

Antimicrobial constituents of *Artemisia afra* Jacq. ex
Willd. against periodontal pathogens

by

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Declaration

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Table of contents

Acknowledgements.....	ii
Declaration.....	iii
List of figures.....	xi
List of tables.....	xvi
List of abbreviations.....	xvii
Abstract.....	xviii
Chapter 1: Literature review: Ethnopharmacology and Periodontology	
1.1 Ethnopharmacology	1
1.2 Traditional medicines in South Africa.....	3
1.2.1 Plants and oral hygiene	4
1.2.2 Plants with activity against oral microorganisms	8

1.2.3 Future prospect of traditional medicine.....	10
1.3 Periodontal diseases.....	12
1.3.1 The structure of the teeth and progression of dental disease.....	16
1.4 Existing antimicrobial drugs.....	18
1.4.1 Bisguanide antiseptics.....	18
1.4.2 Quaternary ammonium compounds.....	19
1.4.3 Phenolic antiseptics.....	20
1.4.4 Triclosan.....	20
1.5 Aims and objectives of this study.....	21
1.6 Scope of the thesis.....	21
1.7 Hypothesis.....	22
1.8 References.....	23

Chapter 2: Antimicrobial activity of *Artemisia afra* extract against oral microorganisms

2.1 Introduction.....	31
2.2 Materials and Methods.....	32
2.2.1 Plant material.....	32
2.2.5 Distribution of <i>A. afra</i>	33
2.3 Preparation of extract.....	34
2.4 Antimicrobial activity.....	35
2.4.1 Microorganisms.....	35
2.4.2 Minimum inhibitory concentration (MIC) assay.....	36
2.5 Results and discussion.....	36
2.6 References.....	40

Chapter 3: Antioxidant activity of *Artemisia afra*

3.1 Introduction.....	44
3.2 Material and methods.....	46
3.2.1 Plant material.....	46
3.2.2 Determination of antioxidant activity.....	46
3.4 Results and discussion.....	47
3.5 References.....	52

Chapter 4: Isolation of compounds from *Artemisia afra* extract

4.1 Introduction.....	57
4.2 Materials and methods.....	61
4.2.1 Preparation of plant extract.....	61
4.2.2 Isolation and identification compounds.....	61

4.3 Results and discussion.....	63
4.3.1 Isolation and identification of isolated compounds.....	63
4.4 References.....	71

Chapter 5: Antibacterial and antioxidant activity of isolated compounds from *Artemisia afra*

5.1 Introduction.....	75
5.2 Antibacterial activity of isolated compounds.....	76
5.3 Antioxidant activity of isolated compounds.....	76
5.4 Results and discussion.....	77
5.4.1 Antimicrobial activity results.....	77
5.4.2 Antioxidant activity results.....	79
5.5 References.....	83

Chapter 6: Cytotoxicity effects of *Artemisia afra* and isolated compounds against McCoy fibroblast cells and extract triggered cytokine release by Hep2 cells

6.1 Introduction.....	87
6.2 Materials and methods.....	88
6.2.1 Cell lines.....	88
6.2.3 Cytotoxicity assay.....	89
6.2.4 Cytokine assay.....	89
6.3 Results and discussion.....	90
6.3.1 Cytotoxicity results on fibroblast cells.....	90
6.3.2 Cytokine assay results on Hep2c cells.....	93
6.4 References.....	97

Chapter 7: General discussions and conclusions

7.1 Introduction.....	101
7.2 Antimicrobial activity of <i>A. afra</i>	102

7.3 Antioxidant activity of <i>A. afra</i>	102
7.4 Isolation of compounds from <i>A. afra</i> extract.....	103
7.5 Antimicrobial and antioxidant activity of isolated compounds.....	103
7.6 Cytotoxicity of the extract and pure compounds on the fibroblast McCoy cells.....	104
7.7 Conclusion.....	104
7.8 References.....	105

Chapter 8: APPENDICES

¹ HNMR and ¹³ CNMR spectrum of compounds isolated from the ethanol extract of <i>A. afra</i>	107
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List of figures

Figure 1.1a: Chewing sticks prepared from (a) <i>Salvadora persica</i> and (b) <i>Diospyros lysiodes</i>	5
Figure 1.2 (a) <i>Salvadora persica</i> perennial evergreen species. It is found growing in tropical Africa, tropical Asia and Arabia. (b) <i>Diospyros lycioides</i> with dull green velvety leaves and red fruit (www.indianaturewatch.net).....	6
Figure 1.3: Mouth cleaning with a chewing stick usually involves the brushing of teeth, gums and tongue.....	6
Figure 1.4: Worldwide prevalence of dental caries among 12 years old in 2004.....	14
Figure 1.5: Worldwide distributions of dental caries among 35-44 years old in 2004.....	15
Figure 1.6: A cross section through the healthy tooth.....	16
Figure 1.7: Comparison of a normal tooth and a tooth with periodontitis anatomy.....	17
Figure 2.1 Leaves and flower heads of <i>Artemisia afra</i>	33

Figure 2.2 Distribution of <i>A. afra</i> along the Southern Cape, northwards to tropical east of South Africa.....	34
Figure 3.1: antioxidant activity of <i>A. afra</i> , a, extract; b, Vitamin C (Positive control); c, solvent (Negative control) and d, Extract only (Blank).....	48
Figure 3.2: Antioxidant activity of <i>A. afra</i> using DPPH assay.....	48
Figure 3.3: Schematic representation of the incidence of local etiologic factors in the inflammatory periodontal disease.....	50
Figure 4.1: Structures of representative flavonoids and coumarins from genus <i>Artemisia</i>	59
Figure 4.2: Structures of representative sesquiterpene lactones from genus <i>Artemisia</i>	60
Figure 4.3: Antibacterial activity of <i>Artemisia afra</i> against <i>S. mutans</i>	63
Figure 4.4: Forty fractions obtained from the first column.....	64
Figure 4.5: Collective fractions from the first column.....	64
Figure 4.6: Schematic presentation of isolation steps followed.....	65

Figure 4.7: Structures of isolated compounds.....	66
Figure 5.1: The DPPH inhibitory activities of the isolated compounds and vitamin C.....	81
Figure 6.1: The cytotoxicity effects of <i>Artemisia afra</i> extract and three selected compounds on the growth of the McCoy fibroblast cells.....	92
Figure 6.2(a): A standard curve showing the optical density of human IL-8 (pg/ml).....	94
Figure 6.3 (b, c, d, e): Comparison of the production of cytokine interleukin-8 (IL-8) in response to <i>Artemisia afra</i> extract and <i>E.coli</i>	95
Figure 8.1.1: ¹ HNMR spectrum of phytol isolated from the ethanol extract of <i>Artemisia afra</i>	108
Figure 8.1.2: ¹³ CNMR spectrum of phytol isolated from the ethanol extract of <i>Artemisia afra</i>	109
Figure 8.1.3: ¹ HNMR spectrum of Betulinic acid isolated from the ethanol extract of <i>Artemisia afra</i>	110

Figure 8.1.4: ^{13}C NMR spectrum of Betulinic acid isolated from the ethanol extract of *Artemisia afra*.....111

Figure 8.1.5: ^1H NMR spectrum of Acacetin isolated from the ethanol extract of *Artemisia afra*.....112

Figure 8.1.6: ^{13}C NMR spectrum of Acacetin isolated from the ethanol extract of *Artemisia afra*.....113

Figure 8.1.7: ^1H NMR spectrum of 1α , 4α -dihydroxybishopsolicepolide isolated from the ethanol extract of *Artemisia afra*.....114

Figure 8.1.8: ^{13}C NMR spectrum of 1α , 4α -dihydroxybishopsolicepolide isolated from the ethanol extract of *Artemisia afra*.....115

Figure 8.1.9: ^1H NMR spectrum of Scopoletin isolated from the ethanol extract of *Artemisia afra*.....116

Figure 8.1.10: ^{13}C NMR spectrum of Scopoletin isolated from the ethanol extract of *Artemisia afra*.....117

Figure 8.1.11: ^1H NMR spectrum of α -Amyrin isolated from the ethanol extract of *Artemisia afra*.....118

Figure 8.1.12: ^{13}C NMR spectrum of α -Amyrin isolated from the ethanol extract of *Artemisia afra*.....119

List of tables

Table 2.1: Traditional use of <i>A. afra</i>	32
Table 2.2: The MIC and MBC (mg/ml) values of plants tested against periodontal pathogens.....	38
Table 4.1: ¹ H and ¹³ C-NMR chemical shifts (δ) for compound 3.....	68
Table 5.1: Table 5.1 The MIC and MBC (mg/ml) values of isolated compounds against periodontal pathogens.....	78
Table 5.2: The IC ₅₀ values of the isolated compounds tested for antioxidant activity.....	80
Table 6.1: The IC ₅₀ values of crude extract and three selected isolated compounds tested for cytotoxicity activity.....	91

List of abbreviations

ATCC	American type culture collection
¹³ CNMR	Carbon nuclear magnetic resonance
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,2-diphenyl-2-picrylhydrazyl
EC ₅₀	Effective concentration at 50 %
¹ HNMR	Proton nuclear magnetic resonance
IC ₅₀	Inhibitory concentration at 50 %
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl
MMC	Minimum microbicidal concentration
MIC	Minimum inhibitory concentration
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
UV-Light	Ultra violet light
WHO	World health organisation
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Abstract

Antimicrobial constituents of *Artemisia afra* against periodontal pathogens

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The phytochemical investigation of an ethanol extract of *Artemisia afra*, led to the isolation of six known compounds, Acacetin (**1**) 12 α ,4 α -dihydroxybishopsolicepolide (**2**), Scopoletin (**3**) α -amyrin (**4**), Phytol (**5**) and a pentacyclic tri-terpenoid Betulinic acid (**6**). The isolated compounds were evaluated for their anti-microbial activity against Gram positive (*Actinomyces naeslundii*, *Actinomyces israelii* and *Streptococcus mutans*), Gram negative bacteria (*Prevotella intermedia*, *Porphyromonus gingivalis* and *Aggregatibacter actinomycetemcomitans* previously known as *Actinobacillus actinomycetemcomitans*) and *Candida albicans*. The crude extract of *A. afra* inhibited the growth of all tested microbial

species at concentration range of 1.6 mg/ml to 25.0 mg/ml. The compounds 1-6 also showed activity range at 1.0 mg/ml to 0.25 mg/ml. Three best compounds which showed good activity were selected for further studies. Cytotoxicity of the extract and compounds was determined using the XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) cell proliferation kit. The antioxidant activity of the extract and compounds was done using the DPPH scavenging method. The extract showed good antioxidant activity with an IC_{50} value of 22.2 μ g/ml. Scopoletin had a strong transformation of the DPPH radical into its reduced form, with an IC_{50} value of 1.24 μ g/ml which was significant to that of vitamin C (1.22 μ g/ml). Acacetin and Betulinic acid exhibited a decreased scavenging activity with the IC_{50} of 2.39 and 2.42 μ g/ml, respectively. The extract and compounds showed moderate toxicity on McCoy fibroblast cell line and the extract influenced the release of cytokine against Hep2 cells. Scopoletin was relatively non-toxic with an IC_{50} value of 132.5 μ g/ml. Acacetin and betulinic acid also showed a smooth trend of non-toxic effects at lower concentrations and toxic at higher concentrations with IC_{50} values of 35.44 and 30.96 μ g/ml. The obtained results in this confirmed the use of *A. afra* in the treatment of microbial infections.

Chapter 1

Literature review: Ethnopharmacology and Periodontology

1.1 Ethnopharmacology

Traditional medicine is widely practiced in the whole world since time immemorial. Reliance on traditional medicines in rural areas is elevated and is accredited to both economic and cultural factors. Medicinal plants which form the backbone of traditional medicine have in the last decades been the subject of very intense pharmacological studies. This was driven by the acknowledgement of the value of medicinal plants as potential source of new compounds of therapeutic importance and drug development in order to provide essential physical and physiological health care (Butler, 2004). The world health organization (WHO) estimates that up to 80 % of African people use traditional medicine. Recently, however, the acceptance of traditional medicine as an alternative form of health care has increased amongst all socio-economic groups of the South African population and phytomedicine has become an important economic sector. In general, the WHO recommends and encourages the use of plants as effective tool for health. Despite the existence of a wide variety of antibacterial agents, the search for new ones is of great importance.

Bacterial infections are the cause of large troublesome diseases and bacteria is listed as common organism responsible for opportunistic disease occurrence. Infectious and inflammatory diseases are among those treated using traditional remedies. Therapy of bacterial disease is a frequent problem due to the emergence of bacterial strains resistant to numerous antibiotics (Marimoto and Fujimoto, 1999). Some of the factors that enhance infectious diseases include poor hygiene, insufficient sanitation and congested conditions (Kerr and Lacey, 1995). Ethnopharmacology provides an opportunity for multidiscipline scientific collaboration between botanists and traditional healers. The classic example is of Dr William withering, who discovered the use of foxglove on the treatment of dropsy which is due to cardiac ailment. The plant was used by old woman in shropshire and Withering combined his medicinal proficiency of botany. He discovered that foxglove was the active ingredient and only dropsy related to heart ailment was curable (Aronson, 1987).

Ethnopharmacology is a field of research that look mainly at the knowledge of indigenous people concerning plant constituents that are used as medicine. Recently, the use of medicinal plants has involved the isolation of active compounds, beginning with the isolation of morphine, cocaine, digitoxin and quinine from opium (Kinghorn, 2001; Newman *et al.*, 2000; Samuelsson, 2004). Reserpine isolated from *Catharanthus roseus* which is now vastly used to in the treatment of childhood leukaemia and Hodgikin's disease serve as an important example of plant-derived medicine. Even when new chemical structures are not found during isolation, known compounds with new biological activity can provide important drug leads (Kramer and Cohen, 2004).

1.2 Traditional medicines in South Africa

There are many disputes between western and traditional medicines in South Africa. These debates have been driven by the fact of healing of which some believe that traditional medicines are capable of healing the spiritual origin of an ailment while the western medicines are labeled as symptom effective. Medicinal practice in South Africa is dated from the ancient times and is still practiced even in the modern years. Traditional medicine is said to involve holistically the body and mind. Physiological bases of the illness can be diagnosed by a traditional healer and treated before the symptoms. According to van Wyk and van Wyk, (1997); WHO, there are about 200 000 indigenous traditional healers in South Africa and almost 60 % of the South African population confer with them. South African traditional healers are divided into two categories inyanga (herbalist), izangoma (diviners) (Pantanowitz, 1994). Herbalist and diviners are said to use plants boiled in water for medicinal purposes (van Wyk and van Wyk, 1997). Interviewing traditional healers for accurate information about their medicinal plants and other uses compose an important activity in ethnopharmacological field. The major challenge has been to translate indigenous diseases in to their modern counter-parts usually confusion in data interpretation occurs (Cox, 1994). Many plants in the field of phytomedicine have been extensively tested for bioactivity against disease causing microorganisms (Balick and Cox, 1997). However, there has been little research carried out on the activity of medicinal plants against oral microorganisms.

1.2.1 Plants and oral hygiene

Toothbrush sticks have been used for many years ago for the maintenance of oral health. Prehistoric Greek and Roman literature discusses the use of tooth sticks that were chewed to cleanse teeth and mouth amongst communities in many developing countries. This is due to the cheaper cost, availability, customs and religious reasons (Carl and Zambon, 1993). The roots, twigs and stems of *Salvadora persica* (Figure 1.2a) have been used in many centuries as oral hygiene tool (Elvin-Lewis, 1980) and are today commonly used as tooth and tongue cleaning sticks by Muslims in the Middle East, as well as in Asia and various African countries (Elvin-Lewis, 1980). Miswak is interpreted as tooth sticks prepared from *S. persica*. In areas where *S. persica* is absent, miswak is prepared from other plants. For instance, in West Africa, *Citrus aurantifolia*, *Citrus sinensis* and *Cassia sieberiana* are commonly used as chewing sticks (Elvin-Lewis, 1980), while in the Indian sub-continent, the neem tree (*Azadirachta indica*) is widely used (Almas and Alla, 1995). *Diospyros lycioides* Desf. (Ebenaceae) (Figure 1.2b), known commonly as muthala is a popular chewing stick used in Namibia (Addo-Yobo *et al.*, 1991).

Rhus natalensis and *Euclea divinorum* are plants used in Kenya as chewing sticks while in Asia, the nut tree (*Juglans regia*) is commonly used. Today, chewing sticks are usually made into bundles and sold in the local market. Although harvesting and trade of chewing sticks threaten some plants. The stick is chewed or tapered at one end until it becomes ragged into a brush (figure 1.1a,b). Soaked in water for a few hours to soften the natural fibres which helps to separate while tapering or chewing. Most of these plants when they are left in the

mouth, they stimulate salivation and therefore there will be a better cleansing effect (Wu *et al.*, 2001). After having been used several times, the chewing stick is either replaced by a new one or its bristles are cut off to expose a fresh end where new bristles are prepared by further chewing or tapering.

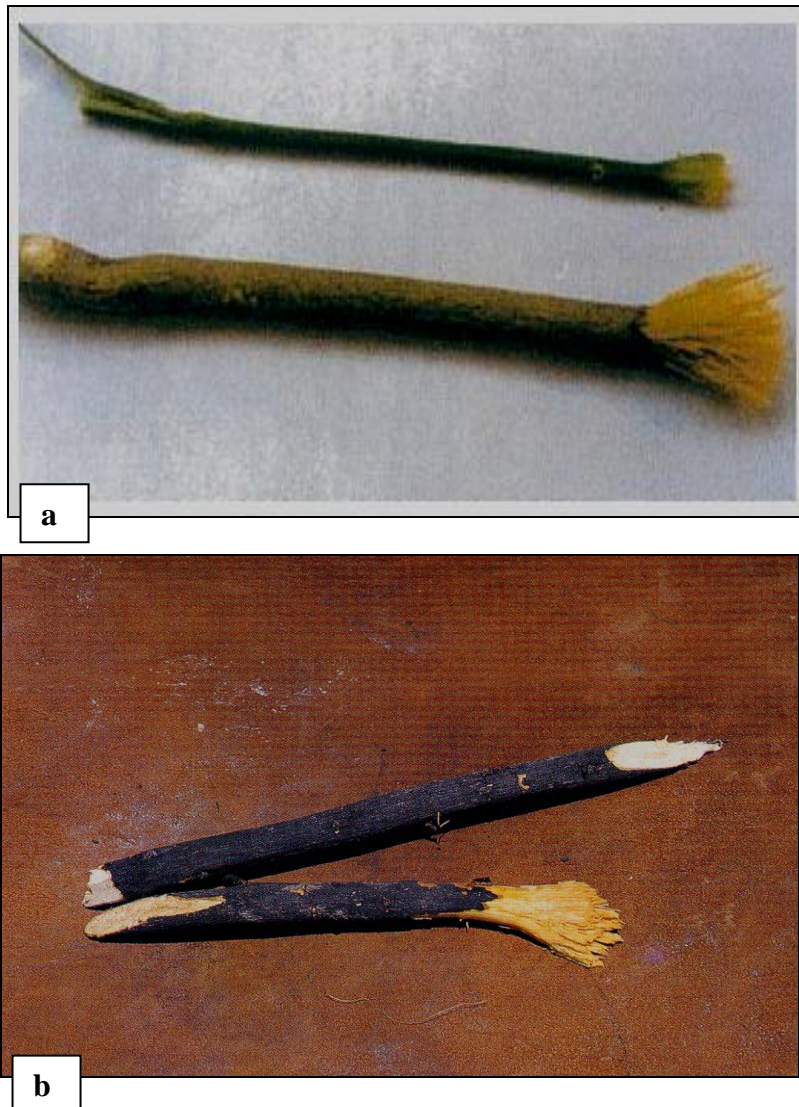


Figure 1.1 (a) Chewing stick prepared from *Salvadora persica* (Wu *et al.*, 2001),
(b) *Diospyros lysioides*. (Van Wyk and Van Wyk., 1997).

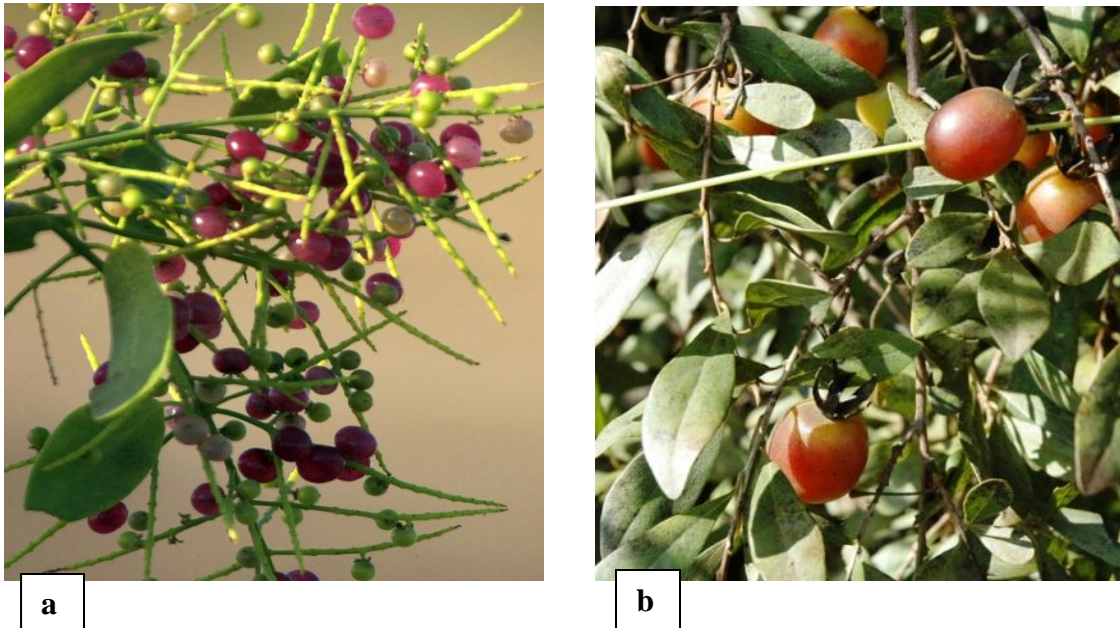


Figure 1.2 (a) *Salvadora persica* perennial evergreen species. It is found growing in tropical Africa, tropical Asia and Arabia. (b) *Diospyros lycioides* with dull green velvety leaves and red fruit (www.indianaturewatch.net).

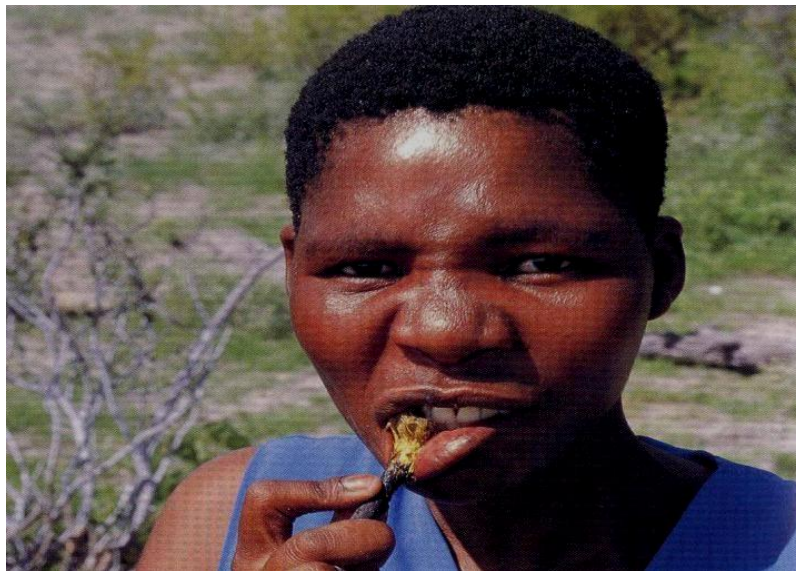


Figure 1.3 Mouth cleaning with a chewing stick usually involves the brushing of teeth, gums and tongue. (Van Wyk and Van Wyk., 1997).

A recent review discussed possible etiological associations between periodontitis (the progressive destruction of the supporting structures of the teeth which is triggered by bacterial plaque) and cardiovascular disease in general and in infective endocarditis specifically, as well as rheumatoid arthritis, pneumonia, preterm birth and low birth weight (Holmstrup *et al.*, 2003). For example, a number of epidemiological studies have associated periodontitis with cardiovascular disease (Holmstrup *et al.*, 2003). Periodontal inflammation facilitates the entrance of bacteria into the bloodstream, especially after chewing food or cleaning teeth. Either direct effects from the bacteremia or secondary effects from the inflammation which their presence may trigger thrombus formation and/or the development of atherosclerotic lesions. Be that as it may, the greatest loss of teeth in adults is caused by periodontal disease, which can be very difficult to treat. This suggests a potentially valuable role for phytotherapy in assisting with the management of this difficult and serious disease.

The evidence and research which supports such a role for a few key plants (or plant products) is reviewed below. The periodontal status of more than 200 adult Sudanese who habitually used either miswak or a toothbrush was compared. Although gingival bleeding and dental calculus were highly prevalent in the test population, miswak users had significantly ($p < 0.05$) lower dental calculus and signs of periodontal disease and a tendency to lower gingival bleeding Darout *et al.*, (2000). A Saudi Arabian study compared the effect of miswak on plaque removal and dental health using a single blind, randomized, crossover design. Compared to the use of a toothbrush, the miswak resulted in significant reduction in plaque ($p < 0.001$) and gingival ($p < 0.01$) indices (Al-Otaibi *et al.*, 2003). Furthermore, image

analysis of the plaque distribution showed a significantly greater reduction in plaque for the miswak users. However, miswak use was associated with greater gum recession in one study, which was attributed to their abrasive properties (Eid *et al.*, 1991). The antimicrobial effects of neem (*Azadirachta indica*) and arak (*Salvadora persica*) were compared *in vitro* against plaque-forming bacteria (Almas *et al.*, 1995).

A mouthwash made from arak was found to significantly reduce gingival bleeding ($p < 0.01$) in an open clinical study although plaque scores were not significantly reduced in the three-weeks study, the use of arak resulted in lower rates of *Streptococcus mutans*, a bacterium associated with dental plaque. A six-weeks clinical trial found that a neem leaf gel significantly ($p < 0.05$) reduced the plaque index and oral bacterial count when compared with a conventional antibacterial mouthrinse (Pai *et al.*, 2004). Although there has been an increase in the number of reports on clinical surveys and epidemiological studies of chewing sticks and their oral health benefits, relatively few studies have looked into the antimicrobial effect of chewing stick extracts against oral pathogens associated with caries and periodontal disease.

1.2.2 Plants with activity against oral microorganisms

Medicinal plants studies have revealed that propolis extract has antimicrobial activity against *A. actinomycetemcomitans*, *P. intermedia*, *P. gingivalis*, and *F. nucleatum*. There is

considerable interest in the development of other classes of antimicrobials for the control of infection. Garlic (*Allium sativum*) has been used as a medicine since ancient times and has long been known to have antibacterial, antifungal and antiviral properties (Bankova *et al.*, 1992). Recently, garlic extract has been shown to be an effective agent for controlling methicillin-resistant *Staphylococcus aureus*, *P. gingivalis* and *A. actinomycetemcomitans*. The main antimicrobial constituent of garlic has been identified as the oxygenated sulphur compound, thio-2-propene-1-sulfinic acid *S*-allyl ester, which is usually referred to as allicin (Bonhevi *et al.*, 1994). Allicin is not present in raw garlic. It is formed rapidly by the action of the enzyme, allinase (alliin lyase) on *S*-allyl-L-cysteine-sulphoxide (alliin) when the garlic is crushed. Filter sterilised, aqueous extract of garlic was tested for ability to inhibit the growth of a range of oral species and to inhibit the trypsin-like and total protease activity *S. aureus*, *P. gingivalis* and *A. actinomycetemcomitans* and the MIC were found to be 17.8 mg/ml for *A. actinomycetemcomitans* with the MBC of 6.87 mg/ml and *P. gingivalis* had the MIC of 4.4 with the MBC of 8.9 mg/ml (Bonhevi *et al.*, 1994).

Lee *et al.*, (2006) studied the inhibitory effects of an acidic polysaccharide purified from the roots of *Panax ginseng* against the adhesion of *Helicobacter pylori* to gastric epithelial cells and *P. gingivalis* and *A. actinomycetemcomitans* to agglutinate erythrocytes. The MIC was found to range from 0.25-0.5 mg/ml with *S. aureus* MIC of 0.01 mg/ml, *P. gingivalis* (0.0001 mg/ml), and *A. actinomycetemcomitans* (MIC= 0.01 mg/ml). Microcarpels, phloroglucinol derived from *Eucalyptus* leaves were tested for antimicrobial activity against oral bacteria, especially *P. gingivalis*, *A. actinomycetemcomitans*, *Prevotella nigrescens* and *Treponema*

denticola (Nagata *et al.*, 2006). Microcarpels inhibited the growth of tested microorganisms with MIC values ranging from 0.5-5 µg/ml for microcarpel A, B, C against *P. gingivalis*, *A. actinomycetemcomitans* MIC of 100 µg/ml. Sanguinarine is a well known natural product extracted from the roots of *Sanguinaria Canadensis*, that grows in the central and South America and Canada has been developed in to commercial teeth cleanser. Sanguinarine is a benzophenanthridine alkaloid which is yellow in colour (Grenby, 1996). Short-term studies have revealed that Sanguinarine has plaque inhibitory effects but the effect of gingivitis appears to be equivocal.

1.2.3 Future prospect of traditional medicine

The prospective future of traditional medicinal plants have shown to be threatened therefore conservation strategies are required to sustain these plants. Overexploitation and intensive harvesting are the main challenges that may cause extinction of medicinal plants (Balick, 1990). Some of these problems include the active trade and marketing of medicinal plants, topography and climate (Mander, 1998). Over 300 tonnes of plants are traded through shops per year with 50 % sold in street traders. An escalating requirement as this is not only seen among the rurals but also in pharmaceutical industries (Mander, 1998). Therefore sustainability has to be taken into consideration. The most common reason individuals choose to utilize herbal therapy is prevention. Several other reasons include ineffectiveness or dissatisfaction with biomedical medicine to enhance the bodies of the immune system, need for personal control over health care decisions, more accessible and less expensive than

biomedical medicine, compatible with the patient's values, and lastly herbs are natural and therefore harmless (Tolstoi, 2001; NCCAM). The WHO formally recognized the importance of collaborating with traditional healers in 1977 (Traditional medicine strategy 2002-2005). It has planned collaborations and guidelines on traditional medicines and biomedicines in order for all known organizations to learn more about biodiversity conservation and indigenous people's rights over their knowledge and resources (Timmermans, 2003).

The UNAIDS appreciates the collaboration with traditional healers, because traditional healers offers a personalized health care that is culturally important, cultural intimacy between clients and healers, which facilitates communication about the disease and associated social problems. Despite the evident successes of drug discovery from medicinal plants, future endeavor face many challenges. Pharmacognosists, phytochemists and other natural scientists will need to endlessly advance the quality and quantity of compounds that enter drug development phase so to keep up with the rapidity of other drug discovery efforts (Butler, 2004). Drug discovery from medicinal plants has been seen to take long periods of time and more complicated than other means of drug discovery, for this reason many pharmaceutical companies have scaled down their natural product research (Butler, 2004; Koehn and Carter, 2005). High technology such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) must be employed to ascertain speedy isolation and identification. Even with all the challenges facing drug development from medicinal plants, natural products have provided numerous clinically used medicines. Herbs have found its place in modern day biomedical medications. About one-fourth of all biomedical medications

commonly prescribed today, contain at least an active ingredient derived from plants and the additional contents are chemically synthesized in the laboratory (McCann, 2004).

1.3 Periodontal diseases

Periodontal disease is an inflammatory change in the periodontium caused by bacterial infection (Samaranayaki, 2000). Commonly known diseases are periodontitis and gingivitis. Gingivitis leads to bleeding, swelling and redness of the marginal gingival. Periodontitis is the progressive stage of gingivitis, is a condition which leads to the distraction of the teeth supporting tissues, both connective tissues and bones eventually leading to tooth loss (Samaranayaki, 2000). More than 500 cultivable bacterial species have been isolated from the gingival crevices of human beings (Moore and Moore, 1994). The subgingival subspecies of healthy oral cavity are facultative anaerobic Gram positive species, but in gingivitis the Gram negative bacteria are elevated (Van Palenstein and Helderman, 1981). Approximately, 10-30 species in periodontitis are mainly putative Gram negative anaerobic bacteria. Recently, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Bacteroides forsythus*, are considered periodontal pathogens and primary etiological agents in periodontitis (World Workshop in periodontics, 1996). Globally, periodontal and dental diseases have been considered the most important oral health problems. WHO has documented that up to 60-90 % of school children and majority of adults are affected by dental caries and periodontal disease. WHO suggested including information on loss of periodontal attachment in oral health surveys (Albandar and Tinoco, 2002). Periodontal disease severity as measured by probing depths and loss of periodontal

attachment has been related to age in numerous studies (Genco, 1996). There have been some major variations currently on the severity of dental diseases world wide in 12 year old children shown in (Figure 1.4). Figure 1.5, shows the distribution of dental caries among 35-44 year old (WHO). Epidemiologic data available at WHO confirm studies which show that periodontal disease prevalence and severity tend to be high in older age groups as compared to younger age groups (Petersen, 2003).

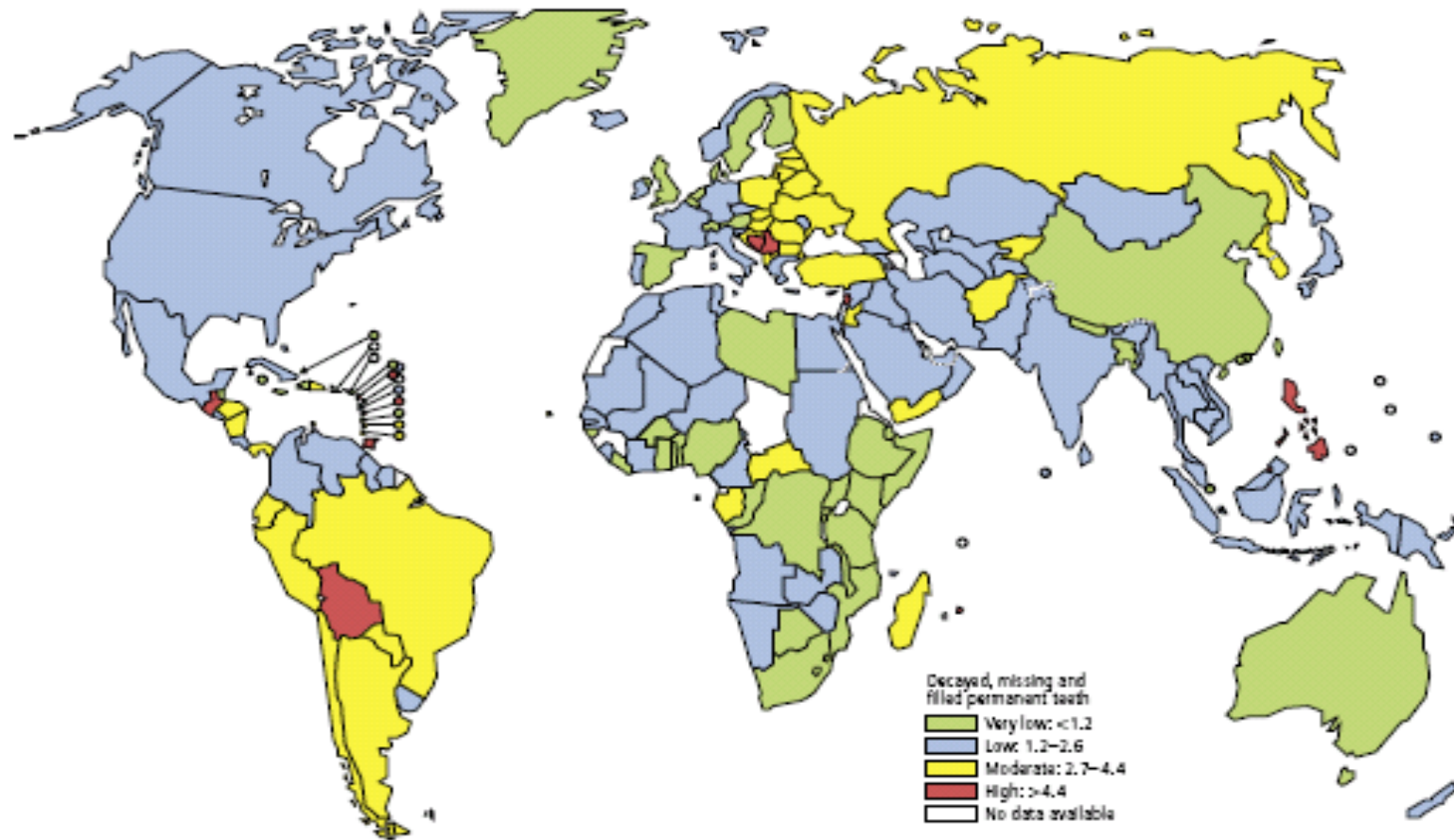


Figure 1.4 Worldwide prevalence of dental caries among 12 years old in 2004 (WHO)

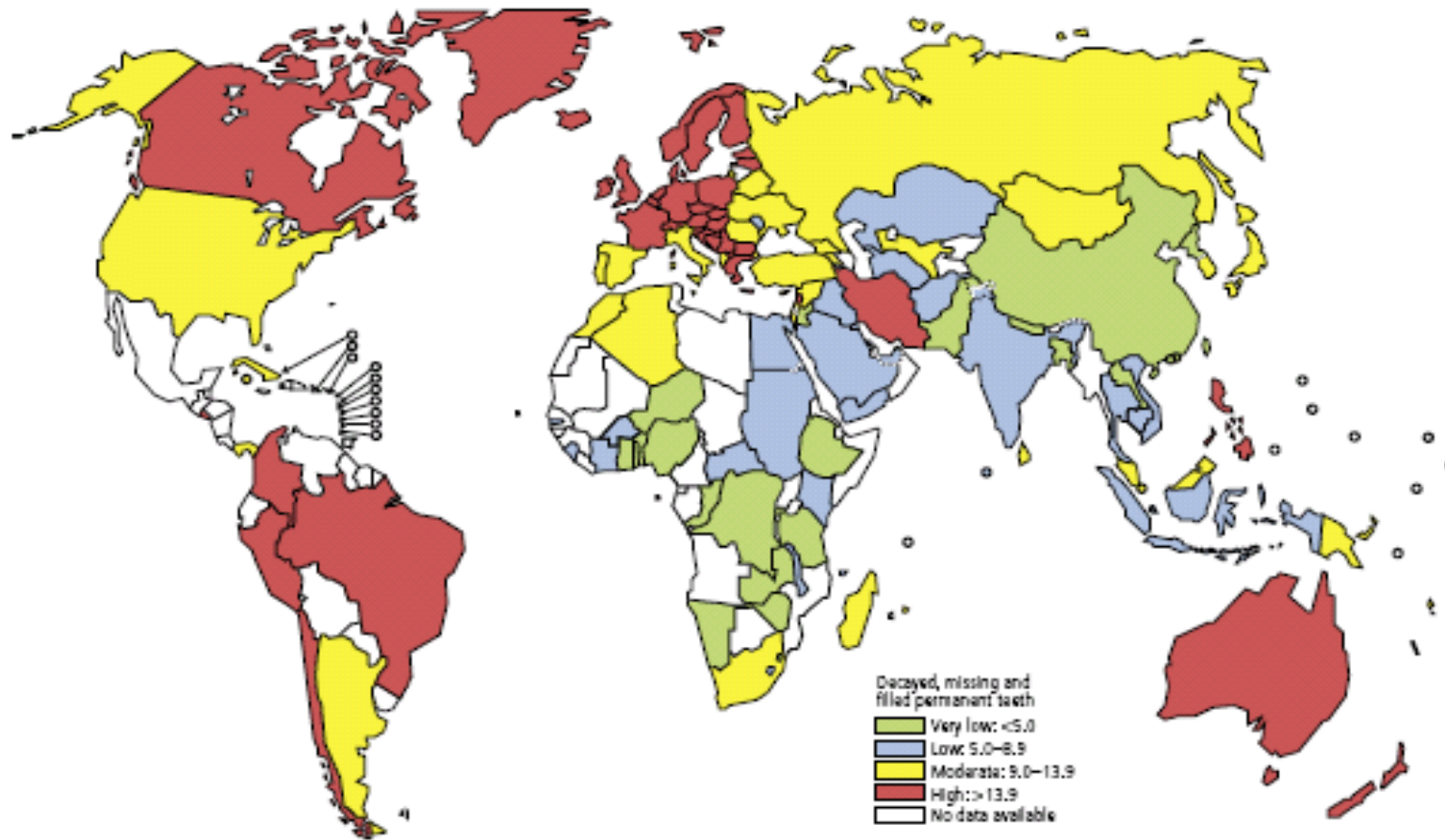


Figure 1.5 World wide distributions of dental caries among 35-44 years old in 2004 (WHO)

1.3.1 The structure of the teeth and progression of dental disease

The tooth (Figure 1.6) can be divided into parts that help to protect defend itself against microbial degradation. The white outermost part is called the enamel, followed by a layer of dentin which resembles true bone (Hunter and Arbona, 1995). Underneath the dentin there is a soft inner zone of dental pulp. The pulp is jelly-like, richly vascularised and has nerves and canals that reach deep into the apex of the jaw and alveolar bone which are responsible for nutrient supplies. Between the crown and root is the neck of the tooth (Hunter and Arbona, 1995). This is a highly vulnerable area for periodontal disease and is vitally protected by healthy gums from the advances of dental decay.

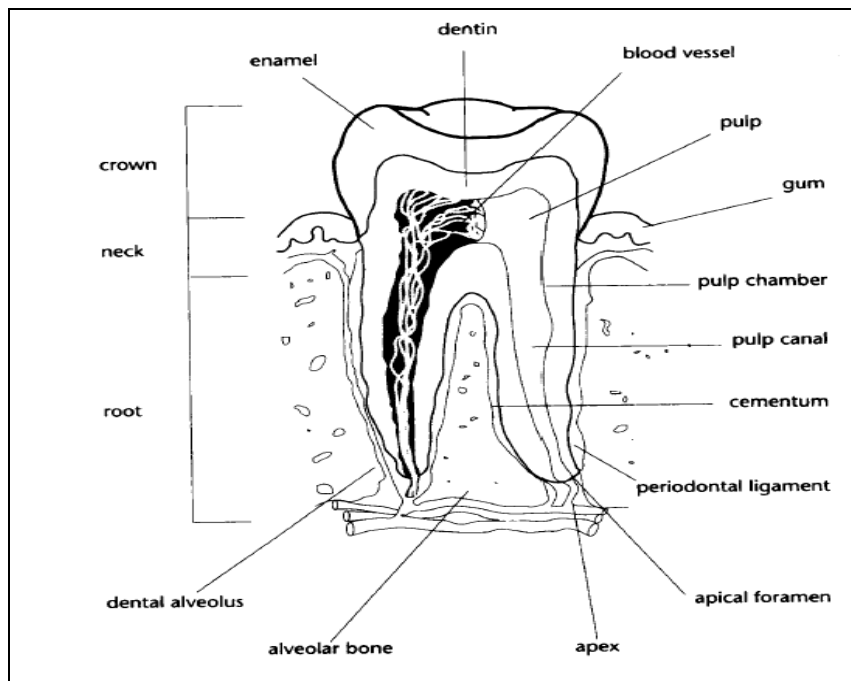


Figure 1.6 A cross section through the healthy tooth (Hunter and Arbona, 1995).

The main cause of tooth decay and tooth loss is a bacterial disease known as caries. The normal, endogenous flora in the mouth can cause disease. The oral cavity microorganisms propagate into a sticky, gel-like film known as plaque (Hunter and Arbona, 1995). Plaque houses polysaccharides, salivary glycoproteins and mucosal cells. Approximately 100 billion bacteria are found per gram of plaque. Carbohydrates and sugars (sucrose, glucose, fructose, maltose and lactose) found in food are the main basis for bacterial inhabitants in dental plaque. Sucrose has been identified mainly as a favourable initiative to dental decay. However, the production of acid prevails in the presence of acidogenic bacteria which then results in solubilization and demineralization of tooth enamel and dentin (Hunter and Arbona, 1995). This is well illustrated by (Figure 1.7 a, b) where a comparison of healthy and diseased teeth shows the progression dental disease from the enamel to dental alveolar and directly to the bone (Salman *et al.*, 1986; Kaplan, 1989).

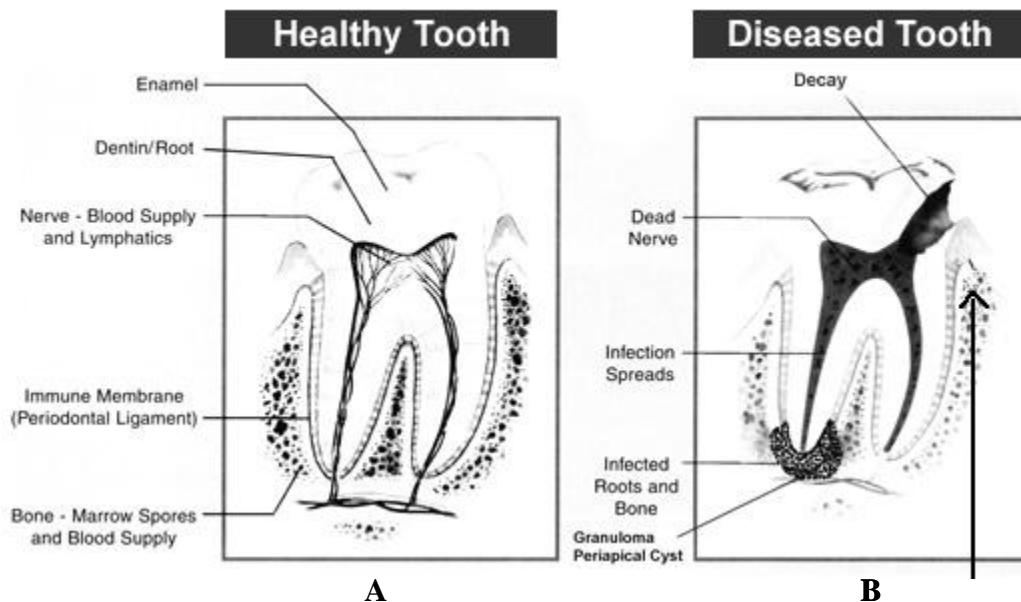


Figure 1.7 Comparison of a normal tooth (A) and a tooth with periodontitis (B) anatomy.

<http://www.biodentistrydrvizcarra.com>

1.4 Existing antimicrobial drugs

Antimicrobial agents have been studied in respect to the control of oral diseases and they can be divided into bisguanide antiseptics, quaternary ammonium antiseptics, phenolic antiseptics and other antiseptics such as oxygenating agents, metal ions and natural products (Addy, 1986).

1.4.1 Bisguanide antiseptics

The bisguanide antiseptic class includes chlorhexidine gluconate, alexidine and octenidine. These antiseptics are able to kill a range of microorganisms by damaging their cell wall. The effects of chlorhexidine are unsurpassed by other agents. Chlorhexidine is a synthetic antibacterial drug which has been used extensively as a broad spectrum antiseptic in clinical and veterinary medicine. It is effective *in vitro* against gram-positive and gram-negative bacteria (Emisilon, 1977; Budtz-Jorgensen and Loe, 1972) including anaerobe and aerobes and yeast (Hennessy, 1977). The antibacterial mode of action of chlorhexidine is due to an increase in cellular membrane permeability followed by coagulation of the cytoplasmic macromolecules (Hennessy, 1977). It has been shown that chlorhexidine can reduce the adhesion capacity of *P. gingivalis* to epithelial cells (Grenier, 1996).

Chlorhexidine has been developed into sugar-free chewing gum which contains 20 mg of chlorhexidine diacetate, mouthwash and placebo gum (Smith *et al.*, 1996). Studies have reported that it is difficult to incorporate chlorhexidine into toothpaste and gels because of its ability to bind to other components of the toothpaste which may lead to inactivity (Addy *et al.*, 1989). Synergistic effects of chlorhexidine and fluoride has been investigated and it was found to reduce the gingival sores and bacterial adherence to oral surfaces (Smith *et al.*, 1996). Some side effects were also studied about chlorhexidine. It has an unpleasant taste and produces brown staining on the teeth. It encourages subgingival calculus formation (Yates *et al.*, 1993). Other rare side effects are mucosal erosion and parotid swelling (Addy, 1986).

1.4.2 Quaternary ammonium compounds

Quaternary ammonium compounds such as cetylpyridinium chloride (CPC) have moderate plaque inhibitory activity (Lobene *et al.*, 1977). It has been found that the antibacterial properties of these compounds is considerably reduced once it is absorbed onto a surface and this may be related to monocationic nature of the compound. CPC is mostly used as an adjunct to mechanically to pre-brushing mouthrinse to maintain the oral hygiene. Synergistic effects of chlorhexidine and CPC was studied. 0.05 % of chlorhexidine and CPC showed poor effects, this is undoubtedly due to the low concentration in these formulations.

1.4.3 Phenolic antiseptics

Phenols have been used in mouthrinses for a considerable time. Listerine is an essential phenolic mouthwash which has moderate plaque inhibitory effects and some anti-gingivitis effects. When Listerine was compared with anti-adhesives and 0.2 % chlorhexidine, it was observed that chlorhexidine has significantly more effects than Listerine which was in turn more effective than the anti-adhesives mouthwashes (Moran *et al.*, 1997). Listerine has showed antioxidative activity (Firatli *et al.*, 1994). The study conducted by Moran *et al.*, (1997) showed that the phenolic antiseptics were found less effective as compared to chlorhexidine mouthwashes.

1.4.4 Triclosan

Triclosan is a non-ionic antiseptic which does not contain staining effects. It has been used as mouthwashes and formulated into toothpastes. Triclosan has been found to produce moderate inhibitory effects on plaque. There is evidence that triclosan may act as anti-inflammatory agent (Kjaerheim *et al.*, 1996). In this way it has been revealed to reduce inflammation on the gingival and skin by sodium lauryl sulphate and nickel hypersensitivity respectively (Barkvoll and Rölla, 1995). Triclosan has further shown to reduce histamine-induced dermal inflammation and reduce severity and healing process aphthous ulceration (Barkvoll and Rölla, 1995). The mechanism of action of triclosan has been investigated *in vitro* and it has been observed that triclosan inhibits both the cyclo-oxygenase

and lipoxygenase therefore reducing the synthesis of prostaglandins and leukotrienes responsible for inflammation.

1.5 Aims and objectives of this study

The purpose of this study is to investigate the effect of ethanol extract of *Artemisia afra* Jacq. ex Willd. widely distributed plants in various countries around the South Africa against oral microorganisms. This plant is used mainly in traditional medicine for the prevention and treatment of various infectious and inflammatory diseases.

- This study aims at examining the antimicrobial activity of *A. afra* against oral microorganisms.
- Determination of antioxidant and cytotoxicity of the extract and isolated compounds on fibroblast cells

1.6 Scope of the thesis

Chapter 1, outlines the relationship between ethnopharmacology and periodontology. The activity of *Artemisia afra* crude extract against periodontal (oral) microorganisms is described in chapter 2. Determination of antioxidant of extract and isolation of the compounds is discussed in chapter 3 and chapter 4 respectively. Chapter 5 will be focusing on the antimicrobial and antioxidant activity of compounds from *A. afra* and cytotoxicity of active compounds in chapter 6. Chapter 7 encompasses general discussion and conclusion.

Chapter 8 is the appendix of the ^1H -NMR and ^{13}C -NMR spectrum of the isolated compounds.

1.7 Hypothesis

Artemisia afra contain antimicrobial phytochemicals that suppress the growth of oral pathogens.

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Chapter 2

Antimicrobial activity of *Artemisia afra* extract against oral microorganisms

2.1 Introduction

Plants have structured the basis for traditional medicine for many years. Recently, some plant extracts have been shown to potentiate the activity of antibiotics against resistant bacterial strains, bringing in the concept of resistance modification (Oluwatuyi *et al.*, 2004). Oral bacterial isolates resistant to penicillin, metronidazole, tetracycline and macrolides have been reported by researchers from different countries. Such resistant bacteria have also been isolated from infected patients (Oluwatuyi *et al.*, 2004). The screening of traditional South African medicinal plants against periodontal microorganisms will be beneficial in providing alternative and affordable medication. These benefits are directed to communities where medicinal plants are used for primary health care.

Belonging to the family of aromatic plants, *Artemisia afra* have been used for many years by different cultures around the world (Stockwell, 1988). Currently, research has revealed some of the essential oils from aromatic plants are useful in food preservation (Tiwari *et al.*, 2009), antibacterial (Cowan, 1999), anti-fungal (Martin and Ernest, 2004), antioxidant (Bettaieb *et al.*, 2010), spasmolytic activity (de Sousa *et al.*, 2008), anti-inflammatory and anticancer activity (Ashour, 2008). These result shows good potential of essential oil replacing synthetic antibiotics

which are responsible for most of an increased resistance of pathogens (Hogberg, 2010). Propolis has also played a crucial role as a potential application in the control of oral caries caused by bacteria (Park *et al.*, 1998) and there is evidence that it has antibacterial (Scazzocchio *et al.*, 2006), antifungal (Silici *et al.*, 2005) and antiviral activity (Amoros *et al.*, 1992). The current study investigates the antibacterial activity of *Artemisia afra* (Table 2.1) against anaerobic Gram positive and Gram negative microorganisms.

2.2 Materials and Methods

2.2.1 Plant material

Plant material was collected based on the traditional uses (Table 2.1) against oral ailments and supported by strong background of literature. The plant investigated (Figure 2.1) was collected from the South African National Biodiversity Institute (SANBI). Voucher specimens were prepared and identified at the H.G.W.J. Schweitkerdt Herbarium, University of Pretoria.

Table 2.1 Traditional use of *A. afra*.

Plant	Plant family	Plant part	Voucher specimen Number	Medicinal use and References
<i>A. afra</i>	Asteraceae	Arial parts	PRU 118251	Cough, colds, sore throat, asthma headache, oral care, gout and intestinal worms (van Vyck and Wink, 2004)

2.2.5 Distribution of *Artemisia afra*

A. afra (Figure 2.1) is widely distributed along the eastern parts of Africa. It grows in thick, bushy areas, usually with tall stems up to 2 m high but sometimes as low as 0.6 m. *A. afra* is a common species in South Africa with a wide distribution from the Cederberg Mountains in the Cape, northwards to tropical East Africa and stretching as far north as Ethiopia (van Wyk and Wink, 2004). In the wild it grows at altitudes between 20-2 440 m on damp slopes, along streamsides and forest margins. *A. afra* is the only indigenous species in this genus. World-wide there are about 400 species of *Artemisia*, mainly from the northern hemisphere. Many of the other *Artemisia* species are aromatic perennials and are used medicinally.



Figure 2.1 Leaves and flower heads of *A. afra* (van Wyk and Wink, 2004)

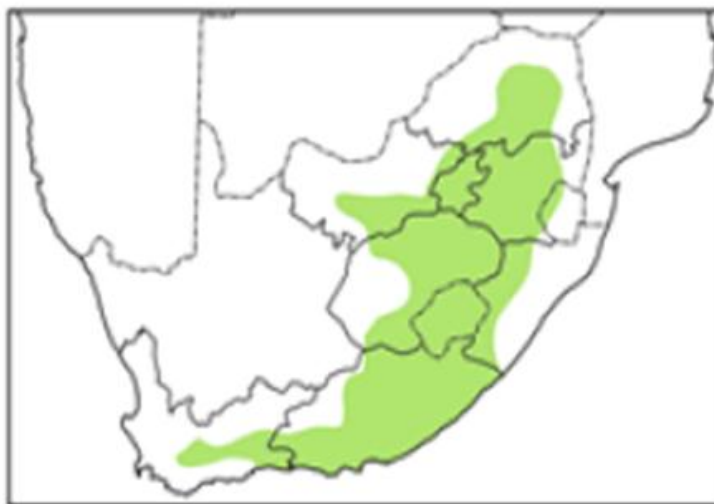


Figure 2.2 Distribution of *A. afra* along the Southern Cape, northwards to tropical east of South Africa.

2.3 Preparation of the extract

Fresh plant material was soaked in 96 % ethanol and homogenized into fine mesh. The sample was then filtered through the Whatman No.1 filter paper. The filtrates were evaporated to dryness in a BUCHI Rotavapor at 40° C.

2.4 Antimicrobial activity

2.4.1 Microorganisms

The microorganisms used in this study were *Actinomyces naeslundii* (ATCC 19039), *Actinomyces israelii* (ATCC 10049), *Actinobacillus actinomycetemcomitans* (ATCC 33384), *Candida albicans* (Med I), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC 25611) and *Streptococcus mutans* (ATCC 25175). Bacteria were grown in the Casein-peptone Soy agar medium (CASO) (Merck SA (Pty) Ltd. Halfway house) under anaerobic condition in anaerobic jar with anaerocult A (Merck SA (Pty) Ltd. Halfway house), at 37 °C for 72 hours. Sabouraud dextrose agar medium (SDA) (Merck SA (Pty) Ltd. Halfway house) was used for yeast (*Candida albicans*) and incubated at 25 °C for 24 hrs under aerobic conditions. Sub-culturing was done once weekly. The microbial growth inhibitory potential of the extracts was determined by using the agar disc diffusion method as described by Washington, (1981). Inocula were prepared by mixing a few microbial colonies with sterile ringer's solution and comparing the turbidity with that of the standard 0.5 McFarland solution (McFarland, 1907) which is equivalent to 1×10^5 CFU/ml. Hundred microlitres of inocula of all tested microorganisms were inoculated on casein-peptone soy agar medium for bacteria and sabouraud dextrose agar medium was used for *Candida*. Extracts were dissolved in 10 % DMSO (Merck SA (Pty) Ltd. Halfway house) to a final concentration of 100 mg/ml. Positive drug control (chlorhexidine 5 %) was prepared and extracts were dissolved in 10 % DMSO to a concentration of 100 mg/ml.

2.4.2 Minimum inhibitory concentration (MIC) assay

A modified method of micro-dilution 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 extract (100 mg/ml) was serially diluted in the 96-well plate with 48 hours old microorganisms (0.3 cfu/ml) grown at 37 °C and the final concentration of extracts and positive control Chlorhexidine (CHX) ranged from 25 mg/ml to 0.2 mg/ml. Microbial growth was indicated by adding 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, South Africa) to micro-plate wells and incubated at 37 °C for 48 hours. 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 assay to 150 µl of fresh broth. These suspensions were re-incubated at 37 °C for 48 h. The MBC was determined as the lowest concentration of extract which inhibited 100 % growth of microorganisms (Cohen *et al.*, 1998).

2.5 Results and discussion

The results of antimicrobial activity are presented in table 2.2. The plant tested in this study exhibited activity against *A. naeslundii*, *A. israelii*, *S. mutans*, *A. actinomycetemcomitans*, *P. intermedia*, *P. gingivalis*, and *C. albicans*. *A. afra* extract showed MIC values ranging from 6.3 mg/ml to 1.6 mg/ml against Gram-positive bacteria while the MIC values ranging from 25.0 mg/ml to 6.3 mg/ml were observed in Gram-negative bacteria including a fungus *C. albicans* which was inhibited at 6.3 mg/ml. The MBC of the tested extract was insignificant when

compared to the MIC results. The positive control (Chlorhexidine) showed to be a stronger control in that its activity was prominent when compared to the MIC of the extract tested (Table 2.2).

Table 2.2 The MIC and MBC (mg/ml) values of plants tested against periodontal pathogens

Plant extract	Microorganisms tested													
	MIC (mg/ml)							MBC (mg/ml)						
	Gram +ve			Gram -ve				Yeast	Gram +ve			Gram-ve		Yeast
	A.n	A.i	S.m	A.a	P.i	P.g	C.a	A.n	A.i	S.m	A.a	P.i	P.g	C.a
<i>A. afra</i>	3.1	1.6	6.3	25.0	6.3	6.3	6.3	1.6	6.3	6.3	Na	12.5	25.0	Na
Chlorexidine	1.6	6.3	6.3	1.6	6.3	1.6	6.3	1.6	6.3	6.3	1.6	6.3	1.6	12.5

A.n, *Actinomyces naeslundii*, A.i, *Actinomyces israelii*, S.m, *Streptococcus mutans*, A.a, *Actinobacillus actinomycetemcomitans*, P.i, *Prevotella intermedia*, P.g, *Porphyromonus gingivalis*, C.a, *Candida albicans*.

The antimicrobial activity of volatile oils found in *A. afra* revealed that the oils have activity against bacteria such as *Acinetobacter calcoaceticus*, *Klebsiella pneumoniae* and *Escherichia coli* (Deans and Svoboda, 1990). These oils may be contributing to the activity observed in this study. *Artocarpus heterophyllus* tested against *Streptococci* and *Actinomyces* species showed inhibition of growth at 25.0 µg/ml (Sato *et al.*, 1996). Isolated compounds from *A. heterophyllus* also inhibited the growth of periodonto-pathogenic bacteria, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* at 3.13 and 12.5 µg/ml respectively. Screening of essential oils from aromatic plants showed excellent antibacterial activity against clinical *Streptococcus aureus* with MIC values ranging from 254 to 1024 mg/ml (Alexopoulos *et al.*, 2011). These results sanction the objectives of this research. More about the potential of active isolated compounds from *A. afra* will be discussed in detail in chapter five.

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Chapter 3

Antioxidant activity of *Artemisia afra*

3.1 Introduction

Periodontal diseases are among the most common chronic diseases affecting children and adults (WHO). Gingivitis is a disease which can be defined as the inflammation of the gums (gingival) mediated by the host parasite interface that is limited to gingival tissues. Gingivitis is mainly caused by the accumulation of microbial plaque in the region of dento-gingival (Gibbons, 1984; Aksoy *et al.*, 2006). Plaque and biofilms are mostly difficult to eradicate and they cause recalcitrant infections (McNeil and Hamilton, 2004). Currently manufactured treatments require reduction and elimination of bacterial accumulation on surfaces of the oral cavity. Products such as Chlorhexidine, fluoride, cetyl pyridinium and phenol derivatives, to mention a few, are capable of inhibiting bacterial growth, however, they have been shown to have undesirable effects such as staining, vomiting and diarrhoea.

Free radicals are implicated in the etiology of several diseases such as stroke, diabetes, cancer, rheumatoid arthritis and coronary artery diseases (Halliwell, 1991). A healthy diet, high in fruit and vegetable consumption is associated with low risk of diseases which is attributed to the antioxidant vitamins and phytochemicals (Prior, 2003). Free radicals not only cause cancer or stroke related diseases but also advances the imbalance between oxidants and antioxidants which lead to periodontal diseases. Besides dental plaque deposition, another mechanism can be implicated in periodontal diseases development. Some studies have

reported that the excess production of reactive oxygen species (ROS) leads to damages of gingival tissues, periodontal ligament and alveolar bone. This occurs due to an increase in free radical production and defect in total antioxidant activity of saliva (Chapple and Matthews, 2007; Alviano *et al.*, 2008).

Reactive oxygen species (ROS) may cause damage either by activating cyclooxygenases and lipoxygenases, protein damage including gingival hyaluronic acid, DNA damage or stimulation of cytokine release by activating KB(NF- κ B) (Staal *et al.*, 1990). Some of the exogenous sources of ROS are cigarette smoke and ionising radiations. Plants have been shown to possess antioxidants. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals. These antioxidants are naturally produced by medicinal and dietary plants which might help to prevent free radical oxidative damage (Silva *et al.*, 2005). The search is therefore on for an antioxidant that could be used to control these diseases, and polyphenolic compounds are likely candidates (Houde *et al.*, 2006). Polyphenols One of the largest group in plants with radical scavenging activity and proven to be more effective *in vitro*. This is due to the high reactivity of donating hydrogen ion, the ability to stabilize unpaired electrons or ability to chelate transition metal ions (Rice-Evans *et al.*, 1997).

3.2 Material and methods

3.2.1 Plant material

Ethanol extract of *A. afra* (2mg) was dissolved with 1.0 ml of 100 % ethanol in an eppendoff to make up a stock solution of 2mg/ml.

3.2.2 Determination of antioxidant activity

The free radical scavenging activity was measured using 1,1 diphenyl-2-picryl-hydraxyl (DPPH) assay as described by Du toit *et al.*, (2001) with slight modifications. The ethanol extract of *A. afra* and Vitamin C (positive control) 2.0 mg/ml (20 μ l) was added to the first three wells of a 96-well plate containing 200 μ l of distilled water to make up a final concentration of 100 μ g/ml. The first row containing the extract was serially diluted to wells which contain 110 μ l of distilled water and the concentration range of the extract was 0.8, 1.563, 3.125, 6.25, 12.5, 25, 50 and 100 μ g/ml. (The concentration of DPPH 90 mM which was added to all wells containing the extract, Vitamin C and Solvent control that is wells with ethanol only). Other wells containing extract only were read as a blank. The plate was incubated at 37 °C for 30 min and the absorbance was measured at 517 nm using the ELISA plate reader. The percent radical scavenging activity by *A. afra* was determined by comparison with ethanol (blank).

3.4 Results and discussion

The basic function of antioxidants is to eradicate detrimental oxidants known as reactive oxygen species (ROS) and to repair damages caused by ROS. Many of the foods and beverages contain phytochemicals that are effective against ROS, for instance wine, Vitamin supplements and green tea (Atsumi *et al.*, 2007). In this study the *A. afra* extract was tested for antioxidant activity, which is said to contain flavonoids that are well known as antioxidants. The ethanol extract of *A. afra* showed effective free radical scavenging activity, visually at 25.0 $\mu\text{g/ml}$. The purple colour of DPPH indicates the effects of free radicals scavenging activity. The extract showed to be capable of scavenging free radicals in various concentrations tested (Figure 3.1 and 3.2).

The scavenging effects of *A. afra* on DPPH radical increases from 1.5625 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ with the percent inhibition of ± 0.261 to 69.894 % respectively. The extract was very competitive to ascorbic acid/Vitamin C in that the concentration of the extract at 100 and 50 $\mu\text{g/ml}$ had high activity than vitamin C. Both were tested at the same concentration range. The effective concentration (EC_{50}) at which the DPPH radicals were scavenged by 50 % of the extract is $\pm 22.2 \mu\text{g/m}$.

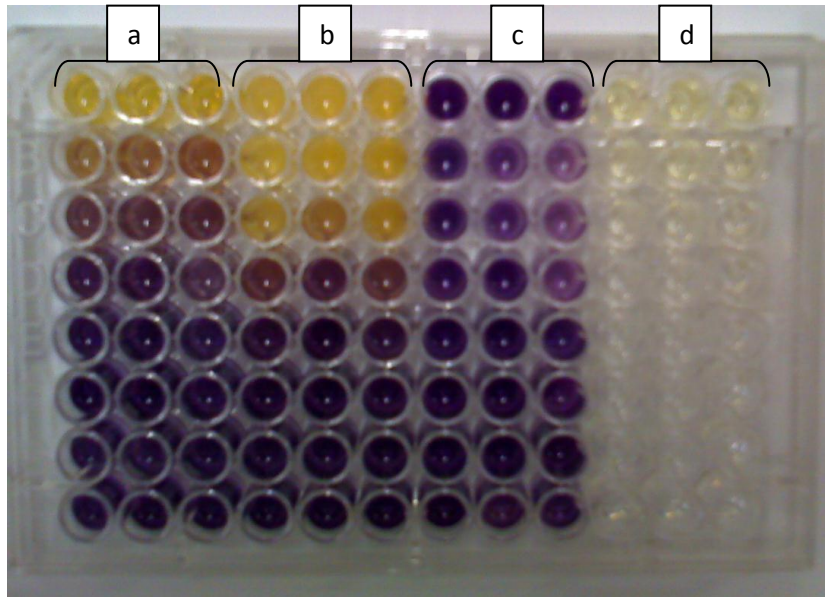


Figure 3.1 Antioxidant activity of *Artemisia afra*, a, extract; b, Vitamin C (Positive control); c, solvent (Negative control) and d, Extract only (Blank)

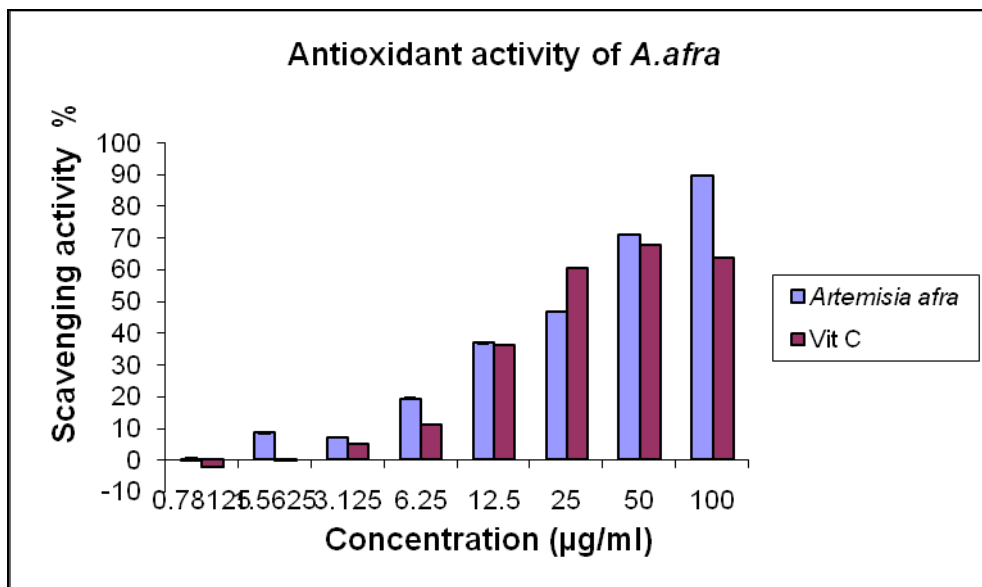


Figure 3.2 Antioxidant activity of *A. afra* using DPPH assay. Means \pm SD = 0.31

The recognition and role of naturally occurring antioxidants is elevating and are currently used to control chronic diseases caused by ROS (Banerjee *et al.*, 2003). Evaluation of various antioxidants in saliva to identify differences between patients with implants diseases has been subjected to antioxidative supplements as a treatment (Alviano *et al.*, 2008). Volatile oils from *A. afra* such as terpinoline, camphor, methyl chavicol and trans-sabinyl acetate to mention a few, are documented to be the main attribute of antibacterial and antioxidant activity (Lopez *et al.*, 2008). The ROS produced by inflammatory and immune cells have been associated with major tissue damage. However, the body then produces defence antioxidants whose role is to protect cells and tissues from radicals of the host cell.

The early onset of periodontitis possess cells that are exhibit increased free radical production and patients with periodontitis shows high prostaglandin E₂ production as compared to patients without periodontitis disease is prompted by the Gram-negative bacterial lipopolysaccharides (Lamont and Jeckinson, 1998). Therefore, there seems to be a delicate balance between inflammatory and immune cell hypofunction where the pathogen cause direct tissue damage and the host defence further elevate the damage. Bacteria which exist in the mouth such as *P. gingivalis*, *A. actinomycetemcomitans* are anaerobic and survive commensally with other aerobic bacteria such as *Streptococci* and *Actimycetes* species (Lamont and Jenkinson, 1998). However, anaerobes survive by interchanging oxygen availability with aerobic bacteria, this is made possible by species such *Streptococci* which reduces Oxygen ion (O₂⁻) tension and provide attachment site for anaerobes. The mechanism of attachment to gingival sites by anaerobes include the utilization of membrane proteins and fimbriae, some provide haem which is required for growth and proteolytic enzymes that degrade substrates such as collagen, fibronectin, fibrinogen, laminin and keratin (Travis *et al.*, 1995). Therefore,

the bacterial defence to avoid oxidative destruction by (O_2^-) released by polymorphonucleocytes (PMN), the bacteria will produce antioxidant superoxide dismutase, which converts the O_2^- to H_2O_2 (Figure 3.3) which acts as a buffer to protect against H_2O_2 (Smalley *et al.*, 2000).

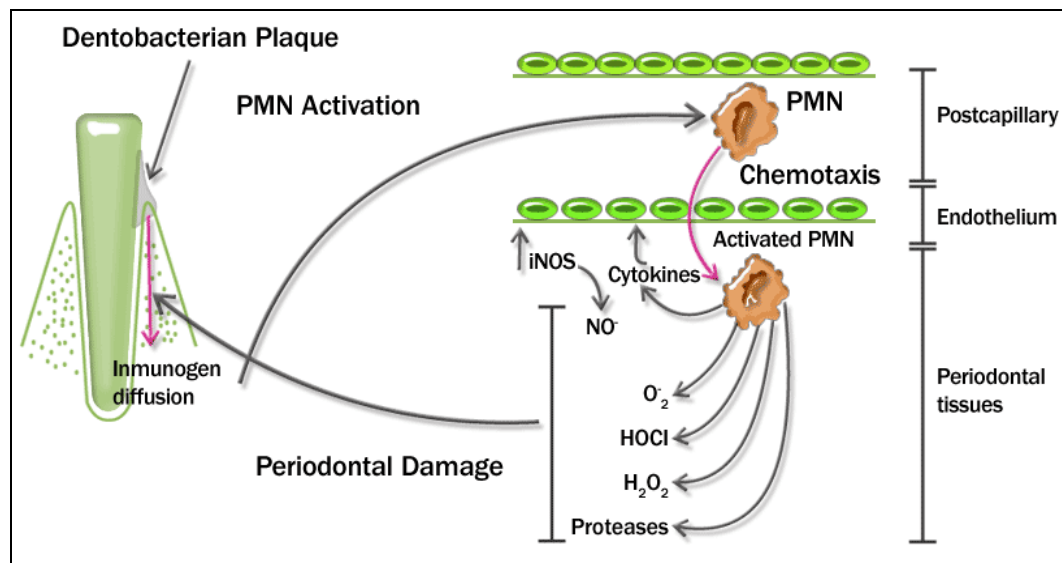


Figure 3.3 Schematic representation of the incidence of local etiologic factors in the inflammatory periodontal disease (www.antioxidantes.com).

The consequences of the presence of ROS, effects of proteases and cytokines in periodontal tissues influence the host response and in the absence of treatment, inflammatory periodontal disease (IPD) becomes reactive and destroy the periodontal tissues (Battino *et al.*, 1999). An increased production of cytokines could lead to bone loss and periodontal tissue damage in local areas. The most common cytokine found in periodontal disease is IL-1. This cytokine concentration has been seen to diminish after the treatment of periodontal disease. One important aspect of the presence of cytokine is to increase the prostaglandins E_2 (PGE_2) in

gingival fibroblasts which trigger bone reabsorption (Battino *et al.*, 1999). Proteases are also an attributing factor that causes the destruction of the extracellular matrix. They are beneficial in combination with ROS which constitute the germicide mechanism of the cells. Therefore the combined action of the cytokines and proteases implicate the induction periodontal damage and tooth loss. Dental plaque, implicated in oral diseases is a very complex biofilm which provides protection to the bacteria against antimicrobial agents (Gilbert *et al.*, 1997; Mah & O'Toole, 2001; Wilson, 1996). These results in the progression of destructive diseases also depend on the abnormal host response (Page and Kornman, 1997).

3.5 References

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Chapter 4

Isolation of compounds from *Artemisia afra* extract

4.1 Introduction

The genus *Artemisia* is one of the largest and most widely distributed genera in the tribe Anthemideae of the Asteraceae (Compositae). Family Asteraceae is characterized by high accumulation of essential oils and terpenoids. Other Asteraceae contains phenolic compounds, together with terpenoids are important in pharmacy and medicine (Wagner, 1977). For example, many studies focused on the antibacterial nature of volatile oils have shown that oils from *Artemisia afra* which contain a mixture of monoterpenes is active against bacteria such as *Acinetobacter calcoaceticus*, *Klebsiella pneumonia*, *Brevibacterium linens* and *Yersinia enterocolitica* including *Escherichia coli* (Deans and Svoboda, 1990). Essential oils from *Artemisia* species are also finding their way in to perfumery industries. The preference of the immature plant parts over mature ones is probably due to the high content of thujone and 1,8-cineole, well known for its psychoactive properties (Shah and Thakur, 1992).

Terpenoids present in *Artemisia* species represent a big class of compound from monoterpenes up to triterpenes, with most of the species characterized by the fragrance of lower terpenoids, such as monoterpenes and sesquiterpenes, these compound are the major reason of the aromatic odour in plants. Among the compounds is camphor, thujone, borneol

and 1,8-cineole. These compounds have been seen to vary with environmental factors. The monoterpenes content of some *Artemisia* species varies seasonally with high content in July (4.18 %) and the lowest during May (0.97 %) (Cedarleaf *et al.*, 1983). *Artemisia* Ketone has been found in the inflorescence of *A. annua* from China (Woerdenbag *et al.*, 1993), while in the mangolian, phenols (thymol and carvacrol) were the main compounds (Satar, 1986). *A. afra* oil was characteristics of the presence of the monoterpenes α - and β -thujone, followed by 1,8-cineole, camphor and α -pinene (Graven *et al.*, 1992). The South African species of *A. afra* is reported to contain scopoletin in flowerheads (Goodson, 1922) and the roots were found to contain acetylene (Kraft *et al.*, 2003). Flavonoids such as luteolin, apigenin and quercetin (Figure 4.1) are the most common compounds in *Artemisia* species (Belenovskaja, 1996). Isolation of *A. giraldii* has lead to two new flavones identified as 4,6,7 trihydroxy-3,5-dimethoxyflavone and 5,5-dihydroxy-3,4,8-trimethoxyflavone (Zheng *et al.*, 1996). Epigenin and 7-methyl ester has been found in *A. afra* (Saleh and Mosharrafa, 1996) and the root extracts contained isofraxidin while flowers contained scopoletin (Murray, 1995).

The common and most important sesquiterpene lactones found in the genus *Artemisia* are compounds such as Dimeric quaianolides (absinthin and anabsinthin) which are responsible for a bitter taste the plant and these kind of compounds are classified as azulenes. Azulenes are compounds which have pharmaceutical importance due to their antiseptic and antibacterial properties (Novotny *et al.*, 1960). Very important among sesquiterpenes from *Artemisia* is a well known antimalarial compound Artemisinin (Figure 4.2), a santanolide of *Artemisia annua*.

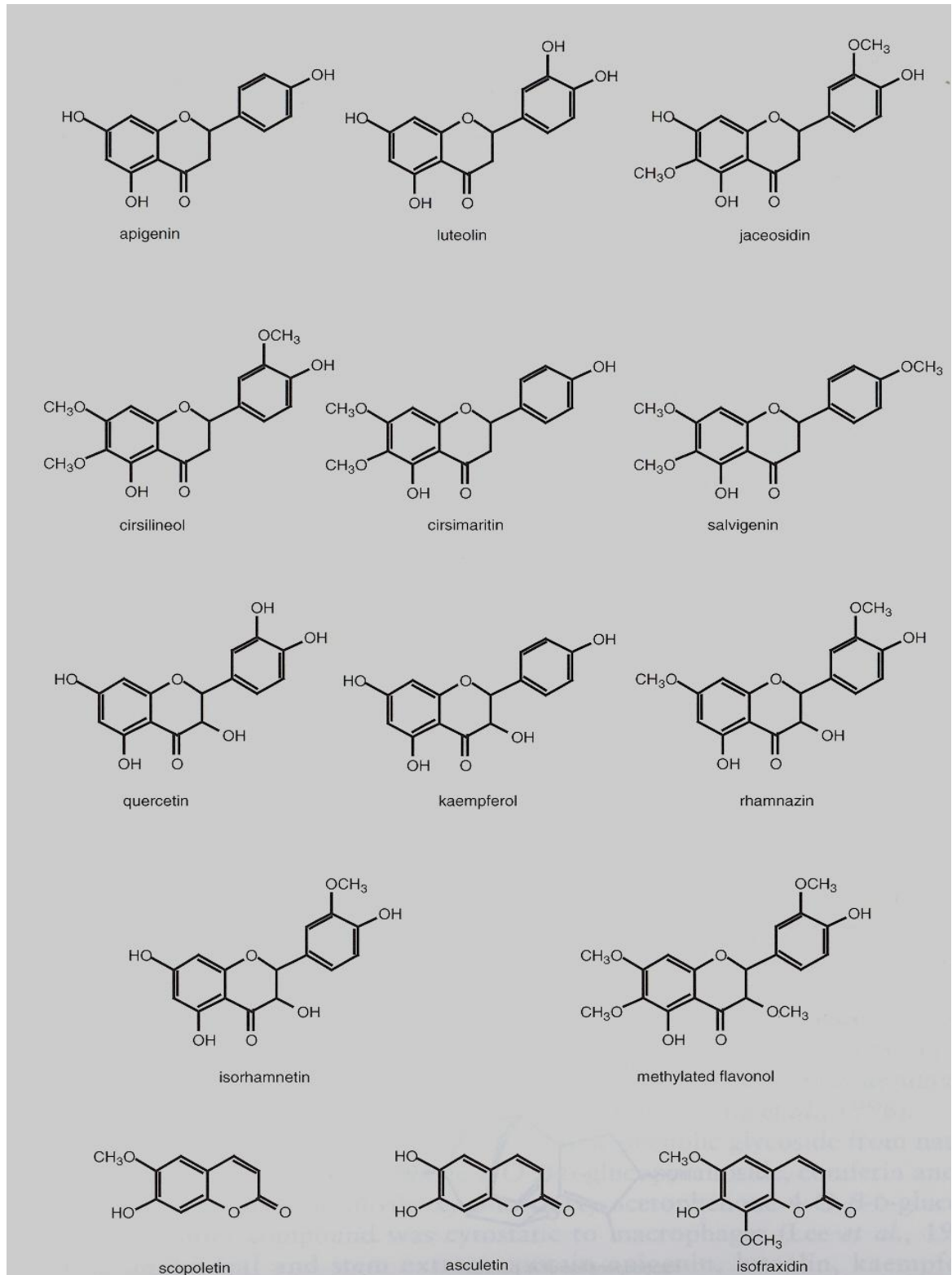


Figure 4.1 Structures of representative flavonoids and coumarins from genus *Artemisia* (Wright, 2002).

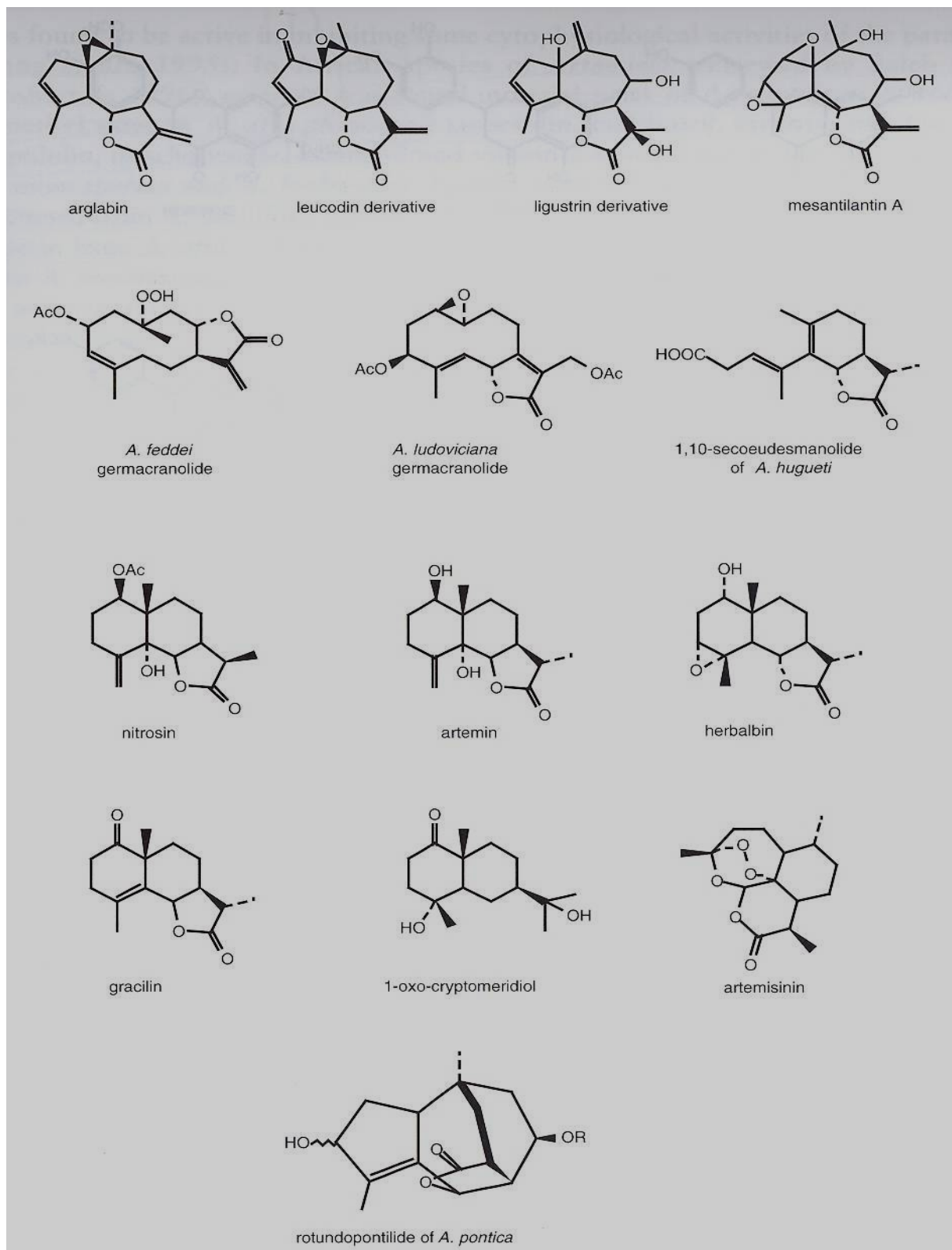


Figure 4.2 Structures of representative sesquiterpene lactones from genus *Artemisia* (Wright, 2002).

4.2 Materials and methods

4.2.1 Preparation of plant extract

Fresh arial parts of *Artemisia afra* were collected and soaked in 96 % ethanol and homogenized. The sample was then filtered through the BUCHI vacuum filter. The filtrates were evaporated to dryness in a BUCHI Rota-vapor at 40° C.

4.2.2 Isolation and identification of compounds

The number of anti-bacterial compounds present was determined by the direct bioautography method of chromatograms using *S. mutans* (Begue and Kline, 1972). The extracts were loaded onto a TLC plate and developed using HEX:EtoAc. The plate was thoroughly dried overnight and then the chromatograms were sprayed with a dense culture of *S. mutans*, incubated overnight at 37 °C and then sprayed with 0.2 mg/ml *p*-iodonitrotetrazolium (INT) (Sigma). Clear zones indicated compounds which inhibited bacterial growth (Figure 4.3).

The isolation of compounds was performed using the column chromatography methods. Fractionation was preceded by using silica gel 60 (70-230 mesh) and Sephadex[®] LH20. Thin layer chromatography (TLC) was performed on aluminium sheets coated with silica gel 60 F₂₅₄ (Merck) and UV light was used to detect compounds. TLC plates were further sprayed with vanillin sulphoxide reagent and spectrums were obtained by Nuclear magnetic resonance (NMR). The Isolation of *A. afra* was started with 200g of ethanol extract on to a

100 mm diameter column. The column was filled with 2 kg silica gel followed by the extract-silica gel mixture and lastly silica gel. The column was eluted first with 3 liters of a non-polar solvent (100 % Hexane) and the polarity was increased gradually with ethylacetate up until a ratio of 0:100. Forty fractions were obtained and combined to make up 12(I-XII) sub-fractions according to similarities of compounds as determined by TLC plate (Figure 4.4 & 4.5). A sephadex column was conducted on the sub-fraction X on a medium column using 100% methanol (MeOH) and it yielded 25 sub-fractions which were combined in to three sub-fractions (L, M, N) Figure 4.6. Fraction N was fractionated using MeOH and a yellow precipitate was obtained which was further fractionated with 100 % Dichloromethane. All the residues that dissolved in DCM were removed, evaporated and spotted on a TLC plate and yielded a pure compound **1**. Fraction M was isolated using a gradient of solvents DCM:MeOH, 9:1 increasing polarity to 3 %. Sub-fractions were obtained, spotted and combined according to TLC profile. Since sub-fraction M₂ was crystallizing, it was dissolved in Dichloromethane:Hexane, 1:1 in order to obtain a florescent blue compound **3**.

A new fraction VII was isolated using a medium column with 100 % MeOH and 40 fractions were obtained and combined to 3 sub-fractions. Sub-fraction G showed an interesting TLC profile. Fraction G was fractionated using DCM:MeOH, 100 %-100 %, on a sephadex column and two fractions were obtained of which one was a pure compound **2**, the second fraction was again fractionated using DCM:MeOH (95:5) and compound **6** was obtained. Compound **5** was isolated using a slow solvent system of Hexane:Ethylacetate, at a ratio of 9:1, using silica gel on a medium column. A succession of a blue coloured compound was observed on a TLC plate after application of vanillin. The pure compound was then obtained after all impurities have eluted. The procedure followed is summarized in (Figure 4.6).

4.3 Results and discussion

4.3.1 Isolation and identification of isolated compounds

The TLC profile gave a clear antibacterial activity of the extract and guide to isolate ideal compounds (Figure 4.3). In all solvent systems tested on TLC, both polar and non-polar bands demonstrated activity by inhibiting the growth of *S. mutans*. Isolation of *A. afra* yielded six compounds (Figure 4.7), one known Flavone (**1**), sesquiterpene (**2**), Diterpene (**5**) two pentacyclic triterpene **4**, **6** and one unknown compound **3**. All these compounds were characterized by their $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra.

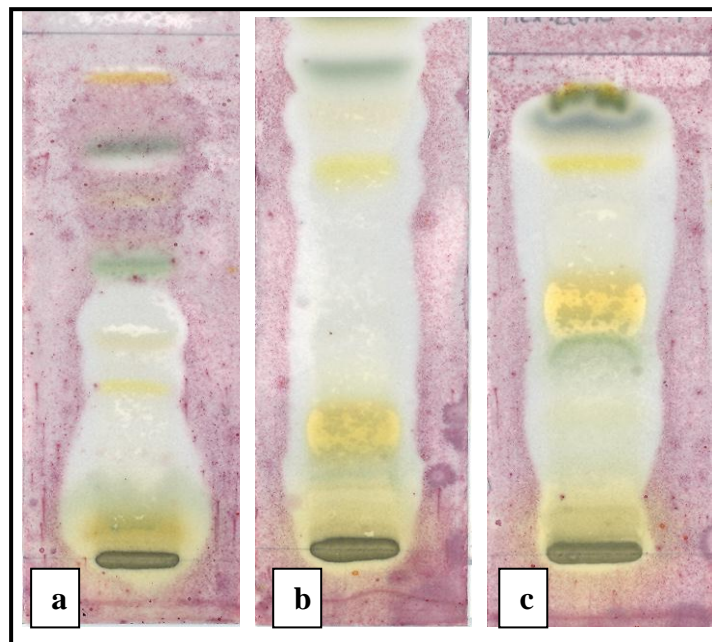


Figure 4.3 Antibacterial activity of *A. afra* against *S. mutans*

- (a) Solvent system of HEX: EtoAc (7:3), (b) Solvent system of HEX: EtoAc (1:1),
 (c) Solvent system of HEX: EtoAc (3:7)

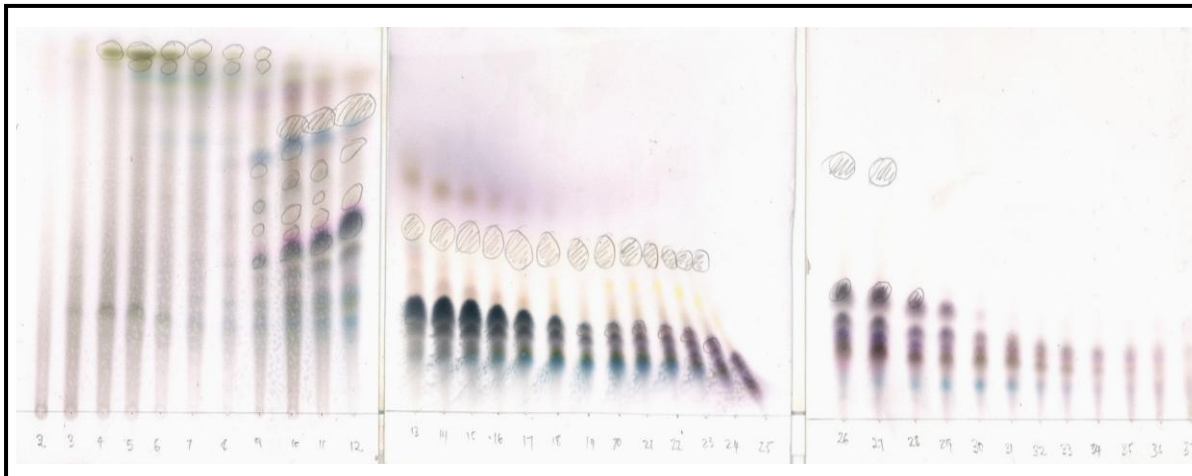


Figure 4.4 Forty fractions obtained from the first column.

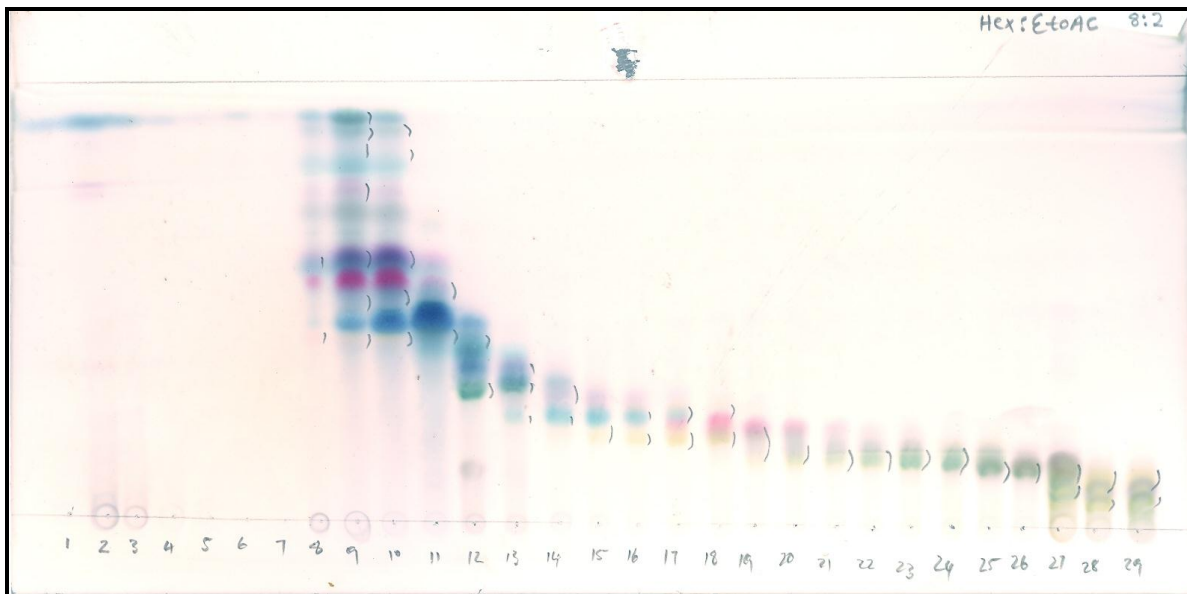


Figure 4.5 Collective fractions from the first column.

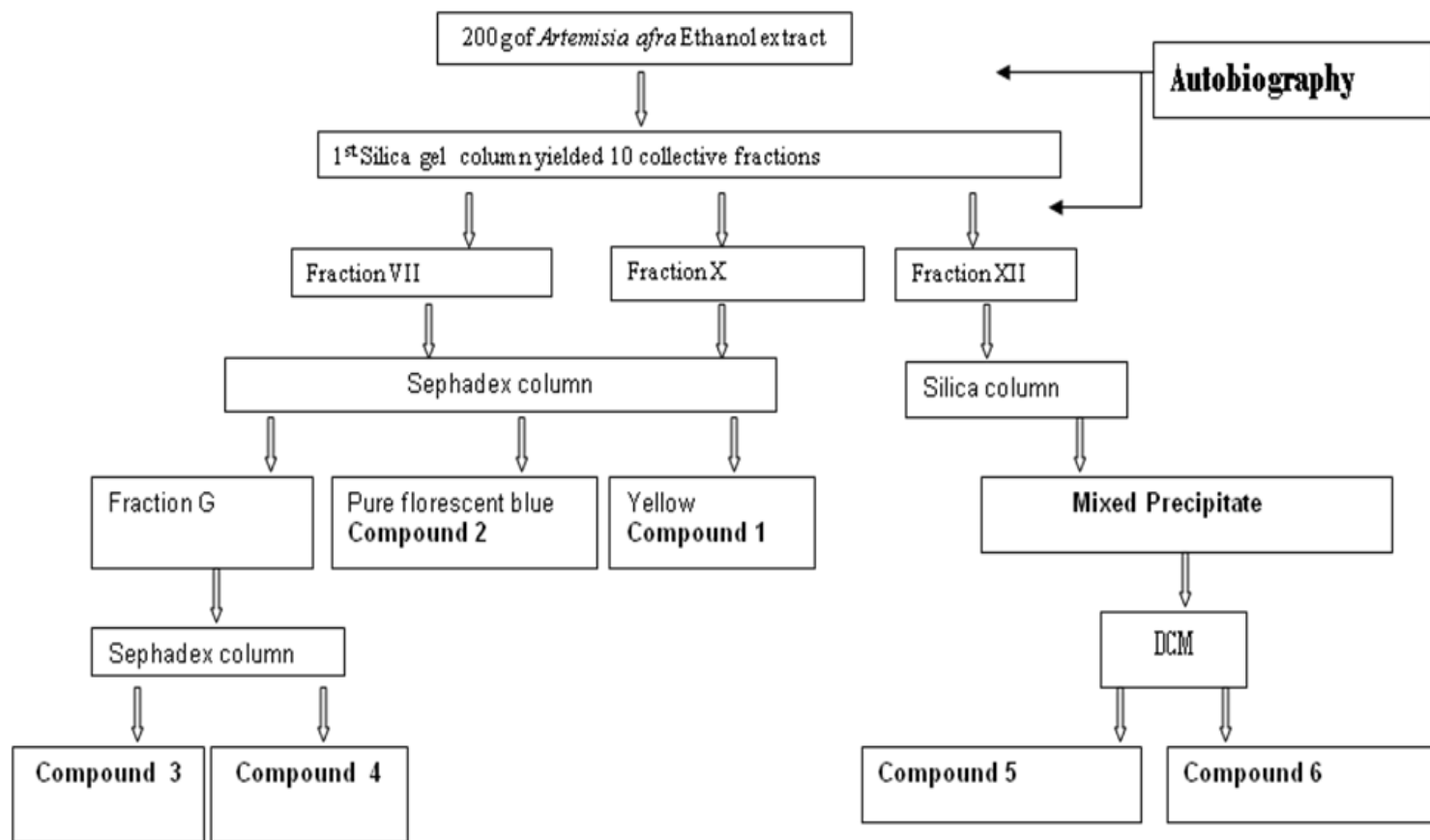


Figure 4.6 Schematic presentation of isolation steps followed

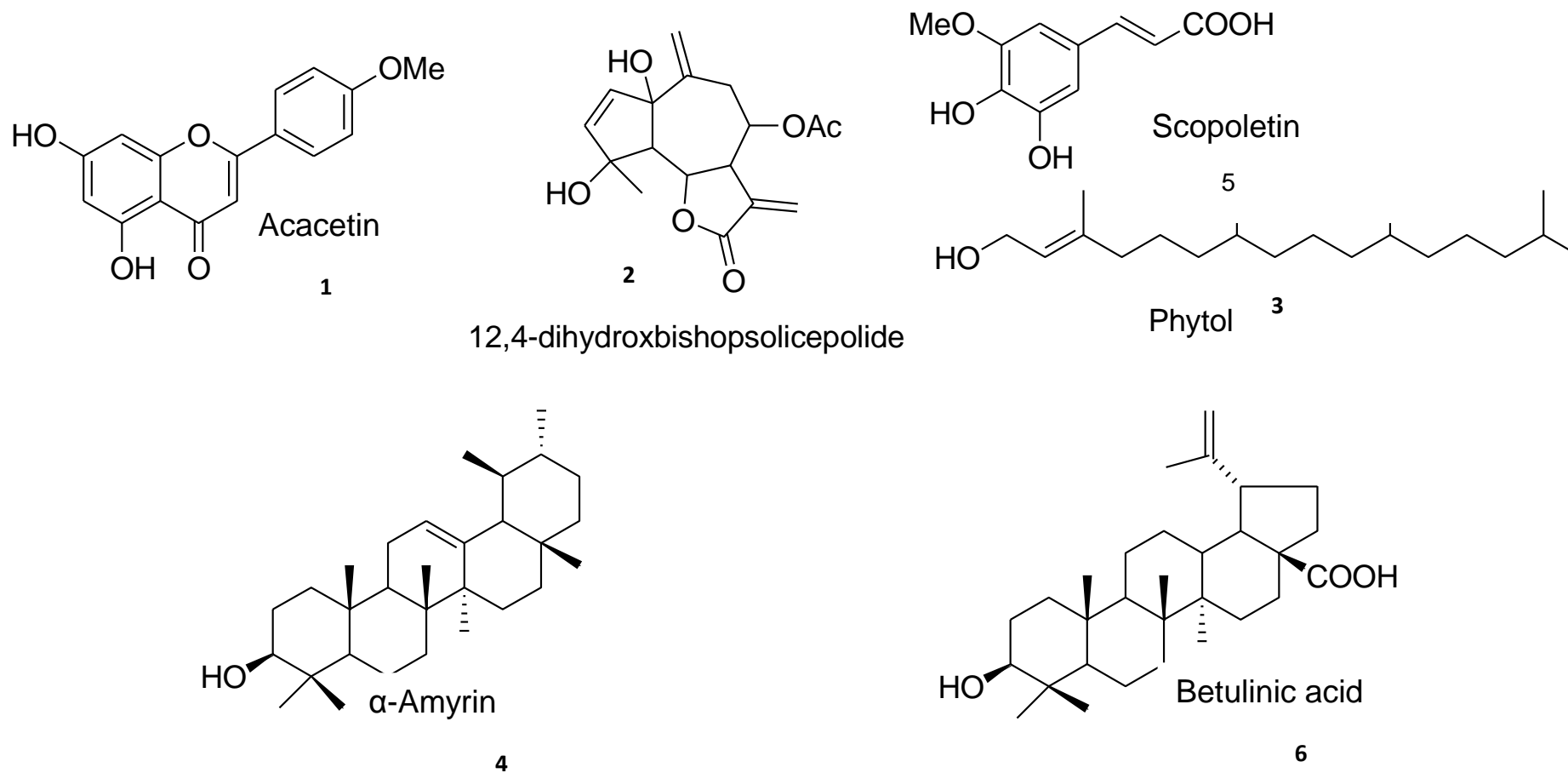


Figure 4.7 Structures of isolated compounds

Compound 3 was identified as phytol. Its structure was elucidated by analysis of ^1H -NMR (Figure 9.1.1 and table 4.1), ^{13}C -NMR (Figure 9.1.2. and table 4.1) and DEPT analysis data as well as by mass spectroscopic technique and confirmed by comparison with literature values.

The ^1H -NMR spectrum showed a doublet at 4.13 ppm for oxygenated methylene group, a triplet at 5.38 ppm for vinyl proton, as well as the expected CH, CH₂, and CH₃ peaks which were characteristic of phytol and in the same relative intensities (Arigoni *et al.*, 1997). Of these the singlet peak at 1.64 ppm for a methyl group attached to a double bond. ^{13}C -NMR and DEPT analysis showed the presence of 20 carbons, 1 quaternary, 4 methines, 10 methylenes and 5 methyl groups. A signal was present at 59.25 ppm for a carbon linked to hydroxyl group, and signals at 123.02 and 140.07 ppm for olefinic carbons (Arigoni *et al.*, 1997).

The geometry of the double bond was confirmed to be the trans isomer, which was deduced from the chemical shifts of both the C-20 protons (δ 1.62 ppm, s) and the C-2 proton (δ 4.05 ppm, d), both showed different values from those reported for the cis isomer, which were δ 1.73 and δ 4.48 ppm respectively. The downfield position of the 1-methylene in cis-phytol with respect to trans-phytol can be explained in terms of intra-molecular van der Waals deshielding between the 1-methylene group and the moderately bulky C₁₆-group which are cis-substituted at the double bond (Sims and Pettus, 1976).

Table 4.1 ^1H and ^{13}C -NMR chemical shifts (δ) for compound 3

δ ppm* (multiplicity**)		
Position	^1H	^{13}C
1	4.13 (d)	35.5
2	5.38 (t)	33.9
3	–	199.4
4	1.97 (m)	125.4
5	1.40, 1.36 (m)	161.0
6	1.24, 1.05 (m)	202.2
7	1.35 (m)	46.8
8	1.23, 1.03 (m)	34.2
9	1.29, 1.15 (m)	51.0
10	1.23, 1.03 (m)	39.4
11	1.35 (m)	20.8
12	1.23, 1.03 (m)	39.1
13	1.25 (m)	42.5
14	1.11, 1.03 (m)	56.5
15	1.50 (m)	23.9
16	0.86 (d)	28.0
17	0.84 (d)	55.9
18	0.83 (d)	11.8
19	0.81 (d)	17.5
20	1.64 (s)	35.6

* Chemical shifts are in ppm downfield from tetramethylsilane, and referenced to solvent signal at 7.26 and 77.00 ppm, respectively. ** Multiplicities of the observed ^1H -NMR are indicated in parentheses (s, singlet; d, doublet; t, triplet; m, multiplet).

The **compound 6** was isolated from the non-polar fractions through different silica gel columns. The compound was obtained as a white powder. The identification of the compound was completed using NMR spectra $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ (Figure 9.1.3 and 9.1.4) and TLC comparison with authentic sample exists in our lab. The presence of seven methyl singlets and an olefinic function in the $^1\text{H-NMR}$ spectrum at 1.63, 1.36, 0.96, 0.95, 0.92, 0.81 and 0.74 revealed that compound **6** may be pentacyclic tri-terpenoidal type in nature. The comparison of $^1\text{H-NMR}$ chemical shift with that of the reported data similar type of compounds and lupeol has led to the conclusion that compound (**6**) is very similar to lupeol and it should be a lupeol derivative. The presence of the signal at 3.0 of H-17 confirms the structure of betulinic acid.

Compound 1 was isolated as faint yellow powder and showed characteristic colour on TLC to flavonoidic nature, this was confirmed using ^1H and ^{13}C NMR spectra (Figure 9.1.5 and 9.1.6), which demonstrate signals at 7.97, 7.05 (d each, 8.8 Hz) of 4 substituted ring B, three signals at 6.80 (s H-3), 6.45, 6.16 (d each 1.8 Hz) of H-6 and H-8 respectively, the spectra showed also a signal at 3.81 of a methoxyl group (d_{C} 56.4). The given data is identical with the known compound 4'-methoxy kaempferol isolated from the same source before.

Compound 2, was identified as 12a,4a-dihydroxybishopsolicepolide using ^1H and ^{13}C NMR (Figure 9.1.7 and 9.1.8) which showed signals at 5.85, 6.29 (each d, $J=2.6$ Hz, CH_2 -13), 5.92, 5.59 (each d, $J=5.4$ Hz, H-2, 3), 5.11, 4.84 (each s, CH_2 -14), 4.84 (m, H-8), 4.12

(dd, $J=8.4, 13$ Hz, H-6), in addition to two methyl singlets at 2.12 (8-OAc), 1.30 (Me-15). The structure of this compound was confirmed also by ^{13}C NMR and DEPT-135 data.

Compound 5, it showed strong fluorescence blue colour under UV_{364} and its ^1H -NMR data demonstrate peaks at 6.25 (d, $J=9.5$, H-3), 7.65 (d, $J=9.5$, H-4), 7.44 (s, H-5), 6-OMe (s, 3.94) and 6.84 (s, H-8). The forgoing data is identical with the known compound scopoletin (Seyed *et al.*, 2008)

Compounds 4, was obtained as white crystals. The ^1H -NMR spectrum of compound, showed the six angular methyl singlet signals at d 1.07, 1.00, 0.99, 0.95 (each 3H, s, H-27,26,23,25) and 0.79 (6H, s, H-28,24), and the two secondary methyl doublet signals at d 0.91 (3H, br s, H-30) and 0.78 (3H, d, $J = 6.0\text{Hz}$, H-29). Careful revision for the literature indicated that the data of this compound typically matched with α -Amyrin, the wide spread triterpene in nature (Kang, 1987; Mahato and Kundu, 1994).

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Chapter 5

Antibacterial and antioxidant activity of isolated compounds from *Artemisia afra*

5.1 Introduction

Aromatic plants are frequently used in traditional medicine as antibacterial agents and their essential oils. Volatile compounds isolated have been known since possess antibacterial and antifungal properties (Benli *et al.*, 2007; Lopes-Lutz *et al.*, 2008). *Artemisia afra* is one of the known aromatic plants that have awakened interest in to investigating the antimicrobial activity of essential oils (Suliman *et al.*, 2010). Current studies have indicated that *A. afra* have potent antimicrobial activity against five pathogens including methicillin-resistant *Staphylococcus aureus* (Tohidpour *et al.*, 2010), *Mycobacterium smegmatis* and *M. tuberculosis*. antioxidants have gained a lot of importance because of their therapeutic potential against many diseases such as cancer, diabetes, cardiovascular diseases, neurodegenerative disorders, and ageing. The discovery of the role played by medicinal plants as therapeutic agents have led to many studies such as isolation of active constituents which are purified and tested for their activity, renewed interest in bioactive compounds from fruits, vegetables and spices. Antioxidants can be classified into enzymatic and non-enzymatic antioxidants.

In this study we focus on non-enzymatic antioxidants which are plant based compounds. However, though plant based antioxidant have many advantages, they also have drawbacks. Many of these antioxidants have poor oral bioavailability which maybe because of low

solubility, permeability and stability. Synthetic antioxidants such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are commonly used as antioxidants in foods to prevent or retard lipid oxidation. However, restrictions on the use of these compounds are being imposed because of their carcinogenicity and some side effects (Veliogluet *al.*, 1998). With regard to natural products, it is generally accepted that phytochemicals are less potent anti-infectives than agents of microbial origin, i.e. antibiotics (Clercq, 2001). Therefore, more in-depth research should be done to come up with new antimicrobial and anti-oxidative natural compounds that can be principal in the illumination of acute and chronic diseases.

5.2 Antibacterial activity of isolated compounds

The antimicrobial activity of isolated compounds was determined as described in chapter 2. All six compounds were tested at the final concentration of between 1000 µg/ml and 0.08 µg/ml.

5.3 Antioxidant activity of isolated compounds

The antioxidant activity of isolated compounds was determined as described in chapter 4. All six compounds were tested against DPPH scavenging activity at a final concentration of 100 µg/ml and 0.80 µg/ml

5.4 Results and discussion

5.4.1 Antimicrobial activity results

The antimicrobial activity of isolated compounds was investigated in terms of MIC and MBC in comparison with the commercially available anti-plaque agent (Chlorhexidine) as a control in this study. As shown in table 5.1, The MIC of the acacetin against *A. israelii* was 0.25 mg/ml. The activity of acacetin was much lower at the highest concentration of 1.0 mg/ml. All tested compound failed to inhibit the Gram negative *A. actinomycetemcomitans* and fungus *C. albicans*. Betullinic acid and 12,4-. dihydroxibishopsolicepolide exhibited weak activity against the Gram positive bacteria *A. naeslundii* and *A. israelii* at the highest of 0.5 and 1.0 mg/ml respectively.

Scopoletin was the best compound with activity range of 1.0 µg/ml for *A. naislundii* to 0.25 mg/ml for *A. israelii* and *Streptococcus mutans*. The three compounds which showed good antimicrobial activities were selected for further investigation into antioxidant and cytotoxicity. Betulinic acid exhibited weak antimicrobial activity against *P. gingivalis* at the concentration of 1.0 mg/ml (Rivero-Cruz *et al.*, 2008). Betulinic acid isolated from the stem bark of *Irvingia gabonensis* has been reported to have antimicrobial activity with MIC values of 156.25 and 78.12 µg/ml against *Escherichia coli* and *Klebsiella pneumoniae*, respectively (Kuate *et al.*, 2007). The study by Shai *et al.*, (2008) reports the antimicrobial activity of betulinic acid isolated from the *Curtisia dentata*, which showed an MIC value of 12 µg/ml against *Microsporium canis* and an MIC value of 250 µg/ml against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Table 5.1 The MIC and MBC (mg/ml) values of isolated compounds against periodontal pathogens

Plant extract	Microorganisms tested											
	MIC (mg/ml)						MBC (mg/ml)					
	Gram +ve		Gram -ve			Yeast	Gram +ve		Gram-ve			Yeast
	A.n	A.i	A.a	P.i	P.g	C.a	A.n	A.i	A.a	P.i	P.g	C.a
1	1.0	0.25	>1.0	1.0	1.0	>1.0	1.0	0.5	>1.0	>1.0	>1.0	>1.0
2	0.5	0.5	>1.0	1.0	>1.0	>1.0	1.0	0.5	>1.0	>1.0	>1.0	>1.0
3	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
4	1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
5	1.0	0.25	>1.0	0.5	>1.0	>1.0	>1.0	0.5	>1.0	1.0	>1.0	>1.0
6	0.25	1.0	>1.0	1.0	1.0	>1.0	0.5	1.0	>1.0	>1.0	>1.0	>1.0

A.n, *Actinomyces naeslundii*, A.i, *Actinomyces israelii*, S.m, *Streptococcus mutans*, A.a, *Actinobacillus actinomycetemcomitans*, P.i, *Prevotella intermedia*, P.g, *Porphyromonus gingivalis*, C.a, *Candida albicans*.

5.4.2 Antioxidant activity results

The antioxidant activity of three selected compounds (scopoletin, acacetin and betulinic acid) revealed that they are effective antioxidant agents. Figure 5.1 shows the DPPH scavenging activity of the tested compounds and vitamin C. Scopoletin had a strong transformation of the DPPH radical into its reduced form, with an IC₅₀ value of 1.24 µg/ml which was significant to that of vitamin C (1.22 µg/ml). Acacetin and Betulinic acid exhibited a decreased scavenging activity with the IC₅₀ of 2.39 and 2.42 µg/ml, respectively. Scopoletin is one of the components of *Artemisia iwayomogi* (Kim *et al.*, 1997) which exhibits relatively high antioxidative activity by virtue of the fact that it has an aromatic hydroxyl group (Chen *et al.*, 1996). In the case of enzyme inhibition, flavonoids have been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and benzopyrone ring (Havsteen, 1986). The antimicrobial mechanism of action associated to each group of compounds such as terpenoids could be membrane disruption (Cowan, 2003; Arvind *et al.*, 2004). The pentacyclic triterpenes (betulinic acid) possesses a carboxylic (-COOH) group which is functional in determining the pharmacological activities (Mallavadhani *et al.*, 2004). Flavonoids such as acacetin which possesses a methoxyl group are reputable for their hydrophobic potential (Baltina *et al.*, 2003) and scopoletin possess both carboxyl group (-COOH) and methoxyl (-OCH₃) which might be the reason why this compound exhibits more antimicrobial and antioxidant activity than acacetin and betulinic acid.

Table 5.2 The IC₅₀ values of the isolated compounds tested for antioxidant activity.

Compounds	IC₅₀ (µg/ml)
Scopoletin	1.24 ± 0.009
Acacetin	2.39 ± 1.090
Betulinic acid	2.42 ± 1.090
Vitamin C	1.22 ± 0.003

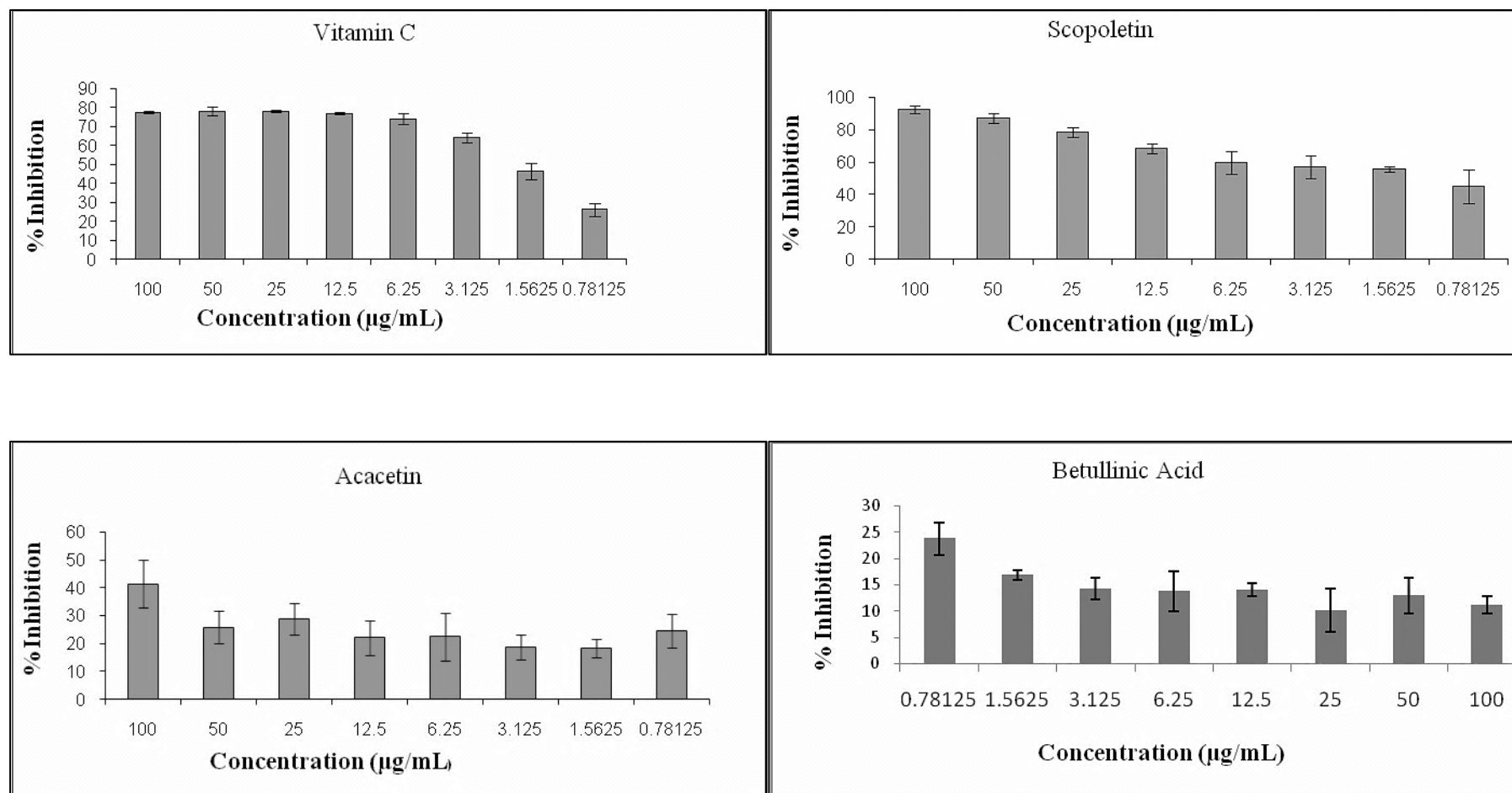


Figure 5.1 The DPPH inhibitory activities of the isolated compounds and vitamin C

There is enough evidence to support the antibacterial and antioxidant potential of the *A. afra* tested. Furthermore, it may be suggested that the *A. afra* compounds possess good antibacterial and antioxidant activities which renders them suitable as potential therapeutics, thus making them excellent candidates for more detailed investigation. Our work has also shown that acacetin and phytol exhibited moderate antimicrobial activities but excellent radical-scavenging properties. Antioxidant and related assays are currently being carried out on the compounds isolated from *A. afra* species and some compounds previously isolated from other *A. afra* species to further establish the correlations proposed.

5.5 References

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Chapter 6

Cytotoxicity effects of *A. afra* and isolated compounds against McCoy fibroblast cells and extract triggered cytokine release by Hep2 cells

6.1 Introduction

The present widespread use of medicinal plants and its derivatives requires a thorough research on their cytotoxicity. Currently medicinal plants and their derivatives are found in many products like antiseptics, soaps, deodorants, flavors and dental products (Sorderberg *et al.*, 1996) of which some if not properly evaluated may result into chronic toxicity. In dentistry many plant-derived products have been used to control oral odor, pain and inflammation associated with periodontal diseases. Derivative class of sesquiterpene lactones have been reported to be potent inhibitors of interleukin-8 gene expression in human respiratory epithelium (Mazor *et al.*, 2000). Plants such as *Hydrastis canadensis* (Goldenseal) have been seen to contain alkaloids active against oral pathogens (Wu *et al.*, 2002) and Capsaicin derived from *Capsicum annum* (Cayenne) has anti-inflammatory properties (Pallevitch and Craker, 1995).

Cytokines have been seen to play a major role in the development of symptoms (Baud *et al.*, 1999). Bacterial pattern recognition molecules (PAMPs) such as lipopeptide, lipopolysaccharides (LPS) and single stranded RNA are recognized by toll-like receptors (TLRs) which are cell-surface receptor. TLR4 was identified as the signal transducer for

LPS, as a major cell-wall component of gram-negative bacteria (Poltorak *et al.*, 1998), TLR2 for lipoproteins and peptidoglycan (Takeuchi *et al.*, 2002), TLR3 for double-stranded RNA, TLR5 for flagellin and TLR7 for single stranded RNA (Takeuchi *et al.*, 2002). Interleukin-8 (IL-8) and interleukin-6 (IL-6) are cytokines that mediate inflammation which are secreted due to the binding of bacterial antigens to toll-like-receptors of immune system (Mohamed *et al.*, 2007). IL-8 is important in acute inflammation and neutrophil chemotaxis and is also involved in degenerative diseases (Elford and Cooper, 1991). IL-8 and IL-6 production makes fibroblast and epithelial cells act as accessory immune cells and contribute to periodontal destruction (Tipton *et al.*, 2003). It is debated whether pro-inflammatory cytokines are produced in response to infection or immune response to different microbial stimuli (Mohamed *et al.*, 2007).

This study focuses on reporting the relative cytotoxic effects of *Artemisia afra* extract and its pure compounds isolated on mouse fibroblast cells (McCoy cells) as these cells have similar susceptibilities as basal keratinocytes, to topical agents (Teepe *et al.*, 1993).

6.2 Materials and methods

6.2.1 Cell lines

Mouse fibroblast cells (McCoy cells) and Hep 2 cells were placed in the culture flasks containing Dulbecco's modified Eagle's medium (DMEM) and 100 units/ml of penicillin and 100 µg/ml of streptomycin. The fragments were grown in DMEM supplemented with 10 %

fetal calf serum and antibiotics. Cultures were maintained at 37°C in humid 5% CO₂. Confluent cell layers were treated with 0.25 % trypsin and 0.05 % EDTA for 5 min.

6.2.3 Cytotoxicity assay

A microtiter plate with Vero cells were used for testing all the ethanol extracts for cytotoxicity following the method of Zheng *et al.*, (2001). Cytotoxicity was measured by the XTT (sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro]benzene sulfonic acid hydrate) method using the cell proliferation kit II (Roche Diagnostics GmbH). Hundred microlitres of Vero cells (1×10^5 ml) was seeded onto a microtiter plate and incubated for 24 h to allow the cells to attach to the bottom of the plate. Dilution series were made of the extracts and the various concentrations (400 to 3.1 µg/ml) were added to the microtitre plate and incubated for 48 h. The XTT reagents were added to a final concentration of 0.3 mg/ml and the cells were incubated for 1-2 hours. The positive drug control (Zelaralene) at concentrations range of 10 µg/ml to 0.6 µg/ml was included in the assay. After incubation the absorbance of the colour was spectrophotometrically quantified using an ELISA plate reader, which measured the optical density at 490 nm with a reference wavelength of 690 nm.

6.2.4 Cytokine assay

The cytokine assay was carried out by following the method by Uehara *et al.*, 2001 with slight modifications. When HEp 2 cells reached 80% confluent they were collected by

trypsinization and washed three times with PBS. Cells were seeded in MEM with RPMI 1640 medium in (96-well microtiter Falcon, Becton Dickinson, Labware tissue culture plates Becton Dickinson, USA) and incubated in 5 % CO₂ at 37 °C overnight. After 24 Hours of incubation the extract, *E. coli* (K12) were added to wells containing cells and plates were further incubated overnight. A negative control of *E. coli* and cells was also prepared. The assay was conducted in duplicates and the concentrations of cytokines in the supernatants were determined using ELISA kit for IL-8.

6.3 Results and discussion

6.3.1 Cytotoxicity results on fibroblast cells

The cytotoxicity effects of the ethanol crude extract of *A. afra* and three selected compound on the growth of Fibroblast cells are shown in Figure 6.1 and table 6.1. The extract as a mixture of different components showed to be non-toxic on lower concentrations of 6.12 and 3.06 µg/ml, with the cell viability of 120 %. However, toxic effects were apparent at higher concentration range of 400 to 12.50 µg/ml, with the cell viability of 60 to 20 %. Acacetin and betulinic acid also showed a smooth trend of non-toxic effects at lower concentrations and toxic at higher concentrations with IC₅₀ values of 35.44 and 30.96 µg/ml respectively. The effect of acacetin on lung cancer (A549) cell proliferation was observed to have a dose-dependent manner with an IC₅₀ value of 9.46 µM (Hsue *et al.*, 2004). Apoptotic activity of betulinic acid against murine melanoma B16 cell line was reported and it was discovered that betulinic acid induces apoptotic effects with an IC₅₀ of 22.5 µg/ml (Liu *et al.*,

2004). Reports postulates that triterpenes with a carboxyl group at c-28 shows more cytotoxic activity against cancer cell lines (Chiang *et al.*, 2005; Banglin *et al.*, 2003) and induce apoptosis (Sakai *et al.*, 2004). Unexpectedly, one out of three compounds tested, scopoletin was relatively non-toxic with an IC₅₀ value of 132.5 µg/ml.

Table 6.1 The IC₅₀ values of crude extract and three selected isolated compounds.

Extract/ compounds	IC₅₀ (µg/ml)	STD
Betulinic Acid	30.96	± 1.95
Acacetin	35.44	± 2.14
Scopoletin	132.5	±1.85
<i>Artemisia afra</i>	16.95	±1.82
Actinomycin-D	0.003364	±0.00002

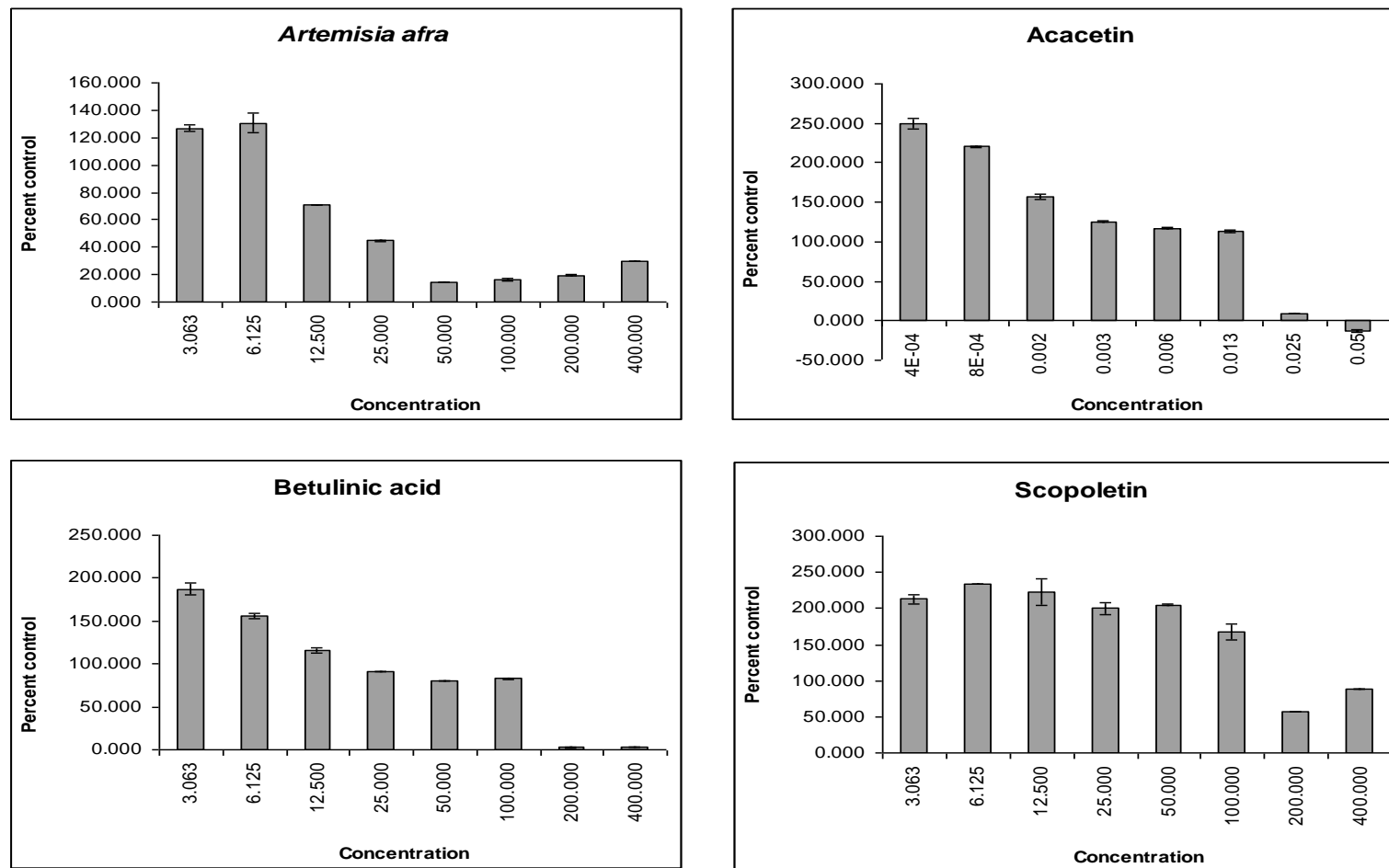


Figure 6.1 The cytotoxicity effects of *A. afra* extract and three selected compounds on the growth of the McCoy fibroblast cells.

6.3.2 Cytokine assay results on Hep2 cells

We examined the production of the interleukin-8 from Hep2 cells on exposure to various concentrations of ethanol extract of *A. afra* and *E. Coli*. The examination of the cells without any treatment was done, in order to check the responsiveness of the cells toward the medium and in order to have a standard curve (Figure 6.2a), that is, the pattern of IL-8 secretion by cells. This will however help to identify the possibility of what stimulates the secretion of IL-8 in the cells. The first treatment was conducted by exposing cells to the extract only and the growth of cells was monitored over 24hours incubation. The second experiment was done by exposing the cells to the extract the later the bacteria was added to examine whether the production of IL-8 is due to the extract or bacterial stimulation and the reverse experiment was also done. Lastly we examined whether the Hep2 cells respond to bacteria and extract added simultaneously. Three concentrations of the sample was tested which are 3.125, 6.25 and 12.5 mg/ml.

When the cells were incubated with the extract for 24 h there was a significant effect on the IL-8 production (Figure 6.3b) as compared to (no treatment) control experiment (Figure 6.2a). At the concentration level of 3.125 mg/ml to 6.25 mg/ml the production of IL-8 was slightly increasing and picked up at the concentration of 12.5 mg/ml to concentration of 5.24 pg/ml. Approximately similar results were obtained when the plant material was inoculated first to the cells and bacteria later. The production was elevated constantly, the concentration of 3.125 mg/ml yielded 1.60 pg/ml, 6.25 mg/ml yielded 2.61 pg/ml and 12.5 mg/ml yielded 5.24 pg/ml (Figure 6.3c).The incubation of cells with both bacteria and extract inoculated at

the same time showed significant production of the IL-8. IL-8 concentrations were escalating with the range of 9.81, 22.8 and 23.10 mg/ml in respect to concentrations tested (Figure 6.3d). The inoculation of the bacteria into the cells and later extract addition increased the production of IL-8 significantly with the difference of the concentrations of IL-8 17.53 pg/ml, 18.00 pg/ml and 24.0 pg/ml at extract concentrations of 3.125 mg/ml, 6.25 mg/ml and 12.5 mg/ml respectively (Figure 6.3e).

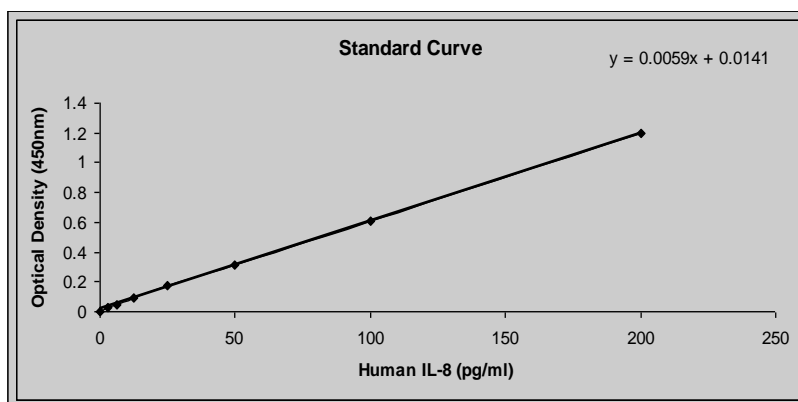


Figure 6.2 (a) A standard curve showing the optical density of human IL-8 (pg/ml)

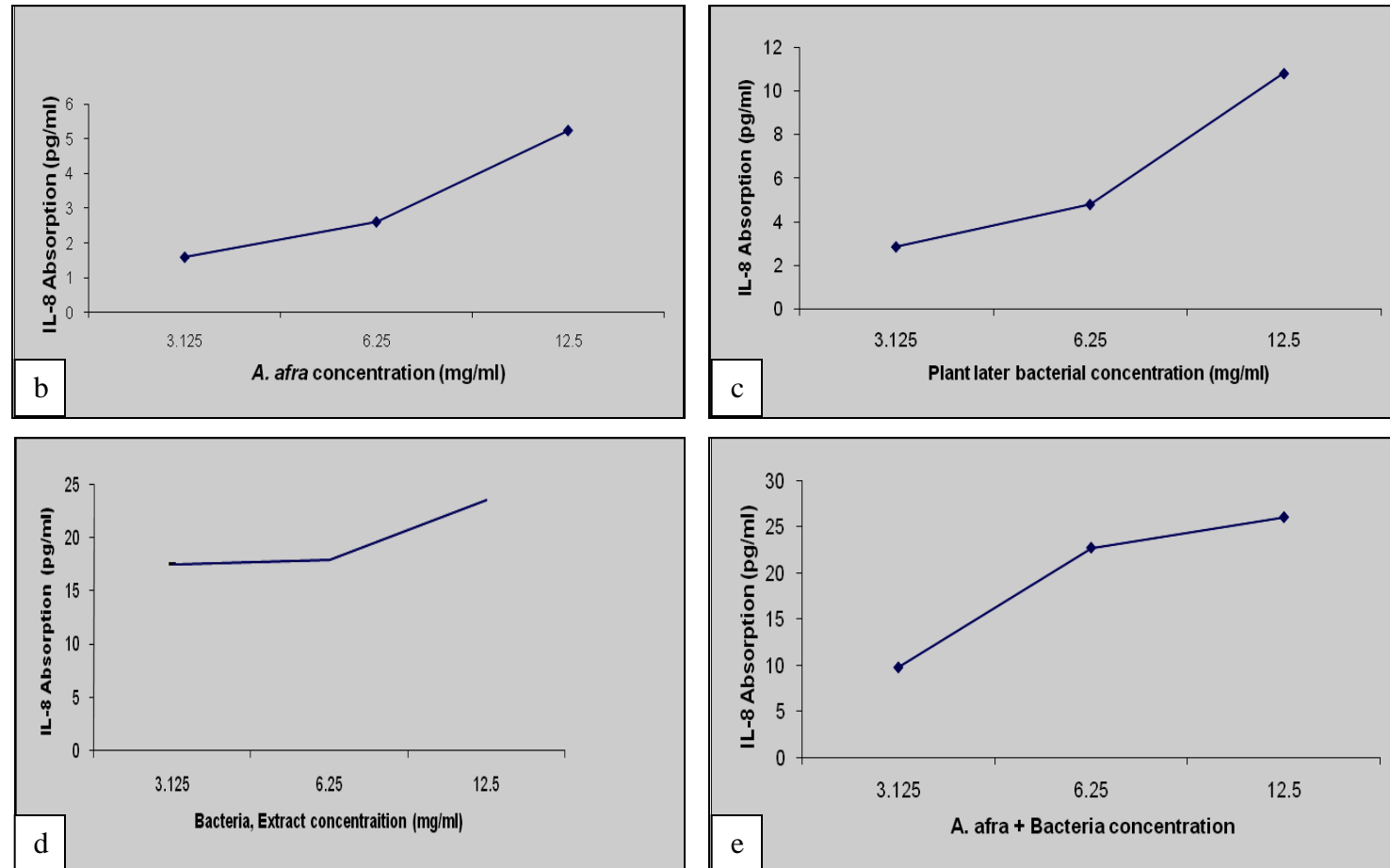


Figure 6.3 Comparison of the production of cytokine interleukin-8 (IL-8) in response to *A. afra* extract and *E.coli*.

The examination of whether the HEP2 cells produced IL-8 in response to *E. coli* and *A. afra* extract revealed that IL-8 secretion was more prominent in the presence of bacteria than in exposure to the extract. These results correlate with the study conducted by Uehjara *et al.*, (2002). In their study they illustrate that a clear dose-dependent of IL-8 secretion by IFN- γ -primed HSC-2 cells was prominent upon stimulation with bacterial cell-surface components.

Mohamed *et al.*, (2007) and Hebra *et al.*, (2001) reported that an increased response of pro-inflammatory cytokines was observed in the presence of a pathogenic *E. coli* produced by the cord blood. IL-6 and IL-8 has been reported to be secreted in response to LPS from gram-negative pathogens (Dembinski *et al.*, 2002). This gives a conformation to the results conducted in this study which show that in the presence of the extract the production of IL-8 was not prominent as compared to the presence of bacteria, therefore the bacteria is responsible for the secretion of cytokines. It has been reported that the sesquiterpene lactones have a potential of inhibiting IL-8 gene expression in the cultured human respiratory epithelium (Mazor *et al.*, 2000). In this case *A. afra* had minimized effect on the production of IL-8.

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Chapter 7

General discussions and conclusions

7.1 Introduction

Ethnopharmacology is the scientific study of traditional medicines, which continue to provide new drugs and guide to molecules for the pharmaceutical industry. There are about 80 % of people living in the developing countries who depend on medicinal plants for their health care (Prozesky *et al.*, 2001). Traditional medicinal plants are often cheaper and readily available. Traditional medicine is currently part of the complementary medicine though in most cases their efficacy and mechanisms of action have not been tested scientifically they still mediate health benefits due to their active chemical constituents (Park and Pezzuto, 2002). Medicinal plant parts (roots, stem, bark, flowers, fruits and leaves) have abundance of compounds such as phenolic, terpenes, flavonoid to mention a few, which play a major role in defence against bacterial, fungal infections, cancer protection and free radicals (Cai *et al.*, 2003).

More research work is needed to elucidate new antibiotics which fight against resistance mechanisms in bacterial, viral and fungal infections. This study focussed more on combating the microorganisms associated with periodontal diseases using a known medicinal plant *Artemisia afra*, well known for its traditional treatment of cough, colds, sore throat, asthma, headache, gout and intestinal worms (van Vyck and Wink, 2004)

Briefly, periodontitis is a disease characterised by infection and inflammation in tooth supporting tissues leading to connective tissue loss and alveolar bone loss. If left untreated, periodontitis results into tooth loss. The bacteria is said to be the primary etiology of periodontitis and the critical role of the host in defence against bacterial challenges (Haffajee and Socransky, 2000).

7.2 Antimicrobial activity of *A. afra*

The *in vitro* antimicrobial activity of the ethanol extract of *A. afra* was evaluated using the 96-well micro plate dilution method. The extract exhibited excellent antimicrobial activity with the MIC values ranging 3.1 and 25.0 mg/ml against six tested microorganisms. Gram-negative bacteria (*Aggregatibacter actinomycetemitans*, *porphyromonus gingivalis*, *Prevotella intermedia* and fungus *Candida albicans* showed resistance against the extract. Similar study by Gursoy *et al.*, (2009) revealed that *Aggrigatusbacter actinomycetemitans*, *porphyromonus gingivalis* and *Prevotella intermedia* are resistant against *Satureja hortensis* extract with an MIC value of 125 µg/ml.

7.3 Antioxidant activity of *A. afra*

Periodontal diseases are associated with an imbalance between reactive oxygen species and antioxidants in favor of the former due to both an increase in free radical production and defect in antioxidant (Diab-Ladki *et al.*, 2003). Some studies have reported that the excess production of reactive oxygen species (ROS) leads to damages of gingival tissues, periodontal ligament and alveolar bone. This occurs due to an increase in free radical

production and defect in total antioxidant activity of saliva (Chapple and Matthews, 2007; Alviano *et al.*, 2008). Reactive oxygen species (ROS) may cause damage either by activating cyclooxygenases and lipoxygenases, protein damage including gingival hyaluronic acid, DNA damage or stimulation of cytokine release by activating KB(NF-KB) (Staal *et al.*, 1990). The antioxidant activity of the *A. afra* was determined using the DPPH scavenging method. The extract was very competitive to ascorbic acid/Vitamin C in that the concentration of the extract at 100 and 50 µg/ml had high activity than vitamin C. Both were tested at the same concentration range. The effective concentration (EC₅₀) at which the DPPH radicals were scavenged by 50 % of the extract is ± 22.2 µg/ml.

7.4 Isolation of compounds from *Artemisia afra* extract

The phytochemical investigation of an ethanol extract of *Artemisia afra*, led to the isolation of six known compounds, Acacetin, 12 α ,4 α -dihydroxybishopsolicepolide, Scopoletin, α -amyrin, Phytol, and Betulinic acid. The isolated compounds were evaluated for their anti-microbial activity against Gram positive bacteria (*Actinomyces naeslundii*, *Actinomyces israelii* and *Streptococcus mutans*), Gram negative bacteria (*Privotella intermedia*, *Porphyromonus gingivalis* and *Aggregatibacter actinomycetemcomitans* previously known as *Actinobacillus actinomycetemcomitans*) and *Candida albicans* and antioxidant activity.

7.5 Antimicrobial and antioxidant activity of isolated compounds

Three out of six compounds were selected for the antioxidant and cytotoxicity based on the best activity shown against microbial screening. The MIC of Acacetin against

A. israelii was 0.5 mg/ml. Scopoletin had a strong antioxidant activity with an IC₅₀ value of 1.24 µg/ml which was significant to that of vitamin C (1.22 µg/ml). Acacetin and Betulinic acid exhibited a decreased scavenging activity with the IC₅₀ of 2.39 and 2.42 µg/ml, respectively.

7.6 Cytotoxicity of the extract and pure compounds on the fibroblast McCoy cells

Periodontal tissue cells stimulated by periodontal disease-associated bacteria secreted inflammatory cytokines. Some cytokines such as Interleukin-8 (IL-8) can modulate bone resorption by activating osteoclast and stimulating Prostaglandin E₂ (PGE₂) synthesis. The extract and compounds showed moderate toxicity on McCoy fibroblast cell line and the extract influenced the release of cytokine against Hep2 cells. Scopoletin was relatively non-toxic with an IC₅₀ value of 132.5 µg/ml. Acacetin and betulinic acid also showed a smooth trend of non-toxic effects at lower concentrations and toxic at higher concentrations with IC₅₀ values of 35.44 and 30.96 µg/ml respectively. The obtained results in this confirmed the use of *A. afra* in the treatment of microbial infections. The adding of the extract and bacteria (*E. coli*) separately and simultaneously to Hep2 cells released the cytokine IL-8. Therefore, the extract can mediate the release of IL-8, of which this mechanism can aid in reducing infection and inflammatory response induced by cytokines.

7.7 Conclusion

In the limits of the present *in vitro* study, we conclude that *A. afra* contain effective antimicrobial phytochemicals that suppress the growth of periodontal pathogens.

7.8 References

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Chapter 8 APPENDICES

^1H NMR and ^{13}C NMR spectrum of compounds isolated from the ethanol extract of

Artemisia afra

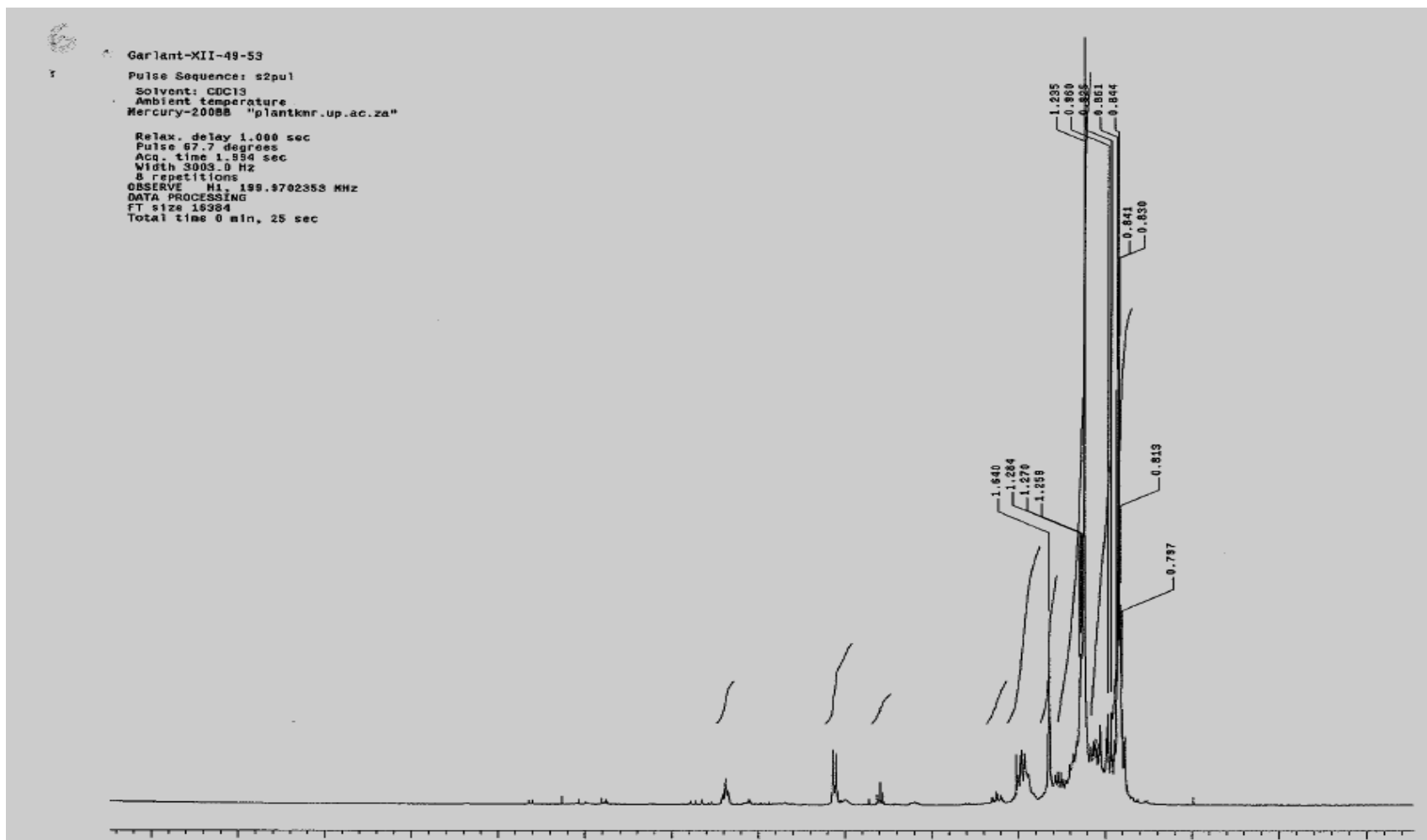


Figure 8.1.1 ¹H NMR spectrum of Phytol isolated from the ethanol extract of *Artemisia afra*

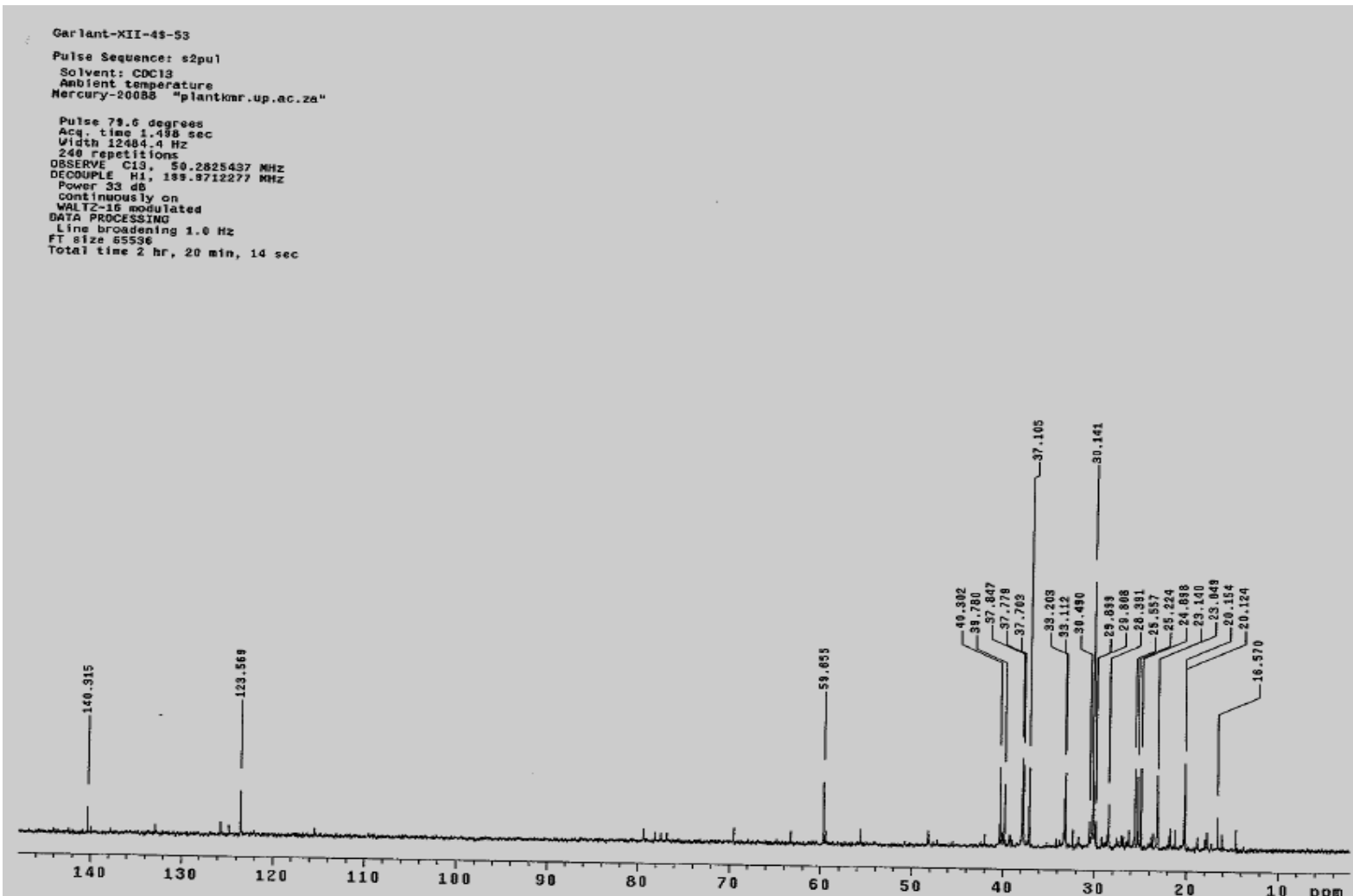


Figure 8.1.2 ¹³CNMR spectrum of Phytol isolated from the ethanol extract of *Artemisia afra*

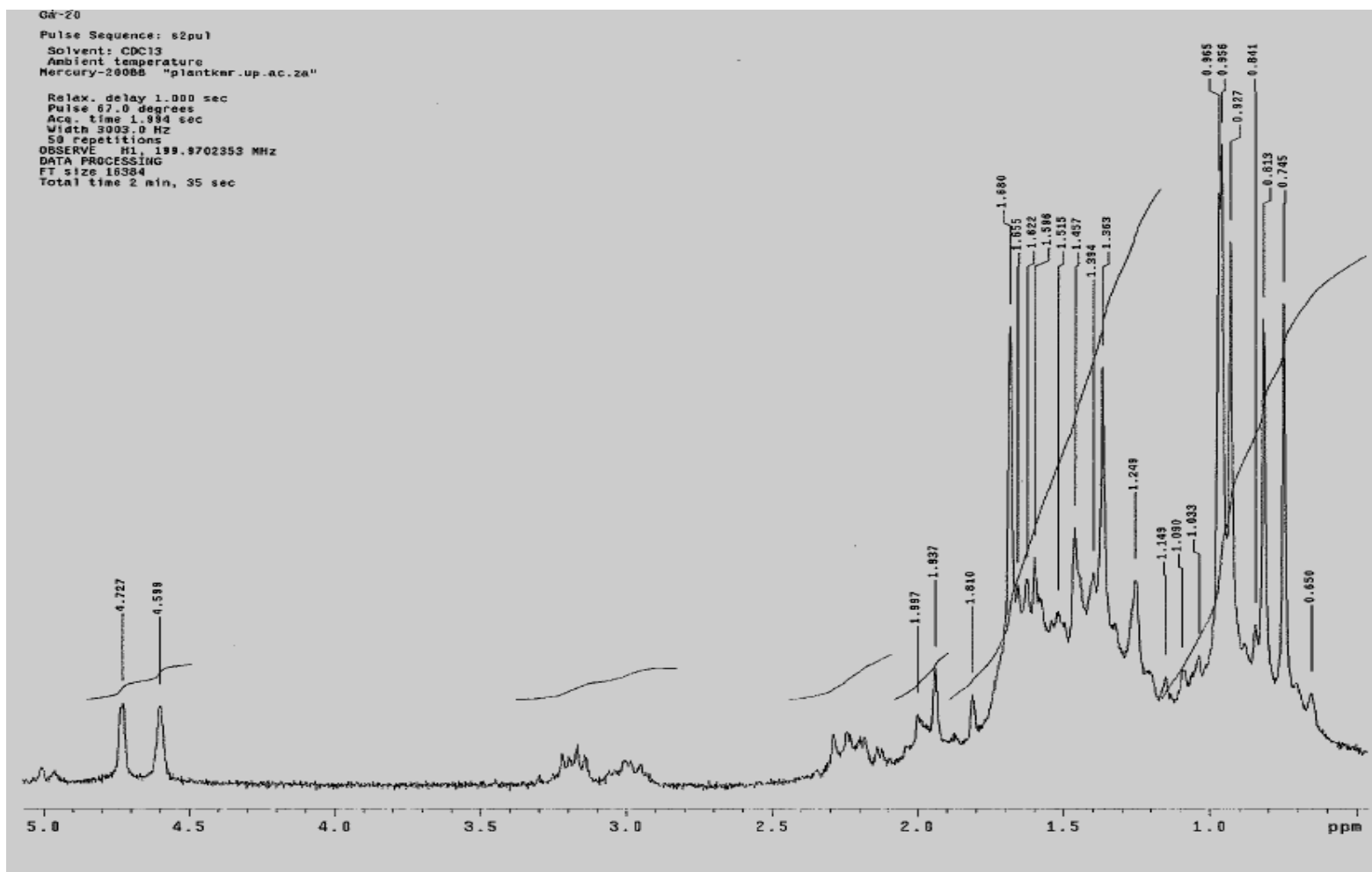


Figure 8.1.3 ^1H NMR spectrum of Betulinic acid isolated from the ethanol extract of *Artemisia afra*

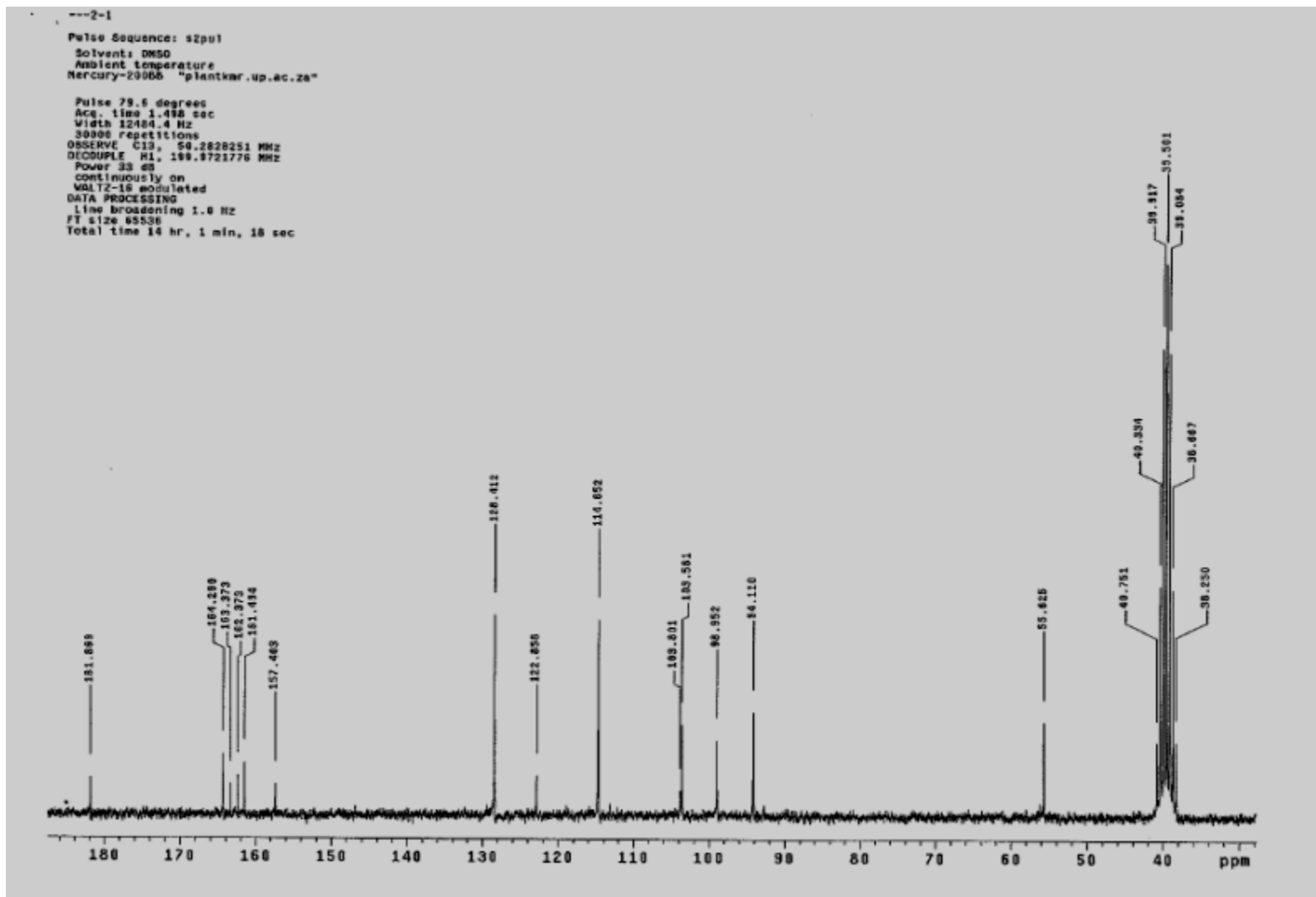


Figure 8.1.4 ^{13}C NMR spectrum of Betulinic acid isolated from the ethanol extract of *Artemisia afra*

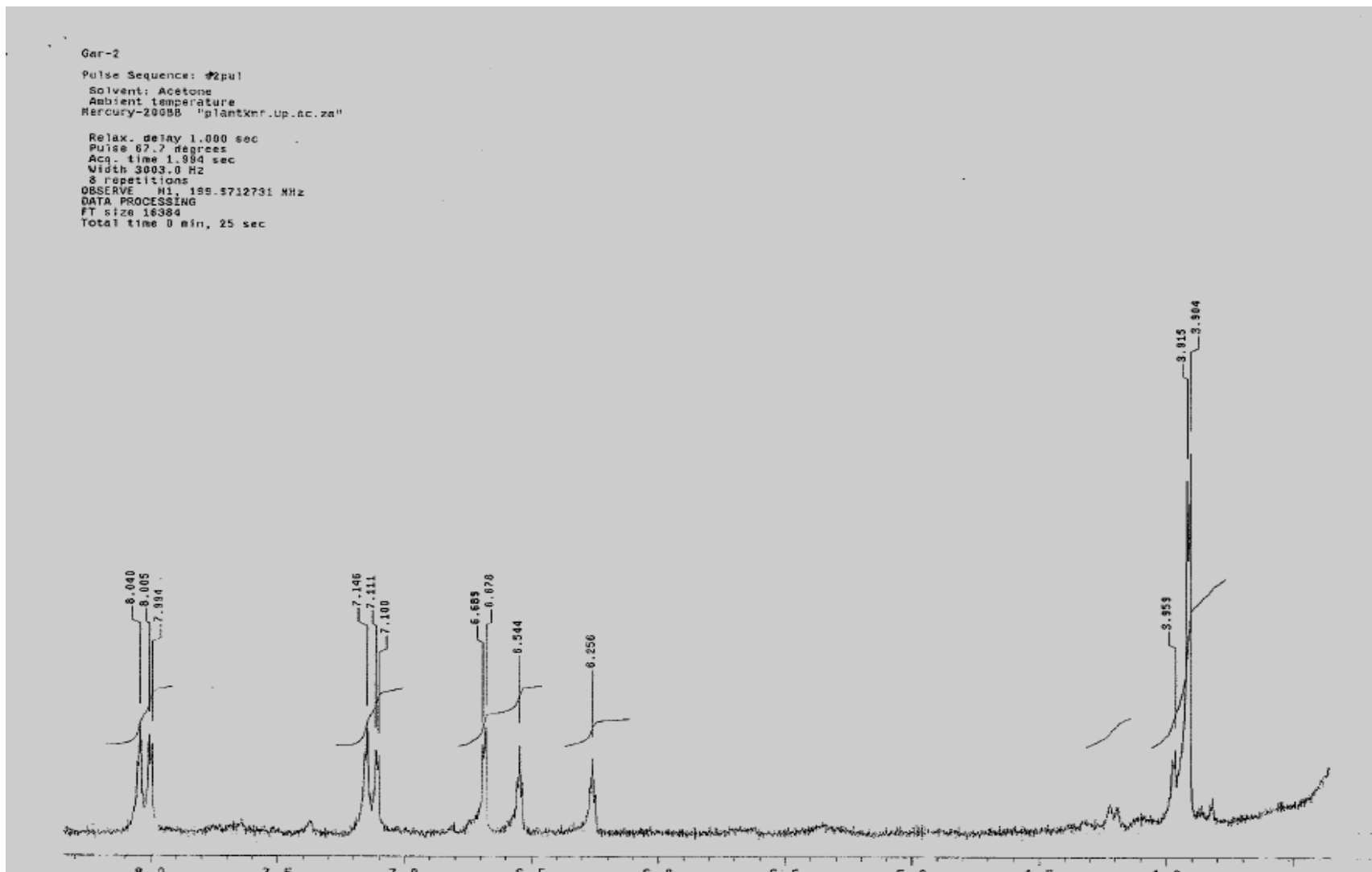


Figure 8.1.5 ^1H NMR spectrum of Acacetin isolated from the ethanol extract of *Artemisia afra*

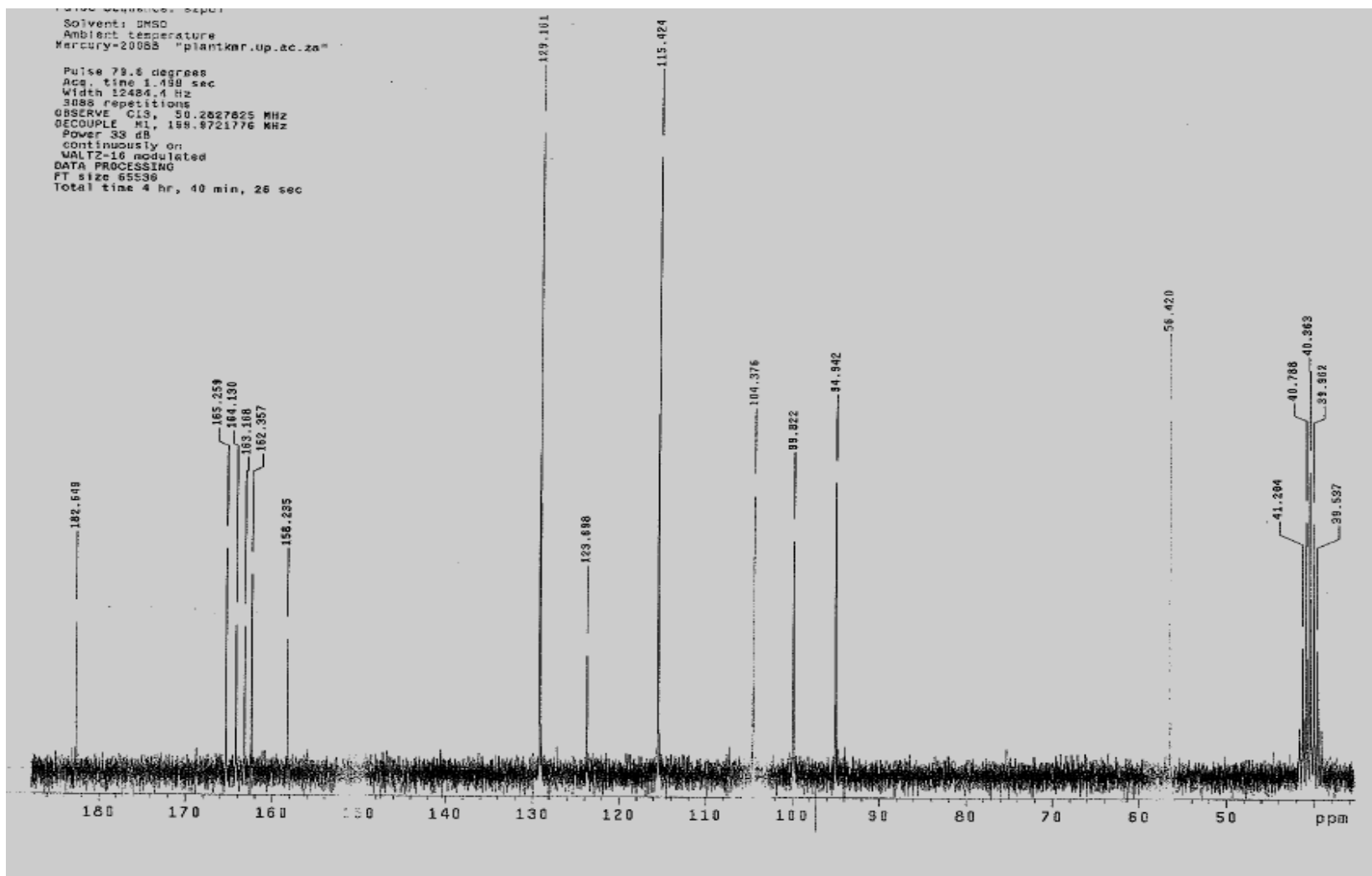


Figure 8.1.6 ^{13}C NMR spectrum of Acacetin isolated from the ethanol extract of *Artemisia afra*

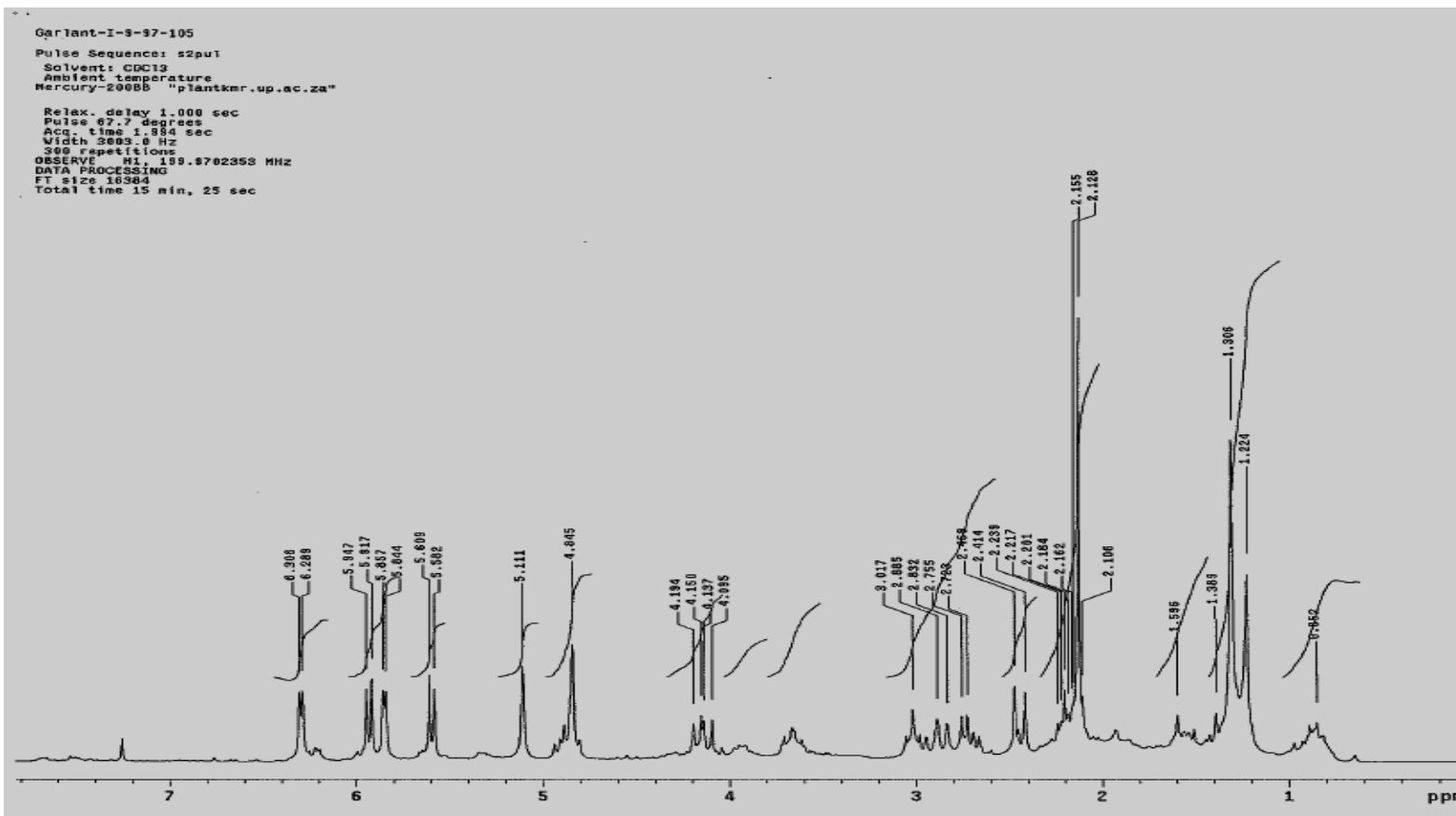


Figure 8.1.7 ¹H NMR spectrum of 1 α , 4 α -dihydroxybishopsolicipolide isolated from the ethanol extract of *Artemisia afra*

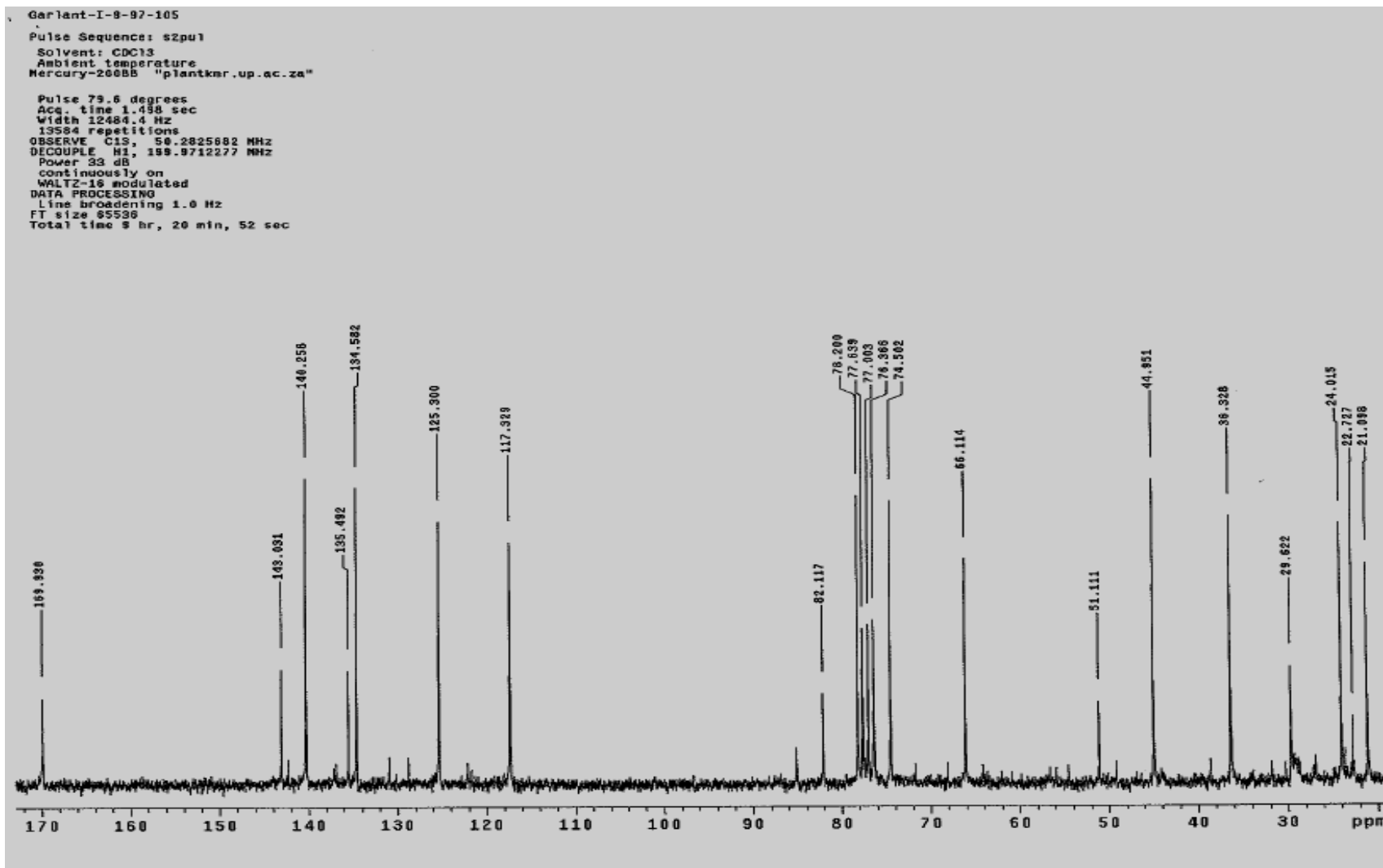


Figure 8.1.8 ^{13}C NMR spectrum of $1\alpha, 4\alpha$ -dihydroxybishopsolicepolide isolated from the ethanol extract of *Artemisia afra*

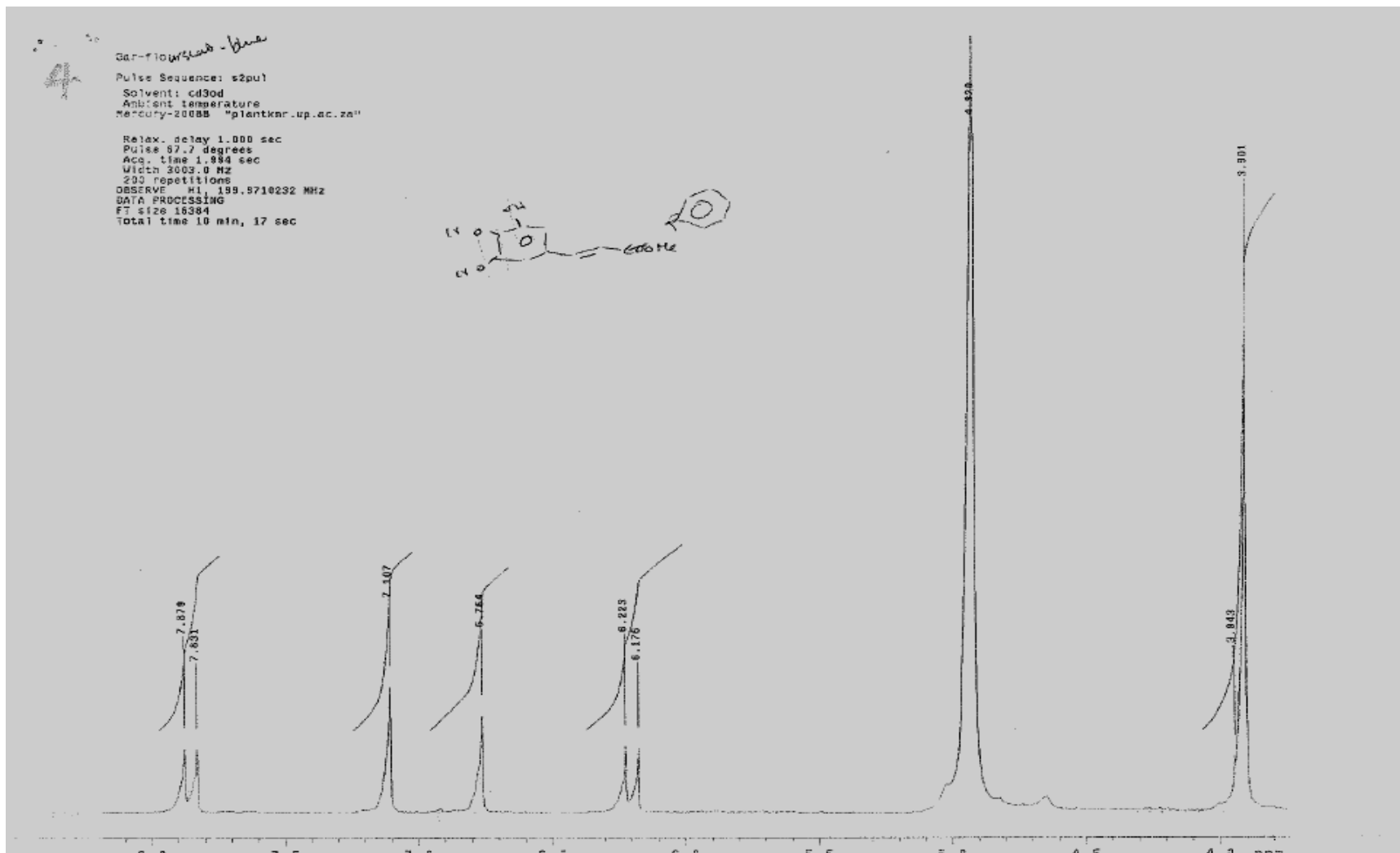


Figure 8.1.9 ^1H NMR spectrum of Scopoletin isolated from the ethanol extract of *Artemisia afra*

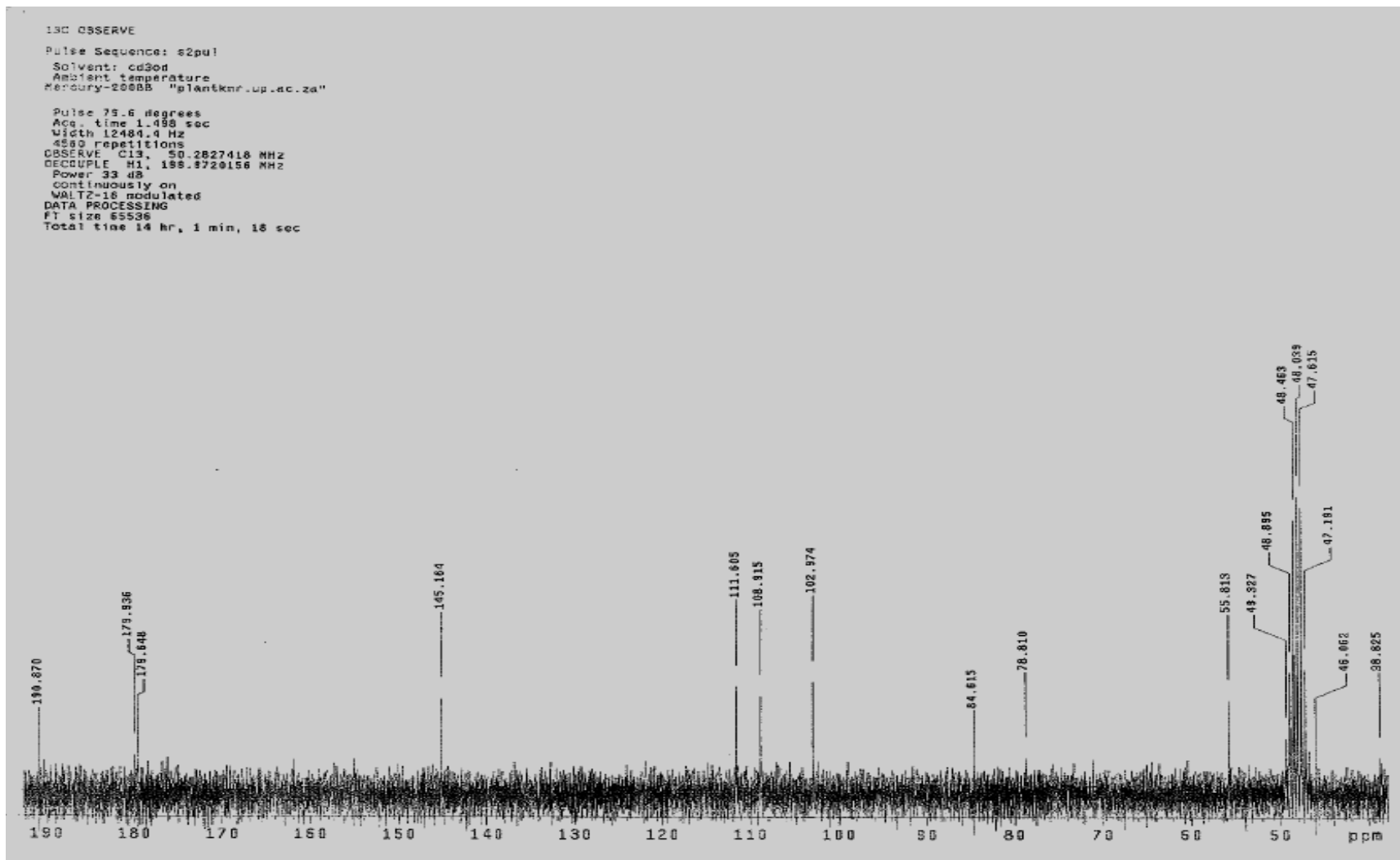


Figure 8.1.10 ^{13}C NMR spectrum of Scopoletin isolated from the ethanol extract of *Artemisia afra*

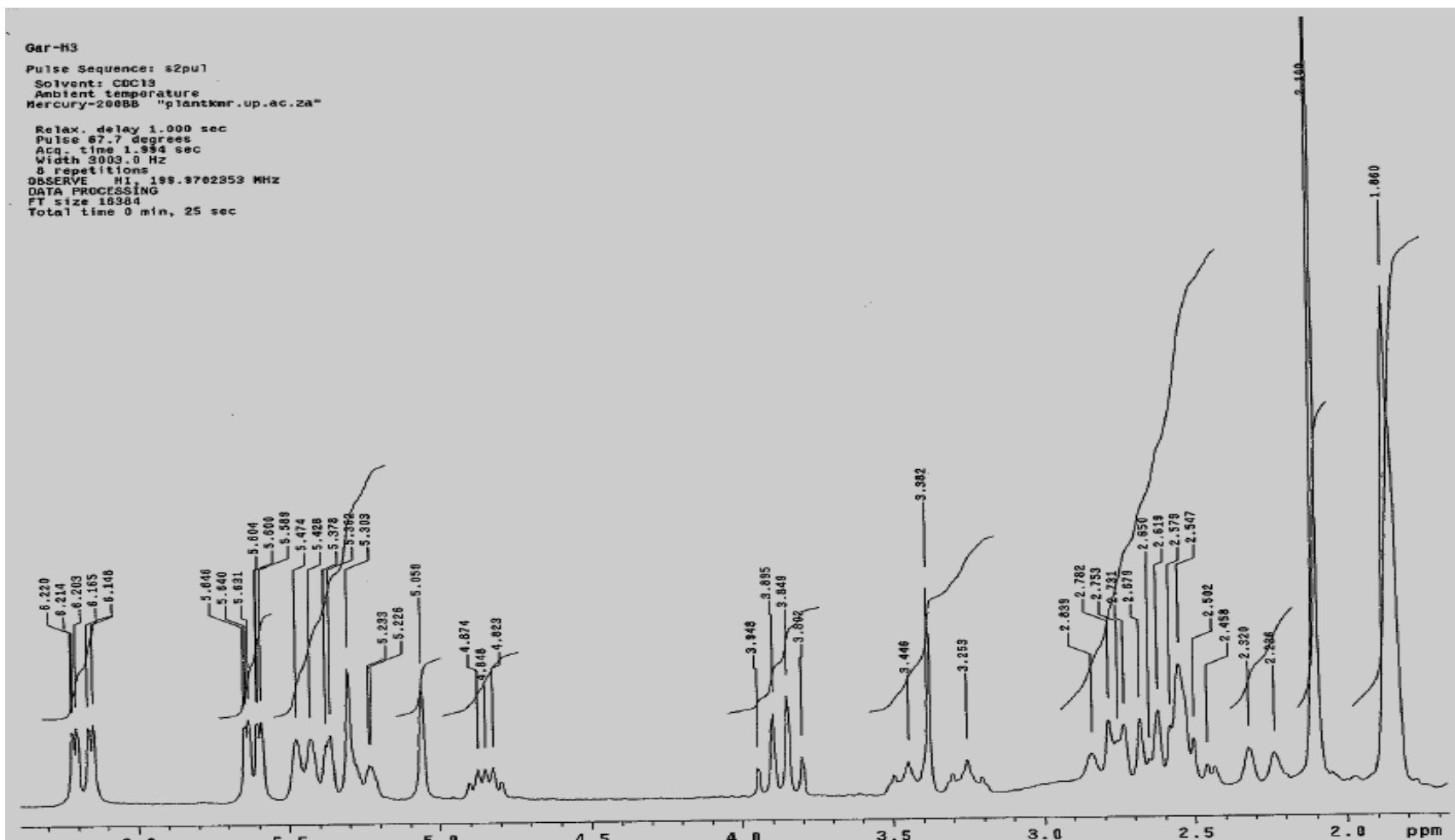


Figure 8.1.11: ^1H NMR spectrum of α -Amyrin isolated from the ethanol extract of *Artemisia afra*

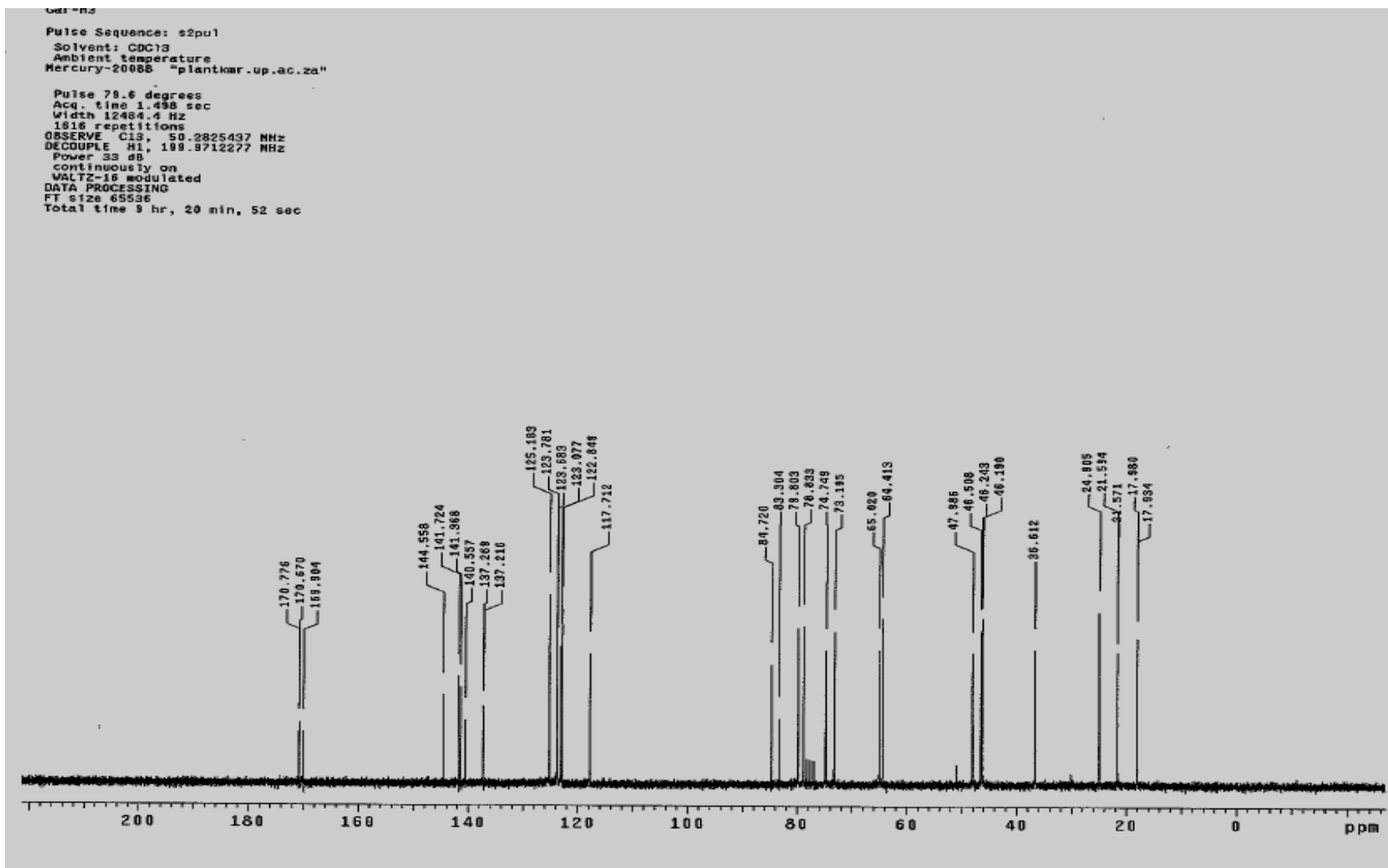


Figure 8.1.12: ^{13}C NMR spectrum of α -Amyrin isolated from the ethanol extract of *Artemisia afra*