Phytochemical analysis of *Dodonaea viscosa* var. *angustifolia* and their beneficial effects against *Streptococcus mutans*

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DECLARATION

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

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

…… (Signature of candidate)

……..day of ……2016

DEDICATION

To my family who gave me a chance….

ABSTRACT

Introduction:

The link between *Streptococcus mutans* and dental caries is well documented. The use of natural plant products in the treatment of oral diseases is gaining popularity. One plant that has gained recognition as a source of traditional medicine is *Dodonaea viscosa* var. *angustifolia.* The aim of this study was to analyse the phytochemical constituents of *D. viscosa var. angustifolia* (DVA) and establish their beneficial effects against *S. mutans.*

Materials and methods

Cultures of *S. mutans* ATCC 10923 and SM1 were obtained from the Oral Microbiology laboratory and the DVA was collected from the Pypeklipberg, Mkhunyane Eco Reserve, South Africa. Dry DVA leaves were extracted with methanol. The crude extract was fractionated into six fractions (F1-F6) using silica gel column chromatography and thin layer chromatography. The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) of the crude extract and six fractions were determined using microtitre plate dilution technique. The effect of the crude extract and fractions on biofilm formation and acid production were investigated using standard techniques. The bioautography technique was also used to identify fractions with bioactive compounds. The most active fraction (F5) was further fractionated and purified into two subfractions, 5.1 and 5.2. Both subfractions were further screened to identify the most beneficial subfraction (5.1). Subfraction 5.1 was identified and elucidated using GC-MS and NMR. The effect of the purified compound on biofilm formation and acid production on *S. mutans* was repeated to establish reproducibility of the results. Cytotoxic effect of the crude extract and identified subfraction (5.1) was studied using human

embryonic kidney cells (HEK). The results were analyzed using Wilcoxon rank-sum test (Mann-Whitney).

Results

The MIC and MBC of the six fractions and crude extract ranged from 0.39 to 12.5 mg/ml. On preliminary screening of 6 fractions, F5 showed lowest MBC of 0.39 mg/ml and highest total activity value of 2000. In addition, at 0.2 mg/ml, F5 reduced biofilm formation by 93.3% and reduced acid production in *S. mutans*. Purification of F5 produced subfraction 5.1 and 5.2. Subfraction 5.1 showed higher antimicrobial activity (MIC-0.05 mg/ml) compared to the crude extract (MIC-0.78 mg/ml) and subfraction 5.2 (MIC-0.78 mg/ml). At a concentration of 0.05 mg/ml, subfraction 5.1 exhibited an inhibitory effect on biofilm formation at both 6 hours (94% reduction) and 24 hours (99% reduction) which was higher compared to the crude extract (87% reduction at 0.78 mg/ml after 6 hours). Subfraction 5.1 also exhibited a higher inhibitory effect on acid production compared to the crude extract. Subfraction 5.1 was identified as, 5,6,8-Trihydroxy-7,4^{l}-dimethoxyflavone. Cytotoxicity analysis of the crude extract and subfraction 5.1 (5,6,8-Trihydroxy-7,4^{l}-dimethoxyflavone) on HEK 293 cells showed IC_{50} values of 0.09 mg/ml and 0.03 mg/ml respectively.

Conclusion

Phytochemical analysis of *D. viscosa* var. *angustifolia* produced an anticariogenic constituent, 5,6,8-Trihydroxy-7,4^{l}-dimethoxyflavone. The compound showed improved antimicrobial and anticariogenic activity at lower concentrations than the crude extract. At subinhibitory concentrations, the compound significantly inhibited biofilm formation and acid production by *S. mutans*. Cytotoxicity analysis established the safe use of this newly isolated compound therefore it has potential to be used in the oral cavity to prevent dental caries.

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To my parents, Ncube family and all the family members who have always catered for my well-being, I will always be grateful.

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API	Analytical Profile Index
ATCC	American Type Culture Collection
CFU/ml	colony forming units per millilitre
C. albicans	*Candida albicans*
CHX	chlorhexidine gluconate
CO_2	carbon dioxide
D:E:F	Dicholoromethane: Ethyl acetate: Formic acid
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E:M:W	Ethyl acetate: Methanol: Water
D. viscosa* var. *angustifolia	*Dodonaea viscosa* var. *angustifolia*
F-ATPase	ATP synthase
g	grams
GC-MS	Gas chromatography-mass spectrometry
GTFs	glucosyltransferases
HEK	Human Embryonic Kidney cells
HIV	Human Immunodeficiency Virus
hrs	hours
INT	Iodonitrotetrazolium
IPS	Intracellular polysaccharides
IR	Infrared

LC	liquid chromatography
LDH	lactate dehydrogenase
MBC	minimum bactericidal concentration
mg	milligrams
mg/ml	milligrams per millilitre
MIC	minimum inhibitory concentration
min	minutes
ml	millilitre
MTT	Dimethythiazole Diphenyltetrazolium Bromide
NPs	Nanoparticles
nm	nanometres
OD	optical density
PBS	phosphate buffered saline
Rf	Retention factor
rpm	revolutions per minute
S. mutans	*Streptococcus mutans*
spp	species
T:E:A	Toluene: Ethanol: Ammonium hydroxide
TLC	Thin layer chromatography
WHO	World Health Organizations
µl	microlitres

CHAPTER 1

Introduction

The impact of oral diseases on mankind continues to be a major health problem. Dental caries and periodontitis are the most prevalent oral ailments. Regardless of advanced developments in the health care system of people living in developed nations, as high as 90% of school aged children have dental caries and a high percentage of adults are also affected (Petersen, 2003). Up to 10% of public health care expenditure in industrialized countries is also related to curative dental care; hence its economic impact factor can hardly be ignored (Rautemaa *et al*., 2007).

The link between oral microorganisms and oral diseases is well documented. The onset of dental caries primarily involves aciduric and acidogenic Gram-positive bacteria collectively called mutans streptococci. These facultative anaerobes metabolize dietary sucrose and produce organic acids, mostly lactic acid, which dissolves the teeth enamel leading to eventual teeth decay (Palombo, 2009).

The use of natural plant products in the treatment of oral diseases is gaining popularity. Henley-Smith *et al* (2013) reports that, in Burkina Faso alone, over 62 plant species which belong to 29 families have been documented to treat oral diseases, which include dental caries. One plant that has been identified as a potential source of traditional medicine in the prevention of dental caries is *Dodonaea viscosa* var. *angustifolia* (DVA).

Various studies have been done on the potential use of *D. viscosa* var. *angustifolia* leaves as an anticariogenic agent. It is important to note though that most of these *D. viscosa* var. *angustifolia* studies have focused mainly on the use of crude extracts. Other studies have established the safe use of *D. viscosa* var. *angustifolia* leaves. The aim of this study therefore was to go beyond the screening of *D. viscosa* var. *angustifolia* as an antimicrobial agent by identifying chemical constituent responsible for its anticariogenic properties against the dental caries causing bacteria, *S. mutans*. Optimized solvent systems were used for extracting constituent phytochemicals followed by bioassays to evaluate the bioactivities of the fractions against *S. mutans* and its virulence factors.

1. Literature Review

1.1 Pharmacognosy

Pharmacognosy is the study of medicines from natural products and their use in the improvement of health. Pharmacognosy used to be focused mainly on the morphological description of plants, but it has now extended to include the molecular science aspect of their active ingredients, mode of action and application. Improvements in isolation and structure determination techniques over the years has facilitated the shift from focusing on plant species used in traditional medicine only, to include exploitation of the huge molecular diversity found in plants, microorganisms and animals (Kato and Pezzuto, 2011).

The demand for phytomedicine is increasing which is supported by the huge investments that are being made by the private sector. Plant-based drugs which include, Camptothecin, Vinblastine, Vincristine, and Paclitaxel exceeded 65 billion dollars in sales in 2003 (Gordazila, 2009). For an industry that is producing billions of dollars per annum, it is interesting to note that as recent as 2008, Africa contributed only 1 % in sales despite the fact that 75 % of the population is still dependent on traditional herbal medicine (Henley-Smith, 2013).

There are three major disciplines in the search for new bioactive molecules from plants. The first discipline is evidence based phytotherapy approach. This technique is simpler in the sense that the search for new lead compounds is guided by the way the plants are used in traditional medicine (Kato and Pezzuto, 2011). According to Fabricant and Fansworth (2001), 88 chemical constituents in modern therapy that have been isolated from 72

medicinal plants possess the same or similar beneficial purpose as their original ethno-medicinal use. The second discipline is chemotaxonomy in which plants that fall in the same taxonomic group as those that have exhibited the presence of specific active compounds are targeted. Finally, the third discipline is high throughput-screening. This is a random and systematic evaluation of vast libraries of chemicals likely to control a specific biological target (Kato and Pezzuto, 2011). The method has several advantages in that it is fast and a vast number of assays can be done in a single day (Bruno *et al*, 2014).

1.1.1 Plant chemistry (phytochemistry)

Phytochemistry refers to the chemistry of natural products. In essence, phytochemistry covers the wide range of different types of organic substances that are produced by plants (Kar, 2007). Prior to the 1800's, relatively small progress had been made in the field of phytochemistry due to the lack of techniques to isolate and elucidate different organic compounds from plants. Compounds like starch, camphor and benzoic acid had been isolated as the techniques involved were simple. Narcotine, the first alkaloid was isolated followed by morphine, strychnine and emetine (Evans, 2002). Currently, with the dawn of advanced analytical procedures, the detailed phytochemical study of an unknown plant can be carried out from elucidation of the structure of the isolated constituents to the detailed study of their biological characteristics (Kar, 2007).

The vast numbers of chemical compounds that exist in the plant-kingdom in one form or the other are called 'constituents'. There are two main categories of constituents, namely, active constituents and inert constituents. Active constituents are chemical compounds that are responsible for the actual therapeutic activities which include pharmacological and anti-

microbial characteristics. Preliminary screening of plant crude extracts is usually done to determine the presence of these active constituents. Active constituents include glycosides alkaloids, steroids and terpenoids. Inert constituents are chemical compounds that exist in plant and animal kingdoms but they not do have any definite therapeutic uses. They can however, serve as adjuncts in drug development or in surgery. Examples are cellulose, starch, albumin, keratin and some chemicals used in food coloring (Kar, 2007). This group of constituents is also known as essential nutrients (Pengelly, 1996). Figure 1.1 gives a general outline of the stages involved in extracting, isolating and elucidating chemical constituents.

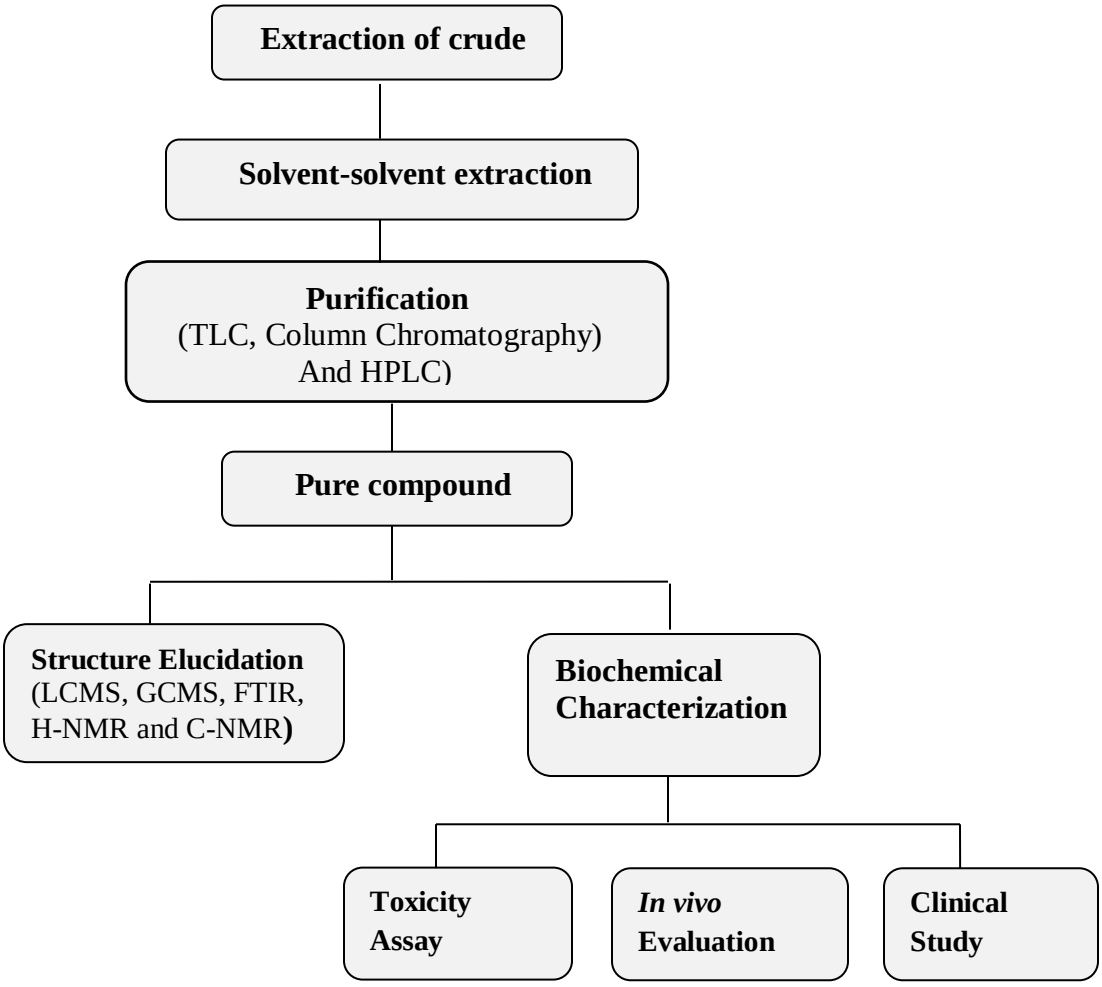

Figure 1.1: General approaches in extraction, isolation and elucidation of chemical constituents (Sasidharan *et al*., 2011).

1.1.2 Drug Biosynthesis (Biogenesis)

Drug biosynthesis is the study of biochemical pathways that lead to the formation of active constituents or secondary constituents. With the dawn of the use of isotopes in labeling organic compounds in the fifties, it became possible to prove scientifically that amino acids together with their corresponding derivatives more or less acted as precursors of complex alkaloids. This had been hypothesized by Trier in 1912 (Kar, 2007). The structure of the plant cells plays an important role in biochemical reactions. The various organelles in the cell and their arrangement promote variability in chemical environments within the cell and increases the surface area needed for biochemical reactions (Evans, 2002).

Pharmacologically active chemical constituents from plants are essentially comprised of large and complex molecules that have one or more chemical functional groups that are responsible for characteristic features of phenols, alcohols aldehydes, esters, oxides, ketones and organic acids (Kar, 2007). The synthesis of these molecules with functional groups is mostly enzyme dependent. These enzymes act as organic catalysts and recently, organic chemists are exploiting their potential ability to catalyze single-step transformations. They have observed that these enzymes can achieve accurate stereo-chemical characteristics which are important for drug synthesis (Evans, 2002). Figure 1.2 gives a summary of the biosynthetic pathways and inter-relationships that lead to the formation of various secondary constituents.

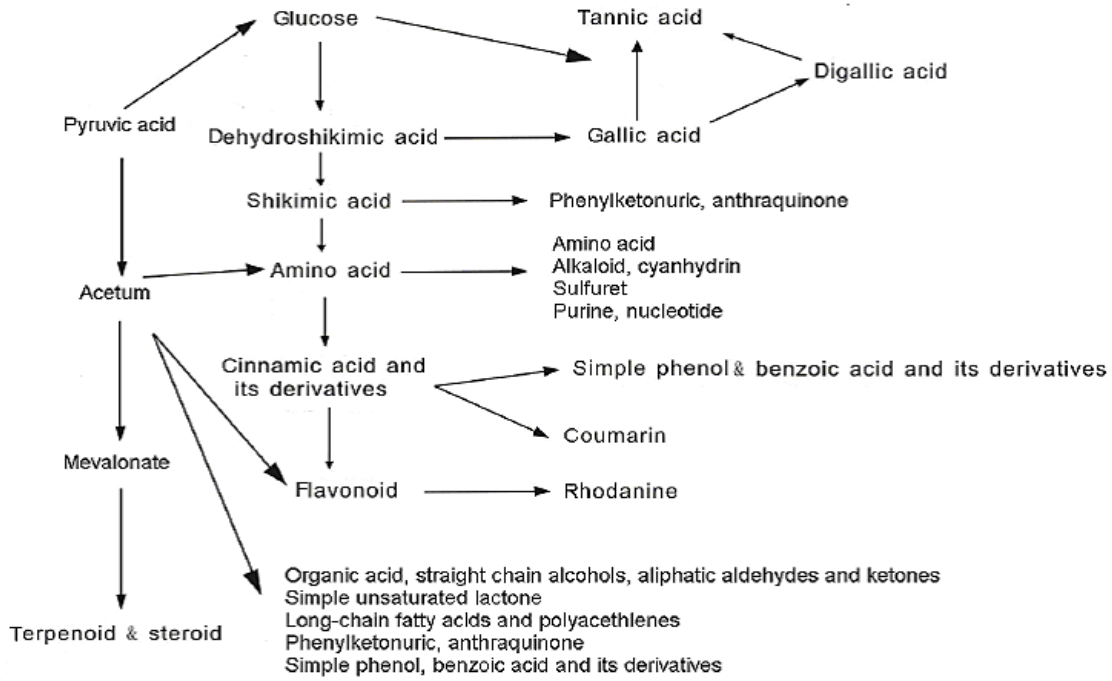

Figure 1.2: Biosynthetic pathways and their interrelationships (El-Hadary and Chung, 2013).

1.2 Phytotherapy in dentistry

In recent years there has been a rise in the global search for alternative medicine in the treatment of oral diseases like dental caries, periodontal diseases, soft tissue conditions and oral candidiasis. The main reason that has been documented is the increase in pathogenic resistance to currently existing antibiotics and chemotherapeutics. There are several synthetic medicines that have been introduced in the market to treat oral diseases, examples being cetylpyridinium chloride, amine fluorides and other products containing these agents (Knoll-Kohler and Stiebel, 2002). However, the use of these products has been shown to have toxic adverse effects which include teeth staining. Ethanol which is found on some of the existing mouth rinses in the market has been linked to oral cancer (Rodrigues *et al*, 2007). The objective of these drug discovery programs in phytotherapy therefore is to produce safe herbal products that are effective and also economically viable for use in developing

countries (Palombo, 2009). Improving drug delivery systems has also been an area of focus. Nanotechnology and phototherapy have shown considerable potential in enhancing drug delivery in the prevention of dental biofilms. When drugs are used in combination with nanoparticles (NPs) which are minute in size, it increases their solubility and ability to adhere to biological surfaces thereby improving bioavailability and therapeutic activity (Bamrungsap *et al*, 2012). Phototherapy is also based on the use of light sensitive nanoparticles that can be activated at the targeted site using laser light. This enhances the efficacy of the drug (Adnan *et al*, 2016).

Herbal products are used in dentistry as anti-inflammatory agents, antibiotics, analgesics, sedative agents and also as endodontic irrigants (Calixto, 2000). There is a wide variety of plant products that are added to dentifrice and mouth rinsing solutions to prevent oral conditions like caries or biofilm formation (Pannuti *et al*, 2003). Parodontax® (GlaxoSmithKline, Middlesex, UK) a herbal medicine widely used in dentistry contains various herbal products that include: *Matricaria chamomilla* (Asteraceae) which possesses anti-inflammatory properties that reduce gingival inflammation; *Echinacea purpurea* (Asteraceae) which stimulates the immune response; *Salvia officinalis* (Lamiaceae) which has anti-hemorrhagic properties; *Commiphora myrrha* (Burseraceae) has natural antiseptic properties and *Mentha piperita* (Lamiaceae) has analgesic, antiseptic and anti-inflammatory properties (Groppo *et al*, 2008).

A noteworthy reduction in gingival index was reported by Pistorius *et al* (2003) from the use of a herbal-based mouth rinse which contained *M. piperita* (menthol), *S. officinalis*, *M. chamomilla*, *C. myrrha*, *Eugenia caryophyllus* (Myrtaceae), *Carum carvi* (Umbelliferae) and

E. purpurea. From this study they concluded that the daily use of this mouth rinse as an adjunctive could reduce gingival inflammation in patients with periodontal ailments (Pistorius *et al*, 2003). *Aloe vera* has been shown to treat aphthous ulcers and is also able to reduce chances of developing alveolar osteitis after third molar extraction surgeries (Wynn, 2005). Scherer *et al* (1998) established that a mouth rinse containing *Aloe vera* was able to reduce gingival bleeding and gingival inflammation significantly. Kaim *et al* (1998) further proved that the mouth rinse was more effective than Listerine® in reducing anaerobic, aerobic and microaerophilic bacterial counts.

In an investigative study done by Almas (2002) to compare the effect of 0.2% chlorhexidine and 50% *Salvadora persia* on human dentine surfaces, he concluded that *Salvadora Persia* was more efficient in removing biofilms from teeth than chlorhexidine. In another comparative clinical study, *Allium sativum* (tea tree) was shown to be more effective as an antimicrobial agent followed by garlic and chlorhexidine which had the same results (Groppo *et al*., 2002). Tea tree oil also displayed antimicrobial activity against a range of oral microorganisms including *Candida albicans* (Wilkinson and Cavanagh, 2005). *Dodonaea viscosa* var. *angustifolia* is another plant that is gaining recognition as a potential source of antimicrobial agents in oral hygiene.

1.3 *Dodonaea viscosa var. angustifolia (DVA)*

Dodonaea viscosa falls under division Angiosperm, subclass Dicotyledonae and Sapindaceae family of plants. The genus *Dodonaea* was named after a Dutch botanist, Rembert Dodoens. The term *viscosa* means sticky and refers to the young growing tips which contain flavonoids on their surface giving them a shiny appearance.

Figure 1.3: *Dodonaea viscosa* var. *angustifolia* flowers and leaves (Ebedes, 2015)

Dodonaea viscosa is also called by other common terms like Giant Hop-Bush, Candlewood, Sand olive, Native Hop Bush and Sticky Hop-Bush (Anbg, 2006). Common vernacular names include 'mutata-vhana' and mutepipuna (Harris, 2012). Figure 1.3 shows a picture of *D. viscosa* var. *angustifolia* leaves and flowers.

Dodonaea viscosa var. *angustifolia* is an ever-green shrub that grows by spreading or trees up to a height of 5 metres long. Its bark is light grey and finely fissured with branchlets that are angled to flattened and are usually covered with minute soft hairs. The leaves are droopy and shiny light green above and paler green below. It has yellowish green flowers that are replaced by yellow or reddish clusters of fruits with papery wings. In a conducive environment, DVA needs minimal supervision to grow and produce flowers especially during the autumn to winter seasons (Harris, 2012). *Dodonaea viscosa* var. *angustifolia* flowers are either unisexual or bisexual, with very short filaments and oblong anthers which range from 3-3.5 mm in length for males, 2-2.5 in bisexual flowers and might be completely absent in females (Walsh and Entwisli, 1996).

Australia has approximately 60 species belonging to the genus *Dodonaea*. The subspecies *D. viscosa* is reported to have seven subspecies which are distinctly geographically located. There are two varieties that are mainly found in tropical Africa, the coastal var. *viscosa* and var. *angustifolia* which is found mainly inland (Turnbull, 1986). *Dodonaea viscosa* survives well in dry, sunny conditions and thrives in sand or loamy soils. It flowers almost throughout the year but mainly in summer and spring. They pollinate using the wind and bees which have been reported to collect the pollen. The fruits reach full maturity approximately 10-11 months following flowering (ICRAf, 1992).

In South Africa, the plant has two recognized taxa, *D. viscosa* var. *angustifolia* and *D. viscosa* var. *viscosa*. It is found in abundance from the Western Cape through to the coast of Namaqualand, from KwaZulu-Natal to Eastern Cape. In general, the plant is widely distributed except for the central part of the country (Harris, 2012).

1.3.1 Biological activity and cultural aspects of *Dodonaea viscosa* var. *angustifolia*

Dodonaea viscosa roots have been reported as having soil-binding properties and are effective in controlling soil erosion and stabilization of sand dunes. *Dodonaea viscosa* var. *angustifolia* is still one of the most widely used medicinal plants especially in rural areas. It has several traditional uses which include treatment of stomach aches, measles, influenza and colds. Early settlers in the Cape region are said to have made decoctions using DVA leaf tips to treat fever. The Khoi-Khoi prepared a concoction using roots to treat influenza and colds. Some people gargle using the decoction from leaves for oral thrush and sore throats. In

Namaqualand, they boil the leaves, let them steep, strain them and use the extract to treat colds, influenza and also induce sweating (Harris, 2012).

The extract from the leaves is also used for conditions like rheumatism, haemorrhoids and skin conditions like rash. The leaves also have pain-killing, wound healing and diaphoretic (sweat-promoting) qualities and are useful for skin rashes. An alcoholic crude extract from DVA leaves has been shown to prevent constriction of coronaries and spasmolytic reflexes on smooth muscles and intestines. These alcoholic extracts from DVA are reported to be more effective than the aqueous extract in minimizing spasms caused by barium chloride, phosphates, histamine acid and acetyl choline (Harbone, 1998). Its leaves are also used as painkillers to alleviate pain from toothaches and headaches. Quershi *et al* (2008) reported the treatment of toothaches using a decoction of *D. viscosa* leaves as a mouth wash by a herbal physician. The traditional use of *Dodonaea viscosa* as a gargle for oral infections was also reported by Van Vuuren (2008). It is also common practice in rural areas to use medicinal plants as toothbrush sticks for cleaning teeth. The leaf juice is effective in treating trachoma and the leaves can be crushed to powder which is used to remove roundworms. *Dodonaea viscosa* var. *angustifolia* leaves are also used to impart a bitter flavour in beer production (Wagner *et al*., 1987).

Khurram *et al* (2009) reported that the crude extracts of ethanol, dichloromethane, n-hexane, n-butanol, ethyl acetate and aqueous extracts of DVA presented inhibition against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Micrococcus luteus.* Patel *at el* (2009) reported activity against *C. albicans*; the crude extract exerted its effect by

inhibiting the adherence of *C. albicans* cells to oral epithelia which is essential for its pathogenicity. Rojas *et al* (1991) observed that *D. viscosa* was able to significantly inhibit the growth of *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Candida albicans* and *Pseudomonas aeruginosa* at a concentration of 20 mg/ml. All parts of *D. viscosa* were able to inhibit the growth of *Vibrio cholerae* with the maximum zone of inhibition of 26mm being observed from the methanol extract (Prakash *et al*., 2012).

Dodonaea viscosa var. *angustifolia* crude extract was shown to have time dependent anti-*S. mutans* properties. Sub-therapeutic concentrations of DVA crude extract inhibited 95%, 97% and 99% of plaque formation after 6, 24 and 30 hours exposure respectively (Naidoo *et al*., 2012). Based on preliminary chemical analysis the authors suggested that polyphenols such as catechin, chalcones with trimethoxyphenyl group and tannin with 4-O-β-D-xylopyranoside may have been responsible for this anti-plaque characteristic.

Patel *et al* (2013) reported that crude extracts from DVA significantly reduced acid production ($P \leq 0.01$) by planktonic *S. mutans* cells. The acid produced by *S. mutans* from metabolising dietary sucrose is the one responsible for decalcification of teeth leading to dental caries. The study aimed to determine which *D. viscosa* var. *angustifolia* phytochemicals were responsible for its antimicrobial properties against *S. mutans.* The results will justify the potential use of *D. viscosa* var. *angustifolia* in the prevention of dental caries.

1.3.2 Major chemical constituents of DVA

It is important to know the individual chemical constituents of plants used for medicinal purposes. The phytochemical knowledge is essential in optimizing the extraction and purification process. It is also important in getting to understand the pharmacological relevance and any toxicity the plant might have. Wagner (2005) mentions the hypothesis that any therapeutic activity the plant might have is due to synergistic combinations of various chemical constituents rather than single purified compounds.

1.3.2.1 Flavonoids

Flavonoid refers to a class of non-nitrogenous phenolic pigments that are mainly found in plants (Gwin *et al*., 1986). Figure 1.4 shows the basic structures of common flavonoids. They form part of natural antioxidants which are biologically active constituents and contribute to the therapeutic characteristics of plants. *Dodonaea viscosa* var. *angustifolia* contains bioflavonoids that are therapeutically used to strengthen capillaries and aid blood circulation.

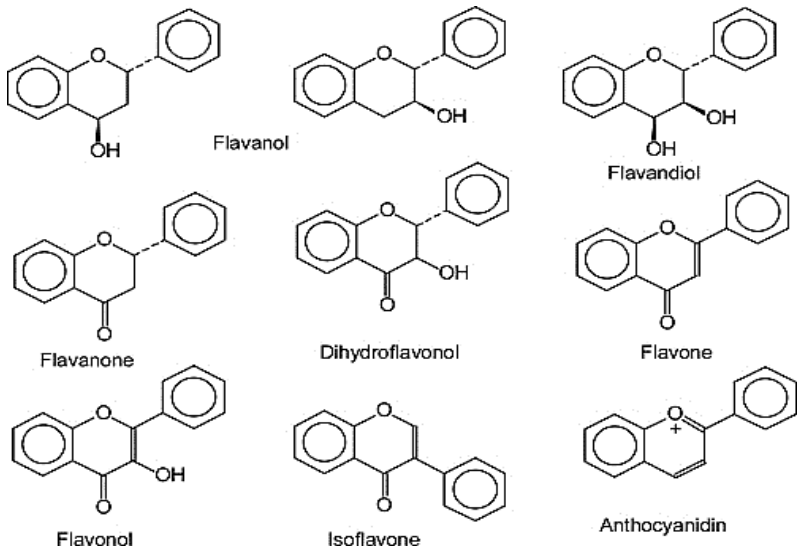

Figure 1.4: Basic structures of common flavonoids (Halbwirth, 2010)

Structurally, flavonoids consist of more than one benzene ring and are derived from parent compounds known as flavans (Shanthi *et al*., 2014). The most common flavonoids in nearly 70% of the plants are quercetin, quercitrin and kaempferol. The less common groups include flavanes, flavones, anthocyanidins, flavonols, proanthocyanidins, catechins and dihydroflavones. Most flavonoids are water soluble and frequently occur in plants as sugar derivatives called glycosides while those that do not have sugar moieties are called aglycones (Doughari, 2012).

Dodonaea viscosa var. *angustifolia* (DVA) leaves are reported to contain a prenylated flavonoid, viscosol. The plant also contains hautriwaic acid, sakunaretin, 5, 6, 4'-trihydroxy-3,7-dimethoxy flavones and ent-15,16-epoxy-3ß,8a-dihydroxy-9a-H-labda-13,14-diene. Isorhamnetin-3-rhamnosyl galactoside which is usually present in the aerial parts of DVA has been shown to lower blood sugar effects (Shanthi, 2014). Methanolic extracts from the plant *Artocarpus heterophyllus* were shown to contain two active isoprenylflavones, artcarpacin and artocarpesin. These flavonoids were able to inhibit the growth of cariogenic bacteria which included *S. mutans* and other oral lactobacilli, Actinomyces at a minimum inhibitory concentration (MIC) of 12.5-3.13 µg/ml (Palombo, 2009).

Flavonone phytoelaxins isolated from the plant *Sophora exigua* (Leguminosae) were reported to have inhibited the growth of various caries causing bacteria with the active flavonoid being 5,7,2,4- tetrahydroxy-8-lavandulylflavanone. The isoflavonoid, erycristagallin isolated from the plant *Erythrina variegata was* shown to inhibit cariogenic bacteria at an MIC value

of 1.56-6.25 µg/ml. Its efficacy as an antimicrobial agent was confirmed by the fact that it inhibited the uptake of glucose and radio-labeled thymidine in *S. mutans* (Palombo, 2009).

Using a bioassay guided fractionation technique; the methanolic extract of the bark of *Morus alba* (Moraceae) showed activity against *S. mutans* and the active ingredient was shown to be the flavoinoid, kuwanon G. It proved to be efficient with an MIC value of 8µg/ml against *S. mutans*. This value is comparable to commercially used oral medicines, chlorhexidine and vancomycin which have an MIC value of 1 µg/ml.

When time-kill assays were conducted using kuwanon G, a concentration of 20 µg/ml was able to completely inactivate *S. mutans* within a minute. More time-kill assays using other bacteria led to the conclusion that kuwanon G showed an antimicrobial activity against cariogenic bacteria. Isopanduratin A, a flavonoid compound isolated from *Kaempferia pandurate* also exhibited the same mode of action (Palombo, 2009).

Clinical studies on the toxicity of flavonoids have concluded that they are toxic to immortalized cells or cancer cells but they are less toxic or non-toxic to normal cells. Research on flavonoids has also shown that they are produced by plants in response to infection from microorganisms (Nijveldt *et al.*, 2001).

1.3.2.2 Terpenes

Terpenes are the most abundant and chemically diverse group of phytochemicals. Figure 1.5 shows the basic structure of terpenes. Their general formula is $(C_5H_8)_n$. Terpenoids exist in

liquid form and are mainly found in essential oils and resins (Firn, 2010). They are unsaturated, flammable hydrocarbons and are classified according to the number of isoprene units they have. Hemiterpenes have one isoprene unit. In fact, the single unit molecule isoprene is regarded as the only hemiterpene. Derivatives that contain oxygen, for example, isovaleric acid and prenol are considered as hemiterpenes. Monoterpenes have two isoprene units. Examples include camphor, menthol and eugenol. Diterpenes have four isoprene units and examples include resins and taxol which is an important anticancer agent (Doughari, 2012). Another important example of a diterpene is hautriwaic acid which was first isolated from *D. viscosa* leaves in 1936 and was characterized as monohydroxy carboxylic acid. Yen Hsti and Yuh Pan Chen determined the full structure in 1970 (Hsu and Chen, 1971). Triterpenes are composed of six isoprene units and examples include sterols, steroids and cardiac glycosides which possess sedative, anti-inflammatory and cytotoxic activity. Sesquaterpenes consist of seven isoprene units and are mainly found in essential oils. They exhibit neurotoxic and antimicrobial action. (Doughari, 2012).

Psoralela corylifolia (Fabaceae) a Chinese medicinal plant was reported to contain a terpenoid, Bakuchiol which showed activity against both Gram-negative and Gram-positive oral bacteria at an MIC value ranging from 1-4 µg/ml. Katsura *et al* (2001) reported that it inhibited *S. mutans* growth when exposed to different ranges of sucrose concentrations and pH values in a temperature dependent manner. Liu *et al* (2006) isolated *ent*-rosane diterpenoids from *Sagittaria sagittifolia* (Alismaceae); four of these diterpenoids showed antimicrobial activity against *S. mutans* with MIC values ranging between 62.5 to 125µg/ml. The same group of researchers isolated new terpenoids from the plant *Sagittaria pygmaea*,

these were also active against *S. mutans* and the active compound was shown to be the terpenoid *β*-d-3, 6-diacetoxyglucopyranosyl-*ent*-kaur-16-ene at an MC value of 15.6 µg/ml. Xanthorrhiol a methanolic extract from the plant roots of *Curcuma xanthorrhiza* (Zingiberaceae) was shown to have antimicrobial activity against a range of oral pathogens, its efficacy against *Streptococcus* spp was comparable to that of chlorhexidine at an MIC value of 2-4 µg/ml. (Palombo, 2009).

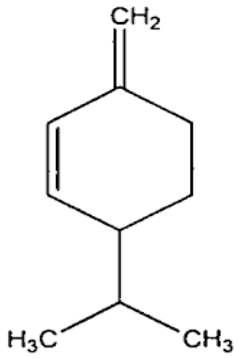

Figure 1.5: Basic structure of terpenes (Kar, 2007)

1.3.2.3 Tannins

Tannins are high molecular weight phenolic compounds that are widely distributed in plants mainly as glycosides. Figure 1.6 shows the basic structure of common tannins. They are mainly found in roots, stem, bark and on the outer layers of the plant tissue. They are soluble in both alcohol and water. Their name 'tannin' was coined from their characteristic feature to tan, that is their ability to produce leather. They are acidic in nature due to the presence carboxylic and phenolic groups. Tannins are able to form complex compounds with carbohydrates, alkaloids, gelatin, proteins and form a bluish-black colour with Ferric salts which are used in ink manufacturing (Kar, 2007). Tannins fall into two main groups, condensed tannis and hydrolysable tannins. Water soluble tannins are called egallitannins and

gallotannins and upon hydrolysis they produce ellagic acid and gallic acid respectively. Tannins are mainly used as antiseptic agents due to their phenolic groups (Doughari, 2012).

Tannin rich formulations from medicinal plants are also used to treat diseases like diarrhea and rhinorrhoea. In China, plant extracts with tannins are used to treat burns (Doughari, 2012). Naidoo *et al* (2012) reported the presence of the tannin, methyl 4-O-methyl-´a-xylopyranoside in DVA. Tannins are known to alter the structure of lipids in the cell membrane of microorganisms which probably provides them their antimicrobial activity (Ishida *et al.*, 2006).

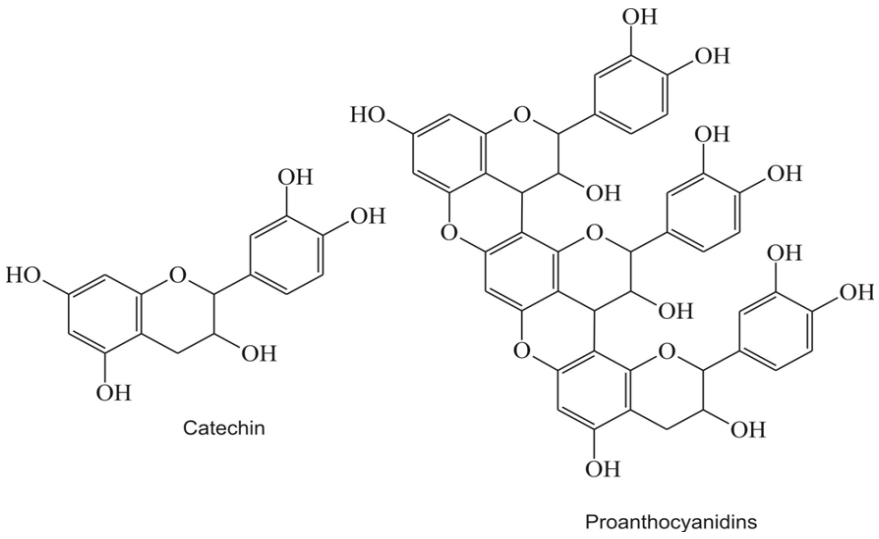

Figure 1.6: Basic structures of common tannins (Amorim *et al.*, 2012)

1.3.2.4 Saponins

Saponins are mainly found in the pericarp of the fruits of *D. viscosa*. Saponin is a term that was derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant with a lot of saponins and was used as a soap in the olden days. Figure 1.7 shows the basic structure of triterpernoids.

Saponins exhibit the same characteristics as soaps in water, they also produce foam. They produce an aglycone called sapogenin on hydrolysis and there are two types of sapogenins; triterpenoidal and steroidal. Saponins are produced from squalene and structurally consist of six isoprene units (Doughari, 2012).

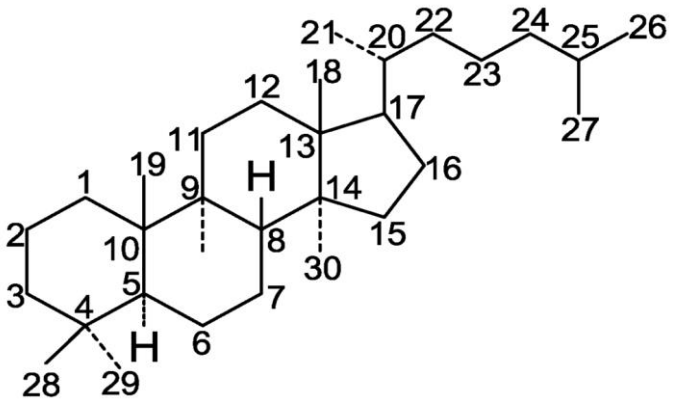

Figure 1.7: Basic triterpernoid structure (Xia et al., 2014)

Various pharmacological properties of saponins have been reported. These include antibiotic, antiviral, antifungal, anti-inflammatory, and anti-ulcer properties (Oakenfull, 1981). Glycyrrhizinic acid, a saponin found in *Glycyrrhiza glabra* was shown to have antiviral and bacteriostatic properties. Ginsenoside, a triterpene saponin found in ginseng (*Panax ginseng*) is believed to have immune-stimulant activity. Saponins are also used commercially for the production of sex hormones (Doughari, 2012). Soetan *et al* (2006) showed that crude saponin extracts from *Sorghum bicolor* inhibited the growth of Gram-positive bacteria. The crude saponins extract also showed activity against *Staphylococcus aureus*, which is a pathogenic microorganism to both animals and humans.

1.4 Isolation, identification and elucidation techniques

1.4.1 Liquid Chromatography

Liquid chromatography is a technique applied to separate a sample into its individual constituents. The separation is achieved by interaction of the sample with a mobile and stationary phase. There are different combinations of mobile/stationary phases that can be used in liquid chromatography; hence it is classified according to the type of phases used. The basic principle of chromatography is based on polarity. The components in a mixture are separated through a column based on each constituent's affinity for the mobile phase. The principle of 'like dissolves like' applies. The components move through the column at different rates to achieve separation. Molecules of the same compound generally move as a group forming distinct bands during separation. In high performance liquid chromatography (HPLC), the bands eluting from the column are detected by means of other instrument analysis techniques like UV-VIS spectroscopy (Betancourt and Gottlieb, 2015).

1.4.2 Thin Layer Chromatography

Thin layer chromatography (TLC) is a technique used to analyze a sample by separating compounds in the sample. The TLC technique can help in determining the purity of a compound. Thin layer chromatography involves three steps; spotting the plate, developing it in solvent and visualizing spots corresponding to constituents. Spotting involves the use of a micro pipette to transfer a minute amount of a dilute solution at one end of the TLC plate. Thin layer chromatography plates are usually composed of a thin layer of silica gel coated onto a metallic or glass sheet. In the next step of development, the end of the TLC that has been spotted is placed into a shallow pool of solvent, which moves up the plate. The

compounds are separated based on their interaction with the solvent and silica gel. Since a lot compounds are colorless, the developed TLC plate is usually sprayed with fluorescent material that can be viewed under ultraviolet light. The distance travelled by the solute in relation to the solute is used to characterize the different compounds. This is called the Rf value. The comparison of these Rf values with those of known compounds can be used to identify unknown compounds (Umass, 2016).

1.4.3 Gas Chromatography-Mass Spectrometry

Gas chromatography (GC) is a widely used technique in various branches of science and technology. It plays a huge role in determining the composition and proportions of chemical mixtures. However, the GC technique has a limitation in identifying the nature and chemical structure of these compounds hence it is attached to a mass spectrometer. The mass spectrometer provides the mass ionization value of the compounds, molecular ionization peaks of the compounds from the sample, the elemental composition and functional groups in cases where high resolution mass spectroscopy is utilized. In some instances, spatial isomerism and geometry of the molecules can be identified (Stashenko and Martinez, 2014).

Both liquid and solid samples can be analyzed in gas chromatography systems; the latter involves the adsorption of molecules onto a surface, for example, the solid-phase micro extraction (SPME) system. During analysis, the sample is first volatilized by exposure to high temperatures (200-300°C) and then mixed with the carrier gases, which could be a mixture of Helium, Argon, Nitrogen or Hydrogen. The gas mixture is pumped into the separation section which consists of a chromatographic column that is usually composed of a fused silica tube that is coated with a thin layer of polymer film. As the analytes are

displaced through the column, the molecules get partitioned between the polymer coated stationary phase and the carrier gas which is the mobile phase. They are then separated according to their chemical structure. At the end of the column, there is a detection system which identifies molecules through thermal conductivity or electron capture and produces an electrical signal related to the amount of molecules with the same identity. The data is then plotted on a graph which shows the variation of the signal with time and this graph is termed a chromatogram (Stashenko and Martinez, 2014).

Gas chromatography-mass spectrometry (GC-MS) is important in phytochemistry in the identification and quantification of bioactive compounds. Plant pigments consist of a vast number of unknown organic compounds which can be identified by GC-MS through interpretation and matching the spectra of those compounds with reference spectra (Ronald, 1997).

1.4.4 Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance (NMR) technology is based on the fact that atoms have nuclei which possess magnetic properties that can be used obtain chemical information. Research groups in the United States first developed the technology in 1946. Over the decades, NMR has developed into the principal organic spectroscopy used by chemists to establish detailed chemical structures of compounds. The development of stabilized permanent magnet technology which allows for high-resolution ^{1}H NMR spectra has facilitated the shift from research laboratory use only, to industrial environments (Edwards, 2013).

The principle of NMR technology uses magnetic properties of atoms. Subatomic particles, which are protons, electrons and neutrons, possess quantum energy and spin. The nucleuses of some atoms have paired spins which tend to cancel each other out leading to no overall spin. These include ^{12}C, ^{32}S and ^{16}O. However, the majority of atoms have nuclei that have an overall spin. These include ^{1}H, ^{19}F, ^{31}P and ^{13}C. There are set rules used to determine the spin of a given nucleus. The nucleus has no overall spin if the number of protons and neutrons are even. If the sum of neutrons and protons is odd, the nucleus is said to possess a half-integer spin (that is ½, $^{3}/_{2}$, $^{5}/_{2}$). If the number of protons and the number of neutrons are both odd, the nucleus possesses an integer spin (that is 1, 2, 3) (Edwards, 2013)

The appropriate radiation frequency necessary for absorption of energy is dependent on three factors, nucleus type (e.g. ^{13}C or ^{1}H), chemical environment of the nucleus (e.g. hydroxyl and methyl protons of methanol absorb differently) and thirdly, the location of the nucleus in space if the magnetic field in not uniform everywhere. It is ideal though to have a magnetic field that is evenly distributed over the sample as much as possible (James, 1998).

1.4.5 Infrared (IR) spectroscopy

Infrared (IR) spectroscopy analysis is based on absorption within the wavelength region of infrared light. The infrared region is associated with light that has a longer wavelength and smaller energy/frequency than visible light. The signal detected through infrared analysis of a sample is then plotted as the percentage transmission of the radiation in comparison to the wavelength of the radiation. A downward peak is plotted which represents a specific wavelength at which absorption occurs. Infrared spectroscopy is important in the identification of different functional groups within a molecule (Stuart, 2005).

1.5 Dental Caries

Dental caries is a multifactorial, chronic endogenous infection caused by bacteria that is part of the normal oral commensal flora. The resulting carious lesion is caused by demineralization and destruction of the enamel and later of dentine, by acids produced from bacterial fermentation of food debris accumulated inside the oral cavity, specifically on teeth surface (Samaranayake, 1996). The actual development of the carious lesion is due to the degradation of hydroxyapatites. Initially, enamel demineralization is at balance with remineralization, cavitation begins when there is an imbalance in the process whereby demineralization occurs faster than remineralization. With the onset of decalcification, the infection progresses to affect the dentine tissue leading to inflammation of the pulp and eventually its necrosis (Karpisnki and Szkaradkiewicz, 2013).

Dental caries is one of the most prevalent diseases worldwide. Every human is susceptible to dental caries throughout their lifetime. It is estimated that 36% of the world population have symptoms of dental caries in their permanent teeth and it affects approximately 9% of the baby population. There are various factors that promote the onset of dental caries; these include biological, environmental, behavioral, physical and lifestyle-related factors. There has been a rise in the consumption of dietary carbohydrates in developing countries which has correlated with an increase in dental caries (Karpisnki and Szkaradkiewicz, 2013).

Current practices that are used in the fight against dental caries are limited conventional preventative methods which are used in combination with restorative procedures. These have proven to be insufficient in the control of dental caries. Novel methods of preventing dental decay of the primary dentition are constantly being developed (Amin, 2003). Transmissibility

of dental caries has also been well documented. It is known that humans do not have cariogenic bacteria at birth; probable sources of transmission have been shown to be through mouth to mouth kissing and sharing of utensils. The general consensus is that children with high counts of mutans streptococci during development of primary teeth have higher chances of developing caries in their permanent teeth (Hurlbutt *et al*., 2010). Extensive research has been done on the pathogenesis of dental caries and one fact that has remained constant is the important role played by cariogenic bacteria in the development of dental caries.

1.5.1 Pathogenesis of dental caries

Generally, dental caries presents itself through local demineralization of enamel due to the onset of hydroxyapatite degradation. When oral biofilms are allowed to mature and inhabit teeth for extended periods of time, caries lesions develop (Karpisnki and Szkaradkiewicz, 2013). Dental caries are classified according to the site of lesion. These include, fissure or pit caries which are found in molars, premolars and also on the surface of maxillary incisors, smooth surface caries which are usually found just below the contact point on approximal tooth surfaces, root surface caries, which are found on dentine or cementum when the roots get exposed to the external oral environment and recurrent caries which exist where restorative procedures have been done (Samaranayake, 1996). Figure 1.8 shows an outline of common caries lesions.

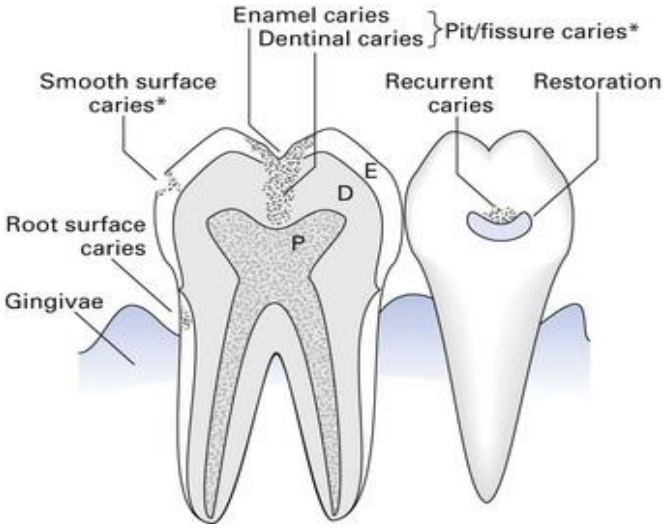

Figure 1.8: Types of caries lesions (Samaranayake, 1996)

The initial presentation of caries is usually seen as a well demarcated chalky-white lesion before the breaching of the enamel continuity. This is termed the 'white spot' lesion and it is able to heal and re-mineralize due to salivary minerals and fluorides. At this stage the disease is reversible. If the lesion progresses, the surface of the teeth become rough and the cavitation process ensues. If the cavitation is not controlled, it spreads to the dentine and most of the time it leads to destruction of the dental pulp. The main factors involved in the development of caries are diet, host factors and plaque microorganisms (Samaranayake, 1996).

1.5.1.1 Host factors

Poor oral health care may result in unsatisfactory cleaning of the mouth and receding gums that are left unattended, leading to exposure of the roots and increasing susceptibility to dental caries development (Schwendicke *et al.*, 2015) The structure of the enamel does not have the same mineral content throughout (especially fluoride); some areas of the teeth are more prone to develop caries than others (Samaranayake, 1996). Preterm infants tend to have

enamel defects which increase their chances of having dental caries (Segura, 2014). The mechanical action and flow rate of saliva has been shown to influence caries development. Saliva possesses buffering activity; it is able to neutralize acids produced by oral bacteria. Its mechanical washing action is efficient in the removal of unattached oral bacteria and food debris from the teeth. Saliva also has a high phosphorous and calcium content which are essential in the remineralization and healing 'white spot' lesions that lead to dental caries (Samaranayake, 1996).

There are conditions that limit the production of saliva which include Sjogren's syndrome, diabetes mellitus and cystic fibrosis. Medications like antihistamines and antidepressants also decrease the rate of saliva production which increases the risk of caries development (Segura, 2014). Individuals who smoke are also at a higher risk of developing dental caries as some brands of tobacco have high sugar content, increasing susceptibility to caries development (Neville *et al*., 2002). Research has shown that there is a link between smoking and root surface caries (U.S. SGA, 2004).

1.5.1.2 Diet

Diet has a big influence on the development of caries and for years the simplified prevention slogan phrase was: 'don't eat too much sugar and sugary foods' (EUFIC, 2003). Research has shown that sucrose rich diets promote the initiation of dental caries. Dental plaques are able to utilize sucrose to produce acid and extracellular polysaccharides because sucrose is very soluble and diffuses easily through plaque (Samaranayake, 1996). It is important to note though that caries levels have decreased over the years but the high consumption of sugar has

remained the same. This implies that the effect of sugar in caries development can be minimized if good oral hygiene is upheld (EUFIC, 2003). The rate of sugar intake rather than amounts that are consumed has been shown to be the decisive factor in caries development. The concentration and stickiness of the sucrose consumed determine how long the sugar remains in contact with the surface of the teeth. Fructose and glucose also promote dental caries but their influence is less than that of sucrose. Xylitol, a polyol carbohydrate is an example of a low cariogenic sugar that is being used as an alternative to sucrose (Samaranayake, 1996).

1.5.2 Microbiology of dental caries

Microorganisms form part of the dental plaque, which is a prerequisite for the onset of dental caries (Samarayanake, 1996). Approximately 700 different species of bacteria have been isolated from the human oral cavity. Extensive research on the oral microbiome has led to the conclusion that the *Mutans streptococci* groups of bacteria are the main factor in the development of caries. *S. mutans* is part of that group of bacteria. The importance of *Lactobacillus* species in further development of dental caries has also been determined. Both *S. mutans* and *Lactobacilli* spp are aciduric; they grow well in acidic environments and are able to rapidly metabolize dietary sugars to organic acids, mainly lactic acid (Karpisnki and Szkaradkiewicz, 2013).

There are various types of microorganisms found in dental lesions. These include many obligate and facultative anaerobic bacteria which belong to different genera such as *Eubacterium, Bifidobacterium, Lactobacillus, Actinomyces, Rothia* and *Parvimonas.*

Different groups of bacteria can cause dental caries and these include different members of the salivarius, mitis, anginosus groups of streptococci. They are part of plaque bacteria and also have inherent biochemical processes factoring in cariogenicity (Karpisnki and Szkaradkiewicz, 2013). Becker *et al* (2002) reported the presence of *S. mutans*, *Streptococcus sanguinis* as well as species of *Bifidobacterium*, *Veillonella*, *Actinomyces* and *Propionibacterium* in the oral cavity.

In the 1920's, a medical microbiologist named Clark reported a bias towards the low pH culture media that was being used to grow oral cultures. He exposed the fact that low pH media favoured *Lactobacillus* growth, which was considered to be the most important caries causative organism at that time. Clark took a different approach and plated his samples at pH 7. He concluded that early caries lesions were caused by *S. mutans*. Clark also suggested the concept of microbial succession, he observed that different types of bacteria were dominant at different stages of caries development, but the main causative organism was *S. mutans* (Russell, 2009). In that regard, it seems plausible that dental caries may be initiated by different combinations of bacteria besides *S. mutans* and *Lactobacillus* species. However, extensive research on dental caries has revealed that *S. mutans* is the predominant species in the initiation of dental caries and that it is involved in almost all caries lesions (Samaranayake, 1996).

1.5.2.1 *Streptococcus mutans*

The role of *S. mutans* in the development of dental caries has been well documented. It is estimated that *S. mutans* is carried by over 98% of adults (Russell, 2009). *Mutans streptococci* is a cluster name that encompasses seven different species; *Stretococcus*

sobrinus, Streptococcus cricetus, Streptococcus mutans, Streptococcus ferus, Streptococcus rattus, Streptococcus macacae and *Streptococcus downei*. These groups of bacteria are highly acidogenic and aciduric, they produce acids which dissolve the enamel hence they are the most cariogenic pathogens (Samaranayaki, 1996).

1.5.2.1.1 Habitat, Morphology and Growth requirements

Streptococcus mutans are Gram positive bacteria with thick cell walls that are composed of a layer of peptidoglycan (murein) and teichoic acids. These are important in preventing osmotic lysis of the cell protoplast and they provide the rigidity needed to maintain the shape of the cell. They thrive in environments that range from 18-40 degrees Celsius. Figure 1.9 shows the colonies of *S. mutans*. They are also composed of a polysaccharide capsule made from dextran glucose subunits. *S. mutans* have circular DNA and closely interrelated plasmids that are about 5.6 kilobases (kb) long.

One of the factors that contribute to the virulence *S. mutans* in causing caries is its ability to attach to teeth surface through the formation of biofilms. It has the ability to adhere to the enamel, produce slime and also divide producing micro-colonies within that layer. This leads to the formation of biofilms. Adherence to the pellicle of the teeth is achieved by proteins on its cell surface. This is followed by its growth while synthesizing the dextran capsule which aids in biofilm formation (Todar, 2012).

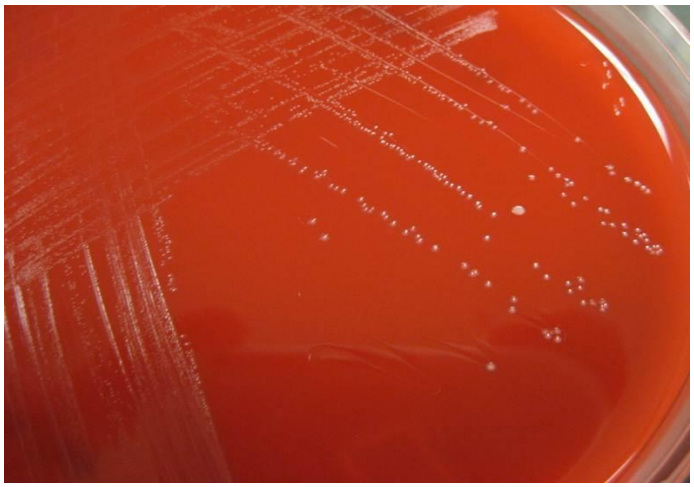

Figure 1.9*: Streptococcus mutans* colonies on blood agar (Morgan, 2015).

Streptococcus mutans are able to breakdown sucrose to glucose and fructose. Fructose is fermented to provide energy while glucose is turned into an extracellular dextran polymer that is essential for the cementation of *S. mutans* to the tooth enamel forming a matrix of dental plaque. The dextran slime layer provides carbon through the de-polymerization of glucose. This process leads to the formation of lactic acid within the plaque which in turn dissolves the enamel leading to caries development. Research has shown that *S. mutans* tend to habitat in pits and fissures, and forms 39% of the total streptococci in the oral cavity and only 2-9% in the buccal surface (Ikeda and Sadham, 1971).

1.5.2.1.2 Pathogenicity of *Streptococcus mutans*

Streptococcus mutans can cause dental caries due to its ability to form biofilm (dental plaque) and its ability to produce lactic acid and intracellular polysaccharides. Prior to the development of caries, *S. mutans* is often isolated from the tooth surface and as the infection progresses, so do the *S. mutans* counts. *Streptococcus mutans* are highly acidogenic, they are

able to metabolize dietary carbohydrates, mainly sucrose and glucose to lactic acid and other organic acids. Among all the plaque bacteria, *S. mutans* are able to lower the pH to levels critical enough for demineralization more rapidly and are also able to continue acid production in that environment making them aciduric. The resulting low pH leads to enamel demineralization and eventual tooth decay (Samaranayake, 1996).

Streptococcus mutans also produce insoluble extracellular polysaccharides from sucrose. These polysaccharides aid in the formation of biofilms on teeth surface. The production of polysaccharides is catalyzed by three glucosyltransferase isoenzymes. They also produce intracellular polysaccharides (IPSs) as glycogen in the presence of extracellular sucrose and glucose. The polysaccharide reserves act as food stores which are used when there are low amounts of dietary carbohydrates. Research has also shown that *S. mutans* produce mutacins (bacteriocins) which play an important role in its colonization and establishment in dental biofilms (Karpisnki and Szkaradkiewicz, 2013).

The main nutritional source for oral bacteria is saliva. Saliva carbohydrate content is generally low; however these levels are increased up to about a 1000 fold after a meal. *Streptococcus mutans* and other oral bacteria have developed regulatory mechanisms that fully exploit these transient increases in carbohydrates. The initial step is the intake of sugar into the organisms. The second step is the breakdown of these carbohydrates through the glycolytic pathway. This stage is critical in the aetiology of dental caries as the acids produced lead to enamel demineralization (Todar, 2012).

In general, bacteria use the Embden-Myerhof pathway to degrade glucose and produce two pyruvate molecules per molecule of glucose. Depending on the existing sugar levels, *Streptococcus mutans* convert pyruvate to ethanol, formate and acetate under low sugar conditions and in excess sugar levels, pyruvate is converted to lactate molecules leading to a fall in pH. This fall in pH is followed by a rise back to its original value in about an hour, giving rise to what is termed the 'Stephan curve' (Samaranayake, 1996). Figure 1.10 gives an outline of the Stephan curve.

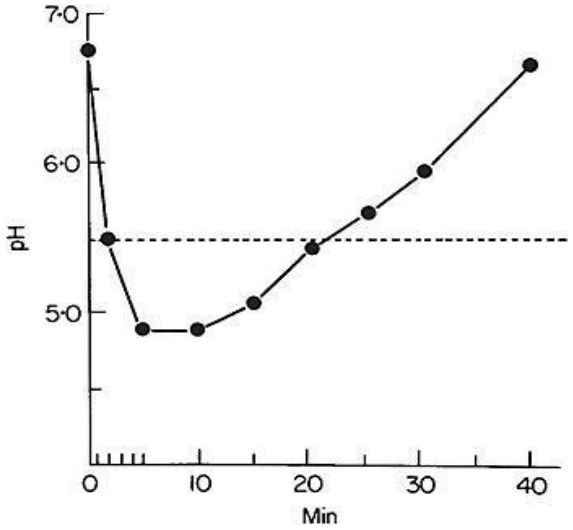

Figure 1.10: The Stephan Curve (Deery and Toumba, 2015).

1.5.3 Management of dental caries

Dental caries is an endemic disease. There is potential to prevent or even cure the disease through early diagnosis and subsequent arrest or reversal of the caries process (Alfano *et al*., 2001). Saliva plays a huge role in preventing caries development. There are also some major approaches that have proved to be successful in the prevention of dental caries. Most are based on the alteration of diet, use of anti-cariogenic agents and protecting susceptible areas.

1.5.3.1 Saliva

The fact that the human tooth lacks its own regulatory mechanism to shed dead surfaces makes it vulnerable to plaque development (Katz, 2015). Saliva plays a huge role in the prevention of dental caries by maintaining some form of equilibrium in the oral cavity. Saliva is composed of proteins, water, mineral elements, electrolytes which determine its pH and organic elements such as immunoglobulin and mucin which are essential for its biological properties. The immunoglobulins and salivary enzymes like amylase hinder the growth, adherence and aggregation of micro-organisms. The presence of mineral elements like phosphates, carbonates together with urea enables its ability to regulate pH, thereby preventing sustained low pH values that promote caries development (Fakhoury and Peraldi, 1996). The washing action of saliva enables its effect as a lubricant and aids in the washing of bacteria from the oral cavity hence limiting their effect.

1.5.3.2 Diet

Metabolism of dietary carbohydrates by oral flora is the initial stage in the development of dental caries. Research has shown that the major factor in the initiation of the disease is the rate of consumption of carbohydrates rather than the amount between meals. The stoppage or reduction of carbohydrate consumption between meals reduces the amount of fermentable sugars that can be accessed by oral flora (Samaranayake, 1996). Use of non-cariogenic artificial sweeteners that are not be easily absorbed and fermented by plaque bacteria is another good alternative. Artificial sweeteners in the market include sorbitol, lycasin and xylitol (NIH, 2001).

1.5.3.3 Antimicrobial agents

There are some antimicrobial agents available commercially that are used to prevent dental caries. Examples of these include oral rinses like iodine, chlorhexidine, triclosan and essential oils. Some of these antimicrobial agents are incorporated into tooth pastes and include polymers, and surfactants that have the ability to reduce biofilm.

1.5.3.3.1 Fluoride

Research data on fluoridation of water and dentifrice material has proved to be effective (Alfano *et al.*, 2001). There are various ways in which fluoride can be delivered to teeth. It can be administered in early childhood by its inclusion during an amelogenesis. The most effective way is via the pipe-borne water supply at 1 ppm. Other ways of delivery include fluoridated tooth paste; fluoridated gel preparation which are topically applied and use of tablets (NIH, 2001) Fluoride ions exert their effect by forming flouroapatite through the substitution of hydroxyl groups from hydroxyapatite. Flouroapatite is less susceptible to being dissolved by acid. Fluoride also aids in remineralization of caries cavities in enamel and also interferes with plaque metabolism by limiting glycolysis thereby inhibiting the production of intracellular polysaccharides (Samaranayake, 1996).

1.5.3.3.2 Chlorhexidine

Chlorhexidine is an antimicrobial agent widely used by most dental professionals in the prevention of oral diseases. It is used as the active ingredient in oral rinses to reduce dental plaque and bacterial build up in the oral cavity. It is also delivered in the form of gels and recently, it is applied as a varnish layer to protect teeth enamel. Its bactericidal action is

immediate and exhibits prolonged bacteriostatic activity. This prolonged bacteriostatic activity characteristic is due to the fact that chlorhexidine adsorbs onto the pellicle coat of teeth enamel. Chlorhexidine gluconate solution (0.12%), which is a combination of chlorhexidine and gluconic acid is used to treat oral diseases and promote regeneration of oral tissues (Jenkins *et al*, 1988).

However, prolonged use has been shown to have adverse effects. It can cause teeth, tongue and gingiva staining. Reduced bitter and salty taste sensations can also be experienced with time. Tooth staining is caused by the breakdown of bacterial membrane and subsequent denaturation of their proteins (Helms *et al*., 1995). Disulfide ions are also reduced to thiol ions which then react with salivary iron (III) ions to form dark complexes (Gilbert, 2006). Some researchers have argued the efficacy of chlorhexidine in the prevention of dental caries claiming that the clinical data is not conclusive (Alfano *et al*., 2001).

1.5.3.3.3 Iodine

Use of iodine solutions as topical applications has been shown to be effective in the suppression of oral *S. mutans.* A study on the use of iodine in dentistry indicated that the application of 0.2% potassium iodine solution (KI) in humans enabled the elimination of *S. mutans* from reachable tooth sites for a period of 13 weeks after treatment (Gibbons *et al*, 1974). Caufield and Gibbons (1979) reported a similar result being observed for 20 to 24 weeks following treatment with 2% iodine-potassium iodide solution. Povidone-iodine (PVP-I) is an effective antimicrobial agent compared to other iodine solutions. The combination of PVP with iodine increases its solubility in both water and alcohol. It also reduces the staining

effect of iodine on teeth. The target sites of iodine are located in the bacterial cytoplasmic membrane and cytoplasm. Its microbial killing action is instant (Amin *et al*., 2004).

1.5.3.3.4 Triclosan

The incorporation of triclosan in toothpaste and its ability to enhance the antimicrobial effects of toothpaste has been well documented. In a study titled the 'Chochrane Review', researchers concluded that continual use of fluoride toothpaste containing triclosan resulted in a 22% reduction in dental plaque when compared to triclosan free fluoride tooth paste. They also concluded that it exhibited a 41% reduction in plaque development when compared to triclosan free fluoride tooth paste (Riley and Lamont, 2013). Davies *et al* (2004) stated that dentifrice which contained triclosan provided a more effective control of dental plaque development compared to conventional fluoride dentifrice. There are some studies however that have shown that triclosan adversely affects the immune system and children exposed to triclosan containing products exhibited more hay fever and allergy symptoms. Some bacteria exposed to triclosan also develop resistance with time (Paleo Personal Care, 2015).

1.6 Anticariogenic activities of plants

The importance of effective antimicrobial agents in the prevention of dental caries has been well-documented. Medicinal plants have been shown to confer significant antimicrobial activity against dental caries causing bacteria, *S. mutans*. Chloroform extracts from the aerial parts (leaves) of the plant *Drosera peltata* which is used traditionally to treat dental caries, were shown to have an MIC of 31.25 µg/ml against *S. mutans*. Phytochemical analysis of the plant showed that plumbagin was the active compound (Didry *et al*, 1998). Aqueous

propanone extracts of red grapes were shown to have antimicrobial activity against *S. mutans* with an MIC of 500 µg/ml. From the same study they also observed that various plant propanone extracts were able to inhibit adhesion of *S. mutans* to glass (Smullen *et al*, 2007).

Ethanol extracts from *Helichrysum italicum* flowers, a plant mainly found by the Mediterranean region was shown to have antimicrobial activity against *S. mutans* and *S. sobrinus* with MIC values ranging from 31.25 to 62.5 µg/ml (Nostro *et al*, 2004). The compound Malvidin-3,5-diglucoside was confirmed to be the active constituent of the plant *Alcea longipedicellata* with MIC values of 160-200 µg/ml against cariogenic oral streptococci. Macelignan, a compound isolated from *Myristia fragans* was shown to have significant antimicrobial activity against *S. mutans* with an MIC value as low as 3.9 µg/ml. Its activity can be compared to Chlorhexidine and it is effective compared to other phytochemical anticariogenic agents. Macelignan also inhibited biofilm formation by *S. mutans* (Rukayadi *et al*, 2008).

The potential use of DVA in the prevention of dental caries has been documented. Naidoo (2012) reported a decrease in biofilm formation by *S. mutans* after exposure to subinhibitory concentrations of DVA. After exposing *S. mutans* for 6, 24 and 30 hours to the crude DVA ethanol extract in a biofilm assay, the bacterial counts were reduced by log 1.3, 0.95 and 1.95 respectively. The author also observed a significant reduction in acid production by *S. mutans* over a 16 hour period when exposed to subinhibitory concentrations of DVA. However, like most studies done to investigate the traditional use of medicinal plants, the potential use of DVA in preventing dental caries has been limited to the use of crude organic

or aqueous solvent extracts and the chemical constituents responsible for the anticariogenic property have not been identified. Therefore, the aim of this study was to identify the anticariogenic chemical constituents present in *D. viscosa var. angustifolia* and establish its safe use in preventing growth of *S. mutans* and dental caries.

1.7 Aim

The aim of this study was to identify the anticariogenic chemical constituents present in *D. viscosa* var. *angustifolia* and establish its safe use in preventing growth of *S. mutans* and dental caries.

1.8 Objectives

- To analyse crude leaf extracts using thin layer chromatography to identify the groups of chemicals present
- To further separate and identify selected groups of chemicals using liquid chromatography, gas chromatography-mass spectrometry (GC-MS) and finally elucidate the structure of isolates using Nuclear Magnetic Resonance (NMR) spectroscopy and Infrared (IR) spectroscopy.
- To determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MIC) of newly isolated and identified chemicals against *S. mutans*
- To determine the inhibitory effect of sub-inhibitory concentrations of newly isolated and identified chemicals on acid production and biofilm formation by *S. mutans*
- To investigate the cytotoxic effect of newly identified beneficial chemical/s

CHAPTER 2

Methods and Materials

2.1 Cultures

Saliva and periodontal pocket debris samples from patients attending the Wits Oral and Dental Clinic at Charlotte Maxeke Johannesburg Academic Hospital were previously collected (Ethical clearance number M10205) and cultured on Mutans Bacitracin agar to isolate *S. mutans. Streptococcus mutans* cultures were then identified using cultural characteristics and a series of biochemical reactions using the Analytical Profile Index (API) 20 Strep standardized system (BioM`erieux, SA). Cultures were then stored at -70^{o}C. *Streptococcus mutans* ATCC 10923 and a clinical strain termed SM 1 were used in the study.

2.2 Plant material

Plant material was collected from the Pypeklipberg, Mkhunyane Eco Reserve in Mpumalanga province of South Africa. Figure 2.1 shows the *D. viscosa* var. *angustifolia* leaves after drying and grinding them to powder.

Figure 2.1: *Dodonaea viscosa* var. *angustifolia* dry leaves and powder

The plant was positively identified by Mrs. Ranee Reddy, a taxonomist from the Herbarium at the University of the Witwatersrand. It was identified as *D. viscosa* var. *angustifolia* Benth which belongs to the Sapindaceae Family. Voucher specimens number J 94882, were previously deposited at this Herbarium (Patel and Coogan, 2008).

2.2.1 Extraction

Dried *D. viscosa* var. *angustifolia* leaves were first milled to a powder (Figure 2.1). The crude extracts were prepared using a method described by Eloff, (1999). Sixty grams of powder was mixed with 600 ml of methanol in a closed container, agitated for 72 hours and centrifuged at 5000 rpm for 20 minutes. The supernatant was then collected in pre-weighed 100 ml beakers. The procedure was repeated three times with the same powder. All the three supernatants were then pooled together in the same beaker and the methanol was allowed to evaporate under a cold air stream. The beaker with the dried extracts was weighed again to calculate the extract yield.

2.3 Column Chromatography

Column chromatography was done to fractionate the dry methanol extract. Sequential fractionation was carried out using a chromatography column packed with a slurry of activated silica gel with mesh ranging from 70 to 230Å (Merck®). The slurry was prepared using 100% hexane. The methanol extract was then dissolved in a 50% v/v mixture of ethyl acetate and hexane. The sample was then loaded onto the silica gel column. Elution was started with hexane (100%). This was followed by increasing gradient polarity through the addition of ethyl acetate up to 100%. Table 1 gives an outline of the solvent system used for elution.

Table 2.1: Solvent gradient used for elution. Analytical grade chemicals from Merck® and Sigma® were used.

Solvent	Proportion (%)
Hexane	100
Hexane/ethyl acetate	90:10
Hexane/ethyl acetate	80:20
Hexane/ethyl acetate	70:30
Hexane/ethyl acetate	30:70
Ethyl acetate	100
Ethyl acetate/methanol	70:30

Fractions were collected in 10 ml vials and were pooled together according to their thin layer chromatography profiles. A total of six fractions labelled fraction 1 to 6 (F1-F6) were collected as represented in Figure 2.2.

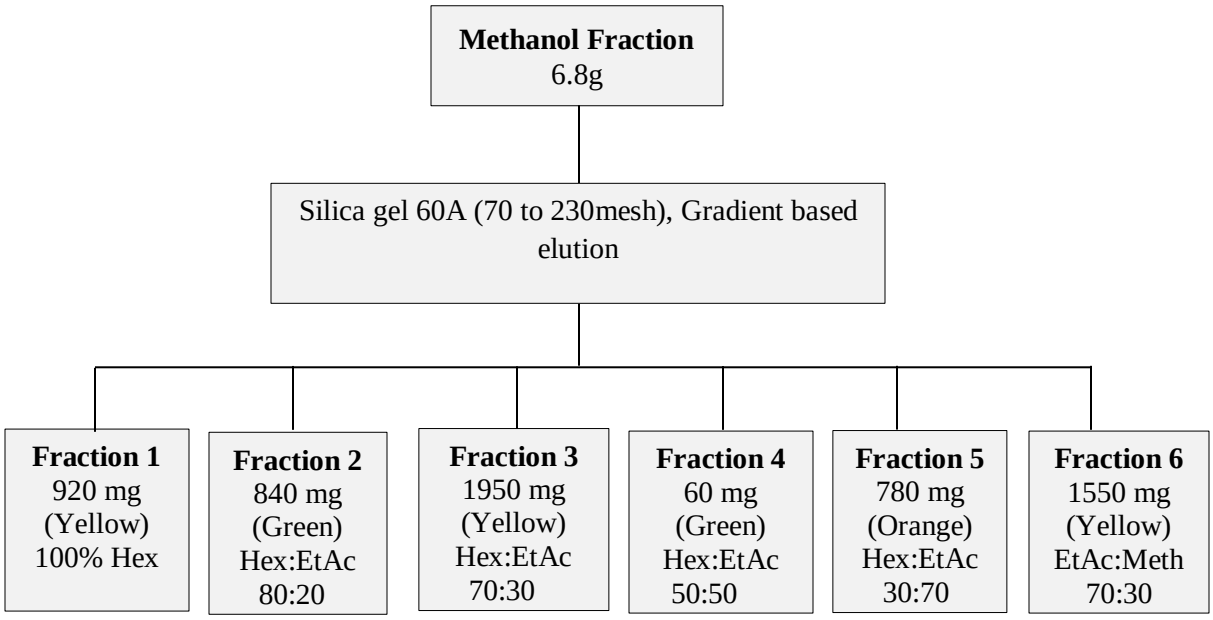

Figure 2.2: Schematic representation of fractions isolated from *D. viscosa* var. *angustifolia* leaves using column chromatography

2.4 Thin layer chromatography (TLC)

Thin layer chromatography plates from Merck® (Silica gel 60 F254) were used to analyse fractions collected from column chromatography according to a technique described by

Kotze and Eloff (2002). Fifty milligrams of the individual fractions were dissolved in 1 ml of solvent (ethyl acetate) and spotted on a line drawn by pencil at one end of the silica gel plate. The TLC plates were then developed using three different solvent systems of polarity containing the mobile phases, toluene/ethanol/ammonium hydroxide (18:2:0.2) [TEA], dichloromethane/ethyl acetate/formic acid (10:8:2) [DEF] and ethyl acetate/methanol/water (40:5.4:5) [EMW]. Thin layer chromatography plates were placed in closed tanks in such a way that the end near the sample application area was in contact with the mobile phase. The chromatograms were then allowed to run for 15 min after which they were dried with a cold air stream. The dry TLC plates were then developed by spraying with the vanillin reagent (0.2 g vanillin + 28 ml methanol + 1 ml sulphuric acid), then dried and placed in an oven at 100°C for optimal colour development. The distance each band travelled was compared to the distance travelled by the solvent reported as the Retention factor (R_f):

$$Rf = \frac{Distance\ migrated\ by\ analyte}{Distance\ migrated\ by\ solvent}$$

Each compound has a characteristic R_f for a particular solvent.

2.5 Preliminary screening for antimicrobial activity

2.5.1 Contact Bioautography

Contact bioautography is a technique used to screen for active compounds on thin layer chromatography plates. A technique described by Suleiman *et al* (2010) was used in the study with some modifications. The six individual fractions were dissolved in methanol at 10 mg/ml. These were then loaded onto TLC plates on a line drawn by pencil in a narrow band. Three solvent systems previously described were used to elute the bands,

toluene/ethanol/ammonium hydroxide (18:2:0.2) [TEA], dichloromethane/ethyl acetate/formic acid (10:8:2) [DEF] and ethyl acetate/methanol/water (40:5.4:5) [EMW]. The plates were then dried under a cold air stream for a week to remove any excess solvent left. One day old *S. mutans* cultures (ATCC 10923) grown on Blood Agar media were used. Using a sterile swab, these were then transferred into 100 ml of freshly prepared Tryptone Soya Broth media. The suspension was then centrifuged at 5000 rpm for 15 minutes. The supernatant was then discarded. The remaining bacterial pellet was then mixed with 50 ml of semi-solid Columbia Agar media and vortexed. The dry chromatograms were then covered with a layer of semi-solid media with bacterial cultures and stored at 4°C for an hour to allow the bands to diffuse into the media. The plates were then incubated for 24 hours at 37°C in a humidified CO_2 jar. After incubation, the TLC plates were sprayed with a solution of p-iodonitrotetrazolium violet (INT) (Sigma®) at 2mg/ml. Areas with growing organisms turned pink after incubation from reduction of the dye while zones of inhibition were clear. This indicated fraction bands with antimicrobial activity. These clear zones were then compared with reference plates to identify the R*f* values of active compounds.

2.6 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

Minimum inhibitory concentration and minimum bactericidal concentration tests were performed. Hundred microliters of double dilutions of constituent plant extracts in dimethyl sulphoxide (DMSO) were added to each well of a 96-well round bottom microtitre plate in varying initial concentrations (25 – 0.0125 mg/ml. Colonies of bacterial cultures were emulsified in Tryptone Soya broth to achieve bacterial counts of approximately 10^7CFU/ml by adjusting the optical density to 0.2 at 405nm. These suspensions were used as inoculum.

Addition of inoculum diluted the plant extracts by half. Hundred microliters of inoculum was transferred into each well of the microtitre plate and incubated anaerobically for 48 hours at 37°C. Dimethyl sulphoxide (DMSO) was used as a solvent and as a control vehicle. Chlorhexidine gluconate, an antimicrobial chemical used for oral hygiene was used as a positive control. After incubation, the lowest concentration that inhibited visible growth was recorded as the MIC. Each dilution was then sub-cultured on blood agar to detect viable bacteria and to determine the MBC

2.7 Effect of chemical constituents on biofilm formation by *S. mutans*

The effect of individual fractions on biofilm formation by *S. mutans* was tested using *D. viscosa* var. *angustifolia* fractions according to a technique described by Limsong *et al* (2004) with some modifications. Sterile 10 ml bijou bottles with two sterile glass slides (25 mm x 12 mm) in an upright position (90^o) were prepared. In one bottle (control), only 2 ml Tryptone Soya broth was added. While in the test bottles, 2 ml Tryptone Soya broth containing sub-inhibitory concentration of the constituent plant extract was added. Hundred microliters of *S. mutans* inoculum was added to each bottle and incubated. Biofilms were grown anaerobically at 37^oC. One glass slide from each of the bottles was removed after 6 hours and rinsed with phosphate buffered saline (PBS) to remove unattached bacterial cells. The attached cells were aseptically scraped off the slides using sterile slides, re-suspended and vortexed in 2 ml PBS. Ten-fold serial dilutions were prepared and 100 µl of each dilution was spread on blood agar. The plates were incubated for 48 hours at 37^oC under CO_2. The numbers of colonies were then quantified and the counts multiplied by the dilution factors to determine viable bacterial count. The same procedure was repeated for the second slides after

24 hours. For preliminary screening, these experiments were done using three different subinhibitory concentrations from each fraction and crude extract. After purification of the bioactive subfractions, the biofilm tests were done using the same technique and were repeated three times per concentration using each of the *S. mutans* strains. The bacterial counts were compared using the Wilcoxon rank-sum test (Mann-Whitney).

2.8 Effect of chemical constituents on acid production by *S. mutans*

The effect of individual fractions on the acid production by planktonic cells of *S. mutans* was studied using a technique described by Nalina and Rahim, (2007) with some modifications. Tryptone Soya broth (4 ml) containing sub-inhibitory concentration of plant extracts was inoculated with 100 µl of culture containing 10^7 CFU/ml of *S. mutans* determined by adjusting the optical density to 0.2 at 405 nm. Similarly, Tryptone Soya broth (4 ml) without extract was inoculated with 100 µl culture of *S. mutans* as a control. The tubes were incubated at 37^oC under CO_2. The pH was read at 0 hours, 8 hours and then every 2 hours for a total exposure period of 20 hours. The bacterial count was done at 0 hours, 8 hours and 10 hours using serial dilution techniques. For preliminary screening, these experiments were done using three different subinhibitory concentrations from each fraction and crude extract. After purification of the bioactive subfractions, the acid tests were done using the same technique and were repeated three times per concentration using each of the *S. mutans* strains. The pH values of the control and tests were then compared for each time interval using the Wilcoxon rank-sum test (Mann-Whitney).

2.9 Purification and identification of fractions showing antimicrobial activity

2.9.1 Liquid chromatography (LC) analysis

After a series of antimicrobial tests, fraction five (F5) exhibited the most significant activity and was chosen for further purification through liquid chromatography. The Cheetah™ MP 100 Flash purification system supplied by Bonna-Agela Technologies was used for liquid chromatography. Two millilitres of sample was injected for each run. Elution was done using a solvent gradient phase of Hexane/Ethyl acetate. Normal phase chromatography was done using silica gel columns. Equilibration of the column was done using a solvent ratio of 90% Hex: Ethyl acetate 10%. The flow rate was set at 10 ml per minute. The peaks were viewed at 270nm. Compounds in the sample had different retention times, which is the time taken between sample injection and a compound reaching a detector at the end of the column. The eluates were collected in 10 ml test tubes and combined according to their TLC profiles.

2.9.2 Gas Chromatography-Mass Spectrometry (GC-MS)

After liquid chromatography analysis and another round of antimicrobial assay tests, two sub fractions were chosen (F5.1 and F5.2) for GC-MS analysis. Gas chromatography-Mass spectrometer analysis was done using an Agilent Technologies 5190-2293 gas chromatography machine with an HP-5ms ultra inert column (30 m x 250µm x 0.25 µm) interfaced with the Agilent Technologies 19091S-433UI mass spectrometer. The initial temperature was set at 50°C and increased at a rate of 5°C per minute up to the set limit of 310°C. The split ratio was set at 1:50 and helium was used the carrier gas. The auxiliary transfer port was set at 280 °C. The ion source (EI) temperature was set at 230 °C with fixed electron energy set at 70 eV. Solvent delay was set at 3 minutes. Mass spectra and the

different retention times were used to identify the compounds through comparison with a catalogue in the Wiley 275 library of compounds.

2.9.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy was done to determine the structure of the compound. ^{1}H NMR and ^{13}C NMR spectra were all recorded using the Bruker AVANCE 500 spectrophotometer. All spectra were recorded in $DMSO_{d6}$. The chemical shift values in all spectra obtained are reported in parts per million and referenced against the internal standard, TMS, which occurs at zero parts per million, in the case for ^{1}H NMR spectra. For ^{13}C NMR spectra, all chemical shift values are relative to the central signal of $DMSO_{d6}$, which occurs at 2.45 parts per million.

2.9.4 Infrared spectroscopy analysis

Infrared analysis was done using the Perkin Elmer FT-IR 100 spectrometer. Ten milligrams per milliliter of subfraction 5.1 was dissolved in ethyl acetate. A small pipette tube was used to draw up the sample from which one drop was placed on the surface plate for analysis.

2.10 Cytotoxicity

Cytotoxicity tests were carried out to investigate the effect of identified potential anticariogenic compounds on human cells. Human embryonic kidney cells (HEK 293) previously frozen at - 80°C were used.

2.10.1 Cell culture passaging

The HEK 293 kidney cell line was routinely sub-cultured and supplemented with fresh medium to cultivate the cells. Dulbeco's modified eagle's medium (88%) supplemented with fetal calf serum (10%), L-glutamine (1%) and penicillin/streptomycin (1%) was used for culturing the cells. Old cell culture medium was first removed from T75 tissue culture flask. Two milliliters of PBS which had been warmed to 37°C was then added to each T75 flask to remove the excess medium and dead cells. After discarding the PBS, 2 ml of Trypsin EDTA was added for cell dissociation. The flasks were incubated at 37°C for 2 minutes. After incubation, the flasks were given a slight knock to aid dissociation of cells and to prevent clumping of cells. Two milliliters of culture medium was then added to the flasks to neutralize trypsin. Two milliliters of resulting cell suspension was transferred to each new T75 flask which was cultured for stock purposes. In the case of T25 flasks, 1 ml was transferred. T25 flasks were used for the cytotoxicity assays. Each T75 flask then received 8 ml of fresh medium and the T25 flasks received 4 ml of medium each. These were then incubated at 37°C under CO_2.

2.10.2 Cytotoxicity assay

The cytotoxicity of subfraction 5.1 which had the highest antimicrobial activity and crude extract were tested using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay technique. The method is based on the ability of the mitochondrial enzyme succinate dehydrogenase from viable cells to reduce MTT into an insoluble purple product called formazan (McCauley, 2013). Human embryonic kidney (HEK) 293 cells were first harvested by trypsinizing the monolayer cell culture grown in T25 tissue culture plates.

2.10.2.1 Determination of cell count and viability

Cell numbers and viability were determined using the Trypan blue method. Twenty microlitres of HEK 293 cells in medium were mixed with 20 µl of Trypan blue inside a vial. Twenty microlitres of this mixture was placed onto a hemocytometer and spread with a cover-slip. Viable cells were counted from the heamocytometer quadrants using a light microscope at x1000 magnification. The number of viable cells needed to get the desired concentration of 111000 cells per ml was then calculated using the following equation:

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

The cell number was then multiplied by the amount of cell suspension needed to fill the applicable wells of a 96 well microtitre plate (McCauley *et al*, 2013).

2.10.2.2 Seeding of cells into the microtitre plate and exposures

Cytotoxicity tests were done from three separate T25 plates in triplicate using 96 well microtitre plates. A hundred microlitres of media only was added to form blank test control wells. The blanks were added to eliminate any interference that might happen during reading of the absorbance values for test wells. Two percent Triton-X was used as a positive control. Dimethyl sulphoxide (DMSO) was used to dissolve the crude extract and subfraction 5.1 hence a DMSO control was added by pipetting 100 µl of the cell solution and later adding 100µl of 1% DMSO. Cell culture controls were also included by pipetting 100 µl of the cell solution. A hundred microlitres of cell suspension was then pipetted into applicable wells to form the sample test wells. The microtitre plates were then incubated for 24 hours at 37°C under 5% carbon dioxide.

After 24 hours of incubation, the tissue culture flasks were viewed for cell growth under the microscope at x1000. The cells had grown and attached to the surface. Media was removed from the plates and replaced with 100 µl of fresh medium. Serial dilutions of the crude extract and subfraction 5.1 were prepared using DMSO diluted with PBS to make 1% DMSO. Hundred microlitres of each sample dilution was added in triplicate to the test wells. Each sample well had 0.5 % DMSO. The concentrations of the crude extract and subfraction in the test wells ranged from 25 mg/ml to 0.0125 mg/ml. Hundred microlitres of 1% DMSO was also added into the DMSO control wells. Two percent Triton-X was added to the positive control wells. The plates were then incubated for 24 hours at 37°C and 5% carbon dioxide.

After incubation, the media with the test samples and controls was removed and replaced with 100 µl of the MTT reagent. The plates were further incubated for 3 hours at 37°C and 5% carbon dioxide. The MTT reagent was pipetted out and replaced with 100% DMSO to dissolve the formazan crystals. After 30 minutes of incubation, the plates were read with a spectrophotometer at 570 nm.

Figure 2.3 gives an outline of the steps taken from extraction, identification and elucidation of the bioactive constituent up to investigating its level of cytotoxicity on human cells.

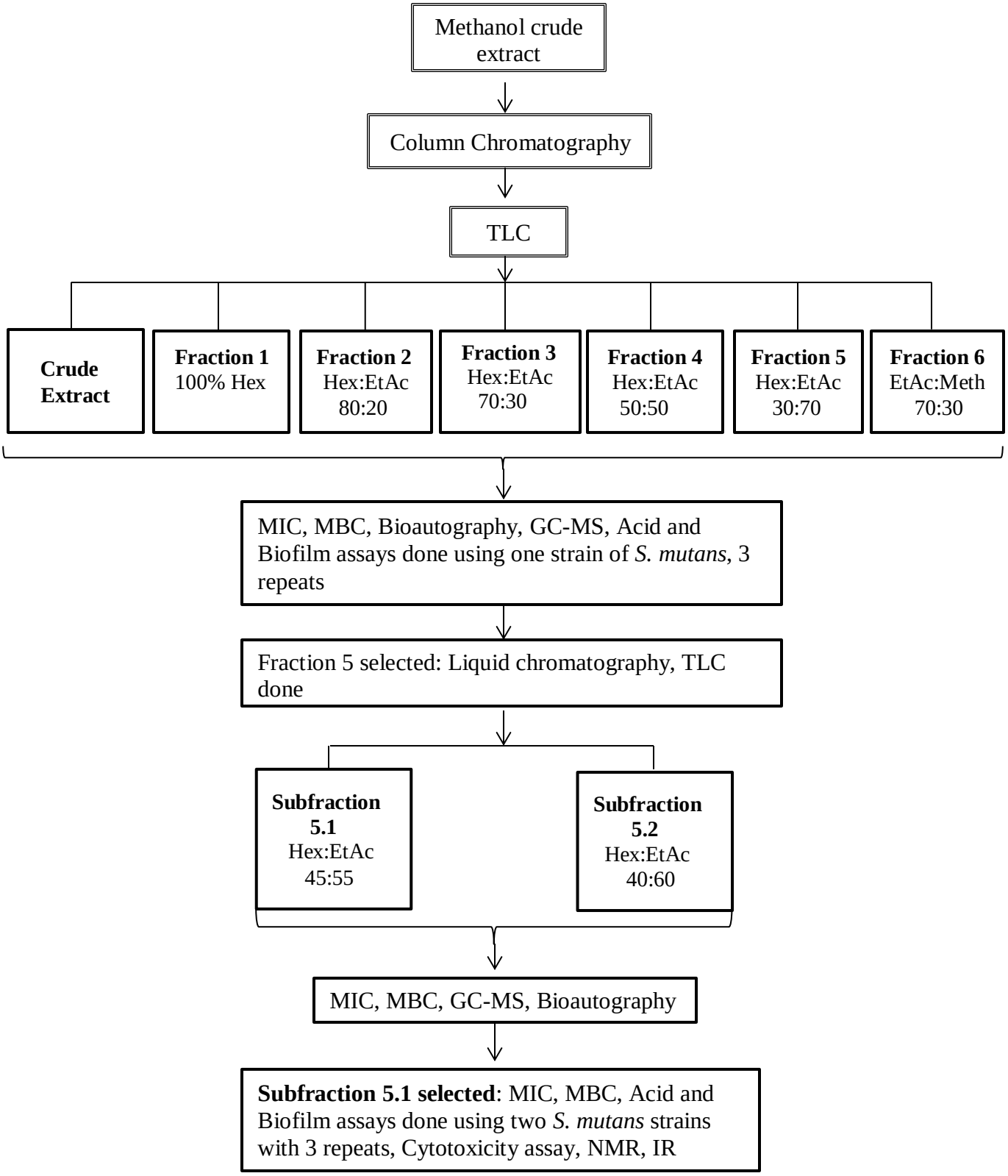

Figure 2.3: Summary of the screening process up to elucidation of the bioactive compound

CHAPTER 3

Results

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

Dodonaea viscosa var. *angustifolia* (DVA) leaves that had been dried and crushed into powder were serially extracted with methanol. Extracts were combined, dried under vacuum and weighed to calculate the yield per given mass of dried leaves. Table 3.1 shows the percentage yield of the methanol extractions.

Table 3.1 Percentage yield from the dry weight of DVA methanol extract

No. of methanol extractions	Mass of DVA leaves (g)	Yield (g)	Yield (%)
1	60 g	3.7	6.18
2	60 g	3.1	5.17
Total yield		**6.8**	**11.4**

Methanol was chosen as a solvent because of its amphiphilic property. It consists of both water soluble and water-insoluble groups which makes it ideal in dissolving both polar and non-polar compounds in plant extracts. It also has a low boiling point which makes it ideal for extraction and concentration of bioactive compounds. The extracts were combined and weighed and a percentage yield of 11.4% was obtained. Column chromatography was used as the first step to fractionate the crude extract.

3.2 Column chromatography

Silica gel chromatography columns were used to fractionate the dry methanol extract. A solvent gradient ratio of hexane and ethyl acetate was used. A total of 9 fractions were obtained. These were combined according to their TLC profiles to give 6 major fractions (F1-F6). Figure 3.1 gives an outline of the fractions and yield.

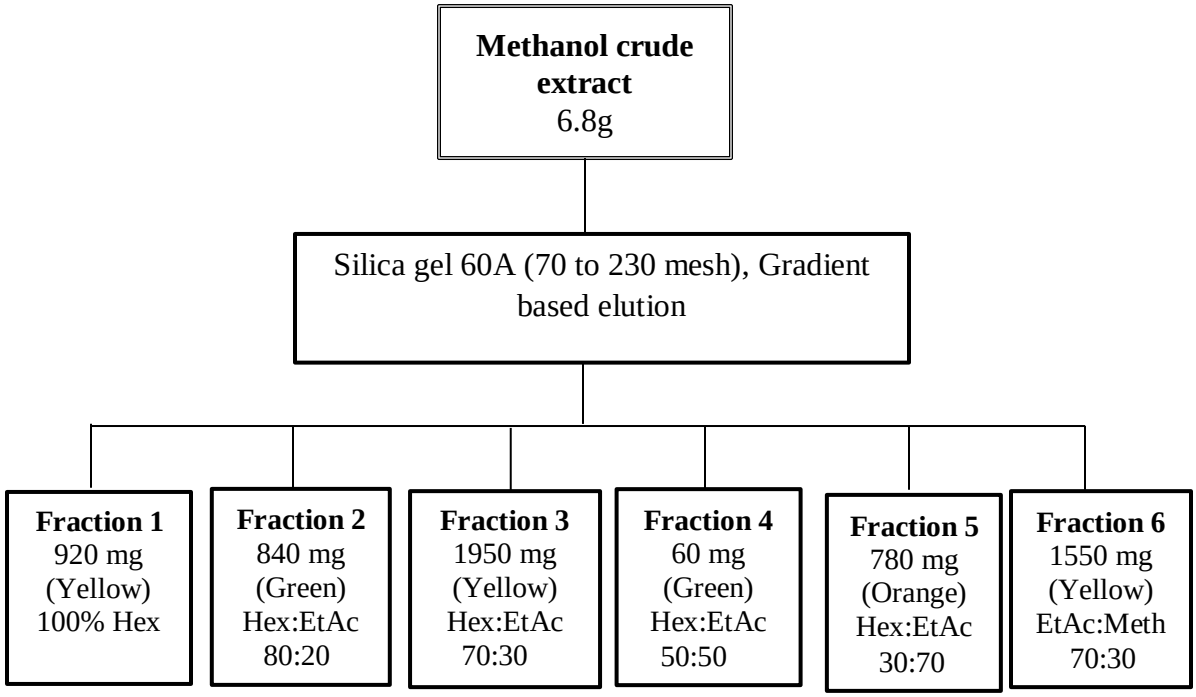

Figure 3.1 Schematic representation of fractions isolated from the *D. viscosa* var. *angustifolia* leaves using column chromatography

Fraction 1 was eluted with 100 % hexane and had deep red-orange colour which is typical of the non-polar β-carotene compounds. Fraction 2 was eluted as a green band from the column which could have been the chlorophyll group of compounds. Fraction 3 was eluted with a more intermediate polar solvent and was the most collected of all fractions with a mass of 1950 mg after drying. Its colour and polarity was typical of xanthophylls or phaeophytin

group of compounds. Fraction 5 and 6 were eluted with polar solvents and had a deep yellow colour which is typical of polar flavonoids. All fractions were dried and weighed.

3.3 Thin layer chromatography (TLC) of the methanol extract

Dried methanol fractions from column chromatography were reconstituted to 10 mg/ml, spotted and ran on TLC. Figure 3.2 shows TLC profiles of the six fractions and the crude extract.

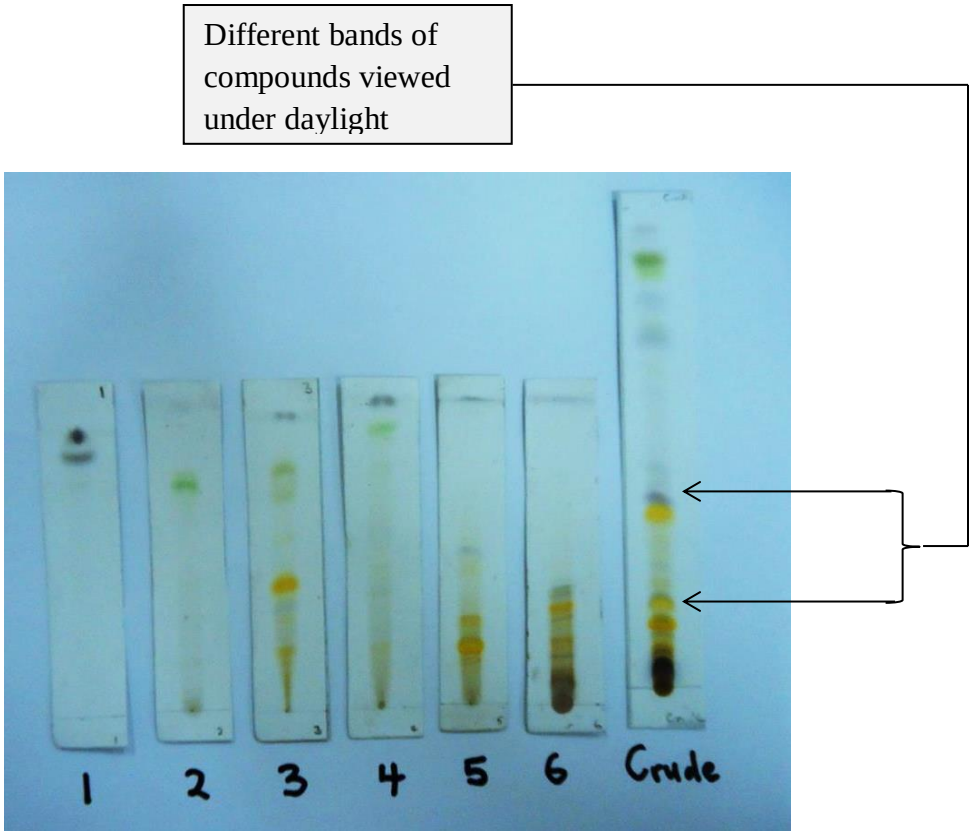

Figure 3.2: TLC plates of the *D. viscosa* var. *angustifolia* crude extract and six fractions after development with 10 % v/v aqueous sulfuric acid

Three solvent systems were used, ethylacetate:methanol:water EMW(40:5:4.5), tetrahydrofouran:ethylacetate:formic acid TEF(10:8:2), and toluene:ethanol:ammonium hydroxide TEA (18:2:0.2). The TEA solvent combination was able to separate non-polar fractions, 1 and 2 with band R_f values ranging from 0.15 and 0.96. Fractions 3 and 4 were run with an intermediate polar solvent combination, TEF, with fraction 4 having the highest band R_f value of 0.88. Fractions 5 and 6 were separated with EMW with R_f values ranging from 0.03 to 0.56. The crude extract was separated with TEA at a concentration of 10 mg/ml. Most of the bands were visible in daylight. Those not visible in daylight were viewed under ultraviolet light at 365 nm.

3.4 Contact Bioautography

Preliminary antimicrobial activity against *S. mutans* of the six fractions was analyzed using the bioautography assay technique. Figure 3.3 shows the autobiograms of the six fractions.

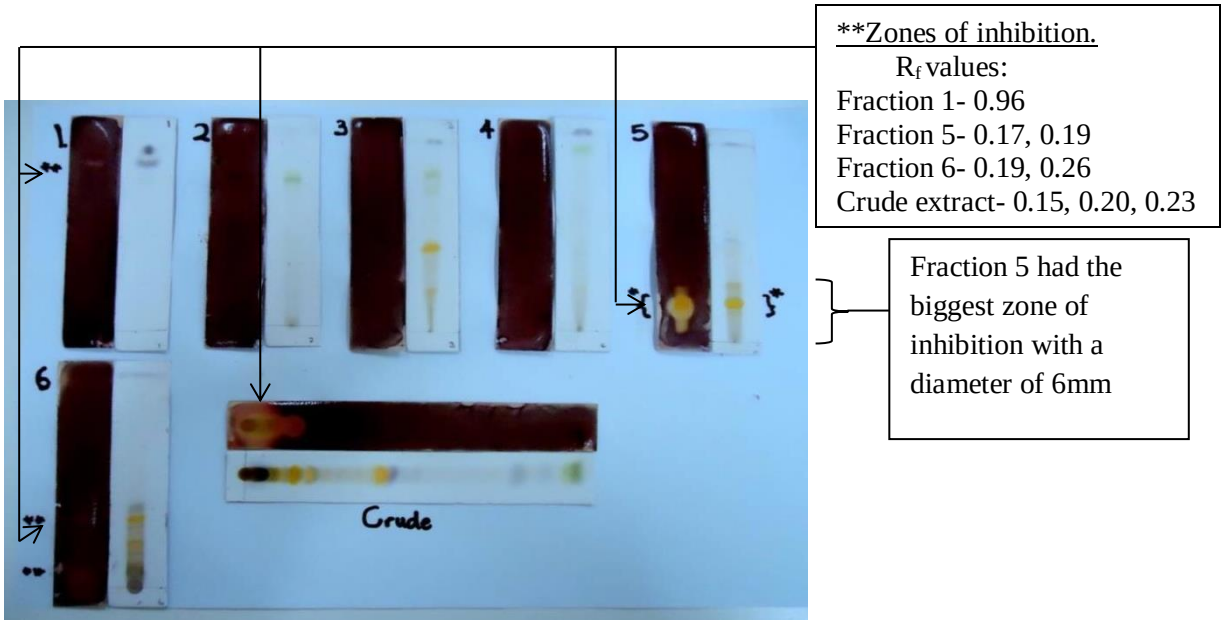

Figure 3.3: TLC bioautograms of the fractions and *D. viscosa* var. *angustifolia* crude extract after incubating them with a layer of semi-solid media with *S. mutans* cultures and spraying with INT dye

Clear areas indicating bacterial inhibition were compared with reference TLC plates using R_f values of the bands to identify compounds with antimicrobial activity. Fraction 1, 5, 6 and the crude extract exhibited zones of inhibition. Fraction 5 had the most active compound as indicated by a bigger zone of inhibition (6 mm) at R_f value 0.17 compared to the other fractions. This preliminary screening showed that fraction 5 was the most bioactive fraction. Therefore fraction 5 was further fractionated and subfractions 5.1 and 5.2 were obtained (Figure 3.4).

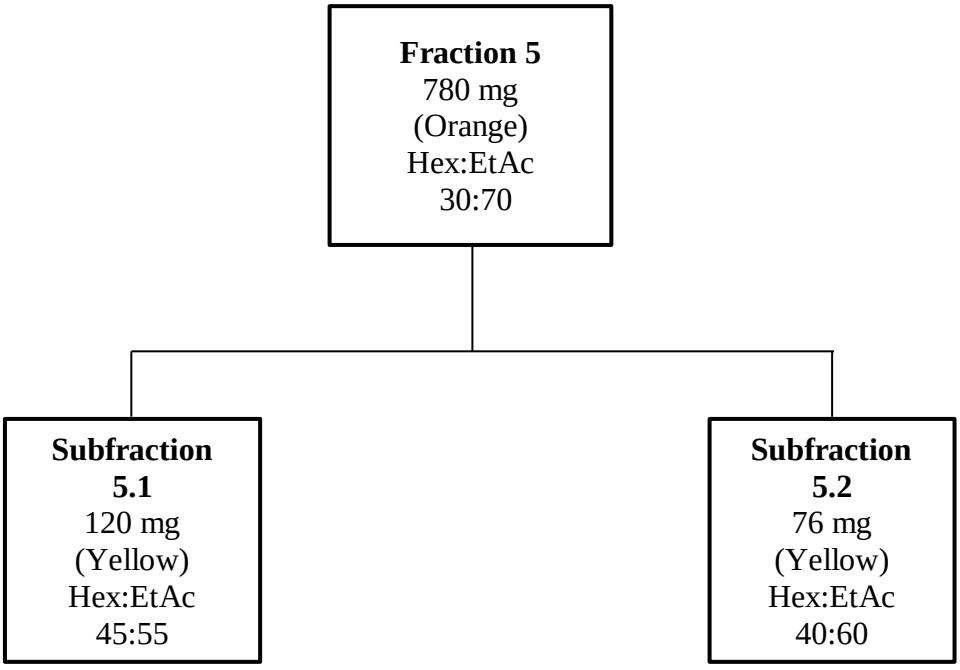

Figure 3.4 Schematic representation of subfractions isolated from the fraction 5 of *D. viscosa* var. *angustifolia* leaves using column chromatography

3.5 Antimicrobial activity assays of fractions (Preliminary screening)

All the six *D. viscosa* var. *angustifolia* fractions (F1-F6) exhibited some degree of antimicrobial activity against the cariogenic bacteria, *S. mutans* (Table 3.2, Figure 3.5). The fractions were tested for activity against two *S. mutans* strains, ATCC 10923 (1) and the clinical strain, SM1 (2). Microtitre plates in Figure 3.5 shows the antimicrobial activity of different fractions after incubation at 37 °C and adding INT dye.

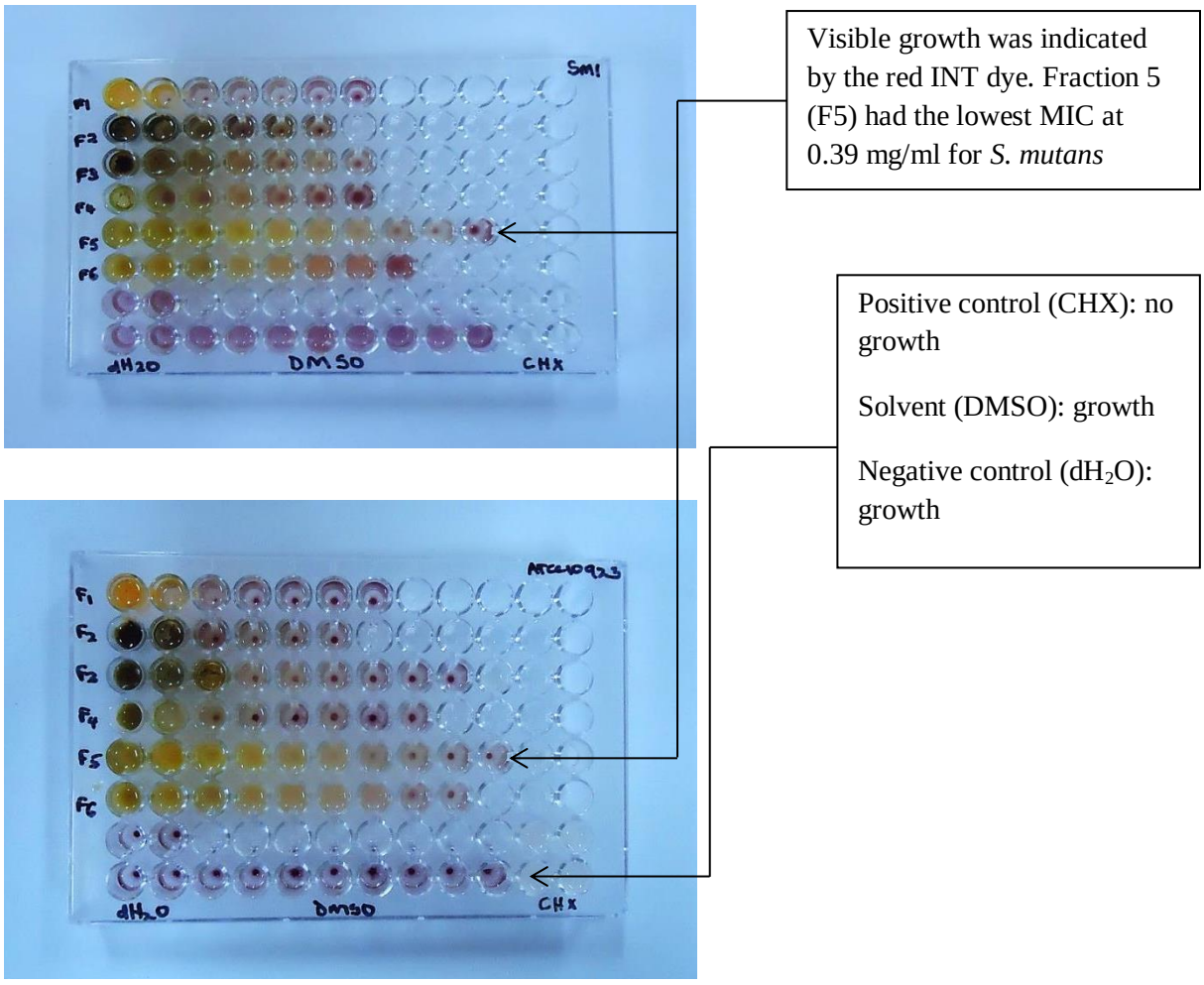

Figure 3.5 Microtitre plates showing antimicrobial activity tests of fractions (F1-F6).

There was no growth observed in the positive control which had chlorhexidine. The negative control with water and the solvent (DMSO) had growth. The lowest minimum inhibitory concentration (MIC) recorded was from fraction 5 with a value of 0.39 mg/ml and with a minimum bactericidal concentration value of 0.78 mg/ml. These results are in congruent with what was observed from bioautography analysis where F5 had a bigger zone of inhibition. The MICs of *D. viscosa* var. *angustifolia* fractions ranged between 0.39-6.25 mg/ml.

Table 3.2 also shows a summary of the antimicrobial activity tests done for screening fractions for their efficacy against *S. mutans.* The concept of total activity is also introduced in the table. When screening fractions for their antimicrobial effect, total activity is used to determine any loss or gain of activity during fractionation of the crude extract. Total activity takes into consideration the MIC of the fraction and its quantity.

$$Total\ activity = \frac{quantity}{MIC}$$

Table 3.2 Summary of the antibacterial activity of *D. viscosa* var. *angustifolia* crude extract and fractions against *S. mutans*

Test		**Crude**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Quantity(mg)		6800	920	840	1950	60	780	1550
MIC	**1**	0.78	12.5	6.25	6.25	12.5	0.39	1.56
mg/ml	**2**	0.78	12.5	12.5	6.25	6.25	0.39	1.56
Total activity		-	73.6	89.6	312	6.4	2000	993.6
MBC	**1**	1.56	12.5	12.5	6.25	12.5	0.78	3.125
mg/ml	**2**	1.56	12.5	12.5	12.5	12.5	0.78	3.125
Bioautogram R_f values		Four bands of activity	One faint band Rf: 0.96	No activity	One faint band Rf: 0.47	No activity	Two clear bands Rf: 0.19 and 0.17	One faint band Rf: 0.14

Fraction 5 had the highest total activity value of 2000 followed by fraction 6 at 993.6. Fraction 4 had the lowest total activity value of 6.4. The minimum bactericidal concentration (MBC) values of the fractions ranged from 0.78-12.5 mg/ml. Fraction 5 exhibited the lowest MBC value of 0.78 mg/ml which had improved compared to the crude extract.

3.6 Effect of *D. viscosa* var. *angustifolia* crude extract and fractions on biofilm formation by *S. mutans* (Preliminary screening).

Depending on the MBC values, three subinhibitory concentrations per fraction were selected and used for the assays as shown in Table 3.3. Bacterial counts in the biofilms were recorded after 6 and 24 hours. Percentage reduction in bacterial counts was calculated using the control counts. Only *S. mutans* strain ATCC 10923 was used for screening and one repeat was carried out per fraction. Table 3.4 shows the results of the effect of fractions on biofilm formation by *S. mutans.*

Table 3.3: Outline of the concentrations used for biofilm and acid production assays

Concentrations used (mg/ml)	3.125	1.56	0.78	0.39	0.2	0.1	0.05
Crude			x	x	x		
F1	x	x	x				
F2	x	x	x				
F3			x	x	x		
F4	x	x	x				
F5					x	x	x
F6			x	x	x		

Table 3.4: Effect of subinhibitory concentrations of *D. viscosa* var. *angustifolia* fractions on biofilm formation by *S. mutans*

Fractions	Growth of *S. mutans* in biofilm (CFU/ml): Strain ATCC 10923						
	Plant (mg/ml)	6 hours			24 hours		
		Control	Plant	%Reduction	Control	Plant	%Reduction
Crude	0.78	$2.2x10^5$	3.9×10^4	82.3	$2.2x10^7$	2.1×10^6	90.5
	0.39	$2.2x10^5$	6.2×10^4	71.8	$2.2x10^7$	4.1×10^6	81.4
	0.2	$2.2x10^5$	9.6×10^4	56.4	$2.2x10^7$	8.7×10^6	60.5
Fraction 1	3.125	$5.5x10^5$	1.3×10^5	76.4	$4.8x10^7$	7.2×10^6	85.0
	1.56	$5.5x10^5$	2.4×10^5	56.4	$4.8x10^7$	9.7×10^6	79.8
	0.78	$5.5x10^5$	3.7×10^5	32.7	$4.8x10^7$	1.8×10^7	62.5
Fraction 2	3.125	$5.5x10^5$	2.1×10^5	61.8	$4.8x10^7$	9.3×10^6	80.6
	1.56	$5.5x10^5$	3.2×10^5	41.8	$4.8x10^7$	1.6×10^7	66.7
	0.78	$5.5x10^5$	3.9×10^5	29.1	$4.8x10^7$	2.9×10^7	35.6
Fraction 3	0.78	$5.5x10^5$	9.1×10^4	83.5	$4.8x10^7$	6.5×10^6	86.5
	0.39	$5.5x10^5$	2.8×10^5	67.3	$4.8x10^7$	8.3×10^6	82.7
	0.20	$5.5x10^5$	3.1×10^5	43.6	$4.8x10^7$	1.5×10^7	68.8
Fraction 4	3.125	$1.2x10^5$	4.8×10^4	60.0	$5.7x10^7$	1.2×10^7	78.9
	1.56	$1.2x10^5$	5.3×10^4	55.8	$5.7x10^7$	2.4×10^7	57.9
	0.78	$1.2x10^5$	6.9×10^4	42.5	$5.7x10^7$	3.4×10^7	40.4
Fraction 5	0.20	$1.2x10^5$	1.5×10^4	87.5	$5.7x10^7$	3.8×10^6	93.3
	0.10	$1.2x10^5$	2.2×10^4	81.7	$5.7x10^7$	4.7×10^6	91.7
	0.05	$1.2x10^5$	3.9×10^4	67.5	$5.7x10^7$	6.2×10^6	89.1
Fraction 6	0.78	$1.2x10^5$	2.5×10^4	79.2	$5.7x10^7$	5.8×10^6	89.8
	0.39	$1.2x10^5$	4.6×10^4	61.7	$5.7x10^7$	8.3×10^6	85.4
	0.20	$1.2x10^5$	6.3×10^4	47.5	$5.7x10^7$	9.2×10^6	83.9

Dodonaea viscosa var. *angustifolia* fractions significantly reduced the attachment of *S. mutans* cells to the glass slides. The biofilm counts of *S. mutans* from the control slides were higher than those exposed to the fractions for all the exposure time intervals. There was an increase in log CFU/ml of *S. mutans* after 24 hours in all the assays but the control biofilm counts were still significantly higher compared to those exposed to the DVA fractions.

Fraction 5 had the highest anticariogenic activity as indicated by its high biofilm inhibition effect of 93.3 % after 24 hours with the lowest subinhibitory concentration of 0.2 mg/ml. The intermediate subinhibitory concentration of 0.1 mg/ml from fraction 5 also exhibited greater biofilm activity of 91.7% after 24 hours compared to all the other fractions. The inhibitory effect was also higher than the crude extract whose subinhibitory concentration value of 0.78 mg/ml had 90.5 % inhibition effect after 24 hours. The biofilm inhibition effect of the plant extracts was shown to be concentration depended with higher concentrations having a more pronounced inhibitory effect. The pronounced inhibitory effect from fraction 5 indicated that it contained an active compound in more concentrated form than in the crude extract.

3.7 Effect of *D. viscosa* var. *angustifolia* crude extract and fractions on acid production by *S. mutans* (Preliminary screening).

Streptococcus mutans cells were incubated in the presence of subinhibitory concentrations of *D. viscosa* var. *angustifolia* fractions. The same three subinhibitory concentrations used for the biofilm inhibition effect were used for the acid production assays (Table 3.3). *Streptococcus mutans* strain ATCC 10923 was used for acid production assays. The pH was measured at beginning of incubation, after 8 hours and then every 2 hours up to 14 hours. The results are presented in Table 3.5. Bacterial counts were also carried out from all the assays at 0, 8 and 10 hours. Bacterial counts were done to prove that any differences in pH observed were due to the inhibitory effect on acid production by fractions on *S. mutans* not due to the differences in bacterial counts. The results are shown in Table 3.6.

Table 3.5 Effect of subinhibitory concentrations of *D. viscosa* var. *angustifolia* crude extract and fractions on acid production by *S. mutans*

Plant	DVA (mg/ml)	Acid production by *S. mutans*: Strain 10923 (pH)										pH Difference after 14 hours	
		0 hours		8 hours		10 hours		12 hours		14 hours			
		Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant
Crude	0.78	7.47	7.47	6.02	7.21	5.81	6.33	4.84	5.98	4.56	5.45	2.91	2.02
	0.39	7.47	7.47	6.02	6.83	5.81	6.08	4.84	5.61	4.56	5.16	2.91	2.31
	0.20	7.47	7.47	6.02	6.75	5.81	5.96	4.84	5.28	4.56	4.93	2.91	2.54
Fraction1	3.125	7.49	7.49	6.22	7.04	5.76	6.68	5.28	6.14	4.81	5.48	2.68	2.01
	1.56	7.49	7.49	6.22	6.93	5.76	6.38	5.28	5.63	4.81	5.11	2.68	2.38
	0.78	7.49	7.49	6.22	6.71	5.76	6.02	5.28	5.52	4.81	4.97	2.68	2.52
Fraction2	3.125	7.49	7.49	6.22	7.02	5.76	6.74	5.28	5.98	4.81	5.43	2.68	2.06
	1.56	7.49	7.49	6.22	6.83	5.76	6.33	5.28	5.61	4.81	5.12	2.68	2.37
	0.78	7.49	7.49	6.22	6.47	5.76	6.08	5.28	5.36	4.81	4.80	2.68	2.69
Fraction3	0.78	7.47	7.47	6.02	6.96	5.81	6.31	4.84	6.06	4.56	5.62	2.91	1.85
	0.39	7.47	7.47	6.02	6.89	5.81	6.04	4.84	5.82	4.56	5.13	2.91	2.34
	0.20	7.47	7.47	6.02	6.86	5.81	5.73	4.84	5.41	4.56	4.94	2.91	2.53
Fraction4	3.125	7.48	7.48	6.14	6.62	5.88	6.28	5.42	5.91	5.04	5.38	2.44	2.10
	1.56	7.48	7.48	6.14	6.54	5.88	6.12	5.42	5.67	5.04	5.14	2.44	2.34
	0.78	7.48	7.48	6.14	6.48	5.88	5.48	5.42	5.28	5.04	4.96	2.44	2.52
Fraction5	0.20	7.47	7.47	6.02	7.38	5.81	6.81	4.84	6.26	4.56	5.96	2.91	1.51
	0.10	7.47	7.47	6.02	6.92	5.81	6.42	4.84	6.02	4.56	5.73	2.91	1.74
	0.05	7.47	7.47	6.02	6.87	5.81	6.11	4.84	5.84	4.56	5.44	2.91	2.03
Fraction6	0.78	7.47	7.47	6.02	6.76	5.81	6.54	4.84	6.07	4.56	5.81	2.91	1.66
	0.39	7.47	7.47	6.02	6.69	5.81	6.36	4.84	5.84	4.56	5.32	2.91	2.15
	0.20	7.47	7.47	6.02	6.63	5.81	5.87	4.84	5.53	4.56	5.11	2.91	2.36

All the fractions inhibited acid production of *S. mutans*. After 10 hours, control cultures had attained the critical pH of 5.5 which is cariogenic. Cultures with plant extract only reached this critical pH after 14 hours. The results were simplified by calculating the difference in pH at 0 and 14 hours as shown in Table 3.5.

A lower difference in the pH recorded at 0 hours and 14 hours indicated that acid production by *S. mutans* was more highly inhibited. The highest subinhibitory concentration of 0.2 mg/ml from fraction 5 had the least change in pH after 14 hours with a calculated value of 1.51. This value was evidently lower when compared to the control which had a pH difference of 2.91 after 14 hours. Among the six fractions, the highest change in pH was observed from the lowest subinhibitory concentration of fraction 2 (0.78mg/ml) which had a pH change of 2.69. However, the inhibitory effect was still evident when compared to the control. Table 3.6 shows the bacterial counts at 0, 8 and 10 hours. *Streptococcus mutans* counts in the exposed cultures and controls increased with time. From observation, bacterial counts between the controls and test cultures were not very different. This indicated that the inhibitory effect in acid production observed between controls and fractions was not due to differences in bacterial counts.

Table 3.6: *Streptococcus mutans* counts (CFU/ml) in the presence and absence of subinhibitory concentrations of *D. viscosa* var. *angustifolia* crude extract and fractions.

Fractions	Growth of *S. mutans* (CFU/ml) in acid production assay : Strain ATCC 10923						
	DVA (mg/ml)	0 hours		8 hours		10 hours	
		Control	Plant	Control	Plant	Control	Plant
Crude	0.78	$9.3x10^5$	$8.8x10^5$	$1.9x10^7$	$6.1x10^6$	$7.4x10^7$	$9.4x10^6$
	0.39	$9.7x10^5$	$7.9x10^5$	$1.9x10^7$	$7.4x10^6$	$7.4x10^7$	$1.5x10^7$
	0.2	$8.4x10^5$	$8.6x10^5$	$1.9x10^7$	$8.2x10^6$	$7.4x10^7$	$3.4x10^7$
Fraction 1	3.125	$8.3x10^5$	$7.6x10^5$	$1.7x10^7$	$5.4x10^6$	$6.9x10^7$	$2.3x10^7$
	1.56	$7.8x10^5$	$8.1x10^5$	$1.7x10^7$	$6.3x10^6$	$6.9x10^7$	$2.9x10^7$
	0.78	$8.6x10^5$	$7.3x10^5$	$1.7x10^7$	$7.6x10^6$	$6.9x10^7$	$3.4x10^7$
Fraction 2	3.125	$8.3x10^5$	$7.6x10^5$	$1.7x10^7$	$6.1x10^6$	$6.9x10^7$	$2.1x10^7$
	1.56	$7.8x10^5$	$8.1x10^5$	$1.7x10^7$	$7.2x10^6$	$6.9x10^7$	$2.4x10^7$
	0.78	$8.6x10^5$	$7.3x10^5$	$1.7x10^7$	$7.9x10^6$	$6.9x10^7$	$3.6x10^7$
Fraction 3	0.78	$9.3x10^5$	$8.8x10^5$	$1.9x10^7$	$5.7x10^6$	$7.4x10^7$	$9.3x10^6$
	0.39	$9.7x10^5$	$7.9x10^5$	$1.9x10^7$	$5.3x10^6$	$7.4x10^7$	$2.6x10^7$
	0.20	$8.4x10^5$	$8.6x10^5$	$1.9x10^7$	$6.9x10^6$	$7.4x10^7$	$3.1x10^7$
Fraction 4	3.125	$1.3x10^6$	$1.6x10^6$	$2.5x10^7$	$7.6x10^6$	$7.8x10^7$	$1.7x10^7$
	1.56	$1.6x10^6$	$1.9x10^6$	$2.5x10^7$	$8.8x10^6$	$7.8x10^7$	$2.8x10^7$
	0.78	$1.1x10^6$	$1.3x10^6$	$2.5x10^7$	$8.1x10^6$	$7.8x10^7$	$3.4x10^7$
Fraction 5	0.20	$9.3x10^5$	$8.8x10^5$	$1.9x10^7$	$5.7x10^6$	$7.4x10^7$	$8.1x10^6$
	0.10	$9.7x10^5$	$7.9x10^5$	$1.9x10^7$	$6.5x10^6$	$7.4x10^7$	$1.8x10^7$
	0.05	$8.4x10^5$	$8.6x10^5$	$1.9x10^7$	$7.7x10^6$	$7.4x10^7$	$2.7x10^7$
Fraction 6	0.78	$9.3x10^5$	$8.8x10^5$	$1.9x10^7$	$7.1x10^6$	$7.4x10^7$	$2.9x10^7$
	0.39	$9.7x10^5$	$7.9x10^5$	$1.9x10^7$	$8.5x10^6$	$7.4x10^7$	$2.3x10^7$
	0.20	$8.4x10^5$	$8.6x10^5$	$1.9x10^7$	$7.8x10^6$	$7.4x10^7$	$3.7x10^7$

3.8 Purification of fraction 5

3.8.1 Liquid chromatography purification

Screening of the six fractions for anticariogenic potential showed that fraction 5 (F5) was the most effective. Fraction 5 was further purified using liquid chromatography which resulted in two subfractions, subfraction 5.1 and 5.2. Figure 3.6 shows the column detection peaks obtained from running the sample on the liquid chromatography machine at 254 nm. Subfractions with two clean peaks were collected and named as subfraction 5.1 and 5.2. A total of 120 mg and 76 mg of extract were collected for subfraction 5.1 and 5.2 respectively. A solvent gradient ratio of hexane and ethyl acetate was used for elution.

Sample name	Fraction 5	User name	Tham
Solvent A	n-Hexane	Solvent B	Ethyl acetate
Flow rate(mL/min)	8.0	Run unit	Min
Rack type	FS-18mm	Fraction size(mL)	10.00
Detection(nm)	254	Monitor(nm)	280
Column		Flash Column 80g	

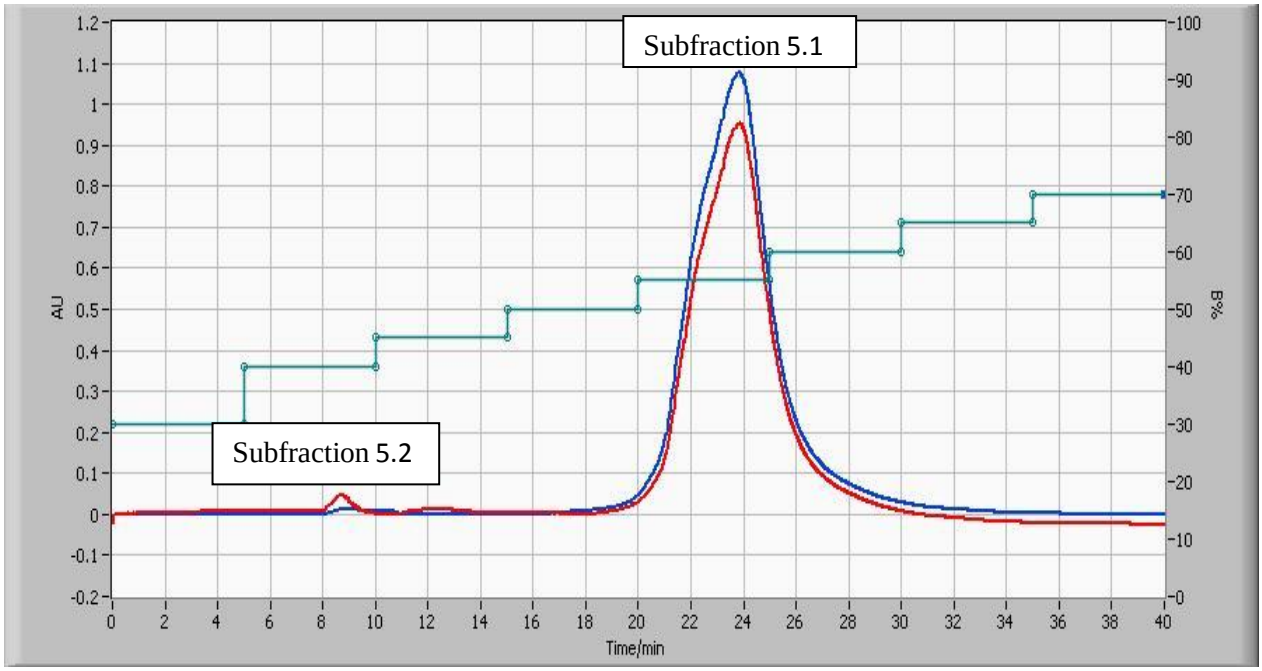

Figure 3.6: Fraction 5 liquid chromatography profile for elution with a solvent gradient ratio of hexane and ethyl acetate.

3.9 Determination of antimicrobial activity of subfraction 5.1 and subfraction 5.2

Both *D. viscosa* var. *angustifolia* subfractions 5.1 and 5.2 exhibited antimicrobial activity against both *S. mutans* strains. The MIC and MBC results for the crude extract, subfraction 5.1 and 5.2 on both *S. mutans* strains are presented in Table 3.7. All the assays were done in triplicate for both strains.

Table 3.7: Minimum inhibitory concentrations and minimum bactericidal concentrations of *D. viscosa* var. *angustifolia* crude extract and subfractions

Cultures	Repeats	Crude Methanol Extract		Sub-fraction 5.1		Sub-fraction 5.2	
		MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S. mutans ATCC 10923	1	0.78	1.56	0.05	0.1	0.78	1.56
	2	0.78	1.56	0.05	0.1	1.56	3.125
	3	0.78	3.125	0.01	0.1	1.56	3.125
Median		0.78	1.56	0.05	0.1	1.56	3.125
S. mutans SM1	1	0.78	1.56	0.1	0.2	0.78	1.56
	2	0.78	1.56	0.1	0.39	1.56	3.125
	3	0.78	1.56	0.1	0.39	1.56	3.125
Median		0.78	1.56	0.1	0.39	1.56	3.125

The crude extract MBCs ranged from 1.56-3.125 mg/ml. The lowest MBC result recorded was observed from subfraction 5.1 at 0.1 mg/ml with *S. mutans* strain ATCC 10923. Subfraction 5.1 also had the lowest MIC recorded of 0.05 mg/ml indicating its significant antimicrobial potential against cariogenic bacteria, *S. mutans* (Table 3.7, Figure 3.7). Subfraction 5.2 did not exhibit significant difference in activity compared to the crude extract with the same MBC results which ranged from 1.56-3.125 mg/ml. Acetone was used to dissolve the extracts and did not have any noteworthy effect on the growth of bacteria. Figure 3.7 shows the microtitre plates with the assays after incubating with INT dye.

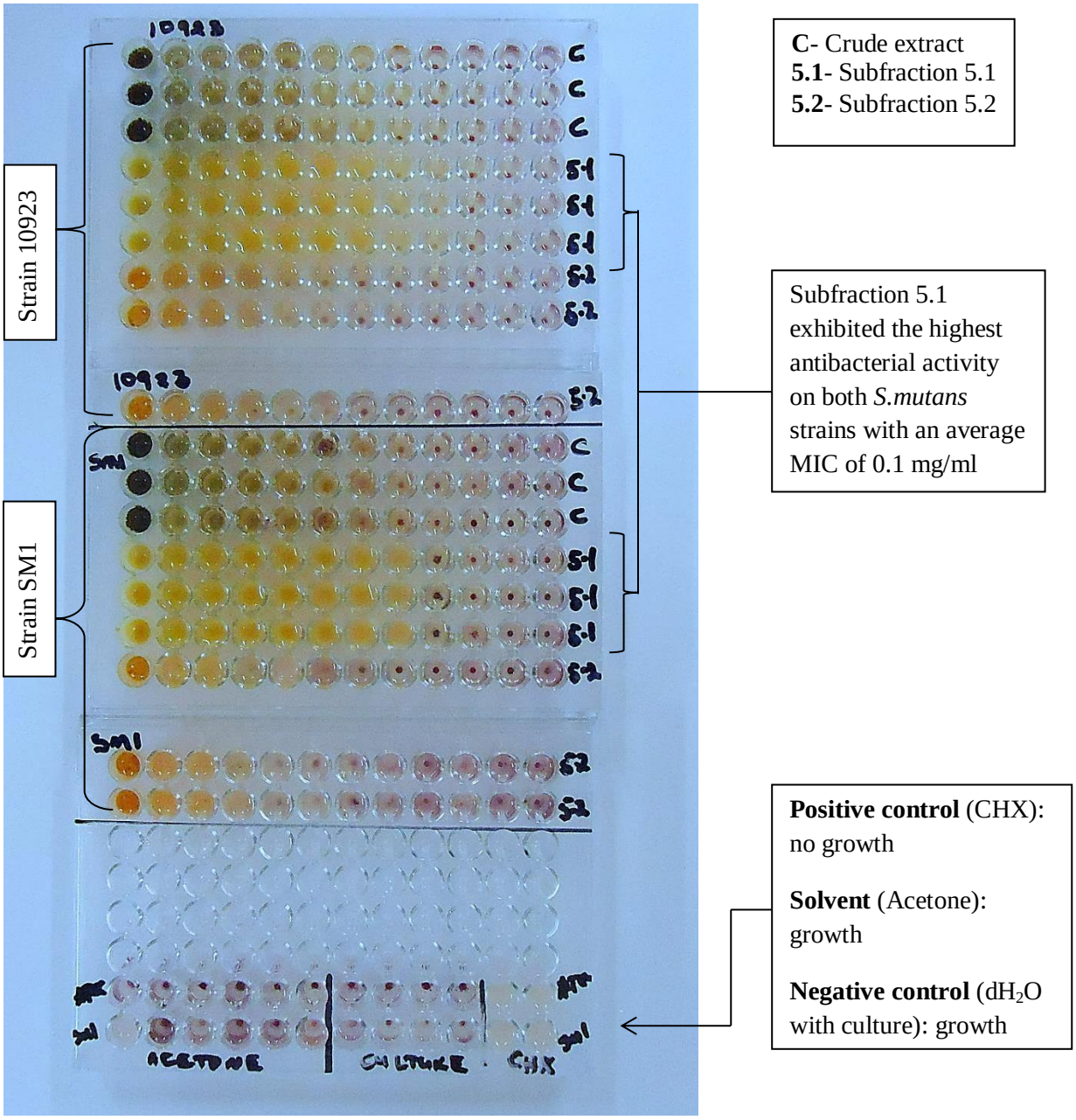

Figure 3.7: Microtitre plates showing the antimicrobial activity tests of *D. viscosa* var. *angustifolia* crude extract and subfractions after incubation with INT dye.

Subfraction 5.1 was the most active of the two subfractions with a higher total activity value of 1200 (120mg/0.1) compared to that of subfraction 5.2 which had a total activity value of 48.7 (76mg/1.56) as recorded in Table 3.7. Subfraction 5.1 was chosen for further

anticariogenic activity assays together with the crude extract for comparison as shown in Table 3.8.

Table 3.8: Outline of the concentrations used for further biofilm and acid production assays.

Concentrations used (mg/ml)	Crude extract	Subfraction 5.1
0.78	**x**	
0.39	**x**	
0.2	**x**	
0.1		
0.05		**x**
0.025		**x**
0.0125		**x**

Biofilm formation and acid production assays were carried out using both *S. mutans* strains, ATCC 10923 and SM1. The experiments were repeated three times producing six results which were statistically analyzed.

3.9.1 Effect of crude methanol extract and subfraction 5.1 on *Streptococcus mutans* biofilm formation

Table 3.9, Figure 3.8 and Figure 3.9 show the results of the effect of *D. viscosa* var. *angustifolia* crude methanol extract and subfraction 5.1 on biofilm formation by *S. mutans*. The effect of *D. viscosa* var. *angustifolia* on *S. mutans* biofilm formation was investigated using three subinhibitory concentrations from the crude extract and subfraction 5.1. The results were recorded after 6 and 24 hours of exposure. The assays were done in triplicate for each of the two strains. Means and standard deviations were calculated.

Both the crude extract and subfraction 5.1 exhibited significant biofilm inhibition after 6 and 24 hours of incubation. The biofilm counts increased with time for all the assays but the slides exposed to *D. viscosa* var. *angustifolia* still had lower counts compared to the control slides. The biofilm reduction effect was shown to be concentration dependent for all the exposure assays. The highest subinhibitory concentration of 0.78 mg/ml from the crude extract reduced biofilm bacterial count by a difference of 0.94 and 1.19 log CFU counts in comparison with the control after 6 and 24 hours respectively. For subfraction 5.1, the highest subinhibitory concentration of 0.05 mg/ml reduced biofilm formation by 1.18 and 2 log CFU counts in comparison with the control after 6 and 24 hours respectively.

The lower subinhibitory test concentrations from subfraction 5.1 (0.05 mg/ml – 0.0125 mg/ml) had a better biofilm inhibitory effect compared to the corresponding crude extract subinhibitory concentrations (0.78 mg/ml – 0.2 mg/ml). This indicated that the purified subfraction had more anticariogenic activity compared to the crude extract. The percentage reduction in biofilm bacterial counts was also calculated for both the crude extract and subfraction using controls. The results are presented in Table 3.10.

Table 3.9 Effect of subinhibitory concentrations of *D. viscosa* var. *angustifolia* crude extract and subfraction 5.1 on *S. mutans* biofilm

Cultures	Repeats	Growth of *S. mutans* in biofilm (CFU/ml): Crude extract											
		6 hours						24 hours					
		0.78 mg/ml		0.39 mg/ml		0.2 mg/ml		0.78 mg/ml		0.39 mg/ml		0.2 mg/ml	
		Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant
***S. mutans* ATCC 10923**	1	$2.2x10^5$	$3.9x10^4$	$2.2x10^5$	$6.2x10^4$	$2.2x10^5$	$9.6x10^4$	$2.2x10^7$	$1.3x10^6$	$2.2x10^7$	$3.6x10^6$	$2.2x10^7$	$8.7x10^6$
	2	$3.6x10^5$	$2.4x10^4$	$3.6x10^5$	$7.8x10^4$	$3.6x10^5$	$2.3x10^5$	$1.9x10^7$	$2.4x10^6$	$1.9x10^7$	$2.8x10^6$	$1.9x10^7$	$6.7x10^6$
	3	$1.7x10^5$	$2.9x10^4$	$1.7x10^5$	$5.1x10^4$	$1.7x10^5$	$8.9x10^4$	$2.8x10^7$	$1.8x10^6$	$2.8x10^7$	$2.4x10^6$	$2.8x10^7$	$6.4x10^6$
	Mean	$2.5x10^5$	$3.1x10^4$	$2.5x10^5$	$6.4x10^4$	$2.5x10^5$	$1.4x10^5$	$2.3x10^7$	$1.8x10^5$	$2.3x10^7$	$2.9x10^6$	$2.3x10^7$	$7.2x10^6$
***S. mutans* SM1**	1	$8.4x10^6$	$8.9x10^5$	$8.4x10^6$	$1.1x10^6$	$8.4x10^6$	$3.2x10^6$	$3.1x10^8$	$2.6x10^7$	$3.1x10^8$	$4.8x10^7$	$3.1x10^8$	$7.9x10^7$
	2	$6.6x10^6$	$7.8x10^5$	$6.6x10^6$	$9.4x10^5$	$6.6x10^6$	$2.9x10^6$	$2.6x10^8$	$1.1x10^7$	$2.6x10^8$	$5.4x10^7$	$2.6x10^8$	$7.1x10^7$
	3	$7.4x10^6$	$9.3x10^5$	$7.4x10^6$	$9.8x10^5$	$7.4x10^6$	$2.5x10^6$	$1.9x10^8$	$1.8x10^7$	$1.9x10^8$	$3.2x10^7$	$1.9x10^8$	$6.7x10^7$
	Mean	$7.5x10^6$	$8.7x10^5$	$7.5x10^6$	$1.0x10^6$	$7.5x10^6$	$2.9x10^6$	$2.5x10^8$	$1.8x10^7$	$2.5x10^8$	$4.5x10^7$	$2.5x10^8$	$7.2x10^7$
Pool mean		**$3.9x10^6$**	**$4.5x10^5$**	**$3.9x10^6$**	**$5.3x10^5$**	**$3.9x10^6$**	**$1.5x10^6$**	**$1.4x10^8$**	**$9.1x10^6$**	**$1.4x10^8$**	**$2.4x10^7$**	**$1.4x10^8$**	**$3.9x10^7$**
±SD		**3646.2**	**420421**	**3646.2**	**116210**	**3646.2**	**137991**	**120338**	**932423**	**120338**	**21878**	**120338**	**327319**

Cultures	Repeats	Growth of *S. mutans* in biofilm (CFU/ml): Subfraction 5.1											
		6 hours						24 hours					
		0.05 mg/ml		0.025 mg/ml		0.0125 mg/ml		0.05 mg/ml		0.025 mg/ml		0.0125 mg/ml	
		Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant
***S. mutans* ATCC 10923**	1	$1.9x10^5$	$1.6x10^4$	$1.9x10^5$	$5.6x10^4$	$1.9x10^5$	$8.4x10^4$	$4.1x10^7$	$3.8x10^5$	$4.1x10^7$	$7.4x10^5$	$4.1x10^7$	$5.2x10^6$
	2	$3.4x10^5$	$1.9x10^4$	$3.4x10^5$	$4.3x10^4$	$3.4x10^5$	$9.8x10^4$	$2.8x10^7$	$4.2x10^5$	$2.8x10^7$	$8.9x10^5$	$2.8x10^7$	$4.8x10^6$
	3	$2.4x10^5$	$1.1x10^4$	$2.4x10^5$	$4.9x10^4$	$2.4x10^5$	$9.2x10^4$	$3.4x10^7$	$3.5x10^5$	$3.4x10^7$	$8.3x10^5$	$3.4x10^7$	$5.6x10^6$
	Mean	$2.6x10^5$	$1.5x10^4$	$2.6x10^5$	$6.6x10^4$	$2.6x10^5$	$9.1x10^4$	$3.4x10^7$	$3.8x10^5$	$3.4x10^7$	$8.2x10^5$	$3.4x10^7$	$5.2x10^6$
***S. mutans* SM1**	1	$3.9x10^6$	$2.1x10^5$	$3.9x10^6$	$3.7x10^5$	$3.9x10^6$	$6.4x10^5$	$3.6x10^8$	$2.3x10^6$	$3.6x10^8$	$6.4x10^6$	$3.6x10^8$	$4.5x10^7$
	2	$1.7x10^6$	$1.4x10^5$	$1.7x10^6$	$1.9x10^5$	$1.7x10^6$	$4.8x10^5$	$2.7x10^8$	$3.1x10^6$	$2.7x10^8$	$5.6x10^6$	$2.7x10^8$	$3.9x10^7$
	3	$2.9x10^6$	$1.8x10^5$	$2.9x10^6$	$2.5x10^5$	$2.9x10^6$	$5.6x10^5$	$2.1x10^8$	$3.4x10^6$	$2.1x10^8$	$5.3x10^6$	$2.1x10^8$	$2.6x10^7$
	Mean	$2.8x10^6$	$1.8x10^5$	$2.8x10^6$	$2.7x10^5$	$2.8x10^6$	$5.6x10^5$	$2.8x10^8$	$2.9x10^6$	$2.8x10^8$	$5.8x10^6$	$2.8x10^8$	$3.7x10^7$
Pool mean		**$1.5x10^6$**	**$9.8x10^4$**	**$1.5x10^6$**	**$1.7x10^5$**	**$1.5x10^6$**	**$3.3x10^5$**	**$1.6x10^8$**	**$1.6x10^6$**	**$1.6x10^8$**	**$3.3x10^6$**	**$1.6x10^8$**	**$2.1x10^7$**
±SD		**143742**	**83208**	**143742**	**122423**	**143742**	**238876**	**1303.4**	**131674**	**1303.4**	**249540**	**1303.4**	**167043**

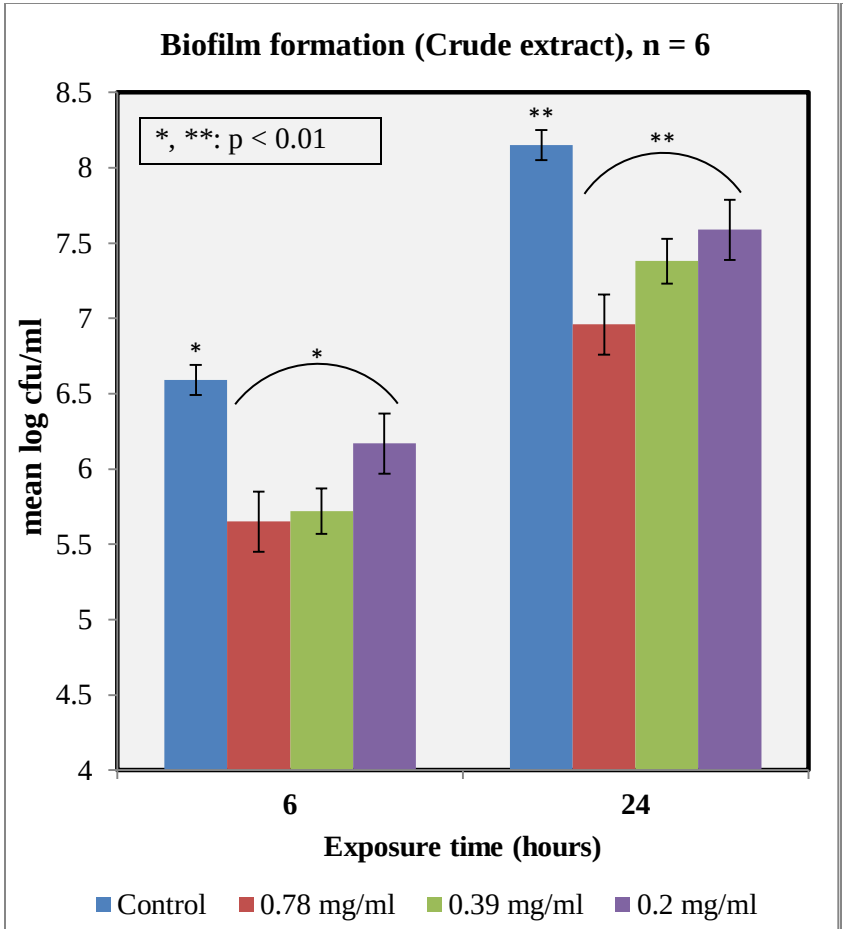

Figure 3.8: Effect of subinhibitory concentrations of crude extract on *Streptococcus mutans* biofilm formation (log CFU/ml) after 6 and 24 hour of exposure. *, **: Comparison between bacterial counts of controls and treated *S. mutans* cultures after 6 and 24 hours using the Wilcoxon rank-sum test (Mann-Whitney).

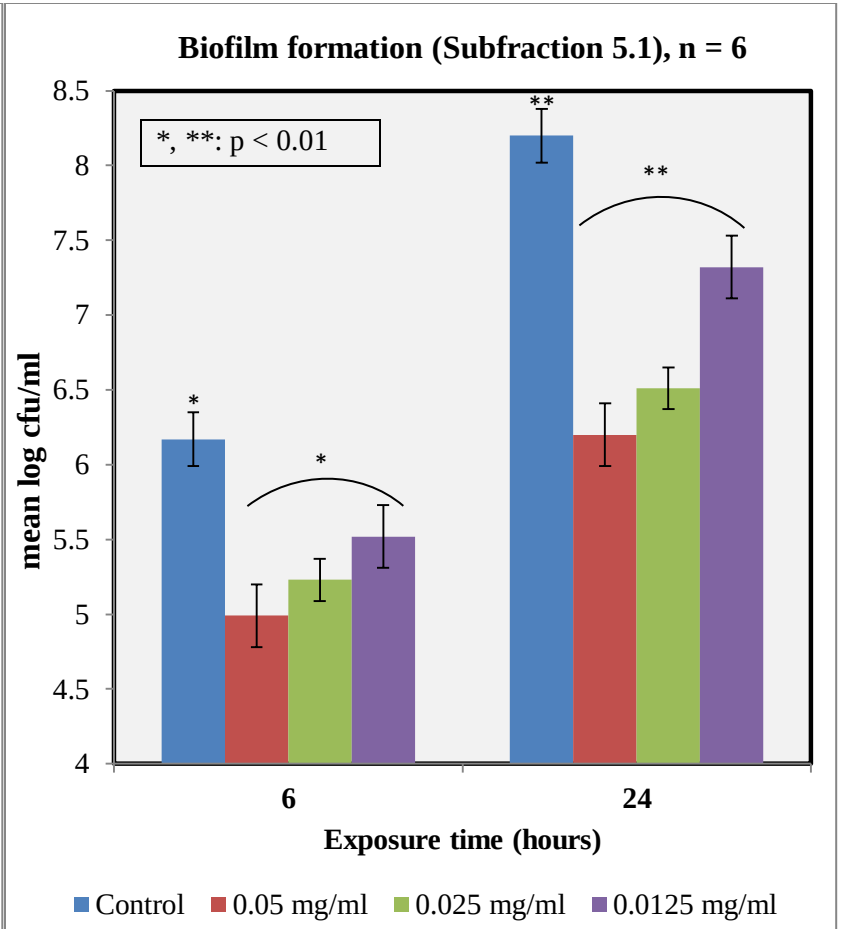

Figure 3.9: Effect of subinhibitory concentrations of subfraction 5.1 on *Streptococcus mutans* biofilm formation (log CFU/ml) after 6 and 24 hours of exposure. *, **: Comparison between bacterial counts of controls and treated *S. mutans* cultures after 6 and 24 hours using the Wilcoxon rank-sum test (Mann-Whitney).

Table 3.10 Percentage reduction in biofilm formation after exposure to subinhibitory concentrations of *D. viscosa* var. *angustifolia* crude extract and subfraction 5.1

Cultures	Repeats	% Reduction in biofilm formation: Crude extract					
		Crude (mg/ml): 6 hours			Crude (mg/ml): 24 hours		
		0.78	**0.39**	**0.2**	**0.78**	**0.39**	**0.2**
***S. mutans* ATCC 10923**	1	82.3	71.8	56.4	94.1	83.6	60.5
	2	93.3	78.3	36.1	87.4	85.3	64.7
	3	82.9	70.0	47.6	93.6	91.4	77.1
	Mean	86.2	73.4	46.7	91.7	86.8	67.4
***S. mutans* SM1**	1	89.4	86.9	61.9	91.6	84.5	74.5
	2	88.2	85.8	56.1	95.7	79.2	72.3
	3	87.4	86.7	66.2	90.6	83.2	64.7
	Mean	88.3	86.5	61.4	92.6	82.3	70.5
	Combined Mean	**87.3**	**79.9**	**54.1**	**92.2**	**84.5**	**68.9**
	Combined ±SD	**4.643544**	**7.026478**	**9.855413**	**2.701029**	**3.624761**	**5.99963**
Wilcoxon-Rank sum test		**P < 0.01**			**P < 0.01**		
Cultures	**Repeats**	**% Reduction in biofilm formation: Sub-fraction 5.1**					
		Sub-fraction 5.1 (mg/ml): 6 hours			**Sub-fraction 5.1 (mg/ml): 24 hours**		
		0.05	**0.025**	**0.0125**	**0.05**	**0.025**	**0.0125**
***S. mutans* ATCC 10923**	1	92.7	74.5	61.8	99.1	98.2	87.3
	2	94.4	87.3	71.2	98.5	96.8	82.9
	3	95.4	79.6	61.6	98.9	97.6	83.5
	Mean	94.2	80.5	64.9	98.8	97.5	84.6
***S. mutans* SM1**	1	94.6	90.5	83.6	99.4	98.2	87.5
	2	91.8	88.8	71.8	98.9	97.9	85.5
	3	93.7	91.4	80.7	98.4	97.4	87.6
	Mean	93.4	90.2	78.7	98.9	97.8	86.9
	Combined Mean	**93.8**	**85.4**	**71.8**	**98.9**	**97.7**	**85.7**
	Combined ±SD	**1.421756**	**7.404372**	**9.68012**	**0.339935**	**0.489898**	**1.920431**
Wilcoxon-Rank sum test		**P < 0.01**			**P < 0.01**		

When the percentage reduction in the biofilm was compared, similar significant reduction was noted with crude extract at 6 and 24 hours ($p<0.01$) and with subfraction 5.1 ($p<0.01$) as shown in Table 3.10. The subinhibitory concentration of 0.78 mg/ml from the crude extract reduced biofilm formation by an average of 87.3 % after 6 hours of incubation. This reduction was less significant when compared to the effect of subfraction 5.1 which reduced biofilm formation by an average of 93.8 % at 0.05 mg/ml after 6 hours of incubation. The same effect was observed after 24 hours of exposure where the crude extract reduced biofilm formation by an average of 92.2 % and subfraction 5.1 reduced biofilm formation by an average of 98.9 %. Overall the subfraction performed better than the crude extract producing greater percentage reduction of biofilm at much lower concentrations.

3.9.2 Effect of crude methanol extract and subfraction 5.1 on acid production by *S. mutans*

Results of the effect of crude extract and subfraction 5.1 on acid production by *S. mutans* are shown in Table 3.11 and 3.12 respectively. Three subsequent subinhibitory concentrations were used in the assays for each *S. mutans* strain. Tests were done in triplicate. Means and standard deviations of the pH values were calculated for each of the *S. mutans* strains. These are represented in Figure 3.10 for the crude extract and Figure 3.11 for subfraction 5.1. The pH was first measured at 0 hours for all the assays and then every 2 hours following an 8 hour incubation period up to 14 hours. There was a gradual drop in pH with time for both the controls and assays indicating bacterial growth (Table 3.11 and Table 3.12). It was important though to prove that the difference in pH between the control and treated assays was not due to significant differences in the number of *S. mutans* cells hence bacterial counts were done

at 0, 8, and 10 hours. *Streptococcus mutans* counts are presented in Table 3.13, Figure 3.10 and Figure 3.11.

Table 3.11 Effect of subinhibitory concentrations of crude extract on acid production by *S. mutans*

Cultures	Plant (mg/ml)	Effect of crude on acid production by *S. mutans* (pH)											pH difference after 14 hours	
		Repeats	0 hours		8 hours		10 hours		12 hours		14 hours			
			Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Crude
***S. mutans* ATCC 10923**	**0.78**	1	7.49	7.48	6.75	7.22	6.12	6.87	5.61	6.64	5.27	6.48	2.22	1
		2	7.49	7.47	6.68	7.26	6.09	6.83	5.58	6.61	5.31	6.45	2.18	1.02
		3	7.49	7.48	6.78	7.24	6.05	6.82	5.67	6.59	5.25	6.51	2.24	0.97
		Mean	7.49	7.48	6.74	7.24	6.08	6.84	5.62	6.61	5.28	6.48	2.21	0.99
	0.39	1	7.49	7.47	6.75	7.11	6.12	6.69	5.61	6.38	5.27	6.23	2.22	1.24
		2	7.49	7.47	6.68	7.14	6.09	7.67	5.58	6.35	5.31	6.19	2.18	1.28
		3	7.49	7.48	6.78	7.09	6.05	6.71	5.67	6.37	5.25	6.20	2.24	1.28
		Mean	7.49	7.48	6.74	7.11	6.09	6.69	5.62	6.36	5.28	6.21	2.21	1.27
	0.2	1	7.49	7.47	6.75	6.94	6.12	6.48	5.61	6.19	5.27	6.01	2.22	1.46
		2	7.49	7.48	6.68	6.92	6.09	6.53	5.58	6.23	5.31	5.98	2.18	1.50
		3	7.49	7.47	6.78	6.89	6.05	6.48	5.67	6.20	5.25	5.97	2.24	1.50
		Mean	7.49	7.47	6.74	6.91	6.09	6.50	5.62	6.21	5.28	5.99	2.21	1.49
***S. mutans* SM1**	**0.78**	1	7.49	7.48	6.87	7.19	6.23	6.82	5.72	6.58	5.24	6.41	2.25	1.07
		2	7.49	7.48	6.79	7.24	6.18	6.80	5.68	6.63	5.18	6.38	2.31	1.10
		3	7.49	7.48	6.84	7.16	6.12	6.77	5.76	6.60	5.12	6.43	2.37	1.05
		Mean	7.49	7.48	6.83	7.20	6.18	6.79	5.72	6.61	5.18	6.41	2.31	1.07
	0.39	1	7.49	7.48	6.87	7.03	6.23	6.66	5.72	6.33	5.24	6.19	2.25	1.29
		2	7.49	7.48	6.79	7.08	6.18	6.63	5.68	6.29	5.18	6.14	2.31	1.34
		3	7.49	7.49	6.84	7.06	6.12	6.62	5.76	6.35	5.12	6.17	2.37	1.32
		Mean	7.49	7.48	6.83	7.06	6.18	6.64	5.72	6.32	5.18	6.16	2.31	1.32
	0.2	1	7.49	7.48	6.87	6.93	6.23	6.45	5.72	6.14	5.24	5.90	2.25	1.58
		2	7.49	7.47	6.79	6.90	6.18	6.47	5.68	6.12	5.18	5.89	2.31	1.57
		3	7.49	7.49	6.84	6.88	6.12	6.51	5.76	6.17	5.12	5.87	2.37	1.62
		Mean	7.49	7.48	6.83	6.90	6.18	6.48	5.72	6.14	5.18	5.89	2.31	1.59

Table 3.12: Effect of subinhibitory concentrations of **subfraction 5.1** on acid production by *S. mutans*

Cultures	Plant mg/ml	Effect of sub-fraction 5.1 on acid production by *S. mutans* (pH)										pH difference after 14 hours	
		Repeats	0 hours		8 hours		10 hours		12 hours		14 hours		
			Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	Plant	Control	5.1
***S. mutans* ATCC 10923**	**0.05**	1	7.49	7.48	6.75	7.38	6.12	7.31	5.61	7.18	5.27	7.11	2.22	0.37
		2	7.49	7.49	6.68	7.35	6.09	7.27	5.58	7.14	5.31	7.05	2.18	0.44
		3	7.49	7.48	6.78	7.41	6.05	7.33	5.67	7.21	5.25	7.08	2.24	0.40
		Mean	7.49	7.48	6.74	7.38	6.08	7.30	5.62	7.18	5.28	7.08	2.21	0.40
	0.025	1	7.49	7.49	6.75	7.28	6.12	7.21	5.61	6.99	5.27	6.84	2.22	0.65
		2	7.49	7.48	6.68	7.25	6.09	7.18	5.58	7.03	5.31	6.91	2.18	0.57
		3	7.49	7.48	6.78	7.31	6.05	7.24	5.67	7.08	5.25	6.87	2.24	0.61
		Mean	7.49	7.48	6.74	7.28	6.09	7.21	5.62	7.03	5.28	6.87	2.21	0.61
	0.013	1	7.49	7.48	6.75	7.18	6.12	6.94	5.61	6.85	5.27	6.69	2.22	0.79
		2	7.49	7.48	6.68	7.13	6.09	6.87	5.58	6.79	5.31	6.64	2.18	0.84
		3	7.49	7.48	6.78	7.23	6.05	6.98	5.67	6.74	5.25	6.61	2.24	0.87
		Mean	7.49	7.48	6.74	7.18	6.09	6.93	5.62	6.79	5.28	6.65	2.21	0.83
***S. mutans* SM1**	**0.05**	1	7.49	7.48	6.87	7.31	6.23	7.25	5.72	7.18	5.24	7.07	2.25	0.41
		2	7.49	7.49	6.79	7.28	6.18	7.21	5.68	7.13	5.18	7.00	2.31	0.49
		3	7.49	7.48	6.84	7.34	6.12	7.27	5.76	7.15	5.12	7.02	2.37	0.46
		Mean	7.49	7.48	6.83	7.31	6.18	7.24	5.72	7.15	5.18	7.03	2.31	0.45
	0.25	1	7.49	7.49	6.87	7.25	6.23	7.14	5.72	7.08	5.24	6.91	2.25	0.58
		2	7.49	7.48	6.79	7.28	6.18	7.18	5.68	7.05	5.18	6.84	2.31	0.64
		3	7.49	7.49	6.84	7.23	6.12	7.10	5.76	6.98	5.12	6.81	2.37	0.68
		Mean	7.49	7.49	6.83	7.25	6.18	7.14	5.72	7.04	5.18	6.85	2.31	0.63
	0.013	1	7.49	7.48	6.87	7.15	6.23	6.94	5.72	6.72	5.24	6.54	2.25	0.94
		2	7.49	7.48	6.79	7.11	6.18	6.87	5.68	6.69	5.18	6.49	2.31	0.99
		3	7.49	7.49	6.84	7.09	6.12	6.83	5.76	6.75	5.12	6.59	2.37	0.90
		Mean	7.49	7.48	6.83	7.12	6.18	6.88	5.72	6.72	5.18	6.54	2.31	0.94

Table 3.13: *Streptococcus mutans* counts (CFU/ml) in the presence and absence of subinhibitory concentrations of crude extract and subfraction 5.1.

Cultures	Repeats	Growth of *S. mutans* in acid production bioassay (CFU/ml)											
		Crude extract											
		0 hours				8 hours				10 hours			
		Control	**0.78**	**0.39**	**0.2**	Control	**0.78**	**0.39**	**0.2**	Control	**0.78**	**0.39**	**0.2**
***S. mutans* ATCC 10923**	1	$4.2x10^4$	$3.5x10^4$	$3.4x10^4$	$4.6x10^4$	$6.5x10^6$	$2.9x10^6$	$3.8x10^6$	$4.6x10^6$	$9.4x10^6$	$4.7x10^6$	$5.9x10^6$	$7.2x10^6$
	2	$3.7x10^4$	$2.9x10^4$	$3.9x10^4$	$3.7x10^4$	$6.1x10^6$	$9.4x10^5$	$4.1x10^6$	$5.1x10^6$	$1.3x10^7$	$3.6x10^6$	$6.3x10^6$	$7.9x10^6$
	3	$5.8x10^4$	$3.9x10^4$	$4.1x10^4$	$4.0x10^4$	$5.8x10^6$	$2.5x10^6$	$3.4x10^6$	$4.3x10^6$	$2.1x10^7$	$4.4x10^6$	$6.6x10^6$	$7.6x10^6$
	Mean	$4.6x10^4$	$3.4x10^4$	$3.8x10^4$	$4.1x10^4$	$6.1x10^6$	$2.1x10^6$	$3.7x10^6$	$4.7x10^6$	$1.5x10^7$	$4.2x10^6$	$6.3x10^6$	$7.5x10^6$
***S. mutans* SM 1**	1	$6.3x10^5$	$5.3x10^5$	$5.7x10^5$	$4.5x10^5$	$3.2x10^7$	$4.4x10^6$	$6.1x10^6$	$9.4x10^6$	$8.1x10^7$	$1.3x10^7$	$4.1x10^7$	$4.9x10^7$
	2	$5.1x10^5$	$4.2x10^5$	$4.9x10^5$	$3.9x10^5$	$2.9x10^7$	$3.9x10^6$	$7.8x10^6$	$8.3x10^6$	$9.9x10^7$	$9.4x10^6$	$3.8x10^7$	$5.7x10^7$
	3	$5.9x10^5$	$4.6x10^5$	$5.2x10^5$	$4.8x10^5$	$3.5x10^7$	$3.5x10^6$	$7.1x10^6$	$9.8x10^6$	$8.6x10^7$	$8.3x10^6$	$4.9x10^7$	$5.9x10^7$
	Mean	**$5.8x10^5$**	**$4.7x10^5$**	**$5.3x10^5$**	**$4.4x10^5$**	**$3.2x10^7$**	**$3.9x10^6$**	**$7.0x10^6$**	**$9.2x10^6$**	**$8.9x10^7$**	**$1.0x10^7$**	**$4.3x10^7$**	**$5.5x10^7$**
	±SD	**267908**	**220211**	**245454**	**201264**	**130503**	**111955**	**170236**	**230609**	**376422**	**333500**	**184948**	**239134**
Cultures	**Repeats**	**Growth of *S. mutans* in acid production bioassay (CFU/ml)**											
		Sub-fraction 5.1											
		0 hours				**8 hours**				**10 hours**			
		Control	**0.05**	**0.025**	**0.0125**	Control	**0.05**	**0.025**	**0.0125**	Control	**0.05**	**0.025**	**0.0125**
***S. mutans* ATCC 10923**	1	$2.7x10^4$	$1.9x10^4$	$3.3x10^4$	$2.5x10^4$	$5.5x10^6$	$3.7x10^6$	$4.1x10^6$	$5.2x10^6$	$1.4x10^7$	$6.1x10^6$	$6.9x10^6$	$7.9x10^6$
	2	$2.2x10^4$	$2.7x10^4$	$2.9x10^4$	$3.1x10^4$	$5.1x10^6$	$9.7x10^5$	$4.6x10^6$	$5.5x10^6$	$2.4x10^7$	$4.9x10^6$	$6.6x10^6$	$8.4x10^6$
	3	$3.1x10^4$	$2.4x10^4$	$1.8x10^4$	$2.0x10^4$	$6.7x10^6$	$3.5x10^6$	$5.1x10^6$	$4.8x10^6$	$2.1x10^7$	$5.8x10^6$	$7.2x10^6$	$8.2x10^6$
	Mean	$2.6x10^4$	$2.3x10^4$	$2.7x10^4$	$2.5x10^4$	$5.8x10^6$	$2.4x10^6$	$4.6x10^6$	$5.1x10^6$	$2.0x10^7$	$5.6x10^6$	$6.9x10^6$	$8.2x10^6$
***S. mutans* SM 1**	1	$4.3x10^5$	$3.9x10^5$	$3.1x10^5$	$2.7x10^5$	$2.7x10^7$	$8.9x10^6$	$9.4x10^6$	$1.2x10^7$	$5.1x10^7$	$1.9x10^7$	$4.9x10^7$	$5.9x10^7$
	2	$3.7x10^5$	$2.8x10^5$	$3.8x10^5$	$2.1x10^5$	$3.9x10^7$	$7.9x10^6$	$8.8x10^6$	$2.1x10^7$	$7.3x10^7$	$9.8x10^6$	$4.4x10^7$	$6.2x10^7$
	3	$4.7x10^5$	$4.1x10^5$	$2.7x10^5$	$1.8x10^5$	$2.5x10^7$	$9.1x10^6$	$9.1x10^6$	$1.8x10^7$	$6.8x10^7$	$2.2x10^7$	$5.2x10^7$	$6.7x10^7$
	Mean	**$4.2x10^5$**	**$3.6x10^5$**	**$3.2x10^5$**	**$2.2x10^5$**	**$3.0x10^7$**	**$8.6x10^6$**	**$9.1x10^6$**	**$1.7x10^7$**	**$6.4x10^7$**	**$1.7x10^7$**	**$4.8x10^7$**	**$6.3x10^7$**
	±SD	**200467**	**173133**	**150215**	**100915**	**130469**	**310510**	**217230**	**648444**	**233339**	**676108**	**208483**	**266108**

Table 3.14: Statistical analysis of the data obtained in the acid production assay

Extract	Test	Comparison	p values
Crude	pH	Control to all concentrations (overall)	0.0002
		Control to 0.78 mg/ml	0.04
		Control to 0.39 mg/ml	0.02
		Control to 0.2 mg/ml	0.03
	Bacterial counts	Control to all concentrations (overall)	0.013
		Control to 0.78 mg/ml	0.031
		Control to 0.39 mg/ml	0.25
		Control to 0.2 mg/ml	0.33
Subfraction 5.1	pH	Control to all concentrations (overall)	<0.0001
		Control to 0.05 mg/ml	0.0002
		Control to 0.025 mg/ml	0.0001
		Control to 0.0125 mg/ml	0.0003
	Bacterial counts	Control to all concentrations (overall)	0.05
		Control to 0.05 mg/ml	0.10
		Control to 0.025 mg/ml	0.35
		Control to 0.0125 mg/ml	0.44

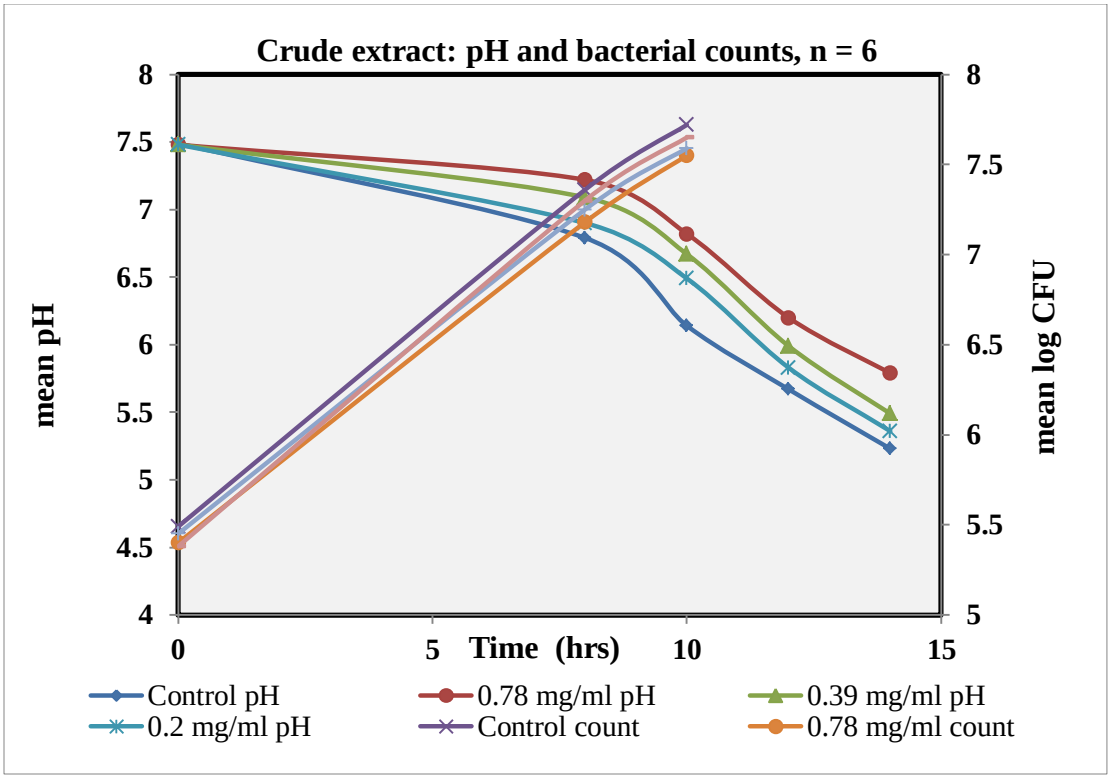

Figure 3.10: *Streptococcus mutans* bacterial counts (log CFU/ml) and mean pH values following exposure to subinhibitory concentrations of the crude extract

The crude methanol extract effectively reduced acid production by *S. mutans*. Notable pH differences were observed after 8 hours of incubation. The average control pH dropped to 5.23 after 14 hours which was lower than that of the exposed assays with the highest subinhibitory concentration (0.78 mg/ml) dropping to an average of 6.45 (Table 3.11). There was not much of a difference in bacterial counts between the controls and treated assays (Figure 3.10). Statistical analysis showed that the inhibitory effect of the overall concentrations was highly significant with a p value of 0.0002 as shown in Table 3.14. Statistical comparison between each of the three subinhibitory concentrations and controls also indicated that all the subinhibitory concentrations significantly reduced acid production by *S. mutans*. The slight difference in bacterial counts proved that the inhibitory effect was due to the plant and not differences in bacterial counts.

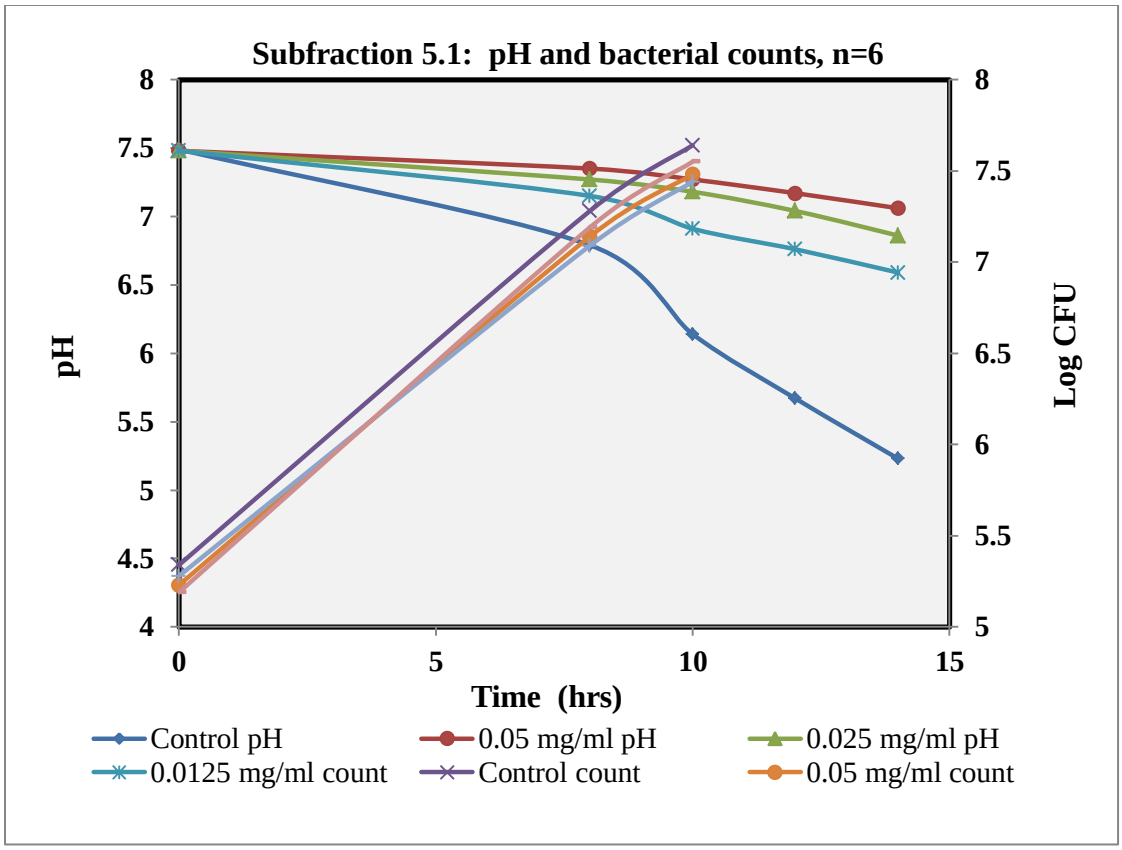

Figure 3.11: *Streptococcus mutans* bacterial counts (log CFU/ml) and mean pH values following exposure to subinhibitory concentrations of subfraction 5.1

Subfraction 5.1 inhibited acid production more effectively at low concentrations compared to crude extract. The drop in pH from the highest subinhibitory concentration of 0.05 mg/ml was 7.05 after 14 hours. That average pH was lower in comparison to the crude extract (0.78 mg/ml) which had dropped to an average pH of 6.45 (Table 3.11). There was no great difference ($p=0.05$) in bacterial counts between the controls and treated assays (Figure 3.11 and Table 3.14). The inhibitory effect of the overall concentrations was highly significant when compared to the controls with a p value of < 0.0001 as shown in Table 3.14. All the three individual subinhibitory concentrations significantly reduced acid production by *S. mutans* ($p<0.01$). In addition, the inhibition was concentration dependent as shown in Figure 3.11.

3.10 Gas Chromatography-Mass Spectrometry analysis of subfraction 5.1

The initial step to identify subfraction 5.1 was through GC-MS analysis. Table 3.15 gives an outline of the main compounds that were identified by GC-MS. Figure 3.12 shows the GC-MS chromatogram for subfraction 5.1. Each discrete chemical compound is represented by a peak. Most of the compounds detected were esters or derivatives of ester compounds. The highest peak was observed at a retention time (RT) of 6.574 which was detected as 2-butoxyethanol which is a glycol ether derivative. This was followed by the peak at RT 35.325 which was detected with 99% probability as 9- Octadecenoic acid (Z), methyl ester. Two more ester compounds were detected at RT 35.218 and 32.005 as 9, 12-Octadecadienoic acid (z,z)-methyl ester and Hexadecanoic acid, methyl ester respectively. The mass to charge ratio of the observed ionization peak 326.9 [M-3H] correlates with the molecular mass (330.28 g/mol) of the compound that was later identified as subfraction 5.1

Table 3.15: Main chemical compounds identified by GC-MS from the internal GC library for subfraction 5.1

Main peaks subfraction 5.1			
Peak	**RT (mins)**	**Area (%)**	**Possible compounds**
1	6.574	6.40	2-butoxyethanol Formic acid, 3,3- dimethylbut-2-yl ester
2	22.720	0.77	1tetradecamethylcycloheptasiloxane Pentasiloxane, dodecamethyl ester
3	32.005	1.57	Hexadecanoic acid, methyl ester Pentadecanoic acid, 14-methyl- methyl ester
4	35.218	3.68	9,12-Octadecadienoic acid (z,z)-, methyl ester 10,13-Octadecadienoic acid , methyl ester 8, 11-Octadecadienoic acid , methyl ester
5	35.325	2.87	11-Octadecenoic acid, methyl ester 6-Octadecenoic acid, methyl ester 9-Octadecenoic acid, methyl ester

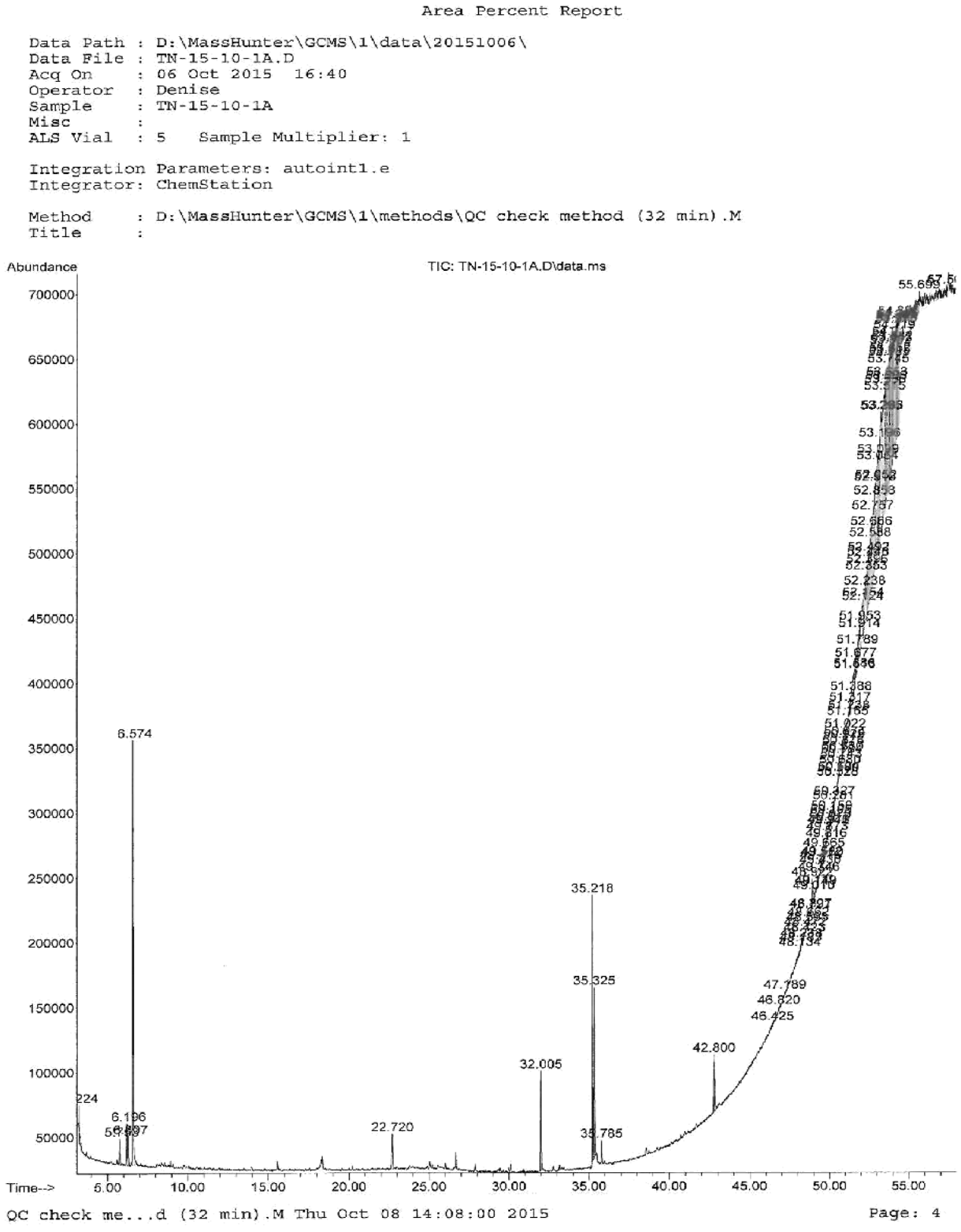

Figure 3.12: Gas Chromatography –Mass Spectrometry chromatogram of subfraction 5.1

3.11 Nuclear magnetic resonance spectroscopy for subfraction 5.1

Subfraction 5.1 was identified as **5,6,8-Trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one** as shown in Figure 3.13 .

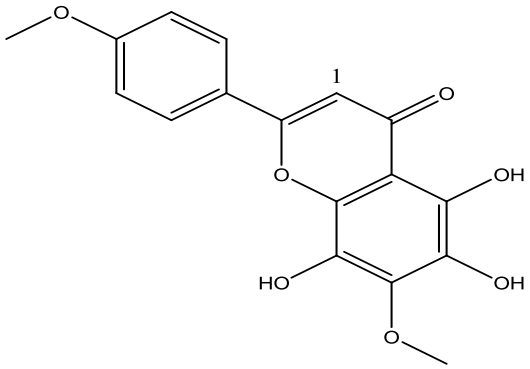

Figure 3.13: 5,6,8-Trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one

Figure 3.14 shows the proton (^{1}H) shift of the compound. Full proton characterization of subfraction 5.1 is outlined in Table 3.16. All the proton chemical shifts were observed in their respective and expected positions.

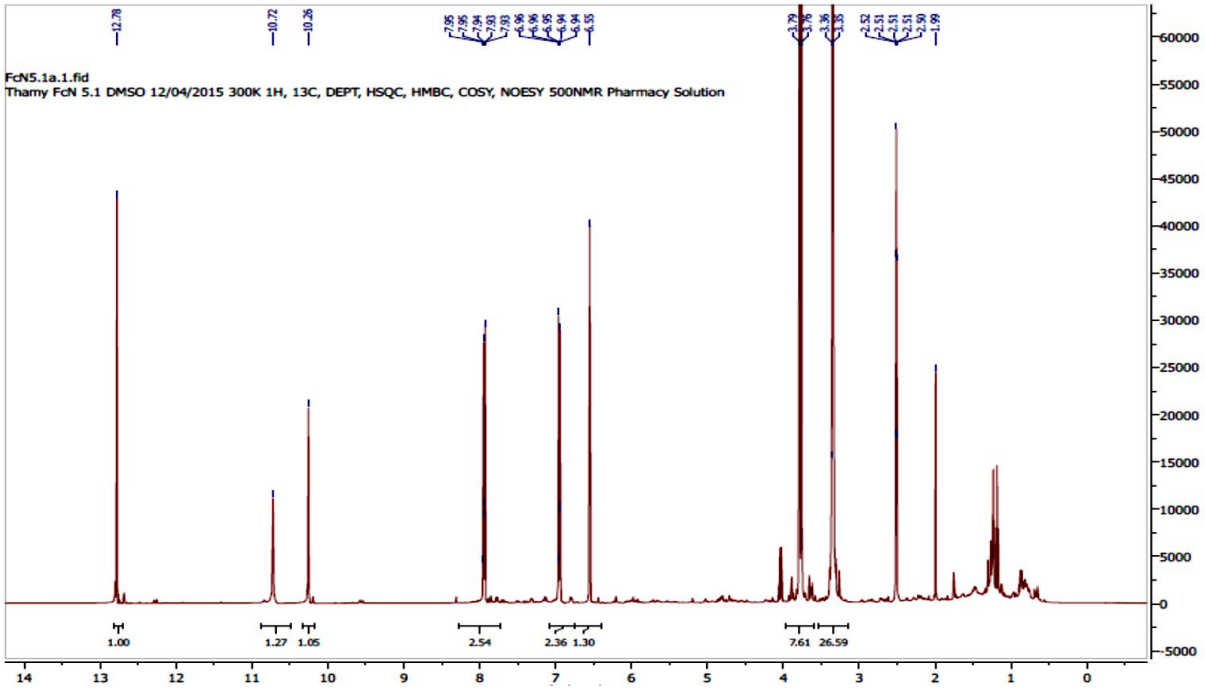

Figure 3.14: Proton chemical shifts for subfraction 5.1

Table 3.16: Subfraction 5.1 proton (H^1) assignment

Chemical shift	Number of protons	Coupling constant and multiplicity	Assignment
12.78	1	S	OH
10.72	1	S	OH
10.26	1	S	OH
7.99-7.92	2	M	ArH
6.98-6.92	2	M	ArH
6.55	1	S	Alkene H-1
3.79	3	S	OMe
3.76	3	S	OMe

The H^1 NMR elucidation yielded chemical shift assignments:

^{1}H NMR (DMSO-d_6): δ ppm: 12.78 (s,1H, Ar-OH), 10.72 (s, 1H, Ar-OH), 10.26 (s, 1H, Ar-OH), 7.99-9.92 (m, 2H, Ar-H), 6.98-6.92 (m, 2H, Ar-H), 6.55 (s, 1H, Alkene H-1), 3.79 (s, 3H, OMe), 3.76 (s, 3H, OMe).

Figure 3.15 shows the carbon (^{13}C) shift of subfraction 5.1. Full carbon characterization is shown in Table 3.17.

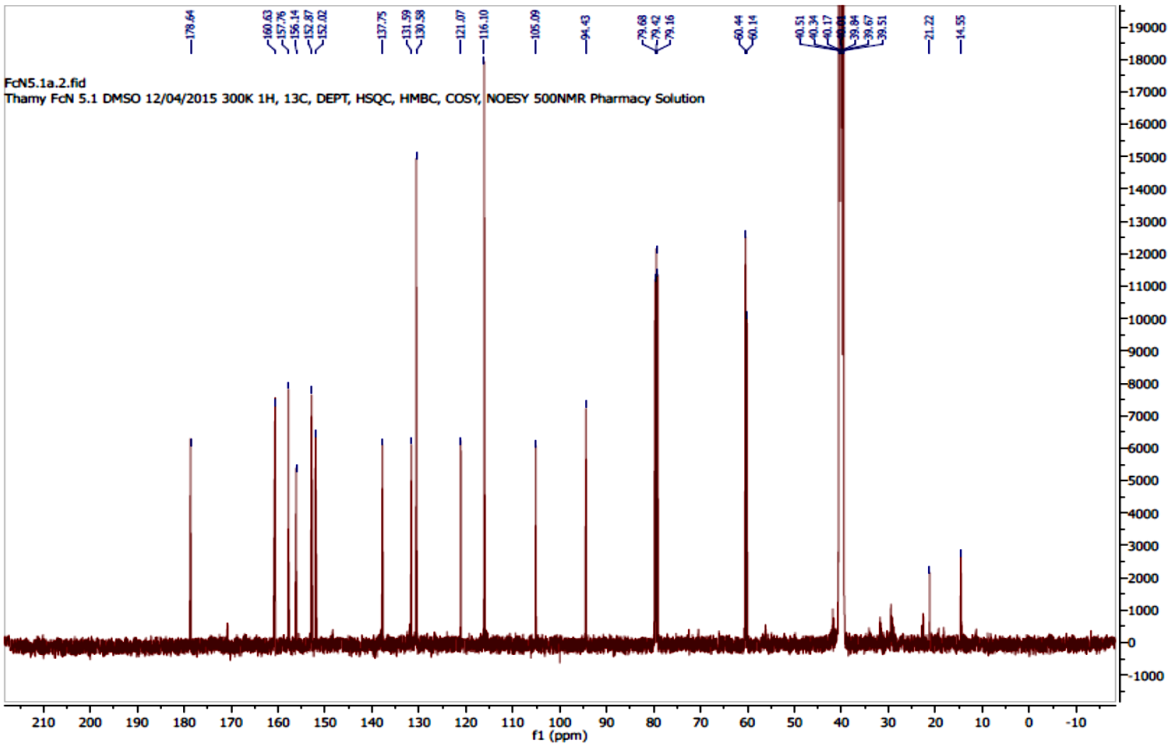

Figure 3.15: Subfraction 5.1 carbon chemical shifts

Table 3.17: Subfraction 5.1 carbon (C^{13}) assignment

Chemical shift	Assignment	Chemical shift	Assignment
178.64	C=O	130.58	ArC
160.63	ArCO	121.07	ArC
157.76	ArCO	116.10	ArC
156.14	ArCO	105.09	ArC
152.87	ArCO	94.43	C-1
152.02	ArCO	60.44	OMe
137.75	ArC	60.14	OMe
131.59	ArC		

The C^{13} chemical shifts were observed in their respective positions;
C^{13} NMR (DMSO-d_6): δ ppm: 178.64 (C=O), 160.63 (ArCO), 157.76 (ArCO), 156.14 (ArCO), 152.87 (ArCO), 152.02 (ArCO), 137.75 (ArC), 131.59 (ArC), 130.58 (Ar), 121.07 (ArC), 116.10 (ArH), 105.09 (ArC), 94.43 (C-1), 60.44 (OMe), 60.14 (OMe).

3.12 Infrared analysis of subfraction 5.1

The results from IR analysis of subfraction 5.1 are shown in Figure 3.16. Table 3.18 gives a summary of the observed infrared absorption bands for subfraction 5.1

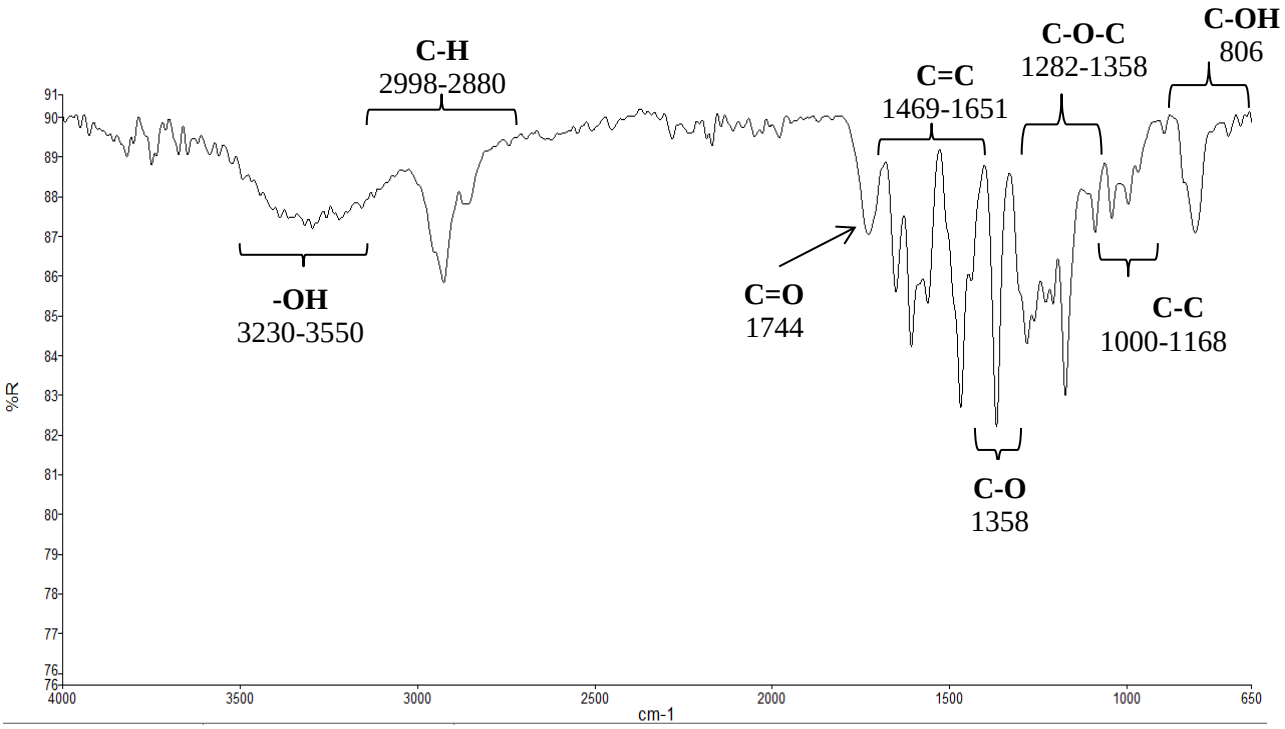

Figure 3.16: Infrared spectrum of subfraction 5.1

Table 3.18: Summary of the observed infrared absorption bands for subfraction 5.1

Wavelength (cm^{-1})	Vibration	Reference
3230-3550	O-H stretch	Clark, 2000
2880-2998	C-H stretch	
1744	C=O stretch	
1469-1651	C=C stretch	
1358	C-O	
1282-1358	C-O-C stretch	
1000-1168	C-C stretch	
806	C-OH	

3.13 Human embryonic kidney epithelial cells cytotoxicity study

The cytotoxicity effect of *D. viscosa* var. *angustifolia* crude extract and purified subfraction were performed on the HEK293 cell line by the MTT assay method. Dose response graphs were constructed for both tests. Figure 3.17 shows the crude extract microtitre plate assay after the addition of 100% DMSO. The absorbance values are presented in Table 3.19

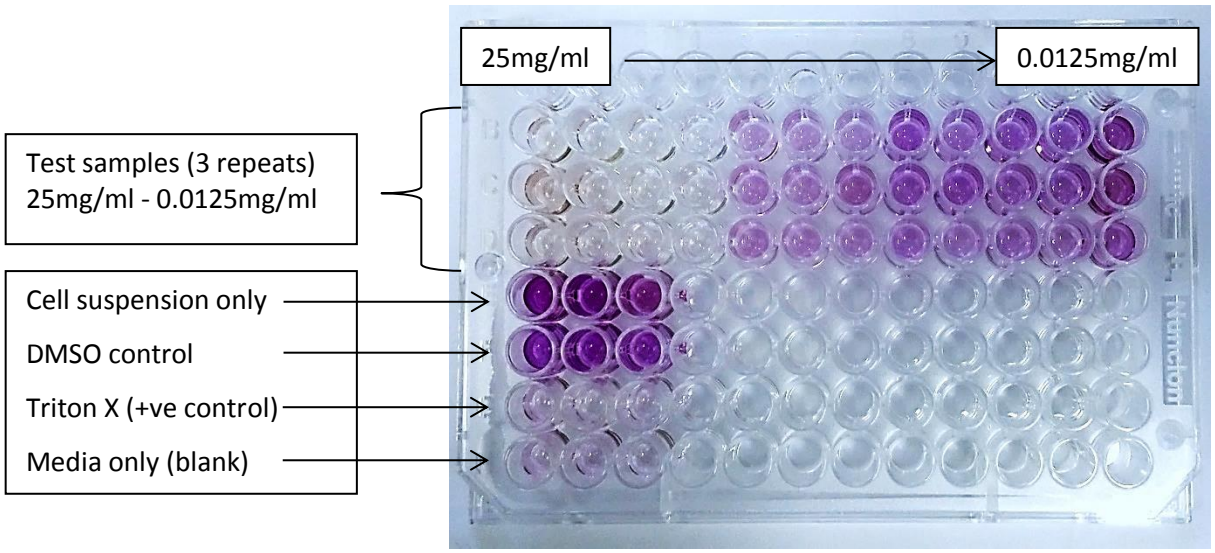

Figure 3.17: Crude extract cytotoxicity assay after the addition of MTT

Table 3.19: Crude extracts cytotoxicity assay absorbance values

Test wells	Media only (blank)	Triton X (+ve control)	DMSO control	Untreated cells (-ve control)	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.12 mg/ml	1.56 mg/ml	0.78 mg/ml	0.39 mg/ml	0.19 mg/ml	0.09 mg/ml	0.05 mg/ml	0.02 mg/ml	0.01 mg/ml
Repeats	0.073	0.091	0.486	0.567	0.074	0.098	0.111	0.132	0.143	0.163	0.187	0.25	0.279	0.316	0.389	0.447
	0.068	0.083	0.51	0.545	0.111	0.085	0.084	0.136	0.159	0.186	0.216	0.247	0.281	0.328	0.372	0.468
	0.078	0.086	0.528	0.427	0.078	0.092	0.128	0.13	0.161	0.178	0.198	0.233	0.294	0.319	0.396	0.438
Avg	0.073	0.087	0.508	0.513	0.088	0.092	0.108	0.132	0.154	0.176	0.2	0.243	0.285	0.321	0.386	0.451
±SD	**0.004**	**0.003**	**0.017**	**0.061**	**0.017**	**0.005**	**0.018**	**0.002**	**0.008**	**0.01**	**0.012**	**0.007**	**0.006**	**0.005**	**0.01**	**0.012**
***I%**	**-**	**97.1**	**1.14**	**-**	**96.6**	**95.7**	**92.1**	**86.6**	**81.59**	**76.59**	**71.14**	**61.36**	**51.88**	**43.64**	**28.86**	**14.1**

* I%- Growth Inhibition (%)

The concentration of the crude extract ranged from 25 mg/ml to 0.0125 mg/ml. Results showed that the antiproliferative effect of *D. viscosa* var. *angustifolia* crude extract was dose dependent. The cytotoxic effect of the crude extract was calculated as the percentage cell growth inhibition using the following formula:

$$\%\ Growth\ inhibition = 100 - \frac{Mean\ OD\ of\ individual\ test\ group}{Mean\ OD\ of\ control\ group} \times 100$$

The level of susceptibility of HEK 293 cells to the crude extract was characterized through the use of IC_{50} values. This is the drug inhibitory concentration that reduces the absorbance of treated cells by 50% with respect to untreated cells. The IC_{50} was initially estimated using Figure 3.18 which shows the viability of cells treated with different concentrations of *D. viscosa* var. *angustifolia* methanol crude extract.

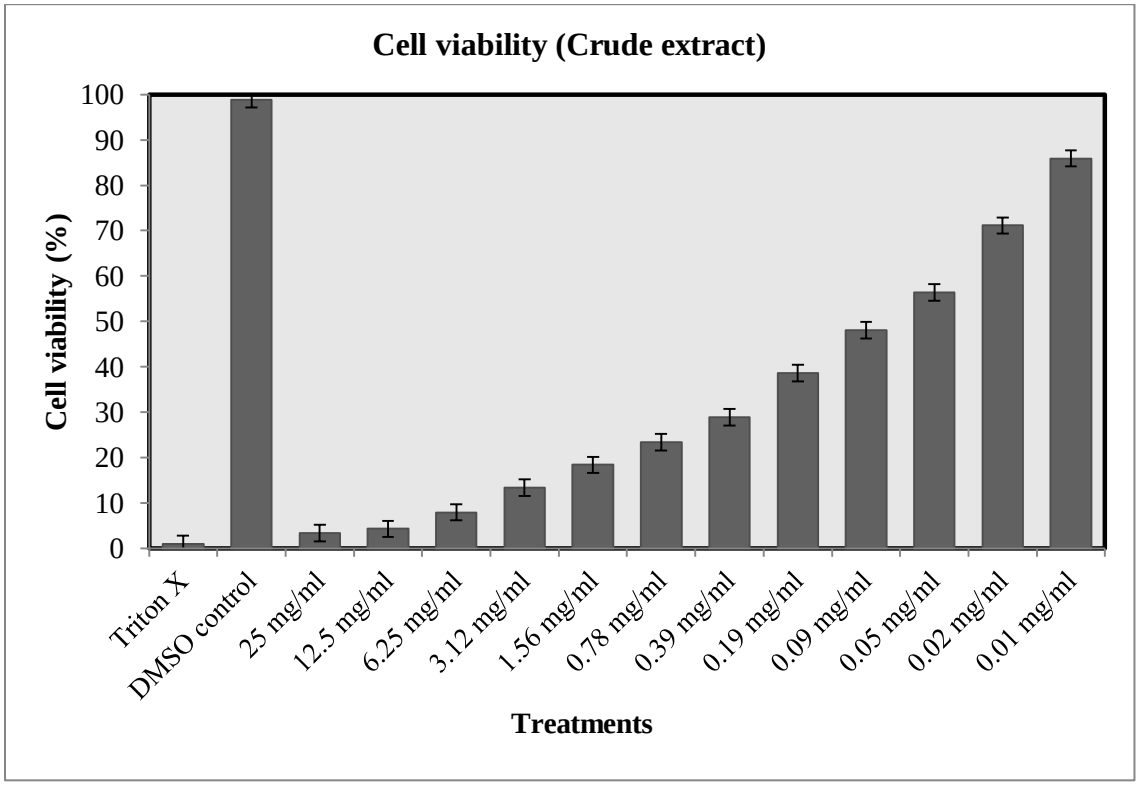

Figure 3.18: Viability of cells treated with *D. viscosa* var. *angustifolia* crude extract

The crude extract concentration that reduced the absorbance of treated cells by nearly 50% in relation to untreated cells (IC_{50}) was estimated from the cell viability graph as 0.09 mg/ml, with a calculated value 51.88% cell growth inhibition.

Preliminary cytotoxicity tests were done for subfraction 5.1 to determine which range of concentration values to use for treating HEK 293 cells. Significant cell death was observed at 0.25 mg/ml of plant extract. Hence a concentration range of 0.25 mg/ml to 0.002 mg/ml was used to determine the IC_{50}. Figure 3.19 shows the cytotoxicity assay of subfraction 5.1 on HEK 293 cells after the addition of 100% DMSO to dissolve the formazan crystals. Table 3.20 gives an outline of the absorbance values recorded from subfraction 5.1 cytotoxicity assays at 570 nm.

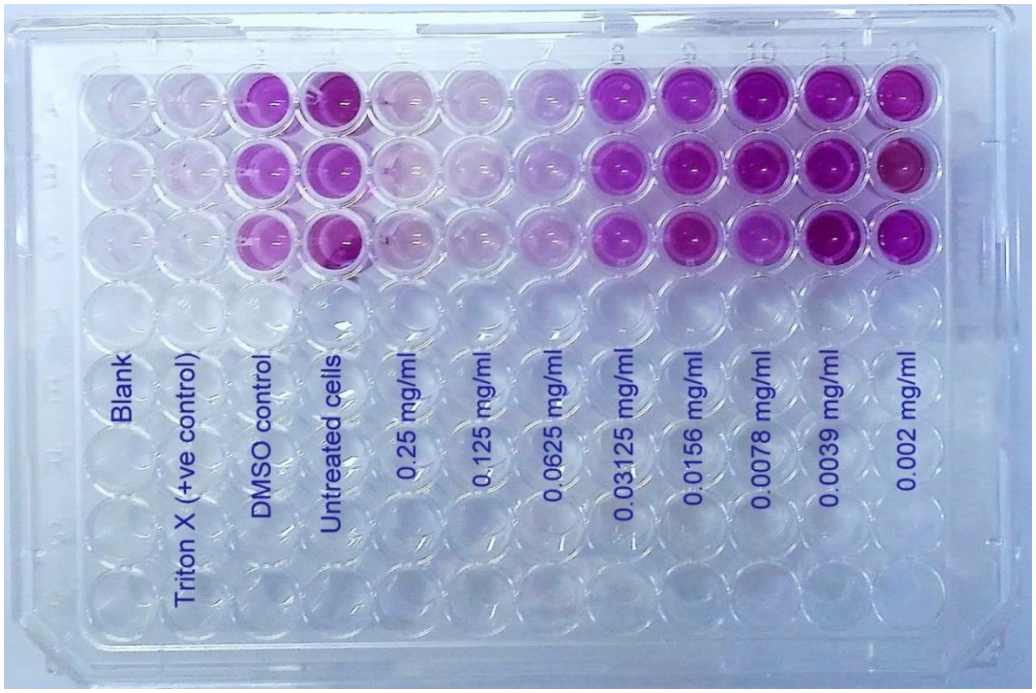

Figure 3.19: Subfraction 5.1 cytotoxicity assay after the addition of MTT

The cytotoxic effect of subfraction 5.1 was calculated as the percentage cell growth inhibition using the following formula:

$$\%\ Growth\ inhibition = 100 - \frac{Mean\ OD\ of\ individual\ test\ group}{Mean\ OD\ of\ control\ group} \times 100$$

Table 3.20: Test absorbance values for subfraction 5.1 cytotoxicity assay.

Test wells	**Blank**	**Triton X (+ve control)**	**DMSO control**	**Untreated cells**	**0.25 mg/ml**	**0.125 mg/ml**	**0.062 mg/ml**	**0.03 mg/ml**	**0.0156 mg/ml**	**0.0078 mg/ml**	**0.0039 mg/ml**	**0.002 mg/ml**
Repeats	0.071	0.086	0.792	0.802	0.094	0.102	0.299	0.439	0.512	0.716	0.557	0.687
	0.076	0.097	0.808	0.797	0.086	0.111	0.294	0.423	0.521	0.548	0.612	0.741
	0.077	0.084	0.798	0.81	0.075	0.111	0.302	0.418	0.498	0.561	0.755	0.784
Avg	**0.075**	**0.089**	**0.799**	**0.803**	**0.085**	**0.108**	**0.298**	**0.427**	**0.510**	**0.608**	**0.641**	**0.737**
±SD	**0.002**	**0.006**	**0.007**	**0.005**	**0.008**	**0.004**	**0.003**	**0.009**	**0.009**	**0.076**	**0.083**	**0.039**
***I%**	**-**	**98.04**	**0.5**	**-**	**98.58**	**95.42**	**69.29**	**48.35**	**40.18**	**26.73**	**22.20**	**9.02**

* I%- Growth Inhibition (%)

The level of susceptibility of HEK 293 cells to the subfraction was characterized through the use of IC_{50} values. The subfraction concentration that reduced the absorbance of treated cells by nearly 50% in relation to untreated cells (IC_{50}) was estimated from the cell viability graph as 0.03 mg/ml (Figure 3.20), with a calculated value 48.35% cell growth inhibition. The antiproliferative effect was shown to be also dose dependent as shown in Table 3.20.

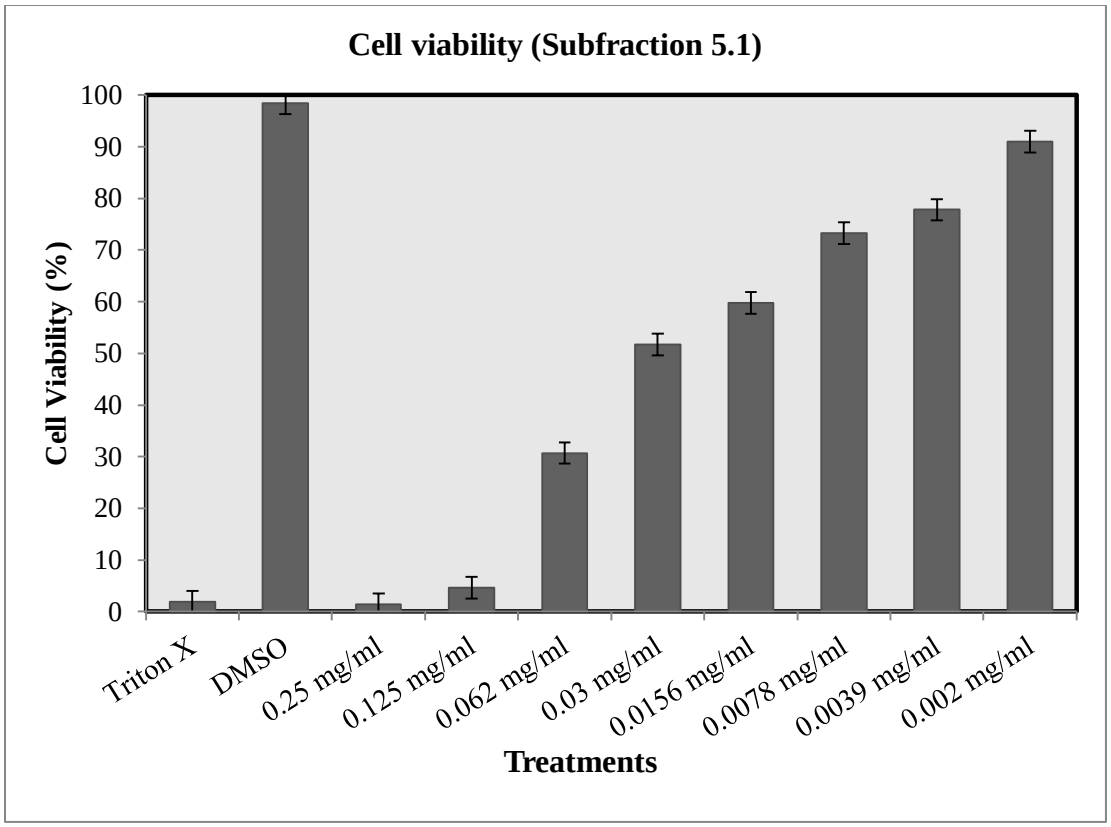

Figure 3.20: Viability of cells treated with *D. viscosa* var. *angustifolia* subfraction 5.1

The IC_{50} values of the methanol crude extract and subfraction 5.1 are presented in Table 3.21. Subfraction 5.1 was more cytotoxic to HEK 293 cells than the crude extract. For both assays, the cytotoxic effect was dose dependent.

Table 3.21: The IC_{50} values of tested *D. viscosa* var. *angustifolia* crude extract and subfraction 5.1

Plant extract	HEK 293 (IC_{50})
Methanol crude extract	0.09 mg/ml ±0.006
Subfraction 5.1	0.03mg/ml± 0.008

SD: n = 3

CHAPTER 4

Discussion

In 2007, the World Health Organization (WHO) declared that, tooth decay ranked as the most expensive infection that most people would have to cope with in their lifetime (Petersen, 2008). Dental treatments and subsequent sick leave cost billions of dollars annually (Samaranayake, 2002). The improvement of livelihood in third world countries as their populations become exposed to various sugary diets, has also correlated with the rise in dental caries worldwide. To avoid the burden of dental caries treatment costs and the amount of manpower involved, the slogan 'prevention is better than treatment' seems more plausible than ever before.

The primary use of effective anticariogenic agents in conjunction with mechanical practices like teeth brushing could be vital in preventing dental caries. There have been various attempts to use antibiotics clinically in the prevention of dental caries. The downside to that have been adverse side effects like hypersensitivity reactions, teeth staining and the fact that most caries causing bacteria have some form of resistance to antibiotics largely due to the indiscriminate use of these antibiotics (Singh *et al.*, 2007). Literature is full of reports concerning the use of natural products in preventing dental caries. As previously mentioned, most of these studies have focused on the use of crude extracts of plants. This quick approach is indeed essential for screening purposes. There is however, the need to better understand the chemistry of the plants to maximize their potential through the isolation of actual

anticariogenic constituents. The purification process also limits any antagonistic effects that may arise from the use of crude extracts.

The potential use of *D. viscosa* var. *angustifolia* in preventing dental caries has been previously reported and the results were positive. Patel *et al* (2013) stated that its crude extract was able to reduce biofilm formation and acid production by the dental caries causing bacteria, *S. mutans*. The authors did highlight the need to identify the active constituents. This suggestion is echoed by most researchers in phytomedicine with the goal of maximizing the potential of medicinal plants. Therefore this study focused on the isolation of the active constituent from *D. viscosa* var. *angustifolia* with the potential to prevent caries causing pathogenic characteristics of *S. mutans*. This was followed by elucidation of the potential active compound and determining its cytotoxic effect on human embryonic kidney cells.

In this study, six fractions were collected from fractionation of the methanol crude extract. These were labeled fraction 1-6 (F1-F6). Preliminary screening of the fractions showed that fraction 5 (F5) had the highest antimicrobial activity as indicated by its lowest minimum inhibitory concentration. The same outstanding effect from F5 was observed when subinhibitory concentrations of the fractions were tested for their effect on biofilm formation and acid production by *S. mutans*. Purification of F5 produced two subfractions, subfraction 5.1 and subfraction 5.2. Further antimicrobial assay tests showed that subfraction 5.1 had more anticariogenic properties. Its efficacy had greatly improved compared to the crude extract. This study also reports the identification of this subfraction 5.1 compound which has

not been previously reported from *D. viscosa* var. *angustifolia*. Each step of this process will be discussed under relevant subheadings.

4.1 Extraction and fractionation

Taking into consideration the rapid growth of the herbal medicine industry in recent years, it has become necessary to establish integrated methods of extracting, purifying and characterizing bioactive compounds and ultimately ways to test their biological activity. Extraction is a very important initial step in analyzing medicinal plants. It facilitates further separation and eventual characterization of the bioactive chemical components. There is always a possibility that potential bioactive constituents are lost, distorted or even degraded during the extraction process. Fabricant and Farnsworth (2001) highlighted that, if the plants are chosen based on the way they are used traditionally, it is important to prepare the extract in relatively the same way as traditional healers in order to mimic the 'herbal drug'.

A large proportion of antimicrobial compounds that have been identified from medicinal plants in literature are either aromatic or saturated organic compounds. For this reason, methanol was chosen as the initial solvent of extraction. Methanol has a polarity index of 5.1 and is ideal for the extraction of both polar and a vast number of non-polar compounds. In addition, its low boiling point of 65°C made it easy to concentrate the bioactive compounds. An average yield of 6.8 grams dried extract from 60 grams (11.33%) of dried *D. viscosa* var. *angustifolia* leaves powder was attained.

Natural products consist of a vast number of constituents and in many cases; the bioactive constituents are present in minute amounts. Column chromatography facilitates the separation of these constituents by utilizing the fact that they migrate differently per given stationary phase and mobile phase used. Since the identity of the active constituents was unknown, gradient elution was used to enable the collection of fractions consisting of compounds that are possibly structurally related. Thin layer chromatography was used to analyze the composition of collected fractions and determine which solvent systems could effectively separate the individual constituents.

4.2 Thin layer chromatography and Bioautography

Thin layer chromatography is widely used during purification of plant constituents because of its low cost and ability to screen many samples at once. Thin layer chromatography was done in conjunction with a technique called contact bioautography. Contact bioautography is technique used to identify any TLC bands that have antimicrobial activity. The technique also aids in determining if any antimicrobial activity observed from the fractions is due to a single constituent or combinations of constituents. Fraction 5 exhibited the biggest zone of inhibition (6 mm) at R_f 0.17 which indicated that the active constituent was a polar compound. The same zone of inhibition was observed when the crude extract was run on TLC though the zone of inhibition was smaller due to the lower quantity of the active constituent.

Contact bioautography has its own limitations. It is more of a qualitative technique. The quantity of constituent that caused the zone of inhibition is not known. That raises the

possibility that there might be some more potent compounds that were present but did not cause any inhibition due to their low concentrations. However, the technique is useful for preliminary screening of antimicrobial activity in phytochemicals. The definitive antimicrobial properties were determined using MIC/MBC techniques.

4.3 MIC/MBC assays

All the six fractions from *D. viscosa* var. *angustifolia* had an antimicrobial effect on the two *S. mutans* test strains. One of the challenges experienced when carrying out MIC tests using crude extracts or fractions from plants is that plants have a variety of pigments. That becomes a problem when one is using dyes like INT or MTT to detect growth. For this particular study, fractions consisted of three colours, green, yellow and orange. The dye chosen, INT turns red when reduced by viable bacteria which facilitated easy detection of bacterial growth.

The median MBC for the crude extract on both *S. mutans* strains was 1.56 mg/ml which was consistent with Naidoo (2012) who reported the same MBC results from *D. viscosa* var. *angustifolia.* Lee *et al,* (2007) reported a growth inhibitory concentration of 1 mg/ml against *S. mutans* from the crude extract of *Saussurea lappa*. More *et al,* (2008) reported MICs greater than 25 mg/ml when testing a series of South African plants used against oral pathogens including *S. mutans*. High values of MICs are usually observed with many crude extracts of plants when tested against *S. mutans* which suggest that these bacteria are difficult to kill compared to many pathogens. Sometimes the active constituents are present in small quantities in crude extracts. Another reason is that there could be antagonistic constituents

that promote growth of bacteria leading to high concentrations of crude extract needed to inhibit growth (Khurram *et al*, 2009). That raises the question though, what is the ideal MIC range of values that can establish a plant as a potential source of compounds that can be used in oral medicine? Unless the antimicrobial activity is due to synergy, fractionating the crude extract and isolating the active constituent does improve MIC values.

Six fractions were collected from the crude extract. Their MICs ranged from 0.39 mg/ml to 12.5 mg/ml. Fraction 5 and 6 had the lowest MICs with 0.39 mg/ml and 1.56 mg/ml respectively. Bioautography showed that the antimicrobial activity observed in fraction 6 could have been same constituent in fraction 5 collected during fractionating. Among the fractions, only fraction 5 had a better MBC value compared to the crude extract. This indicated that the dilution effect with the other chemical present in the crude extract was removed by purifying the fraction. Screening plants for antimicrobial activity and isolating active constituents raises the issue of quantity. In some cases, the most active constituent is only present in very minute quantities compared to less active constituents. Therefore, Kotze and Eloff (2002) suggested the concept of total activity. Total activity is calculated by dividing the mass of the fraction or constituent by its MIC. A high mass and low MIC gives a high total activity value. Fraction 5 had the highest total activity value of 2000 with fraction 4 having the lowest at 3.2. Nevertheless, all the fractions were then tested for their anticariogenic activity (biofilm formation and acid production).

4.4 Fractions and biofilm formation

The basis for the development of dental caries is the initial accumulation of endogenous acid producing bacteria on teeth surfaces. Micro colonies of bacteria live on teeth forming part of dental biofilms also known as dental plaque. A number of techniques have been performed to determine the potential of phytochemicals in reducing or eliminating oral biofilms. Most of these methods are focused more on preventing the cariogenic properties of *S. mutans* like biofilm formation than killing the bacteria. These anti-pathogenic phyto-compounds are preferred to those that kill bacteria. Bactericidal activity leads to selective pressure and subsequent resistance (Palombo, 2009). The same concept was applied for this particular study, only subinhibitory concentrations were used for the assays. The idea is to target *S. mutans* virulence rather than killing the bacteria.

Depending on the individual MBCs, three subinhibitory concentrations per fraction were used for the biofilm assays. All the fractions had an inhibitory effect when compared to the controls. Fraction 5 had the highest biofilm inhibitory effect with 87.5% and 93.3% at 0.2 mg/ml concentration after 6 and 24 hours respectively. This was followed by the crude extract which had 82.3% and 90.5% inhibitory effect at 6 and 24 hours respectively. The high inhibition effect from fraction 5 is even more relevant by virtue of the fact that it had the highest biofilm inhibitory effect at the lowest concentration. Similarly, Lee *et al*, (2007) reported that at 1 mg/ml, crude ethanol extracts from *Saussurea lappa* inhibited the adherence of *S. mutans*. It limited its ability to form the water insoluble glucan which is essential for biofilm development. Recent studies have shown that polyphenols found in cranberry juice inhibit glucosyltransferase thereby reducing insoluble glucan content

(Johnson-White *et al*, 2006). Dextran found in the flowering plant, *Helichrysum italicum* was shown to be essential in reducing cell aggregation and adherence of *S. mutans* to glass (Osaka *et al*, 2000).

4.5 Fractions and acid production

Acid production by *S. mutans* is essential for the onset of dental caries. Organic acids produced by plaque bacteria as by-products lead to dissolution of the teeth's crystalline surface. *Streptococcus mutans* is part of acidogenic and aciduric bacteria. They thrive in acidic environments. Phytochemicals that are able to inhibit the glycolytic pathway in bacteria are of interest. The study therefore also investigated the effect of fractions on acid production by *S. mutans*.

Streptococcus mutans cells were incubated in the presence of subinhibitory concentrations of *D. viscosa* var. *angustifolia* fractions. The pH was measured at 0 and 8 hours, then every 2 hours up to 14 hours in total. All the fractions inhibited acid production by *S. mutans*. After 10 hours of incubation, the control cultures attained the critical pH of 5.5 which promotes cariogenicity. When the pH levels drop below 5.5, the process of enamel demineralization ensues. Fraction 5 exhibited its high anticariogenic effect by having the least change in pH after 14 hours and the bacterial counts showed that the change in the pH was not count dependent. The assumption is that there was a constituent in the fraction that had a high inhibitory effect on the *S. mutans* glycolytic pathway.

Green tea has been shown to interfere with acid production by *S. mutans*. It exerts its anticariogenic effect by inhibiting the bacterial enzyme lactate dehydrogenase. That effect stops the production of lactic acid. The inhibitory agent was reported to be a catechin compound (Hamilton-Miller, 2001). Catechin is reported to be present in *D. viscosa* var. *angustifolia* (Naidoo *et al*, 2012). Hamilton-Miller (2001) does however, highlight that this antibacterial activity from tea can decrease because catechins have an affinity for proteins which leads to the loss of their activity.

Fraction 5 certainly had the beneficial chemical constituent and therefore was purified further.

4.6 Sub-fractionation of fraction 5

Successful isolation of active constituents from plants is very much dependent on the solvent combination used. When screening and isolating active compounds from plants, it is important to preserve the integrity of the sample through the different purification stages. Liquid chromatography is done at room temperature, which makes it ideal for the isolation of compounds that may be thermally labile. However, liquid chromatography analysis is slow and in some instances, it does not provide optimum resolution of the band. The sample had to be re-run a couple of times to achieve complete resolution of the two subfraction peaks.

The solvent gradient combination of hexane and ethyl acetate separated the crude extract into fractions effectively. For further purification of fraction 5, the same solvent gradient combination was used starting with 100% hexane to 100% ethyl acetate. Sequential liquid chromatography fractionation in conjunction with thin layer chromatography and

bioautography lead to the isolation of two subfractions (5.1 and 5.2) that showed antimicrobial activity.

4.7 MIC/MBC of subfraction 5.1 and 5.2

Both the subfractions exhibited antimicrobial activity against *S. mutans.* Subfraction 5.1 showed a pronounced inhibitory effect with an MIC of 0.1 mg/ml compared to the crude extract (0.78 mg/ml) and fraction 5 (0.39 mg/ml). In addition, subfraction 5.1 had high total activity. In some cases, potential activity can be lost when compounds are separated due to synergy. Bioassay guided purification steps like the utilization of bioautography make it possible to determine loss or gain of activity during the isolation process. The subfractions were analyzed for any loss or gain in activity after liquid chromatography analysis. The results indicated that the active compound was now present at higher concentrations in subfraction 5.1 than for subfraction 5.2. This conclusion was drawn from the lower MIC value against *S. mutans* compared to the crude extract and fraction 5. Bioautography analysis of subfraction 5.1 also showed a bigger zone of inhibition compared to fraction 5. If synergy was a key factor in the antimicrobial activity of *D. viscosa* var. *angustifolia*, purification of the fractions showing antimicrobial potential might have led to loss of activity.

Therefore subfraction 5.1 was further purified and identified.

4.8 Identification and elucidation of subfraction 5.1

Subfraction 5.1 was selected for GC-MS analysis based on its antimicrobial activity against *S. mutans* and purity. The main chemical compounds that were identified by GC-MS analysis

are outlined in Table 3.15. Gas chromatography-mass spectrometry analysis is an important step towards the elucidation of an isolated compound. The molecular ion data from GC-MS analysis of subfraction 5.1 provided guidance as to the molecular weight and elemental composition of the compound. In essence, GC-MS fragments the molecule and this is termed electron ionization (EI). The detected EI fragment ions provide daughter ion peaks with corresponding mass to charge ratio (McShane, 2011). Hence the interpretation of the GC-Ms data involves working backwards and putting the 'pieces' back together to get a result.

Interpretation of the GC-MS results from analysis of subfraction 5.1 gave an indication that the compound could be a flavonoid. Retention values of subfraction 5.1 using solvents reported in literature and the colour of the dried extract also pointed towards that conclusion. There are different types of flavonoids. The list includes flavones, flavonols and catechins. All these compounds have different absorbance spectrums. Subfraction 5.1 analysis using the ultra-violet visible spectrometer showed two peaks at 330nm (band A) and 270nm (band B). The results narrowed the possible identity of the compound to a group of flavonoids called flavones. Their absorption spectra fall within the range of 310 nm-350 nm for band A and 250nm-290nm for band B (Tsimogiannis, 2007).

Nuclear magnetic resonance spectroscopy was used for final characterization of subfraction 5.1. The isolated compound was identified as a flavone. Spectral data was attained for ^{1}H NMR (Table 3.16 and Figure 3.13) and ^{13}C NMR (Table 3.17 and Figure 3.14). Subfraction 5.1 was identified as 5,6,8-Trihydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one with an empirical formula of $C_{17}H_{14}O_{7}$. Further NMR spectral data was collected which

included NOESY, HMBC and HSQC correlations (See appendix). Naidoo *et al* 2012 reported the presence of 3-Hydroxy-5,7-dimethoxy-2-(4-methoxyphenyl)chroman-4-one from *D. viscosa* var. *angustifolia*.

The crude extract of *D. viscosa* var. *angustifolia* is known to contain a lot of methylated flavonols and flavones (Omosa *et al*, 2010). The flavone from this study was eluted with a high concentration of ethyl acetate. The results are in correlation with the findings of Sachdev and Kulshreshtha (1982) who isolated two flavones, penduletin and 5,7,4'-trihydroxy-3,6-dimethoxyflavone from the ethyl acetate fraction of *D. viscosa*. Ghilbert (1998) reported the presence of 23 flavones from flowers, seeds, leaves and bark of *D. viscosa*. Close to 50% of those flavones were methoxylated at C-6. The flavone isolated from this study is methoxylated at C-7 and C-4^1. Getie *et al*, (2000) were able to isolate relatively large quantities of the flavones, kaempferol and isorhamnetin from *D. viscosa* crude leaf extract. Teffo *et al* (2009) reported the presence of four flavones from *D. viscosa* var. *angustifolia*. They tested the susceptibility of four pathogens to the isolated compounds and the MIC's ranged from 23 to 250µg/ml. The isolated flavone from this study, 5,6,8-Trihydroxy-7-4^1-dimethoxyflavone has not been previously identified from *D. viscosa var. angustifolia*.

This identified compound was subjected to biofilm and acid inhibition assays. Reproducibility of the results was also established.

4.9 Subfraction 5.1 (5,6,8-Trihydroxy-7-4^{1}-dimethoxyflavone) and biofilm formation

The effect of *D. viscosa* var. *angustifolia* on *S. mutans* biofilm formation was investigated using three subinhibitory concentrations from the crude extract and subfraction 5.1. The highest subinhibitory concentration from subfraction 5.1 (0.05 mg/ml) exhibited a statistically significant biofilm inhibitory effect ($p < 0.01$). The purified subfraction showed a great improvement in anti-cariogenicity potential compared to the crude extract and fraction 5 at lower concentrations. This indicated that the flavone was the active agent and the activity was not reliant on synergy.

There are various reports that have discussed the effect of flavones on glucosyltransferase (GTF) enzymes found in *S. mutans.* Glucosyltransferase (GTF) enzymes are essential for development of biofilm. They catalyze the conversion of sucrose to α- linked glucans which are insoluble. Glucans facilitate the attachment accumulation of *S. mutans* onto the teeth surface leading to biofilm formation (Koo *et al*, 2002). Koo *et al* (2002) also investigated the effect of flavonols and flavones on GTF enzymes. They reported a 40 to 90% inhibition of GTF enzymes in solution and 15 to 60% of inhibition on GTF enzymes attached to the surface at a concentration of 0.135 mg/ml. These results explain the findings of Naidoo *et al*. (2012) who showed that the crude extract of *D. viscosa* var. *angustifolia* can inhibit the biofilm formation rather than remove the fully formed biofilm.

The isolated flavone apigenin was able to inhibit 90.5 to 95% of GTF activity at a low concentration of 0.135 mg/ml (Koo *et al*, 2002). The authors also reported that the flavone did not have much of an effect on surface adsorbed GTF enzymes compared to those in

solution. The inhibitory effect was concentration dependent. For this particular study, 0.05 mg/ml of the isolated flavone, 5,6,8-Trihydroxy-7,4^1-dimethoxyflavone reduced *S. mutans* biofilm formation by 94% and 98.9% after 6 and 24 hours of incubation respectively. The inhibitory effect was also concentration dependent.

There are relatively few studies that have reported on the mechanisms by which flavonoids exert their antibacterial effect. The general consensus is that individual flavonoids with antimicrobial activity target various sites than one specific location of activity. Osawa *et al* (1992) examined the activity of various flavones on *S. mutans* and *S. sobrinus*. They observed that 5-hydroxyflavones and 5-hydroxyisoflavones with more hydroxyl groups on C-7 and C-4^1 did not have any inhibitory effect. However, Sato *et al* (1996) tested two isoflavones hydroxylated at 5, 2^1 and 4^1 positions using agar well diffusion assays. A strong inhibitory effect was observed against various streptococcal species. The assumption then is that C-2^1 hydroxylation is vital for activity. The flavone from this study had high antimicrobial activity and is hydroxylated at 5,6 and 8. This highlights how the structure of the compound is important for activity.

Other ways in which flavones exert their antimicrobial effect include the inhibition of DNA gyrase and rigidifying the membranes of bacteria. Fourteen flavonoids were screened for their effect on *Escherichia coli* DNA gyrase. DNA gyrase reduces the strain on DNA while it is being unwound. It was observed that seven of the selected compounds including the flavone apigenin inhibited DNA gyrase. However, there was no correlation between antimicrobial activity and enzyme inhibition which suggested that other mechanisms might

be involved (Ohemeng *et al*, 1993). Inhibition of cell membrane and cell wall synthesis are other modes of mechanisms that have been suggested for flavones (Zhang and Rock, 2004). Another biofilm inhibitory effect mechanism that has been reported is that flavonoids form complexes with cell surface proteins which lead to the reduction in overall cell hydrophobicity. Cell surface hydrophobicity is essential for biofilm formation (Prabu, 2005).

4.10 Subfraction 5.1 (5,6,8-Trihydroxy-7-4^{1}-dimethoxyflavone) and acid production

Streptococcus mutans has the ability metabolize dietary carbohydrates to produce acid which forms the basis of its virulence and pathogenesis of dental caries. The results showed that both the crude extract and subfraction 5.1 inhibited acid production by *S. mutans*. The same high inhibitory effect from subfraction 5.1 in comparison to the crude extract was observed. In addition, subfraction 5.1 inhibited acid production more effectively at low concentrations compared to crude extract. The inhibition effect was concentration depended. The bacterial counts which could have been the reason for the difference in pH between the controls and exposed assays were shown not to be very different. This indicated that there was a mechanism of activity being exerted by the crude extract and subfraction 5.1 that was not bactericidal in nature.

In addition to the fact that *S. mutans* is acidogenic, it is also acid tolerant with the ability to preserve its glycolytic capabilities at pH levels of up to 4.4 (Bender *et al*, 1985). *Streptococcus mutans* is able to survive in a low pH environment by increasing the activity of its F-ATPase enzyme which pumps protons out of the bacterial cell (Lemos and Burne,

2008). The activity of the F-ATPase enzyme makes the intracellular pH of the bacteria to be more than alkaline than the surrounding environment. This characteristic of *S. mutans* plays a crucial role in the development of dental caries (Banas, 2004). Guan *et al* (2011) therefore suggesting that to combat the caries causing ability of *S. mutans*, strategies that inhibit its acidogenic and acid tolerant properties would be the ideal solution. Any chemical that inhibits F-ATPase activity and the enzyme lactate dehydrogenase (LDH) which facilities the fermentation of dietary glucose to lactic acid would achieve that goal.

Guan *et al* (2011) investigated the effect of two flavonoids, quercetin and kaempferol, isolated from the crude extract of *Nidus vespae* on the activity of LDH and F-ATPase found in *S. mutans*. They observed that when *S. mutans* cultures are exposed to 1/8-1/2 of the flavonoids concentrations, there was less than 10% antimicrobial activity on LDH. However, the same flavonoid concentrations were able to significantly inhibit F-ATPase activity. Quercetin reduced F-ATPase activity by 47.37% at half its *S. mutans* MIC. Kaempferol reduced F-ATPase activity by 49.66% also at half its *S. mutans* MIC. Some authors have suggested that the high inhibitory activity exhibited by flavonoids which include flavones is due to the fact that they are able to bind to bacterial extracellular proteins and form complexes. These complexes then bind to the bacterial cell wall (Cowan, 1999). By disrupting F-ATPase activity, the proton pumping ability of *S. mutans* is affected leading to an increase in acidity of the intracellular pH. That would affect the *S. mutans* pH sensitive glycolytic enzymes and subsequent loss of its acidogenic and aciduric properties (Guan *et al*, 2011). Similarly, Prabu *et al* (2005) investigated the effect of the flavonoid guaijverin on acid production by *S. mutans*. At subinhibitory concentration, the flavonoid compound reduced

acid production by both *S. mutans* strains used. However, they postulated that the inhibitory effect may have been due to the bacteriostatic effect of the compound.

Fluoride, a widely used anticariogenic chemical affects enzyme enolase which is responsible for the conversion of 2 phosphoglycerate to phosphoenolpyruvate which is responsible for the subsequent production of acid in the glycolytic pathways. The newly identified flavone may intercept this glycolytic pathway.

The isolated flavone could have exerted its acid inhibitory effect through any of these mechanisms outlined. What is evident is that the structure of the compound plays a crucial role in the bioactivity. The positions of the hydroxyl groups and presence of methoxy groups do determine their antimicrobial and anticariogenic potential through structure-activity relationships.

4.11 Cytotoxicity of crude extract and subfraction 5.1 (5,6,8-Trihydroxy-7-4^1-dimethoxyflavone)

The determination of cell viability is important in establishing the pharmaceutical toxicity of potential active ingredients for medicine (Komissarova *et al*, 2004). The effect of *D. viscosa* var. *angustifolia* crude extract and subfraction 5.1 on HEK 293 cells was investigated. The results were expressed as the concentration that inhibits 50% of cell growth in relation to untreated cells. According to IC_{50} values, HEK 293 cells were more susceptible to subfraction 5.1 than the crude extract after 24 hours of exposure. The results exhibited a dose

dependent cytotoxic effect for both assays. The methanol crude extract had an IC_{50} value of 0.09 mg/ml and the purified subfraction had an IC_{50} value of 0.03 mg/ml.

Beneficial effects of flavones have been reported in literature. Matsuo *et al* (2005) investigated the cytotoxicity effect of flavonoids including the flavone 4,5,7-trihydroxyflavone (apigenin) on human lung embryonic fibroblasts (TIG-1) and human umbilical vein endothelial (HUVE) cells. The flavone apigenin had an IC_{50} value of 0.03 mg/ml on both types of cells. The same IC_{50} result was reported in this study for the isolated flavone, 5,6,8-Trihydroxy-7,4^1-dimethoxyflavone.

Drug availability is a key factor for the success of any medicine. The washing action of saliva has to be considered when determining drug concentrations that will be effective in the oral cavity. The MIC of the isolated flavone from *D. viscosa* var. *angustifolia* on *S. mutans* was 0.1 mg/ml. All three subinhibitory concentrations from this compound (0.05, 0.025 and 0.0125 mg/ml) showed inhibition of biofilm formation and acid production. The implication is that if the isolated flavone is used as the active component of any oral medicine at subinhibitory concentration, it would still exert its effect. When taking into consideration its IC_{50} value of the compound (0.03 mg/ml), the subinhibitory concentrations of the flavone active component would not be harmful for two reasons. The first reason is that, saliva would dilute the active ingredient to safe concentrations and that concentration would still be effective. The washing action of saliva also limits the exposure time of the chemical, which would also prevent any harmful activity on oral epithelial cells.

4.12 Clinical implications

Cytotoxicity study has established the safe use of crude extract as well as the newly identified beneficial compound. Therefore both can be developed into an anticariogenic, oral hygiene product. These products can be toothpaste, mouthrinses and chewing gums. Identified chemical would be advantageous because the required concentrations would be lower. If the product is used at MIC/MBC concentrations it would reduce the number of *S. mutans* in the oral cavity. However, the constant flow of saliva would dilute this active ingredient gradually to subinhibitory concentrations represented in the acid and biofilm assays. At these concentrations the surviving *S. mutans* would not be able to form biofilm. In addition, they will not be able to produce acids. These two activities would reduce the process of development of dental caries. Recommendation of the usage of this compound in the form of toothpaste and mouth rinse would impart day and night protection.

Chapter 5

Conclusion, Future research and limitations to the study

5.1 Conclusion

On fractionation of *D. viscosa* var. *angustifolia* crude extract, fraction 1, 2, 3, 4, 5 and 6 were identified and all the fractions showed varying levels of antimicrobial activity against *S. mutans* and inhibited biofilm formation and acid production. Among all the fractions, fraction 5 proved to have higher antimicrobial activity as well as high ability to reduce biofilm and acid production compared to the other fractions and crude extract. On subfractionation of fraction 5, subfractions 5.1 and 5.2 were generated, of which subfraction 5.1 proved to be the most active with an MIC of 0.1 mg/ml compared to the crude extract (0.78 mg/ml) and subfraction 5.2 (1.56 mg/ml). In addition at subinhibitory concentrations, subfraction 5.1 significantly inhibited biofilm formation and acid production at lower concentrations compared to the crude extract. These results suggested that subfraction 5.1 contained an active anticariogenic compound which was originally detected in the crude extract. Gas chromatography-mass spectrometry and NMR analysis identified that compound in subfraction 5.1 as the flavone, 5,6,8 Trihydroxy-7,4^{l}-dimethoxyflavone.

Cytotoxicity analysis of this flavone on HEK 293 cells revealed that the IC_{50} values are higher than the MIC and therefore it is safe to use in the oral cavity. The results in this study have shown that compound 5,6,8 Trihydroxy-7,4^{l}-dimethoxyflavone present in the extracts of *D. viscosa* var. *angustifolia* is responsible for its potent anticariogenic activity and therefore it has potential to be developed as a anticariogenic therapeutic product.

5.2 Limitations of the study

1. Dental plaque is composed of a variety of microorganisms and therefore ideally biofilm studies should be performed on mixed flora. However, it is difficult to study the effect on the virulence of any organism in a mixed flora environment. Therefore the *S. mutans* was used which is implicated in the development of dental caries and is the pioneer species in the development of plaque.
2. The oral cavity has saliva which contains glycoproteins and antimicrobial compounds which may have effect on the biofilm. Saliva was not used in the study which would have been ideal to mimic the oral cavity environment due to the lack of availability of sterile clarified saliva.
3. *Streptococcus mutans* produces extracellular polysaccharides which play a role in biofilm development. The effect of 5,6,8 Trihydroxy-7,4^{l}-dimethoxyflavone on extracellular polysaccharide production was not done in the study due to the limited study time available to complete the degree. However, in future it will be pursued.
4. Extract is lost during the purification process which might affect the yield attained.
5. The pure form of the isolated compound was not available commercially for comparison of its antimicrobial and cytotoxicity effect.
6. Other active compounds might not have been detected because they were present in minute amounts.
7. Some constituents are volatile and light sensitive. It is possible that other potential active compounds might have degraded, losing their activity in the process.

5.3 Future research

1. *In vitro* analysis does not provide a complete representation of what happens in the oral cavity. Clinical trials would be ideal in testing the isolated compound by incorporating it into a mouth rinse or toothpaste and determining its efficacy in preventing dental caries.
2. Dental plaque is composed of a variety of organisms. Investigating the effect of the isolated compound on other microorganisms that are also found in dental plaque would be a step forward.
3. Structure-activity relationships play a key role in the efficacy of compounds against microorganisms. Determining how the isolated compound exerts its effect against cariogenic bacteria at the biochemical level would be an important study. Plant compounds cannot be patented, hence if any analogues are to be developed; the biochemical mechanism by which the compound functions has to be known.
4. The use of antimicrobial agents in conjunction with nanoparticles has gained popularity. The isolated compound can be coated onto nanoparticles to enhance its activity as well as retentiveness.
5. Enzymes like glucosyltransferase, F-ATPase contribute to the pathogenicity of *S. mutans*. A study could be done to investigate the effect of the compound on such enzymes.
6. The effect of the compound on the production of extracellular polysaccharides that contribute to development of dental plaque could be investigated.
7. Phytochemical studies on *D. viscosa* var. *angustifolia* plants from different locations can be done as geographical variability can have an influence on chemical composition.

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Appendices

Appendix 1: Composition and preparation of media

Blood agar

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

39 g of Columbia agar base was suspended in 1000 ml distilled water. The suspension was boiled to dissolve completely. It was sterilized by autoclaving at 121 °C for 10 minutes. After cooling to below 50 °C, 5 % sterile defibrinated blood was added. The media was then poured into petri dishes and allowed to set. The plates were then refrigerated.

Human cell culture medium

88%	Dulbeco's modified eagle's medium
10%	Fetal calf serum
1%	L-glutamine
1%	Streptomycin

Dulbecos's modified was supplemented with Fetal calf serum, L-glutamine and the antibiotic streptomycin according to the proportions mentioned above

1X Phosphate buffered saline

8 g	sodium chloride
0.24g	potassium hydrogen phosphate
0.2g	potassium chloride
1.44g	sodium hydrogen phosphate
800 ml	distilled water

The solids were suspended in 800 ml distilled water and autoclaved for 15 minutes

1X Trypsin-EDTA solution

0.05 %	Typsin
0.5 mM	EDTA
1X	PBS

Tryptone Soya broth

30 g	Tryptone Soya broth
500 ml	distilled water

The media was dissolved in distilled water and autoclaved at 121 °C for 15 minutes

5 % Sucrose Broth

30 g	Tryptone
0.6 g	sucrose
1000 ml	distilled water

The solids were first boiled in 500 ml distilled water. The remaining water was then added, boiled and then autoclaved at 121 °C for 15 minutes.

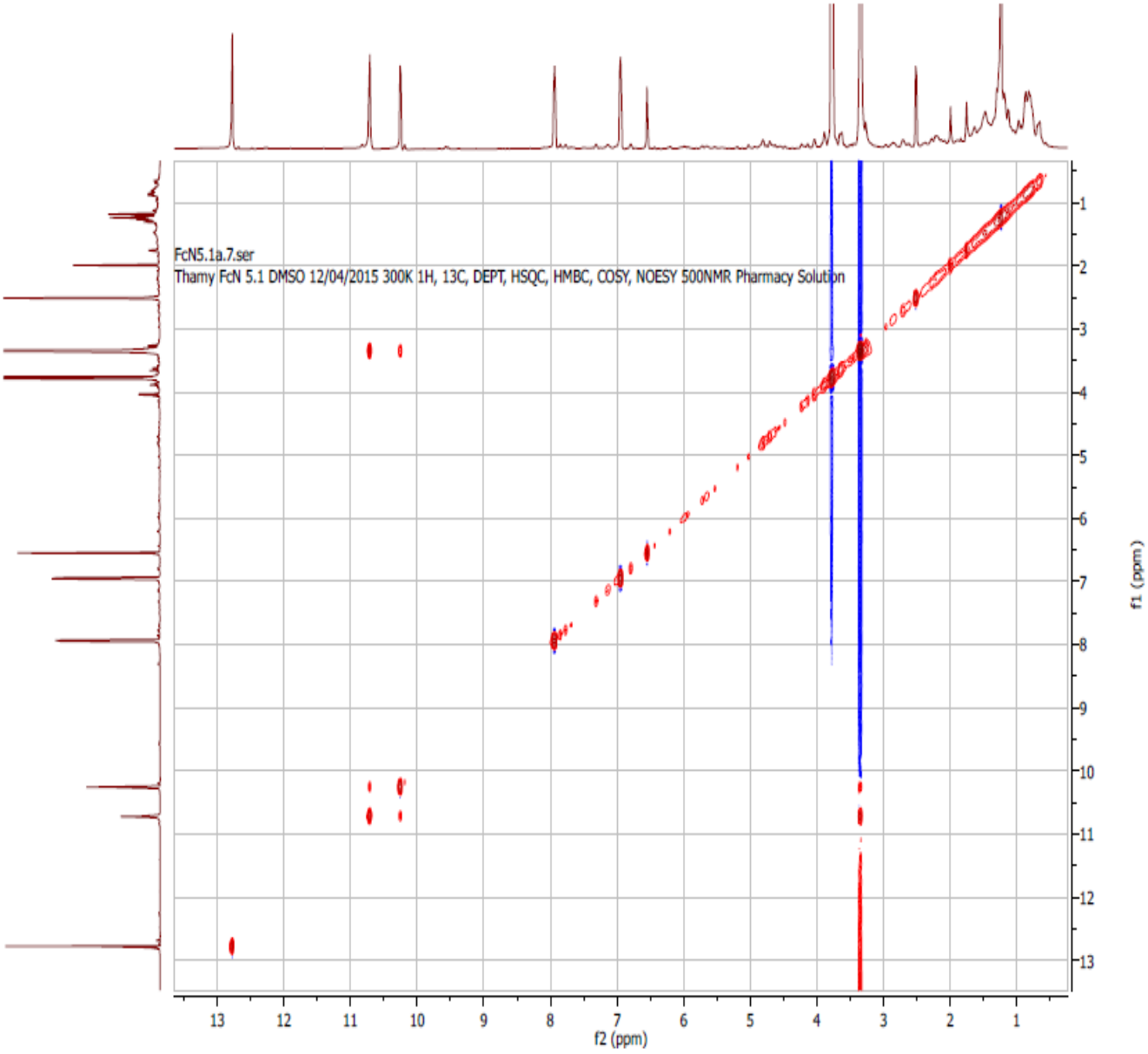
Semi-solid media

1.125g	Columbia agar base
100ml	Distilled water

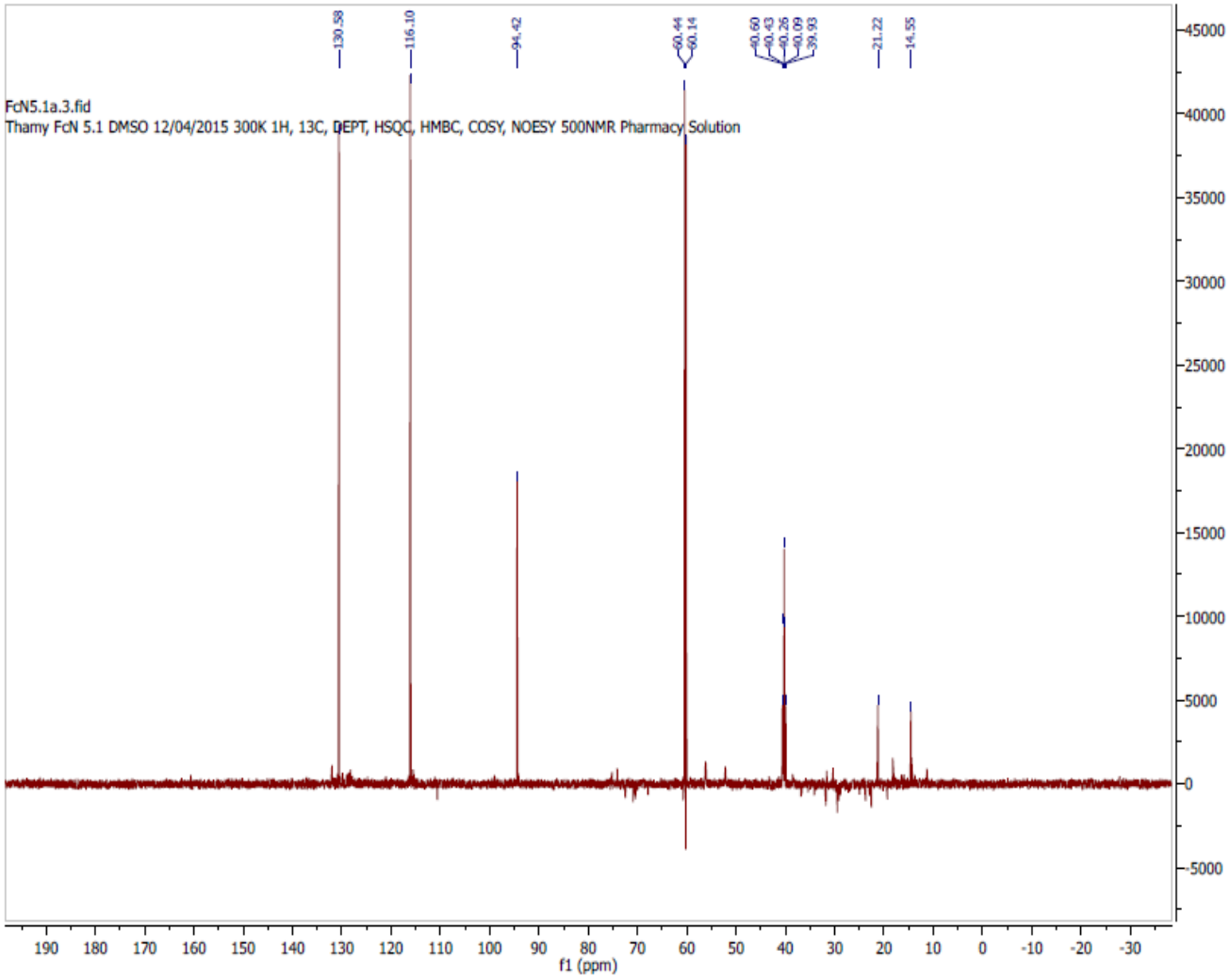
1.125g of Columbia agar base media was dissolved in 100 ml of distilled water, boilthen autoclaved at 121 °C for 15 minutes.

Appendix 2: NMR analysis of 5,6,8 Trihydroxy-7,4^{1-} dimethoxyflavone

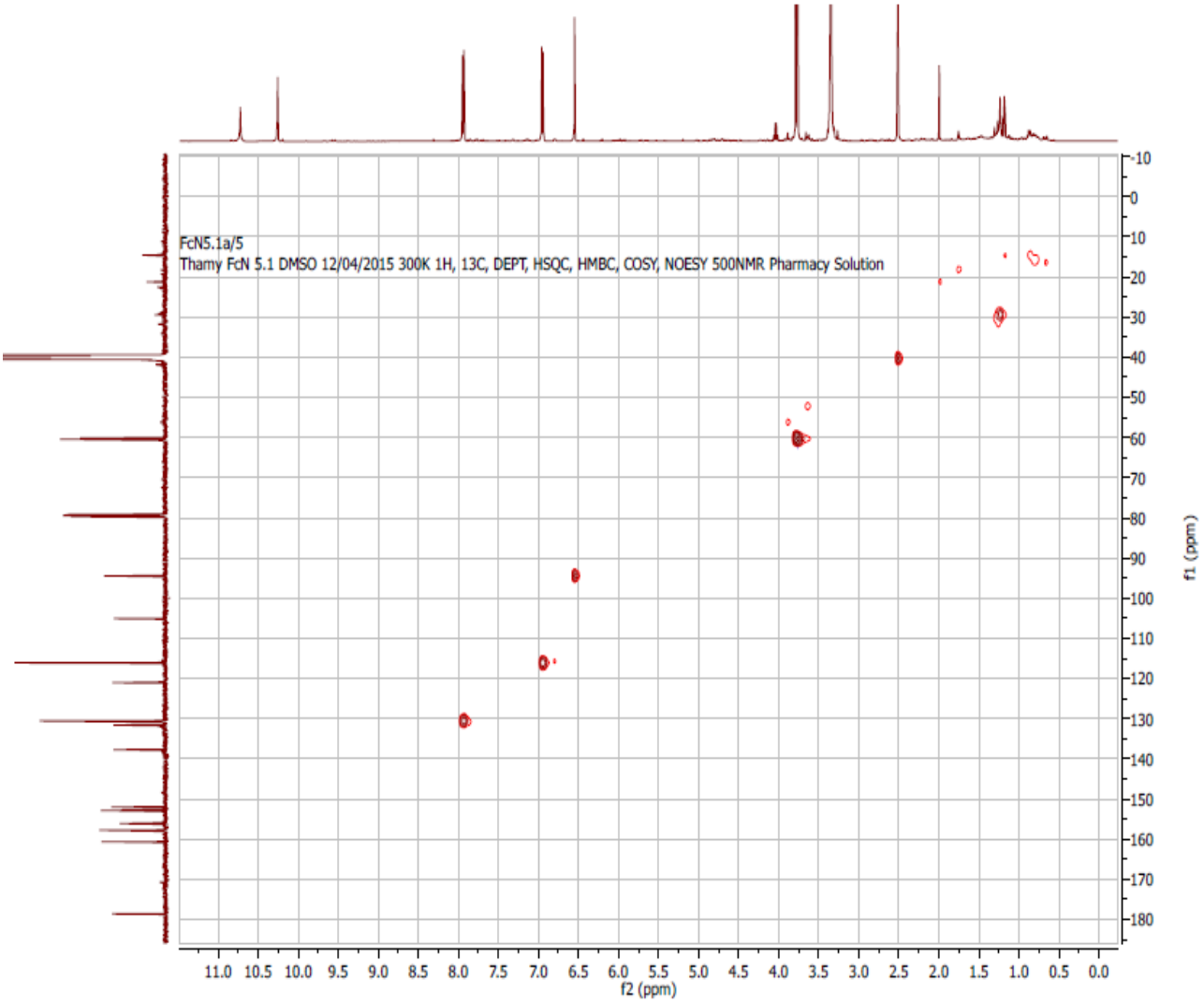
2.1: COSY NMR (5,6,8 Trihydroxy-7,4^{1}- dimethoxyflavone)



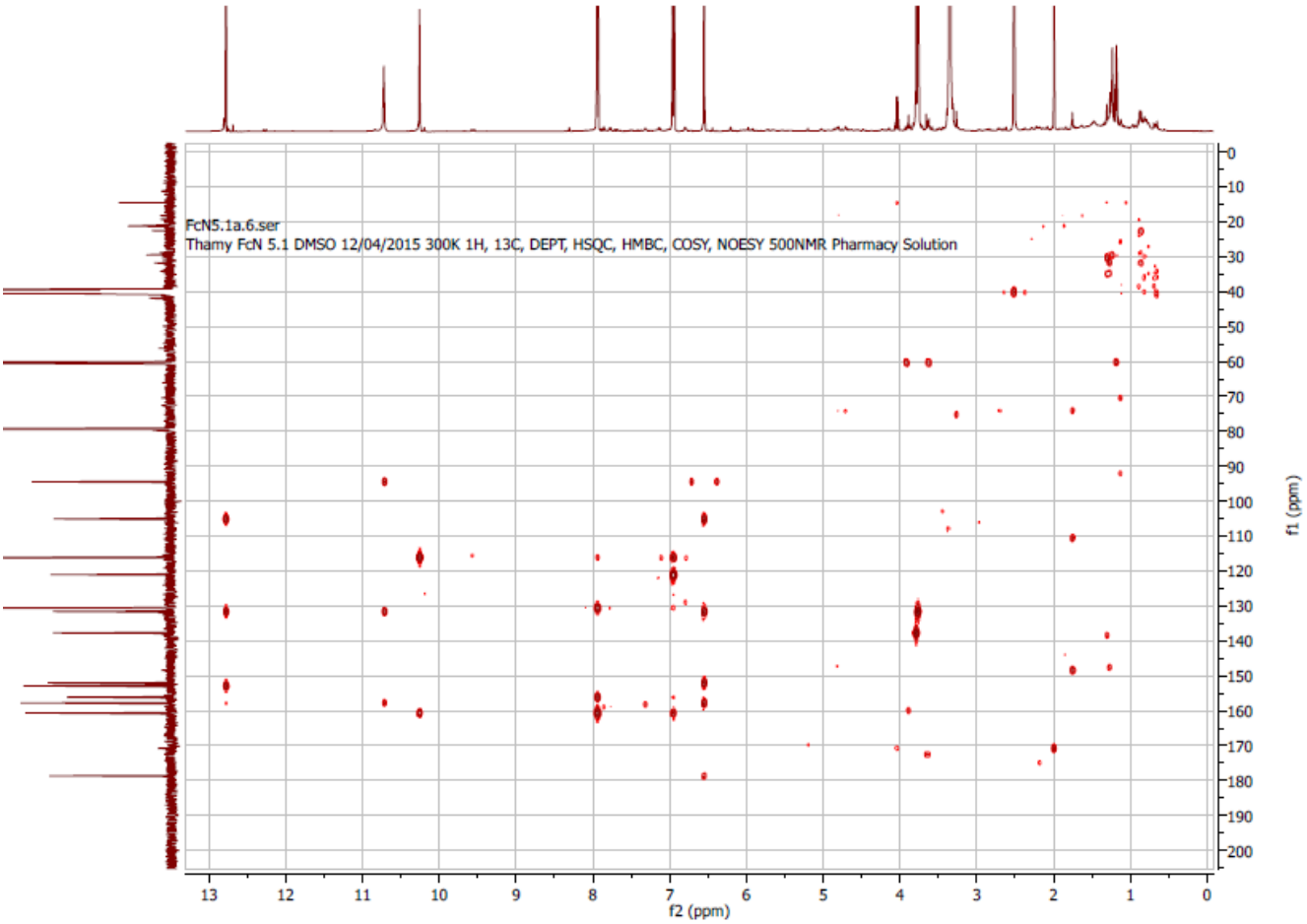
2.2: DEPT NMR (5,6,8 Trihydroxy-7,4[1]- dimethoxyflavone)



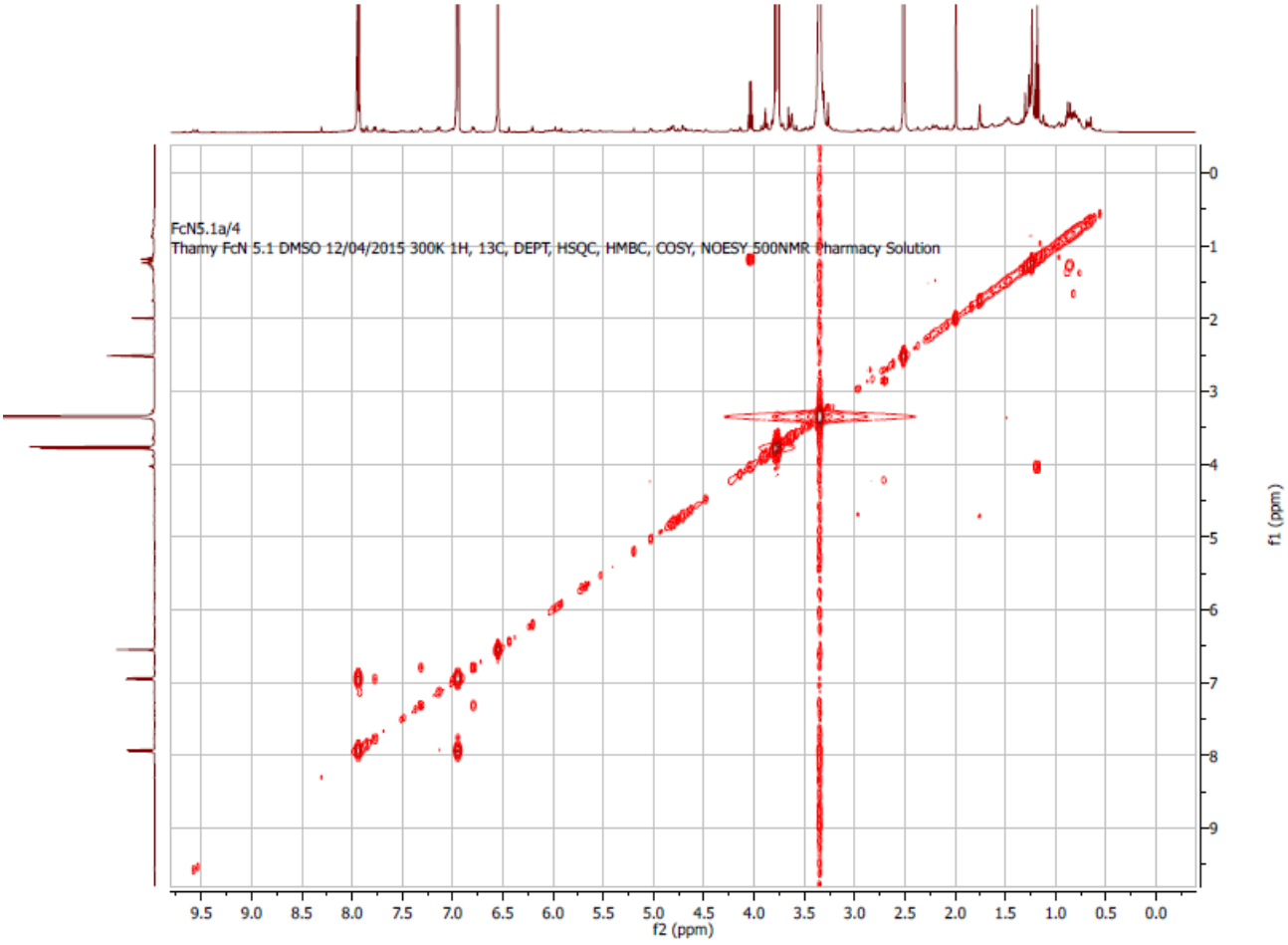
2.3: HMBC NMR (5,6,8 Trihydroxy-7,4[1]- dimethoxyflavone)



2.4: HSQC NMR (5,6,8 Trihydroxy-7,4[1]- dimethoxyflavone)



2.5: NOESY NMR (5,6,8 Trihydroxy-7,4[1]- dimethoxyflavone)



Appendix 3: Ethical clearance

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: P V Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za Fax +27 (0)11-717-1265

Ref: W-CJ-141201-1 01/12/2014

TO WHOM IT MAY CONCERN:

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

Investigator: **Thamsanqa Ngabaza (student no 999123).**

Project title: **Phytochemical analysis of *Dodonaea viscosa* var. *augustifolia* and their beneficial effects against *Streptococcus mutans*.**

Reason: This is a laboratory study using existing stock cultures of *Streptococcus mutans* isolated under ethics clearance M10205. No human participants are in the study.

UNIVERSITY OF THE WITWATERSRAND
PROFESSOR CLEATON-JONES
RECEIVED
2014 -12- 01
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu.

Appendix 4: Statistics

4.1: Statistics for effect of *D. viscosa* var. *angustifolia* crude extract and 5,6,8 Trihydroxy-7,4[1]-dimethoxyflavone on biofilm formation by *S. mutans*

Biofilm study – % reduction. (1)

User: Foluso

```
. ********************************************************************************
. *PERCENTAGE REDUCTION
.
. clear

. use "Thami reduction.dta"

.                                   Crude extract - overall.
. *Compare % reductions between concentrations a, b, c at 6 hours and at 24 hours
. table conc time if group==1, c( mean reduction sd reduction median reduction)
```

Conc	Time 6	24
1	87.75	92.1667
	5.086748	2.958827
	87.8	92.6
2	79.9167	84.5333
	7.69712	3.970728
	82.05	84.05
3	54.05	68.9667
	10.79606	6.572266
	56.25	68.5

```
. kwallis reduction if group==1 & time==6, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum
1	6	87.00
2	6	63.00
3	6	21.00

```
chi-squared =     13.053 with 2 d.f.
probability =      0.0015

chi-squared with ties =     13.053 with 2 d.f.
probability =      0.0015

. kwallis reduction if group==1 & time==24, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum

1	6	91.00
2	6	59.00
3	6	21.00

chi-squared = 14.363 with 2 d.f.
probability = 0.0008

chi-squared with ties = 14.377 with 2 d.f.
probability = 0.0008

```
12 .
13 . *"REDUCTION" REDUCED FROM CONC 1 TO 3 FOR BOTH TIME PERIODS AND BOTH SIGNIFICAN
   >  THE VALUES
14 .
15 .
16 . *GROUP 2
17 . table conc time if group==2, c( mean reduction sd reduction median reduction)
```

Conc	Time 6	24
1	93.5833	98.8667
	1.557455	.3723797
	94.05	98.9
2	84.6833	97.7
	8.111084	.5366541
	88.1	97.8
3	70.6833	85.7167
	10.60404	2.103727
	71.5	86.4

```
18 .
19 . kwallis reduction if group==2 & time==6, by(conc)
```

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	93.00
2	6	51.00
3	6	27.00

chi-squared = 13.053 with 2 d.f.
probability = 0.0015

```
chi-squared with ties =     13.053 with 2 d.f.
probability =      0.0015
```

```
20 . kwallis reduction if group==2 & time==24, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum
1	6	93.00
2	6	57.00
3	6	21.00

```
chi-squared =     15.158 with 2 d.f.
probability =      0.0005

chi-squared with ties =     15.189 with 2 d.f.
probability =      0.0005
```

```
21 .
22 . *CONCLUSIONS SIMILAR TO GROUP 1 BUT IT APPEARS IT IS MORE PRONOUNCED HERE. PLEA
23 .
24 . ********************************************************************************
25 . *BIOFILM FORMATION — counts.
26 .
27 . clear

28 .
29 . use "Thami biofilm formation.dta"        Crude extract.

30 .
31 . * Questions: Does plant have effect at 6 hours - overall?
32 . table plant time if group==1, c( mean biofilmformaiton sd biofilmformaiton medi
```

Plant	Time 6	24
0	3.9e+06	1.4e+08
	3751858	1.24e+08
	3.5e+06	1.1e+08
1	706000	2.5e+07
	1043595	2.71e+07
	210000	9.9e+06

```
33 .
34 . *THE DIFFERENCE BETWEEN PLANT AND CONTROL IS OBVIOUS AT BOTH TIMES (6 & 24 HRS)
35 .
36 . bysort time: ranksum biofilmformaiton if group==1, by(plant)
```

4

```
-> time = 6

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

      plant |      obs     rank sum     expected
------------+---------------------------------
          0 |       18          417          333
          1 |       18          249          333
------------+---------------------------------
   combined |       36          666          666

unadjusted variance      999.00
adjustment for ties       -3.09
                        --------
adjusted variance        995.91

Ho: biofil~n(plant==0) = biofil~n(plant==1)
                 z =    2.662
    Prob > |z| =    0.0078

-> time = 24

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

      plant |      obs     rank sum     expected
------------+---------------------------------
          0 |       18          435          333
          1 |       18          231          333
------------+---------------------------------
   combined |       36          666          666

unadjusted variance      999.00
adjustment for ties       -3.21
                        --------
adjusted variance        995.79

Ho: biofil~n(plant==0) = biofil~n(plant==1)
                 z =    3.232
    Prob > |z| =    0.0012

37 .
38 . bysort time: ranksum biofilmformaiton if group==2, by(plant)

-> time = 6

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

      plant |      obs     rank sum     expected
```

Subtraction - overall.

5

0	18	448.5	333
1	18	217.5	333
combined	36	666	666

unadjusted variance 999.00
adjustment for ties -3.86

adjusted variance 995.14

Ho: biofil~n(plant==0) = biofil~n(plant==1)
z = 3.661
Prob > |z| = 0.0003

-> time = 24

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

plant	obs	rank sum	expected
0	18	480	333
1	18	186	333
combined	36	666	666

unadjusted variance 999.00
adjustment for ties -3.21

adjusted variance 995.79

Ho: biofil~n(plant==0) = biofil~n(plant==1)
z = 4.658
Prob > |z| = 0.0000

```
39 . *SIGNIFICANT EFFECT AT BOTH TIMES IN BOTH GROUPS
40 .
41 .
42 . *Which concentration is effective at 6 hours?
43 .
44 . kwallis biofilmformaiton if group==1 & time==6 & plant==1, by(conc)
```

Crude extract.

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	48.00
2	6	47.00
3	6	76.00

chi-squared = 3.170 with 2 d.f.

probability = 0.2050

chi-squared with ties = 3.170 with 2 d.f.
probability = 0.2050

45 . kwallis biofilmformaiton if group==1 & time==6 & plant==0, by(conc)

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	57.00
2	6	57.00
3	6	57.00

chi-squared = 0.000 with 2 d.f.
probability = 1.0000

chi-squared with ties = 0.000 with 2 d.f.
probability = 1.0000

46 . kwallis biofilmformaiton if group==1 & time==24 & plant==1, by(conc)

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	39.50
2	6	56.50
3	6	75.00

chi-squared = 3.687 with 2 d.f.
probability = 0.1583

chi-squared with ties = 3.691 with 2 d.f.
probability = 0.1580

47 . kwallis biofilmformaiton if group==1 & time==24 & plant==0, by(conc)

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	57.00
2	6	57.00
3	6	57.00

(7)

```
chi-squared =     0.000 with 2 d.f.
probability =     1.0000

chi-squared with ties =     0.000 with 2 d.f.
probability =     1.0000

48 .
49 . kwallis biofilmformaiton if group==1 & time==6, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum
1	12	214.00
2	12	204.00
3	12	248.00

```
chi-squared =     0.799 with 2 d.f.
probability =     0.6707

chi-squared with ties =     0.801 with 2 d.f.
probability =     0.6699

50 . kwallis biofilmformaiton if group==1 & time==6, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum
1	12	214.00
2	12	204.00
3	12	248.00

```
chi-squared =     0.799 with 2 d.f.
probability =     0.6707

chi-squared with ties =     0.801 with 2 d.f.
probability =     0.6699

51 .
52 . *CONCENTRATION DOES NOT APPEAR TO MAKE A DIFFERENCE WHEN PLANTS AND CONTROLS AR
53 .
54 . *WE DO THE SAME FOR GROUP 2
55 . kwallis biofilmformaiton if group==2 & time==6 & plant==1, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum

1	6	40.00
2	6	56.00
3	6	75.00

chi-squared = 3.591 with 2 d.f.
probability = 0.1661

chi-squared with ties = 3.591 with 2 d.f.
probability = 0.1661

56 . kwallis biofilmformaiton if group==2 & time==6 & plant==0, by(conc)

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	57.00
2	6	57.00
3	6	57.00

chi-squared = 0.000 with 2 d.f.
probability = 1.0000

chi-squared with ties = 0.000 with 2 d.f.
probability = 1.0000

57 . kwallis biofilmformaiton if group==2 & time==24 & plant==1, by(conc)

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	30.00
2	6	55.50
3	6	85.50

chi-squared = 9.026 with 2 d.f.
probability = 0.0110

chi-squared with ties = 9.036 with 2 d.f.
probability = 0.0109

58 . kwallis biofilmformaiton if group==2 & time==24 & plant==0, by(conc)

Kruskal-Wallis equality-of-populations rank test

conc	Obs	Rank Sum
1	6	57.00
2	6	57.00
3	6	57.00

```
chi-squared =     0.000 with 2 d.f.
probability =     1.0000

chi-squared with ties =     0.000 with 2 d.f.
probability =     1.0000

59 .
60 . kwallis biofilmformaiton if group==2 & time==6, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum
1	12	192.50
2	12	222.00
3	12	251.50

```
chi-squared =     1.307 with 2 d.f.
probability =     0.5203

chi-squared with ties =     1.312 with 2 d.f.
probability =     0.5190

61 . kwallis biofilmformaiton if group==2 & time==6, by(conc)

Kruskal-Wallis equality-of-populations rank test
```

conc	Obs	Rank Sum
1	12	192.50
2	12	222.00
3	12	251.50

```
chi-squared =     1.307 with 2 d.f.
probability =     0.5203

chi-squared with ties =     1.312 with 2 d.f.
probability =     0.5190

62 .
63 . *CONCENTRATION HAD AN EFFECT ON PLANTS IN 24 IN GROUP 2
64 . log close
```

(10)

```
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Thami.log
  log type:  text
 closed on:   7 Mar 2016, 15:44:04
-------------------------------------------------------------------------------

65 .
66 .
67 . set more on

68 .
   end of do-file

69 .
```

4.2: Statistics for effect of *D. viscosa* var. *angustifolia* crude extract and 5,6,8 Trihydroxy-7,4[1]- dimethoxyflavone on acid production by *S. mutans*

Counts & pH

(1)

Crude :— Counts.

```
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Thami.log
  log type:  text
 opened on:  29 Feb 2016, 17:54:58

. use "Thami.dta"

.
. *Compare control to plant overall difference at concentration 1+2+3 Q 1 Does plant have effect on coun
> ts?
. table plant if group==1, c( mean count sd count median count)

----------------------------------------------------
    Plant | mean(count)   sd(count)   med(count)
----------+-----------------------------------------
        0 |     2.4e+07     3.16e+07       8.0e+06
        1 |     8.8e+06     1.49e+07       4.2e+06
----------------------------------------------------

. ranksum count if  group==1, by(plant)

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

       plant |      obs    rank sum    expected
-------------+---------------------------------
           0 |       54      3349.5        2943
           1 |       54      2536.5        2943
-------------+---------------------------------
    combined |      108        5886        5886

unadjusted variance    26487.00
adjustment for ties      -13.88
                       ----------
adjusted variance      26473.12

Ho: count(plant==0) = count(plant==1)
             z =   2.498
    Prob > |z| =   0.0125

.
. *SIGNIFICANT REDUCTION IN COUNT BY PLANT
.
. * Compare control to plant at concentration 1, 2 OR 3- Q 2 which concentration affects the counts?
. table plant conc if group==1, c( mean count sd count median count)

----------------------------------------
           |                Conc
    Plant  |         1          2          3
-----------+----------------------------------
         0 |  2.4e+07   2.4e+07   2.4e+07
           | 3.22e+07  3.22e+07  3.22e+07
           |  8.0e+06   8.0e+06   8.0e+06
           |
         1 |  3.5e+06   1.0e+07   1.3e+07
           |  3620407  1.54e+07  1.98e+07
           |  3.2e+06   5.0e+06   6.2e+06
----------------------------------------

.
. bysort conc:ranksum count if  group==1, by(plant)

-------------------------------------------------------------------------------------------------------------------------------
-> conc = 1

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

       plant |      obs    rank sum    expected
-------------+---------------------------------
           0 |       18         401         333
           1 |       18         265         333
-------------+---------------------------------
    combined |       36         666         666

unadjusted variance      999.00
adjustment for ties       -0.39
                       ----------
adjusted variance        998.61

Ho: count(plant==0) = count(plant==1)
             z =   2.152
    Prob > |z| =   0.0314

-------------------------------------------------------------------------------------------------------------------------------
-> conc = 2

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

       plant |      obs    rank sum    expected
-------------+---------------------------------
           0 |       18       369.5         333
```

(2)

```
          1 |       18       296.5         333
------------+---------------------------------
    combined |       36         666         666

unadjusted variance      999.00
adjustment for ties       -0.13
                       ----------
adjusted variance        998.87

Ho: count(plant==0) = count(plant==1)
             z =   1.155
    Prob > |z| =   0.2481

-------------------------------------------------------------------------------------------------------------------
-> conc = 3

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

       plant |      obs    rank sum    expected
-------------+---------------------------------
           0 |       18         364         333
           1 |       18         302         333
-------------+---------------------------------
    combined |       36         666         666

unadjusted variance      999.00
adjustment for ties       -0.26
                       ----------
adjusted variance        998.74

Ho: count(plant==0) = count(plant==1)
             z =   0.981
    Prob > |z| =   0.3266

.
. *Concentration 1 was significant
.
.
. *SIMILAR ANALYSIS FOR GROUP 2
. *Compare control to plant overall difference at concentration 1+2+3 Q 1 Does plant have effect on coun
> ts?
. table plant if group==2, c( mean count sd count median count)

----------------------------------------------------
    Plant | mean(count)    sd(count)   med(count)
----------+-----------------------------------------
        0 |     2.0e+07     2.32e+07      1.0e+07
        1 |     1.1e+07     1.68e+07      5.4e+06
----------------------------------------------------

. ranksum count if  group==2, by(plant)

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

       plant |      obs    rank sum    expected
-------------+---------------------------------
           0 |       54      3259.5        2943
           1 |       54      2626.5        2943
-------------+---------------------------------
    combined |      108        5886        5886

unadjusted variance    26487.00
adjustment for ties      -13.25
                       ----------
adjusted variance      26473.75

Ho: count(plant==0) = count(plant==1)
             z =   1.945
    Prob > |z| =   0.0517

.
. *REDUCTION IN COUNT BY PLANT BUT NOT STATISTICALLY SIGNIFICANT
.
. * Compare control to plant at concentration 1, 2 OR 3- Q 2 which concentration affects the counts?
. table plant conc if group==2, c( mean count sd count median count)

-------------------------------------------
          |              Conc
    Plant |         1         2         3
----------+--------------------------------
        0 |  2.0e+07   2.0e+07   2.0e+07
          | 2.37e+07  2.37e+07  2.37e+07
          |  1.0e+07   1.0e+07   1.0e+07
          |
        1 |  5.7e+06   1.2e+07   1.5e+07
          |  6418175  1.73e+07  2.20e+07
          |  4.3e+06   5.9e+06   6.7e+06
-------------------------------------------

.
. bysort conc:ranksum count if  group==2, by(plant)
```

Subfraction 5.1 - count.

```
-> conc = 1

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       18       384.5         333
          1 |       18       281.5         333
------------+---------------------------------
   combined |       36         666         666

unadjusted variance      999.00
adjustment for ties       -0.13
                        --------
adjusted variance        998.87

Ho: count(plant==0) = count(plant==1)
             z =   1.629
    Prob > |z| =   0.1032

-------------------------------------------------------------------------------
-> conc = 2

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       18       362.5         333
          1 |       18       303.5         333
------------+---------------------------------
   combined |       36         666         666

unadjusted variance      999.00
adjustment for ties       -0.13
                        --------
adjusted variance        998.87

Ho: count(plant==0) = count(plant==1)
             z =   0.933
    Prob > |z| =   0.3506

-------------------------------------------------------------------------------
-> conc = 3

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       18       357.5         333
          1 |       18       308.5         333
------------+---------------------------------
   combined |       36         666         666

unadjusted variance      999.00
adjustment for ties       -0.39
                        --------
adjusted variance        998.61

Ho: count(plant==0) = count(plant==1)
             z =   0.775
    Prob > |z| =   0.4382

.
. *NO Concentration was significant
.
.
. ********************************************************************************
. *ACID PRODUCTION                                                    Crude - pH .
.
. clear

.
. use "Thami acid.dta"

.
. *Compare pH in control to plant overall difference at concentration 1+2+3 Q 1 Does plant have effect o
> n pH7
. table plant if group==1, c( mean ph sd ph median ph)

-------------------------------------
    Plant |  mean(ph)    sd(ph)  med(ph)
----------+--------------------------
        0 |     6.261  .8085207     6.12
        1 |     6.733  .5696785    6.675
-------------------------------------

. ranksum ph if  group==1, by(plant)

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

plant	obs	rank sum	expected
0	90	6843	8145
1	90	9447	8145
combined	180	16290	16290

unadjusted variance 122175.00
adjustment for ties -214.82
adjusted variance 121960.18

Ho: ph(plant==0) = ph(plant==1)
z = -3.728
Prob > |z| = 0.0002

```
. *MEAN pH higher in plant and significant
.
.
. * Compare control to plant at concentration 1, 2 OR 3- Q 2 which concentration affects the ph?
. table plant conc if group==1, c( mean ph sd ph median ph)
```

Plant	Conc 1	Conc 2	Conc 3
0	6.279	6.247	6.257
	.8130078	.8126845	.8271686
	6.175	6.135	6.085
1	6.72767	6.75467	6.71667
	.5579808	.5973605	.5718472
	6.675	6.715	6.665

```
.
. bysort conc:ranksum ph if  group==1, by(plant)
```

-> conc = 1

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

plant	obs	rank sum	expected
0	30	781.5	915
1	30	1048.5	915
combined	60	1830	1830

unadjusted variance 4575.00
adjustment for ties -12.46
adjusted variance 4562.54

Ho: ph(plant==0) = ph(plant==1)
z = -1.976
Prob > |z| = 0.0481

-> conc = 2

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

plant	obs	rank sum	expected
0	30	760.5	915
1	30	1069.5	915
combined	60	1830	1830

unadjusted variance 4575.00
adjustment for ties -10.42
adjusted variance 4564.58

Ho: ph(plant==0) = ph(plant==1)
z = -2.287
Prob > |z| = 0.0222

-> conc = 3

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

plant	obs	rank sum	expected
0	30	772.5	915
1	30	1057.5	915

5

```
    combined |        60        1830        1830

unadjusted variance     4575.00
adjustment for ties      -16.27
                        ---------
adjusted variance       4558.73

Ho: ph(plant==0) = ph(plant==1)
           z =  -2.111
    Prob > |z| =   0.0348

.
. *pH higher in plants with all concentrations
.
.
. *Group 2
. *Compare pH in control to plant overall difference at concentration 1+2+3 Q 1 Does plant have effect o
> n pH?
. table plant if group==2, c( mean ph sd ph median ph)

-------------------------------------------------
    Plant |  mean(ph)     sd(ph)     med(ph)
----------+--------------------------------------
        0 |      6.261  .8085207         6.12
        1 |   7.13544  .2660369        7.165
-------------------------------------------------

. ranksum ph if  group==2, by(plant)

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       90      5860.5        8145
          1 |       90     10429.5        8145
------------+---------------------------------
   combined |      180       16290       16290

unadjusted variance   122175.00
adjustment for ties     -354.34
                        ---------
adjusted variance     121820.66

Ho: ph(plant==0) = ph(plant==1)
           z =  -6.545
    Prob > |z| =   0.0000

.
.
. * Compare control to plant at concentration 1, 2 OR 3- Q 2 which concentration affects the ph?
. table plant conc if group==2, c( mean ph sd ph median ph)

----------------------------------------
          |                Conc
    Plant |         1         2         3
----------+-----------------------------
        0 |     6.279     6.247     6.257
          |  .8130078  .8126845  .8271686
          |     6.175     6.135     6.085
          |
        1 |   7.14667   7.12133   7.13833
          |     .2595   .272444  .2744032
          |      7.18     7.135      7.18
----------------------------------------

.
. bysort conc:ranksum ph if  group==2, by(plant)

-------------------------------------------------------------------------------
-> conc = 1

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30         663         915
          1 |       30        1167         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance     4575.00
adjustment for ties      -16.91
                        ---------
adjusted variance       4558.09

Ho: ph(plant==0) = ph(plant==1)
           z =  -3.733
    Prob > |z| =   0.0002

-------------------------------------------------------------------------------
-> conc = 2
```

Subtraction 5.1 – pH.

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30       655.5         915
          1 |       30      1174.5         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance     4575.00
adjustment for ties      -17.42
                       ----------
adjusted variance       4557.58

Ho: ph(plant==0) = ph(plant==1)
            z =  -3.844
    Prob > |z| =   0.0001

-------------------------------------------------------------------------------
-> conc = 3

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30         669         915
          1 |       30        1161         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance     4575.00
adjustment for ties      -16.40
                       ----------
adjusted variance       4558.60

Ho: ph(plant==0) = ph(plant==1)
            z =  -3.644
    Prob > |z| =   0.0003

.
. *SIMILAR OBSERVATIONS IN GROUP 2 AND SIGNIFICANT
. log close
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Thami.log
  log type:  text
 closed on:  29 Feb 2016, 17:54:58
-------------------------------------------------------------------------------
```

Appendix 5: Subfraction 5.1 (5,6,8 Trihydroxy-7,4[1]-methoxyflavone) mass spectra