Identification of bioactive compounds of a South African plant extract for combating potentially pathogenic oral microorganisms

by

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Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

Medicinal Plant Science

in the

Faculty of Natural and Agricultural Sciences

University of Pretoria

May 2011

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"*I declare that this dissertation/thesis, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution*."

Cynthia Joan Henley-Smith
2011

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List of Abbreviations

A431	Human skin carcinoma cells
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
C	Catechin
CASO	Casein-peptone Soymeal-peptone Agar medium
CC_{50}	Modelling of cytotoxicity data
CFU	Colony forming units
CHX	Chlorhexidine gluconate
^{13}C-NMR	Carbon nuclear magnetic resonance
CO_2	Carbon dioxide
Cu^+	Copper
DEPT	Distortionless Enhancement by Polarization
DHCo	Dental Herb Company
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FE-SEM	Ultra high resolution scanning electron microscope
GBP	Glucan binding proteins
GCF	Gingival crevicular fluid
GMK	Green monkey kidney cells
GTFs	Glucosyltransferases
H_2SO_4	Sulfuric acid
HeLa	Human cervical carcinoma cells
HepG2	Human hepatoma cells
HEp-2	Human laryngeal epidermoid carcinoma cells
HIV	Human immunodeficiency virus

^{1}H-NMR	Proton nuclear magnetic resonance
HSV	Herpes simplex virus
HT29	Human colon adenocarcinoma cells
IC_{50}	50% inhibitory concentration
IL-8	Interleukin 8
INT	*p*-iodonitrotetrazolium violet
LPS	Lipopolysaccharide
MEM	Minimum essential medium
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MDCK	Madin-Darby canine kidney cells
MRSA	Methicillin-resistant *Staphylococcus aureus*
NMR	Nuclear magnetic resonance spectroscopy
OsO_4	Osmiumtetroxide
SDA	Sabouraud Dextrose 4% Agar
SEM	Scanning electron microscope
PBS	Phosphate buffer saline
pH	Potential Hydrogen
PRU	H.G.W.J. Schwelcherdt Herbarium
PVP	Polyvinyl-pirrolidone
TLC	Thin layer chromatography
TTO	Tea tree oil
Vero	Kidney epithelial cells of the African Green Monkey
V/v	Volume per volume
WHO	World Health Organization
W/v	Weight per volume
XTT	Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolim]-bis-[4 methoxy-6-nitro] benzene sulfonic acid hydrate

Abstract

Seven plants generally used for traditional oral care namely, *Barleria albostellata, Cotyledon orbiculata, Dichrostachys cinerea, Heteropyxis natalensis, Carpobrotus edulis, Zanthoxylum capense and Dodonaea viscosa* were investigated for antimicrobial activity and safety. Four pathogenic microorganisms, *Actinomyces israelii*, *Streptococcus mutans*, *Prevotella intermedia* and *Candida albicans*, were selected that represented the diversity of microbial flora in the oral cavity.

No evidence could be found in the literature on the activity of the selected plant extracts against *A. israelii*, *P. intermedia* and *S. mutans*. Only *H. natalensis* exhibited activity against the Gram-positive microorganisms, *A. israelii* and *S. mutans*; minimum inhibitory concentration (MIC) was found to be 0.88 mg/ml and 1.82 mg/ml respectively. The MIC against the Gram-negative bacteria, *P. intermedia* was found to be 3.13 mg/ml. *Dichrostachys cinerea* exhibited activity towards a drug-sensitive stain of *C. albicans* (MIC of 10.71 mg/ml) and against a drug-resistant (polyene and azole resistant) strain of *C. albicans* (MIC of 10.42 mg/ml). *Dichrostachys cinerea* was the least toxic to both the Kidney epithelial cells of the African Green Monkey (Vero) and Human laryngeal epidermoid carcinoma cells (HEp-2) cell lines with 50% inhibitory concentrations (IC_{50}) of 204 ± 0.13 µg/ml and 224 ± 0.1 µg/ml respectively. *Heteropyxis natalensis* was selected for further study as it exhibited moderate cytotoxicity (IC_{50} of 33.66 ± 0.04 µg/ml) on HEp-2 cells and the best antibacterial activity as compared to the other plant extracts investigated in this study. When *H. natalensis* was incorporated in a synergistic combination with the essential oils *Melaleuca alternifolia* (Tea tree) and *Mentha piperita* (peppermint); a fourfold reduction in the MIC of *A. israelii* was exhibited.

Gingivitis, the infection of the gums, induces inflammation. To attract the white blood cell, leukocytes, to the site of infection; a chemokine known as Interluekin-8 (IL-8) is released. These cytokine, IL-8, levels were not reduced when the extract of *H. natalensis* was utilized to prevent the interaction of *A. israelii* with the epithelial cells, HEp-2. A Scanning Electron Microscopy (SEM) study to determine bacterial adhesion in the presence of *H. natalensis* indicated that the plant extract interferes with pellicle formation and glucan binding of *S. mutans* to the enamel surface of the tooth.

Five known compounds were identified from the ethanolic extract of *H. natalensis* leaves and twigs. The compounds were identified as Aurentiacin A (**1**), Cardamomin (**2**), 5-hydroxy-7-methoxy-methylflavanone (**3**), Quercetin (**4**) and 3,5,7-trihydroxyflavan (**5**). The MICs of the compounds **1** and **4** were found to be 0.063 mg/ml and 1.0 mg/ml respectively against *A. israelii*. Compounds **2** and **5** exhibited no inhibitory activity at 1.0 mg/ml (the highest

concentration tested) against *A. israelii*. This is the first report of the isolation of the five compounds and their activity against *A. israelii*.

Chapter 1

Introduction

1.1 Background and motivation of study

Biofilms (plaque) are formed from the extensive growth of microorganisms, resulting from changes in the oral bacterial ecosystem. Once a biofilm is established it may lead to the formation of dental caries (tooth decay) or even more severe periodontal diseases. Dental caries and periodontal diseases in humans have an astonishing impact on the health and welfare of communities (Samaranayake, 2002). Sick leave, due to oral infections, and the consequent cost of dental treatment results in costing billions of dollars each year (Samaranayake, 2002). In 2007, the World Health Organization (WHO) stated that 5-10% of public health expenditure was related to dental care. 'Tooth decay and, to a lesser extent periodontal infections, are perhaps the most expensive infections that most individuals have to contend with, during a lifetime' (Loesche, 1986). Natural plant products are becoming increasingly popular treatments, even for oral health care.

One of the fastest growing sectors in the agribusiness industry is the natural plant products which led to worldwide sales of $ 23 billion in 2002 alone. In 2008, there were approximately 85,000 medicinally useful plant species; however Africa only contributed 1% to the market even though 75% of the African population still relies on traditional herbal medicine (Makunga *et al*., 2008).

In this study, selected plants from Southern Africa have been evaluated for their bioactivity against pathogens which cause periodontal diseases. Identification of the bioactive principles from the best plant has also been attempted.

1.2 Plants in oral care

Civilizations throughout history have been using plants as traditional medicine to cure various ailments, including toothache. Principle plant parts used in remedies to treat toothache, gingivitis, loose teeth, dental abscesses and general mouth sores, were fresh or dried roots, stems, leaves and bark. Traditional preparation of the plant material is generally a decoction, used to rinse the mouth, gargling or inhalation (Tapsoba & Deschamps, 2005). Plants can also be used as chewing sticks to form basic toothbrushes and dental floss. Over time, people discovered that chewing the twigs or leaves of certain plants alleviate mouth sores, infections and toothache. The fibrous texture and a palatable taste, combined with antibacterial properties

make good chewing sticks (Van Wyk & Gericke, 2000). There are over 62 plant species belonging to 29 families documented to treat oral diseases in Burkina Faso, West Africa, alone (Tapsoba & Deschamps, 2005). Some of these plants used for various tooth problems in various forms are as follows:

1.2.1 Toothache

Plants used to treat toothache include *Acokanthera oppositifolia*; *Albizia adianthifolia* (leaves and roots); *Annona senegalensis* (bark); *Barleria prionitis*; *Carissa bispinosa* (root); *Dicoma anomala* (root); several *Cassia*, *Acacia* and *Ficus* species (Tapsoba & Deschamps, 2005; Van Wyk & Gericke, 2000). *Zea mays*, more commonly known as maize, is part of the staple diet in South Africa; however in Burkina Faso, the decoction made from the flowers are used to treat toothache (Tapsoba & Deschamps, 2005).

1.2.2 Gingivitis

Plants utilized to treat gingivitis include *Alternanthera pungens* (leaves); *Ceiba pentandra* (bark); *Boswellia dalzielii* (bark and roots); *Maytenus senegalensis* (leaves, bark and roots); *Anogeissus leiocarpus* (bark and roots); *Pteleopsis suberosa* (bark); *Diospyros mespiliformis* (leaves); *Indigofera tinctoria* (leaves and roots); *Ximenia americana* (leaves, bark and roots); *Myrothamnus flabellifolius* (leaves); *Pinus pinaster* (bark); *Bauhinia*, *Acacia* and *Cassia* species (Tapsoba & Deschamps, 2005; Van Wyk & Gericke, 2000).

1.2.3 Chewing sticks

African, Middle Eastern and Asian communities often make use of chewing sticks, or miswak, as an oral hygiene aid. The World Health Organization is still encouraging this practice for those communities who do not have professional dental care. Various plant species are selected due to availability, long bristle-like fibres and also for pleasant taste. The activity within these natural toothbrushes may often be diverse (Cowan, 1999; Wu *et al*., 2001).

Some of the plants used as chewing sticks include *Acacia mellifera* (twigs); *Diospyros lycioides* (roots and twigs); *Jasminum fluminense* (branches), *Salvadora persica* (roots) and *Azadirachta indica* (twigs) (van Wyk & Gericke, 2000; Wolinsky *et al*., 1996). In West Africa, the twigs from citrus trees such as *Citrus aurantafolia*, *Citrus sinensis* and *Cassia sleberlanba* are used as chewing sticks. A popular Nigerian chewing stick is *Fagara zanthoxyloides*. In Kenya, twigs from *Rhus natalensis* and *Euclea divinorum* are preferred. In the United States, *Betula lenta*, *Gaultheria procumbens*, *Liquidambar stryaciflua*, *Sassafras albidum* and *Populus* species are utilized. *Faidherbia albida* bark strips are used in the same manner as dental floss (van Wyk & Gericke, 2000; Wu *et al*., 2001).

Several of these chewing sticks have been tested for bioactivity against oral pathogens. A phosphate-buffered saline (PBS) extract from the bark of *Serindeia warneckei* showed activity against *Porphyromonas gingivalis* and *Bacteroides melaninogenicus* exhibiting an MIC of 0.25 µg/ml and 0.5 µg/ml respectively. Aqueous and tannic extracts of the twigs exhibited activity at 2.5% v/v against *Candida albicans* (Cowan, 1999; Rotimi *et al*., 1988; Wu *et al*., 2001). Aqueous and alcohol extracts of *Juglandaceae regia* at 2-8% w/v and 10% w/v respectively, inhibited *in vitro* growth, adherence, acid production and glucan-induced adherence of *Streptococcus mutans*. *Streptococcus mutans* utilizes an enzyme termed Glucosyltransferases (GTFs) to synthesize extracellular glucans with different solubility's in water, which aid in adherence of the bacteria to the tooth surface and other bacteria nearby. *Rhus natalensis* and *E. divinorum* was shown to inhibit the extracellular peptidase and glycosidase activities of *P. gingivalis*, *Prevotella intermedia* and *Treponema denticola* (Taubman & Nash, 2006; Wu *et al*., 2001). When chewing sticks are used effectively, they are at least as efficient as modern toothbrushes (Wu *et al*., (2001).

1.2.4 Chewing gum

Research has shown that some chewing gums containing plant components can treat gingival bleeding and plaque formation. One such example is the chewing gum PYCNOGENOL®, which contains phytochemicals extracted from the bark of the French maritime pine, '*Pinus pinaster'*. PYCNOGENOL® has been shown to be a potent antioxidant-phytochemical, with anti-inflammatory properties (Kimbrough *et al*., 2002). Chewing PYCNOGENOL® reduced gum bleeding by 50% and may suppress biofilm formation (Kimbrough *et al*., 2002). Chewing gum, containing mastic gum, derived from the resin of the *Pistacia lentiscus* tree had significant antibacterial activity against *S. mutans*. It may prove to be a useful adjunct in the prevention of dental caries (Aksoy *et al*., 2006).

1.2.5 Oral rinses

Oral rinses deliver their therapeutic ingredients and benefits to all accessible surfaces of the oral cavity. Depending on their composition, oral rinses can remain active for an extended period of time. Clinicians often recommend oral rinses to patients to reduce biofilm formation, which aids in controlling gingivitis. Recent studies compared the antimicrobial action of a herbal mouth rinse (The Natural Dentist Healthy Gums Daily Oral Rinse); an essential oil oral rinse (Listerine Cool Mint) and an established 0.12% chlorhexidine gluconate oral rinse (Peridex), against predominant oral bacteria. 'The Natural Dentist' contained extracts of *Aloe barbadensis*, *Echinacea angustifolia*, *E. purpurea*, *Hydrastis canadensis*, *Calendula officinalis* and *Citrus paradisi*. Although 'The Natural Dentist' was found to be less potent than the chlorhexidine gluconate rinse, Peridex, it inhibited the growth of 40 bacterial species tested. When compared to Listerine, 'The Natural Dentist' exhibited significantly lower minimum inhibitory concentrations

(MIC's) for *Actinomyces* species, periodontal pathogens *Eubacterium nodatum*, *Tannerella forsythia* and *Prevotella* species, as well as the cariogenic *S. mutans* (Haffajee *et al*., 2008).

1.2.6 Halitosis

Halitosis is generally caused by Gram-negative anaerobic bacteria such as *P. gingivalis*, *Fusobacterium nucleatum* and *P. intermedia*. A herbal formulation comprising of herbs, known to contain antimicrobials, such as sage; echinacea; lavender and mastic gum was formulated as a mucoadhesive tablet. The clinical trials showed a significant decrease in halitosis assessments, and may be an effective means of treatment for halitosis (Sterer *et al*., 2008).

1.2.7 Antimicrobial activity shown by phyto-derived samples

Plant extracts have been screened for their antibacterial activity against several oral microorganisms, such as *S. mutans*. Garlic juice from *Allium sativum* has shown impressive inhibition of *S. mutans*, considering that this microorganism is resistant to antibacterial agents such as penicillin, amoxicillin, tetracycline and erythromycin (Xavier & Vijayalakshmi, 2007). *Saussurea lappa* is traditionally used in the treatment of halitosis, dental caries and periodontal disease. An ethanolic extract of the roots of *S. lappa*, at a concentration of 1 mg/ml, inhibited the growth of *S. mutans*; the acid production; as well as lowered the adherence of *S. mutans*. It also inhibited the formation of water-insoluble glucan by *S. mutans*, an essential component of biofilm formation (Yu *et al*., 2007). *Myristica fragrans*, known as 'nutmeg', has a wide variety of uses, including being anti-inflammatory and anti-fungal. Macelignan, an isolated compound from *M. fragrans*, exhibited an inhibitory activity of 3.9 µg/ml against *S. mutans*. Macelignan also showed preferential activity against *Streptococcus salivarius*, *S. sobrinus*, *S. sanguis*, *Lactobacillus acidophilus* and *L. casei* with an MIC range of 2-31.3 µg/ml (Chung *et al*., 2006). Four traditional Brazilian plants used in oral care were tested against *P. intermedia*, *P. gingivalis*, *F. nucleatum* and *S. mutans*. The bacteria were susceptible ethanolic extracts of the stem of *Aristolochia cymbifera*; aqueous extracts of the leaves of *Caesalpinia pyramidalis* and aqueous extracts from the husk fibre of *Cocos nucifera*. Aqueous extracts from the inner bark of *Ziziphus joazeiro* were not as effective (Alviano *et al*., 2008).

The inhibiting effect of aqueous twig extracts of *Azadirachta indica* (Neem) on the *in vitro* biofilm formation by bacteria was investigated (Wolinsky *et al*., 1996). No inhibition of *Streptococcus cricetus*, *S. sobrinus*, *S. mutans* and *S. sanguis*, was observed in the presence of ≤ 320 µg/ml Neem extract. Pre-treatment of *S. sanguis* with Neem extract resulted in the significant inhibition of bacterial adhesion to saliva-conditioned hydroxyapatite, a composite of bone and enamel. Neem extract also inhibited insoluble glucan synthesis, suggesting that Neem has the ability to reduce the adherence of streptococci to tooth surfaces (Wolinsky *et al*., 1996). *Melaphis chinensis* (Chinese nutgall) extracts are rich in gallotannins. These extracts have anti-

adherence properties as well as the ability to inhibit insoluble glucan production among streptococci (Wolinsky *et al*., 1996). A high-molecular weight constituent, isolated from *Vaccinium macrocarpon* (cranberry juice), reduced and even reversed bacterial co-aggregation of *F. nucleatum*, *Actinomyces naeslundii* and *Escherichia coli* in dental biofilms and reduced the enzymatic activity of glucosyltransferase within the biofilm (Nowack & Schmitt, 2008). A clinical trial showed a significant reduction of *S. mutans*. *Vaccinium macrocarpon* macromolecules have been shown to halt lipopolysaccharide (LPS), as well as induced the bacterial production of pro-inflammatory cytokines and proteolytic enzymes by *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola*, thereby aiding in the reduction of periodontal diseases (Nowack & Schmitt, 2008).

1.2.8 Plant-derived substances in oral care products

Oral rinses and toothpaste manufacturers have incorporated plant-derived antimicrobials into their formulations. Miswak extracts, tea tree oil (as Melafresh T96), peppermint, green tea, *Aloe vera*, manuka honey oil (from the manuka tree flowers in New Zealand), and several more essential oils such as eucalyptus, lavandula and rosmarinus are a few of the plants incorporated into oral formulas (Figure 1.1) (Allaker & Douglas, 2009).

These essential oils are active against several cariogenic and periodontopathic bacteria. Tea tree oil and manuka oil inhibited *P. gingivalis* adhesion as well. Listerine™ is a well-known oral rinse containing active ingredients such as thymol, eucalyptol (antimicrobials), methyl salicylate (a cleaning agent) and menthol (a local anaesthetic) (Allaker & Douglas, 2009). There are several more natural oral care product ranges.

The Dental Herb Company (DHCo), in the United States of America, manufactures a line of natural oral health care products. ‘Tooth & Gum Tonic’; ‘Under The Gum Irrigant’; ‘Tooth & Gum Paste’ and ‘Tooth & Gum Spritz’ formulated from a various combination of red thyme, cinnamon bark, eucalyptus, lavender and peppermint essential oils; as well as echinacea, *Gota kola* and green tea extracts (www.wholebodymed.com).

‘LOGONA Naturkosmetik’, in Germany, develops oral care products for adults and children known as LOGODENT. Herbal ingredients such as echinacea, anise, green tea, clove oil and a saccharose ester are included to prevent plaque formation and tooth decay. To strengthen the gums and prevent infection, myrrh, witch hazel and chamomile are included (www.logona.com).

The Natural Dentist range, from the United States of America, formulated for adults and children contains echinacea, goldenseal, calendula, *Aloe vera* gel, blood root and grapefruit seed extract (sale.dentist.net). In India, Himalaya and oral care, HiOra, is formulated and contains extracts of

Symplocos racemosa (Lodh tree), *Vitis vinifera* (grapes), *Cinnamomum zeylanicum* (cinnamon) and *Carica papaya* (papaya) (apnnews.com).

Figure 1.1: Herbal toothpastes available. a) Natural dentist range (sale.dentist.net); b) HiOra (apnnews.com); c) Dabur herbal toothpaste (www.emporiumonnet.com); d) LOGODENT oral care products (www.logona.com); e) Dental Herb Company (www.wholebodymed.com).

Although many herbal/natural toothpastes are available worldwide, few if any of those toothpastes apart from Colgate and Aquafresh are manufactured in South Africa. Among herbal toothpastes produced in South Africa five are commonly used, namely; Colgate Herbal, Dabur herbal toothpaste, Miswak, Nature Fresh and Aloe. Colgate and Aquafresh brands are readily available in stores (www.alibaba.com; www.apnnews.com www.emporiumonnet.com; www.naturalwise.co.za, www.pricecheck.co.za; www.sale.dentist.net www.tradekey.com; www.wholebodymed.com).

Dabur herbal toothpaste has three herbal toothpastes formulated with clove, basil or neem as the main herbal ingredient. Other herbal ingredients include *Anacyclus pyrethrum*, *Acacia arabica*, *S. racemosa*, *Mimuspos elengi* and *Eugenia jambolana*. The benefits stated, indicate that there is an analgesic action for sensitive teeth and it relieves toothache; prevents tooth decay and cavities; removes plaque and refreshes breath. No additives, preservatives, chemicals or fluoride are stated to be present (naturalwise.co.za).

No ingredients are listed for the Miswak herbal toothpaste, Nature Fresh or Aloe toothpaste. The aloe toothpaste indicates that it stimulates the healing of gums and sores in the mouth (www.alibaba.com; www.pricecheck.co.za; www.tradekey.com).

1.2.9 Southern African plants in oral care

Of the estimated 3000 medicinal plant species that are regularly used in South African traditional medicine, only 38 indigenous species have been commercialized to some extent (Van Wyk, 2008). The natural product industry as well as local communities are benefiting from the trade of indigenous medicinal plants. Due to national and international research institutions focusing on South African plants, several phytochemical products have already been marketed. Some plants, such as *Aspalathus linearis* (Rooibos) and *Aloe ferox*, have a greater international status as their trade outside South Africa becomes more prominent (Makunga *et al*., 2008).

Southern Africa plants used in traditional oral care include *Carpobrotus edulis*; *Dodonaea viscosa* (gargles for oral infections); *Euclea natalensis*; *Securidaca longepedunculata*; *Artemisia afra*; *Dalbergia obovata* and *Warburgia salutaris* (toothache). *Siphonochilus aethiopicus*, *Polygala myrtiflia*, *Glycrrhiza glabra* and *W. salutaris* are used for symptoms of *C. albicans* infections (Rabe & van Staden, 1997; Van Vuuren, 2008; Van Wyk, 2008). Some of these plants have been reported to have antibacterial activity *in vitro*.

Antimicrobial activity of the methanol and aqueous extracts of *A. afra* and *D. obovata* against *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus subtilis*, *E. coli* and *Klebsiella pneumonia* were determined. Only the methanol extract of *A. afra* showed activity against *S. aureus* and *B. subtilis* (Rabe & van Staden, 1997). The aqueous extracts of *P. myrtiflia* and *G. glabra* showed activity against *C. albicans* (Van Vuuren, 2008).

1.3 Selected plants

Seven plants, based on their traditional use in South Africa for oral care, and/or antibacterial properties exhibited by the plants themselves or related species, were selected for this study. The detailed description of each plant is as follows:

1.3.1 *Barleria albostellata* C.B.Clarke

Barleria albostellata belongs to the Acanthaceae family and is more commonly known as Grey Barleria (Figure 1.2). In South Africa *B. albostellata* is distributed in the Limpopo province (Van Wyk & van Wyk, 1997).

1.3.1.1 Traditional use

No traditional uses of *B. albostellata* have been found during literature surveys

Figure 1.2: *Barleria albostellata* (commons.wikimedia.org/wiki/Image)

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1.3.1.2 Phytochemicals

Several phytochemicals have been isolated from the *Barleria* species. Iridoid glucosides and a shanzhiside methyl ester and its derivatives 6-*O*-acyl, 8-*O*-acetyl and 6, 8-*O*-diacetyl have been isolated from *Barleria lupulina* (Yoosook *et al*., 1999). Sankaranin, an antibiotic and immunostimulant protein was isolated from several *Barleria* species and *B. lupulina* (Yoosook *et al*., 1999).

1.3.1.3 Bioactivity

Recent studies found that ethanol, petroleum ether and dichloromethane extracts of *B. albostellata* exhibited antibacterial activity against *B. subtilis*, *S. aureus* and *K. pneumoniae* (Amoo *et al*., 2009).

The related species, *B. lupulina,* has shown to have anti-herpes simplex virus 2 (anti-HSV-2) activity; an anti-inflammatory effect associated by the inhibition of neutrophil responsiveness and was also reported to have strong inhibitory effects against acne-inducing bacteria *Propionibacterium acnes* (Chomnawang *et al*., 2005; Wanikiat *et al*., 2008; Yoosook *et al*., 1999).

1.3.2 *Cotyledon orbiculata* L. var. *orbiculata*

Cotyledon orbiculata from the family Cassulaceae is known as *Plakkie* in Afrikaans or Pig's Ear (Figure 1.3). It is distributed in the grassland, fynbos and karoo regions of southern Africa (Van Wyk & van Wyk, 1997).

1.3.2.1 Traditional use

Cotyledon orbiculata is traditionally used for tooth problems. The warmed leaf juice is applied as drops for toothache and hot poultices are applied on boils and inflamed areas (Amabeoku *et al*., 2007; Van Wyk & Gericke, 2000).

Figure 1.3: *Cotyledon orbiculata* (www.plantzafrica.com/plantcd/cotyledorbic.htm)

1.3.2.2 Phytochemicals

Phytochemicals such as cardiac glycosides, saponins, tannins, alkaloids, anthraquinones, flavonoids, reducing sugars and triterpene steroids have been isolated from the leaves of *C. orbiculata* (Amabeoku *et al*., 2007).

1.3.2.3 Bioactivity

No medicinal activity, other than the traditional use, of *C. orbiculata* has been found recorded in the literature.

1.3.3 *Dichrostachys cinerea* L. Wright & Arn. subsp. *africana* Brenan & Brummitt var. *africana*

The Sickle Bush, *Dichrostachys cinerea* from the family Fabaceae, occurs in Limpopo, North West Province, Gauteng, Free State and KwaZulu Natal (Figure 1.4) (Van Wyk & van Wyk, 1997).

1.3.3.1 Traditional use

The leaves of *D*. *cinerea* are traditionally used to treat toothache and oral candidiasis (Runyoro *et al*., 2006; van Wyk & Gericke, 2000).

1.3.3.2 Phytochemicals

Phytochemicals such as friedelan-3β-ol, α-amyrin were isolated from the benzene extract of the bark; while octacosanol was isolated from the benzene extract of the heartwood; hentriacontanol and β-amyrin were isolated from benzene extracts of the leaves. Sitosterol was isolated from the bark, heartwood and leaves (Joshi & Sharma, 1974). Eisa *et al*. (2000) reported the isolation of sterols and tannins from the plant.

Figure 1.4: *Dichrostachys cinerea* (commons.wikimedia.org/wiki/Image)

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1.3.3.3 Bioactivity

Chloroform, methanol and aqueous extracts of the fruits and leaves of *D. cinerea* exhibited antibacterial properties against *B. subtilis*, *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Pasturella pestis* and *Proteus vulgaris* (Eisa *et al*., 2000). The dried stem extract of *D. cinerea* has antifungal activity against *Aspergillus niger* and *C. albicans* (Runyoro *et al*. 2006)

Tannins extracted from the ethanolic extract of the powdered root of *D. cinerea* showed antibacterial activity against *S. aureus*, *Shigella boydii*, *Sh. flexneri*, *E. coli* and *P. aeruginosa* with MIC's ranging between 4.0-5.5 mg/ml (Banso and Adeyemo, 2007).

1.3.4 *Heteropyxis natalensis* Harv.

Heteropyxis natalensis, belonging to the family Heteropyxidaceae, is better known as the Lavender Tree (Figure 1.5). It is distributed in Limpopo, Gauteng and KwaZulu Natal (Van Wyk & van Wyk, 1997).

Figure 1.5: *Heteropyxis natalensis* (Plantzafrica.com)

1.3.4.1 Traditional use

Decoctions of the leaves and twigs of *H. natalensis* are traditionally used by Venda and Zulu communities as an oral rinse for toothache and for oral and gum infections (Van Wyk & Gericke, 2000). Traditionally the inhalation of the smoke, created by burning medicinal plants, is a practiced route of administration of drugs for the treatment and prophylaxis of airway diseases (Braithwaite *et al*., 2008).

1.3.4.2 Phytochemicals

The essential oil of *H. natalensis* contains antibacterial monoterpenoids. The major oil constituents of the essential oil are 1,8-cineole, limonene, β-myrcene, α-phellandrene and α-pinene (Van Vurren *et al*., 2007; Van Wyk & Gericke, 2000). Seasonal and geographical variation of *H. natalensis* essential oil was conducted. There was little seasonal variation as the essential oils extracted from samples grown in four different seasons had similar chemical profiles. However, one sample from Lagalametse (Northern Province) showed distinct geographical variation in samples collected from the Gauteng, Nelspriut and Waterberg regions. (*Z*)-3-hexenyl nonanoate and linalool were found to be the major constituents in the essential oil of the sample from Lagalametse (Gundidza *et al*., 1993; Van Vurren *et al*., 2007).

1.3.4.3 Bioactivity

The antibacterial activity of the essential oil, acetone and methanol extracts and the 'smoke-extract' of *H. natalensis* against four microorganisms, *S. aureus*, *Bacillus cereus*, *K. pneumoniae* and the fungus *Cryptococcus neoformans* were compared. The inhalation extract showed the most significant activity against *K. pneumoniae* as compared to the essential oil, methanol and acetone extracts (Braithwaite *et al*., 2008). Essential oil studies showed moderate antimicrobial activity, exhibiting MICs ranging between 3-16 mg/ml against *S. aureus*, *B. cereus*, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Moraxella catarrhalis* and *C. neoformans* (Van Vuuren *et al*., 2007). The essential oil was tested against 25 Gram-positive and Gram-negative bacteria, including food-poisoning and food spoilage bacteria. Four fungi known to cause food spoilage from the *Aspergillus* species were also included in the test. The essential oil proved to have significant activity against all the microorganisms (Gundidza *et al*., 1993). The essential oil also showed some activity against *C. albicans* with an MIC of 4.5 mg/ml (Van Vuuren & Viljoen, 2006).

1.3.5 *Carpobrotus edulis* (L.) L.Bolus

The sour fig, *Carpobrotus edulis*, belongs to the Mesembryanthemaceae family (Figure 1.6). It is distributed in the Western Cape (Van Wyk & van Wyk, 1997).

Figure 1.6: *Carpobrotus edulis* (commons.wikimedia.org/wiki/Image)

1.3.5.1 Traditional use

The leaves of *C. edulis* are traditionally used as an astringent. The leaf juice is an effective medicine for throat infections and oral thrush. The leaf juice also possesses antibacterial properties against *Moraxella catharralis* (Van der Watt & Pretorius, 2001; van Wyk & Gericke, 2000).

1.3.5.2 Phytochemicals and bioactivity

Isolated phytochemicals from the methanolic extract of the leaves of *C. edulis* are the flavonoids: rutin, neohesperidin, hyperoside, cactichin and ferulic; which were found to be active against *S. epidermis* and *S. aureus* and *P. aeruginosa* (Van der Watt & Pretorius, 2001). Ethanol, ethyl acetate and hexane extracts of *C. edulis* had activity against *C. albicans*, while an aqueous extract exhibited no antifungal activity (Motsei *et al*., 2003).

1.3.6 *Zanthoxylum capense* (Thunb.) Harv.

Zanthoxylum capense, Small Knobwood (Figure 1.7a,b), belongs to the Rutaceae family and is generally found throughout South Africa in Limpopo, North West Province, Gauteng, Free State, KwaZulu Natal, Western Cape and the Eastern Cape (Van Wyk & van Wyk, 1997).

a)

b)

Figure 1.7: *Zanthoxylum capense* (a) flowers; (b) fruit (Van Wyk and van Wyk, 1997)

1.3.6.1 Traditional use

The bark is removed from the ends of twigs, which is then beaten flat to make toothbrushes. The bark and powdered root are used to treat toothache, and infusions are used as an oral rinse (Van Wyk & Gericke, 2000). *Zanthoxylum capense* was a popular treatment in South Africa during the influenza epidemic of 1918, and was subsequently used against colds and flu (Steyn *et al*., 1998).

1.3.6.2 Phytochemicals

Isolated phytochemicals from the chloroform:methanol extract of the stems, twigs and leaves of *Z. capense* include pellitorine, xanthoxylum-γ, γ-dimethylallyl ether, β-sitosterol and sitosterol-β-D-glucoside. Pellitorine and sitosterol-β-D-glucoside have been reported to have biological activity (Steyn *et al*., 1998). The related species, *Zanthoxylum clava-herculis*, possesses benzophenanathridine and aporphine alkaloids, chelerythrine and magnoflorine (Gibbons *et al*., 2003).

1.3.6.3 Bioactivity

The related species, *Zanthoxylum chalybeum,* is traditionally used in the treatment of oesophageal candidiasis and showed activity against *B. subtilis*, *Penicillium crustosum* and *Saccharomyces cerevisiae* (Runyoro *et al*. 2006). *Zanthoxylum clava-herculis* showed activity against clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) (Gibbons *et al*., 2003).

1.3.7 *Dodonaea viscosa* Jacq. var. *angustifolia* (L.f.) Benth.

The Sand Olive, *Dodonaea viscosa*, from the family Sapindaceae (Figure 1.8), is distributed in Limpopo, North West Province, Gauteng, KwaZulu Natal, Western and the Eastern Cape (Van Wyk & van Wyk, 1997).

Figure 1.8: *Dodonaea viscosa* (www2.hawaii.edu)

1.3.7.1 Traditional use

The leaves of *Dodonaea viscosa* are traditionally used as a treatment for sore throats and oral thrush (Getie *et al*., 2003; Van Wyk, 2008).

1.3.7.2 Phytochemicals

Isolated phtochemicals from the methanolic extract of the leaves of *D. viscosa* include quercetin, kaemferol and isorhamnetin. Dodonic acid and hautriwaic acid have been isolated from *D. viscosa* including various other *Dodonaea* species. Isolated compounds from related *D. viscosa* species include pinocembrin, santin and penduletin and coumarins and steroids (Getie *et al*., 2003; Van Heerden *et al*., 2000).

1.3.7.3 Bioactivity

According to previous studies, the crude extract of the leaves of *D. viscosa* showed antibacterial activity against *Streptococcus pyogenes* and *S. aureus* and had strong antiviral activity against Coxsackie virus B3 and influenza A virus (Getie *et al*., 2003). Acetone, ethanol, ethyl acetate and hexane extracts of *D. viscosa* had activity against *C. albicans*, while an aqueous extract exhibited no activity against *C. albicans* (Motsei *et al*., 2003; Patel & Coogan, 2008). Acetone extracts of *D. viscosa* significantly inhibited the adherence of *C. albicans* to buccal oral epithelial cells at a sub-inhibitory concentrations; suggesting that *D. viscosa* has therapeutic properties at low concentrations (Patel *et al*., 2009).

1.4 Microbial targeting

Oral pathogenic bacteria attach or adhere to the tooth surface which leads to pathogenesis. In response to the infection of soft tissues, oral epithelial cells produce various pro-inflammatory cytokines such as interleukin-8 (IL-8). The adherence of bacteria and the infection of the gums, leading to the production of cytokines are discussed below:

1.4.1 Adherence

To cause an infection microorganisms first need to enter or attach to a cell or hard surface. Not all bacteria are capable of attaching to a surface, such as the tooth, which is shielded by saliva. *Streptococcus* and *Lactobacillus* species are some of the bacteria that have the ability to adhere to the tooth surface creating an environment in which other bacteria can then attach. As this attachment stage is critical to the bacteria it is worthwhile investigating whether plants can interfere with the attachment process and thereby reduce the risk of infection, activating inflammation in the gums and disease (Madigen *et al*., 2003; Taweechaisupapong *et al*., 2005).

1.4.2 Host response to infection

Once attachment of the microorganism occurs, infection in the gums induces the activation of the host's response system. In humans, the first line of defence is known as innate immunity. White blood cell leukocytes are a key component of innate immunity as they attack pathogens. In order to find the pathogens, the cells release a cytokine called 'interleukin-8'. The highest concentration of IL-8 is at the site of infection; leukocytes follow the concentration gradient of IL-8 to the point of infection. This process can be utilized in determining whether or not the plant helps prevent adherence of the bacteria as infection only occurs after attachment. Therefore monitoring the levels of IL-8 released by cells can be a means of determining the effectiveness of the plant to prevent microbial adherence (Samaranayake, 2002; Silverthorn, 2004; Uehara *et al*., 2008).

1.4.3 Synergism

The immune system is not infallible, and treatment for infections is often required. However, multi-drug-resistant pathogens are emerging due to the indiscriminate use of antibiotics. By combining active plant extracts and antibiotics, pathogens previously non-susceptible to the antibiotics are now susceptible. This concept is known as synergy. As oral pathogens are becoming increasing resistant to treatment this may be a means of countering resistant organisms (Hemaiswarya *et al*., 2008).

1.4.4 Isolation of active compounds

Plants utilize the concept of synergy to combat microbial infections by producing small molecule antibiotics, which work together to fight off infection. These compounds are often isolated and developed into chemical drugs for human consumption. If a plant exhibits high antimicrobial activity, it may be worth isolating the compounds that infer activity to the plant (Hemaiswarya *et al*., 2008).

1.5 Hypotheses

The selected plants will have antibacterial activity against the pathogenic microorganisms that occur in the oral cavity. The extract with the best bioactivity and least cytotoxicity should reduce cytokine levels. The isolated compounds' efficacy is better as compared to the crude extract.

1.6 Aim

The aim of this study was to:

- Collect the selected plant material and to determine the antimicrobial activity of the plant extracts

- investigate the cytotoxicity of the extracts using cell lines
- evaluate the anti-adherence capabilities using the cytokine, IL-8
- determine the ultrastucture of selected bacterial species on exposure to the extracts of plants
- evaluate synergism of the best plant extract with other extracts selected for their known fluoride content and anti-adherence
- isolation of active compounds from the best extract

1.7 Structure of the thesis

Chapter 1 A concise review of plants in oral care, plants used for toothache, gingivitis, halitosis, chewing sticks, chewing gum, oral rinses and the antimicrobial studies done using medicinal plants. A brief overview of plant-derived substances in oral care products. Selected plants, their traditional usage, phytochemicals and their bioassays. An introduction to microbial targeting by plants including microbial adherence, the host response to infection, synergism and isolation of active compounds.

Chapter 2 An introduction to periodontal diseases; the cause, pathogenesis of disease, plaque-mediated diseases, host response and treatments. Treatment failures, side effects of treatment and solutions to combat resistance to treatment. The conventional treatments used for the pathogenesis caused by the microorganisms selected for the study.

Chapter 3 The antimicrobial activity of the selected plant are determined against Gram-positive (*Actinomyces israelli* and *Streptococcus mutans*) and Gram-negative (*Prevotella intermedia*) organisms as well as an opportunistic pathogen (*Candida albicans*). The cytotoxicity of selected plants against Human laryngeal epidermoid carcinoma cells (HEp-2) and Kidney epithelial cells of the African Green Monkey (Vero) cell lines.

Chapter 4 The synergistic effect of the best plant extract, *Heteropyxis natalensis*, with essential oils, *Melaleuca alternifolia* and *Mentha piperita* and green tea against *A. israelli* and *C. albicans*.

Chapter 5 The cytokine evaluation of the anti-adherence effect of *H. natalensis* on *A. israelli* using HEp-2 cells. The ultra-structure determination of *S. mutans* on exposure the extract of *H. natalensis* using scanning electron microscopy.

Chapter 6 The isolation and identification of five compounds from *H. natalensis* using various isolation procedures such as column chromatography, thin layer chromatography and nuclear magnetic resonance. The cytotoxicity and antibacterial activities of the isolated compounds.

Chapter 7 The general discussion and conclusions from the study are provided to enable a complete understanding of the results of the study, with recommendations to future work.

Chapter 8 Acknowledgements

Chapter 9 Appendices

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Chapter 2

Periodontal diseases:

Cause, host response and treatments

2.1 Introduction

The oral cavity of humans is a habitat for Gram-positive and Gram-negative bacteria, as well as certain yeast and fungi, making it one of the most complex microbial habitats in the body. Although saliva contains lysozyme and lactoperoxidase, which are both antibacterial agents, the presence of food particles and shedded epithelial cells, makes the oral cavity a favourable microbial habitat (Madigan *et al*., 2003).

Living cells are classified as either prokaryotic, which are simple cells without internal membranes or organelles, such as bacteria; or eukaryotic which contain a nucleus and organelles such as mitochondria and internal membranes. Humans and fungi are examples of eukaryotes. Bacteria and fungi are however unicellular or simple multicellular organisms unlike the more complex, multicell plants and animals (Samaranayake, 2002).

2.2 Bacteria

Bacteria have a nucleoid with DNA, instead of a nucleus, which is surrounded by the cytoplasm in which proteins are synthesized and energy is generated. Bacterial reproduction is termed binary fission where a parent cell divides into two similar cells and can replicate extracellularly – outside the host's cells. As one bacterium can produce 16 bacteria over four generations, a logarithmic growth rate is established. The doubling or mean generation time of each bacterium varies, but the shorter the doubling time the faster the rate of multiplication. Factors that may also influence the doubling time are temperature and pH. The optimum temperature is required for the efficient activity of the bacteria's enzymes to take place. The bacteria and fungi used in the present study, namely *Streptococcus mutans*, *Prevotella intermedia*, *Actinomyces israelii* and *Candida albicans*; are mesophiles that grow best at a body temperature of 37°C. As these organisms grow best in a human host it would make sense that the hydrogen ion concentration of the environment should be the physiological pH of 7.2-7.4 (Samaranayake, 2002).

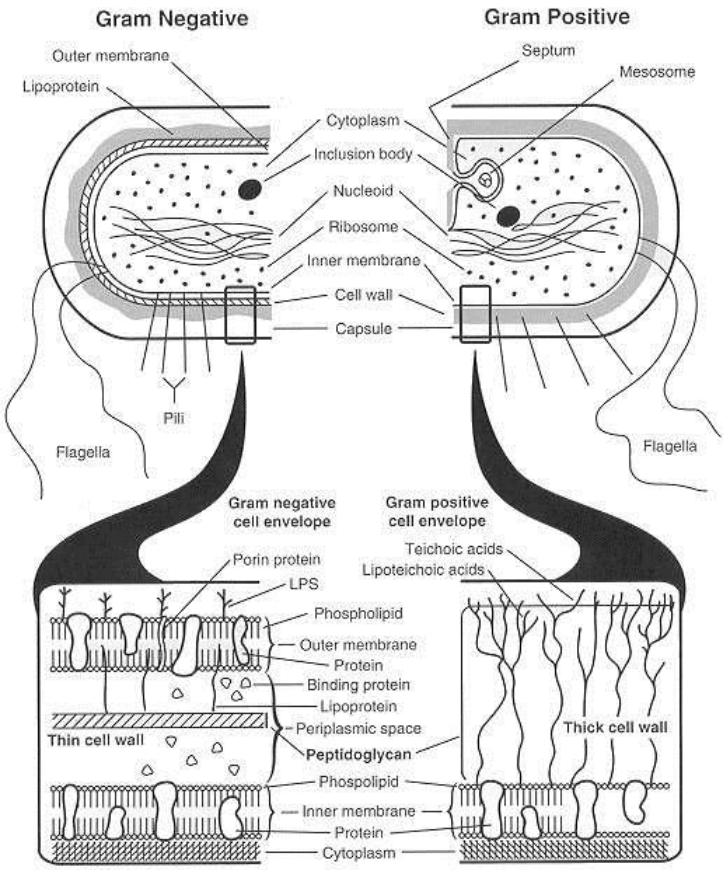

Figure 2.1: Cell membrane structure of Gram-positive and Gram-negative bacteria (ncbi.nlm.nih.gov)

Bacteria are divided into two classes with the use of the Gram stain: Gram-positive and Gram-negative, for example *S. mutans* and *P. intermedia* respectively. If the Gram stain is purple the bacteria are Gram-positive, while Gram-negative bacteria stain pink (Figure 2.2). The grouping depends on their staining characteristics which are due to their different cell wall composition (Figure 2.1). Both Gram-positive and Gram-negative bacteria's cell walls consist of peptidoglycan. Peptidoglycan is comprised of *N*-acytyl-muramic acid and *N*-acetyl-glucosamine cross linked by peptide side chains and cross-bridges; however peptidoglycan is thicker in Gram-positive bacteria. Gram-negative bacteria also possess a periplasmic space which lies between the outer membrane and the cytoplamic membrane. It is within this space that some Gram-negative bacteria produce the lactamase enzyme that can destroy drugs such as penicillin. Gram staining is not only useful as a diagnostic tool between Gram-positive and Gram-negative bacteria but can also be useful to determine the necessary therapy as Gram-positive bacteria are more susceptible to penicillin's due to their cell wall composition (Bauman, 2006; Samaranayake, 2002).

The cell wall determines the shape of the bacteria. There are three basic shapes: cocci (spherical), bacilli (rod-shaped) and spirochaetes (helical). However, some bacteria are pleomorphic and have the ability to vary their shape appearing as both coccal and bacillary.

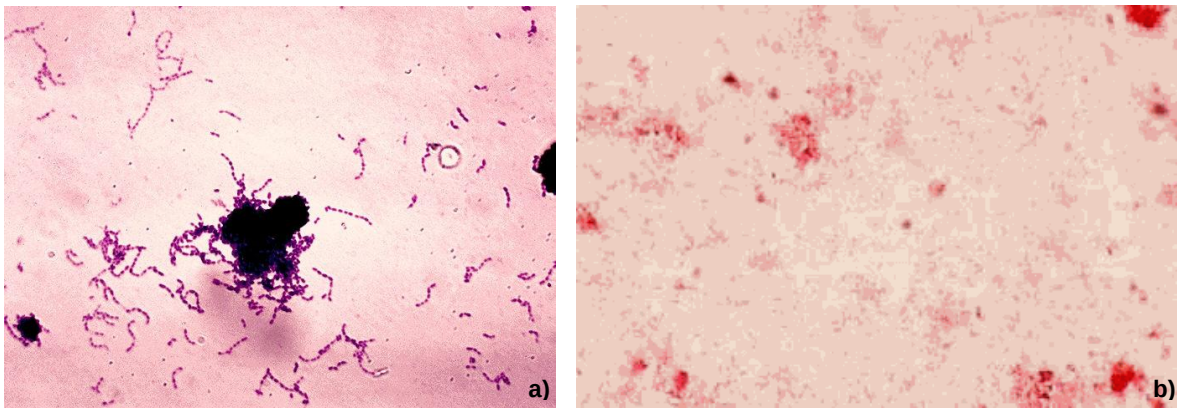

Figure 2.2: a) Gram stain of *Streptococcus mutans* (bioweb.uwlax.edu); b) Gram stain of *Prevotella intermedia* (lookfordiagnosis.com)

Bacterial arrangement usually depends on the plane of successive cell division and may therefore, be arranged in pairs, chains, grape-like clusters or as angled pairs (palisades). Bacteria sizes generally range from 0.2 µm to 5 µm (Samaranayake, 2002).

Bacteria are further classified depending on their oxygen requirements. In the final steps of energy production by bacteria, oxygen is the hydrogen acceptor. If a bacteria's ATP-generating system is reliant on oxygen as the hydrogen acceptor, they are known as strict or obligate aerobes. However if a bacteria is able to use oxygen for respiration and utilize a fermentation pathway to produce ATP then the bacteria is classified as a facultative anaerobe. Those bacteria that cannot grow in the presence of oxygen at all because they lack either one or both of the enzymes necessary to breakdown the end products hydrogen peroxide and the free radical superoxide, namely catalase and superoxide dismutase, are known as obligate anaerobes (Samaranayake, 2002).

2.3 The oral cavity

In humans, stratified squamous epithelium lines the oral cavity. The tongue is a modification of the squamous epithelium with structures such as teeth and salivary ducts disrupting the continuity. A cuff is formed around each tooth by gingival tissue forming a gingival crevice. A continuous flow of crevicular fluid is released from the gingival crevice. This flow increases during inflammation (Samaranayake, 2002). Saliva is composed of several functional components which aid in lubrication, enamel remineralisation, digestion, aggregation and provides oral buffering (Devine & Cosseau, 2008).

Oral commensal bacteria are important as they can regulate the expression of immune mediators, suppress cytokine responses in epithelial cells and prevent colonization by exogenous organisms. *Streptococcus mitis*, *S. oralis*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae*, *Eikenella corrodens* and certain *Prevotella* species

may be oral commensals as they are isolated from healthy but not diseased sites (Divine & Cosseau, 2008).

There are four natural major habitats in the oral cavity, namely the buccal mucosa, dorsum of the tongue, tooth surfaces and crevicular epithelium. Prosthodontic and orthodontic appliances make up a fifth habitat. The diversity of the oral micro flora is intimately linked with the habitats of the oral cavity. The dorsum of the tongue, with its papillary surface, is highly colonized as it provides refuge for the microorganism between the papillae. A low redox potential promotes the growth of anaerobic flora and serves as a potential reservoir for some Gram-negative anaerobes associated with periodontal disease. The only non-shedding surface in the body that harbours a microbial population is the surfaces of the teeth. Dental plaque is produced by the large masses of bacteria and their products accumulating on the surface of the teeth. There are two main habitats on the tooth's surfaces where plaque accumulate, supragingival and subgingival. Supragingival plaque is further divided into two locations, smooth surface plaque on the crown of the tooth above the gingival tissue and approximal surface plaque (the surface area between teeth). Subgingival plaque occurs below the surface of the gingival epithelium in the shallow crevice surrounding the teeth (Loesche, 1986; Samaranayake, 2002).

Protective calcium phosphate enamel surrounds the living tissue matrix of the tooth, dentin and pulp. Most bacteria found in the oral cavity are facultative anaerobes, which are specifically adapted to grow on the non-shedding surfaces of teeth and in anaerobic gingival crevices (where food particles accumulate). The gingival crevice is the area where the enamel protrudes from the gingiva (gum). Acidic glycoproteins found in saliva form a thin film on the tooth surface, which allows single bacterial cells to attach. There are only a few bacteria that can attach to the acidic glycoprotein film, namely *Streptococcus sanguis, S. sobrinus, S. mutans, S. mitis* and *Lactobacillus* species. Biofilms are formed due to extensive growth of these acidogenic bacteria and their by-products. Other bacteria such as filamentous *Fusobacterium* and anaerobic *Actinomyces* species are then able to attach to these areas, forming even larger biofilms (Madigan *et al*., 2003).

The facultative anaerobic nature of oral bacteria in a somewhat aerobic cavity is due to facultative bacteria, which grow aerobically, forming a dense biofilm and decreasing the amount of oxygen which reaches the tooth surface, thereby forming an anaerobic microenvironment (Madigan *et al*., 2003).

2.4 Pathogenesis of dental disease are as follows:

Several stages occur during plaque formation. As dental caries are generally associated with *S. mutans*, the process of biofilm formation of the bacteria on tooth surfaces will be used to clarify

the description (Figure 2.3). Firstly, sucrose and the glucosyltransferases (GTFs) enzymes are required for the accumulation of *S. mutans*. Saliva of the oral cavity produces a film on the tooth surface. This film contains glycoprotein constituents and forms a pellicle on the tooth surface. *Streptococcus mutans* interacts with the α-galactosides in the saliva-derived glycoprotein of the pellicle using an adhesion known as antigen I/II. The cell membrane of *S. mutans* also possesses glucanbinding protein (GBP), serotype carbohydrates and GTFs (Taubman & Nash, 2006).

Streptococcus mutans cleaves dietary sucrose into its component saccharides, glucose and fructose. Glucosyltransferases synthesize extracellular glucans with different solubilities in water, i.e. water soluble and water insoluble glucans; as GTFs possess a glucan-binding site they can also bind with the glucan products. Glucan binding protein is a receptor-like protein which specifically binds glucans. This allows for the accumulation of *S. mutans*. Co-aggregation or co-adhesion then takes place as new bacteria attach to those bacteria already attached to the tooth's surface. Bacteria from the same or different genus will co-aggregate. These steps lead to the formation of a biofilm. Over time bacteria from the biofilm will detach and become suspended in saliva where they can be transported to new colonization sites or swallowed (Samaranayake, 2002; Taubman & Nash, 2006).

Bacteria adherent to surfaces have a higher resistance to clearance by normal cleansing methods as well as to bacteriolytic enzymes and antibiotics. The adherent state is therefore advantageous to survival and a key step in pathogenesis. By preventing microbial adhesion, disease formation can be prevented as well (Ofek *et al*., 2003).

If plaque is allowed to grow undisturbed calculus may form. Saliva contains calcium and phosphate ions, which may become deposited within deeper layers of undisturbed dental plaque. Bacterial enzymes such as phosphatases and proteases degrade calcification inhibitors also contained within saliva which leads to the formation of insoluble calcium phosphate crystals which combines and forms a calcified mass of plaque, termed calculus. Supragingival plaque and calculus contains more Gram-positive organisms, such as *Streptococcus* and *Actinomyces* species, while subgingival contains more anaerobic Gram-negative species. Considerable amounts of metabolic by-products such as lactic acid accumulate in plaque. This is a result of the metabolism of sucrose, glucose and fructose by the bacteria. The lactic acid produced in plaque leads to caries formation (Loesche & Grossman, 2001; Samaranayake, 2002; Taubman & Nash, 2006).

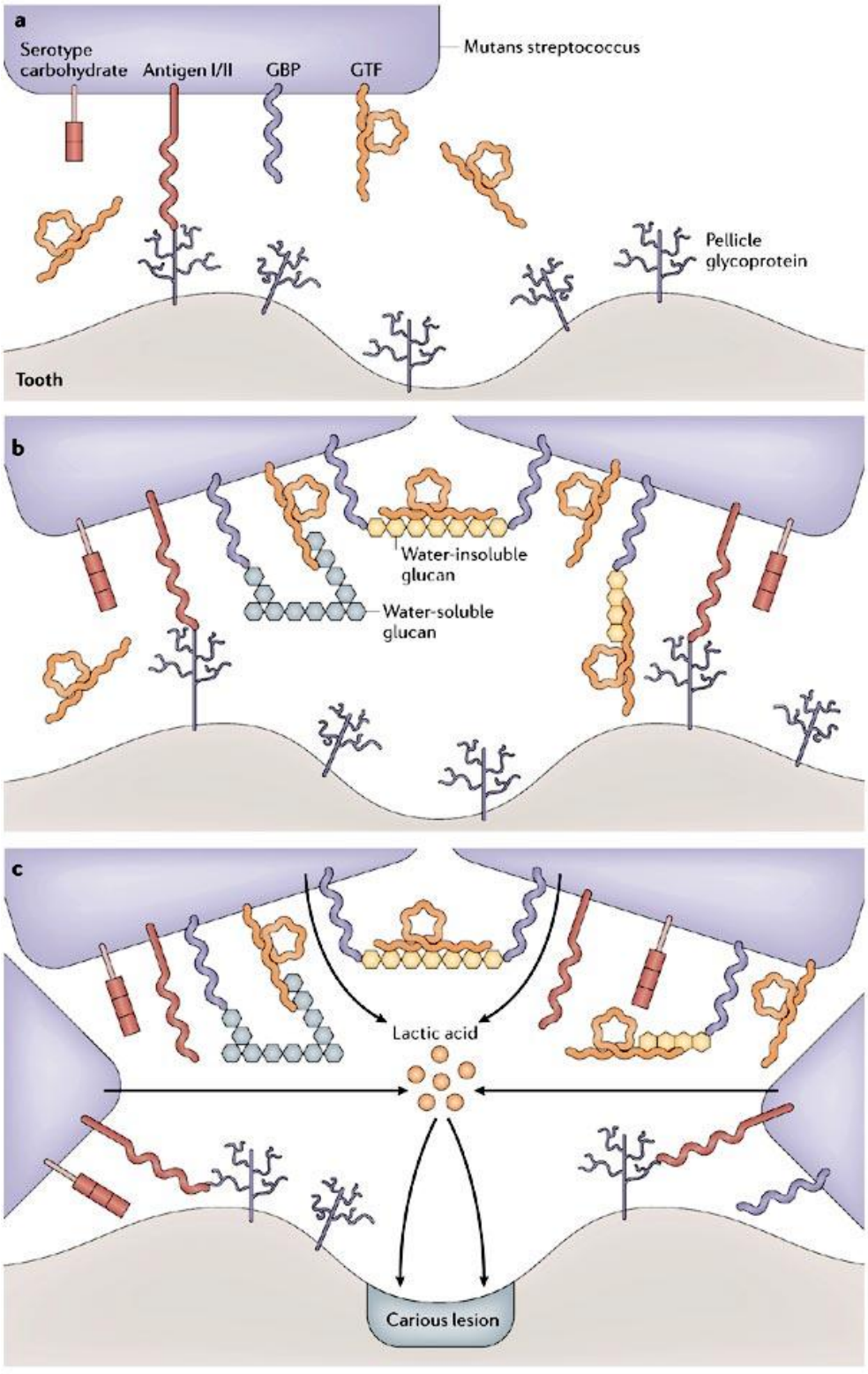

Figure 2.3: Formation of a caries lesion by *Streptococcus mutans*. a) Initial attachment of the bacterial involves the dental pellicle which is mediated by an adhesion of *S. mutans* known as antigen I/II. b) *S. mutans* glucosyltransferases (GTFs) synthesize glucans with different water solubilities. c) The synthesised glucans interact with the GTFs and glucan-binding proteins (GBP) of *S. mutans* allowing for aggregation and multiplication of the bacteria. Lactic acid is a metabolic by-product and induces carious lesions (Taubman & Nash, 2006).

'Caries' is defined as localized destruction of the tissues of the tooth by bacterial fermentation of dietary carbohydrates. First the enamel is demineralised and then the dentin by the acid by-products of microbial metabolism of carbohydrates. However, demineralization is followed by remineralisation. Cavities occur when the demineralization overtakes remineralisation. Streptococci such as *S. mutans* are acidogenic and aciduric (acid tolerant) and reduce plaque pH levels encouraging conditions for other plaque bacteria. Once the pH level falls below 5.5, enamel demineralisation occurs. Fluoride promotes remineralisation and may be one of the mechanisms in which it protects against tooth decay (Loesche & Grossman, 2001; Samaranayake, 2002).

2.5 Plaque-mediated diseases

Dental caries is an infectious disease caused by microorganisms when they produce acidic by-products in the increasing dental plaque. The acids produced cause decalcification of the enamel. Once the enamel has been breached, protein breakdown of the tooth matrix occurs, through proteolytic enzymes produced by the microorganisms. Two of the main bacterial species responsible for lactic acid production and dental caries are *Streptococcus* (*S. sobrinus*a and *S. mutans*) and *Lactobacillus* species (Madigan *et al*., 2003).

Three distinct processes are involved in how microorganisms cause human infections (pathogenesis). Firstly, adherence of the bacteria to the tooth surface; secondly, the bacteria produce a sticky glucan using the enzyme glucosyl transferase on sucrose, leading to the formation of a glycocalyx. A glycocalyx is a network of polysaccharides that project from a bacteria's cellular surface. It both protects the bacteria and allows the bacteria to attach to inert surfaces or other bacteria. Lastly the formation of biofilms allows for the production of lactic and acetic acid which enables the bacteria to metabolise carbohydrates at a low pH, which consequently also demineralises the enamel of teeth (Hamilton-Miller, 2001).
Sucrose, the favoured carbohydrate substance for oral bacteria, is made available either directly by food ingested or by the action of bacterial or salivary amylases on dietary starch. The trapping of carbohydrates in food particles remaining in the mouth for considerable periods is of particular relevance here. Sucrose is required for the last two processes involved in the formation of dental caries (Hamilton-Miller, 2001).

In the oral cavity, transition from a predominantly normal Gram-positive facultative microbia, associated with health, to plaque consisting of obligately anaerobic, proteolytic Gram-negative rods and spirochetes, will give rise to diseases of the soft tissues (Devine & Cosseau, 2008).

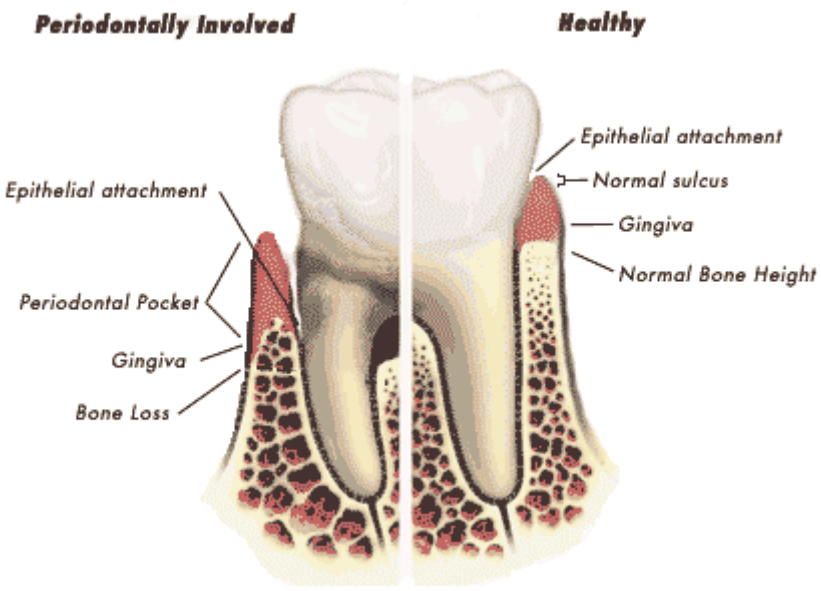

Figure 2.4: Periodontitis (www.kazmerperio.com/disease.html)

The total number of bacteria will increase from 10^2-10^3, normally found in healthy individuals, to 10^4-10^8 organisms during gingivitis and as many as 10^5-10^8 organisms during periodontitis (Dixon *et al*., 2004).

2.5.1 Periodontal diseases

Periodontal diseases are: 'a collective term ascribed to several pathological conditions characterized by degeneration and inflammation of gums, periodontal ligaments, alveolar bone and dental cementum' (Jain *et al*., 2008). Gingivitis is the inflammation of the periodontal ligament (areas of the periodontal membrane around the gingival crevice) forming the periodontal pocket. Clinical features include redness, swelling and bleeding of the gums. Periodontitis usually develops from untreated gingivitis and can involve loss of bone and tissue decay (Figure 2.4 & 2.5). The combined activities of microorganisms within the subgingival biofilms and the host responses to them, lead to the progression of the disease and tissue damage (Devine & Cosseau, 2008; Madigan *et al*., 2003; Samaranayake, 2002). Periodontopathogens include Gram-negative bacteria such as *Porphyromonas gingivalis*, *P. intermedia*, *Bacteroidis forsythus*, *Aggregatibacter actinomycetemcomitans*, *F. nucleatum* and *Capnocytophaga* species.

The pathogenesis of periodontal diseases may fluctuate from slow, chronic progressive destruction of collagen and aggressive tissue degeneration, to brief and acute with varying intensities and durations. Treatment of periodontal diseases includes biofilm control, root surface debridement or root scaling, surgery and the use of antimicrobial agents (Samaranayake, 2002).

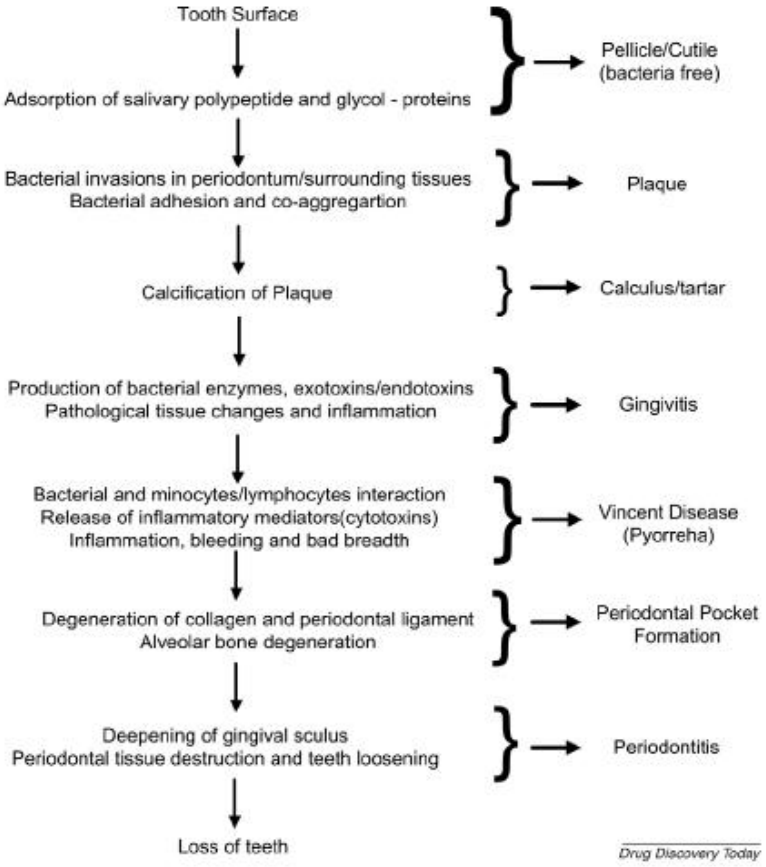

Figure 2.5: Flow chart representing the pathogenesis of periodontal diseases (Jain *et al*., 2008)

2.5.2 *Candida albicans*

Candidiasis has become a major public health concern as *Candida* species are opportunistic pathogens associated with immunocompromised individuals, especially in those affected with the acquired immunodeficiency syndrome (AIDS). Oral candidiasis is most commonly characterized by the development of oral thrush. Up to 90% of individuals with human immunodeficiency virus (HIV) suffer from at least one episode of candidiasis, making candidiasis the leading oral fungal infection in immunocompromised individuals (Runyoro *et al*., 2006). It has been determined that isolates of *C. albicans* are more virulent and genetically altered in HIV-positive patients than those strains encountered in HIV-negative patients (Patel & Coogan, 2008). *Candida albicans* also causes denture-associated stomatitis as it is capable of colonizing polymethyl methacrylate materials (Devine & Cosseau, 2008).

The cell wall of *Candida* is composed of polysaccharides, mannan, glucan and chitin. There are three virulence factors of *C. albicans*. The ability of the organism to adhere to oral epithelial cells; enzyme secretion such as proteinase and phospholipase that hydrolyse peptide bonds and phospholipids respectively, causing tissue invasion and damage; and a change in

phenotypic expression and morphology (Patel *et al*., 2009). The oral cavity has to cope with not only bacterial and yeast infections, but with viral infections as well.

Viruses such as the papilloma virus and herpes simplex virus (HSV) types 1 and 2, as well as other herpes viruses are commonly isolated from oral tissues. It has been suggested that herpes viruses may play a significant role in periodontal diseases as they disrupt host defence mechanisms and thereby facilitate bacterial infection (Devine & Cosseau, 2008).

The human body is capable of responding to and fighting off moderate infection through its adaptable defence system, the innate and adaptive immune response.

2.6 Host response

The body's non-specific immune response to invasion is known as innate immunity. The innate immune response is comprised of patrolling and stationary leukocytes that attack and destroy pathogens. The innate immune response is considered to be non-specific as the leukocytes respond in the same manner to any foreign matter. As the innate immune response does not target a particular pathogen, it begins within hours or minutes of exposure to a pathogen. Components of the innate immune system include phagocytes, natural killer cells, the alternative complement pathway and inflammation, which is the characteristic reaction of innate immunity (Samaranayake, 2002; Silverthorn, 2004). Innate defences include saliva, gingival crevicular fluid (GCF), epithelial cells and neutrophils (Devine & Cosseau, 2008).

A healthy gingival epithelium is characterized by the presence of human β-defensin-2, which is a protective antimicrobial peptide, and a gradient of interleukin 8 (IL-8), which is a chemokine that guides the transmigration of leukocytes. Chemokines are defined as a family of low molecular weight, structurally related cytokines that promote adhesion of cells to endothelium, chemotaxis and activation of leucocytes. The main producers of IL-8 are macrophages (Samaranayake, 2002).

In gingivitis, the gingival epithelium becomes inflamed, and the epithelium becomes infiltrated with leukocytes around the periodontal pocket. This process is accompanied by the elevated expression of IL-8. Interleukin-8 is part of the first line of defence as it increases phagocytosis, bacterial killing, and induces the release of lysosomal enzymes and generates superoxide anions (Uehara *et al*., 2008).

2.6.1 Systemic diseases

The immune response to oral infections may itself damage host tissues; for example, epitopes from certain streptococci stains are cross-reactive to the epitopes present in heart tissue. The antibodies required to combat the infecting bacteria can cause rheumatic heart diseases by binding to the heart tissue and inducing complement-mediated lysis and antibody-dependent cellular toxicity. Deposits of circulating immune complexes in the synovial can cause complementary-mediated joint pain. Other examples include subacute bacterial endocarditis, infected ventriculoarterial shunts, secondary syphilis and gonococcal and meningococcal septicaemia (Samaranayake, 2002). In a literature review to determine whether oral disease was a risk factor or causal agent of cardiovascular disease, the authors came to the conclusion that 'the cumulative evidence presented supports, but does not prove, a causal association between causal periodontal infection and atherosclerotic cardiovascular disease' (Beck & Offenbacher, 2005).

Local inflammatory reactions due to chronic periodontitis and bacterial by-products generally induce high levels of pro-inflammatory cytokines. This can lead to pathophysiological processes and result in preterm labour as the maternal inflammatory cells respond and cytokine cascades activate during periodontitis. Enough evidence for the association between periodontal disease and adverse pregnancy outcomes especially in disadvantaged populations are available; although the researchers admit that the limited studies on the association prevent a clear conclusion from being obtained (Xiong *et al*., 2006).

Investigations in recent years have also implicated oral bacteria as the causal agent of certain systemic diseases, such as pneumonia and cardiovascular disease (Takarada *et al.,* 2004).

The following conclusion can be drawn, that the release of IL-8 when bacteria cause disease may prove a useful tool in determining the anti-adhesion capabilities of plants and thereby their protective effect against microbial establishment.

2.7 Treatment

There are several characteristics of an ideal antibiotic. Activity against the microorganisms involved in the infection; good penetration and diffusion at the infection site; it must be well tolerated with few or no adverse effects and should allow for patient compliance. As most infections are not just due to one microorganism, the antibiotic should be active against both Gram-positive and Gram-negative microorganisms, and it is often necessary to use a combination of antibiotics to achieve a spectrum of activity (Bascones *et al*., 2004).

Antibiotics such as penicillin are narrow-spectrum antibiotics as they are generally active against Gram-positive microorganisms, with the exceptions of ampicillin and amoxicillin which are broad-spectrum and active against Gram-negative bacteria as well. Metronidazole is also a narrow-spectrum antibiotic as it only acts on obligate anaerobes. Tetracycline and ampicillin are broad spectrum antibiotics active against a wide range of Gram-positive and Gram-negative microorganisms. They were often utilized when the causative pathogen was unknown, which lead to the frequent use of the antibiotics resulting in the emergence of resistant pathogens that were once sensitive to the treatment. As yeast and fungi share similarities to human cells, selective toxicity is more difficult to achieve (Samaranayake, 2002).

The over usage and misuse of antibiotics has encouraged alternative methods of treatment, such as treatment delivery vehicles that can release antimicrobial agents directly into the periodontal pocket (Loesche & Grossman, 2001). Intra-pocket delivery systems place delivery vehicles in or around the periodontal pocket. The delivery vehicles such as fibres, strips, films and injectable gels, are composed of a variety and different combination of drugs such as tetracycline, chlorhexidine, metronidazole and amoxicillin. More advanced delivery systems include the microparticle, nanoparticle or vesicular system (Jain *et al*., 2009).

2.7.1 Drug interactions

Drug interactions should also be kept in mind when antibiotics are taken. Penicillin and erythromycin can reduce the effectiveness of oral contraceptives, while probenecid and neomycin may potentiate the effect of penicillin and reduce absorption. Erythromycin taken with theophylline will increase theophylline levels and may lead to potential toxicity. If cephalosporins are taken with gentamicin an additive effect occurs leading to nephrotoxicity. If cephalosporins are taken with frusemide a possibility of nephrotoxicity may occur. Any of the following: antacids, dairy products, oral iron and zinc sulphate lead to reduced absorption of tetracycline. Alcohol consumed with metronidazole results in 'antabuse' effect, while disulfiram, phenobarbitone and phenytoin reduces the effectiveness of metronidazole (Samaranayake, 2002).

2.7.2 Treatment failure

Drug resistance in microorganisms is becoming a major problem. Mechanisms of antibiotic resistance include inactivation of the drug, altered uptake and modification of the active site of the drug, acquisition of new genetic material via horizontal transfer or phentotypic variation. The most common mechanism is inactivation of the drug, such as the production of β-lactamase by staphylococci. This plasmid-coded enzyme breaks down the β-lactam ring, which is responsible for converting the antibacterial activity in penicillin’s into an inactive form. Microorganisms such as *Pseudomonas aeruginosa* can alter the uptake of the drug by either reducing the amount of

drug, such as tetracycline, in reaching the microorganism by altering the permeability of the cell wall or completely inhibiting the drug by pumping the drug out of the cell (Ofek *et al*., 2003; Samaranayake, 2002).

Current treatments for periodontis include the use of the antibiotics penicillin, amoxicillin, tetracycline and erythromycin against bacteria occurring in the oral cavity. Resistance to penicillin's (including amoxicillin) occurs in β-lactamase producing bacteria (*Prevotella*, *Porphyromonas* and *Fusobacterium* genera among others), which inactivate penicillin by acting on the β-lactam ring. If potassium clavulanate is incorporated with amoxicillin the combination is resistant to β-lactamase activity. Should the antibiotic be given by mouth it is required that the drug must be acid-stable to ensure stability on the stomach (Samaranayake, 2002).

As already mentioned there are current treatments available for candidiasis, however resistant strains of *Candida* are emerging which complicates the treatment process. Due to resistance it is not uncommon for a relapse infection to occur. New antifungal agents are required to both treat candidal infections and to curb the growing resistance of these organisms (Runyoro *et al*., 2006).

2.7.3 Treatment side effects

Administration of erythromycin in high doses causes nausea, and use for longer than 14 days may be hepatotoxic. Bacterial resistance to tetracycline's has increased, and due to the deposition of tetracycline within bone and developing teeth, before the eruption of secondary teeth normally occurring after the age of 13 years; it cannot be given to children under the age of 13 or in pregnant and lactating women. Diarrhoea and nausea may occur after oral administration of tetracycline due to the disturbance of intestinal flora. Excessive intravenous doses of tetracycline may be seriously hepatotoxic (Bascones *et al*., 2004; Samaranayake, 2002). Prolonged administration of ampicillin and amoxicillin frequently leads to nausea and, less so with amoxicillin, diarrhoea. There is also the risk that superinfection and colonization with ampicillin-resistant bacteria, coliforms and fungi may occur (Samaranayake, 2002).

For fungal infections, such as candidiasis caused by *C. albicans*, polyenes, azoles and DNA analogues are used. Polyenes such as nystatin and amphotericin have rare side effects but nystatin may induce nausea, vomiting and diarrhoea. The azoles include miconazole, fluconazole and intraconazole. Resistance occurs rarely with miconazole, but fluconazole may induce minor gastrointestinal irritation and an allergic rash. It also interacts with anticoagulants, terfenadine (taken for allergies), cisapride (used to prevent migraine attacks) and astermizole (an antihistamine). All azoles elevate liver enzymes. Resistance to azoles occurs in *Candida* species with prolonged treatment (Samaranayake, 2002). The DNA analogue, flucytosine may

induce anaemia, a decrease in leucocytes (leucopenia) and a decrease in platelets (thrombocytopenia) (Sommers, 2002; Stedman's, 2001).

Chlorhexidine gluconate (CHX) has broad spectrum antimicrobial activity and is generally more effective than either nystatin or amphotericin B in anti-*Candidal* activity (Pusateri *et al*., 2009). Most mouthwashes contain CHX due to its antibacterial properties. *In vitro* studies have shown that CHX also possesses antifungal activity and anti-adherence against *Candida* species by affecting its structural integrity which leads to fragmentation of the cell wall. However, although CHX reduces the incidence of oral candidiasis, clinical trials have shown that it does not eradicate oral yeasts. Chlorhexidine gluconate has been shown to be inactivated by food and saliva; it causes taste disturbances and mucosal irritation as well as staining of the teeth and tongue (Patel & Coogen, 2008; Patel *et al*., 2009; Pusateri *et al*., 2009).

2.8 Synergism

Antibiotics appeared to be the cure for most infections; however the indiscriminate use of antibiotics has led to emergence of multidrug-resistant pathogens. Novel therapeutics have always been found in plants. Plants produce small molecule antibiotics, which are generally weaker than those produced by bacteria and fungi; however plants are still able to fight infections successfully. Plants appear to use a concept known as synergy to combat infections. The synergistic interactions of secondary plant metabolites with antibiotics were examined in the treatment of infectious diseases. For example, butylated hydroxyanisole (BHA) green tea with the antibiotic vancomycin was found to be effective against *S. mutans*, non-susceptible *Escherichia coli* and *C. albicans* (Hemaiswarya *et al*., 2008).

Tea tree oil from *Melaleuca alternifolia*, peppermint essential oil (*Mentha piperita*) and the beneficial properties of green tea (*Camellia sinensis*) have already been widely studied. They have been reported to have good antimicrobial activity as individual remedies. The question arises if the combined activity of these established products can be any better? In the present study it was decided to investigate the synergistic activity of the following essential oils with the plant extract which showed the best activity against the microorganisms tested.

2.8.1 Essential oil – Tea tree oil

Melaleuca alternifolia, a tree indigenous to Australia, produces an essential oil that is more commonly known as tea tree oil (TTO) (Figure 2.6). The tree has been used medicinally for 220 years, but the oil was only commercially produced by steam distillation around 80 years ago. In the 1930's TTO was already recognized as having potential in oral hygiene (Carson *et al.*,

1993). Tea tree oil has approximately 100 components and has shown broad-spectrum antimicrobial and anti-inflammatory properties *in vitro* (Hammer *et al.*, 2003).

Figure 2.6: *Melaleuca alternifolia* (Tea tree oil) (dkimages.com)

The essential oil contains around 100 components, most of which are monoterpenes. Uses of TTO range from cuts and insect bites, to acne and tinea, a fungus infection of the keratin component of hair, skin, or nail (Hammer *et al*., 2003 and Stedman's, 2001). Infections caused by viruses, bacteria and fungi respond to clinical treatment with TTO. Scientific evidence has now indicated that a wide variety of oral bacteria are susceptible to TTO (Hammer *et al*., 2003). The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of *Actinomyces* species, *C. albicans*, *P. intermedia* and *S. mutans* has been determined (Table 2.1) (Hammer *et al*., 2003). The anti-adhesion capability of TTO has also been determined using *S. mutans* (Takarada *et al*., 2004). The safety of TTO has also been determined on cultured human umbilical vein endothelial cells and at a concentration of 0.2% they have little adverse effect on the viability of cells at this concentration (Takarada *et al*., 2004).

Melafresh T-96, is a toothpaste produced by the Australian company Southern Cross Botanicals, which incorporates TTO into the formula at a concentration of 0.2%. Even after loss of activity during manufacturing it still possess a high potency and a broad spectrum of antibacterial activity of Gram-positive and Gram-negative organisms (Bolel, 2009).

Table 2.2: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of *Melaleuca alternifolia* against selected microorganisms

Microorganism	MIC (%v/v)	MBC (%v/v)
Actinomyces species	0.1 – 1	0.1 – 2[b]
Candida albicans	0.5[a]	Na[c]
Prevotella intermedia	0.003 - 0.1	0.003 - 0.1[b]
Streptococcus mutans	0.25 – 2	0.25 – 2[b]

[a] Bagg *et al*., 2006; [b] Hammer *et al*., 2003; [c] Na: Not active

2.8.2 Peppermint essential oil

Mentha piperita L., a perennial herb, is a hybrid between spearmint (*M. spicata* L.) and water mint (*M. aquatica* L.) more commonly known as peppermint. It has been utilized in many foods, cosmetic and pharmaceutical products (Figure 2.7) (McKay & Blumberg, 2006).

Figure 2.7: *Mentha piperita* essential oil (www.myalchemilla.com)

Mentha piperita essential oil contains the terpenoid, methanol, as its main compound, menthone; isomenthone; 1,8-cineole; menthyl acetate; menthofuran; limonene; β-myrcene; β-carophyllene; pulegone and carvone (McKay & Blumberg, 2006).

Evidence that four *M. piperita* essential oils from various sources, and its components, menthol and methone are active against *Staphylococcus aureus*, *S. epidermis*, *Klebsiella pneumoniae*, *E. coli* and *C. albicans*, was found. The antibacterial activity of the essential oils and methanol was 0.63 mg/ml, 2.5 mg/ml, 1.25-2.5 mg/ml and 0.63-2.5 mg/ml for *S. aureus*, *K. pneumoniae*, *E. coli* and *S. epidermis* respectively. Menthone was generally less active than menthol; however against *S. mutans* menthol showed weak activity at 400 µg/ml. An MIC of 0.31-0.63 mg/ml was obtained for the essential oils and methanol against *C. albicans*. However, menthone exhibited an MIC of 2.5 mg/ml against *C. albicans*. Previous investigations of the oil composition were consistent with these results (İşcan *et al*., 2002).

In vitro activity of *M. piperita* essential oil against *C. albicans* with an MIC of 0.5% v/v was exhibited (Hammer *et al*., 1998). The free radical scavenging capacity of *M. piperita* essential oil has been noted to be stronger than either *M. aquatica* or *M. longifolia*, reducing the radical generator 2,2-diphenyl-1-picrylhydrazyl (DPPH) by 50% with a 50% inhibitory concentration (IC_{50}) value of 2.53 µg/ml. The antiviral activity of HSV-1 and HSV-2 have also been noted when the viruses were pre-treated with the oil prior to adsorption into the host cell, with IC_{50} values of 0.002% and 0.0008% for HSV-1 and HSV-2 respectively (McKay & Blumberg, 2006).

Mentha piperita essential oil is approved for internal as well as external use. Internal uses include treatment of spastic discomfort of the upper gastrointestinal tract and bile ducts and inflammation of the oral mucosa. It can be used externally for myalgia (muscular pain), neuralgia (pain associated with nerves) and as an antimicrobial and antiseptic. Although the oil, leaf extract and aqueous extracts are considered safe, the concentration of pulegone should be limited to 1% (İşcan *et al*., 2002; McKay & Blumberg, 2006).

2.8.3 Tea – *Camellia sinensis*

In Japan, green tea has been traditionally used to cleanse the mouth. It is also believed that those who consume large amounts of green tea have less tooth decay. Recent studies have shown that tea has potential anti-cariogenic properties (Hamilton-Miller, 2001).

Camellia sinensis (family Theaceae) (Figure 2.8) selectively absorbs fluoride from the soil and stores the fluoride in the leaves as an anion. Acidic soils are conducive to the increased uptake of fluoride in *C. sinensis*. Fluoride levels also increase within leaves and stems of the plant as the plant ages. Leaves store up to 98% of fluoride in plants (Cao *et al*., 2006; Malinowska *et al*., 2008).

Fluoride plays a vital role in bone and teeth mineralization and is therefore an essential element in our diet. It also has inhibitory or stimulatory effects on many soft tissue enzymes and plays a role in dental caries resistance. However, exposure to too much fluoride may result in skeleton and dental fluorosis. The recommended safe daily intake of fluoride for adults ranges from 1.5–4 mg, while for children and teenagers it ranges from 1.5–2.5 mg (Malinowska *et al*., 2008). A high level of fluoride exposure leads to tooth discolouration in children and severe osteoarthritis in adults (Cao *et al*., 2006).

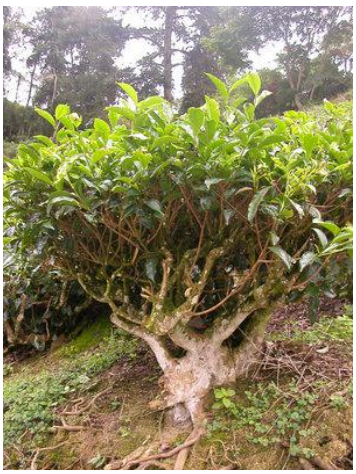

Figure 2.8: *Camellia sinensis* (philisha.net/pics/tea_plant)

There are many forms of tea available on the market these days, from green tea and black tea to herbal tea, instant teas and ready made to drink teas. Only green and black tea will be discussed here. Green tea is produced from the bud and young leaves of *C. sinensis*, while black tea undergoes a manufacturing process known as fermentation and possesses a naturally higher level of fluoride (Hamilton-Miller, 2001; Malinowska *et al*., 2008). The fluoride concentrations in green tea are lower than those in black. Green tea fluoride concentrations range from 0.59–1.83 mg/l after a five minute infusion. While for black tea the concentrations range from 0.32–2.76 mg/l (Malinowska *et al*., 2008).

Apart from the different fluoride content the main difference between green and black tea is that green tea contains five major catechins. Catechins are polyphenolic (flavan-3-ols) antioxidant plant metabolites which exert antimicrobial and antiviral activity against a variety of organisms such as *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* and *C. albicans* (Song *et al*., 2007). Black tea undergoes fermentation, in which many catechins are oxidised and theaflavins and thearubigins are produced. A few simple catechins, such as epicatechin (EC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) are still found in black tea, while catechin (C) and epigallocatechin (EGC) also occur in green tea. An infusion of green tea with hot water contains 0.5–1 g of catechins/l. Black tea prepared in the same manner only produces one-third of the catechins in green tea (Hamilton-Miller, 2001; Song & Seong, 2007).

Green tea catechins also possess powerful antioxidant properties; better than those of the antioxidant vitamin C. Epicatechin gallate is the most effective antioxidant, followed by EGCG and EGC. These catechins act as nitrogen scavengers and chelating agents to copper (Cu^{2+}) (Song & Seong, 2007).

Green tea interferes with the three processes involved in the pathogenesis of dental caries; namely adherence, glycocalyx formation and acid production. At 100 mg/l, which is less than a ‘cup of tea’ concentration, tea caused inhibition of adherence of *S. mutans* to saliva-coated hydroxyapatite (component of enamel and dentin). Tea catechins also inhibit *S. mutans* and *S. sobrinus* glucosyl transferase activity, with EGCG showing the greatest inhibition. Green tea halts the production of lactic acid by inhibiting bacterial lactate dehydrogenase. Green tea has also been found to inhibit salivary amylase, while black tea inhibits both salivary amylase and the bacterial amylase of *S. mutans*, which may be due to theaflavins found in black tea (Hamilton-Miller, 2001; Song & Seong, 2007).

The antibacterial activity of tea is somewhat decreased due to catechins affinity for proteins. This property is known as astringency and contributes to the sensation, known as ‘mouthfeel’,

experienced when drinking tea. Scientists theorise that the inhibition of amylases and glucosyl transferase and the adherence of *S. mutans* might be due to the interactions of catechins such as EGCG and related compounds with these proteins, ultimately resulting in a loss of function. However no experimental evidence has as yet been produced (Hamilton-Miller, 2001).

Pharmacokinetic studies have revealed that after rinsing the mouth with tea that catechins are found in the saliva for up to 60 minutes; while the rinsing with a tea catechin 0.25% solution had an anti-plaque effect for up to 90 minutes. The enzymatic breakdown of starch in food particles trapped in the mouth was also remarkably reduced (Hamilton-Miller, 2001).

Due to the lower fluoride content and high catechin activity in green tea, it is conclusive to incorporate green tea into a mouthwash or toothpaste, when compared to black tea. It may be worth considering combining a strong antibacterial sample with green tea in toothpastes or mouthwashes, thereby creating a product that contains antibacterial properties as well as being capable of inhibiting bacterial adherence and providing a source of natural fluoride.

The use of antimicrobial agents may prevent the formation of biofilms and the development of periodontal diseases. The antimicrobial agents of plants therefore, need to be capable of combating periodontal microorganisms and thereby preventing periodontal diseases.

2.9 Selected microorganisms

The following three bacteria and yeast which are most commonly found in the diseased oral cavity were selected for the present study.

2.9.1 Actinomyces israelii

2.9.1.1 Properties

Actinomyces israelii is Gram-positive, anaerobic bacteria, with filamentous branching rods (bacilli) that occurs in the normal oral flora but which can cause oral-cervicofacial infections and is a major agent of actinomycosis. Infections are chronic, granulomatous and endogenous. The lesions present themselves as chronic abscess usually after trauma to the mouth such as a blow to the jaw or tooth extraction (Pinilla *et al*., 2006; Samaranayake, 2002).

Actinomyces species are mainly situated at approximal sites of teeth; are a major component of dental plaque and their numbers are known to increase during gingivitis. An association is found between root surface caries and the presence of *Actinomyces* species (Samaranayake, 2002).

2.9.1.2 Treatment

Penicillin and amoxicillin are used to treat infections, but for chronic infections penicillin is required for a period of 6 weeks. Tetracycline is also utilized for its good bone penetration. Surgical intervention may be required in chronic jaw lesions. The endogenous nature of the infection makes prevention difficult (Samaranayake, 2002).

2.9.2 Prevotella intermedia

2.9.2.1 Properties

Anaerobic, Gram-negative, rod shaped bacterium. It is a periodontopathogen contributing to human gingivitis, periodontitis, periimplantitis and endodontitis. Periodonto pathogens release proteases as virulence factors that cause deterrence of host defences and tissue destruction. *Prevotella* produce acetic and succinic acid from glucose (Bascones *et al*., 2004; Mallorqui-Fernández *et al*., 2008; Takahashi *et al*., 2006).

2.9.2.2 Treatment

Treatments for the *P. intermedia* include the use of erythromycin, oleandromycin, spiramycin, clindamycin, spiramycin, metronidazole and tetracycline. Treatments with penicillins may induce resistance as it is a β-lactamase producing bacteria (Andrés *et al*., 1998; Samaranayake, 2002).

2.9.3 Streptococcus mutans

2.9.3.1 Properties

Streptococcus mutans is Gram-positive cocci that occur in chains. *Streptococcus mutans* is one of the major agents of dental caries and is able to produce large amounts of extracellular polysaccharides from dietary carbohydrates enabling binding to enamel and each other (Samaranayake, 2002).

Roughly one-quarter of the total cultivable flora form supragingival and gingival plaque and half the isolates from the tongue and saliva are streptococci. Initial exposures to streptococci are vertical transmission from mother to child. During intraoral surgical procedures such as tooth extraction (and at times during tooth brushing) these streptococci can enter the bloodstream and cause infective endocarditis – 60% of cases are due to this organism (Samaranayake, 2002).

2.9.3.2 Treatment

Antibiotic treatments for *S. mutans* include penicillin, ampicillin, cephalothin, methicillin and erythromycin (Ferretti & Ward, 1976; Samaranayake, 2002).

2.9.4 Candida albicans

2.9.4.1 Properties

Candida albicans is an aerobic, eukaryotic yeast with spherical to oval blastospores and pseudohyphae and stains positively with the Gram stain method. *Candida* is an oral commensal and is prevalent in about half the general population as an opportunistic pathogen that can cause infections in healthy patients when the immune system is attacked by other infections. *Candida albicans* can also cause infections in immunocompromised patients (Samaranayake, 2002).

Virulent attributes of *Candida* include the ability to adhere to host tissues and prostheses, to modify their surface antigens, form hyphae (which aids in tissue invasion) and their ability to break down physical defence barriers of the host with the production of extracellular phospholipase and proteinases. *Candida* infections are usually endogenous in origin and can cause either superficial infections of the skin and/or mucosa or systemic candidiasis (Samaranayake, 2002).

2.9.4.2 Treatment

Three groups of agents can treat *Candida* infections. Polyenes such as nystatin and amphotericin, azoles such as imidazole and DNA analogues such as flucytosine. Superficial infections can be topically treated with polyenes or azoles with polyenes effective in treating oral infections as well. Systemic infections require intravenous amphotericin with or without fluconazole. Chlorhexidine gluconate and amphotericin are also commonly used (Samaranayake, 2002).

New treatments are required to counter the increasing incidences of resistance and sometimes harsh side effects of treatments, while still combating the microorganisms that cause caries and periodontal diseases. Plants and essential oils, such as tea tree oil, are becoming renowned as alternative and sometimes better cures than established drugs. Resistance also occurs more slowly with natural products. It is estimated that a quarter of all prescriptions in industrialised countries contain one or more components derived from plants (Runyoro *et al*., 2006). The challenge is to find plants capable of preventing or minimising the effects of dental and other oral diseases. These plants should therefore, contain antimicrobial properties, without being cytotoxic to human epithelial cells.

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Chapter 3

Antimicrobial activity

3.1 Introduction

Aromatic and saturated organic compounds in plants generally possess antimicrobial activity and are initially extracted from plants with ethanol or methanol (Cowan, 1999). The antimicrobial activities of a drug/extract/compound against an organism are usually tested using a disc diffusion test and/or micro titre dilution tests. The disc diffusion tests are a qualitative measure of antimicrobial activity while the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests are quantitative measures (Samaranayake, 2002).

Seven plants, used in South African traditional medicine for oral care, namely *Barleria albostellata, Cotyledon orbiculata, Dichrostachys cinerea, Heteropyxis natalensis, Carpobrotus edulis, Zanthoxylum capense* and *Dodonaea viscosa* were screened in the present study against oral pathogens involved in caries formation, gingivitis, periodontitis and candidiasis. To ensure safety, the cytotoxicity of the plant extracts with the highest inhibitory effect was determined against kidney epithelial cells of the African Green Monkey (Vero) and human laryngeal epidermoid carcinoma cells (HEp-2). It is considered necessary to use more than one cell line in the detection of cytotoxic compounds, as each cell line may exhibit different sensitivities to the cytotoxic compound (Kamuhabwa *et al*., 2000).

3.2 Materials and methods

3.2.1 Plant material

Aerial plant parts, comprising of leaves and twigs of seven plants, were collected. The plants were collected from the University of Pretoria's Botanical Garden during January. Voucher specimens were prepared and identified at the H.G.W.J. Schwelcherdt Herbarium (PRU), University of Pretoria (Table 3.1).

Table 3.1: Plant parts used for the present study:

Plants	Plant family	Plant part	Voucher specimen
Barleria albostellata	Acanthaceae	Leaves and twigs	PRU 096399
Cotyledon orbiculata	Cassulaceae	Leaves	PRU 096402
Dichrostachys cinerea	Fabaceae	Leaves and twigs	PRU 096403

Heteropyxis natalensis	Heteropyxidaceae	Leaves and twigs	PRU 096405
Carpobrotus edulis	Mesembryanthemaceae	Leaves	PRU 096398
Zanthoxylum capense	Rutaceae	Leaves and twigs	PRU 096406
Dodonaea viscosa	Sapindaceae	Leaves and twigs	PRU 096404

3.2.2 Preparation of extracts

The plant material was air dried at room temperature (25ºC), and ground to a fine powder using a Janke & Kunkel (IKA Labortechnik, Germany) grinder. The powdered material was extracted by shaking with 400 ml ethanol (Merck Chemicals (Pty) Ltd Wadeville, South Africa) (Labcon, Lab Design Engineering, Maraisburg, South Africa). The samples were filtered through a Whattman No.1 (110 mm diameter) (Merck Chemicals (Pty) Ltd Wadeville, South Africa) filter paper using a vacuum filter (Merck Chemicals (Pty) Ltd Wadeville, South Africa). The process was repeated several times. The solvent was evaporated in a BÜCHI Rotavapor (Labotec (PTY) Ltd. Halfway House, South Africa) under reduced pressure of 40°C. The extracts were further dried at room temperature after which they were subjected to antimicrobial tests.

3.2.3 Antibacterial and antifungal screening

3.2.3.1 Microbial strains

The microorganisms used in this study included *Actinomyces israelii* (ATCC 10049), *Prevotella intermedia* (ATCC 25611), *Streptococcus mutans* (ATCC 25175), *Candida albicans* (ATCC 10231) and a strain of *Candida albicans* resistant to polyenes and azoles (1051604). The bacteria were grown on Casein-peptone Soymeal-peptone Agar medium (CASO) (Merck Chemicals (Pty) Ltd Wadeville, South Africa) under anaerobic conditions in an anaerobic jar with Anaerocult® A (Merck KGaA Darmstadt, Germany), at 37 ºC for 72 hours. *Actinomyces israelii* and *S. mutans* had CASO agar enriched with 1% sucrose (Merck Chemicals (Pty) Ltd Wadeville, South Africa). The drug susceptible and drug resistant strains of *C. albicans* were grown on Sabouraud Dextrose 4% Agar (SDA) (Merck Chemicals (Pty) Ltd Wadeville, South Africa), at 37 ºC for 72 hours. Sub-culturing was done every second week. Inocula were prepared by suspending bacterial test organisms in quarter strength sterile Ringer solution (Merck KGaA Darmstadt, Germany) until turbidity was compatible with McFarland Standard 1 (Merck Chemicals (Pty) Ltd Wadeville, South Africa). Yeast test organisms were suspended in sterile distilled water until turbidity was compatible with McFarland Standard 1 (McFarland, 1907).

3.2.3.2 Gram stain for the determination of the identity of bacteria

Gram staining was used to determine pure cultures and the shape and cell-wall properties of the microorganisms. The microorganisms were fixed to a glass slide by heating. Crystal violet (Merck Chemicals (Pty) Ltd Wadeville, South Africa) was used as the initial stain. Iodine resublimed (Merck Chemicals (Pty) Ltd Wadeville, South Africa) solution was used as a mordant. A mixture of acetone and ethyl alcohol was used as a decolourant. Safranine O (Merck Chemicals (Pty) Ltd Wadeville, South Africa) was used as a counter-stain (Gerhardt *et al*., 1981).

The cell wall structure determines the appearance of the microorganism stained by the Gram method. As Gram-positive bacteria possess a thick peptidoglycan layer, which retains the crystal violet dye during decolourization, the organisms appear purple. Gram-negative bacteria possess a thinner peptidoglycan layer in their cell walls and therefore do not retain the crystal violet. They therefore showed the pink colouring of the counter-stain. Due to their cell wall composition yeast and fungi stained purple as well (Gerhardt *et al*., 1981).

3.2.3.3 Preparation of McFarland 1 standards

Before inoculation, the microorganisms were standardised to an absorbency equal to McFarland Standard 1 (Barium Sulfate turbidity). For these purposes cell suspensions were prepared in sterile quarter-strength Ringers solution (Merck KGaA Darmstadt, Germany) and calibrated by spectrophotometry at an absorbance of 540 nm (McFarland, 1907).

3.2.3.4 Cell counts

To confirm the cell concentration which was inoculated, the cell suspensions of each culture, which matched the absorbency of the standard, was plated by the spread plate technique (Gerhardt *et al*., 1981) after a dilution series was prepared from 100 µl of inoculum equal to McFarland Standard 1.

McFarland standards were plated and incubated in the same way, bearing in mind their dilution of 1% when used as inoculums for use as a comparison. Ten-fold dilutions were made in sterile quarter-strength Ringers solution. One hundred µl of 10^{-3} to 10^{-6} of these dilutions were plated in duplicate onto agar plates. The yeast plates were incubated at 37˚C for 5 days. The bacteria were incubated at 37˚C for 5 days in anaerobic conditions stimulated by the use of Anaerocult® A (Merck KGaA Darmstadt, Germany). The colony forming units (CFU) were determined by counting the colony growth after 5 days (Table 3.2).

Table 3.2: Colony forming units (CFU/ml) of each microorganism used during bioassays

Microorganisms	Colony forming unit (CFU)/ml
Actinomyces israelii	8×10^7
Prevotella intermedia	40×10^7
Streptococcus mutans	30×10^7
Candida albicans	4×10^7
Polyene and azole resistant *C. albicans*	4×10^7

3.2.3.5 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The microdilution technique using 96-well micro-plates, as described by Eloff (1998) was used to obtain the MIC and MBC values of the crude extracts against the microorganisms under study. The extracts, dissolved in 10% dimethyl sulphoxide (DMSO) (Merck Chemicals (Pty) Ltd), were serially diluted in broth (enriched for *A. israelii* and *S. mutans*) for the bacteria and sterile water for the *Candida* species; in the 96-well plate adding 48 hour old microorganisms grown at 37˚C. The final concentration of extracts ranged from 12.5–0.10 mg/ml and the positive control, 5% chlorhexidine gluconate (CHX) (Dental Warehouse, Sandton, South Africa), ranged from 12.5–3.8 x 10^{-4} mg/ml. Amphotericin B (Davis Diagnostics, Gauteng) an established antifungal drug, ranging from 0.2 mg/ml to 1.5 x 10^{-3} mg/ml, was included for the *Candida* assays. The highest concentration of the solvent DMSO (2.5%) was found to be non-toxic to the microorganisms tested. *Actinomyces israelii* and *S. mutans* were incubated at 37˚C, under anaerobic conditions, for 24 hours; *P. intermedia* was incubated at 37˚C, under anaerobic conditions, for 48 hours; and *C. albicans* was incubated at 37˚C with *p*-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, South Africa) already added, under moist aerobic conditions, for 24 hours.

To indicate bacterial growth, 50 µl of (0.2 mg/ml) INT, was added to micro-plate wells and incubated at 37˚C, under anaerobic conditions, for 20–60 minutes until a red colour developed. The MIC was defined as the lowest concentration that inhibited the colour change of INT. The MBC was determined by adding 50 µl of the suspensions from the wells, which did not show any growth after incubation during MIC assays, to 150 µl of fresh broth. These suspensions were reincubated at 37˚C for 28 hours (48 hours for *P. intermedia*), under anaerobic conditions. The MBC was determined as the lowest concentration of extract which inhibited 100% growth of microorganisms (Cohen *et al*., 1998).

3.3 Determination of cytotoxicity

Microtitre plates with Vero and HEp-2 cells (Highveld Biological, Gauteng) were used for testing the four best ethanol extracts for cytotoxicity following the method of Basson (2005). Cytotoxicity was measured by the XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolim]-bis-[4 methoxy-6-nitro] benzene sulfonic acid hydrate) method using the cell proliferation kit II (Roche Diagnostics GmbH). A hundred microliters of Vero and HEp-2 cells (1 x 10^5 ml) were seeded onto micro-plates and incubated for 24 hrs to allow the cells to attach to the bottom of the plate. Dilution series were made of the extracts and the various concentrations (400–3.1 µg/ml) were added to the micro-plate and incubated for 72 hours. The XTT reagents were added to a final concentration of 0.3 mg/ml and the cells were incubated for one to two hours. The positive drug controls Doxorubicin HCl (Sigma-Aldrich, South Africa) and Actinomycin D (Sigma-Aldrich, South Africa), at concentration ranges of 0.78–0.01 µg/ml, were included in the assay. After incubation the absorbance of the colour was spectrophotometrically quantified using an enzyme-linked immunosorbent assay (ELISA) plate reader (BIO-TEK Power-Wave XS, Weltevreden Park, South Africa), which measured the optical density at 490 nm with a reference wavelength of 690 nm. The assay was carried out in triplicate.

3.4 Results and discussion

The MICs, MBCs and 50% inhibition concentration (IC_{50}) of the cytotoxicity assays of each plant extract against the microorganisms are tabulated in Table 3.3.

3.4.1 Antibacterial screening

Van Vuuren (2008) states that 'extracts having activities where MIC values are below 8 mg/ml are considered to possess some antimicrobial activity and natural products with MIC values below 1 mg/ml are considered noteworthy.'

Only the extract of *H. natalensis* showed positive inhibitory activity against all three the bacteria tested in this study. No other plant tested showed activity against the Gram-negative bacterium *P. intermedia*. *Dichrostachys cinerea* showed moderate activity against the Gram-positive organism, *S. mutans*, exhibiting an MIC of 6.64 mg/ml. The MBC exhibited by the ethanol extract of *H. natalensis* was found to be 3.13 mg/ml and 3.32 mg/ml against *S. mutans* and *A. israelii* respectively (Figure 3.1).

Table 3.3: The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and 50% inhibitory concentration (IC_{50}) of the ethanol extracts of selected plants on oral microorganisms

Plant extract	Microorganism tested											
	MIC (mg/ml)					MBC (mg/ml)					Cytotoxicity IC_{50} (µg/ml) + Standard deviation	
	Gram +ve		Gram -ve	Yeast		Gram +ve		Gram -ve	Yeast		Vero	HEp-2
	A.i	S.m	P.i	C.a	C.a (res)	A.i	S.m	P.i	C.a	C.a (res)		
Heteropyxis natalensis	0.88	1.82	3.13	10.94	12.5	3.32	3.13	>12.5	>12.5	>12.5	147 ± 0.150	33.66 ± 0.04
Dichrostachys cinerea	4.84	6.64	>12.5	10.71	10.42	6.25	12.5	Na[a]	>12.5	>12.5	204 ± 0.125	224.1 ± 0.1
Dodonaea viscosa	4.69	10.16	>12.5	11.25	>12.5	6.25	>12.5	Na	>12.5	Na	137 ± 0.067	47.96 ± 0.11
Barleria albostellata	4.1	12.5	>12.5	>12.5	>12.5	6.77	>12.5	Na	Na	Na	114.1 ± 0.163	160.8 ± 0.6
Carpobrotus edulis	11.46	6.25	>12.5	>12.5	12.5	12.5	12.5	Na	Na	>12.5		
Cotyledon orbiculata	5.21	12.5	>12.5	>12.5	>12.5	12.5	>12.5	Na	Na	Na		
Zanthoxylum capense	9.38	12.5	>12.5	>12.5	>12.5	>12.5	>12.5	Na	Na	Na		
Positive controls	0.033[b]	0.061[b]	0.26[b]	0.352[b]	0.26[b]	0.039[b]	0.415[b]	1.042[b]	0.293[b]	0.293[b]		
				>0.2[c]	0.013[c]					0.013[c]	0.06 ± 2.44[d]	$8.5x10^{-3}$ ± 9.[95x10-5(e)]

[a] Na: not active; [b] Chlorhexidine; [c] Amphotericin B; [d] Doxorubicin; [e]Actinomycin D; A.i: *Actinomyces israelii*; S.m: *Streptococcus mutans*; P.i: *Prevotella intermedia*; C.a: *Candida albicans*; C.a (res): Polyene and azole resistant *C. albicans*

Plants such as *C. edulis*, *D. cinerea* and *D. viscosa* have been investigated for their antibacterial activity. According to van der Watt and Pretorius (2001), ethanolic and methanolic crude extracts of *C. edulis* showed significant activity against the Gram-negative *Moraxella catharralis* and Gram-positive *Staphylococcus epidermidis* and *S. aureus* using the agar plate diffusion method. Results obtained in this study showed that an ethanol extract of *C. edulis* had weak antibacterial activity against *A. israelii* and *P. intermedia* but the extract exhibited an MIC of 6.25 mg/ml against *S. mutans*. Unlike the investigation by van der Watt and Pretorius (2001), *C. edulis* did not show any activity against the Gram-negative *P. intermedia*. This may be due to the high pathogenicity of *P. intermedia*. In a study conducted by Eisa *et al*. (2000), chloroform, methanol and aqueous extracts of *D. cinerea* showed activity against several bacteria such as *Bacillus subtilis*, *S. aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The MICs obtained ranged from 0.27 to over 90 mg/ml. Our results for *D. cinerea* correlated with these findings as the MICs ranged from 4.84 to over 12.5 mg/ml against *S. mutans*, *A. israelii* and *P. intermedia*.

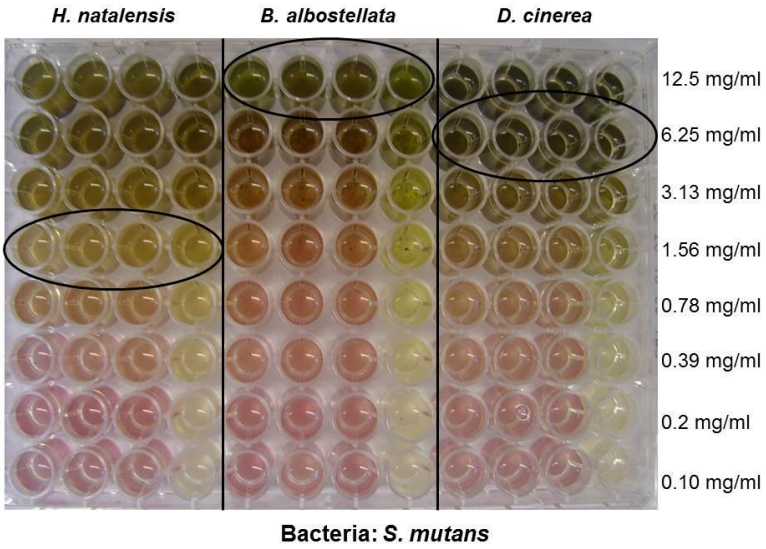


Figure 3.1: Microtitre plate showing the MICs of *Heteropyxis natalensis*, *Barleria albostellata* and *Dichrostachys cinerea* against *Streptococcus mutans*

Similar to the studies carried out by Getie *et al*. (2003), where it was found that the methanol extract of the leaves of *D. viscosa* exhibited no activity against the Gram-negative bacteria *P. aeruginosa* and *E. coli*, using the agar plate diffusion method, *D. viscosa* did not show inhibitory activity in the present study against the Gram-negative bacteria *P. intermedia* either. *Dodonaea viscosa* showed better activity against Gram-positive microorganisms in this investigation exhibiting MIC values of 4.69 and 10.16 mg/ml for *A. israelii* and *S. mutans* respectively.

Plant species belonging to the genus *Barleria* and *Zanthoxylum* have shown antibacterial activity in previous studies. Recent studies by Chromnawang *et al*. (2005) showed that *Barleria*

lupulina had strong inhibitory effects against *Propionibacterium acnes* and *S. epidermidis* with MICs of 1.25 and 2.5 mg/ml respectively. *Barleria albostellata* in the present study showed inhibitory action for the Gram-positive bacteria *A. israelii*, with an MIC of 4.10 mg/ml but showed no activity against *P. intermedia*. A study conducted by Gibbons *et al*. (2003) on *Zanthoxylum clava-herculis* showed resistance to methicillin-resistant *S. aureus* but its related species *Z. capense* did not show significant antibacterial activity in this study. *Zanthoxylum capense* may not possess the phytochemicals which imbue its related species *Z. clava-herculis* with antibacterial activity.

Little is known about the antibacterial activity of *C. orbiculata* as it has been mainly tested for anticonvulsant activity (Amabeoku *et al*., 2007). This study showed that *C. orbiculata* has antibacterial activity against *A. israelii* at 5.21 mg/ml; however it showed no activity against the Gram-negative bacteria, *P. intermedia*. In a study by Iauk *et al*. (2003), the antibacterial activity of the methanol extracts of six plants against *P. intermedia* was conducted. *Hamamelis virginiana*, and *Arnica montana* had MIC values of 0.512 mg/ml; while the other four plants *Althaea officinalis*, *Melissa officinalis*, *Calendula officinalis* and *Illicium verum* had MIC values of 2.05 mg/ml at a bacterial concentration of 5 x 10^5 CFU/ml. *Heteropyxis natalensis* exhibited a MIC value of 3.13 mg/ml against *P. intermedia* when the bacterial concentration used was 40 x 10^7 CFU/ml; hence, the results of the present study compares well with that of Iauk *et al*. (2003).

3.4.2 Antifungal screening

Only *D. cinerea* showed activity towards both *C. albicans* strains with an MIC of 10.71 mg/ml for *C. albicans* and 10.42 mg/ml for the drug resistant *C. albicans*. In this investigation the resistant strain of *C. albicans* was more susceptible to the positive controls CHX and Amphotericin B. The resistant *C. albicans* was also more susceptible to *D. cinerea* but not to the extracts of *H. natalensis* and *D. viscosa*.

Several studies have been reported earlier on the antimicrobial activity of *D. cinerea*. According to Steenkamp *et al*. (2007a, b) the methanol and aqueous extracts of *D. cinerea* were tested at a concentration of 100 mg/ml against *S. epidermidis* and *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans* (ATCC 10231) and various *C. albicans* clinical isolates. No zone of inhibition was observed when using the plate-hole diffusion assay and the MIC was not determined. In a study conducted by Runyoro *et al*. (2006), a 80% methanol extract of *D. cinerea* (200 µg/µl) was tested against *C. albicans* (ATCC 90028) at a CFU of 2 x 10^7 cells/ml in a bioautography agar overlay method but an inhibition zone was not obtained.

The antifungal properties of the aqueous, ethanol, ethyl acetate and hexane extracts of *D. angustifolia*, *Z. capense* and *C. edulis* were screened against *C. albicans* (ATCC 10231) and two clinical isolates (CFU/ml equivalent to 0.5 McFarland's solution). The aqueous extracts showed no inhibition. The ethyl acetate extract of *C. edulis* showed activity at 2.09 mg/ml. The ethanol extracts of *D. angustifolia* showed activity at 2.09 mg/ml and the ethyl acetate MIC values ranged between 1.04-2.09 mg/ml. None of the extracts of *Z. capense* showed any activity (Motsei *et al*., 2003). Acetone extract of *D. viscosa* was tested for antifungal activity against *C. albicans* (ATCC 90028) inocula with 10^6-10^7 cells/ml. The MIC ranged from 6.25-25 mg/ml (Patel & Coogan, 2008).

As Amoo *et al*. (2009) states, 'weak *in vitro* activity is not necessarily weak *in vivo* due to metabolic transformation'; this needs to be taken into consideration when testing a plant with traditional usage as an experiment covering one aspect of infection cannot convey the intricacies of the human body as a whole.

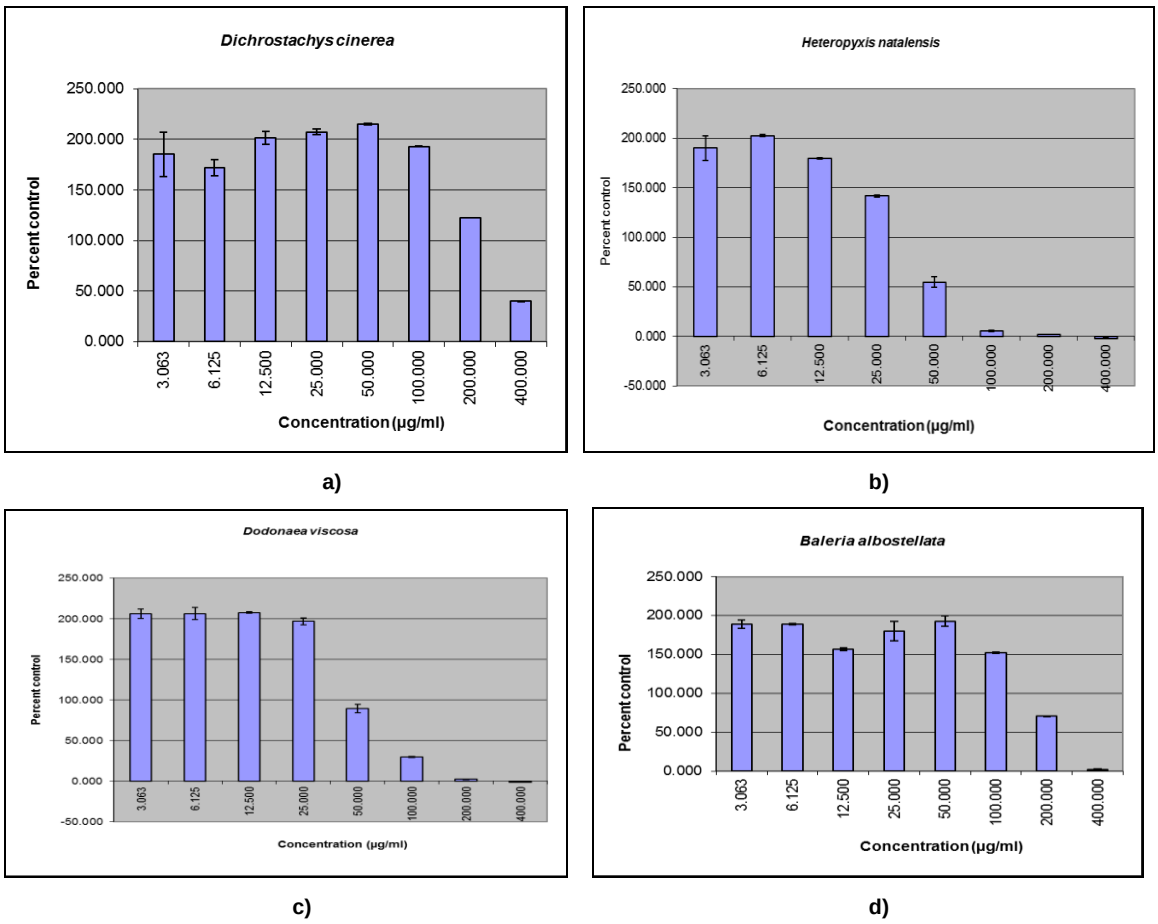
3.4.3 Cytotoxicity

The plant extracts which demonstrated the best results were selected for cytotoxicity. Four plant extracts, namely *B. albostellata, D. cinerea, H. natalensis* and *D. viscosa* were considered for cytotoxicity. The cytotoxicity of each extract was determined as a percentage of the positive control, which were cells grown in medium only.

The extracts on the HEp-2 cell line showed a trend of increase in cell viability at lower concentrations, with a decrease in cell viability as the concentration increased (Graph 3.1(a–d)). Fifty percent inhibitory concentration (IC_{50}) of all the plants namely *B. albostellata, D. cinerea, H. natalensis* and *D. viscosa* were found to range from 33.66 to 224 µg/ml (Table 3.3). The IC_{50} value of the positive control, Actinomycin D, for HEp-2 cells was found to be 0.008 µg/ml.

Two of the plant extracts showed moderate toxicity on the HEp-2 cell line, namely *H. natalensis* and *D. viscosa*, while *D. cinerea* and *B. albostellata* appeared to be relatively non-toxic.

Of the extracts tested for cytotoxicity only *D. cinerea* and *D. viscosa* had been previously tested. Cancer cell lines HeLa (human cervical carcinoma), HT29 (human colon adenocarcinoma) and A431 (human skin carcinoma), treated with methanolic extracts of *D. cinerea* had up to 75–100% cell proliferation as the percentage of control (Kamuhabwa *et al*., 2000).



Graph 3.1: Dose response curve of cytotoxicity results of plant extracts against HEp-2 cell line; a) *Dichrostachys cinerea*; b) *Heteropyxis natalensis*; c) *Dodonaea viscosa*; d) *Barleria albostellata*

Modelling of cytotoxicity data (CC_{50}) by Getie *et al*. (2003), with a methanolic extract of the leaves of *D. viscosa* exhibited a CC_{50} of 96.17 (± 13.86), 170.95 (± 74.3) and 25.7 (± 5.8) µg/ml with HeLa, Madin-Darby canine kidney (MDCK) and green monkey kidney (GMK) cells respectively.

3.5 Conclusion

The antimicrobial activity and safety of seven plants generally used for traditional oral care was analysed using the Gram-positive bacteria, *A. israelii* and *S. mutans*; the Gram-negative bacteria, *P. intermedia*, the yeast, *C. albicans* and two cells lines, Vero and HEp-2. Of the seven extracts tested, *H. natalensis* was the only plant to exhibit activity against both Gram-positive and negative bacteria. With moderate cytotoxicity, *H. natalensis* shows potential for further analysis and was selected for further study.

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Chapter 4

Synergism

4.1 Introduction

The synergism of plant extracts with established antibiotics could be a means to overcome resistance in pathogens and should undergo further investigation (Hemaiswarya *et al*., 2008). The combinations of 7-methyljugone, an isolate from *Euclea natalensis*, with anti-tuberculosis drugs isoniazid or rifampicin resulted in a four to six-fold reduction in the minimum inhibitory concentration (MIC) of each compound. Incorporations of plant extract with established drugs increases the efficacy of the drug and reduces microbial resistance (Bapela *et al*., 2006). As drugs for oral care are also encountering increasing microbial resistance, this may be a means to improve treatment.

Heteropyxis natalensis, selected for its high antibacterial activity and moderate toxicity to cells, was combined with the already established plant oils for antimicrobial activity, *Melaleuca alternifolia* essential oil and *Mentha piperita* essential oil. Green tea was selected for its natural fluoride content and ability to interfere with the processes involved in the pathogenesis of dental caries – adherence, glycocalyx formation and acid production (Hamilton-Miller, 2008).

4.2 Materials and methods

Heteropyxis natalensis, *Melaleuca alternifolia* essential oil (Holistic Emporium cc, Gauteng, South Africa), *Mentha piperita* essential oil (Holistic Emporium cc, Gauteng, South Africa), 30 g of Five roses green tea (South Africa) and 40 g of Thé Vert, Chinese green tea (Guangdong Chine, China), available from local shops, were selected for this investigation.

Actinomyces israelii, a Gram-positive microorganism, which has been linked to root surface caries and *Candida albicans* an opportunistic pathogen were selected.

4.2.1 Antimicrobial susceptibility testing

To determine the effects of combinations of *H. natalensis*, *M. alternifolia* essential oil, *M. piperita* essential oil and green tea, the MIC of each component was determined first using the anti-microbial method described previously. A stock solution of the ethanol extract of *H. natalensis* was prepared as previously described in 10% DMSO. The essential oils were dissolved in 25% Tween (80) (Merck Chemicals (Pty) Ltd Wadeville, South Africa) and aqueous decoctions were prepared from the selected green tea tea-bags. The final concentration tested of the essential

oils ranged from 125-0.98 mg/ml and 30-0.47 mg/ml and 40-0.63 mg/ml for Five Roses and Thé Vert green teas respectively. Chlorhexidine gluconate (5%) was incorporated as the positive control. The highest concentration of the solvent Dimethyl sulphoxide (DMSO) (2.5%) and Tween 80 (6.25%) was found to be non-toxic to the microorganisms tested. *Actinomyces israelli* and *C. albicans* were incubated at 37ºC, under anaerobic and aerobic conditions respectively for 24 hours.

4.2.2 Determination of *in vitro* synergic activity

The synergistic activity of the samples was determined using a slightly modified method. The activity of the combination of the four components was evaluated in Casein-peptone Soymeal-peptone medium broth for the selected bacteria, *A. israelii*, and Sabouraud Dextrose 4% broth for the yeast, *C. albicans*, at sub-MIC levels (below original MIC values) so that each component was present at concentrations corresponding to ½, ¼, $^1/_8$, $^1/_{16}$ and $^1/_{32}$ of the MIC. Analysis of the extract combination data was achieved by calculating the fractional inhibitory concentration (FIC) index as follows: FIC = ($MIC_{a\ combination}$/ $MIC_{a\ alone}$) + ($MIC_{b\ combination}$/ $MIC_{b\ alone}$) *etc*. where *a* = first compound and *b* = second compound used in synergistic study *etc*. The FIC was interpreted as: FIC < 1, synergistic activity; FIC = 1, indifference; FIC > 1, antagonistic activity (Bapela *et al*., 2006; De Logu *et al*., 2002).

4.3 Results and discussion

The results for the MICs of each component alone and in combination with other components, and the calculated FICs of the essential oils of *M. alternifolia* and *M. piperita* and *H. natalensis* are depicted in Table 4.1.

As neither of the green teas, Five Roses or Thé Vert, showed any inhibitory effect against *A. israelii* or *C. albicans* at the highest concentration of 30mg/ml and 40 mg/ml respectively, they were not included for the synergism assay (Figure 4.1). The FIC thus obtained with *H. natalensis*, *M. alternifolia* and *M. piperita* essential oils was 0.75 which indicated synergism (Figure 4.2 & 4.3). The FIC thus obtained with *H. natalensis*, tea tree and peppermint essential oil was 1.5 which indicated antagonism.

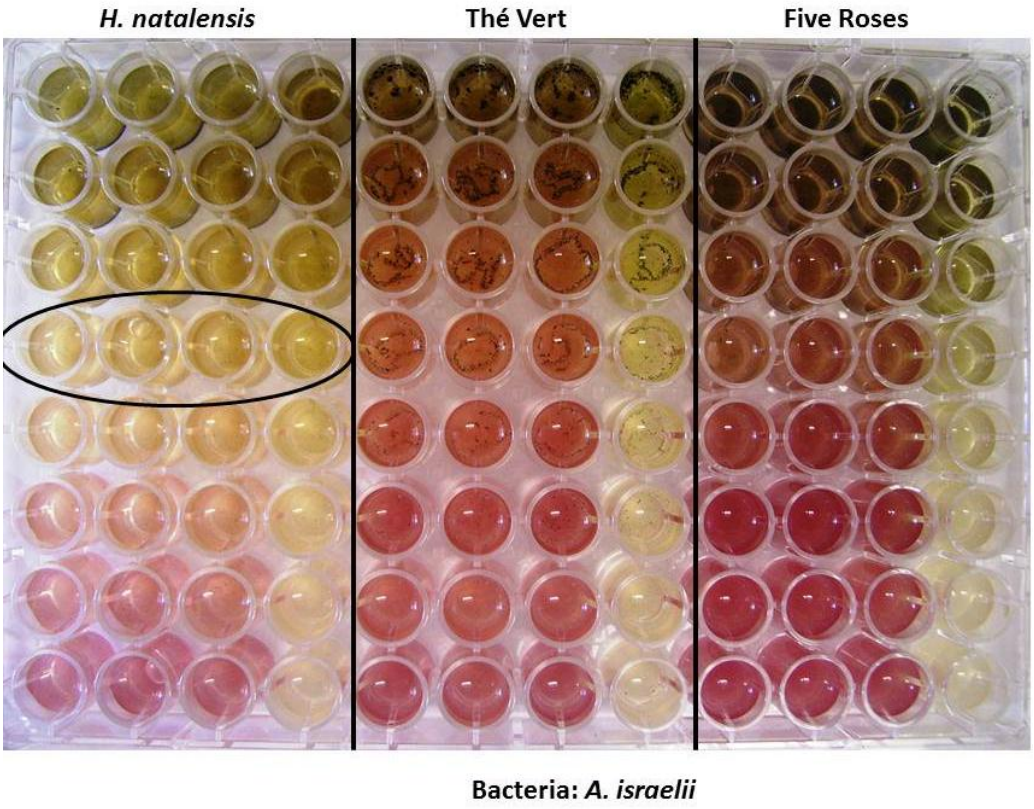

Figure 4.1: Microtitre plate showing the MICs of *Heteropyxis natalensis*, Thé Vert green tea and Five Roses green tea against *Actinomyces israelii*

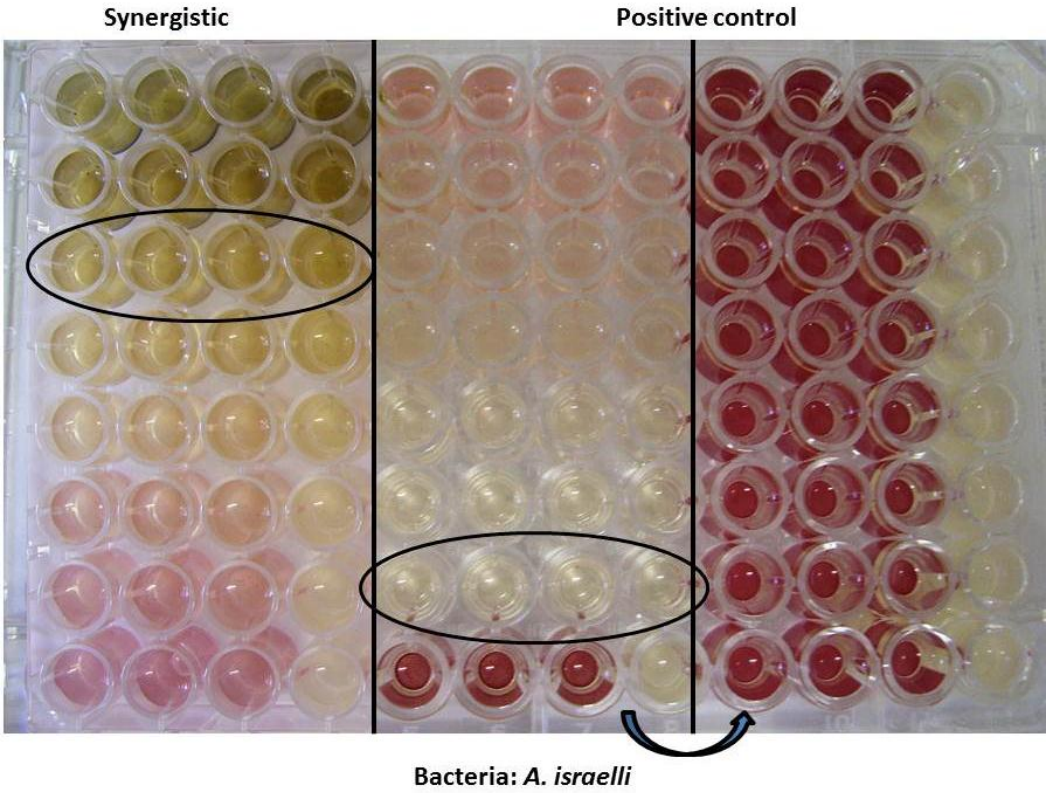

Figure 4.2: Microtitre plate showing the MICs of the synergistic combination of *H. natalensis*, *M. alternifolia* and *M. piperita*, as well as the positive control, CHX against *Actinomyces israelii*

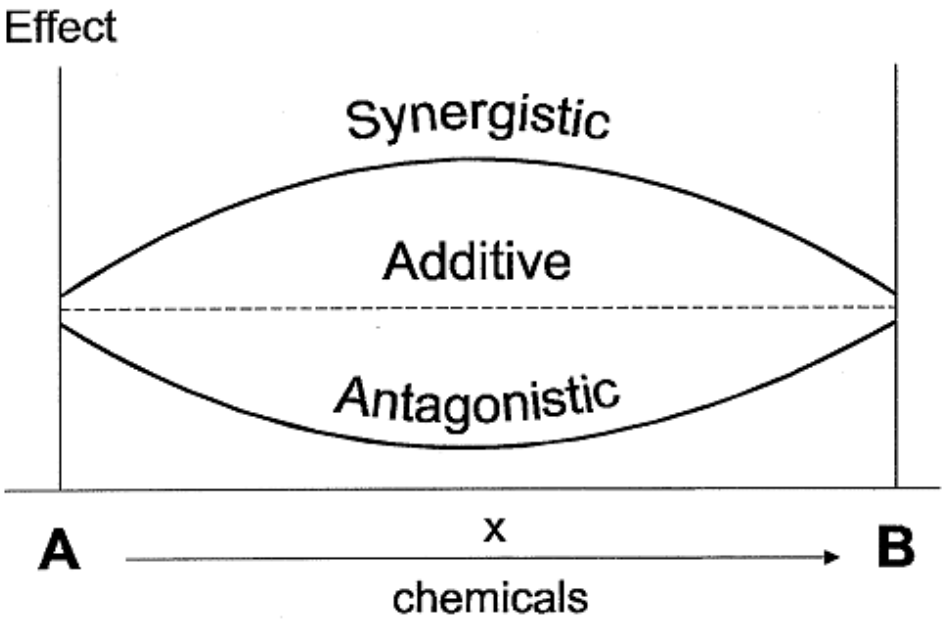

Figure 4.3: Antagonism and synergism (www.balticuniv.uu.se)

Table 4.1: Synergistic activity of *Heteropyxis natalensis* with established plant oils and green tea

Plant extract	Microorganism tested					
	MIC (mg/ml)			MIC (mg/ml)		
	A.i			C.a		
	Alone	In combination[b]	FIC	Alone	In combination[b]	FIC
Heteropyxis natalensis	0.88	0.22	0.75	10.94	5.47	1.5
Melaleuca alternifolia essential oil	9.12	2.28	0.75	1.47	0.73	1.5
Mentha piperita essential oil	3.26	0.81	0.75	0.98	0.49	1.5
Five Roses Green tea	>30	Na[a]		>30	Na	
Thé Vert Green tea	>40	Na		>40	Na	
Chlorhexidine	0.02	Na		0.24	Na	

[a] Na: Not active; A.i: *Actinomyces israelii*; C.a: *Candida albicans*; Combination of *Heteropyxis natalensis*, *Melaleuca alternifolia* essential oil and *Mentha piperita* essential oil

The anti-fungal activities of *M. alternifolia* and *M. piperita* essential oil have been previously investigated with *C. albicans*. Chlorhexidine gluconate MIC values correlated with the earlier studies in this investigation.

In a study conducted by Van Vuuren & Viljoen (2006) *M. alternifolia* and *M. piperita* essential oils were tested for inhibitory activity against *C. albicans* (ATCC 10231) with an approximate inoculum size of 1 x 10^6 CFU/ml. MIC values of 3.7 and 2.4 mg/ml were obtained for *M. alternifolia* and *M. piperita* essential oils respectively using the micro plate method. *Heteropyxis natalensis* essential oil was also tested in the study which exhibited an MIC value of 4.5 mg/ml

obtained. Although the results are not directly comparable to the present study, it would seem that *H. natalensis* essential oil is somewhat more effective against *C. albicans*.

A dichloromethane:methanol (1:1) extract of the *M. piperita* herb was investigated for inhibitory activity against *C. albicans* (ATCC 10231) and a clinical isolate (CI002). The inoculums were 1 x 10^6 CFU/ml. Minimum inhibitory concentrations of 2.50 and 1.0 mg/ml were obtained for *C. albicans* and the clinical isolate, respectively (Sandasi *et al*., 2011). Although the herb and not the essential oil of *M. piperita* was investigated, these results correlated with the results of Van Vuuren and Viljoen (2006) for the standard strain of *C. albicans*. In this study the activity of the essential oil of *M. piperita* exhibited much higher activity against *C. albicans* (MIC of 0.98 mg/ml).

Catechins from green tea have been extensively studied. It has been reported that these compounds exhibited good inhibitory activity against *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus*, *Mycobacterium tuberculosis, Escherichia coli, Streptococcus mutans* and *C. albicans* (Hamilton-Miller, 2001; Song & Seong, 2007). Cathechins belong to the flavones and flavonoids major group of antimicrobial compounds from plants. These compounds are only extracted by alcohols such as acetone and not through aqueous extraction (Cowan, 1999). This may possibly account for lack of activity found in the present study. According to Hamilton-Miller (2001) a cup of green tea contains 0.5-1 g of catechins/L and that a ‘cup of tea’ is inhibitory and often bactericidal. It was found in the present study that a ‘cup of tea’ of the two green teas studied do not have an inhibitory effect.

In another study, green and black tea was firstly macerated with two litres of methanol. Chloroform, n-hexane and ethyl acetate were added to three separate funnels containing the methanol macerate. An aqueous extraction was also performed. The extracts were tested against *S. mutans* and *E. coli*. *Streptococcus mutans* and *E. coli* were only sensitive to the ethyl acetate extract using the disc diffusion method. Black tea showed no inhibitory activity (Rasheed and Haider, 1998). These results correlated to the one observed in the present study.

In the present study there was a fourfold reduction in the MIC value of *A. israelii* with the combination of *M. alternifolia oil*, *M. piperita* and *H. natalensis* and according to the FIC indicates synergistic activity. Although there was a twofold reduction in the MIC value for *C. albicans*, according to the FIC value there was antagonistic activity between the products in combination. This may be due to the eukaryotic nature of *C. albicans* which are complex multicellular microorganisms. *Melaleuca alternifolia* essential oil and *M. piperita* essential oil in particular show noteworthy activity in this study compared to other investigations and may show synergism against *C. albicans* if *H. natalensis* is not included, which did not exhibit anti-fungal

activity. *Melaleuca alternifolia* has previously been incorporated in a toothpaste, Melafresh T-96, at 0.2% which roughly correlated to the active amount (2.28 mg/ml) within the combination of *H. natalensis*, *M. alternifolia* and *M. piperita*. The active amount of *H. natalensis* (0.75 mg/ml) in this combination is much less (Bolel, 2009).

4.4 Conclusion

The synergistic activity of the essential oils *M. alternifolia* and *M. piperita* with the ethanolic extract of *H. natalensis* resulted in a fourfold reduction in the MIC of *A. israelii*. An antagonistic rather than synergistic activity was obtained for *C. albicans*.

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Chapter 5

Anti-adherence

5.1 Introduction

Microorganisms rely on the successful attachment of the pellicle for adhesion to the hosts mucosal or enamel surface to enable colonization and thereby biofilm formation. Subsequent infection occurs and the body's non-specific immune response, innate immunity, releases a cytokine known as Interluekin-8 (IL-8). Interleukin-8 is just one of the components involved in the body's first line of defence by increasing phagocytosis, bacterial killing, and inducing the release of lysosomal enzymes. The infective stage of the microorganism is a critical stage in the pathogenesis of microorganisms (Taweechaisupapong *et al*., 2005; Uehara *et al*., 2008). To reduce contact between the host tissues and pathogens by either preventing or reversing the adhesion of infectious agents are the purpose of anti-adhesion therapy and anti-adhesion immunity (Ofek *et al*., 2003).

Bacteria that are adherent to surfaces are more resistant to removal and treatment, therefor prevention of adhesion at an early stage, following initial exposure of the host to pathogens should prevent disease (Ofek *et al*., 2003). A certain incidence of resistance to anti-adhesion agents may emerge but as 'these agents do not act by killing or arresting growth of the pathogen it is reasonable to assume that any strains resistant to anti-adhesion agents will be diluted with the sensitive bacteria whose adhesion is inhibited and are shed out of the host' (Ofek *et al*., 2003).

In the present study the ability of the plant, *H. natalensis*, to prevent the adhesion of caries producing bacteria to the tooth's surface and prevent the release of IL-8, and thereby prevent infection of epithelial cells will be conducted.

5.2 Materials and method

Actinomyces israelii and *Streptococcus mutans* were selected for this investigation as *H. natalensis* exhibited good antimicrobial activity against these organisms with MICs of 0.88 mg/ml and 1.82 mg/ml obtained respectively. Both microorganisms are linked to caries formation with *S. mutans* producing extracellular polysaccharides from dietary carbohydrates enabling binding to enamel and each other. *Actinomyces israelii* is linked to the inflammation of the soft tissues of the gums in gingivitis (Samaranayake, 2002). As *S. mutans* does not infect

soft tissues and therefore does not induce a cytokine response, *A. israelii* was utilized in the cytokine assay. Human laryngeal epidermoid carcinoma (HEp-2) cells were obtained from Highveld Biological (Johannesburg, South Africa) for the study. The larynx is connected to the oral cavity and possesses the same epithelial cells that will release IL-8 under infection. As oral epithelial cells lines are difficult to maintain, hence laryngeal cells were utilized instead.

5.2.2 Cytokine evaluation

Levels of IL-8 in the supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Pharmigen, OptEIA Human IL-8 Set, catalog no. 555244) obtained from BD Bioscience.

Cells were grown in pre-coated T-75 flasks in Eagle's minimum essential medium (MEM) (Sigma-Aldrich, South Africa) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, South Africa) and re-fed every 2-3 days until confluent. The confluent, adherent monolayers were then released from the plastic surface after treatment with polyvinyl-pirrolidone (PVP)-trypsin-EDTA (Sigma-Aldrich, South Africa) and were seeded to 24-well plates for 24 hrs before receiving the treatments. The cells were rinsed three times with phosphate-buffered saline (PBS) buffer (Sigma-Aldrich, South Africa) and 1 ml antibiotic free medium was added to the cells which were then treated with *H. natalensis* at non-cytotoxic concentrations established previously in cell culture studies.

To determine if the plant extract had an effect on IL-8 release from oral epithelial cells, extracts were added to the cells at concentrations varying from 12.5-200 µg/ml. Time-dependent studies were carried out. Plant extracts were added to HEp-2 cells and one hour later *A. israelii* was added. Plant extracts and *A. israelii* were added to HEp-2 cells together and lastly *A. israelii* was added to HEp-2 cells with the plant extracts being added one hour later.

Heteropyxis natalensis extracts were added in duplicate to each well. A negative control of only *A. israelii* and HEp-2 cells was included. The plates were incubated overnight at 37ºC in 5% CO_2.

After the treatment described above, the supernatants were collected to determine IL-8 released using a commercially available ELISA kit. Briefly, 96-well plates were coated with 100 µl of capture antibody (anti-human IL-8 monoclonal antibody) incubated overnight, washed three times with 0.05% Tween-20 in FBS and coated with PBS with 10% FBS in order to block non-specific binding . Known concentrations of IL-8 (standard) and the samples containing the IL-8 released by the cells after treatment (supernatant) were added as aliquots into appropriate wells, incubated for two hours and decanted from the wells. Aspirated and washed five times.

Biotinylated (the attachment of a biotin residue to a biological macromolecule in order to label it) anti-human IL-8 monoclonal antibody and streptavidin-horseradish peroxidase conjugate were added and incubated for one hour. After washing the plate, a solution containing a substrate for the enzyme (3,3',5,5' tetramethylbenzidine-peroxide chromogen) present in the anti-IL-8 and enzyme reagent mixture was added and the plate was incubated for 30 minutes. The reaction was stopped using a 2N sulfuric acid (H_2SO_4) solution and the absorbance was read at 450 nm using an ELISA plate reader (BIO-TEK Power-Wave XS, Weltevreden Park, South Africa). The absorbencies were then used to calculate the IL-8 concentration from the standard curve (Vilela *et al*., 2006).

5.3 Ultrastructure

To determine whether or not the plant extract affected the attachment of *S. mutans* to the enamel of human teeth, time-dependent studies were carried out. Firstly *H. natalensis* extract, at an MIC concentration of 1.82 mg/ml and sub-MIC value of 0.91 mg/ml, were added to the prepared enamel fragments and one hour later *S. mutans* was added (Treatment 1). Secondly plant extracts and *S. mutans* were added to the enamel fragments together (Treatment 2) and lastly *S. mutans* was added to the enamel fragments with the plant extracts being added one hour later (Treatment 3).

The plant extract was added in duplicate to each well. The positive control consisted of enamel fragments being coated with a chemical that prevents adhesion of bacteria. A negative control of only *S. mutans* and enamel fragments was included. The plates were incubated overnight.

5.3.1 Collection of Material

Teeth collected for this study had been extracted from human patients for reasons other than the purpose of this study. Each patient who attended the extraction clinic of the University of Pretoria must complete and sign a patient information leaflet and informed consent form.

Non-carious, recently extracted human teeth, were collected from Dental Clinics. Only teeth which had been extracted for periodontal or orthodontic reasons were used. Ethical and safety guidelines for the handling of human teeth and laboratory research were strictly followed. Immediately after extraction the teeth were rinsed in running water. Thereafter the teeth were placed in distilled water in an ultrasonic water bath and sonicated for periods of fifteen minutes in clean distilled water until all loose biological material was removed and stored at 4 ºC. The crowns of the teeth were removed by horizontally sectioning at the cemental-enamel junction

with a diamond wafering blade in an Isomet 11-1180 low speed saw (Buehler Ltd., Lake Bluff, Illinois, USA) under permanent water irrigation. The crowns were further cut into blocks; the samples were placed in sterile Ringer's solution (Merck SA (Pty) Ltd., Halfway House, South Africa) and sterilized at 125 ºC for 15 min.

The rest of the procedure was carried out under sterile conditions. Sterility was maintained for the duration of the entire experiment that was conducted in a positive sterile airflow laboratory, using sterile instruments as well as gloves and masks. Before sterilisation some of the enamel blocks used as samples were cleaned, dried and coated with 2% Dimethyl dichlorosilane in 1,1,1-trichloroethane (Repelcote® - Saarchem-Holpro Analytic (Pty.) Ltd., 40 Fransen Street, Chamdor, Krugersdorp) in order to create a repellent surface that would prevent organisms attachment, utilized as a positive control.

All samples were placed in 24 well tissue culture plates containing 1 ml CASO broth and incubated at 37˚C for 1 hour. For Treatment 1, 1ml of the different plant extracts were added and incubated at 37˚C for 1 hour, after which 1% MacFarland Standard-1 bacterial suspension was added. For Treatment 3, the bacterial suspension was added to the enamel blocks and incubated for an hour, after which the different plant extracts were added. For Treatment 2, the different plant extracts and the 1% MacFarland Standard-1 bacterial suspension were added at the same time, the plates incubated anaerobically as described in a shake incubator for 24 and 48 hours. One enamel block with the organism tested was used as a positive control and all the samples were prepared for the Scanning Electron Microscope (SEM).

5.3.2 Preparation for Scanning Electronmicroscopy (SEM)

One sample was collected from each of the tissue culture wells containing the different plant extract concentrations at 24 and 48 hrs for SEM to determine the colonization of the organisms on enamel. The samples were prepared according to standard methods for biological SEM evaluation according to Glauert (1975) and Hayat (1981) as follows:

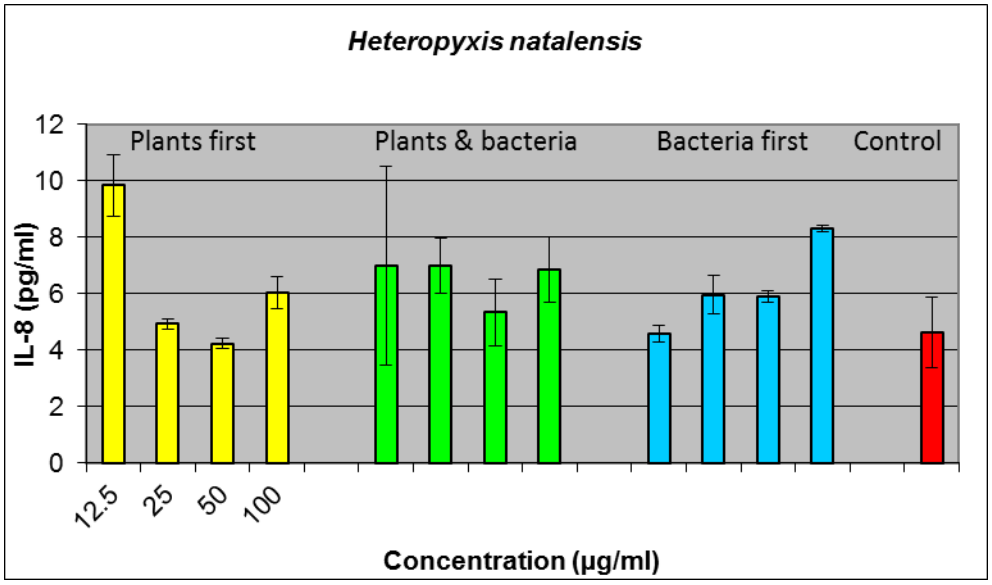
The samples were fixed in 2% gluteraldehyde (Electron Microscopy Sciences, Washington DC, USA) for 1 hour. The gluteraldehyde was siphoned off with a pipette and rinsed 3 times with PBS (Whittaker MA Bioproducts, Walkersville, USA) for 5 minutes each time. Samples were then fixed in 0,25% Osmiumtetroxide (OsO_4) (Merck, Darnstadt, Germany) for 30 minutes and again rinsed for 3 times with PBS for 5 minutes each time. Thereafter the samples were rinsed for 5 minutes each time in increasing concentrations of ethanol (Merck, Darnstadt, Germany), namely 30%, 50%, 70% and 3 times in 100%. Samples were then stored in 100% ethanol before they were dried in a critical point dryer (Poloron, Oxford, England) until dry (± 8 hours). All the prepared samples were then coated with gold in a sputter-coater (polaron E5200, Whatford, England) before they were examined in a Scanning Electron Microscope (JEOL JSM

840 Scanning Electron Microscope, Tokyo, Japan) and Ultra High Resolution FE-SEM for Nano-scale Compositional Analysis (Carl Zeiss, South Africa, 363 Oak Avenue, 2194 Ferndale, Randburg).

5.4 Results and discussion

5.4.1 Cytokine evaluation

The results of the IL-8 assay for *H. natalensis* with *A. israelii* are summarized in Graph 5.1.



Graph 5.1: The effect of *Heteropyxis natalensis* on the release of IL-8, taken in a time-dependent manner at various concentrations; as compared to the control of *Actinomyces israelii*

There is a general trend in the graph that the higher the concentration of the plant (µg/ml) the more IL-8 is released at each stage of testing. Although the plants themselves did not induce a release of IL-8 (not shown on graph) there seems to be a negative interaction between *H. natalensis* and *A. israelii* that induces the release of IL-8, as all the IL-8 readings are higher than that of the negative control of the bacteria and cells only, at 4.6 pg/ml. Although *H. natalensis* inhibited *A. israelii* growth at 0.88 mg/ml and is bacteriostatic at 3.32 (mg/ml); it does not appear to hinder the adhesion mechanism of *A. israelii* and must therefore affect the microorganism in another way.

In *Candida* studies carried out by Taweechaisupapong *et al*. (2005) the importance of *Candida* adhering to the mucosal cells and their ability to cause infection has been established in both *in vitro* and animal studies. The ability of *Steblus asper*, a plant utilized for pharmaceutical purposes, to reduce the adherence of *C. albicans* to human buccal epithelial cells was investigated with SEM. It was found that not only did *S. asper* significantly reduce the

adherence of *C. albicans* to buccal cells but it also reduced *C. albicans* germ tube formation which allows for more efficient adherence (Taweechaisupapong *et al*., 2005). In a similar study conducted by Patel *et al*. (2009) results showed that acetone extracts of *Dodonaea viscosa* could significantly inhibit the adherence of *C. albicans* to buccal epithelial cells compared to the control, water. *Candida albicans*, 10^6 CFU/ml, was pre-treated with 0.4 mg/ml plant extract before exposure to the oral epithelial cells.

Most pathogens possess genes that encode for more than one type of adhesion which are expressed during the infectious process. Other factors are involved in adhesion such as hydrophobic and other non-specific interactions under different shear-forces. This may be a draw back in anti-adhesion therapy which only targets one adhesion factor. To ensure effective anti-adhesion therapy, multiple agents may be required which target different adhesion factors of infecting pathogens; a single agent that exhibits a broad spectrum anti-adhesion activity would be preferable.

5.4.2 Ultrastructure

For the initiation of carious lesions on smooth enamel surfaces, dietary sucrose is essential. *Streptococcus mutans* is able to synthesize extracellular glucans specifically from sucrose. The synthesis of these glucans is associated with their ability to accumulate on solid surfaces. Glucan synthesis by *S. mutans* further involves a glucan-binding protein (GBP) and at least two types of glucosyltransferase (GTFs). The interaction between these ligands and soluble and insoluble glucan molecules is thought to promote the cohesion between streptococcal cells after they have become attached to the pellicle formed on the enamel surface of teeth (Gibbons, 1984).

With the exception of the image of negative control (Figure 5.5a) and the image of the salivary pellicle (Figure 5.1a) after 24 hrs of growth, all images where taken after allowing bacterial growth for 48 hrs. Each image characterizes the most representative field of five scans for each sample tested. The images are also visual comparisons at the same magnification of x 5000. Each sample was further analysed with an Ultra High Resolution FE-SEM (see Appendix A).

Figure 5.1a, b and c represents the comparison of the pellicle as formed on the enamel surface of teeth. Treatment 1 is represented by Figures 5.1b and 5.2; Treatment 2 by Figure 5.3a and b and Treatment 3 by Figure 5.4.

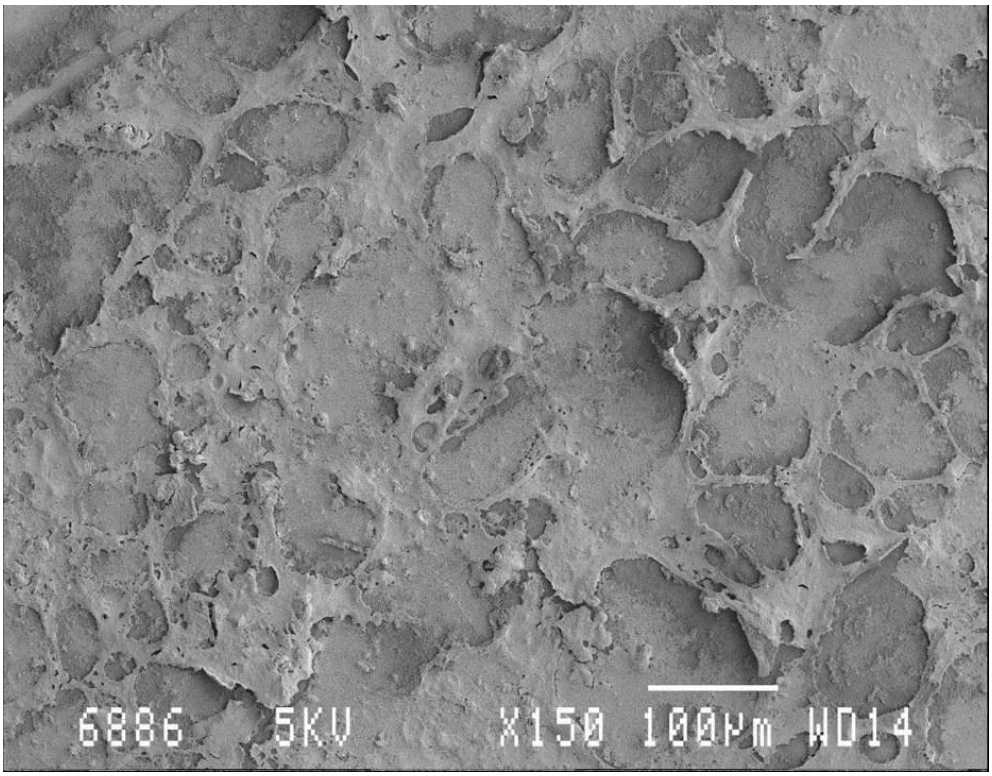


Figure 5.1a): Image of enamel surface of the extracted tooth was exposed to stimulated saliva to form a pellicle on the enamel. Image courtesy of Dr F.S. Botha, Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria.

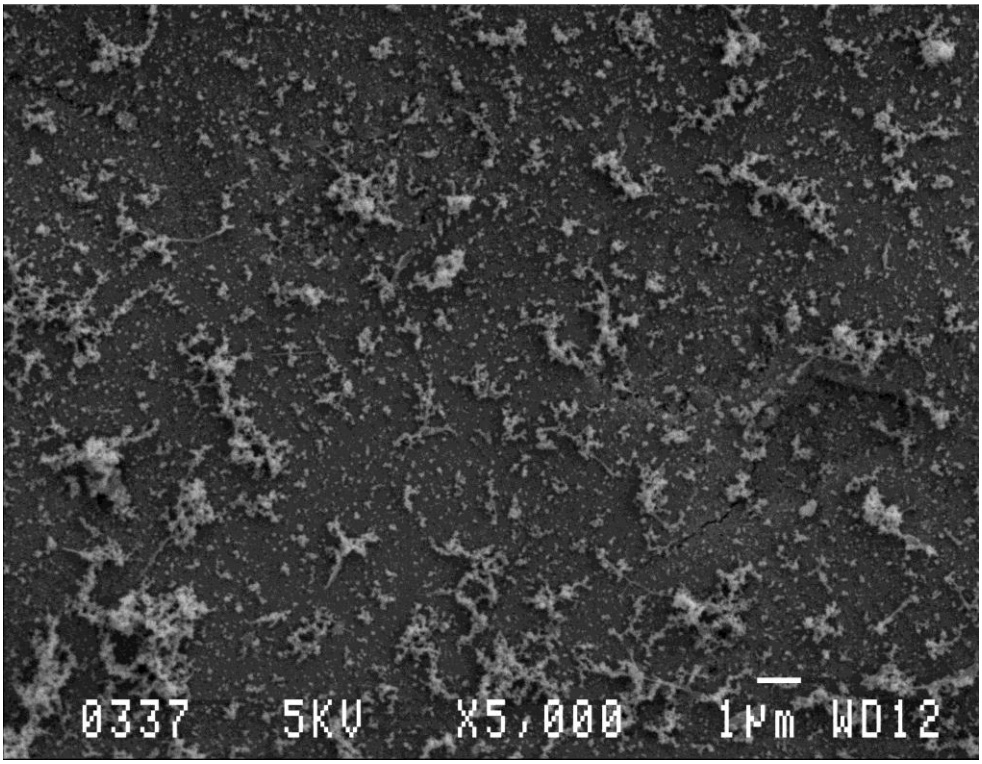


Figure 5.1b): Enamel surface was exposed to the plant extract in a carbohydrate containing growth medium (CASO), to determine if a coating similar to a normal pellicle, could be formed on the enamel (Treatment 1). This image shows the coating of the enamel surface of teeth by the plant extract.

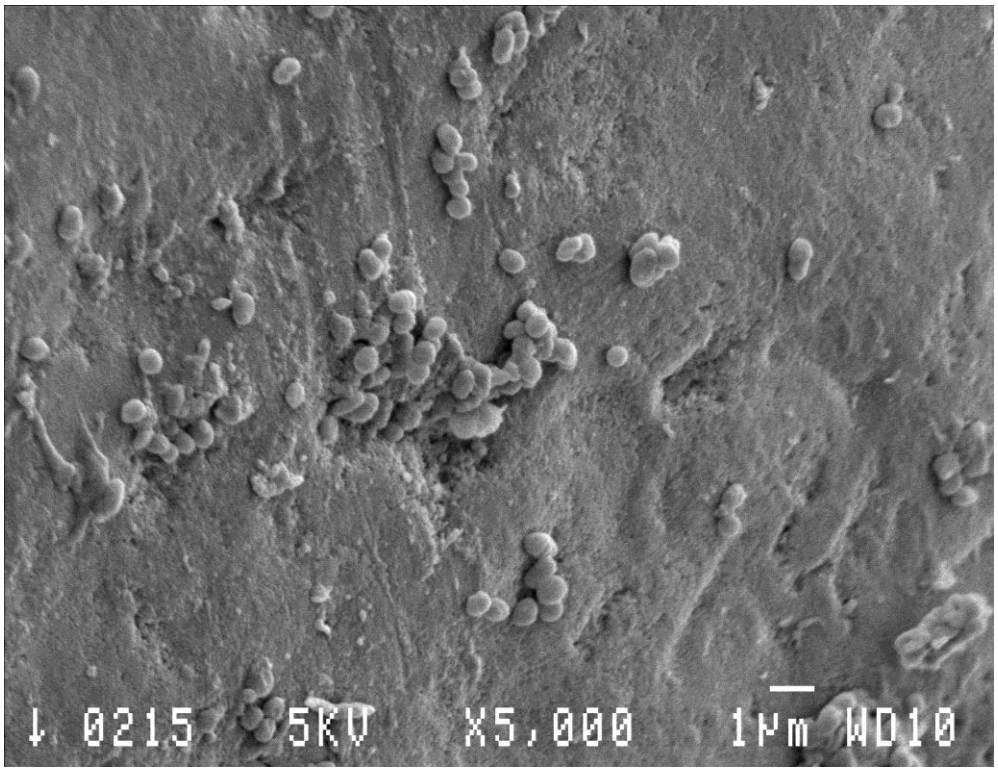


Figure 5.1c): Image of enamel surface coated with the chemical substance, Repelcote® that prevents adhesion of bacterial cells to a surface. The enamel was exposed to a carbohydrate medium to determine if a 'pellicle' could be formed on enamel.

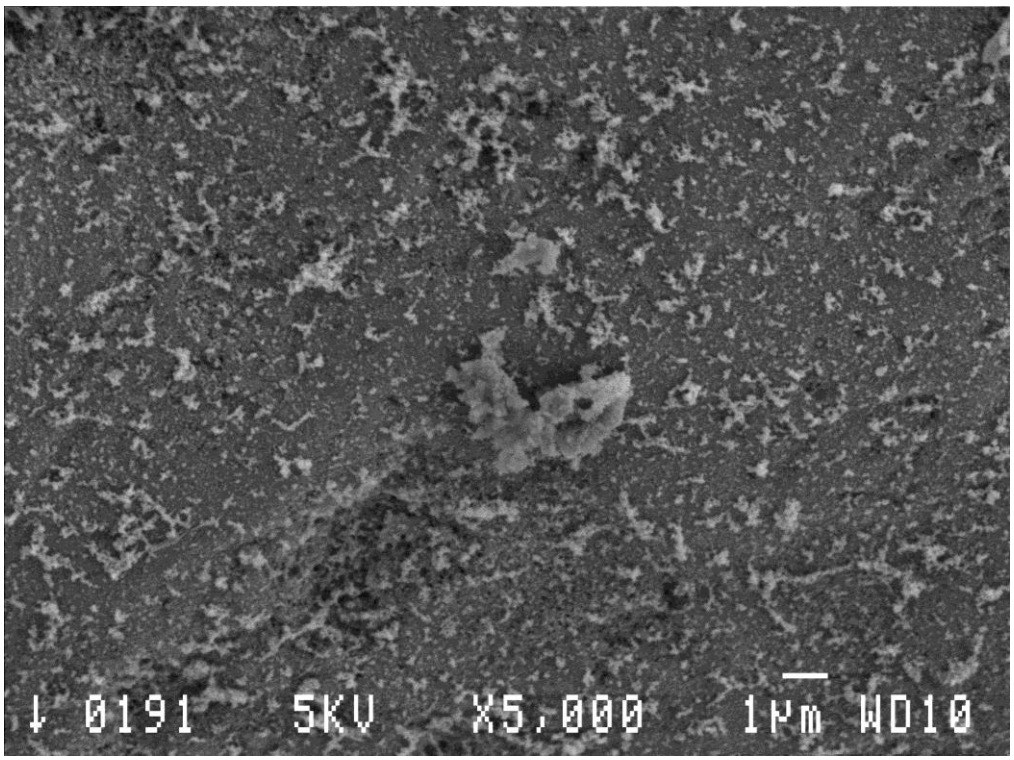


Figure 5.2: Image of enamel after Treatment 1 and 48 hrs of growth and exposure to *Heteropyxis natalensis* at a sub-MIC of 0.9 mg/ml added 1h before *Streptococcus mutans*. The plant extract can be seen coating the enamel but no bacteria adhering.

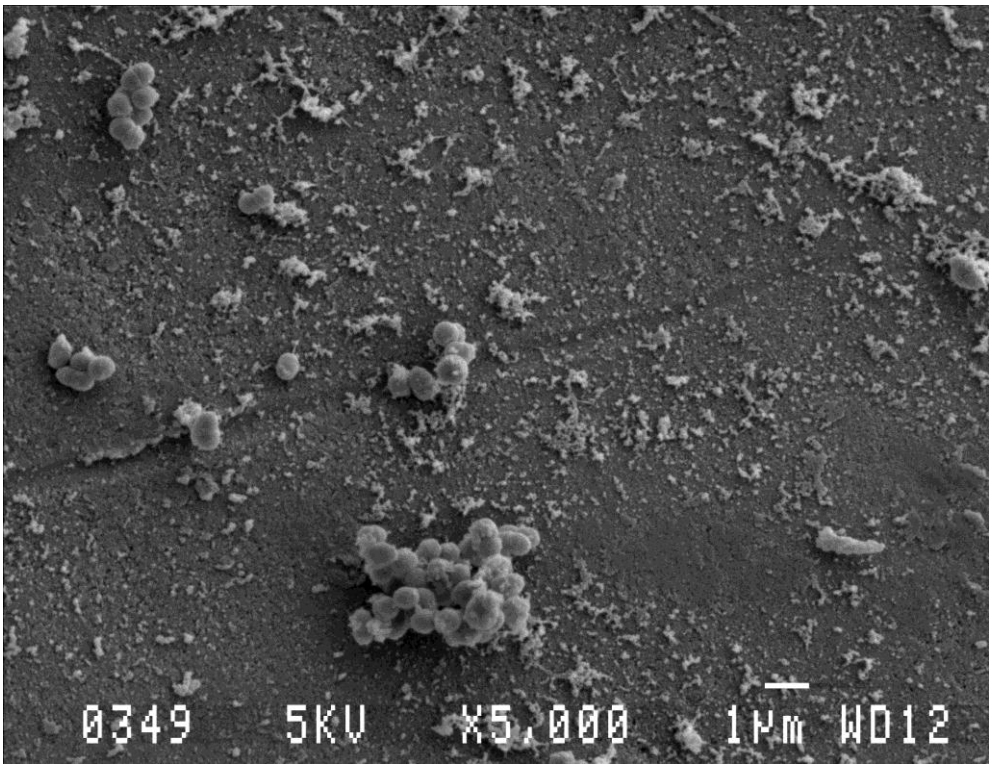


Figure 5.3a): Image of enamel after Treatment 2 and 48 hrs of growth; *Heteropyxis natalensis* at an MIC of 1.82 mg/ml added together with *Streptococcus mutans*. The plant extract can be seen coating the enamel and colonies of streptococci adhering to each other and the enamel surface through pellicle formation.

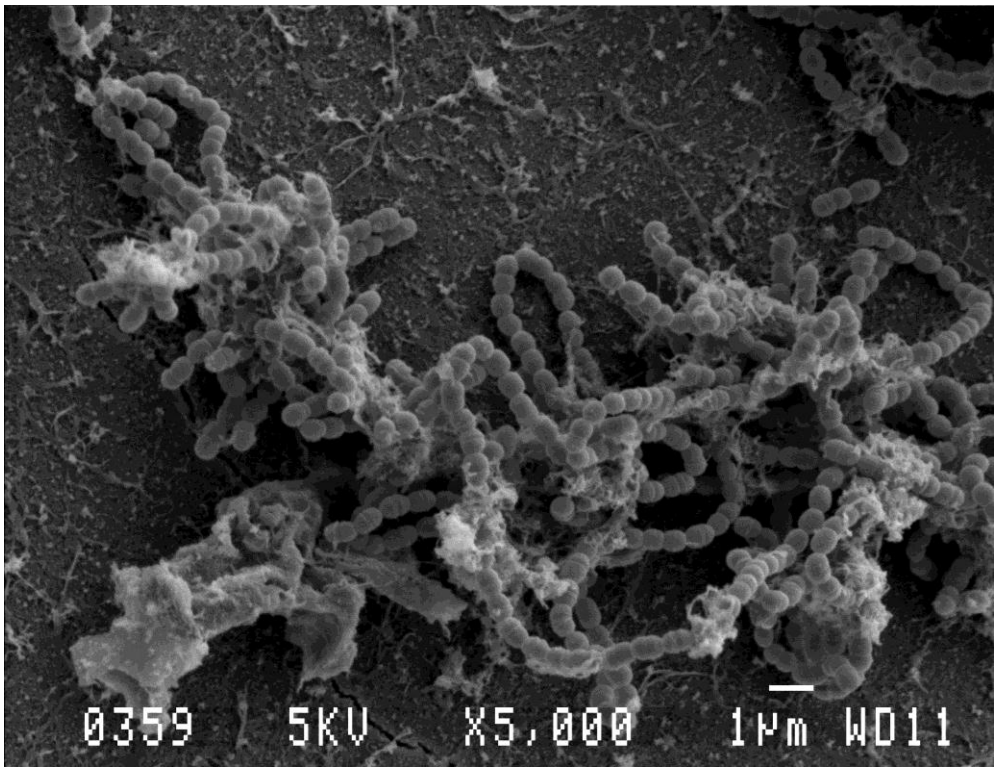


Figure 5.3b): Image of enamel after Treatment 2 and 48 hrs of growth; *Heteropyxis natalensis* at a sub-MIC of 0.9 mg/ml added together with *Streptococcus mutans*. Extensive growth of the streptococci can be seen with the characteristic chain formation occurring.

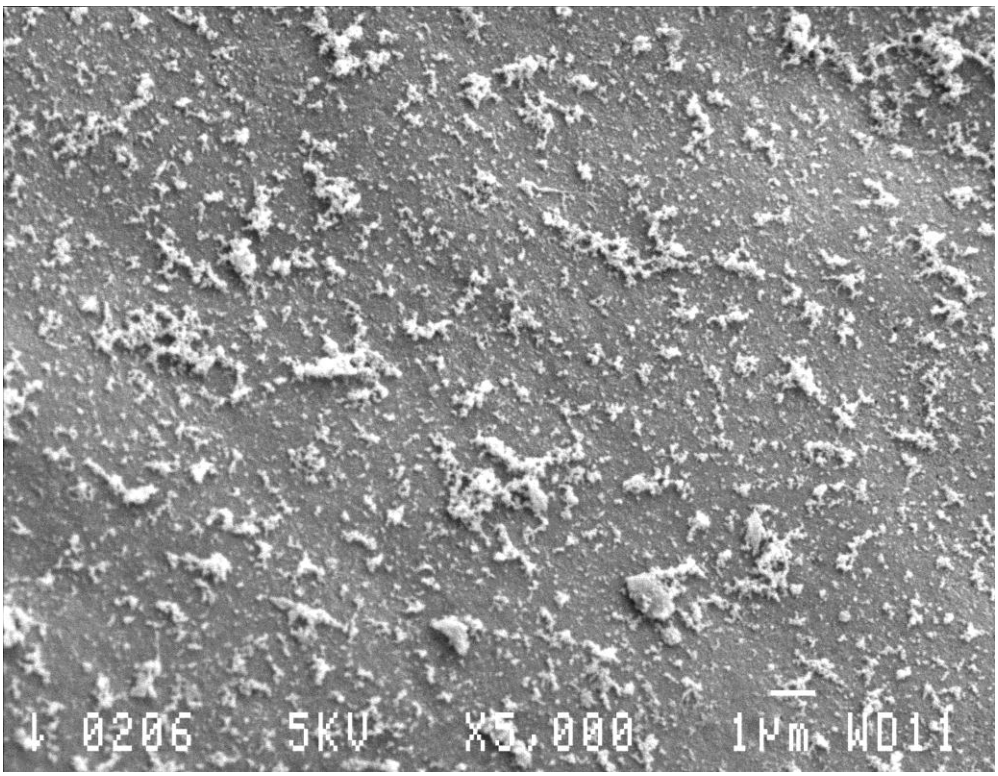


Figure 5.4: Image of enamel after Treatment 3 after 48 hrs of growth; *Streptococcus mutans* was added 1h before *Heteropyxis natalensis* at an MIC of 1.82 mg/ml. The plant extract can be seen coating the enamel and a single cocci adhering to the enamel surface.

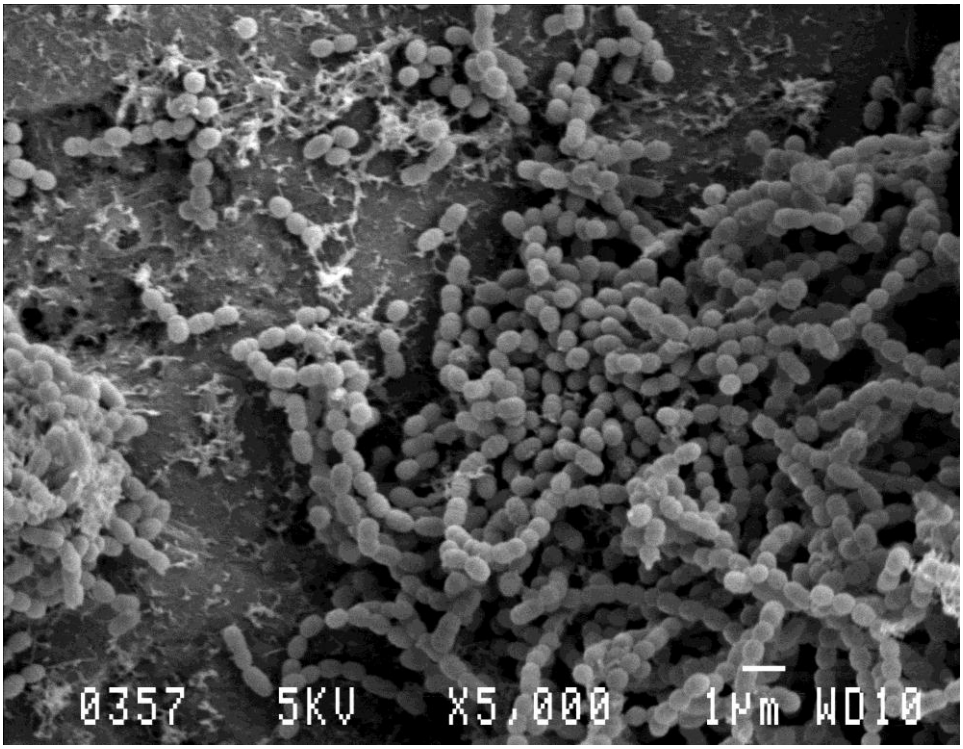


Figure 5.5a): Image of enamel after 24 hrs growth; a negative control of *Streptococcus mutans*. Chain formation of the streptococci is already established. Bacterial by-product is seen to accumulate and collect on the enamel.

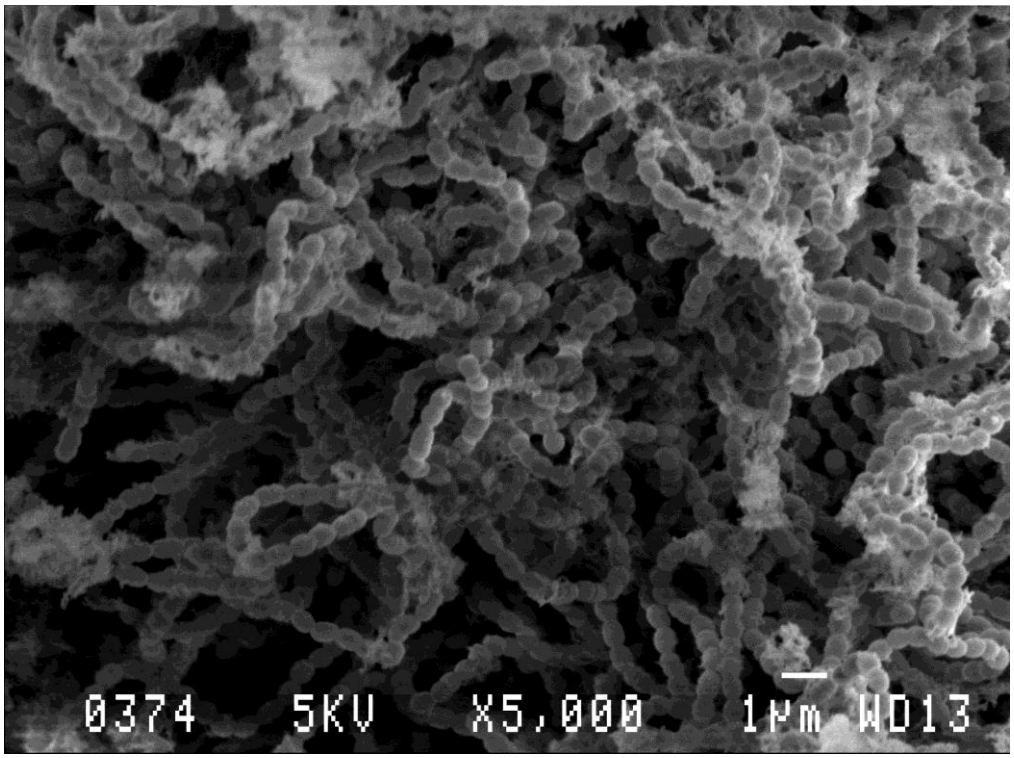


Figure 5.5b): Image of enamel after 48 hrs growth; a negative control of *Streptococcus mutans*. Extensive growth and clumping of the streptococci chains can be seen adhering both to the enamel surface and each other through pellicle and glucan binding. Due to the extent of the growth metabolic by-products can be seen accumulating.

In comparison to the negative control of *S. mutans* only, after both 24 and 48 hrs (Figures 5.5a & 5.5b) there seems to be a significant reduction in the ability of the bacteria to adhere to the tooth's surface. It may be that the plant extract forms a protective coat on the tooth's surface and prevents the bacteria from binding to the pellicle (Figures 5.1b, 5.2 & 5.4). The extract appears to be even more effective than the positive control, Repelcote® (Figure 5.1c), as seen in Figures 5.1b, 5.2, 5.3a & 5.4.

5.5 Conclusion

The anti-adherence effect of *H. natalensis* was investigated in two ways: through cytokine evaluation as epithelial cells release IL-8 upon infection with bacteria, and ultra-structure determination. The cytokine, IL-8, levels were not reduced when the extract of *H. natalensis* was added before, together and after *A. israelii*; indicating that *H. natalensis* was unable to prevent an interaction between the HEp-2 cells and the bacteria and therefore unable to prevent adherence. The highest concentration of *H. natalensis* extract utilized was 12.5 µg/ml. According to the dose response curve, (Figure 3.1b) 50 µg/ml of the plant extract leads to the inhibition of growth of HEp-2 cells. A higher plant extract of 25 µg/ml could be safely utilized in future investigations; as it is still well below the obtained MIC of 0.88 mg/ml.

Although *H. natalensis* was unable to prevent *A. israelii* from infecting cells it appears to be able to prevent *S. mutans* from adhering to pellicle on the tooth surface as well as interfering with the glucan binding of *S. mutans* inhibiting the bacteria from adhering to each other as well. This indicates that *H. natalensis* worked on an infectious mechanism of *S. mutans* other than that of *A. israelii*.

5.6 References

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Chapter 6

Isolation and identification of bioactive compounds of *Heteropyxis natalensis*

6.1 Introduction

Heteropyxis natalensis exhibited activity against both Gram-positive microorganisms, *Actinomyces israelii* and *Streptococcus mutans* and the Gram-negative bacteria, *Prevotella intermedia*. With moderate cytotoxicity (33.66 ± 0.04 µg/ml), best antibacterial activity and the ability to interfere with pellicle formation and glucan binding of *S. mutans* to the enamel surface of the tooth, *H. natalensis* was selected for bioassay guided fractionation. However, researches have previously isolated a few compounds from this plant.

The leaves of *H. natalensis* were examined for their phytochemical potential. A dichloromethane extract was eluted through silica gel from which a yellowish crystalline compound was obtained. It was determined to be a chalcone isomer of aurentiacin A and triangularin previously isolated from *Myrica serrate* and *Pityrogramma triagularis*. Spectroscopic data established the compound to be (*E*)-1-(2',4'-dihyroxy, 5'-methoxy, 3'-methylphenyl)-3-phenylprop-2-en-1-one (Adesanwo *et al*., 2009). Preliminary results from a study showed that (2E)-2-[(2E)-1-hydroxy-3-phenylprop-2-en-1-ylidene]-5-methoxy-6,6-dimethylcyclohex-4-ene-1,3-dione, commonly known as ceroptin was also present in the leaf extract of *H. natalensis* (Shode *et al*., 2005)

The twigs and roots of *H. natalensis* were also investigated for phytochemicals. A CH_2Cl_2 extract of the twigs yielded 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone; 3',4',5'-tri-*O*-methyl-3,4-methylenedioxyellagic acid and lupane derivatives betulinic acid. Hexane extracts of the twigs yielded lupenone and lupeol; and from the roots 3β-hydroxylup-20(29)-en-28-al and sitost-4-en-3-one (Mohammed *et al*., 2009).

6.2 Material and methods

6.2.1 Preparation of extract

Two kilograms of air-dried leaves and twigs of the plant were milled into a fine powder using a commercial grinder. The powder was extracted three times, each time with 2.5 L of ethanol for two days. The total ethanolic extracts were combined and concentrated under reduced pressure at 40° C.

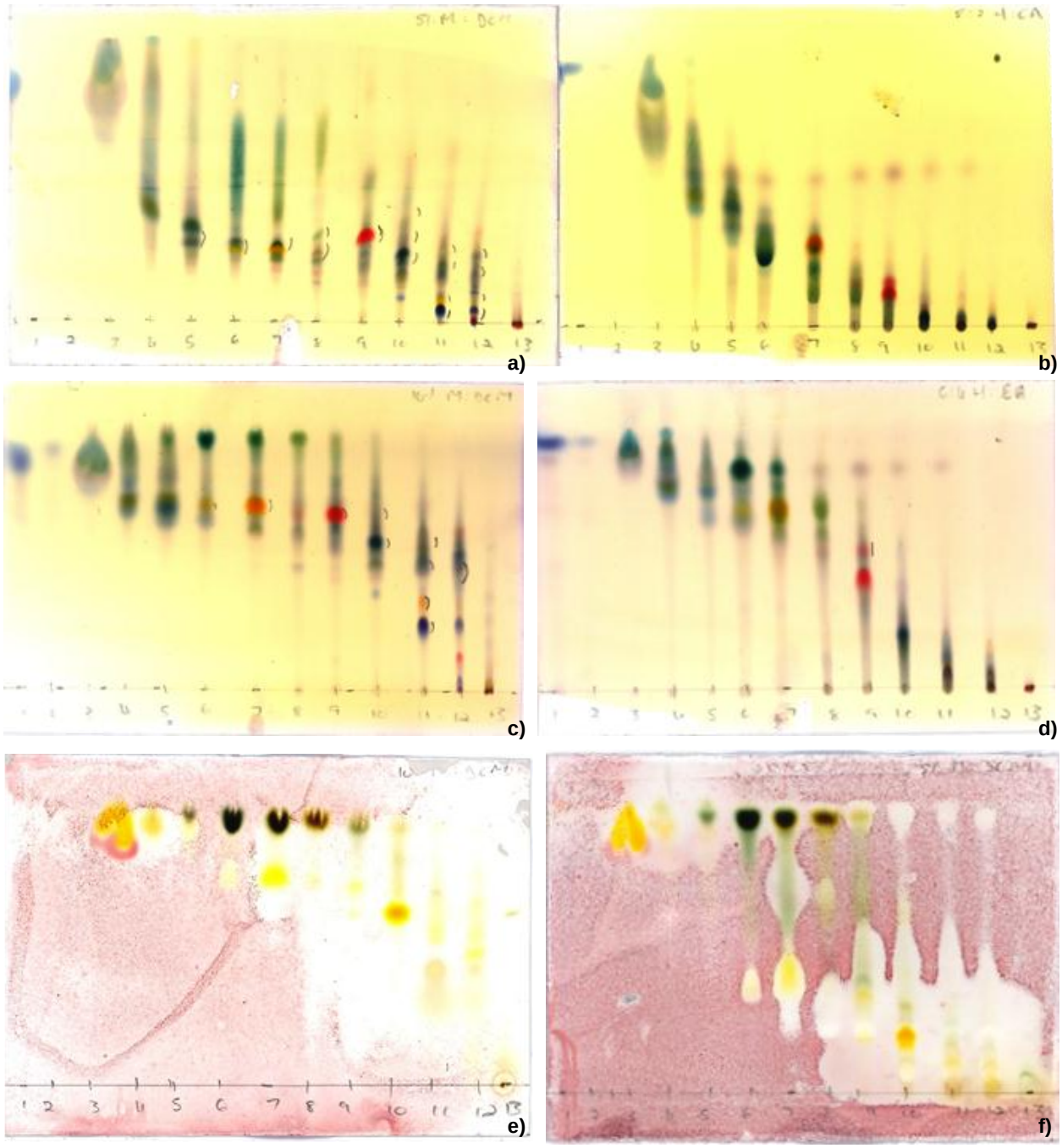

Figure 6.1: Thin layer chromatography of the 13 pooled fractions developed in a) dichloromethane:methane (99.5:0.5); b) hexane:ethyl acetate (8:2); c) dichloromethane:methane (99:1); d) hexane:ethyl acetate (6:4). The pooled fractions were developed in e) dichloromethane:methane (99:1), and f) dichloromethane:methane (99.5:0.5) sprayed with *Actinomyces israelii* and developed with INT.

6.2.2 Purification of active compounds

The dried ethanolic extract (60 g) was subjected to fractionation on a silica column (10 x 70 cm) using a gradient of hexane:ethyl acetate of increasing polarity (0% to 100% ethyl acetate) as eluent. Twenty-nine fractions were collected and those with similar thin layer chromatography (TLC) profiles were combined together (TLC plates were developed using hexane:ethyl acetate (6:4); hexane:ethyl acetate (8:2); dichloromethane:methane (99.5:0.5) and dichloromethane:methane (99:1) as eluent. Acidic vanillin; 0.34% vanillin in 3.5% sulphuric acid

in methanol; was used for detection). Thirteen major fractions (1B to 13B) were obtained and tested for antibacterial activity against *A. israelii*, as *H. natalensis* exhibited the best antimicrobial activity against *A. israelii* (0.88 mg/ml) in this study (Figure 6.1 and Table 6.1).

Table 6.1: Average minimum Inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the Fractions against *Actinomyces israelii*

Fraction	MIC (mg/ml)	MBC (mg/ml)
1B	>12.5	Na[a]
2B	>12.5	Na
3B	>12.5	Na
4B	>12.5	Na
5B	9.375	9.375
6B	9.375	9.375
7B	0.830078	1.611328
8B	0.830078	2.34375
9B	1.5625	2.34375
10B	2.213542	8.203125
11B	4.25	12.5
12B	12.5	Na
13B	1.822917	5.46875
Positive control[b]	0.018311	0.048828

[a]Na: Not active; [b] Chlorhexidine

Based on the antibacterial results, availability of substantial amounts of the fractions and preliminary TLC profiles; Fractions 7B (2 g), 9B (3 g), 11B (0.8 mg) and 12B (1 g) were chromatographed separately using Sephadex columns (Sigma-Aldrich, South Africa). Fraction 7B was chromatographed on a sephadex column eluted with dichloromethane:methane (99.5:0.5). Sixty-four subfractions were collected, spotted on TLC plates and developed in dichloromethane:methane (99:1) to yield pure compound **1** (87 mg, 0.15%). Fraction 9B was chromatographed on a sephadex column eluted with 100% ethanol. Ninety-one subfractions were collected, spotted on TLC plates and developed in dichloromethane:methane (99.5:0.5) as eluent which yielded pure compounds **2** (2 mg, 0.003%) and **3** (22 mg, 0.037%). Fraction 11B was chromatographed on a sephadex column eluted with dichloromethane:methane (99:1). Fifty-five subfractions were collected, spotted on TLC plates and developed in dichloromethane:methane (99:1) eluent, yielding pure compound **4** (75 mg, 0.13%). Fraction 12B was chromatographed on a sephadex column eluted with dichloromethane:methane (99:1). Seventy-one subfractions were collected, spotted on TLC plates and developed in dichloromethane:methane (99:1), yielding pure compound **5** (7mg, 0.012%) (Figure 6.2).

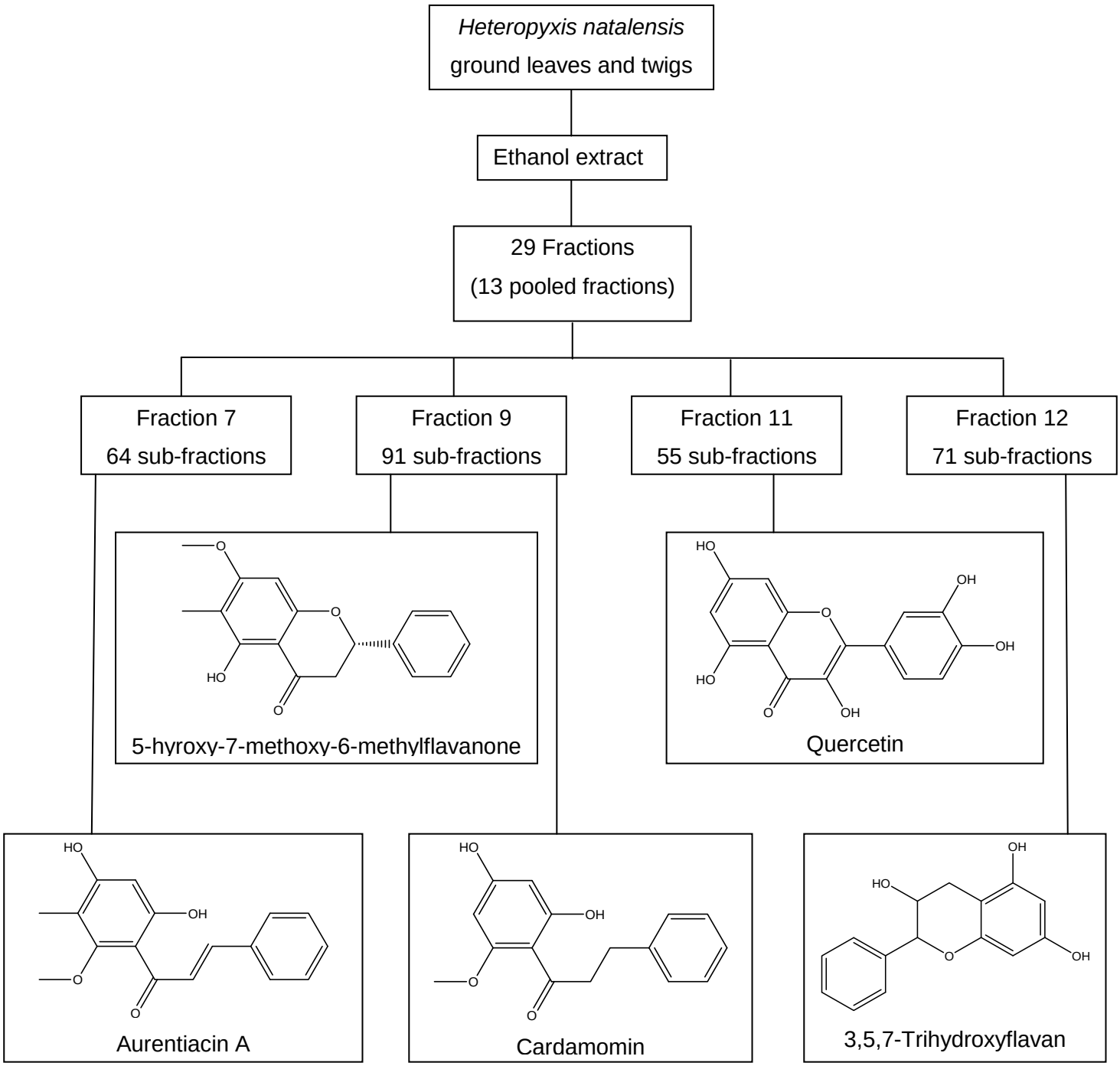

Figure 6.2: Isolation of compounds from the ethanol extract of *Heteropyxis natalensis*

6.2.3 Results and discussion

The identification of the five isolated compounds was done by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and distortionless enhancement by polarization (DEPT). Bioassay-guided isolation of an ethanolic extract of *H. natalensis* leaves and twigs, using bioautographic TLC antibacterial assays, led to the isolation of compounds aurentiacin A (**1**) and quercetin (**4**) as active principles. In addition, compounds cardamomin (**2**) and 3,5,7-trihydroxyflavan (**5**) were isolated as inactive constituents.

All compounds were tested for antibacterial activity against *A. israelii* and cytotoxicity on Human laryngeal epidermoid carcinoma (HEp-2) cells using the methods previously discussed in chapter 3.

Table 6.2: The minimum inhibitory concentration (MIC), minimum bactericidal concentrations (MBC) and 50% inhibition of cell growth (IC_{50}) of the isolated compounds against *Actinomyces israelii* and on HEp-2 cells

Isolated compounds	MIC(mg/ml)	MBC (mg/ml)	IC_{50} (µg/ml) + S.D.
Heteropyxis natalensis	1.5625	1.5625	33.66 ± 0.04
5-hydroxy-7-methoxy-methylflavanone (**3**)	-	-	-
Aurentiacin A (**1**)	0.0625	0.0625	6.36 ± 5.095
Cardamomin (**2**)	>1	NA[a]	52.12 ± 9.41
3,5,7-trihydroxyflavan (**5**)	>1	NA	>200
Quercetin (**4**)	1	1	186.7 ± 42.605
Positive control	0.024[b]	0.024[b]	$9.6x10^{-3}$ ± 0.0003[c]

[a] NA: Not active; [b] Chlorhexidine; [c] Actinomycin D

Only one of the compounds showed activity below 1 mg/ml against *A. israelii*, namely aurentiacin A, at 62.5 µg/ml (Table 6.2). The positive control, chlorhexidine gluconate, activity is not much further below aurentiacin A's activity (MIC of 24 µg/ml). Cardamomin (**2**) and 3,5,7-trihydroxyflavan (**5)** suggested activity above 1 mg/ml and quercetin (**4**) showed activity at 1 mg/ml. Due to the small quantity of 5-hydroxy-7-methoxy-methylflavanone (**3**) obtained, further analysis of properties could not take place.

6.2.3.1 Fraction 9B subfraction 2-5, sub-subfraction 4

Compounds **2** and **3** were identified as chalcones with different substitution patterns in ring B, while ring C is not substituted. Compound **3** was identified based on the spectral data of ^{1}H and ^{13}C NMR. It showed in ^{1}H NMR signals of an unsubstituted ring C at 7.72 (2H 2, 6), 7.45 (3H, 3, 4, 5). It also showed signals of trans alkene at 8.05 and 7.73 (1H, d each, J=15.4 Hz) and two aromatic protons at 6.08, 6.02 (*s* each, H3' and 5') in addition to a methoxyl signal at 3.97.

The ^{13}C NMR data showed 16 carbon signals including a carbonyl group at 193.0 ppm. DEPT-135 showed 10 protonated carbons one of them is the methoxyl group at δ_C 56.4 and two α,β double bonds adjacent to the carbonyl group at 142.6 and 128.4; while the other signals were attributed to the aromatic carbons of both ring B and ring C (Figures 9.9 & 9.10, Appendix B).

The data given established the structure of Cardamomin, or cardamonin, as compound **2**. Cardamomin is a 2'- Me ether derivative of 2',4',6'-Trihydroxychalcone and has been previously isolated from *Alpinia katsumadai*, *Boesenbergia pandurata*, *Comptonia peregrina*, *Myrica*

pensylvanica, *Piper* sp., *Populus* sp. and *Dracaena draco* (Dictionary of Natural Products, 2011).

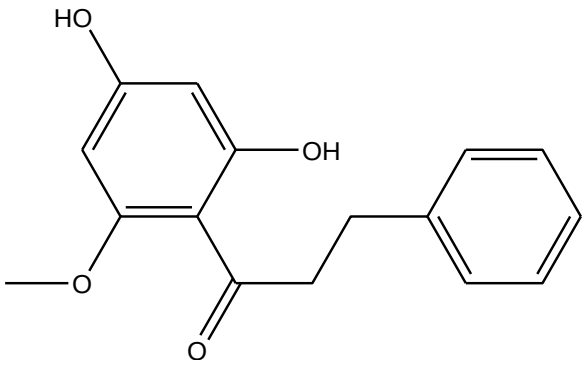

Figure 6.3: Cardamomin

Cardamomin, from the *B. pandurata* rhizome, is traditionally used in Southeast Asia for the treatment of dry mouth, stomach discomfort, leukorrhea and dysentery. Cardamomin exhibited inhibition of nitric oxide (NO), an inorganic free radical and inflammatory mediator. An IC_{50} of 24.7 µM was obtained on lipopolysaccharide (from *Salmonella enteritidis*) induced NO release from murine macrophage cells (Dictionary of Natural Products, 2011; Tewtrakul *et al*., 2009). The anti-oxidative activity of cardamomin was also quantified from the scavenging ability of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and the superoxide anion, O_2 but no anti-oxidative activity was exhibited under these circumstances. Isolated from *Zingiberacea* species, cardamonin exhibited strong anti-platelet aggregation (Jantan *et al*., 2008).

In the present study cardamomin (**2**) suggested antibacterial activity above 1 mg/ml against *A. israelii*, which may be comparative to the results obtained by cardamomin, isolated from the seeds of *A. katsumadai*, which exhibited antibacterial activity against *Helicobacter pylori*, *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli* with MICs ranging between 0.12-2.56 mg/ml (Huang *et al*., 2006). Cardamomin exhibited strong cytotoxicity effects against HEp-2 cells with an IC_{50} of 52.12 ± 9.41 µg/ml which correlated with the cytotoxicity of cardamomin investigated on human normal hepatocytes (L-02) and human hepatoma cells (HepG2). Cardamomin exhibited strong cytotoxicity effects on L-02 and HepG2 with IC_{50} of 30.90 ± 4.03 and 22.63 ± 3.00 µM respectively (Li *et al*., 2008).

6.2.3.2 Fraction 7B subfraction 26 sub-subfraction 30

^{1}H NMR showed downfield signals at δ_H 13.51 ppm of a chelated hydroxyl at C-6'; signals of monosubstituted benzene ring at 7.40 (*s*, 3H), 7.67 (*s*, 2H) and another singlet signal of an aromatic proton at 6.31, two doublets at 8.02, 7.79 (d, J=15.8 Hz), a low field methyl group at 2.09 and a methoxyl group at 3.67 (Figure 9.11, Appendix B).

The ^{13}C NMR data showed 17 signals; two methyls (60.9, 7.2), eight methines at 127.1 (C-2,6), 128.3 (C-3,5), 125.9 (C-4), 142.0 (C-7), 129.6 (C-8) and 98.6 (C-3) and a carbonyl group at 191.9 (C-9) in addition to seven quaternary carbons (Figure 9.12, Appendix B).

The above data indicated the presence of a chalcone derivative with no oxygenation at ring B. Ring A showed only one signal of an aromatic proton (δ_H 6.31) which showed correlation heteronuclear multiple bond correlation (HMBC) to C-1' (107.3) and C-3' (110.1). The hydroxyl proton (δ_H 13.51*)* showed correlation with C-1' and C-5' H-5' / C6', C1', C3' and C4'. This relation could establish the substitution pattern of ring A to 2'-methoxy,4',6' dihydroxy. The compound (**1**) was identified as 2'-Me ether derivative of 2',4',6'-trihydroxy-3'-methylchalcone; or more commonly known as aurentiacin A.

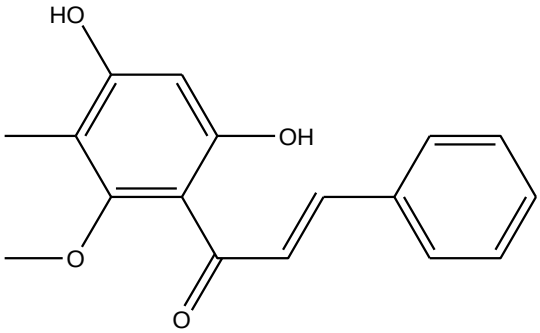

Figure 6.4: Aurentiacin A

Aurentiacin A has previously been isolated from *Didymocarpus aurentiacum*, *C. peregrina*, *Dalea* species including *Dalea scandens* var. *paucifolia* (medicinal value in Mexico), *M. pensylvanica* and also from *Dracaena* species (Dictionary of Natural Products, 2011; Dominguez *et al*., 1980).

Aurentiacin A (**1**) was the only isolated compound from *H. natalensis* to exhibited antibacterial activity under 1 mg/ml (62.5 µg/ml). Gafner *et al*., (1996) indicated that Aurentiacin A, inhibited the growth of *Cladosprium cucumerinum*, *Bacillus subtilis* and *E. coli*. This compares favourably with the results obtained in this study. The cytotoxicity of this compound on HEp-2 cells appears to be very toxic (IC_{50} value of 21.05 µg/ml).

6.2.3.3 Fraction 9B subfraction 2-5, sub-subfraction 2

The compound showed to be C-flavonoid from its spectroscopic data. The ^{1}H NMR showed signals 5.44 (d, J=14.2 Hz) of H2. Two H-3 protons appeared at 2.64 (d, J=16.6 Hz) and 3.04 (dd, 16.6, 14.2 Hz). The signal at 6.35 (*s*) was attributed to H-8 and those at 7.40 and 7.53 attributed to monosubstituted ring B, methyl (attached at C-6) appears at 2.04 and a methoxyl group at 3.76 (Figure 9.13, Appendix B).

^{13}C NMR and DEPT-135 showed a two methyl signals at 61.0 and 8.0, a methylene group at 45.9 and 5 methine signals at 79.3 (C2), 100.0 (C8), 126.6, 129.1 and 129.2 of ring B. Other signals for quaternary carbons belong to C-4 (188.0), C-7(163.4), C-5(162.9), C-9(116.5), C1' (140.4) and C-6 (113.7) (Figure 9.14, Appendix B).

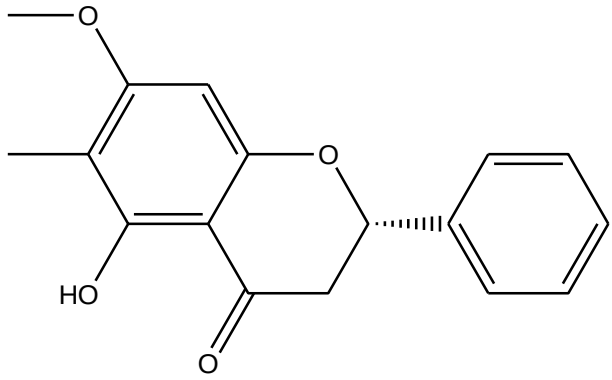

Figure 6.5: 5-hyroxy-7-methoxy-6-methylflavanone

The above mentioned data established the structure of the compound (**3**) as 5-hyroxy-7-methoxy-6-methylflavanone. Previously isolated from *Leptospermum scoparium* (used in Australian and New Zealand traditional medicine), *Leptospermum recurvum*, *Piper carniconnectivum*, *Pseudotsuga wilsoniana* (used as building wood in Taiwan), the seeds of *Myrica gale* (fruit is a beer additive, essential oil is an insect repellent) and a trace constitute of *P. triangularis* (Dictionary of Natural Products, 2011; Facundo & Braz-Filho, 2004; Fang *et al*., 2011; Hsieh *et al*., 1997; Mayer, 1989; Mustafa *et al*., 2003).

According to literature, no medicinal activity of this compound has been reported and due to the small quantity of 5-hydroxy-7-methoxy-methylflavanone (**3**) obtained, further analysis was not done.

6.2.3.4 Fraction 12B subfraction 56, sub-subfraction 70

The ^{1}H NMR showed five aromatic signals at 6.80 (m), two meta coupled protons at 5.92, 5.84 (br. *s* each), two protons germinal to hydroxyl groups at 4.55 (d, J=7.0 Hz), 3.36 (m) [C-2, C-3], in addition to methylene protons at 2.84 (dd, J=16.1, 4.8 Hz) and 2.49 (dd, J=16.1, 8.4 Hz) (Figure 9.15, Appendix B). This compound has two possibilities, depending on the alpha D value.

The above ^{1}H NMR data established the structure of compound **5** as 3,5,7-Trihydroxyflavan. There are two variants of the compound, namely the 2*R*, 3*R* and 2*R*, 3*S* forms. The 2*R*, 3*R* form is known as distenin, previously isolated from *Dennstaedtia distenta*; while the 2*R*, 3*S* form is known as 3-Oxykoaburagenin, previously isolated from the leaves of *Enkianthus nudipes* (Dictionary of Natural Products, 2011).

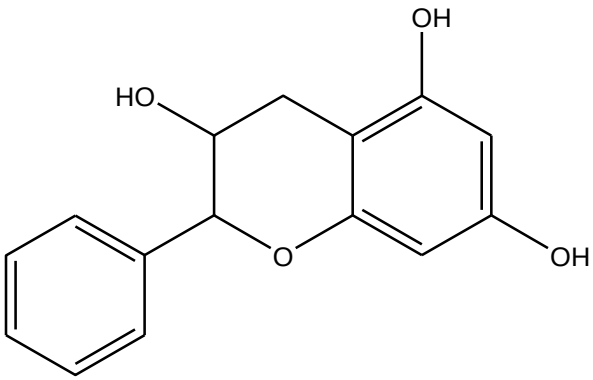

Figure 6.6: 3,5,7-Trihydroxyflavan

In the present study, 3,5,7-trihydroxyflavan (**5**) indicated activity above 1 mg/ml against *A. israelii* and little toxicity against HEp-2 cells at >200 µg/ml. According to literature, no medicinal activity of this compound has been reported.

6.2.3.5 Fraction 11 subfraction 10

This compound was identified as the well-known flavonoid, quercetin. The ^{1}H NMR showed a typical quercetin signal at 7.82 (br. *s*, H-2'), 7.70 (br. d, J=8.0 Hz, H-6'), 6.99 (br. d, J=8.0 Hz, H-5'), 6.52 (br. *s*, H-8) and 6.26 (br. *s*, H-6) (Figure 9.16, Appendix B).

The above mentioned data established the structure of the compound (**4**) as 3,3',4',5,7-pentahydroxyflavone, or more commonly known as quercetin. This compound has been previously isolated from many plant species, especially fruits, such as *Helichrysum*, *Euphorbia* and *Karwinskia* spp. The compound is present in almost all species of the Umbelliferae family and is present in the Solanaceae, Rhamnaceae and Passifloraceae families. In Brazil, leaves of *Desmodium adscenens*, known to contain quercetin, are used in traditional medicine to treat body aches and pains, leucorrhoea, excessive urination, gonorrhoea and diarrhoea. The United States Food and Drug Administration (FDA) has approved quercetin as an over-the-counter supplement. Quercetin may support cardiovascular function, reduces cholesterol, promotes prostate health and joint function as well as alleviate allergies and improve the immune system (Dictionary of Natural Products, 2011; Geoghegan *et al*., 2010; Muanda *et al*., 2011).

Quercetin is a general flavonoid pigment in propolis, a resinous substance manufactured by honeybees. Propolis has renowned medical properties and quercetin was investigated for its anti-inflammatory properties in mouse induced oedema and antibacterial activity towards *S. aureus*. Quercetin reduced inflammation by 50% in the mouse model after three hours with a decrease in anti-inflammatory activity after six hours. The growth of *S. aureus* was inhibited by 20% (Du Toit *et al*., 2009).

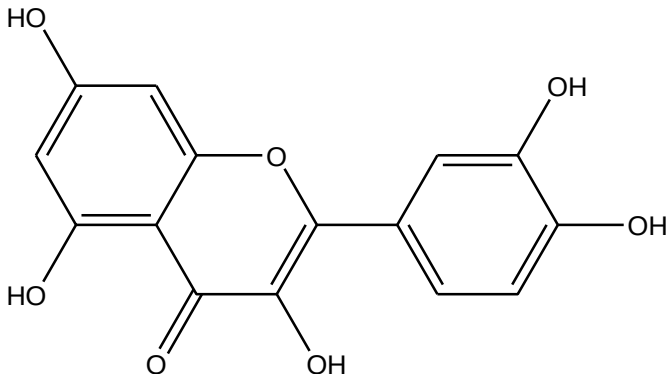

Figure 6.7: Quercetin

Quercetin (**4**) exhibited antibacterial activity against *A. israelii* with an MIC of 1 mg/ml. This result is significantly lower than the MIC obtained in a study conducted by Geoghegan *et al*. (2010), against *P. gingivalis* and *A. actinomycetemcomitans*. Quercetin was tested against *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Pseudomonas cepacia*, methicillin-resistant *Staphylococcus aureus*, *Candida albicans*, *C. glabrata* and *C. krusei*. Only two of the eight microorganisms were inhibited by quercetin, namely *P. gingivalis* and *A. actinomycetemcomitans* at MIC's of 0.0125 and 0.1 g/ml respectively (Geoghegan *et al*., 2010).

In a study by Badria & Zidan (2004), quercetin was tested against four strains of *S. mutans* and exhibited an MIC of 12.5 µg/ml for each strain. An adherence inhibition test was also conducted by examining the adherence of heat-treated cells to glass or smooth tooth surfaces and examines the presence of quercetin at 10 µg/ml. No inhibition of adherence was observed for any of the *Streptococcus* strains tested (Badria & Zidan, 2004). A greater inhibition activity is exhibited against the four strains of *S. mutans* than what was obtained in the present study against *A. israelii* (1 mg/ml).

In this study there is weak toxicity of quercetin (**4**) against HEp-2 cells as an IC_{50} of 186.7 ± 42.605 µg/ml was obtained. These results are in agreement with the results obtained by Li *et al*., (2008) where quercetin exhibited relatively weak cytotoxicity on human normal hepatocytes (L-02) and human hepatoma cells (HepG2). Cytotoxicity effects on L-02 and HepG2 with IC_{50} of 113.03 ± 6.02 and 188.84 ± 0.03 µM respectively. The anti-oxidative activity of quercetin was also quantified from the scavenging ability of DPPH and the superoxide anion, O_2. A 50% inhibition of O_2 (EC_{50}) was obtained at 6.15 ± 0.03 and 0.87 ± 0.01 µM for DPPH and O_2 respectively (Li *et al*., 2008).

6.3 Conclusion

Bioassay-guided isolation of an ethanolic extract of *H. natalensis* leaves and twigs, using bioautographic TLC antibacterial assays, led to the isolation of compounds aurentiacin A (**1**) and quercetin (**4**) as active principles. In addition, compounds cardamomin (**2**) and 3,5,7-trihydroxyflavan (**5**) were isolated as inactive constituents.

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Chapter 7

General discussion and conclusion

7.1 Discussion and conclusion

- The antimicrobial activity and safety of seven plants, *Barleria albostellata, Cotyledon orbiculata, Dichrostachys cinerea, Heteropyxis natalensis, Carpobrotus edulis, Zanthoxylum capense, Dodonaea viscosa*, generally used for traditional oral care was determined using *A. israelii*, *S. mutans*, *P. intermedia*, *C. albicans* and two cells lines, Vero and HEp-2.
- No evidence could be found in the literature of the selected plant extracts against *A. israelii*, *P. intermedia* and *S. mutans*.
- Only *H. natalensis* exhibited activity against the Gram-positive microorganisms, *A. israelii* and *S. mutans* and the Gram-negative bacteria, *P. intermedia*.
- Only *D. cinerea* exhibited activity towards *C. albicans* with an MIC of 10.71 mg/ml for *C. albicans* and 10.42 mg/ml for the polyene and azole resistant *C. albicans*.
- *Dichrostachys cinerea* was the least toxic to both the Vero and HEp-2 cell lines.
- *Heteropyxis natalensis exhibited* moderate cytotoxicity and the best antibacterial activity, hence it was selected for further study.
- *Heteropyxis natalensis* exhibited a fourfold reduction in the MIC of *A. israelii*, when used in combination with the essential oils *M. alternifolia* and *M. piperita*.
- No evidence could be found in the literature of the inhibitory concentrations of *M. piperita* essential oil and green teas Five Roses and Thé Vert on *A. israelii*.
- The cytokine, IL-8, levels were not reduced when the extract of *H. natalensis* was utilized to prevent the interaction of *A. israelii* with the epithelial cells, HEp-2.
- *Heteropyxis natalensis* interferes with pellicle formation and glucan binding of *S. mutans* to the enamel surface of the tooth.
- Five previously isolated compounds were identified for the first time from the ethanolic extract of *H. natalensis* leaves and twigs. The compounds were identified as Aurentiacin A (**1**), Cardamomin (**2**), 5-hydroxy-7-methoxy-methylflavanone (**3**), Quercetin (**4**) and 3,5,7-trihydroxyflavan (**5**). The MICs of the compounds **1** and **4** were found to be 0.0625 mg/ml and 1 mg/ml respectively against *A. israelii*. Compounds **2** and **5** exhibited no activity under 1 mg/ml against *A. israelii*. This is the first report of the isolation of the five compounds and their activity against *A. israelii*.

7.2 Recommendations for future work

- The antioxidant activity of the *B. albostellata*, *H. natalensis*, *D. cinerea* and *D. viscosa* should be investigated as evidence suggests an association between periodontal diseases and an imbalance between oxidants and antioxidants due to both an increase in free radical production and a decrease in the antioxidant activity of saliva. Reactive oxygen species (ROS) have been linked to the destruction of periodontal tissues (Alviano *et al*., 2008). A plant with antibacterial as well as antioxidant capabilities, would substantially improve oral health.
- The cytotoxicity of the synergistic combination of *M. alternifolia* and *M. piperita* essential oils with the extract of *H. natalensis* should be investigated further.
- 'Problems that still need to be addressed are stability, selectivity and bioavailabity of these natural products' as these samples (combination of *H. natalensis, M. alternifolia* and *M. piperita)* can be considered for their inclusion into an oral care formulation (Hemaiswarya *et al*., 2008).

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Chapter 8

Acknowledgements

I would like to thank my colleagues and friends, especially Karlien le Roux and Elizabeth Mogapi for their assistance with the cytotoxicity analysis; Rene Swart and Garland More for their support during my studies.

A special Thank You goes to my supervisors Prof Namrita Lall and Dr Francien Botha for their support, care and understanding.

I would like to thank the funding organization NRF/Royal Society for providing me the opportunity to go to London for training with regard to anti-adherence research. Prof Peter Houghton and Barry Hudspith, thank you for your support while I was overseas.

Thank you, Sean Gordon-Davis, for editing my dissertation.

Lastly I would like to thank Brenda and Stephen Henley-Smith, my Mom and Dad for their never ending support throughout my degree and post-graduate. This would not have been possible without you.

Chapter 9

Appendices

Appendix A

Ultra High Resolution FE-SEM

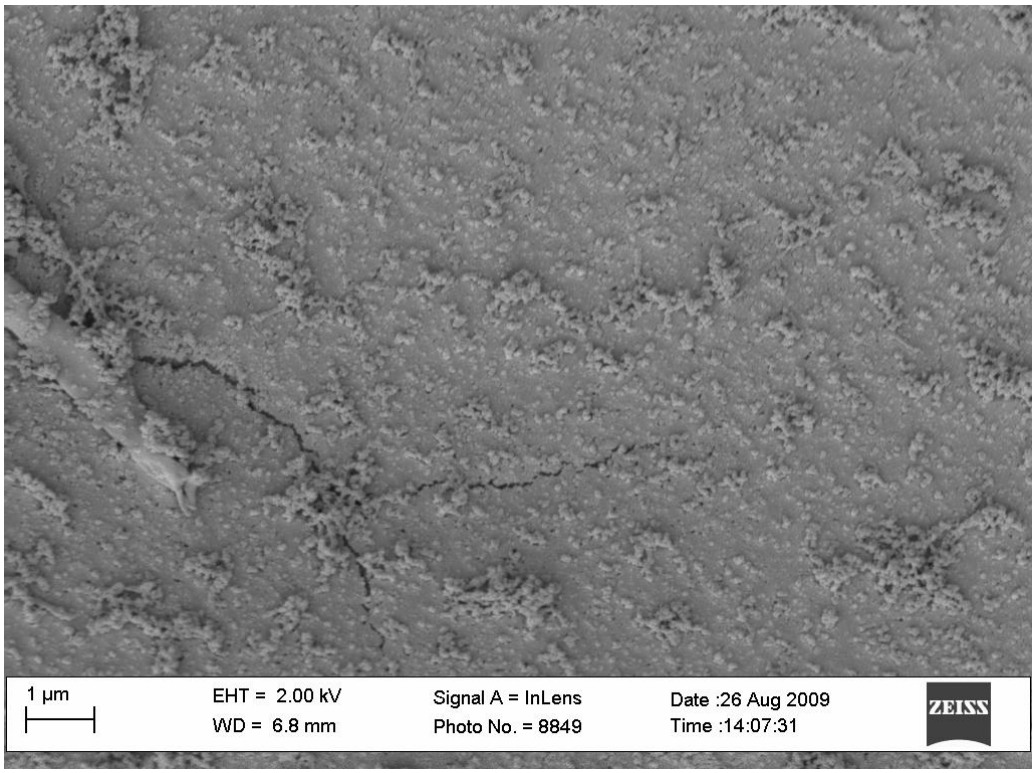

Figure 9.1: Image of enamel after Treatment 1, and 48 hrs of growth; *Heteropyxis natalensis* at an MIC of 1.82 mg/ml added 1h before *Streptococcus mutans*. The plant extract can be seen coating the enamel.

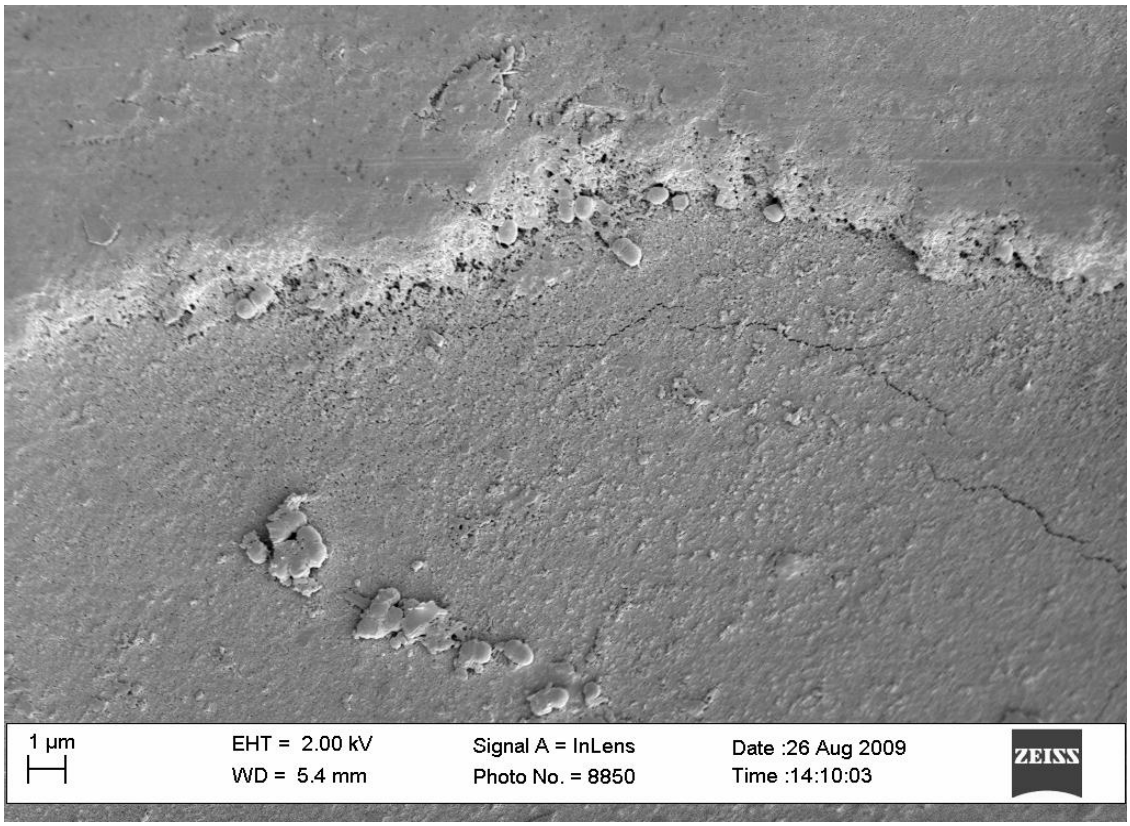

Figure 9.2: Image of enamel after Treatment 1, and 48 hrs of growth; *Heteropyxis natalensis* at a sub-MIC of 0.9 mg/ml added 1h before *Streptococcus mutans*. Cocci can be seen adhering to the enamel next to a layer formed by the plant extract.

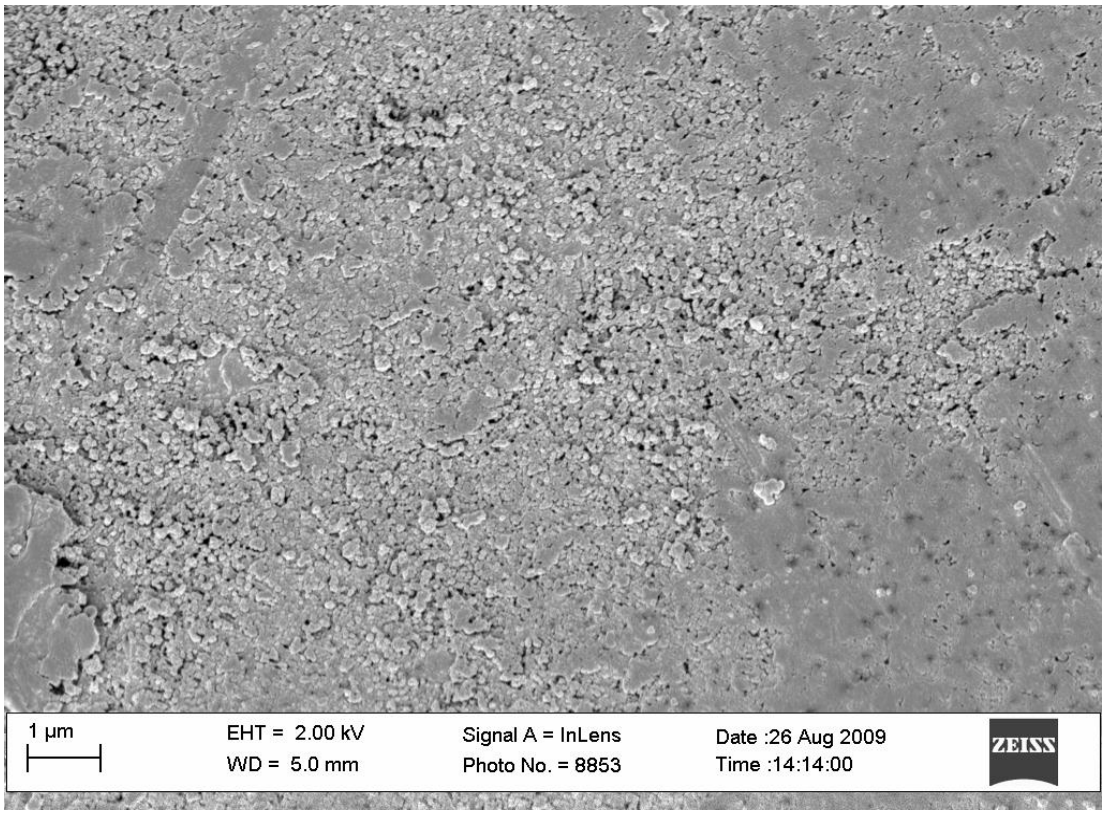

Figure 9.3: Image of enamel after Treatment 2 and 48 hrs of growth; *Heteropyxis natalensis* at an MIC of 1.82 mg/ml added together with *Streptococcus mutans*. The plant extract can be seen forming a broken layer the enamel with bacterial cocci adhering to the enamel surface. The bacterial membrane appears uneven.

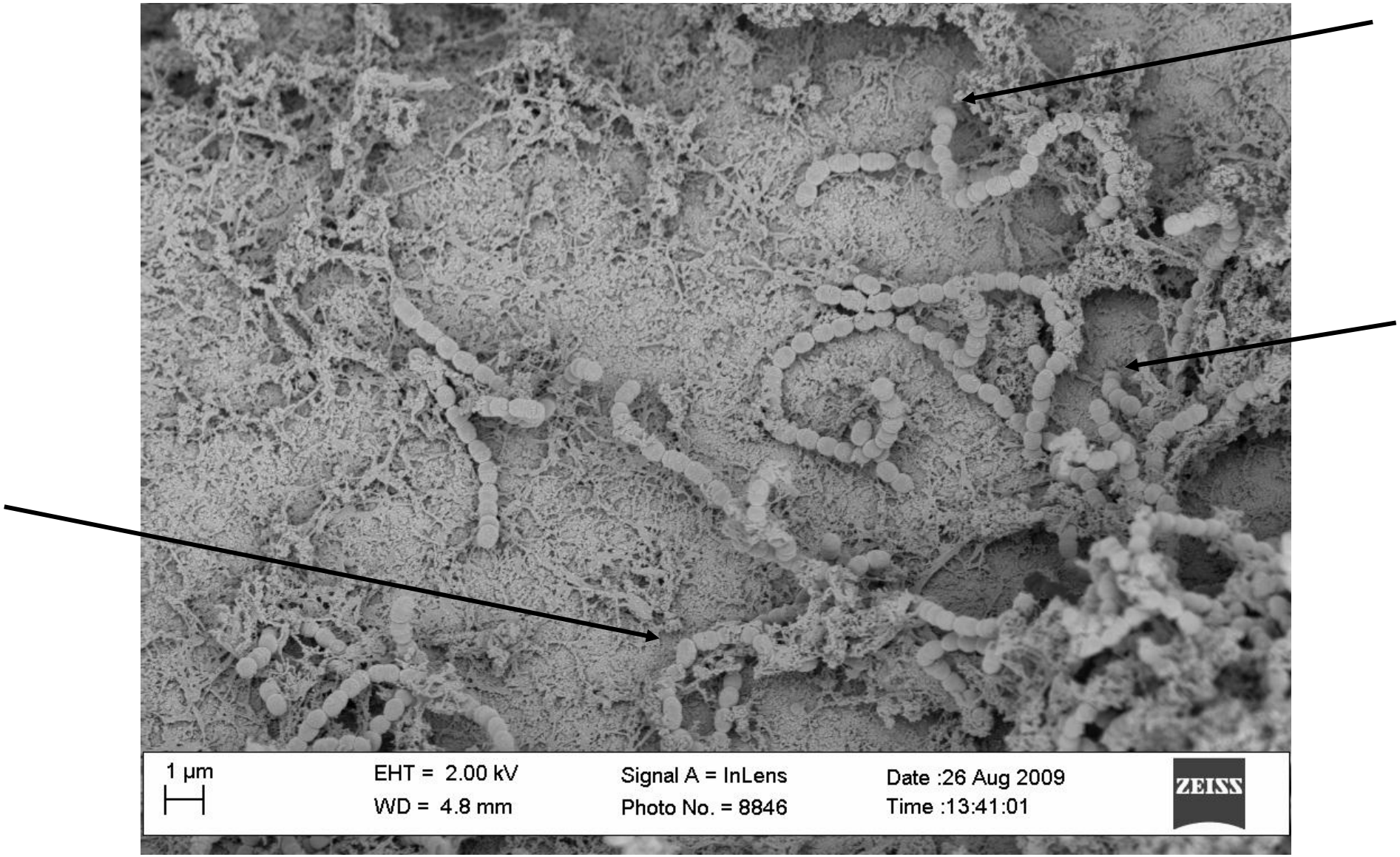

Figure 9.4: Image of enamel after Treatment 2, after 48 hrs of growth; *Heteropyxis natalensis* at a sub-MIC of 0.9 mg/ml added together with *Streptococcus mutans*. A uniform pellicle (as in Fig 9.5) was not established before bacteria were added. Some bacteria adhere to the enamel surface and then to each other to form a more solid biofilm (as in Figure 9.5). The arrows indicate formation of adhesion structure (pilli).

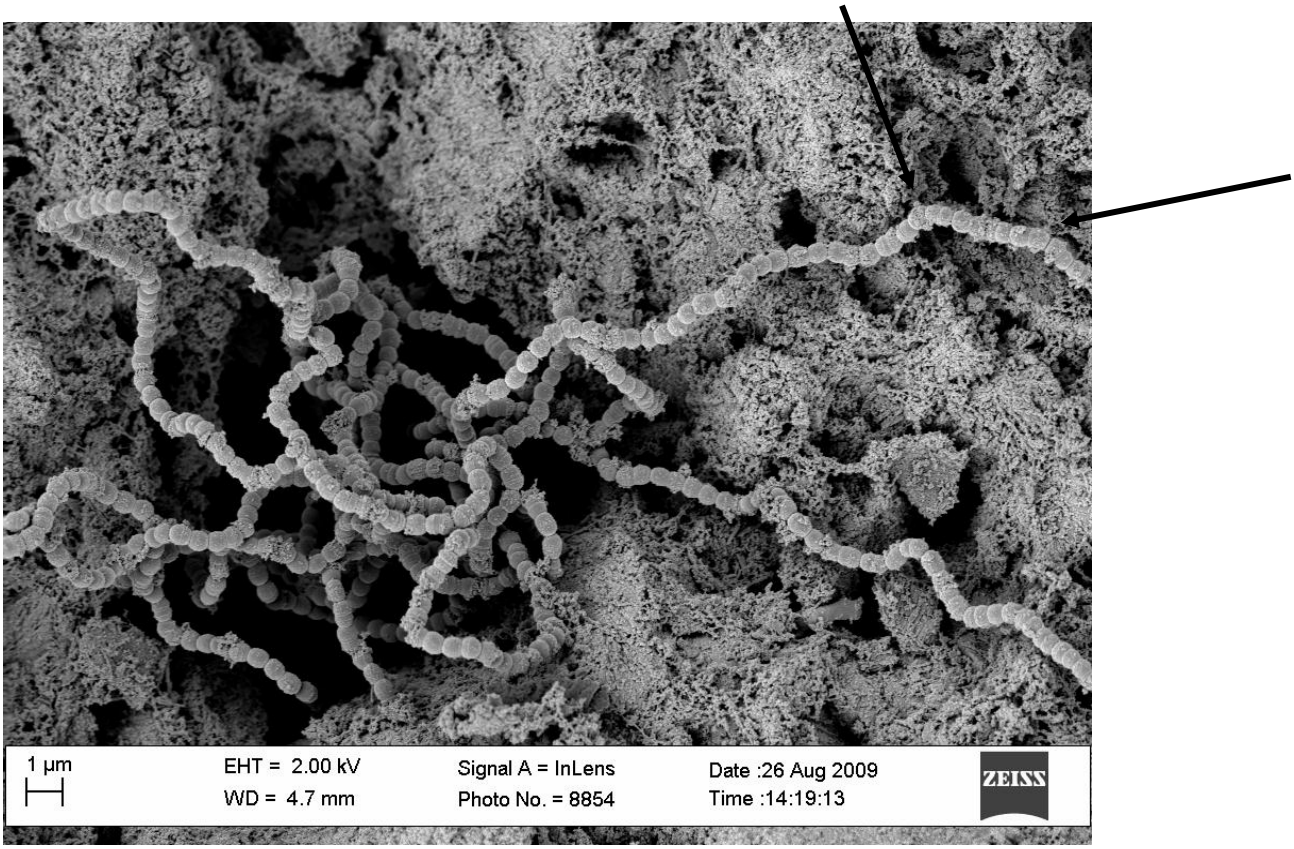

Figure 9.5: Image of enamel after Treatment 3, and 48 hrs of growth; *Streptococcus mutans* was added 1h before *Heteropyxis natalensis* at an MIC of 1.82 mg/ml. It was observed that streptococci chains adhere to each other because the plant extract prevents adhesion to the enamel. The arrows indicate that very few adhesion structures (pilli) were formed.

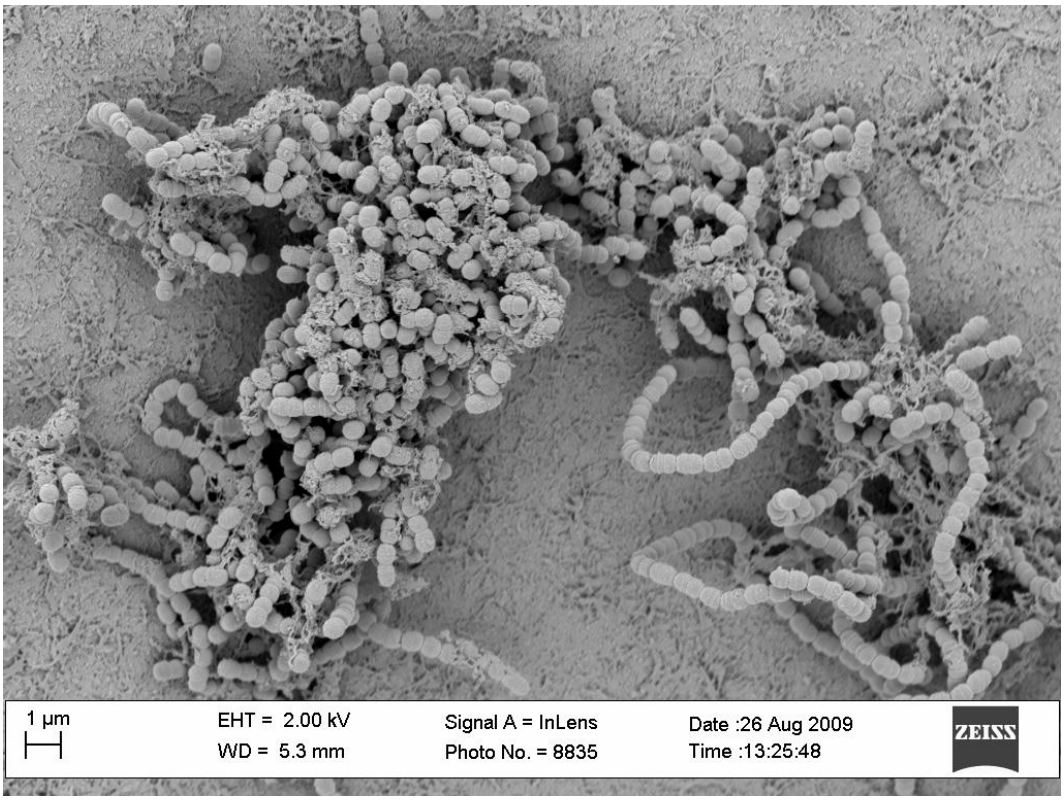

Figure 9.6: A negative control of Streptococcus mutans after 24 hrs of growth. Chain formation of the cocci is already established. Bacterial by product is seen to accumulate and collect on the enamel.

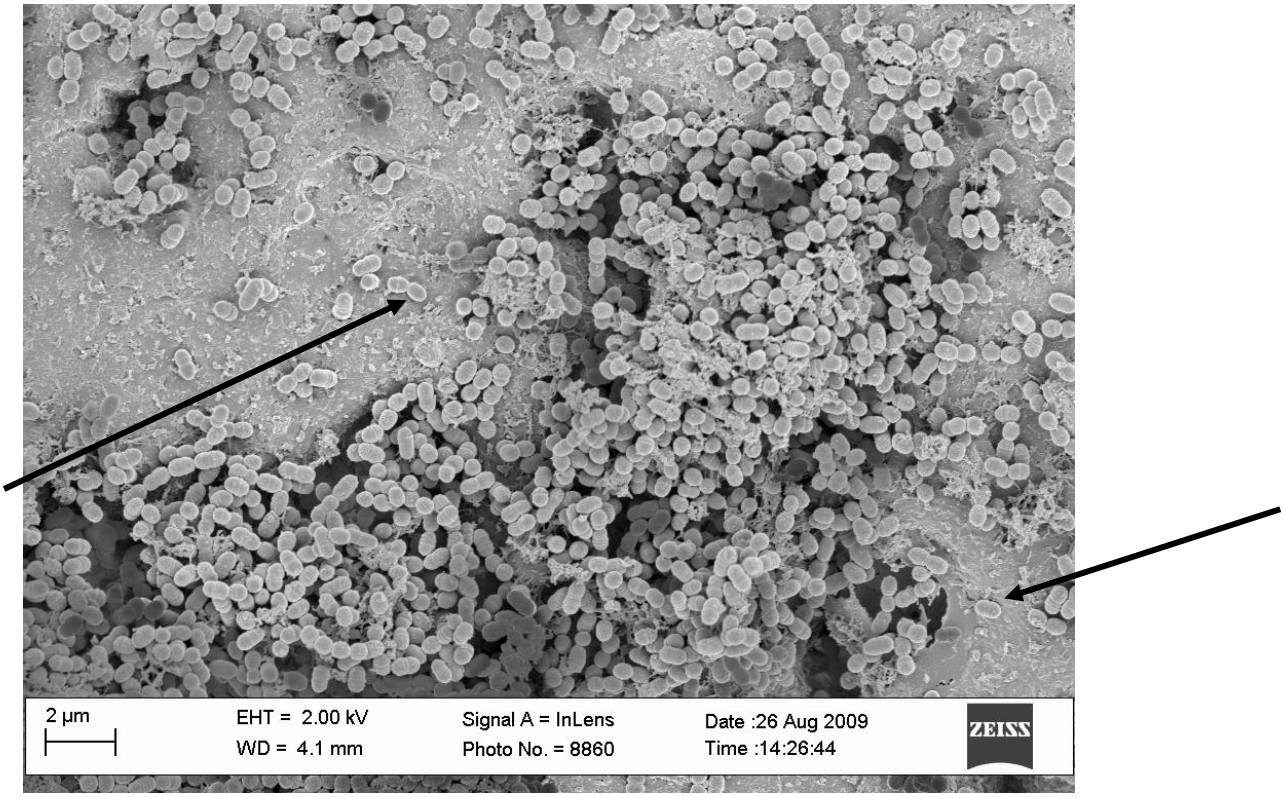

Figure 9.7: A negative control of Streptococcus mutans after 48 hrs of growth. Adhesion structures (pilli) found between bacteria and enamel.

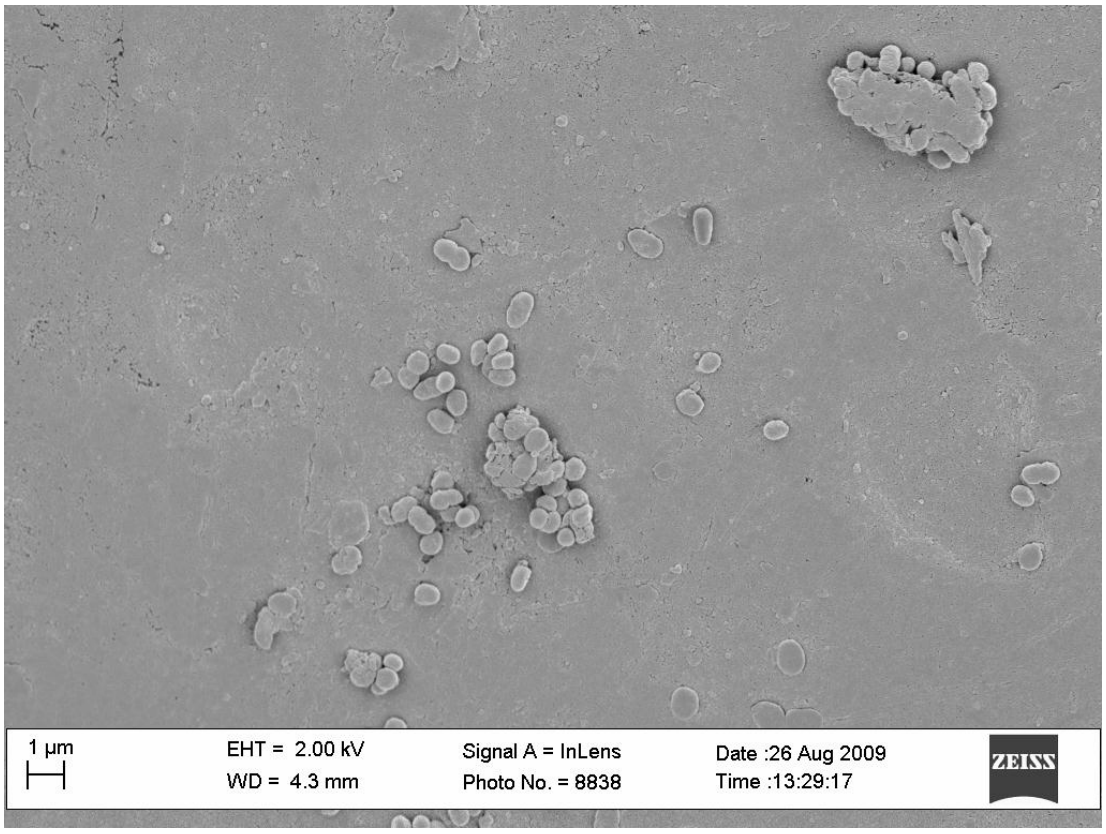

Figure 9.8: A positive control of Streptococcus mutans with the Repelcote® after 48 hrs. Only single cocci are visible on the enamel surface due the lack of pellicle formation on the smooth surface created by the Repelcote®. No clear adherence structures can be seen as in Fig 9.7.

Appendix B

^{1}H-NMR and ^{13}C-NMR of purified compounds

from *Heteropyxis natalensis*

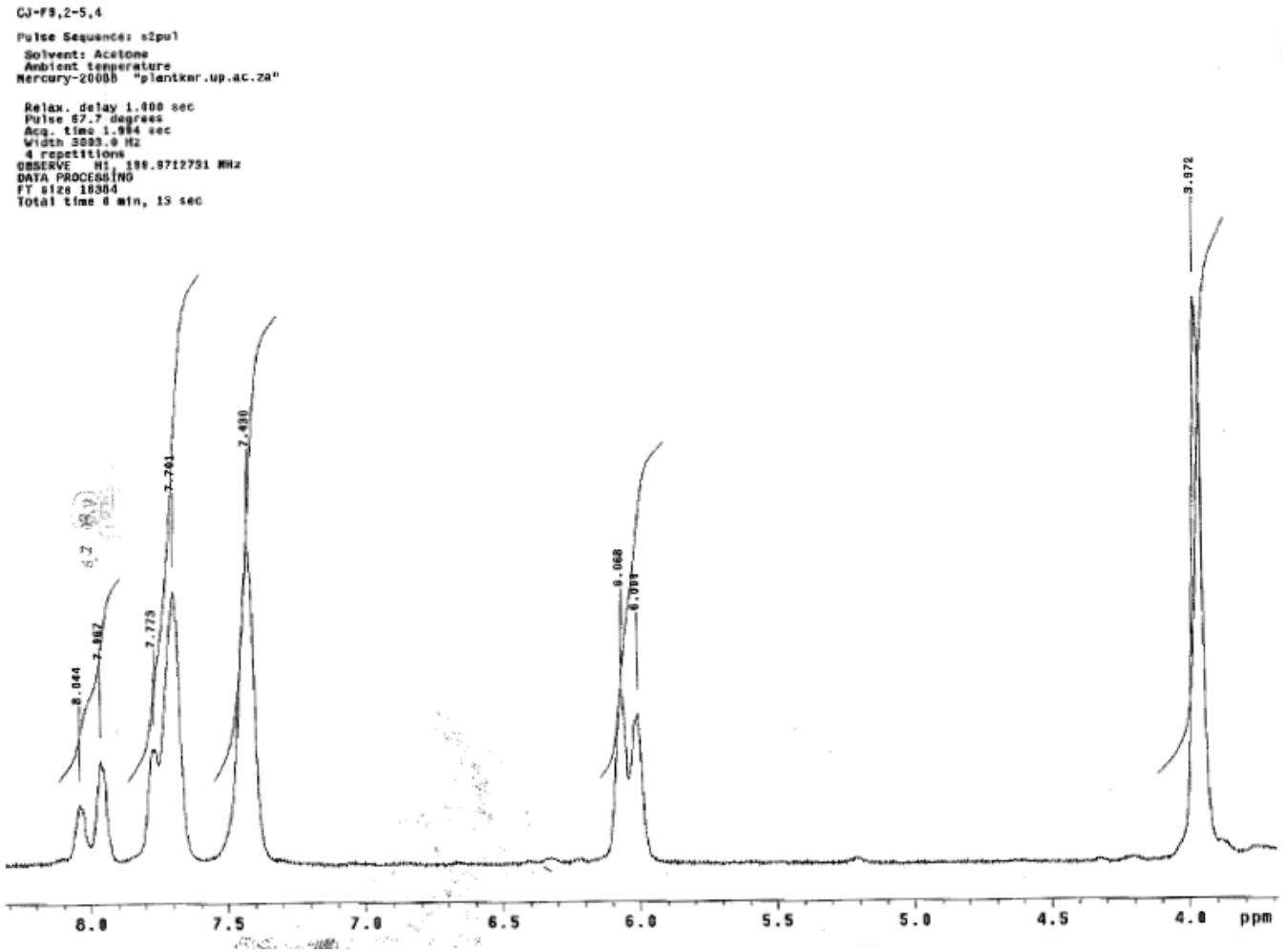

Figure 9.9: ^{1}H NMR spectra for Cardamomin, isolated from *Heteropyxis natalensis*

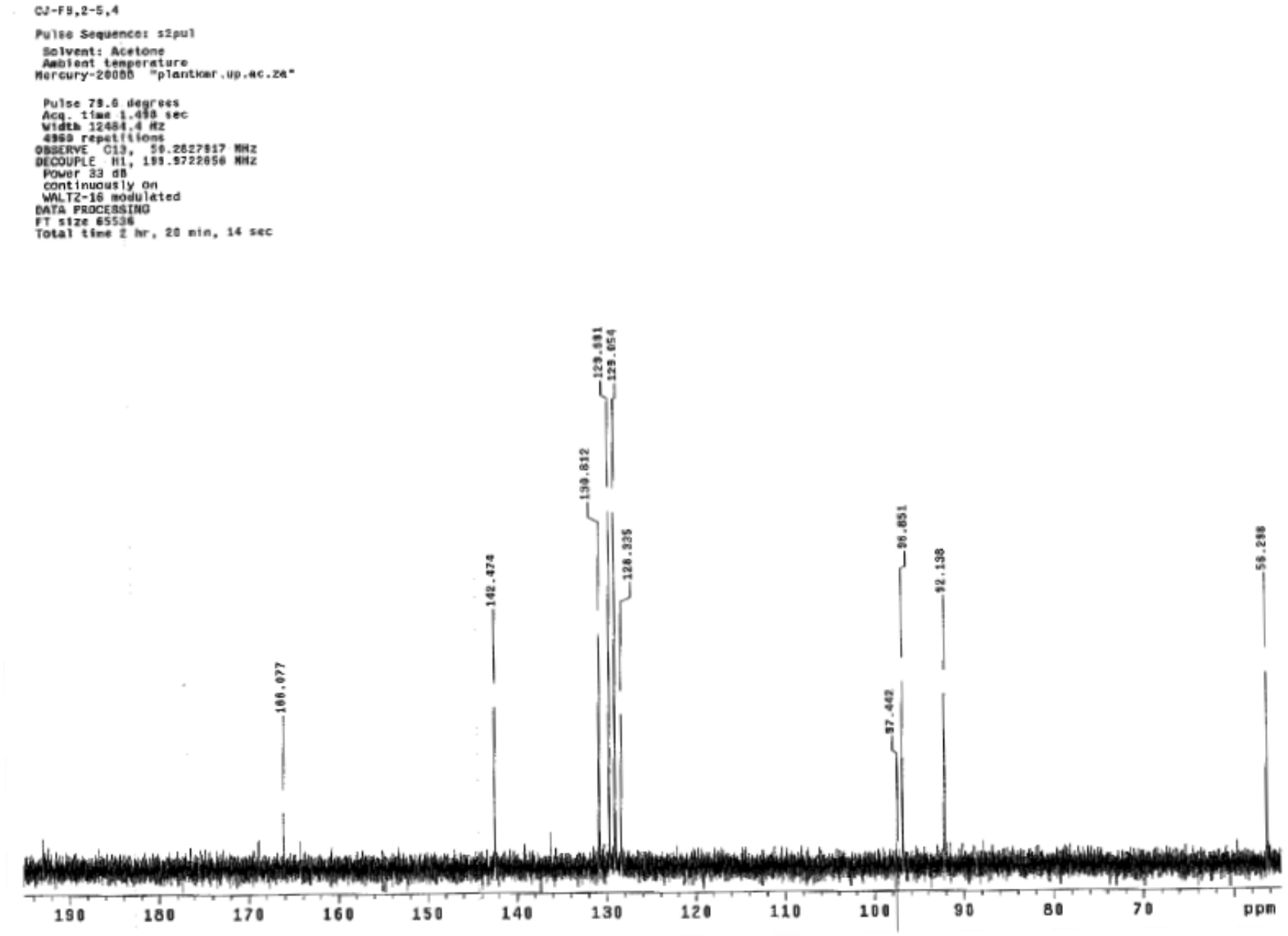

Figure 9.10: ^{13}C NMR spectra for Cardamomin, isolated from *Heteropyxis natalensis*

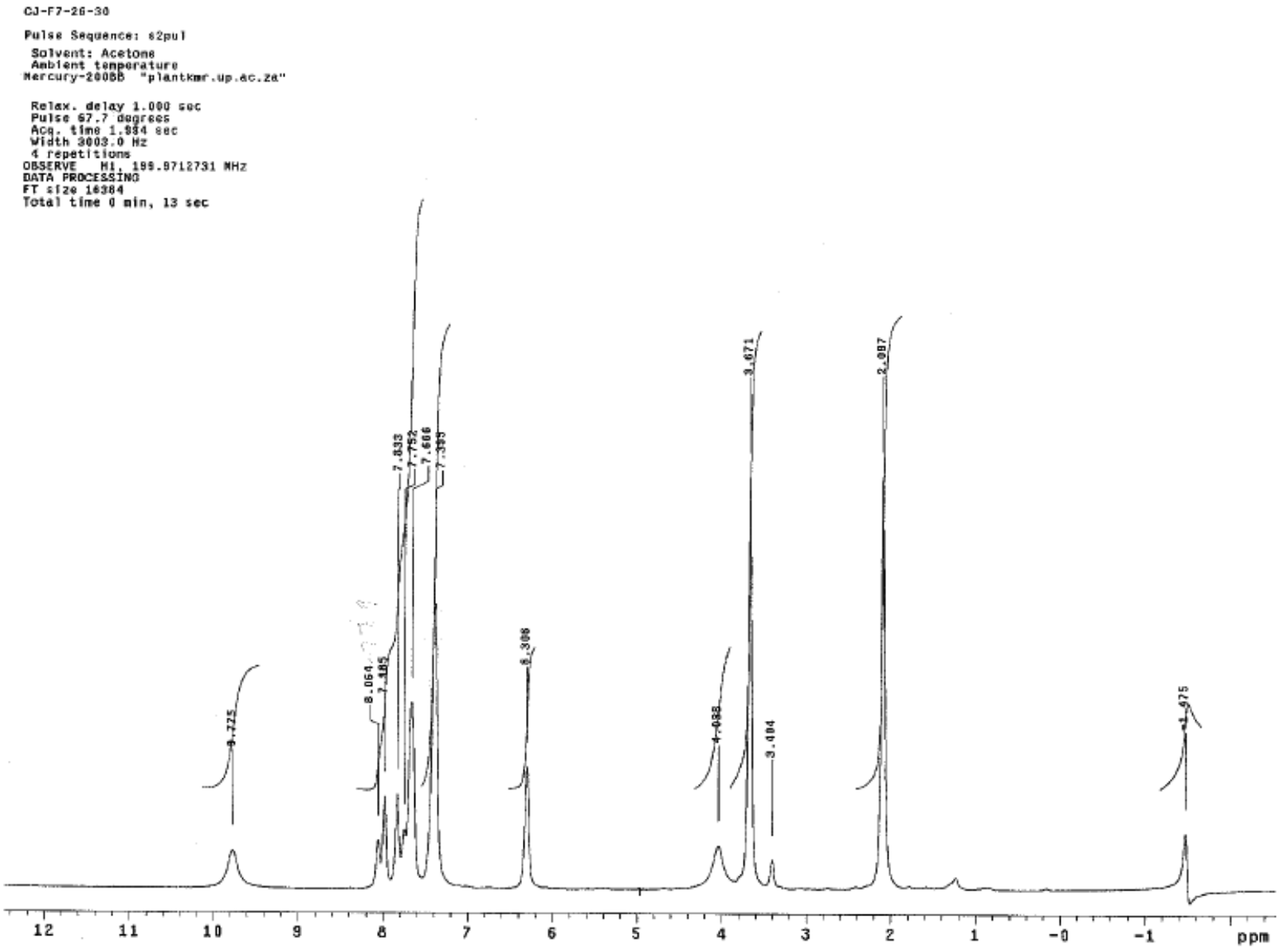

Figure 9.11: ^{1}H NMR spectra for Aurentiacin A, isolated from *Heteropyxis natalensis*

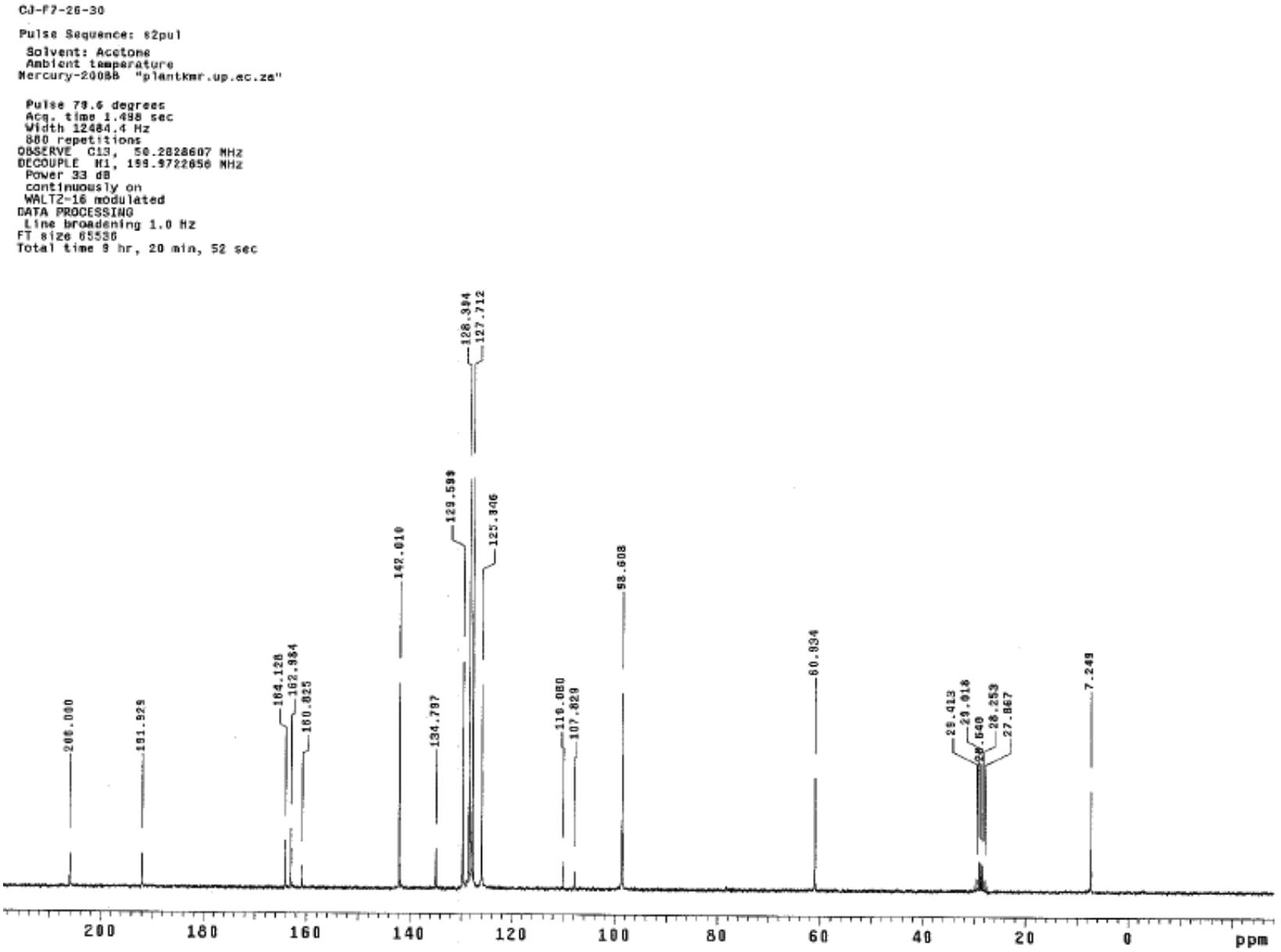

Figure 9.12: ^{13}C NMR spectra for Aurentiacin A, isolated from *Heteropyxis natalensis*

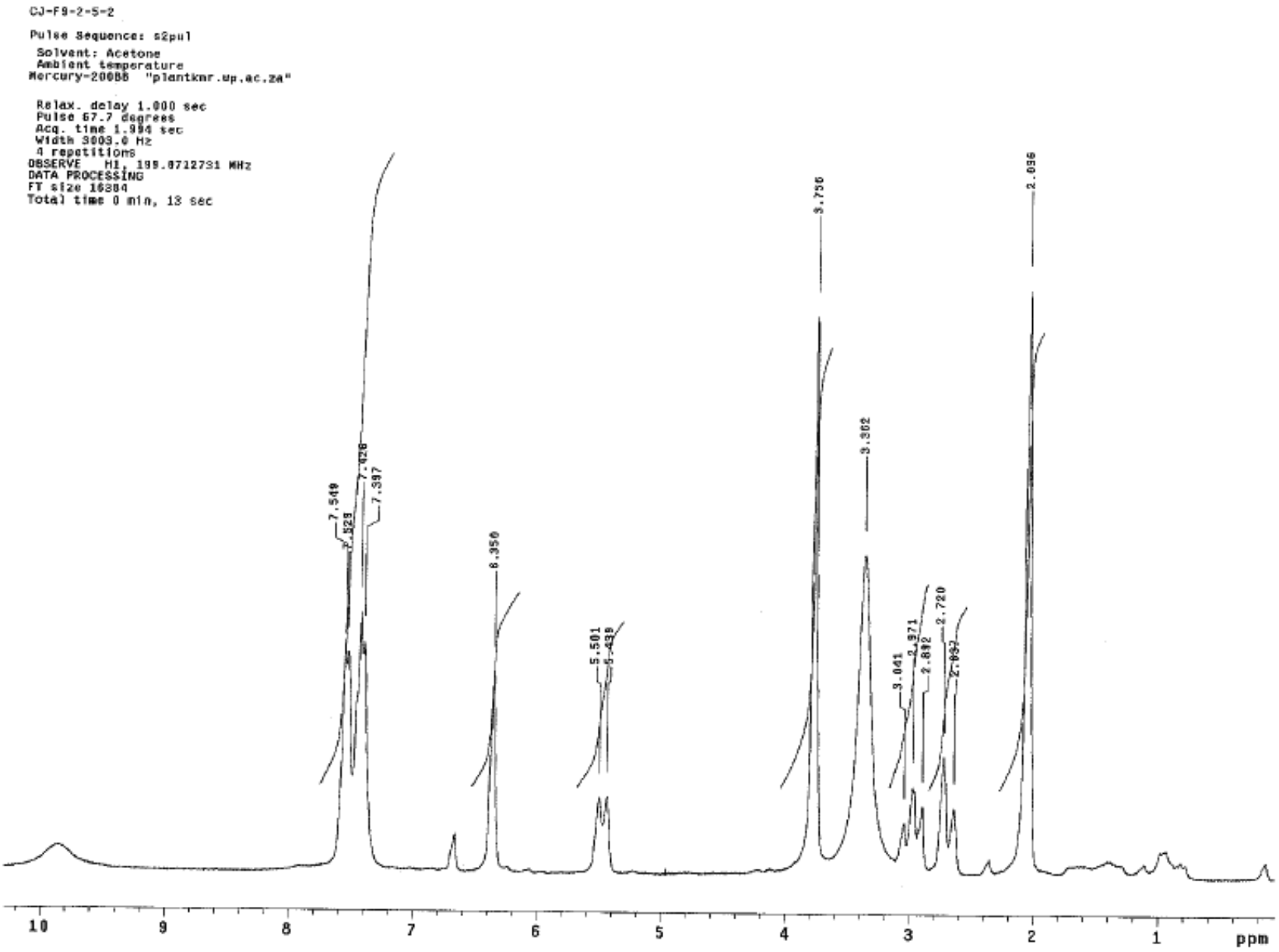

Figure 9.13: ^{1}H NMR spectra for 5-hydroxy-7-methoxy-6-methylflavone, isolated from *Heteropyxis natalensis*

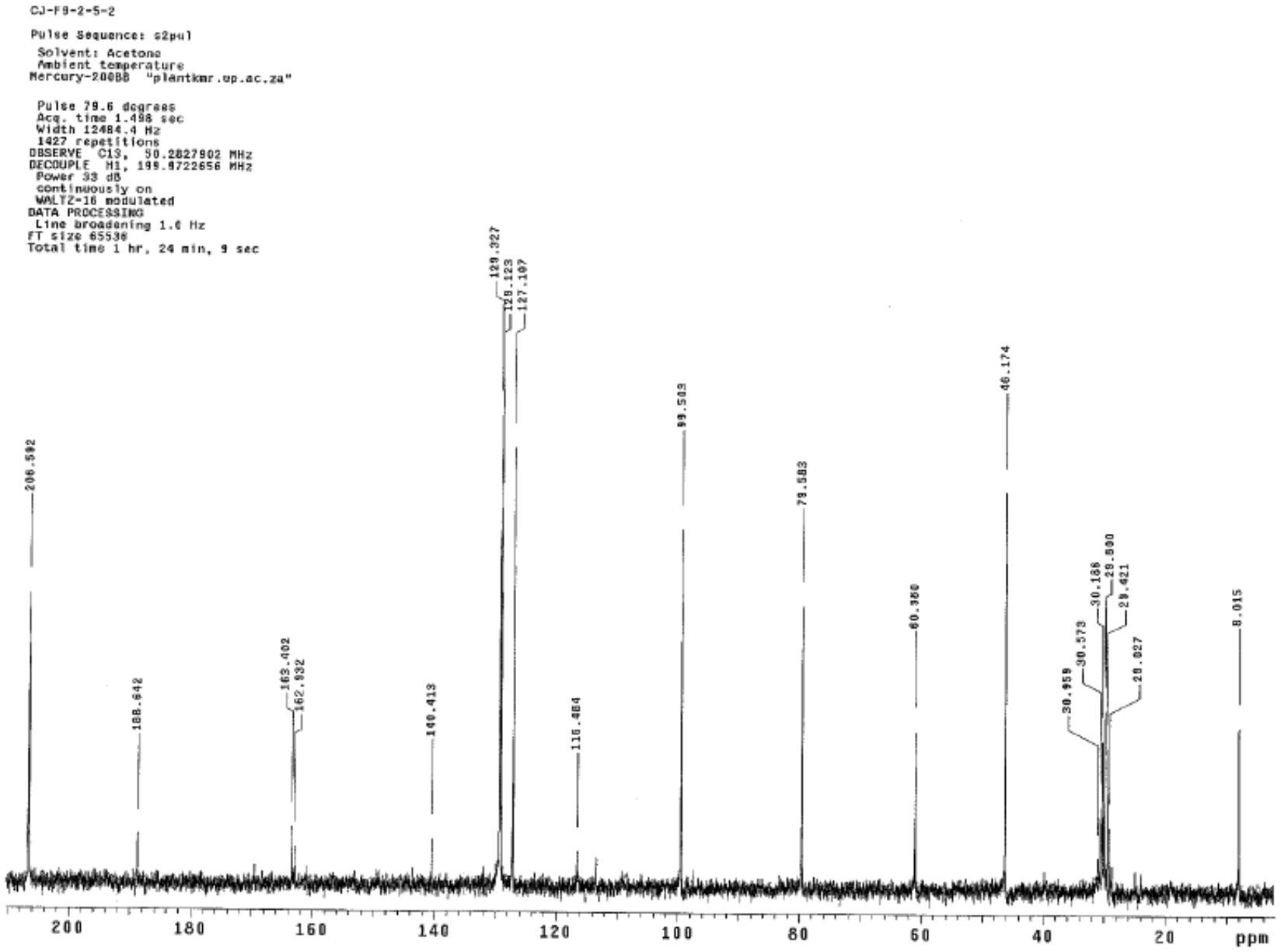

Figure 9.14: ^{13}C NMR spectra for 5-hydroxy-7-methoxy-6-methylflavone, isolated from *Heteropyxis natalensis*

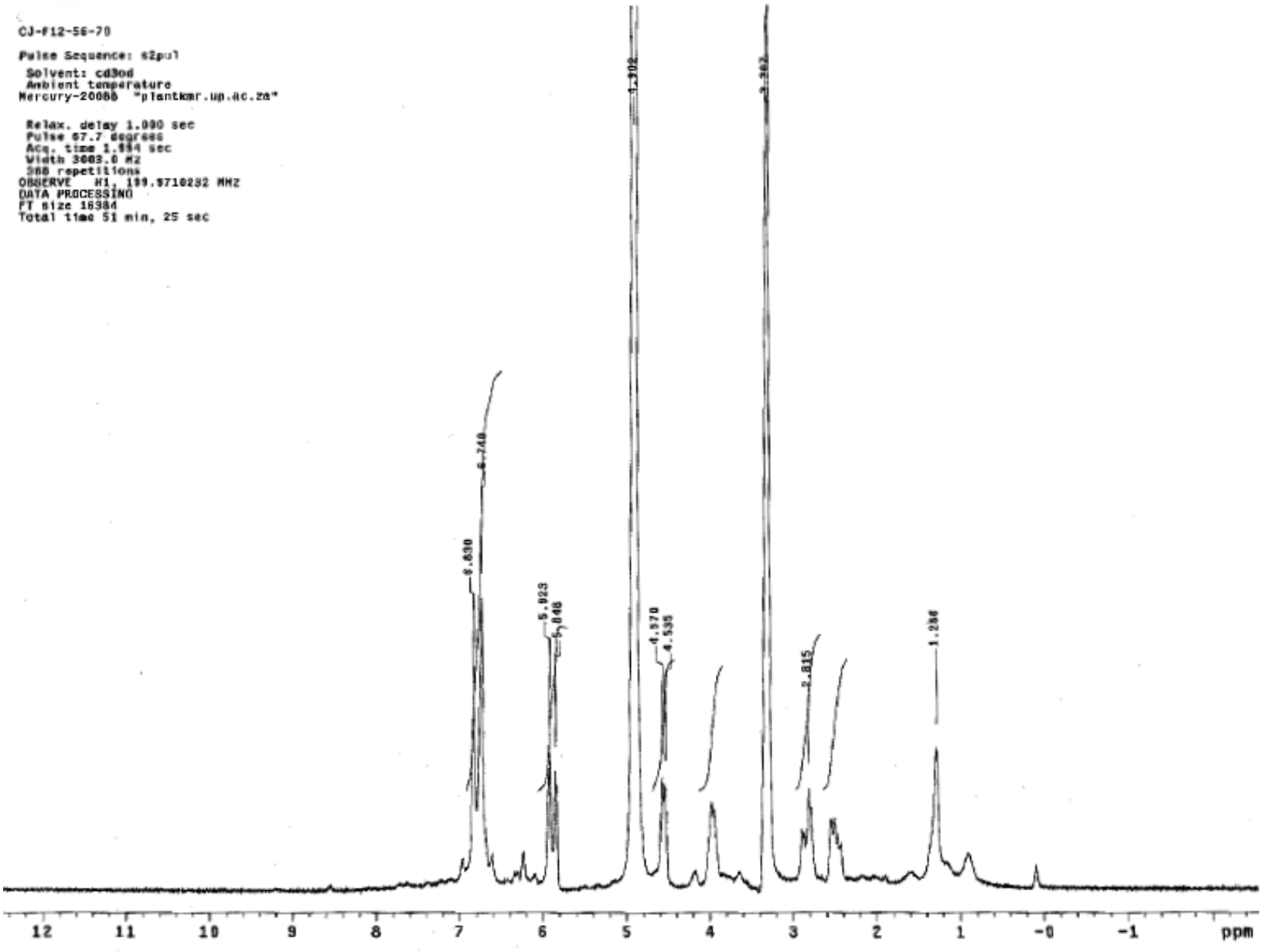

Figure 9.15: ^{1}H NMR spectra for 3,5,7-Trihydroxyflavan, isolated from *Heteropyxis natalensis*

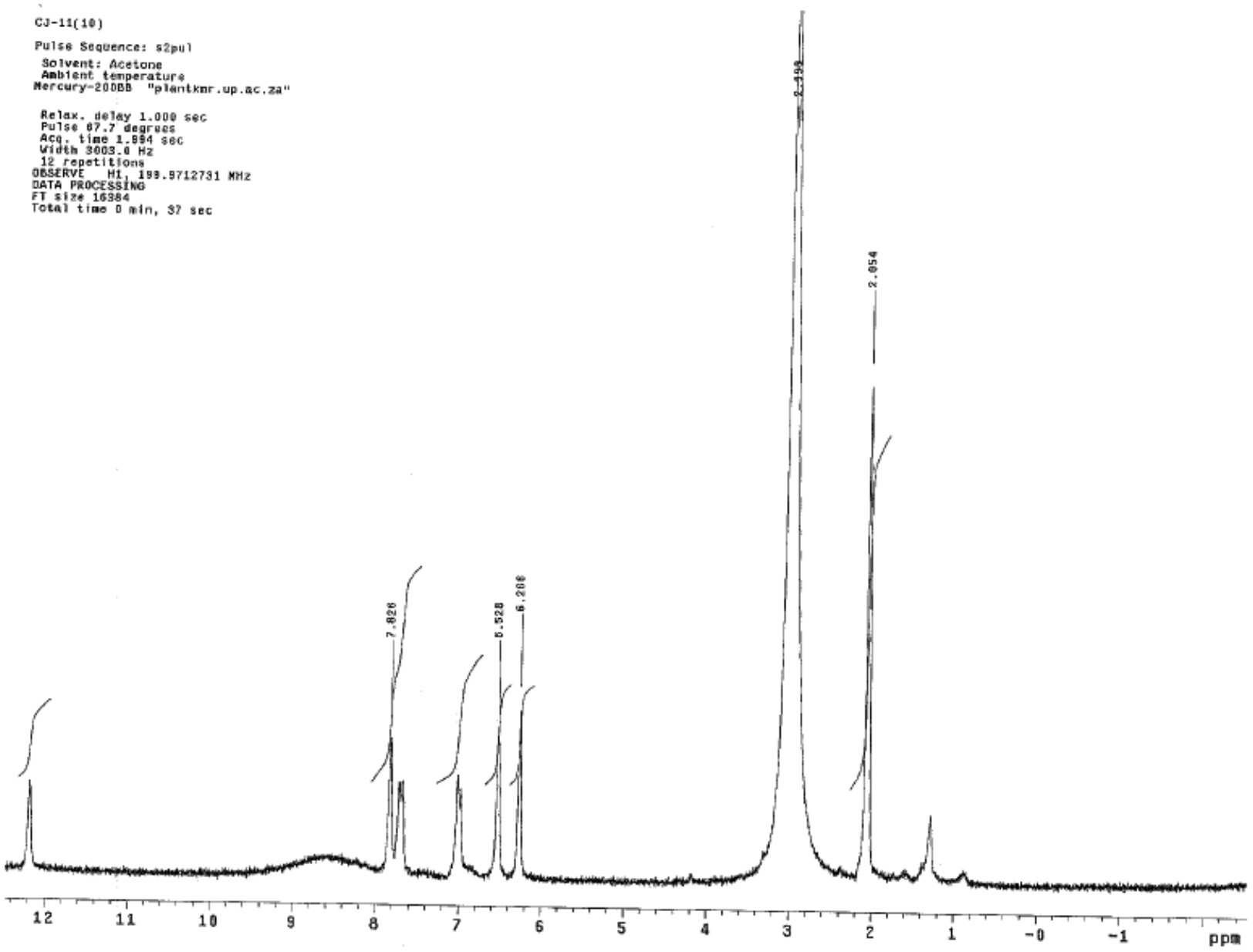

Figure 9.16: ^{1}H NMR spectra for Quercetin, isolated from *Heteropyxis natalensis*

Appendix C
Patent/publication/conference presentations resulting from this thesis

Conference presentations

Henley-Smith, C.J., Lall, N. & Botha, F.S. South Africa's solution to plaque, gingivitis and periodontitis. Coschem Conference. Society of Cosmetic Chemistry. Bytes Conference Centre, Midrand, 2-3 September 2009.

Henley-Smith, C.J., Lall, N. & Botha, F.S. South African plant extracts in combating periodontal microorganisms. Fanie de Meillon Post-Graduate Symposium, 20 November 2009, Faculty of Natural and Agricultural Science Department of Botany and Department of Medicinal Plant Science, School of Biological Sciences, University of Pretoria.

Awarded Best Masters presentation at the Fanie de Meillon Post-graduate Symposium, 2009.

Henley-Smith, C.J., Lall, N. & Botha F.S. South African plant extracts in combating potentially pathogenic oral microorganisms. SAAB Conference 36th Annual Conference of the South African Association of Botanists (SAAB), 11 - 14 January 2010. North-West University, Potchefstroom, 2010.

Awarded Best Masters Presentation at The South African Association of Botany, 2010.

Awarded Third Prize For Young Botanist at The South African Association of Botany, 2010.

Henley-Smith, C.J., Lall, N., Botha F.S. & Hussein, A. Synergistic activity of essential oils with a South African plant against *Actinomyces israelii*. Coschem Conference. Society of Cosmetic Chemistry. Bytes Conference Centre, Midrand, 25-26 August 2010; and Seminar of South Africa Indigenous Knowledge System (IKS) Bio-prospecting Platform, Shanghai, China, 28-29 September 2010 (International).

Publications

Manuscripts under publication:

Henley-Smith, C.J., Lall, N., Botha, F.S., Hussein, A. (June 2011). Identification of bioactive flavonoids from an extract of a South African plant in combating potentially pathogenic oral microorganisms. Preparation for Phytomedicine.

Henley-Smith, C.J., Botha, F.S., Hall, A., Botha, A. (July 2011). A Scanning Electron Microscopy study to determine bacterial adhesion in the presence of *Heteropyxis natalensis*. Preparation for Journal of Ethnopharmacology.

Patent

The following article is considered for a National Patent:

Henley-Smith, C.J., Lall, N., Botha, F.S., Hussein, A. (June 2011). Identification of bioactive flavonoids from an extract of a South African plant in combating potentially pathogenic oral microorganisms. Method Patent document. Department of Plant Science, University of Pretoria, Pretoria, 0002, South Africa.