Anti-*Streptococcus mutans* property of *Uvaria chamae,* and its anticariogenecity

Madiba Mukonazwothe

Degree of Master of Science in Medicine by research only

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

Johannesburg, 2022

DECLARATION

I, Madiba Mukonazwothe declare that this dissertation is my own. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University and has been complemented by referenced works duly acknowledged.

I further declare that this dissertation has the approval by The Human Research Ethics Committee, University of The Witwatersrand. Ethical waiver (W-CBP-200529-03)

m.madiba

Signature of candidate

12/04/2022

Date

DEDICATION

I dedicate this dissertation to my family. Thank you for investing in my future. Your words of encouragement will forever be remembered.

PUBLICATIONS AND PRESENTATIONS

Poster presentation

Anti-*Streptococcus mutans* property of *Uvaria chamae*, and its anticariogenicity. The International Association for Dental Research (IADR) South African Division, 51 st Scientific meeting, Cape town (online), 20-21 October 2021

Oral presentation

Anti-*Streptococcus mutans* property of *Uvaria chamae*, and its anticariogenicity. The International Association for Dental Research (IADR), South African Division, 51 st Scientific meeting, Cape town (online), 20-21 October 2021

ACKNOWLEDGMENT

This dissertation is a result of the combined efforts of many people and institutions who made invaluable contributions to make sure it was a success. I wish to express my sincere gratitude to my supervisors Prof. M Patel and Dr. ZM Gulube. Thank you for your commitment in guiding me through the journey of completing this dissertation. I will forever be grateful for your constant supervision, constructive criticism, expert guidance, and enthusiastic encouragement to pursue new ideas and never-ending inspiration. I would also like to express my gratitude to Dr. MP Ngoepe for assistance with the statistical analysis of the results and Dr. MD Marimani for assistance with the RT-qPCR experiment. To Sibongile Nciki, thank you for all the assistance and support provided during my study. I would also like to acknowledge my colleagues in the Department of Oral Biological Sciences and Clinical Microbiology and Infectious Diseases for the fruitful atmosphere you accorded me with. I would also like to appreciate my sponsor from the Council of Scientific and Industrial Research (CSIR) for financial support.

ABSTRACT

Introduction

Dental caries is the most prevalent oral infection. *Streptococcus mutans* is a major cause of dental caries in humans. These bacteria form biofilms and produce acids and extracellular polysaccharides which contribute to the development of dental caries. Many oral hygiene products containing antimicrobial chemicals have been used to prevent dental caries. In recent years, medicinal plants have been researched for their beneficial properties in the prevention of dental caries. *Uvaria chamae* have been used to treat various infections. It has proven antiparasitic, antiplasmodial, antidiabetic, antimicrobial, and antioxidant properties. Although the anti-*S. mutans* activity of this plant has been reported, its effect on the virulence properties has not been studied. Therefore, this study aimed to investigate the antimicrobial activity of *U. chamae* roots extracts on *S. mutans* virulence factors.

Methods and materials

Stock cultures of *S. mutans* were obtained from the Oral microbiology laboratory, the University of the Witwatersrand, and the plant extracts were provided by Dr. Ogunyemi Olajide Oderinlo from Nigeria. The plant extracts were prepared using methanol, dichloromethane, hexane, ethanol, and methanol: water. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the microdilution technique. Based on the MIC, the solvent with the best results was selected and three different concentrations were evaluated for their effect on biofilm formation, acid, and extracellular polysaccharides production in *S. mutans*. The effect of the plant extract on the expression of virulence genes (*gtfB, gtfC, spaP, IDH, atpD, vicR, brpA,* and gbpB) was also investigated using RT-qPCR. The results were analyzed using the one-way ANOVA and Wilcoxon Rank Sum Test.

Results

The mean MIC of *U. chamae* roots extracts against *S. mutans* ranged between 0.02 and 1.25 mg/ml and the MBC ranged between 0.04 and 1.25 mg/ml. The dichloromethane plant extract showed the best antibacterial activity against all the five cariogenic *S. mutans* strains with an average MIC and MBC of 0.02 and 0.04 mg/ml respectively and was used in the subsequent experiments such as the biofilm, acid, EPS, and RT-qPCR assay. At 6 hours, exposure to 0.005, 0.01, and 0.02 mg/ml of the plant extract reduced biofilm formation by 39.70, 59.17, and 76.82 % respectively. At 24 hours, the percentage reduction of the biofilm counts significantly improved up to 91 %. Not much difference in the test results was observed between 24 and 30 hours. The plant extract also significantly inhibited acid production *(*$p < 0.01$). The roots extract did not inhibit the production of soluble and insoluble extracellular polysaccharides. Furthermore, a significant decline in the transcription of virulence genes (*gbpB, vicR, brpA, spaP, gtfB, gtfC, atpD,* and *IDH)* was observed in the presence of the plant extract.

Conclusion

The dichloromethane extracts showed the best antibacterial activity. At subinhibitory concentrations, this plant extract significantly inhibited biofilm formation, acid production, and virulent gene expression by *S. mutans*. Therefore, this suggests that *U. chamae* has the potential to control and prevent dental caries.

TABLE OF CONTENT

DECLARATION ..... **i**

DEDICATION ..... **ii**

PUBLICATIONS AND PRESENTATIONS ..... **iii**

ACKNOWLEDGMENT ..... **iv**

ABSTRACT ..... **v**

TABLE OF CONTENT ..... **vii**

LIST OF TABLES ..... **xii**

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

LIST OF ABBREVIATIONS AND ACRONYMS ..... **xvi**

Chapter 1: Introduction and literature review **1**

Introduction 1

1. Literature review 2

1.1. Dental caries 2

1.2. Classification of dental caries 4

1.3. Microbiology of dental caries 7

1.3.1. *Streptococcus mutans* 7

1.4. Virulence factors of *Streptococcus mutans* 9

1.4.1. Biofilm production 10

1.4.2. Acid production 11

1.4.3. Extracellular polysaccharides (EPS) production 13

1.5. *Lactobacillus spp.* 15

1.6. The role of diet in the occurrence of dental caries 15

1.7. Role of saliva in the caries process 16

1.8. Prevention of dental caries 17

1.9. *Uvaria chamae* 18

1.9.1. Taxonomy of *Uvaria chamae* 18

1.9.2. Description of *Uvaria chamae* 18

1.9.3. Origin and distribution of *Uvaria chamae* 19

1.9.4. Traditional uses of *Uvaria chamae* 19

1.9.5. Major chemical constituents of *Uvaria chamae* 20

1.10. The role of medicinal plants in oral diseases 24

1.11. Anticariogenic effects of plant extracts against *Streptococcus mutans* 25

1.11.1. *Artemisia princeps* 25

1.11.2. Ethyl gallate 26

1.11.3. *Rhodiola rosea* 26

1.11.4. *Chamaecyparis obtusa* 27

1.11.5. *Prangos acaulis Bornm.* 27

1.12. Aim 27

1.13. Objectives 28

Chapter 2: The antibacterial effect of *Uvaria chamae* dichloromethane extract against *Streptococcus mutans* 29

2.1. Introduction 29

2.2. Methods and materials 30

2.2.1. Plant materials 30

2.2.2. Bacterial cultures 30

2.2.3. Antimicrobial activity 31
2.3. Results 32
2.4. Discussion 34
2.5. Conclusion 35
Chapter 3: The effect of *Uvaria chamae* dichloromethane extract on the biofilm formation by *Streptococcus mutans* 36
3.1. Introduction 36
3.2. Methods and materials 37
3.2.1. Bacterial cultures 37
3.2.2. Plant materials 38
3.2.3. Biofilm assay 38
3.2.4. Statistical analysis 39
3.3. Results 39
3.4. Discussion 44
3.5. Conclusion 45
Chapter 4: The effect of *Uvaria chamae* dichloromethane extract on *Streptococcus mutans* acid production 46
4.1. Introduction 46
4.2. Method and materials 48
4.2.1. Plant extractions 48
4.2.2. Bacterial cultures 48
4.2.3. Acid production assay 48
4.2.4. Statistical analysis 49
4.3. Results 49

4.4. Discussion 57

4.5. Conclusion 58

Chapter 5: The effect of *Uvaria chamae* dichloromethane extract on soluble and insoluble extracellular polysaccharides production by *Streptococcus mutans* 60

5.1. Introduction 60

5.2. Method and materials 62

5.2.1. Bacterial cultures 62

5.2.2. Plant materials and extraction 62

5.2.3. Soluble extracellular polysaccharide production assay 63

5.2.4. Insoluble Extracellular Polysaccharide production assay 63

5.2.5. Statistical analysis 64

5.3. Results 64

5.4. Discussion 67

5.5. Conclusion 68

Chapter 6: The effect of *Uvaria chamae* extract on the expression of virulence genes in *Streptococcus mutans* 69

6.1. Introduction 69

6.2. Methods and materials 71

6.2.1. Plant materials and preparation 71

6.2.2. Bacterial cultures and growth conditions 71

6.2.3. Total RNA extraction from microbial cultures 71

6.2.4. Generation of complementary DNA by reverse transcription 72

6.2.5. Examining the expression level of virulence genes by RT-qPCR 72

6.3. Data analysis 73

6.4. Results 74

6.4.1. Variable virulence gene expression in treated and untreated *S. mutans* strains 74

6.5. Discussion 78

6.6. Conclusion 79

Chapter 7: Overall discussion, conclusion, limitations of the study, and future research ... 80

7.1. Overall discussion 80

7.2. Clinical implications 81

7.3. Conclusion 82

7.4. Limitations to the study 82

7.5. Future research 83

References 84

Appendices 99

Appendix I: Media composition and preparation 99

Appendix II: Primer preparation 101

Appendix III: Statistical analysis 104

Appendix IV: Ethical clearance 116

Appendix VI: Turn it in report 117

LIST OF TABLES

Table 2.1	MIC and MBC of *U. chamae* roots extract against *S. mutans.*	33
Table 2.2	Summary results of mean MBC and MIC of *U. chamae* roots extract against *S. mutans*	34
Table 3.1	The effect of 0.005, 0.01, and 0.02 mg/ml of *U. chamae* roots extract on *S. mutans* biofilm formation	41
Table 3.2	Statistical analysis of the data obtained in the biofilm formation assay	42
Table 4.1	The effect of 0.005, 0.01 and 0.02 mg/ml of *U. chamae* extract on *S. mutans* acid production	51
Table 4. 2	The effect of 0.005 mg/ml of *U. chamae* on *S. mutans* counts in acid assay	52
Table 4.3	The effect of 0.01 mg/ml of *U. chamae* on *S. mutans* counts in acid assay	53
Table 4.4	The effect of 0.02 mg/ml of *U. chamae* extract on *S. mutans* counts in acid assay	54
Table 4.5	Statistical analysis of the data obtained in the acid production assay	55
Table 5.1	The effect of 0.005, 0.01, and 0.02 mg/ml of *U. chamae* on the production of soluble and insoluble extracellular polysaccharides by planktonic cells of *S. mutans*.	65
Table 5.2	Statistical analysis of the data obtained in the extracellular polysaccharides production assay	66
Table 6.1	Primer sequences used to amplify the virulence genes and the 16 S ribosomal RNA reference genes in *S. mutans* RT-qPCR	73

Table 6.2 Fold change in gene expression in SM1 strain 75

Table 6.3 Fold change in gene expression in SM12 strain 75

Table 6.4 Statistical analysis of SM1 and SM12 strains obtained in RT-qPCR 76

LIST OF FIGURES

Figure 1.1	a) Carious lesions on different sites of teeth and b) patient with white spot lesions at the Bucco-cervical surfaces (Deveci et al., 2018)	4
Figure 1.2	Classification of dental caries according to the American Dental Association Classification System (Ng and Fida, 2016).	6
Figure 1.3	Microscopic depiction of *S. mutans* (Ranganathan and Akhila, 2019b).	9
Figure 1.4	Schematic illustration of cariogenic biofilm formation in the presence of fermentable sugar (Kalesinskas et al., 2014).	11
Figure 1.5	Schematic illustration of sucrose metabolism by *S. mutans* (Lemos et al., 2019).	13
Figure 1.6	The percentage composition of polysaccharide matrix (Krzyściak et al., 2014)	14
Figure 1.7	Stephan's curve illustrating the changes in plaque pH over time following a sucrose rinse (Bilbilova, 2020).	16
Figure 1.8	*U. chamae* tree and fruits (Teanpaisan et al., 2014)	19
Figure 1.9	Basic structure of alkaloids (Achilonu and Umesiobi, 2015)	21
Figure 1.10	Basic structure of triterpenoid (Xia et al., 2014).	21
Figure 1.11	Basic structure of common tannins (Amorim et al., 2012)	22
Figure 1.12	Basic structure of flavonoid (Nishiumi et al., 2011)	23

Figure 3.1	Biofilm assay (a) Beaker containing glass slides after incubation at 37^{o}C (b) Washing of biofilm c) Removal of the biofilm by scraping off the attached cells with a clean slide, (d) *S. mutans* colonies on blood agar.	39
Figure 3.2	The effect of *U. chamae* on *S. mutans* biofilm formation	42
Figure 3.3	Percentage reduction in *S. mutans* biofilm formation by *U. chamae*	43
Figure 4.1	Acid production assay a) Grow *S. mutans* at 37^{o}C for 48 hours b) pH measurements c) *S. mutans* colonies on blood agar	49
Figure 4.2	The effect of *U. chamae* on *S. mutans* acid production in acid assay	55
Figure 4.3	The effect of *U. chamae* on the growth of *S. mutans* in acid production assay	56
Figure 5.1	Illustration of the efficient metabolism of sucrose by *S. mutans*	62
Figure 5.2	The effect of *U. chamae* on soluble and insoluble extracellular polysaccharides by *S. mutans*	66
Figure 5.3	Percentage reduction of *S. mutans* soluble and insoluble extracellular polysaccharides production by *U. chamae*	67
Figure 6.1	Virulence genes in *Streptococcus mutans*	70
Figure 6.2	Target gene expression in treated and untreated *S. mutans* (SM1) strain	76
Figure 6.3	Target gene expression in treated and untreated *S. mutans* (SM12) strain	77

LIST OF ABBREVIATIONS AND ACRONYMS

µl	Microliters
ABS	ATP-binding cassette
*brp*A	Biofilm regulatory protein
CDC	Centre for disease control and prevention
cDNA	Complementary DNA
CFU	Colony Forming Unit
CHX	Chlorohexidine
CO_2	Carbon dioxide
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
eDNA	Extracellular DNA
EPS	Extracellular polysaccharides
F-ATPase	ATP synthase
Fruct-6-P	Fructose-Phosphate
gbpA	Glucan binding protein A
gbpD	Glucan binding protein D
GlcN-6-P	Glucosamine-6-phosphate Glucosyltransferase B
gtfB	Glucosyltransferase B
gtfC	Glucosyltransferase C
gtfD	Glucosyltransferase D
Hrs	hours
ICDAS	International Caries Detection and Assessment System
INT	Iodonitrotetrazolium chloride
IPS	Intracellular polysaccharides
LDH	Lactate dehydrogenase
LTA	Lipoteichoic acid

MBC	Minimum Bactericidal Concentration
mg	Milligrams
mg/ml	Milligrams per milliliter
MIC	Minimum Inhibitory Concentration
mins	Minutes
mL	Milliliter
nm	Nanometers
OD	Optical density
PBS	Phosphate Buffered Saline
QS	Quorum sensing
RNA	Ribonucleic acid
RT-qPCR	Real-Time Polymerase Chain Reaction
S. mutans	*Streptococcus mutans*
spp	Species
TB	Tryptone Broth
TSB	Tryptone Soy Broth
U. chamae	*Uvaria chamae*

Chapter 1: Introduction and literature review

Introduction

The human oral cavity harbour up to 700 different types of commensal bacteria, and only a few cause infections such as dental caries (Abid et al., 2015). Dental caries is the most common chronic infectious disease in the oral cavity and the most prevalent bacterial infection affecting all age groups (Yactayo-Alburquerque et al., 2021). In South Africa, 60% of 6 years old have had dental caries (Bhayat and Chikte, 2019). Factors such as the inadequate ratio of dentists per 1,000 population, cost of service, lack of oral health service providers due to geographic isolation, and shortage of healthcare providers play an important role in delayed diagnosis and compromised health status (Tiwari et al., 2021).

Dental caries is caused by the oral bacteria *Streptococcus mutans* which ferments dietary carbohydrates and produces organic acids that demineralize the enamel of the tooth (Culp et al., 2021). This bacterium alters the ecology of dental plaque primarily due to its acidic metabolic products. In addition, *S. mutans* may express certain virulence factors to maintain their ecological niche in the oral cavity (Li et al., 2016). These bacteria form biofilms and produce extracellular polysaccharides which also contribute to the development of caries. Although dental caries is not a life-threatening infection, it has become a major financial burden to the public health system due to its costly and ongoing treatment, which consumes between 5 to 10 % of the health budget in developed countries (Ngabaza et al., 2018;Lee et al., 2019).

The prevention of dental caries is based on reducing oral bacteria, maintaining an alkaline environment, and providing appropriate dietary advice (Jacob and Nivedhitha, 2018). Antibacterial agents are recommended for the prevention of dental caries. Chlorhexidine (CHX) is an antibacterial compound against most bacterial species found in the oral cavity. However, there is controversy on the use of chlorhexidine for caries prevention due to common side effects such as irritation of the mucosa, tooth staining, taste alteration, and formation of calculus on the tooth surfaces (Moghadam et al., 2020). Fluoride is widely used as a highly effective anticaries

agent (Villa et al., 2018) because it promotes caries lesion remineralization and inhibits demineralization of tooth surfaces subjected to organic acids (Almohefer et al., 2018).

In contrast, natural products have been proven to be safe and they contain bioactive compounds with potential therapeutic applications in dentistry (Jacob and Nivedhitha, 2018). *Uvaria chamae* have been used throughout the world to treat infections. This plant synthesizes various secondary metabolites from its root, stem, leaf, and fruit which has beneficial medicinal properties (Abu et al., 2018). It has proven antiparasitic, antiplasmodial, antidiabetic, antimicrobial, and antioxidant activity. Although the anti-*S. mutans* activity of this plant has been reported, its effect on the virulence properties has not been studied.

Therefore, this study aimed to investigate the antimicrobial effect of *U. chamae* roots extracts on acid production, biofilm formation, extracellular polysaccharides production, and virulence gene expressions by *S. mutans*. Antibacterial activity was studied using a microdilution technique. Biofilm assay was performed using a glass slide technique. In the acid assay, sequential pH measurements were performed. Extracellular polysaccharides assay was performed using the phenol-sulphuric acid technique. The expression of virulence genes was quantified using Real-Time Polymerase Chain Reaction (RT-qPCR).

1. Literature review

1.1. Dental caries

Dental caries as shown in Figure 1.1a is a chronic bacterial disease that involves the destruction of tooth hard tissue structure. The term dental caries originates from the Latin word “caries”, which means decay (Rathee and Sapra, 2019). Untreated dental caries in permanent teeth affect 2.3 billion people, and it is the most prevalent non-fatal non-communicable disease worldwide (Chikte et al., 2020). Dental caries is caused by acid-producing bacteria e.g *Streptococcus mutans, Lactobacillus acidophilus, Actinomyces viscosus,* and *Nocardia spp* that ferment dietary carbohydrates in the dental plaque and produces organic acids such as lactic, acetic, formic, and propionic acids (Chu et al., 2016;Kabra et al., 2012). These acids have been reported to readily dissolve the enamel and

dentine of the teeth (Featherstone, 2008). Lifestyle behavioral factors such as poor oral hygiene, frequent consumption of refined carbohydrates, frequent use of oral medications that contain sugar, and inappropriate methods of feeding infants contribute to the risk of developing dental caries (Pitts et al., 2017a).

Figure 1.1b presents the first clinical sign of dental caries called the “white spot”. This is the first sign that can be seen with human eyes and as the disease progress into the enamel, they can be detected by radiographs (Featherstone, 2008). During the disease process, the organic substances on the tooth surfaces are broken down and demineralization of the calcified tissues of the tooth occurs (El Sherbiny, 2014). If caries progresses it can lead to cavitation, the condition can cause considerable pain and discomfort. Dental caries can also spread to the dental pulp and cause infection and ultimately sepsis and tooth loss (Peres et al., 2019). The progression of dental caries is determined by the equilibrium status between protective factors which are components of the saliva e.g carbonate (Ca^{2+}), phosphates, fluoride, protective proteins of the pellicle, saliva antibacterial components, and pathological factors (cariogenic bacteria, a dysfunction of the salivary glands, frequent consumption of carbohydrates). A preponderance of pathological factors results in the processes of demineralization and dental caries (Strużycka, 2014). Over time, this process leads to either caries lesions- or the repair and reversal of a lesion. Reversal of the lesions occurs through the process of remineralization, in which calcium, phosphate, and fluoride are incorporated in the areas damaged due to demineralization processes, resulting in a stronger, fluoridated mineral (Pitts et al., 2017a).

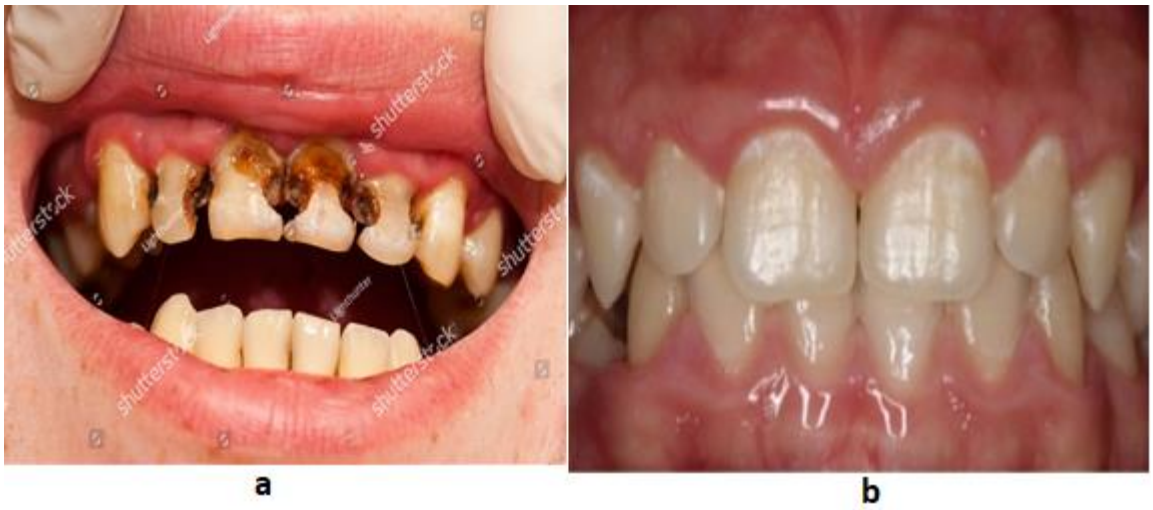

Figure 1.1 a) Carious lesions on different sites of teeth adapted from https://image.shutterstock.com/z/stock-photo-neglected-teeth-cleaned-for-restoration-98850518.jpg and b) patient with white spot lesions at the Bucco-cervical surfaces (Deveci et al., 2018).

1.2. Classification of dental caries

The oldest dental caries lesions classification is the G.V Blacks classification. The G.Vs Black classification was first introduced in the year 1908 (Macri and Chitlall, 2017). This classification was based on the type of tooth and the tooth surface where caries was more likely to begin e.g occlusal fissures, proximal contacts, and cervical areas. Based on these observations, he developed a classification of caries lesions dependent on the position of a lesion and prescribed a cavity design regardless of the size and extent of the lesion (Nagarajan and Anjaneyulu, 2019). Other epidemiologic studies have used the modified versions of Klein and colleagues, decayed, missing, and filled (DMF) method to measure the prevalence and severity of caries. The Decayed-Missing-Filled (DMF) was proposed by the World Health Organisation (WHO) and it is the most common method used in oral health epidemiology for assessing and measuring dental caries (Campus et al., 2019). This method records both current and past caries experiences for an individual or group by numerically counting affected teeth per individual collected at either tooth (DMFT) or tooth surface level (DMFS) (Tellez and Lim, 2020).

Other indexes were designed to describe additional stages of the caries (Young et al., 2015). The International Caries Detection and Assessment System (ICDAS) was developed to create a caries detection method that might be used universally to measure caries at different stages (Pradeep, 2020). This method assesses both the presence of caries lesions and their severity from initial and reversible states to cavitated lesions, and classify them in increasing severity codes from 0 to 6 (Coelho, 2020). In 2008, the American Dental Association (ADA) convened a group of experts and stakeholders to begin the development of a Caries Classification System (CCS) that would be useful in clinical practice while incorporating up-to-date scientific evidence (Campus et al., 2019). The ADA CCS scores each surface of the dentition based on the tooth surface, presence or absence of a caries lesion, anatomic site of origin, the severity of the change, and estimation of lesion activity. The ADA CCS uses categories such as sound, initial, moderate, advanced to score a tooth surface's clinical appearance. The initial, moderate, and advanced categories are each subdivided to account for variations in appearance- Figure 1.2.(Young et al., 2015).

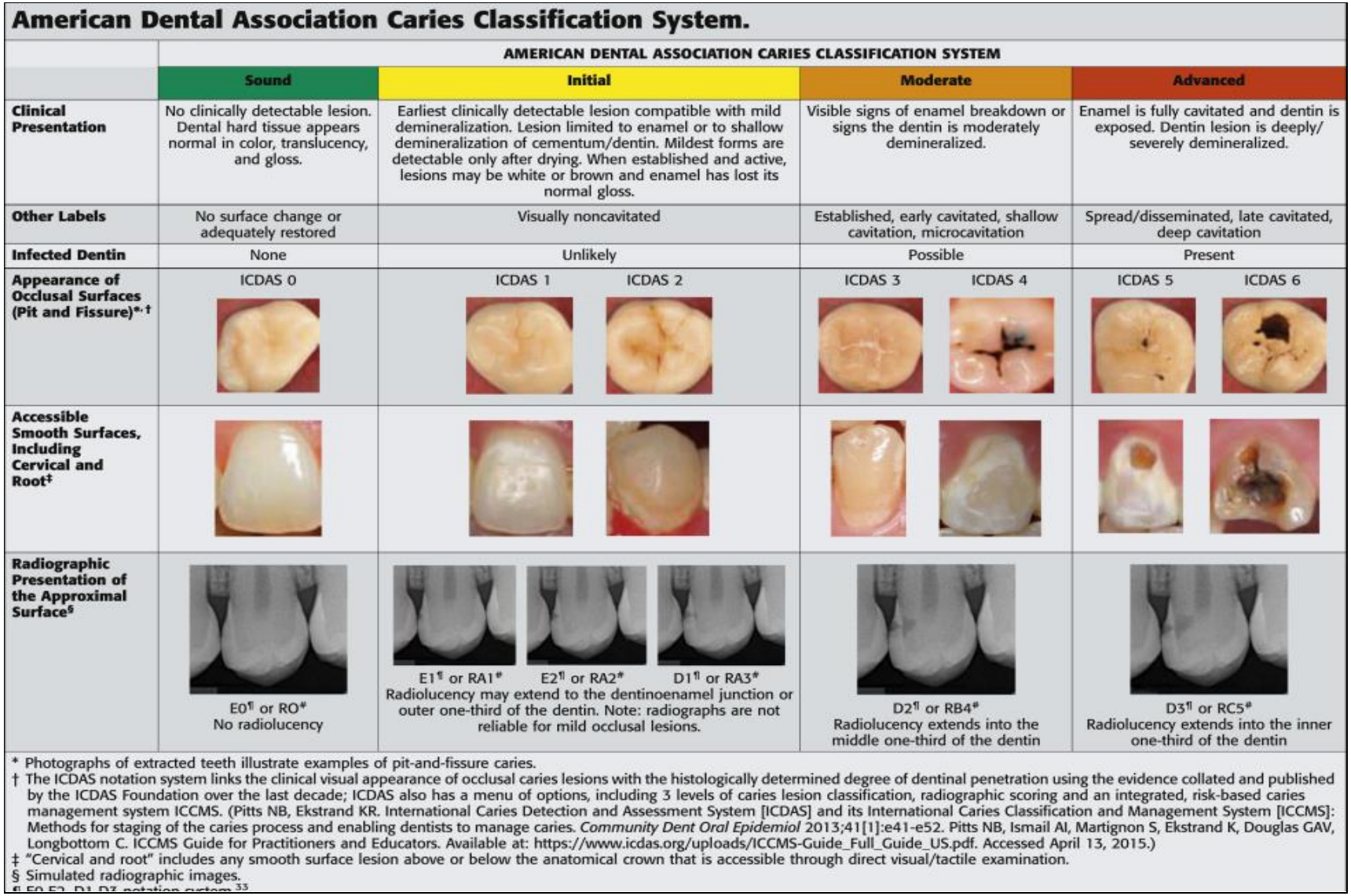

Figure 1.2: Classification of dental caries according to the American Dental Association Classification System (Ng and Fida, 2016).

1.3. Microbiology of dental caries

Dental caries was believed to be caused by only a few Gram-positive bacterial species, such as *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus* spp. These findings were based on cultivation studies and the determination of the cariogenecity of these bacteria. This is termed the specific plaque hypothesis. Recent studies have shown that different population groups and individuals are susceptible to dental caries even when they have a low level of *S. mutans*, and vice versa. This led to the formulation of the ecological plaque hypothesis which was proposed by Phil Marsh with an attempt to explore dental caries etiology (Al-Shahrani, 2019). According to the "Ecological Plaque Hypothesis", the disease is caused by an imbalance in the total microflora due to ecological stress, resulting in an enrichment of oral pathogens (Rosier et al., 2014). Recently, the ecological hypothesis was extended and highlighted the role of the metabolic activity of the oral microbiota rather than its composition as the principal modulator of the environment (Nyvad and Takahashi, 2020). According to the extended ecological plaque hypothesis, changes in the local environment, e.g. frequent carbohydrate availability, assumedly favour certain representatives of the oral biofilm and thus lead to a distinct shift in the microbiota composition towards a higher proportion of acidogenic and acid-tolerant species (Anderson et al., 2018). Previous studies suggested that the main pathogenic bacteria in dental caries are *Streptococcus mutans* and *Lactobacillus* spp (Ahirwar et al., 2019a).

1.3.1. *Streptococcus mutans*

Streptococcus mutans is a Gram-positive bacterium with thick cell walls that are composed of a layer of peptidoglycan (murein) and teichoic acids. These thick walls are important in preventing osmotic lysis of the cell protoplast and they provide the rigidity needed to maintain the shape of the cell (Ramawat and Mérillon, 2015). *S. mutans* are commonly found in the human oral cavity and contribute significantly to tooth decay (David et al., 2011). These bacteria generally appear as pairs or chains: spherical under a light microscope (Hardie and Whiley, 1997). Their diagram is presented in Figure 1.3. On Mitis Salivarius Agar (MSA), they grow as highly convex colonies. Unlike other oral streptococci, majority of *S. mutans* strains can grow on a selective media, Mitis Salivarius agar containing 20% sucrose and 0.2% units/ml of bacitracin (Rajendran, 2009).

Streptococcus mutans was first isolated from human cavities and received the name *S. mutans* because Clarke believed that it was a mutant version of *Streptococcus* (Clarke, 1924). The mutans streptococci comprise a group of seven species, of which *S. mutans* and *S. sobrinus* are the predominant species isolated from human saliva and dental plaque (Banas, 2004a). This bacterium is first acquired by infants soon after their first tooth emerges and mothers are considered to be a major source since identical genotypic profiles of the isolated strains are shared between mother and child (Matsui and Cvitkovitch, 2010). In some cases, *S. mutans* can coexist with *S. sobrinus*, and their collaboration leads to various oral clinical conditions (Ranganathan and Akhila, 2019a).

Strains of *S. mutans* are classified into four different serological groups, which are *c, e, f,* and *k*. This classification is based on the composition of the cell-surface rhamose-glucose polysaccharide, which is composed of a backbone of rhamnose and side chains of *a*1,2 (serotype *c*), *b*1,2-(serotype *e*), and *a*1,3-(serotype *f*) glucosidic residues (Nakano et al., 2008). Serotype *c* accounts for approximately 70 to 80% of *S. mutans* strains in the oral cavity, followed by *e* (20%) and less than 5% of serotype *f* (Nakano et al., 2010). Up to date, the genome of only two serotypes of *S. mutans* are known. The genome analysis provides further insight into how *S. mutans* have adapted to surviving the oral environment through resource acquisition, defense against host factors, and the use of gene products that maintain its niche against microbial competitors. The recent completion of three *S. mutans* genome sequences (UA159, NN2025, and LJ23) indicates a large degree of diversity and genome rearrangement within the species (Al-Shahrani, 2019). Of the four *S. mutans* serotypes, serotype c is the most predominant oral isolate, with over 70% of strains isolated from dental plaque (Al-Shahrani, 2019). The cariogenic potential of *S. mutans* includes the ability to metabolize numerous dietary carbohydrates into organic acids, and survive under low pH environmental stress conditions. This bacterium also synthesizes extracellular polysaccharides polymers which promote bacterial adherence and accumulation on tooth surfaces (Garcia et al., 2021).

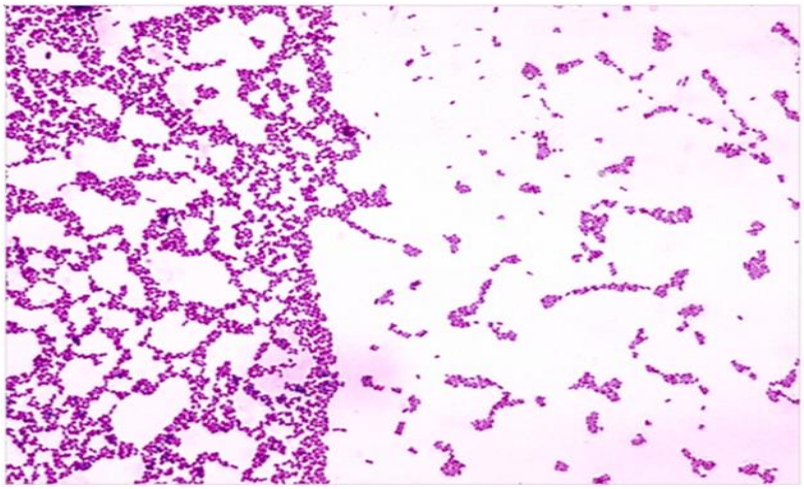

Figure 1.3: Microscopic depiction of *Streptococcus mutans* (Ranganathan and Akhila, 2019b).

1.4. Virulence factors of *Streptococcus mutans*

Streptococcus mutans possess various virulence factors that enable them to ferment numerous carbohydrates and produce organic acids. Unlike other oral plaque, *S. mutans* can accumulate in large numbers in the presence of sucrose (Alejandra and Daniel, 2020). These bacteria secrete glucosyltransferase on their cell wall, which allows the bacteria to produce polysaccharides from sucrose. The sticky, highly hydrated extracellular matrix created by dental plaque bacteria, largely comprised of glucans, which act as a glue that encourages plaque formation (Chen et al., 2016). Glucans mediate the attachment of bacteria to the tooth surface and other members of the oral biota and form biofilm (Argimón et al., 2013). Each of these virulence factors works coordinately to change dental plaque ecology. The ecological changes are characterized by increased proportions of *S. mutans* and other species that are similarly acidogenic and aciduric. The selection for a cariogenic flora increases the magnitude of the drop in pH following the fermentation of available carbohydrates and increases the probability of enamel demineralization (Banas, 2004b). The main virulence factors associated with cariogenicity include adhesion, biofilm formation, acidogenicity, acid tolerance, and extracellular polysaccharides production (Li et al., 2020).

1.4.1. Biofilm production

Biofilm is an organized aggregate of microorganisms living within an extracellular polymeric matrix that they produce and irreversibly attached to the living surface which will not be cleared unless rinsed quickly (Jamal et al., 2018). The formation of cariogenic biofilm is regulated by genes that are responsible for microbial adhesion and biofilm formation. The genes *gbpB*, *sacB* (*ftf*), and *vicR* are involved in sucrose-dependent adhesion, and *spaP* is involved in sucrose-independent adhesion. In addition to the genes, *S. mutans* encode several surface-associated glucan-binding proteins, gbpA, gbpB, gbpC, and gbpD (Gabe et al., 2019). The major role of the gbpB includes promoting bacterial adhesion, cell aggregation, and biofilm maturation process (Fujita et al., 2011). The gene *ftf* catalyzes the formation of fructans from sucrose. Fructan polymers serve as the storage of extracellular nutrients, which may be utilized during periods of nutrient deprivation. The *vicR* gene encodes a *vicR* response regulator, which is an essential part of VicRKX TCS. It is known to regulate a set of genes (*gtfB, gtfC, gtfD,* and gbpB) that code for the synthesis of glucan matrix, which is crucial for adhesion to a smooth tooth surface (Senadheera et al., 2005). *spaP* gene participates in bacterial adherence to teeth via interaction with the salivary pellicle (Lemos et al., 2019).

The formation of biofilm involves several stages as presented in Figure 1.4. In the beginning, salivary proteins are selectively adsorbed to the tooth enamel. This forms an initial layer of material called salivary pellicle, which acts as a substrate for the fixation of cariogenic bacteria such as *S. mutans*. The salivary pellicle is formed by salivary components namely proline-rich proteins, amylase, lysozyme, histatin, peroxidase, mucin, and bacterial components, e.g., *ftf, Gtf* and lipoteichoic acid (Krzyściak et al., 2014;Castro et al., 2006a). The second step is bacterial adhesion to the pellicle. Early colonizers such as oral streptococci and *Actinomyces spp* recognize the binding proteins i.e., α-amylase and proline-rich glycoproteins in the acquired pellicle and bind to the pellicle. The early attachments are primarily based on hydrogen bonds, hydrophobic interactions, calcium bridges, van der Waals forces, acid-base interactions, and electrostatic interactions. Chemical forces become predominant at a later stage (Huang et al., 2011).

Specific interactions between the pathogen and components of the salivary coating are mediated by the streptococcal antigen I/II protein. This polypeptide plays an important role in the binding activities, such as binding with the salivary glycoproteins, host cell receptors, and soluble extracellular matrix glycoproteins (Xu et al., 2007). Secondary colonizer species such

as *Prevetella intermedia*, *Prevetella loescheii*, *Capnocytophaga* spp, and *Fusobacterium nucleatum* adhere to the microbes that are already present in the dental plaque. After the adherence of the oral pathogen into the tooth surfaces, the dental biofilm undergoes maturation and numerous microbial interactions such as synergistic and antagonistic microbial interactions occur (Pitts et al., 2017a;Aruni et al., 2015).

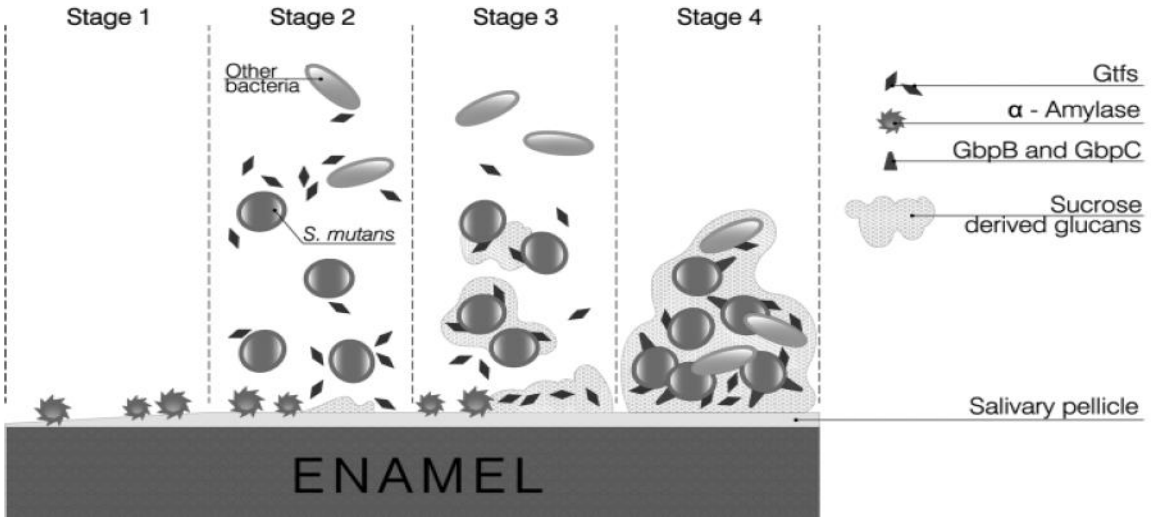

Figure 1.4: Schematic illustration of cariogenic biofilm formation in the presence of fermentable sugar (Kalesinskas et al., 2014).

1.4.2. Acid production

The production of organic acids such as lactic acid from dietary carbohydrates metabolism is another major virulence factor in *S. mutans*. Lactic acid is produced via the glycolysis pathway, a biochemical reaction that occurs in the cytoplasm of a cell (Gomes et al., 2018). This acid erodes the hydroxyapatite of dentin and enamel thereby leading to the initiation and progression of caries. When acid is released into the environment, it dissociates as anions and conjugates protons, thereby reducing the pH of the environment (Kianoush et al., 2014). These protons may also diffuse into the cytoplasm of a cell through bacterial membranes, thereby acidifying the cytoplasm (Quivey et al. 2001). As *S. mutans* carry out glycolysis at a pH lower than 4.0, the organism protects its acid-sensitive glycolytic enzymes by transporting the protons across the cell membrane through the membrane-associated F-ATPase (Matsui and Cvitkovitch, 2010). *S. mutans* is also capable of surviving at low pH and continue with metabolic activities.

This is termed aciduricity (Alejandra and Daniel, 2020). The genes *atpD, aguD, brpA*, and *relA* in *S. mutans* enable it to overcome an acidic environment (You, 2019). The gene *atpD* in *S. mutans* encodes the F1F0ATPase. F1F0-ATPase is a proton pump that discharges H+ from within the bacteria to the outside, to overcome acid stress and maintain acid tolerance, which may confer a selective advantage over other members of dental biofilm (Argimón and Caufield, 2011). The gene *relA* and *brpA* are involved in the formation of dental plaque, acid, and oxidative stress tolerance mechanisms (He et al., 2019). In addition, *aguD* encodes the agmatine deiminase system, which produces alkali by converting agmatine to carbon dioxide, ammonia, and putrescine, enabling it to overcome acid stress and maintain acid tolerance (Griswold et al. 2006).

During sucrose metabolism (Figure 1.5), sucrose is broken down into fructans and glucans by the action of fructosyltransferase and glucosyltransferase enzymes respectively. The enzyme DexA breaks glucans down the α1, 6-linkage thereby yielding maltodextrans whereas fructans are degraded by the enzyme fructanase enzyme FruA to yield fructose which is used for energy production (Lemos et al., 2019). After being transported into the cell, oligosaccharides (e.g maltodextrans) are degraded into monosaccharides by the enzyme DexB glucosidase. The transport of oligosaccharides is primarily conducted by the activity of ATP binding cassette (ABC) transporters encoded in the genome of *S. mutans*. The ATP binding cassette (ABC) transporters include multiple sugar metabolism (MSM) and malXFGK transport systems. The predominant route for the uptake of mono- and disaccharides by *S. mutans* is the phosphoenolpyruvate: sugar phosphotransferase system (Moye et al., 2014). In the intracellular environment, carbohydrates are phosphorylated and processed to fructose-6-phosphate (Fru-6-P) and fermented by glycolysis with the production of organic acids, mainly lactic acid. Furthermore, glucosamine-6-phosphate (GlcN-6-P) is synthesized from Fru-6-P, which is a precursor for cell wall biosynthesis. When carbohydrates are abundant, cells can produce an intracellular polysaccharide (IPS), a glycogen-amylopectin polymer that can be stored as intracellular granules and is used as an energy source reserve during starvation (Lemos et al., 2019).

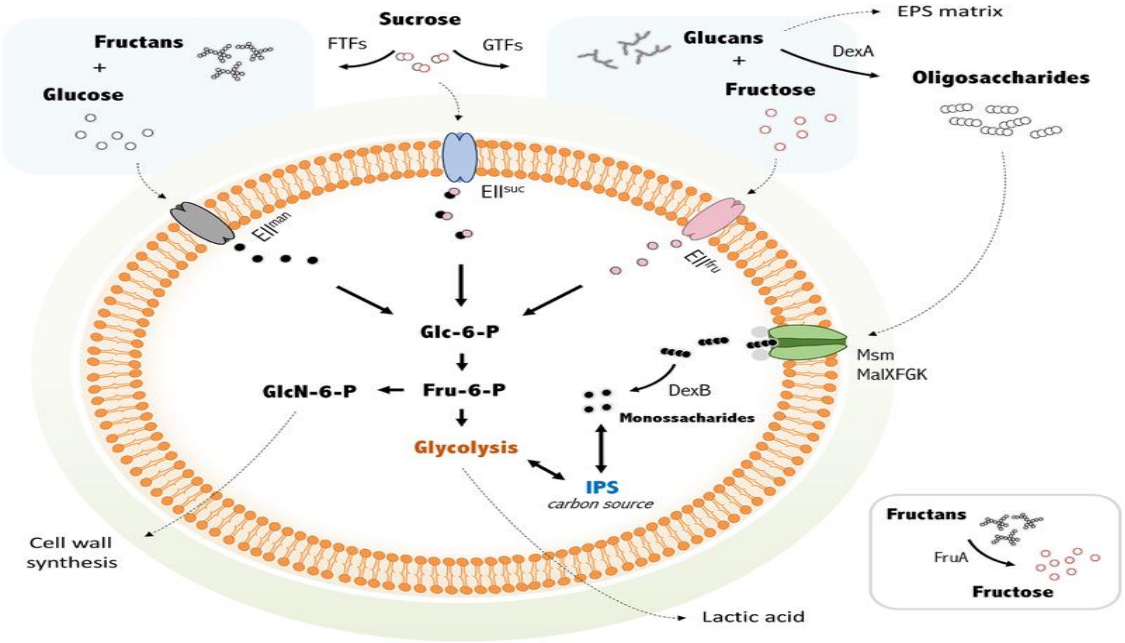

Figure 1.5: Schematic illustration of sucrose metabolism by *S. mutans* (Lemos et al., 2019).

1.4.3. Extracellular polysaccharides (EPS) production

Extracellular polysaccharides are high molecular weight sugar-based polymers that are synthesized and secreted by different microbes. These sugar-based polymers are a critical virulence factor in the production of biofilm formed in the presence of sucrose (Bowen and Koo, 2011). The composition of the polysaccharide matrix is shown in Figure 1.6 (Krzyściak et al., 2014). The production of EPS promotes the cariogenic potential of dental biofilms and their resistance to oral hygiene measures (Decker et al., 2014). Various genes are involved in the formation of extracellular polysaccharides. The genes gtfs, *gtfB, gtfC,* and *gtfD* encode glycosyltransferase (GTF) B, GTF C, and GTF D respectively whereas *ftf* encode fructosyltransferase (Shemesh et al., 2007). The gene *gtfB* synthesizes water-insoluble polysaccharides containing α1,3-linked glucans, which contributes to the scaffolding of the EPS matrix and facilitates cell aggregation in stable biofilms. The gene *gftC* catalyzes the synthesis of a mixture of water-insoluble and alkali-soluble glucan from sucrose, with both α-1,3 and α-1,6-linked glucans, which are required for plaque formation and structurally stable biofilms. GtfD forms a soluble polysaccharide that acts as a primer for GtfB (De et al., 2018). The gene *ftf* catalyzes the formation of fructans from sucrose. Fructan polymers serves as the

storage of extracellular nutrients, which may be utilized during periods of nutrient deprivation. In addition, EPS serves as a storage nutrient for the bacteria (Lemos et al., 2005).

Streptococcus mutans decomposes sucrose in the oral cavity into glucose and fructose using bacterial invertase and then synthesizes glucan by polymerizing glucose using the enzyme glycosyltransferase whereas fructosyltransferase synthesizes polysaccharides such as fructan by polymerizing fructose. The Gtfs secreted by *S. mutans* binds avidly to the pellicle formed and bacterial surfaces. In the presence of sucrose, glucans are formed in situ within minutes (Leme et al., 2006b). The exopolymers contribute to the bulk and physical stability of the biofilm matrix. The glucan-mediated processes promote tight adherence and coherence of bacterial cells bound to each other and to the apatitic surface, which leads to the formation of microcolonies and thereby modulates the initial steps of cariogenic biofilm development (Kooi et al., 2010). As the biofilm develops, the EPS formed in situ enmeshes and surrounds the microorganisms while forming an insoluble matrix facilitating the assembly of spatially heterogeneous yet cohesive 3D multicellular structures. The spatial heterogeneities shaped by EPS synthesis form a complex 3D matrix architecture and create environmental and protective niches within biofilms that can directly modulate caries pathogenesis (Klein et al., 2015).

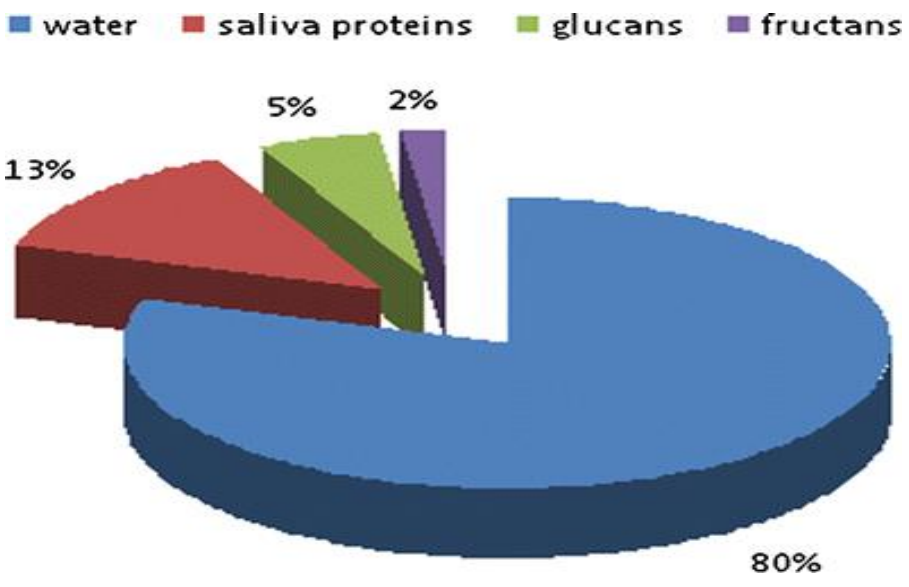

Figure 1.6: The percentage composition of polysaccharide matrix (Krzyściak et al., 2014)

1.5. *Lactobacillus spp.*

Lactobacillus spp. is considered the second most cariogenic bacteria of oral microflora and it plays a major role in the progression of caries. These bacteria are part of the normal flora of the oral cavity and they are present in a large number on the saliva, surfaces of mucous membranes, and tooth surfaces (Ahirwar et al., 2019b). The presence of lactobacilli in the oral cavity depends on the presence of ecological niches such as natural anfractuosities of the teeth, partly erupted third molars, or orthodontic devices (Badet and Thebaud, 2008). The main virulence property of *Lactobacillus spp.* includes adhesion, biofilm formation, and their ability to ferment glucose and produces lactic acid that demineralizes the enamel of the tooth. The dominant *Lactobacillus* species associated with the pathogenesis of dental caries are *Lactobacillus gassier*, *Lactobacillus fermentum*, and *Lactobacillus casei*. These bacteria use two types of metabolism methods including homo-fermentative and heterofermentative to produce lactic acid and acetic acid (Sachidananda and Mallya, 2020). *Lactobacillus spp.* carry out glycolysis at pH as low as 3. After colonizing the established dental plaque, the lactobacilli can further acidify the plaque and suppress the acid susceptible microorganism, further enriching acidogenic and aciduric bacteria (Salvetti et al., 2012).

1.6. The role of diet in the occurrence of dental caries

Dental diet and nutrition play a crucial role in childhood caries (Tungare and Paranjpe, 2018). The consumption of fermentable carbohydrates such as sucrose, glucose, and starch have both local and systemic effects on dental caries. The local dietary effect is dependent on individual diet and it is influenced by factors such as overall dietary habits, biofilm composition, saliva, and fluoride (Hujoel and Lingström, 2017). Numerous studies conducted within different population groups have demonstrated the role of high intake of sugars and carbohydrates in the occurrence of dental caries (Palacios et al., 2016;Touger-Decker and Van Loveren, 2003;van Loveren, 2019). The World Health Organization (WHO) guideline recommends reducing the consumption of free sugars below 10% of the energy intake and below 5% of the total diet. Free sugars are defined as all monosaccharides and disaccharides added to foods by the manufacturer, cook, or consumer, and sugars naturally present in honey, syrups, fruit juices, and fruit juice concentrates (van Loveren, 2019). Figure 1.7 presents the changes in plaque pH following a sucrose rinse. This phenomenon creates a Stephan curve. After sucrose rinse, the plaque pH was reduced to less than 5.0. When the pH of the enamel is below the critical pH of

5.5, demineralization of the enamel occurs. The plaque pH stays below the critical pH for 15-20 and then returns to a normal pH of 6.5 after 40 min. In the presence of saliva, the enamel is remineralized when the plaque pH recovers to a level above the critical pH (Bilbilova, 2020).

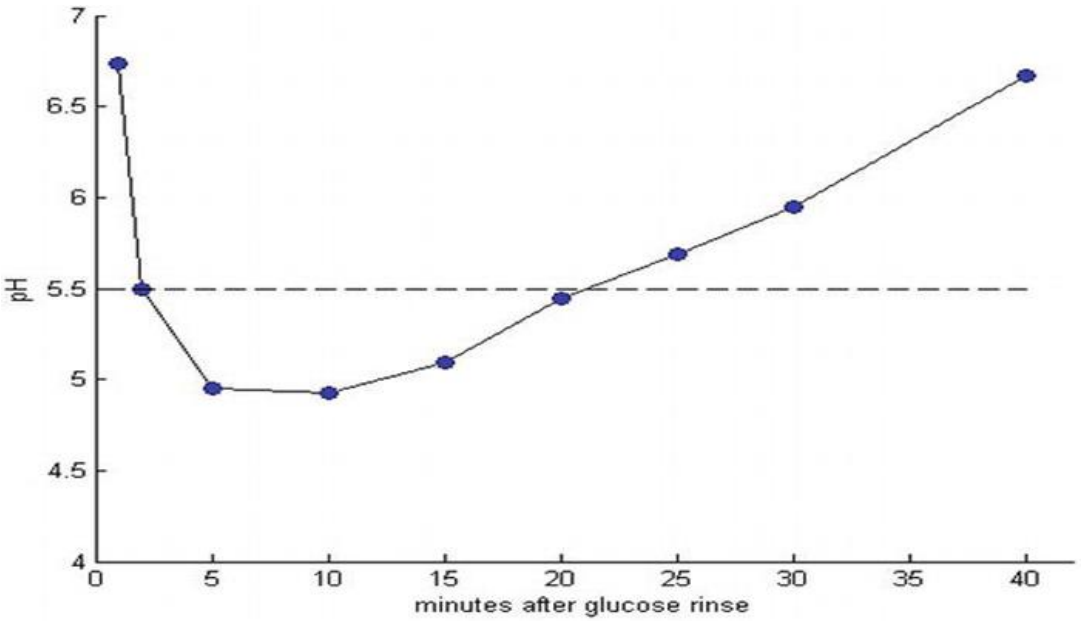

Figure 1.7: Stephan's curve illustrating the changes in plague pH over time following a sucrose rinse (Bilbilova, 2020).

1.7. Role of saliva in the caries process

Saliva plays a significant role in maintaining oral health (Dodds et al., 2015). It contains water (99.5%), protein (0.3%), and inorganic and organic substances (0.2%). Organic constituents of the saliva include amylases, growth factors, peroxidases, lipases, lysozyme, kallikreins, mucins cystatins, lactoferrin, hormones, and, whereas inorganic constituents include salt, potassium, calcium, magnesium, chlorides, and carbonates (Marsh et al., 2016;Kubala et al., 2018). The functions of the saliva in the mouth include antibacterial action, buffering properties, cleansing effect, and maintenance of saliva supersaturated in calcium phosphate. Importantly, saliva has been recognized as having the ability to reduce the incidence of dental caries due to its antimicrobial compounds such as immunoglobulins, lactoperoxidases, and lactoferrin which inhibit the growth of bacteria (Kertiasih and Artawa, 2015). The presence of calcium, phosphate, and fluoride within the saliva enhances the resistance of the tooth surfaces to a cariogenic attack, therefore decreasing the chances of demineralization and promoting remineralization of previously demineralized enamel and root surfaces (Hicks et al.,

2004;Stookey, 2008). Several salivary proteins attach to hydroxyapatite and help maintain saliva supersaturated state. This allows calcium and phosphate-containing mineral components in solution at saliva's resting pH (close to neutral) and prevents precipitation out of solution (Hicks et al., 2004). A dry mouth exposes the teeth to acidic challenges from food, drinks, and organic acids produced by acidogenic bacteria. When the pH in the oral cavity falls below the critical pH≤5.5, demineralization occurs. In a dry mouth, natural remineralization and protection may not occur. These may be due to a lack or inadequate salivary calcium and phosphate ions (Su et al., 2011).

1.8. Prevention of dental caries

Oral hygiene, healthy eating, increasing fluoride availability, and the placement of fissures and sealants are the four main principles to prevent dental caries (Morgan, 2008). Mechanical plaque control by brushing and flossing is the most widely used method in disrupting and eliminating oral biofilm on tooth surfaces (Hughes and Dean, 2015). However, mechanical plaque control alone may be insufficient in preventing the development or recurrence of caries (Figuero et al., 2019). With more than 50 years of clinical success, fluoride serves as the gold standard agent for preventing caries (Karlinsey and Pfarrer, 2012). Animals and in vitro systems have demonstrated that dairy products have hypoacidogenic, anti-acidogenic properties, and prevent demineralization, and enhance remineralization (Walsh, 2006). Consumption of sugar-free gums containing xylitol and sorbitol can reduce the acidogenic potential of dental plaque and neutralize lactase produced by dental plaque thereby promoting enamel remineralization. Xylitol reduces caries by keeping sucrose molecules from binding to the *S. mutans,* thereby blocking sucrose metabolism. Chewing gum can also prevent dental caries by stimulating salivary flow and also enhancing salivary function, especially for those people with low flow rates (Shen et al., 2001). Increasing availability of fluoride using methods such as water fluoridation, fluoride toothpaste, fluoride mouth rinse, dietary fluoride supplements, and professionally applied fluoride compounds such as gels and varnishes also reduces dental caries. Mechanisms of action of fluoride for caries control are based on inhibiting demineralization of the crystal structures inside the tooth and enhancing remineralization. Additionally, fluoride inhibits bacterial enzymes (Lee, 2013). Placement of fissure sealants is one of the methods which can be used to prevent and control dental caries. By restoring teeth with fissure sealants, occlusal pits and fissures become less morphologically

susceptible. This preventive measure is recommended in young patients with erupting teeth and adults with a high caries index. Sealants prevent food from collecting in molar pits and fissures and, therefore, prevent dental caries (Goršeta, 2015).

Many medicinal plants have been studied for their anticariogenic activity. *Punica granatum, Dodoneae viscosa var. angustifolia, Cedrus deodara, Terminalia chebula, Psidium guajava, Azadirachta indica* and *Pongamia pinnata* are some of the plants that have shown a very good anticariogenic potential. The present study examined *Uvaria chamae*.

1.9. *Uvaria chamae*

1.9.1. Taxonomy of *Uvaria chamae*

The name "*Uvaria chamae"* is derived from the Greek word *chamai*, which means "on the ground" (Bongers et al., 2005). This plant is a member of the Annonaceae family. The Annonaceae family consists of flowering plants such as trees, shrubs, and lians, and it contains 2106 species and over 130 genera. Several genera, most notably *Annona, Anonidium, Asimina, Rollinia*, and *Uvaria*, produce edible fruits (Tamokou et al., 2017).

1.9.2. Description of *Uvaria chamae*

Uvaria chamae is an evergreen plant that grows to a height of 3.6-4.5 m. The leaves are stipulate, the leaf apex cuminate, and the leaf vestiture is glabrous (Monon et al., 2015). The fruit carpels are in finger-like clusters, the shape giving rise to many vernacular names translated as a bush banana. The fruit is yellow when ripe and has a sweet pulp which is widely eaten-Figure 1.8 (Abu et al., 2018).

Figure 1.8: *Uvaria chamae* tree and fruits (Teanpaisan et al., 2014)

1.9.3. Origin and distribution of *Uvaria chamae*

Uvaria chamae is a Nigerian medicinal plant. It is commonly called Ayiloko by the Igala people of Kogi State, Kaskaifi by the Hausas, Oko Oja by the Yorubas in Nigeria as well as Akotompo by the Fula-fainte of Ghana (James et al., 2013). This plant is native to tropical West and Central Africa. It has been introduced to other parts of Africa and elsewhere in the tropics as a curiosity plant because of its finger-like, ornamental fruits (Lim, 2012a).

1.9.4. Traditional uses of *Uvaria chamae*

In West Africa, *U. chamae* is mainly used to treat jaundice and intermittent fevers. The root bark is used for respiratory catarrh and dysentery (Kadiri et al., 2014). A root infusion of this plant is used to treat severe abdominal pain. The root decoction is also administered as a purgative for the treatment of hepatitis. The juice of the fresh leaves is applied to fresh wounds, sores, and into the eyes to treat conjunctivitis (Jalil et al., 2020). An alcoholic extract prepared from root bark, stem, or dried leaves is taken to treat inflammatory condition known as "Calabar swelling" (Iwu, 2014). The roots, bark, and leaves extracts are used to treat inflamed gums, gastroenteritis, malaria, fever, vomiting, wounds, and sore throat. The roots powder is consumed to treat hyperprolactinemia (Yakubu and Fayemo, 2021).

1.9.5. Major chemical constituents of *Uvaria chamae*

The medicinal properties of a plant depend on its bioactive phytochemical constituents (Sheikh et al., 2013). The composition of essential bioactive compounds in medicinal plants depends on the plant species, the soil type, and as well as their association (Egamberdieva et al., 2017). Secondary metabolites isolated and characterized from the Annonaceae family include monoterpenes, diterpenes, triterpenes, lignans, flavonoids, asarone-derived phenylpropanoids, acetogenins, and primarily typical isoquinoline-derived alkaloids. Some of these secondary metabolites have been shown to have important biological activities, such as antimicrobial, anti-inflammatory, anticarcinogenic and urease-inhibiting properties (Costa et al., 2021). Phytochemical analysis of *U. chamae* reported the presence of bioactive compounds with potential antimicrobial activity against a variety of pathogens. The ethanolic roots extracts of *U. chamae* contain flavonoids, alkaloids, cardiac glycosides, terpenes, saponins, and tannins.

1.9.5.1. Alkaloids

Alkaloids are low molecular weight compounds with a nitrogen atom in a heterocyclic ring. Alkaloids include neuroactive molecules like caffeine and nicotine, anti-tumoral vincristine, vinblastine as well as emetine, which is used to treat oral intoxication (Matsuura and Fett-Neto, 2015). Figure 1.9 shows the basic structures of common alkaloids. Alkaloids are classified as isoquinolines, quinolines, indoles, piperidine alkaloids, etc. This classification is based on the chemical core structures of these alkaloids. Various pharmacological properties such as anticancer, antiviral, anti-inflammatory, and antibacterial activities have been reported (Yan et al., 2021). The phytochemical analysis of *U. chamae* leaves extract resulted in the isolation of *Uvaria* of benzylisoquinoline alkaloids (+)-armepavine and racem O, O-dimethyl coclaurine. The aporphines nornantenine, nantenine, and corydine have been isolated for the first time for the species (Thomas et al., 2018).

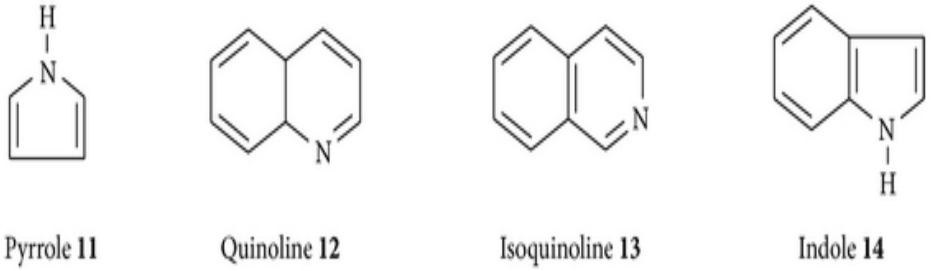

Figure 1.9: Basic structure of alkaloids (Achilonu and Umesiobi, 2015)

1.9.5.2. Saponins

Saponins are a group of naturally occurring plant glycosides. They are distinguished by their high foaming properties in aqueous solutions. Saponins have been isolated from over 100 plant families, and at least 150 different types of natural saponins have been shown to have significant anti-cancer properties. Saponins are classified into more than 11 distinct classes, including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroids (Man et al., 2010). Figure 1.10 shows the basic structures of common saponins. The majority of plants used in traditional medicine worldwide contain saponins. Saponins are widely distributed in higher plants have a wide range of biological properties such as antimicrobial, anti-tumor, and anti-inflammatory properties (Sparg et al., 2004).

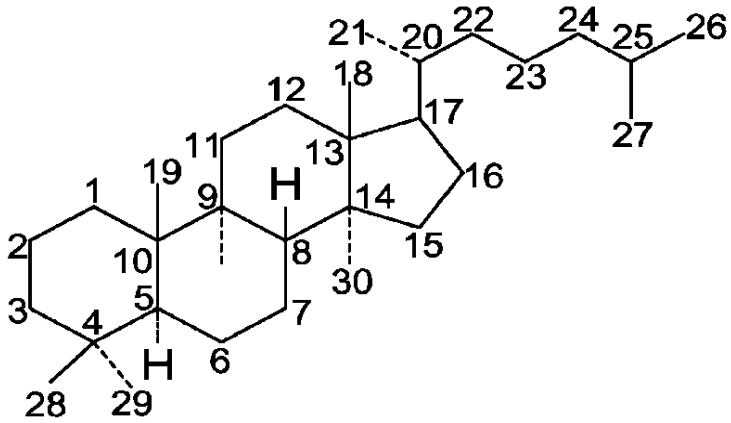

Figure 1.10: Basic structure of triterpenoid (Xia et al., 2014).

1.9.5.3. Tannins

Tannins are present in a wide range of fruits and vegetables and they have been isolated and characterized from several medicinal herbs such as cinnamon, thyme, black cohosh, and feverfew. Tannins are a common phenolic antioxidant that has been shown to have medicinal and therapeutic properties. Tannic acid is one such magical molecule with strong antioxidant properties. Tannic acid is one such magic molecule with potent antioxidant activity (Ghosh, 2015). Figure 1.11 shows a basic structure of common tannins. Their mechanism of action includes the inhibition of extracellular microbial enzymes, the deprivation of microbial growth substrates, or direct action on microbial metabolism via oxidative phosphorylation inhibition (Haslam, 1989).

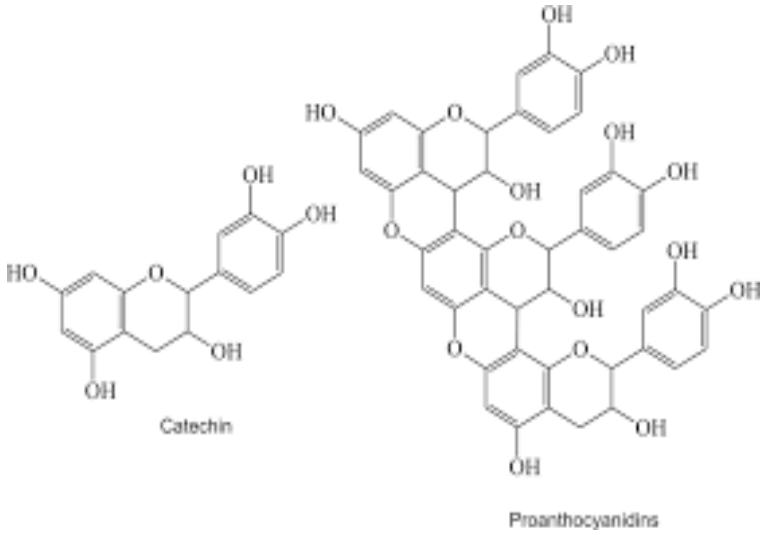

Figure 1.11: Basic structure of common tannins (Amorim et al., 2012)

1.9.5.4. Flavonoids

Flavonoids are a group of natural substances that contains various phenolic structures and they are commonly found in fruits, vegetables, grains, bark, roots, stems, flowers, and wines. Flavonoids have long been recognized for their beneficial effects in nutraceutical, pharmaceutical, medicinal, and cosmetic applications (Panche et al., 2016). Figure 1.12 shows the basic structures of common flavonoids. The novel C- benzylated flavanones and C-benzylated dihydrochalcones have been obtained from several *U. chamae* (Thomas et al., 2018). The flavonoid pinocembrin, chamanetin, uvaretin, uvarinol, and pinostrobin isolated

from stem bark and root bark (Enin et al., 2021). The drug benzyl benzoate used as antifungal preparation has a mutagenic compound, chamuveritin, a benzyldihydrochalcone that was isolated from *U. chamae* (Olumese and Onoagbe, 2019). Chalcones isolated from *Uvaria chamae* have been found to have strong antimicrobial activity against Gram-positive cocci (*Staphylococcus aureus* and *Streptococcus pyogenes*) compared to Gram-negative bacteria (*Salmonella typhimurium* and *Escherichia coli* (Koudokpon et al., 2018).

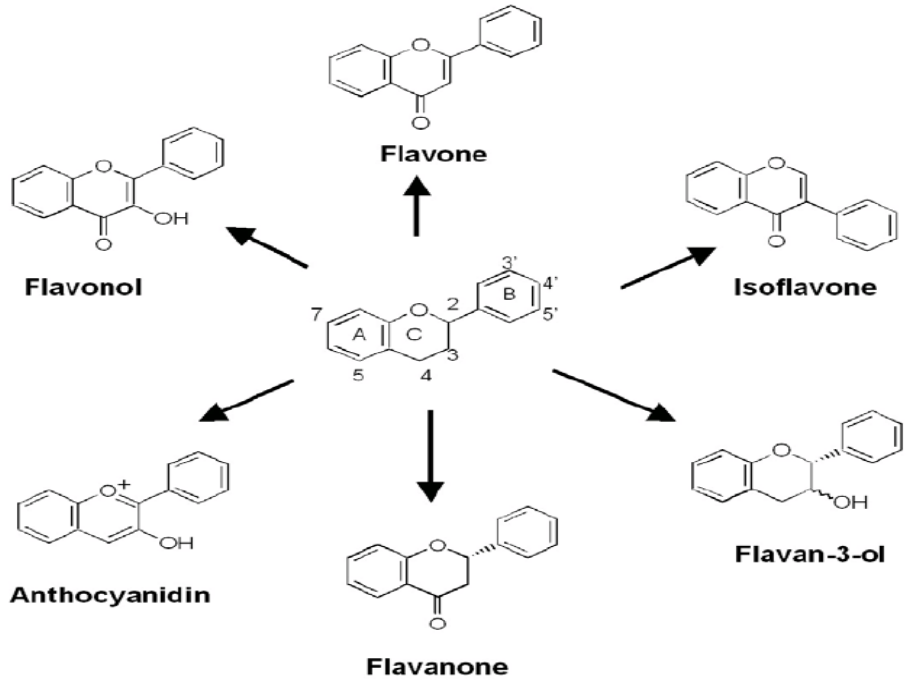

Figure 1.12: Basic structure of flavonoid (Nishiumi et al., 2011)

1.9.5.5. Terpenes

Terpenes are the largest and the most diverse group of naturally occurring secondary metabolites. They are commonly found in plants but larger classes of terpenes such as sterols and squalene can be found in animals (Cox-Georgian et al., 2019). Terpenes are classified based on their distinct carbon skeleton. It consists of a basic five-carbon isoprene unit (2-methyl-1,3-butadiene). Terpenes are typically composed of two, three, four, or six isoprene units. These are known as monoterpenes, sesquiterpenes, diterpenes, and triterpenes respectively (Man et al., 2010). Sesquiterpenes were reported as the predominant constituents of *U. chamae* leaf. The major constituent of *U. chamae* leaf oil was D-cadinene while thymoquinoldimethyl ether

and benzyl benzoate were the major components of the root oil. Two benzyldihydrochalcones and the known benzyl benzoate were identified in the roots of *U. chamae*. A monobenzylated monoterpene, chamanen, and the dimethyl ether of thymoquinol were isolated from the root bark of *U. chamae* (Lim, 2012b). Essential oil constituents from the leaves and root also revealed the presence of terpenes of 1-Nitro-2-pheneylethane, Linalool, Germacrene D, (E)-Caryophyllene, (E)-β-Ocimene, (E)- erolidol, 1,8-Cineole, 1-epi-Cubebol, α-Humulene, α-Copaene and others in trace quantities (Enin et al., 2021).

1.10. The role of medicinal plants in oral diseases

Medicinal plants include a variety of plants that are used in herbalism (Rasool Hassan, 2012). In developing countries, medicinal plants are used as a primary source of medicine (Palombo, 2011). These plants have been used throughout the world to treat various diseases since ancient times and they continue to play a vital role in the health care systems in many regions, especially Africa where modern drugs are not affordable (Agbor and Naidoo, 2019). Medicinal plants are inexpensive and available through traditional knowledge of medicinal plants that is passed from generation to generation (Nemudzivhadi and Masoko, 2015). The traditional healers typically diagnose and treats the psychological basis of an illness before prescribing medicines, particularly medicinal plants to treat the symptoms. The profound knowledge of herbal remedies in traditional cultures was developed through trial and error over many centuries, along with the most important cures was carefully passed on verbally from one generation to another (Mahomoodally, 2013). Other factors such as poverty, the inadequacy of health services, shortage of health care workers, and rampant shortage of drugs and equipment in existing health care facilities make traditional medicine an important component of health in Africa (Ashu Agbor and Naidoo, 2015). Various parts of these plants used in the preparation of traditional medicine include root, stem, seed, flower, fruit, and twig exudates (Kalaivani et al., 2012).

A pilot study conducted in Limpopo, South Africa reported the use of more than 41 plant species belonging to 30 botanical families, mainly the Asteraceae and Solanaceae as remedies for different oral diseases. The results showed that the most common medicinal plants used to treat oral diseases in Limpopo province, Lepelle Nkumpi Municipality are *Artemisia afra, Cannabis sativ, Carpobrotus edulis, Mentha longifolia*, *Nicotianatabacum*, *Punica granatum, Ricinus communis, Solanum panduriforme, Zanthoxylum capense,* and *Ziziphus mucron*. In

some regions, medicinal plants are used traditionally as a toothbrush or chewing sticks. The plants such as *Diospyros lycioides*, the sticks of *Salvadora persica* (miswak), *Salvadora persica* leaves, *Acacia mellifera, Jasminum Fluminense*, *Azadirachta indica* as toothbrush or chewing sticks (Bodiba et al., 2018). Tapsoba and Deschamps. (2006) reported the use of 62 plant species belonging to 29 families. The study reported that these plant species are effective in the management of toothache, gingivitis, acute necrotizing gingivitis, loose teeth, dental abscesses, sores on the tongue and lips. All the plant parts are used as remedies and they are prepared in various ways. Vegetable materials are often boiled for drinking, mouth washing, gargling, or inhalation. The principal plant parts include fresh or dried roots, stems, leaves, and barks. Plants were used either alone or in association with other species. For instance, a decoction made with roots of *Capparis tomentosa Lam*., *Cassia sieberiana DC.,* and *Indigofera tinctoria L*. is used for mouthwash against toothache (Tapsoba and Deschamps, 2006).

1.11. Anticariogenic effects of plant extracts against *Streptococcus mutans*

There are nearly 500,000 species of plants that occur in all parts of the world, and only 1% has been investigated phytochemically (Agbor and Naidoo, 2019). In recent years, oral care products and medicinal plant extracts are gaining high interest because they are less toxic and have fewer side effects compared to synthetic drugs (Şener and Kiliç, 2019). With the increase in the development of resistant strains, there is a need for an alternative therapy since resistance microbes are difficult to treat and require a higher dose which is more expensive and toxic to the human body (Adedayo et al., 2020). These findings have led to the screening of natural products for possible pharmaceutical value, particularly for anti-inflammatory, cytotoxic, antimicrobial, and antioxidant properties. Several studies have investigated the activity of traditional plants against oral pathogens and the examination was mainly to validate the traditional use of the medicinal plants.

1.11.1. *Artemisia princeps*

Artemisia princeps is originally native to eastern Asia (China, Japan, and Korea). It has been present for at least two decades in several localities in Belgium and the Netherland. This plant is frequently used as a medicine, culinary herb, essential oil, and for re-vegetation (Verloove

and Andeweg, 2020). *Artemisia princeps* extract shows antibacterial activity against *S. mutans* at a concentration of 0.4 mg/mL. The bacterial activity was observed at a concentration range of 0.05-0.4 mg/mL. This extract also reduces the expression of the gene *gftB, gtfD,* and *relA* at a concentration greater than 0.1 mg/mL, *gtfC,* and *vicR* at a concentration greater than 0.2 mg/mL, *spaP*, and *brpA* at the concentration greater of 0.005 mg/ml (Yang et al., 2019).

1.11.2. Ethyl gallate

Ethyl gallate is a phenolic compound richly contained in Longan. Longan (also known as Dimocarpus longan) is a member of the soapberry family (Sapindaceae). It is commonly regarded as a "hot" fruit in traditional Chinese medicine because it induces inflammatory immunological responses. It is grown extensively in China and Southeast Asia, as well as in Australia, Florida, southern Europe, and southern Africa (Wang et al., 2018). Ethyl gallate significantly suppressed *S. mutans* biofilm build-up on polystyrene and glass surfaces by 68% and more than 91% and inhibit acidogenicity by 95% of *S. mutans*. The study also demonstrated that this extract produced a significant gene expression change in the genes *gtfB* and gbpB at the concentration of 3.53 mM (Gabe et al., 2019).

1.11.3. *Rhodiola rosea*

Rhodiola rosea is a flowering biennial grown in high latitude and altitude regions of the world. It has been a part of traditional medicine systems in parts of Europe, Asia, and Russia for centuries. The traditional use of *R. rosea* as a treatment of cancer and Tuberculosis, and as a fertility booster has been documented previously in countries such as Mangolia and Siberia. In Norway, it has been used as food and hair wash (Ishaque et al., 2012). The extract significantly decreased the expression of the *gft* genes and slightly decreased the expression of *comD* and *comE* genes. Biofilm formation was strongly inhibited at a concentration of 0.25 μg/μL and 0.50 μg/μL on ex vivo bovine enamel (Zhang et al., 2020a).

1.11.4. *Chamaecyparis obtusa*

Chamaecyparis obtusa is a tropical tree species found in Japan and the southern region of South Korea, and the essential oil is extracted from the leaves and twigs of the *C. obtusa* tree. The essential oil has several types of terpenes and has been commercially used in soaps, toothpaste, and cosmetics as a functional additive (Bae et al., 2012;Hong et al., 2004). *Chamaecyparis obtusa* oil extract significantly inhibited organic acid production from 5.30-7.40 produced by *S. mutans* at a concentration range of 0.025 mg/mL-0.2 mg/mL. The expression of virulence genes *brpA*, *gbpA*, *gtfC*, and *gtfD* were significantly decreased at a concentration of 0.025 mg/mL of *C. obtuse* (Kim et al., 2016).

1.11.5. *Prangos acaulis Bornm.*

Prangos acaulis Bornm. is one of the important species of genus Prangos in Iran that is used in folk medicine as a sedative and anti-infective agent. The use of *Prangos acaulis Bornm*. as a traditional medicine for tooth whitener and pain relievers have been validated (Rustaiyan et al., 2006). The roots extracts showed a greater antibacterial activity compared to flowers, leaf, stem, and seed extracts. The MIC and MBC for the roots and seeds extract against *S. mutans* ranged between 500-1000 and 2000-3000 with the greatest antibacterial activity at 500-1000 and 2000-3000 µl/mL. The extract significantly reduced biofilm formation by 66.40% and 22 +/- 0.20% (Nosrati et al., 2018).

Although the antibacterial activity of *U. chamae* against *S. mutans* is known, its effect on biofilm formation and the production of acid and extracellular polysaccharides has not been studied.

1.12. Aim

This study aimed to evaluate the anti-*S. mutans* property of *U. Chamae* and its antivirulence activity.

1.13. Objectives

a) To investigate the antibacterial effect of methanol, dichloromethane, ethanol, hexane, and methanol: water crude roots extracts against *S. mutans* using microdilution technique.
b) To determine the effect of subinhibitory concentrations of the dichloromethane crude roots extracts on *S. mutans* biofilm formation using a glass slide technique.
c) To determine the effect of subinhibitory concentrations of the dichloromethane crude roots extracts on *S. mutans* acid production using sequential pH measurements.
d) To determine the effect of subinhibitory concentration of dichloromethane crude roots extract on *S. mutans* extracellular polysaccharides (EPS) production using phenol sulfuric acid assays.
e) To analyze the effect of the dichloromethane crude roots extract on the expression of virulence genes using Real-Time Polymerase Chain Reaction (RT-qPCR).

Chapter 2: The antibacterial effect of *Uvaria chamae* dichloromethane extract against *Streptococcus mutans*

2.1. Introduction

Streptococcus mutans, a Gram-positive, facultatively anaerobic bacterium is generally known as a major pathogen of dental caries and a possible causative agent of bacteremia and infective endocarditis (Nakano et al., 2008;Nakano and Ooshima, 2009). It is a part of the normal flora of the oral cavity and it resides primarily in the biofilm, also called plaque formed on the teeth surfaces (Metwalli et al., 2013;Lemos et al., 2013). In the prevention and control of dental caries, it is important to reduce the number of cariogenic bacteria such as *S. mutans*. There are several commercially available oral hygiene products such as toothpaste and mouth rinses containing fluoride, triclosan, and chlorhexidine with varied efficacy and a few have been shown to reduce dental plaque formation (Prasanth, 2011).

The use of medicinal plants in the treatment and prevention of oral diseases including dental caries has been well documented in recent years. Herbal extracts have been used in dentistry for many years to reduce inflammation, inhibit the growth of oral pathogens, prevent the release of histamine, and as antiseptics, antioxidants, and analgesics (Megersa et al., 2019). These extracts consist of many bioactive compounds that are considered good alternatives in the management and treatment of various oral diseases (Semenya et al., 2019). In some countries, many synthetic and routine drugs are inaccessible. In addition, there is an increase in the loss of effectiveness and potency to multidrug resistance organisms to oral antibacterial agents. Therefore there is a need to source locally available drugs and alternative medicine to treat ailments (Udoh et al., 2019).

Uvaria chamae is used in many African countries to treat bacterial infections. In addition to its antimicrobial activity, the plant has also been reported to have anti-inflammatory properties (Emordi et al., 2018;Abachi et al., 2016). Ogbulie et al. (2007) demonstrated that cold water extracts of *U. chamae* fresh leaves can moderately inhibit the growth of *Staphylococcus aureus* and *Streptococcus pyogenes*. The cold and hot ethanol extract profoundly inhibited *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli,* and *Salmonella typhi* (Ogbulie et al., 2007). Stem bark extracts were found to have the greatest antimicrobial activity

against *Escherichia coli,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella spp,* and *Proteus spp*, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pneumonia aeruginosa*. Leaf extracts showed the least antimicrobial activity against *Escherichia coli, Klebsiella spp,* and *Proteus spp* with MIC of 250 mg/mL (Oluremi et al., 2010).

This chapter aimed to determine antimicrobial activity of *U. chamae* against *S. mutans* by investigating the minimum inhibitory concentration (MIC) and Minimal Bactericidal Concentrations (MBC).

2.2. Methods and materials

2.2.1. Plant materials

Uvaria chamae roots extracts were provided by Dr. Ogunyemi Olajide Oderinlo from the Department of Chemistry, Faculty of Science, Federal University, Otuoke, Bayelsa, Nigeria. The plant was identified and authenticated by a qualified botanical expert in Nigeria. The voucher specimen number of the plant was FHI 107901. Plant extracts were prepared using methanol, methanol: water, hexane, ethanol, and dichloromethane. Briefly, the roots were harvested, washed, dried, milled, and stored at 4^{o}C until required for use. The crude extracts were obtained by mixing 1g of the root powder separately with 10 ml of each solvent. The solution was shaken at 120 rpm at room temperature overnight and centrifuged at 10000 rpm for 10 minutes using a microcentrifuge. This procedure was done 3 times. Thereafter, the solvents were allowed to evaporate under a cold air stream and stored at 4^{o} C until required. The crude extracts were weighed and reconstituted in 10% Dimethyl sulfoxide (DMSO) to obtain a final concentration of 50 mg/ml.

2.2.2. Bacterial cultures

Five clinical strains (SM1, SM6, SM7, SM12, and SM13) of cariogenic *S. mutans* were obtained from the Oral Microbiology laboratory, University of Witwatersrand. An ethics

waiver (W-CBP-200529-03) for the use of stock cultures was granted by The Human Research Ethics Committee, University of The Witwatersrand. Cultures were grown on blood agar at 37^{o}C for 48 hours. For each experiment, fresh cultures were used. To prepare inoculum which was used in this experiment, colonies were emulsified in phosphate-buffered saline and the suspension was adjusted to 0.5 McFarland standard containing approximately 10^5-10^6 cfu/ml.

2.2.3. Antimicrobial activity

Antimicrobial activity of *U. chamae* roots extracts was performed using the microdilution technique as previously described by Gulube and Patel (2016) and Kuete et al (2012) with modifications. Dry *U. chamae* extracts were reconstituted in DMSO to obtain a concentration of 50 mg/ml. Two-fold dilutions of *U. chamae* roots extracts were prepared using tryptone broth to obtain concentrations of 5 mg/ml to 0.02 mg/ml. Thereafter, 50 µl of *S. mutans* inoculum prepared as a 0.5 McFarland standard containing approximately 10^5-10^6 cfu/ml cells was added to all the wells of the 96-microtiter plate containing 50 µl diluted plant extract. A well-containing 50 µl tryptone soy broth, inoculated with 50 µl *S. mutans* was used as a positive control, while different wells containing 50 µl of *S. mutans* inoculum and 50 µl of chlorohexidine was used as a negative control. The effect of 10 % DMSO on *S. mutans* growth was determined by adding 50 µl of the inoculum in a well containing 50 µl of DMSO. The 96-microtiter plates were incubated at 37^{o}C for 48 hours under CO_2 -enriched environment. After incubation, 5µl from each microtiter well was aliquoted on blood agar plates to determine the MBC. Thereafter, 0.2 % iodonitrotetrazolium chloride (INT) was added to each well. The microtiter plates were incubated for an additional 48 hours. The MIC was interpreted as the sample concentrations that prevent change in colour of the medium from colourless to pink and completely prevent microbial growth. The MBC was defined as the lowest concentration of the extract which did not produce any growth after 48 hours of incubation. The experiment was performed in triplicates for all 5 clinical strains of *S. mutans*. Based on the MIC concentrations, the solvent with the best results was selected and two subinhibitory concentrations were used for further studies, such as biofilm, acid assay, and extracellular polysaccharides study.

2.3. Results

The results of the MIC/MBC of *U. chamae* roots extracts are presented in Tables 2.1 and the summarized results are presented in Table 2.2. A total of five extracts, extracted using different solvents of varying polarity (ethanol, dichloromethane hexane, methanol, and methanol: water) were investigated. All five crude extracts had a degree of antibacterial activity against *S. mutans*. The MIC values of *U. chamae* against *S. mutans* ranged from 0.02 to 1.25 mg/ml whereas the MBC ranged from 0.04 to 1.25 mg/ml. The dichloromethane extracts of the roots showed the best antibacterial activity against all the five *S. mutans* strains with a MIC of 0.02 mg/ml and MBC of 0.04 mg/ml (Table 2.2). Ethanol and methanol showed a moderate activity with MIC of 0.63 mg/ml. Hexane and methanol: water extract showed the minimum antimicrobial activity with a MIC of 1.25 mg/ml. The positive control (chlorhexidine) killed *S. mutans*, and the negative control (tryptone soy broth) supported the growth of *S. mutans*. Dimethyl sulfoxide (DSMO) did not affect the *S. mutans* bacterial growth.

Table 2.1: Minimum Inhibitory concentrations (MIIC) and Minimum Bactericidal Concentrations (MBC) of *U. chamae* roots extract against *S. mutans.*

Clinical strains	**Repeats**	***Uvaria chamae* MIC and MBC (mg/ml)**									
		Ethanol		**Dichloromethane**		**Hexane**		**Methanol**		**Methanol: water**	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
SM1	1	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	1.25
	2	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	1.25
	3	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	1.25
SM6	1	0.63	2.5	0.02	0.04	1.25	125	0.63	1.25	1.25	1.25
	2	0.63	2.5	0.02	0.04	1.25	1.25	0.63	1.25	1.25	1.25
	3	0.63	2.5	0.02	0.04	1.25	1.25	0.63	1.25	1.25	1.25
SM7	1	0.63	2.5	0.02	0.02	1.25	1.25	0.63	0.63	1.25	1.25
	2	0.63	2.5	0.02	0.02	1.25	1.25	0.63	0.63	1.25	1.25
	3	0.63	1.25	0.02	0.02	1.25	1.25	0.63	0.63	1.25	1.25
SM12	1	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	1.25
	2	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	1.25
	3	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	1.25
SM13	1	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	2.5
	2	0.63	2.5	0.02	0.04	1.25	1.25	0.63	0.63	1.25	2.5
	3	0.63	1.25	0.02	0.04	1.25	1.25	0.63	0.63	1.25	2,5
Mean	**n=15**	**0.63**	**2.33**	**0.02**	**0.04**	**1.25**	**1.25**	**0.63**	**0.75**	**1.25**	**1.50**
±SD		**0.00**	**0.44**	**0.00**	**0.01**	**0.00**	**0.00**	**0.26**	**0.26**	**0.00**	**0.52**

Table 2.2: Summary results of the mean MIC and MBC of *U. chamae* roots extract against *S. mutans*.

Extraction solvents	Mean MIC (mg/ml) n=15	Mean MBC (mg/ml) n=15
Ethanol	0.63 (moderate)	2.33
Dichloromethane	0.02 (good)	0.04
Hexane	1.25 (weak)	1.25
Methanol	0.63 (moderate)	0.75
Methanol: water	1.25 (weak)	1.50

2.4. Discussion

It is well established that medicinal plants have antimicrobial activity against oral bacteria (Ghamari et al., 2017). The efficacy of natural products is significantly affected by the solvent and method used for extraction (Miliauskas et al., 2004). In this study, the MIC and MBC of *U. chamae* roots extracts against *S. mutans* were determined. The results showed that methanol: water, hexane, methanol, dichloromethane, and ethanol of *U. chamae* roots extracts had antimicrobial activity against *S. mutans* (Table 2.2). Antimicrobial activity of plant extracts has been classified previously as good (MIC< 0.1 mg/mL), moderate (0.1 ≤ MIC ≤0.625 mg/mL) and weak (MIC >0.625 mg/mL) (Famuyide et al., 2019). Based on these criteria, ethanol and methanol showed moderate antimicrobial activity against *S. mutans*. Hexane and methanol: water extracts showed weak antimicrobial activity against *S. mutans*. Dichloromethane extract showed a good activity with an average MIC of 0.02 mg/ml. These results suggest that bioactive compounds that inhibit the growth of *S. mutans* were better extracted with dichloromethane compared to other solvent. Earlier studies investigating aqueous root bark extracts of *U. chamae* have shown poor antimicrobial activity against *S. mutans*. They reported the MIC of 400 mg/ml (Amadi et al., 2007). The present study demonstrated that the mean MIC of *U. chamae* root extract against *S. mutans* ranged between 0.02 to 1.25 mg/ml. The difference in results may be due to the season of plant harvesting, solvents used for extraction, location, growth conditions, and storage duration of the plant extracts (Seleshe and Kang, 2019).

Generally, Gram-positive bacteria are more susceptible to antimicrobial drugs than Gram-negative bacteria (Kapoor et al., 2017). This can be attributed to the presence of an outer membrane layer that is rigid and rich in lipopolysaccharide (LPS) in Gram-negative bacteria. This protective layer limits the diffusion of hydrophobic compounds through it. Gram-positive bacteria cell is surrounded by thick peptidoglycan walls which are not dense enough to resist small antimicrobial molecules, thereby facilitating access to the cell membrane. Additionally, the presence of the lipophilic ends of lipoteichoic acids present in the cell membrane of Gram-positive may ease the infiltration of the hydrophobic bioactive compounds present in this plant extract (Chouhan et al., 2017)

The activity of this plant extract may be attributed to alkaloids, tannins, steroids, terpenes, and flavonoids known to be present in the family Annonaceae. Annonaceae plants have been shown to possess antibacterial activities (Tamfu et al., 2019). A previous study by Lindsey reported that when active compounds are found in one species, the majority of species within the same genus contain active compounds of a similar nature (Lindsey et al., 1998). Similarly, the antimicrobial effect of this plant may be affiliated with the bioactive compounds present within the family Annonaceae (Attiq et al., 2017). These bioactive substances have different functions and mechanisms of action. For instance, the antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall while tannins may inactivate microbial adhesions, enzymes and cell envelop proteins (Mogana et al., 2020). A recent study (2019) reported that 2,4,60-trihydroxy-30-methylchalcone flavonoids lead *S. mutans* to leak intracellular substances such as protein and ions (Górniak et al., 2019).

2.5. Conclusion

Uvaria chamae dichloromethane extract had the best antimicrobial activity followed by ethanol and methanol. Methanol: water and hexane extract had the least activity with MIC of 1.25 mg/ml. At a low MIC of 0.02 mg/ml, dichloromethane extract completely inhibited the growth of *S. mutans*. This study demonstrated that *U. chamae* dichloromethane extracts have the potential to be used to prevent dental caries. Further studies can be undertaken to study the effect of *U. chamae* on *S. mutans* virulence factors as well as its specific mechanism of action. These results also encourage further investigations on the active chemical compounds responsible for the observed antibacterial effect in this study.

Chapter 3: The effect of *Uvaria chamae* dichloromethane extract on the biofilm formation by *Streptococcus mutans*

3.1. Introduction

Dental caries is a biofilm-induced oral disease that results due to a disruption of the microbial ecological balance in the oral cavity. This disturbance results in a population shift leading to the over-representation of pathogenic species in the oral cavities. Additionally, normal biota may also become opportunistic pathogens by the acquisition of *gtfs* genes through horizontal gene transfer thereby contributing to the onset and establishment of dental caries (Chen et al., 2016;Adedayo et al., 2020;Hoshino et al., 2012). Dental plaque is an oral microbial biofilm that forms on exposed tooth surfaces and is characterized by species diversity (Yu et al., 2017). Dental plaque comprises surface-attached bacterial communities encased in extracellular polysaccharides, proteins, and DNA (Davies, 2003) and they form through sequential events which may result in a structurally and functionally organized species-rich microbial community. The species composition of a plaque at a site is characterized by a degree of stability among the component species once it has formed. This stability is a result of a balance imposed by biochemical interactions where complex host glycoproteins catabolize to develop food chains and cell to cell signalling which leads to coordinated gene expression within the microbial community (Yadav and Prakash, 2017).

Streptococcus mutans has been discovered as the primary etiological bacteria that predominantly proliferates in the dental biofilm (Akhalwaya et al., 2018). The pathogen *S. mutans* is a major bacterium producing the extracellular polysaccharide matrix in dental biofilms. During this biological process, *S. mutans* secretes the enzyme glucosyltransferases (GTFs) to synthesize glucans and binds them to the interface of bacteria and teeth leading to bacterial adhesion and biofilm formation. Bacteria in the plaque biofilm respond to many factors, such as cellular recognition of specific or non-specific attachment sites on a surface and nutritional signal using quorum sensing mechanisms (Yu et al., 2017;Yoshida and Kuramitsu, 2002;Zhang et al., 2020b). Quorum sensing plays a critical role in the formation of biofilm with its surrounding extracellular matrix (Munir et al., 2020;Lu et al., 2019). There are many treatments plans available in treating oral biofilms. These treatments include mechanical and chemical strategies. Mechanical strategies include physical removal of the dental plaque

by brushing and scrubbing. Chemical strategies include the use of mouthwashes and other products that contain chlorhexidine, stannous fluoride, and conventional antimicrobials (Sandasi et al., 2011).

To date, several studies have investigated the effect of plant extracts and their products against different oral pathogens. Other researchers focused on the ability of the medicinal products to inhibit the formation of dental biofilms by reducing the adhesion of microbial pathogens to the tooth surface, which is a primary event in the formation of dental plaque and the progression to tooth decay (Palombo, 2011;Gabe et al., 2019;Nosrati et al., 2018;Teanpaisan et al., 2014). Many medicinal plants including *Dodonaea viscosa var. angustifolia* (Ngabaza et al., 2018), Curcuma (Kim et al., 2008), *Prangos acaulis Bornm* (Nosrati et al., 2018), *Rhodiola rosea* (Zhang et al., 2020b), Mikania (Yatsuda et al., 2005) have been shown to inhibit biofilm formation by *S. mutans*. The ability of these plant extracts to suppress and destruct mature biofilms has the potential to reduce microbial colonization of tooth surfaces and epithelial mucosa.

In this chapter, the antibiofilm effects of *U. chamae* on *S. mutans* were investigated. Antibacterial effects of *U. chamae* have already been studied by some authors, but no report on the antibiofilm activity is available. Therefore, this chapter was to examine the effect of *U. chamae* extract on *S. mutans* biofilm formation.

3.2. Methods and materials

3.2.1. Bacterial cultures

Five clinical isolates of cariogenic *S. mutans* (SM1, SM6, SM7, SM12, and SM13) were revived by culturing on blood agar plates. These cultures were grown at 37^{o}C in a CO_2-enriched environment for 48 hours. The inoculum was prepared by suspending the freshly grown *S. mutans* cells in phosphate buffered saline.

3.2.2. Plant materials

The dichloromethane extract was selected based on its antimicrobial activity against *S. mutans* described in chapter 2. The results for antimicrobial activity can be found in chapter 2 (Table 2.1). The two subinhibitory concentrations were selected for the biofilm assay.

3.2.3. Biofilm assay

A biofilm assay was performed to determine the effect of *U. chamae* roots extracts on *S. mutans* biofilm formation. This was done using the glass slide technique adapted from (Gulube and Patel, 2016) with modifications. Briefly, three glass slides (25×77mm) were held side by side in an upright (90^{o}C) position with an autoclave tape in four separate 100 ml beakers. The beakers were autoclaved at 121^{o}C for 2 hours before use. Tryptone broth was used as a media broth for this assay. Fifty milliliters of tryptone soy broth were added to each of the four beakers. One beaker was used as a control with no plant extract. The other three beakers were used to test the effect of the three concentrations of the *U. chamae* extracts. The plant extract with the concentration of 0.005, 0.01, and 0.02 mg/ml was added in each of the three beakers respectively. One milliliter of *S. mutans* culture containing approximately 10^{5}-10^{6} CFU/ml was added to each of the four beakers. The four test beakers were then incubated at 37^{o}C under CO_2. After 6, 24, and 30 hours, one glass slide was removed from each of the four beakers and rinsed with 50 ml distilled water to remove unattached cells and media. The attached cells were removed from the glass slide by scrapping off the biofilm using a sterile glass slide. The cells were resuspended and vortexed in 5 ml of PBS. Ten-fold serial dilutions from 1:10, 1:100, and 1:1000 were performed in bijou bottles. Hundred microliters of each dilution were spread on blood agar and colony count was obtained after 48 hours. The number of colonies on each plate was quantified and the counts were multiplied by dilution factors to determine viable bacterial counts (CFU/ ml). The experiment was performed in triplicates using 5 strains of *S. mutans*.

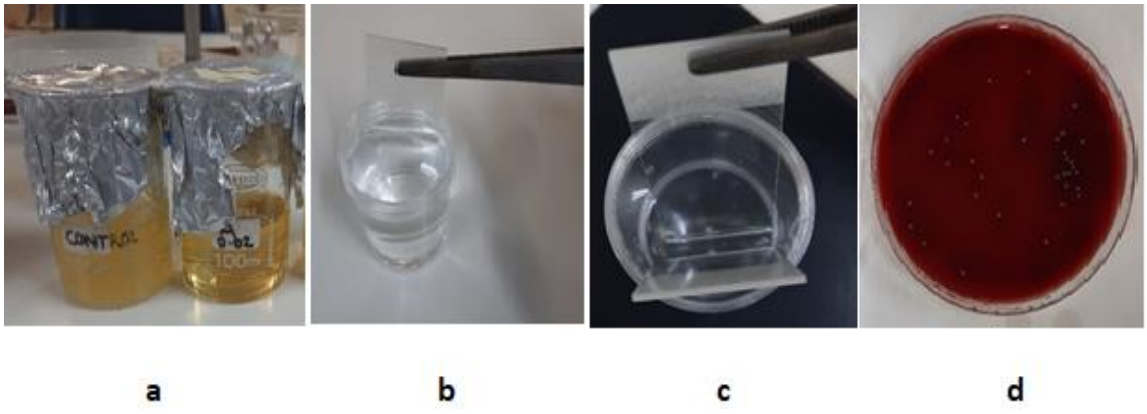

Figure 3.1: Biofilm assay (a) Beaker containing glass slides after incubation at 37°C (b) Washing of biofilm c) Removal of the biofilm by scraping off the attached cells with a clean slide, (d) *S. mutans* colonies on blood agar.

3.2.4. Statistical analysis

The efficacy of the plant extract on *S. mutans* biofilm formation was compared using GraphPad Prism software. To determine the effect of different concentrations of *U. chamae* extract on biofilm formation, bacterial counts in the test biofilms were compared to the control using Wilcoxon Rank Sum Test. P values of <0.05 were considered significant. The mean and standard deviations were calculated using Microsoft Excel (Table 3.1). The percentage reduction in the biofilm formation was calculated after 6, 24, and 30 hours at all test concentrations. The formula used was $(x-y/x) \times 100$ where x is the control while y is the test biofilm counts.

3.3. Results

At 3 concentrations, the effect of *U. chamae* on the biofilm formation by 5 strains of *S. mutans* was studied. The results in the form of viable bacterial counts in the biofilms are shown in Table 3.1. Bacterial counts were converted into logs and the graph (Figure 3.1) was prepared. Using the control and the test results, percentage reductions were calculated which are depicted in Table 3.1 and Figure 3.2. The results showed that the biofilm formation was inhibited by all three test concentrations in all the test strains of *S. mutans*. The reduction in the biofilm increased with time. However, not much difference in the results from 24 hours and 30 hours

was found. At 6 hours, the average percentage reduction at 0.005, 0.01 and 0.02 mg/ml was 39.7, 59.17 and 76.82 % respectively. At 24 hours, the average percentage reduction at 0.005, 0.01 and 0.02 mg/ml was 83.63, 90.75 and 91.12 % respectively. At 30 hours, the average percentage reduction at 0.005, 0.01 and 0.02 mg/ml was 94.73, 98.46 and 97.82 % respectively. When the test results of the bacterial biofilm counts were compared to the test counts it was found that all the test concentrations at all the exposure time period significantly reduced the biofilm formation with p values of $\leq$0.01 (Table 3.2).

Table 3.1: The effect of 0.005, 0.01, and 0.02 mg/ml of *U. chamae* roots extract on *S. mutans* biofilm formation

		6 hours				**24 hours**				**30 hours**			
		S. mutans cfu/ml in various concentrations (mg/ml)				*S. mutans* cfu/ml in various concentrations (mg/ml)				*S. mutans* cfu/ml in various concentrations (mg/ml)			
Strains	**Rep.**	**Control**	**0.005**	**0.01**	**0.02**	**Control**	**0.005**	**0.01**	**0.02**	**Control**	**0.005**	**0.01**	**0.02**
SM1	1	28650	16300	16250	3850	47200	7600	7400	300	47200	8950	1700	12150
	2	37200	25100	13450	8250	76800	4900	800	100	40000	2300	400	100
	3	36700	24750	16600	7800	39400	33600	3300	1000	47400	2000	400	300
SM6	1	19700	4050	4300	3200	66800	85000	60000	46800	197000	5400	1300	4650
	2	20300	25100	16700	15100	127867	3100	1600	1500	136000	30000	21500	11200
	3	16950	9750	6750	1100	128100	6550	6950	35350	97500	3400	800	600
SM7	1	39500	23000	18800	21850	87500	5750	3750	2450	132300	4500	2300	1200
	2	39000	20000	22450	18200	72800	3450	3750	1950	210000	1800	1700	1200
	3	33000	23900	800	1900	71550	8000	3100	800	145900	11000	1450	400
SM12	1	30950	22500	23200	5900	63200	800	400	100	200867	1600	600	18700
	2	32250	10600	2100	5150	41200	2600	1100	200	112500	1200	500	100
	3	30500	16950	9250	1100	56200	1400	400	200	850000	1050	2200	200
SM 13	1	30700	27400	10500	3150	74100	2000	2400	1650	13900	3350	400	200
	2	35850	3300	12000	5000	56400	4100	850	200	75000	44500	100	100
	3	22250	20750	12000	3550	39000	2750	1100	500	49150	3000	1000	100
Combined mean		**30233**	**18230**	**12343**	**7007**	**69874**	**11440**	**6460**	**6207**	**156981**	**8270**	**2423**	**3413**
Combined ±SD		**7317**	**7852**	**6880**	**6376**	**27751**	**21830**	**14972**	**14340**	**201754**	**12405**	**5322**	**5814**
% Reduction			**39.70**	**59.17**	**76.82**		**83.63**	**90.75**	**91.12**		**94.73**	**98.46**	**97.82**

Table 3.2: Statistical analysis of the data obtained in the biofilm formation assay

Time (hours)	Comparison	p-value
6	Control to all concentrations (overall)	0.0079
	Control to 0.005 mg/ml	0,0024
	Control to 0.01 mg/ml	<0,0001
	Control to 0.02 mg/ml	<0,0001
24	Control to all concentrations (overall)	<0,0001
	Control to 0.005 mg/ml	<0,0001
	Control to 0.01 mg/ml	<0,0001
	Control to 0.02 mg/ml	<0,0001
30	Control to all concentrations (overall)	0.0079
	Control to 0.005 mg/ml	0,0108
	Control to 0.01 mg/ml	0,0082
	Control to 0.02 mg/ml	0,0086

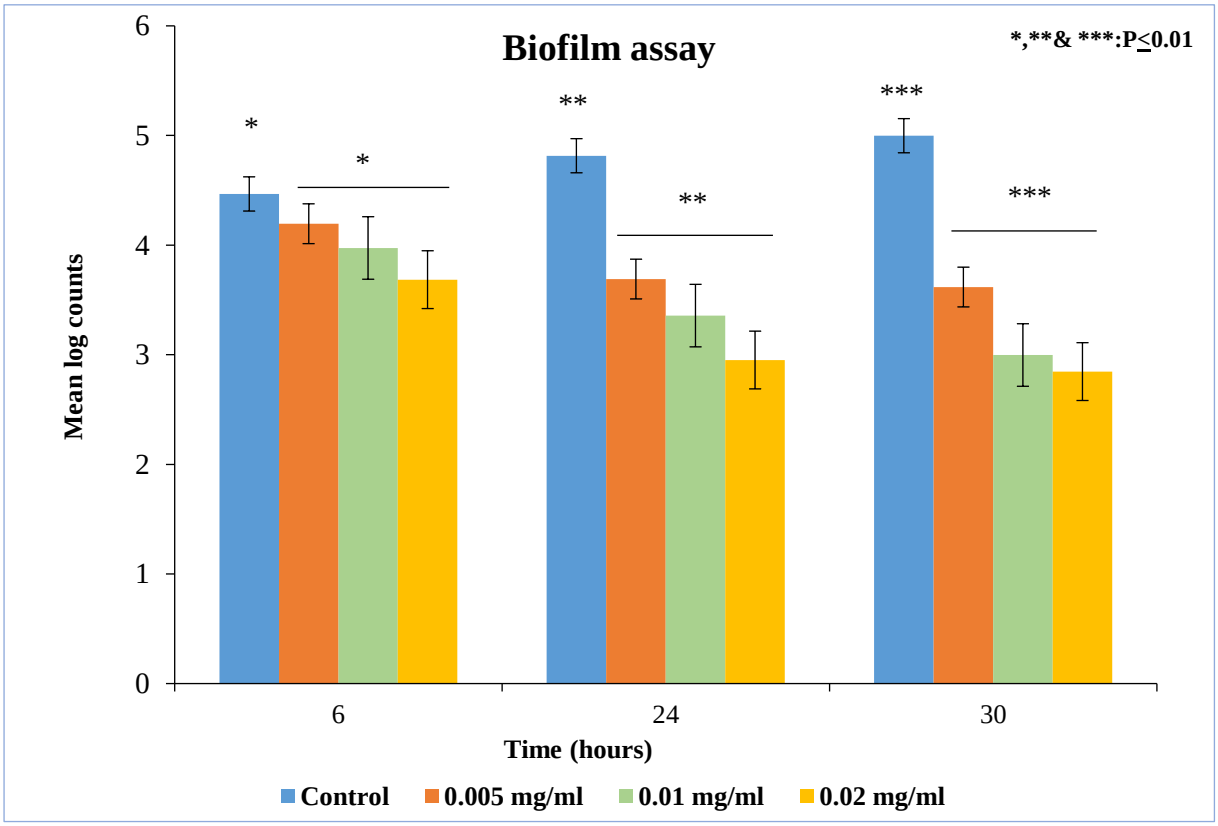

Figure 3.2: The effect of *Uvaria chamae* on *Streptococcus mutans* biofilm formation

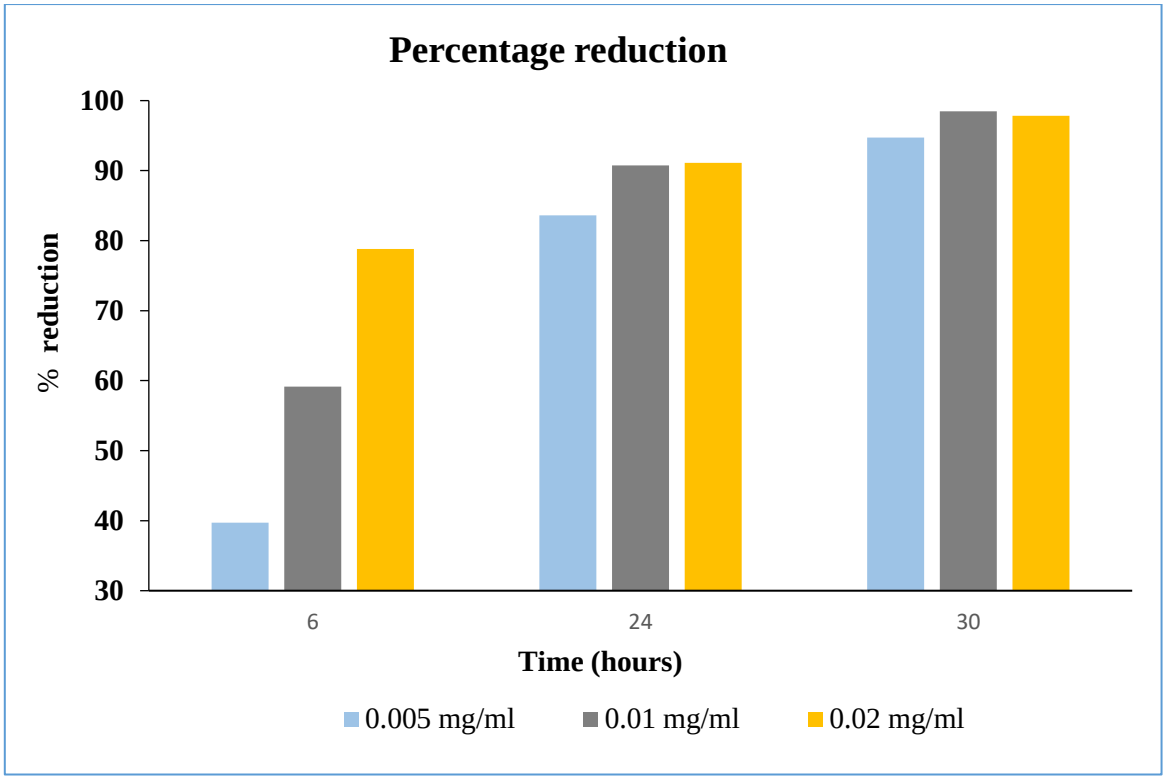

Figure 3.3: Percentage reduction in *Streptococcus mutans* biofilm formation by *Uvaria chamae*

3.4. Discussion

Oral biofilm has been recognized as a major virulence factor in the development of dental caries (Kuang et al., 2018). One of the best strategies to control and prevent dental caries involves the reduction and elimination of dental plaque on the tooth surfaces (Ramalingam and Amaechi, 2018). In this study, the anti-biofilm activity of the *U. chamae* extract against *S. mutans* was investigated. This plant extract significantly inhibited the adherence and biofilm formation of *S. mutans* to a glass slide. The ability of this extract to reduce biofilm formation in the initial attachment phase at 6 hours and plateau accumulated phase at 24 hours suggests that this extract does not only inhibit mature biofilm formation but also its maturation at critical developmental stages. Additionally, this plant extract does not only inhibit planktonic *S. mutans* but also biofilm formation. These findings suggest that *S. mutans* that survive exposure to the plant extract may be unable to adhere to the acquired pellicle and form dental plaque. The antimicrobial effect of *U. chamae* extract might be attributed to interference with the adhesion forces such as Brownian, sedimentation, Lifshitz–Van der Waals, and electrostatic interaction forces. These adhesive forces favour the deposition and adherence of bacteria to the tooth surfaces. Additionally, the plant extract might have affected the cell surface protein adhesion P1, also known as antigen I/II which are responsible sucrose–independent bacteria adherence to the tooth surfaces.

Phytochemical screening of *U. chamae* root extracts revealed the presence of flavonoids, saponins, phenols, and tannins (Ogbuanu and Emmanuel, 2020). These bioactive compounds, especially flavonoids have been found to inhibit *S. mutans* adhesion and biofilm formation. A study reported that a flavone (5,6,8-Trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one) isolated from *Dodonaea viscosa var. angustifolia* has been identified as the main compound exhibiting anti-*S. mutans* and antibiofilm properties against *S. mutans* (Ngabaza et al., 2018). Furthermore, the flavonoids, guaijaverin, and quercetin isolated from guava leaf have been reported to significantly inhibit *S. mutans* biofilm formation. Guaijaverin inhibits *S. mutans* surface adherence by reducing the bacterial surface hydrophobicity. Additionally, it also inhibits sucrose-dependent and sucrose-independent surface adherences of *S. mutans* (Phaiboon et al., 2019). Das et al. (2011) discovered that eDNA is involved in enhanced bacterial adhesion, aggregation, architecture, and biofilm mechanical stability (Das et al., 2011). Thus, *U. chamae* extract might have interfered with the eDNA and exopolysaccharides by increasing the hydrophobicity and decreasing the aggregation of the

cells to adhere to the glass surface, thereby reducing the biofilm formation (He et al., 2019). In addition to flavonoids, tannins are generally responsible for the decreased adherence property because they bind to bacterial cell surface lipoteichoic acid. The tannin, 4-O-β-D-xylopyranoside was associated with decreased adherence properties of *S. mutans* on a glass slide (Naidoo et al., 2012). Therefore, tannins might be responsible for the reduced adherence of *S. mutans*. The plant might have also affected the production of glucosyltransferases especially GTF B and C enzymes which are required for maximum biofilm formation through the production of insoluble extracellular polysaccharides by *S. mutans* (Ansari et al., 2017;Koo et al., 2010).

The oral cavities are constantly exposed to secretions and maintaining the correct concentrations of the antimicrobial compounds is thus challenging unless the compound's substantivity is effective (Patel et al., 2009). In the presence of this extract, *S. mutans* will be unable to form biofilm and it will prevent further colonization of the *S. mutans* by preventing their adhesion to the host tissues once the concentration is reduced. The beneficial effects of this plant extract will be increased at night due to reduced salivary flow, and slower dilution. Continuous exposure to the *U. chamae* will be necessary to reduce bacterial counts and prevent bacterial growth. The diminishing antimicrobial effect of the extract might be due to the accumulation of bacterial by-products that might have neutralized the plant extract.

3.5. Conclusion

This study has demonstrated that *U. chamae* extracts can be used as anti-cariogenic agents. In this experiment, *U. chamae* significantly reduced biofilm formation at all three concentrations. At 6 hours, exposure to 0.005, 0.01 and 0.02 mg/ml reduced bacterial count by 39.70, 59.17, and 76.82 % respectively. At 24 hours, exposure to 0.005, 0.01 and 0.02 mg/ml reduced bacterial count by 83.63, 90.75, and 91.12 % respectively. At 30 hours, exposure to 0.005, 0.01 and 0.02 mg/ml reduced bacterial count by 94.73, 98.46, and 97.82 % respectively. This suggests that if this plant extract is used in the form of oral hygiene products such as toothpaste, mouth rinses, and chewing gums, it can provide long-term protection against dental plaque. Therefore, *U. chamae* have the potential to be used in the prevention of dental caries.

Chapter 4: The effect of *Uvaria chamae* dichloromethane extract on *Streptococcus mutans* acid production

4.1. Introduction

Acid production is another major virulence factor responsible for dental caries. *Streptococcus mutans* can generate acidic end products from dietary carbohydrates (Zayed et al., 2021). Upon ingestion of food, the level of sugar available to oral bacteria may increase from the normal resting level of 5-40 µM of salivary secretions to 5-40 mM. Under these conditions, the *S. mutans* fermentation pattern shift from the production of mixed acid end-products to lactic acid (Dashper and Reynolds, 1996). It has been reported that lactic acid reduces the pH of the saliva from 7.0 to 5.0 (Dashper and Reynolds, 2000). Glycolysis is the main pathway for organic acid production. The bacteria carry out glycolysis continuously by metabolizing a wide range of dietary carbohydrates and produces organic acids such as lactic, acetic, propionic, and formic acid. These organic acids are produced as fermentation products during carbohydrate metabolism through the Embed-Meyerhof-Parnas pathway (Kawada-Matsuo et al., 2017). Lactic acid is a major product of the fermentation process when sucrose is present in large quantities (Walsh, 2006). The critical pH value of 5.0–5.5 is important for the balance between demineralization and remineralization of tooth enamel. If the surrounding solution pH caused by the accumulation of acid is less than the critical pH, tooth demineralization and subsequent initiation of dental caries occur (He et al., 2019).

Organic acids drive the dissolution of critical ions such as calcium and phosphate, which are involved in the demineralization and remineralization of enamel and dentin in the oral environment (Vaillancourt et al., 2021;Cai et al., 2016). The deprivation of these minerals in the hydroxyapatite may result in increased porosity and softening of the tooth structure. Additionally, the space between the enamel crystal may also widen. As a consequence, the acids diffuse deeper into the tooth resulting in demineralization of the mineral below the surface (Pitts et al., 2017b). The demineralization phases are followed by a period of remineralization through the presence of calcium, phosphate, and fluoride found in saliva (Featherstone, 2008). When acidification phases exceed the neutralizing capacity of both alkali-producing bacteria and the saliva, dental caries can occur (Du et al., 2020;Burne and Marquis, 2000). The acidic environment promotes the growth of aciduric species e.g *S. mutans*

and *Lactobacillus*. Aciduric strains of non-mutans such as streptococci, Actinomyces, bifidobacteria, and yeasts facilitate the progression and formation of dental caries (Nyvad et al., 2013;Du et al., 2020).

The oral pathogen *S. mutans* surpasses the rates of acid production by other oral streptococci due to its acidogenicity and acid tolerance mechanisms (Guo et al., 2013). The membrane-bound F1F0-ATPase system protects *S. mutans* against environmental acid stress by regulating pH homeostasis (Hasan et al., 2014). FIF0-ATPase pump maintains the intracellular pH at 7.5 (Ajdić et al., 2002). *Streptococcus mutans* constitute an acid tolerance response (ATR) to combat the destructive nature of the acidic environment it produces. ATR is characterized by the induction of multiple cellular pathways upon exposure to mildly acidic conditions to allow cells to adapt to the acid challenge. In addition to this mechanism, some oral streptococci use the arginine deiminase pathway to survive a low pH environment (Banas, 2004b).

Amongst dietary carbohydrates, sucrose is the most cariogenic carbohydrate because it is easily fermentable and also serves as a substrate for extracellular enzymes (GTF) of plaque bacteria. Plaque bacteria synthesize sucrose-derived polymers such as soluble and insoluble extracellular polysaccharides (Krzyściak et al., 2016). Sucrose fermentation by oral bacteria can rapidly reduce the pH in dental biofilms, which results in a shift in the balance of resident plaque microflora to become more cariogenic (Cai et al., 2016). In vivo studies showed that sucrose treatment produced more severe caries lesions than glucose, fructose, maize starch, and amylopectin (Du et al., 2020). Prevention of acid production by *S. mutans* using a therapeutic agent may reduce the development of dental caries.

Uvaria chamae has antibacterial properties against *S. mutans.* Although the anti-*S. mutans* activity of this plant has been reported, its effect on acid production by *S. mutans* has not been studied. Therefore, this study aimed to investigate the effect of *U. chamae* roots extracts on acid production by planktonic *S. mutans*.

4.2. Method and materials

4.2.1. Plant extractions

The extracts were prepared and provided by our collaborator. The extraction procedure is described in Chapter 2, section 2.2.1.

4.2.2. Bacterial cultures

Isolation of *S. mutans* strains is described in Chapter 2, section 2.2.2.

4.2.3. Acid production assay

The effect of the *U. chamae* roots extracts on acid production by the *S. mutans* was studied using pH sequential technique as previously described by (Gulube and Patel, 2016). Five hundred microlitres of *S. mutans* inoculum containing approximately 10^5-10^6 cfu/ml were transferred into a specimen jar containing 25 ml of 5 % sucrose tryptone broth and *U. chamae* extract at different concentrations of 0.005, 0.01, and 0.02 mg/ml respectively. A specimen jar containing *S. mutans* and 5 % sucrose tryptone broth with no plant extract was used as an experimental control. The specimen jars were incubated at 37^{o}C for 16 hours. At 0, 10, 12, 14, and 16 hours, 2 ml of *S. mutans* culture was removed and the pH was measured with a pH glass electrode. In addition, bacterial counts were obtained by plating 100 µl of the serially diluted *S. mutans* culture into blood agar at each incubation period. These experiments were repeated three times using five strains of *S. mutans*.

a b c

Figure 4.1: Acid production assay a) Grow *S. mutans* at 37°C for 48 hours b) pH measurements c) *S. mutans* colonies on blood agar

4.2.4. Statistical analysis

The measured pH readings of the test results were compared with the experimental control readings using the Wilcoxon Rank Sum Test. The test bacterial counts were compared with the experimental control using ordinary one-way ANOVA. P values <0.05 were considered significant.

4.3. Results

The effects of *U. chamae* on *S. mutans* acid production were evaluated using pH measurements at 3 different concentrations. The results in a form of pH readings are shown in Table 4.1. The results of the bacterial counts are shown in Table 4.2-4.4. Bacterial counts were converted into logs and the graph showing time vs pH readings and bacterial counts were prepared (Figure 4.1). The results showed that all three concentrations inhibited acid production and bacterial counts in all the five strains of *S. mutans* in a concentration-dependent manner. The reduction in bacterial counts and acid production increased with time. At 10 hours, the pH of the control experiment significantly decreased from pH 6.62 to pH 5.41 and it was not due to bacterial counts because the test counts were not significantly different from the control. In the presence of the plant extract, the pH of the culture did not drop below 6.50 after 16 hours. The percentage reduction of the bacterial counts was 66.66, 77.37 and 86.39 % in 0.005, 0.01, and 0.02 mg/ml respectively after 10 hours. After 12 hours, *S. mutans* counts were reduced by 79.42, 81.15 and

87.13 % at 0.005, 0.01, and 0.02 mg/ml respectively. At 14 hours, the percentage reduction was 90.10, 89.69 and 94.34 % in 0.005, 0.01, and 0.02 mg/ml respectively. Percentage reduction was 89.68, 88.41 and 93.91 at 0.005, 0.01, and 0.02 mg/ml respectively at 16 hours. Statistical analysis showed that all the test concentrations significantly reduced acid and bacterial counts with $p<0.01$.

Table 4.1: The effect of 0.005, 0.01 and 0.02 mg/ml of *U. chamae* extract on *S. mutans* acid production

		0 hours				10 hours				12 hours				14 hours				16 hours			
Strains	**Repeats**	**control**	**0.005**	**0.01**	**0.02**	**Control**	**0.005**	**0.01**	**0.02**	**Control**	**0.005**	**0.01**	**0.02**	**Control**	**0.005**	**0.01**	**0.02**	**control**	**0.005**	**0.01**	**0.02**
SM1	1	6.73	6.75	6.72	6.77	6.09	6.45	6.54	6.61	5.33	6.23	6.56	6.42	4.82	6.07	6.28	6.56	4.70	5.66	6.36	6.47
	2	6.57	6.53	6.52	6.57	6.12	6.48	6.48	6.67	5.56	6.31	6.40	6.43	5.00	6.37	6.40	6.26	4.76	6.39	6.39	6.41
	3	6.58	6.53	6.50	6.55	6.14	6.47	6.48	6.55	5.54	6.33	6.38	6.49	5.09	6.26	6.43	6.43	4.68	6.36	6.44	6.44
SM6	1	6.51	6.61	6.66	6.73	4.83	6.35	6.25	6.48	4.53	6.19	6.64	6.62	4.49	6.59	6.45	6.59	4.52	6.31	6.48	6.55
	2	6.52	6.57	6.64	6.66	4.70	6.40	6.43	6.47	4.72	6.54	6.53	6.59	4.52	6.4	6.37	6.50	4.47	6.36	6.40	6.55
	3	6.54	6.57	6.61	6.67	4.42	6.36	6.51	6.49	4.59	6.40	6.53	6.59	4.49	6.61	6.52	6.58	4.50	6.40	6.53	6.54
SM7	1	6.73	6.82	6.70	6.82	5.62	6.54	6.63	6.67	4.83	653	6.82	6.78	4.77	6.81	6.89	6.86	4.75	6.86	6.92	6.9
	2	6.74	6.78	6.81	6.69	5.66	6.60	6.64	6.69	4.98	6.83	6.74	6.76	4.76	6.82	6.89	6.88	4.63	6.91	6.93	6,91
	3	6.72	6.69	6.70	6.70	5.56	6.63	6.65	6.75	4.96	6.83	6.8	6.76	4.81	6.85	6.8	6.96	4.66	6.89	6.93	6.9
SM12	1	6.58	6.82	6.65	6.77	5.86	6.67	6.5	6.81	5.37	6.47	6.55	6.54	6.67	6.75	6.71	6.67	4.78	6.82	6.62	6.66
	2	6.74	6.81	6.70	6.82	5.78	6.77	6.76	6.54	5.64	6.81	6.18	6.56	4.49	6.75	6.63	6.79	4.78	6.65	6.34	6.81
	3	6.72	6.70	6.75	6.65	5.79	6.63	6.57	6.53	5.31	6.55	6.54	6.52	5.03	6.73	6.61	6.78	4.75	6.73	6.88	6.72
SM13	1	6.57	6.61	6.56	6.58	4.82	6.51	6.47	6.45	4.61	6.45	6.42	6.47	4.47	6.39	6.46	6.45	4.44	6.36	6.46	6,41
	2	6.44	6.62	6.46	6.52	4.93	6.49	6.48	6.48	4.49	6.45	6.45	6.49	4.44	6.39	6.35	6.42	4.44	6.38	6.45	6.44
	3	6.59	6.65	6.62	6.61	4.80	6.44	6.48	6.46	4.56	6.44	6.44	6.44	4.45	6.36	6.37	6.31	4.45	6.45	6.43	6.44
Mean		**6.62**	**6.67**	**6.64**	**6.67**	**5.41**	**6.52**	**6.52**	**6.58**	**5.00**	**6.49**	**6.53**	**6.56**	**4.82**	**6.52**	**6.54**	**6.60**	**4.62**	**6.50**	**6.57**	**6.61**
±SD		**0.10**	**0.10**	**0.10**	**0.10**	**0.59**	**0.12**	**0.12**	**0.12**	**0.42**	**0.20**	**0.17**	**0.12**	**0.56**	**0.23**	**0.20**	**0.21**	**0.14**	**0.32**	**0.23**	**0.19**

Table 4.2: The effect of 0.005 mg/ml of *U. chamae* on *S. mutans* counts in acid assay

Strains	Repeats	0 hours		10 hours		12 hours		14 hours		16 hours	
		Control	**Plant**	**Control**	**Plant**	**Control**	**Plant**	**Control**	**Plant**	**Control**	**Plant**
SM1	1	435000	371500	133000	220900	276000	227000	892000	230300	1216000	302600
	2	335000	345000	1056000	380000	226800	214400	3240000	285600	1368000	304000
	3	418000	323000	1536000	341600	2136000	463000	4800000	196600	341600	144200
SM6	1	480000	472000	185000	265600	3540000	192000	5600000	444000	5640000	248000
	2	372000	172400	5680000	264000	3384000	369600	5600000	461600	4095000	270400
	3	318400	605600	444000	362400	1992000	395200	454200	278200	4812000	304000
SM7	1	286400	936000	1870000	384000	2160000	344000	578000	288800	3370000	420000
	2	358800	346000	2460000	216000	3400000	252000	560000	284800	5760000	282400
	3	456000	345000	1568000	384000	216000	234400	3674000	228000	2736000	280000
SM12	1	864000	1170000	2460000	525600	152950	744000	2540000	294000	726000	423600
	2	856000	774000	482000	420400	2160000	257600	840000	257600	428800	422400
	3	672000	784000	340000	362400	3420000	464000	3670000	204000	3612000	344000
SM13	1	354000	218400	128000	364800	390000	312800	7680000	269600	4200000	372000
	2	435000	371500	133000	220900	276000	227000	892000	230300	1216000	302600
	3	309600	384000	4220000	2400000	540000	297600	4836000	588000	5832000	260000
Mean		**463347**	**507893**	**1513000**	**474173**	**1617983**	**332973**	**3057080**	**302760**	**3023560**	**312013**
±SD		**186297**	**285598**	**1645285**	**539618**	**1377127**	**143517**	**2314712**	**109637**	**2017434**	**75509**
% Reduction				**66.66**		**79.42**		**91.00**		**89.68**	

Table 4.3: The effect of 0.01 mg/ml of *U. chamae* on *S. mutans* counts in acid assay

Strains	Repeats	0 hours		10 hours		12 hours		14 hours		16 hours	
		Control	**Plant**	**Control**	**Plant**	**Control**	**Plant**	**Control**	**Plant**	**Control**	**Plant**
SM1	1	435000	361500	133000	267800	276000	210600	892000	191300	1216000	218000
	2	335000	311000	1056000	228000	226800	206800	3240000	285500	1368000	254100
	3	418000	300000	1536000	289200	2136000	143500	4800000	226200	341600	240500
SM6	1	480000	240800	185000	112000	3540000	374400	5600000	284000	5640000	312800
	2	372000	360000	5680000	275200	3384000	236000	5600000	376800	4095000	282400
	3	318400	268000	444000	239200	1992000	202000	454200	305200	4812000	256000
SM7	1	286400	580000	1870000	240800	2160000	309600	578000	268000	3370000	471000
	2	358800	264000	2460000	254400	3400000	116800	560000	131200	5760000	188800
	3	456000	4110000	1568000	360000	216000	231200	3674000	81600	2736000	182400
SM12	1	864000	640000	2460000	754200	152950	664000	2540000	804000	726000	372000
	2	856000	682000	482000	444000	2160000	680000	840000	242800	428800	600000
	3	672000	656000	340000	525600	3420000	388000	3670000	560800	3612000	580000
SM13	1	354000	318000	128000	421600	390000	398400	7680000	434400	4200000	230000
	2	435000	361500	133000	267800	276000	210600	892000	191300	1216000	218000
	3	309600	456000	4220000	456000	540000	202800	4836000	344000	5832000	850000
Mean		**463347**	**660587**	**1513000**	**342387**	**1617983**	**304980**	**3057080**	**315140**	**3023560**	**350400**
±SD		**186297**	**966370**	**1645285**	**157280**	**1377127**	**171169**	**2314712**	**180189**	**2017434**	**192692**
% Reduction				**77.37**		**81.15**		**89.69**		**88.41**	

Table 4.4: The effect of 0.02 mg/ml of *U. chamae* extract on *S. mutans* counts in acid assay

Strains	Repeats	0 hours		10 hours		12 hours		14 hours		16 hours	
		Control	**Plant**	**Control**	**Plant**	**Control**	**Plant**	**Control**	**Plant**	**Control**	**Plant**
SM1	1	435000	420000	133000	143550	276000	135000	892000	119900	1216000	110300
	2	335000	345000	1056000	127900	226800	151500	3240000	164000	1368000	79450
	3	418000	205000	1536000	148000	2136000	77600	4800000	141500	341600	190500
SM6	1	480000	994400	185000	235600	3540000	180800	5600000	74800	5640000	172800
	2	372000	344000	5680000	143000	3384000	87200	5600000	124400	4095000	50800
	3	318400	322400	444000	215000	1992000	226800	454200	113200	4812000	96000
SM7	1	286400	2440000	1870000	176000	2160000	176000	578000	288800	3370000	222200
	2	358800	410400	2460000	215200	3400000	176000	560000	240800	5760000	192000
	3	456000	287000	1568000	220000	216000	242400	3674000	148800	2736000	82400
SM12	1	864000	602000	2460000	301600	152950	230400	2540000	138200	726000	204900
	2	856000	1640000	482000	444000	2160000	218400	840000	240400	428800	262000
	3	672000	868000	340000	164100	3420000	300000	3670000	218800	3612000	260000
SM13	1	354000	361200	128000	301600	390000	562000	7680000	269600	4200000	320800
	2	435000	420000	133000	143550	276000	135000	892000	119900	1216000	110300
	3	309600	288000	4220000	110300	540000	224000	4836000	189700	5832000	408000
Mean		**463347**	**663160**	**1513000**	**205960**	**1617983**	**208207**	**3057080**	**172853**	**3023560**	**184163**
±SD		**186297**	**618262**	**1645285**	**88321**	**1377127**	**114772**	**2314712**	**64558**	**2017434**	**100171**
% Reduction				**86.39**		**87.13**		**94.34**		**93.91**	

Table 4.5: Statistical analysis of the data obtained in the acid production assay

Test	Comparison	P value
pH	Control to all concentrations	<0.0001
	Control to 0.005 mg/ml	0,0004
	Control to 0.01 mg/ml	0,0004
	Control to 0.02 mg/ml	0,0003
Bacterial counts	Control to all concentrations	<0.0001
	Control to 0.005 mg/ml	0,0060
	Control to 0.01 mg/ml	0,0052
	Control to 0.02 mg/ml	0.0034

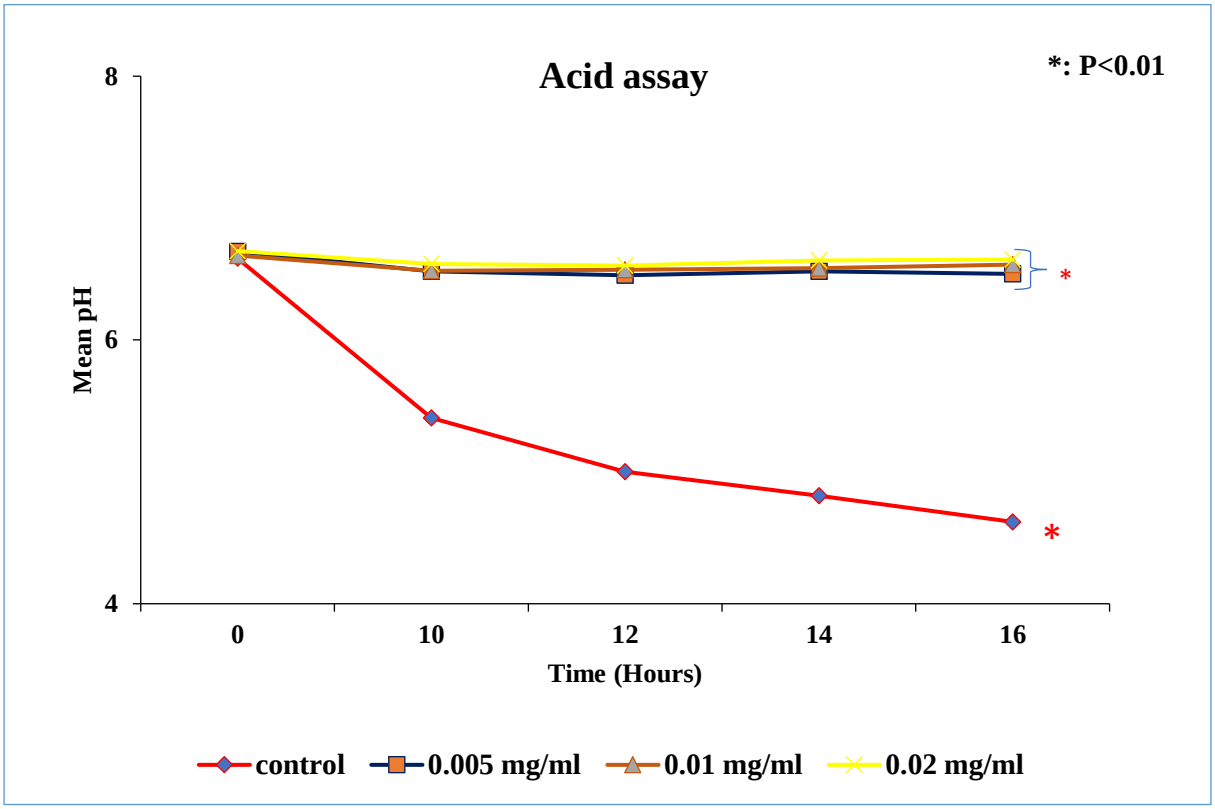

Figure 4.2: The effect of *U. chamae* on *S. mutans* acid production in acid assay

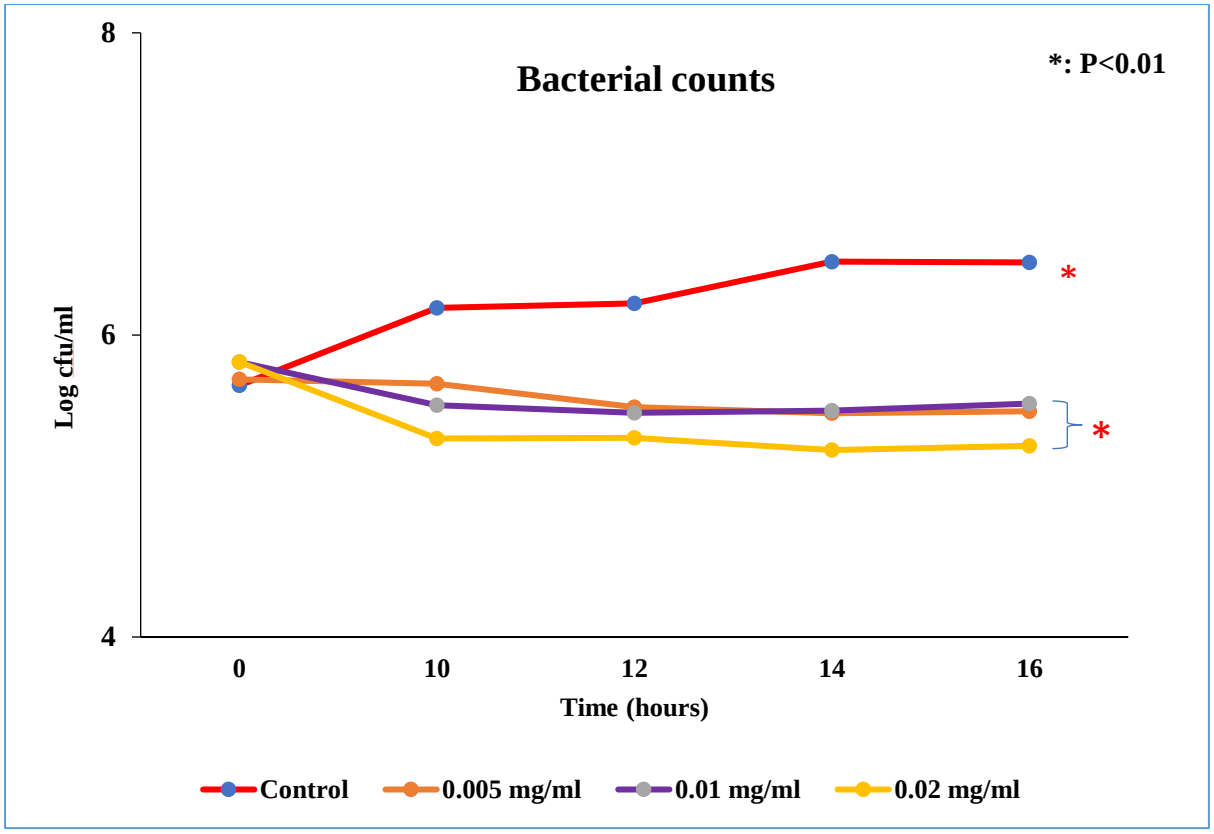

Figure 4.3: The effect of *U. chamae* on the growth of *S. mutans* in acid production assay

4.4. Discussion

Acid production is another important virulence factor in *S. mutans* (Xu et al., 2014). Natural plant products, mainly phytochemicals, and their derivates have been used as major sources of effective therapeutic agents throughout history and are considered as an alternative to antimicrobial agents (Jeon et al., 2011). Phytochemicals that can inhibit the glycolytic pathway in oral bacteria are of interest. In the present study, *U. chamae* significantly reduced the level of acid produced by *S. mutans*. At 10 hours, the control experiment produced a critical pH below 5.5 in which demineralization of tooth enamel occurs and it was independent of the bacterial counts. The presence of the plant extract did not allow the pH to drop below 6.50. The plant extract might have interfered with the glycolytic pathway e.g F-ATPase which is responsible for the aciduric and acidogenic properties of *S. mutans*. Additionally, the plant extract might have affected the catalytic enzymes lactate dehydrogenase involved in the conversion of glucose to pyruvate and subsequently into lactic acid, and/or the enolase enzyme which is responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate and subsequent production of acid. After 10 hours, the inhibitory effect of the plant extract was due to antibacterial activity rather than the direct effect on the glycolytic pathway and/or disruption on the membrane-associated F-ATPase activity since the bacterial counts were significantly reduced.

Medicinal plants and their compounds are known to inhibit acid production of cariogenic streptococci and some of them appear to reduce bacterial growth rather than inhibiting the glycolytic activity and/ or aciduricity of the cariogenic organisms. For example, a study by Prabu et al. (2006) found that subinhibitory concentrations of guaijaverin were associated with a gradual decrease in the acid production of *S. mutans*. The effect of the guaijaverin on the acid production of *S. mutans* was related to the bacteriostatic effect of the bioactive compounds present on the plant extract (Prabu et al., 2006). Moreover, Gregoire et al. (2007) has shown that methanolic extracts of *Polygonum cuspidatum* can inhibit glycolytic acid production thereby reducing *S. mutans* acid production. The author suggested that the inhibitory effect may be due to the presence of bioactive compounds in the extract (Palombo et al., 2011). Smullen et al. (2007) have shown that 70% aqueous propanone extracts of fermented cocoa, unfermented cocoa, cocoa liquor, and several polyphenol fractions derived from unfermented

cocoa extract inhibited the growth and acid production by *S. mutans*. The effect of these extracts was associated with the bacteriostatic effect of the extracts (Smullen et al., 2007).

Recent studies have shown that some medicinal plants and their metabolites affect the acidogenicity of *S. mutans* by disrupting the membrane proton motive force, enzymatic activity, and expression of specific enzymes related to sugar transport, glycolysis, and general metabolism. For example,7-epiclusianone isolated from *Rheedia garneriana*, and tt -farnesol has been reported to increase the proton permeability of *S. mutans* cells, causing cytoplasmic acidification, and thereby inhibiting the acid-sensitive intracellular glycolytic enzymes (Jeon et al., 2011). Moreover, Gregoire et al., (2007) has shown that the cranberry flavonols disrupt the membrane-associated F-ATPase activity of *S. mutans*, thereby reducing acid production (Gregoire et al., 2007). The fruit extracts such as cranberry, cocoa, babchi, mangosteen, and grape have shown inhibitory effects on the F0-ATPase, F1-ATPase, and acid production activities of *S. mutans*. In addition to the F1–F0-ATPase, the flavones have also been shown to interact with the Ca^{2+}-ATPase and Na+/K+-ATPase. The α-mangostin has been shown to inhibit the *S. mutans* glycolysis thereby leading to indirect inhibition of respiration (Abachi et al., 2016). The propolis extracts have been found to inhibit the acidogenicity and aciduricity of *S. mutans.* Furthermore, apigenin and tt -farnesol significantly reduce caries development in rats with minimal effects on the population of viable cells in the plaque. The antimicrobial effect of this extract was attributed to inhibition of the bacterial glycolysis (Jeon et al., 2011). A crude extract of *Psidium cattleianum* and epigallocatechin gallate (isolated from the green tea extracts) has been shown to disrupt the expression and activity of lactate dehydrogenase enzyme, which is involved in the glycolytic pathway of *S. mutans* (Hirasawa et al., 2006;Brighenti et al., 2008).

4.5. Conclusion

The concentrations of 0.005, 0.01, and 0.02 mg/ml of *U. chamae* extract reduced the bacterial counts and acid production of *S. mutans*. The activity of *U. chamae* extract on acid production was due to the antibacterial effect of the extract. This extract did not target the virulence of *S. mutans* acid production but affected the number of infectious agents thereby reducing acid production. These results are significant as acid production has a direct influence on the

aetiology of dental caries and advocates this extract as a candidate for the development of a natural product-based caries prevention product.

Chapter 5: The effect of *Uvaria chamae* dichloromethane extract on soluble and insoluble extracellular polysaccharides production by *Streptococcus mutans*

5.1. Introduction

Extracellular polysaccharides are recognized as an essential virulence factor associated with dental caries (Szkaradkiewicz-Karpińska and Szkaradkiewicz, 2021). The ability of *S. mutans* to produce extracellular polysaccharides has a significant impact on the development and composition of pathogenic biofilms (Lins de Sousa et al., 2015). Extracellular glucans are involved in the pathogenesis of dental caries and they are synthesized by the enzyme glucosyltransferases (Gtfs). These glucans promote adherence and accumulation of cariogenic bacteria to the tooth surfaces (Chen et al., 2016). Although *S. mutans* accounts for only a small fraction of the oral microflora in healthy conditions, this cariogenic oral pathogen can rapidly assemble virulence biofilms through the production of exopolysaccharides in the presence of high exposure to dietary sugar (Palmer et al., 2019). Other microbial species e.g *Actinomyces viscosus*, *Lactobacillus casei*, and *Candida albicans* become extracellular glucan producers when they are bound to *S. mutans* Gtfs and contribute to the growing multispecies biofilm (Cugini et al., 2019).

Sucrose is considered the most cariogenic dietary carbohydrate, and also serves as a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides (Oliveira et al., 2021;Leme et al., 2006a). *Streptococcus mutans* utilize dietary sucrose to synthesize soluble and insoluble exopolysaccharides (EPS) by glucosyltransferases (GtfBCD, forming homopolymers of glucose called glucans) and fructosyltransferase (Ftf, forming homopolymers of fructose termed fructans) secreted by *S. mutans* and can be internalized for fermentation through the sugar: phosphotransferase system-Figure 5.1(Cai et al., 2016;Napimoga et al., 2005). The glucans promote bacterial attachment and biofilm accumulation, whereas fructans serve primarily as extracellular storage polysaccharides that contribute to the persistence and cariogenicity of *S. mutans* (Oliveira et al., 2021). The glucan-binding domain (GLU) is responsible for the composition of the glucans and promotes adherence to the tooth surface, forming compact dental plaque (Huang et al., 2013). EPS and IPS are storage nutrients for the bacteria (Costa Oliveira et al., 2017).

In addition to GTFs, another two cell-associated antigens (Ags) are correlated directly with the ability of *S. mutans* to adhere to EPS and accumulate in the tooth surface. These are antigen I/II (AgI/II) as well as the glucan-binding protein (Smith, 2002). Glucan binding protein A contains carboxyl-terminal repeats similar to the glucan-binding domain of glucosyltransferase enzymes and it contributes to the development of optimal plaque biofilm. Furthermore, gbpA also plays an important role in binding proteins and exopolysaccharides during the construction of the biofilm. A deficiency of gbpA results in loose binding to the EPS matrix, resulting in a weak non-uniform biofilm structure (Matsumoto-Nakano, 2018).

Extracellular polysaccharides contain biologically active components, such as antimicrobial enzymes. The biological function of these enzymes is to protect the biofilm community against noxious environmental stimuli (Seneviratne et al., 2011). Due to the presence of LTA and possibly eDNA, EPS from *S. mutans* may be charged. The presence of negatively charged EPS affects the diffusion and antimicrobial activity of positively charged chlorohexidine. This results in acid accumulation and hinders neutralization by buffering saliva that surrounds the teeth (Klein et al., 2015). Furthermore, EPS could constitute an extracellular energy reserve in which soluble glucans and fructans are degraded by specific hydrolases, releasing glucose and fructose to be metabolized, resulting in acids production. While EPS degradation may occur during "famine" periods, the acid produced by a non-removed biofilm may prolong enamel demineralization, particularly at night when the salivary flow rate is low (Costa Oliveira et al., 2017).

The widespread usage of fluoride has had a significant impact on caries prevention (Thurnheer and Belibasakis, 2018). Fluoride plays an important role in preventing the prevalence and severity of dental caries. It is a well-known cariostatic agent that inhibits demineralization while promoting remineralization. It also plays an important role in caries prevention by inhibiting the synthesis of extracellular polysaccharides (EPS) by reducing the secretion of the enzyme glucosyltransferase (Li et al., 2020;Nassar and Gregory, 2017). However, excess use of fluoride results in adverse effects such as fluorosis. Therefore, the development of an alternative cariostatic agent with minimal side effects is urgent. Nowadays, oral care products and medicinal plant extracts are gaining high interest because they are less toxic and have fewer side effects compared to synthetic drugs (Şener and Kiliç, 2019). Therefore, this study aimed to investigate the effect of *U. chamae* on the production of soluble and insoluble extracellular polysaccharides by *S. mutans.*

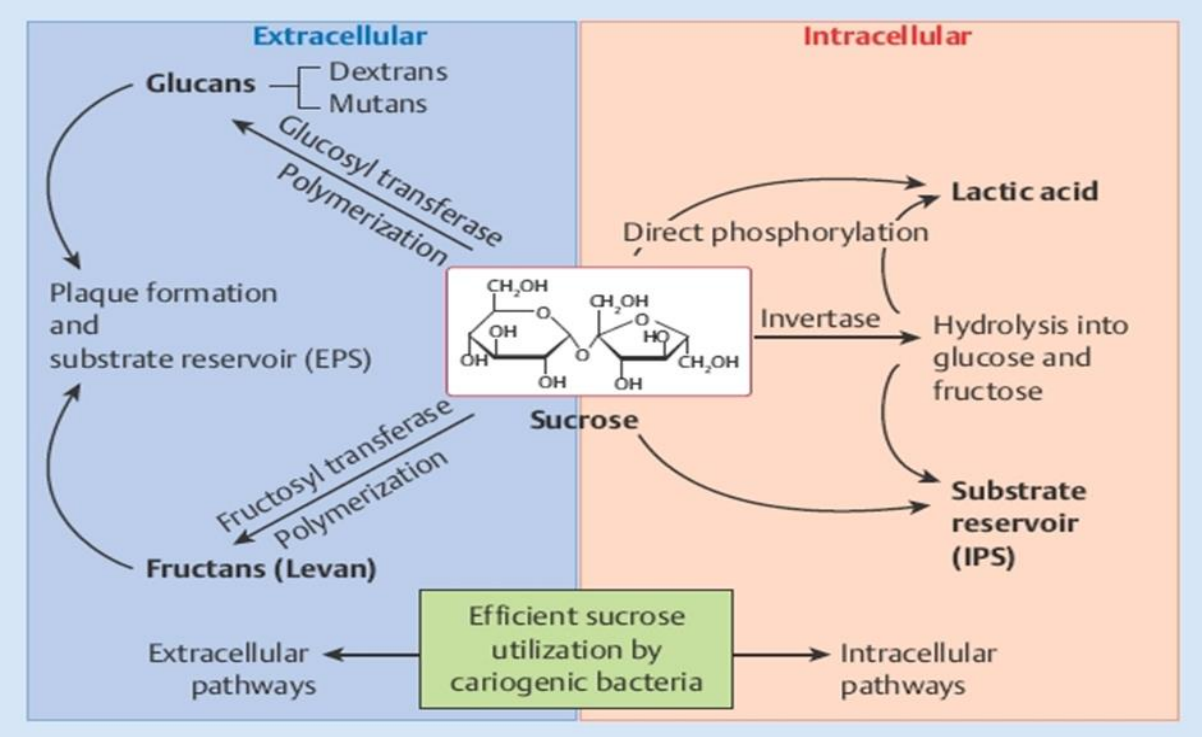

Figure 5.1: Illustration of the efficient metabolism of sucrose by cariogenic *S. mutans.*

5.2. Method and materials

5.2.1. Bacterial cultures

Five cariogenic *S. mutans* clinical strains were obtained and isolated as described in Chapter 2, section 2.2.2.

5.2.2. Plant materials and extraction

Uvaria chamae extracts were prepared and provided by our collaborator from Nigeria. The extraction procedure is described in Chapter 2, section 2.2.1.

5.2.3. Soluble extracellular polysaccharide production assay

The effect of *U. chamae* on the SEPS production was studied using a technique as previously described by (Koo et al., 2002). The *S. mutans* was grown in 5% sucrose broth for 48 hours. Thereafter, 5 ml of the resulting culture was centrifuged at 5000×g for 10 minutes. The procedure was repeated 3 times. After centrifugation, the supernatant was collected and placed on a clean 50 millilitres centrifuge tube. Pellets were used in the subsequent assay. Three volumes of absolute ethanol were added into the supernatant and placed at -20^{o}C for 30 minutes to precipitate polysaccharides. The resulting mixture was centrifuged at 5000×g for 5 minutes followed by washing the pellets with 70% ethanol. The precipitate was resuspended in a 5 ml sodium hydroxide (NaOH). Two ml of the 5% aqueous phenol and 5 millilitres of the concentrated sulphuric acid were added to the mixture and allowed to stand at room temperature in dark for 10 minutes. Thereafter, the mixture was vortexed for 30s then placed in a water bath at the temperature of 25^{o}C for 20 minutes. The absorbance was read at 490 nm. This study was performed in triplicates using 5 strains of *S. mutans*.

5.2.4. Insoluble Extracellular Polysaccharide production assay

Five millilitres of 1 M sodium hydroxide was added to the pellets (described above). The suspension was vortexed for 30 s, agitated for 15 minutes, and centrifuged for 5 minutes at 5000×g. The supernatant was transferred to a clean 50 ml centrifuge tube. This step was repeated three times. The supernatant was pooled together and three times the volume of cold absolute ethanol was added. The polysaccharides were allowed to precipitate at -20^{o}C for 30 minutes. The mixture was centrifuged at 5000×g for 5 minutes and the pellet was washed with 70% ethanol. The precipitate was then resuspended in 5 ml of NaOH. Two millilitres of 5% aqueous solution of phenol and 5 mL of concentrated sulphuric acid were added rapidly to the mixture and were left to stand at room temperature in the dark for 10 minutes. Thereafter, it was vortexed for 30 s, placed in the water bath at 25^{o}C for 20 minutes and the absorbance was read at 490 nm. These experiments were repeated three times for 5 strains of *S. mutans.*

5.2.5. Statistical analysis

The effect of different concentrations of *U. chamae* extract on SEPS and IEPS production by *S. mutans* was determined by comparing optical density (OD) readings of the test results to the controls using the Wilcoxon Rank Sum Test. The mean and standard deviations were calculated using Microsoft Excel. The percentage reduction in the SEPS and IEPS was calculated using the formula $(x-y/x) \times 100$ where x is the control while y is the test EPS optical density.

5.3. Results

The amount of SEPS and IEPS produced by *S. mutans* were quantitatively measured using the phenol sulphuric acid technique. The results in a form of optical density readings are shown in Table 5.1 and are presented in the graphs (Figure 5.1). Additionally, the percentage reduction was calculated and a graph is presented in Figure 5.2. The percentage reduction in this test results ranged between 14.29 to 36.19% for SEPS and 0 to 46.67% for IEPS. The percentage reduction of the test results on IEPS was similar at approximately 0.005 and 0.02 mg/ml. At 0.01 mg/ml, the percentage reduction of SEPS was 0 %. The statistical analysis showed that the test concentrations of *U. chamae* extract had no significant effect on the SEPS and IEPS production in the planktonic growth of *S. mutans* with the p value < 0.05.

Table 5.1: The effect of 0.005, 0.01, and 0.02 mg/ml of *U. chamae* on the production of soluble and insoluble extracellular polysaccharides by planktonic cells of *S. mutans*.

Optical density at 490 nm									
		SEPS				**IEPS**			
Strain	Repeats	Control	0.005	0.01	0.02	Control	0.005	0.01	0.02
SM1	1	0.441	0.599	0.724	0.559	0.182	0.066	0.057	0.059
	2	0.596	0.666	0.167	0.491	0.175	0.055	0.06	0.059
	3	0.264	0.179	0.162	0.178	0.243	0.065	0.058	0.062
SM6	1	1.625	0.648	1.064	2.231	0.130	0.071	0.083	0.083
	2	1.625	0.939	0.382	0.386	0.120	0.081	0.101	0.079
	3	0.722	0.948	0.762	1.132	0.108	0.106	0.053	0.083
SM7	1	2.466	0.737	2.348	0.406	0.207	0.085	0.093	0.086
	2	2.712	0.929	1.822	0.742	0.150	0.087	0.589	0.082
	3	2.578	1.508	1.405	1.001	0.321	0.153	0.094	0.108
SM12	1	0.325	0.239	0.315	0.087	0.097	0.064	0.080	0.055
	2	0.234	0.087	0.113	0.112	0.125	0.058	0.059	0.059
	3	0.298	0.111	0.741	0.155	0.126	0.058	0.061	0.083
SM13	1	0.532	0.975	1.175	0.815	0.092	0.090	0.089	0.105
	2	0.718	1.253	1.035	0.889	0.116	0.071	0.097	0.075
	3	0.67	0.255	1.213	1.34	0.100	0.080	0.703	0.084
Mean		**1.05**	**0.67**	**0.90**	**0.71**	**0.15**	**0.080**	**0.150**	**0.08**
±SD		**0.90**	**0.43**	**0.64**	**0.57**	**0.06**	**0.02**	**0.20**	**0.02**
% Reduction			**36.19**	**14.29**	**32.38**		**46.67**	**0**	**46.67**

Table 5.2: Statistical analysis of the data obtained in the extracellular polysaccharides production assay

Test	Comparison	P value
SEPS	Control to all concentrations (overall)	0,3125
	Control to 0.005 mg/ml	0,6672
	Control to 0.01 mg/ml	0.9596
	Control to 0.02 mg/ml	0.7222
IEPS	Control to all concentrations (overall)	0,3125
	Control to 0.005 mg/ml	0,2194
	Control to 0.01 mg/ml	>0,9999

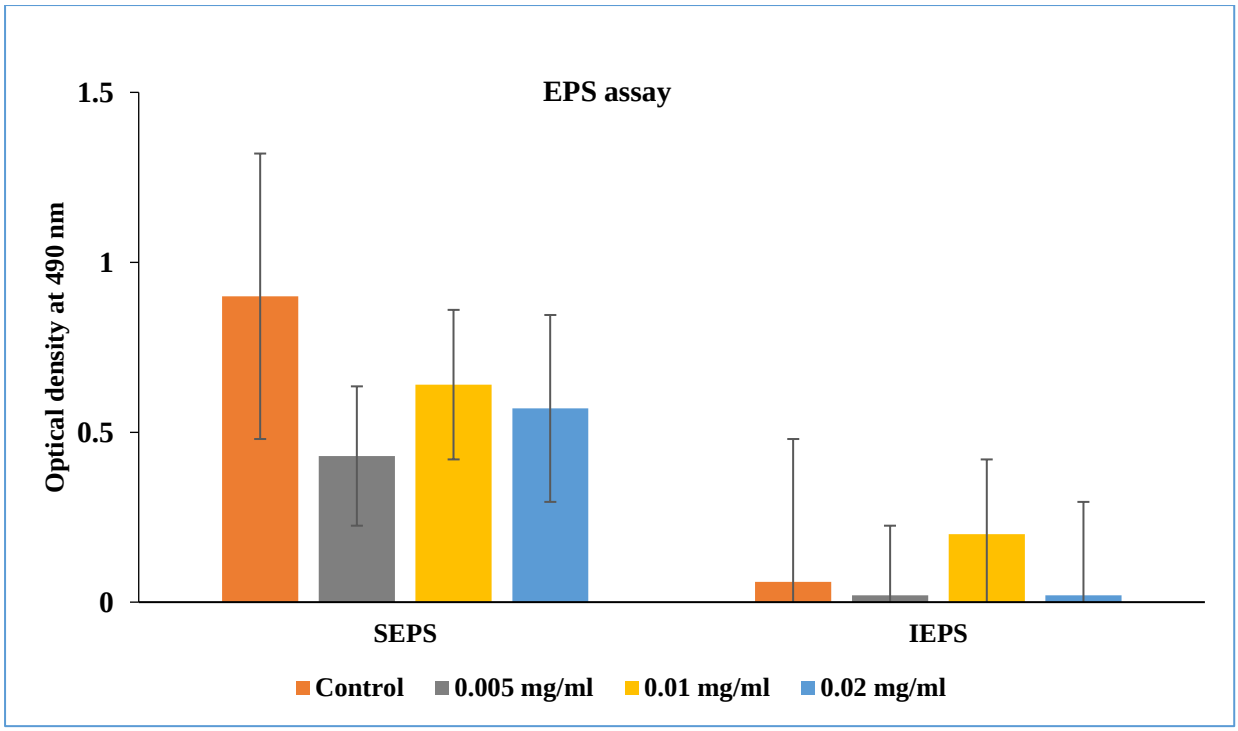

Figure 5.2: The effect of *Uvaria chamae* on soluble and insoluble extracellular polysaccharides by *Streptococcus mutans*

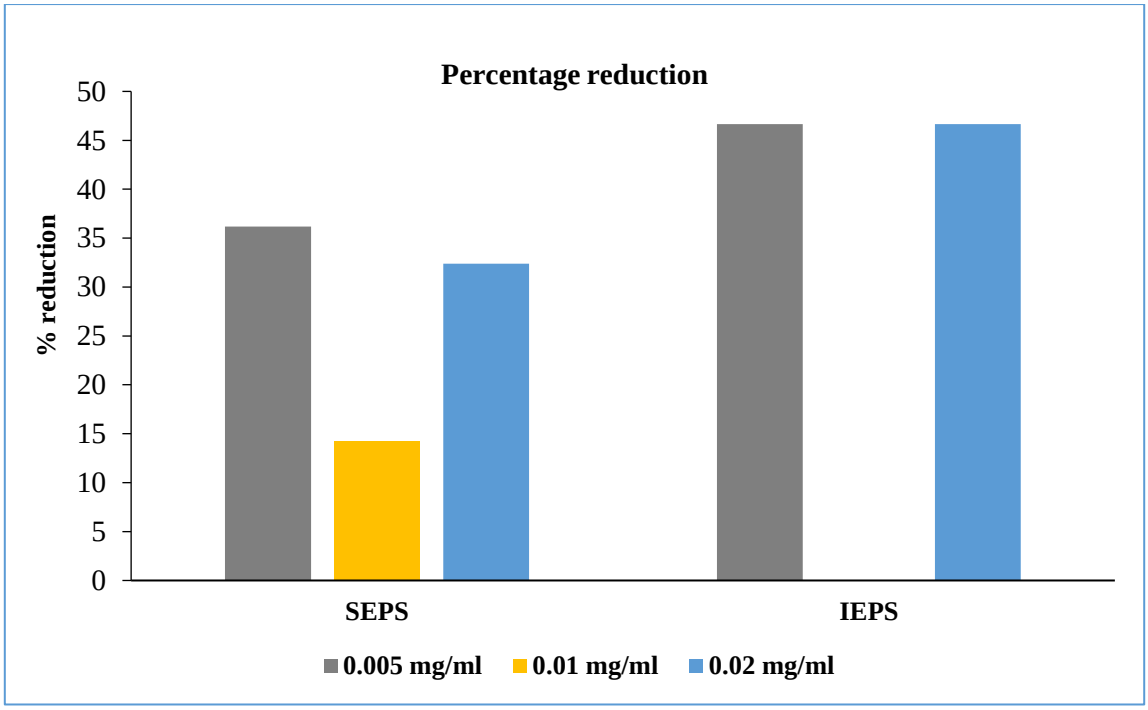

Figure 5.3: Percentage reduction of *S. mutans* soluble and insoluble extracellular polysaccharides production by *U. chamae*

5.4. Discussion

The production of extracellular polysaccharides by *S. mutans* contributes to the cariogenic potential of dental biofilms and its disruption is important for the control of dental caries (Mandava et al., 2019). Water-soluble polysaccharides serve as a source of energy for plaque bacteria when nutrient conditions become limited, and thus support cariogenic attack at the enamel surface. The water-insoluble glucans promote the adhesive interactions of bacteria with the tooth surfaces and contribute to the formation of dental biofilm. Therefore, this study investigated the effect of *U. chamae* extracts on the synthesis of SEPS and IEPS by *S. mutans*. The results showed that *U. chamae* partially inhibited the synthesis of SEPS and IEPS production. However, the reduction was not statistically significant. These results suggest that EPS might have prevented the penetration of bioactive compounds present in the plant extract due to the presence of negatively charged LTA and possibly eDNA (Klein et al., 2015). Both eDNA and LTA influence diffusion properties and thereby affect the activity of cationic antimicrobial or anti-biofilm agents (Castillo Pedraza et al., 2017). The detailed mechanisms involved in limiting diffusion remain unclear (Klein et al., 2015). In contrast, EPS were

metabolized in a small quantity, enough to maintain the bacterial basal metabolism, and were not detected by the phenol sulfuric acid method.

Medicinal plants and their products have been reported to partially inhibit the activity of the *gtfB* and *gtfC*. For example, aqueous-ethanol extracts of propolis, have shown to be effective *gtfB* and *gtfC* inhibitors, and the effect was dependent on the geographical origin and the biodiversity of the sample collected. The inhibitory effect of propolis on Gtf activity was due to the presence of flavones and flavonols compounds (Bowen and Koo, 2011). Rahim and Thurairajah has shown that the *Piper betle L.* extract affects the adhering capacity of *S. mutans* by inhibiting the activity of the GTF and hence the EPS formation (Rahim and Thurairajah, 2011). Tea polyphenols and the grape extracts inhibited EPS synthesis. Cranberry proanthocyanidins inhibited the activities of GTFs and EPS biovolumes (Lin et al., 2021). An in vivo study has shown that the polymeric polyphenols Oolong tea inhibited both the glucosyltransferase production and the sucrose-dependent cell adherence of the *S. mutans* thus preventing plaque accumulation and the development of dental caries (Gulube and Patel, 2016). The chloroform/methanol fractions of *Nidus Vespae* extract and their chemical fractions inhibited the enzymatic activity of cell-associated and extracellular GTFs by *S. mutans* at a sub-MIC concentration (Xiao et al., 2007). The *B. crassifolia* water leaves extract and sub extracts has been shown to inhibit water-insoluble glucans more than the water-soluble glucans (Liu et al., 2017). *Punica granatum* methanolic extracts have been shown to reduce insoluble EPS production by *S. mutans*. The author suggested that the plant extract might have affected the production of the enzyme glucosyltransferase, which is an enzyme responsible for the production of EPS (Gulube and Patel, 2016).

5.5. Conclusion

The results showed that *U. chamae* had no significant effect on SEPS and IEPS production in the planktonic growth. Therefore, future studies using a longer period of starvation or a methodology using radiolabelled sucrose in the fructosyl (3H) and glucosyl (14C) moieties, instead of the phenol sulfuric method, might be useful to clarify EPS metabolization. Further research was performed to fully elucidate the effect of *U. chamae* on the gene expression of GTFs (following chapter).

Chapter 6: The effect of *Uvaria chamae* extract on the expression of virulence genes in *Streptococcus mutans*

6.1. Introduction

Streptococcus mutans is the most common pathogen associated with dental caries through products of multiple genes involved in bacterial adhesion, extracellular polysaccharide formation, sugar uptake and metabolism, acid tolerance, biofilm formation, and regulation (Lee et al., 2019;You, 2019;Schneider-Rayman et al., 2021). The summary of virulence genes in *S. mutans* is shown in Figure 6.1. The first step in the development of caries is initial adherence to a solid surface, which is followed by the formation of dental plaque (Krzyściak et al., 2014). This process is regulated by several genes including the *gbpB*, *sacB* (*ftf*), *vicR*, and *wapA*. These genes are involved in the sucrose-dependent adhesion, and *spa*P is involved in sucrose-independent adhesion. The genes *atlA*, *sacB* (*ftf*), SMU.609, *vic*R, and *wapA* play a significant role in the formation of biofilms. Other genes such as the *gtfB*, *gtfC*, *gtfD*, *ftf*, and *vic*R are involved in the formation of extracellular polysaccharides (Gabe et al., 2019). The *gtfB* promotes cell aggregation and cohesion of various bacterial strains and can convert non glucan-producing bacteria into glucans producers (Wu et al., 2020). These genes also encode several groups of proteins and enzymes, including glucosyltransferases (GTFs) GTFB and GTFC, which synthesize water-insoluble glucans, and GTFD, which synthesizes water-soluble glucans, and glucan-binding proteins (Zain, 2011).

Another virulence factor that is essential for the onset of dental caries is acid production via the glycolytic pathway (Walsh, 2006). During this process, glucose is converted into organic acids such as pyruvic acid, lactic acid, and formic acid. Sugars are processed via the Embden Meyerhof pathway and metabolic degradation, in which the phosphorylated glucose is metabolized to form fructose-6-phosphate, which is then used to produce organic acids (Kawada-Matsuo et al., 2017). Lactic acid is the major end product of glycolysis when sugar levels are high (Dashper and Reynolds, 1996). Lactate dehydrogenase (LDH), encoded by the *IDH* gene, catalyzes the conversion of pyruvate to lactic acid (Guan et al., 2020;Walther et al., 2021). This causes rapid acidification of the dental biofilm, which is then sustained by the proton-translocating F-type ATPase (Gabe et al., 2020). The F-ATPase regulates the internal

pH of the cell by pumping protons out of the cell, resulting in an internal pH that is more basic than that of the plaque environment, thereby protecting relatively acid-sensitive glycolytic enzymes (Kuhnert and Quivey Jr, 2003). The expression of several genes, including the *gtfB*, *gtfC* and *gtfD*, *ftf*, and gbpB are regulated by the gene *vicR* (Guan et al., 2020).

This chapter investigated the effect of *U. chamae* on the expression of virulence genes (gbpB, *vicR*, *brpA*, *spaP*, *gtf*B, *gtf*C, *atpD*, and *IDH)*. This study may aid the development of a natural product as a novel therapeutic agent to counteract the virulence effect of *S. mutans*.

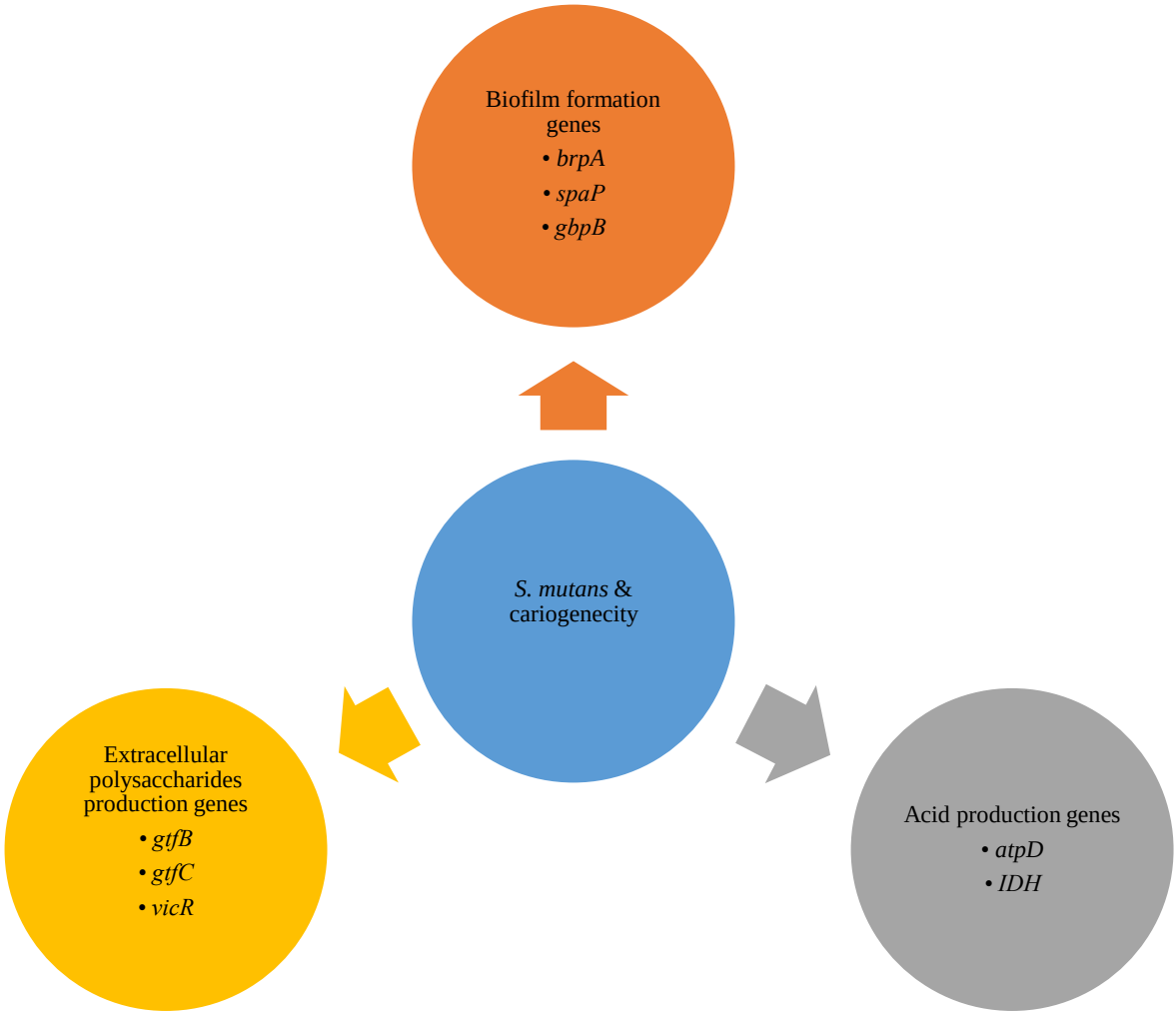

Figure 6.1: Virulence genes in *Streptococcus mutans*

6.2. Methods and materials

6.2.1. Plant materials and preparation

The extract was prepared by our collaborator from Nigeria. The extraction procedure is described in Chapter 2, section 2.2.1.

6.2.2. Bacterial cultures and growth conditions

Five cariogenic *S. mutans* clinical strains were obtained and isolated as described in Chapter 2, section 2.2.2. *Streptococcus mutans* strains SM1 and SM12 were independently grown in culture plates. These strains were used to perform acid production, biofilm formation, and secretion of extracellular polysaccharides (EPS) assays. For all assays, both strains prepared as 0.5 MacFarland standard containing approximately 10^5 to 10^6 cfu/ml were treated with *U. chamae* at a ½ MIC concentration of 0.01 mg/ml, while untreated samples were used as negative controls. For extraction of biofilm cells, the attached cells were removed from the glass slide by scrapping off the biofilm using a sterile glass slide. The cells were resuspended and vortexed in 5 ml phosphate buffered saline (pH 7.0). For the acid assay, the culture was grown in 25 ml of tryptone broth containing 5 % sucrose. For EPS assay, the culture was grown in 5% sucrose broth at 37^{o}C.

6.2.3. Total RNA extraction from microbial cultures

The total RNA was isolated at 10 hours after treatment with *U. chamae* for acid production assay and 24 hours post administration for biofilm and EPS experiments. Subsequently, 200 µl of the *S. mutans* culture was transferred to a sterile 1.5 ml Eppendorf tube and mixed with 200 µl TRI Reagent (Sigma-Aldrich, MI, USA). The solutions were mixed by vortexing and incubated at room temperature for 5 minutes. Fifty microliters of chloroform and an equal volume of acid-phenol (pH 4.5) were dispensed and mixed by shaking. The mixtures were centrifuged (Eppendorf centrifuge 5424, Merck NJ, USA) at 12 000 × g for 15 minutes, and 150 µl of the upper aqueous phase containing nucleic acids was transferred to a new 1.5 ml Eppendorf tube. Thereafter, 150 µl isopropanol was added to each sample and mixed by

inverting the tube. Samples were incubated at -70°C for 1 hour followed by centrifugation at 12 000 × g for 20 minutes. The resulting supernatants were discarded and the pellets were washed with 100 µl 70% ethanol. The ethanol was removed by centrifuging at 7500 × g for 5 minutes and the supernatants were removed. The RNA pellets were resuspended in 50 µl nuclease-free water and stored at -70°C until use.

6.2.4. Generation of complementary DNA by reverse transcription

To allow amplification and quantification by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique, total RNA was subjected to reverse transcription to generate complementary DNA (cDNA). For each RNA sample, 1 µg was used to produce cDNA and the reaction was performed in a total volume of 20 µl (ProtoScript®II First Strand cDNA Synthesis Kit, New England BioLabs, MA, USA). Reaction mixtures were incubated at room temperature for 5 minutes followed by reverse transcription at 42°C for 1 hour. Enzyme activity was inactivated by incubating samples at 80°C for a minute. The concentration and purity of the synthesized cDNA samples were evaluated by measuring the absorbance ratio at A260/A280 using the Nanodrop ONE instrument (Thermo Fisher Scientific, MA, USA).

6.2.5. Examining the expression level of virulence genes by RT-qPCR

The RT-qPCR method was used to determine the level of target gene expression in treated and untreated *S. mutans* strains. This assay was applied to determine the impact of administering the plant extract *U. chamae* on the expression of various virulence genes. Specifically, genes involved in acid production *atpD* and *IDH*, and biofilm formation *brpA*, *gbpB*, *spaP*, and *vicR* were investigated. Additionally, the transcription profile of *gtfB* and *gtfC* candidate genes implicated in the secretion of EPS was also evaluated. The RT-qPCR reactions were prepared by using 2× Luna® Universal qPCR Master Mix (New England BioLabs, MA, USA), 10 µM of each gene-specific primer and 2 µl cDNA (100 ng/µl). The nucleotide sequence of the primers employed for amplifying the target genes and the *16S ribosomal RNA* reference gene are shown in Table 6.1. Negative control samples lacking cDNA and water blanks were included in all gene expression experiments for quality control validation.

The thermal cycling conditions used for all amplifications consisted of hot start at 95°C for 60 seconds; followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 60°C for 30 seconds. Amplification reactions were conducted using the LightCycler® 480 II (Roche Diagnostics, Germany). The mRNA concentration of each gene was determined by crossing-point analysis and the specificity of gene expression data was verified by melting curve analysis. Data were analysed from three replicates representing treated and untreated *S. mutans* samples. The gene expression level was calculated by applying the formula: $\Delta Cq=2^{Cp\ target\ gene-Cp\ reference\ gene}$, where the crossing point (Cp) was the average Cp value of the target gene minus the mean of the *16S* reference gene. In addition, fold change in gene expression was determined by relativizing the mean $2^{Cp\ target\ gene}$ in treated samples to the average $2^{Cp\ target\ gene}$ in untreated samples.

Table 6.1: Primer sequences used to amplify the virulence genes and the 16 S ribosomal RNA reference genes in *S. mutans* RT-qPCR

Gene	Gene description	Forward primer	Reverse primer
atpD	ATP-binding protein	CCAGGCGGTTCATTCATCTGAC	GGCGGGATTTCGGTATTTACTG
brpA	Biofilm regulatory protein A	GGAGGAGCTGCATCAGGATTC	AACTCCAGCACATCCAGCAAG
gbpB	Glucan binding protein B	ATGGCGGTTATGGACACGTT	TTTGGCCACCTTGAACACCT
gtfB	Water insoluble glucan production	AGCAATGCAGCCAATCTACAAAT	ACGAACTTTGCCGTTATTGTCA
gtfC	Water soluble and insoluble glucan production	GGTTTAACGTCAAAATTAGCTGTATTAGC	CTCAACCAACCGCCACTGTT
IDH	Lactate dehydrogenase	TTGCTCGTATCACTAAGGCTATTC	GGGCTGACCGATAAAGACTTC
spaP	Surface protein attachment	TCCGTGCCGATAATCCAAGA	CGCCTGTTTGTCCCATTTGT
vicR	Response regulator	GCATCACTTAGCGACACACA	CAGACGACGAACAGTAACATCAA
16S	Normalizing internal standard	CCATGTGTAGCGGTGAAATGC	TCATCGTTTACGGCGTGGAC

6.3. Data analysis

Gene expression data were expressed as the mean ± standard error of the mean (±SEM). Statistical analysis was carried out using the non-parametric test (GraphPad Software Inc, CA, USA) and data were considered statistically significant when $p < 0.05$.

6.4. Results

6.4.1. Variable virulence gene expression in treated and untreated *S. mutans* strains

Gene expression experiments were performed to ascertain if application of the natural test compound *U. chamae* would result in a significant change in the expression of candidate genes that advance virulence of *S. mutans*. Accordingly, total RNA was isolated at 10 hours following drug treatment for acid production test and at 24 hours post-treatment for biofilm and EPS experiments. The extracted RNA was converted to cDNA by reverse transcription to allow amplification by PCR. The concentration of cDNA samples ranged from 1000-1850 ng/µl. These DNA samples also had high purity with an A260/280 ratio of between 1.8 and 2.0. Thereafter, RT-qPCR was applied to assess and quantify the relative expression of *atpD* and *IDH* genes involved in acid production, and *brpA*, *gbpB*, *spaP*, and *vicR* that induce biofilm formation. Moreover, the expression level of *gtfB* and *gtfC* genes that play a crucial role in EPS secretion was also determined.

Compared to untreated cells, administration of *U. chamae* triggered a considerable decrease in the expression of target genes that promote virulence of *S. mutans* SM1 (Figure 6.1) and SM12 strains (Figure 6.2). *Uvaria chamae* exerted an antibacterial efficacy ranging from 35% in *brpA* to 67% in *vicR* gene in the SM1 pathogen (Table 6.2). Interestingly, both genes are implicated to promote biofilm formation in *S. mutans*. Furthermore, a significant decline in the transcription of virulence genes was also observed following treatment of the SM12 strain with the natural antibacterial agent (Figure 6. 2). Delivery of *U. chamae* to SM12 markedly induced the lowest gene repression of 30% in *brpA* gene and the highest downregulation of 75% in the *vicR* target gene (Table 6.3). Similarly to SM1 gene expression data, both *brpA* and *vicR* in SM12 facilitate bacterial virulence by inducing biofilm formation. Importantly, the transcription profile of all virulence genes was dramatically decreased after treating both *S. mutans* SM1 and SM12 strains with a single ½ MIC dosage of 0.01 mg/ml.

Table 6.2: Fold change in gene expression in SM1 strain

Target gene	Untreated fold change	Treated fold change	% Reduction
brpA	1	0.654	34.6
gbpB	1	0.590	41.0
spaP	1	0.449	55.1
vicR	1	0.338	66.2
atpD	1	0.688	31.2
IDH	1	0.361	63.9
gtfB	1	0.361	63.9
gtfC	1	0.579	42.1

Table 6.3: Fold change in gene expression in SM12 strain

Target gene	Untreated fold change	Treated fold change	% Reduction
brpA	1	0.700	30.0
gbpB	1	0.526	47.4
spaP	1	0.499	50.1
vicR	1	0.235	76.5
atpD	1	0.468	53.2
IDH	1	0.281	71.9
gtfB	1	0.272	72.8
gtfC	1	0.392	60.8

Table 6.4: Statistical analysis of SM1 and SM12 strains obtained in RT-qPCR

Target gene	Comparison	*P-value*
brpA	Untreated *brpA* to treated *brpA*	0.0383
gbpB	Untreated gbpB to treated gbpB	0.0383
spaP	Untreated *spaP* to treated *spaP*	0.0383
vicR	Untreated *vicR* to treated *vicR*	0.0383
atpD	Untreated *atp*D to treated *atpD*	0.0383
IDH	Untreated *IDH* to treated *IDH*	0.0383
gtfB	Untreated *gtfB* to treated *gtfB*	0.0383
gtfC	Untreated *gtfC* to treated *gtfC*	0.0383

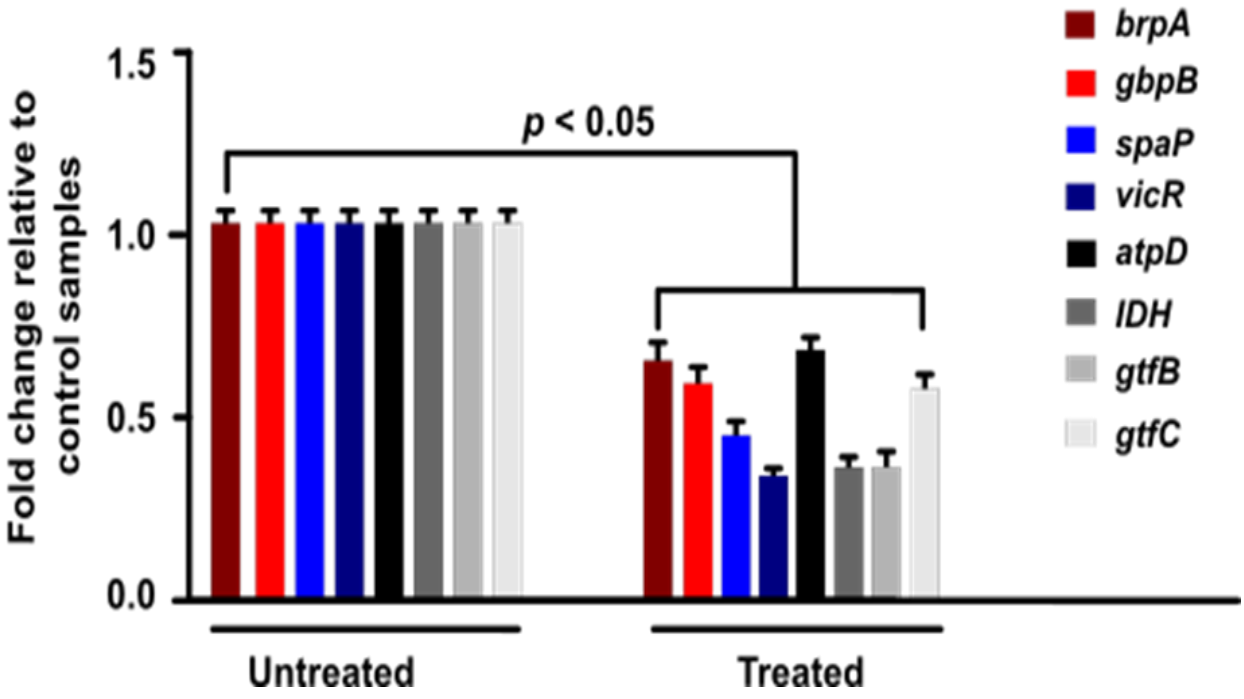

Figure 6.2: Target gene expression in treated and untreated *Streptococcus mutans* SM1 strain

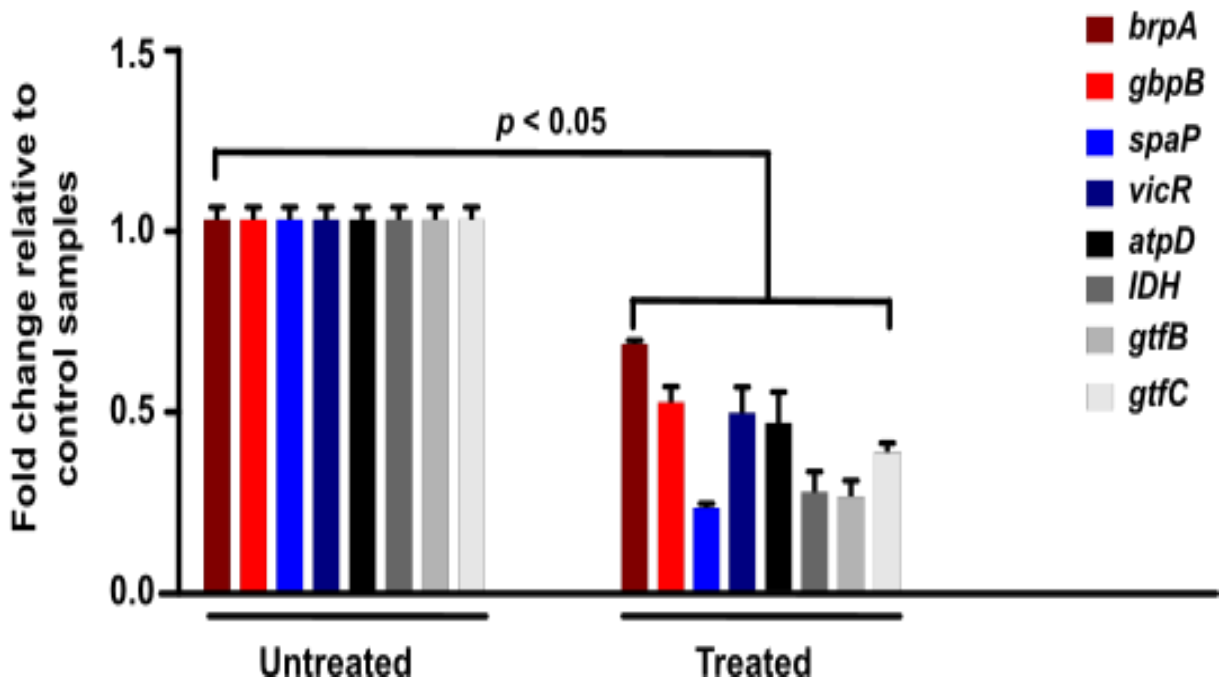

Figure 6.3: Target gene expression in treated and untreated *Streptococcus mutans* SM12 strain

6.5. Discussion

Administration of *U. chamae* to *S. mutans* (SM1) strain-induced considerable downregulation of virulence genes associated with biofilm production (*brpA, gbpB, spaP,* and *vicR*), acid production (*atpD* and *IDH*), and EPS secretion (*gtfB* and *gtfC*). Impressively, treatment of the SM12 strain with *U. chamae* also caused significant decrease in the transcription of mRNAs encoding *brpA, gbpB, spaP*, *vicR*, *atpD*, *IDH*, *gtfB,* and *gtfC* genes that drive the pathology of *S. mutans*. These results were consistent with previous studies that documented a significant decline in the transcription of *atpD* and *IDH* virulence after treatment of *S. mutans* with baicalein, a natural extract obtained from the roots of *Scutellaria lateriflora* and *Scutellaria baicalensis* (Vijayakumar et al., 2021). In addition, these results were also congruent with observations reported by Yoo *et al.,* 2018, Park *et al.*,2019 and Elango *et al*., 2021 that demonstrated a significant downregulation of *atpD, brpA, gtfB, gtfC,* and *vicR* candidate genes following delivery of antibacterial agents baicalin, β-caryophyllene essential oil refined from clove and sabinene herbal essential oil extracted from medicinal plant *Chamaecyparis obtusa* to *S. mutans* respectively (Yoo and Jwa, 2018;Park et al., 2019;Elango et al., 2021).

In contrast, a drastic increase in the expression of *gbpB, gtfB,* and *gtfC* was observed after treating *S. mutans* with Ethyl gallate (Gabe et al., 2019). Interestingly, although the introduction of the natural extract oxyresveratrol to *S. mutans* cultures triggered downregulation of *gtfB* and *gtfC,* pronounced upregulation of *atpD, comDE, gtfD, IDH, liaR,* and *vicR* genes was also noted (Wu et al., 2020). The discrepancy observed in target gene transcription profiles may be due to the efficacy, specificity, and mechanism of action of the administered antimicrobial agent. It is well documented that the genes *gtfB* and *gbpB* are essential components of the sucrose-dependent pathway in biofilm formation (Viszwapriya et al., 2017;El-Ezmerli and Gregory, 2019). The inhibition of these genes may decrease the production of extracellular polysaccharides and cell aggregation, thus preventing biofilm formation. The downregulation of *gtfB* and *gbpB* explain the reduction in *S. mutans* adherence and preformed biofilm as demonstrated in Chapter 3.

Another cariogenic virulence factor in *S. mutans* is acid production. Organic acids are produced during the glycolysis pathway. During this process, *IDH* catalyzes the conversion of pyruvate to lactic acid, which in turn reduces the pH of the environmental medium (Wu et al., 2020). Lactic acid from dietary carbohydrates dissolves tooth minerals and causes dental caries. The suppression of the *IDH* and *atpD* gene impaired acid production and result in decreased

virulence expressions of these genes. VicRKX, encoded by the *vicR* gene is one of the response regulatory systems that are essential for the adaptation to environmental stresses. This system regulates the expression of genes responsible for biofilm formation, gtfBCD, oxidative stress, bacteriocin production, genetic competence, cell death, cell envelope stress, and many other external environmental stimulants (Wu et al., 2020;Chakraborty and Burne, 2017). The downregulation of this gene influenced the expression of *gbpB*, and all three glycosyltransferases *gtfBC* which are critical for the adherence to the tooth surfaces.

Overall, current and previous studies have highlighted the advantage of using natural compounds to treat various human diseases (Oliva et al., 2018;Jafri et al., 2019;Chowdaiah et al., 2019;Shlezinger et al., 2019;Nagasawa et al., 2020). Some of these natural extracts target and inhibit a single or multiple therapeutic targets in the infecting microbial pathogen. These targets mainly include DNA, RNA, virulence proteins, and associated signalling pathways. Natural compounds are convenient for therapeutic application as they are freely available in nature, generally cheaper to produce compared to commercially produced medicines, and often less toxic due to the absence of harmful artificial chemicals. These attributes promote the use of medicinal plant extracts for therapeutic purposes, especially in developing and underdeveloped countries with limited economic resources.

6.6. Conclusion

The plant extract showed a potential to act as an alternative anticaries agent. It inhibited *S. mutans* biofilm, EPS, and acid production in vitro by reducing the activity of related enzymes, downregulating virulence genes, and inactivating specific regulatory systems. Considering the complexity of gene regulation in *S. mutans*, further studies with transcriptomic and proteomic approaches should be carried out.

Chapter 7: Overall discussion, conclusion, limitations of the study, and future research

7.1. Overall discussion

In recent years, the use of natural plant products and their derivates for oral diseases has been well documented (Tapsoba and Deschamps, 2006;Bairwa et al., 2012;Fatima et al., 2018;Akhalwaya et al., 2018). Natural plant products are now considered as an alternative to antibacterial agents because they are readily available, low levels of cytotoxicity, high chemical diversity and biochemical specificity, and are less prone to developing resistance to antibiotics compared to synthetic drugs (Karimi et al., 2015;Nisar et al., 2018). Antimicrobial activity of plant extracts has been classified previously as good (MIC< 0.1 mg/mL), moderate (0.1 ≤ MIC ≤0.625 mg/mL) and weak (MIC>0.625 mg/mL) (Famuyide et al., 2019). This study demonstrated that *U. chamae* dichloromethane extract showed the best antibacterial activity and was used in the subsequent experiments. Other extracts showed weak to moderate activity. These results suggest that bioactive compounds that inhibit the growth of *S. mutans* were better extracted with dichloromethane compared to other extracts.

The formation of biofilm is divided into several stages including initial adherence to a solid surface, reversible attachment to that surface, production of extracellular polymeric substance (EPS), irreversible attachment, and maturation into a complex three-dimensional architecture (Castro et al., 2006b;He et al., 2019). The plant extract significantly reduced the initial adherence and maturation of biofilms into complex 3-dimensional architecture. The extract might have interfered with the cell surface protein antigen I/II (*spaP*) and the Brownian, sedimentation, Lifshitz–Van der Waals, and electrostatic interaction forces which are responsible for the adherence of the bacteria to the surfaces. Additionally, the extract might have interfered with the bacterial properties such as cell aggregation and cell hydrophobicity e.g sucrose-independent mode, which depends on hydrogen bonding and hydrophobic interactions between bacteria and the adhering surface. Based on the RT-qPCR results, the inhibitory effect of this extract was due to the significant downregulation of the *brpA*, *spaP*, *vicR*, and *gbpB* at the transcriptional level. The gene *brpA* has been shown to contribute to biofilm formation and plays a major role in cell envelope biogenesis. The *gbpB* mediates the binding of bacteria to glucans and enables the development of biofilm (Kim et al., 2016).

Downregulation of these genes could decrease the production of extracellular polysaccharides and oral bacterial aggregation, thus inhibiting biofilm formation. The anti-biofilm activity of plant extracts and natural products against the biofilm of *S. mutans* is well documented. The medicinal plants such as *Myracrodruon urundeuva* All., *Qualea grandiflora* Mart. Leaves, green tea extract, and the guava leave extract have been shown to reduce the *S. mutans* counts in dental plaque, showing the potential use of plant extracts in the clinical prevention of caries (Prabu et al., 2006;Pires et al., 2018;Yabuta et al., 2018).

The ability to ferment dietary carbohydrates and produce organic acid are key physiological factors of *S. mutans* for the demineralization of the tooth surface (Matsui and Cvitkovitch, 2010;Kawada-Matsuo et al., 2017). The plant extract significantly inhibited acid production at subinhibitory concentrations. These findings suggest that this plant extract impairs acid tolerance and prevents tooth demineralization. The plant extract interfered with the *IDH* and the *atpD* genes responsible for aciduricity or acid tolerance properties in *S. mutans*. This plant extract will not only inhibit biofilm formation but also inhibit the production of organic acids from dietary carbohydrates. The production of EPS from sucrose contributes to microbial attachment and may also act as an extracellular energy reserve (Costa Oliveira et al., 2017). Based on the EPS assay, no significant reduction in the amount of soluble and insoluble EPS production was noted. These results suggest that EPS were metabolized in a small quantity, enough to maintain the bacterial basal metabolism, and were not detected by the phenol sulfuric acid method. However, molecular analysis using the RT-qPCR showed significant downregulation of the GTFs gene. Gtfs are a family of enzymes that catalyze the transformation of glucosyl groups from one chemical component to another and contribute to *S. mutans* cariogenicity. Their collective function includes the synthesis of glucans from dietary carbohydrates (Huang et al., 2015). Downregulation of GTFs genes disrupted both the adherence of *S. mutans* into the glass surface and the structure of biofilm.

7.2. Clinical implications

Based on these observations, this plant extract can be incorporated into toothpaste, mouth rinses, and chewing gums. If the product is used at MIC of 0.02 mg/ml, it will completely inhibit the growth of *S. mutans*. At subinhibitory concentrations, it will reduce biofilm formation, virulence gene expressions, and acid production. These plants will prevent further colonization of the *S. mutans* by preventing their adhesion, acid production, and virulence gene

expression once the concentration is reduced. The beneficial effects of this extract will be increased at night due to reduced salivary flow, and slower dilution. Continuous exposure of this plant extract will be necessary to reduce bacterial counts and prevent bacterial growth. Additionally, the extract can be coated onto nanoparticles and used as anti-cariogenic agents. The use of nanoparticles will prevent dilutions and washing away the effect of the extract by the saliva. The extract will become absorbed onto the oral mucosa, and released slowly providing long-lasting effects by inhibiting virulence factors of *S. mutans*.

7.3. Conclusion

All five crude extracts had a degree of antimicrobial activity against *S. mutans*. Dichloromethane showed the best antimicrobial activity against all five cariogenic *S. mutans* strains with the mean MIC of 0.02 mg/ml and it was used for subsequence studies. Other extracts such as hexanol, ethanol, methanol, and methanol: water showed weak to moderate activity with a mean MIC ranging between 0.63 to1.25 mg/ml. The mean MBC ranged between 0.04 to 1.25 mg/ml. These results suggest dichloromethane solvent extract to be more active anti-cariogenic compounds with potential therapeutic application in dentistry compared to the other four extracts. At a concentration of 0.02 mg/ml, the dichloromethane extract completely inhibited the growth of *S. mutans*. At subinhibitory concentrations, this plant extract significantly inhibited biofilm formation, acid production, and virulence gene expressions. This suggests that if this extract is used in the form of oral hygiene products such as toothpaste and mouthwash, it may provide a long-lasting anti-cariogenic effect. Therefore, this plant extract has the potential to be developed as a novel anti-cariogenic agent for the prevention and treatment of dental caries. However, studies are required to characterize the active chemical compounds responsible for the observed antibacterial effect in this study.

7.4. Limitations to the study

- Dental biofilms are formed through the interaction between different microbial species, the host, diet, and other external factors instead of by an individual bacterium. However, only a single bacterial species (*S. mutans*) was investigated in this study.

- The antimicrobial activity of natural products depends on the combined effect of all the bioactive phytochemicals in them. The biological functions of each chemical compound in this plant responsible for antimicrobial activity are still unknown.
- Glass slides surfaces were used instead of hydroxyapatite discs to study the effect of *U. chamae* on biofilm formation.
- The biocompatibility of this plant extract against human cells was not studied.

7.5. Future research

- Future studies should investigate and characterize the active chemical compounds responsible for the observed antibacterial effect in this study.
- Prospective investigations should investigate the specificity and toxicity upon delivery into drug-susceptible and resistant *S. mutans* strains and other pathogenic microorganisms.
- An ex vivo assay based on bovine dental enamel biofilm formation should be performed for better simulation of the real situation
- Besides, experimental evaluations and rigorous analysis in mammalian cell cultures and animals are required to examine the activity, cytotoxicity, substantivity, and specificity of the natural plant extracts.
- The discovery of the cellular signalling pathways targeted by these compounds and a comprehensive understanding of their mode of action is essential for gaining insight into their pharmacodynamics.
- Animal experiments, in vivo experiments, and large clinical trials also need to be performed to ascertain the effect of *U. chamae* on dental caries.

References

Abachi, S., Lee, S. & Rupasinghe, H. 2016. Molecular mechanisms of inhibition of Streptococcus species by phytochemicals. *Molecules,* 21**,** 215.

Abid, A., Maatouk, F., Berrezouga, L., Azodo, C., Uti, O., El-Shamy, H. & Oginni, A. 2015. Prevalence and severity of oral diseases in the Africa and Middle East Region. *Advances in Dental Research,* 27**,** 10-17.

Abu, T., Rex-Ogbuku, E. M. & Idibiye, K. 2018. A review: secondary metabolites of *Uvaria chamae* p. Beauv. (Annonaceae) and their biological activities. *International Journal of Agriculture, Environment and Food Sciences,* 2**,** 177-185.

Achilonu, M. C. & Umesiobi, D. O. 2015. Bioactive phytochemicals: bioactivity, sources, preparations, and/or modifications via silver tetrafluoroborate mediation. *Journal of Chemistry,* 2015**,** 629085.

Adedayo, M., Ajiboye, A. & Adetula, F. 2020. Antibacterial screening of *Phoenix dactylifera* L.(Date palm) seed extracts on some bacterial isolates associated with dental caries. *Bio-Research,* 18**,** 1127-1134

Agbor, A. M. & Naidoo, S. 2019. Plants used by African traditional healers in the management of oral diseases: A review. *International Journal of Research-Granthaalayah,* 7**,** 273-286.

Ahirwar, S. S., Gupta, M. & Snehi, S. K. 2019a. Dental caries and lactobacillus: Role and ecology in the oral cavity. *International Journal of Pharmaceutical Sciences and Research,* 10**,** 4818-4829.

Ahirwar, S. S., Gupta, M. & Snehi, S. K. 2019b. Dental caries and lactobacillus: role and ecology in the oral cavity. *International Journal of Pharmaceutical Sciences and Research,* 10**,** 4818-4829.

Ajdić, D., Mcshan, W. M., Mclaughlin, R. E., Savić, G., Chang, J., Carson, M. B., Primeaux, C., Tian, R., Kenton, S. & Jia, H. 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proceedings of the National Academy of Sciences,* 99**,** 14434-14439.

Akhalwaya, S., Van Vuuren, S. & Patel, M. 2018. An in vitro investigation of indigenous South African medicinal plants used to treat oral infections. *Journal of Ethnopharmacology,* 210**,** 359-371.

Al-Shahrani, M. A. 2019. Microbiology of dental caries: A literature review. *Annals of Medical and Health Sciences Research,* 9**,** 655-659.

Alejandra, B. M. & Daniel, O. M. 2020. Virulence Factors of *Streptococcus mutans* Related to Dental Caries. *Staphylococcus and Streptococcus.* IntechOpen.

Almohefer, S. A., Levon, J. A., Gregory, R. L., Eckert, G. J. & Lippert, F. 2018. Caries lesion remineralization with fluoride toothpastes and chlorhexidine-effects of application timing and toothpaste surfactant. *Journal of Applied Oral Science,* 26**,** 1-8.

Amadi, E., Oyeka, C., Iroha, I., Orji, J. & Onyeagba, R. 2007. The effects of indigenous medicinal plants on dental caries organisms. *World,* 8**,** 1385-1389.

Amorim, E., Castro, V., Melo, J., Corrêa, A. & Peixoto Sobrinho, T. 2012. Standard operating procedures (SOP) for the spectrophotometric determination of phenolic compounds contained in plant samples. *Latest Research Into Quality Control***,** 47-66.

Anderson, A. C., Rothballer, M., Altenburger, M. J., Woelber, J. P., Karygianni, L., Lagkouvardos, I., Hellwig, E. & Al-Ahmad, A. 2018. In-vivo shift of the microbiota in oral biofilm in response to frequent sucrose consumption. *Scientific Reports,* 8**,** 1-13.

Ansari, J. M., Abraham, N. M., Massaro, J., Murphy, K., Smith-Carpenter, J. & Fikrig, E. 2017. Anti-biofilm activity of a self-aggregating peptide against *Streptococcus mutans*. *Frontiers in Microbiology,* 8**,** 1-12

Argimón, S., Alekseyenko, A. V., Desalle, R. & Caufield, P. W. 2013. Phylogenetic analysis of glucosyltransferases and implications for the coevolution of mutans streptococci with their mammalian hosts. *PloS one,* 8**,** e56305.

Argimón, S. & Caufield, P. W. 2011. Distribution of putative virulence genes in *Streptococcus mutans* strains does not correlate with caries experience. *Journal of Clinical Microbiology,* 49**,** 984-992.

Aruni, A. W., Dou, Y., Mishra, A. & Fletcher, H. M. 2015. The biofilm community: rebels with a cause. *Current Oral Health Reports,* 2**,** 48-56.

Ashu Agbor, M. & Naidoo, S. 2015. Ethnomedicinal plants used by traditional healers to treat oral health problems in Cameroon. *Evidence-Based Complementary and Alternative Medicine,* 2015.

Attiq, A., Jalil, J. & Husain, K. 2017. Annonaceae: breaking the wall of inflammation. *Frontiers in Pharmacology,* 8**,** 752.

Badet, C. & Thebaud, N. 2008. Ecology of lactobacilli in the oral cavity: a review of literature. *The Open Microbiology Journal,* 2**,** 38.

Bae, D., Seol, H., Yoon, H.-G., Na, J.-R., Oh, K., Choi, C. Y., Lee, D.-W., Jun, W., Youl Lee, K. & Lee, J. 2012. Inhaled essential oil from *Chamaecyparis obtus*e ameliorates the impairments of cognitive function induced by injection of β-amyloid in rats. *Pharmaceutical Biology,* 50**,** 900-910.

Bairwa, R., Gupta, P., Gupta, V. K. & Srivastava, B. 2012. Traditional medicinal plants: use in oral hygiene. *International Journal of Pharmaceutical Chemistry,* 1**,** 1529-38.

Banas, J. A. 2004a. Virulence properties of *Streptococcus mutans*. *Frontiers in Bioscience,* 9**,** 1267-77.

Banas, J. A. 2004b. Virulence properties of *Streptococcus mutans*. *Frontiers Bioscience,* 9**,** 1267-77.

Bhayat, A. & Chikte, U. 2019. Human resources for oral health care in South Africa: a 2018 update. *International Journal of Environmental Research and Public Health,* 16**,** 1668.

Bilbilova, E. Z. 2020. Dietary factors, salivary Parameters, and Dental Caries. *Dental caries.* IntechOpen.

Bodiba, D., Szuman, K. M. & Lall, N. 2018. The Role of medicinal plants in oral care. *Medicinal plants for holistic health and well-being.* Elsevier.

Bowen, W. & Koo, H. 2011. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Research,* 45**,** 69-86.

Brighenti, F. L., Luppens, S., Delbem, A. C. B., Deng, D., Hoogenkamp, M., Gaetti-Jardim Jr, E., Dekker, H., Crielaard, W. & Ten Cate, J. 2008. Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production. *Caries Research,* 42**,** 148-154.

Burne, R. A. & Marquis, R. E. 2000. Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiology Letters,* 193**,** 1-6.

Cai, J.-N., Jung, J.-E., Dang, M.-H., Kim, M.-A., Yi, H.-K. & Jeon, J.-G. 2016. Functional relationship between sucrose and a cariogenic biofilm formation. *PLoS One,* 11**,** e0157184.

Campus, G., Cocco, F., Ottolenghi, L. & Cagetti, M. G. 2019. Comparison of ICDAS, CAST, Nyvad's criteria, and WHO-DMFT for caries detection in a sample of Italian

schoolchildren. *International journal of environmental research and public health,* 16**,** 4120.
Castillo Pedraza, M. C., Novais, T. F., Faustoferri, R. C., Quivey Jr, R. G., Terekhov, A., Hamaker, B. R. & Klein, M. I. 2017. Extracellular DNA and lipoteichoic acids interact with exopolysaccharides in the extracellular matrix of *Streptococcus mutans* biofilms. *Biofouling,* 33**,** 722-740.
Castro, P., Tovar, J. A. & Jaramillo, L. 2006a. Adhesion of *Streptococcus mutans* to salivary proteins in caries-free and caries-susceptible individuals. *Acta odontologica latinoamericana: AOL,* 19**,** 59.
Castro, P., Tovar, J. A. & Jaramillo, L. 2006b. Adhesion of Streptococcus mutans to salivary proteins in caries-free and caries-susceptible individuals. *Acta odontologica latinoamericana: AOL,* 19**,** 59-66.
Chakraborty, B. & Burne, R. A. 2017. Effects of arginine on *Streptococcus mutans* growth, virulence gene expression, and stress tolerance. *Applied and Environmental Microbiology,* 83**,** e00496-17.
Chen, L., Ren, Z., Zhou, X., Zeng, J., Zou, J. & Li, Y. 2016. Inhibition of *Streptococcus mutan*s biofilm formation, extracellular polysaccharide production, and virulence by an oxazole derivative. *Applied Microbiology and Biotechnology,* 100**,** 857-867.
Chikte, U., Pontes, C. C., Karangwa, I., Kimmie-Dhansay, F., Erasmus, R., Kengne, A. P. & Matsha, T. E. 2020. Dental caries in a South African adult population: findings from the Cape Town vascular and metabolic health study. *International Dental Journal,* 70**,** 176-182.
Chouhan, S., Sharma, K. & Guleria, S. 2017. Antimicrobial activity of some essential oils—present status and future perspectives. *Medicines,* 4**,** 58.
Chowdaiah, M., Sharma, P. & Dhamodhar, P. 2019. A study on phytochemicals from medicinal plants against multidrug resistant *Streptococcus mutans*. *International Journal of Peptide Research and Therapeutics,* 25**,** 1581-1593.
Chu, J., Zhang, T. & He, K. 2016. Cariogenicity features of *Streptococcus mutans* in presence of rubusoside. *BMC Oral Health,* 16**,** 54.
Clarke, J. K. 1924. On the bacterial factor in the aetiology of dental caries. *British Journal of Experimental Pathology,* 5**,** 141.
Coelho, M. a. G. 2020. ICDAS and dmft/DMFT. Sensitivity and specificity, the importance of the index used: a systematic review. *Journal of Dentistry & Public Health (inactive/archive only),* 11**,** 176-187.
Costa, E. V., Soares, L. D. N., Chaar, J. D. S., Silva, V. R., Santos, L. D. S., Koolen, H. H., Silva, F., Tavares, J. F., Zengin, G. & Soares, M. B. 2021. Benzylated Dihydroflavones and isolated-derived alkaloids from the bark of *Diclinanona calycina* (Annonaceae) and their cytotoxicities. *Molecules,* 26**,** 3714.
Costa Oliveira, B. E., Cury, J. A. & Ricomini Filho, A. P. 2017. Biofilm extracellular polysaccharides degradation during starvation and enamel demineralization. *PLoS One,* 12**,** e0181168.
Cox-Georgian, D., Ramadoss, N., Dona, C. & Basu, C. 2019. Therapeutic and medicinal uses of terpenes. *Medicinal Plants.* Springer.
Cugini, C., Shanmugam, M., Landge, N. & Ramasubbu, N. 2019. The role of exopolysaccharides in oral biofilms. *Journal of Dental Research,* 98**,** 739-745.
Culp, D. J., Robinson, B. & Cash, M. N. 2021. Murine salivary amylase protects against *Streptococcus mutans*-induced caries. *Frontiers in Physiology,* 12**,** 1-19.
Das, T., Sharma, P. K., Krom, B. P., Van Der Mei, H. C. & Busscher, H. J. 2011. Role of eDNA on the adhesion forces between *Streptococcus mutans* and substratum surfaces:

influence of ionic strength and substratum hydrophobicity. *Langmuir,* 27**,** 10113-10118.
Dashper, S. & Reynolds, E. 2000. Effects of organic acid anions on growth, glycolysis, and intracellular pH of oral streptococci. *Journal of Dental Research,* 79**,** 90-96.
Dashper, S. G. & Reynolds, E. C. 1996. Lactic acid excretion by *Streptococcus mutans*. *Microbiology,* 142**,** 33-39.
David, B., Linda, O. & Charles, O. 2011. Isolation, characterization and antibiotic susceptibility studies of clinical isolates of *Streptococococcus mutans* obtained from patients visiting major dental clinics in Nsukka. *African Journal of Pharmaceutical Sciences and Pharmacy,* 2**,** 1-15.
Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug discovery,* 2**,** 114-122.
De, A., Jorgensen, A. N., Beatty, W. L., Lemos, J. & Wen, Z. T. 2018. Deficiency of MecA in *Streptococcus mutans* causes major defects in cell envelope biogenesis, cell division, and biofilm formation. *Frontiers in Microbiology,* 9**,** 2130.
Decker, E.-M., Klein, C., Schwindt, D. & Von Ohle, C. 2014. Metabolic activity of *Streptococcus mutans* biofilms and gene expression during exposure to xylitol and sucrose. *International Journal of Oral Science,* 6**,** 195-204.
Deveci, C., Çınar, Ç. & Tirali, R. E. 2018. Management of white spot lesions. *Dental Caries-Diagnosis, Prevention and Management. InTech***,** 129-65.
Dodds, M., Roland, S., Edgar, M. & Thornhill, M. 2015. Saliva: A review of its role in maintaining oral health and preventing dental disease. *Bdj Team,* 2**,** 15123.
Du, Q., Fu, M., Zhou, Y., Cao, Y., Guo, T., Zhou, Z., Li, M., Peng, X., Zheng, X. & Li, Y. 2020. Sucrose promotes caries progression by disrupting the microecological balance in oral biofilms: an in vitro study. *Scientific Reports,* 10**,** 1-12.
Egamberdieva, D., Wirth, S., Behrendt, U., Ahmad, P. & Berg, G. 2017. Antimicrobial activity of medicinal plants correlates with the proportion of antagonistic endophytes. *Frontiers in Microbiology,* 8**,** 199.
El-Ezmerli, N. F. & Gregory, R. L. 2019. Effect of nicotine on biofilm formation of *Streptococcus mutans* isolates from smoking and non-smoking subjects. *Journal of Oral Microbiology,* 11**,** 1662275.
El Sherbiny, G. M. 2014. Control of growth *Streptococcus mutans* isolated from saliva and dental caries. *International Journal of Current Microbiology and Applied Sciences,* 3**,** 1-10.
Elango, A. V., Vasudevan, S., Shanmugam, K., Solomon, A. P. & Neelakantan, P. 2021. Exploring the anti-caries properties of baicalin against *Streptococcus mutans*: an in vitro study. *Biofouling,* 37**,** 267-275.
Emordi, J. E., Agbaje, E. O., Oreagba, I. A. & Iribhogbe, O. I. 2018. Antidiabetic effects of the ethanolic root extract of *Uvaria chamae* P. Beauv (Annonaceae) in alloxan-induced diabetic rats: a potential alternative treatment for diabetes mellitus. *Advances in Pharmacological Sciences,* 2018**,** 1314941.
Enin, G. N., Shaibu, S. E., Ujah, G. A., Ibu, R. O. & Inangha, P. G. 2021. Phytochemical and nutritive composition of *Uvaria chamae* P. Beauv. leaves, stem bark and root bark. *ChemSearch Journal,* 12**,** 9-14.
Famuyide, I. M., Aro, A. O., Fasina, F. O., Eloff, J. N. & Mcgaw, L. J. 2019. Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated south African Eugenia and Syzygium (Myrtaceae) species and their selectivity indices. *BMC Complementary and Alternative Medicine,* 19**,** 1-13.

Fatima, A., Ahmad, M., Zafar, M., Yaseen, G., Khan, M. P. Z., Butt, M. A. & Sultana, S. 2018. Ethnopharmacological relevance of medicinal plants used for the treatment of oral diseases in Central Punjab-Pakistan. *Journal of Herbal Medicine,* 12**,** 88-110.

Featherstone, J. D. 2008. Dental caries: a dynamic disease process. *Australian Dental Journal,* 53**,** 286-291.

Figuero, E., Herrera, D., Tobías, A., Serrano, J., Roldan, S., Escribano, M. & Martin, C. 2019. Efficacy of adjunctive anti-plaque chemical agents in managing gingivitis: A systematic review and network meta-analyses. *Journal of Clinical Periodontology,* 46**,** 723-739.

Fujita, K., Takashima, Y., Inagaki, S., Nagayama, K., Nomura, R., Ardin, A., Grönroos, L., Alaluusua, S., Ooshima, T. & Matsumoto-Nakano, M. 2011. Correlation of biological properties with glucan-binding protein B expression profile in *Streptococcus mutans* clinical isolates. *Archives of Oral Biology,* 56**,** 258-263.

Gabe, V., Kacergius, T., Abu-Lafi, S., Kalesinskas, P., Masalha, M., Falah, M., Abu-Farich, B., Melninkaitis, A., Zeidan, M. & Rayan, A. 2019. Inhibitory effects of ethyl gallate on *Streptococcus mutans* biofilm formation by optical profilometry and gene expression analysis. *Molecules,* 24**,** 529.

Gabe, V., Zeidan, M., Kacergius, T., Bratchikov, M., Falah, M. & Rayan, A. 2020. Lauryl gallate activity and *Streptococcus mutans*: its effects on biofilm formation, acidogenicity and gene expression. *Molecules,* 25**,** 3685.

Garcia, B., Acosta, N., Tomar, S., Roesch, L., Lemos, J., Mugayar, L. & Abranches, J. 2021. Association of Candida albicans and Cbp+ Streptococcus mutans with early childhood caries recurrence. *Scientific reports,* 11**,** 1-11.

Ghamari, S., Mohammadrezaei-Khorramabadi, R., Mardani, M. & Shahsavari, S. 2017. An overview of the most important medicinal plants with anti-toothache property based on ethnobotanical sources in Iran. *Journal of Pharmaceutical Sciences and Research,* 9**,** 796.

Ghosh, D. 2015. Tannins from foods to combat diseases. *International Journal of Pharmaceutical Sciences Review and Research,* 4**,** 40-44.

Górniak, I., Bartoszewski, R. & Króliczewski, J. 2019. Comprehensive review of antimicrobial activities of plant flavonoids. *Phytochemistry Reviews,* 18**,** 241-272.

Goršeta, K. 2015. Fissure sealing in occlusal caries prevention. *Emerging Trends in oral Health Sciences and Dentistry***,** 1.

Gregoire, S., Singh, A., Vorsa, N. & Koo, H. 2007. Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. *Journal of Applied Microbiology,* 103**,** 1960-1968.

Guan, C., Che, F., Zhou, H., Li, Y., Li, Y. & Chu, J. 2020. Effect of rubusoside, a natural sucrose substitute, on *Streptococcus mutans* biofilm cariogenic potential and virulence gene expression in vitro. *Applied and Environmental Microbiology,* 86**,** e01012-20.

Gulube, Z. & Patel, M. 2016. Effect of Punica granatum on the virulence factors of cariogenic bacteria Streptococcus mutans. *Microbial pathogenesis,* 98**,** 45-49.

Guo, L., Hu, W., He, X., Lux, R., Mclean, J. & Shi, W. 2013. Investigating acid production by *Streptococcus mutans* with a surface-displayed pH-sensitive green fluorescent protein. *PloS one,* 8**,** e57182.

Hardie, J. & Whiley, R. 1997. Classification and overview of the genera *Streptococcus* and *Enterococcus*. *Journal of Applied Microbiology,* 83**,** 1S-11S.

Hasan, S., Singh, K., Danisuddin, M., Verma, P. K. & Khan, A. U. 2014. Inhibition of major virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a synergistic approach of infection control. *PLoS one,* 9**,** e91736.

Haslam, E. 1989. *Plant polyphenols: vegetable tannins revisited,* CUP Archive.

He, Z., Huang, Z., Jiang, W. & Zhou, W. 2019. Antimicrobial activity of cinnamaldehyde on *Streptococcus mutans* biofilms. *Frontiers in Microbiology,* 10**,** 2241.
Hicks, J., Garcia-Godoy, F. & Flaitz, C. 2004. Biological factors in dental caries: role of saliva and dental plaque in the dynamic process of demineralization and remineralization (part 1). *Journal of Clinical Pediatric Dentistry,* 28**,** 47-52.
Hirasawa, M., Takada, K. & Otake, S. 2006. Inhibition of acid production in dental plaque bacteria by green tea catechins. *Caries Research,* 40**,** 265-270.
Hong, E.-J., Na, K.-J., Choi, I.-G., Choi, K.-C. & Jeung, E.-B. 2004. Antibacterial and antifungal effects of essential oils from coniferous trees. *Biological and Pharmaceutical Bulletin,* 27**,** 863-866.
Hoshino, T., Fujiwara, T. & Kawabata, S. 2012. Evolution of cariogenic character in *Streptococcus mutans*: horizontal transmission of glycosyl hydrolase family 70 genes. *Scientific Reports,* 2**,** 1-7.
Huang, L., Xu, Q.-A., Liu, C., Fan, M.-W. & Li, Y.-H. 2013. Anti-caries DNA vaccine-induced secretory immunoglobulin A antibodies inhibit formation of *Streptococcus mutans* biofilms in vitro. *Acta Pharmacologica Sinica,* 34**,** 239-246.
Huang, R., Li, M. & Gregory, R. L. 2011. Bacterial interactions in dental biofilm. *Virulence,* 2**,** 435-444.
Huang, R., Li, M. & Gregory, R. L. 2015. Nicotine promotes *Streptococcus mutans* extracellular polysaccharide synthesis, cell aggregation and overall lactate dehydrogenase activity. *Archives of Oral Biology,* 60**,** 1083-1090.
Hughes, C. V. & Dean, J. A. 2015. Mechanical and chemotherapeutic home oral hygiene. *McDonald and Avery's Dentistry for the Child and Adolescent-E-Book***,** 120.
Hujoel, P. P. & Lingström, P. 2017. Nutrition, dental caries and periodontal disease: a narrative review. *Journal of Clinical Periodontology,* 44**,** S79-S84.
Ishaque, S., Shamseer, L., Bukutu, C. & Vohra, S. 2012. Rhodiola rosea for physical and mental fatigue: a systematic review. *BMC Complementary and Alternative Medicine,* 12**,** 70.
Iwu, M. M. 2014. *Handbook of African medicinal plants,* CRC press.
Jacob, B. & Nivedhitha, M. 2018. Comparative assessment of the antibacterial efficacy of natural products and chlorhexidine mouthwash against *Streptococcus mutans*: A systematic review. *Journal of Clinical & Diagnostic Research,* 12.
Jafri, H., Khan, M. S. A. & Ahmad, I. 2019. In vitro efficacy of eugenol in inhibiting single and mixed-biofilms of drug-resistant strains of *Candida albicans* and *Streptococcus mutans*. *Phytomedicine,* 54**,** 206-213.
Jalil, J., Attiq, A., Hui, C. C., Yao, L. J. & Zakaria, N. A. 2020. Modulation of inflammatory pathways, medicinal uses and toxicities of uvaria species: Potential role in the prevention and treatment of inflammation. *Inflammopharmacology,* 28**,** 1195-1218.
Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M. & Kamil, M. A. 2018. Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association,* 81**,** 7-11.
James, O., Godwin, E. U. & Otini, I. G. 2013. *Uvaria chamae* (Annonaceae) plant extract neutralizes some biological effects of Naja nigricollis snake venom in rats. *British Journal of Pharmacology and Toxicology,* 4**,** 41-50.
Jeon, J.-G., Rosalen, P., Falsetta, M. & Koo, H. 2011. Natural products in caries research: current (limited) knowledge, challenges and future perspective. *Caries Research,* 45**,** 243-263.
Kabra, P., Loomba, K., Kabra, S. K., Majumdar, D. S. P. & Kumar, N. 2012. Medicinal plants in the treatment of dental caries. *Asian Journal of Oral Health & Allied Sciences Volume,* 2**,** 13.

Kadiri, M., Ojewumi, A., Amuda, A., Ogunderu, O. & Fadimu, O. 2014. Ethnobiological survey of indigenous flora used for management of catarrh and arthritis in Abeokuta. *International Journal of Green and Herbal Chemistry,* 3**,** 1690-1704.

Kalaivani, R., Devi, V. J., Umarani, R., Periyanayagam, K. & Kumaraguru, A. 2012. Antimicrobial activity of some important medicinal plant oils against human pathogens. *Journal of Biologically Active Products from Nature,* 2**,** 30-37.

Kalesinskas, P., Kačergius, T., Ambrozaitis, A., Pečiulienė, V. & Ericson, D. 2014. Reducing dental plaque formation and caries development. A review of current methods and implications for novel pharmaceuticals. *Stomatologija,* 16**,** 44-52.

Kapoor, G., Saigal, S. & Elongavan, A. 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology, Clinical Pharmacology,* 33**,** 300.

Karimi, A., Majlesi, M. & Rafieian-Kopaei, M. 2015. Herbal versus synthetic drugs; beliefs and facts. *Journal of Nephropharmacology,* 4**,** 27.

Kawada-Matsuo, M., Oogai, Y. & Komatsuzawa, H. 2017. Sugar allocation to metabolic pathways is tightly regulated and affects the virulence of *Streptococcus mutans*. *Genes,* 8**,** 11.

Kertiasih, N. L. P. & Artawa, I. M. B. 2015. THE FUNCTION OF SALIVA IN CARIES PREVENTION. *Jurnal Kesehatan Gigi (Dental Health Journal),* 3**,** 56-60.

Kianoush, N., Adler, C. J., Nguyen, K.-a. T., Browne, G. V., Simonian, M. & Hunter, N. 2014. Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. *PloS one,* 9**,** e92940.

Kim, E.-H., Kang, S.-Y., Park, B.-I., Kim, Y.-H., Lee, Y.-R., Hoe, J.-H., Choi, N.-Y., Ra, J.-Y., An, S.-Y. & You, Y.-O. 2016. *Chamaecyparis obtusa* suppresses virulence genes in *Streptococcus mutans*. *Evidence-Based Complementary and Alternative Medicine: eCAM,* 2016.

Kim, J.-E., Kim, H.-E., Hwang, J.-K., Lee, H.-J., Kwon, H.-K. & Kim, B.-I. 2008. Antibacterial characteristics of *Curcuma xanthorrhiza* extract on *Streptococcus mutans* biofilm. *The Journal of Microbiology,* 46**,** 228-232.

Klein, M. I., Hwang, G., Santos, P. H., Campanella, O. H. & Koo, H. 2015. *Streptococcus mutan*s-derived extracellular matrix in cariogenic oral biofilms. *Frontiers in Cellular and Infection Microbiology,* 5**,** 10.

Koo, H., Rosalen, P. L., Cury, J. A., Park, Y. K. & Bowen, W. H. 2002. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrobial Agents and Chemotherapy,* 46**,** 1302-1309.

Koo, H., Xiao, J., Klein, M. & Jeon, J. 2010. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *Journal of Bacteriology,* 192**,** 3024-3032.

Koudokpon, H., Armstrong, N., Dougnon, T., Fah, L., Hounsa, E., Bankolé, H., Loko, F., Chabrière, E. & Rolain, J. 2018. Antibacterial activity of chalcone and dihydrochalcone compounds from *Uvaria chamae* roots against multidrug-resistant bacteria. *BioMed Research International,* 2018.

Krzyściak, W., Jurczak, A., Kościelniak, D., Bystrowska, B. & Skalniak, A. 2014. The virulence of *Streptococcus mutans* and the ability to form biofilms. *European Journal of Clinical Microbiology & Infectious Diseases,* 33**,** 499-515.

Krzyściak, W., Jurczak, A. & Piątkowski, J. 2016. The role of human oral microbiome in dental biofilm formation. *Microbial Biofilms—Importance and Applications. InTech***,** 329-382.

Kuang, X., Chen, V. & Xu, X. 2018. Novel approaches to the control of oral microbial biofilms. *BioMed Research International,* 2018.

Kubala, E., Strzelecka, P., Grzegocka, M., Lietz-Kijak, D., Gronwald, H., Skomro, P. & Kijak, E. 2018. A review of selected studies that determine the physical and chemical properties of saliva in the field of dental treatment. *BioMed research international,* 2018.

Kuhnert, W. L. & Quivey Jr, R. G. 2003. Genetic and biochemical characterization of the F-ATPase operon from *Streptococcus sanguis* 10904. *Journal of Bacteriology,* 185**,** 1525-1533.

Lee, Y.-C., Cho, S.-G., Kim, S.-W. & Kim, J. N. 2019. Anticariogenic potential of korean native plant extracts against *Streptococcus mutans*. *Planta medica,* 85**,** 1242-1252.

Lee, Y. 2013. Diagnosis and prevention strategies for dental caries. *Journal of Lifestyle Medicine,* 3**,** 107.

Leme, A. P., Koo, H., Bellato, C., Bedi, G. & Cury, J. 2006a. The role of sucrose in cariogenic dental biofilm formation-new insight. *Journal of Dental Research,* 85**,** 878-887.

Leme, A. P., Koo, H., Bellato, C., Bedi, G. & Cury, J. 2006b. The role of sucrose in cariogenic dental biofilm formation—new insight. *Journal of dental research,* 85**,** 878-887.

Lemos, J., Palmer, S., Zeng, L., Wen, Z., Kajfasz, J., Freires, I., Abranches, J. & Brady, L. 2019. The biology of *Streptococcus mutans*. *Gram-Positive Pathogens***,** 435-448.

Lemos, J. A., Abranches, J. & Burne, R. A. 2005. Responses of cariogenic streptococci to environmental stresses. *Current Issues in Molecular Biology,* 7**,** 95-108.

Lemos, J. A., Quivey Jr, R. G., Koo, H. & Abranches, J. 2013. *Streptococcus mutans*: a new Gram-positive paradigm? *Microbiology,* 159**,** 436.

Li, J., Wu, T., Peng, W. & Zhu, Y. 2020. Effects of resveratrol on cariogenic virulence properties of *Streptococcus mutans*. *BMC Microbiology,* 20**,** 1-11.

Li, M., Huang, R., Zhou, X., Qiu, W., Xu, X. & Gregory, R. L. 2016. Effect of nicotine on cariogenic virulence of *Streptococcus mutans*. *Folia Microbiologica,* 61**,** 505-512.

Lim, T. 2012a. *Uvaria chamae*. *Edible Medicinal and Non-Medicinal Plants.* Springer.

Lim, T. K. 2012b. *Edible medicinal and non-medicinal plants,* Springer.

Lin, Y., Chen, J., Zhou, X. & Li, Y. 2021. Inhibition of *Streptococcus mutans* biofilm formation by strategies targeting the metabolism of exopolysaccharides. *Critical Reviews in Microbiology***,** 1-11.

Lindsey, K., Jäger, A. K., Raidoo, D. M. & Van Staden, J. 1998. Screening of plants used by Southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin-synthesis inhibitors and uterine relaxing activity. *Journal of Ethnopharmacology,* 64**,** 9-14.

Lins De Sousa, D., Araújo Lima, R., Zanin, I. C., Klein, M. I., Janal, M. N. & Duarte, S. 2015. Effect of twice-daily blue light treatment on matrix-rich biofilm development. *PloS one,* 10**,** e0131941.

Liu, Y., Xu, Y., Song, Q., Wang, F., Sun, L., Liu, L., Yang, X., Yi, J., Bao, Y. & Ma, H. 2017. Anti-biofilm activities from *Bergenia crassifolia* leaves against *Streptococcus mutans*. *Frontiers in Microbiology,* 8**,** 1738.

Lu, L., Hu, W., Tian, Z., Yuan, D., Yi, G., Zhou, Y., Cheng, Q., Zhu, J. & Li, M. 2019. Developing natural products as potential anti-biofilm agents. *Chinese Medicine,* 14**,** 1-17.

Macri, D. V. & Chitlall, A. 2017. Caries classification. *Dimensions of Dental Hygiene,* 15**,** 17-18, 21.

Mahomoodally, M. F. 2013. Traditional medicines in Africa: an appraisal of ten potent African medicinal plants. *Evidence-Based Complementary and Alternative Medicine,* 2013.

Man, S., Gao, W., Zhang, Y., Huang, L. & Liu, C. 2010. Chemical study and medical application of saponins as anti-cancer agents. *Fitoterapia,* 81**,** 703-714.

Mandava, K., Batchu, U. R., Kakulavaram, S., Repally, S., Chennuri, I., Bedarakota, S. & Sunkara, N. 2019. Design and study of anticaries effect of different medicinal plants against *Streptococcus mutans* glucosyltransferase. *BMC Complementary and Alternative Medicine,* 19**,** 1-8.

Marsh, P. D., Do, T., Beighton, D. & Devine, D. A. 2016. Influence of saliva on the oral microbiota. *Periodontology 2000,* 70**,** 80-92.

Matsui, R. & Cvitkovitch, D. 2010. Acid tolerance mechanisms utilized by *Streptococcus mutans*. *Future Microbiology,* 5**,** 403-417.

Matsumoto-Nakano, M. 2018. Role of *Streptococcus mutan*s surface proteins for biofilm formation. *Japanese Dental Science Review,* 54**,** 22-29.

Matsuura, H. N. & Fett-Neto, A. G. 2015. Plant alkaloids: main features, toxicity, and mechanisms of action. *Plant Toxins***,** 1-15.

Megersa, M., Jima, T. T. & Goro, K. K. 2019. The use of medicinal plants for the treatment of toothache in Ethiopia. *Evidence-Based Complementary and Alternative Medicine,* 2019**,** 2645174.

Metwalli, K. H., Khan, S. A., Krom, B. P. & Jabra-Rizk, M. A. 2013. *Streptococcus mutans, Candida albican*s, and the human mouth: a sticky situation. *PLoS pathogens,* 9**,** e1003616.

Miliauskas, G., Venskutonis, P. & Van Beek, T. 2004. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry,* 85**,** 231-237.

Mogana, R., Adhikari, A., Tzar, M., Ramliza, R. & Wiart, C. 2020. Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. against bacterial clinical isolates. *BMC Complementary Medicine and Therapies,* 20**,** 1-11.

Moghadam, E. T., Yazdanian, M., Tahmasebi, E., Tebyanian, H., Ranjbar, R., Yazdanian, A., Seifalian, A. & Tafazoli, A. 2020. Current herbal medicine as an alternative treatment in dentistry: In vitro, in vivo and clinical studies. *European Journal of Pharmacology***,** 173665.

Monon, K., Abdoulaye, T., Karamoko, O. & Adama, C. 2015. Phytochemical composition, antioxidant and antibacterial activities of root of *Uvaria chamae* P. Beauv.(Annonaceae) used in treatment of dysentery in North of Côte d'Ivoire. *International Journal of Pharmacognosy and Phytochemical Research,* 7**,** 1047-1053.

Morgan, A. 2008. Vital guide to preventing dental caries. *Vital,* 5**,** 21-24.

Moye, Z. D., Zeng, L. & Burne, R. A. 2014. Fueling the caries process: carbohydrate metabolism and gene regulation by *Streptococcus mutans*. *Journal of Oral Microbiology,* 6**,** 24878.

Munir, S., Shah, A. A., Shahid, M., Manzoor, I., Aslam, B., Rasool, M. H., Saeed, M., Ayaz, S. & Khurshid, M. 2020. Quorum sensing interfering strategies and their implications in the management of biofilm-associated bacterial infections. *Brazilian Archives of Biology and Technology,* 63.

Nagarajan, K. & Anjaneyulu, K. 2019. Awareness of GV Black, Mount's and ICDAS scoring systems of dental caries amongst dental practitioners in Chennai: A survey. *Drug Invention Today,* 12.

Nagasawa, R., Sato, T., Nomura, N., Nakamura, T. & Senpuku, H. 2020. Potential risk of spreading resistance genes within extracellular-DNA-dependent biofilms of *Streptococcus mutans* in response to cell envelope stress induced by sub-MICs of bacitracin. *Applied and Environmental Microbiology,* 86**,** e00770-20.

Naidoo, R., Patel, M., Gulube, Z. & Fenyvesi, I. 2012. Inhibitory activity of *Dodonaea viscosa var. angustifolia* extract against *Streptococcus mutan*s and its biofilm. *Journal of Ethnopharmacology,* 144**,** 171-174.

Nakano, K., Nomura, R., Matsumoto, M. & Ooshima, T. 2010. Roles of oral bacteria in cardiovascular diseases-from molecular mechanisms to clinical cases: cell-surface structures of novel serotype k *Streptococcus mutans* strains and their correlation to virulence. *Journal of Pharmacological Sciences,* 113**,** 120-125.

Nakano, K., Nomura, R. & Ooshima, T. 2008. *Streptococcus mutans* and cardiovascular diseases. *Japanese Dental Science Review,* 44**,** 29-37.

Nakano, K. & Ooshima, T. 2009. Serotype classification of *Streptococcus mutans* and its detection outside the oral cavity. *Future Microbiology,* 4**,** 891-902.

Napimoga, M. H., Höfling, J. F., Klein, M. I., Kamiya, R. U. & Gonçalves, R. B. 2005. Transmission, diversity and virulence factors of *Streptococcus mutans* genotypes. *Journal of Oral Science,* 47**,** 59-64.

Nassar, H. M. & Gregory, R. L. 2017. Biofilm sensitivity of seven *Streptococcus mutans* strains to different fluoride levels. *Journal of Oral Microbiology,* 9**,** 1328265.

Nemudzivhadi, V. & Masoko, P. 2015. Antioxidant and antibacterial properties of Ziziphus mucronata and Ricinus communis leaves extracts. *African Journal of Traditional, Complementary and Alternative Medicines,* 12**,** 81-89.

Ng, M. W. & Fida, Z. 2016. Dental hygienist–led chronic disease management system to control early childhood caries. *Journal of Evidence Based Dental Practice,* 16**,** 20-33.

Ngabaza, T., Moeno, S. & Patel, M. 2018. Anti-acidogenic and anti-biofilm activity of 5, 6, 8-trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one. *Microbial Pathogenesis,* 123**,** 149-152.

Nisar, B., Sultan, A. & Rubab, S. 2018. Comparison of medicinally important natural products versus synthetic drugs-a short commentary. *Natural Products Chemistry and Research,* 6**,** 308.

Nosrati, M., Behbahani, M. & Mohabatkar, H. 2018. Antibacterial and antibiofilm activities of *Prangos acaulis Bornm*. extract against *Streptococcus mutans*: an in silico and in vitro study. *Journal of Herbmed Pharmacology,* 7**,** 176-184.

Nyvad, B., Crielaard, W., Mira, A., Takahashi, N. & Beighton, D. 2013. Dental caries from a molecular microbiological perspective. *Caries Research,* 47**,** 89-102.

Nyvad, B. & Takahashi, N. 2020. Integrated hypothesis of dental caries and periodontal diseases. *Journal of Oral Microbiology,* 12**,** 1710953.

Ogbuanu, C. & Emmanuel, S. A. 2020. Secondary metabolites investigation and TLC analysis of leaves, stem back and root extracts of *Uvaria chamae* (UDAGU).

Ogbulie, J., Ogueke, C. & Nwanebu, F. 2007. Antibacterial properties of *Uvaria chamae, Congronema latifolium, Garcinia kola, Vemonia amygdalina* and *Aframomium melegueta*. *African Journal of Biotechnology,* 6.

Oliva, A., Costantini, S., De Angelis, M., Garzoli, S., Božović, M., Mascellino, M. T., Vullo, V. & Ragno, R. 2018. High potency of *Melaleuca alternifolia* essential oil against multi-drug resistant gram-negative bacteria and methicillin-resistant *Staphylococcus aureus*. *Molecules,* 23**,** 2584.

Oliveira, B. E. C., Ricomini Filho, A. P., Burne, R. A. & Zeng, L. 2021. The route of sucrose utilization by *Streptococcus mutans* affects intracellular polysaccharide metabolism. *Frontiers in Microbiology,* 12.

Olumese, F. E. & Onoagbe, I. O. 2019. Proximate analysis and phytochemical composition of *Uvaria chamae* root. *Society for Experimental Biology of Nigeria* 17.

Oluremi, B., Osungunna, M. & Omafuma, O. 2010. Comparative assessment of antibacterial activity of *Uvaria chamae* parts. *African Journal of Microbiology Research,* 4**,** 1391-1394.

Palacios, C., Rivas-Tumanyan, S., Morou-Bermúdez, E., Colon, A. M., Torres, R. Y. & Elías-Boneta, A. R. 2016. Association between type, amount, and pattern of carbohydrate

consumption with dental caries in 12-year-olds in Puerto Rico. *Caries Research,* 50**,** 560-570.

Palmer, S. R., Ren, Z., Hwang, G., Liu, Y., Combs, A., Söderström, B., Lara Vasquez, P., Khosravi, Y., Brady, L. J. & Koo, H. 2019. *Streptococcus mutans* yidC1 and yidC2 impact cell envelope biogenesis, the biofilm matrix, and biofilm biophysical properties. *Journal of Bacteriology,* 201**,** e00396-18.

Palombo, E. A. 2011. Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. *Evidence-Based Complementary and Alternative Medicine,* 2011**,** 680354

Panche, A., Diwan, A. & Chandra, S. 2016. Flavonoids: an overview. *Journal of Nutritional Science,* 5.

Park, B.-I., Kim, B.-S., Kim, K.-J. & You, Y.-O. 2019. Sabinene suppresses growth, biofilm formation, and adhesion of *Streptococcus mutans* by inhibiting cariogenic virulence factors. *Journal of Oral Microbiology,* 11**,** 1632101.

Patel, M., Gulube, Z. & Dutton, M. 2009. The effect of *Dodonaea viscosa var. angustifolia* on *Candida albicans* proteinase and phospholipase production and adherence to oral epithelial cells. *Journal of Ethnopharmacology,* 124**,** 562-565.

Peres, M. A., Macpherson, L. M., Weyant, R. J., Daly, B., Venturelli, R., Mathur, M. R., Listl, S., Celeste, R. K., Guarnizo-Herreño, C. C. & Kearns, C. 2019. Oral diseases: a global public health challenge. *The Lancet,* 394**,** 249-260.

Phaiboon, N., Pulbutr, P., Sungthong, B. & Rattanakiat, S. 2019. Effects of the ethanolic extracts of guava leaves, licorice roots and cloves on the cariogenic properties of *Streptococcus mutans*. *Pharmacognosy Journal,* 11.

Pires, J. G., Zabini, S. S., Braga, A. S., De Cássia Fabris, R., De Andrade, F. B., De Oliveira, R. C. & Magalhães, A. C. 2018. Hydroalcoholic extracts of *Myracrodruon urundeuva* All. and Q*ualea grandiflora Mar*t. leaves on *Streptococcus mutans* biofilm and tooth demineralization. *Archives of Oral Biology,* 91**,** 17-22.

Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F., Tagami, J., Twetman, S., Tsakos, G. & Ismail, A. 2017a. Dental caries. *Nature Reviews Disease primers,* 3**,** 1-16.

Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F., Tagami, J., Twetman, S., Tsakos, G. & Ismail, A. 2017b. Dental caries. *Nature Review Disease Primers,* 3**,** 17030.

Prabu, G., Gnanamani, A. & Sadulla, S. 2006. Guaijaverin–a plant flavonoid as potential antiplaque agent against *Streptococcus mutans*. *Journal of Applied Microbiology,* 101**,** 487-495.

Pradeep, G. 2020. Comparison of efficacy of ICDAS, CAST, Nyvad's criteria, SCI and WHO–DMFT in caries identification among six to twelve year old children. *International Journal of Oral Health Dentistry,* 6**,** 267-272.

Prasanth, M. 2011. Antimicrobial efficacy of different toothpastes and mouthrinses: an in vitro study. *Dental Research Journal,* 8**,** 85.

Rahim, Z. H. A. & Thurairajah, N. 2011. Scanning electron microscopic study of *Piper betle* L. leaves extract effect against *Streptococcus mutans* ATCC 25175. *Journal of Applied Oral Science,* 19**,** 137-146.

Rajendran, R. 2009. *Shafer's textbook of oral pathology*, Elsevier India.

Ramalingam, K. & Amaechi, B. T. 2018. Antimicrobial effect of herbal extract of *Acacia arabica* with triphala on the biofilm forming cariogenic microorganisms. *Journal of Ayurveda and Integrative Medicine***,** 322-328.

Ramawat, K. G. & Mérillon, J.-M. 2015. *Polysaccharides: bioactivity and biotechnology*, Springer.

Ranganathan, V. & Akhila, C. 2019a. *Streptococcus mutans* has it become prime perpetrator for oral manifestations. *Journal of Microbiology & Experimentation,* 7**,** 322-328.
Ranganathan, V. & Akhila, C. 2019b. Streptococcus mutans: has it become prime perpetrator for oral manifestations. *Journal of Microbiology & Experimentation,* 7**,** 207-213.
Rasool Hassan, B. 2012. Medicinal plants (Importance and uses). *Pharmaceutica Analytica Acta* 3**,** 2153-2435.
Rathee, M. & Sapra, A. 2019. Dental Caries. *StatPearls Publishing***,** 1-10.
Rosier, B. T., De Jager, M., Zaura, E. & Krom, B. P. 2014. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Frontiers in Cellular and Infection Microbiology,* 4**,** 92.
Rustaiyan, A., Mazloomifar, H., Masoudi, S. & Aghjani, Z. 2006. Volatile oils of *Ducrosia assadii Alava*. and *Prangos acaulis* (DC.) Bornm. from Iran. *Journal of Essential Oil Research,* 18**,** 682-684.
Sachidananda, M. P. & Mallya, S. 2020. Microbiology and Clinical Implications of Dental Caries-A Review. *Journal of Evolution of Medical and Dental Sciences,* 9**,** 3670-3676.
Salvetti, E., Torriani, S. & Felis, G. E. 2012. The genus Lactobacillus: a taxonomic update. *Probiotics and antimicrobial proteins,* 4**,** 217-226.
Sandasi, M., Leonard, C., Van Vuuren, S. & Viljoen, A. 2011. Peppermint (Mentha piperita) inhibits microbial biofilms in vitro. *South African Journal of Botany,* 77**,** 80-85.
Schneider-Rayman, M., Steinberg, D., Sionov, R. V., Friedman, M. & Shalish, M. 2021. Effect of epigallocatechin gallate on dental biofilm of *Streptococcus mutans*: An in vitro study. *BMC Oral Health,* 21**,** 1-11.
Seleshe, S. & Kang, S. N. 2019. In vitro antimicrobial activity of different solvent extracts from Moringa stenopetala leaves. *Preventive nutrition and food science,* 24**,** 70.
Semenya, S. S., Mokgoatšana, S. & Maroyi, A. 2019. Medicinal plants used to treat oral diseases in the Lepelle-Nkumpi Municipality, Limpopo Province, South Africa. *Journal of Pharmacy and Nutrition Sciences,* 9**,** 000-000.
Senadheera, M. D., Guggenheim, B., Spatafora, G. A., Huang, Y.-C. C., Choi, J., Hung, D. C., Treglown, J. S., Goodman, S. D., Ellen, R. P. & Cvitkovitch, D. G. 2005. A VicRK signal transduction system in *Streptococcus mutans* affects gtfBCD, gbpB, and ftf expression, biofilm formation, and genetic competence development. *Journal of Bacteriology,* 187**,** 4064-4076.
Şener, B. & Kiliç, M. 2019. Herbal extracts used in dental disorders. 14107-14111.
Seneviratne, C. J., Zhang, C. F. & Samaranayake, L. P. 2011. Dental plaque biofilm in oral health and disease. *Chinese Journal of Dental Research,* 14**,** 87.
Sheikh, N., Kumar, Y., Misra, A. & Pfoze, L. 2013. Phytochemical screening to validate the ethnobotanical importance of root tubers of Dioscorea species of Meghalaya, North East India. *Journal of Medicinal Plants,* 1**,** 62-69.
Shemesh, M., Tam, A. & Steinberg, D. 2007. Expression of biofilm-associated genes of *Streptococcus mutans* in response to glucose and sucrose. *Journal of Medical Microbiology,* 56**,** 1528-1535.
Shen, P., Cai, F., Nowicki, A., Vincent, J. & Reynolds, E. 2001. Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *Journal of Dental Research,* 80**,** 2066-2070.
Shlezinger, M., Coppenhagen-Glazer, S., Gelman, D., Beyth, N. & Hazan, R. 2019. Eradication of vancomycin-resistant enterococci by combining phage and vancomycin. *Viruses,* 11**,** 954.
Smith, D. 2002. Dental caries vaccines: prospects and concerns. *Critical Reviews in Oral Biology & Medicine,* 13**,** 335-349.

Smullen, J., Koutsou, G., Foster, H., Zumbé, A. & Storey, D. 2007. The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*. *Caries Research,* 41**,** 342-349.
Sparg, S., Light, M. & Van Staden, J. 2004. Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology,* 94**,** 219-243.
Stookey, G. K. 2008. The effect of saliva on dental caries. *The Journal of the American Dental Association,* 139**,** 11S-17S.
Strużycka, I. 2014. The oral microbiome in dental caries. *Polish Journal of Microbiology,* 63**,** 127-135.
Su, N., Marek, C. L., Ching, V. & Grushka, M. 2011. Caries prevention for patients with dry mouth. *The Journal of the Canadian Dental Association,* 77**,** 1-8.
Szkaradkiewicz-Karpińska, A. K. & Szkaradkiewicz, A. 2021. Effect of exopolysaccharides from cariogenic bacteria on human gingival fibroblasts. *International Journal of Medical Sciences,* 18**,** 2666.
Tamfu, A. N., Tagatsing Fotsing, M., Talla, E., Jabeen, A., Mbafor Tanyi, J. & Shaheen, F. 2019. Bioactive constituents from seeds of *Annona senegalensis Persoon* (Annonaceae). *Natural Product Research***,** 1-6.
Tamokou, J., Mbaveng, A. & Kuete, V. 2017. Antimicrobial activities of African medicinal spices and vegetables. *Medicinal spices and vegetables from Africa.* Elsevier.
Tapsoba, H. & Deschamps, J.-P. 2006. Use of medicinal plants for the treatment of oral diseases in Burkina Faso. *Journal of Ethnopharmacology,* 104**,** 68-78.
Teanpaisan, R., Senapong, S. & Puripattanavong, J. 2014. In vitro antimicrobial and antibiofilm activity of *Artocarpus lakoocha* (Moraceae) extract against some oral pathogens. *Tropical Journal of Pharmaceutical Research,* 13**,** 1149-1155.
Tellez, M. & Lim, S. 2020. ICDAS is widely used as standardized and reliable caries detection criteria, but its reporting varies widely. *Journal of Evidence Based Dental Practice,* 20**,** 101409.
Thomas, A., Edia, R.-O. & Idibiye, K. 2018. A review: secondary metabolites of *Uvaria chamae* p. Beauv.(Annonaceae) and their biological activities. *International Journal of Agriculture Environment and Food Sciences,* 2**,** 177-185.
Thurnheer, T. & Belibasakis, G. N. 2018. Effect of sodium fluoride on oral biofilm microbiota and enamel demineralization. *Archives of Oral Biology,* 89**,** 77-83.
Tiwari, R., Bhayat, A. & Chikte, U. 2021. Forecasting for the need of dentists and specialists in South Africa until 2030. *Plos one,* 16**,** e0251238.
Touger-Decker, R. & Van Loveren, C. 2003. Sugars and dental caries. *The American Journal of Clinical Nutrition,* 78**,** 881S-892S.
Tungare, S. & Paranjpe, A. G. 2018. Diet and nutrition to prevent dental problems.
Udoh, D. I., Ekong, U. S., Etang, B. B. & Udo, N. a. E. 2019. Qualitative assessment of *Uvaria chamae* (bush banana) crude extracts against wound isolated strains of *Pseudomonas aeruginosa* and *Proteus mirabilis* alongside resistance and plasmid profiles determination of the isolates. *African Journal of Microbiology Research,* 13**,** 520-530.
Vaillancourt, K., Lebel, G., Pellerin, G., Ben Lagha, A. & Grenier, D. 2021. Effects of the licorice isoflavans licoricidin and glabridin on the growth, adherence properties, and acid production of *Streptococcus mutans*, and assessment of their biocompatibility. *Antibiotics,* 10**,** 163.
Van Loveren, C. 2019. Sugar restriction for caries prevention: amount and frequency. Which is more important? *Caries Research,* 53**,** 168-175.
Verloove, F. & Andeweg, R. 2020. *Artemisia princeps* L.(Asteraceae), an overlooked invasive far Eastern weed in Western Europe. *Gorteria,* 42**,** 1-18.

Vijayakumar, A., Sarveswari, H. B., Vasudevan, S., Shanmugam, K., Solomon, A. P. & Neelakantan, P. 2021. Baicalein inhibits *Streptococcus mutans* biofilms and dental caries-related virulence phenotypes. *Antibiotics,* 10**,** 215.

Villa, O., Ramberg, P., Fukui, H., Emilson, C.-G., Papanikolaou, G., Heijl, L. & Birkhed, D. 2018. Interaction between chlorhexidine and fluoride in a mouthrinse solution-a 4-day and 6-week randomized clinical pilot study. *Clinical Oral Investigations,* 22**,** 1439-1448.

Viszwapriya, D., Subramenium, G. A., Radhika, S. & Pandian, S. K. 2017. Betulin inhibits cariogenic properties of *Streptococcus mutans* by targeting vicRK and gtf genes. *Antonie Van Leeuwenhoek,* 110**,** 153-165.

Walsh, L. J. 2006. Dental plaque fermentation and its role in caries risk assessment. *International Dentistry South Afria (Australasian Edition),* 1**,** 4-13.

Walther, C., Zumbülte, S., Faerber, C. M., Wierichs, R. J., Meyer-Lueckel, H., Conrads, G., Henne, K. & Esteves-Oliveira, M. 2021. Analysis of relative bacterial activity and lactate dehydrogenase gene expression of caries-associated bacteria in a site-specific natural biofilm: an ex vivo study. *Clinical Oral Investigations,* 25**,** 3669-3679.

Wang, H. R., Sui, H. C., Ding, Y. Y. & Zhu, B. T. 2018. Stimulation of the production of prostaglandin E2 by Ethyl Gallate, a natural phenolic compound richly contained in Longan. *Biomolecules,* 8**,** 91.

Wu, J., Fan, Y., Wang, X., Jiang, X., Zou, J. & Huang, R. 2020. Effects of the natural compound, oxyresveratrol, on the growth of *Streptococcus mutans*, and on biofilm formation, acid production, and virulence gene expression. *European Journal of Oral Sciences,* 128**,** 18-26.

Xiao, J., Zuo, Y., Liu, Y., Li, J., Hao, Y. & Zhou, X. 2007. Effects of *Nidus vespae* extract and chemical fractions on glucosyltransferases, adherence and biofilm formation of Streptococcus mutans. *Archives of Oral Biology,* 52**,** 869-875.

Xu, C.-P., Van De Belt-Gritter, B., Dijkstra, R. J., Norde, W., Van Der Mei, H. C. & Busscher, H. J. 2007. Interaction forces between salivary proteins and *Streptococcus mutans* with and without antigen I/II. *Langmuir,* 23**,** 9423-9428.

Xu, J.-S., Cui, Y., Liao, X.-M., Tan, X.-B. & Cao, X. 2014. Effect of emodin on the cariogenic properties of *Streptococcus mutans* and the development of caries in rats. *Experimental and Therapeutic Medicine,* 8**,** 1308-1312.

Yabuta, Y., Mukoyama, H., Kaneda, Y., Kimura, N., Bito, T., Ichiyanagi, T., Ishihara, A. & Watanabe, F. 2018. A lemon myrtle extract inhibits glucosyltransferases activity of *Streptococcus mutans*. *Bioscience, Biotechnology, and Biochemistry,* 82**,** 1584-1590.

Yactayo-Alburquerque, M. T., Alen-Méndez, M. L., Azañedo, D., Comandé, D. & Hernández-Vásquez, A. 2021. Impact of oral diseases on oral health-related quality of life: A systematic review of studies conducted in Latin America and the Caribbean. *PloS one,* 16**,** e0252578.

Yadav, K. & Prakash, S. 2017. Dental caries: A microbiological approach. *Journal of Clinical Infectious Diseases & Practice* 2**,** 1-15.

Yakubu, M. & Fayemo, H. 2021. Anti-hyperprolactinemic activities of aqueous extract of *Uvaria chamae* (P. Beauv) roots and associated biochemical changes in chlorpromazine-induced hyperprolactinemic female Wistar rats. *Journal of Ethnopharmacology,* 271**,** 113863.

Yan, Y., Li, X., Zhang, C., Lv, L., Gao, B. & Li, M. 2021. Research progress on antibacterial activities and mechanisms of natural alkaloids: A review. *Antibiotics,* 10**,** 318.

Yang, Y., Hwang, E.-H., Park, B.-I., Choi, N.-Y., Kim, K.-J. & You, Y.-O. 2019. *Artemisia princeps* inhibits growth, biofilm formation, and virulence factor expression of *Streptococcus mutans*. *Journal of Medicinal Food,* 22**,** 623-630.

Yatsuda, R., Rosalen, P., Cury, J., Murata, R., Rehder, V., Melo, L. & Koo, H. 2005. Effects of Mikania genus plants on growth and cell adherence of mutans streptococci. *Journal of Ethnopharmacology,* 97**,** 183-189.

Yoo, H.-J. & Jwa, S.-K. 2018. Inhibitory effects of β-caryophyllene on *Streptococcus mutans* biofilm. *Archives of Oral Biology,* 88**,** 42-46.

Yoshida, A. & Kuramitsu, H. K. 2002. Multiple *Streptococcus mutans* genes are involved in biofilm formation. *Applied and Environmental Microbiology,* 68**,** 6283-6291.

You, Y.-O. 2019. Virulence genes of *Streptococcus mutans* and dental caries. *International Journal of Oral Biology,* 44**,** 31-36.

Young, D. A., Nový, B. B., Zeller, G. G., Hale, R., Hart, T. C., Truelove, E. L., Ekstrand, K. R., Featherstone, J. D., Fontana, M. & Ismail, A. 2015. The American Dental Association caries classification system for clinical practice: a report of the American Dental Association Council on Scientific Affairs. *The Journal of the American Dental Association,* 146**,** 79-86.

Yu, O. Y., Zhao, I. S., Mei, M. L., Lo, E. C.-M. & Chu, C.-H. 2017. Dental biofilm and laboratory microbial culture models for cariology research. *Dentistry Journal,* 5**,** 21.

Zain, N. B. M. Differential expression of gene of *Streptococcus mutans* in response to treatment with *Piper Betle* aqueous extract–A research framework. Proceedings of International Conference on Bioscience, Biochemistry and Bioinformatics 2011.

Zayed, S. M., Aboulwafa, M. M., Hashem, A. M. & Saleh, S. E. 2021. Biofilm formation by *Streptococcus mutans* and its inhibition by green tea extracts. *AMB Express,* 11**,** 1-10.

Zhang, Z., Liu, Y., Lu, M., Lyu, X., Gong, T., Tang, B., Wang, L., Zeng, J. & Li, Y. 2020a. *Rhodiola rosea* extract inhibits the biofilm formation and the expression of virulence genes of cariogenic oral pathogen *Streptococcus mutans*. *Archives of Oral Biology***,** 104762.

Zhang, Z., Liu, Y., Lu, M., Lyu, X., Gong, T., Tang, B., Wang, L., Zeng, J. & Li, Y. 2020b. Rhodiola rosea extract inhibits the biofilm formation and the expression of virulence genes of cariogenic oral pathogen Streptococcus mutans. *Archives of oral biology,* 116**,** 104762.

Appendices

Appendix I: Media composition and preparation

Blood agar

39 g of Columbia agar base

1000 ml distilled water

Dissolve by stirring and heating

Autoclave at 121 ^{o}C for 2 hours

Allow media to cool to 42.5^{O}C

Add 5 % horse blood

Pour plates

Allow them to cool and set

Refrigerate until ready to use

Tryptone Broth

12.5 g tryptone powder

7.5 g yeast extracts

5 g sucrose

500 ml distilled water

Sterilise by autoclaving in 121 ^{o}C for 15 min

Tryptone Soya Broth

30 g tryptone soya broth

1000 ml of distilled water

Dissolve 30 g in 1 litre

Sterilise by autoclaving in 121 ºC for 15 min

Phosphate buffered saline

Phosphate buffered saline tablet

250 ml of distilled water

Dissolve 1 PBS tablet in 250 ml of distilled water

Dissolve by stirring and heating

Autoclave at 121 ºC for 2 hours

5 % sucrose broth

10 g tryptone

5 g protease peptone no.3

5 g protease peptone

1 g glucose

50 g sucrose

4 g dipotassium phosphate

1000 ml distilled water

Autoclave at 150 kPa, 121 ^{o} C for 10 minutes

5 % Aqueous phenol

5 g phenol crystals

Dissolve in 100 ml of distilled water

Dissolve by stirring and heating

Acid phenol

Dissolve 25 g phenol crystals in 25 ml absolute ethanol

Mix thoroughly by stirring

70 % ethanol

Add 700 ml of absolute ethanol and top up with 300 ml of distilled water

Appendix II: Primer preparation

16 s RRNA-F

For 100 µM stock solution, add 825.05 µl of nuclease free water

16 S RRNA-R

For 100 µM stock solution, add 671.74 µl of nuclease-free water

GBPB-F

For 100 µM stock solution, add 377.15 µl of nuclease-free water

GBPB-R

For 100 µM stock solution, add 773.39 µl of nuclease-free water

SPAP-F

For 100 µM stock solution, add 521.17 µl of nuclease-free water

SPAP-R

For 100 µM stock solution, add 779.28 µl of nuclease-free water

BRPA-F

For 100 µM stock solution, add 692.32 µl of nuclease-free water

BRPA-R

For 100 µM stock solution, add 748.78 µl of nuclease-free water

IDH-F

For 100 µM stock solution, add 689.7 µl of nuclease-free water

IDH-R

For 100 µM stock solution, add 842.88 µl of nuclease-free water

GTFB-F

For 100 µM stock solution, add 825.05 µl of nuclease-free water

GTFB-R

For 100 µM stock solution, add 823.13 µl of nuclease-free water

GTFC-F

For 100 µM stock solution, add 594.83 µl of nuclease-free water

GTFC-R

For 100 µM stock solution, add 679.0 µl of nuclease-free water

VICR-F

For 100 µM stock solution, add 670.23 µl of nuclease-free water

VICR-R

For 100 µM stock solution, add 596.43 µl of nuclease-free water

ATPD-F

For 100 µM stock solution, add 600.83 µl of nuclease-free water

ATPD-R

For 100 µM stock solution, 582.66 µl of nuclease-free water

Preparation of 100 µl: 10 µl of each primer into 90 µl of nuclease-free water. Then mix by centrifuging at 500×g for 2 seconds. Store at -20 ^{O}C until required for use.

Appendix III: Statistical analysis

Based on the arrangement of data, Dunnett's Multiple Comparison was used. Dunnett's is used after the ANOVA has been run to identify the pairs with significant differences. A one-way ANOVA was used to compare two means from two independent (unrelated) groups using the F-distribution. The null hypothesis for the test is that the two means are equal. Therefore, a significant result means that the two means are unequal.

Biofilm Control vs Each Concentration

Dunnett's multiple comparisons test	*Mean Diff,*	*95,00% CI of diff,*	*Significant?*	*Summary*	*Adjusted P Value*	*A-?*	
Control (6 Hrs) vs. 0.005 mg/ml (6 Hrs)	12003	4390 to 19616	Yes	**	0,0024	B	0,005 mg/ml (6 Hrs)
Control (6 Hrs) vs. 0.01 mg/ml (6 Hrs)	17890	10277 to 25503	Yes	****	<0,0001	C	0,01 mg/ml (6 Hrs)
Control (6 Hrs) vs. 0.02 mg/ml (6 Hrs)	23226	15613 to 30839	Yes	****	<0,0001	D	0,02 mg/ml (6 Hrs)
Control (24 Hrs) vs. 0,005 mg/ml (24 Hrs)	58434	33453 to 83415	Yes	****	<0,0001	F	0,005 mg/ml (24 Hrs)
Control (24 Hrs) vs. 0,01 mg/ml (24 Hrs)	63414	38433 to 88395	Yes	****	<0,0001	G	0,01 mg/ml (24 Hrs)
Control (24 Hrs) vs. 0,02 mg/ml (24 Hrs)	63667	38686 to 88648	Yes	****	<0,0001	H	0,02 mg/ml (24 Hrs)
Control (30 Hrs) vs. 0,005 mg/ml (30 Hrs)	148714	33786 to 263642	Yes	*	0,0108	J	0,005 mg/ml (30 Hrs)
Control (30 Hrs) vs. 0,01 mg/ml (30 Hrs)	154551	39623 to 269479	Yes	**	0,0082	K	0,01 mg/ml (30 Hrs)
Control (30 Hrs) vs. 0,02 mg/ml (30 Hrs)	153568	38640 to 268496	Yes	**	0,0086	L	0,02 mg/ml (30 Hrs)

Biofilm control vs all concentration

Table Analyzed	Biofilm Con vs All
Column B	All Concentrations
vs.	vs,
Column A	Control
Mann Whitney test	
P value	0,0079
Exact or approximate P value?	Exact
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	40 , 15
Mann-Whitney U	0
Difference between medians	
Median of column A	90024, n=5
Median of column B	7757, n=5
Difference: Actual	-82267
Difference: Hodges-Lehmann	-81887

Extracellular Polysaccharides Control vs Each Concentration

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value	A-?	
Control (SEPS) vs. 0,005 mg/ml (SEPS)	0,3825	-0,6603 to 1,425	No	ns	0,6672	B	0,005 mg/ml (SEPS)
Control (SEPS) vs. 0,01 mg/ml (SEPS)	0,1588	-0,8840 to 1,202	No	ns	0,9596	C	0,01 mg/ml (SEPS)
Control (SEPS) vs. 0,02 mg/ml (SEPS)	0,3488	-0,6940 to 1,392	No	ns	0,7222	D	0,02 mg/ml (SEPS)
Control (IEPS) vs. 0,005 mg/ml (IEPS)	0,07347	-0,03386 to 0,1808	No	ns	0,2194	F	0,005mg/ml(IEPS)
Control (IEPS) vs. 0,01 mg/ml (IEPS)	0,001000	-0,1063 to 0,1083	No	ns	>0,9999	G	0,01 mg/ml (IEPS)
Control (IEPS) vs. 0,02 mg/ml (IEPS)	0,07533	-0,03200 to 0,1827	No	ns	0,2038	H	0,02 mg/ml (IEPS)

Control vs All

SEPS Con vs All

Wilcoxon matched-pairs signed rank test	
P value	0,3125
Exact or approximate P value?	Exact
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Sum of positive, negative ranks	3,000, -12,00
Sum of signed ranks (W)	-9,000
Number of pairs	5
Number of ties (ignored)	0

How effective was the pairing?	
rs (Spearman)	0,9000
P value (one tailed)	0,0417
P value summary	*

Was the pairing significantly effective?	Yes

IEPS Con vs All

Wilcoxon matched-pairs signed rank test	
P value	0,3125
Exact or approximate P value?	Exact
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Sum of positive, negative ranks	3,000, -12,00
Sum of signed ranks (W)	-9,000
Number of pairs	5
Number of ties (ignored)	0

How effective was the pairing?	
rs (Spearman)	-0,3000
P value (one tailed)	0,3417
P value summary	ns
Was the pairing significantly effective?	No

Acid assay

Dunnett's multiple comparisons test	**Mean Diff,**	**95,00% CI of diff,**	**Significant?**	**Summary**	**Adjusted P Value**	**A-?**	
Control (0 Hrs) vs. 0,005 mg/ml (0 Hrs)	-0,05200	-0,1903 to 0,08635	No	ns	0,6516	B	0,005 mg/ml (0 Hrs)
Control (0 Hrs) vs. 0,01 mg/ml (0 Hrs)	-0,02100	-0,1593 to 0,1173	No	ns	0,9599	C	0,01 mg/ml (0 Hrs)
Control (0 Hrs) vs. 0,02 mg/ml (0 Hrs)	-0,05500	-0,1933 to 0,08335	No	ns	0,6142	D	0,02 mg/ml (0 Hrs)
Control (10 Hrs) vs. 0,005 mg/ml (10 Hrs)	-1,111	-1,651 to -0,5711	Yes	***	0,0002	F	0,005 mg/ml (10 Hrs)
Control (10 Hrs) vs. 0,01 mg/ml (10 Hrs)	-1,117	-1,657 to -0,5771	Yes	***	0,0002	G	0,01 mg/ml (10 Hrs)
Control (10 Hrs) vs. 0,02 mg/ml (10 Hrs)	-1,169	-1,709 to -0,6291	Yes	***	0,0001	H	0,02 mg/ml (10 Hrs)
Control (12 Hrs) vs. 0,005 mg/ml (12 Hrs)	-1,490	-1,913 to -1,067	Yes	****	<0,0001	J	0,005 mg/ml (12 Hrs)

Control (12 Hrs) vs. 0,01 mg/ml (12 Hrs)	-1,531	-1,954 to -1,108	Yes	****	<0,0001	K	0,01 mg/ml (12 Hrs)
Control (12 Hrs) vs. 0,02 mg/ml (12 Hrs)	-1,563	-1,986 to -1,140	Yes	****	<0,0001	L	0,02 mg/ml (12 Hrs)
Control (14 Hrs) vs. 0,005 mg/ml (14 Hrs)	-1,723	-2,172 to -1,274	Yes	****	<0,0001	N	0,005 mg/ml (14 Hrs)
Control (14 Hrs) vs. 0,01 mg/ml (14 Hrs)	-1,724	-2,173 to -1,275	Yes	****	<0,0001	O	0,01 mg/ml (14 Hrs)
Control (14 Hrs) vs. 0,02 mg/ml (14 Hrs)	-1,783	-2,232 to -1,334	Yes	****	<0,0001	P	0,02 mg/ml (14 Hrs)
Control (16 Hrs) vs. 0,005 mg/ml (16 Hrs)	-1,881	-2,247 to -1,515	Yes	****	<0,0001	R	0,005 mg/ml (16 Hrs)
Control (16 Hrs) vs. 0,01 mg/ml (16 Hrs)	-1,950	-2,316 to -1,584	Yes	****	<0,0001	S	0,01 mg/ml (16 Hrs)
Control (16 Hrs) vs. 0,02 mg/ml (16 Hrs)	-1,989	-2,355 to -1,623	Yes	****	<0,0001	T	0,02 mg/ml (16 Hrs)
Combined times							
Control vs. 0.005 mg/ml	-1,251	-1,908 to -0,5943	Yes	***	0,0004	B	0,005 mg/ml
Control vs. 0,01 mg/ml	-1,269	-1,926 to -0,6115	Yes	***	0,0004	C	0,01 mg/ml
Control vs. 0,02 mg/ml	-1,312	-1,969 to -0,6547	Yes	***	0,0003	D	0,02 mg/ml

Acid Assay Control vs All

Wilcoxon matched-pairs signed rank test	
P value	0,0625
Exact or approximate P value?	Exact
P value summary	Ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Sum of positive, negative ranks	15,00, 0,000
Sum of signed ranks (W)	15,00
Number of pairs	5
Number of ties (ignored)	0

How effective was the pairing?	
rs (Spearman)	0,1000
P value (one tailed)	0,4750
P value summary	ns
Was the pairing significantly effective?	No

Bacterial Counts

Dunnett's multiple comparisons test	Mean Diff	95,00 CI of diff	Significant?	Summary	Adjusted P value	A-? Summary
Control (0 Hrs) vs. 0,005 mg/ml (0 Hrs)	-44546	-1025755 to 936663	No	ns	0.9988	B 0,005 mg/ml (0 Hrs)
Control (0 Hrs) vs. 0,01 mg/ml (0 Hrs)	-197240	-1178449 to 783969	No	ns	0.9152	C 0,01 mg/ml (0 Hrs)
Control (0 Hrs) vs. 0,02 mg/ml (0 Hrs)	-199813	-1181022 to 781396	No	ns	0.9124	D 0,02 mg/ml (0 Hrs)
Control (10 Hrs) vs. 0,005 mg/ml (10 Hrs)	1038827	-388427 to 2466081	No	ns	0.1821	F 0,005 mg/ml (10 Hrs)
Control (10 Hrs) vs. 0,01mg/ml (10 Hrs)	1170613	-256641 to 2597867	No	ns	0.1199	G 0,01 mg/ml (10 Hrs)
Control (10 Hrs) vs. 0,02 mg/ml (10 Hrs)	1307040	-120214 to 2734294	No	ns	0.0760	H 0,02 mg/ml (10 Hrs)
Control (12 Hrs) vs. 0,005 mg/ml (12 Hrs)	1285010	137362 to 2432658	Yes	*	0.0270	J 0,005 mg/ml (12 Hrs)
Control (12 Hrs) vs. 0,01 mg/ml (12 Hrs)	1313003	165355 to 2460651	Yes	*	0.0238	K 0,01 mg/ml (12 Hrs)
Control (12 Hrs) vs. 0,02 mg/ml (12 Hrs)	1409776	262128 to 2557424	Yes	*	0.0153	L 0,02 mg/ml (12 Hrs)
Control (14 Hrs) vs. 0,005 mg /ml (14 Hrs)	2754320	848022 to 4660618	Yes	**	0.0048	N 0,005 mg/ml (14 Hrs)
Control (14 Hrs) vs. 0,01/ml mg (14 Hrs)	2741940	835642 to 4648238	Yes	**	0.0050	O 0,01 mg/ml (14 Hrs)
Control (14 Hrs) vs. 0,02/ml mg (14 Hrs)	2884227	977929 to 4790525	Yes	**	0.0033	P 0,02 mg/ml (14 Hrs)
Control (16 Hrs) vs. 0,005 mg/ml (16 Hrs)	2711547	1046860 to 4376234	Yes	**	0.0018	R 0,005 mg/ml (16 Hrs)
Control (16 Hrs) vs. 0,01 mg/ml (16 Hrs)	2673160	1008473 to 4337847	Yes	**	0.0020	S 0,01 mg/ml (16 Hrs)
Control (16 Hrs) vs. 0,02 mg/ml (16 Hrs)	2839397	1174710 to 4504084	Yes	**	0.0012	T 0,02 mg/ml (16 Hrs)

Overall comparison

	Control	vs	all concentrations
Theoretical mean	0.000		0.000
Actual mean	1934994		355843
Number of values	75		225
One sample t test			
t, df	t=8.769, df=74		t=14.23, df=224
P value (two tailed)	<0.0001		<0.0001
P value summary	****		****
Significant (alpha=0.05)?	Yes		Yes
How big is the discrepancy?			
Discrepancy	1934994		355843
SD of discrepancy	1911030		375146
SEM of discrepancy	220667		25010
95% confidence interval	1495306 to 2374682		306559 to 405128
R squared (partial eta squared)	0.5096		0.4747

RT-qPCR assay

Streptococcus mutans 1 (SM1)

Column A	Untreated brpA
Vs	Vs
Column B	Treated brpA
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	15 , 6
Mann-Whitney U	0.0

Column C	Untreated gbpB
Vs	Vs
Column D	Treated gbpB
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column C,D	15 , 6
Mann-Whitney U	0.0

Column E	Untreated spaP
Vs	Vs
Column F	Treated spaP
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column E,F	15 , 6
Mann-Whitney U	0.0

Column G	Untreated vicR
Vs	Vs
Column H	Treated vicR
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column G,H	15 , 6
Mann-Whitney U	0.0

Column A	Untreated atpD
Vs	Vs
Column B	Treated atpD
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column A, B	15 , 6
Mann-Whitney U	0.0

Column C	Untreated IDH
Vs	Vs
Column D	Treated IDH
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column C,D	15 , 6
Mann-Whitney U	0.0

Column A	Untreated gtfB
Vs	Vs
Column B	Treated gtfB
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes

One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	15 , 6
Mann-Whitney U	0.0

Column C	Untreated gtfC
Vs	vs
Column D	Treated gtfC
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column C,D	15 , 6
Mann-Whitney U	0.0

***Streptococcus mutans* 12 (SM12)**

Column A	Untreated brpA
Vs	Vs
Column B	Treated brpA
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	15 , 6
Mann-Whitney U	0.0

Column C	Untreated gbpB
Vs	Vs
Column D	Treated gbpB
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column C,D	15 , 6
Mann-Whitney U	0.0

Column E	Untreated spaP
Vs	Vs
Column F	Treated spaP
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column E,F	15 , 6
Mann-Whitney U	0.0

Column G	Untreated vicR
Vs	Vs
Column H	Treated vicR
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column G,H	15 , 6
Mann-Whitney U	0.0

Column A	Untreated atpD
Vs	Vs
Column B	Treated atpD
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	15 , 6
Mann-Whitney U	0.0

Column C	Untreated IDH
Vs	Vs
Column D	Treated IDH
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*

Column E	Untreated spaP
Vs	Vs
Column F	Treated spaP
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column E,F	15 , 6
Mann-Whitney U	0.0

Column G	Untreated vicR
Vs	Vs
Column H	Treated vicR
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column G,H	15 , 6
Mann-Whitney U	0.0

Column A	Untreated atpD
Vs	Vs
Column B	Treated atpD
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	15 , 6
Mann-Whitney U	0.0

Column C	Untreated IDH
Vs	Vs
Column D	Treated IDH
Mann Whitney test	
P value	0.0383
Exact or approximate P value?	Gaussian Approximation
P value summary	*

Appendix IV: Ethical clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Human Research Ethics Committee (Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
Private Bag 3, Wits 2050
Office email: HREC-Medical.ResearchOffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-200529-03 29/05/2020

TO WHOM IT MAY CONCERN:

Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)
Investigator:	Miss Mukonazwothe Madiba
Supervisor:	Prof M Patel and Dr Zandiswa Dube
Department:	Clinical Microbiology and Infectious Diseases
Project title:	Anti- *Streptococcus mutans* property of *Uvaria chamae*, and its anticariogenicity
Reason:	*In vitro* laboratory study using stock cultures of bacteria. No human participants, human data or human tissues will be used.

Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Ms Zanele Ndlovu, Ms Mapula Ramaila and Mr Rhulani Mkansi

Appendix VI: Turn it in report

Ms

by Mukonazwothe Madiba

Submission date: 18-Nov-2021 12:43PM (UTC+0200)
Submission ID: 1706486963
File name: Madiba_Mukonazwothe_Dissertation_18_Oct_2021.docx (4.03M)
Word count: 31518
Character count: 176935

Ms

ORIGINALITY REPORT

13%	8%	7%	6%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of Witwatersrand Student Paper	4%
2	new.esp.org Internet Source	2%
3	hdl.handle.net Internet Source	1%
4	Zandiswa Gulube, Mrudula Patel. "Effect of Punica granatum on the virulence factors of cariogenic bacteria Streptococcus mutans", Microbial Pathogenesis, 2016 Publication	1%
5	www.frontiersin.org Internet Source	1%
6	wiredspace.wits.ac.za Internet Source	1%
7	J.A. Lemos, S.R. Palmer, L. Zeng, Z.T. Wen, J.K. Kajfasz, I.A. Freires, J. Abranches, L.J. Brady. " The Biology of ", American Society for Microbiology, 2019 Publication	1%

8	Zhong Zhang, Yiduo Liu, Miao Lu, Xiaoying Lyu, Tao Gong, Boyu Tang, Liu Wang, Jumei Zeng, Yuqing Li. "Rhodiola rosea extract inhibits the biofilm formation and the expression of virulence genes of cariogenic oral pathogen Streptococcus mutans", Archives of Oral Biology, 2020 Publication	1 %
9	www.karger.com Internet Source	1 %
10	ir.amu.ac.in Internet Source	1 %

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		