PHYTOCHEMICAL AND ANTIBACTERIAL ANALYSIS OF INDIGENOUS CHEWING STICKS, *DIOSPYROS LYCIOIDES* AND *EUCLEA DIVINORUM*

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MOOLA MARITHA NYAMBE

(200028170)

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Main Supervisor: Dr Renate Hans (Department of Chemistry and Biochemistry, University of Namibia)

Co-Supervisors: Dr Mervyn Beukes (Department of Biochemistry, University of Pretoria)

Prof. Jane Morris (African Centre for Gene Technologies (ACGT), and Department of Biochemistry, University of Pretoria)

Dr Martha Kandawa-Schulz (Department of Chemistry and Biochemistry, University of Namibia)

ABSTRACT

Diospyros lycioides and *Euclea divinorum* commonly known as muswiti and mutakula respectively in Zambezi region are indigenous to Namibia and belong to the family Ebenaceae. The twigs and roots from these plants are commonly used as chewing sticks in Namibia. If properly used, the chewing sticks have proven to be effective in removing dental plaque due to mechanical cleaning and enhanced salivation. Chewing sticks from other plants have been shown to display antimicrobial activities against a broad spectrum of microorganisms. However, there is limited information available in Namibia on the chemical composition, antimicrobial properties and the ability of the plants under study to prevent bacterial adhesion to tooth surface. Therefore, the purpose of this study was to ascertain the phytochemical and antibacterial properties of *D. lycioides* and *E. divinorum* and correlate the results obtained to their ethnomedicinal uses as chewing sticks. Powdered twigs and roots of the two plants were exhaustively extracted using dichloromethane/methanol mixture (1:1) at room temperature for 48 hours. Fractions were obtained from crude extracts using vacuum liquid chromatography with solvents of increasing polarity. Antimicrobial activities of the crude extracts and fractions were assessed using the agar overlay, disc diffusion and agar dilution methods against the oral pathogens, *Streptococcus mutans* and *Streptococcus sanguinis*. In addition, the effect of fractions on the attachment of oral pathogens to tooth surface were also analyzed using saliva-coated hydroxyapatite beads (S-HA) as a model. Phytochemical screen tests revealed the presence of the following secondary metabolites in the twigs and roots of both plants: anthraquinones, cardenolides, saponins, tannins, polyphenols and terpenoids.

Alkaloids were detected only in the roots of both plants. Root fractions from both plants displayed higher antibacterial activity than twig fractions. This supports the preference of roots over twigs by stick users. *D. lycioides* root fraction displayed minimum inhibitory concentrations (MICs) of 0.625 mg/mL and 1.25 mg/mL against *S. sanguinis* and *S. mutans*, respectively. *E. divinorum* root fraction showed MICs of 1.25 mg/mL and 2.5 mg/mL against the aforementioned organisms. Adherence of the bacteria to S-HA was reduced more by root fractions from *D. lycioides* while twig fractions from both plants also showed significant anti-adhesive properties. Since fractions from both plants inhibited the growth of bacteria and reduced attachment to S-HA, it is an indication that these plants are potential sources of antibacterial and anti-adhesive agents and their use should be encouraged. The anti-adhesive activity of extracts from these plants has not yet being reported. Further research on isolation and characterization of the active compounds from fractions that showed antibacterial and anti-adhesive activity is recommended.

Keywords: Chewing stick, oral hygiene, natural products, *Diospyros lycioides*, *Euclea divinorum*, antibacterial activity, adhesion assay.

TABLE OF CONTENTS

ABSTRACT……………………………………………………………………………ii

ABBREVIATIONS……………………………………………………………….....ix

LIST OF FIGURES……………………………………………………………….....xii

LIST OF TABLES……………………………………………………………….…....xvi

ACKNOWLEDGEMENTS…………………………………………………………xvii

DECLARATIONS……………………………………………………………….....xix

1. CHAPTER ONE: INTRODUCTION 1

1.1 Orientation of the study 1

1.2 Statement of the problem 3

1.3 Objectives 4

1.4 Research hypothesis 5

1.5 Significance of the study 6

2. CHAPTER TWO: LITERATURE REVIEW 8

2.1 Natural products 8

2.1.1 Phytochemistry 9

2.1.2 Medicinal plants used in oral health 12

2.2 Ebenaceae family 15

2.2.1 *Diospyros lycioides* 15

2.2.2 *Euclea divinorum* 22

2.3 Chromatographic methods 27

2.3.1 Thin layer chromatography (TLC).. 28

2.3.2 Vacuum Liquid Chromatography (VLC)... 29

2.3.3 Column chromatography (CC) 30

2.4 Biological testing... 30

2.4.1 Oral pathogens 30

2.4.2 Susceptibility tests 33

2.4.3 Bacterial adhesion 34

3. CHAPTER THREE: RESEARCH METHODOLOGY 37

3.1 Collection and pretreatment of plant material 37

3.2 Phytochemical screening 38

3.2.1 Alkaloids 39

3.2.2 Anthraquinones 39

3.2.3 Cardenolides 40

3.2.4 Flavonoids 40

3.2.5 Saponins 40

3.2.6 Tannins 41

3.2.7 Polyphenols 41

3.2.8 Terpenoids 41

3.3 Extraction and fractionation of extracts 42

3.3.1 *D. lycioides* roots 42

3.3.2 *E. divinorum* roots 45

3.3.3 *D. lycioides* twigs 45

3.3.4 *E. divinorum* twigs 47

3.3.5 Leaves 48

3.4 Bioassay 49

3.4.1 Microorganisms and growth conditions.. 49

3.4.2 Determination of antimicrobial activity against oral pathogens 49

3.4.3 Determination of minimum inhibitory concentration (MIC)...................... 52

3.4.4 Adhesion assay.. 53

4. CHAPTER FOUR: RESULTS AND DISCUSSION 58

4.1 Phytochemical screening 58

4.2 Antimicrobial activity against oral pathogens.. 60

4.2.1 Growth inhibition.. 60

4.2.2 Minimum inhibitory concentration (MIC)... 70

4.2.3 Inhibition of cell attachment to a glass surface.. 72

4.2.4 Inhibition of cell attachment in microtiter plates....................................... 74

4.2.5 Adhesion of cells to S-HA 85

5. CHAPTER FIVE: CONCLUSIONS..90

5.1 Recommendation and future work 91

6. CHAPTER SIX: REFERENCES 92

LIST OF ABBREVIATIONS

AR	Analytical reagent
AST	Antimicrobial susceptibility test
ATCC	American Type Culture Collection
$BaCl_2.H_2O$	Barium chloride dihydrate
BHI	Brain heart infusion
CC	Column chromatography
CFU	Colony forming unit
$CHCl_3$	Chloroform
CPM	Counts per minute
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
EA	Ethyl acetate
EPS	Extracellular polymeric substances

GC	Gas chromatography
GPS	Global positioning satellites
H_2O	Water
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hex	Hexane
HPLC	High performance liquid chromatography
IR	Infrared
KOH	Potassium hydroxide
LPS	lipopolysaccharides
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MS	Mass spectroscopy
Na_2CO_3	Sodium carbonate

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBRI	National Botanical Research Institute
NMR	Nuclear Magnetic Resonance
OD_{595}	Optical density at 595 nm
R_f	Retention factor
S-HA	Saliva coated hydroxyapatite beads
SFC	Supercritical fluid chromatography
Subsp	Subspecies
TLC	Thin layer chromatography
TSB	Trypticase Soy Broth
UPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
VLC	Vacuum Liquid Chromatography
WHO	World Health Organization

LIST OF FIGURES

Figure 1: Structures of phytochemical 11

Figure 2: *Diospyros lycioides* (A) leaves and fruits, (B) a young shrub 16

Figure 3: Distribution and abundance of *Diospyros lycioides* in Namibia 17

Figure 4: Chemical structures of naphthoquinones isolated from *Diospyros lycioides* ... 21

Figure 5: Chemical structures of triterpenes isolated from *Diospyros* genus. A) Taraxerol, and B) Taraxerone 22

Figure 6: *Euclea divinorum* shrub 23

Figure 7: Distribution and abundance of *Euclea divinorum* in Namibia 24

Figure 8: Chemical structure of triterpenes (3β-(5-hydroxyferuloyl)lup-20(30)-ene) isolated from the twig**s** of *Euclea* species 27

Figure 9: Biofilm formation 35

Figure 10 Map of Namibia showing sample collection site in the Zambezi region. Collection site is shown as a red dot 38

Figure 11: Schematic diagram of the extraction and fractionation of crude extract from the roots of *Diospyros lycioides* 44

Figure 12: Schematic diagram of the extraction and fractionation of crude extract from the twigs of *Diospyros lycioides* 46

Figure 13: A TLC showing the DCM/MeOH extracts (1 and 3) and MeOH extracts (2 and 4) of the twigs of *Diospyros lycioides* with the UV active spots circled with a pencil 47

Figure 14: Schematic diagram of the extraction and fractionation of crude extract from the twigs of *Euclea divinorum* 48

Figure 15: Distribution of test extracts at various concentration in a microtiter plate wells 55

Figure 16: Inhibition effect of DR2 on (a) *Streptococcus mutans*, (b) unspecified oral microorganism. (c) negative control DMSO and (d) positive control ampicillin............. 64

Figure 17: Antibacterial activity of extracts against *Streptococcus mutans*, *Streptococcus sanguinis* and two unidentified oral microorganisms 66

Figure 18: Schematic diagram of the extraction and bioassay of fractions from the roots of *Diospyros lycioides*.. 67

Figure 19: Schematic diagram of the extraction and bioassay of fractions from the twigs of *Diospyros lycioides*.. 68

Figure 20: Schematic diagram of the extraction and bioassay of fraction from the twigs of *Euclea divinorum* 69

Figure 21: The effect of crude extracts from *Diospyros lycioides* and *Euclea divinorum* roots and leaves on *Streptococcus mutans* and *Streptococcus sanguinis* cell adhesion to a glass surface 73

Figure 22: Representative destaining solution indicating the inhibition of *Streptococcus mutans* attachment to the polystyrene plate. 77

Figure 23: Percentage adhesion of A) *Streptococcus mutans* and B) *Streptococcus sanguinis* on polystyrene plates in the presence of crude extracts from *D. lycioides* and *E. divinorum* 78

Figure 24: Percentage adhesion of C) *Streptococcus mutans* and D) S*treptococcus sanguinis* on polystyrene plates in the presence of root fractions of *Diospyros lycioides* 79

Figure 25: Percentage adhesion of E) S*treptococcus mutans* and F) *Streptococcus sanguinis* on polystyrene plates in the presence of the twig fractions of *Diospyros lycioides* 80

Figure 26: Percentage adhesion of G) *Streptococcus mutans* and H) *Streptococcus sanguinis* on polystyrene plates in the presence of the twig fractions of *Euclea divinorum* 81

Figure 27: Actual OD 595 values indicating cell attachment of *Streptococcus mutans* to polystyrene plates in the presence of crude extracts at different concentrations. 82

Figure 28: Cell attachment of *Streptococcus mutans* to polystyrene plates in the presence of all fractions and crude extracts at different concentrations. 83

Figure 29: Cell attachment of *Streptococcus sanguinis* to polystyrene plates in the presence of all fractions and crude extracts at different concentrations. 84

Figure 30: Inhibition of bacterial cell adhesion to saliva-coated hydroxyapatite beads by crude extracts and fractions. 87

LIST OF TABLES

Table 1: Plants used traditionally in oral hygiene in Namibia 14

Table 2: Phytochemical constituents of *Diospyros lycioides* and *Euclea divinorum* 60

Table 3: Growth inhibition effect of *Diospyros lycioides* and *Euclea divinorum* crude extracts and fractions dissolved in DMSO at 20 mg/mL on *Streptococcus mutans*, *Streptococcus sanguinis* and two isolates from the teeth surface. 65

Table 4: Minimum inhibitory concentration (MIC) of crude extracts and fractions with inhibition activity against the tested microbes 71

Table 5: Percentage of *Streptococcus mutans* and *Streptococcus sanguinis* cells attached to three different surfaces... 89

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DECLARATION

I Moola Maritha Nyambe, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Moola M. Nyambe

1. CHAPTER ONE: INTRODUCTION

1.1 Orientation of the study

The phytochemical analysis and biological testing of traditionally used medicinal plants has become an important research area as it promotes the use of herbal medicine through confirmation of their efficacy and safety. It also determines their potential as sources of new drugs. Only a small fraction of plant species have been subjected to phytochemical analysis and of these only 5-15% have been investigated for biologically or pharmacologically active compounds (Hostettmann, *et al*., 1996; Balandrin, *et al*., 1993). Research on antimicrobial and other drugs derived from plants has gained much interest recently. This is because traditional antibiotics have become ineffective since many pathogens develop resistance against them. More research is therefore needed on the extraction and identification of bioactive compounds from plant materials to develop new drugs with hopefully new mechanism of action and better activity profiles.

For many decades now, plants have played an important role in oral hygiene. Most societies recognize that the cleaning of teeth is a desirable social habit and also a means of keeping diseases away. Plants have been used in oral health as chewing sticks, mouth wash, dental floss and to cure tooth ache and infections (van Wyk & Gericke, 2000). Many rural communities in Africa still depend mainly on locally available plants for oral

hygiene. Chewing sticks are sourced from the roots and/or twigs of some plants and have played an important role in preventing tartar build-up which is caused by plaque accumulation on the surface of teeth (Wu *et al*., 2001). This plaque is a type of biofilm which can cause tooth decay and gum disease. Chewing sticks effect the mechanical removal of plaque from the surface of the teeth and may also exhibit antibacterial activity (Almas & Al-Lafi, 1995).

Different methods of oral hygiene are used in different countries and cultures. In Namibia, the two preferred methods used are the toothbrush-toothpaste and the use of chewing sticks which are used by both adults and children. The two common plants from which chewing sticks are sourced in Namibia are *Diospyros lycioides* and *Euclea divinorum*. The choice between these methods is influenced by personal preferences, perceived effectiveness, availability and the medicinal properties (Aderinokun *et al*., 1999), whereas agreeable taste and anti-plaque activity are also considered by many. A study by Odongo *et al*., 2011 revealed that the choice of plants used as sources of chewing stick is mainly driven by the high fiber content of their stems and branches. Chewing stick users mention various reasons for choosing a stick over the toothbrush and toothpaste method. Among the reasons are: ease of access (proximity), availability, reliability, efficiency, no cost and age long practice (David *et al*. 2010, Odongo *et al*., 2011). Moreover, the use of chewing sticks has been recommended by the World Health Organization (WHO) as an effective tool for oral hygiene (Cai *et al*., 2000). The sticks

can be used fresh or may be dried and kept for later use. To prepare a stick, the plant part is cut into a suitable size, cleaned and the bark removed on the side to be used for cleaning. When the stick is chewed, the fibers at the end become loose, forming a rough “brush” which cleans the teeth surface. Continued chewing on the stick dislodges particles between the teeth and stimulates blood circulation in the gums. Chewing also increases saliva production; the later acts as a natural mouthwash that rinses away bacteria and creates an inhospitable environment for them to flourish (Hoque *et al.*, 2007).

1.2 Statement of the problem

Chewing stick users in the Sangwali area (Zambezi region, Namibia) where the plants under study were collected, generally believe that these sticks promote oral health and hygiene. They also mention other medicinal properties of the plants from which the sticks are sourced. Previous studies on chewing sticks primarily focused on the antimicrobial activity of extracts and/or the major metabolites. The existence of other mechanisms by which these extracts exert antimicrobial activity is relatively unexplored. To our knowledge, no study on the anti-adhesive activity of extracts from *Diospyros lycioides* and *Euclea divinorum* has been done. Chewing stick from the roots of *Diospyros lycioides* is preferred by users over those from the twigs. The phytochemical profile of the two plant parts is not known.

This study therefore focused on the preparation of extracts from the twigs, roots and leaves of *D. lycioides* and *E. divinorum* the most common plant species from which chewing sticks are sourced in Namibia. The study also evaluated the antimicrobial and anti-adhesive properties of the above mentioned extracts. Antibacterial studies were done using oral pathogens *Streptococcus mutans* and *Streptococcus sanguinis*, due to the role they play in carie formation (Li *et al*., 1998).

1.3 Objectives

The goal of this research was to contribute to the preservation of indigenous knowledge and to add value to the plants used as chewing sticks. The project aimed to determine the presence of biologically active compounds and identify the major active compounds in the selected plants. The specific objectives of the study were:

1. To determine the classes of phytochemicals present in crude extract from *D. lycioides* and *E. divinorum*
2. To perform antimicrobial testing of crude extracts and fractions from the roots and twigs of *D. lycioides* and *E. divinorum* and correlate these to the ethnomedicinal use of the plants as chewing sticks.
3. To test the crude extracts and fractions from *D. lycioides* and *E. divinorum* for inhibition of adhesion of microorganisms to dental enamel using saliva-coated hydroxyapatite beads as a model.

1.4 Research hypothesis

This study investigated the following hypothesis:

1. H_o: Crude extracts from *D. lycioides* and *E. divinorum* contains the same classes of phytochemicals

   H_a: Crude extracts from *D. lycioides* and *E. divinorum* do not contain the same classes of phytochemicals

2. H_o: Crude extracts and fractions from *D. lycioides* and *E. divinorum* do not exhibit antimicrobial activities against oral pathogens

   H_a: Crude extracts and fractions from *D. lycioides* and *E. divinorum* exhibit antimicrobial activities against oral pathogens

3. H_o: Crude extracts and fractions from *D. lycioides* and *E. divinorum* do not inhibit adhesion of microorganisms to dental enamel

   H_a: Crude extracts and fractions from *D. lycioides* and *E. divinorum* do inhibit adhesion of microorganisms to dental enamel

1.5 Significance of the study

For many years, plants have been used in traditional medicine and as teeth cleaning agents. Other plants used as chewing sticks have been shown to have the ability to act on plaque, bacterial infection and inflammation. This is an indication that plants in general contain beneficial compounds which need to be identified scientifically. Despite the extensive use of *D. lycioides* and *E. divinorum* in oral health in Namibia, details of phytochemical investigation of the leaves, twigs and roots are very scanty. According to a literature review, no study has been done to explore the anti-adhesive properties of the plants under study. The display of anti-adhesive properties of plant extracts can be ascribed to the presence of phytochemicals that disrupts the mechanism of plaque formation and thus prevent tooth decay. The scientific knowledge on the biological activity of extracts from the plants under study can be used to add value to the plants and promote the use of chewing sticks. Some clinical studies have shown that chewing sticks, when properly used, can be as effective as the toothbrush and toothpaste method in maintaining oral hygiene due to the combined mechanical cleaning and enhancement of salivation it offers. The phytochemical and antimicrobial analysis of extracts from these plants can serve as the baseline for identifying the active compounds responsible for this activity. These in turn can be used as substrates in the formation of new antimicrobial agents. Validation of these plants can also encourage the use of chewing sticks and this can benefit many societies because of their easy availability, low cost and simplicity. With added value, these plants can be marketed and thus be a source of income. This can contribute to improved oral health in communities. The selected plants are also used

medicinally and the biological activity, particularly antimicrobial activity exerted by extracts from various plant parts were investigated and compared to their traditional medicinal use as chewing sticks.

2. CHAPTER TWO: LITERATURE REVIEW

2.1 Natural products

Natural products have played an important role as a source of drugs for thousands of years and many useful drugs have been derived from phytochemicals (Hostettmann *et al*., 1996). Traditional healers, herbalists and individuals use a variety of wild roots, leaves and other plant parts to cure many illnesses. Plants have also been used for food, for example, functional foods, flavours, spices etc. as well as for materials like rubber, craft works and clothing.

The World Health Organization (WHO) has shown that 80% of the world's population, mostly from developing countries still rely on traditional medicine for the treatment of common ailments (Antherton, 1994; Shagal *et al*., 2012). According to Van Wyk (2011), 13.8% of the Southern African flora is known to be used in traditional medicine and 14 species are partially or fully commercialized. Developed countries also use traditional medicine especially in the form of herbs. Many people use plant extracts and other alternative forms of medical treatments rather than conventional therapy (Cowan, 1999). This is an indication that plants are still an important and reliable source of drugs. This reliance on plants as medicines calls for scientific validation of their safety and effectiveness. The development of new bioassays also adds to the potential of discovering new compounds from natural product sources.

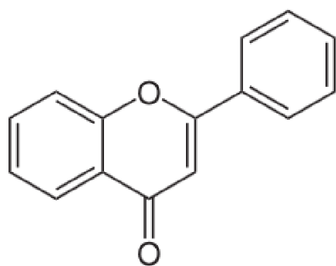
Plants have also been used to produce products such as toothpicks, concoctions, decoction, pastes or powders which have also been used to keep the oral cavity clean and to cure oral and other diseases. Chewing sticks have more advantages because unlike tooth picks that are disposed of after one use, the former can be used several times before being disposed. Although brushing with fluoridated toothpaste, inter-dental cleaning using dental floss and dental attendances can reduce caries formation, affordability poses a challenge to many low income populations. Another advantage is that the practice of preparing chewing sticks from twigs is considered bio-conservation friendly because it is unlikely to threaten survival of the plants used. However, many people consider the use of the roots from *D. lycioides* to be more effective which could threaten the species' survival (Odongo *et al*., 2011).

2.1.1 Phytochemistry

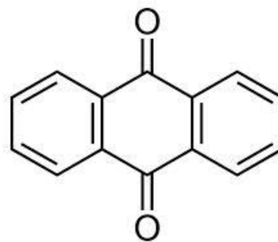
Secondary metabolites play an important role in the treatment of diseases and in the ecological interaction of plants with other organisms. Each plant species, family or genus produces a characteristic mixture of these secondary metabolites (Dewick, 2001). Thousands of phytochemicals have been discovered in several major classes but the health properties of only a few have been investigated (Mata, Rivero-Cruz & Chavez, 2001). Many of the known phytochemicals that belong to several chemical classes have inhibitory effect on a wide range of microorganisms (Talukdar, *et al*., 2010; Khan *et al*. 2011). They have been used as medicines, flavouring and colouring agents. Some of the

well-known phytochemicals are: alkaloids, flavonoids, saponins, tannins polyphenols and terpenoids (Figure 1). Alkaloids are known to possess many pharmacological properties like analgesic (morphine), stimulants (caffeine), antitumor (vinblastine) antimalaria (quinine), antibacterial (berberine) and amoebicide (emetine) (Dewick, 2001). Flavonoids are polyphenolic compounds with strong antioxidant activity. Biological activities ascribed in this class include: antifungal, antiviral, antibacterial, anti-inflammatory and anticarcinogenic activity (Okwu, 2005). Anthraquinones have laxative effect, anti-inflammatory, anti-cancer and antimalarial properties (Sakulpanich and Gritsanapan, 2009; Choi *et al*. 2013). Saponins are known to be immune boosters. They are also known to demonstrate anti-inflammatory, anti-haemolytic, cholesterol lowering and anticancer properties (Sauvaire *et al.,* 1996; Sowmya *et al.,* 1999; Mandeaau *et al.,* 2005). Tannins display good antimicrobial and antiviral activity (Bruneton, 1995; Cowan, 1999) and play a role in inhibiting the growth of bacteria by reacting with protein on the cell wall (Dangoggo *et al*. 2012). Terpenes are an abundant class of natural products that are responsible for many fragrances. They are made up of C5 isoprene units and possess strong antimicrobial properties (Das *et al*. 2010; Talukdar *et al*. 2010)

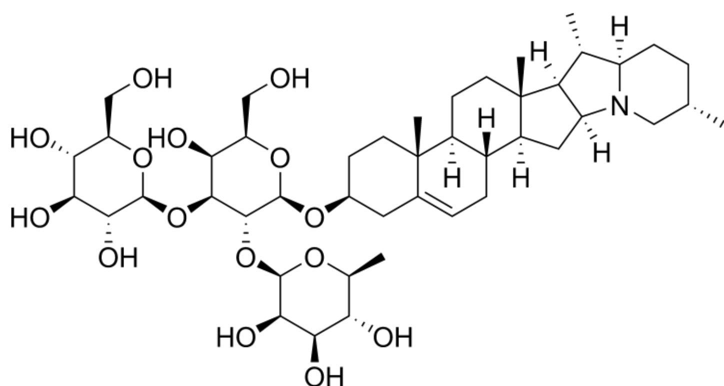
In the Ebanaceae family, naphthoquinones are found in several organs. These compound is active against fungi, bacteria, mollusks, insects, worms and termites. Derivatives and oxidative decomposition products from naphthoquinones are responsible for the dark colour in the bark and other parts. Terpenoids, benzopyrones, polyphenols and tannins are also common in this family (Wallnöfer, 2001). The fruits are rich in vitamins and tannins.



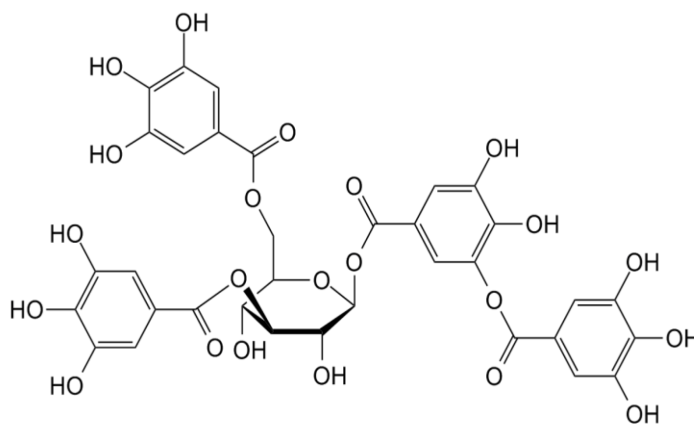
Flavonoid (flavone backbone)



Anthraquinone



Saponin (solanine)



Tannin (tanic acid)

Figure 1: Chemical structures of selected phytochemical classes

2.1.2 Medicinal plants used in oral health

A study by Chinsembu *et al*. (2011) and Chinsembu and Hedimbi (2010) revealed that 12% of plants used medicinally in Kavango region and 19% in Zambezi region are members of the Ebanaceae family. The mode of administration varies from using it as an oral wash, chewing it, applying it as a paste or by rubbing it on the affected area. It can also be by preparing a decoction and an infusion which is drunk. The study by Chinsembu and Hedimbi (2010) also reported on plant species used to treat oral candidiasis in the Zambezi region. These include: *Sclerocarya birrea, Lannea stuhlmannii, Capparia tomentosa, Antidesma venosum, Abrus precatorius, Vangueria infausta* and *Ximenia american.* The last is also used to treat toothache (Chinsembu and Hedimbi, 2010). Table 1 list plants used in oral health in Namibia. Leaves, seeds, bark and gum from most of the plants used as chewing sticks are also used to cure oral diseases and other ailments. In East Africa nearly 300 different species of trees and shrubs are used to make chewing sticks (Masalu *et al*., 2009). Many studies on various chewing sticks have reported that chewing sticks have medicinal properties associated with gum healing, analgesia and antimicrobial and plaque inhibiting effect (Rotimi & Mosadomi, 1987; Odongo *et al.*, 2011, Muhammad & Lawal, 2010, Adeniyi *et al*., 2010, Cai *et al*., 2000, Wu *et al.,* 2001).

Claims supported by scientific studies are that, using chewing sticks affords an extract which contains compounds that have an effect on oral pathogens and other ailments and thus prevent tooth decay and gum diseases (Akande and Hayashi, 1998). Apart from

mechanical effects, chewing sticks from various plant species have been shown to have significant antimicrobial activity against a broad spectrum of microorganisms (More *et al*, 2008; Adeniyi *et al*. 2010; Wu *et al*, 2001 and Cai *et al*, 2000). Many of these bioactive compounds extracted from plants have also been incorporated in the preparation of toothpaste. For example, essential oils of *Eucalyptus*, tea tree, clove, cinnamon and mint (Muhammad & Lawal, 2010). This is mainly because these plants contain useful compounds which exhibit antibacterial or anti-inflammatory activity (David *et al.,* 2010).

Cai *et al*. (2000) isolated naphthalene glycosides, diospyrosides, naphthoquinones, juglone and 7-methyljuglone from the methanol extracts of the twigs of *D. lycioides* that inhibited growth of oral microoganisms such as *Streptococcus mutans, Streptococcus. sanguinis, Porphyromonas gingivalis* and *Prevotella intermedia*. These active compounds showed inhibition at a minimum inhibitory concentration ranging from 0.019 to 1.25 mg/mL.

Table 1: Plants used traditionally in oral hygiene in Namibia

Family	Scientific name	Common name	Use
Boraginaceae	*Cordia sinensis*	Omusepa (Herero)	Young branches are used as chewing sticks
Burseraceae	*Commiphora multijuga*	Omuzumba (herero)	Twigs are used as a tooth brush
Ebenaceae	***Diospyros lycioides***	Bluebush, omuzeme (Herero), oshimumu (Kwanyama), sihorowa (Rukwangali)	Roots and twigs are used as chewing sticks
Ebenaceae	***Euclea divinorum***	Magic guarri (English), Towerghwarrie (Afrikaans), mutakula (Lozi), mpumutwi (Rukwangali)	The twigs and root bark are chewed as a tooth and mouth cleanser.
Fabaceae	*Colophospermum mopane*	Mupani (Lozi), omutati (Herero), omusati (Ndonga), //gais, tsaurahais (Nama/damara)	Twigs are used for chewing sticks. Leaves are used to treat lower jaws when teeth are removed in children
Fabaceae	*Indigofera cryptantha*	Orukohatjinyo (Herero)	Twigs are used as toothbrushes. The sap is used as a mouthwash
Fabaceae	*Mundulea sericea*	Cork bush, omukeka(Herero)	Twigs are used as toothbrushes
Melianthaceae	*Berchemia discolor*	Brown ivory, motsentsila (Tswana)	The fruit juice treats bleeding gums
Oleaceae	*Jasminum fluminense*	Okarondo (Herero)	Branches are used as a chewing stick

(Curtis & Mannheimer, 2005; Von Koenen, 1996; Van Wyk & Gericke, 2000, http://biodiversity.org.na)

2.2 Ebenaceae family

The Ebenaceae family includes the genera *Diospyros* and *Euclea* and consists of about 500 - 600 species (Wallnöfer, 2001). The genus *Euclea* is the smaller one of the two and is only found in Africa and south Arabia. The family is distributed mainly in tropical Africa, South East Asia, Madagascar and South America (Wallnöfer, 2001). Members of this family are trees and shrubs most of which are evergreen plants with few deciduous species. Various species in the family are valued for their fruits and timber. Some are cultivated on a large and small scale for fruits. Different regions use various species of *Diospyros* and *Euclea* as food.

2.2.1 *Diospyros lycioides*

The genus *Diospyros* consists of about 400 species, 7 of which are found in Namibia (Utsunomiya *et al*., 1998; Namibia Biodiversity Database). This genus has been classified as the most important genus of the Ebanaceae family due to its economic and medicinal uses (Amar dev and Rajarajeshwari, 2013). There are two subspecies of *D. lycioides* in Namibia, subsp. *sericea* and subsp *lycioides*; with the latter being the most common and widespread of the two (Curtis & Mannheimer, 2005, p.520). The two are very similar, but differ in the smoothness of the leaves. Subspecies *sericea* have leaves that are densely hairy whereas subspecies *lycioides* have smooth leaves.

The plant is commonly known as bluebush, monkey plum (English); bloubos, jakkalsbessie, karoobloubos (Afrikaans); Schakalbeere, Zanhnburstnstrauch (German); omuzeme, omundumbiri, omuryambandje (Herero); oshimumu, oshilugulu (Oshiwambo); sihorowa (Rukwangali); muswiti (Silozi); karema (Siyeyi), muvitji (Mbukushu). It is a semi-deciduous shrub or small tree which grow up to 6 m high. (Curtis & Mannheimer, 2005, p.520; von Koenen, 1996 p.112). The leaves are small, finely silky and clustered towards the twig ends. It bears red round berries (Figure 2).

Figure 2: *Diospyros lycioides* (A) leaves and fruits, (B) a young shrub

2.2.1.1 Geographic distribution

D. lycioides is distributed throughout Namibia, except the Namib Desert (Figure 3). It grows mostly along river banks of dry rivers and on flood plains.

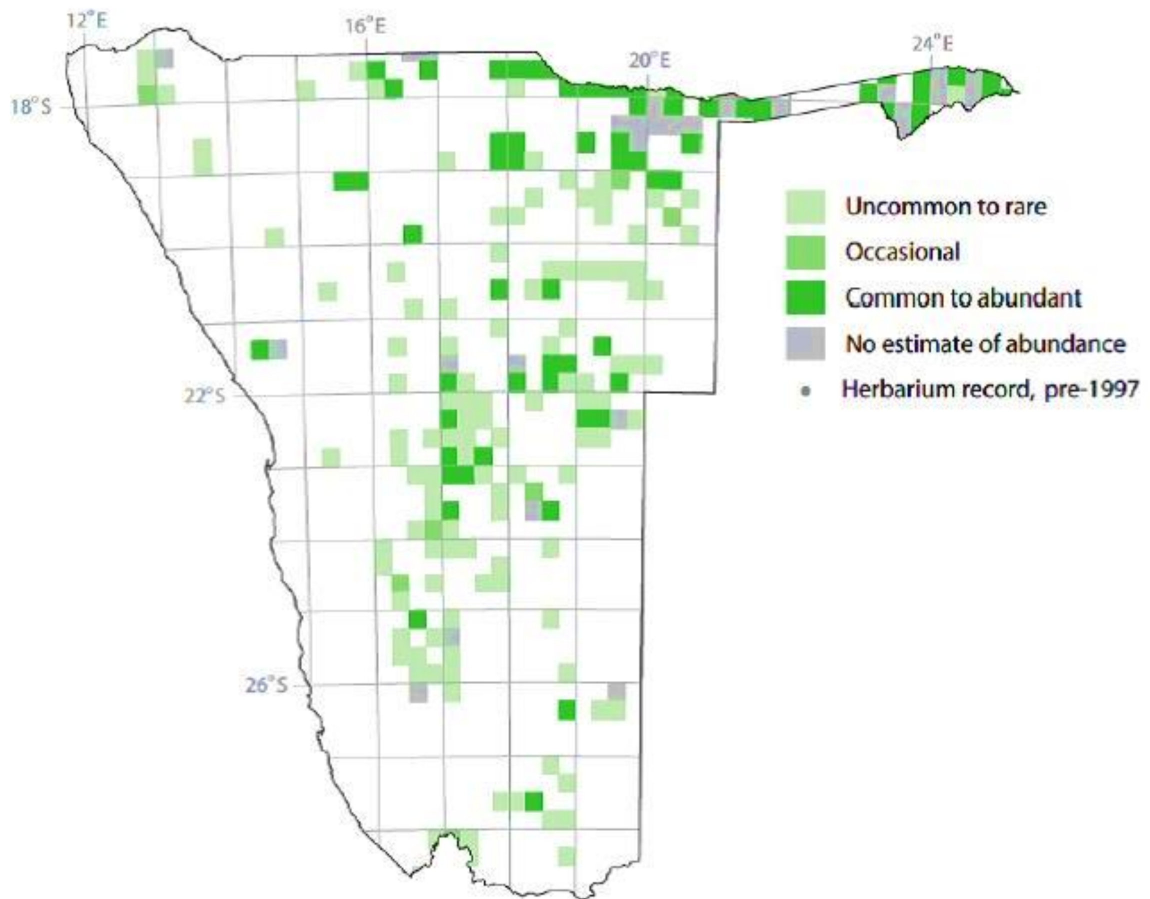


Figure 3: Distribution and abundance of *Diospyros lycioides* in Namibia (Curtis & Mannheimer, 2005)

2.2.1.2 Ethnobotanical uses of *D. lycioides*

D. lycioides is widely known and used in Namibia as a chewing stick made from the roots or twigs. Sticks made from the roots are considered more effective by users; however, the twigs are easier to collect. The effectiveness of this plant as a teeth cleansing agent can be observed from the users. An oral health survey conducted in Namibia involving 2394 subjects in the age group 12 to 44 years, showed that approximately 20% of the participants examined in this study used chewing sticks from *D. lycioides* as a tooth cleaning agent. The study further revealed that those who used the sticks had fewer cavities compared to non-users (Li *et al*, 1998).

Although most valued for its use as a chewing stick, *D. lycioides* has other uses. Roasted ground seeds were once used as a coffee substitute (Joffe, 2001). A yellowish brown dye is obtained from the roots and the bark is used for tanning skins. The fruit is eaten by animals and humans, but has a laxative effect when consumed in large quantities. When chewed, the roots leave a yellowish-brown dye in the mouth and this could be attributed to naphthoquinones such as diospyrin and 7-methyljuglone in the roots. This is believed to function as a mouth wash and keep the mouth clean. Von Koenen (1996) reported that when used for a long time as chewing stick, the root can remove both plaque and tartar. Other uses of *D. lycioides* in Namibia includes its use to cure toothache by keeping the root decoction in the mouth for ten minutes or by chewing the root intensely thereby bringing the sap into contact with the diseased tooth. In the Okavango region of Namibia, the roots are cleaned, chopped into small pieces and mixed with water. A water extract of

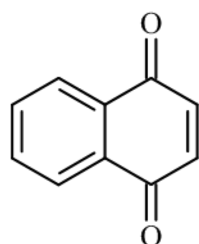
the cleaned and chopped roots is an effective eye medication, achieving a cure after two to three days of application. In Kavango, the root peel is used to cause abortions in the first two months of pregnancy. It is pulverized, mixed with water and made into a large ball, which is inserted into the vagina thus inducing labour. This can cause death from hemorrhages (Von Koenen, 1996, p. 112). According to van Wyk & Gericke (2000) the plant has an effect on digestion by either causing nausea or stopping diarrhea depending on the dosage taken. The fruits are sometimes used to distill alcohol. Menstrual problems can be treated by taking the root decoction, to which stem and leaves may be added. Cheikhyoussef *et al*. (2011) reported that the plant is used to treat bleeding, worms and high fever by people in the Oshikoto region of Namibia.

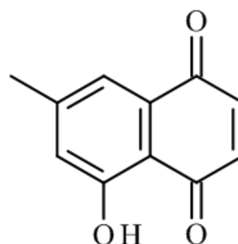
2.2.1.3 Chemistry of *Diospyros* species

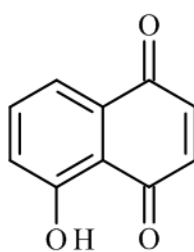
Diospyros species are a rich source of biologically active compounds and almost all parts of plants in this genus have been used as traditional medicine (Akak *et al.* 2010). Plants in this genus are well documented, and are reported to contain naphthoquinones, including 7-methyljuglone, diospyrin, isodisopyrin (Figure 4) (Li *et al*, 1998) and triterpenes of the lupine series. The latter have been found to exhibit ichthyotoxic, antimicrobial and antitumor activities (Chen *et al*, 2007; Zhong *et al*., 1984). Naphthoquinones produced by this genus are usually in the form of dimers (Zhong *et al*., 1984). Other biologically active compounds that have been reported from *Diospyros* species are coumarin, flavonoids and other phenolic compounds (Akak *et al*. 2010). Cai

et al. (2001) analysed extracts of the twigs from *D. lycioides*, and isolated six chemical compounds (juglones, 7-methyljuglone and diospyroside A, B, C, and D) that demonstrated antimicrobial activity. They showed that these compounds can kill the oral pathogens responsible for periodontal disease and slow formation of plaque. Figure 4 shows chemical structures of some of the isolated compounds. Plant species from this genus are used as medicinal agents for various ailments which includes anthelmintic, anti-inflammatory, antibacterial, antifungal, antioxidant, anticancer, antiviral, molluscicidal and piscicidal activities (Amar dev & Rajarajeshwari, 2013). The fruit from various *Diospyros* species has been used as an anti-inflammatory and antipyretic drug in many local traditional medicines, for example, Chinese herbal medicine, Tibetan medicine, and Ayurvedic medicine (Maridass, *et al.,* 2008).

Zakaria *et al*. (1984) reported three triterpenes, lupeol, betulm and betulmic acid that were isolated from all plant parts of the nine *Diospyros* species investigated. The olean-14-ene derivatives taraxerol and taraxerone are common in this genus (Figure 5). Species from Asia appear to have high diversity of triterpenes compared to species indigenous to Africa (Zakaria *et al*., 1984).







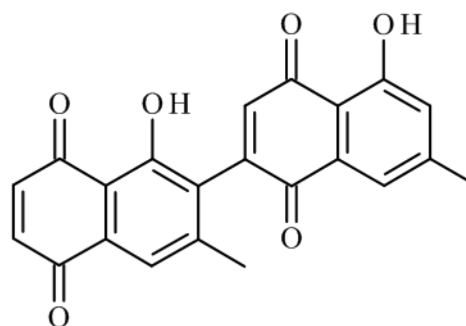


Figure 4: Chemical structures of naphthoquinones isolated from *Diospyros lycioides*

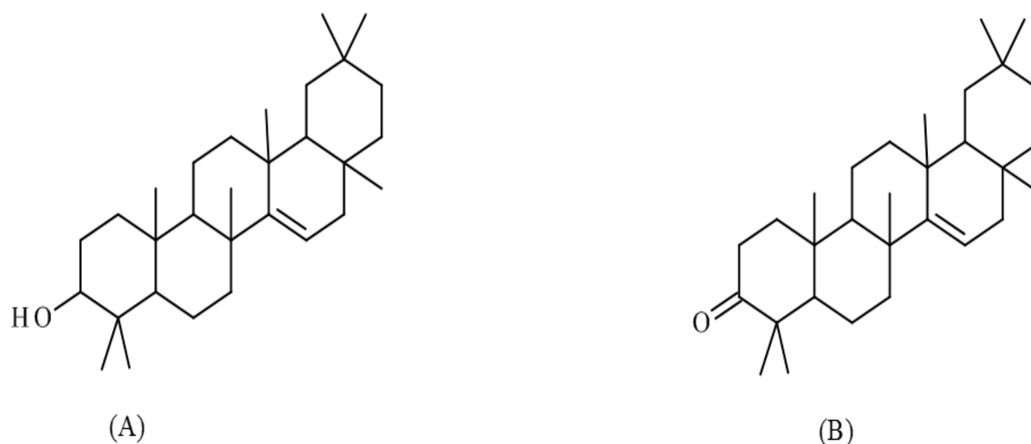


Figure 5: Chemical structures of triterpenes isolated from *Diospyros* genus. A) Taraxerol, and B) Taraxerone

2.2.2 *Euclea divinorum*

Euclea divinorum is another member of the Ebenaceae family. It is an evergreen shrub or small tree which grows up to 3 m high, often branching from the base or sometimes having a single stem (Figure 6). It has rough bark that cracks and scales off with age and dull green stiff leaves. The fruits are purple roundish berries. In Namibia, the plant is commonly known as magic guarri, wild ebony (English); towerghwarrie, gwarriesbos (Afrikaans); Wildes Ebonholz (German); omuryambambi, omuzema (Herero); omudime (Oshiwambo); mushitondo (Mbukushu), mutakula (Silozi), mpumutwi (Rukwakangali) (Curtis & Mannheimer, 2005; Von Koenen 1996).

Figure 6: *Euclea divinorum* shrub

2.2.2.1 Geographic distribution

E. divinorum is indigenous to southern Africa. In Namibia, it is found in the northern and northeastern part (Figure 7). It usually grows on termite mounds and along river banks

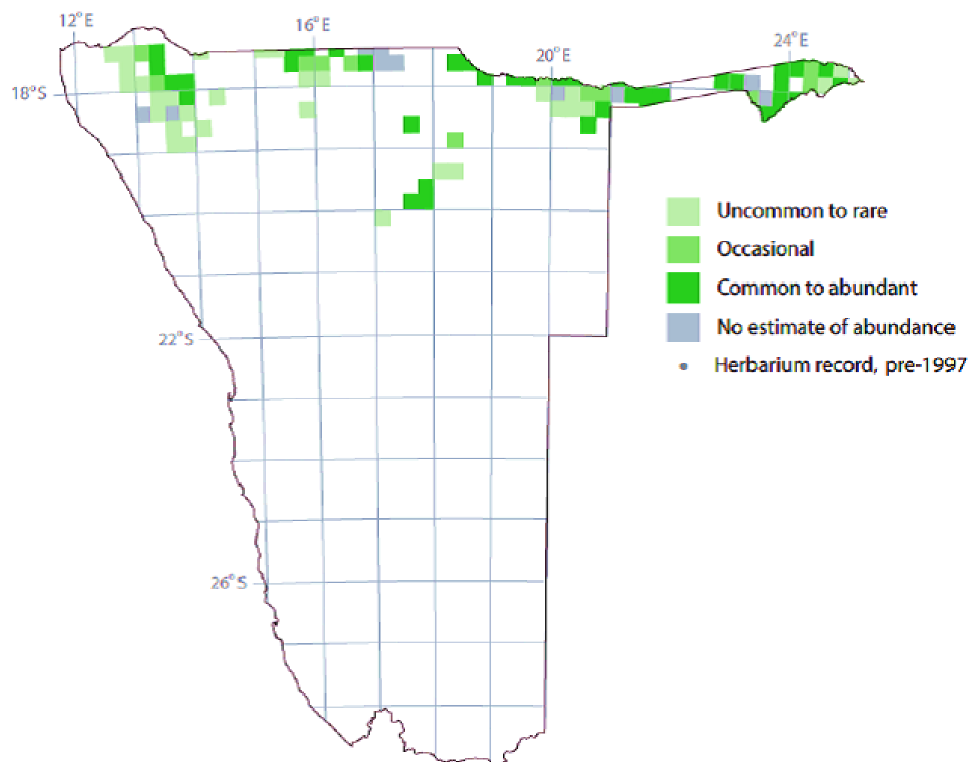


Figure 7: Distribution and abundance of *Euclea divinorum* in Namibia (Curtis & Mannheimer, 2005)

2.2.2.2 Ethnobotanical uses of *E. divinorum*

The roots and twigs are used as toothbrushes. This plant has been used successfully in the leather production industry and offers a sustainable source of natural tannin (van Grinsven *et al*., 1999). The root is a source of traditional dye for commercial craftwork production and gives a dark brown to black colour depending on how long the roots are boiled (Cunningham, 1994, van Wyk & Gericke 2000). The fruits give a red sap which is used for dyeing clothes, baskets and mats. It is known for its economic and cultural values by people in the north and northeastern regions in Namibia as it is used in traditional religious rites. The name *Euclea divinorum* and its common name magic guarri is derived from its use in divination (van Wyk & Gericke 2000). The Ovambo people use it to treat nose bleed where they administer it for a period of 1-4 days (Cheikhyoussef *et al*., 2011; Von Koenen, 1996). The location where the tree grows serves as a burial place for babies since it is believed that babies must be buried under this plant species or the plant must be present at the burial site (Hainduwa, 2013). In the Zambezi region they use the leaves and roots to perform ritual cleansings of homes, for good luck and in field cultivation for protection against evil spirits. (Manga Sangwali, pers comm.). Wood from this plant is considered unsuitable for firewood.

The root bark is used in treating diarrhoea, convulsion, cancer, skin disease and gonorrhoea (Mebe *et al*. 1998; Luleka *et al*. 2008). A root infusion is dropped in the ear to treat headache and an ointment is rubbed on the body to treat convulsions. The bark is

chewed to serve as a mouth wash. In the north-east of Namibia, especially the Kavango region, an infusion is made from the roots and drunk to treat ailments such as malaria, fevers and venereal diseases. The roots are also chewed and rubbed onto sores and wounds as well as to treat toothache and fungal diseases (Chinsembu *et al*., 2011). The Ovahimba people apply squelched leaves to a rash to treat a condition called "ombargwa", pustules (Von Koenen, 1996). A root decoction is cooled and drunk for bile problems by people in Zambezi.

2.2.2.3 Chemistry of *E. divinorum*

A number of studies have reported the presence of naphthoquinones, triterpenes and flavonoids in the *Euclea* species. Mebe *et al*. (1998) reported the isolation of lupeole, lupine, botulin, 7-methyljuglone, isodiospyrin, shinalene, catechin and 3β-(5-hydroxyferul) lup-20(30)-ene from the root bark of *E. divinorum* (Figure 8).

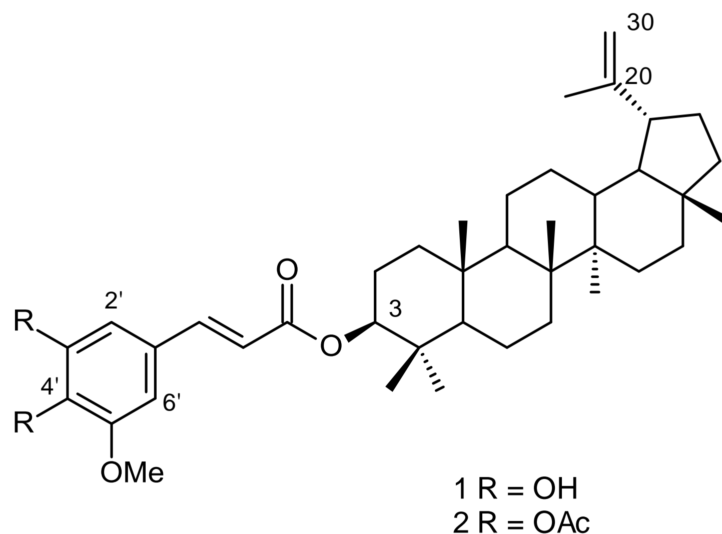


Figure 8: Chemical structure of triterpenes (3β-(5-hydroxyferuloyl)lup-20(30)-ene) isolated from the twig**s** of *Euclea* species

2.3 Chromatographic methods

Chromatography is one of different methods that can be used to separate and purify the chemical components in a mixture, for example plant extracts. The separation is based on the difference in the migration rates of the components between the stationary phase and the mobile phase. Distribution may take place on the basis of component structure, size, shape, charge or molecular weight. The stationary phase can be a solid like silica gel, or a liquid supported on a solid or a gel (Braithwaite & Smith, 1985; Parriott, 1993). It may be

packed in a column, spread as a layer, or distributed as a film. The mobile phase is either a liquid or gas. Various types of chromatographic techniques are used, these include: high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and ultra high performance liquid chromatography (UPLC). HPLC is a highly improved form of column chromatography. It is common in natural product for separation and purification of compounds and can be applied as both analytical and preparative technique. SFC provides fast and simple procedures for the determination of lipophilic-amphiphilic natural compounds, which often created problems in their isolation and analytical determination. The classification of the methods depends on the type of stationary phase, medium of the mobile phase and mechanism of separation.

2.3.1 Thin layer chromatography (TLC)

Thin layer chromatography is widely used to detect the presence of organic compounds in an extract and to separate them. It can be used as a preparative separation method to separate small quantities of partially purified extracts. The technique is widely applied in natural product extract analysis, stability tests of extracts and the final product. TLC is preferred over other chromatographic techniques because the technique is simple, quick, inexpensive and requires very little sample and equipment. It is a useful technique to determine the proper solvent system for performing separations using column chromatography. It utilizes a solid stationary phase usually highly polar medium like silica gel and alumina (standard) or non-polar medium like C18 bonded to silica (reverse

phase) and a liquid mobile phase whose polarity is carefully selected based on the compound of interest. The identification of the separated compound can be achieved on the basis of the retention factor (R_f) and colour of spots. Visualization of separated compounds can be done with the naked eye and for colourless spot, by using a UV lamp, exposing it to iodine vapour and by using spray reagents such as vanillin and anisaldehyde.

2.3.2 Vacuum Liquid Chromatography (VLC)

Vacuum liquid chromatography is a separation technique that works similarly to preparative TLC but is run as a column and the flow is sped up by a vacuum pump connected to it. It is used for fractionation of crude extracts prior to other separation techniques and other analysis. This technique has increasingly been used because it is simple, quick and economical since it uses less amount of chromatographic support. Another advantage is that it can use a variety of chromatographic support such as silica gel, aluminium oxide, cyanide, diol and polyamides (Sticher, 2008). Subjecting crude plant extracts to VLC afford a collection of partially purified fractions which can be tested for biological activities. These fractions contains simpler mixture of compounds that are more concentrated than they are in a crude extract. Further purification of the fractions can be guided by biological screening (Quinn, 2012).

2.3.3 Column chromatography (CC)

Column chromatography separates or isolate pure chemical compounds from a mixture of compounds. The stationary phase like silica gel and Sephadex is packed into a vertical glass column. When using Sephadex as a stationary phase, separation is obtained on the basis of molecular size and the substances are eluted in order of decreasing molecular size. The eluent is selected so that it allows separation of compounds effectively. The mobile phase is then collected in a series of receiving vials.

2.4 Biological testing

2.4.1 Oral pathogens

Oral health is fundamental to the general wellbeing and relates to the quality of life that affects the functions of the oral cavity, dental and soft tissues of the face (Palombo, 2009). Millions of people around the world are affected by diseases and conditions of the oral cavity. These effects include pain, bad breath, difficulty in speaking, chewing and swallowing and in some cases death can result (Wu, 2009). Pathogens causing oral diseases are evolving at a faster rate and most have developed resistance to drugs used clinically. Because of this, oral diseases continue to be a major health problem worldwide. Dental caries and periodontal diseases are among the most important global oral health problems. Although gum disease is the most common cause of tooth loss in

adults, the early signs and symptoms which include bleeding gums and loose teeth are often seen by many as part of the aging process (Griffith & Boyle, 1993).

Naturally, humans are a host of a variety of microorganisms. Over 750 species of bacteria are found in the oral cavity alone and many of these play a role in causing oral diseases. While some of these microorganisms are harmful, others help in preventing diseases by fighting diseases causing germs in the mouth. There is a link between oral diseases and the activities of microbial species that form part of the microbiota of the oral cavity. Among other microoragnisms involved in the development of dental caries are *Streptococcus mutans*, *S. sanguinis*, *S. sobrinus* and *Lactobacilli* (Palombo, 2009). Periodontal diseases have mainly been associated with *Actinomyces*, *Actinobacillus*, *Streptococcus* and C*andida* species (More *et al*., 2008). The *S. mutans* strains make up at least 90% of what affects the oral cavity negatively (Wu, 2009) and it is also known to cause pneumonia, sinusitis, *otis media* and meningitis. S. *mutans* and *S. sanguinis* are members of the human indigenous biota belonging to the phylum Firmicutes and genus *Streptococcus* of spherical Gram-positive bacteria. They form part of the bacterial community in plaque which is the main cause of dental disease. On average, *Streptococci* bacteria account for less than 10% of bacteria species in the plaque, indicating that other bacterial species play a role in caries formation (Ge *et al*. 2008). *Streptococcus* and other bacterial species metabolize sugar in the mouth to produce organic acids, mainly lactic acid that dissolves the calcium phosphate in teeth which leads to tooth decay (More *et al*.

2008; Palombo, 2009). This process is affected by the amount of food particles trapped on the surface of the teeth that can be used by the bacteria as a source of fermentable carbohydrates. The *mutans Streptococci* group also have the ability to synthesize adherent glucan from dietary sucrose and this facilitates plaque formation on teeth surfaces (Wu, 2009).

Streptococcus sanguinis reportedly plays an antagonistic role in dental caries and periodontal diseases. It has been shown that, in the presence of *S. sanguinis*, other oral bacteria such as *Tannerella forsythia* and *Prevotella intermedia* are reduced (Stingu *et al*., 2008). *S. sanguinis* and *S. gordonii* can also keep the number of *S. mutans* in the mouth low but the latter can become dominant in the oral biofilms leading to dental caries development (Kreth *et al*. 2008). The cariogenic potential of *S. sanguinis* is considered low compared to that of *S. mutans*. Both species require the presence of teeth (the right environment) for colonization. Oral colonization with *S. sanguinis* comes first before that with *S. mutans*, however, it has been reported that after *S. mutans* colonize the oral cavity, the levels of *S. sanguinis* decreases (Coufield *et al.,* 2000). Poor oral health has been linked to chronic conditions, for example, infection by *S. sanguinis* in the blood stream has been associated with heart attack and stroke (Bahekar *et al*., 2007; Wu, 2009). Also reported on is the strong association between severe periodontal disease and diabetes (Palombo, 2009).

2.4.2 Susceptibility tests

A number of assays have been developed over the years to measure the antimicrobial activity of plant products or plant extracts. The antimicrobial susceptibility test (AST) is widely used to test for resistance of certain microorganisms to different antimicrobial agents. There are different AST methods, these include: agar disk diffusion assay, agar well diffusion assay, agar dilution, broth microdilution, broth macrodilution technique, poison food technique and bioautograph (Tenover *et al.*, 1995). The broth dilution method is preferred by many over the agar dilution because some compounds do not diffuse into the agar (Tenover *et al.*, 1995). Disk diffusion method seems to be the preferential method to test the antimicrobial activity of plant extracts. It is simple, inexpensive and allows for a large number of extracts to be tested. However, no standard interpretive criterion of disk diffusion results is currently in place to support natural antimicrobials susceptibility testing. Also, this method only give qualitative data as the minimum inhibitory concentration (MIC) values cannot be determined (Dickert *et al*, 1981). On the other hand, the agar dilution and microdilution methods allows the determination of MIC values for antimicrobial agents. Agar dilution offers the advantage that a number of different microorganisms can be tested simultaneously in one plate. Compared to the agar based methods, broth microdilution requires less time and labour. Its disadvantage, however, is that anaerobic microorganisms grow poorly with this method. For this study the disk diffusion and agar dilution methods were used.

2.4.3 Bacterial adhesion

Another factor that plays a role in the formation of dental caries and development of periodontal disease is the existence of microorganisms in a biofilm structure. Oral biofilms are commonly known as plaque. Microorganisms colonise and build up on the tooth surface and initiate plaque formation. If not controlled, this kind of biofilm can cause diseases in the oral cavity (Rahim & Khan, 2006).

In nature, most microorganisms are believed to exist in biofilms that develop on biotic or abiotic surfaces (Costerton *et al*., 1987). These biofilms may be composed of single or multiple microbial species depending on the intra- and/or intercellular interactions and communication (Davey & O'Toole, 2000). Biofilm formation begins with the attachment of one or more bacterial cells, to a substratum, and is followed by irreversible binding and secretion of extracellular polymeric substances (EPS) which in turn serve as a substratum for adhesion of additional bacterial species. The biofilm matures, increases in biomass and changes in architecture forming mushroom-like structures before it finally disperses (Ofek *et al*., 2003; Thormann *et al*., 2004) (Figure 9, www.uweb.engr.washington.edu/research/tutorials/biofilm.htmL).

Biofilm growth is stimulated by factors such as unfavourable environmental conditions like high salt concentration, temperature or toxic substances. The ability of bacteria to form biofilms is attributed to their ability to produce different adhesins, usually proteins (pili, fimbriae) and lipopolysaccharides (LPS). These adhesins mediate interbacterial associations, thus promoting coaggregation of bacterial species (Mulvey & Hurtgren,

2000). Biofilms commonly grow on surfaces that are often concentrated with organic and inorganic nutrients (Masak *et al*., 2003).

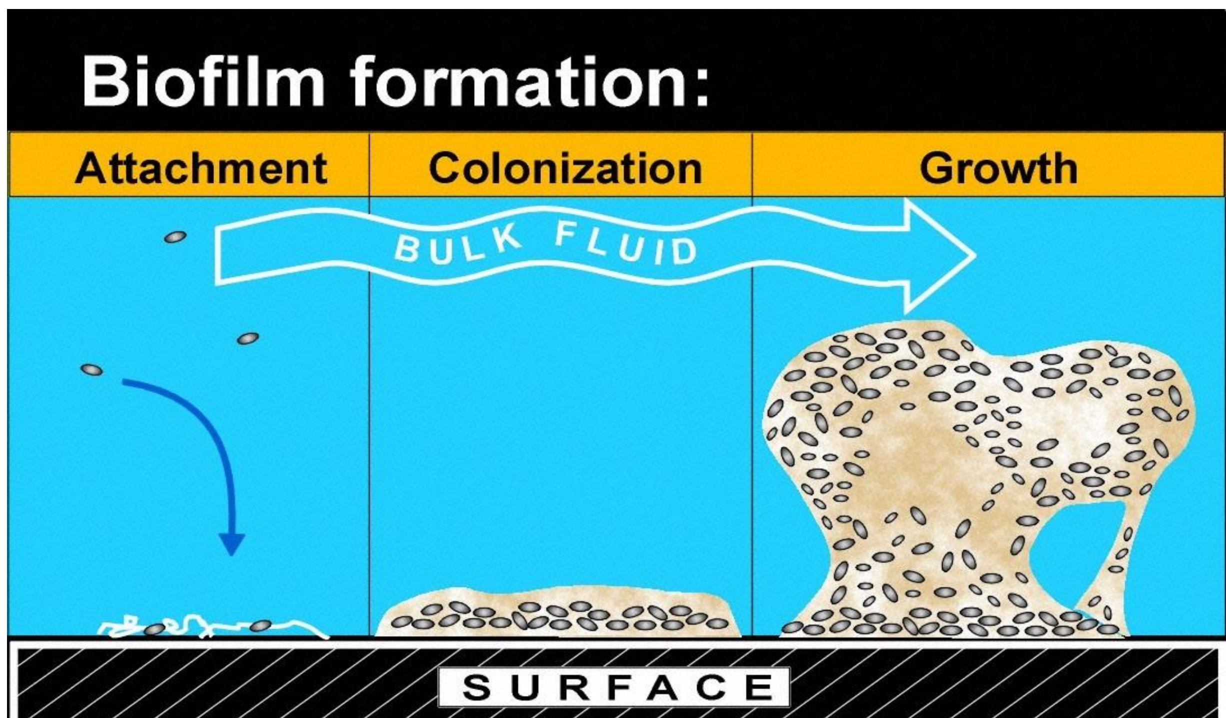


Figure 9: Biofilm formation

Bacteria are more resistant to antimicrobial agents in biofilm forms, than when moving freely (Ehrlich *et al*., 2004). Organisms within biofilms can readily acquire resistance against antimicrobial agents and disinfectants through the transfer of plasmids encoding resistance to multiple antimicrobial agents, which are carried by many enteric microorganisms (Donlan, 2002). However, dispersed biofilm cells become susceptible to

antimicrobial agents, indicating that the ability of the microorganisms in a biofilm state to resist antimicrobial agents is not as a result of mutations (Ehrlich *et al*., 2004). Susceptibility is influenced by factors such as biofilm thickness, age, cell diversity, antimicrobial dose concentration, species composition and genotype (Stewart *et al*., 2004). In the oral cavity, bacteria adhere to the teeth surface and subsequently form a biofilm, and this gives them resistance to salivary flow (Babu *et al*. 2011). Extracts from different plants and plant parts have shown the ability 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 and periodontal diseases (Palombo, 2009).

3. CHAPTER THREE: RESEARCH METHODOLOGY

3.1 Collection and pretreatment of plant material

Fresh plant parts (leaves, twigs and roots) were collected from Zambezi region, (Sangwali area). The plants were taxonomically identified at the National Botanical Research Institute (NBRI) of Namibia and confirmed to be *Diospyros lycioides Desf. subspecies lycioides* and *Euclea divinorum* Hiern. Collection was done in the same area (Figure 10) on different trees during the rainy season (December – February). The plant material was air-dried and then ground in a blender to a uniform powder which was stored in dark bottles until use. The different parts of the plant were analyzed separately.

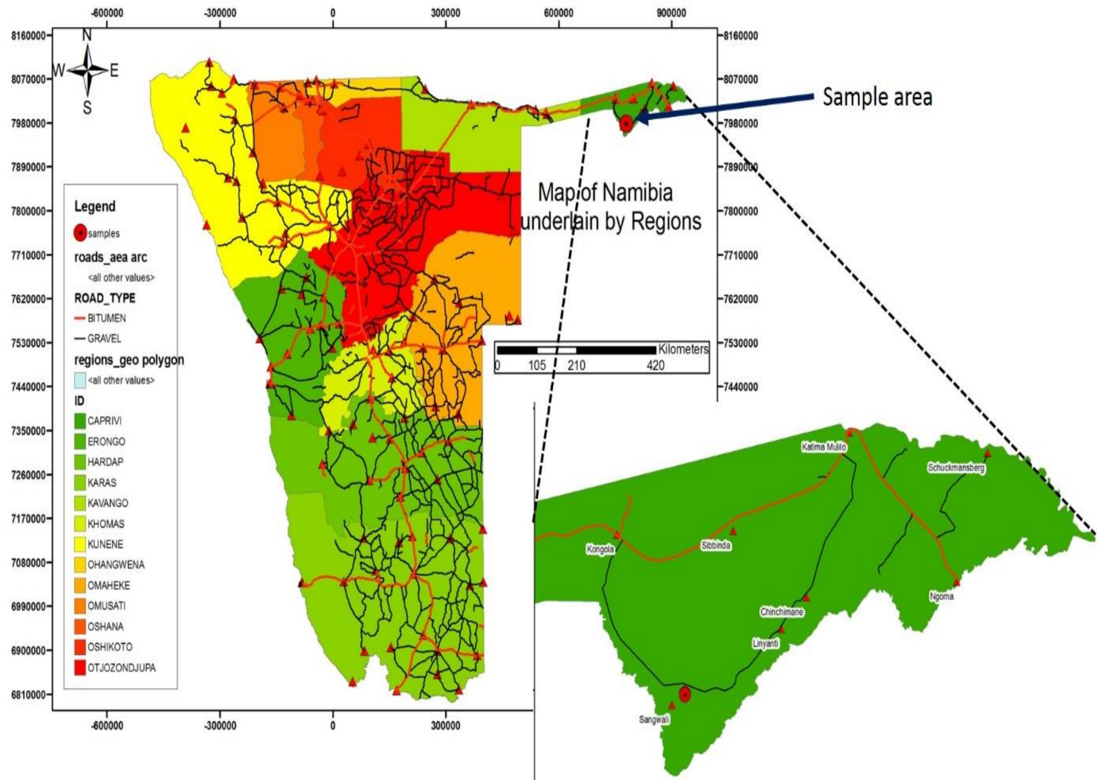


Figure 10 Map of Namibia showing sample collection site in the Zambezi region. Collection site is shown as a red dot

3.2 Phytochemical screening

Qualitative chemical tests were carried out on the roots, twigs and leaves of *D. lycioides* and *E. divinorum* in order to detect the presence or absence of certain classes of compounds. These tests were based on the methods described by Farnsworth & Euler (1962) and were done in duplicate. The plant material was ground to a fine powder. All reagents and solvents used were of Analytical Reagent (AR) grade.

3.2.1 Alkaloids

Two grams of ground plant material, was treated with 25 mL of 0.3 M HCl for 15 minutes in a water bath at 100 ºC. The suspension was filtered with Whatman No. 1 filter paper. Five drops of Dragendorff's reagent (Solution A = 0.17 g bismuth nitrate in 2 mL acetic acid and 8 mL water; solution B = 4 g potassium iodide in 10 mL acetic acid and 20 mL water. Solution A and B were mixed and diluted to 100 mL with water) was added. The formation of a reddish-brown precipitate indicated the presence of alkaloids. To confirm the results, saturated Na_2CO_3 solution was added to a new suspension drop wise until the universal indicator paper turned blue (pH 8-9). Four milliliter of chloroform was added to the mixture. On separation of the layers, the upper layer was removed and treated with acetic acid until a drop gave a yellow-brown colour with the universal indicator paper. Then 5 drops of Dragendorff's reagent was added. A precipitate confirmed presence of alkaloids.

3.2.2 Anthraquinones

One gram of plant material was extracted with 10 mL ether-chloroform (1:1) for 15 minutes and filtered using Whatman No. 1 filter paper. One milliliter of the extract was treated with 1 mL of 10% (w/v) NaOH solution. A red coloration indicated the presence of anthraquinones. A weak coloration was assigned + while a strong colouration got +++.

3.2.3 Cardenolides

Two grams of plant material was extracted for 2 hours with 20 mL of distilled water at room temperature. Four drops of Kedde reagent (4 mL of 3,5-dinitrobenzoic acid and 0.6 mL of 1N KOH in methanol) was added to the filtrate. A blue-violet colour appears in the presence of cardenolides.

3.2.4 Flavonoids

One gram of plant material was extracted with 15 mL water and methanol mixture (2:1) and filtered using Whatman No. 1 filter paper. Magnesium turnings were added to the filtrate and then concentrated HCl was added dropwise. Development of colours indicated the presence of flavonoids.

3.2.5 Saponins

One gram of plant material was mixed with 15 mL water and heated in a water bath at 100 ºC and then filtered through Whatman No. 1 filter paper. The filtrate was left to cool to room temperature. In a 16×160 mm test tube the filtrate was shaken for 10 seconds. The height of the honeycomb froth which persists was measured. Froth higher than 1 cm confirms the presence of saponins.

3.2.6 Tannins

Two grams of plant material was boiled with 15 mL water for 5 minutes and allowed to cool before being filtered through Whatman No. 1 filter paper. Five milliliter of 2 % (w/v) NaCl solution was added to the filtrate. One percent gelatin (5 mL) was added, precipitation confirmed the presence of tannins.

3.2.7 Polyphenols

One gram of plant material was boiled with 15 mL water for 15 minutes and the resulting solution was filtered through Whatman N0. 1 filter paper. Three drops of 1% ferric cyanide solution [1 mL of 1% (w/v) $FeCl_3$ and 1 mL of $K_3Fe(CN)_6$] was added to the filtrate. The formation of a blue-green colour indicated the presence of polyphenols.

3.2.8 Terpenoids

About 0.5 g plant material was extracted with 3 mL chloroform at room temperature. Ten drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. The formation of a reddish brown colouration at the interface indicated positive results for the presence of terpenes

3.3 Extraction and fractionation of extracts

3.3.1 *D. lycioides* roots

Solvents used in this study were AR grade, purchased from Sigma-Aldrich and the water used was distilled. The ground roots of *D. lycioides* (494 g) were soaked in a 1:1 mixture of dichloromethane and methanol (DCM:MeOH) solution (1L) to allow for the extraction of a wide range of polar and non-polar compounds. The mixture was occasionally stirred and filtered after 48 hours. The residue was rinsed with an aliquot of the same solvent mixture and the rinse added to the first extract. To ensure an exhaustive extraction, the residue was extracted further with MeOH (400 mL) for another 48 hours. TLC profiles of the DCM/MeOH and MeOH extracts obtained using (ethyl acetate:hexane 1:1) as solvent system, were compared and appeared similar. The extracts were therefore combined and concentrated using a rotory evaporator at 50 ºC. The concentrated crude extract (*D. lycioides* 12.5 g) was adsorbed on silica gel 60-200 mesh (20 g) and allowed to dry to constant weight at room temperature. The adsorbed crude extract was subjected to VLC where a sintered glass funnel (75 mm wide and 50 mm long) was packed with Silica gel 60, 0.04-0.06 mm; 230-400 mesh as a stationary phase. The extract adsorbed onto silica gel 60-200 mesh was applied uniformLy at the top. Solvents in order of increasing polarities: EA/Hex (0:1 → 1:0), EA/Acetone (8:1 → 0:1), Acetone/MeOH (8:1 → 0:1), MeOH/water (8:1 → 0:1) were introduced in 100 mL volumes. Each solvent combination was used to extract three fractions of 100 mL each resulting in a total of 45 fractions collected. Commercially available TLC aluminium sheets (20 x 20 cm), coated with Silica gel 60 F_{254} were purchased from Merck and used in this study. The extracts were

spotted at about 0.5 cm from the base of the plate. Different solvent mixtures at various ratios (e.g Hexane/Ethyl acetate, Chloroform/Methanol/H_2O, Methanol/Ethyl acetate, Acetone/Ethyl acetate, Petroleum ether/Ethyl acetate/Methanol etc.) were used to develop the plates at all stages of TLC profile studies. Spots were visualized using a UV lamp to show any UV-active spots and by using spray reagents, vanillin and anisaldehyde to try and detect the maximum number of compounds in each fraction. Fractions with similar TLC profiles (1-6, 7-12, 13-15, 16-27, 28-34, 35-36, 37-45) were pooled resulting in 7 fractions which were named DR1-DR7 (Figure 18). Fractionations and treatment of the combined crude extracts is outlined in Figure 11. The fractions together with the crude extract were tested for antimicrobial and anti-adhesive activities. Fraction DR7, which was obtained using aqueous solvent was not tested due to microbial contamination.

Fraction DR2 from the roots of *D. lycioides*, that showed the highest activity against unspecified oral microorganisms and against the oral pathogens *S. mutans* and *S. sanguinis*, was subjected to column chromatography to purify the fraction. A glass column (30 mm in diameter and 60 cm long) was used for CC using Sephadex LH-20 as the stationary phase. A smaller column (20 mm wide and 30 cm long) was used when Silica gel (70-230 mesh) was used as a stationary phase. The column was packed with 50 g Sephadex and 1% methanol in chloroform was used as the starting mobile phase. The polarity of the mobile phase was increased by increasing the amount of methanol (1%-10%). DR2 was separated on Sephadex and collected in fractions. Out of the 42 fractions

collected, fractions 1-8 were fairly pure giving a single spot on TLC analysis developed with EA/Ace (2:1) solvent system. This fraction was named DLR1 (0.28 g) and all other fractions (0.4997 g) were combined and purified further on Silica gel. A partially purified fraction obtained from silica gel column was named DLR2 (0.1538 g) (Figure 11).

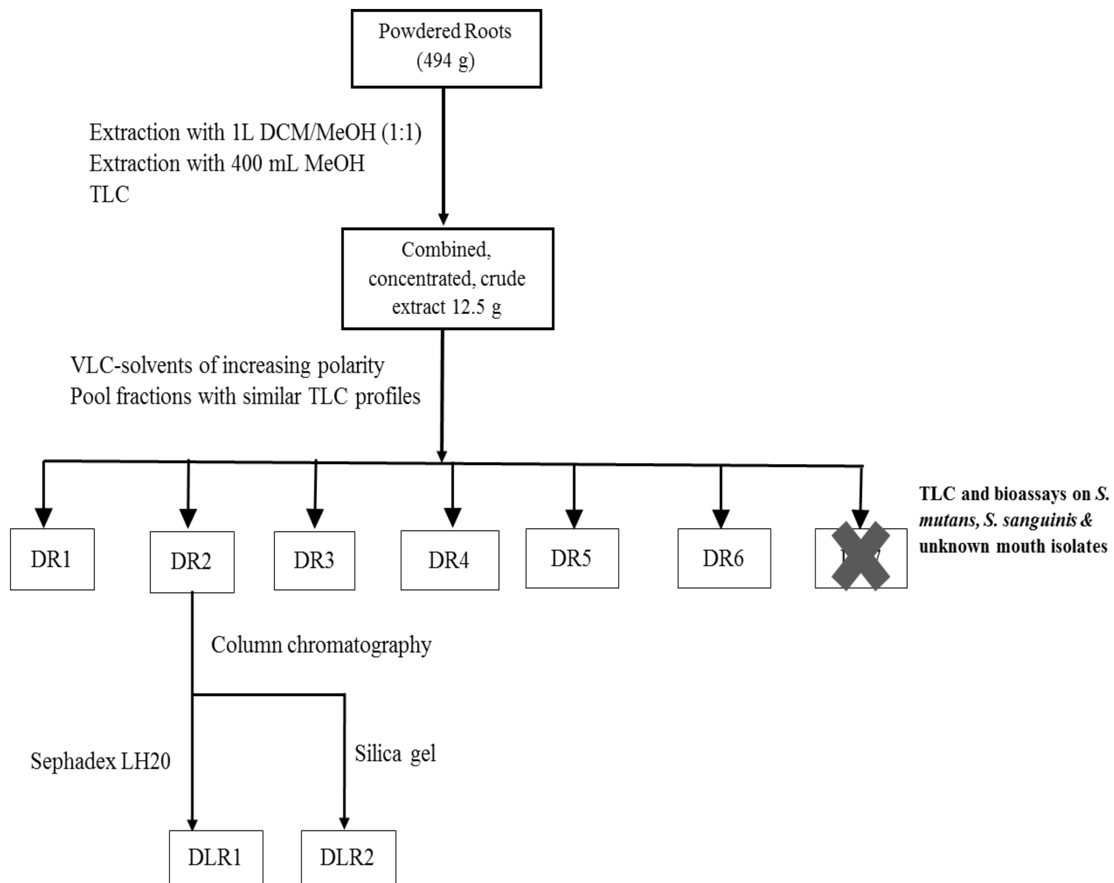


Figure 11: Schematic diagram of the extraction and fractionation of crude extract from the roots of *Diospyros lycioides*
DR = *D. lycioides* root fractions
DLR1 = Partially purified fraction from Sephadex column
DLR2 = Partially purified fraction from Silica gel column
✖ = Contaminated fraction

3.3.2 *E. divinorum* roots

A crude root extract of *E. divinorum* (17.9 g) was prepared using DCM:MeOH (1:1) and MeOH as described in section 3.3.1. This crude extract was not subjected to VLC due to time constraints and because people in the area, where the plants were collected, do not often use the roots of *E. divinorum* as chewing sticks. The crude root extract for *E. divinorum* was only tested for antibacterial and anti-adhesive activities.

3.3.3 *D. lycioides* twigs

Powdered twigs (215 g) were extracted with 700 mL DCM/MeOH mixture (1:1) as described in section 3.3.1. The TLC profile of the DCM/MeOH and MeOH extracts were compared and appeared to be similar (Figure 13), thus the extracts were combined. The extract was then concentrated using a rotory evaporator at 50 ℃. The concentrated crude extract (*D. lycioides* 4.8 g) was adsorbed on silica gel 60-200 mesh (20 g) and allowed to air dry. This adsorbed crude extract was subjected to VLC using solvents of increasing polarity: EA/Hex (0:1 → 1:0), EA/Acetone (8:1 → 0:1), Acetone/MeOH (8:1 → 0:1), MeOH/water (8:1 → 0:1). Fractions with similar TLC profiles were pooled, resulting in a total of 6 fractions coded DT1 - DT6 (Figure 12). Each of these pooled fractions were tested for presence of antibacterial and anti-adhesive activities.

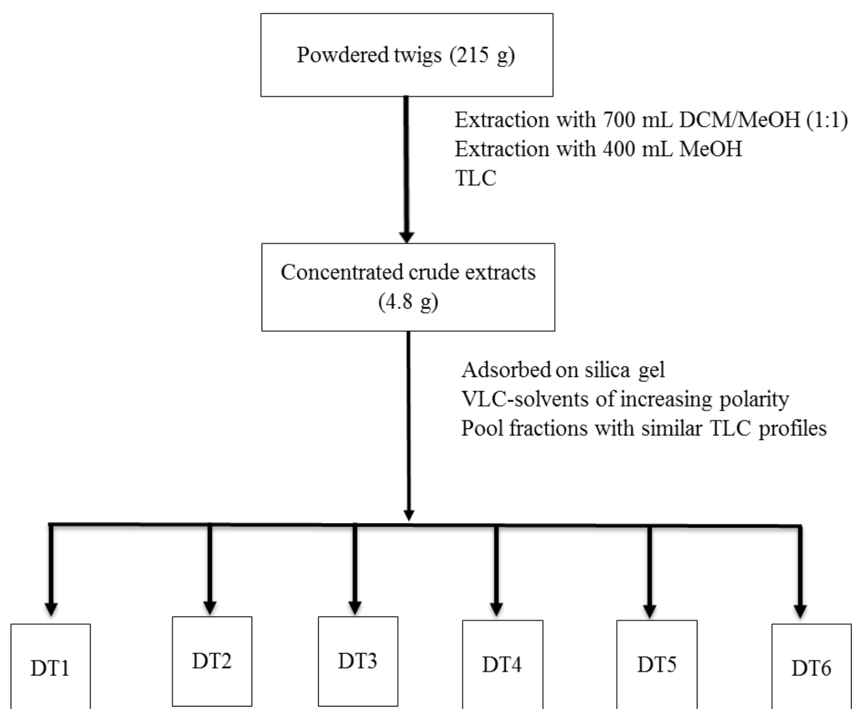


Figure 12: Schematic diagram of the extraction and fractionation of crude extract from the twigs of *Diospyros lycioides*
DT = *D. lycioides* twig fractions

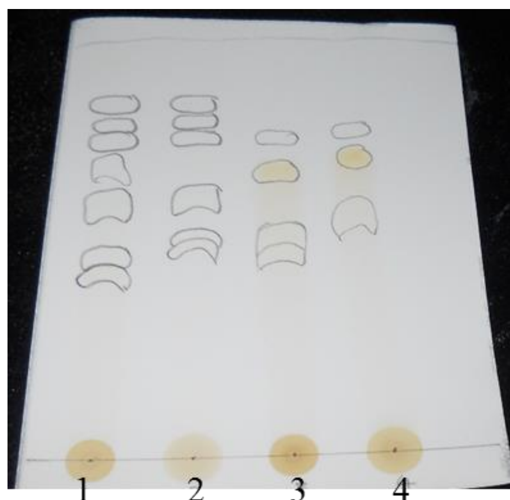


Figure 13: A TLC showing the DCM/MeOH extracts (1 and 3) and MeOH extracts (2 and 4) of the twigs of *Diospyros lycioides* with the UV active spots circled with a pencil

3.3.4 *E. divinorum* twigs

E. divinorum twigs (196 g) were extracted separately with 700 mL DCM/MeOH mixture (1:1) as described in section 3.3.1 The TLC profile of the DCM/MeOH and MeOH extracts were compared and appeared to be similar, thus the extracts were combined. The concentrated crude extract (*E. divinorum* 13.8 g) was subjected to VLC as described in section 3.3.3. A total of 7 fractions were obtained and coded EDT1 – EDT7 (Figure 14). Each of these pooled fractions were tested for presence of antibacterial and anti-adhesive activities.

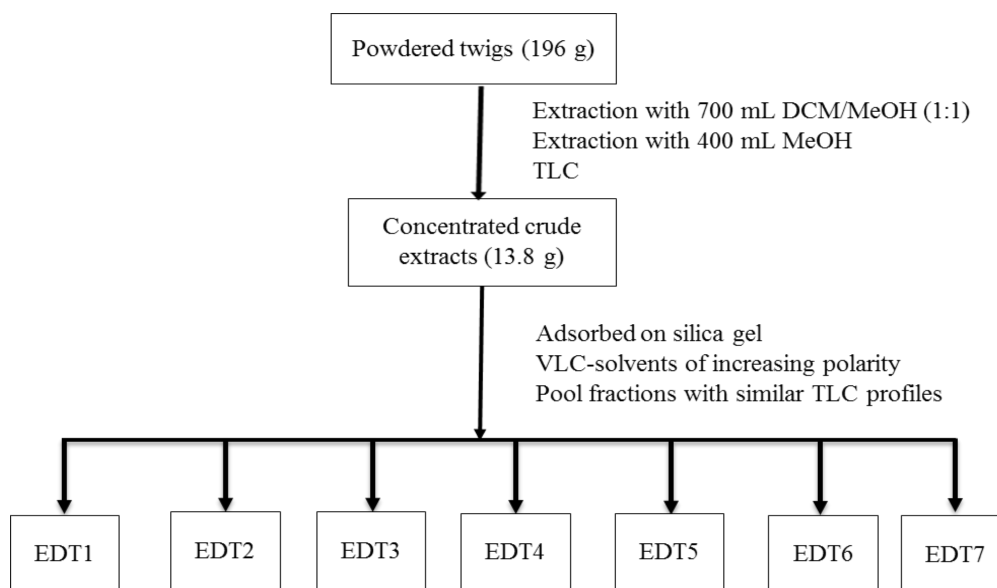


Figure 14: Schematic diagram of the extraction and fractionation of crude extract from the twigs of *Euclea divinorum*
EDT = *E. divinorum* twig fractions

3.3.5 Leaves

The extraction method outlined in section 3.3.1 afforded the crude leaf extract of *D. lycioides* (11.07 g) and *E. divinorum* (15.02 g) with the exception that 215 g and 284 g of powdered leaves were extracted respectively with 1 L of DCM/MeOH. The resulting crude extracts were tested for antibacterial and anti-adhesive activities.

3.4 Bioassay

3.4.1 Microorganisms and growth conditions

The microorganisms used for this study, *Streptococcus mutans* ATCC® 25175™ and *Streptococcus sanguinis* ATCC® 10556™ were purchased from American Type Culture Collection at Manassas through the University of Pretoria, Department of Biochemistry, South Africa. They were rehydrated in Trypticase Soy Broth (TSB) and TSB supplemented with 0.3% yeast extract respectively and grown at 37 ºC for 48 hours. Stock cultures were prepared and kept at -70 ºC until needed. Unless specified *S. mutans* was grown in Brain heart infusion (BHI) agar/broth at 37 ºC for all experiments and *S. sanguinis* was grown in Tryptic soy agar/broth supplemented with 0.3% yeast extract at 37 ºC. To standardize the inoculum, the culture was grown overnight on an agar plate. One or two colonies was transferred into a test tube containing 6 mL broth and incubated at 37 ºC until the absorbance of 0.1 (about 2 x 10^8 CFU/mL) at 625 nm.

3.4.2 Determination of antimicrobial activity against oral pathogens

3.4.2.1 Agar overlay

Growth inhibitory effects of all fractions obtained from VLC for all plant parts were tested against oral microorganisms *S. mutans* and *S. sanguinis* which are known to cause dental caries. The Agar overlay method as described by Akande & Hayashi (1998) was used with modification. Bacterial cells were grown overnight in BHI for *S. mutans* and

TSB supplemented with 0.3% yeast extract for *S. sanguis* at 37 ℃. Overlay agar was prepared and dispensed into 2 mL portions in test tubes with caps. The tubes were maintained at 45 ℃, a temperature that keeps the agar melted. Each of the 2 mL melted soft agar was seeded with 100µL of the overnight culture. The agar/bacterial culture mixture was mixed by vortex and poured over a pre-warmed agar as overlay on assay plates containing the base medium (BHI agar for *S. mutans* and TSA with 0.3% yeast extract for *S. sanguinis*). This allowed for a uniform growth of bacteria. The preparation was left to gel and dry under a fume hood. Paper discs (6 mm in diameter) were placed on the surface of the gelled agar and 10µL of plant extract and fractions (20 mg/mL) was spotted onto the disc and the plate was allowed to stand for 1 hour before inverting the plates to allow the extract and fractions to diffuse into the medium. They were then incubated at 37 ℃ for 24 hours. Each extract and fraction was spotted in triplicate and the experiment was done twice for each extract and each fraction. DMSO and ampicillin (1mg/mL) were used as the negative and positive controls respectively. Clear inhibition zones around the discs indicated the presence of antibacterial activity. The zone of inhibition was measured. The strength of activity was classified based on the method described by Nematollahi *et al*., 2011 as follows: for diameter ≥ 25 mm (very strong), diameters ranging from 24 – 15 mm (strong), diameters ranging from 14 – 11 (moderate) and diameters ranging from 10 – 8 mm (weak), while diameter ≤ 8 mm (negative).

3.4.2.2 Agar disc diffusion

The disc diffusion method was used to test the antimicrobial activity of the extracts as described by Nematollahi *et al*., 2011. This method was used on unidentified oral microorganisms. Using a cotton swab, microorganisms were collected from the teeth surface and grown at 37 ℃ in nutrient broth (10 mL) for 48 hours. A loopful of the growth was streaked onto Mueller-Hilton agar and allowed to grow for 48 hours. Using a microscope, colonies with different morphological type were observed showing that the plate contained different microorganisms. Using a loop, a single colony of each from the mixture was isolated on a separate plate to get pure cultures.

A loopful of colonies from pure cultures was placed into 9 mL nutrient broth and incubated at 37 ℃ for 24 and 48 hours when the bacterial suspension obtained a turbidity visually comparable to that of 0.5 McFarland standard (Mixing 0.05 mL 1.18% (w/v) barium chloride dehydrate ($BaCl_2.2H_2O$) with 9.95 mL of 1% (v/v) sulphuric acid (H_2SO_4). Using a sterile swab, the bacterial suspension of each isolate was spread onto Mueller-Hinton agar plates. Paper discs (6 mm) were dipped into extracts dissolved in DMSO at a concentration of approximately 10 mg/mL and incubated at 37 ℃. The discs were placed on inoculated agar. Discs impregnated with DMSO were used as negative control. The plates were incubated at 37 ℃ for 24 hours. Clear inhibition zones around the discs indicated the presence of antibacterial activity. The zone of inhibition was measured. The test was done in triplicates and was repeated twice. The strength of activity was classified as follows: for diameter ≥ 25 mm (very strong, ++++), diameters ranging from 24 – 15 mm

(strong, +++), diameters ranging from 14 – 11 (moderate, ++) and diameters ranging from 10 – 8 mm (weak), while diameter ≤ 8 mm (negative).

3.4.3 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the extracts that showed growth inhibition activity against the selected organism was determined by the agar dilution method as described by Adeniyi *et al.* (2000) with modifications. Briefly, BHI agar for *S. mutans* and TSA supplemented with 0.3% yeast extract for *S. sanguinis* was prepared as per manufacturer's instruction. After autoclaving, 19 mL of the media was dispensed into sterile test tubes with caps and maintained at 45 ºC. To each test tube 1 mL of the extracts and fractions already diluted to give the following concentrations 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL and 0.625 mg/mL was added. They were properly mixed for even distribution of the extracts within the agar, and the agar was poured into plates and allowed to set. The plates were then dried in a fume hood to remove steam. Each plate was divided into four sections. A loopful of the undiluted overnight broth culture was used to inoculate each section of the solidified agar/drug mixture in duplicates. A media/extract plate without an organism and a plate containing only the organism served as positive and negative control respectively. The plates were incubated at 37 ºC for 24 hours. They were then examined for the presence of colonies after the incubation period. The lowest concentration that gave no visible colonies was taken as

the minimum inhibitory concentration of the crude extract and fraction for the particular organism.

3.4.4 Adhesion assay

3.4.4.1 Inhibition of cell attachment in test tubes

Two methods were used to determine the *in vitro* effect of extracts on *S. mutans* and *S. sanguinis* cell adhesion. The first method was described by Rahim and Khan (2006) and was used to test only crude extracts of both plants. The second method involved the use of microtiter plates. A standardized overnight culture was diluted 100 times in 3 mL BHI broth containing 1% (w/v) sucrose in a test tube and grown at 37 ºC held at an angle of 30 degrees for 18 hours. After the incubation period, cells were washed twice with 1 mL 0.9% (w/v) NaCl to remove the non-adherent cells. Adhering cells were then collected by sonication. The turbidity was determined at OD_{550nm} . A test tube containing media and cell culture gave the total cell number and represented 100% adhesion. A test tube containing media, cell culture and DMSO served as a negative control. Other test tubes contained media, cell culture and different extracts (crude root extract from *D. lycioides* and *E. divinorum*, crude leaves extract from *D. lycioides* and *E. divinorum*). This gave the total cell number adhering in the presence of different extracts and this was expressed as a percentage of the total cell number in the absence of the extract.

3.4.4.2 Inhibition of cell attachment in microtiter plates

The ability of extracts from *D. lycioides* and *E. divinorum* to prevent formation of biofilm on teeth surface was tested using a 96 well polystyrene microtiter plate from Nunc, as a model. The inoculum was prepared by diluting an overnight standardized culture 100 times. The test extracts were prepared at a concentration of 20 mg/mL. To each well, 100 µL broth (BHI for *S. mutans* and TSB with 0.3% yeast extract for *S. sanguinis*) was added. Then 100 µL of the test extract in the first column, resulting in a final concentration of 10 mg/mL. Six extracts were tested per plate as shown in Figure 15 and each extract was tested in duplicate. The experiment was done twice. The extracts were then serially diluted from A – H obtaining a dilution from a concentration of 10 mg/mL – 0.078 mg/mL. A standardized culture (100 µL) was added to all the wells. The plate was covered and incubated at 37 ºC for 24 hours. After incubation period, the plate was washed 3 times with sterile distilled water to remove loosely associated cells. The plate was air dried and then oven dried at 60 ºC for 45 minutes. After drying, the wells were stained with 100 µL of 1% (w/v) crystal violet and incubated at room temperature for 15 minutes. The plate was then washed 5 times with sterile distilled water to remove unabsorbed stain. To destain the wells, 125 µL of ethanol was added. The destaining solution was then transferred to a new plate and the absorbance was determined at 595 nm using a microplate reader and the mean absorbance was determined.

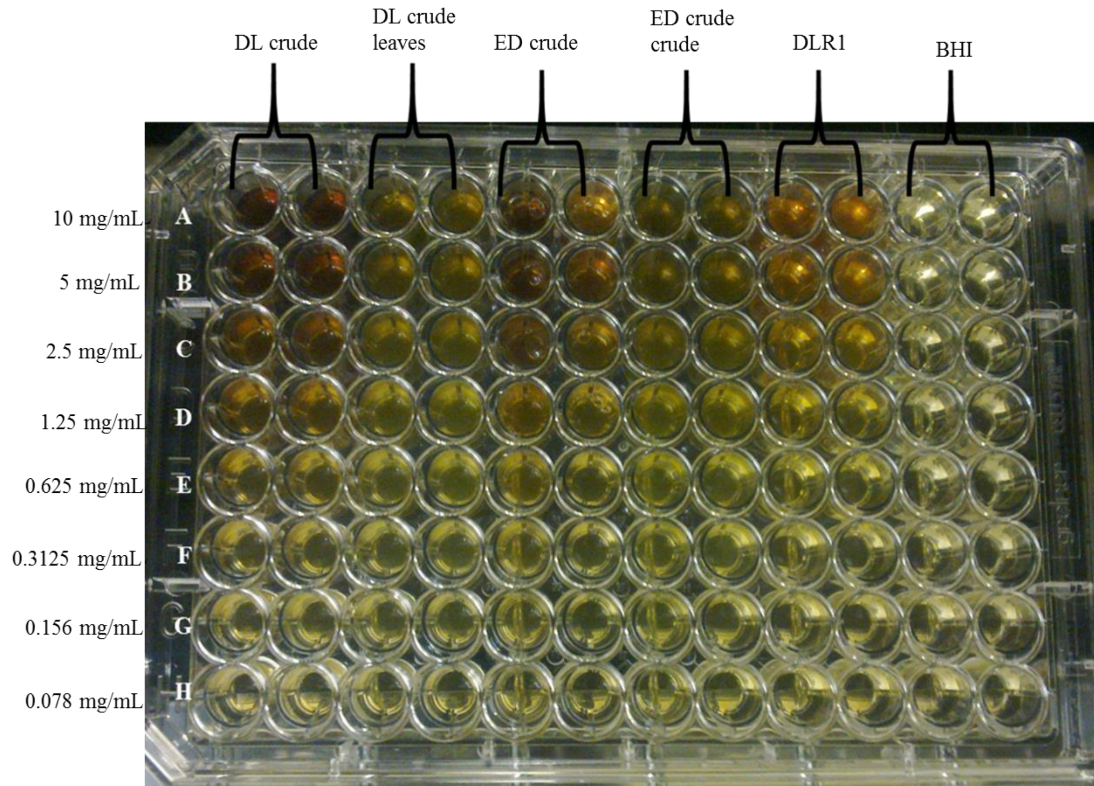


Figure 15: Distribution of test extracts at various concentration in a microtiter plate wells
BHI = Brain heart infusion broth, the negative control
DLR1 = Partially purified fraction from *D. lycioides* root extract

3.4.4.3 Bacterial adherence to saliva coated hydroxyapatite beads (S-HA)

A more sensitive method to test the amount of cells that adhere to the surface was performed using saliva-coated hydroxyapatite beads (Morris and McBride, 1984; Fachon-Kalweit *et al.* 1985; Cimasoni *et al.,* 1987).

Preparing the saliva: Parafilm stimulated saliva was collected from a single donor at 0 ºC and clarified by centrifugation at 8000 rpm for 15 minutes. The clarified saliva was heated at 60 ºC for 30 minutes to destroy endogenous enzymes and then centrifuged to remove precipitated materials. The prepared saliva was stored at -20 ºC until required and centrifuged again before use.

Saliva-coated hydroxyapatite beads: Hydroxyapatite beads (Sigma-Aldrich), were washed extensively with sterile distilled water to remove fine particles and dried at 60 ºC before use. Beads (40 mg) were dispensed into plastic 2 mL microtubes to which 1 mL of saliva was added. The mixture was shaken gently at room temperature for 2 hours and then kept at 4 ºC overnight. The excess coating solution was then removed by using an aspirating micropipette. The beads were washed twice with 1 mL of sterile distilled water and once with 1 mL of 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH7.2).

Bacterial suspension: ^{3}H Thymidine (ART 0178A Thymidine[methyl-^{3}H] with 84 Ci/mmol activity and 1 mCi/mL concentration) was purchased from the American Radiolabeled Chemicals, Inc, U.S.A. Bacterial cells for *S. mutans* and *S. sanguinis* were

labeled in broth by adding 1 μCi of [3H] thymidine per mL to the culture medium and grown overnight at 37 ºC. Cells were allowed to grow until they reached a bacterial suspension with optical density of 2.0 at 580 nm. Cells were then pelleted by centrifugation at 8000 rpm and 4 ºC for 15 minutes. They were washed three times in 0.05 M HEPES buffer (pH 7.2). Cells were then resuspended (absorbance at 660 nm, 1.5) in HEPES. The cells were briefly sonicated (30 sec) to break chains to ensure that only singles or pairs are present.

Adhesion assay: Samples of 1 mL of cells were added to duplicate tubes of S-HA and incubated for 2 hours at 37 ºC for 2 hours with gently shaking. The beads were allowed to settle and unattached bacteria were removed by aspiration. The beads were washed with 1 mL of HEPES buffer. The positive control tube was then dried at 60 ºC. To the test samples, 70 μL of the extracts were added and the mixture was further incubated at 37 ºC for 2 hours with gently shaking. Another control was included to which 70 μL of sterile distilled water was added. After the incubation period, the beads were washed three times with 1 mL HEPES buffer and transferred to 20 mL scintillation vials (plastic cap, PerkinElmer, Inc.). They were dried at 60 ºC. 5 mL scintillation fluid (ultra gold, PerkinElmer, Inc.). Cells were counted using a Liquid scintillation counter (QuantaSmart^{TM} Tri-Carb) to determine the number of cells that remained bound to S-HA. The mean and standard deviation of bacterial cells from two trial were calculated

4. CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Phytochemical screening

Ground plant parts (roots, twigs and leaves) of *D. lycioides* and *E. divinorum* were screened separately for the presence of alkaloids, anthraquinones, cardenolides, flavonoids, saponins, tannins, terpenoids and polyphenols. These qualitative tests are helpful in detecting the presence or absence of certain phytochemicals and their distribution in different plant parts. Additionally, they allow for the semi-quantitative analysis of phytochemicals and aid in locating potentially pharmacologically active compounds. The phytochemical profiles of the two plants under study were very similar. The phytochemical profile of the extracts from the leaves, twigs and roots of both plants showed the presence of flavonoids, saponins, polyphenols, tannins and terpenoids with varying concentrations (Table 2). These chemical compounds have been associated with antimicrobial activities. Flavonoids in particular are known to possess numerous biological activities. They have anti-inflammatory activities. The phytochemical test results show that the plant parts examined from both *D. lycioides* and *E. divinorum* may be rich sources of phytochemicals, which can be isolated and further screened for different kinds of biological activities.

The roots and twigs of both plants are rich in anthraquinones and cardenolides, but these chemicals were absent in the leaves of *E. divinorum*. The roots of both plants showed presence of alkaloids. Other studies reported the presence of alkaloids in *Euclea* and

Diospyros species (Mbanga *et al*., 2013). Shagal *et al.* (2012) also reported the presence of alkaloids in the roots, stem bark and leaves of *Diospyros mespiliformis*. Maridass *et al*. (2008) showed that 11 *Diospyros* species have alkaloids in the fruit extract. The main difference in the phytochemical profile of the roots and twigs was the presence of alkaloids in the roots. Alkaloids are well known for their wide range of pharmacological activities so this finding confirms the potential of both plants as potential antimicrobial agents. *E. divinorum* leaves, twigs and roots displayed a higher saponins content compared with the same plant parts of *D. lycioides*. The presence of saponins in both plants is of importance because saponins are known to be immune boosters. Results of the phytochemical screening are in accordance with the popular use of the *D. lycioides* and *Euclea divinorum* as chewing sticks since the results show the presence of various secondary metabolites that are associated with various pharmacological effects. The presence of saponins in *D. lycioides* roots, twigs and leaves, supports its traditional use for procuring abortion since saponins are linked to the sex hormone oxytocin involved in controlling the onset of labour (Njoku & Akumefula, 2007). The presence of tannins suggest the growth inhibitory effect of these plant extracts on bacteria. However further work on quantitative analyses of these compounds need to be done.

Table 2: Phytochemical constituents of *Diospyros lycioides* and *Euclea divinorum*

Phytochemicals	*D. lycioides*	*E. divinorum*	*D. lycioides*	*E. divinorum*	*D. lycioides*	*E. divinorum*
	Leaves		**Twigs**		**Roots**	
Alkaloids	-	-	-	-	+	+
Anthraquinones	+	-	++	+	+++	+++
Flavonoids	+	+	+	+	+	+
Saponins	+	++	+	++	+	+++
Polyphenols	+++	+++	+++	+++	+++	+++
Tannins	+++	+++	++	++	+	+
Cardenolides	+	-	++	+	+++	+++
Terpenoids	+	+	+	+	+	+

Tests done in duplicate
+ = Presence of constituent and intensity
- = Absence of constituent

4.2 Antimicrobial activity against oral pathogens

4.2.1 Growth inhibition

In accordance with the objectives of the study, crude extracts from *D. lycioides* (leaves, twigs, roots), and fractions from the roots (DR1-DR6) were evaluated for antimicrobial activity against unidentified microorganisms isolated from the mouth and Gram positive bacteria *S. mutans* and *S. sanguinis* using the disk diffusion and agar overlay methods (Figure 16, Figure 18). Crude extracts from *E. divinorum* (leaves, twigs, roots), and fractions from the twigs (EDT1 - EDT6) were also evaluated using the same method (Figure 20). The zone of inhibition of the test samples (crude extracts and fractions) were

compared to the antibiotic standard ampicillin. An inhibition zone ≥ 8 mm was taken as the break-point of bacterial susceptibility of the extracts (Akande & Hayashi, 1998).

The result of the antimicrobial susceptibility test showed that crude extracts had antimicrobial activity against the two characterized microorganisms and against unidentified microorganisms isolated from the mouth. This was reflected in the varying zones of inhibition of the individual extracts on the oral pathogens *in vitro* with mean inhibition diameters that ranged from 8 – 25 mm (Figure 17; Table 3). The crude root extracts from both plants inhibited the growth of all test organisms. At 20 mg/mL concentration, the crude root extract from *D. lycioides* produced zones of inhibition of 17 mm and 12 mm for *S. mutans* and *S. sanguinis*, respectively. This same extract gave a zone of inhibition of 12 mm and 10 mm for unknown isolates 1 and 2, respectively at the same concentration. Antibacterial activity of the crude root extract of *E. divinorum* showed zone of inhibition of 9 mm and 13 mm for *S. mutans* and *S. sanguinis* respectively, while isolate 1 and 2 were 8 mm each at 20 mg/mL concentration. The means for the zone of inhibition of crude extracts from both plants were significantly different ($p = 0.043$, $t = -2.696$). Surprisingly, the leaf extract of *E. divinorum* only showed activity against *S. sanguinis*. No zone of inhibition was observed for the crude leaf extract of *D. lycioides* for all test organisms (Table 3).

D. lycioides root fractions DR2 and DR3 inhibited the growth of all test organisms (Table 3). This suggests that these fractions have a high antibacterial activity against the tested organisms. The root fraction DR2 that was extracted with ethyl acetate/hexane mixture

(1:1 → 1:0) by VLC showed the best growth inhibitory activity on the tested organisms with the inhibition zones ranging from 17 - 25 mm at a concentration of 20 mg/mL. Fraction DR4 and DR5 from *D. lycioides* roots gave a zone of inhibition of 9 mm on isolate 1 but no zone of inhibition was observed for the other three test organisms. Selection of fractions DR2 for further purification was based on the observed antibacterial activity against the unidentified isolates from the mouth and activity against *S. mutans* and *S. sanguinis*. The fraction from *D. lycioides* twigs, DT2 displayed strong inhibitory activity against *S. mutans* and *S. sanguinis* with zones of inhibition ranging from 9 – 14 mm (Table 2 and Figure 19). This is in agreement with previous studies that reported on the antimicrobial and inhibitory effects of compounds from *D. lycioides* twigs against *S. mutans* and *S. sanguinis* (Li *et al.,* 1998). Fraction EDT1 from *E. divinorum* twigs inhibited the growth of *S. sanguinis* (Figure 20) with an inhibition zone of 11 mm, but had no effect on *S. mutans*. The activity in these fractions can be attributed to the presence of various secondary metabolites in the extracts. Where the crude extracts and fractions inhibited growth of both *S. mutans* and *S. sanguinis*, the latter appeared to be more susceptible. It was susceptible to 8 extracts compared to only 5 for *S. mutans* (Table 3). The difference in susceptibility can possibly be attributed to the nature of the active ingredients as well as the level of concentration in the extracts. DMSO showed no antibacterial effect. Although the zone of inhibition for DR crude was higher than that of DT2, the mean zone of inhibition were not statistically different. There was a significant difference in the mean zone of inhibition between DR2 and DR crude ($p = 0.005$, -0.491) and between EDT1 and crude E. divinorum root extract ($p = 0.013$, $t = -3.796$).

The results for this study shows that at least one fraction from each plant part (i.e roots, twigs and leaves) for both plants shows activity against one or all of the organisms tested (Figure 17). This is an indication that the extracts possess substances that can inhibit the growth of microorganisms, particularly oral pathogens. Although the phytochemical screen test showed that all plant parts from both plants contain polyphenols, saponins and tannins which are potentially good antimicrobial agents (Duru & Onyedineke, 2010), most *E. divinorum* fractions and leaf extract from *D. lycioides* did not have an effect on the growth of the tested microbes. This could be a result of incomplete extraction of the active ingredients such as polyphenols and tannins and possibly a concentration factor. Contamination of the aqueous extract could also have led to loss of active compounds as this extract was not tested. The crude twig extracts for both plants were not tested for antibacterial and anti-adhesive activity because of very little extract was kept from VLC fractionation and due to time constraints. The omission of the aqueous extract from the roots of *D. lycioides* due to contamination was a limitation of this study. The reported antibacterial activity of *D. lycioides* roots is therefore not a complete representation. Mbanga *et al*., (2013) reported that the aqueous extracts from *D. lycioides* were active against multidrug sensitive and multidrug resistant *S. mutans* isolates.

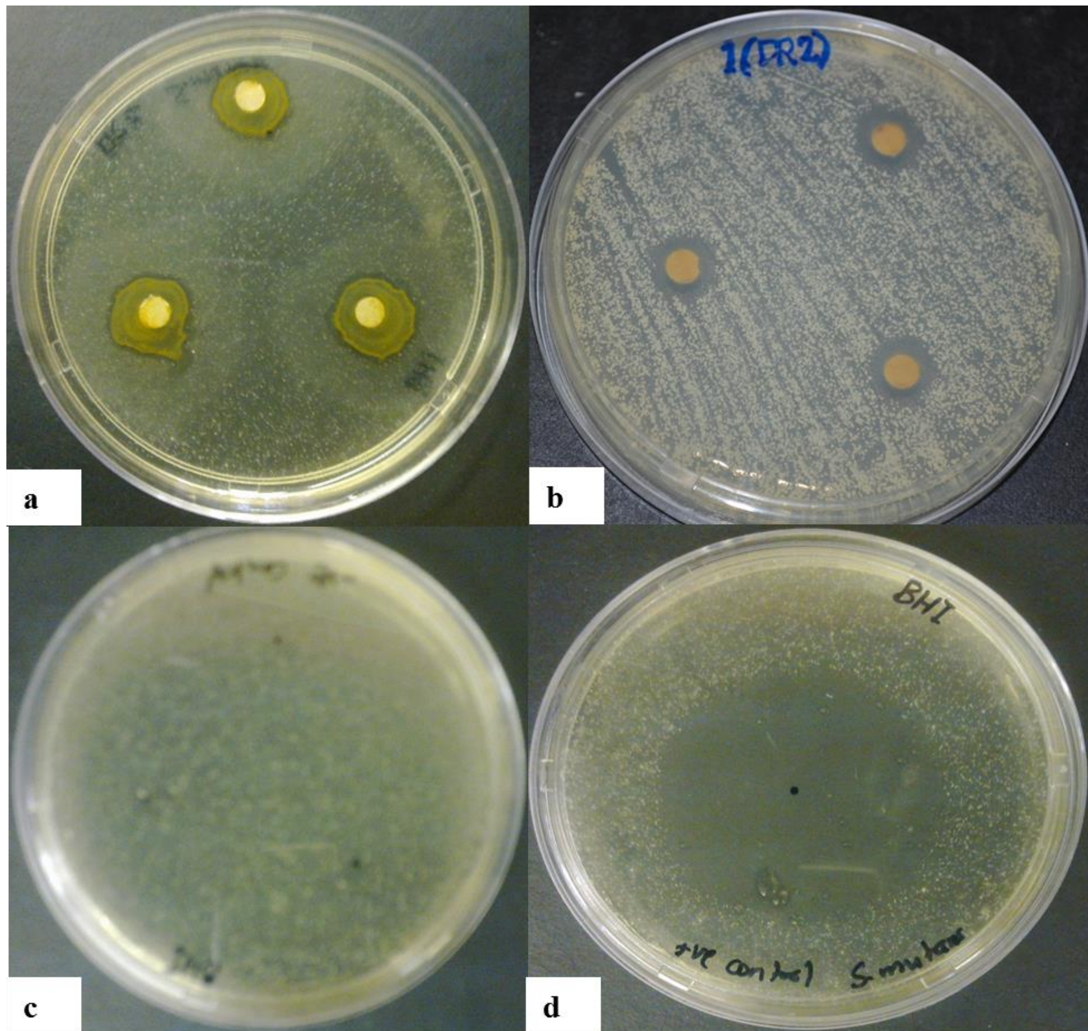


Figure 16: Inhibition effect of DR2 on (a) *Streptococcus mutans*, (b) unspecified oral microorganism. (c) negative control DMSO and (d) positive control ampicillin

Table 3: Growth inhibition effect of *Diospyros lycioides* and *Euclea divinorum* crude extracts and fractions dissolved in DMSO at 20 mg/mL on *Streptococcus mutans*, *Streptococcus sanguinis* and two isolates from the teeth surface.

				Unidentified oral microorganisms	
		S. mutans	***S. sanguinis***	**Isolate 1**	**Isolate 2**
Plant name&part	**Extracts & fractions**	**Inhibition zone**			
D. lycioides roots	Crude	17 mm ± 2	12 mm ± 1	12 mm ± 2	10 mm ± 2
	DR1	-	-	-	-
	DR2	25 mm ± 3	17 mm ± 2	18 mm ± 2	11 mm ± 0
	DR3	9 mm ± 1	8 mm ± 1	12 mm ± 0	8 mm ± 1
	DR4	-	6 mm ± 5	9 mm ± 0	-
	DR5	-	-	9 mm ± 1	-
	DR6	-	-	-	-
D. lycioides twigs	DT1	-	-	-	-
	DT2	9 mm ± 2	14 mm ± 4	-	-
	DT3	-	-	-	-
	DT4	-	-	-	-
	DT5	-	-	-	-
D. lycioides leaves	Crude	-	-	-	-
E. divinorum twigs	EDT1	-	11 mm ± 2	-	-
	EDT2	-	-	-	-
	EDT3	-	-	-	-
	EDT4	-	-	-	-
	EDT5	-	-	-	-
	EDT6	-	-	-	-
E. divinorum leave	Crude	-	10 mm ± 1	-	-
E. divinorum roots	Crude	9 mm ± 1	12 mm ± 1	8 mm ± 1	8 mm ± 0
Control	Ampicillin*	45 mm ± 4	48 mm ± 5	22 mm ± 1	22 mm ± 0
	DMSO	-	-	-	-

The results are mean of two replicate experiments using three disks for each experiment; mean ± standard deviation; - = No bacterial growth inhibition,
* = Ampicillin concentration is 1 mg/mL

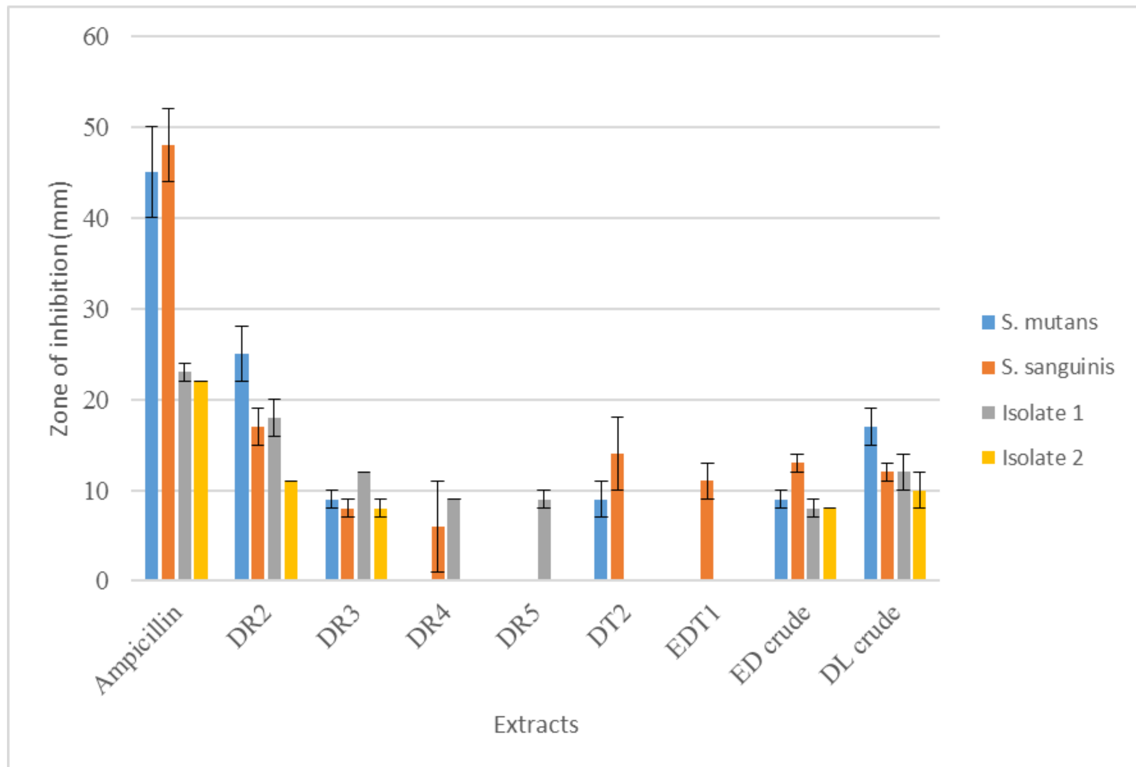


Figure 17: Antibacterial activity of extracts against *Streptococcus mutans*, *Streptococcus sanguinis* and two unidentified oral microorganisms

DR (2, 3, 4, 5) = fractions from *D. lycioides* root crude extract; DT2 = fraction from *D. lycioides* twigs crude extract; EDT1 = fraction from *E. divinorum* twig crude extract; DL crude = crude extract from *D. lycioides* roots and ED crude = crude extract from *E. divinorum* roots.

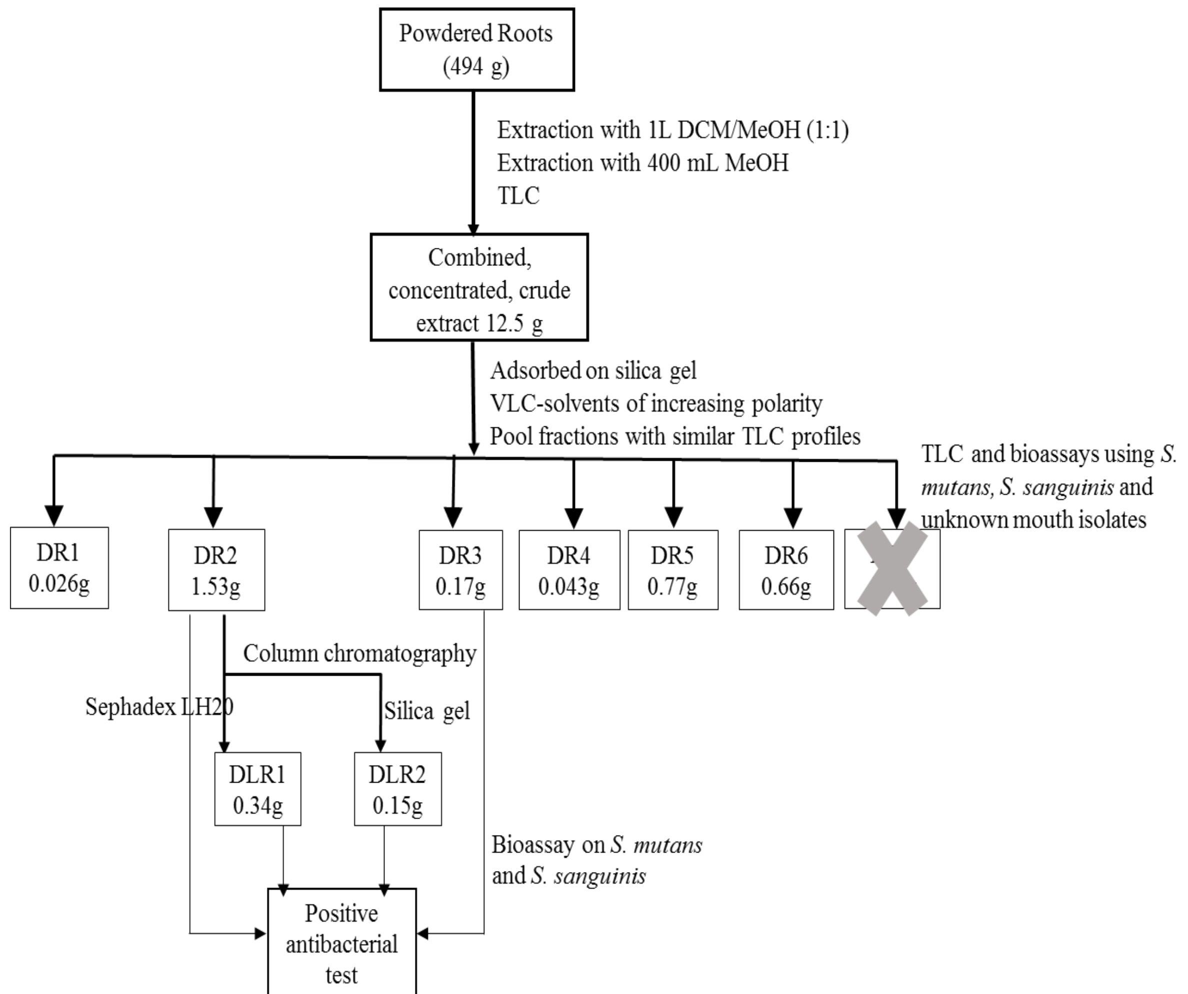


Figure 18: Schematic diagram of the extraction and bioassay of fractions from the roots of *Diospyros lycioides*

DR = *D. lycioides* root fractions; DLR1 = partially purified fraction from Sephadex column; DLR2 = partially purified fraction from Silica gel column;

✖ = Contaminated fraction

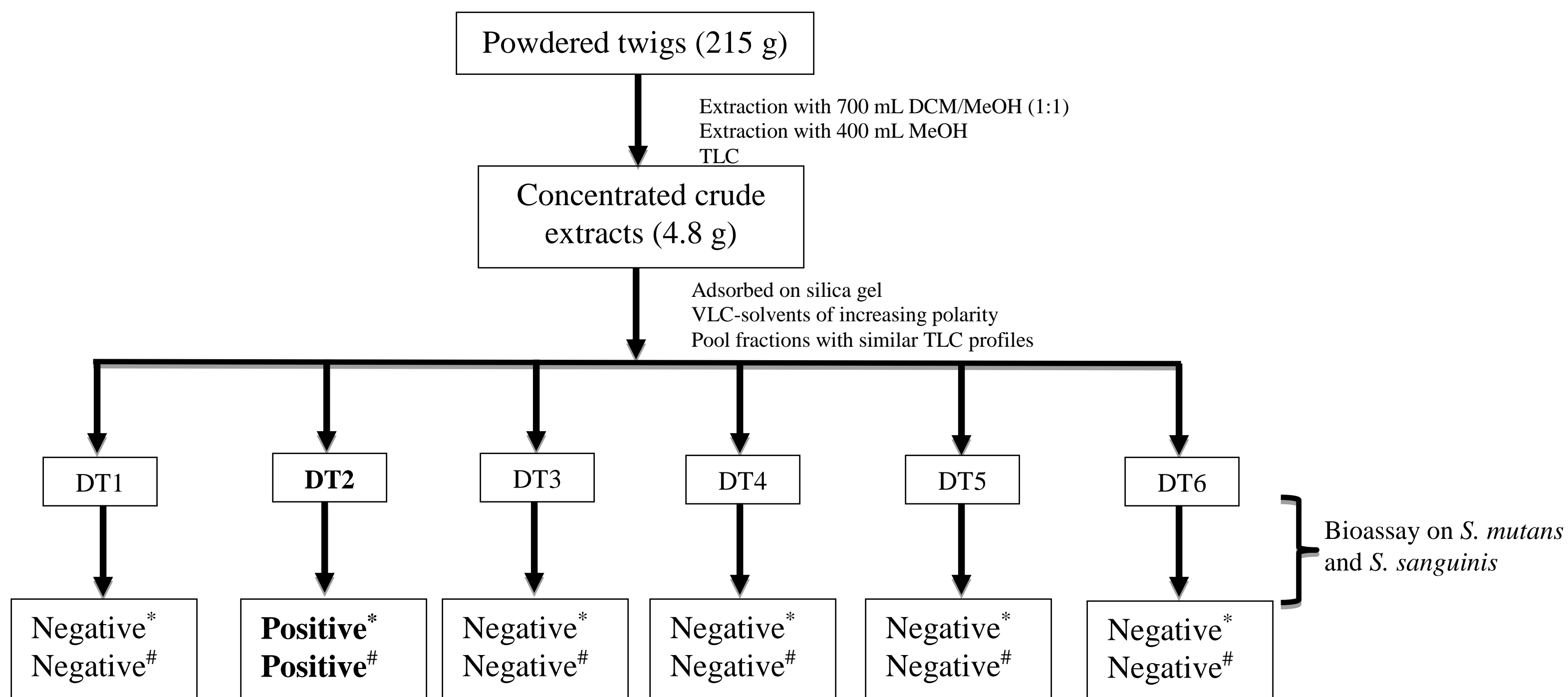


Figure 19: Schematic diagram of the extraction and bioassay of fractions from the twigs of *Diospyros lycioides*
DT = Fractions from the twig crude extract of *D. lycioides*; * = Test result of *S. sanguinis;* # = Test results of *S. mutans*

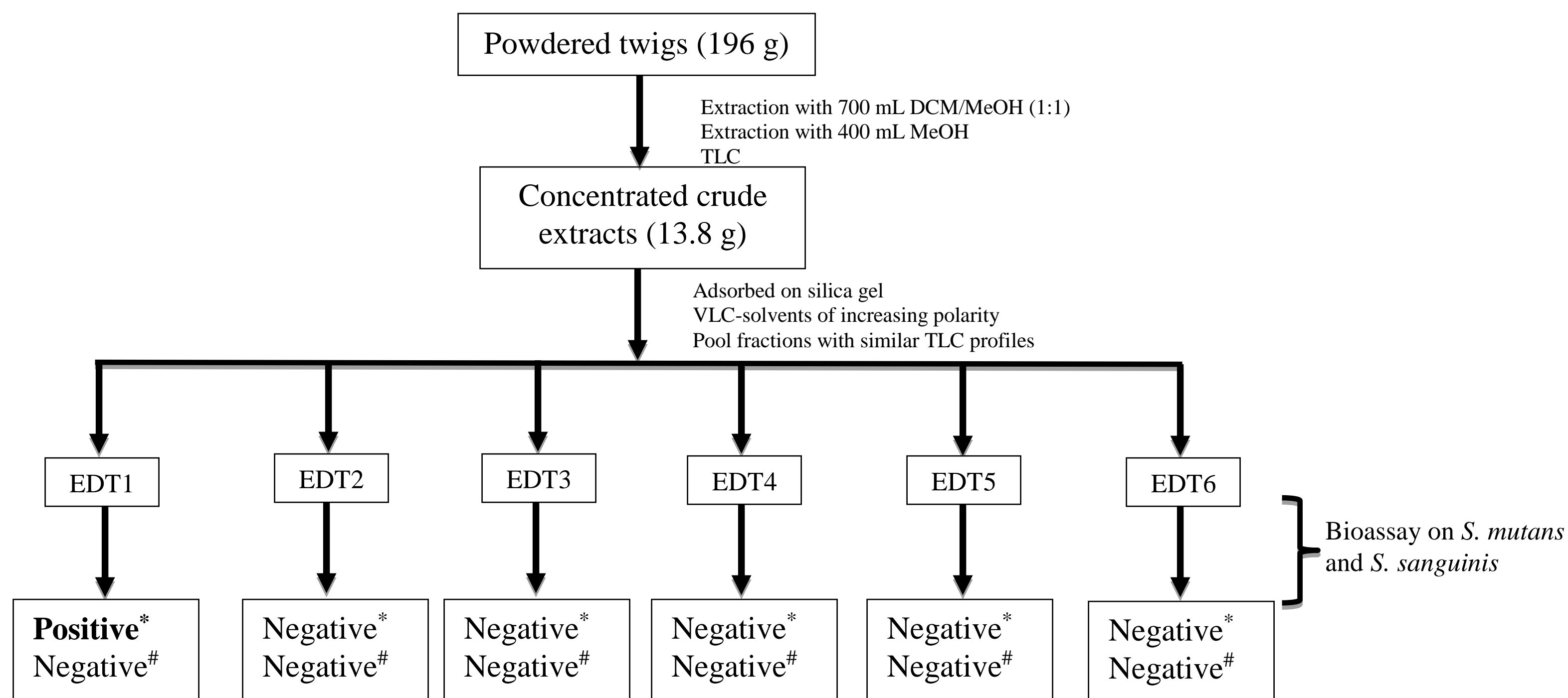


Figure 20: Schematic diagram of the extraction and bioassay of fraction from the twigs of *Euclea divinorum*
EDT = Fractions from the twig crude extract of *E. divinorum*; * = Test result of *S. sanguinis*; # = Test results of *S. mutans*

4.2.2 Minimum inhibitory concentration (MIC)

The MICs for the plant extracts that showed growth inhibition activity are shown in Table 4. *S. sanguinis* was inhibited at the lowest concentrations of all the crude extracts and fractions that showed activity. These are: crude root extract of *D. lycioides*, crude root extract of *E. divinorum*, fractions DR2, DR3, DT2, EDT1 and crude leaf extract from *E. divinorum*. This indicate that *S. sanguinis* is more susceptible to these extracts and fractions compared with *S. mutans*. MIC also shows that DR2 of *D. lycioides* roots was the most active fraction as it inhibited both test microbes at an MIC of 2.5 mg/mL for *S. mutans* and 0.165 mg/mL for *S. sanguinis*. This indicates that DR2 contains a high amount of active compounds and therefore only a small amount of it is needed to kill these microorganisms after 24 hours incubation. This low MIC exhibited by DR2 is of great significance because it suggests that this extract can be used in the prevention of dental caries. It also suggests that using *D. lycioides* roots as teeth cleaning agents can kill oral pathogens. The low MIC also indicate that chewing of the roots of *D. lycioides* can be an effective teeth cleaning agent and thus can be an alternative to conventional toothbrush and toothpaste method since it is cheaper. *D. lycioides* twig fraction DT2 displayed a moderate activity with MIC value of 10 mg/mL against both organisms. These results show that the root extract from *D. lycioides* displays better antimicrobial activity than its twigs. This supports the preference that stick users have for roots over twigs. *E. divinorum* twig extract EDT1 inhibited the growth of *S. sanguinis* at an MIC of 20 mg/mL but had no effect on *S. mutans* thus displaying poor activity. This is in agreement with a study by Mbanga *et al*., (2013) who also showed that *E. divinorum* has

limited antibacterial activity against *S. mutans*. Both plants however, have active ingredients against both organisms although at different concentrations.

Table 4: Minimum inhibitory concentration (MIC) of crude extracts and fractions with inhibition activity against the tested microbes

	Microorganisms	
Extracts	***S. mutans***	***S. sanguinis***
DL crude	2.5 mg/mL	1.25 mg/mL
ED crude	2.5 mg/mL	1.25 mg/mL
DR2	1.25 mg/mL	0.625 mg/mL
DR3	20 mg/mL	1.25 mg/mL
DT2	10 mg/mL	10 mg/mL
E. divinorum leaves (crude)	-	20 mg/mL
EDT1	-	20 mg/mL

The results are mean of two replicate experiments.
- No inhibition; DL crude = crude extract from *D. lycioides* roots; ED crude = crude extract from *E. divinorum* roots; EDT1 = fraction from *E. divinorum* twig extract; DT2 = fraction from *D. lycioides* twigs extract; DR (2, 3) = fractions from *D. lycioides* roots crude extract

4.2.3 Inhibition of cell attachment to a glass surface

Figure 21 show the ability of tested crude extracts to prevent the attachment of *S. sanguinis* more than *S. mutans* to glass surface. The crude extract from the roots of *E. divinorum* had the highest activity against both organisms, preventing the attachment of *S. mutans* and *S. sanguinis* cells by 51% and 85%, respectively. Although *D. lycioides* leaf extract did not prevent the growth of any of the organisms, it was able to inhibit their attachment to glass by 45% (*S. sanguinis*) and 24% (*S. mutans*). The effect of *D. lycioides* root extract was very weak on *S. mutans*, inhibiting its attachment by only 10%, but very high for *S. sanguinis* inhibiting its attachment by 77%. The statistical analysis showed that there was a significant difference between the percentage inhibition of extracts from the two plants ($p = 0.029$). *E. divinorum* leaves showed moderate cell attachment inhibition of 31% for *S. mutans* and 38% for *S. sanguinis*. Statistically, there was no significant difference between the percentage inhibition from the crude leaf extracts of both plants ($p = 0.867$). Consistent with the growth inhibition test and the MIC, *S. sanguinis* showed more susceptibility to all the crude extracts. Inhibition of bacterial adhesion may be associated with the modification of the receptors on the cell surface of the bacteria (Rahim & Khan, 2006). This implies that the crude extracts from the roots and leaves of both *D. lycioides* and *E. divinorum* have components that reduce the adhesion ability of the cells. The root extracts from both plants seem to contain more of these components since they inhibited attachment of *S. sanguinis* by 70 - 80%. This is an important factor to consider because it means that using these plants as chewing sticks

can reduce the adhesion of pathogenic oral microorganisms to the teeth surface and thus limit plaque formation.

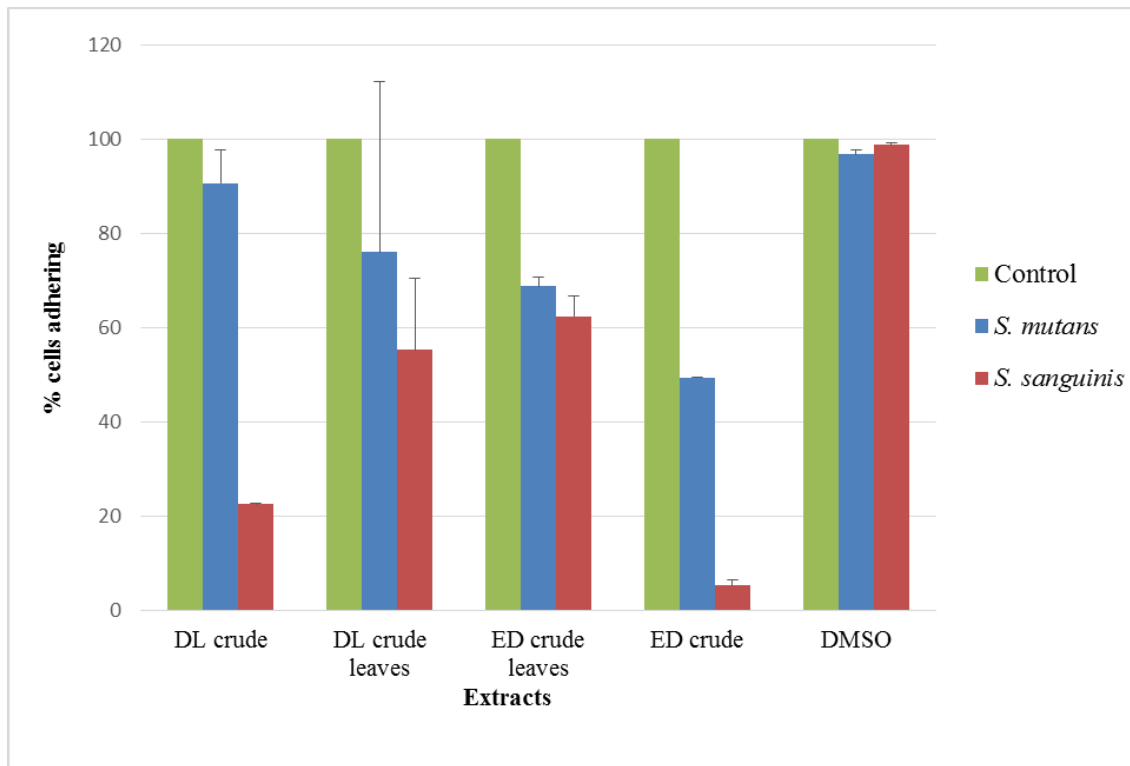


Figure 21: The effect of crude extracts from *Diospyros lycioides* and *Euclea divinorum* roots and leaves on *Streptococcus mutans* and *Streptococcus sanguinis* cell adhesion to a glass surface

The graph shows percentage adherence of bacterial cells expressed relative to the control. DL crude = crude extract from *D. lycioides* roots; ED crude = crude extract from *E. divinorum* roots; DL crude leaves = crude extract from *D. lycioides* leaves; n = 2

4.2.4 Inhibition of cell attachment in microtiter plates

This assay tested the ability of extracts to prevent adhesion of bacterial cells to the polystyrene plate and to test for the lowest concentration at which the extracts inhibit attachment. This adhesion is the initial stage in biofilm formation. Bacterial cells that adhered to the plate were visible after staining with crystal-violet (Figure 22). All crude extracts displayed inhibition of cell attachment of both organisms by over 80% at concentrations tested, ranging from 0.156 mg/mL – 10 mg/mL. The crude root extract from *D. lycioides* showed the highest inhibition activity since it prevented attachment of cells for both organisms by 88% (Figure 23) at a concentration of 0.313 mg/mL *S. mutans* and 0.156 mg/mL *S. sanguinis*. This was followed by the crude extract from *E. divinorum* roots which prevented attachment by 88% at 1.25 mg/mL for *S. mutans* and 0.625 mg/mL for *S. sanguinis.* The leaf extract from both plants showed moderate activity with *D. lycioides* leaf extract inhibiting attachment by 88% only at 2.5 mg/mL *S. mutans* and 1.25 mg/mL *S. sanguinis*, while *E. divinorum* leaf extract was the least active with activity observed at 5 mg/mL for both organisms (Figure 23). Consistent with the growth inhibition test results for the crude extracts, the roots fraction from *D. lycioides* showed better attachment inhibition compared to the twigs fraction. At a concentration of 2.5 mg/mL, the inhibition effect of all the root fractions on the attachment of *S. mutans* was the same as the positive control, ampicillin. The same effect was observed on *S. sanguinis*, except that DR4 and DR6 showed slightly low activity (Figure 24).

Therefore, the activity of extracts from *D. lycioides* roots can be comparable to commercially available toothpastes which when used as directed help to prevent tooth decay. *D. lycioides* twig fractions DT4 and DT5 inhibited attachment of *S. mutans* at a concentration as low as 0.313 mg/mL by over 50% and by more than 80% at a higher concentration of 1.25 mg/mL. On the other hand, DT2 which has growth inhibition activity, only prevented attachment at a concentration of 2.5 mg/mL (Figure 25). The fraction DT4 inhibited attachment of *S. sanguinis* by over 80% at 0.625 mg/mL, while DT2 and DT3 inhibited at 1.25 mg/mL (Figure 25). *E. divinorum* twig fractions EDT2 and EDT3 prevented attachment of *S. mutans* by over 70% at a low concentration of 0.313 mg/mL. However, EDT3 only prevented attachment of *S. sanguinis* by 50% at 5 mg/mL (Figure 26). Fraction EDT1 showed activity against both organism. The fact that the extracts or fractions prevent attachment of *S. mutans* more than *S. sanguinis* is encouraging since *S. mutans*' cariogenic potential is reported to be higher than that of *S. sanguinis* (Coufield *et al*., 2000). These results indicate that although some extracts from the plants selected do not kill the bacteria, they can prevent their attachment to surfaces. This prevention of attachment is an important activity because bacteria tend to be more resistant when they are in biofilm structure than when they exist as single cells (Ehrlich *et al.*, 2004).

Figure 27 shows that all extracts and fractions inhibited attachment of *S. mutans* at a concentration of 2.5 mg/mL giving an OD_{595} value less than 0.3 except EDT3. Root

extract of *D. lycioides* inhibited attachment with OD_{595} less than 0.5 up to a concentration of 0.313 mg/mL while fractions obtained from this extract only inhibited with the same OD_{595} value at 1.25 mg/mL. The crude extract thus showed higher inhibition activity compared to the fractions. This could be due to synergistic interaction of the compounds in the crude fraction. All extracts and most fractions prevented attachment of both test organisms by over 60% at concentrations from 10 mg/mL to 1.25 mg/mL for *S. mutans* and from 10 mg/mL to 0.625 mg/mL for *S. sanguinis* (Figure 28, Figure 29)

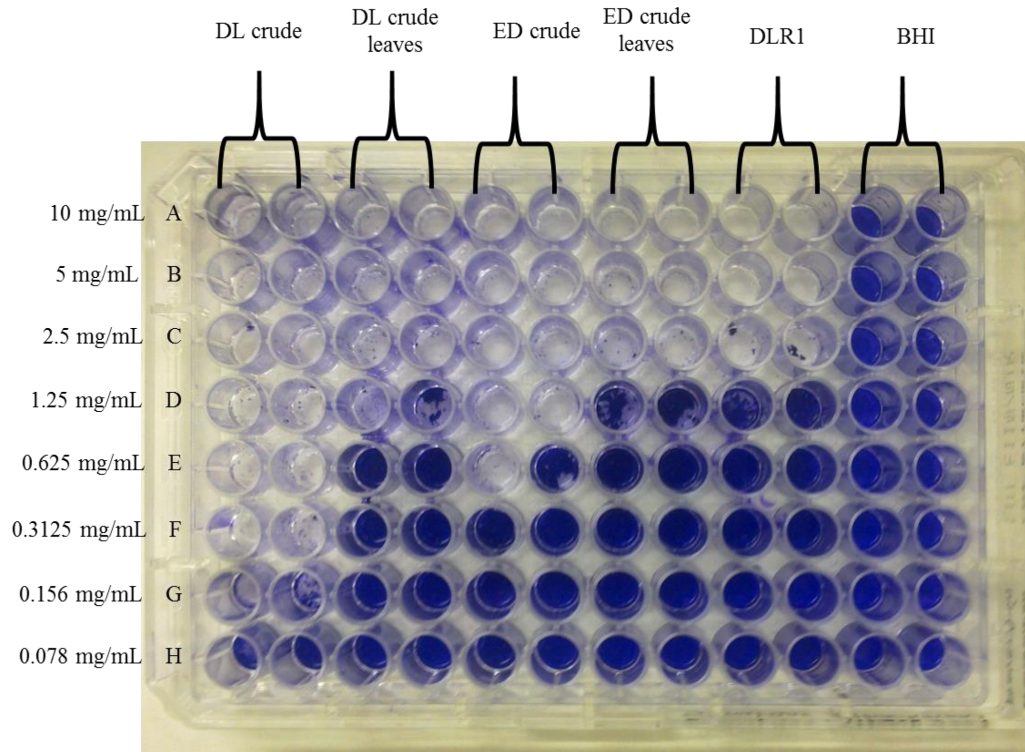


Figure 22: Representative destaining solution indicating the inhibition of *Streptococcus mutans* attachment to the polystyrene plate.

Dilution of extracts from A-H. DL crude = crude extract from *D. lycioides* roots; ED crude = crude extract from *E. divinorum* roots; DL crude leaves = crude extract from *D. lycioides* leaves; DRL1 = partially purified fraction from Sephadex column
Mean OD 595 nm values of bacterial cells bound to the plate are indicated in Figure 27.

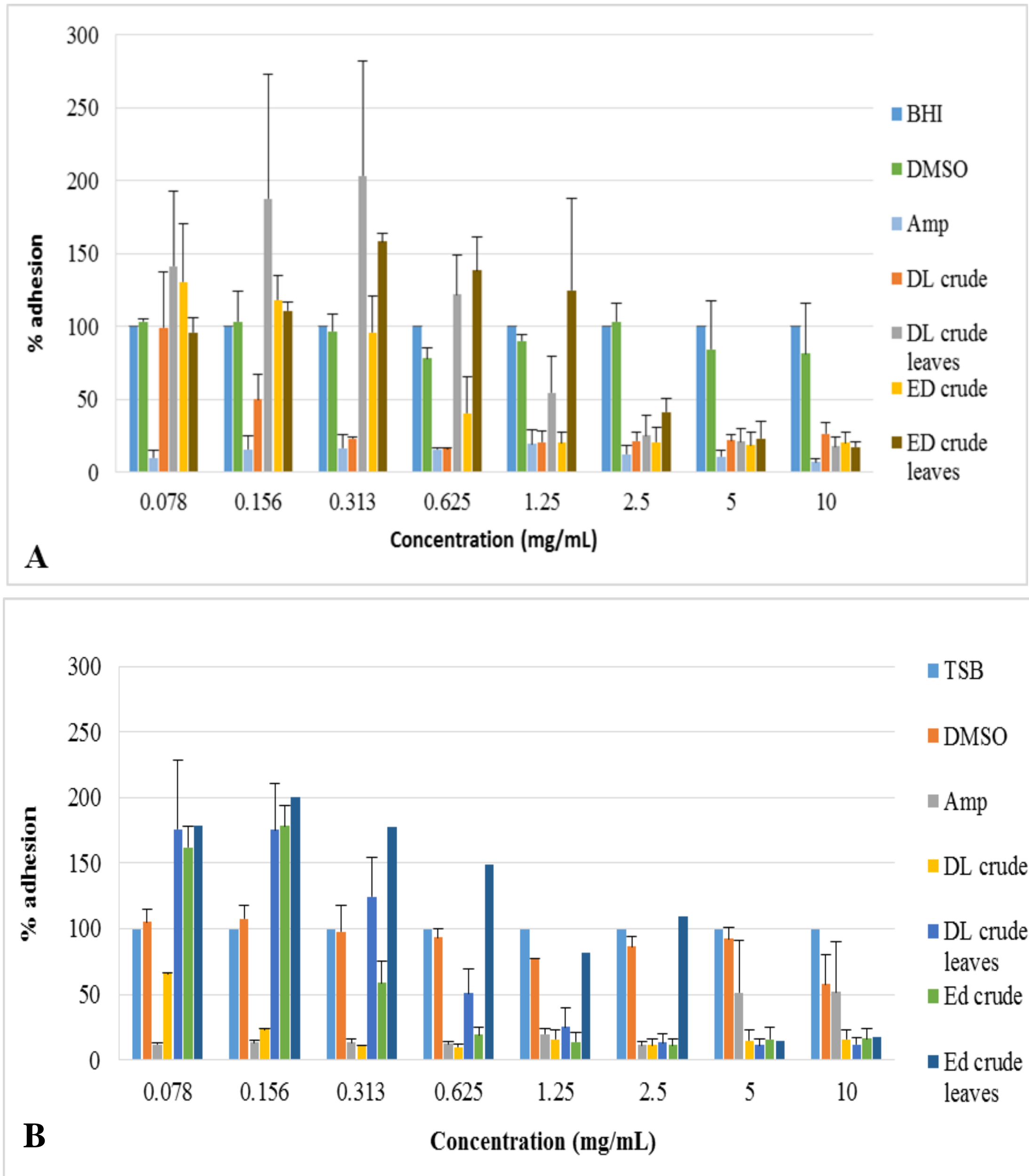
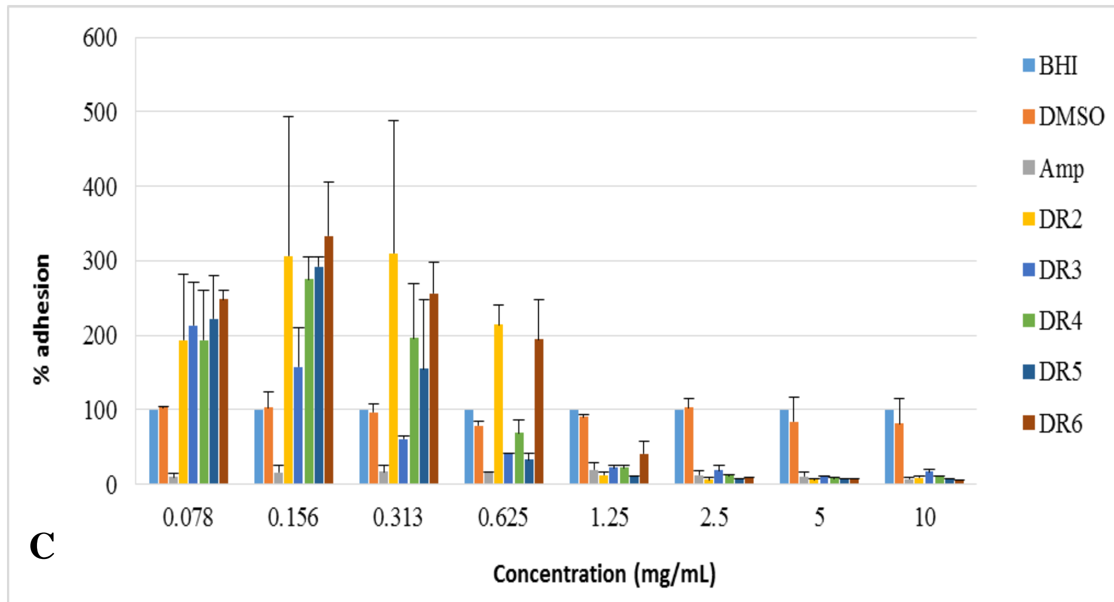


Figure 23: Percentage adhesion of A) *Streptococcus mutans* and B) *Streptococcus sanguinis* on polystyrene plates in the presence of crude extracts from *D. lycioides* and *E. divinorum*

Percentage adherence is relative to the control. DL crude = crude extract from *D. lycioides* roots; ED crude = crude extract from *E. divinorum* roots; DL crude leaves = crude extract from *D. lycioides* leaves; ED crude leaves = crude extracts from E. divinorum leaves; n = 2



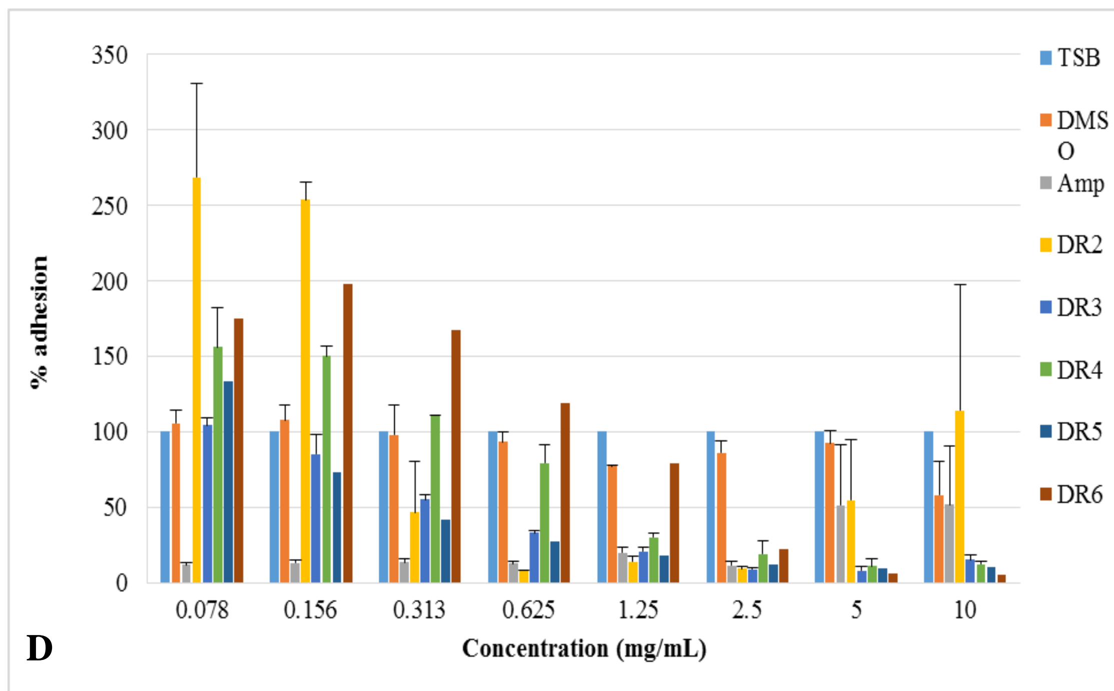
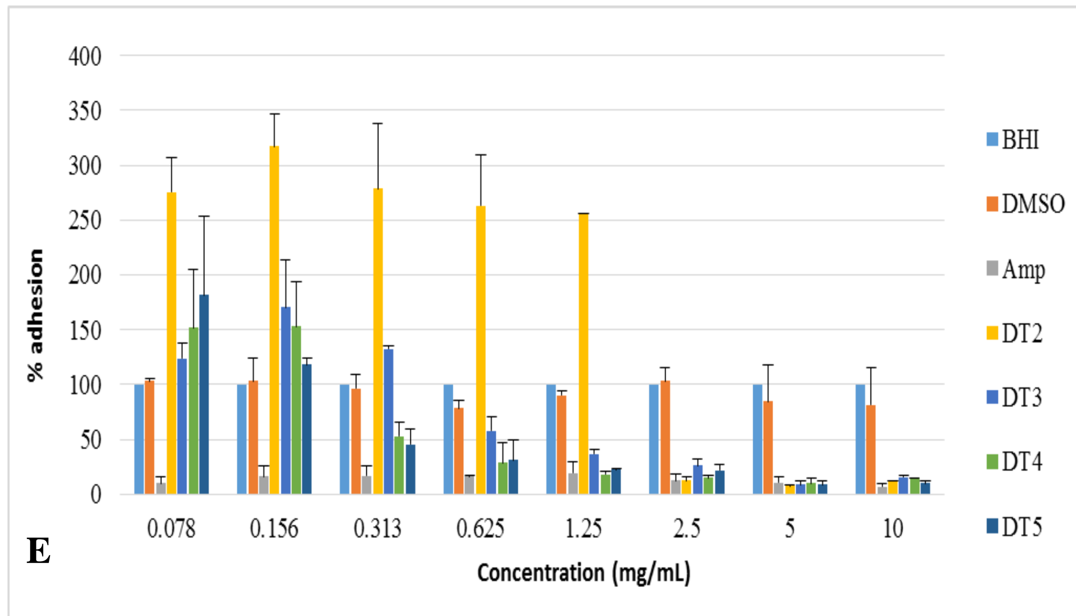


Figure 24: Percentage adhesion of C) *Streptococcus mutans* and D) S*treptococcus sanguinis* on polystyrene plates in the presence of root fractions of *Diospyros lycioides* Percentage adherence is relative to the control. DR = Fractions from the root crude extract of *D. lycioides*



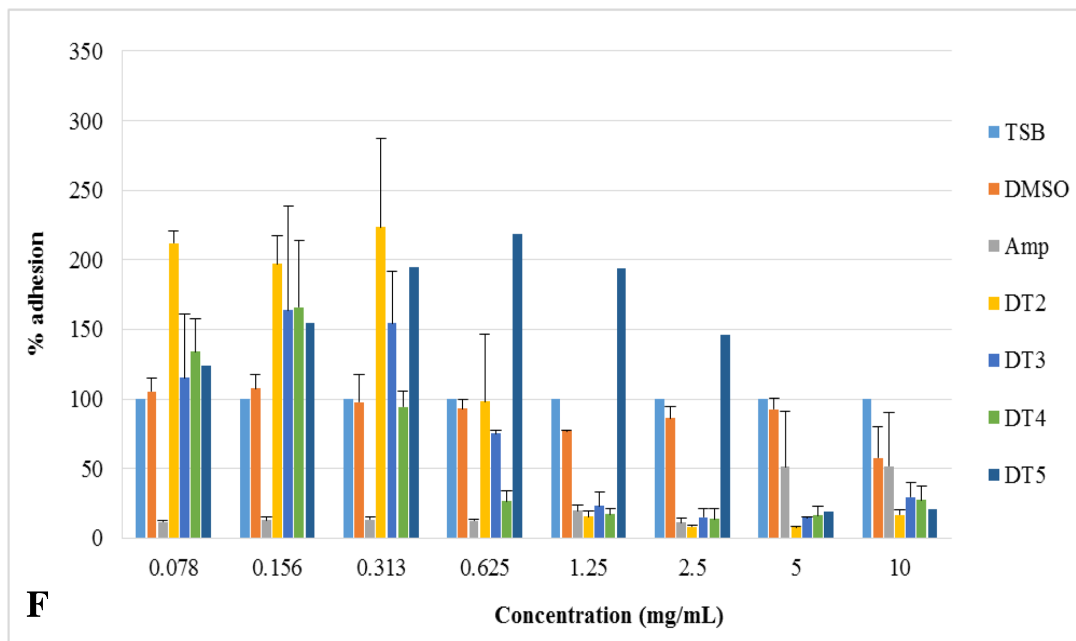
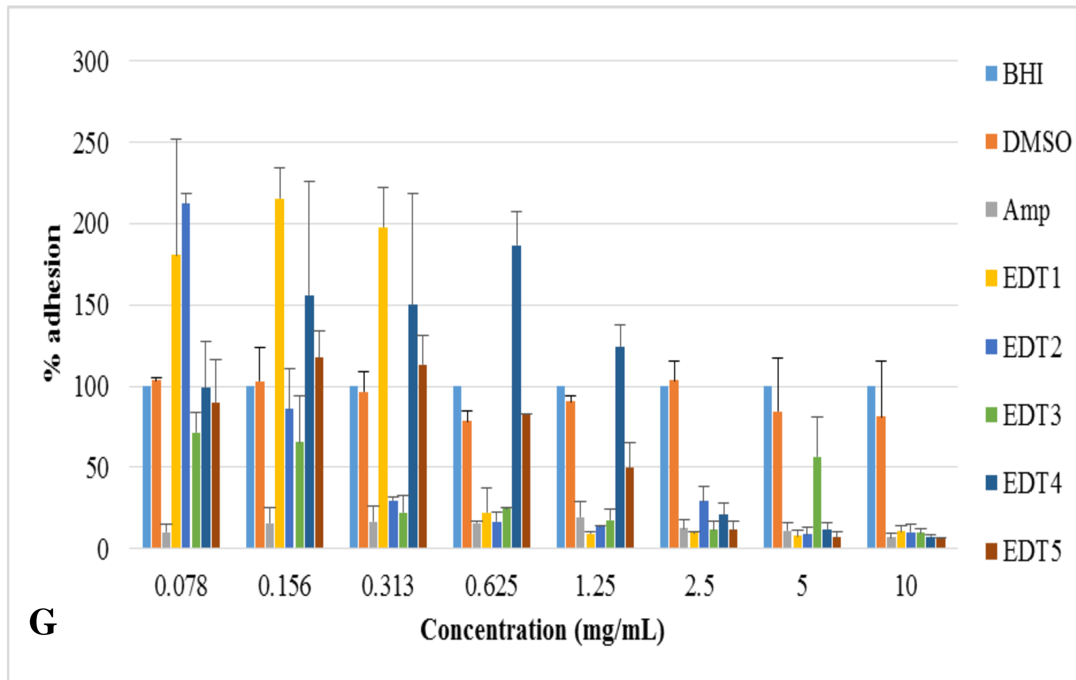


Figure 25: Percentage adhesion of E) S*treptococcus mutans* and F) *Streptococcus sanguinis* on polystyrene plates in the presence of the twig fractions of *Diospyros lycioides*

Percentage adherence is relative to the control. DT = Fractions from the twig crude extract of *D. lycioides*



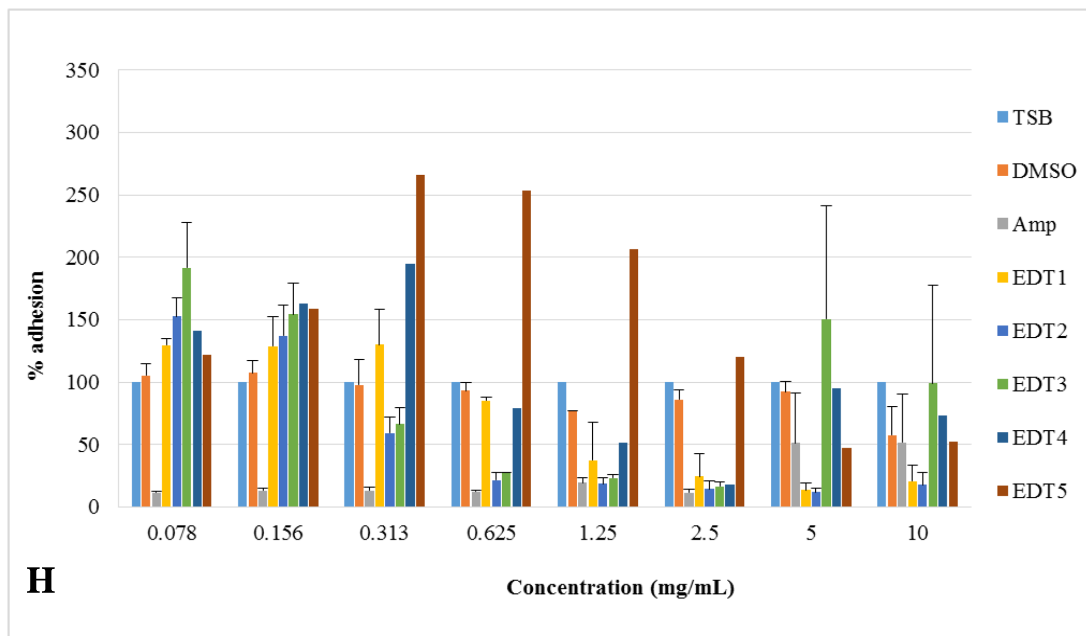


Figure 26: Percentage adhesion of G) *Streptococcus mutans* and H) *Streptococcus sanguinis* on polystyrene plates in the presence of the twig fractions of *Euclea divinorum* Percentage adherence is relative to the control. EDT = Fractions from the twig crude extract of E. divinorum

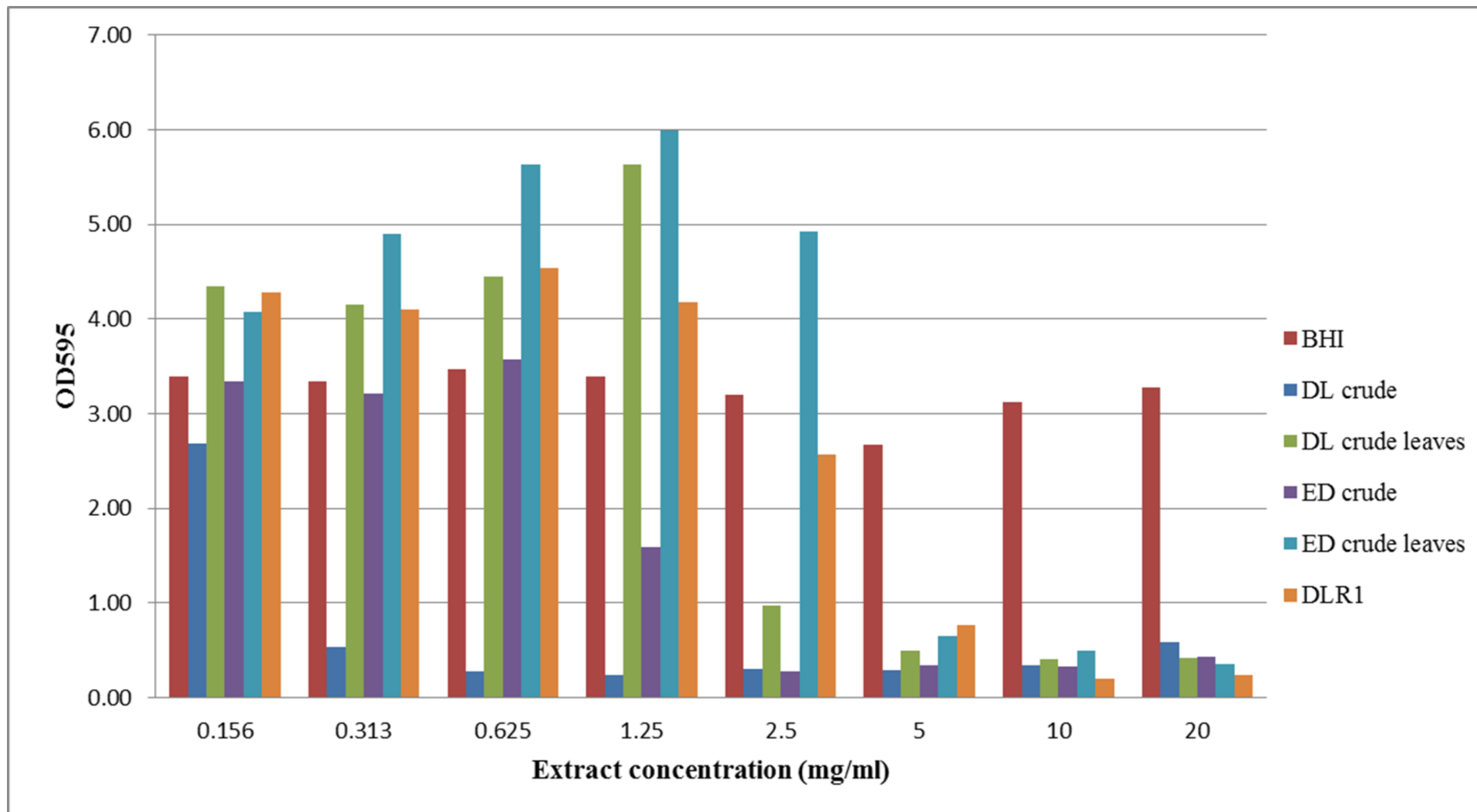


Figure 27: Actual OD 595 values indicating cell attachment of *Streptococcus mutans* to polystyrene plates in the presence of crude extracts at different concentrations.

OD_{595} = Optical density at 595 nm; n = 2; DL crude = crude extract from *D. lycioides* roots; ED crude = crude extract from *E. divinorum* roots; DL crude leaves = crude extract from *D. lycioides* leaves; ED crude leaves = crude extract from E. divinorum leaves

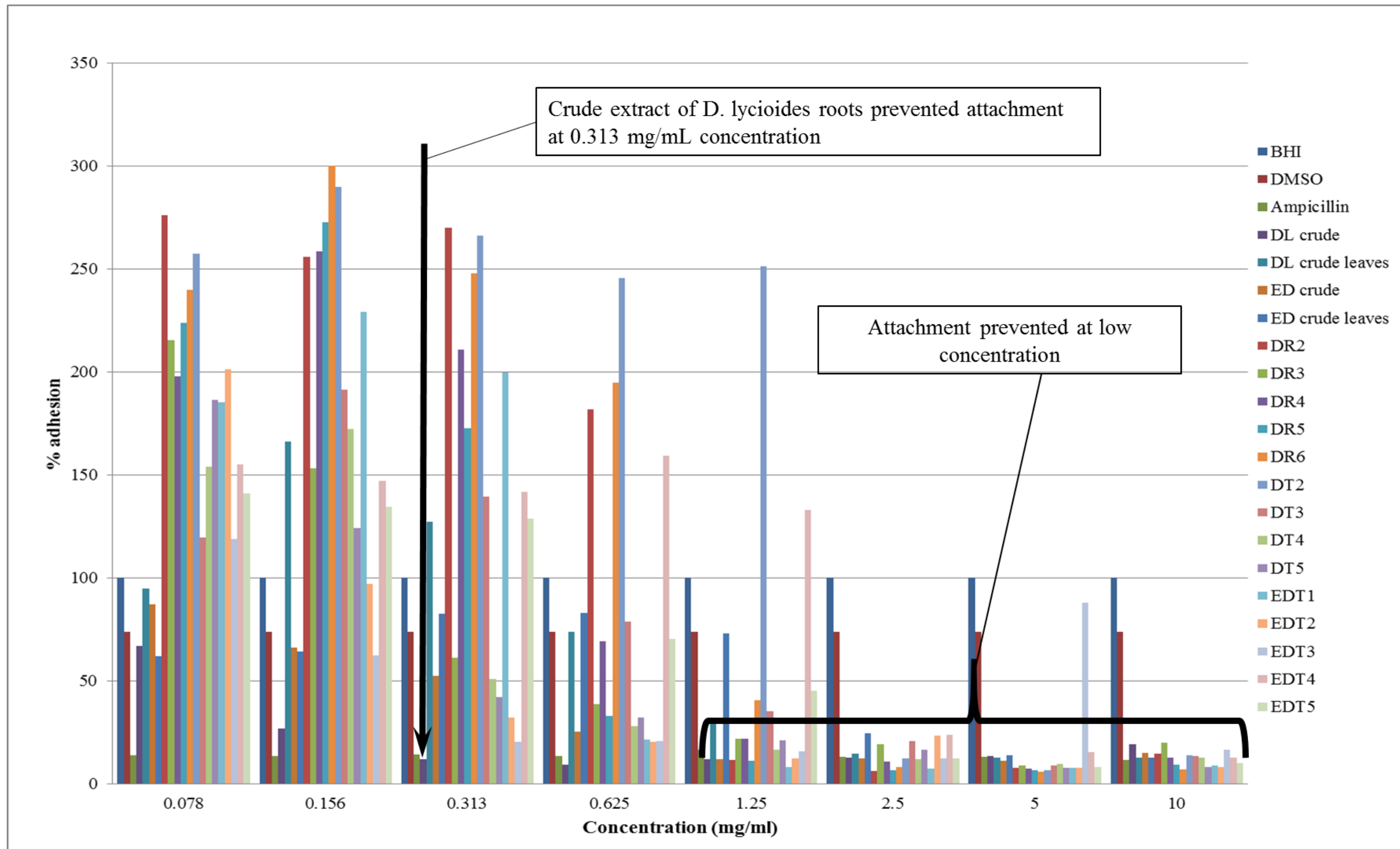


Figure 28: Cell attachment of *Streptococcus mutans* to polystyrene plates in the presence of all fractions and crude extracts at different concentrations.

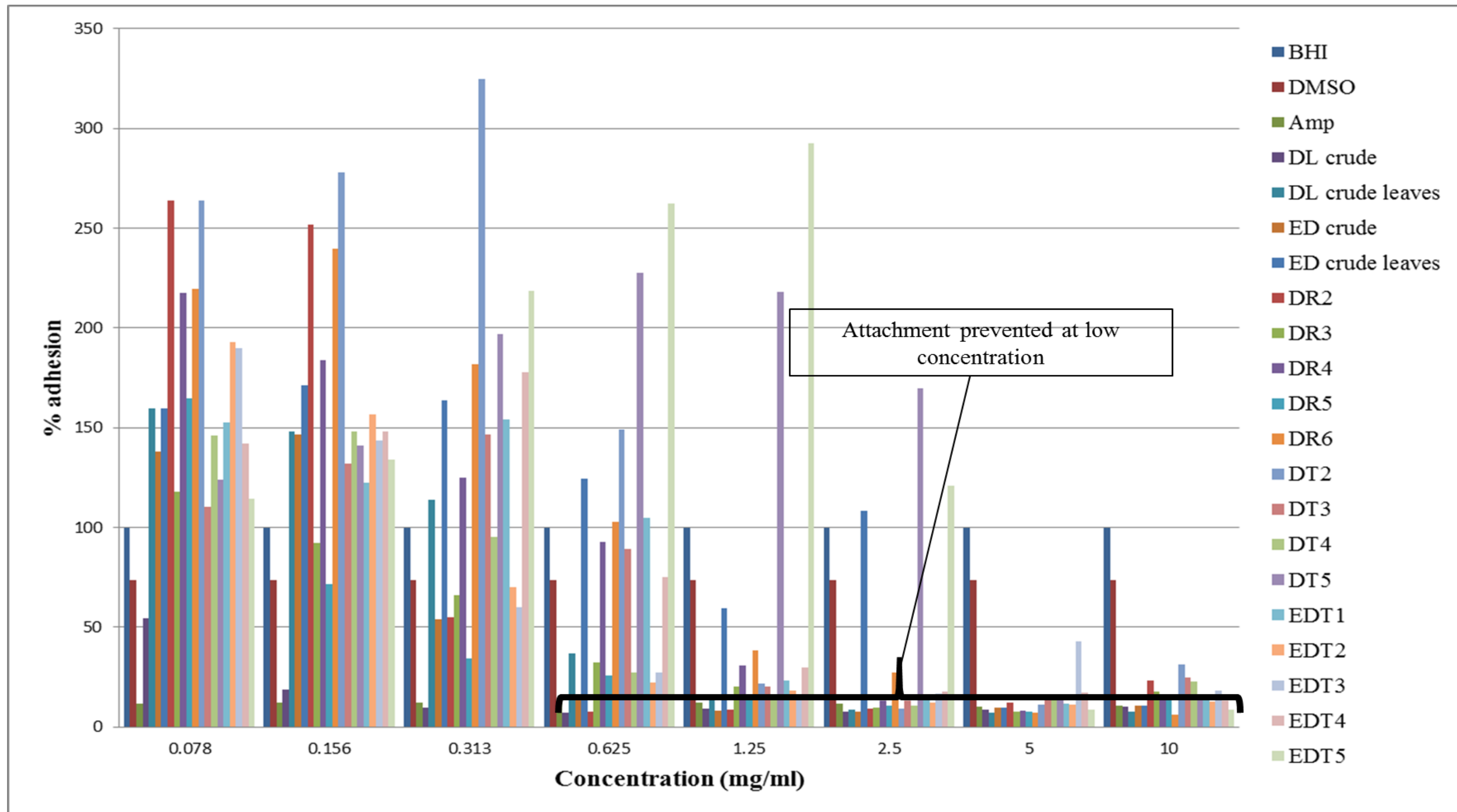


Figure 29: Cell attachment of *Streptococcus sanguinis* to polystyrene plates in the presence of all fractions and crude extracts at different concentrations.

4.2.5 Adhesion of cells to S-HA

S-HA was used as a model of bacterial attachment to teeth since it has been confirmed that S-HA pellicles are similar to natural pellicles in the salivary macromolecules they contain as well as the amino acid and carbohydrate composition (Gibbons et al. 1983). S-HA surface also has multiple binding sites for various bacteria. Cells were allowed to attach to the saliva-coated beads and then extracts and fractions were tested for their ability to remove attached cells. For S-HA the count of 10000 counts per minute (CPM) was taken as the cut off point for activity.

All crude extracts from both plants showed activity on the attached cells of both test organisms (Figure 30). In most cases, drugs are designed for a particular therapeutic application where as many biologically active natural products exhibit a wide range of activity (Dev, 2010). The results in this study shows that extracts from both plants have a range of activities because many fractions that did not affect the growth of the test organisms (DR4, DR5, DR6, DT3, DT4, DT5, EDT2, EDT3, EDT4 and EDT5), inhibited adherence of cells to the S-HA beads. All the root fractions from *D. lycioides* removed bacterial cells of both organisms that attached to the beads. Fractions DR4 and DR5 displayed the highest anti-adhesive property with a cell count of 3198 and 3737 CPM, respectively for *S. mutans*. The same fractions DR4 and DR5 gave a cell count of 3937 and 2833 CPM, respectively for *S. sanguinis*. The statistical analysis showed that there was a strong correlation between the adhesion activity of fractions DR4 and DR5, $r = 0.995$, $n = 4$, $p = 0.005$. Fraction DR6 showed poor activity when compared to other root

fractions from *D. lycioides*. Additionally Fraction DT2, the only twig fraction from *D. lycioides* that showed activity on the growth of the organisms, did not have an effect on the attached cells for both test organisms. However, all the other twig fractions of *D. lycioides* that did not prevent the growth of the organisms, removed attached cells from the beads. Fraction DT4 gave a cell count of 4473 and 3943 CPM for *S. mutans* and *S. sanguinis,* respectively (Figure 30). This suggests that extracts from these two plants are able to release intact living cells from a biofilm matrix, and thus could have an effect on the cell-to-cell and/or cell-to-surface attachment. Further, the results show that attachment of *S. sanguinis* was reduced significantly compared to that of *S. mutans* at the same concentration of bacterial cells of $OD_{660} = 1.4$. Many bacteria have several types of adhesins on their surface, therefore attachment of oral bacteria to the teeth involves interaction of these adhesins and the receptors on the teeth (Gibbons *et al*. 1983). These results suggest that the extracts contain compounds that affect the structure of the adhesion protein on the bacterial surface and thereby reducing the bacteria's adhesion to the surface. These compounds could also affect the salivary receptors on the S-HA beads and may even compete with the bacteria to bind to the S-HA beads. The crude extracts removed more attached cells than the fractions, this suggest that constituents in the extract are more effective when in combination than in isolation, suggesting a synergistic action between the fractions. These results can be used to encourage the use of chewing sticks in their natural form. Phytochemical screen results showed that both plants are rich in polyphenols, this could explain the inhibition of *S. mutans* as phenolics have been reported to inhibit biological activity of this pathogen (Yano *et al*. 2012).

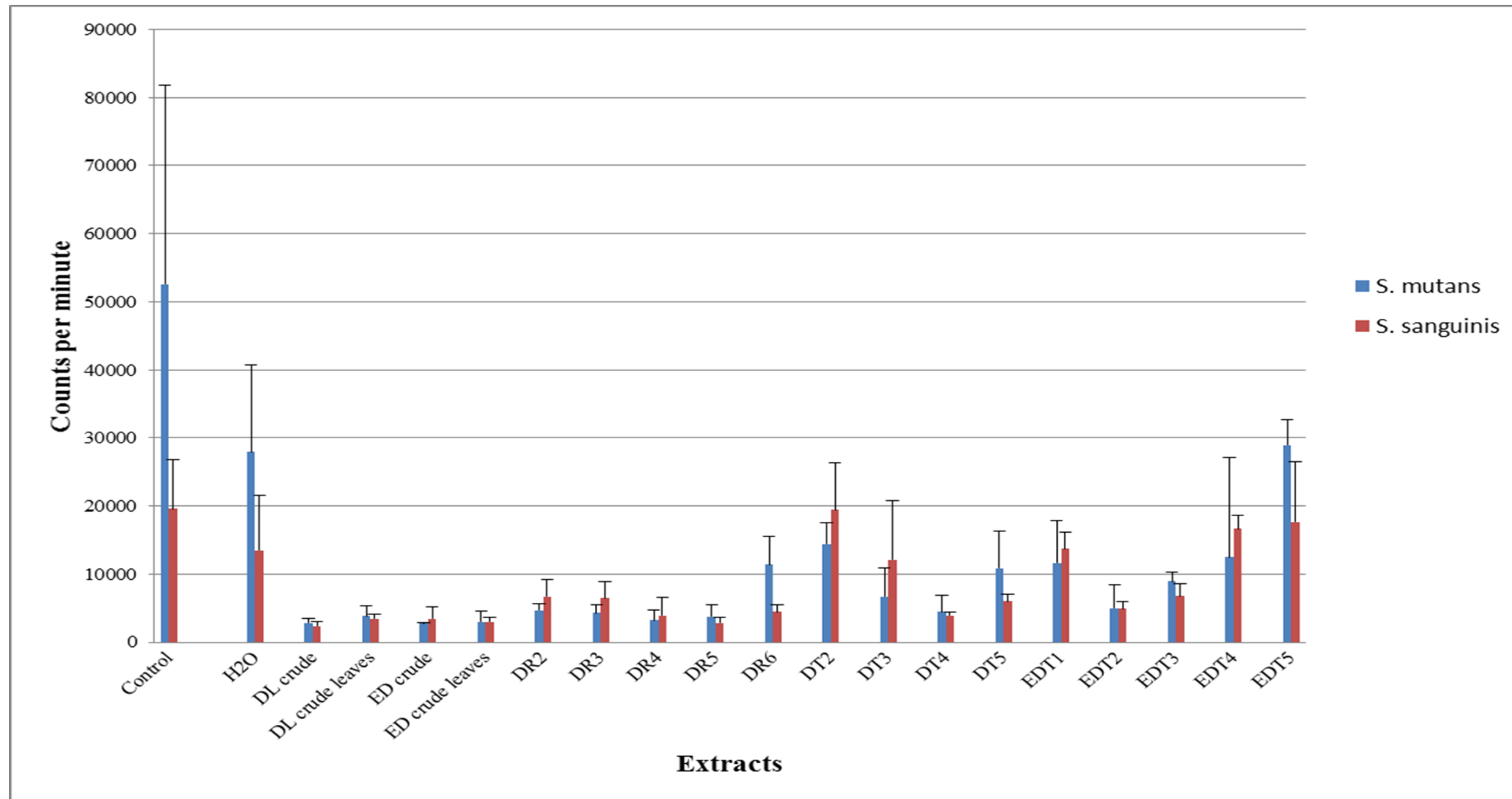


Figure 30: Inhibition of bacterial cell adhesion to saliva-coated hydroxyapatite beads by crude extracts and fractions.
The graph is a plot of actual bacterial cell count. Experiment was done in duplicate and repeated twice. Error bars indicate standard deviations; n = 2

The ability of bacteria to attach to and colonize surfaces increase their survival in different environments. Table 5 compare the tread of attachment of *S. mutans* and *S. sanguinis* to three different surfaces namely, glass (using test tubes), polystyrene (microtiter plates) and S-HA beads. The experiments were performed as outlined in sections 3.5.4.1, 3.5.4.2 and 3.5.4.3. The glass surface appeared to be a more favorable surface for *S. mutans* as a high percentage of these cells attached to it compared to the polystyrene and S-HA beads. All four crude extracts had less effect on the attachment of *S. mutans* to glass with percentage adhesion ranging between 49-91%. The results in Table 5 shows that all extracts had more effect on the attachment of *S. sanguinis* to glass and polystyrene surface compare to *S. mutans*. However, the former attached more on S-HA bead compared to *S. mutans* in the presence of these extracts (Table 5; Figure 30).

The root extract from *E. divinorum* showed high anti-adhesive property on all surfaces, preventing 95% of *S. sanguinis* and 51% *S. mutans* attaching to the glass surface. In contrast, the leaf extract from *E. divinorum* displayed very low anti-adhesive property on the glass and polystyrene surface, but prevented attachment on S-HA beads by 89% for *S. mutans* and 78% for *S. sanguinis*. The percentage value of *S. mutans* adhering to polystyrene surface in the presence of *E. divinorum* leaf extract at 1.25 mg/mL was higher than that of the control suggesting that this extract stimulated adhesion. This could also be as a result of increased binding of the crystal violet to attached cells. The root extract from *D. lycioides* also showed better anti-adhesive property than the leaf extract from the same plant. Attachment of bacterial cells to a glass surface and to polystyrene

surface in the presence of tested crude extracts showed a similar pattern, as high percentage of *S. muatans* adhered compared with *S. sanguinis*. In contract, the crude extracts removed more *S. mutans* cells from S-HA beads compared with *S. sanguinis*. Since S-HA mimics the tooth surface more than the glass and polystyrene surface, these results supports the use of these plants as teeth cleaning agents.

Table 5: Percentage of *Streptococcus mutans* and *Streptococcus sanguinis* cells attached to three different surfaces

	% adhesion					
	glass		**polystyrene plate**		**Saliva-coated hydroxyapatite beads**	
	S. mutans	*S. sanguinis*	*S. mutans*	*S. sanguinis*	*S. mutans*	*S. sanguinis*
DL crude	91	23	21	16	10	18
DL crude leaves	76	55	54	25	14	26
ED crude	49	5	20	14	10	25
ED crude leaves	69	62	125	82	11	22

Tests were done at different times with the following concentrations of extracts: Glass surface (1.4 mg/mL), polystyrene plate (1.25 mg/mL) and S-HA (1.3 mg/mL). The calculated percentage is the number of attached cells after washing off non adhering cells relative to the control. n = 2 for each experiment. DL crude = crude extract from *D. lycioides* roots; ED crude = crude extract from *E. divinorum* roots; DL crude leaves = crude extract from *D. lycioides* leaves; ED crude leaves = crude extract from E. divinorum leaves

5. CHAPTER FIVE: CONCLUSIONS

The study was done to test extracts from *D. lycioides* and *E. divinorum* for the presence of phytochemicals, antibacterial and anti-adhesive activity. The qualitative study of secondary metabolites showed the presence of flavonoids, saponins, polyphenols, tannins and terpenoids. Biological tests on extracts and fractions from both plants showed that *D. lycioides* and *E. divinorum* contain biologically active compounds which prevent the growth and/or attachment of oral pathogens *S. mutans* and *S. sanguinis*. The study confirms the use of these two plants as chewing stick in removing plaque not only mechanically but also with antibacterial and anti-adhesion effects. Furthermore, the results show that extracts from both plants can disrupt the bacteria-SHA interaction, suggesting that using chewing sticks from these plants can disrupt the attachment of bacteria to teeth surface which is the initial phase of biofilm formation and therefore prevent plaque formation. The root extracts for both plants displayed higher growth inhibition activity, with *D. lycioides* exhibiting higher activity than *E. divinorum* ($p < 0.05$). This finding supports the use of *D. lycioides* as a teeth cleaning agent since this plant species is widely distributed throughout Namibia and thus available to most people. This also supports the ethnomedicinal use of *D. lycioides* as stick users prefer using the roots over twigs.

The presence of antibacterial and anti-adhesive properties in the twigs and roots extracts strongly supports the use of these plants as teeth cleaning agents. This indicates that using chewing sticks obtained from the roots and twigs of *D. lycioides* and *E. divinorum* can

help control infections caused by *S. mutans*, *S. sanguinis* and other oral pathogens causing periodontal disease and teeth decay in humans. It is possible that compounds in these plants can disrupt interbacterial association in the mouth and thus prevent plaque formation. This study revealed the beneficial property of extracts from *D. lycioides* and *E. divinorum* roots and twigs in reducing the attachment of *S. mutans* and *S. sanguinis* to S-HA. It is the first time that the anti-adhesive activity of *D. lycioides* and *E. divinorum* is reported. Additionally, the study shows that the selected plants are more effective as anti-adhesion agents than anti-microbial agents since crude extracts and more fractions prevented adhesion of the bacteria.

5.1 Recommendation and future work

A more detailed phytochemical analysis should be done to determine the bioactive compounds present. Further investigation is needed to isolate compounds responsible for preventing attachment of cells and the removal of attached bacterial cells. Further work must be done to purify fractions DR2 and DT2 from the roots and twigs respectively, of *D. lycioides* and fraction EDT1 from the twigs of *E. divinorum*. The synergistic effect of compounds should also be studied. The mechanisms in which these extracts and fractions inhibit adherence of the study microorganisms to S-HA should also be studied. Extracts and fractions should also be tested against a large number of microorganisms to give a representative picture of the antimicrobial activity. Based on the results in this study, it can be recommended that the use of chewing sticks be encouraged.

6. CHAPTER SIX: REFERENCES

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