimicrobial agent that inhibits fungi, gram-positive and gram-negative bacteria present in the oral cavity. It targets the membrane structure of bacteria through electrostatic forces that allow the binding to the negatively charged sites on the bacterial wall causing leakage of intracellular components (Dogan *et al,* 2003). It is used as a mouthwash and has proven to be effective on fully formed plaque and in the inhibition of plaque formation. Oral surfaces also have the ability to absorb CHX which is later slowly released (substantivity) for a prolonged effect (Dogan *et al,* 2003).

##### 1.1.2.6.2 Fluoride

The most effective and widely used chemotherapeutic method involves the utilization of fluorides. Fluoride exerts a topical and systemic effect after ingestion (Marsh and Martin, 1999). Topical mechanisms of action involve the inhibition of bacterial metabolism due

to the diffusion of the hydrogen fluoride molecule into bacterial cells when plaque is acidified, prevention of demineralization at the crystal surfaces and remineralization by functioning as a catalyst allowing the diffusion of phosphate and calcium into the tooth (Featherstone, 2000). This produces a rebuilt crystalline structure more resistant to acid (Selwitz *et al,* 2007). The systemic effect is achieved through supplementation by tablets. Oral fluids generally have low levels of fluoride which forms the thermodynamically stable fluorapatite when it interacts with the surface of the enamel or erupted teeth. Fluorapatite displays high resistance to acid dissolution. Fluoride also has the ability to inhibit the metabolism of plaque bacteria by reducing glycolysis through inhibition of enolase, inhibiting sugar transport, inactivating key metabolic enzymes by acidifying the interior of cells, inhibiting the synthesis of intracellular storage compounds and altering the bacterial membrane permeability thereby interfering with the structural integrity of the plaque biofilm (Marsh and Martin, 1999).

#### 1.1.2.6.3 Iodine

Iodine is a widely used effective antiseptic. It is active against a variety of microorganisms such as bacteria, fungi, protozoa, viruses and yeasts (Zinner *et al,* 1961). However, its use intraorally is more limited due to its insolubility and instability as well as its staining and irritating properties. It has been successfully used for the preparation of oral mucosal sites before surgery. Iodine has been proven to exhibit antiplaque activity against preformed plaque. Studies have shown that the number of viable microorganisms in saliva and gingival crevices are reduced by the administration of prophylactic topical iodine (Tanzer *et al,* 1977).

#### 1.1.2.6.4 Triclosan

Triclosan is a lipophilic, water insoluble chemical used as an antibacterial agent in toothpastes, and other oral related products (Loftsson *et al*, 1999). Triclosan is approximately 65 % as effective as CHX and studies have shown a reduction in plaque ranging between 0 and 30 % over a period of six months (Nisengard and Newman, 1994). Also, the availability and substantivity of triclosan is significantly less than that of CHX. The antiplaque effect of triclosan can be improved by the addition of other compounds such as triclosan containing liposomes and triclosan/copolymer dentrifice (Loftsson *et al*, 1999).

#### 1.1.2.6.5 Essential oils

Essential oils are natural, complex, multi-component systems composed of a mixture of phenolic compounds and methylsalicylate contained in mouthwashes which exhibit moderate antiplaque activity resulting in plaque reduction as proven by clinical trials (Edris, 2007; Nisengard and Newman, 1994). Essential oils are usually extracted from different parts of the aromatic plant by distillation (Edris, 2007). They have been used against a wide variety of bacteria and fungi including oral pathogens (Filoche, 2005). Essential oils have been used for many years in oral hygiene products such as the mouthrinse Listerine, whose efficacy has been reported since the 1890s.

### 1.1.3 Periodontal diseases

The prevalence of periodontal diseases (disease of the supporting structure of teeth) is relatively high and affects up to 90 % of the population (Pihlstrom *et al*, 2005). The

progression of plaque formation around the gingival margin and the subgingival region leads to periodontal diseases (Koo *et al,* 2000). Periodontal diseases are usually classified into two groups, those that affect only the gingiva causing gingivitis and those that cause the destruction of the underlying structures on the periodontium including bone causing periodontitis (Williams, 1990).

Gingivitis, the mildest form of periodontal disease, is the inflammation of the gingiva which causes it to undergo changes in appearance and can be characterized by bleeding of gingival crevices (Nisengard and Newman, 1994). It is characterized by a change in the position of the gingival margin, redness and swelling (Figure 1.5, page 17). It is often a direct immune response due to the microbial plaque build-up on the teeth surface and is reversible as it does not affect the underlying supporting structures of the teeth (Kinane, 2001; Pihlstrom *et al,* 2005). A severe form of gingivitis is acute necrotizing ulcerative gingivitis (trench mouth or Vincent's infection) which is often seen in adolescents and immunocompromised individuals. This is characterized by pain and tissue necrosis (Williams, 1990). Gingivitis is influenced by several factors such as hormonal disturbances, smoking, drugs, diseases such as diabetes and medication that reduces salivary flow.



**Figure 1.5:** Redness and swelling of gingiva and plaque build-up on teeth surfaces (Wen, 1985).

Periodontitis arises when inflammation of the deeper structures of the periodontium occurs (Figure 1.6, page 18). This results due to the spread of infection from the gingiva to the periodontal ligament and alveolar bone and is characterized by the formation of periodontal pockets, mobility of teeth and eventually loss of bone. Periodontal pockets are formed due to the destruction of the periodontal ligament and the resorption of the alveolar bone which causes the gingiva to migrate along the root surface (Williams, 1990). Progression of the disease involves the rapid destruction of tissue followed by some repair and disease remission periods (Listgarten, 1986). The initiation of disease development is dependent upon the formation of plaque above and below the gingival margin (Koo *et al*, 2000). This plaque contains capnophilic and obligatory anaerobic bacteria.




**Figure 1.6:** Severe periodontitis: Swelling of gingiva due to the plaque formation above and below gingival margin (Wen, 1985).

#### 1.1.3.1 Microorganisms of periodontal diseases

Many anaerobic oral bacteria cause periodontal diseases but the primary causative gram-negative anaerobic bacteria are *Porphyromonas gingivalis, Prevotella intermedia, Capnocytophaga* spp. and *Fusobacterium nucleatum.* These are opportunistic pathogens which do not readily induce disease in a healthy host but rather in an immunocompromised one. Therefore, they are present in periodontally healthy subjects in the sub- and supragingival plaques (Cortelli *et al,* 2008).

##### 1.1.3.1.1 *Porphyromonas gingivalis*

*Porphyromonas gingivalis* are obligate anaerobes that grow only in the absence of oxygen (Hansen *et al,* 2000). They are most commonly found at subgingival sites in patients with advanced periodontal diseases and are very rarely recovered from the

tongue and tonsils. These bacteria are non-motile, short, pleomorphic gram-negative rods that grow with dark pigmentation on media containing lysed blood (Figure 1.7, page 20). Identification is usually based on biochemical characteristics, although DNA and molecular probing has been successfully implemented to identify these organisms from plaque samples (Samaranayake, 2002). They are the key causative agents of adult periodontitis (Brochu *et al,* 2001).

Pathogenic factors include fimbriae which mediates adhesion to sites to facilitate colonization and a capsule to protect it against phagocytosis. The main virulence factor is the production of gingipains such as Arg-gingipain and Lys-gingipain which are cysteine proteases responsible for the trypsin-like activity of the bacterium (Lourbakos *et al*, 2010). There are three different genes present that are responsible for encoding arginine – X-specific cysteine proteases and lysine-X-specific cysteine proteases and these are responsible for most of the proteolytic activity. Two genes code for an Arg-specific (RgpA and RgpB) and one for a Lys-specific (Kgp) protease (Potempa *et al,* 2003). They are responsible for the degradation of human plasma and tissue component proteins including immunoglobulin G, haemoglobin, bio-active peptides, fibrinogen and collagen and the modulation of host cytokine networks (Brochu *et al,* 2001; Yamanaka *et al*, 2007). Other virulence factors include, lipopolysaccharides, haemagglutinin, complement and haem-sequestering proteins (Samaranayake, 2002). *Porphyromonas gingivalis* also has the ability to inhibit the accumulation of IL-8 which is dependant upon the invasion of the epithelial cells by the bacteria itself (Lourbakos *et al,* 2010).

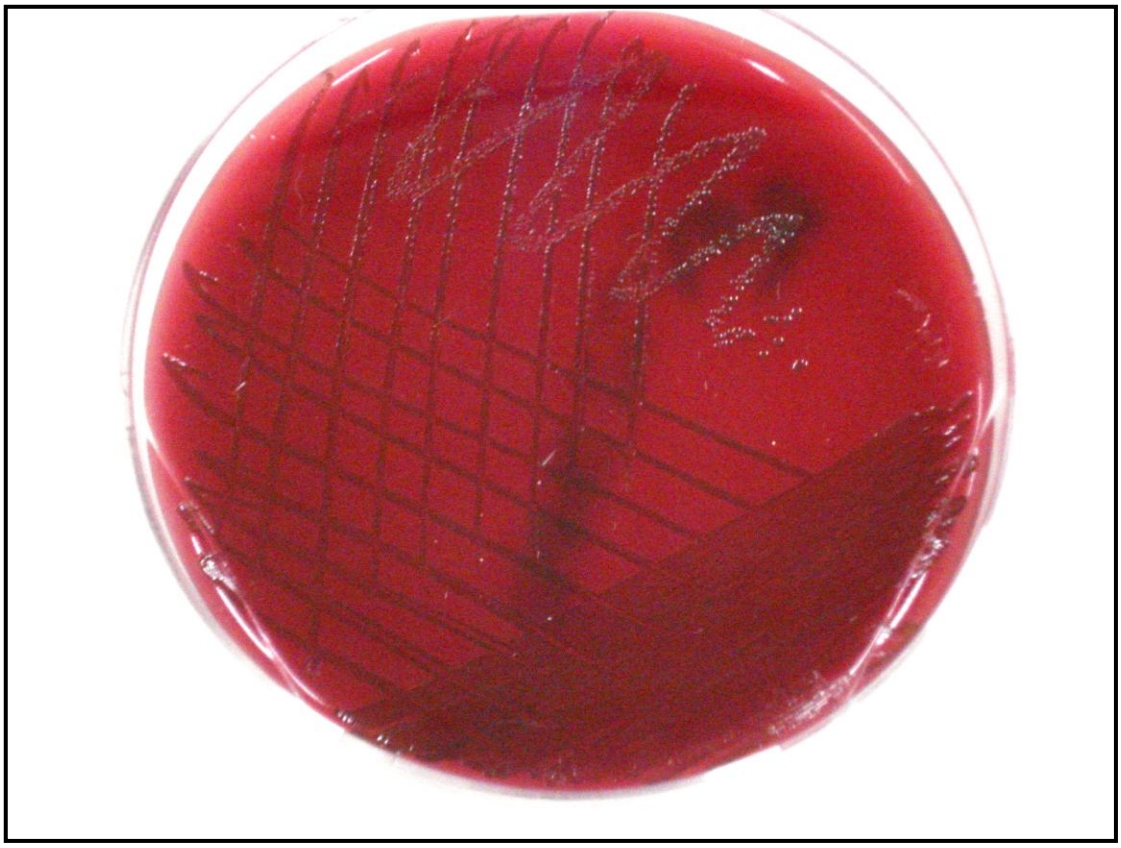


**Figure 1.7:** The black pigmented colonies of *Porphyromonas gingivalis* cultured on blood agar for 7 days at 37 °C under anaerobic condition.

#### 1.1.3.1.2 *Prevotella intermedia*

*Prevotella intermedia* are predominantly present in the human oral cavity. They are non-motile, gram-negative rods that are identified as brown-black colonies on blood agar (Figure 1.8, page 21) and give off a brick red fluorescence when exposed to ultraviolet light (Samaranayake, 2002; Nisengard and Newman, 1994). Identification is usually achieved by the use of molecular techniques (Samaranayake, 2002). *Prevotella intermedia* ferment sugars by producing β-galactosidase. They are usually dominant in individuals suffering from moderate-to-severe gingival inflammation, acute necrotizing ulcerative gingivitis and chronic adult periodontitis (Nisengard and Newman, 1994).

Pathogenicity of *P. intermedia* involves the degradation of immunoglobulins, invasion of eukaryotic cells and immunosupression of B and T cells (Silva *et al,* 2003). Tissue destructive factors include the production of various enzymes such as acid and alkaline phosphatase and a trypsin-like enzyme and gelatinase. Toxic factors include, ammonia, epitheliotoxins, endotoxins, fibroblast growth inhibitors and indole. It is also responsible for the production of IgG and C3 proteases and superoxide dismutase to counteract host defenses. Colonization by the bacterium is facilitated by a capsule and pili (Nisengard and Newman, 1994).

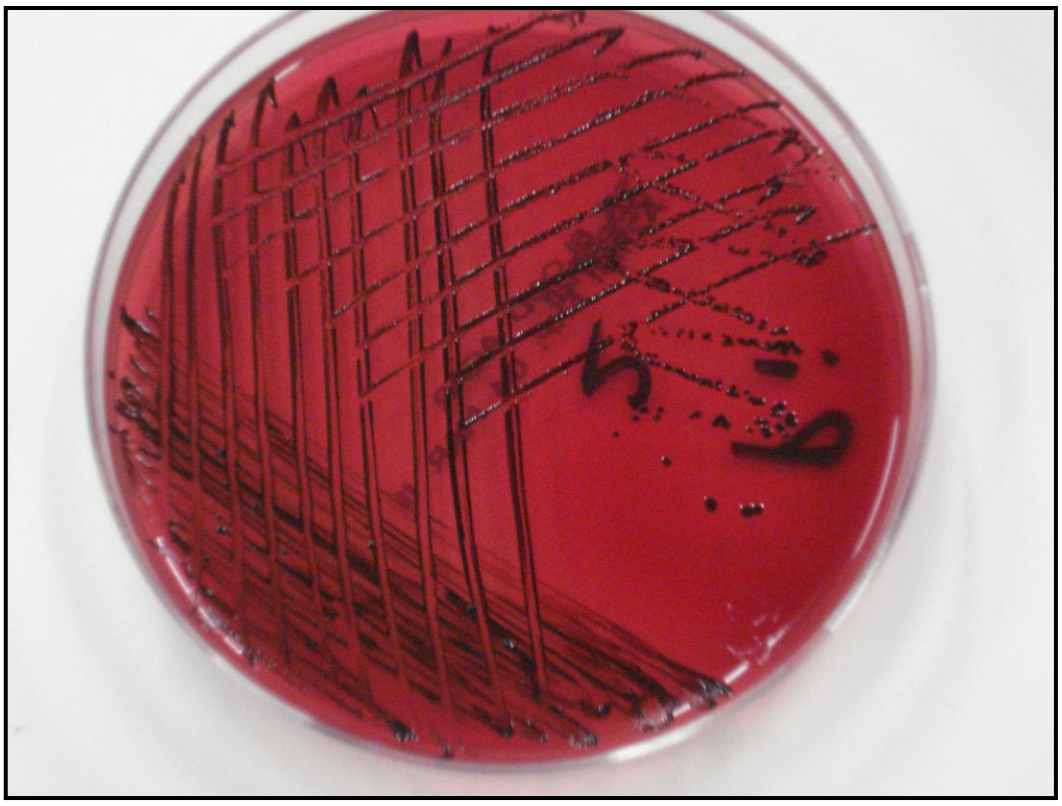


**Figure 1.8:** Black pigmented colonies of *Prevotella intermedia* cultured on blood agar for 7 days at 37 °C under anaerobic condition.

#### 1.1.3.1.3 *Fusobacterium nucleatum*

Several subspecies of *F. nucleatum* have been identified. The most common are those that may be found in healthy gingival crevices (*F. nucleatum* subsp. *polymorphum*) and in periodontal pockets (*F. nucleatum* subsp. *nucleatum*). These bacteria are anaerobic, non-sporing, non-motile, gram-negative cigar-shaped bacilli with pointed ends (Bolstad *et al,* 1996; Samaranayake, 2002). Cells are 5 to 10 µm long and are most commonly isolated from dental plaque. They require rich media for growth such as blood agar (Figure 1.9, page 23) or media containing trypticase, peptone or yeast extract and may be identified by dull, granular colonies with an irregular rhizoid edge (Samaranayake, 2002).

*Fusobacterium nucleatum* is characterized by its ability to produce an endotoxin that is involved in pathogenesis (Bakken *et al,* 1990). Also produced are butyrate, propionate and ammonium ions which inhibit the proliferation of human gingival fibroblasts thus compromising the potential for rapid wound healing. They have the ability to penetrate the gingival epithelium and are present in high amounts in plaque associated with periodontitis. These factors provide *F. nucleatum* with an etiological role in periodontal diseases (Bartold *et al,* 1991; Singer and Buckner, 1981). It is the causative agent of halitosis as it is capable of removing sulphur from cysteine and methionine which results in the production of odoriferous hydrogen sulphide and methylmercaptan (Samaranayake, 2002).

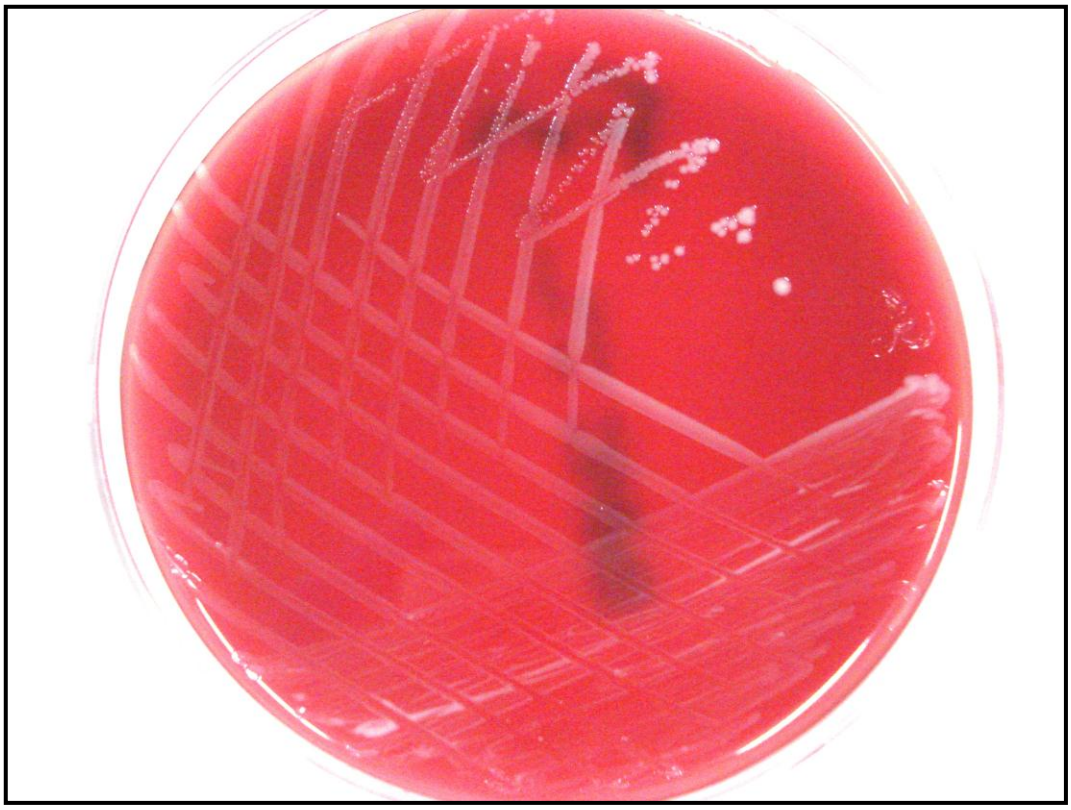


**Figure 1.9:** Dull, white granular colonies with irregular rhizoid edges of *Fusobacterium nucleatum* cultured on blood agar for 7 days at 37 °C under anaerobic condition.

#### 1.1.3.1.4 *Capnocytophaga* species

The genus *Capnocytophaga* consists of gram-negative, fermentative, facultative anaerobes that grow under capnophilic conditions (Socransky *et al,* 1979). They include three species namely, *C. ochracea, C. sputigena* and *C. gingivalis* (Sinnott *et al*, 1988). Their natural habitat is the human oropharynx and they are predominantly isolated from the subgingival area, characterized by long, thin fusiform cells with gliding motility. Colonies of pink, yellow or white are usually spread over the agar surface and are characteristic of *Capnocytophaga* spp. (Figure 1.10, page 24). They are identified by their cell morphology and gliding motility as well as by biochemical reactions and acid end-

products (Samaranayake, 2002). Studies have shown that these bacteria are responsible for alveolar bone loss in gnotobiotic rats and are known to cause human neutrophil and fibroblast dysfunction *in vitro* (Crawford *et al*, 1977; Irving *et al*, 1976; Stevens *et al*, 1980). They are the major cause of sepsis in individuals with malignancy complicated by profound granulocytopenia and oral mucositis. Species belonging to this genus have also been implicated in juvenile periodontal diseases and periodontal diseases associated with insulin-dependent diabetes mellitus (Forlenza *et al*, 1981).

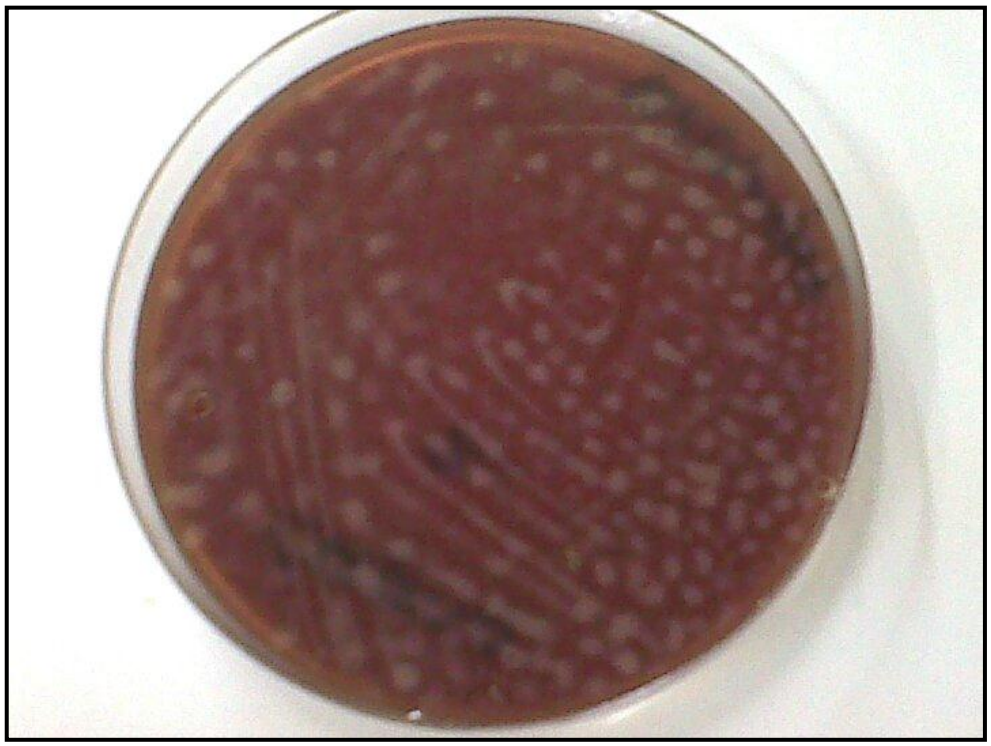


**Figure 1.10:** White spreading colonies of *Capnocytophaga* species cultured on blood agar for 7 days at 37 °C under $CO_2$.

Surface attachment may be mediated by external appendages, membrane capsules and vesicles and endotoxic lipopolysaccharides. Colonization of the oral cavity then takes place through bacterial interactions, host, nutritional and physical and chemical factors.

The unique gliding motility serves as a transport system to transport black-pigmented organisms such as *Bacteroides* and other non-motile pathogenic organisms from one oral site to another playing a role in pathogenesis (Slots and Genco, 1984). They produce a dialyzable heat stable protein that induces abnormalities in neutrophil morphology and inhibits chemotaxis and a protease that is capable of cleaving human IgAI in the oral cavity (Sinnott *et al,* 1988).

#### 1.1.3.2 Pathogenic characteristics

Periodontal pathogens have virulence factors that cause direct damage to the periodontium (Rose *et al,* 2000). Infection by these pathogens causes an imbalance in host machinery performing protective and destructive functions. The common virulence factor among the pathogens is the production of proteases (Nisengard and Newman, 1994). However, both the host and the bacteria in the periodontal biofilm are responsible for the release of proteolytic enzymes that cause tissue destruction (Pihlstrom *et al*, 2005). These proteases are responsible for the destruction of the gingival tissue and of the collagen bridges in the gingival crevices.

Direct toxic effects may also be due to the production of toxins, mitogens and antigens that trigger host cells (Rose *et al,* 2000). Periodontal pathogens have the ability to kill neutrophils thus evading the neutrophil-protective response exhibited by the host. Bacteria produce chemotactic peptides which recruit neutrophils to the sites of infection; they damage the epithelial cells resulting in the release of cytokines that further attract neutrophils. When these neutrophils become congested with bacteria, they explode

resulting in the release of toxic enzymes that cause tissue damage (Kinane, 2001). The induction of macrophages and fibroblasts leads to the production of metalloproteinases which destroy the extracellular matrix components of the gingiva including the periodontal ligament, inflammatory cytokines such as IL-1 and reactive oxygen species which in turn lead to the destruction of connective tissue and to the resorption of the alveolar bone. They are able to penetrate the gingival epithelium by secreting endotoxins, immunologically active compounds and cytotoxic enzymes directly to host inflammatory cells (Loesche, 2010). These are usually accompanied by the release of bacterial leucotoxins, collagenases, fibrinolysins and other proteases (Pihlstrom *et al*, 2005).

#### 1.1.3.3 Control and Prevention

There are primarily two factors that need to be targeted for the control and prevention of periodontal diseases. These include the removal of plaque and calculus around the teeth and the maintenance of good oral hygiene practices (Ismail *et al*, 1993).

##### 1.1.3.3.1 Mechanical control

Clinical trials confirm that effective plaque removal by brushing every 12 - 24 hours is associated with healthy periodontium tissues. Professional care such as scaling and polishing of teeth may also prove to be effective. Scaling involves the use of sharp dental instruments to remove calcified dental plaque and bacteria around gingival tissues and inside the gingival pocket. Removal of stains and plaque is then achieved by addition of an abrasive. Another method of prevention is root planing. Root planing may involve the

surgical exposure of the root through the opening of the gingival flap (Pihlstrom *et al*, 2005).

#### 1.1.3.3.2 Chemical control

An alternative to these procedures involves the use of antimicrobial agents. Many products are commercially available containing different types of antimicrobial compounds. These include the use of oral rinses that may contain antimicrobials such as CHX, iodine, triclosan and essential oils that also control dental caries (see 1.1.2.6). Mouthwashes and toothpastes may also contain biocides, surfactants and polymers that have the potential to reduce biofilm (Pihlstrom *et al*, 2005).

Another alternative is systemic antibiotic therapy aimed at combating severe periodontal infections (Slots and Ting, 2002). Antibiotics may be defined as synthetic organic substances that have the potential to kill or inhibit selective microorganisms when used in low concentrations (Slots, 2004). Systemic antibiotics target the periodontal tissues and pockets and can affect organisms that are usually not reached by cleaning instruments or topical anti-infective chemotherapeutics. Therapy may also suppress periodontal pathogens present on the tongue and other oral surfaces. The most common antibiotics used in the treatment of periodontal diseases are metronidazole, clindamycin, penicillins/amoxicillin and tetracycline/doxycycline (Slots, 2004). These antibiotics may also be used in combination with each other. This is referred to as combination therapy (Slots and Ting, 2002). This is often used in periodontitis caused by a variety of

periodontal pathogens that differ in antimicrobial susceptibilities and to cover aerobic and anaerobic bacteria.

### 1.1.4 Medicinal Plants

Natural products have been used for thousands of years in folk medicine for various human diseases in many parts of the world (Koo *et al,* 2000). The high incidence of oral diseases and the increased resistance by bacteria to antibiotics are the main reasons for the development of alternative prevention and treatment options (Palombo, 2009). Synthetic chemical antimicrobial agents such as CHX and triclosan have broad and strong antimicrobial properties but also have long term side effects such as the disturbance of the oral bacterial ecology and intra oral staining, thus providing another reason for the development of alternative treatments (Ciancio, 2007; Phan and Marquis, 2006). The use of indigenous medicinal plant extracts and natural products as alternative antimicrobial agents in the treatment of oral diseases has been reported (Tapsoba and Deschamps, 2005). These natural products serve as a useful source of biologically active compounds which have been involved in the development of new chemicals for pharmaceuticals.

Of the 500 000 plant species occurring worldwide, only 1 % has been phytochemically investigated, therefore there is still great potential for the discovery of more useful bioactive compounds. Studies have investigated the effect of plant extracts and plant products against oral pathogens and the ability of the plant to inhibit the adhesion of

pathogenic microorganisms to the surface of the tooth thereby inhibiting biofilm formation (Palombo, 2009).

An example of such an agent is sanguinarine, an extract from the rhizome of the *Sanguinaria canadensis* plant (Dzink and Socransky, 1985). This has been used in oral rinses that are commercially available and has shown to be effective against a wide range of organisms such as fungi, yeasts and phages. Another natural agent that has been used for thousands of years is the chewing stick and its extracts (Almas and Al-Lafi, 1995; Kassu *et al,* 1999; Lafl and Ababneh, 1995). The most common source of chewing sticks is the *Salvadora persica* plant. These sticks contain large amounts of fluoride and silica which are beneficial and help reduce plaque. Other substances that have proven to exhibit antibacterial activity include, spice and herb extracts like cinnamon bark oil, papua-mace extracts and clove bud oil (Palombo, 2009).

Propolis has been extensively researched and it is commercially available. Propolis is a resinous hive product from tree buds, collected by bees and mixed with beeswax (Koo *et al,* 2000). Its pharmacological activities have been proven as an ethanol extract of propolis was able to serve as an anti-inflammatory, anaesthetic and cytostatic in studies performed and cited by Ghisalberti (1979), Bankova *et al,* (1989) and Grange and Davey (1990). It serves as a successful antimicrobial as it is active against a wide range of oral bacteria and also affects the adherence of *S. mutans* and *S. sobrinus* to glass and inhibits the synthesis of water soluble glucan (Koo *et al,* 2000).

### 1.1.5 *Dodonaea viscosa* var. *angustifolia*

*Dodonaea viscosa* var. *angustifolia* is a dioecious or monoecious indigenous South African plant that grows mainly in the coastal regions (Figure 1.11, page 31) belonging to the Sapindaceae family (Van Wyk *et al*, 2002). The long, narrow leaves and tips are the main components of the plant that are used for medicinal purposes (Figure 1.12 and 1.13, page 32). It has traditionally been used for the treatment of fever, colds, influenza, stomach trouble, oral thrush, pneumonia, tuberculosis, measles and has been used as an antipyretic (Van Wyk *et al*, 2002). The stem or leaf infusions are often used to treat sore throats, fever, rheumatism and aches. The origin of *D. viscosa* is said to be Australia. It is also found in regions such as India, New Zealand, Mexico, Northern Mariana Islands, Virgin Islands, South America and Florida (Reddy, 2009). It is most popularly known as the sand olive, hop bush or hop seed bush. The roots along with other plant parts may be used to treat sprains, bruises, burns and wounds (Rani *et al*, 2009). Digestive system disorders such as indigestion, ulcers, constipation and diarrhea are also treated using the leaves and roots.

This plant contains diterpenoids, triterpenes, flavonoids, saponins and a complex mixture of other phenolic compounds (Rani *et al*, 2009; Van Wyk *et al*, 2002). The therapeutic activity is associated with polyvalent pharmacological effects occurring due to a synergistic combination of several constituents instead of a single isolated one (Reddy, 2009). Four kaempferol methyl ethers that showed antibacterial and antioxidant activities have been isolated from *D. viscosa* Jacq. var. *angustifolia* leaf extracts (Teffo *et al*, 2010). *Dodonaea viscosa* var. *angustifolia* is known to have antimicrobial and anti-

inflammatory activity (Pirzada *et al*, 2010 and Getie *et al*, 2003). The antibacterial and antifungal properties of this plant have been investigated and was found to inhibit gram-positive bacteria such as *Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa* and fungi such as *Candida albicans* (Khurram *et al*, 2009; Khurram *et al*, 2011). The essential oils and extracts obtained from the leaves are responsible for this activity. The plant has also proven to have antiviral activity against HIV types 1 and 2 (Asres *et al*, 2001). Derivatives from the seeds, bark and leaves such as 3-methoxy flavones exhibit antiviral activity against poli-, rhino- and picorna-viruses (Rani *et al*, 2009). Its anticandida activity has also been proven by the performance of *in vitro* studies by Patel and Coogan (2008), including the ability to inhibit the adherence of *C. albicans* to oral epithelial cells thereby inhibiting the initial stage of colonization and infection (Patel *et al*, 2009).

**To date nothing is known about the effect of this plant on oral opportunistic pathogens which coexist with *C. albicans* in the oral cavity.**

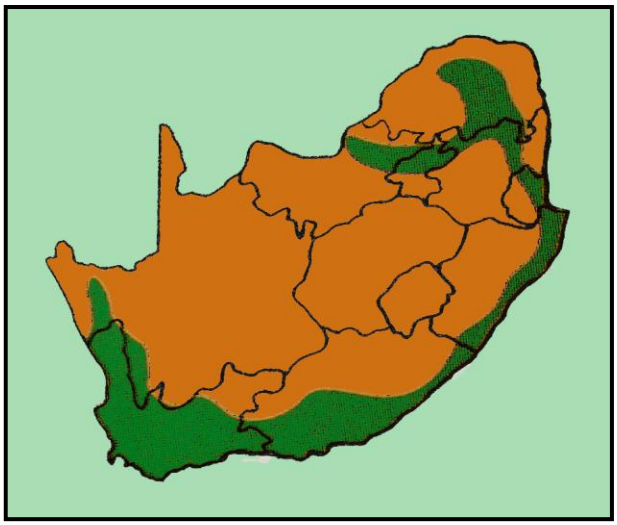


**Figure 1.11:** The indigenous plant *Dodonaea viscosa* var. *angustifolia* may be found in these regions (green on map) in South Africa (Van Wyk *et al*, 2002).



**Figure 1.12:** The dioecious or monoecious plant *Dodonaea viscosa* var. *angustifolia*.



**Figure 1.13:** The long, narrow leaves and flowers of *Dodonaea viscosa* var. *angustifolia*.

## 1.2 Aim

The purpose of this study was to investigate the antimicrobial effect of *D. viscosa* var. *angustifolia* on oral pathogens that cause dental caries and periodontal diseases and the effect of *D. viscosa* var. *angustifolia* on the pathogenicity of *S. mutans* and *P. gingivalis*.

## 1.3 Specific Objectives

- To determine the most effective *D. viscosa* var. *angustifolia* crude extract and minimum bactericidal concentration against *S. mutans, Lactobacillus* spp., *P. gingivalis, P. intermedia, F. nucleatum* and *Capnocytophaga* spp.
- To determine the effect of the subinhibitory concentration of crude plant extract on preformed biofilms of *S. mutans.*
- To determine the effect of the subinhibitory concentration of crude plant extract on *S. mutans* biofilm formation.
- To analyze the extracellular polysaccharide production by *S. mutans* biofilm and planktonic cells after treatment with subinhibitory concentration of crude plant extract.
- To determine the effect of the subinhibitory concentration of crude plant extract on the acid production by *S. mutans* biofilm and planktonic cells.
- To study the effect of the subinhibitory concentration of crude plant extract on the production of proteases by *P. gingivalis.*

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Plant Material

Plant material harvested in April 2010 was collected from the Pypeklipberg, Mkhunyane Eco Reserve in Mpumalanga, South Africa. The plant was identified as *Dodonaea viscosa* var. *angustifolia* Benth. belonging to the family Sapindaceae by Mrs. Ranee Reddy, a taxonomist, from the Herbarium at the University of the Witwatersrand. Voucher specimens number J 94882, were previously deposited in this Herbarium (Patel and Coogan, 2008).

## 2.2 Plant extract

Leaves of *D. viscosa* var. *angustifolia* were dried in the shade and milled to a fine powder. Extracts were prepared using a method described by Eloff, (1999). One gram of powder was mixed with 10 ml of acetone (Merck Chemicals Pty. Ltd, SA), vortexed for 5 minutes using Genie 2 (Lasec, SA) and centrifuged at 10000 rpm for 10 minutes using a micro centrifuge 5424 (Merck Chemicals Pty. Ltd, SA). The supernatant was collected in a pre-weighed 100 ml beaker. The above procedure was repeated three times using the same powder. All three supernatants were pooled together in the same beaker and acetone was allowed to evaporate under a cold air stream. The beaker was weighed again with the dried plant extract. A yield of dried extract was calculated by subtracting the weight of the empty beaker from the weight of the beaker with the plant extract. Similarly extractions were prepared using two other solvents; methanol (Merck Chemicals Pty. Ltd, SA) and ethanol (Sigma-Aldrich, SA). The crude extracts were then re-dissolved in

dimethyl sulphoxide (Sigma-Aldrich, SA) to yield a final solution containing 50 mg of crude plant extract per ml of solution. Fresh plant extracts were prepared for each experiment.

### 2.3 Bacterial cultures

*Streptococcus mutans* NCTC 10919 and clinical strains of *S. mutans, Lactobacillus casei, Lactobacillus* spp., *P. gingivalis, P. intermedia, F. nucleatum* and *Capnocytophaga* spp. were used throughout the study. All the test strains were isolated from the oral cavities of patients attending clinics at Wits Oral and Dental teaching Hospital, Johannesburg. Ethical clearance, clearance number (M10205) was obtained from The Committee for Research on Human Subjects (Medical), University of the Witwatersrand (Appendix 3, page 135). *Streptococcus mutans* and *Lactobacillus* spp. were isolated from saliva and *P. gingivalis, P. intermedia, F. nucleatum* and *Capnocytophaga* spp. from periodontal pocket debris samples. Four strains of *S. mutans* and *Lactobacillus* spp. were randomly selected, identified and used in the study. *Lactobacillus* spp. strains were identified using a gram reaction and cultural characteristics. *Streptococcus mutans* strains were identified using cultural characteristics and a series of biochemical reactions using the Analytical Profile Index (API) 20 Strep standardized system (BioMerieux, SA).

This system comprised of 20 biochemical reactions that allow for identification of streptococci and enterococci. The biochemical reactions took place on cardboard strips that contain dehydrated substances incorporated into microtubules. The substances were rehydrated with the addition of inoculum. The microtubules of the strip containing the

tests based on enzymatic activity were inoculated with a dense suspension of a pure culture of organism which reconstituted the dehydrated enzymatic substrates. Fermentation tests were inoculated with an enriched medium that rehydrates the sugar substrates and carbohydrate fermentation was detected by a shift in the pH indicator.

Strips were incubated for 4 hours to obtain the first reading and a further 24 hours to obtain a second reading according to a reading table supplied by the manufacturer (Table 2.3.1, page 37). The metabolism of these substrates during incubation produced spontaneous colour changes or colour changes that were only revealed by the addition of reagents. Identification was obtained through the use of an API or identification software whereby a numerical profile was recorded on a results sheet. The tests were separated into groups of three and a value of one, two or four was indicated for each group. The values corresponding to positive reactions within each group were added together to obtain a 7-digit profile number.

*Streptococcus mutans* NCTC 10919 and *Lactobacillus casei* were used as control strains. *Streptococcus mutans* was cultured on blood agar (Oxoid Ltd, UK) and *Lactobacillus* spp. on Rogosa agar (Condalab, SA). These were incubated in a candle jar ($CO_2$) for 48 hours. *Porphyromonas gingivalis, P. intermedia* and *F. nucleatum* were identified using a polymerase chain reaction (PCR) technique as described by Ashimoto *et al*, (1996) and Avila-Campos *et al*, (1999) by the staff of the Oral Microbiology Laboratory. *Capnocytophaga* spp. was identified by a gram reaction, morphology and cultural

characteristics. These strains were cultured on blood agar in an anaerobic jar for 7 days at 37 °C and used throughout the study.

**Table 2.3.1** Reading table for API 20 Strep standardized system (BioMerieux, SA)

| Tests | Active ingredients | QTY (mg/cup.) | Reactions/Enzymes | Results | | | |
|---|---|---|---|---|---|---|---|
| | | | | Negative | | Positive | |
| VP | sodium pyruvate | 1.9 | acetoin production (Voges Proskauer) | VP 1 + VP 2/ wait 10 min | | | |
| | | | | Colourless | | Pink-red | |
| HIP | hippuric acid | 0.4 | hydrolysis (HIPpuric acid) | NIN/ wait 10 min | | | |
| | | | | Colourless/pale blue Bluish-grey | | Dark-blue/violet | |
| ESC | esculin<br>ferric acid | 1.16<br>0.152 | ß-glucosidase hydrolysis (ESCulin) | **4 hrs** | **24 hrs** | **4 hrs** | **24 hrs** |
| | | | | Colourless<br>Pale yellow | Colourless<br>Pale yellow<br>light grey | Black<br>Grey | Black |
| PYRA | pyroglutamic acid-ß-naphthylamide | 0.0256 | PYRrolidonyl Arylamidase | ZYM A + ZYM B/ 10 min (PYRA to LAP) if necessary decolourise with intense light | | | |
| | | | | Colourless or very pale orange | | Orange | |
| αGAL | 6-bromo-2-naphthyl-αD-galactopyranoside | 0.0376 | α-GALactosidase | Colourless | | Violet | |
| ßGUR | naphthol ASBI-glucuronic acid | 0.0537 | ß-GlUcuRonidase | Colourless | | Blue | |
| ßGAL | 2-naphthyl-ßD-galactopyranoside | 0.0306 | ß-GALactosidase | Colourless or very pale violet | | Violet | |
| PAL | 2-naphthyl phosphate | 0.0244 | Alkaline Phosphatase | Colourless or very pale violet | | Violet | |
| LAP | L-leucine-ß-naphthylamide | 0.0256 | Leucine AminoPeptidase | Colourless | | Orange | |
| ADH | L-arginine | 1.9 | Arginine DiHydrolase | Yellow | | Red | |
| | | | | **4 hrs** | **24 hrs** | **4 hrs** | **24 hrs** |
| RIB | D-ribose | 1.4 | acidification (RIBose) | Red | Orange/Red | Orange/Yellow | Yellow |
| ARA | L-arabinose | 1.4 | acidification (ARAbinose) | Red | Orange/Red | Orange/Yellow | Yellow |
| MAN | D-mannitol | 1.36 | acidification (MANnitol) | Red | Orange/Red | Orange/Yellow | Yellow |
| SOR | D-sorbitol | 1.36 | acidification (SORbitol) | Red | Orange/Red | Orange/Yellow | Yellow |
| LAC | D-lactose (bovine origin) | 1.4 | acidification (LACtose) | Red | Orange/Red | Orange/Yellow | Yellow |
| TRE | D-trehalose | 1.32 | acidification (TREhalose) | Red | Orange/Red | Orange/Yellow | Yellow |
| INU | inulin | 5.12 | acidification (INUlin) | Red | Orange/Red | Orange/Yellow | Yellow |
| RAF | D-raffinose | 3.12 | acidification (RAFfinose) | Red | Orange/Red | Orange/Yellow | Yellow |
| AMD | starch | 2.56 | acidification (AmiDon) | Red | Orange/Red | Orange/Yellow | Yellow |
| GLYG | glycogen | 1.28 | acidification (GLYcoGen) | Red or Orange | | Bright yellow | |

## 2.4 Antibacterial activity assay

Minimum bactericidal concentration tests (MBC) were performed using a microtitre double dilution technique (Koo *et al*, 2000). These tests were carried out to obtain the lowest concentration of crude plant extract that resulted in the complete elimination of growth of bacteria. Generally minimum inhibitory concentration (MIC) is obtained to determine antibacterial properties. In this study, it was not possible to determine MIC because the crude plant extract precipitated with the culture medium. Instead MBC was determined where the concentration that caused complete killing of bacteria was recorded.

Two-fold dilutions of crude plant extracts were prepared using brain heart infusion (BHI) broth (Biolab Diagnostics Pty. Ltd, SA) for *S. mutans* and Rogosa broth for *Lactobacilli.* One hundred microliters of crude plant extract was added to each well of a 96-well round bottom microtitre plate (Corning Incorporated, US) in varying initial concentrations of 50, 25, 12.5, 3.125, 1.56, 0.78 mg/ml. For anaerobic bacteria, plant extracts were diluted with tryptone soy broth (Biolab Diagnostics Pty. Ltd, SA) containing haem and menadione which was used for *P. gingivalis* and *P. intermedia.* Similarly, dilutions were prepared with tryptone soy broth for *Capnocytophaga* spp. and Fusi Form medium for *F. nucleatum*. Plant extracts were diluted 15 times (50 - 0.0006 mg/ml) and 100 µl of each concentration was added to each well (Figure 2.1, page 39).

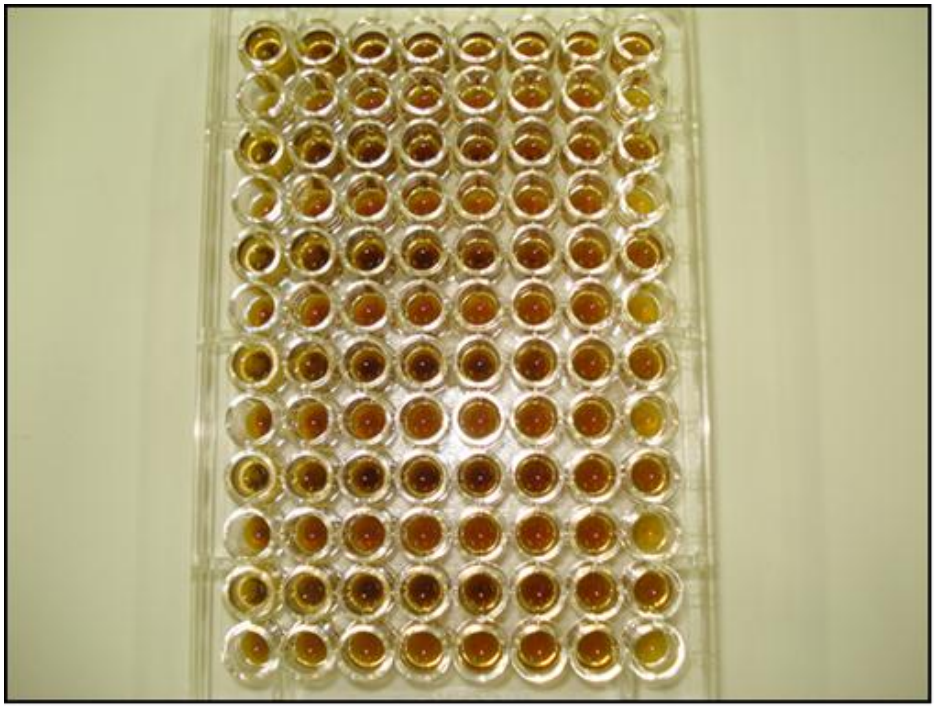


**Figure 2.1:** Microtitre plate showing the growth of *Prevotella intermedia* after an incubation period of 7 days at 37 °C under anaerobic condition with various concentrations of crude plant extract.

Colonies of bacterial cultures were emulsified in the respective media in which the plant dilutions were made for each organism. Optical density was measured at 405 nm and adjusted to 0.2 which had approximately $10^7$ CFU/ml. These suspensions were used as inoculums. One hundred microliters of inoculums were transferred into each well of a microtitre plate and was incubated for 48 hours at 37 ºC under $CO_2$ (*S. mutans* and *Lactobacillus* spp.), 4 days at 37 °C under $CO_2$ (*Capnocytophaga* spp.) and 7 days at 37 °C under anaerobic condition (*P. gingivalis, P. intermedia, F. nucleatum*). The addition of inoculum diluted the plant extract by half giving the first well a concentration of 25 mg/ml and subsequent wells had two-fold dilutions. After incubation each dilution was

subcultured on blood agar (*S. mutans* and periodontal pathogens) and Rogosa agar (*Lactobacillus* spp.) to detect the viable bacteria. The lowest concentration that had no growth or viable bacteria was recorded as MBC for that test organism. Minimum bactericidal concentration experiments for acetone, methanol and ethanol extracts were repeated three times for each of the five *S. mutans* and *Lactobacillus* spp. test strains. For *P. gingivalis, P. intermedia, F. nucleatum* and *Capnocytophaga* spp. minimum bactericidal concentration experiments were repeated three times using the three extracts. Median MBC was calculated for each organism and extract.

Crude extracts prepared using the solvent ethanol was used in subsequent experiments. From the results, subinhibitory concentrations of crude ethanol plant extract for *S. mutans* and *P. gingivalis* were selected for the subsequent experiments. For *S. mutans* the subinhibitory concentration was 0.78 mg/ml and for *P. gingivalis* they were 0.02, 0.01, 0.006, 0.003, 0.001 mg/ml. With each test dimethyl sulphoxide (DMSO) was used as a vehicle fluid control, water as a negative control and CHX as a positive control.

### 2.5 Biofilm Assay

#### 2.5.1 Effect of crude ethanol plant extract on *Streptococcus mutans* biofilm (preformed)

These experiments were carried out to study the effects of the crude ethanol plant extract on the adherence of *S. mutans* in preformed biofilms. The biofilms were grown in sterile 250 ml beakers on five sterile glass slides placed at an angle of 90 ° in 150 ml tryptone broth containing 1 ml *S. mutans* inoculum. Inoculum was prepared by suspending freshly

grown *S. mutans* colonies in phosphate buffered saline (PBS) and adjusting optical density (OD) to 0.2 at 405 nm to obtain approximately $10^7$ CFU/ml. Glass slides were held in place by autoclave tape. Beakers and slides were covered with foil and autoclaved before use for all experiments. The cultures were incubated for 48 hours at 37 ºC under $CO_2$ to allow bacterial deposition and biofilm formation according to a method described by Koo *et al,* (2003) with some modifications. During incubation media was changed once after 24 hours. One slide containing two day old biofilm was taken as the initial number of cells in the biofilm. The attached cells were aseptically scraped off the slide using a sterile glass slide and suspended in 5 ml phosphate buffered saline (Calbiochem, US). Ten-fold dilutions from 1:10 to 1:10000 were prepared using PBS and 20 µl of each dilution was spread on blood agar. Plates were incubated for 48 hours at 37 °C under $CO_2$. The number of colonies on each plate was quantified and the counts were multiplied by dilution factors and 50 to determine viable bacterial count (CFU/ml). The resultant count was taken as the initial bacterial count in biofilm.

The second slide containing two day old biofilm was then exposed to 0.78 mg/ml of crude ethanol plant extract diluted in tryptone broth (150 ml tryptone broth, 2.34 ml concentrated crude plant extract) by placing into the mixture for 1 minute and the third slide with medium (150 ml) and water (2.34 ml) which served as a control. After 1 minute slides were removed, gently rinsed with PBS and transferred to a fresh tryptone broth medium and incubated for 6 hours. The above 1 minute exposure to 0.78 mg/ml crude ethanol plant extract (test) and media and water (control) was repeated after 6 hours, the second and third slides were then removed and bacterial counts were obtained

as described for the first slide. The fourth slide with biofilm was further incubated overnight with the mixture containing 0.78 mg/ml crude ethanol plant extract diluted with medium and the fifth slide with medium and water only (control) to obtain complete 24 hours exposure.

After incubation biofilm was removed from the medium, rinsed and the count of surviving bacteria was obtained as described for the first slide. These experiments were performed three times for each of the five *S. mutans* test strains. The bacterial counts of the controls (untreated – slide three and five) biofilm were compared with the bacterial counts of biofilm treated with the crude ethanol plant extract during two 1 minute exposures (slide two) and a 24 hour exposure (slide four) using the Wilcoxon rank-sum test (Mann-Whitney).

In summary biofilms incubated for 6 hours were exposed to the crude ethanol plant extract twice at 0 hours and 6 hours for 1 minute. Whereas biofilms that were incubated for 24 hours were exposed to the crude ethanol plant extract for a full 24 hours.

### 2.5.2 Effect of crude ethanol plant extract on biofilm formation by *Streptococcus mutans*

These experiments were performed to study the formation of *S. mutans* biofilm in the presence of crude ethanol plant extract. Effect of crude ethanol plant extract on biofilm formation was studied using a technique described by Limsong *et al*, (2004) with some modifications. Two sterile 250 ml beakers with three glass slides held in an upright

position (90°) by autoclave tape were prepared and autoclaved. In one beaker (control) 150 ml tryptone broth and 2.34 ml water in the second beaker 150 ml tryptone broth containing 2.34 ml of crude ethanol plant extract (subinhibitory concentration of 0.78 mg/ml) was added. One milliliter of *S. mutans* inoculum was added to each beaker and incubated for 6 hours at 37 ºC under $CO_2$. Inoculum was prepared by suspending freshly grown *S. mutans* colonies in PBS and adjusting OD to 0.2 at 405 nm to obtain approximately $10^7$ CFU/ml. One glass slide from each of the beakers were removed and rinsed with PBS. The attached cells were aseptically scraped off the slides using sterile slides and the cells were resuspended and vortexed in 5 ml PBS. Ten-fold serial dilutions from 1:10 to 1:10000 were prepared using PBS and 20 µl of each dilution was spread on blood agar. Plates were incubated for 48 hours at 37 °C under $CO_2$, the number of colonies on each plate was quantified and the counts were multiplied by the dilution factors and 50 to determine viable bacterial count (CFU/ml).

The resultant count from the control beaker was taken as the 6 hour control and the count from the beaker with plant extract was taken as the 6 hour biofilm count in the presence of crude ethanol plant extract. Beakers with the remaining slides were re-incubated. The above procedure was repeated after 24 hours for one slide from each of the beakers and after 30 hours for the last slides. The media was changed after 24 hours maintaining sterile conditions. These experiments were repeated three times for each of the five *S. mutans* test strains. The bacterial counts of the control and tests for each time interval (6 hours, 24 hours, 30 hours) was compared using the Wilcoxon rank-sum test (Mann-Whitney).

## 2.6 Effect of crude ethanol plant extract on the production of extracellular polysaccharides

These experiments were carried out to study the mass of polysaccharides produced by *S. mutans* biofilm and planktonic cells in the presence and absence of crude ethanol plant extract. The effect of the crude plant extract on the production of extracellular polysaccharides by *S. mutans* biofilm was studied using a technique described by Koo *et al*, (2003) with some modifications. Biofilms were grown in 250 ml beakers on two glass slides at an angle of 90 ° in 150 ml tryptone broth containing 1 ml *S. mutans* inoculum for 48 hours at 37 °C under $CO_2$. Inoculum was prepared by suspending freshly grown *S. mutans* colonies in PBS and adjusting OD to 0.2 at 405 nm to obtain approximately $10^7$ CFU/ml.

During incubation, media was changed once after 24 hours. Slides with biofilms were rinsed with PBS and aseptically scraped using sterile glass slides to harvest adherent cells. Adherent cells were transferred to two McCartney bottles, one containing 19.7 ml 5 % sucrose broth and 0.312 ml concentrated crude ethanol plant extract to obtain a subinhibitory concentration of 0.78 mg/ml and one containing 19.7 ml 5% sucrose broth and 0.312 ml water (control). Both bottles containing biofilm cultures were then incubated for 48 hours at 37 °C under $CO_2$. After 48 hours, the bacterial cultures were diluted one in ten in 10 % sodium acetate (Saarchem Pty. Ltd, SA) to determine EPS production. Two milliliters of each culture was then placed into two separate 100 ml pre-weighed beakers. Into the first beaker, 2.4 ml of absolute alcohol was added in order to precipitate glucans and into the second beaker 5 ml of absolute alcohol was added to

precipitate levans (Beighton *et al*, 1981). This was allowed to stand at room temperature for 48 hours.

The polysaccharides were precipitated, the solvent was allowed to evaporate under a cold air stream and the precipitate was quantified by measuring the dry mass using a standard laboratory scale (Denver Instrument S-403). These experiments were repeated three times for each of the five *S. mutans* test strains. Dry mass of glucans and levans produced by cells exposed to the crude ethanol plant extract was compared to the dry mass of controls individually and collectively (total polysaccharides) using a student's *t*-test (two-sample). Bacterial counts of the control were compared to the counts of the cells exposed to the crude ethanol plant extract using the Wilcoxon rank-sum test (Mann-Whitney).

Similar experiments were performed to study the effect of crude ethanol plant extract on the production of extracellular polysaccharides by *S. mutans* planktonic cells. Nineteen milliliters 5 % sucrose broth was inoculated with 1 ml of bacterial suspension of *S. mutans* in PBS with OD of 0.2 at 405 nm and 0.78 mg/ml (0.312 ml) of crude ethanol plant extract in McCartney bottles. Controls containing 19 ml 5 % sucrose broth, 1 ml bacterial suspension of *S. mutans* and 0.312 ml water were also included. Glucan and levan production was detected as described above in biofilm experiments. These experiments were repeated three times for each of the five *S. mutans* test strains. Dry mass of glucans and levans produced by cells exposed to the crude ethanol plant extract was compared to the dry mass of controls individually and collectively (total polysaccharides) using a student's *t*-test (two-sample). Bacterial counts of the control

were compared to the counts of the cells exposed to the crude ethanol plant extract using the Wilcoxon rank-sum test (Mann-Whitney).

## 2.7 Acid production

### 2.7.1 Effect on the acid production by biofilms of *Streptococcus mutans*

The effect of the crude ethanol plant extract on the acid production by *S. mutans* biofilm was studied using a technique described by Kim *et al*, (2008) with modifications. These experiments were carried out to study the effect of the crude ethanol plant extract on the production of acid by cells of *S. mutans* in the biofilm form. Biofilms were allowed to grow in 250 ml beakers on two glass slides at 90 ° in 150 ml tryptone broth containing 1 ml *S. mutans* inoculum for 48 hours at 37 °C under $CO_2$ with a change of medium after 24 hours. Inoculum was prepared by suspending freshly grown *S. mutans* colonies in PBS and adjusting OD to 0.2 at 405 nm to obtain approximately $10^7$ CFU/ml. One slide with biofilm was then exposed to 0.78 mg/ml of crude ethanol plant extract for a period of 30 minutes (150 ml tryptone broth, 2.34 ml concentrated crude ethanol plant extract). The second slide with biofilm which served as a control was exposed to media (150 ml) and water (2.34 ml). Slides were rinsed in PBS, scraped using a sterile glass slide and adherent cells were transferred to 16 ml tryptone broth and incubated at 37 °C under $CO_2$. The pH of both the cultures was measured every 2, 4, 6, 8, 13 and 22 hours. These experiments were repeated three times for each of the five *S. mutans* test strains. The pH values of the control and the tests were compared for each time interval using the Wilcoxon rank-sum test (Mann-Whitney).

### 2.7.2 Effect on the acid production by planktonic cells of *Streptococcus mutans*

The effect of crude ethanol plant extract on the acid production by planktonic cells of *S. mutans* was studied using a technique described by Nalina and Rahim, (2007) with some modifications. These experiments were performed to determine the effect of the crude ethanol plant extract on the production of acid by cells of *S. mutans* in the planktonic form. Tryptone broth (16 ml) containing 0.25 ml concentrated crude ethanol plant extract (final concentration 0.78 mg/ml) was inoculated with 100 µl of culture containing $10^7$ CFU/ml of *S. mutans* in PBS with OD of 0.2 at 405 nm. Similarly, tryptone broth (16 ml) with water (0.25 ml) inoculated with 100 µl of a culture of *S. mutans* was also included as a control. Cultures were incubated at 37 °C under $CO_2$ and allowed to grow for a period of 10 hours. The pH was read at 0 hours, 10 hours and every 2 hours thereafter for 16 hours. The bacterial count was done at 0 hours, 12 hours and 16 hours using a serial dilution technique described in 2.5.1. These experiments were repeated three times for each of the five *S. mutans* test strains. The pH values of the control and tests were compared for each time interval using the Wilcoxon rank-sum test (Mann-Whitney).

## 2.8 Effect on protease activity by *Porphyromonas gingivalis*

The effect of the crude ethanol plant extract on Arg-gingipain and Lys-gingipain activity was evaluated as detailed by Yamanaka *et al*, (2007) with modifications. These tests were carried out to determine the effect of the crude ethanol plant extract on the activity of the two major proteases produced by *P. gingivalis. Porphyromonas gingivalis* was grown for 7 days in 20 ml tryptone soy broth with supplements (0.02 ml haem and 0.02 ml menadione) at 37 °C under anaerobic condition. Seven day old *P. gingivalis* cultures

were harvested by centrifugation at 5000 g for 20 minutes using Centrifuge 5804 (Merck Chemicals Pty. Ltd, SA), washed three times, resuspended and vortexed in 5 ml of 50 mM PBS (pH 7.4) to an optical density of 2.0 at 660 nm. Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (Figure 2.2) and N-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (Figure 2.3, page 49) in 0.1 M Tris-HCL (Calbiochem, US), pH 8.0 containing 1 mM dithiothreitol (Merck Chemicals Pty. Ltd, SA) was used as substrates for Arg-gingipain and Lys-gingipain, respectively.

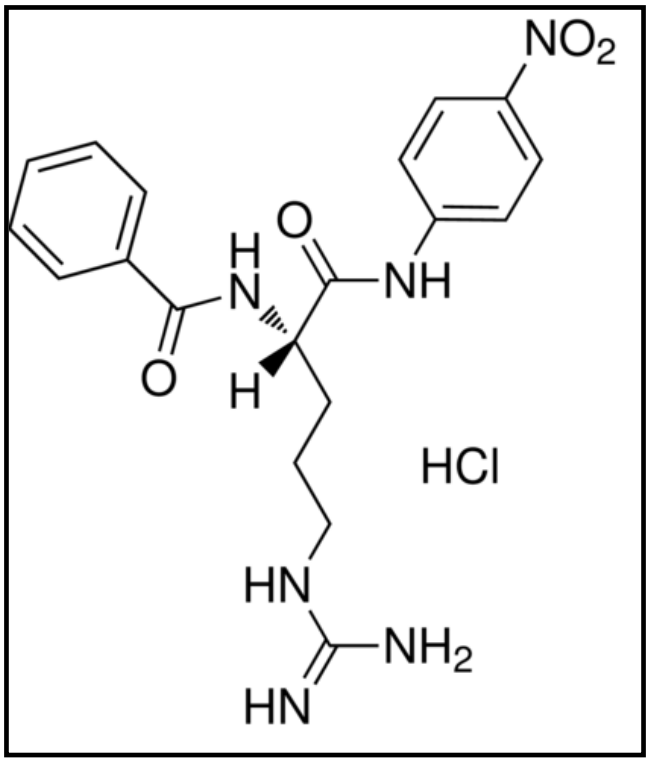



**Figure 2.2:** Chemical structure of Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride used as a substrate for Arg-gingipain obtained from Sigma-Aldrich, SA.

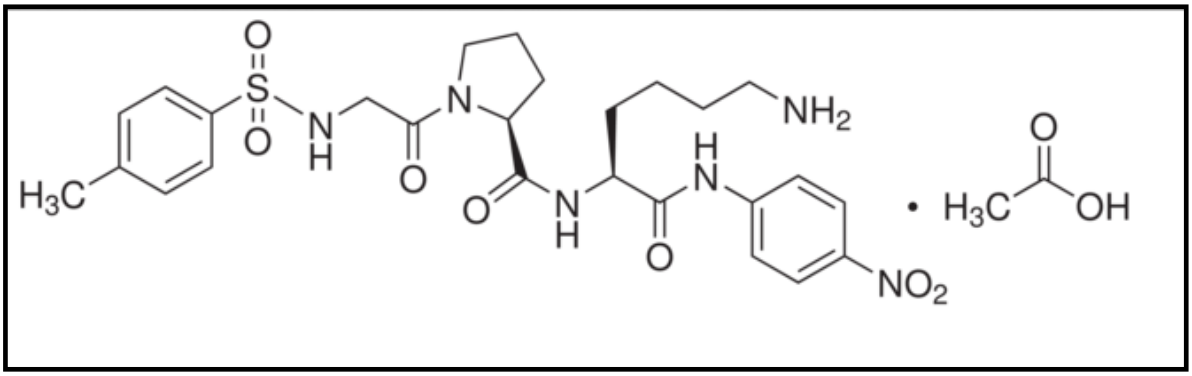



**Figure 2.3:** Chemical structure of N-(*p*-Tosyl)-Gly-Pro-Lys 4 nitroanilide acetate salt used as a substrate for Lys-gingipain obtained from Sigma-Aldrich, SA.

One hundred and sixty microlitres of the two substrates were dispensed into five wells of a microtitre plate. Twenty microlitres of different concentrations of crude ethanol plant extract diluted in DMSO (0.02, 0.01, 0.006, 0.003, 0.001 mg/ml) were added to each of the five wells. The sixth well was inoculated with 180 µl substrate and 20 µl inoculum and the seventh well with 160 µl substrate and 40 µl 0.01 g/ml proteinase K (Merck Chemicals Pty. Ltd, SA). These wells served as the positive controls. Bacterial cell suspensions (20 µl) were added to the first six wells and incubated aerobically at 37 º C for 20 minutes (Figure 2.4A, page 51). Controls were set up for each concentration of plant extract without the addition of substrates to determine any colour change by DMSO. These readings were called control readings and were subtracted from the test readings to obtain correct enzymatic activity (Figure 2.4B, page 51)

For each well adsorption at a wavelength of 405 nm was determined using a spectrophotometer (Jenway 6300 Spectrophotometer). This experiment was repeated 21 times for Arg-gingipain and 18 times for Lys-gingipain. Experiments were repeated 18

times only for Lys-gingipain due to inadequate quantity of substrate for analysis. Relative enzymatic activity was determined as follows: [($A_{405}$ with bacterial cells and plant extract – $A_{405}$ of DMSO control)/($A_{405}$ with bacterial cells – $A_{405}$ of DMSO control)] x 100 (Figure 2.4C, page 51). The protease inhibition for both substrates was statistically compared using the student's *t*-test (one-sample).

### 2.9 Statistical Analysis

A statistician was consulted with regards to the number of strains and repeats to be used for each experiment to allow for adequate degrees of freedom so that hypotheses may be tested accordingly using the appropriate statistical techniques. Statistical significance was referred at $p < 0.05$. All results were analyzed using the Stata 10 software (StataCorp. College Station, Texas).

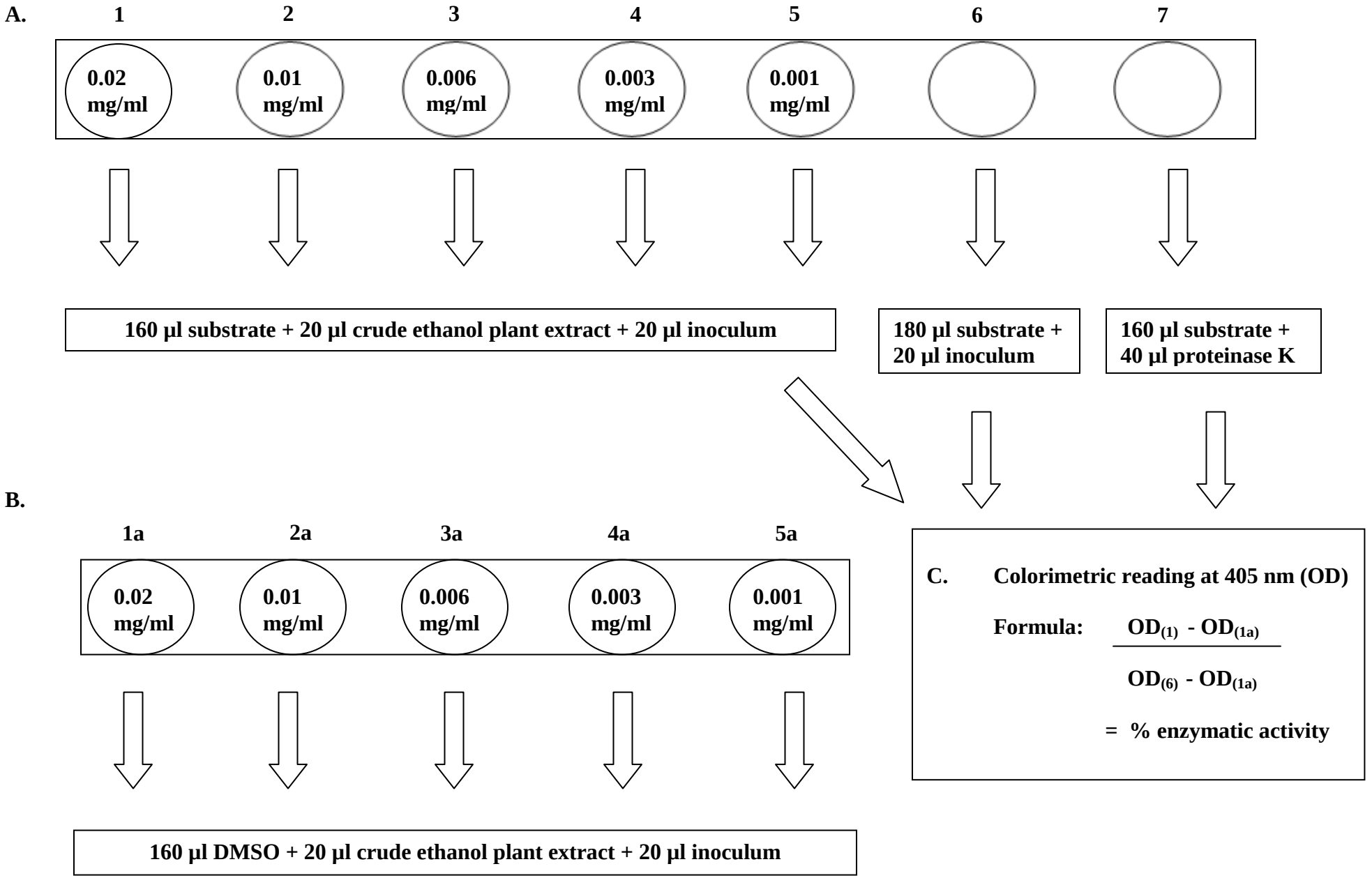



**Figure 2.4:** (A) Wells showing tests and positive controls, (B) wells showing dimethyl sulphoxide controls for each concentration of crude ethanol plant extract, (C) the formula used to calculate percentage enzymatic activity

# CHAPTER 3: RESULTS

## 3.1 *Dodonaea viscosa* var. *angustifolia* crude extract yield

Plant leaves were extracted in acetone, methanol and ethanol and the dried extracts were reconstituted in DMSO. All extracts were successfully used for all MBC experiments against cariogenic bacteria and periodontal pathogens. The crude extract yield of dried leaves for all extracts is shown in Tables 3.1.1 - 3.1.3.

**Table 3.1.1** The dry weight of acetone extracts of *Dodonaea viscosa* var. *angustifolia*

| No. of acetone extractions | Yield per gram of dried leaves (g) |
|---|---|
| 1 | 0.169 |
| 2 | 0.142 |
| 3 | 0.156 |
| 4 | 0.159 |
| 5 | 0.157 |
| 6 | 0.187 |
| **Average yield** | **0.2** |

**Table 3.1.2** The dry weight of methanol extracts of *Dodonaea viscosa* var. *angustifolia*

| No. of methanol extractions | Yield per gram of dried leaves (g) |
|---|---|
| 1 | 0.314 |
| 2 | 0.248 |
| 3 | 0.345 |
| 4 | 0.354 |
| 5 | 0.361 |
| **Average yield** | **0.3** |

**Table 3.1.3** The dry weight of ethanol extracts of *Dodonaea viscosa* var. *angustifolia*

| No. of ethanol extractions | Yield per gram of dried leaves (g) |
|---|---|
| 1 | 0.147 |
| 2 | 0.247 |
| 3 | 0.212 |
| 4 | 0.218 |
| 5 | 0.208 |
| 6 | 0.186 |
| 7 | 0.202 |
| 8 | 0.197 |
| 9 | 0.189 |
| 10 | 0.188 |
| 11 | 0.214 |
| 12 | 0.241 |
| 13 | 0.228 |
| 14 | 0.213 |
| 15 | 0.123 |
| 16 | 0.226 |
| 17 | 0.256 |
| 18 | 0.207 |
| 19 | 0.255 |
| 20 | 0.2 |
| 21 | 0.185 |
| 22 | 0.207 |
| 23 | 0.215 |
| 24 | 0.201 |
| 25 | 0.197 |
| 26 | 0.209 |
| 27 | 0.209 |
| 28 | 0.201 |
| 29 | 0.216 |
| 30 | 0.211 |
| 31 | 0.204 |
| 32 | 0.164 |
| **Average yield** | **0.2** |

The highest yield was obtained when the plant leaves were extracted using methanol. Acetone and ethanol produced the same quantity of plant extract, however, ethanol was chosen for all subsequent experiments to examine the effect of the plant on the pathogenic characteristics of *S. mutans* and *P. gingivalis.* Ethanol extracts were selected for the subsequent experiments because they produced the lowest minimum bactericidal

concentrations, meaning the extraction of an active ingredient was the highest with this solvent.

### 3.2 Identification of *Streptococcus mutans* using the API 20 Strep standardized system

Strains of *S. mutans* were identified using an API 20 strep strip containing substrates for the analysis of enzymatic activity and the fermentation of sugars. The results were read based on colour reactions (Figure 3.1). A numerical profile was recorded on a results sheet to obtain a 7-digit profile (Figure 3.2, page 55).

The colour reactions are those which have been obtained after a 24 hour incubation period. These colour reactions are characteristic of species that have been identified by the API 20 strep system as *S. mutans.* Each colour reaction was given a score according to the identification software. Clinical strains 1, 6 and 13 were all identified as 99. 9 % *S. mutans.* Clinical strain 7 and NCTC 10919 were identified as 93.2 % and 51.1 % *S. mutans* respectively. These results were further verified using a Microscan (Walkaway) SI40 (Siemens, US) that confirmed all cultures as *S. mutans* based on a series of biochemical reactions.




**Figure 3.1:** The 20 biochemical reactions characteristic of species that gave 99.9 % *Streptococcus mutans* match on an API 20 strep strip.

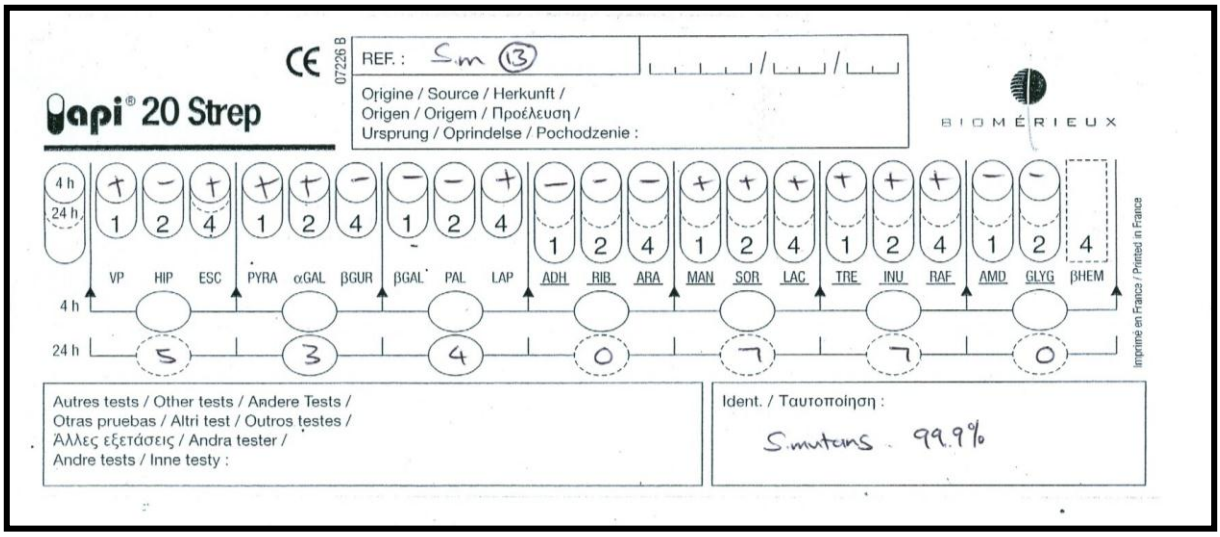



**Figure 3.2:** The results sheet showing the numerical profile of species that gave 99.9 % *Streptococcus mutans* match on an API 20 strep strip.

### 3.3 Antibacterial activity assay

*Dodonaea viscosa* var. *angustifolia* exhibited bactericidal activity against cariogenic bacteria and periodontal pathogens. The median MBC of three plant extracts for all bacterial strains under investigation are represented in Tables 3.3.1, page 56, 3.3.2, page 57, 3.3.3, page 58. The positive control (CHX) showed no growth, the negative control (water) and vehicle fluid control (DMSO) should growth for all strains under investigation. The effect of DMSO on all periodontal pathogens are represented separately in Table 3.3.4, page 59. A summary of the median MBC of three plant extracts for both cariogenic bacteria and periodontal pathogens are represented in Table 3.3.5, page 60. All experiments were performed in triplicate and the median MBC was determined.

**Table 3.3.1** Minimum bactericidal concentrations of crude plant extracts of *Dodonaea viscosa* var. *angustifolia* against *Streptococcus mutans*

| Cultures | Repeats | MBC mg/ml | | |
|---|---|---|---|---|
| | | Acetone extract | Ethanol extract | Methanol extract |
| *S. mutans* | 1 | 0.78 | 1.56 | 3.125 |
| Clinical strain 1 | 2 | 3.125 | 3.125 | 6.25 |
| | 3 | 3.125 | 6.25 | 6.25 |
| **Median** | | **3.125** | **3.125** | **6.25** |
| *S. mutans* | 1 | 3.125 | 6.25 | 6.25 |
| Clinical strain 6 | 2 | 6.25 | 6.25 | 12.5 |
| | 3 | 6.25 | 6.25 | 12.5 |
| **Median** | | **6.25** | **6.25** | **12.5** |
| *S. mutans* | 1 | 3.125 | 6.25 | 6.25 |
| Clinical strain 7 | 2 | 6.25 | 6.25 | 6.25 |
| | 3 | 6.25 | 6.25 | 6.25 |
| **Median** | | **6.25** | **6.25** | **6.25** |
| *S. mutans* | 1 | 0.78 | 0.78 | 1.56 |
| Clinical strain 13 | 2 | 3.125 | 1.56 | 3.125 |
| | 3 | 3.125 | 6.25 | 6.25 |
| **Median** | | **3.125** | **1.56** | **3.125** |
| *S. mutans* | 1 | 6.25 | 6.25 | 12.5 |
| NCTC 10919 | 2 | 6.25 | 6.25 | 12.5 |
| | 3 | 12.5 | 6.25 | 12.5 |
| **Median** | | **6.25** | **6.25** | **12.5** |

Positive control: CHX – no growth

Negative control: water – growth

Vehicle fluid control: DMSO – growth

Median MBCs of acetone extracts for *S. mutans* ranged from 3.125 - 6.25 mg/ml. Median MBCs of ethanol extracts ranged from 1.56 - 6.25 mg/ml. This extract produced the lowest MBC (0.78 mg/ml) as seen for *S. mutans* clinical strain 13. Median MBCs of methanol extracts, which produced the highest yield, ranged from 3.125 - 12.5 mg/ml,

producing the highest MBC when compared to the other two extracts. In each experiment the positive control showed no growth, the negative control and vehicle fluid control showed growth.

**Table 3.3.2** Minimum bactericidal concentrations of crude plant extracts of *Dodonaea viscosa* var. *angustifolia* against *Lactobacillus* species.

| **Cultures** | **Repeats** | **MBC (mg/ml)** | | |
|---|---|---|---|---|
| | | **Acetone extract** | **Ethanol extract** | **Methanol extract** |
| *Lactobacillus* spp. | 1 | 6.25 | 6.25 | 12.5 |
| Clinical strain 1 | 2 | 6.25 | 12.5 | 12.5 |
| | 3 | 12.5 | 12.5 | 12.5 |
| **Median** | | **6.25** | **12.5** | **12.5** |
| *Lactobacillus* spp. | 1 | 1.56 | 1.56 | 0.78 |
| Clinical strain 4 | 2 | 3.125 | 1.56 | 0.78 |
| | 3 | 3.125 | 3.125 | 0.78 |
| **Median** | | **3.125** | **1.56** | **0.78** |
| *Lactobacillus* spp. | 1 | 12.5 | 12.5 | 12.5 |
| Clinical strain 5 | 2 | 12.5 | 12.5 | 12.5 |
| | 3 | 12.5 | 12.5 | 12.5 |
| **Median** | | **12.5** | **12.5** | **12.5** |
| *Lactobacillus* spp. | 1 | 6.25 | 6.25 | 6.25 |
| Clinical strain 8 | 2 | 6.25 | 6.25 | 12.5 |
| | 3 | 12.5 | 12.5 | 12.5 |
| **Median** | | **6.25** | **6.25** | **12.5** |
| *Lactobacillus casei* | 1 | 12.5 | 12.5 | 12.5 |
| | 2 | 12.5 | 12.5 | 12.5 |
| | 3 | 12.5 | 12.5 | 12.5 |
| **Median** | | **12.5** | **12.5** | **12.5** |

Positive control: CHX – no growth

Negative control: water – growth

Vehicle fluid control: DMSO – growth

Median MBCs for *Lactobacillus* spp. ranged from 0.78 - 12.5 mg/ml. The lowest MBC (0.78 mg/ml) was obtained for *Lactobacillus* clinical strain 4 using the methanol extract.

All other strains were killed at a relatively high concentration of methanol extract (12.5 mg/ml). Ethanol proved to be the most effective solvent yet again, killing *Lactobacillus* spp. at concentrations ranging from 1.56 - 12.5 mg/ml. Acetone proved to be the second most effective extract producing MBCs ranging from 3.125 - 12.5 mg/ml.

**Table 3.3.3** Minimum bactericidal concentrations of crude plant extracts of *Dodonaea viscosa* var. *angustifolia* against periodontal pathogens

| Cultures | Repeats | MBC mg/ml | | |
|---|---|---|---|---|
| | | Acetone extract | Ethanol extract | Methanol extract |
| *P. intermedia* | 1 | 0.04 | 0.04 | 0.04 |
| | 2 | 0.04 | 0.09 | 0.04 |
| | 3 | 0.04 | 0.09 | 0.04 |
| **Median** | | **0.04** | **0.09** | **0.04** |
| *P. gingivalis* | 1 | 0.04 | 0.09 | 0.09 |
| | 2 | 0.04 | 0.09 | 0.09 |
| | 3 | 0.04 | 0.09 | 0.09 |
| **Median** | | **0.04** | **0.09** | **0.09** |
| *F. nucleatum* | 1 | 0.09 | 0.04 | 0.02 |
| | 2 | 0.09 | 0.09 | 0.02 |
| | 3 | 0.09 | 0.09 | 0.04 |
| **Median** | | **0.09** | **0.09** | **0.04** |
| *Capnocytophaga* spp. | 1 | 0.02 | 0.01 | 0.02 |
| | 2 | 0.04 | 0.02 | 0.02 |
| | 3 | 0.04 | 0.02 | 0.04 |
| **Median** | | **0.04** | **0.02** | **0.02** |

Positive control: CHX – no growth

Negative control: water – growth

Vehicle fluid control: DMSO (see Table 3.3.4 on page 59)

All MBCs obtained for periodontal pathogens were lower when compared to MBCs obtained for cariogenic bacteria. The lowest MBC (0.02 mg/ml) was observed for *Capnocytophaga* spp. using both ethanol and methanol extracts. The highest MBC (0.09

mg/ml) was obtained for *P. intermedia, P. gingivalis* and *F. nucleatum* using the ethanol extracts. Acetone and methanol proved to be the most effective extracts against *P. intermedia,* killing this bacterium at a low concentration of 0.04 mg/ml. Acetone extract killed *P. ginigivalis* at a concentration of 0.04 mg/ml, proving to be more effective than ethanol and methanol (0.09 mg/ml). Methanol proved to be the most effective extract against *F. nucleatum,* killing this bacterium at 0.04 mg/ml.

**Table 3.3.4** Minimum bactericidal concentrations of dimethyl sulphoxide against periodontal pathogens

| **Cultures** | **Repeats** | **% solution** |
|---|---|---|
| | | **DMSO - diluent** |
| *P. intermedia* | 1 | 12.5 |
| | 2 | 12.5 |
| | 3 | 12.5 |
| **Median** | | **12.5** |
| *P. gingivalis* | 1 | 12.5 |
| | 2 | 6.25 |
| | 3 | 6.25 |
| **Median** | | **6.25** |
| *F. nucleatum* | 1 | 12.5 |
| | 2 | 12.5 |
| | 3 | 12.5 |
| **Median** | | **12.5** |
| *Capnocytophaga* spp. | 1 | 25 |
| | 2 | 50 |
| | 3 | 50 |
| **Median** | | **50** |

All periodontal pathogens tested were killed at relatively high concentrations of DMSO. The DMSO, however, does not have any influence on the elimination of both the cariogenic bacteria and periodontal pathogens by the crude plant extract as it was used in the concentrated form for all experiments and was further diluted by the respective media and the inoculum used in each experiment. Therefore, the concentration of DMSO would

have been too low to have a killing effect in all MBC experiments testing the effect of the crude plant extract on cariogenic bacteria and periodontal pathogens. These results showed that the slight antibacterial effect of DMSO did not influence the MBC results of test organisms.

**Table 3.3.5** Summary of median minimum bactericidal concentrations of acetone, ethanol and methanol extracts of *Dodonaea viscosa* var. *angustifolia* against cariogenic bacteria and periodontal pathogens

| Cultures | | Median MBC in mg/ml (n=3) | | |
|---|---|---|---|---|
| | | Acetone extract | Ethanol extract | Methanol extract |
| *S. mutans*: | clinical strain 1 | 3.125 | 3.125 | 6.25 |
| | clinical strain 6 | 6.25 | 6.25 | 12.5 |
| | clinical strain 7 | 6.25 | 6.25 | 6.25 |
| | clinical strain 13 | 3.125 | 1.56 | 3.125 |
| | NCTC 10919 | 6.25 | 6.25 | 12.5 |
| *Lactobacillus*: | clinical strain 1 | 6.25 | 12.5 | 12.5 |
| | clinical strain 4 | 3.125 | 1.56 | 0.78 |
| | clinical strain 5 | 12.5 | 12.5 | 12.5 |
| | clinical strain 8 | 6.25 | 6.25 | 12.5 |
| | *L. casei* | 12.5 | 12.5 | 12.5 |
| *P. intermedia* | | 0.04 | 0.09 | 0.04 |
| *P. gingivalis* | | 0.04 | 0.09 | 0.09 |
| *F. nucleatum* | | 0.09 | 0.09 | 0.04 |
| *Capnocytophaga* spp. | | 0.04 | 0.02 | 0.02 |

## 3.4 *Streptococcus mutans* biofilm assay

### 3.4.1 Effect of crude ethanol plant extract on preformed biofilm of *Streptococcus mutans*

Results of the effect of the crude ethanol plant extract on *S. mutans* biofilm are represented in Table 3.4.1.1, page 61. Each culture was treated with the subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract by two 1 minute exposures and 6 hour incubation and a 24 hour exposure. Experiments were performed in triplicate and

the bacterial counts (CFU/ml) in the biofilm after the two exposure times are listed in the table. The mean and standard deviation is also represented for each strain. The bacterial counts in the biofilm after the two exposures to the crude ethanol plant extract are also represented graphically as log CFU/ml and exposure time (Figure 3.3, page 62).

**Table 3.4.1.1** Effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on preformed biofilm of *Streptococcus mutans*

| **Cultures** | **Repeats** | **Initial bacterial** | **Growth of *S. mutans* in biofilm (cfu/ml)** | | | |
|---|---|---|---|---|---|---|
| | | **count in biofilm** | **6 hrs (two, 1 min exposures)** | | **24 hrs treatment** | |
| | | **cfu/ml** | **control** | **plant extract** | **control** | **plant extract** |
| *S. mutans* | 1 | $7.6 \times 10^{8}$ | $1.9 \times 10^{10}$ | $8.0 \times 10^{10}$ | $3.6 \times 10^{9}$ | $2.1 \times 10^{7}$ |
| Clinical strain 1 | 2 | $1.6 \times 10^{9}$ | $1.6 \times 10^{10}$ | $1.2 \times 10^{9}$ | $1.3 \times 10^{9}$ | $2.4 \times 10^{4}$ |
| | 3 | $1.9 \times 10^{9}$ | $1.4 \times 10^{10}$ | $5.5 \times 10^{9}$ | $3.8 \times 10^{9}$ | $1.2 \times 10^{7}$ |
| | **Mean** | **$3.7 \times 10^{8}$** | **$1.6 \times 10^{10}$** | **$2.9 \times 10^{10}$** | **$2.9 \times 10^{10}$** | **$1.1 \times 10^{7}$** |
| | **±SD** | **338082830.1** | **2516611478** | **44306094389** | **1389244399** | **10523126.53** |
| *S. mutans* | 1 | $2.6 \times 10^{7}$ | $6.5 \times 10^{8}$ | $2.4 \times 10^{7}$ | $1.6 \times 10^{8}$ | $5.0 \times 10^{5}$ |
| Clinical strain 6 | 2 | $1.5 \times 10^{7}$ | $7.0 \times 10^{7}$ | $1.7 \times 10^{8}$ | $1.2 \times 10^{8}$ | $1.0 \times 10^{5}$ |
| | 3 | $2.3 \times 10^{7}$ | $1.9 \times 10^{8}$ | $1.4 \times 10^{8}$ | $7.7 \times 10^{8}$ | $9.0 \times 10^{5}$ |
| | **Mean** | **$2.1 \times 10^{7}$** | **$3.0 \times 10^{8}$** | **$1.1 \times 10^{8}$** | **$3.5 \times 10^{8}$** | **$5.0 \times 10^{5}$** |
| | **±SD** | **5686240.703** | **306159000.1** | **77105987.66** | **364280112** | **400000** |
| *S. mutans* | 1 | $1.1 \times 10^{8}$ | $5.6 \times 10^{8}$ | $2.4 \times 10^{8}$ | $1.3 \times 10^{9}$ | $9.0 \times 10^{6}$ |
| Clinical strain 7 | 2 | $1.4 \times 10^{8}$ | $3.0 \times 10^{8}$ | $1.1 \times 10^{9}$ | $1.4 \times 10^{9}$ | $4.5 \times 10^{6}$ |
| | 3 | $2.3 \times 10^{8}$ | $1.9 \times 10^{10}$ | $4.7 \times 10^{8}$ | $1.5 \times 10^{8}$ | $9.2 \times 10^{6}$ |
| | **Mean** | **$1.6 \times 10^{8}$** | **$6.6 \times 10^{9}$** | **$6.0 \times 10^{8}$** | **$9.5 \times 10^{8}$** | **$7.6 \times 10^{6}$** |
| | **±SD** | **62449979.98** | **10722182614** | **445234020.9** | **694622199** | **2657693.235** |
| *S. mutans* | 1 | $2.9 \times 10^{8}$ | $9.3 \times 10^{8}$ | $5.3 \times 10^{8}$ | $5.4 \times 10^{8}$ | $1.5 \times 10^{8}$ |
| Clinical strain 13 | 2 | $4.2 \times 10^{8}$ | $1.2 \times 10^{9}$ | $4.2 \times 10^{8}$ | $1.6 \times 10^{9}$ | $1.5 \times 10^{7}$ |
| | 3 | $3.8 \times 10^{8}$ | $9.4 \times 10^{8}$ | $1.0 \times 10^{9}$ | $6.5 \times 10^{8}$ | $1.5 \times 10^{8}$ |
| | **Mean** | **$3.6 \times 10^{8}$** | **$1.0 \times 10^{9}$** | **$6.5 \times 10^{8}$** | **$9.3 \times 10^{8}$** | **$1.1 \times 10^{8}$** |
| | **±SD** | **66583281.18** | **153079500** | **308058436** | **582837885** | **77942286.34** |
| *S. mutans* | 1 | $1.9 \times 10^{8}$ | $8.0 \times 10^{8}$ | $1.5 \times 10^{8}$ | $2.9 \times 10^{9}$ | $2.9 \times 10^{4}$ |
| NCTC 10919 | 2 | $7.2 \times 10^{7}$ | $1.4 \times 10^{7}$ | $4.4 \times 10^{7}$ | $5.8 \times 10^{7}$ | $4.3 \times 10^{6}$ |
| | 3 | $1.2 \times 10^{8}$ | $2.0 \times 10^{7}$ | $2.1 \times 10^{7}$ | $7.4 \times 10^{7}$ | $1.1 \times 10^{5}$ |
| | **Mean** | **$1.3 \times 10^{8}$** | **$2.8 \times 10^{8}$** | **$7.2 \times 10^{7}$** | **$1.0 \times 10^{9}$** | **$1.5 \times 10^{6}$** |
| | **±SD** | **59340823.5** | **452075215** | **68806491.94** | **1636230220** | **2442816.066** |
| **Combined Mean** | | **$2.1 \times 10^{8}$** | **$4.9 \times 10^{9}$** | **$6.1 \times 10^{9}$** | **$1.2 \times 10^{9}$** | **$2.5 \times 10^{7}$** |
| **Combined ±SD** | | **195385260.4** | **7638431859** | **20498316797** | **1266707423** | **51100132.2** |
| **Wilcoxon rank-sum test (p values)** | | | | **0.3614** | | **$p < 0.01$** |

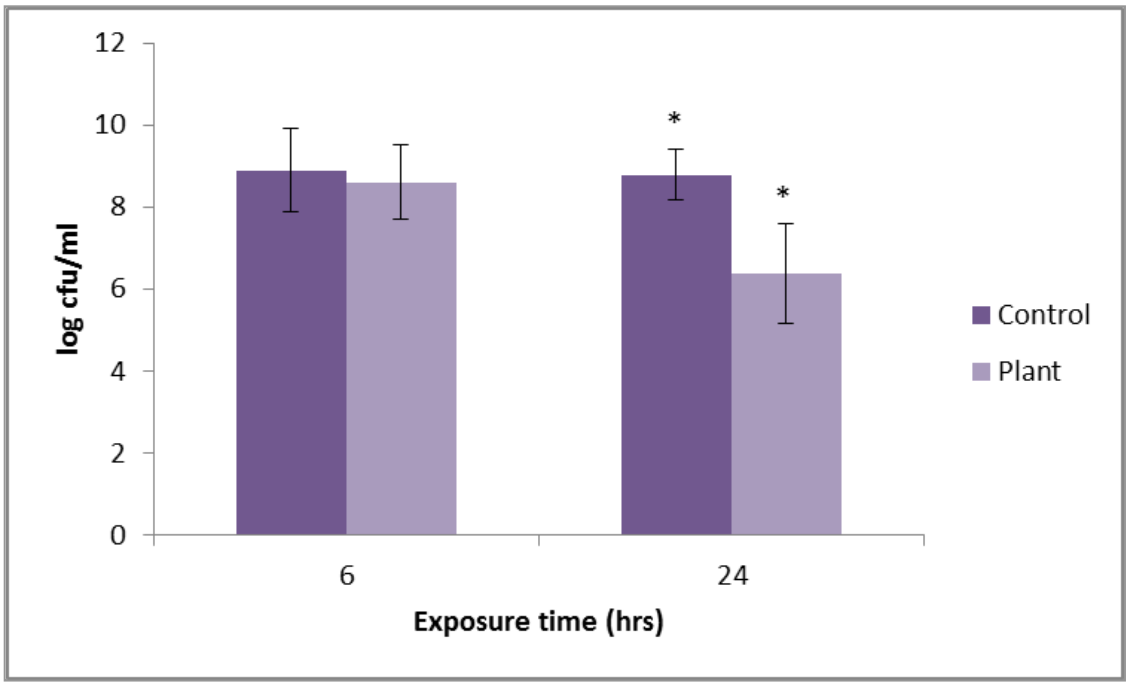



**Figure 3.3:** Bacterial counts (log CFU/ml) in *Streptococcus mutans* biofilm after 6 hours (two 1 minute exposures) and 24 hour exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract. * = p: < 0.01.
*: Comparison between bacterial counts of control and plant extract exposed biofilms of *Streptococcus mutans* after 24 hour exposure to subinhibitory concentration of crude ethanol plant extract using the Wilcoxon rank-sum test (Mann-Whitney).

The initial count is representative of the number of bacteria that constituted the biofilm after 48 hours of incubation before treatment with crude ethanol plant extract (Table 3.4.1.1). The bacterial counts of the control and the biofilm exposed to the crude ethanol plant extract during the two 1 minute exposures were not significantly different ($p > 0.05$). The bacterial counts in the control were slightly high but not significantly higher, for all strains, when compared to those of the plant extract treated biofilm. The plant extract, therefore, had no effect on the number of cells in the biofilm after 6 hours with two exposures to subinihibitory concentration of crude ethanol plant extract for 1 minute.

However, biofilm exposed to the plant extract for a period of 24 hours showed a significant difference ($p < 0.01$) compared to the control. The bacterial counts in the biofilms treated with subinhibitory concentration of crude ethanol plant extract were lower than the control biofilms for all the test strains. The crude ethanol plant extract was able to reduce 1.68 log CFU of *S. mutans* present in biofilm.

### 3.4.2 Effect of crude ethanol plant extract on *Streptococcus mutans* biofilm formation

The results of the effect of the crude ethanol plant extract on the growth of *S. mutans* biofilms are represented in Table 3.4.2.1, page 64 and Figure 3.4, page 65. The bacterial counts (CFU/ml) in biofilm developed for 6, 24 and 30 hours with subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract are depicted. Experiments were performed in triplicate for each strain and a mean and standard deviation were calculated.

The crude ethanol plant extract significantly reduced the adherence of *S. mutans* biofilms to the glass slides showing a reduced number of bacteria (Table 3.4.2.1). This was observed for all tested strains. The log CFU/ml of *S. mutans* were higher for all control slides for all the exposure time intervals when compared to biofilms developed in the presence of crude ethanol plant extract. The number of *S. mutans* adhering to the glass slides increased with time but control slides were still significantly higher than plant extract exposed slides ($p < 0.01$). The crude ethanol plant extract reduced the bacterial counts in biofilm by 1.3, 0.95 and 1.95 logs after 6, 24 and 30 hours respectively. The

results were statistically significant at each test time. These results showed that the crude ethanol plant extract reduced the biofilm formation by *S. mutans.*

**Table 3.4.2.1** Effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on biofilm formation by *Streptococcus mutans*

| **Cultures** | **Repeats** | **Growth of *S. mutans* in biofilm (cfu/ml)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **6 hrs** | | **24 hrs** | | **30 hrs** | |
| | | **control** | **plant** | **control** | **plant** | **control** | **plant** |
| *S. mutans* | 1 | $7.9 \times 10^5$ | $4.3 \times 10^3$ | $9.7 \times 10^6$ | $5.0 \times 10^4$ | $2.7 \times 10^7$ | $4.5 \times 10^5$ |
| Clinical strain 1 | 2 | $1.4 \times 10^5$ | $6.7 \times 10^3$ | $5.1 \times 10^6$ | $7.7 \times 10^4$ | $1.8 \times 10^7$ | $2.0 \times 10^5$ |
| | 3 | $1.3 \times 10^5$ | $8.7 \times 10^3$ | $1.0 \times 10^6$ | $8.6 \times 10^4$ | $2.2 \times 10^7$ | $2.1 \times 10^5$ |
| | **Mean** | $\mathbf{3.5 \times 10^5}$ | $\mathbf{6.6 \times 10^3}$ | $\mathbf{5.3 \times 10^6}$ | $\mathbf{7.1 \times 10^4}$ | $\mathbf{2.2 \times 10^7}$ | $\mathbf{2.9 \times 10^5}$ |
| | **±SD** | **378197.5** | **2203.028** | **4352394** | **18734.99** | **4509250** | **141539.2** |
| *S. mutans* | 1 | $8.3 \times 10^4$ | $6.7 \times 10^3$ | $4.2 \times 10^6$ | $1.1 \times 10^5$ | $1.6 \times 10^7$ | $1.9 \times 10^5$ |
| Clinical strain 6 | 2 | $8.7 \times 10^4$ | $6.1 \times 10^3$ | $6.2 \times 10^6$ | $7.3 \times 10^4$ | $5.8 \times 10^7$ | $4.0 \times 10^5$ |
| | 3 | $7.7 \times 10^4$ | $8.4 \times 10^3$ | $7.4 \times 10^6$ | $1.6 \times 10^5$ | $1.1 \times 10^8$ | $2.4 \times 10^5$ |
| | **Mean** | $\mathbf{8.2 \times 10^4}$ | $\mathbf{7.1 \times 10^3}$ | $\mathbf{5.9 \times 10^6}$ | $\mathbf{1.1 \times 10^5}$ | $\mathbf{6.1 \times 10^7}$ | $\mathbf{2.8 \times 10^5}$ |
| | **±SD** | **5033.223** | **1193.035** | **1616581** | **43661.58** | **47088569** | **109696.6** |
| *S. mutans* | 1 | $2.6 \times 10^4$ | $4.1 \times 10^3$ | $1.5 \times 10^7$ | $1.8 \times 10^5$ | $1.3 \times 10^8$ | $4.5 \times 10^6$ |
| Clnical strain 7 | 2 | $3.8 \times 10^4$ | $3.0 \times 10^3$ | $1.8 \times 10^7$ | $3.0 \times 10^5$ | $1.6 \times 10^8$ | $3.7 \times 10^6$ |
| | 3 | $4.0 \times 10^4$ | $2.8 \times 10^3$ | $1.0 \times 10^7$ | $3.5 \times 10^5$ | $1.2 \times 10^8$ | $7.1 \times 10^5$ |
| | **Mean** | $\mathbf{3.5 \times 10^4}$ | $\mathbf{3.3 \times 10^3}$ | $\mathbf{1.4 \times 10^7}$ | $\mathbf{2.8 \times 10^5}$ | $\mathbf{1.4 \times 10^8}$ | $\mathbf{5.1 \times 10^6}$ |
| | **±SD** | **7571.878** | **700** | **4041452** | **87368.95** | **20816660** | **1997674** |
| *S. mutans* | 1 | $1.7 \times 10^4$ | $2.7 \times 10^3$ | $1.3 \times 10^7$ | $7.7 \times 10^5$ | $1.5 \times 10^8$ | $9.0 \times 10^5$ |
| Clinical strain 13 | 2 | $2.9 \times 10^4$ | $2.4 \times 10^3$ | $1.4 \times 10^7$ | $4.1 \times 10^5$ | $2.3 \times 10^8$ | $6.1 \times 10^5$ |
| | 3 | $4.5 \times 10^4$ | $8.5 \times 10^2$ | $1.3 \times 10^7$ | $6.2 \times 10^5$ | $1.2 \times 10^8$ | $8.7 \times 10^5$ |
| | **Mean** | $\mathbf{3.0 \times 10^4}$ | $\mathbf{2.0 \times 10^3}$ | $\mathbf{1.3 \times 10^7}$ | $\mathbf{6.0 \times 10^5}$ | $\mathbf{1.7 \times 10^8}$ | $\mathbf{7.9 \times 10^5}$ |
| | **±SD** | **14047.54** | **992.8914** | **577350.3** | **180831.4** | **56862407** | **159478.3** |
| *S. mutans* | 1 | $1.7 \times 10^4$ | $8.3 \times 10^3$ | $2.5 \times 10^6$ | $6.8 \times 10^4$ | $1.6 \times 10^7$ | $1.8 \times 10^5$ |
| NCTC 10919 | 2 | $8.8 \times 10^4$ | $7.1 \times 10^3$ | $5.0 \times 10^6$ | $2.5 \times 10^5$ | $4.4 \times 10^7$ | $4.5 \times 10^5$ |
| | 3 | $2.0 \times 10^4$ | $9.8 \times 10^3$ | $3.3 \times 10^6$ | $1.8 \times 10^5$ | $4.2 \times 10^7$ | $6.1 \times 10^5$ |
| | **Mean** | $\mathbf{4.2 \times 10^4}$ | $\mathbf{8.4 \times 10^3}$ | $\mathbf{3.6 \times 10^6}$ | $\mathbf{1.7 \times 10^5}$ | $\mathbf{3.4 \times 10^7}$ | $\mathbf{4.1 \times 10^5}$ |
| | **±SD** | **40153.87** | **1352.775** | **1276715** | **91804.14** | **15620499** | **217332.3** |
| **Combined mean** | | $\mathbf{1.1 \times 10^5}$ | $\mathbf{5.5 \times 10^3}$ | $\mathbf{8.5 \times 10^6}$ | $\mathbf{2.5 \times 10^5}$ | $\mathbf{8.4 \times 10^7}$ | $\mathbf{9.5 \times 10^5}$ |
| **Combined ±SD** | | **192691.4** | **2761.629** | **5175694** | **214457.4** | **66315480** | **1310666** |
| **Wilcoxon rank-sum test (p values)** | | | **$p < 0.01$** | | **$p < 0.01$** | | **$p < 0.01$** |

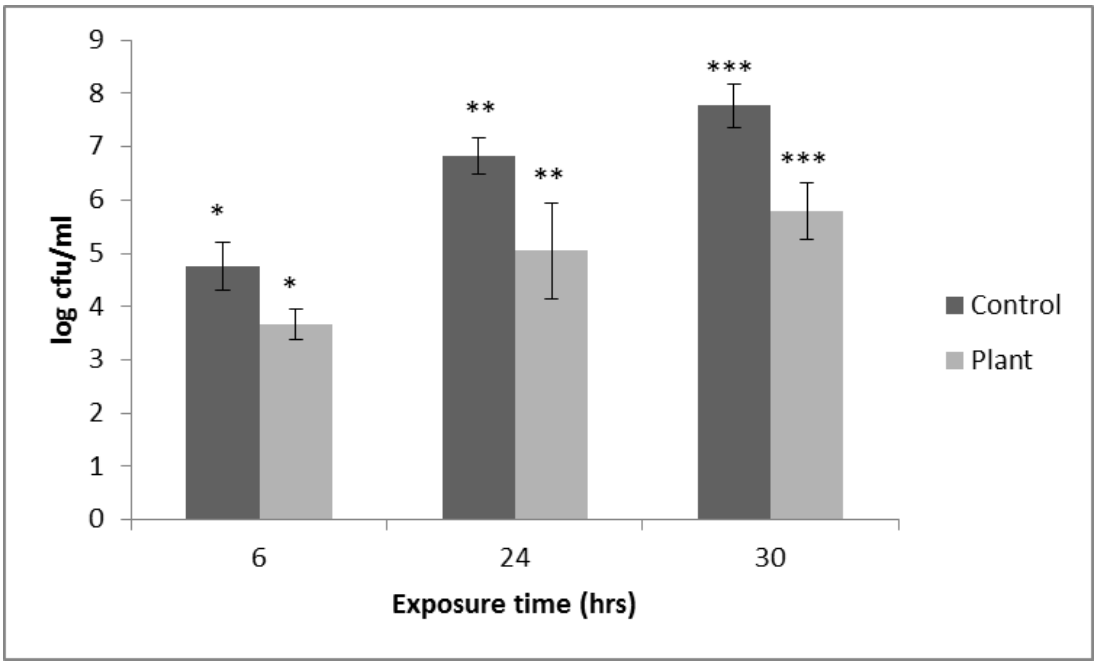



**Figure 3.4:** Growth of *Streptococcus mutans* biofilm in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract (log CFU/ml) in biofilm after 6, 24 and 30 hour exposures to plant extract. *, **, *** = p: < 0.01.
*, **, ***: Comparison between bacterial counts of control and plant extract exposed biofilms of *Streptococcus mutans* after 6, 24 and 30 hours using the Wilcoxon rank-sum test (Mann-Whitney).

### 3.5 Extracellular polysaccharide analysis

The results of the EPS produced by bacteria in the biofilm and planktonic form of *S. mutans*, unexposed and exposed to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract are represented in Table 3.5.1 - 3.5.2, pages 66 and 68 and Figures 3.5 and 3.6, pages 67 and 69. For each strain experiments were performed in triplicate. Mean and standard deviations were calculated for the mass of polysaccharides produced. Polysaccharides are represented as glucans and levans separately and as total polysaccharides (glucans and levans).

**Table 3.5.1** Extracellular polysaccharide production by *Streptococcus mutans* biofilm after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract

| Cultures | Repeats | Polysaccharide production by *S. mutans* biofilm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | | | | Plant | | | |
| | | Final bacterial count cfu/ml | Glucans (g) | Levans (g) | Total Polysaccharides (g) | Final bacterial count cfu/ml | Glucans (g) | Levans (g) | Total Polysaccharides (g) |
| *S. mutans* | 1 | $4.5 \times 10^6$ | 0.148 | 0.142 | 0.29 | $1.3 \times 10^6$ | 0.148 | 0.148 | 0.296 |
| Clinical strain 1 | 2 | $7.0 \times 10^6$ | 0.151 | 0.146 | 0.297 | $1.2 \times 10^6$ | 0.152 | 0.142 | 0.294 |
| | 3 | $4.1 \times 10^7$ | 0.143 | 0.144 | 0.287 | $6.4 \times 10^5$ | 0.146 | 0.159 | 0.305 |
| | **Mean** | **$1.8 \times 10^7$** | **0.147333** | **0.144** | **0.291333333** | **$1.0 \times 10^6$** | **0.1486667** | **0.1496667** | **0.298333333** |
| | **±SD** | **20389948.5** | **0.004041** | **0.002** | **0.005131601** | **355715.2419** | **0.0030551** | **0.0086217** | **0.005859465** |
| *S. mutans* | 1 | $7.6 \times 10^7$ | 0.158 | 0.143 | 0.301 | $7.2 \times 10^5$ | 0.143 | 0.153 | 0.296 |
| Clinical strain 6 | 2 | $1.6 \times 10^7$ | 0.142 | 0.151 | 0.293 | $1.7 \times 10^5$ | 0.162 | 0.15 | 0.312 |
| | 3 | $2.8 \times 10^7$ | 0.153 | 0.151 | 0.304 | $6.4 \times 10^5$ | 0.147 | 0.16 | 0.307 |
| | **Mean** | **$4.0 \times 10^7$** | **0.151** | **0.14833333** | **0.299333333** | **$5.1 \times 10^5$** | **0.1506667** | **0.1543333** | **0.305** |
| | **±SD** | **31749015.73** | **0.008185** | **0.0046188** | **0.005686241** | **297153.1592** | **0.0100167** | **0.0051316** | **0.008185353** |
| *S. mutans* | 1 | $3.7 \times 10^5$ | 0.144 | 0.157 | 0.301 | $1.5 \times 10^7$ | 0.111 | 0.156 | 0.267 |
| Clinical strain 7 | 2 | $1.1 \times 10^7$ | 0.164 | 0.153 | 0.317 | $8.0 \times 10^6$ | 0.15 | 0.166 | 0.316 |
| | 3 | $8.3 \times 10^6$ | 0.145 | 0.16 | 0.305 | $5.7 \times 10^6$ | 0.141 | 0.153 | 0.294 |
| | **Mean** | **$3.1 \times 10^7$** | **0.151** | **0.15666667** | **0.307666667** | **$9.6 \times 10^6$** | **0.134** | **0.1583333** | **0.292333333** |
| | **±SD** | **44953149.31** | **0.011269** | **0.00351188** | **0.008326664** | **4843896.503** | **0.0204206** | **0.0068069** | **0.02454248** |
| *S. mutans* | 1 | $3.6 \times 10^6$ | 0.139 | 0.143 | 0.282 | $4.0 \times 10^5$ | 0.162 | 0.155 | 0.317 |
| Clinical strain 13 | 2 | $1.5 \times 10^6$ | 0.142 | 0.133 | 0.275 | $8.5 \times 10^5$ | 0.141 | 0.14 | 0.281 |
| | 3 | $3.4 \times 10^6$ | 0.138 | 0.138 | 0.276 | $5.5 \times 10^5$ | 0.147 | 0.138 | 0.285 |
| | **Mean** | **$2.8 \times 10^6$** | **0.139667** | **0.138** | **0.277666667** | **$6.0 \times 10^5$** | **0.15** | **0.1443333** | **0.294333333** |
| | **±SD** | **1159022.577** | **0.002082** | **0.005** | **0.003785939** | **229128.7847** | **0.0108167** | **0.0092916** | **0.019731531** |
| *S. mutans* | 1 | $1.6 \times 10^6$ | 0.152 | 0.143 | 0.295 | $9.7 \times 10^4$ | 0.151 | 0.144 | 0.295 |
| NCTC 10919 | 2 | $1.5 \times 10^6$ | 0.146 | 0.15 | 0.296 | $4.2 \times 10^5$ | 0.145 | 0.148 | 0.293 |
| | 3 | $3.5 \times 10^4$ | 0.147 | 0.145 | 0.292 | $2.1 \times 10^5$ | 0.153 | 0.136 | 0.289 |
| | **Mean** | **$1.0 \times 10^6$** | **0.148333** | **0.146** | **0.294333333** | **$2.4 \times 10^5$** | **0.1496667** | **0.1426667** | **0.292333333** |
| | **±SD** | **876113.5771** | **0.003215** | **0.00360555** | **0.002081666** | **163909.5279** | **0.0041633** | **0.0061101** | **0.00305505** |
| **Combined Mean** | | **$1.9 \times 10^7$** | **0.147467** | **0.1466** | | **$2.4 \times 10^6$** | **0.1466** | **0.1498667** | **0.296466667** |
| **Combined ±SD** | | **27303801.14** | **0.00712** | **0.00710935** | **0.011215211** | **4153269.811** | **0.0117218** | **0.0087167** | **0.013495325** |
| | | # | * | ** | | # | * | ** | |
| **p values** | | | | | | # **0.01** | * **0.7085** | ** **0.3393** | |

*, **: Comparison between the mass of glucans and levans produced by the control and plant exposed cells using the student's *t*-test

# : Comparison between the bacterial counts of the control and plant exposed cells using the Wilcoxon rank-sum test (Mann-Whitney)

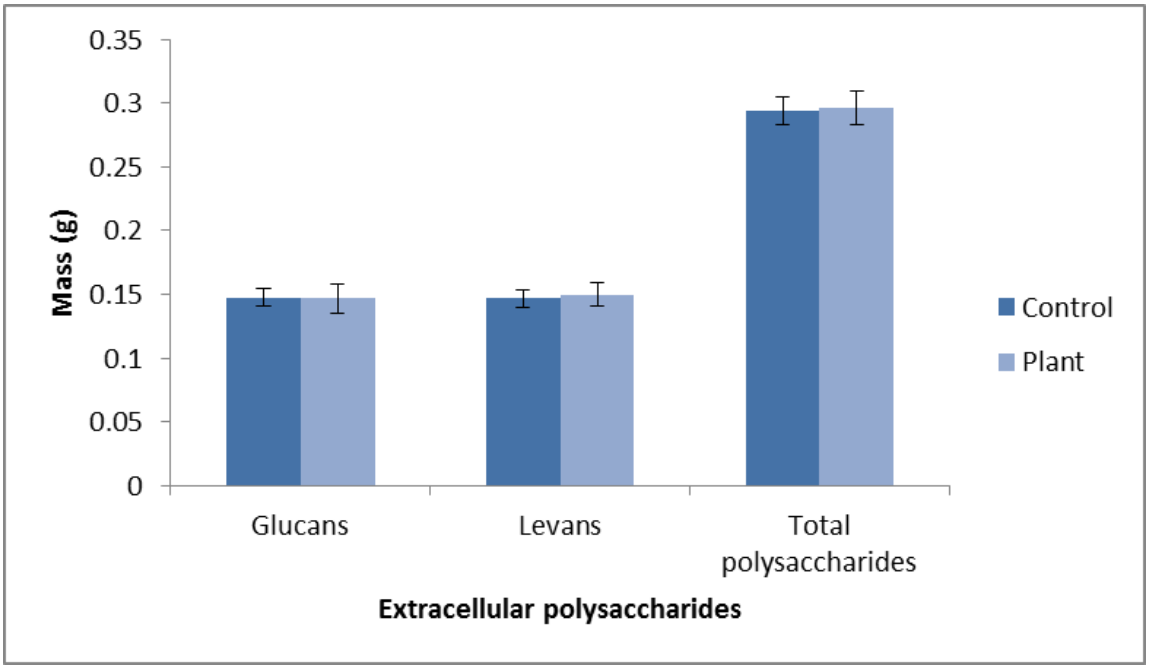



**Figure 3.5**: Extracellular polysaccharides produced by *Streptococcus mutans* in biofilm after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract.

The crude ethanol plant extract had no effect on the production of both the polysaccharides by *S. mutans* biofilms compared to the controls. All the test strains behaved similarly. Although bacterial counts were higher in control samples ($p = < 0.01$), the amount of polysaccharides produced were similar to the plant extract treated biofilms. Therefore, no significant difference can be seen between control and plant extract treated biofilms in terms of the mass of polysaccharides produced ($p > 0.05$). The plant extract therefore, had no effect on the amount of polysaccharides produced by *S. mutans* in the biofilm form. The subinhibitory concentration of crude plant extract may have killed some of the bacteria but the reduction in the number of bacteria did not affect the EPS production.

**Table 3.5.2** Extracellular polysaccharide production by planktonic cells of *Streptococcus mutans* in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract

| Cultures | Repeats | Polysaccharide production by *S. mutans* planktonic cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | | | | Plant | | | |
| | | Final bacterial count cfu/ml | Glucans (g) | Levans (g) | Total Polysaccharides (g) | Final bacterial count cfu/ml | Glucans (g) | Levans (g) | Total Polysaccharides (g) |
| *S. mutans* | 1 | $6.6 \times 10^7$ | 0.145 | 0.139 | 0.284 | $8.4 \times 10^5$ | 0.151 | 0.158 | 0.309 |
| Clinical strain 1 | 2 | $1.1 \times 10^7$ | 0.144 | 0.15 | 0.294 | $4.0 \times 10^3$ | 0.148 | 0.147 | 0.295 |
| | 3 | $5.5 \times 10^7$ | 0.143 | 0.147 | 0.29 | $9.8 \times 10^5$ | 0.159 | 0.145 | 0.304 |
| | **Mean** | **$4.4 \times 10^7$** | **0.144** | **0.1453333** | **0.289333333** | **$6.0 \times 10^5$** | **0.152666667** | **0.15** | **0.302666667** |
| | **±SD** | **29103264.42** | **0.001** | **0.0056862** | **0.005033223** | **527742.3614** | **0.005686241** | **0.007** | **0.007094599** |
| *S. mutans* | 1 | $8.0 \times 10^5$ | 0.143 | 0.145 | 0.288 | $2.1 \times 10^4$ | 0.132 | 0.149 | 0.281 |
| Clinical strain 6 | 2 | $3.0 \times 10^4$ | 0.141 | 0.147 | 0.288 | $2.0 \times 10^4$ | 0.154 | 0.144 | 0.298 |
| | 3 | $2.4 \times 10^4$ | 0.139 | 0.146 | 0.285 | $1.8 \times 10^4$ | 0.139 | 0.146 | 0.285 |
| | **Mean** | **$2.8 \times 10^5$** | **0.141** | **0.146** | **0.287** | **$2.0 \times 10^4$** | **0.141666667** | **0.14633333** | **0.288** |
| | **±SD** | **446301.8411** | **0.002** | **0.001** | **0.001732051** | **1527.525232** | **0.01123981** | **0.00251661** | **0.008888194** |
| *S. mutans* | 1 | $1.6 \times 10^7$ | 0.164 | 0.16 | 0.324 | $8.4 \times 10^5$ | 0.15 | 0.158 | 0.308 |
| Clinical strain 7 | 2 | $6.0 \times 10^6$ | 0.156 | 0.158 | 0.314 | $1.0 \times 10^7$ | 0.16 | 0.158 | 0.318 |
| | 3 | $8.9 \times 10^7$ | 0.161 | 0.153 | 0.314 | $2.6 \times 10^6$ | 0.16 | 0.157 | 0.317 |
| | **Mean** | **$3.7 \times 10^7$** | **0.16033333** | **0.157** | **0.317333333** | **$4.5 \times 10^6$** | **0.156666667** | **0.15766667** | **0.314333333** |
| | **±SD** | **45310043.04** | **0.00404145** | **0.0036056** | **0.005773503** | **4860781.83** | **0.005773503** | **0.00057735** | **0.005507571** |
| *S. mutans* | 1 | $1.9 \times 10^6$ | 0.151 | 0.148 | 0.299 | $3.3 \times 10^5$ | 0.152 | 0.159 | 0.311 |
| Clinical strain 13 | 2 | $2.0 \times 10^6$ | 0.163 | 0.157 | 0.32 | $5.5 \times 10^5$ | 0.158 | 0.16 | 0.318 |
| | 3 | $2.3 \times 10^6$ | 0.158 | 0.158 | 0.316 | $8.2 \times 10^5$ | 0.16 | 0.153 | 0.313 |
| | **Mean** | **$2.1 \times 10^6$** | **0.15733333** | **0.1543333** | **0.311666667** | **$5.7 \times 10^5$** | **0.156666667** | **0.15733333** | **0.314** |
| | **±SD** | **208166.5999** | **0.00602771** | **0.0055076** | **0.011150486** | **245424.8018** | **0.004163332** | **0.00378594** | **0.003605551** |
| *S. mutans* | 1 | $5.8 \times 10^5$ | 0.132 | 0.132 | 0.264 | $2.1 \times 10^5$ | 0.142 | 0.131 | 0.273 |
| NCTC 10919 | 2 | $2.4 \times 10^5$ | 0.133 | 0.141 | 0.274 | $2.5 \times 10^5$ | 0.139 | 0.14 | 0.279 |
| | 3 | $2.1 \times 10^5$ | 0.143 | 0.144 | 0.287 | $1.8 \times 10^5$ | 0.144 | 0.13 | 0.274 |
| | **Mean** | **$3.4 \times 10^5$** | **0.136** | **0.139** | **0.275** | **$2.1 \times 10^5$** | **0.141666667** | **0.13366667** | **0.275333333** |
| | **±SD** | **205507.5019** | **0.00608276** | **0.006245** | **0.011532563** | **35118.84584** | **0.002516611** | **0.00550757** | **0.00321455** |
| **Combined Mean** | | **$1.7 \times 10^7$** | **0.14773333** | **0.1483333** | **0.296066667** | **$1.2 \times 10^7$** | **0.149866667** | **0.149** | **0.298866667** |
| **Combined ±SD** | | **28693081.84** | **0.010464** | **0.0078801** | **0.017806366** | **2529212.324** | **0.009006876** | **0.00987059** | **0.016539419** |
| | | # | * | ** | | # | * | ** | |
| **p values** | | | | | | # **0.03** | * **0.6328** | ** **0.7235** | |

*, **: Comparison between the mass of glucans and levans produced by the control and plant exposed cells using the student's *t*-test

# : Comparison between the bacterial counts of the control and plant exposed cells using the Wilcoxon rank-sum test (Mann-Whitney)

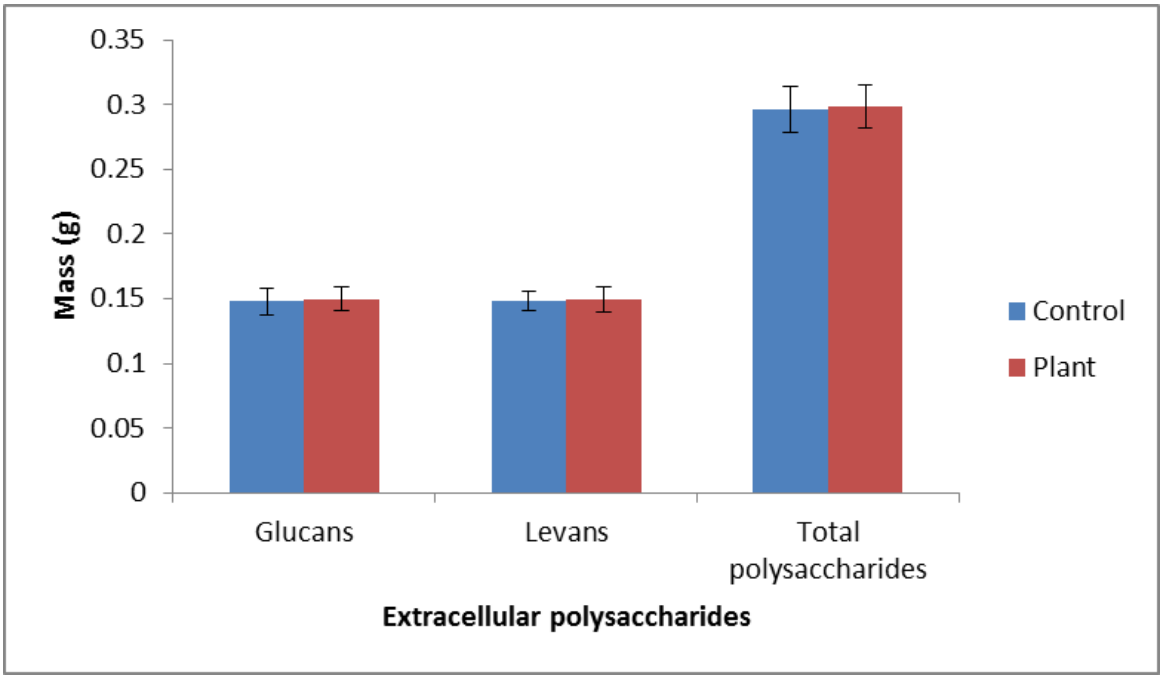



**Figure 3.6:** Extracellular polysaccharides produced by *Streptococcus mutans* planktonic cells in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract.

Although cells in the planktonic form behave differently than those in biofilm form, no effect of crude ethanol plant extract on polysaccharide production in planktonic cells were observed. The mass of glucans and levans produced were similar for both the control and test, although bacterial counts were higher in control samples ($p < 0.05$). Therefore, no significant difference in mass of polysaccharides produced can be seen between control and test for all strains ($p > 0.05$). The crude ethanol plant extract therefore, had no effect on the amount of polysaccharides produced by *S. mutans* in the planktonic form. The subinhibitory concentration of crude ethanol plant extract may have killed some of the bacteria but the reduction in the number of bacteria did not affect the EPS production.

## 3.6 Acid production

### 3.6.1 Effect of crude ethanol plant extract on the acid production by *Streptococcus mutans* in biofilms

The results of acid production by biofilms of *S. mutans* in the presence and absence of crude ethanol plant extract are represented in Table 3.6.1.1, page 71. The amount of acid produced by biofilms exposed to the crude ethanol plant extract and those that remained unexposed are represented as a means of pH (Figure 3.7, page 72). The means and standard deviations of pH were calculated for each test strain.

The biofilms treated with the crude ethanol plant extract and the untreated biofilms showed a gradual drop in pH from time 0 to 22 hours and the gradual drop was similar in both the groups. The crude ethanol plant extract had no effect on the amount of acid produced by biofilm. At time 0, pH values for all strains were approximately neutral and dropped to approximately 4 over a period of 22 hours which suggests that the cells in the biofilm were viable and metabolically active. Therefore, the crude ethanol plant extract had no effect on the production of acid by *S. mutans* in biofilms.

**Table 3.6.1.1.** The effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on acid production by biofilms of *Streptococcus mutans*

| Cultures | Repeats | Acid production by *S. mutans* in biofilm (pH values) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 hrs | | 2 hrs | | 4 hrs | | 6 hrs | | 8 hrs | | 13 hrs | | 22 hrs | |
| | | control | plant | control | plant | control | plant | control | plant | control | plant | control | plant | control | plant |
| *S. mutans* | 1 | 7 | 7 | 6.78 | 6.8 | 6.53 | 6.4 | 6.06 | 5.74 | 5.56 | 5.06 | 4.42 | 4.2 | 3.97 | 3.89 |
| Clinical strain 1 | 2 | 7 | 7 | 6.76 | 6.83 | 6.54 | 6.38 | 6.02 | 5.58 | 5.16 | 4.93 | 4.19 | 4.19 | 3.9 | 3.84 |
| | 3 | 7 | 7 | 6.68 | 6.85 | 6.35 | 6.64 | 5.69 | 6.18 | 4.98 | 5.54 | 4.19 | 4.34 | 3.89 | 3.94 |
| | **Mean** | **7** | **7** | **6.74** | **6.83** | **6.47** | **6.47** | **5.92** | **5.83** | **5.23** | **5.18** | **4.27** | **4.24** | **3.92** | **3.89** |
| | **±SD** | **0** | **0** | **0.0529** | **0.02517** | **0.1069** | **0.1447** | **0.2031** | **0.3107** | **0.2969** | **0.3213** | **0.1328** | **0.0839** | **0.0436** | **0.05** |
| *S. mutans* | 1 | 7 | 7 | 6.72 | 6.82 | 6.04 | 6.48 | 5.15 | 5.78 | 4.6 | 5.07 | 4.27 | 4.36 | 3.94 | 4.05 |
| Clinical strain 6 | 2 | 7 | 7 | 6.82 | 6.83 | 6.45 | 6.4 | 5.61 | 5.52 | 4.78 | 4.64 | 4.25 | 4.19 | 3.97 | 3.98 |
| | 3 | 7 | 7 | 6.74 | 6.76 | 6.18 | 6.32 | 5.23 | 5.51 | 4.51 | 4.66 | 4.2 | 4.2 | 3.97 | 3.99 |
| | **Mean** | **7** | **7** | **6.76** | **6.8** | **6.22** | **6.4** | **5.33** | **5.6** | **4.63** | **4.79** | **4.24** | **4.25** | **3.96** | **4** |
| | **±SD** | **0** | **0** | **0.0529** | **0.03786** | **0.2084** | **0.08** | **0.2458** | **0.1531** | **0.1375** | **0.2427** | **0.0361** | **0.0954** | **0.0173** | **0.0379** |
| *S. mutans* | 1 | 7 | 7 | 6.89 | 6.85 | 6.45 | 6.52 | 5.85 | 5.88 | 5.09 | 5.12 | 4.41 | 4.41 | 4.08 | 4.12 |
| Clinical strain 7 | 2 | 7 | 7 | 6.73 | 6.88 | 6.14 | 6.48 | 5.35 | 5.76 | 4.8 | 4.94 | 4.33 | 4.32 | 4.1 | 4.09 |
| | 3 | 7 | 7 | 6.8 | 6.86 | 6.38 | 6.52 | 5.66 | 5.8 | 4.93 | 4.95 | 4.33 | 4.3 | 4.1 | 4.09 |
| | **Mean** | **7** | **7** | **6.81** | **6.86** | **6.32** | **6.51** | **5.62** | **5.81** | **4.94** | **5** | **4.36** | **4.34** | **4.09** | **4.1** |
| | **±SD** | **0** | **0** | **0.0802** | **0.01528** | **0.1626** | **0.0231** | **0.2524** | **0.0611** | **0.1453** | **0.1012** | **0.0462** | **0.0586** | **0.0115** | **0.0173** |
| *S. mutans* | 1 | 6.98 | 6.98 | 6.83 | 6.83 | 6.7 | 6.54 | 6.24 | 5.97 | 5.75 | 5.06 | 4.33 | 4.21 | 3.84 | 3.83 |
| Clinical strain 13 | 2 | 6.98 | 6.98 | 6.79 | 6.83 | 6.62 | 6.56 | 6.16 | 5.93 | 5.23 | 5.04 | 4.26 | 4.19 | 3.83 | 3.85 |
| | 3 | 6.98 | 6.98 | 6.85 | 6.83 | 6.54 | 6.65 | 5.95 | 6.23 | 5.11 | 5.43 | 4.42 | 4.5 | 3.86 | 3.8 |
| | **Mean** | **6.98** | **6.98** | **6.82** | **6.83** | **6.62** | **6.58** | **6.11** | **6.04** | **5.36** | **5.18** | **4.34** | **4.3** | **3.84** | **3.83** |
| | **±SD** | **0** | **0** | **0.0306** | **0** | **0.08** | **0.0586** | **0.1498** | **0.1629** | **0.3402** | **0.2196** | **0.0802** | **0.1735** | **0.0153** | **0.0252** |
| *S. mutans* | 1 | 7 | 7 | 6.9 | 6.83 | 6.45 | 6.32 | 5.68 | 5.49 | 4.87 | 4.8 | 4.44 | 4.38 | 3.99 | 4.01 |
| NCTC 10919 | 2 | 7 | 7 | 6.87 | 6.84 | 6.45 | 6.29 | 5.64 | 5.29 | 4.81 | 4.63 | 4.37 | 4.32 | 4 | 3.91 |
| | 3 | 7 | 7 | 6.86 | 6.87 | 6.34 | 6.4 | 5.35 | 5.56 | 4.64 | 4.78 | 4.33 | 4.38 | 3.99 | 3.97 |
| | **Mean** | **7** | **7** | **6.88** | **6.85** | **6.41** | **6.34** | **5.56** | **5.45** | **4.77** | **4.74** | **4.38** | **4.36** | **3.99** | **3.96** |
| | **±SD** | **0** | **0** | **0.0208** | **0.02082** | **0.0635** | **0.0569** | **0.1801** | **0.1401** | **0.1193** | **0.0929** | **0.0557** | **0.0346** | **0.0058** | **0.0503** |
| **Combined Mean** | | **6.99** | **6.99** | **6.8** | **6.83** | **6.41** | **6.46** | **5.71** | **5.74** | **4.99** | **4.98** | **4.31** | **4.3** | **3.96** | **3.96** |
| **Combined ±SD** | | **0.0083** | **0.0083** | **0.0664** | **0.02874** | **0.1803** | **0.1128** | **0.3382** | **0.2634** | **0.3432** | **0.2643** | **0.0863** | **0.0983** | **0.0876** | **0.1028** |
| **Wilcoxon rank-sum test (p values)** | | | **1** | | **0.0914** | | **0.3766** | | **0.7294** | | **0.92** | | **0.6256** | | **0.8945** |

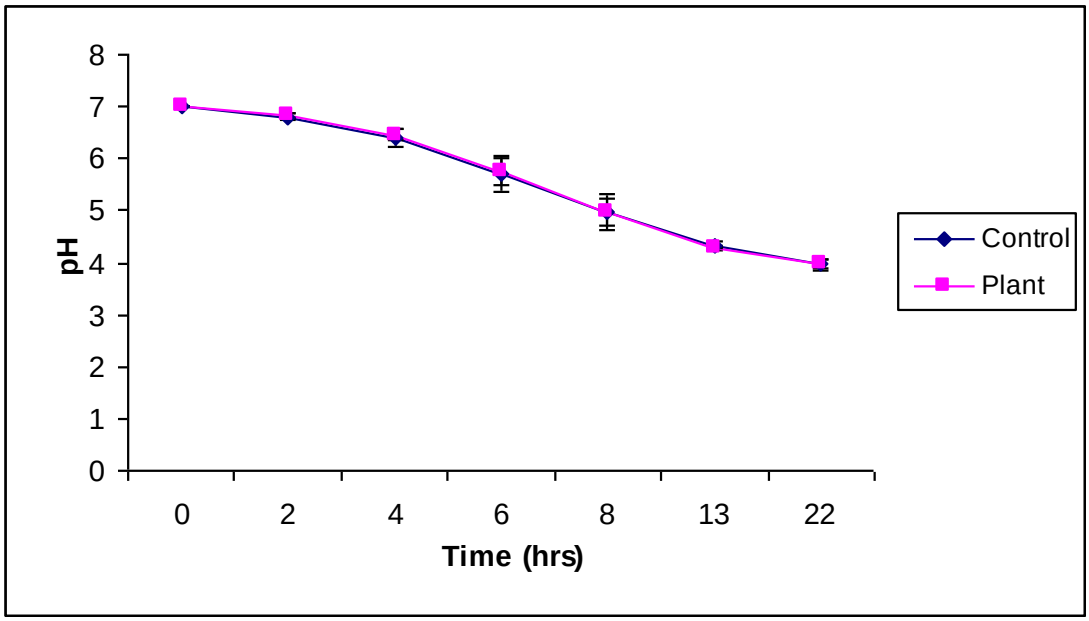



**Figure 3.7:** Mean pH values of *Streptococcus mutans* in biofilm after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract.

### 3.6.2 Effect of crude ethanol plant extract on the acid production by planktonic cells of *Streptococcus mutans*

The results of the acid produced by planktonic cells of *S. mutans* in the presence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract are represented in Table 3.6.2.1, page 73. The acid production was measured by reading pH every 2 hours following a 10 hour incubation period after inoculation. The bacterial counts at 0, 12 and 16 hours are represented in Table 3.6.2.2, page 74. The mean pH values and bacterial counts (log CFU/ml) are represented together in Figure 3.8, page 75. Mean and standard deviation values are shown for each time interval.

**Table 3.6.2.1.** The effect of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract on the acid production by planktonic cells of *Streptococcus mutans*

| Cultures | Repeats | Acid production by *S. mutans* in planktonic cells (pH values) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0hrs | | 10hrs | | 12hrs | | 14hrs | | 16 hrs | |
| | | control | plant | control | plant | control | plant | control | plant | control | plant |
| *S. mutans* | 1 | 7 | 7 | 4.84 | 6.98 | 4.49 | 6.56 | 4.41 | 6.11 | 4.3 | 5.65 |
| Clinical strain 1 | 2 | 7 | 7 | 4.9 | 6.99 | 4.5 | 6.54 | 4.41 | 6.06 | 4.32 | 5.61 |
| | 3 | 7 | 7 | 4.9 | 6.97 | 4.51 | 6.6 | 4.4 | 6.11 | 4.33 | 5.63 |
| | **Mean** | **7** | **7** | **4.88** | **6.98** | **4.5** | **6.57** | **4.41** | **6.09** | **4.32** | **5.63** |
| | **±SD** | **0** | **0** | **0.034641** | **0.01** | **0.01** | **0.0305505** | **0.005774** | **0.0288675** | **0.0152753** | **0.02** |
| *S. mutans* | 1 | 6.98 | 6.98 | 5.26 | 6.66 | 4.56 | 6.16 | 4.28 | 5.59 | 4.1 | 5.15 |
| Clinical strain 6 | 2 | 6.98 | 6.98 | 5.03 | 6.66 | 4.47 | 6.2 | 4.24 | 5.59 | 4.06 | 5.13 |
| | 3 | 6.98 | 6.98 | 5.1 | 6.69 | 4.48 | 6.1 | 4.26 | 5.71 | 4.06 | 5.22 |
| | **Mean** | **6.98** | **6.98** | **5.13** | **6.67** | **4.5** | **6.15** | **4.26** | **5.63** | **4.07** | **5.17** |
| | **±SD** | **0** | **0** | **0.1178983** | **0.017321** | **0.049329** | **0.0503322** | **0.02** | **0.069282** | **0.023094** | **0.047258** |
| *S. mutans* | 1 | 7 | 7 | 5.6 | 6.94 | 4.7 | 6.59 | 4.4 | 6.13 | 4.3 | 5.67 |
| Clinical strain 7 | 2 | 7 | 7 | 5.56 | 6.95 | 4.69 | 6.62 | 4.38 | 6.2 | 4.25 | 5.71 |
| | 3 | 7 | 7 | 5.62 | 6.95 | 4.64 | 6.63 | 4.38 | 6.19 | 4.24 | 5.67 |
| | **Mean** | **7** | **7** | **5.59** | **6.95** | **4.68** | **6.61** | **4.39** | **6.17** | **4.26** | **5.68** |
| | **±SD** | **0** | **0** | **0.0305505** | **0.005774** | **0.032146** | **0.0208167** | **0.011547** | **0.0378594** | **0.0321455** | **0.023094** |
| *S. mutans* | 1 | 7 | 7 | 5.42 | 6.75 | 4.59 | 6.69 | 4.34 | 6.38 | 4.18 | 5.97 |
| Clinical strain 13 | 2 | 7 | 7 | 5.46 | 6.77 | 4.58 | 6.74 | 4.33 | 6.46 | 4.12 | 5.98 |
| | 3 | 7 | 7 | 5.32 | 6.77 | 4.56 | 6.72 | 4.33 | 6.48 | 4.1 | 5.96 |
| | **Mean** | **7** | **7** | **5.4** | **6.76** | **4.58** | **6.72** | **4.33** | **6.44** | **4.13** | **5.97** |
| | **±SD** | **0** | **0** | **0.072111** | **0.011547** | **0.015275** | **0.0251661** | **0.005774** | **0.052915** | **0.0416333** | **2.973464** |
| *S. mutans* | 1 | 7 | 7 | 5.91 | 6.69 | 4.88 | 6.44 | 4.4 | 5.97 | 4.15 | 5.34 |
| NCTC 10919 | 2 | 7 | 7 | 5.63 | 6.69 | 4.64 | 6.45 | 4.27 | 5.97 | 4.05 | 5.39 |
| | 3 | 7 | 7 | 5.84 | 6.71 | 4.71 | 6.5 | 4.27 | 6.07 | 4.1 | 5.46 |
| | **Mean** | **7** | **7** | **5.79** | **6.7** | **4.74** | **6.46** | **4.31** | **6** | **4.1** | **5.4** |
| | **±SD** | **0** | **0** | **0.1457166** | **0.011547** | **0.123423** | **0.0321455** | **0.075056** | **0.057735** | **0.05** | **0.060277** |
| **Combined Mean** | | **6.99** | **6.99** | **5.36** | **6.81** | **4.6** | **6.5** | **4.34** | **6.07** | **4.18** | **5.57** |
| **Combined ±SD** | | **0.0082808** | **0.008281** | **0.3449941** | **0.133088** | **0.11244** | **0.2014755** | **0.061991** | **0.2757898** | **0.103058** | **0.283006** |
| **Wilcoxon rank-sum test (p values)** | | | **1** | | **p < 0.01** | | **p < 0.01** | | **p < 0.01** | | **p < 0.01** |

**Table 3.6.2.2** The bacterial counts (CFU/ml) of *Streptococcus mutans* planktonic cells in the presence and absence of subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract

| **Cultures** | **Repeats** | **Growth of *S. mutans* planktonic cells (cfu/ml)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **0 hrs** | | **12 hrs** | | **16 hrs** | |
| | | **control** | **plant** | **control** | **plant** | **control** | **plant** |
| *S. mutans* | 1 | $1.1 \times 10^7$ | $1.2 \times 10^7$ | $5.5 \times 10^8$ | $1.6 \times 10^8$ | $1.1 \times 10^9$ | $2.5 \times 10^8$ |
| clinical strain 1 | 2 | $1.1 \times 10^7$ | $1.1 \times 10^7$ | $3.5 \times 10^8$ | $2.1 \times 10^8$ | $4.9 \times 10^8$ | $2.6 \times 10^8$ |
| | 3 | $1.0 \times 10^7$ | $1.0 \times 10^7$ | $3.4 \times 10^8$ | $3.1 \times 10^7$ | $4.7 \times 10^8$ | $1.9 \times 10^8$ |
| | **Mean** | **$1.1 \times 10^7$** | **$1.1 \times 10^7$** | **$4.1 \times 10^8$** | **$2.3 \times 10^8$** | **$6.9 \times 10^8$** | **$2.3 \times 10^8$** |
| | **±SD** | **577350.3** | **1000000** | **118462371** | **92359803.7** | **358096821** | **40414519** |
| *S. mutans* | 1 | $1.2 \times 10^7$ | $1.4 \times 10^7$ | $2.3 \times 10^8$ | $1.9 \times 10^8$ | $3.6 \times 10^8$ | $8.5 \times 10^8$ |
| clinical strain 6 | 2 | $1.3 \times 10^7$ | $1.2 \times 10^7$ | $1.7 \times 10^8$ | $1.9 \times 10^8$ | $1.5 \times 10^8$ | $2.6 \times 10^8$ |
| | 3 | $1.3 \times 10^7$ | $1.4 \times 10^7$ | $2.1 \times 10^8$ | $7.5 \times 10^8$ | $8.2 \times 10^8$ | $8.2 \times 10^8$ |
| | **Mean** | **$1.3 \times 10^7$** | **$1.3 \times 10^7$** | **$2.0 \times 10^8$** | **$3.8 \times 10^8$** | **$4.4 \times 10^8$** | **$6.4 \times 10^8$** |
| | **±SD** | **577350.27** | **1154700.5** | **30550505** | **323316151** | **342685473** | **332315112** |
| *S. mutans* | 1 | $1.5 \times 10^7$ | $1.3 \times 10^7$ | $2.2 \times 10^8$ | $8.4 \times 10^7$ | $4.5 \times 10^8$ | $2.0 \times 10^8$ |
| clinical strain 7 | 2 | $1.2 \times 10^7$ | $1.3 \times 10^7$ | $1.7 \times 10^8$ | $2.1 \times 10^8$ | $2.5 \times 10^9$ | $2.3 \times 10^8$ |
| | 3 | $1.5 \times 10^7$ | $1.3 \times 10^7$ | $1.5 \times 10^8$ | $1.2 \times 10^8$ | $4.8 \times 10^8$ | $1.6 \times 10^8$ |
| | **Mean** | **$1.4 \times 10^7$** | **$1.3 \times 10^7$** | **$1.8 \times 10^8$** | **$1.4 \times 10^8$** | **$1.1 \times 10^9$** | **$2.0 \times 10^8$** |
| | **±SD** | **1732050.8** | **0** | **36055513** | **64899923** | **1175003546** | **35118846** |
| *S. mutans* | 1 | $4.8 \times 10^6$ | $4.5 \times 10^6$ | $6.5 \times 10^7$ | $1.6 \times 10^7$ | $1.1 \times 10^8$ | $1.8 \times 10^8$ |
| clinical strain 13 | 2 | $4.0 \times 10^6$ | $4.8 \times 10^6$ | $1.1 \times 10^7$ | $8.1 \times 10^7$ | $1.2 \times 10^8$ | $1.7 \times 10^8$ |
| | 3 | $4.7 \times 10^6$ | $3.7 \times 10^6$ | $4.7 \times 10^7$ | $2.9 \times 10^7$ | $5.5 \times 10^8$ | $1.1 \times 10^8$ |
| | **Mean** | **$4.5 \times 10^6$** | **$4.3 \times 10^6$** | **$4.1 \times 10^7$** | **$4.2 \times 10^7$** | **$2.6 \times 10^8$** | **$1.5 \times 10^8$** |
| | **±SD** | **435889.89** | **568624.07** | **27495454** | **34394767** | **251197134** | **37859389** |
| *S. mutans* | 1 | $2.6 \times 10^6$ | $2.4 \times 10^6$ | $1.5 \times 10^7$ | $1.1 \times 10^7$ | $9.7 \times 10^7$ | $1.3 \times 10^7$ |
| NCTC 10919 | 2 | $2.4 \times 10^6$ | $3.3 \times 10^6$ | $4.4 \times 10^7$ | $1.1 \times 10^7$ | $6.2 \times 10^7$ | $5.8 \times 10^7$ |
| | 3 | $2.2 \times 10^6$ | $2.8 \times 10^6$ | $1.0 \times 10^7$ | $1.6 \times 10^7$ | $7.1 \times 10^7$ | $4.2 \times 10^7$ |
| | **Mean** | **$2.4 \times 10^6$** | **$2.8 \times 10^6$** | **$2.3 \times 10^7$** | **$1.3 \times 10^7$** | **$7.7 \times 10^7$** | **$3.8 \times 10^7$** |
| | **±SD** | **200000** | **450924.98** | **18357560** | **2886751.35** | **18175074.5** | **22810816** |
| **Combined Mean** | | **$8.9 \times 10^6$** | **$8.9 \times 10^6$** | **$1.7 \times 10^8$** | **$1.4 \times 10^8$** | **$5.2 \times 10^8$** | **$2.5 \times 10^8$** |
| **Combined ±SD** | | **4799831.3** | **4639273.3** | **153685145** | **185654749** | **623265250** | **248827211** |
| ***t*-test** | | | | | | | |
| **p values** | | | **0.944639** | | **0.3778766** | | **0.1325282** |

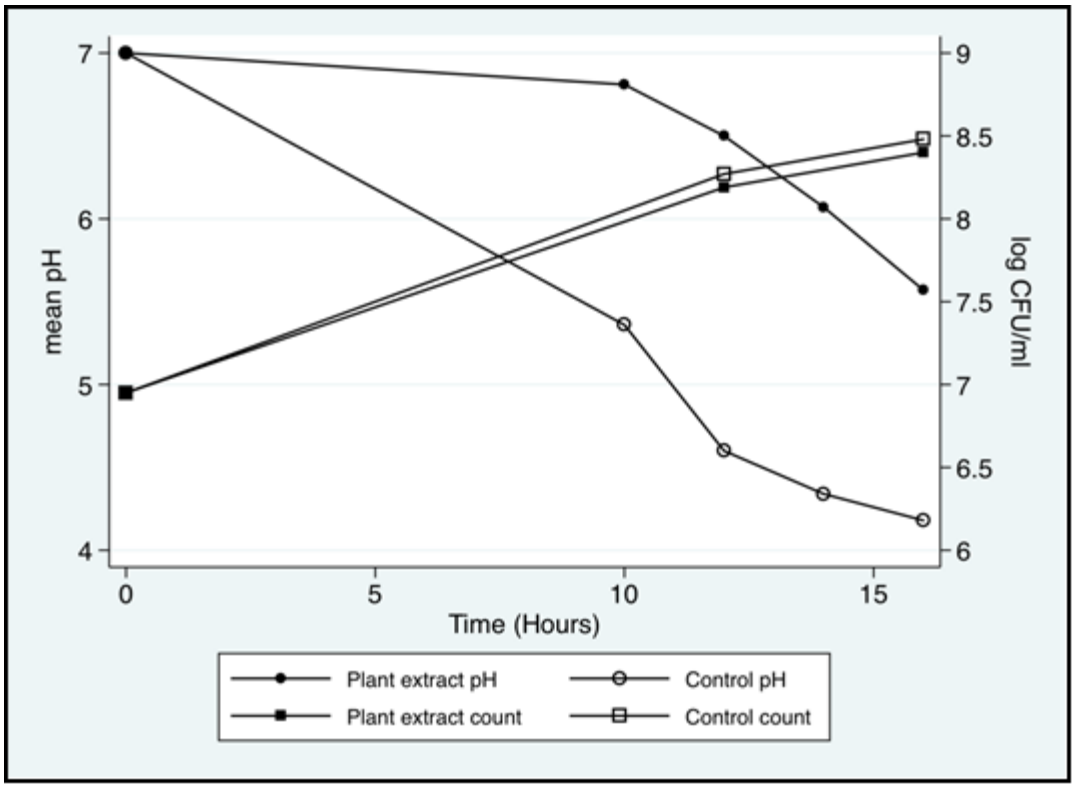



**Figure 3.8:** Mean pH values and the bacterial counts (log CFU/ml) of *Streptococcus mutans* in planktonic form after exposure to subinhibitory concentration (0.78 mg/ml) of crude ethanol plant extract.

The crude ethanol plant extract effectively reduced the production of acid by planktonic cells of *S. mutans* (Table 3.6.2.1). All the test strains behaved similarly. The control cultures experienced a significant drop in pH ($p < 0.01$) over a 16 hour period (pH 7 to 4.18) compared to the cultures exposed to plant extract (7 to 5.57). The bacterial counts for both control and plant extract exposed cultures were not significantly different ($p > 0.05$) from each other indicating that the growth and number of organisms had no effect on the pH over a period of time (Table 3.6.2.2). Thus, the difference in pH is due to the effect of the plant extract on the metabolism of the bacteria and not on the number of bacterial cells.

### 3.7 Effect of the crude ethanol plant extract on the activity of proteases of *Porphyromonas gingivalis*

The effect of the crude ethanol plant extract on the activity of the specific gingipains, Arg-gingipain and Lys-gingipain by *P. gingivalis* was evaluated. Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride and N-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt were used as substrates for Arg-gingipain and Lys-gingipain respectively. *Porphyromonas gingivalis* was exposed to five different subinhibitory concentrations of crude ethanol plant extract (0.02, 0.01, 0.006, 0.003, 0.001 mg/ml). These concentrations were chosen based on MBC results.

Crude ethanol plant extract was diluted in DMSO (1:2) and used as controls. It was also used in the actual experiments together with the various substrates and inoculum to examine protease activity. Therefore a control containing DMSO and substrate only was used to evaluate the effect of DMSO on the substrate itself. It was found that DMSO had no significant effect on substrate degradation. A positive control containing proteinase K and substrate only was used to check if the actual method worked. The results showed that proteinase K was able to degrade both substrates. The complete degradation of substrates by protease produced by *P. gingivalis* was given a value of 100 % (control). Values lower than 100 % were taken as inhibition of protease activity. The results of the percentage of activity of Arg-gingipain and Lys-gingipain produced by *P. gingivalis* exposed to the various subinhibitory concentrations of crude ethanol plant extract are represented in Table 3.7.1, page 77 and Figure 3.9, page 78 and Table 3.7.2, page 79 and Figure 3.10, page 80 respectively. The results as absorbances at 405 nm are represented

in Table 2.1, page 133 and Table 2.2, page 134 (Appendix 2). The activities of the two gingipains are represented together in Figure 3.11, page 81.

**Table 3.7.1** Arg-gingipain activity (%) by *Porphyromonas gingivalis* exposed to subinhibitory concentrations of crude ethanol plant extract

| **Repeats** | | **Protease activity (%)** | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **Crude plant extract concentrations** | | | | |
| | | **0.02 mg/ml** | **0.01 mg/ml** | **0.006 mg/ml** | **0.003 mg/ml** | **0.001 mg/ml** |
| **1** | 100 | 90 | 66 | 93 | 75 | 63 |
| **2** | 100 | 81 | 74 | 82 | 76 | 99 |
| **3** | 100 | 71 | 80 | 63 | 93 | 90 |
| **4** | 100 | 91 | 104 | 88 | 85 | 84 |
| **5** | 100 | 96 | 120 | 73 | 79 | 86 |
| **6** | 100 | 112 | 107 | 90 | 72 | 75 |
| **7** | 100 | 84 | 85 | 92 | 96 | 88 |
| **8** | 100 | 84 | 82 | 90 | 102 | 77 |
| **9** | 100 | 95 | 76 | 96 | 107 | 101 |
| **10** | 100 | 148 | 52 | 49 | 53 | 54 |
| **11** | 100 | 50 | 33 | 76 | 41 | 53 |
| **12** | 100 | 60 | 71 | 64 | 35 | 50 |
| **13** | 100 | 52 | 77 | 59 | 83 | 62 |
| **14** | 100 | 57 | 71 | 83 | 60 | 67 |
| **15** | 100 | 65 | 70 | 64 | 72 | 68 |
| **16** | 100 | 61 | 66 | 69 | 81 | 58 |
| **17** | 100 | 69 | 47 | 67 | 57 | 64 |
| **18** | 100 | 70 | 66 | 63 | 64 | 58 |
| **19** | 100 | 68 | 62 | 61 | 64 | 65 |
| **20** | 100 | 39 | 80 | 63 | 64 | 69 |
| **21** | 100 | 62 | 63 | 63 | 55 | 59 |
| **Mean** | **100** | **76** | **74** | **74** | **72.1** | **70.95** |
| **±SD** | **0** | **0.24254** | **0.196975** | **0.137882** | **0.188783** | **0.151244** |
| ***t*-test** | | | | | | |
| **(p values)** | | **p < 0.01** | **p < 0.01** | **p < 0.01** | **p < 0.01** | **p < 0.01** |

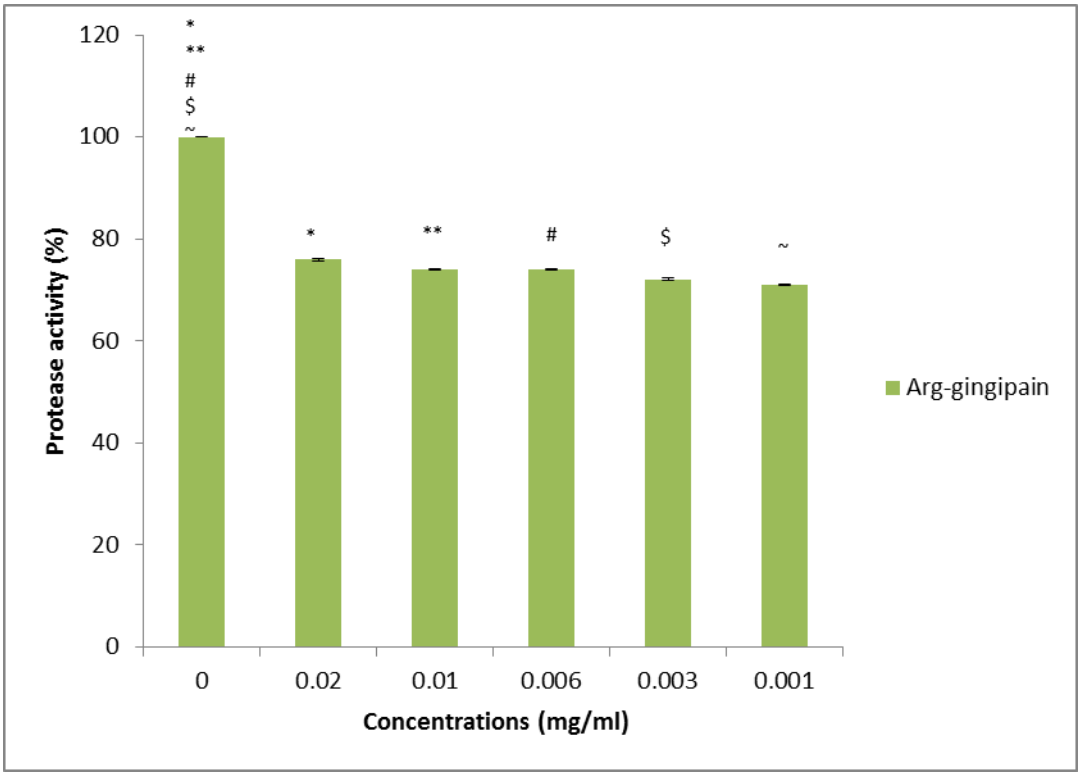



**Figure 3.9:** The protease activity of Arg-gingipain after exposure to different subinhibitory concentrations of crude ethanol plant extract. *, **, #, $, ~ = p: < 0.01.
*, **, #, $, ~: Comparison between the protease activity of Arg-gingipain in cells of *Porphyromonas gingivalis* unexposed and exposed to subinhibitory concentrations of crude ethanol plant extract.

The control containing no crude ethanol plant extract showed 100 % degradation of Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride. The different subinhibitory concentrations of crude ethanol plant extract significantly reduced ($p < 0.01$) the activity of Arg-gingipain as degradation at all concentrations was less than 100 % (Table 3.7.1). Activity was inhibited by approximately 24 - 28 % for all tested concentrations. All tested concentrations produced similar inhibition of activity.

**Table 3.7.2** Lys-gingipain activity (%) by *Porphyromonas gingivalis* exposed to subinhibitory concentrations of crude ethanol plant extract

| Repeats | Protease activity (%) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Crude plant extract concentration | | | | |
| | | 0.02 mg/ml | 0.01 mg/ml | 0.006 mg/ml | 0.003 mg/ml | 0.001 mg/ml |
| **1** | 100 | 72 | 79 | 71 | 98 | 79 |
| **2** | 100 | 77 | 68 | 72 | 75 | 73 |
| **3** | 100 | 79 | 72 | 70 | 77 | 71 |
| **4** | 100 | 48 | 41 | 49 | 54 | 44 |
| **5** | 100 | 44 | 42 | 47 | 56 | 61 |
| **6** | 100 | 49 | 37 | 42 | 42 | 35 |
| **7** | 100 | 45 | 41 | 45 | 32 | 37 |
| **8** | 100 | 42 | 44 | 37 | 36 | 44 |
| **9** | 100 | 49 | 40 | 45 | 43 | 47 |
| **10** | 100 | 44 | 46 | 48 | 47 | 53 |
| **11** | 100 | 51 | 50 | 49 | 51 | 40 |
| **12** | 100 | 37 | 38 | 42 | 35 | 47 |
| **13** | 100 | 35 | 39 | 30 | 30 | 36 |
| **14** | 100 | 37 | 41 | 44 | 44 | 45 |
| **15** | 100 | 47 | 44 | 40 | 51 | 44 |
| **16** | 100 | 43 | 42 | 38 | 47 | 58 |
| **17** | 100 | 42 | 40 | 40 | 40 | 43 |
| **18** | 100 | 43 | 48 | 38 | 36 | 43 |
| **Mean** | **100** | **49** | **47** | **47** | **50** | **50** |
| **±SD** | **0** | **0.131547** | **0.124097** | **0.12017** | **0.176868** | **0.131597** |
| ***t*-test** | | | | | | |
| **(p values)** | | **$p < 0.01$** | **$p < 0.01$** | **$p < 0.01$** | **$p < 0.01$** | **$p < 0.01$** |

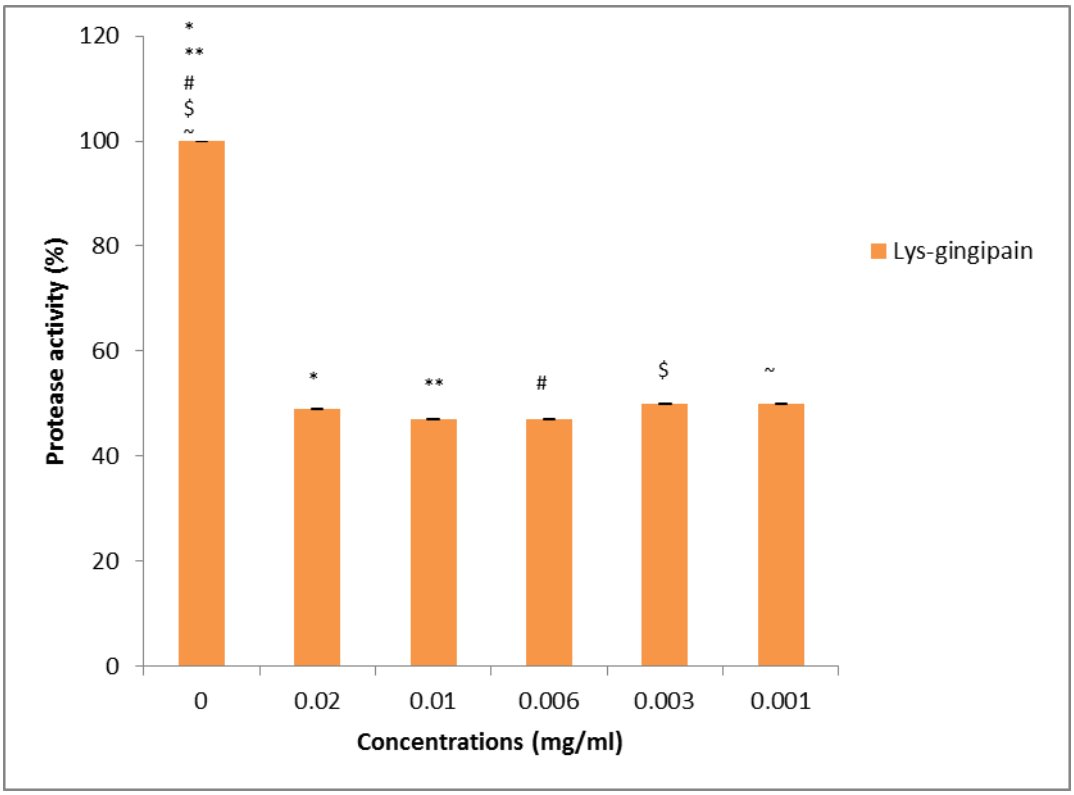



**Figure 3.10:** The protease activity of Lys-gingipain after exposure to different subinhibitory concentrations of crude ethanol plant extract. *, **, #, $, ~ = p: < 0.01
*, **, #, $, ~: Comparison between the protease activity of Lys-gingipain in cells of *Porphyromonas gingivalis* unexposed and exposed to subinhibitory concentrations of crude ethanol plant extract.

The control containing no crude ethanol plant extract showed 100 % degradation of N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt. The different subinhibitory concentrations of crude ethanol plant extract significantly reduced ($p < 0.01$) the activity of Lys-gingipain as degradation at all concentrations was less than 100 % (Table 3.7.2). Activity was reduced by approximately 50 % for all tested concentrations of crude ethanol plant extract.

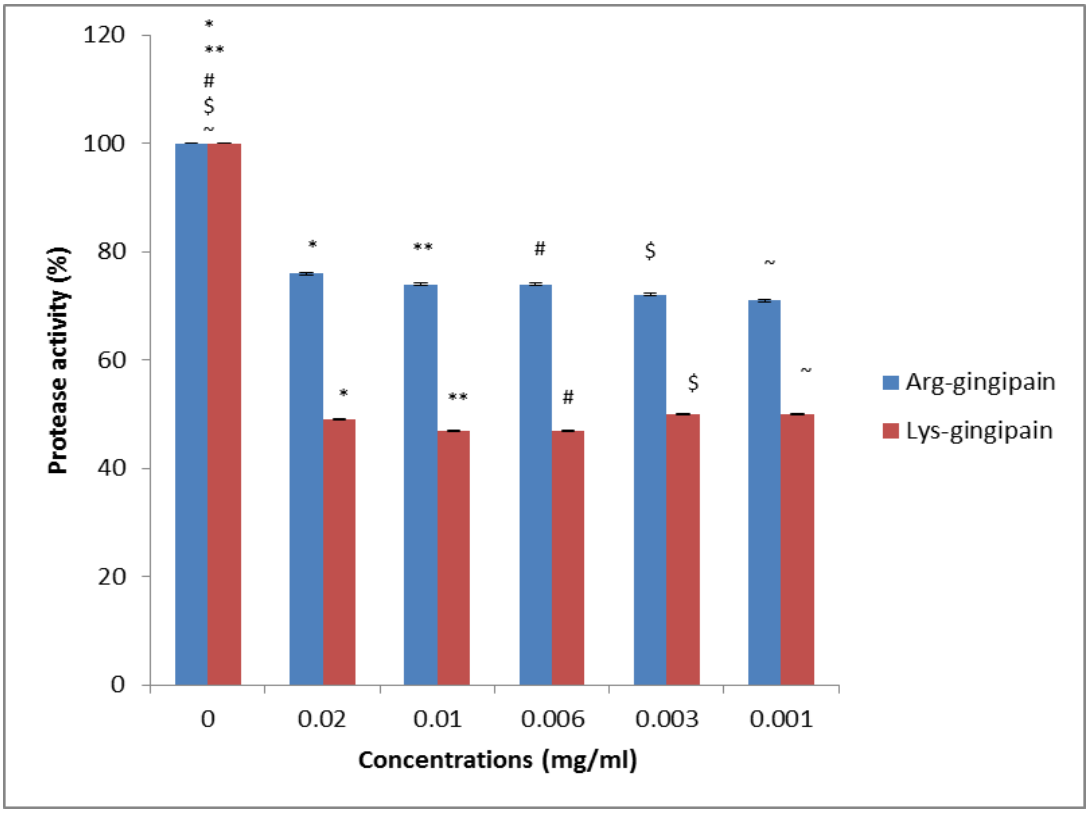



**Figure 3.11:** The protease activity of Arg-gingipain and Lys-gingipain produced by *Porphyromonas gingivalis* exposed to various subinhibitory concentrations of crude ethanol plant extract. *, **, #, $, ~ = p: < 0.01
*, **, #, $, ~: Comparison between the protease activity of Arg-gingipain and Lys-gingipain in cells of *Porphyromonas gingivalis* unexposed and exposed to subinhibitory concentrations of crude ethanol plant extract.

Degradation of substrates in the absence of crude ethanol plant extract was given a value of 100 % (control). Results show that the crude ethanol plant extract causes greater inhibition of Lys-gingipain activity compared to Arg-gingipain. The plant extract, however, significantly reduces both the activities of the respective gingipains. Highest inhibition of Arg-gingipain was obtained at the lowest concentration (0.001 mg/ml) of crude ethanol plant extract, whereas the highest inhibition of Lys-gingipain was obtained at 0.01 and 0.006 mg/ml crude ethanol plant extract.

# CHAPTER 4: DISCUSSION

Dental caries and periodontal diseases are the most common bacterial oral infections in humans. According to the World Health Organization (WHO) report, dental caries remains a major public health problem worldwide, affecting 60 - 90% of school children and the vast majority of adults. Although it appears to be less common and less severe in the greater part of Africa, the report anticipates that in light of changing living conditions and dietary habits, the incidence of dental caries will increase in many of the continent's developing countries (Petersen and Lennon, 2004). Periodontal diseases contribute significantly to the global burden of oral diseases. Limited attention has been given to this disease by providers and public health administrators (Petersen and Ogawa, 2005). Their significance in overall human health has been minimized due to their non-life-threatening nature. However, economic burden for treatment can be staggering.

Good oral hygiene is the common preventative measure for both the diseases which can be achieved by mechanical and chemical application. Among many chemicals, plant and plant products have been used for centuries for the management of oral health-related problems throughout the world (Tapsoba and Deschamps, 2005). These chemicals either target the actual number of causative organisms or their virulence to render them inactive. *Dodonaea viscosa* var. *angustifolia* one of the medicinal plants has antifungal activity against *C. albicans* and the authors suggest its use in the oral cavity where other oral pathogens coexist (Patel *et al*, 2009). Therefore this study investigated the antibacterial

and anti-virulence effect of *D. viscosa* var. *angustifolia*, a medicinal plant on the cariogenic bacteria and the causative organisms of periodontal diseases.

The results showed that at high concentrations of crude extract, *D. viscosa* var. *angustifolia* has bactericidal effect on cariogenic bacteria such as *S. mutans* and *Lactobacillus* spp. At subinhibitory concentrations, this plant extract reduces acid production and biofilm formation by *S. mutans*, the two key pathogenic characteristics responsible for the development and progression of dental caries. However extracts of *D. viscosa* var. *angustifolia* had no effect on the production of extracellular polysaccharides by *S. mutans* and it could only remove fully developed biofilm after 24 hour exposure. On the other hand periodontal pathogens were killed at very low concentrations and at subinhibitory concentrations it inhibited the protease activity of *P. gingivalis* leaving them avirulent. Therefore this plant has a potential to be developed into a therapeutic agent for prevention of dental caries and periodontal diseases. These results are discussed under separate sections.

### 4.1 Minimum bactericidal concentration assay

*Dodonaea viscosa* var. *angustifolia* was successfully extracted using acetone, methanol and ethanol with average yield of 0.2 g from 1 g of dried leaves powder (Tables 3.1.1, 3.1.2 and 3.1.3). Clinical strains of *S. mutans* were successfully identified using API 20 Strep standardized system which is based on the method described by Facklam *et al*, (1984). This is a cheap, easy and effective method of identification compared to the genetic methods with DNA probes (Cangelosi *et al*, 1994) and immunological methods

(Hamada and Slade, 1980). These results were further verified by using a Microscan (Walkaway) that confirmed the identification of all *S. mutans* strains using biochemical reactions.

Instead of MIC, MBC were determined for all the test organisms because the crude extract of *D. viscosa* var. *angustifolia* precipitated with the growth medium and the visible growth of the test organisms was not possible. The crude extracts completely killed *S. mutans* (1.56 – 12.5 mg/ml) and *Lactobacillus* spp. (0.78 – 12.5 mg/ml) but the concentrations were high (Table 3.3.1 - 3.3.2). Cariogenic bacteria are generally resistant to medicinal plant extracts. When four Brazilian medicinal plants were tested by Alviano *et al,* (2008), the MICs for cariogenic bacteria ranged from 1.6 to 16 mg/ml. If this plant extract is developed into an oral hygiene product such as a mouth rinse, gel or toothpaste with these high doses as shown in this study it could reduce the number of both of these cariogenic bacteria in the saliva. Reduction in the salivary bacterial count may reduce the time required to develop dental plaque which is a prerequisite in the development of dental caries. However, such effective high concentrations are difficult to maintain in the mouth because of the constant salivary flow.

Similar results with *C. albicans* and *D. viscosa* var. *angustifolia* were reported where it was found that at high concentrations *C. albicans* was completely killed but at subinhibitory concentrations which are generally found in the oral cavity due to the dilution with saliva, the adherence of this organism to oral epithelial cells was inhibited rendering them avirulent (Patel *et al*, 2009). This led to further investigations on the

effects of the subinhibitory concentration of crude plant extract on the pathogenic characteristics of *S. mutans* which is the primary causative organism of dental caries.

Periodontal pathogens proved to be more sensitive to *D. viscosa* var. *angustifolia* giving MBC ranges from 0.02 to 0.09 mg/ml. Similar results were found by Alviano *et al,* (2008) with four Brazilian medicinal plants, where lower MIC values were noted for the periodontal pathogens compared to the cariogenic bacteria. Santos *et al,* (2002) tested fractions of propolis and found MICs between 0.064 and 0.1 mg/ml. All the periodontal pathogens tested here gave MBC results similar to the propolis and Brazilian plant study. Rios *et al,* (1988) demonstrated that natural crude extracts which have the ability to exhibit antimicrobial activity at concentrations lower than 100 µg/ml are most likely to possess great antimicrobial potential due to the fact that the active compounds can be isolated and used at lower concentrations. *Dodonaea viscosa* var. *angustifolia* caused the complete elimination of *P. intermedia, P. gingivalis, F. nucleatum* and *Capnocytophaga* spp. at concentrations lower than 100 µg/ml, therefore it has great potential as an antimicrobial agent.

The active constituent having antimicrobial effect in *D. viscosa* var. *angustifolia* extract remains unknown. However, its beneficial effect is well known, dating as far back as the 1700s. Oral infections were and still are treated using a decoction of the leaves (Van Wyk *et al,* 2002). Amabeoku *et al,* (2001) used high performance liquid chromatography (HPLC) finger printing and identified the presence of flavonoids, reducing sugars, alkaloids, saponins and tannins. Among these chemical components flavonoids could be

responsible for the antibacterial effect because flavonoids are phenolic compounds that are known to have medicinal properties including antibacterial and antiviral properties (Dixon *et al*, 1983). They are usually synthesized by plants in response to microbial infection and their antimicrobial activity has been proven *in vitro* against a wide variety of microorganisms. A major leaf flavonoid from *D. viscosa* var. *angustifolia* has already been identified as 5,7,4'-trihydroxy-3,6-dimethoxyflavone by Van Heerden *et al*, (2000).

The ethanol extract was chosen for all subsequent experiments investigating the effect of the crude plant extract on the pathogenic characteristics of *S. mutans* because the MBC results suggested that the active ingredient responsible for the antibacterial effect may be better extracted in ethanol as this produced the lowest MBC values particularly for *S. mutans*, when compared to the other two extracts. Ethanol extracts are often used in dilution assays and have shown successful antimicrobial activity against various bacteria such as, *Staphylococcus aureus* and *Mycobacterium smegmalis* (Reddy, 2009).

### 4.2 Inhibitory effects on *Streptococcus mutans* biofilm

Many different types of oral bacteria are present in a mature thick biofilm called dental plaque. Since *S. mutans* is the pioneer species that initiates biofilm formation, only *S. mutans* was used to produce biofilms. The biofilms were grown in the presence of plant extract and the growth was measured in all three phases shown by Li *et al*, (2001) which are the adherent phase (0-4 hours), the active accumulated phase (4-20 hours) and the slow or plateau accumulated phase (after 20 hours). The results showed that the plant extract reduced the biofilm formation and all three phases were affected, 6 hours being

the adherent, 24 hours being the active accumulative and 30 hours being the plateau accumulated phase (Table 3.4.2.1). However, a short exposure (two, 1 minute exposures) of the crude ethanol plant extract did not have any effect on the 48 hour mature biofilm but a longer exposure (24 hours) with subinhibitory concentration (0.78 mg/ml) significantly removed the mature biofilm (Table 3.4.1.1). This is generally acceptable because several different approaches have been aimed at controlling biofilm such as prevention of biofilm formation, disruption of existing biofilm, prevention of further biofilm growth and killing microorganisms in the biofilm (Sbordone and Bortolaia, 2003) but generally commercially available oral hygiene chemicals have only one target property.

*Dodonaea viscosa* var. *angustifolia* has proven to target the biofilm formation and further re-growth rather than disruption of existing biofilm. This exposure times were chosen so as to mimic the usual routine brushing of an individual, during a 24 hour period. These results suggest that the crude ethanol plant extract affects the adherence mechanism of *S. mutans* to surfaces or the extract has a good substantivity, forming a film on the glass slide and not allowing the adherence of *S. mutans*. This effect was observed at the subinhibitory concentration meaning it could not have been due to the bacterial cell death. The plant extract therefore prevents the adherence of some of the bacteria to the glass surface. Possible explanations for these results are, (1) the plant extract may have formed a film on the glass slide, (2) disrupted the synthesis of adhesins by the bacteria preventing them from adhering to the glass surface or, (3) the plant extract may have caused the mechanical disruption of the adhesins already present. Similar suggestions

have been made by Jagtap and Karkera (2000) where an extract of *Juglandaceae regia* was found to inhibit the adherence of *S. mutans* to a glass slide due to its effect on the activity of glucosyltransferase which is one of the adhesins that facilitate with the binding of *S. mutans* to a surface.

The three major protein antigens that facilitate the binding of *S. mutans* to surfaces are the surface fibrillar adhesins (AgI/II), glucosyltransferases (GTFs) and the glucan-binding protein (Russell *et al*, 2004) which may have been affected by the plant extract. Since the crude ethanol plant extract has no effect on the production of polysaccharides by *S. mutans*, the plant extract has no effect on GTFs which are responsible for the production of polysaccharides (glucans). Therefore, the plant extract may have either blocked the binding site of AgI/II or may have prevented the formation of this antigen by *S. mutans.* Since this experiment did not involve the presence of saliva for ultimate binding, it is more likely that the plant extract may have prevented the formation of this antigen by the bacteria or the antigen already present could not bind due to blockage of enzyme-binding site as AgI/II usually binds to the salivary component or salivary agglutinin glycoprotein (SAG) in a lectin-like interaction (Demuth *et al*, 1990).

The crude ethanol plant extract may have also prevented glucan-induced aggregation which is an important mechanism that results in the accumulation of *S. mutans* on the surface of the tooth which ultimately leads to the formation of dental plaque. *Streptococcus mutans* aggregate on the tooth surface by means of a cell-surface glucan-binding protein that binds high molecular weight glucan (Jagtap and Karkera, 2000).

Since sucrose was present in the growth medium, the bacteria were most likely able to synthesize glucan using GTF at subinhibitory concentration but the binding of the protein to glucan was probably inhibited by the plant extract. Such as the Chinese Nutgall, some plants are known to exhibit strong inhibition of glucan-induced aggregation of *S. mutans* (Wu-Yuan *et al,* 1988).

Since there is no effect on the fully formed biofilm, perhaps the plant extract is not capable of penetrating the slimy biofilm matrix which consists of bacteria, salivary compounds and the bacterial byproducts. However the 24 hour results were significant which suggests that the bacteria were either killed within the surface layer of the biofilm, or the plant extract may have caused the detachment of the bacteria from the biofilm thereby reducing the bacterial count. Similar results were found by Rukayadi and Hwang (2005) with a chemical xanthorrizol (XTZ) isolated from the java turmeric (*Curcuma xanthorrizol* Roxb.). They showed that exposure time and the phase of growth of bacteria are two important factors when studying the effects on cells in biofilm form. The older biofilms (> 20 hours) require a higher concentration of antimicrobials over a short period of time or a low concentration of antimicrobials over a long period of time.

If a toothpaste and mouth rinse containing *D. viscosa* var. *angustifolia* is used in the morning, the tooth brushing technique will remove the plaque layer and the high concentration of plant extract will kill the bacteria. As the concentrations drop due to the saliva flow the plant extract will still have an additional effect on reduction in new plaque formation. Although this is an *in vitro* study, if the low concentrations are maintained,

plaque that was not removed in the morning will also be reduced. Further research is required to test the substantivity of this plant extract. Also the biofilm formed in the actual oral cavity will be chemically and microbiologically different and consistency will also be different. This biofilm will have mix flora containing aerobic and anaerobic bacteria. Therefore, a mixture of byproducts and salivary glycoproteins will be different making it more tenacious which will reduce the penetrability.

Similar studies were carried out by Limsong *et al*, (2004) investigating the effect of six herbal extracts on the adherence of *S. mutans* to a glass slide showing similar results with Chinese black tea and guava being the superior. The inhibition of *S. mutans* by oolong tea is also known (Ooshima *et al*, 1993; Nakahara *et al*, 1993). These herbal extracts are rich in polyphenols, majority of them being tannins of the catechin group and their efficacy towards *S. mutans* adherence has been established.

A number of polyphenols have been isolated and reported for *D. viscosa* var. *angustifolia* (Rani *et al*, 2009). It is possible that the inhibition of adherence of *S. mutans* to the glass slides is due to the presence and action of phenolic compounds present in the plant extract. *Dodonaea viscosa* var. *angustifolia* contains tannins which can bind to proline-rich proteins present on the salivary pellicle or with the cell-surface lipoteichoic acid, thereby reducing the attachment of *S. mutans* (Hogg and Embery, 1982; Wolinsky and Sote, 1984)**.** Tannins have the ability to cause changes to the insoluble enzyme structure or bind to the active site. Plant tannins may also prevent the adsorption of GTF to bacterial cells thereby preventing the production of glucans that may facilitate in the

attachment of bacteria to the tooth surface (Jagtap and Karkera, 2000). Bacterial membranes may also be damaged by tannins belonging to the catechin group. Catechins interact with bacterial surface proteins causing distortion and loss of tertiary structure resulting in loss of function (Ikigai *et al*, 1993; Hamilton-Miller, 2001). These results suggest that an *in vivo* study should be considered.

### 4.3 Effect on extracellular polysaccharide production in planktonic and biofilm forms

*Streptococcus mutans* has an adherence mechanism due to the adhesins present on the cell wall which allow them to adhere to the tooth surface. The results in the adherence study showed that this mechanism may have been impaired by *D.viscosa* var. *angustifolia.* Production of extracellular polysaccharides (EPS) by these organisms also contribute to the adherence of *S. mutans* not only to the tooth but also to each other (Freedman and Tanzer, 1982), and in the process contribute to the structural integrity of the plaque which makes the plaque more resistant to penetration. In addition EPS acts as a food reserve (Guggenheim, 1970).

As suggested by some of the researchers, reduction in the production of EPS should also be one of the targets for caries development and prevention (Matos-Graner *et al*, 2000; Nobre dos Santos *et al*, 2002). Therefore this study examined the effect on the ability of *S. mutans* to produce EPS in the presence of *D.viscosa* var. *angustifolia*. The results showed that subinhibitory concentration had no effect on the production of both the types of polysaccharides, glucans and levans in biofilm and planktonic forms of *S. mutans*

(Tables 3.5.1 - 3.5.2) and further suggests that the subinhibitory concentration of plant extract may not have affected glucosyltransferase which is responsible for the synthesis of EPS as shown by studies with propolis.

Koo *et al*, (2003) isolated two compounds from propolis, apigenin and *tt*-farnesol, which have two distinct mechanisms of action with respect to the reduction of polysaccharide production. Apigenin was found to inhibit the production of glucans by targeting the GTF enzymes, GTF B and C which are responsible for the production of insoluble glucans. The compound *tt*-farnesol affects the production of glucans by causing damage to the bacterial cell membrane. By inflicting damage to the cell membrane *tt*-farnesol may reduce bacterial metabolism and glucan synthesis by *S. mutans* (Bowen, 2002; Marquis *et al*, 2002). However, propolis had weak anti-*S. mutans* properties. Similarly defatted cocoa bean extract inhibited the EPS production but not the growth of oral bacteria (Paolino and Kashket, 1985). In this study *D. viscosa* var. *angustifolia* does not inhibit the production of polysaccharides, however, it reduces the number of bacteria in the planktonic and biofilm form and due to some other mechanism such as interference of adhesins, the adherence is also reduced. However high sucrose intake is one of factors implicated in the development of dental caries and therefore *S. mutans* that survived the antibacterial property of *D. viscosa* var. *angustifolia* if provided with sucrose, will still produce polysaccharides normally.

### 4.4 Acid production in biofilms and planktonic cells

The definition of dental caries is "demineralization of mineralized area of the tooth due to the microbial (*S. mutans,* usually in a biofilm form) action (acids and EPS production) on the fermentable carbohydrate (sucrose). This study discussed the antibacterial effect of *D. viscosa* var. *angustifolia* on *S. mutans,* and effect on the biofilm and EPS production by this organism. However, the virulence of *S. mutans* is directly related to its acid producing ability through glycolysis in dental plaque (Sturr and Marquis, 1992). Therefore, the effect of this study plant on the acid production by planktonic and biofilm forms of *S. mutans* were investigated.

The results showed that *D. viscosa* var. *angustifolia* inhibits the acid production significantly by *S. mutans* cells if they are in a planktonic form. However once they are in a biofilm form embedded in an extracellular matrix *D. viscosa* var. *angustifolia* has no effect on the acid production by this organism (Table 3.6.1.1). The difference in the pH of the controls and the tests could have been due to the reduction of the number of *S. mutans* due to some antibacterial effect of the plant extract. This phenomenon was verified by performing bacterial counts at an initial point, at 12 hours and at 16 hours which were found to be comparable to the control (Figure 3.8), suggesting that the effect may have been on the glycolysis of carbohydrates which is responsible for the acid production and not due to antibacterial effect of the plant.

This plant extract was able to inhibit the conversion of sucrose to lactic acid by possibly acting on the acid sensitive glycolytic enzymes responsible (Nalina and Rahim, 2007).

Acid sensitization in *S. mutans* can readily be seen in glycolytic pH-drop experiments in which cells are supplied with excess glucose. Cells can then rapidly degrade glucose causing a drop in the pH of the suspension to a minimum value at which the bacteria can no longer maintain a cytoplasmic pH compatible with glycolytic enzymes (Duarte *et al*, 2006). In the results the final pH values after a 16 hour period was approximately 5.2 - 5.9 compared with 4.1 - 4.3 for cells not exposed to the plant extract (control). Thus *D. viscosa* var. *angustifolia* has the potential to prevent enamel dissolution even after 16 hours as pH values of approximately 5.2 - 5.9 are still above the critical pH for enamel dissolution which is 5.5 and below. The maintenance of higher pH values in bacterial cells treated with the plant extract may also be due to the possibility that the plant may be preventing the uptake of sucrose via the phosphotransferase system which is usually active at a high pH (Marsh *et al,* 1984).

The exercise of determining bacterial cells in the biofilm throughout the incubation period was not possible and it was not necessary because the results showed that the amount of acid produced by the control and the test was exactly the same. This suggests that the number of organisms were the same in both the test and the control. The quantity of acid was increasing in the medium of both the test and the control meaning the organisms were metabolically active in these biofilms (Figure 3.7). However the initial count of the biofilms in the control as well as test was performed to standardize the initial stage and the results showed similar bacterial counts. During the experiment, biofilm bacterial counts were not possible because the cultures were not homogenized due to the biofilm clumps and a pipetting error could occur.

In the experiments with planktonic cells, *S. mutans* was exposed to the plant extract throughout the experiment period which was 16 hours, meaning the extract was constantly harming the cells. Whereas in the biofilm study, the biofilms were exposed to the plant extract for 30 minutes only which may not have been enough to penetrate the cells in the biofilm to cause an effect on the metabolism of the bacteria. The pH values of controls dropped to 4.5 which is a highly acidic environment for any cells to survive and proliferate. *Streptococcus mutans* are known to be acidogenic and aciduric meaning they are capable of producing acid and successfully tolerating the acidic environment. *Streptococcus mutans* are acid-tolerant organisms that have developed mechanisms to alleviate the influences of acidification. These mechanisms include increasing proton-translocating F-ATPase activity in response to low pH (Sturr and Marquis, 1992; Quivey *et al*, 2000).

The role of F-ATPase is to transport protons out of cells through adenosine triphosphate (ATP) hydrolysis in order to maintain an intracellular pH that is more alkaline than the extracellular environmental pH (Sturr and Marquis, 1992). The detrimental pH for the demineralization of teeth is 5.5 and below (Marsh and Martin, 1999). Figure 3.8 shows that the control attained that pH only after 8 hours but the test containing plant extract reach that pH only after 16 hours meaning double the amount of time. This suggests that if this plant extract has a good substantivity, low concentrations will be maintained in the oral cavity and the beneficial effects will continue not only on the biofilm formation but also on the acid production.

Investigations carried out by Duarte *et al*, (2006), have identified cranberry as an inhibitor of acid production by biofilms of *S. mutans* due to the presence of polyphenols. They identified flavonols, anthocyanins and proanthocyanidins that alone or in combination significantly affected the production of acid by *S. mutans* biofilms. Similar chemicals identified from green propolis and *Baccharis dracunculifolia* have been suggested to be responsible for the inhibition of acid production (Leitao *et al*, 2004). Tannins such as catechin are also known to affect the acid production by *S. mutans* biofilm (Maeyama *et al*, 2005). A preliminary gas chromatography-mass spectrometry (GC-MS) analysis of *D. viscosa* var. *angustifolia* has revealed the presence of polyphenols such as catechin or chromene group, calcones with trimethoxyphenyl group, tannin with 4-O-β-D-xylopyranoside and stigmasterol (Patel M, 2011). Catechins usually have better antibacterial properties against gram-positive than gram-negative bacteria (Toda *et al*, 1992) because of the repulsion between catechins and the surface of gram-negative bacteria which is coated with lipopolysaccharides. The test microorganism was gram-positive which suggests catechins may have been responsible for the antibacterial effect. Catechin penetrates the lipid bilayer of bacterial cells and causes leakage of small molecules (Ikigai *et al*, 1993). This may have reduced the adherence of *S. mutans* to glass surfaces and the acid production.

The neutralization of pH within biofilms is difficult due to the limited access of saliva and the presence of EPS, thus low pH values are easily maintained (Dibdin and Shellis, 1988, Duarte *et al*, 2006). Biofilms are physiologically different from cells in the planktonic form and have a higher resistance to antibiotics and are also able to better

withstand the defense mechanisms of the host (Gillbert *et al*, 1997). Therefore, the effect on the acid production by planktonic cells has an important implication. It is not known if the damage is temporary or permanent to the cells of *S. mutans* but if the damage is permanent in the production of acid, the biofilm formed by *S. mutans* may be impaired in acid production which will eventually prevent the development of caries.

### 4.5 Inhibition of proteolytic activity of *Porphyromonas gingivalis*

Many anaerobic gram-negative bacteria are implicated in periodontal diseases. Among these organisms *P. gingivalis* is one of the most aggressive pathogens because it produces lipopolysaccharides, fimbriae, hemagglutinin and proteases. The proteolytic activity of *P. gingivalis* is due to gingipains, collegenase and dipeptidyl aminopeptidase IV (Potempa *et al*, 1995; Grenier and La, 2011). The new drug target approaches have been suggested to target these proteases. Among these proteases, extracellular Arg-gingipain and Lys-gingipain are the most important virulence factors because they down regulate polymorphonuclear neutrophils, modulate host cytokine networks and degrade extracellular proteins which are the gingival tissues (Brochu *et al*, 2001; Yamanaka *et al*, 2007). Because the gingipains are the major contributors in the pathophysiology of periodontitis, therapeutic agents may be developed to either inhibit the growth of this pathogen or inhibit the production of gingipains. Inhibitors of periodontal proteases may reduce bacterial pathogenicity and in turn have a positive effect on periodontal diseases. This study investigated the potential of *D. viscosa* var. *angustifolia* not only in the elimination of *P. gingivalis* but also the effect on the production of Arg-gingipain and Lys-gingipain.

The results showed that the five subinhibitory concentrations of crude ethanol plant extract of *D. viscosa* var. *angustifolia* significantly inhibited the production of both the gingipains (Table 3.7.1 - 3.7.2). Inhibition of Lys-gingipain was better than Arg-gingipain by 20%. However, there was no difference in the effect of various concentrations of the plant extract. This inhibition could be due to the effect of the plant extract on these enzymes, causing structural changes resulting in less cleavage of substrate which was detected colorimetrically in this test. It is also possible that the crude ethanol plant extract may have affected the substrate by blocking the enzyme-binding site thus preventing cleavage.

Gingipains may be essential to *P. gingivalis* as they provide iron, peptides and amino acids from environmental proteins (Grenier *et al,* 2001.). Iron is important for the growth and virulence of *P. gingivalis.* One of the major sources of iron is human transferrin which is contained in high amounts in the gingival crevicular fluid. Gingipains are responsible for the cleavage of human transferrin which promotes growth and formation of hydroxyl radicals that play an important role in tissue destruction during periodontitis (Bodet *et* al, 2006). They may also contribute to the processing and maturation of several cell-surface proteins of *P. gingivalis* (Kadowaki *et al,* 2000). Therefore, inhibition of gingipains by *D. viscosa* var. *angustifolia* will also deprive *P. gingivalis* of available iron and prevent some of the tissue destruction due to the lack of hydroxyl radicals.

Degradation of substrates in the absence of *D. viscosa* var. *angustifolia* was given a value of 100 % (Yamanaka et al, 2007). The crude ethanol plant extract significantly reduced

the activity of Arg-gingipain by approximately 24 - 28 % for all tested concentrations (Figure 3.9). Arg-gingipain is an arginine-specific enzyme and is a high molecular mass form of gingipain. It consists of a 50-kDa gingipain noncovalently complexed with 44-kDa binding proteins specifically hemagluttinins (Pike *et al*, 1994). It can either occur as a single chain, part of a proteinase-hemagluttinin complex or a free protease (Potempa *et al,* 1995). Arg-gingipain is usually activated by cysteine which is the most effective reducing agent. Activation of this enzyme is also brought about by the activity of dithiothreitol (Pike *et al*, 1994) and therefore dithiothreitol was used in experiments examining the effect of *D. viscosa* var. *angustifolia* on Arg-gingipain as well as Lys-gingipain.

Arg-gingipain often cleaves very long substrates containing extracellular matrix components, various plasma proteins, cytokines and their receptors and various host cell surface proteins (Potempa *et al*, 2000). Their specific activity however is very limited as they mimic the action of highly regulated host proteins (Potempa *et al*, 2003). Since Arg-gingipain specifically cleaves after arginine residues, the plant extract therefore inhibited cleavage by 24 - 28 % after the L-arginine residue in the substrate used for analysis. Therefore, cleavage was not completely inhibited at the tested concentrations of plant extract. Higher concentrations of plant extract could possibly result in higher inhibition of activity but concentrations for experiments were kept at a minimum as the plant showed bactericidal activity at fairly low concentrations (0.04 - 0.09 mg/ml) and the purpose of this experiment was to investigate the effect of subinhibitory concentrations of this plant extract which could normally be found in the oral cavity due to the saliva flow.

The crude ethanol plant extract significantly reduced the activity of Lys-gingipain by approximately 50 % for all tested concentrations (Fig. 3.9). Lys-gingipain is a 60-kDa lysine-specific protease where cysteine is the most effective reducing agent in the activation of the enzyme followed by dithiothreitol (Pike *et al*, 1994). Therefore it was used to measure the enzyme activity in this study. The enzyme is most stable and active under conditions dominating advanced *P. gingivalis* colonization of periodontal pockets such as pH values of approximately 7.5 - 8.0 and higher and in the presence of reducing agents (Bickel and Cimasoni, 1985). In this investigation, pH was maintained at 8.0 to allow for stabilization and activation of the enzyme. The plant extract therefore inhibited cleavage by approximately 50 % after the lysine residue in the substrate used for analysis.

The plant extract showed greater inhibition of Lys-gingipain activity (50 %) when compared to Arg-gingipain (25 %). This may be due to the fact that Lys-gingipain has proven to be a less stable, poor protease when compared to Arg-gingipain (Abe *et al*, 1997; Potempa *et al*, 2003). Lys-gingipain is however, a more important virulence factor when compared to Arg-gingipain (Potempa *et al*, 2003). It is also greatly affected by residues more upstream from the scissile bond than penultimate lysine. Both gingipains, however, are similar in terms of enzymatic properties and structural features (Abe *et al*, 1997; Okamoto *et al*, 1996). However, much of the data obtained with respect to molecular mass, substrate specificity, hemagluttinin activity and sensitivity of these enzymes to various inhibitors have been unclear (Okamoto *et al*, 1996).

The results obtained in this study are comparable to similar studies carried out by Yamanaka *et al*, (2007), in which the inhibitory effect of cranberry polyphenol fractions on the cysteine proteases of *P. gingivalis* was investigated. They found that the cranberry polyphenol inhibited the activities of the two gingipains and the effect was dose dependent. The polyphenol fraction at 100 µg/ml produced 87 - 91 % inhibition of gingipain activities. The cranberry polyphenol preparation significantly reduced the activities of the gingipains at a low concentration suggesting that small molecules of polyphenols are capable of producing an inhibitory effect on proteases. Investigations conducted by Bodet *et al*, (2006) also showed that the non-dialyzable material prepared from cranberry efficiently inhibited the activities of both Arg-gingipain and Lys-gingipain respectively. Catechins have also been suggested to be responsible for the antibacterial and antiproteolytic effects against *P. gingivalis* (Okamoto *et al*, 2004). Thus, it is possible that at low concentrations the polyphenols such as catechin present in *D. viscosa* var. *angustifolia* may be responsible for the inhibitory effect on the gingipains tested.

The results of this study have shown that if *D. viscosa* var. *angustifolia* is developed into a mouth rinse, gel or a toothpaste, the high concentrations and much diluted concentrations (0.09 mg/ml) will eliminate four periodontal pathogens including *P. gingivalis* and as the concentrations are reduced (0.02 - 0.001 mg/ml) further due to the salivary flow and depending on the substantivity of this plant extract it will render *P. gingivalis* avirulent. This validates the potential of this plant to be used as a preventive

and therapeutic agent of periodontal diseases. **A summary of the results obtained in this research project are represented in Table 4.1, page 103.**

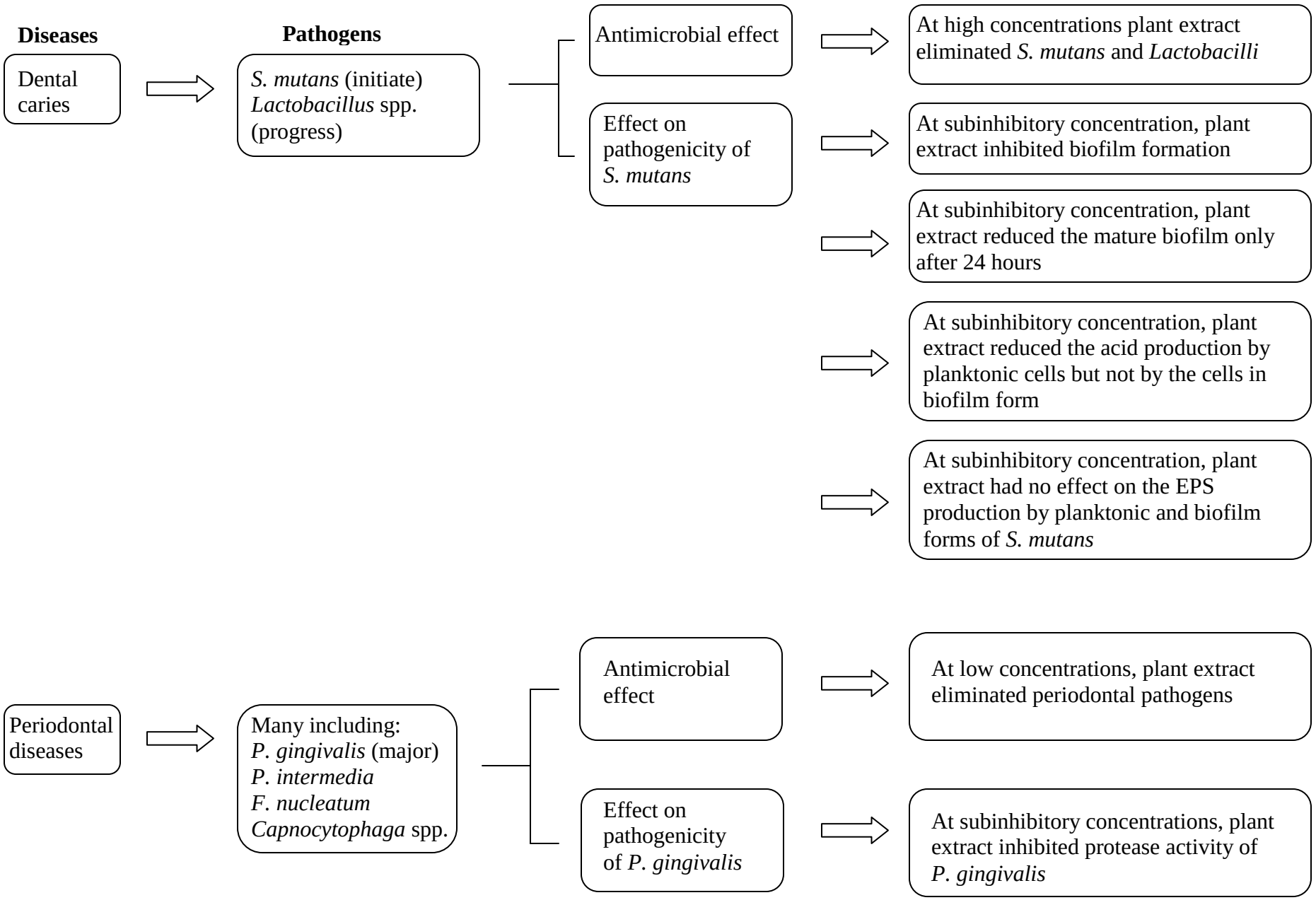



**Figure 4.1** Effect of *Dodonaea viscosa* var. *angustifolia* on the cariogenic bacteria and periodontal pathogens: A summary of the findings

# CHAPTER 5: CONCLUSION, FUTURE RESEARCH AND LIMITATIONS TO THE STUDY

## Conclusion

The crude extracts of medicinal plant *D. viscosa* var. *angustifolia* have shown to effectively eliminate cariogenic bacteria at high concentrations of 1.56 - 12.5 mg/ml (*S. mutans*) and 0.78 - 12.5 mg/ml (*Lactobacillus* spp.). At a subinhibitory concentration of 0.78 mg/ml *D. viscosa* var. *angustifolia* inhibited the dental plaque biofilm formation within 6 hours and continued to inhibit up to 30 hours. However, *D. viscosa* var. *angustifolia* had no significant effect on the fully developed mature biofilm that received two 1 minute exposures to the plant extract but the same biofilm incubated for 24 hours with the plant extract showed significantly reduced bacterial growth. Subinhibitory concentration of this plant extract also inhibited the acid production by the planktonic cells of *S. mutans* but not by the cells in a biofilm form. *D. viscosa* var. *angustifolia* had no effect on the extracellular polysaccharides production.

At low concentrations, the crude extract of *D. viscosa* var. *angustifolia* eliminated four periodontal pathogens. At subinhibitory concentrations it also inhibited the protease production by one of the major periodontal pathogens, *P. gingivalis* rendering them avirulent.

There are three major oral diseases that commonly occur in all age populations. They are oral candidiasis, dental caries and the periodontal diseases. The pathogens coexist in the

oral cavity as commensals. The beneficial effect of *D. viscosa* var. *angustifolia* has been established. Therapeutic agents may target the pathogenic organisms by eliminating them or their ability to cause infection by inhibiting the virulence factors. *Dodonaea viscosa* var. *angustifolia* if regularly used in the oral cavity, at high concentrations will kill cariogenic bacteria and the periodontal pathogens. As the saliva flow reduces the concentrations it will continue to kill periodontal pathogens and when the concentrations are even lower it will render *P. gingivalis* avirulent. At low concentrations the plant extract will not allow biofilm formation and acid production which are the major virulent factors in the development of dental caries. This study has shown that *D. viscosa* var. *angustifolia* has a potential to be developed into a preventative and therapeutic agent for commonly occurring oral diseases. However, further research is required to establish its safety and *in vivo* activity.

**Future research**

1. Extraction, isolation and identification of chemical constituents of *D. viscosa* var. *angustifolia* responsible for the beneficial effects on the oral pathogens and their virulence requires extensive research involving chromatography. Essential oils of this plant can also be extracted and obtained from distillation to study their antimicrobial effect.
2. Responsible chemicals can be cheaply synthesized and commercialized for the prevention of dental caries and periodontal diseases, as well as treatment of periodontal diseases. Threads impregnated with plant extract can be developed

which are placed in the periodontal pockets and sockets of tooth extracted areas. Tetracycline and clove oil impregnated threads are used regularly.

3. Although *D. viscosa* leaves are toxic in cattle if ingested in a large quantity (Colodel *et al*, 2003), the plant extract has been proven to be safe in mice (Khalil *et al*, 2006). Therefore cytotoxicity and carcinogenic effects of the plant extract in human cells can be established to determine the safe usage.
4. Clinical trials can be performed using the plant extract in either a mouth rinse, gel or a toothpaste form and monitor the effect on the oral hygiene and the plaque and bleeding index which are responsible for both the diseases.
5. Substantivity meaning absorption to oral surfaces, slow release and maintenance of low concentration of this plant extract can be determined.
6. Periodontal diseases are caused by byproducts of anaerobic bacteria and the host response itself. It is a chronic inflammatory disease. Recently some medicinal plants have been explored for their effect on the host response such as secretion of various cytokines, matrix metalloproteinase and interleukin (Yanti *et al*, 2009; La *et al*, 2009; La *et al*, 2010[a]; La *et al*, 2010[b]). Similarly efficacy of *D. viscosa* var. *angustifolia* can be studied.
7. Mechanism responsible for the inhibition of adherence of *S. mutans* to surfaces can be investigated by studying the cell surface adhesins and glucan binding proteins.
8. Mechanism responsible for the inhibition of acid production by *S. mutans* requires further investigation. Each step of glycolysis can be monitored for the effect on any of the enzymes responsible for the glycolytic pathways and acid production.

For example fluoride inhibits the acid production by *S. mutans* by acting on the enzyme enolase which is responsible for the conversion of phosphoenole to phosphoenole pyruvate which subsequently prevents acid production.

9. Effect of *D. viscosa* var. *angustifolia* on collagenase can be studied and the beneficial effect on the gingipains which was established in this study can be further analysed in terms of responsible mechanism.
10. Since only the MBC values were studied, a percentage-kill study can be performed. This can be done by determining number of surviving bacteria at various concentrations and exposure time.

**Limitations to the study**

1. Minimum bactericidal concentration values were determined for all the organisms instead of MIC values. *Dodonaea viscosa* var. *angustifolia* precipitates with culture medium used in the study therefore visible growth was not possible which is required for the determination of MIC values. P-Iodonitrotetrazolium violet that detects the metabolic activity of cells could have been used but the intensity in the colour change would be difficult due to the heavy precipitation and pigment production by anaerobes. Minimum bactericidal concentration values are the ultimate results and it is generally known that MIC values falls few dilutions below the MBC values.
2. In the study where effect on the acid production by biofilm was established, bacterial counts at various time intervals similar to the planktonic study would have been ideal. However, it was not possible because the cultures with biofilms

were not homogenous but rather full of clumps and pipetting would not give comparable results.

3. Although an acceptable and referenced method was used for the experiments analyzing extracellular polysaccharide production, it could have been improved by adding positive controls such as synthetic glucans and levans. This could not be done as these were not available in the lab.

4. The effects of volatiles could not be tested as these were lost during the plant extraction procedure where air caused oxidation.

# CHAPTER 6: REFERENCES


- Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., Dewhirst, F, E., 2005. Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology* **43**, 5721-5732.
- Abe, N., Kadowaki, T., Okamoto, K., Nakayama, K., Ohishi, M., Yamamoto, K., 1997. Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. *Journal of Biochemistry* **123**, 305-312.
- Almas, K., Al-Lafi, T. R., 1995. The Natural Toothbrush. *World Health Forum* **16**, 206-210.
- Alviano, W. S., Alviano, D. S., Diniz, C. G., Antoniolli, A. R., Alviano, C. S., Farias, L. M., Carvalho, M. A. R., Souza, M. M. G., Bolognese, A. N., 2008. *In vitro* antioxidant potential of medicinal plant extracts and their activities against oral bacteria based on Brazilian folk medicine. *Archives of Oral Biology* **53**, 545-552.
- Amabeoku, G. J., Eagles, P., Scott, G., Mayeng, I., Springfield, E., 2001. Analgesic and antipyretic effects of *Dodonaea angustifolia* and *Salvia Africana-lutea*. *Journal of Ethnopharmacology* **75**, 117-124.
- Ashimoto, A., Chen, C., Bakker, I., Slots, J., 1996. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis

and advanced periodontitis lesions. *Oral Microbiology and Immunology* **11**, 266-273.

- Asres, K., Bucar, F., Kartnig, T., Witvrouw, M., Pannecouque, C., De Clercq, E., 2001. Antiviral activity against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) of ethnobotanically selected Ethiopian medicinal plants. *Phytotherapeutic Research* **15**, 62–69.
- Avila-Campos, M. J., Sacchi, C. T., Whitney, A. M., Steigerwalt, A. G., Mayer, L. W., 1999. Arbitrarily primed-polymerase chain reaction for identification and epidemiology subtyping of oral isolates of *Fusobacterium nucleatum. Journal of Periodontology* **70**, 1202-1207.
- Bakken, V. B. T. H., Jensen, H. B., 1990. Growth conditions and outer membrane proteins of *Fusobacterium nucleatum* **98**, 215-224.
- Bankova, V. S., Popov, S. S., Marekov, N. L., 1989. Isopentenyl cinnamates from poplar buds and propolis. *Phytochemistry* **28**, 871-873.
- Bartold, P. M., Gully, N. J., Zilm, P. S., and Rogers, A. H., 1991. Identification of components in *Fusobacterium nucleatum* chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation. *Journal of Periodontal Research*. 26, 314–322.
- Beighton, D., Russell, R. R. B., Hayday, H., 1981. The isolation and characterization of *Streptococcus mutans* serotype *h* from dental plaque of monkeys (*Macaca fascicularis*). *Journal of General Microbiology* **124**, 271-279.
- Bickel, M., Cimasoni, G., 1985. The pH of human crevicular fluid measured by a new microanalytical technique. *Journal of Periodontal Research* 20, 35-40.

- Bodet, C., Piche, M., Chandad, F., Grenier, D., 2006. Inhibition of periodontopathogen-derived proteolytic enzymes by high-molecular weight fraction isolated from cranberry. *Journal of Antimicrobial Chemotherapy* **57**, 685-690.
- Bolstad, A. I., Jensen, H. B., Bakken, V., 1996. Taxonomy, Biology, and Periodontal aspects of *Fusobacterium nucleatum. Clinical Microbiology Reviews* **9**, 55-71.
- Bowen, W. H., 2002. Do we need to be concerned about dental caries in the coming millennium? *Critical Reviews in Oral Biology and Medicine* **13**, 126-131.
- Brochu, V., Grenier, D., Nakayama, K., Mayrand, D., 2001. Acquisition of iron from human transferrin by *Porphyromonas gingivalis:* a role for Arg- and Lys-gingipain activities. *Oral Microbiology and Immunology* **16**, 79-87.
- Byun, R., Nadkarni, M. A., Chhour, K. L., Martin, F. E., Jacques, N. A., Hunter, N., 2004. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *Journal of Clinical Microbiology* **42**, 3128-3136.
- Cangelosi, G. A., Iversen, J. M., Zuo, Y., Oswald, T. K., Lamont, R. J., 1994. Oligonucleotide probes for mutans streptococci. *Molecular Cellular Probes* **8**, 73-80.
- Caufield, P. W., Dasanayake, Y. L. A., Saxena, D., 2007. Diversity of Lactobacilli in the oral cavities of young women with Dental caries. *Caries Research.* **41,** 1-8.
- Ciancio, S. G., 2007. Improving our patients' oral health: the role of a triclosan/copolymer/fluoride dentrifice. *Compendium of Continuing Education in Dentistry* **28**, 178-183.

- Clarkson, B. H., Hall, D., Heilman, J. R., Wefel, J. S., 1986. Effect of proteolytic enzymes on caries lesion formation *in vitro*. *Journal of Oral Pathology* **15**, 423-429.
- Colby, S. M., Russell, R, R. B., 1997. Sugar metabolism by mutans streptococci. *Journal of Applied Microbiology Symposium Supplement* **83**, 80S-88S.
- Colodel, E. M., Traverso, S. D., Seitz, A. L., Correa, A., Oliveira, F. N., Driemeier, D., Gava, A., 2003. Spontaneous poisoning by *Dodonaea viscosa* (Sapindaceae) in cattle. *Veterinary and Human Toxicology* **45**, 147-148.
- Cortelli, J. R., Aquino, D. R., Cortelli, S. C., Fernandes, C. B., Carvalho-Filho, J., Franco, G. C. N., Costa, F. O., Kawai, T., 2008. Etiological analysis of initial colonization of periodontal pathogens in oral cavity. *Journal of Clinical Microbiology* **46**, 1322-1329.
- Crawford, A. C. R., Socransky, S. S., Smith, E., and Phlllips, R., 1977. Pathogenicity testing of oral isolates in gnotobiotic rats. *Journal of Dental Research* **56**, 275.
- Demuth, D. R., Golub, E. E., Malamud, D., 1990. Streptococcal-host interactions. Structural and functional analysis of a *Streptococcus sanguis* receptor for a human salivary glycoprotein. *Journal of Biological Chemistry* **265**, 7120-7126.
- Dibdin, G. H., Shellis, R. P., 1988. Physical and biochemical studies of *Streptococcus mutans* sediments suggest new factors linking the cariogenicity of plaque with its extracellular polysaccharide content. *Journal of Dental Research* **67**, 890-895.

- Dixon, R. A., Dey, P. M., Lamb, C. J., 1983. Phytoalexins: enzymology and molecular biology. *Advances in Enzymology and Related Areas of Molecular Biology* **55**, 1-136.
- Dogan, S., Gunay, H., Leyhausen, G., Geurtsen, W., 2003. Effects of low-concentrated chlorhexidine on growth of *Streptococcus sobrinus* and primary human gingival fibroblasts. *Clinical Oral Investigations* **7**, 212-216.
- Duarte, S., Gregoire, S., Singh, A. P., Vorsa, N., Schaich, K., Bowen, W. H., Koo, H., 2006. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. *Federation of European Microbiological Sciences Microbiology Letters* **257**, 50-56.
- Duarte, S., Klein, M. I., Aires, C. P., Cury, J. A., Bowen, W. H., Koo, H., 2008. Influences of starch and sucrose on *Streptococcus mutans* biofilms. *Oral Microbiology Immunology* **23**, 206-212.
- Dzink, J. L., Socransky, S. S., 1985. Comparative *in vitro* activity of sanguinarine against oral microbial isolates. *Antimicrobial Agents and Chemotherapy*. **4,** 663-665.
- Ebisu, S., Kato, K., Kotani, S., 1975. Structural differences in fructans elaborated by *Streptococcus mutans* and *Streptococcus salivarius. Journal of Biochemistry* **78**, 879-887.
- Edris, A. E., 2007. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: A review. *Phytotherapy Research* **21**, 308-323.

- Eloff, J. N., 1999. The antimicrobial activity of 27 southern African members of the Combretaceae. *South African Journal of Science* **95,** 148-152.
- Emilson, C. G., Bratthall, D., 1976. Growth of *Streptococcus mutans* on various selective media. *Journal of Clinical Microbiology* **4**, 95-98.
- Facklam, R., Bosley, G. S., Rhoden, D., Franklin, A. R., Weaver, N., Schulman, R., 1984. Comparative evaluation of the API 20S and AutoMicrobic gram-positive identification systems for non-beta-hemolytic Streptococci and Aerococci. *Journal of Clinical Microbiology* **21**, 535-541.
- Featherstone, J. D. B., 2000. The science and practice of caries prevention. *The Journal of the American Dental Association* **131,** 887-899.
- Featherstone, J. D. B., 2004. The Continuum of Dental caries-Evidence for a dynamic disease process. *Journal of Dental Research* **83**, 39-42.
- Fejerskov, O., Kidd, E. A. M., 2008. Dental caries. The Disease and its Clinical Management Second Edition. Blackwell Munkgaard, 17, Iowa.
- Filoche, S. K., Soma, K., Sissons, C. H., 2005. Antimicrobial effects of essential oils in combination with chlorhexidine digluconate. *Oral Microbiology and Immunology* **20**, 221-225.
- Forlenza, S. W., Newman, M. G., Horikoshi, A. L., Blachman, U., 1981. Antimicrobial susceptibility of *Capnocytophaga. Antimicrobial Agents and Chemotherapy* **19**, 144-146.
- Freedman, M. L., Tanzer, J. M., 1982. The use of mutants to study the glucan-associated pathophysiology of *Streptococcus mutans. In Microbiology* **11**, 186-190.

- Getie, M., Gebre-Mariam, T., Rietz, R., Hohne, C., Huschka, C., Schmidtke, M., Abate, A., Neubert, R. H. H., 2003. Evaluation of the anti-microbial and anti-inflammatory activities of the medicinal plants *Dodonaea viscosa, Rumex, nervosus* and *Rumex abyssinicus. Fitoterapia* **74**, 139-143.
- Ghisalberti, E. L., 1979. Propolis: a review. *Bee World* **60**, 59-84.
- Gillbert, P., Das, J., Foley, I., 1997. Biofilm susceptibility to antimicrobials. *Advances in Dental Research* **11**, 160-167.
- Grange, J. M., Davey, R. W., 1990. Antibacterial properties of propolis (bee glue). *Journal of the Royal Society of Medicine* **83**, 159-160.
- Grenier, D., Imbeault, S., Plamondon P., 2001. Role of gingipains in growth of *Porphyromonas gingivalis* in the presence of human serum albumin. *Infection and Immunology* **69**, 5166-5172.
- Grenier, D., La, V. D., 2011. Proteases of *Porphyromonas gingivalis* as important factors in periodontal disease and potential targets for plant-derived compounds: a review article. *Current Drug Targets* **3**, 322-331.
- Guggenheim, B., 1970. Extracellular polysaccharides and microbial plaque. *International Journal of Dentistry* **20**, 657-678.
- Hamada, S., Slade, H. D., 1980. Biology, immunology and cariogenicity of *Streptococcus mutans. Microbiological Reviews* **44,** 331-384.
- Hamilton-Miller, J. M. T., 2001. Anti-cariogenic properties of tea (*Camellia sinensis*). *Journal of Medical Microbiology* **50**, 299-302.

- Hansen, M. C., Palmer, R. J., White, D. C., 2000. Flowcell culture of *Porphyromonas gingivalis* biofilms under anaerobic conditions. *Journal of Microbiological Methods* **40**, 233-239.
- Hogg, S. D., Embery, G., 1982. Blood-group-reactive glycoprotein from human saliva interacts with lipoteichoic acid on the surface of *Streptococcus sanguis* cells. *Archives of Oral Biology* **27**, 261-268.
- Humphrey, S. P., Williamson, R. T., 2001. A review of saliva: Normal composition, flow and function. *Journal of Prosthetic Dentistry* **85**, 162-169.
- Ikigai, H., Nakae, T., Hara, Y., Shimamura, T., 1993. Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta* **1147**, 132-136.
- Irving, J. T., Socransky, S. S., Newman, M. G and E. Savitt., 1976. Periodontal destruction induced by *Capnocytophaga* in gnotobiotic rats. *Journal of Dental Research.* **55**, 783-789
- Ismail, A. I., Lewis, D. W., Dingle, J. L., 1993. Prevention of Periodontal disease. Unpublished report, Dalhousie University, Halifax, Nova Scotia, 420-431.
- Jagtap, A. G., Karkera, S. G., 2000. Extract of *Juglandaceae regia* inhibits growth, in-vitro adherence, acid production and aggregation of *Streptococcus mutans. Journal of Pharmacy and Pharmacology* **52**, 235-242.
- Jenkinson, H. F., Lamont, R. J., 2005. Oral microbial communities in sickness and in health. *Trends in Microbiology*. **13**, 589-595.
- Kadowaki, T., Nakayama, K., Okamoto, K., 2000. *Porphyromonas gingivalis* proteinases as virulence determinants in progression of periodontal diseases. *Journal of Biochemistry* **128**, 153-159.

- Kassu, A., Dagne, E., Abate, D., De Castro, A., van Wyk, B. E., 1999. Ethnomedical aspects of the commonly used toothbrush sticks in Ethiopia. *East African Medical Journal* **11**, 651-653.
- Kawasaki K., Featherstone J. D., 1997. Effects of collagenase on root demineralization. *Journal of Dental Research* **76**, 588-595.
- Khalil, N. M., Sperotto, J. S., Manfron, M. P., 2006. Antiinflammatory activity and acute toxicity of *Dodonaea viscosa. Fitoterapia* **77**, 478-480.
- Khurram, M., Khan, M. A., Hameed, A., Abbas, N., Qayum, A., Inayat, H., 2009. Antibacterial activities of *Dodonaea viscosa* using contact bioautography technique. *Molecules* **14,** 1332-1341.
- Khurram, M., Hameed, A., Amin, M. U., Gul, A., Ullah, N., Hassan, M., Qayum, A., Manzoor, W., 2011. Phytochemical screening and *in vitro* evaluation of anticandida activity of *Dodonaea viscosa* (L.) Jaeq. (Sapindaceae). *African Journal of Pharmacy and Pharmacology* **5**, 1422-1426.
- Kim, J. E., Kim, H. E., Hwang, J. K., Lee, H. J., Kwon, H. K., Kim, B., 2008. Antibacterial characteristics of *Curcuma xanthorrhiza* extract on *Streptococcus mutans* biofilm. *The Journal of Microbiology.* **46,** 228-232.
- Kinane, D. F., 2001. Causation and pathogenesis of periodontal disease. *Periodontology* **25**, 8-20.
- Koo, H., Gomes, B. P. F. A., Rosalen, P. L., Ambrosano, G. M. B., Park, Y. K., Cury, J. A., 2000. *In vitro* antimicrobial activity of *propolis* and *Arnica Montana* against oral pathogens. *Archives of Oral Biology* **45,** 141-148.

- Koo, H., Rosalen, P. L., Cury, J. A., 2002. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrobial Agents and Chemotherapy* **46**, 1302-1309.
- Koo, H., Pearson, S. K., Scott-Anne, K., Abranches, J., Cury, J. A., Rosalen, P. L., Park, Y. K., Marquis, R. E., Bowen, W. H., 2003. Effects of apigenin and *tt*-farnesol on glucosyltransferase activity, biofilm viability and caries development in rats. *Oral Microbiology and Immunology* **17,** 337-343.
- La, V. D., Bergeron, C., Gafner, S., Grenier, D., 2009. Grape seed extract suppresses lipopolysaccharides-induced matrix metalloproteinase (MMP) secretion by macrophages and inhibits human MMP-1 and -9 activities. *Journal of Periodontology* **11**, 1875-1882.
- La, V. D., Howell, A. B., Grenier, D., 2010[a]. Anti-*Porphyromonas gingivalis* and anti-inflammatory activities of A-type cranberry proanthocyanidins. *Antimicrobial Agents and Chemotherapy* **54**, 1778-1784.
- La, V. D., Tanabe, S., Bergeron, C., Gafner, S., Grenier, D., 2010[b]. Modulation of matrix metalloproteinase and cytokine production by licorice isolates Licoricidin and Licorisoflavan A: Potential therapeutic approach for periodontitis. *Journal of Periodontology* **82**, 122-128.
- Lafl, T. A., Ababneh, H., 1995. The effect of the extract of the Miswaki (chewing sticks) used in Jordan and the Middle East on oral bacteria. *International Dental Journal* **45**, 218-222.
- Leitao, D. P., Filho, A. A., Polizello, A. C. M., Bastos, J. K., Spadaro, A. C. C., 2004. Comparative evaluation of *in vitro* effects of Brazilian green propolis and

*Baccharis dracunculifolia* extracts on cariogenic factors of *Streptococcus mutans. Biology and Pharmacology Bulletin* **27**, 1834-1839.

- Lemos, J. A., Burne, R, A., 2008. A model of efficiency: stress tolerance by *Streptococcus mutans. Microbiology* **154,** 3247-3255.
- Li, Y. H., Hanna, M. N., Svensater, G., Ellen, R. P., Cvitkovitch, D. G., 2001. Cell density modulates acid adaptation in *Streptococcus mutans*: implication for survival in biofilm. *Journal of Bacteriology* **183**, 6875–6884.
- Limsong, J., Benjavongkulchai, E., Kuvatanasuchati, J., 2004. Inhibitory effect of some herbal extracts on adherence of *Streptococcus mutans. Journal of Ethnopharmacology* **92**, 281-289.
- Listgarten, M. A., 1986. Pathogenesis of periodontitis. *Journal of Clinical Periodontology* **13**, 418-425.
- Loesche, W. J., 2010. Bacterial mediators in periodontal disease. *Clinical Infectious Diseases* **16**, 203-210.
- Loftsson, T., Leeves, N., Bjornsdottir, B., Duffy, L., Masson, M., 1999. Effect of cyclodextrins and polymers on triclosan availability and substantivity in toothpastes *in vivo*. *Journal of Pharmaceutical Sciences* **88**, 1254-1258.
- Lourbakos, A., Potempa, J., Travis, J., D'Andrea, M. R., Andrade-Gordon, P., Santulli, R., Mackie, E. J., Pike, R, N., 2010. Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin -6 secretion. *Infection and Immunity* **69**, 5121-5130.

- Maeyama, R., Kwon, I. K., Mizunoe, Y., Anderson, J. M., Tanaka, M., Matsuda, T., 2005. Novel bactericidal surface: Catechin-loaded surface-erodible polymer prevents biofilm formation. *Journal of Biomedical Materials Research Part A* **75**, 146-155.
- Marquis, R. E., Clock, S. A., Mota-Meira, M., 2002. Fluoride and organic weak acids as modulators of microbial physiology. *Federation of European Microbiological Sciences Microbiology Reviews* **760**, 1-18.
- Marsh, P. D., Keevil, C. W., Ellwood, D. C., 1984. Relationship of bioenergetic processes to the pathogenic properties of oral bacteria. *Journal of Dental Research* **63**, 401-406.
- Marsh, P. D., 1994. Microbial ecology of dental plaque and its significance in health and disease. *Advances in Dental Research* **8**, 263-271.
- Marsh, P. D., Martin, M. V., 1999. Oral Microbiology Fourth Edition. *Wright*, Oxford, United Kingdom.
- Marsh, P. D., Martin, M. V., 2009. Oral Microbiology Fifth Edition. *Churchill Livingstone Elsevier*, London, United Kingdom.
- Matos-Graner, R. O., Smith, D. J., King, W. F., 2000. Water-insoluble glucan synthesis by mutans streptococci strains correlates with caries incidence in 12- to 30-month-old children. *Journal of Dental Research* **79,** 1371-7.
- Nakahara, K., Kawabata, S., Ono, H., Ogura, K., Tanaka, T., Ooshima, T., Hamada, S., 1993. Inhibitory effect of oolong tea polyphenols on glucosyltransferases of mutans streptococci. *Applied and Environmental Microbiology* **59**, 968-973.

- Nalina, T., Rahim, Z. H. A., 2007. The crude aqueous extract of *Piper betle* L. and its antibacterial effect towards *Streptococcus mutans. American Journal of Biotechnology and Biochemistry* **3**, 10-15.
- Nisengard, R. J., Newman, M. G., 1994. Oral Microbiology and Immunology Second Edition, *WB Saunders Company*, Philadelphia.
- Nobre dos Santos, M., Melo dos Santos, L., Francisco, S. B., 2002. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. *Caries Research* **36**, 347-52.
- Okamoto, K., Kadowaki, T., Nakayama, K., Yamamoto, K., 1996. Cloning and sequencing of the gene encoding a novel Lysine-specific cysteine proteinase (Lys-gingipain) in *Porphyromonas gingivalis:* Structural relationship with the Arginine-specific cysteine proteinase (Arg-gingipain). *Journal of Biochemistry* **120**, 398-406.
- Okamoto, M., Sugimoto, A., Leung, K. P., Nakayama, K., Kamaguchi, A., Maeda, N., 2004. Inhibitory effect of green tea catechins on cysteines proteinases in *Porphyromonas gingivalis. Oral Microbiology and Immunology* **19**, 118-120.
- Ooshima, A. M., Minami, T., Aono, W., Izumitani, A., Sobue, S., Fujiwara, T., Kawabata, S., Hamada, S., 1993. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. *Caries Research* **27**, 124-129.
- Palombo, E. A., 2009. Traditional medicinal plant extracts and natural products with activity against oral bacteria: Potential application in the prevention and treatment of oral diseases. *Oxford Journal* **10**, 1-15.

- Paolino, V. J., Kashket, S., 1985. Inhibition by cocoa extracts of biosynthesis of extracellular polysaccharide by human oral bacteria. *Archives of Oral Biology* **30**, 359-363.
- Patel, M., Coogan, M. M., 2008. Antifungal activity of the plant *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* from HIV-infected patients. *Journal of Ethnopharmacology* **118,** 173-176.
- Patel, M., Gulube, Z., Dutton, M., 2009. The effect of *Dodonea viscose var. angustifolia* on *Candida albicans* proteinase and phospholipase production and adherence to oral epithelial cells. *Journal of Ethnopharmacology* **625**, 562-565.
- Patel, M., 2011. Personal communication.
- Petersen, P. E., Lennon, M. A., 2004. Effective use of fluorides for the prevention of dental caries in the 21st century: the WHO approach. *Community Dentistry and Oral Epidemiology* **32**, 319-321.
- Petersen, P. E., Ogawa, H., 2005. Strengthening the prevention of periodontal disease: the WHO approach. *Journal of Periodontology* **76**, 2187-2193.
- Phan, T. N and Marquis, R. E., 2006. Triclosan inhibition of membrane enzymes and glycolysis of *Streptococcus mutans* in suspensions and biofilms. *Canadian Journal of Microbiology* **52**, 977-983.
- Pihlstrom, B. L., Michalowicz, , S., Johnson, N. W., 2005. Periodontal Diseases. *The Lancet* **366**, 1809-1820.
- Pike, R., McGraw, W., Potempa, J., Travis, J., 1994. Lysine- and Arginine-specific proteinases from *Porphyromonas gingivalis. The Journal of Biological Chemistry* **269**, 406-411.

- Pirzada, A. J., Shaikh, W., Usmanghani, K., Mohiuddin, F., 2010. Antifungal activity of *Dodonaea viscosa* Jacq. extract on pathogenic fungi isolated from superficial skin infection. *Pakistan Journal of Pharmaceutical Sciences* **23**, 337-340.
- Potempa, J., Pike, R., Travis, J., 1995. The Multiple forms of Trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. *Infection and Immunity* **63**, 1176-1182.
- Potempa, J., Banbula, A., Travis, J., 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontology* **24**, 153-192.
- Potempa, J., Sroka, A., Imamura, T., Travis, J., 2003. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis:* Structure, Function and Assembly of Multidomain protein complexes. *Current Protein and Peptide Science* **4**, 397-407.
- Quivey, R. G., Faustoferri, R., Monakan, K., Marquis, R., 2000. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans. Federation of European Microbiological Sciences Microbiology Letters* **189**, 89-92.
- Rani, M. S., Pippalla, R. S., Mohan, K., 2009. *Dodonaea viscosa* Linn. An Overview. *Journal of Pharmaceutical Research and Health Care* **1,** 97-112.
- Reddy, B. A., 2009. Preparation, Characterization and Biological Evaluation of some overview of *Dodonaea viscosa* Linn. *Journal of Pharmaceutical Science and Technology* **1**, 1-9.

- Rios, J. L., Recio, M. C., Villar, A., 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology* **23**, 127-149.
- Rosan, B., Lamont, R. J., 2000. Dental plaque formation. *Microbes and Infection* **2**, 1599-1607.
- Rose, L. F., Genco, R. J., Cohen, D. W., Mealey, B. L., 2000. Periodontal Medicine. B.C Decker Inc. London.
- Rukayadi, Y., Hwang, J. K., 2005. *In vitro* activity of xanthorrhizol against *Streptococcus mutans* biofilms. *Letters in Applied Microbiology* **42**, 400-404.
- Russell, M. W., Childers, N. K., Michalek, S. M., Smith. D. J., Taubman, M. A., 2004. A Caries Vaccine? *Caries Research* **38**, 230-235.
- Samaranayake, L., 2002. Essential Microbiology for Dentistry. Second Edition. Churchill Livingstone. London.
- Santos, F. A., Bastos, E. M., Uzeda, M., Carvalho, M. A., Farias, L. M., Moreira, E. S., Braga, F. C., 2002. Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria. *Journal of Ethnopharmacology* **80**, 1-7.
- Sbordone, L., Bortolaia, C., 2003. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clinical Oral Investigations* **7**, 181-188.
- Selwitz, R. H., Ismail, A., Pitts, N. B., 2007. Dental caries. *The Lancet* **369,** 51-59.
- Shimotoyodome, A., Koudate, T., Kobayashi, H., Nakamura, J., Tokimitsu, I., Hase, T., Inoue, T., Matsukubo, T., Takaesu, Y., 2007. Reduction of

*Streptococcus mutans* adherence and dental biofilm formation by surface treatment with phosphorylated polyethylene glycol. *Antimicrobial agents and Chemotherapy* **51**, 3634-3641.

- Silva, T. A., Rodrigues, P. H., Ribeiro, R. N., Noronha, F. S. M., Farais, L. M., Carvalho, M. A. R., 2003. Hemolytic activity of *Prevotella intermedia* and *Prevotella nigrescens* strains: Influence of abiotic factors in solid and liquid assays. *Research in Microbiology* **154**, 29-35.
- Singer, R. E., and Buckner, B. A., 1981. Butyrate and propionate: important components of toxic dental plaque extracts. *Infection and Immununity*. **32**, 458–463.
- Sinnott, J. T., Cullison, J. P., Blanco, P. J., 1988. *Capnocytophaga. Infection Control and Hospital Epidemiology* **9**, 170-173.
- Slots, J., Genco, R. J., 1984. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in Human Periodontal Disease: Virulence Factors in Colonization, Survival, and Tissue Destruction. *Journal of Dental Research* **63**, 412-421.
- Slots, J., Ting, M., 2002. Systemic antibiotics in the treatment of periodontal disease. *Periodontology* **28**, 106-176.
- Slots, J., 2004. Systemic antibiotics in periodontics. *Journal or Periodontology* **75**, 1553-1565.
- Smelcerovic, A., Jugovic, Z. K., Petronijervic, Z., 2008. Microbial polysaccharides and their derivatives as current and prospective pharmaceuticals. *Current Pharmaceutical Design* **14**, 3168-3195.

- Socransky, S. S., Holt, S. C., Leadbetter, E. R., Tanner, A. C. R., Savitt, E., Hammond, B. F., 1979. *Capnocytophaga:* New genus of gram-negative gliding bacteria . III Physiological characterization. *Archives of Microbiology* **122**, 29-33.
- Song, J. H., Yang, T. C., Chang, K. W., Han, S. K., Yi, H. K., Jeon, J. G., 2007. *In vitro* effects of a fraction separated from *Polygonum cuspidatum* root on the viability, in suspension and biofilms, and biofilm formation of mutans streptococci. *Journal of Ethnopharmacology* **112**, 419-425.
- Stevens, R. H., Sela, M. N., Shapira, J and Hammond, B. F., 1980. Detection of a fibroblast proliferation inhibitory factor from *Capnocytophaga sputigena. Infection and Immunology* **27**, 271-275.
- Sturr, M. G., Marquis, R. E., 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. *Applied Environmental Microbiology* **58**, 2287-2291.
- Susumu, K., Hiroshi, M., 2005. Oral microflora and their relation to health and disease. *Foods and Food Ingredients Journal Japan* **4,** 348-360.
- Tanzer, J. M., Slee, A. M., Kamay, B., Scheer, E. R., 1977. *In vitro* evaluation of three iodine-containing compounds as antiplaque agents. *Antimicrobial Agents and Chemotherapy.* **12,** 107-113.
- Tapsoba, H., Deschamps, J. P., 2005. Use of medicinal plants for the treatment of oral diseases in Burkina Faso. *Journal of Ethnopharmacology* **104,** 68-78.
- Teffo, L. S., Aderogba, M. A., Eloff, J. N., 2010. Antibacterial and antioxidant activities of four kaempferol methyl ethers isolated from *Dodonaea viscosa* Jacq. var. *angustifolia* leaf extracts. *South African Journal of Botany* **76**, 25-29.

- Toda, M., Okubo, S., Ikigai, H., Suzuki, T., Suzuki, Y., Hara, Y., Shimamura, T., 1992. The protective activity of tea catechins against experimental infection by *Vibrio cholerae O1*. *Microbiology and Immunology* **36**, 999-1001.
- Van Heerden, F. R., Viljoen, A. M., Van Wyk, B. E., 2000. The major flavonoid of *Dodonaea angustifolia. Fitoterapia* **71**, 602-604.
- Van Wyk, B. E., Van Oudtshoorn, B., Gericke, N., 2002. Medicinal Plants of South Africa. Briza Publications. Pretoria, South Africa.
- Wen, C. R., 1985. A Colour Atlas of Periodontics. Ishiyaku EuroAmerica, Inc. St. Louis, America, 25-26.
- Wiater, A., Szczodrak, J., Rogalski, J., 2004. Hydrolysis of mutan and prevention of its formation in streptococcal films by fungal α-D-glucanases. *Process Biochemistry* **39**, 1481-1489.
- Williams, R. C., 1990. Periodontal Disease. *The New England Journal of Medicine* **322**, 373-382.
- Wolinsky, L. E., Sote, E. O., 1984. Isolation of natural plaque-inhibiting substances from Nigerian chewing sticks. *Caries Research* **18**, 216-225.
- Wu-Yuan, C. D., Chen, C. Y., Wu, R. T., 1988. Gallotannins inhibit growth, water-insoluble glucan synthesis, and aggregation of Mutans Streptococci. *Journal of Dental Research* **67**, 51-55.
- Yamanaka, A., Kouchi, T., Kasai, K., Kato, T., Ishihara, K., Okuda, K., 2007. Inhibitory effect of cranberry polyphenol on biofilm formation and cysteine proteases of *Porphyromonas gingivalis. Journal of Periodontal Research* **42,** 589-592.

- Yanti., Oh H. I., Anggakusuma., Hwang, J. K., 2009. Effects of panduratin A isolated from Kaempferia pandurata ROXB. on the expression of matrix metalloproteinase-9 by *Porphyromonas gingivalis* supernatant-induced KB cells. *Biology and Pharmacology Bulletin* **1**, 110-115.
- Yoo, S. Y., Kim, P. S., Hwang, H. K., Lim, S. H., Kim, K. W., Choe, S. J., Min, B. M., Kook, J. K., 2005. Identification of Non-mutans Streptococci organisms in dental plaques recovering on Mitis-Salivarius Bacitracin Agar Medium. *The Journal of Microbiology* **43**, 204-208.
- Zinner, D. D., Jablon, J. M., Saslaw, M. S., 1961. Bactericidal properties of povidone-iodine and its effectiveness as an oral antiseptic. *Oral Surgery, O Medicine* and *Oral Pathology* **14**, 1377-1382.

# CHAPTER 7: APPENDICES

## Appendix 1

## Composition and preparation of media

### Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA)

| | |
|---|---|
| 0.02 g | BAPNA |
| 100 ml | distilled water |

Dissolved in water

### Blood agar

| | |
|---|---|
| 39 g | Columbia agar (Oxoid Ltd, UK) |
| 1000 ml | distilled water |
| 5 g | sterile defibrinated blood |

39 g Columbia agar base was suspended in 1000 ml distilled water. It was dissolved completely by bringing to the boil. It was sterilized by autoclaving at 151b and 121 °C for 10 minutes. Allowed to cool to 50 °C and 5 % sterile defibrinated blood was added. It was poured into plates and allowed to set. Plates were labeled and refrigerated until use.

### Brain Heart Infusion Broth

| | |
|---|---|
| 37 g | brain heart powder (Biolab Diagnostics Pty. Ltd, SA) |
| 1000 ml | distilled water |

These were mixed, dissolved by heating the solution, dispensed into bottles and autoclaved at 151b and 121 °C for 15 minutes.

### Dithiothreitol (DTT)

| | |
|---|---|
| 0.154 g | DTT |
| 1000 ml | distilled water |

Dissolved in water.

**Fusi Form Medium**

| | |
|---|---|
| 37 g | brain heart infusion (Biolab Diagnostics Pty. Ltd, SA) |
| 3 g | yeast extract |
| 2 g | soluble starch |
| 1000 ml | distilled water |
| pH 7.6 | |

Medium was dissolved in water and autoclaved at 151b and 121 °C for 15 minutes.

**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 |

Dissolved in water.

**Phosphate buffered saline**

| | |
|---|---|
| 4.2 g | sodium chloride |
| 0.078 g | sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) |
| 0.64 g | sodium hydrogen phosphate ($NaHPO_4$) |
| 500 ml | distilled water |

These were suspended in water and autoclaved at 151b and 121 °C for 15 minutes.

**Proteinase K**

| | |
|---|---|
| 0.01 g | proteinase K |
| 1 ml | distilled water |

Dissolved in water.

**Tryptone Broth**

| | |
|---|---|
| 12.5 g | tryptone powder (Biolab Diagnostics Pty. Ltd, SA) |
| 7.5 g | yeast extract |
| 5 g | sucrose |
| 500 ml | distilled water |
| pH 7.0 | |

Medium was dissolved in water and autoclaved at 151b and 121 °C for 15 minutes.

**Tris-HCL**

| | |
|---|---|
| 15.76 g | Tris-HCL |
| 1000 ml | distilled water |

Dissolved in water.

**Tryptone Soy Broth**

| | |
|---|---|
| 30 g | Tryptone soy broth (Biolab Diagnostics Pty. Ltd, SA) |
| 1000 ml | distilled water |

Medium was dissolve in water and autoclaved at 151b and 121 °C for 15 minutes.

**Tryptone soy broth**

| | |
|---|---|
| 15 g | tryptone soy broth |
| 0.5 ml | haem |
| 0.5 ml | menadione |
| 500 ml | distilled water |

Medium was dissolved in water and autoclaved at 151b and 121 °C for 15 minutes.

**5 % Sucrose Broth**

| | |
|---|---|
| 10 g | tryptone |
| 5 g | protease peptone no. 3 (Difco Laboratories, USA) |
| 5 g | protease peptone (Difco Laboratories, USA) |
| 1 g | glucose |
| 50 g | sucrose |
| 4 g | dipotassium phosphate |
| 1000 ml | distilled water |

Half the water was heated to boiling point. The solids were dissolved in a small amount of water and added to boiling water. The remaining water was added and heated to boiling point. Mixture was poured into large bottles and autoclaved at 151b and 121 °C for 15 minutes.

**10 % Sodium Acetate**

| | |
|---|---|
| 50 g | sodium acetate |
| 500 ml | distilled water |

Dissolved in water.

**Rogosa Agar**

| | |
|---|---|
| 82 g | Rogosa agar (Condalab, SA) |
| 1000 ml | distilled water |

Half the water was heated to boiling point. The powder was dissolved in water and added to the remaining water. Mixture was poured into large bottles. Sterilized at 151b and 121 °C for 15 minutes. Plates were labeled and refrigerated until use.

**Rogosa Broth**

| | |
|---|---|
| 10 g | tryptone |
| 5 g | yeast extract |
| 20 g | glucose |
| 1 g | tween |
| 6 g | potassium dihydrogen phosphate |
| 2 g | ammonium citrate |
| 25 g | sodium acetate |
| 1.32 ml | glacial acetic acid |
| 0.58 g | magnesium sulphate |
| 0.34 g | ferrous sulphate |
| 0.02 g | bromocresol green |
| 1000 ml | distilled water |

Ingredients were mixed until dissolved. The solution was dispensed into bottles and autoclaved at 101b and 121 ºC for 20 minutes.

## Appendix 2: Results of *Porphyromonas gingivalis* protease activity assay

**Table 2.1** The protease activity (OD) of Arg-gingipain exposed to subinhibitory concentrations of *Dodonaea viscosa* var. *angustifolia* where controls are those for each concentration of crude plant extract using dimethyl sulphoxide (Figure 2.7B)

| Repeats | Protease activity at 405 nm (OD) at various subinhibitory concentrations of crude plant extract | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.02 mg/ml | | 0.01 mg/ml | | 0.006 mg/ml | | 0.003 mg/ml | | 0.001 mg/ml | | *P.gingivalis* + | Proteinase K + |
| | control | plant | control | plant | control | plant | control | plant | control | plant | substrate | substrate |
| 1 | 0.661 | 2.847 | 0.742 | 2.287 | 0.683 | 2.929 | 0.676 | 2.484 | 0.637 | 2.18 | 2.623 | 0.188 |
| 2 | 0.612 | 2.636 | 0.833 | 2.505 | 0.704 | 2.672 | 0.769 | 2.53 | 0.53 | 3.083 | 3.1 | 0.22 |
| 3 | 0.618 | 2.386 | 0.528 | 2.587 | 0.54 | 2.152 | 0.624 | 2.927 | 0.57 | 2.848 | 2.718 | 0.174 |
| 4 | 0.44 | 1.676 | 0.458 | 1.856 | 0.443 | 1.641 | 0.46 | 1.594 | 0.412 | 1.576 | 1.8 | 0.234 |
| 5 | 0.506 | 1.753 | 0.455 | 2.08 | 0.485 | 1.45 | 0.447 | 1.51 | 0.424 | 1.602 | 1.422 | 0.216 |
| 6 | 0.404 | 1.969 | 0.397 | 1.895 | 0.394 | 1.661 | 0.339 | 1.39 | 0.407 | 1.455 | 1.505 | 0.187 |
| 7 | 0.542 | 2.541 | 0.656 | 2.569 | 0.677 | 2.738 | 0.681 | 2.824 | 0.555 | 2.627 | 2.911 | 0.207 |
| 8 | 0.432 | 2.505 | 0.699 | 2.519 | 0.572 | 2.675 | 0.708 | 2.955 | 0.704 | 2.403 | 2.374 | 0.192 |
| 9 | 0.512 | 2.783 | 0.472 | 2.338 | 0.627 | 2.82 | 0.556 | 3.085 | 0.537 | 2.927 | 2.175 | 0.185 |
| 10 | 0.363 | 2.101 | 0.334 | 0.954 | 0.358 | 0.934 | 0.336 | 0.976 | 0.303 | 0.973 | 1.456 | 0.219 |
| 11 | 0.262 | 0.905 | 0.384 | 0.759 | 0.33 | 1.248 | 0.335 | 0.827 | 0.311 | 0.964 | 1.349 | 0.21 |
| 12 | 0.398 | 1.087 | 0.362 | 1.193 | 0.331 | 1.1 | 0.348 | 1.124 | 0.306 | 0.924 | 1.413 | 0.179 |
| 13 | 0.345 | 0.97 | 0.323 | 1.263 | 0.29 | 1.03 | 0.352 | 1.333 | 0.341 | 1.082 | 1.319 | 0.262 |
| 14 | 0.375 | 1.038 | 0.339 | 1.187 | 0.35 | 1.33 | 0.319 | 1.045 | 0.326 | 1.141 | 1.519 | 0.278 |
| 15 | 0.321 | 1.11 | 0.329 | 1.162 | 0.317 | 1.097 | 0.269 | 1.176 | 0.322 | 1.148 | 1.537 | 0.197 |
| 16 | 0.346 | 1.077 | 0.286 | 1.111 | 0.347 | 1.169 | 0.32 | 1.305 | 0.321 | 1.032 | 1.536 | 0.226 |
| 17 | 0.329 | 1.164 | 0.323 | 0.865 | 0.327 | 1.137 | 0.329 | 1.012 | 0.271 | 1.08 | 1.41 | 0.245 |
| 18 | 0.316 | 1.168 | 0.319 | 1.121 | 0.291 | 1.082 | 0.274 | 1.088 | 0.294 | 1.013 | 1.535 | 0.263 |
| 19 | 0.302 | 1.141 | 0.281 | 1.064 | 0.34 | 1.069 | 0.337 | 1.1 | 0.3 | 1.098 | 1.422 | 0.241 |
| 20 | 0.449 | 0.869 | 0.338 | 1.295 | 0.377 | 1.103 | 0.329 | 1.1 | 0.304 | 1.152 | 1.242 | 0.25 |
| 21 | 0.334 | 1.089 | 0.237 | 1.061 | 0.326 | 1.083 | 0.273 | 0.963 | 0.303 | 1.029 | 1.348 | 0.24 |
| **Mean** | **0.422238** | **1.6578571** | **0.4330952** | **1.603381** | **0.4337619** | **1.6247619** | **0.43242857** | **1.63561905** | **0.40371429** | **1.58747619** | **1.795904762** | **0.219666667** |
| **±SD** | **0.113682** | **0.7089286** | **0.1666076** | **0.6489186** | **0.1410067** | **0.71088697** | **0.16395535** | **0.78567032** | **0.13069206** | **0.74920702** | **0.589304752** | **0.030254476** |

**Table 2.2** The protease activity (OD) of Lys-gingipain exposed to subinhibitory concentrations of *Dodonaea viscosa* var. *angustifolia* where controls are those for each concentration of crude plant extract using dimethyl sulphoxide (Figure 2.7B)

| Repeats | Protease activity at 405 nm (OD) at various subinhibitory concentrations of crude plant extract | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.02 mg/ml | | 0.01 mg/ml | | 0.006 mg/ml | | 0.003 mg/ml | | 0.001 mg/ml | | *P.gingivalis* + | Proteinase K + |
| | control | plant | control | plant | control | plant | control | plant | control | plant | substrate | substrate |
| 1 | 0.532 | 2.224 | 0.535 | 2.386 | 0.601 | 2.217 | 0.683 | 2.328 | 0.551 | 2.398 | 2.608 | 1.494 |
| 2 | 0.466 | 2.33 | 0.742 | 2.188 | 0.64 | 2.252 | 0.609 | 2.325 | 0.566 | 2.268 | 2.882 | 1.517 |
| 3 | 0.568 | 2.402 | 0.546 | 2.228 | 0.652 | 2.223 | 0.572 | 2.36 | 0.575 | 2.221 | 2.841 | 1.526 |
| 4 | 0.423 | 1.599 | 0.524 | 1.489 | 0.421 | 1.626 | 0.449 | 1.753 | 0.422 | 1.495 | 2035 | 1.836 |
| 5 | 0.427 | 1.516 | 0.528 | 1.505 | 0.437 | 1.582 | 0.523 | 1.854 | 0.444 | 1.92 | 2.133 | 1.929 |
| 6 | 0.374 | 1.612 | 0.487 | 1.368 | 0.485 | 1.48 | 0.412 | 1.453 | 0.462 | 1.306 | 2.09 | 1.935 |
| 7 | 0.444 | 1.534 | 0.438 | 1.447 | 0.434 | 1.539 | 0.359 | 1.177 | 0.459 | 1.356 | 2.396 | 1.972 |
| 8 | 0.473 | 1.481 | 0.497 | 1.555 | 0.482 | 1.358 | 0.443 | 1.323 | 0.448 | 1.527 | 2.144 | 1.959 |
| 9 | 0.409 | 1.623 | 0.436 | 1.419 | 0.384 | 1.503 | 0.476 | 1.509 | 0.407 | 1.579 | 2.036 | 1.991 |
| 10 | 0.49 | 1.539 | 0.544 | 1.624 | 0.447 | 1.622 | 0.524 | 1.631 | 0.495 | 1.751 | 2.017 | 1.95 |
| 11 | 0.45 | 1.69 | 0.455 | 1.673 | 0.43 | 1.638 | 0.444 | 1.685 | 0.469 | 1.431 | 2.136 | 1.98 |
| 12 | 0.438 | 1.348 | 0.455 | 1.381 | 0.448 | 1.461 | 0.446 | 1.293 | 0.414 | 1.58 | 2.049 | 1.986 |
| 13 | 0.475 | 1.314 | 0.409 | 1.378 | 0.507 | 1.214 | 0.45 | 1.172 | 0.44 | 1.327 | 1.924 | 1.925 |
| 14 | 0.408 | 1.323 | 0.463 | 1.455 | 0.471 | 1.529 | 0.46 | 1.52 | 0.494 | 1.575 | 1.923 | 1.938 |
| 15 | 0.398 | 1.571 | 0.426 | 1.511 | 0.361 | 1.378 | 0.363 | 1.648 | 0.378 | 1.473 | 1.909 | 1.994 |
| 16 | 0.419 | 1.485 | 0.485 | 1.491 | 0.402 | 1.349 | 0.435 | 1.574 | 0.421 | 1.837 | 2.495 | 1.971 |
| 17 | 0.436 | 1.465 | 0.47 | 1.433 | 0.42 | 1.416 | 0.442 | 1.418 | 0.415 | 1.479 | 2.693 | 2.033 |
| 18 | 0.428 | 1.473 | 0.412 | 1.591 | 0.465 | 1.379 | 0.394 | 1.279 | 0.407 | 1.48 | 2.629 | 1.999 |
| **Mean** | **0.4476667** | **1.6405** | **0.491778** | **1.61789** | **0.4715** | **1.59811** | **0.471333** | **1.62789** | **0.459278** | **1.66683** | **115.2169444** | **1.885277778** |
| **±SD** | **0.0476285** | **0.329391** | **0.076753** | **0.31225** | **0.082144** | **0.31166** | **0.083548** | **0.3776** | **0.057111** | **0.3327** | **479.1147992** | **0.176625162** |

## Appendix 3

## Ethical clearance

**UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG**
Division of the Deputy Registrar (Research)

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)**
R14/49 Dr M Patel/Ms R Naidoo

| | |
|---|---|
| **CLEARANCE CERTIFICATE** | **M10205** |
| **PROJECT** | Effect of Dodonaea viscosa var. angustifolia on the oral pathogens |
| **INVESTIGATORS** | Dr M Patel/Ms R Naidoo. |
| **DEPARTMENT** | Clinical Microbiology & Infectious Diseases |
| **DATE CONSIDERED** | 26/02/2010 |
| **DECISION OF THE COMMITTEE*** | Approved unconditionally |

**Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.**

**DATE** 26/02/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : M Patel

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**DECLARATION OF INVESTIGATOR(S)**

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...