Isolation, Characterisation of Terpenoids and Biosynthesis of Silver Nanoparticles of *Acacia Mearnsii* De Wild and *Acacia Karroo* Hayne and Their Bioassays

By

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DECLARATION

I, the undersigned, declared that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Chemistry in the Faculty of Science & Agriculture, Department of Pure & Applied Chemistry, and the work contained herein is my original work with exemptions to citations, that this work has not been submitted at any other University in partial or entirety for the award of any degree.

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Prof. A.O. Oyedeji.

DEDICATION

This thesis is dedicated to the memories of my late parent-

Mr & Mrs Christopher Hodonu Avoseh

Just as your sayings goes “As you make your bed, so you lie on it”.

Thank you for laying my “bed” for me; it all begins with you.

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"Success is the ability to go from one failure to another with no loss of enthusiasm." – Winston Churchill.

My PhD experience is a memoir that I can never do away with most especially with those that made the dream come true. I will like to say a heartfelt thank you to the following people;

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ABSTRACT

Great wealth of traditional knowledge about the use of plants had been transferred from generation to generations leading to the present day drug discovery and invention of new scientific methods of isolation, purification and identification. With the discovery of new diseases and drug-resistant organisms, there is no other source or deposit of lead compounds or drugs than the plant kingdom. As a result of this, about 25% of the current drug administered owe their origin to plant sources with the view to reduce the carcinogenic effect of synthetic drugs.

Volatile terpenoids among other broad spectrum of natural product had been implicated to show high therapeutic properly. In the present study, selected locally-used medicinal plants were exploited for the presence of potent bioactive compounds and ability to form nanoparticles with distinctive property for use as chemoprotective agent against inflammation, tumors, cancer and other chronic diseases.

Acacia mearnsii De Wild and *Acacia karroo* Hayne studied in this report are known to be invasive species with no proper regulation to conserve and preserve them. However, ethnopharmacology report of these plant species in the Southern Africa region reveals that they are good antiseptic, anti-diarrhea, anti-inflammation and a forage for livestock.

These plants were subjected to volatile extraction protocol of some parts of the plants (stem and leaves) followed by examination of the anti-inflammation capacity of the extracts using an animal model. In addition, the bye-product (hydrosol) from the stem bark of each species possess a high reducing and stabilizing property leading to synthesis of silver nanoparticles, followed by investigation of the anti-inflammation potential of the synthesized silver nanoparticles using animal model.

The volatile oils of the leaves and stem bark of *Acacia mearnsii* De Wild obtained by hydro-distillation were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). Twenty, Thirty-Eight, Twenty-nine and Thirty-Eight components accounting for 93.8%, 92.1%, 78.5% and 90.9% of the total oils of the fresh, dry leaves and fresh, dry stem bark respectively. The major components of the oil were octadecyl alcohol (25.5%) and phytol (10.5%); cis-verbenol (29.5%); phytol (10.1%) and phytol (23.4%) for the fresh leaves, dried leaves, and fresh stem, dry stem bark respectively. Oral administration of essential oils at the dose of 2% showed significant ($p<0.05$) anti-inflammatory properties in the albumin induced

test model in rats. Oils from the fresh leaves and dry stems inhibited inflammation beyond 4 h post treatment.

Furthermore, the chemical composition of the essential oils obtained by hydro-distillation from the leaves and stem bark (dry and fresh) of *Acacia karroo* Hayne, analysed by GC-MS, shows that hexanal (10.67%) and ß-ionone (9.74%) were dominant in the dried leaves, β-pinene (14.30%), and (Z)-2-Hexen-1-ol (10.21%) in the fresh leaves while Octacosane (10.59%) and phytol (23.38%) were dominant in the dry and fresh stem respectively. The anti-inflammation ability of these oils after an albumin-induced inflammation on wistar rats, shows a significant effect at the 1^{st} h of treatment with a significance of $P< 0.01$ for all part plants, while the fresh leaves shows further inhibitory activities at the 2^{nd} h of analysis.

Silver nanoparticles (AgNPs) were successfully synthesized from $AgNO_3$ through a green route using the aqueous extract (hydrosols) of *Acacia mearnsii* De Wild and *Acacia karroo* Hayne as reducing agent and as well as capping agent. The *Acacia*-mediated AgNPs were characterized with the use of UV-vis absorption spectroscopy, Fourier Transform Spectroscopy (FT-IR), Transmission electron microscope (TEM), Scanning Electron Microscope (SEM), Energy Dispersive Spectroscopy (EDX), and X-ray Diffractometry (XRD). A spherical, 10-40 nm diameter silver nanoparticles were synthesized with very low level of stability for the AMDS and the AKDS-AgNPs. In addition, nociceptive activity with a mice rat reveals higher inhibition at the neurogenic phase for the AKDS-AgNPs, while AMDS-AgNPs exhibited a high inhibition at the inflammatory phase.

The potent anti-inflammatory activity of essential oils of *A. mearnsii* De Wild and *A. karroo* Hayne hereby confirmed its traditional use in treating various inflammatory diseases, while the inflammatory studies on the synthesized AgNPs reveals a very active compound which can be used as a potent opioid or non-steroidal anti-inflammatory drug (NSAID).

PUBLICATIONS AND CONFERENCES ATTENDED

Publication 1: **Avoseh, Opeyemi N.**, Oyedeji Ope-oluwa O., Aremu Kayode, Nkeh-Chungag,

Benedicta N., Songca Sandile P., Oluwafemi Samuel O., and Oyedeji. Adebola. O. Chemical composition and anti-inflammatory activities of the essential oils from *Acacia mearnsii* de Wild. ***Nat. Prod. Res.*** 1–5 (2014). doi:10.1080/14786419.2014.983504

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Conferences

2013 South African Chemical Institute (SACI) International Convention, East London 1st -6th December, 2013.

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2014 SACI Eastern Cape Regional Conference. Nelson Mandela Metropolitan University, Port Elizabeth, South Africa. Oral presentation on Anti-inflammatory Potential of the volatiles and synthesized Silver nanoparticles of *Acacia mearnsii*

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LIST OF ABBREVIATIONS

US	United States
NP’s	Nanoparticles
P450	Cytochrome
CYP cells	cyclophosphamide
HMGCoA	3-hydroxy-3-methylglutaryl acetyl CoA
MVA	Mevalonate Acid
NADPH	Nicotinamide adenine dinucleotide phosphate
DMAPP	Dimethyl pyrophosphate
IPP	Isopentenyl pyrophosphate
HMGR	HMGCoA-Reductase
GGPP	Geranylgeranyl pyrophosphate
FPP	Farnesyl pyrophosphate
GPP	Geranyl pyrophosphate
MEP	Methylerythritol phosphate
Pyr	Pyruvate
G3P	Glyceraldehyde -3-phosphate
POH	Perillyl alcohol
MAHD	Microwave Assisted Hydro distillation
GHz	Giga hertz
SPME	Solid Phase Micro-extraction
GC	Gas Chromatography
H-GC	Headspace Gas chromatography
GC-O	Gas chromatography Olfactometry
GC-FID	Gas chromatography Flame Ionization Detector
MS	Mass spectrometry
M/z	mass-to- charge ratio
GC-MS	Gas chromatography mass spectrometry

GC/TOF-MS	Gas Chromatography time-of-flight Mass spectrometry
Rt	Retention time
NO	Nitric oxide
COX-2	Cyclooxygenase
RO's	Reactive oxygen's
IFN-γ	Interferons gamma
MCP-1	Monocyte Chemotactic Protein 1
GM-CSF	Granulocyte Macrophage colony- stimulating factor
IL	Interleukin
IFNs	Interferons
IGIF	Interferon-gamma Inducing factor
TGF-*β*	Transferring growth factor-beta
TNF-α	Tumor necrosis factor-alpha
NF-kB	Nuclear factor kappa B
*i*NOs	Inducible nitric oxide synthase
LTB4	Leukotriene B4
NSCLC-N6	Non-small cell lung cancer cell line- N6
VL-MSD	VL- Mass spectrometry Detector
HP-5	Hewlett Packard – 5 column
SAVP	South African Vaccine Producers
AMDL	*Acacia mearnsii* Dry Leaves
AMDS	*Acacia mearnsii* Dry stems
AMFL	*Acacia mearnsii* fresh leaves
AMFS	*Acacia mearnsii* fresh stem
AKDL	*Acacia karroo* Dry leaves
AKDS	*Acacia karroo* Dry stems
AKFL	*Acacia karroo* fresh leaves
AKFS	*Acacia karroo* fresh stem

S.E.M.	Standard Error of mean
AgNPs	Silver nanoparticles
SPR	Surface Plasmon Resonance
$NaBH_4$	Sodium borohydride
HDA	Hexadecylamine
TOP	tri-n-octylphosphine
PVP	poly (vinyl) pyrolidine
EWP-64	Egg White Protein-46
GRA	Grahams town
XRD	X-ray diffractometer
TEM	Transmission Electron Microscope
SEM	Scanning Electron Microscope
EDXRF	Energy Dispersive X-Ray Fluorescence Spectroscopy
FT-IR	Fourier Transform Infra-Red Spectroscopy
NSAIDs	Non-steroidal anti-inflammatory drugs
DNA	Deoxyribonucleic acids
CMCTS	Carboxymethylated chitosan
ANOVA	Analysis of variance

CHAPTER 1

GENERAL INTRODUCTION

1.0 Exploration of plant-originated drugs

1.0.1 Overview of plant-originated drugs

Plants play an indispensable part in human existence as he's the main support for feeding, disease alleviation, shelter, life support and other uses[1]. Plants contribution to health care delivery is dependent on the properties and activity of plant-originated drugs. Plants immense and complex biosynthetic route leads to formation of biologically and pharmaceutically potent substances, that are isolated from plants, purified to act as drug precursors[2].

At present time, 40% of most prescriptions in North America contain not less than one plant-derived drug. Similarly it is reported that physicians in Europe routinely recommend concoction mixture of herbal origins to their sick clients. Plant use as medicine by humans has been an age-long event which is sometimes mixed with mineral water or animal produce, serving as principal drugs[3]. However the synthesis of urea by Friedrich Wohler in 1828 ushered a period when the use of synthetic compounds gained popularity and eventually replaced plant based products. The tide of events was reversed with the isolation of penicillin from *Penicillium notatum* leading to the rediscovery of plant derived drugs.

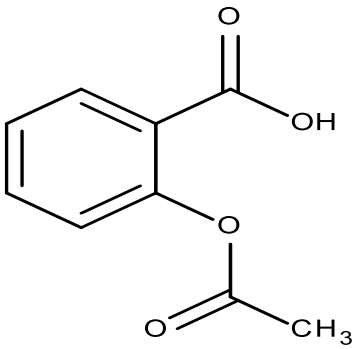
Vincristine, morphine, artemisinin, quinine, aspirin and vinblastine are some commercially available drugs with plant origin currently on sale[4]. Aspirin (**1.1**), with an annual consumption of about 70 million tablets yearly in US only, was first isolated in *Salix Alba* (willow bark). A non-steroidal anti-inflammatory drugs (NSAIDs), aspirin has been used predominantly for supressing fever, pain and inflammations[5]. *Atropa belladonna*, is a historic plant from which atropine (**1.2**) is isolated had been remarkable for the treatment of asthma,

pain, anticholinergic use and for paralysis related to Parkinson's disorder. Quinine (**1.3**), a versatile and effective drug used for the treatment of malaria was discovered from *Cinchona officinalis*, *Cinchona pubescens* bark and *Cinchona legeriana*. Morphine (**1.4**), CNS-active drugs, an opioid alkaloids from *Papaver somniferum* used for treatment of pain related conditions[6]. Nevertheless, wrong prescription and use of morphine may be fatal to health and could result in addiction.

The last decade has seen an increased interest and rapid development in the discipline of medicinal chemistry. In spite of this swift development, synthetic manufacturing of some plant derived drugs has been limited for two main reasons. High cost of synthesis of the drugs (reserpine and atropine) and the safety/regulation concern of other drugs such as morphine, cocaine (**1.5**), ergotamine (**1.6**) and digitalis (**1.7**)[7]. Thus, plant source remain the only alternative for effective isolation of plant derived drugs and plays a pressing role in drug discovery. Whenever, a new drug is isolated and pharmaceutically confirmed, it may be used as lead compound becoming a good starting material in synthesising new drugs. This procedure opens up a new room of opportunities for designing and bio mimetic synthesis of drugs with new pharmacological activity not yet connected to the lead compounds[8]. Salicylic acid which was formally prepared to replace phenol as antiseptic drug has been proven to be useful for analgesic purpose.

Natural products has become an integral part in drug discovery and manufacturing providing and inspiring several lifesaving medicines and advancement in medical frontiers, most especially in the alleviation of epidemic and pandemic diseases such as cancer, HIV/AIDS, malaria, leprosy, hypercholesterolemia and age-related disorders. In the space 2003 to 2008 alone, about twenty one (21) drugs authorised for marketing were sourced from natural product out of the actinomycete, bacterial, fungal and plant sources[9].

Cancer treatment via the use of plants had been in existence for a long time[3] and the vinca-alkaloids are example of anticancer drug under clinical use; they includes the vinblastine (**1.8**) and vincristine (**1.9**). They are isolated from Madagascan periwinkle, a vinca alkaloids, *Catharanthus roseus* (Linn.), Apocynaceae, popularly called "old maid". Vinblastine and vincristine were first uncover during the screening of the plant for latent dental hypoglycaemic activity. Etoposide (**1.10**) and teniposide (**1.11**), derivatives synthesized from epipodophyllotoxin which has been isolated from *Podophyllum peltatum*, are clinically active agents used for the treatment of cancer[10].



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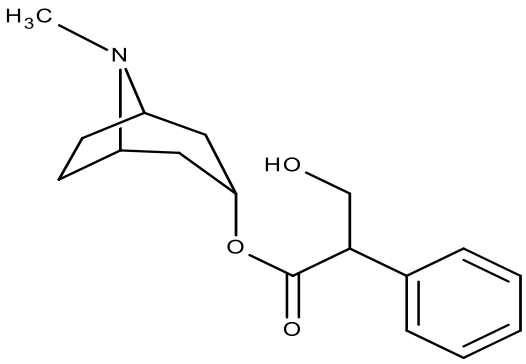

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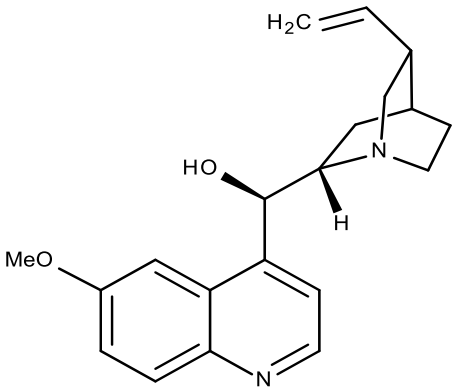

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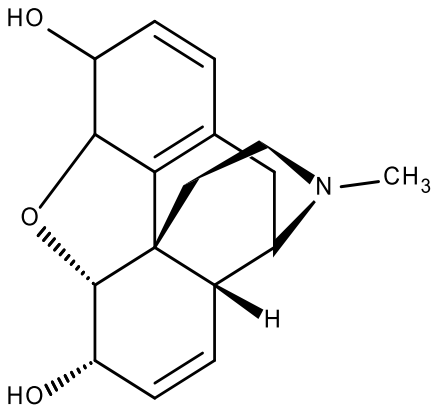

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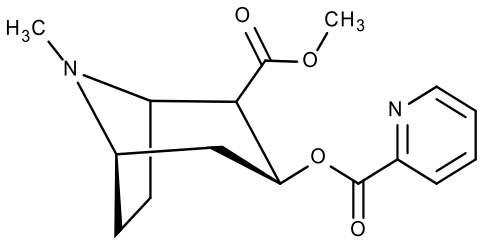

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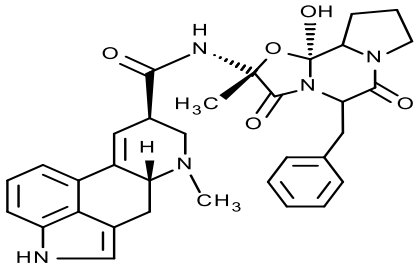

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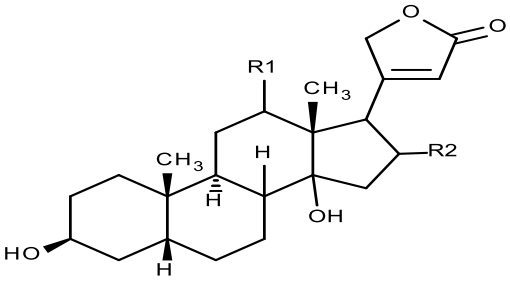

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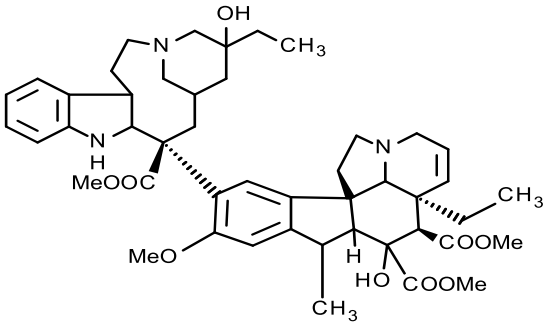

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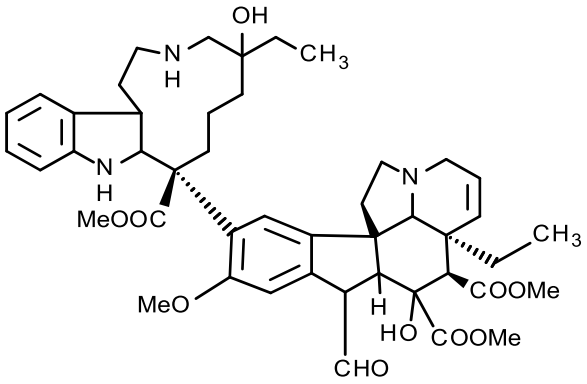

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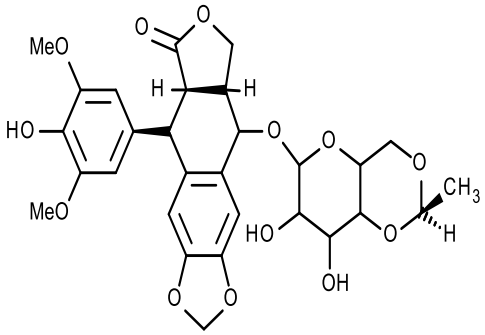

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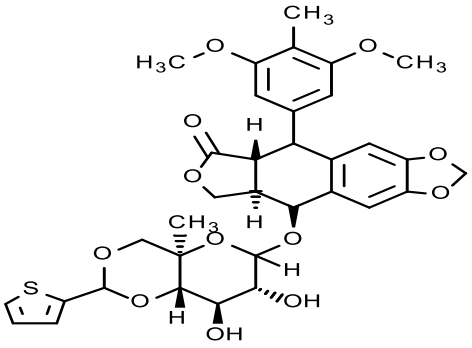

1.11

Figure 1-1: Some natural compounds isolated from natural sources.

The use of traditional medicines in South Africa is historical, owing to the abundance of about 30,000 flowering plants species[11]. Several plant derived drugs had been reported which owes their origin to the traditional use by locals and traditional healers. The trade in plant medicines in South Africa had been on the rise because large population still depend on prescription by the traditional healers[12]. Ethno-pharmacology had revealed the use of *Acacia* species as source of treatment of dysentery among locals in South Africa[13].

Figure 1-2: A vendor chopping plant roots for sale at a local medicinal market in Kwazul-natal

Acacia plant species has been shown to be highly rich in pentacyclic triterpenoids and their derivatives[14,15]. The application of these triterpenoids as potential lead compounds for drug synthesis had also been reported. *Acacia* species reported in South Africa are shown to be widely distributed as invasive plants, but no adequate reports of their bioactive compounds had so far been reported. Therefore, this research work aims to explore their rich chemical constituents and propose their use through bioassay analysis.

Greener chemistry had posed to be a remarkable means of producing compounds/substances (drugs) with less toxicity and with cost effective materials. Nanotechnology involves a

discipline of science that entails production, manipulation and utilisation of materials in nanometres scale. The application of this concept intermingles with many fields due to its wide range of applications which includes bio-medical, sensors, antimicrobials, optical fibres, agricultural, drug synthesis, electronics, polymers and in other numerous disciplines [16]. The synthesis of nanoparticles in recent years had been characterised with generation of hazardous chemicals, tedious procedures and cost. These limitations had paved way for a more eco-friendly, cheaper and safe methods which employs the use of natural products from plants and organism as starting material because of their ability to produce precise shape and structure[17]. Plant extracts, isolates and whole plant had been studied for the bio-reduction of metal nanoparticles because of its simplicity, practical, scalable and application[18]. Several investigations had shown the use of the leaves[19,20], latex[21,22], roots[23], fruits[24], stem bark[25] and buds of plants[26].

Furthermore, biological properties of this plant NP's had shown them to be a good bioactive agents and had been implicated as anti-diabetics, anti-inflammatory, anti-microbial, as sunscreen[27] and as a potential source of numerous anticancer agent[22].

1.1 Motivation

Acacia species reported in South Africa are shown to be widely distributed, but no adequate reports of their bioactive compounds had so far beendocumented. In some local communities where medical facilities are beyond rich, *Acacia* plants offers a wide range of application. The research work aims to confirm the Ethnopharmacology, isolate active components, synthesize metallic nanoparticles and establish their bioactive components.

1.2 Hypothesis

Several active medicinal compounds are present in plants; thus *Acacia mearnsii* and *Acacia karroo* species contains antimicrobial, antitumor, anti-inflammatory, analgesic and anticancer compounds that can be isolated and their activity tested for *in-vitro* and *in-vivo*

1.3 Problem Statement

In earlier report, plant metabolites isolated from plants has been used for disease control and cure. With little or no scientific proof, ethnobotanical use of plant species has been the major source of drug and presently had shown to be a leading source of drug discovery. *Acacias* are woody legumes of the Mimosaceae family, inhabiting the entire continents except in Europe and is majorly known for its large deposit of tannins used in pharmaceuticals, oil industry, leather industry and wine industry.

Acacia mearnsii and *Acacia karroo* widely distributed in South Africa had been termed an invasive or alien species irrespective of its traditional use as forage, anti-inflammation and as wound healing concoction. As a result of this practise, many pharmacologically important plant species had been destroyed which led to eradication of the Indigenous knowledge of such plant.

Systematic approach incorporating plant terpenoids from these plant species as a potential anti-inflammation remedy and consequently evaluating their reductive capacity of metal salts leads to the formation of silver nanoparticles. This concise approach will further improve the pharmacological potential of these plant species and unavoidably improve their economic importance.

1.4 Aim of research

To isolate the essential oils and triterpenoids present in some South African species of *Acacia* plants, semi-synthesis of isolated triterpenoids, synthesis of Plant-Metallic nanoparticle and to characterise these compounds chemically and biologically.

1.5 Specific Objectives

i. Plant collection and identification with voucher number.
ii. Plant preparation followed by extraction
iii. Isolation of the essential oils present in the plants
iv. Silver nanoparticles synthesis from *Acacia mearnsii* and *Acacia karroo* hydrosols
v. Characterisation of Silver nanoparticles using several techniques
vi. Anti-inflammation potential of the essential oils and the synthesized silver nanoparticles.

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CHAPTER 2

LITERATURE REVIEW

2.1 Plant kingdom as source of Lead drugs

2.1.1 Introduction

About 8,700,000 species had been estimated to exist on earth including plant, animals, and micro-organisms[1]. Plants species alone account for approximately 298,000 species having different uses ranging from shelter to food and medicine. Having a large variety of plant species with difference in components due to environment, age, climate and parts of the plant creates room for a whole lot of discoveries in the application of plants especially for medicinal use.

Out of the estimated number of plant species, about 70,000 species had been estimated to be used in folk medicine[2]. Traditional healers in many Africa countries depend largely on the regional plant species and over 5,000 plant species are known to be used[3]

2.2 Procedure for plant selection leading to drug discovery.

There are enormous resources present in the plant kingdom thus presenting a great potential as source of medicine[4]. Pharmaceutical activity of plants can be attribute to the existence of potent compounds called Natural Products or Secondary Metabolites in the organs. Medicines from natural products had emanated from various source materials including terrestrial and marine plants and organisms[5]. Though, synthetic drugs had been on the rise this century, secondary metabolites from natural sources still holds a strong promise to disease control mainly because they tend to show "drug likeness and biological friendliness than totally synthetic drugs''[6] .

Random method of plant collection for drug discovery involves a complete screening of herbs, trees and shrubs available in a particular vicinity. This method leads to collection of bulk quantity within a short time; this type of method is likely to generate compound novelty[7]. The second approach involves target of compounds from plant with previous biological and phytochemical knowledge. A far more enriching method is the approach of ethno botanical, where plant selection and analysis is based on information from local people about medicinal uses by the Indigenes[8]. Lastly, Bioassay-guided fractionation or isolation combines the stepwise separation of plant matrix based on results acquired from biological screening. These procedure incorporates simultaneous separation and biological screening and could lead to pure compounds of definite biological activity[9] .

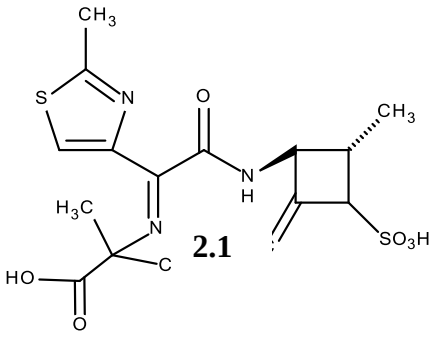
2.3 Secondary Metabolites in Drug discovery

Secondary metabolites are deposited in specialised cells located in cell cavity of plant walls. They equip plants to adapt to their environment and also help to provide clinical effect for their host organisms. This type of metabolite unlike the primary metabolite (responsible for metabolism and cell reproduction) are secreted in little amounts and they basically exert their biological activities by regulating interactions between the plants and other plants, organisms and humans[10]

Many pure compounds which are secondary metabolites had been isolated from different sources which include plants, fungi, marine environment, marine algae and marine sponges. The application of these sets of isolates had been severally reported and some of the drugs are administered for treatment of a large class of epidemic such as; HIV/AIDS, malaria, inflammations, analgesic, diabetes etc.

Penicillin (**2.1**), isolated from the fungus, *Penicillium notatum* had proved to be the best antibiotic drug in previous years[11]. Erythromycin (**2.2**) produced from *Saccharopolyspora*

erythraea and its derivatives cethromycin (**2.3,** under trial) had shown a myriad of activity against the cocci bacteria and rod-shaped bacteria (bacilli) leading to its application for lower respiratory tract infections[12]



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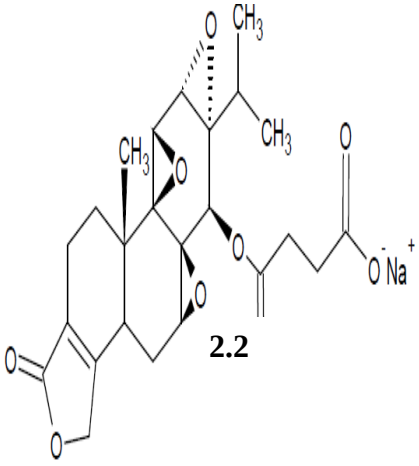

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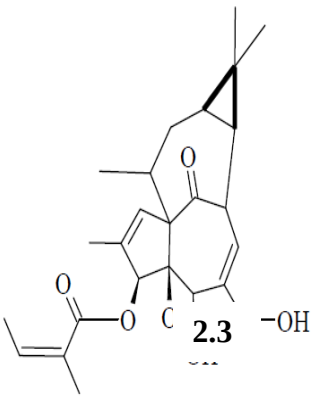

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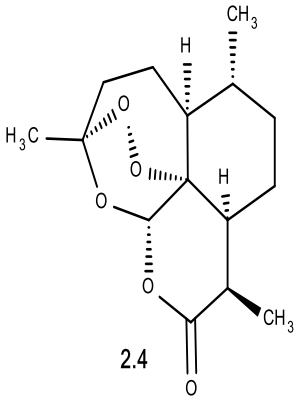

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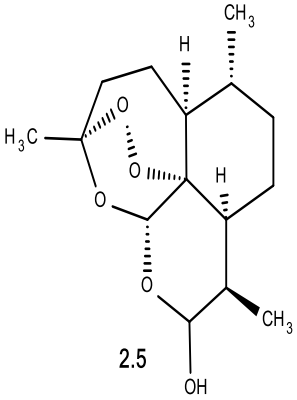

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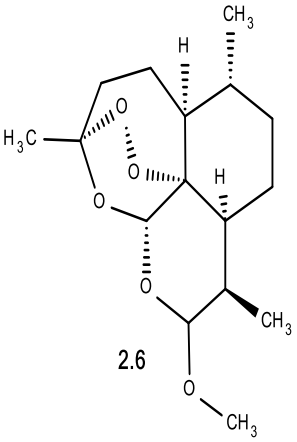

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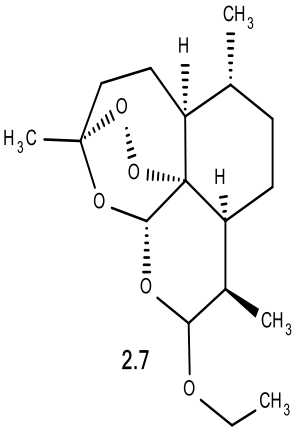

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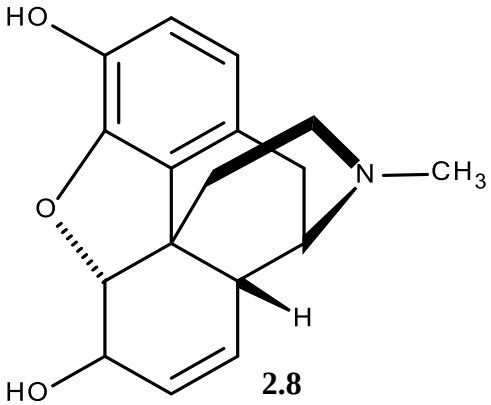
Artemisinin (**2.4**), a Sesquiterpene Lactone (SL's) extracted from wormwood (*Artemisia annua* L.) (Asteraceae) discovered by Chinese chemist, was introduced into the rest of the world in 1972[13]. Its application as anti-malarial drug had well been proven and several derivatives such as Dihydroartemisinin (**2.5**), Artemether (**2.6**), Arteether (**2.7**) and others had as well been synthesised from Artemisinin to improve its potency.

Secondary metabolites or bioactive compounds in living things include the Alkaloids, Non protein amino acids, Amines, Cyanogenic glycosides, Glucosinolates (Nitrogen containing); Terpenoids, steroids, saponines and the phenolics groups (Flavonoid, Polyacetylens, Polyketides and Phenylpropanoids) which are the non-nitrogen's.

Other biologically important chemotherapeutic agents isolated from plants are Morphine (**2.8**) alkaloids from *Papaver somniferum* apply mostly for analgesic purpose, quercetin (**2.9**), a flavonoid isolated from onion, broccoli and berries had been shown to inhibit breast cancer cells by inhibiting cytochrome P450 (CYP) cells[14].



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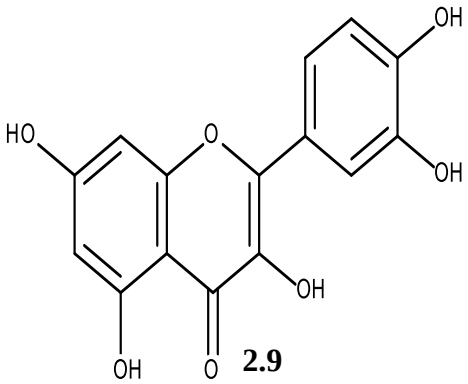

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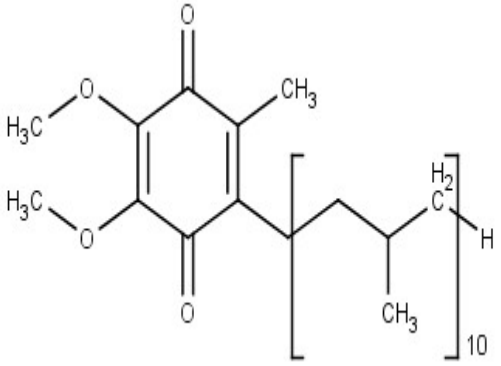
2.3.1 Terpenoids and Steroids

Terpenoids are derived from the linear arrangements of repeated units of isoprene supervened by various cyclisation and rearrangements of the carbon backbone. Terpenoids and steroids

can be broken down into their respective units (C_5 isoprene units) connected in a head-to-tail manner according to Ruzicka[15]. They are termed as the oldest and the largest family of natural products constituting about 55,000 individual natural compounds already described. Certain isolated terpenoids had shown to play a definite roles in plant which can be generalised to these metabolites; such roles includes electron carriers (ubiquinone (**2.10**), plastoquinone (**2.11**)), hormones (gibberellins (**2.12**) , abscisic acid (**2.13**)), photosynthethic pigments (phytol (**2.14**), lycopenes[16] (**2.15**)), polysaccharide mediators and defence mechanism[17].



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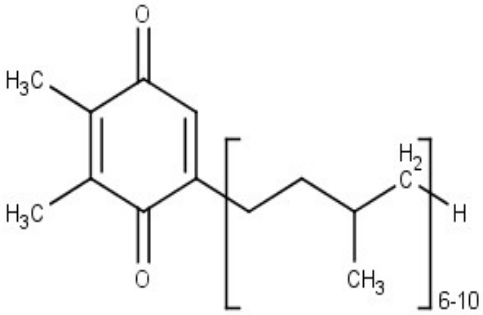

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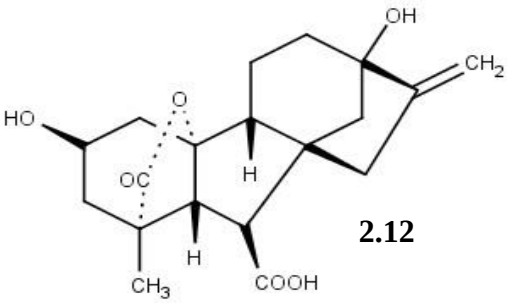

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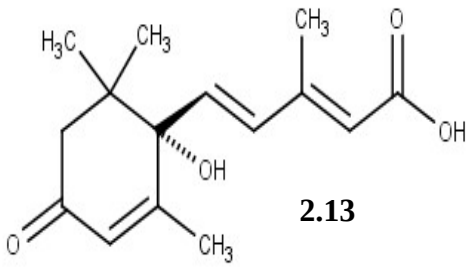

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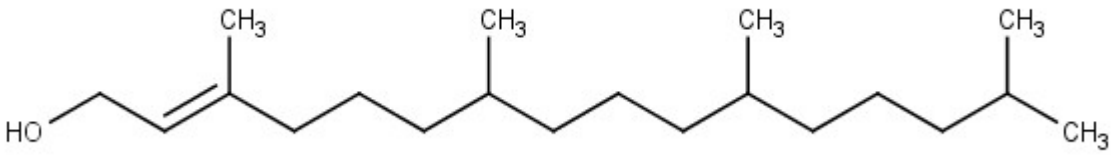

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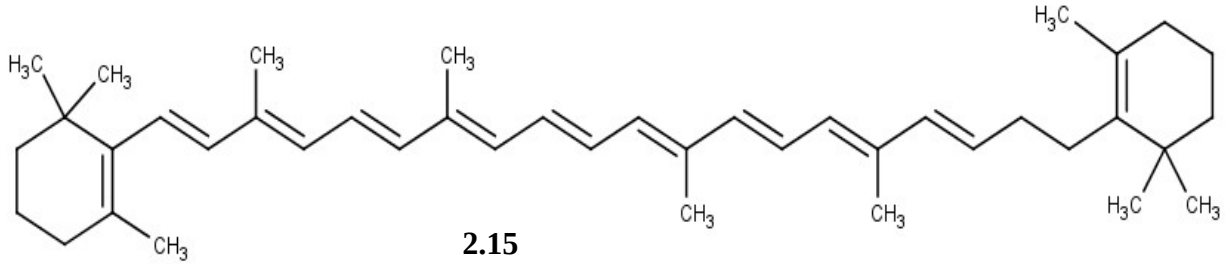

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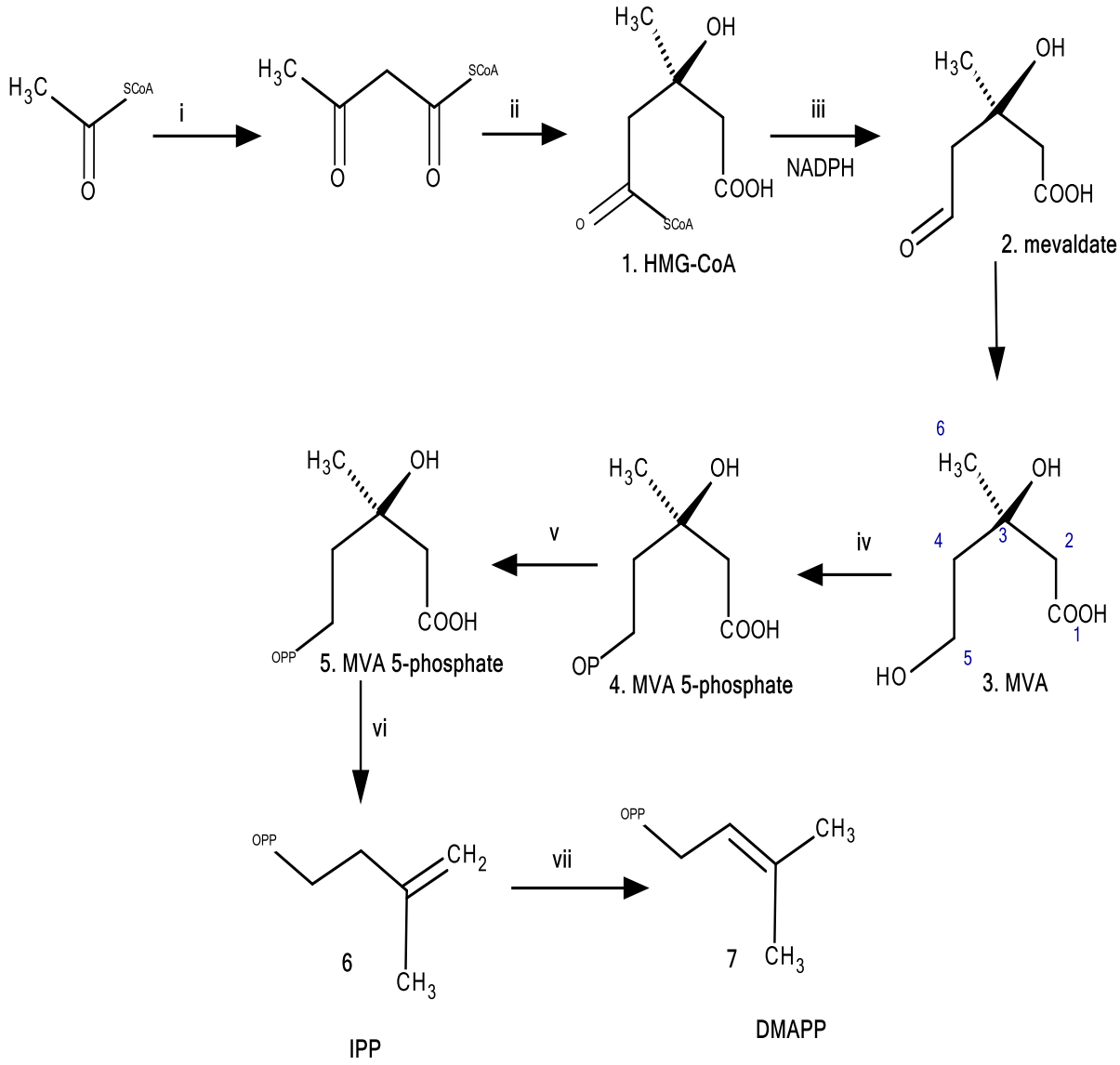
Volatile terpenoids play a central role in plant pollination and seed dispersal as a result of the release of aroma from some specialised cells located in different parts of the plant. A large class of terpenoids had shown antimicrobial, antifungal, anti-parasitic, anti-plasmodic, anti-inflammatory and several therapeutic properties.

2.3.2 Biosynthesis of Terpenoids

Terpenes are generally known to be the building block of terpenoids. Two pathways are known for terpenoids synthesis; the Mevalonate and non-Mevalonate pathway[18].

2.3.2.1 Mevalonate acid pathways:

This mechanism involves the aldol condensation of acetyl-CoA with Acetoacetyl-CoA giving rise to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is catalysed by the enzyme HMG-CoA synthase. As shown in the scheme below, HMG-CoA reductase enzyme further catalyses HMG-CoA to Mevalonic acid (MVA) using the mevaldate intermediate, accompanying with two mole equivalents of NADPH as reducing agents. Further transformation and conversion leads to the formation of Isopentenyl pyrophosphate (IPP) and Dimethylallyl pyrophosphate (DMAPP*).*



KEY: Enzymes: i: acetoacetyl-CoA thiolase (AACT); ii: HMG-CoA synthase; iii: HMG-CoA reductase (HMGR); iv: mevalonate kinase; v: phosphomevalonate kinase: vi: mevalonate 5-diphosphate decarboxylase; vii: IPP isomerase

Figure 2-1 Biosynthesis of Isoprene via the mevalonate pathway

The polymerization of IPP, a five carbon unit is the starting point for terpenoids biosynthesis. Thereafter, the successive head to tail joining of this monomer of IPP forms GPP, FPP, GGPP terpenes. The higher classes of C-30 is formed by joining of two FPP moiety as shown in the scheme above, while the joining of two GGPP leads to Tetraterpenes, C-40 family.

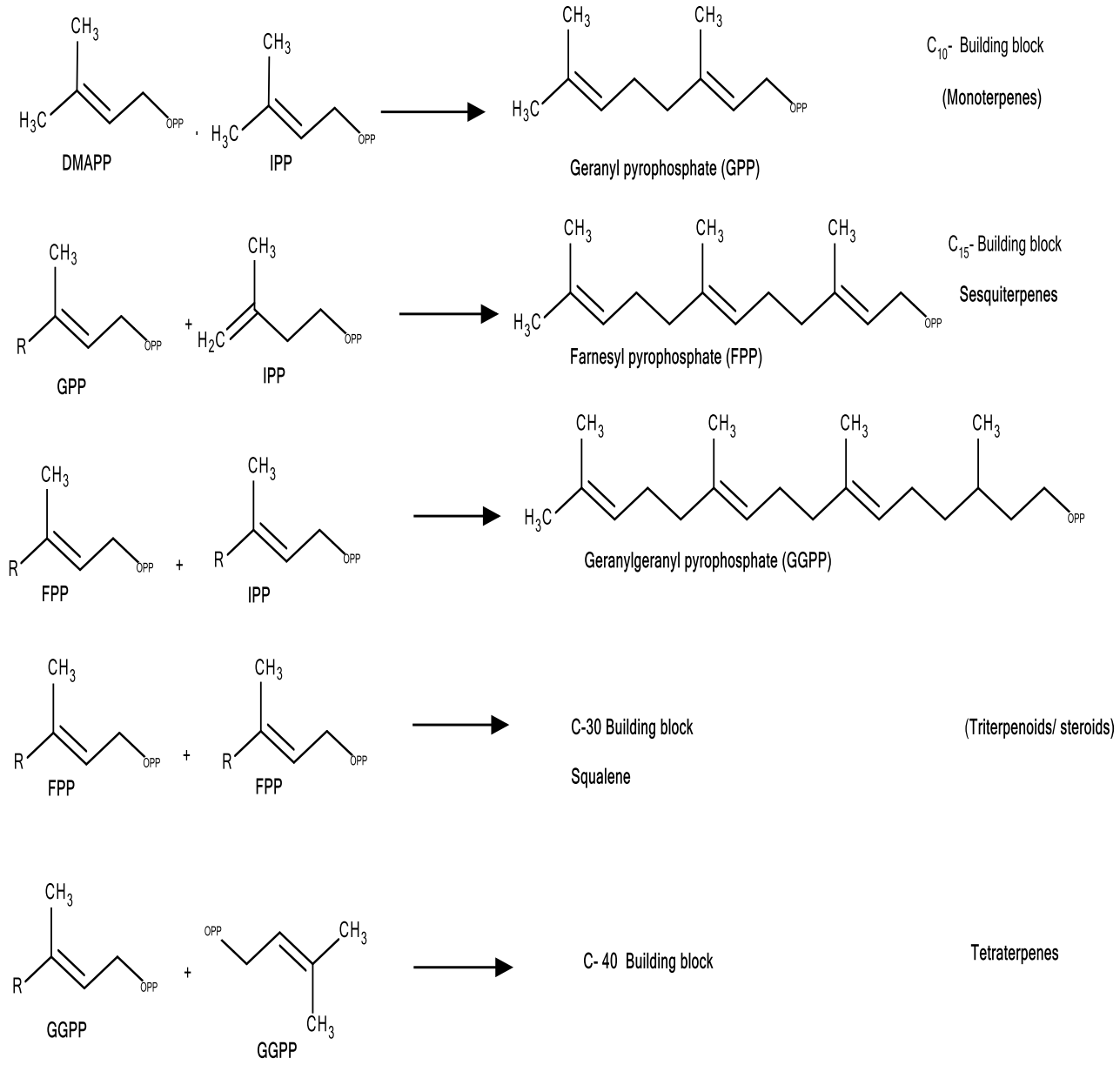


Figure 2-2: Polymerisation of terpenes isomer

2.3.2.2 The non-mevalonate pathway

The Mevalonate-independent route of isoprenoid biosynthesis, also known as the methylerythritol phosphate (MEP) pathway involves 8 enzymes unlike the Mevalonate pathway that involves 7 enzymes. This pathway is utilized by a wide variety of plant

chloroplasts, algae, cyanobacteria, parasites and higher plants. This biosynthetic procedure involves different cofactors and metal ions which leads to formation of IPP and DMAPP.

The scheme below shows the biosynthetic route of the MEP pathway.

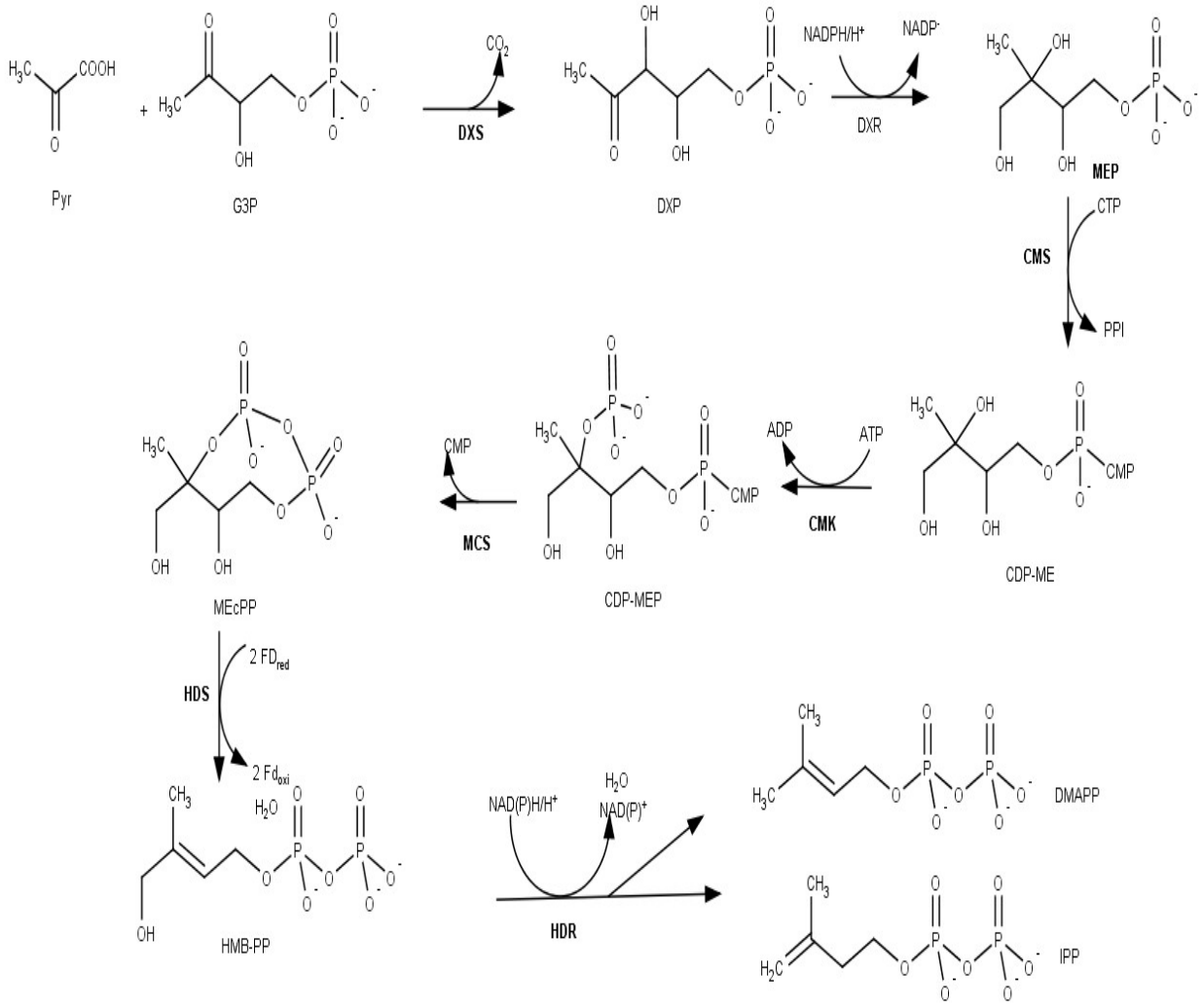


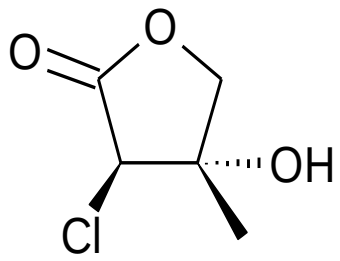
Figure 2-3: Biosynthesis of terpenoids (IPP) using the non-Mevalonate pathway.

This pathway begin via the condensation of pyruvate (Pyr) with glyceraldehyde 3-phosphate (G3P) followed sequentially by series of enzymatic reactions to yield the Isopentyl pyrophosphate (IPP) and Dimethyl Allyl Phosphate (DMAPP) which are precursors to the terpenes monomers of the terpenoids [19].

2.4 Classes of Terpenoids

2.4.1 Hemiterpenes

These are the simplest form of terpenes. Most of this family occur as oils and are mostly soluble in water. Isoprene, which is the basic unit of all terpenes is the only class of hemiterpene. Chlorinated hemiterpenes, utililactone (**2.16**) and epiutililactone (**2.17**) were found in the leaves of *Prinsepia utilis*[20]. Tiglic (**2.18**), angelic acid (**2.19**) and isovaleric acids (**2.20**) are other examples of hemiterpenes.



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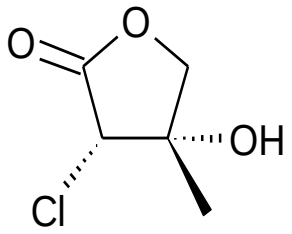

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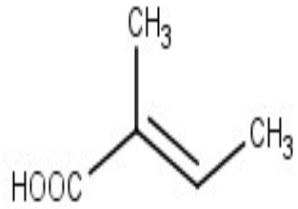

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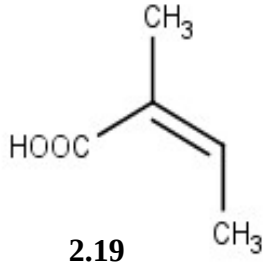

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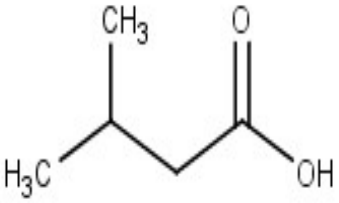

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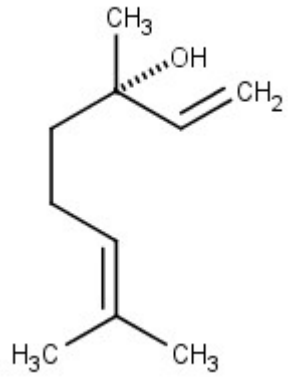
2.4.2 Monoterpenes

Monoterpenes, (C_{10}) represent one of the most studied classes of the terpenoids. It accumulates at the time of root and shoot growth/development in numerous plant species and had shown to play a defensive role against a variety of herbivores and pathogens[21]. Monoterpenes are colourless, hydrophobic, gaseous and volatile compounds exhibiting a

strong aroma and are the main constituents of volatile compounds known as Essential Oils. Its presence had been reported in Euphorbiaceous, Lamiaceae, Apocynaceae, Oleaceae, Myrtaceae, Frankeniaceae, Asteraceae and several plant families and genres.

Different forms of monoterpenes are formed when DMAPP and IPP joined together to form a C_{10} compound. Characteristics of these bye-products are the acyclic monoterpenes (Linalool derivatives (**2.21**)), monocyclic (Carvacrol and its analogues (**2.22**)), bicyclic monoterpenes (α and β pinene (**2.23, 2.24)**) and 1,8-cineole (**2.25**) etc. and tricyclic monoterpenes (tricyclene (**2.26**) and teresantanol (**2.27**)) [22].



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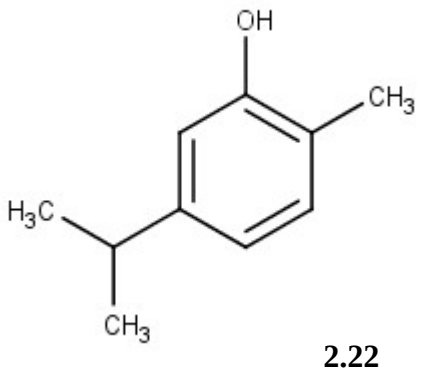

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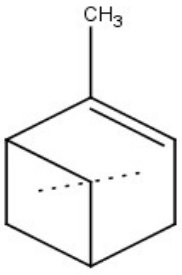

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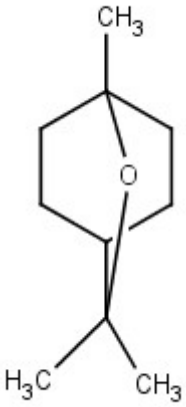

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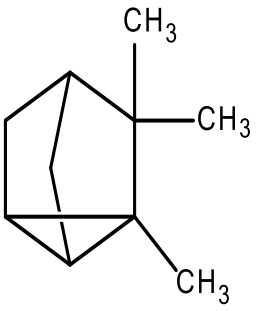

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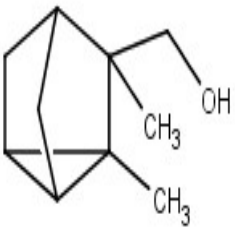


In addition to the major classes and skeletons of monoterpenoids available, essential oil components easily undergo intra/inter-conversion enzymatically and chemically via isomerization, cyclization, oxidation and dehydrogenation giving rise to a new set of moieties with new pharmacological potential[23].

A well-studied example of such transformation is observed in the biotransformation of the limonene present in Cymbopogon species. Scheme below shows the transformation of limonene (**2.28**) to D-limonene (**2.29**), - (-) *trans*-Carveol (**2.30**) and (-)-Carvone (**2.31**).

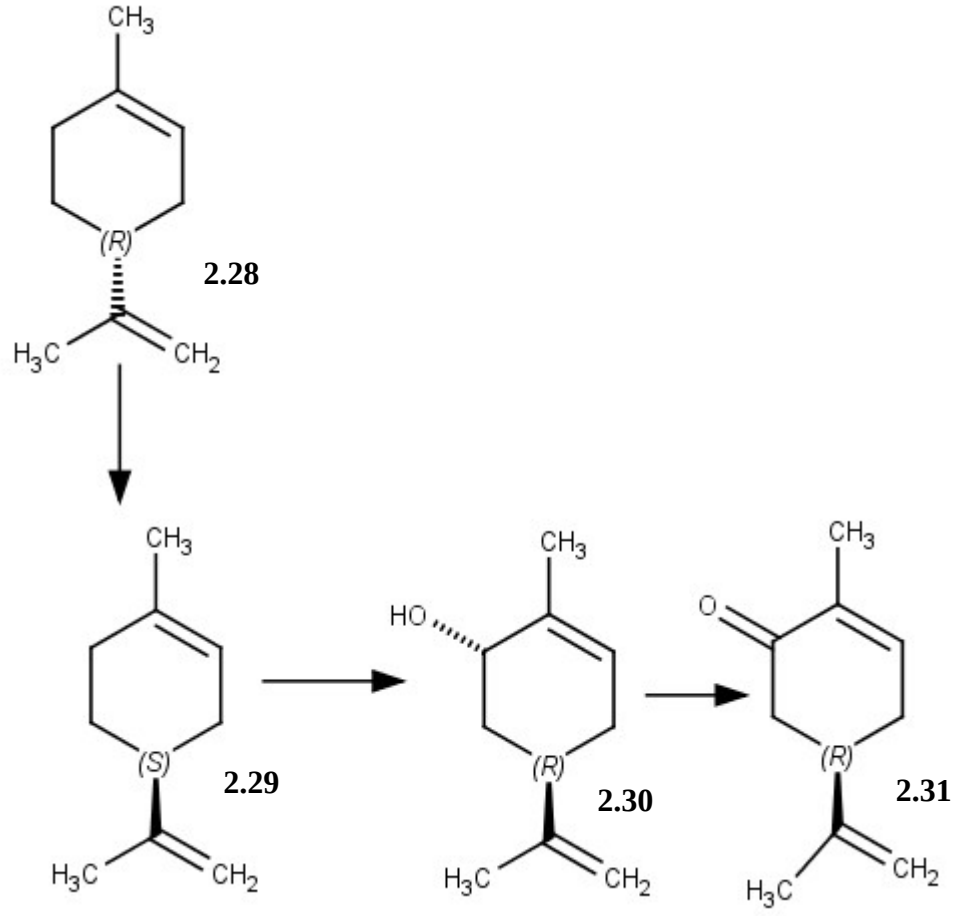
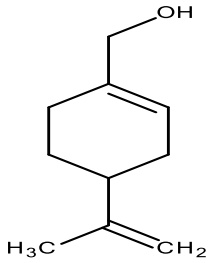


Figure 2-4: Limonene transformation.

Monoterpenes, exhibit a variety of medicinal properties which had been investigated. The monoterpenes, Perillyl alcohol (POH) (**2.32**) which is present in most cherries and mints, exhibits a very high anticancer potential[24]. Other pharmacological effect of monoterpenes are analgesic, anti-inflammation, antiseptic, anti-insecticide, etc.



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2.4.3 Sesquiterpenoids

FPP, a major product from the combining of the GPP with IPP, is the major precursor for a wide variety of sesquiterpenoids. In addition to the formation of FPP, certain enzymes are involve in the cyclization and transformation to form other members of the C_{15} family. An example is the enzymatic transformation of (–)-(Z)-α-bisabolene (**2.33**) to Cuprenene (**2.34**), Trichodene (**2.35**) and Isochamigrene (**2.36**) [25] as shown in the scheme below;

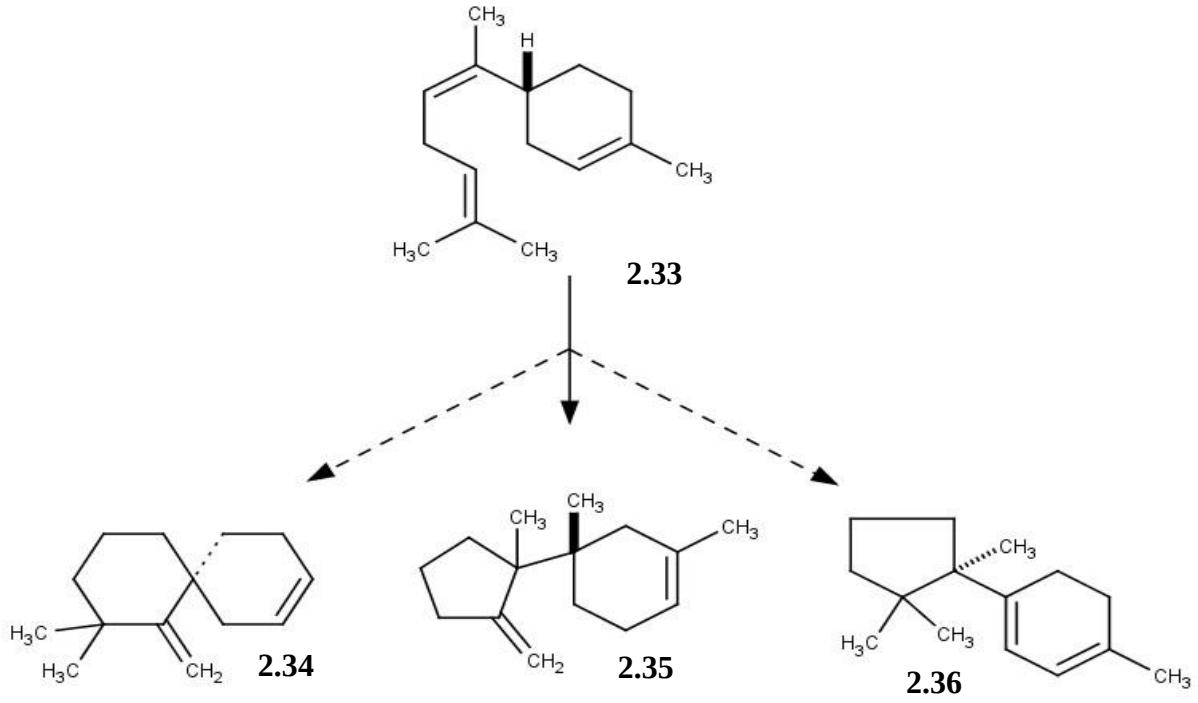
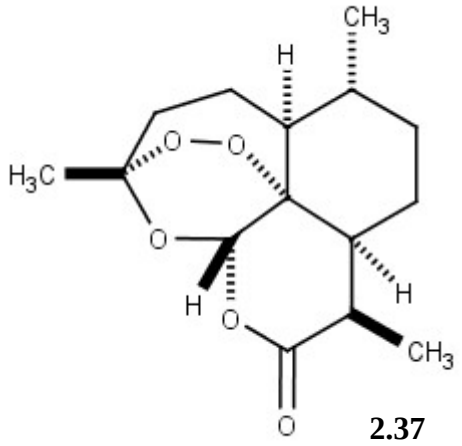


Figure 2-5: Enzymatic transformation of (–)-(Z)-α-bisabolene to Trichodene , Cuprenene and Isochamigrene

Sesquiterpene are characterised with bitter taste, volatiles and non-volatiles (sesquiterpene lactones) and has been shown to exhibit a myriad of pharmacological effect. Arteminisin (**2.37**) (antimalarial lead drug), Pathenolide (**2.38**) (anticancer drug)[26], and eremophilanolide (**2.39**) (anti-diabetic drugs)[27] shows few examples of the application of this groups of terpenoids



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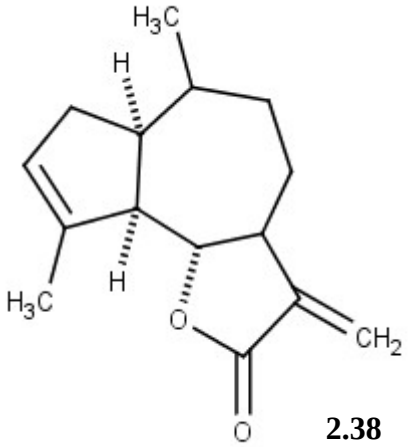

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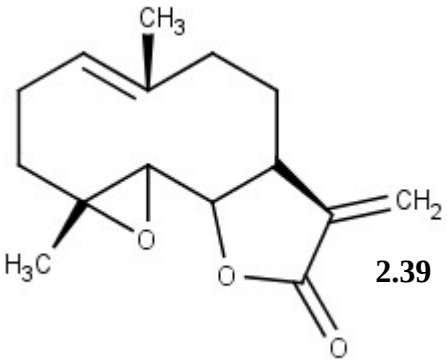
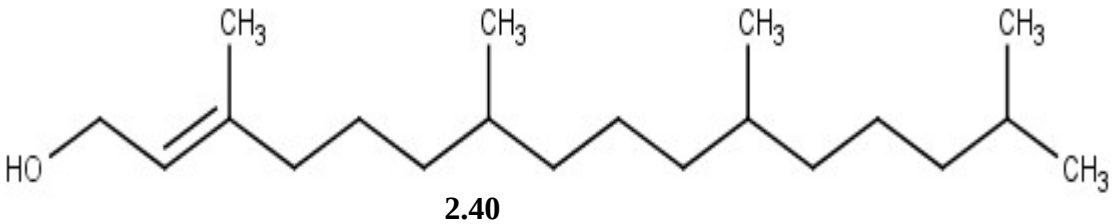


2.39

2.4.4 Diterpenes

The geranylgeranyl diphosphate (GGPP) is the parent source of the diterpenes. These contain 20 carbons (four C5 units), and they are also responsible for chemical defence in plants. Major classes of diterpenes are the acyclic (phytanes), bicyclic (labdanes, haliman), tricyclic (cassanes, rosanes), tetracyclic (kauranes, gibberelanes) and the macrocyclic diterpenes (taxanes, tiglianes, cembranes). Phytol (**2.40**), an acyclic diterpenoids is a precursor for the manufacture of synthetic forms of Vitamin E and K1. Jatrophone (**2.41**), isolated from *Jatrapus (J.) gossypifolia* and *J. elliptica*, a macrocyclic diterpenes was shown to inhibit insulin release and tumor cells[28]. Other activity includes anti-protozoal, molluscidal, gastroprotective and inhibition of lymphocytes activity. Use of taxol (**2.42**) and forskolin

(**2.43**) (commercial anti-obesity drug) as anticancer and glaucoma agent respectively had been reported[29,30].



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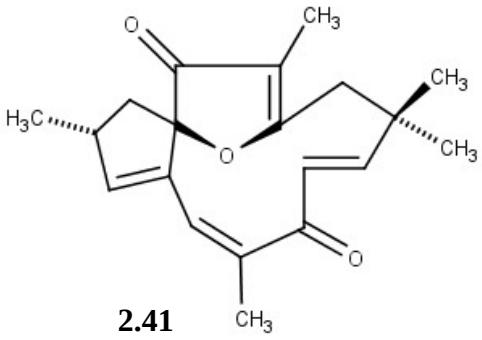

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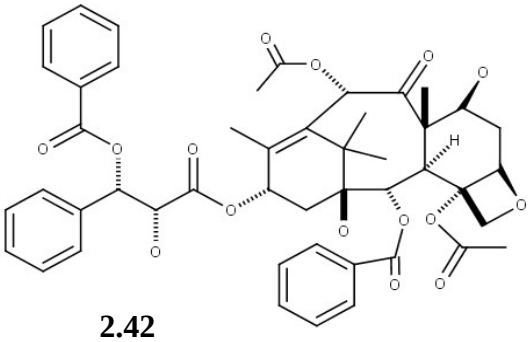

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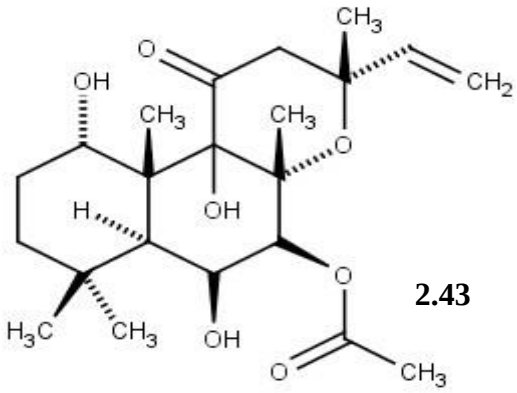
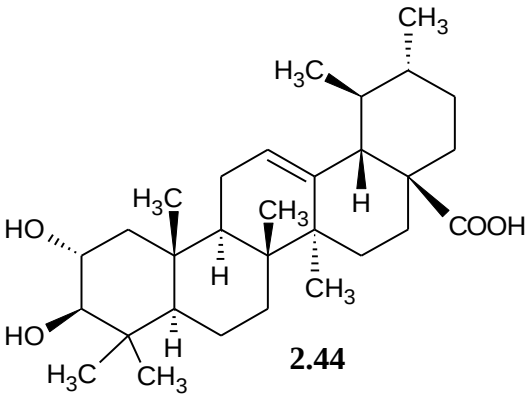


2.43

2.4.5 Triterpenoids

The universal precursor of all triterpenes is the squalene and also steroids. More than 20,000 triterpenes have been isolated, with tetracyclic triterpenoids and pentacyclic triterpenes being the major. They arise biosynthetically through enzymatic action of six (6) isoprene units leading to a 30 carbons unit combined with oxygen atoms; these polycyclic terpenoids are referred to as triterpenoids. Naturally, they can be found in their free form as sapogenins or

bound to glycosides. The class pentacyclic triterpenes are further divided into gammaceranes, hopanes, lupanes, oleananes, ursanes, etc. They occur readily in fruit peel, leaves and stem bark and had been shown to possess a very high pharmacological properties (antitumor, antiviral, antidiabetic, anti-inflammatory…) and offers a huge therapeutic potential. Corosolic acid (**2.44**) used as dietary supplement against diabetes is already on the market while several others are under clinical trial[31]. Betulinic acid (**2.45**), Oleanolic acid (**2.46**) and Ursolic acids (**2.47**) also shown to be a selective inhibitor of human melanoma, and also shows anti-HIV activity by inhibiting the maturation of the virus[32]. Oyedeji et al; synthesised derivatives of oleanolic acids, which displays varying degree of anti-inflammation and anticancer potential[33]. According to the report, 3-acetoxyoleanolic acid (**2.48**) and 3-acetoxy-28-methylester oleanolic acid (**2.49**) exhibited a very high anti-inflammatory and membrane stabilising effect using wistar rats[34].



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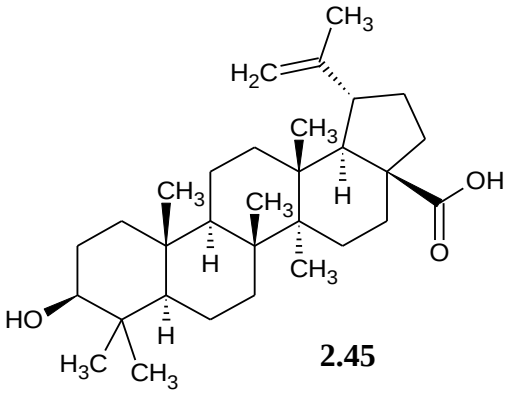

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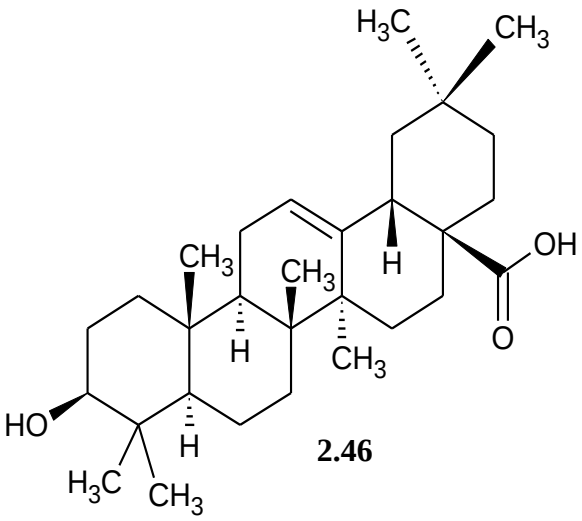

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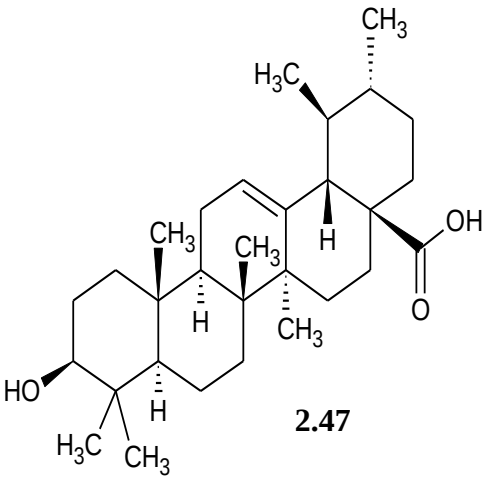

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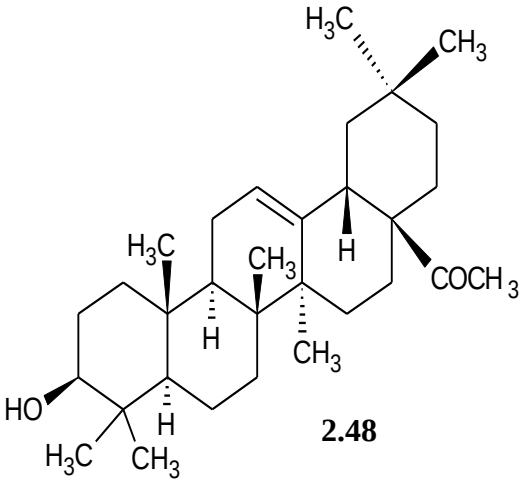

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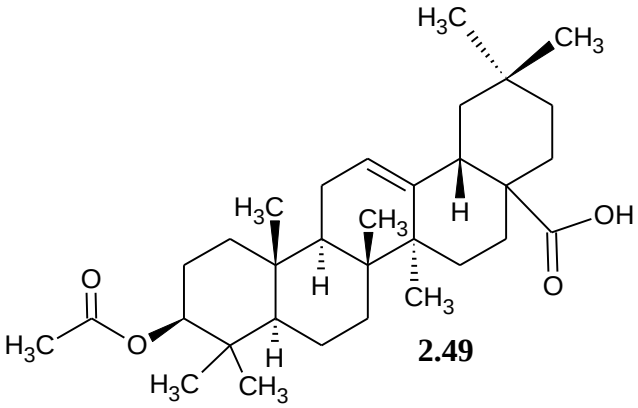
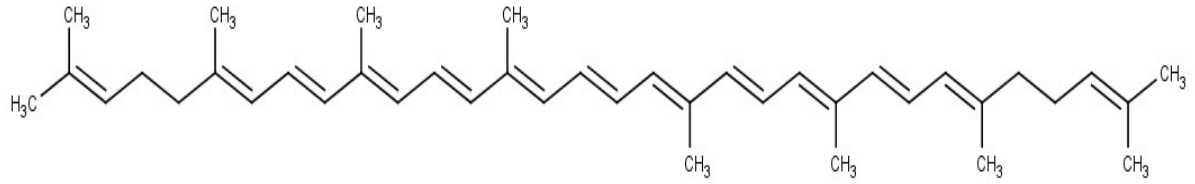


2.49

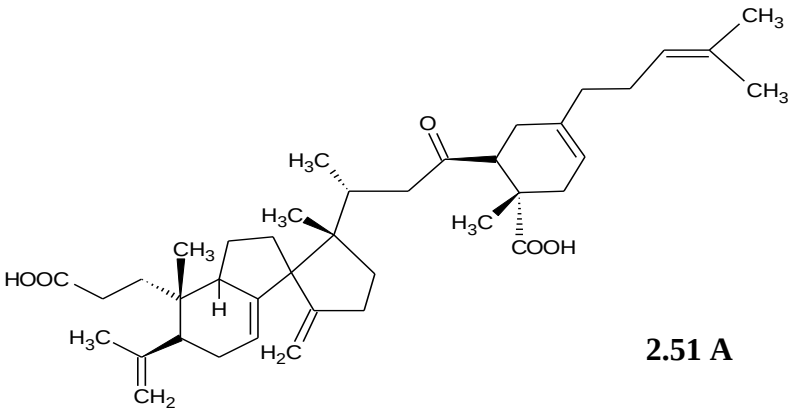
2.4.6 Tetraterpenes

Tetraterpenes, otherwise known as carotenoids are C-40 polyolefinic metabolites derived from mevalonic acid. They are basically coloured pigments and absorb light at the visible region of the spectrum; thereby they have yellow to orange or red colour. Major compounds of this group are acyclic or have chains that terminate with one or two 6-membered rings. They occur in all plants, fungi, and bacteria. They are involved in many important processes such as photosynthesis and mammalian vision[16]. They serve mostly as vegetable, fruit

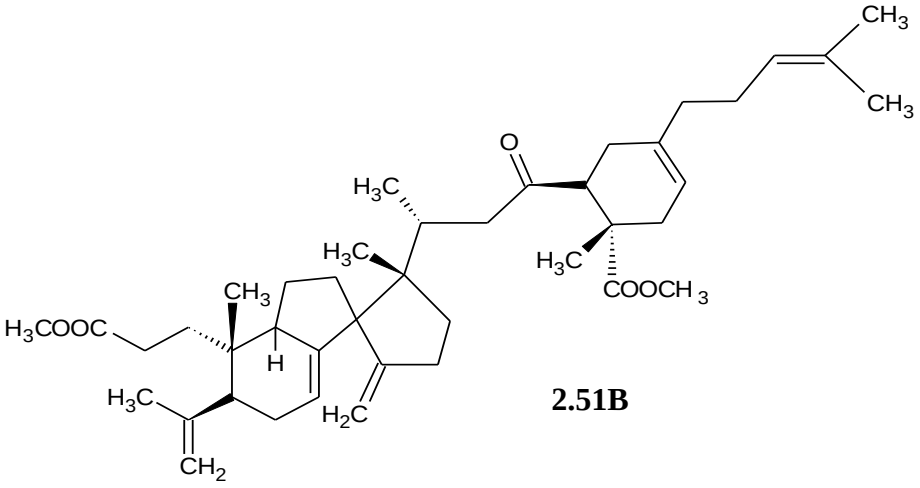
pigments, skins of fish and as floral. Lycopene (**2.50**) had been isolated from tomatoes, the orange colour of carrot is due to the presence of 5-carotene. Abibalsamins A and B (**2.51, 2.52**) were recently isolated from *Abies balsemea* plant and exhibited significant cytotoxic activity against cancer cell lines[35].



2.50



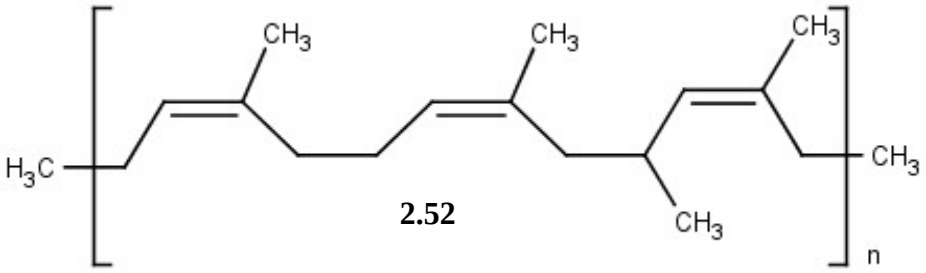
2.51 A



2.51B

2.4.7 Higher terpenoids

Terpenoid fragments containing a large amount of isoprene units are found in plants. Ubiquinone typically contains C40-C50 side chains, menaquinones-8 ($C_{51}H_{72}O_2$), C-65. Natural rubber (**2.52**), a component from rubber tree (*Hevea brasiliensis)* is composed of 1, 4-polyisoprene with molecular mass of about 10,000 to 100,000 g/mol and the isoprene units runs to 100 or more.



2.52 n > 100

2.5 ESSENTIAL OILS

An essential oil is a hydrophobic natural occurring substance in plant with characteristic odour obtained by mechanical means (heating, drying, pressing etc.) from plant components or membranous tissue[23]. They are fragrant, odoriferous free flowing oils, volatile and are characterised by sweet smelling scent, and having lesser density that water. They are called "oils" because they are liquid at room temperature. They are complex mixtures containing among others volatile terpenes. They are bio-synthesised in the whole plant tissues including the fruits, flowers, petals, buds, stem bark, leaves, bulbs, twigs, root and seeds. In the ecosystem, essential oils function as agent of pollination by attracting insects for seed dispersal. Furthermore, they act as antibacterial, antifungals, herbicides, insecticides and deters predators' effect on herbivores by reducing their appetite for such plant. Allelopathic transmission or communication between plants is also a role played by essential oils.

The complex compositions obtained from essential oils may contain from a dozen to several hundred of constituents. As mentioned under monoterpenes, the building block for most essential oil components is mostly terpenes. Thus, constituents may include hydrocarbons, carbonyl groups and several phenylpropanoids[36]. Essential oils trivial names are sometimes from the source or plant where they are isolated. For instance, rose oil (genus Rosa, from *Rosa bracteata*) or peppermint oil (genus Menta, from *Menta piperita*) or lavender oil from (*Lavandula angustifolia* Mill. Lamiaceae).

The influenced seasonal deviations, growing conditions and climate on essential oil compositions had resulted in a more defined concept of classification that focuses on the specific chemical found in a plant. The concept of chemotypes has gradually gained ground in the classification of essential oils, becoming increasingly important in discerning the properties of the oil and defining its applications.

Plant metabolic engineering approaches for terpenoids is a recent technological approach that helps in mitigating the extinction of plant species and enriching certain metabolites in plant. Such improvements and enhancement include disease resistant plants, rapid plant growth, new floral colours and novel blends of aroma from fruits, leaves and flowers. For example, level of linalool were elevated in, potato and Arabidopsis plants[37,38]. Approximately 9-fold increase in terpenoids content were observed in tobacco leaves and flowers after expression with (+)-limonene, γ-terpinene and β-pinene[39].

2.5.1 Extraction, Analysis and Identification of Essential Oils.

As a result of the structural similarity within similar class of terpenoids, chemical transformation resulting from cyclization, oxidation, dehydrogenation and isomerization reactions occur in essential oils. These reactions are triggered by enzymatic, thermal and

chemical effects. The quality and quantity of essential oils composition relies on the processing and isolation method as well as subsequent handling of the oil itself[23,40].

2.5.2 Methods of Isolating Essential Oil from Plant Material

2.5.2.1 Hydro-distillation

This is one of the oldest method used for essential oil extraction. It is applied to plant material that is dried or fresh and is thermally stable. The general procedures involves the immersion of plant samples from which the essential oil is to be extracted into a distillation flask, and the vaporized water with the essential oils is separated by cooling off with a cold condenser. Employed for Cinnamon oil and Clove oil as example.

2.5.2.2 Microwave Assisted Hydro distillation (MAHD)

Microwave energy corresponds to energy frequency of 2.45 GHz, which had been used widely in extraction processes and chemical synthesis. Domestic and industrial microwaves commonly operate at this frequency and unlike the traditional mode of heating, microwaves extraction results in disruption of weak hydrogen bonds which results in faster heating times[41]. To reduce a significant damage to active molecules, MAHD method is highly useful. It is rapid, consumes less energy and offers essential oils of relatively low volatile fractions. Unlike the Microwave Assisted extraction that incorporates use of organic solvents, MAHD involves the use of water as extraction medium [42–44].

2.5.2.3 Steam Distillation

In this process, the water and plant are placed separately in different chambers. Steam is instead injected through the plant material placed on a perforated tray. This method reduces alteration to the constituents of the oils; thus suitable for fresh plants e.g. peppermint.

2.5.2.4 Hydrolytic Maceration Distillation

Certain volatiles are glycosidically bound, thus maceration in hot water prior to distillation is preferred. In the oils of *Gaultheria procumbens*, certain enzyme acts on the oils to bring about release of methyl salicylate and primeverose[45] while the release of amygdalin in bitter almonds is enhanced by this method[46].

2.5.2.5 Solvent Extraction

This technique is used in order to increase yield of oil, or to extract products that cannot be obtained by any other processes. The plants are immersed in a suitable solvent and the separation is performed by distillation at special temperatures that condense the oil but not the solvent. In addition, such solvent must not react with the oil. Diethyl ether, petroleum ether, ethanol, pentane, dichloromethane are the common solvents used. The products of such solvent extraction are called resinoids or concretes. Resinoids are products of hydrocarbon extraction of essential oil-bearing resinous plant exudates, such as benzoin (*Styrax tonkinensis*), myrrh (*Commiphora molmol*)[47] and labdanum (*Cistus ladaniferus*).

Concretes are liquid, semi-liquid or solid materials, which contain up to and in some cases more than 50 % of odourless fat or wax, together with varying amounts of natural pigment. The treatment of concretes with ethanol affords absolutes.

2.5.2.6 Expression or Cold Pressing

These are processes of forcibly separating liquids from solids. Expression can only be done when the plant material has a naturally high content of oil. It is used to obtain citrus fruits oils. The oil are liberated by bursting of the oil glands through mechanical puncturing, collected as paste together with the plant debris. The obtained slurry can be separated using decantation, mechanical agitation, filtration or other similar process.

2.5.2.7 Extraction by Fat

Fat readily absorbs perfume when brought in contact with fragrant flowers be of its high absorption potential. The outcome of the method depends solely on degree of purity of the fat used. Recent combination involves use of a mixture of tallow and lard fat.

2.5.2.8 Effleurage and Maceration

Highly important and special flowers with low amounts of and sensitive essential oils from Jasmin and tuberose, are treated specially with avoidance of heating which can ruin the blossoms. This is a process whereby the odorous principles in fresh flowers are adsorbed in purified cold fat. Upon placing of the petals on the fat as shown in Fig.2-6, they are removed and replaced with fresh ones daily or hourly after absorption by the fat.

The fat is thereby dissolved in alcohol or organic solvents, the solvent is evaporated leaving behind the essential oils. However, in maceration, the petals of the flower are immersed in fat and heated within 45 – 60^0C for 2 h according to the type of flower. Upon immersion, the extracted fat is filtered and then extracted with alcohol, followed by concentration of the essential oil.

Figure 2-6: Processing of plant material for Effleurage (a). Spreading on fat (b) Handpicking of plant material after extraction.

2.5.2.9 Solid-Phase Micro extraction

This method is easy to perform and does not involve organic solvents. A simple SPME method involves a fused silica fibre coated on a stationary phase. Equilibrium is reached between the essential oils and the fiber by exposing the plant material to an aqueous or gaseous sample. The essential oil is thereby thermally desorbed from the fibre surface into the injection unit of Gas chromatography for further analysis[48]. The technique incorporates sample extraction, purification, sampling and analysis into a single step. Durant et al incorporated Headspace into the SPME for essential oil extraction[49].

2.5.3 Chemical analysis of Essential Oils.

Analysis of essential oil is a rigorous process because of the matrices of components they contain. A typical essential oil may contain about 20 to 150 components; thus a well-defined spectroscopic analysis must be employed. In addition, the volatility of the oils is also an important factor to be considered in method selection. Technological advancement had opened room for reliable method for essential oils analysis. They include;

2.5.3.1 Gas Chromatography (GC)

After its introduction as method for the separation of volatile substances by James and Martin[50], Gas chromatography rapidly proved to be an efficient method for the analysis of essential oils. The rapidity, very high separation capacity and its high sensitivity makes GC methods more preferable to other analytical methods; Chromatographic separations allow the quantification (GC-FID) of specific compounds that may be indicative of positive or negative quality notes in oil.

The main components of a functional GC are the inlet, column (stationary phase), mobile phase and the detector. A typical GC elutes components based on their molecular masses and

the class of compounds. Thus, monoterpenes, C_{10} constituents are eluted in their right order before the sesquiterpene, C_{15}.

Sample preparation prior to analysis by GC is incorporated for well resolved separation and identification of components even at lower concentrations. Headspace GC (H-GC), is a technique that employ the use of polymeric coatings as an adsorbent before injection into the septum[51]. Incorporation of odour detecting or GC-olfactometry technique (GC-O) is another development to the contemporary GC method. The matching of essential oils component peaks with definite odours which affords exact retention indices [52].

Hyphenated and multidimensional techniques

A hyphenated method involves coupling of either many chromatographic technique or spectroscopic detection methods to improve separation power and identification of components from analysis.

2.5.3.2 Mass Spectrometry (MS)

Mass spectrometry technique involve determination of the molecular mass of substances/ components. The basic principle is based on mass-to-charge ratio of charged particles. Basic mass spectrometer is composed of three different components with distinctive functions:

An Ion Source: This part converts the neutral atoms into ions. As gas molecules enter into source chamber under high vacuum, they are bombarded by a stream of electron beam (70 eV) leading to fragment ions.

A Mass Analyser: Using combination of electric and magnetic fields, a filter separates ions by mass. The physical property of ions measured by a mass analyser is their mass-to-charge ratio (*m/z*).

Detector or Detector Array: As the ions pass through the mass analyser, they are detected and transformed into a signal based on the quantity of the ions.

The mass spectrum of the molecule thus produced represent a plot of ion signal versus mass-to-charge ratio (*m/z*). A compound's mass spectrum is a unique fingerprint of the compound. The most intense peak is called the base peak and the parent molecule or molecular ion, $(M^{+}*)$[53,54].

2.5.3.3 Gas chromatography-mass spectrometry (GC-MS)

The essential feature of this technique is that it integrates separation, identification and confirmation of the molecular formula of a detected peak/ component. Structural differences of components exists as isomers, these close resemblance are detected by MS and separated accordingly. For example, α-pinene and β-pinene, are eluted and identified at different times. An improvement to the sensitivity of MS is the new technique of Gas Chromatography Time-of-flight Mass Spectrometry (GC/TOF-MS) technology offers high resolution, high mass accuracy and fast scan speeds. Analysis of the essential oils of *Lonicerae japonicae* GCTOF-MS shows a higher resolving power and peak capacity[55].

2.5.4 Identification of Essential Oil components.

2.5.4.1 Retention time

Gas chromatographic separation for analyte separation and identification is dependent on polarity strength and vaporizing ability of the analyte. Separated components elutes at different time which is known as Retention time (Rt). Retention time derived from gas chromatography is a valuable tool for characterising the compound because it is reproducible under the same analytical condition.

Furthermore, such characterisation relies on comparism of the retention time of analyte with reference compounds which must be measured under the same conditions. Naturally, reference sample of the whole essential oils components are hard to come by and also analysis at the exact condition may not be possible due to human and instrumental error. Though Rt offers valuable data for characterising and identifying compounds, reproducibility is poor over a long range.

2.5.4.2 Kovats Index

To surpass this setback, Érvin Kováts[56] developed a retention indices (RI) series by comparing the test compound with peaks from homologous series of normal-alkanes as reference standard. Under an isothermal GC conditions, as the homologues elutes, retention times increases exponentially[57]. This practical parameter is as a result of interpolation of analyte between those of two standard compounds that bracket its retention time, n-paraffins are used extensively. Furthermore, calculation of KI for a given compound is derived from its linear interpolation of its position in the homologous series of the two bracketing paraffins[58].

$$I^{T}_{column} = 100.z + 100\frac{\log tR'(x) - \log tR''(nPz)}{\log tR'(nP_{z+1}) - \log tR'(nPz)} \qquad \text{Eqn. 2-1}$$

Where

x is the analyte of interest eluting between nPz and nP_{z+1}

tR' is the retention time of the analyte

tR''(nPz) retention time of the alkane eluted before the analyte

tR'(nP$_{z+1}$) retention time of the alkane eluted after the analyte

z is the carbon number of the alkane eluted before the analyte

Using a series of alkane standards, the retention time can be converted to a retention index which can be compared to literature values. Therefore, retention indices of analyte differs based on different columns used. Essential oils components are therefore compared from one

column to the other. The difference in nature of stationary phases coating determines the separation efficiency of the column. Basic differences between columns are their different stationary phases. For example Table 2-1 shows comparism in sample KI values from different columns coated with different stationary phases.

Table 2-1: Compounds Kovats index on different columns[59,60].

Compounds	DB5	OV17	C20M	OV101
Hexane	600	600	600	600
Heptane	700	700	700	700
α-pinene	939	945	1032	926
β-pinene	981	994	1116	985
Myrcene	992	1020	1145	990
1,4-cineole	1018	929	1186	1001
p-cymene	1027	1076	1261	1025
limonene	1033	1056	1178	1022
(Z)-ocimene	1043	1134	1245	1026

2.6 Role of natural products in Inflammation mediation

Natural product had been shown to behave synergistically, more exquisitely and widely in an array of steps in the anti-inflammatory mechanisms compared to a single step of synthesized drug[61]. It had been shown that the effective anti-inflammation effects ability of natural products are reliant on dosage, treated cells and tissues and medium of treatment[62]. Inflammation can be the response of a tissue or cell to pathogenic invasion, host to invading foreign pathogens, to tissue lesions, or reaction to a range of external stimuli (physical, biological or chemical). Protective response of inflammation are mostly induced by tissue destruction and restoration by tissue healing. A typical inflammatory stimuli is extensively limited, and involves the ultimate cellular decrease of expression of inflammatory mediators (some chemokines, kinin system and chemokines) [63].

2.6.1 Acute Inflammation

This is an important and composite response leading to the protection of the body system towards injurious stimuli. It also involves the physiological condition occurring at the very beginning of the inflammatory process. The entire process for the acute inflammation takes from 1-2 days accompanied by blood vessels widening and enlarged vascular porosity brought about by inflammation mediators. The release of Nitric oxide (NO), bradykinases, serotonin, prostaglandins, thromboxane, leukotriene, COX-2 genes, histamines and platelet-activating factor) mediators enhances chemo attraction, rolling, tight and endothelial adhesion and transmigration process of the mediators to the wounded site[64]. The COX-2 genes had become a reference for anti-inflammatory remedy because it is highly inductive and are controlled by divergent transcription agents. However, the actual release of the mediators are time-dependent in an animal model; Edema growth is a tri-phasic process – the liberation of histamine and serotonin in the first phase (0–2 h), cytokines, at the 2nd phase (3rd h) and prostaglandin in the 3rd phase (>4 h).

2.6.2 Chronic anti-inflammation

Chronic inflammation has many features of acute inflammation but is usually of low grade and persistent, resulting in responses that lead to tissue degeneration. It is the type of inflammation that lasts for a long period of time. There are several possible triggering force for chronic inflammations. They could arise from untreated acute inflammation, thereby lasting for weeks, years and may lead to arthritis. However, chronic inflammation may develop insidiously without any acute inflammation symptoms. Development of microphages, lymphocytes, tissue destruction angiogenesis leading to tissue repair are characteristic of chronic inflammations [65].

The general mechanism of chronic inflammation follows three (3) modes of action;

Continuous production of reactive molecules (NOs, ROS[66]; etc) by surrounding leukocytes designed to kill pathogens which eventually damages the structural and cellular elements of tissues;

Non-immune and activated immune cells which had been damaged leads to the production of cytokines that modulate the inflammatory response and alter the phenotypes of nearby cells, often to the detriment of normal tissue function.[67]

Chronic inflammation results in cytokine reactions resulting in mononuclear cells migration to inflammation sites where IFN-γ and MCP-1 attracts and activates the macrophages but their migration are hindered at the inflammation site by migration inhibition factors (MIF), like GM-CSF and IFN-γ[68]. Cytokines responsible for chronic inflammation can be broadly categorised into several classes.

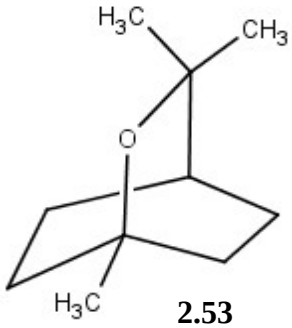
- Humoral inflammation mediated by Interleukin (IL)-3 (eosinophils activator), IL-4 (collagen activator), IL-5 (intensification of T cells), IL-7 (activates thymocytes), and IL-9 regulates T cells and the TGF-β.

- Cellular inflammation such as IL-2, IL-12, IL-15, interferons (IFNs), IFN-γ inducing factor (IGIF), and TNF-α and -β.

Several studies had reported the efficacy of secondary metabolites like phenolics, flavonoids, alkaloids, terpenoids, essential oils and tannins in showing anti-inflammatory properties by regulating the degree of several inflammatory cytokines or inflammatory intermediate such as prostaglandin E_2, IL-1, TNF-α, NO, COX-2, IL-6, NK, NF-κB, and iNOS [69].

2.6.3 Essential oils as anti-inflammation Inhibitors

The oil of Salvia essential oils from South Africa had been reported to inhibit 5-lipoxygenase inhibitors which were attributed to 1,8-cineole (**2.53**), an α-pinene (**2.54**) with β-Carryophyllene (**2.55**) [70]. Chamazulene (**2.56**) and α-bisabolol (**2.57**) isolated from the oils of chamomile, exhibits anti-inflammatory properties by inhibiting leukotriene synthesis because of their ability to supress the 5-lipoxygenase mediators.



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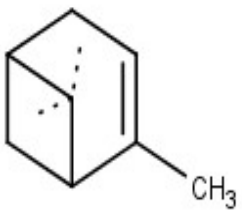

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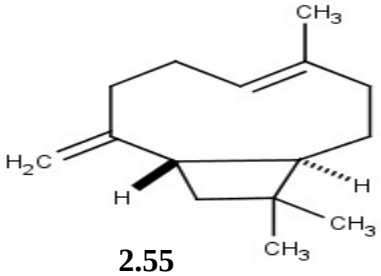

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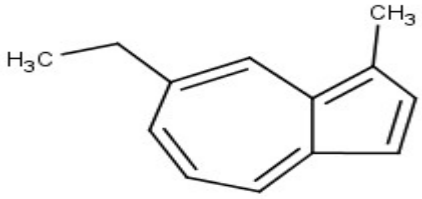

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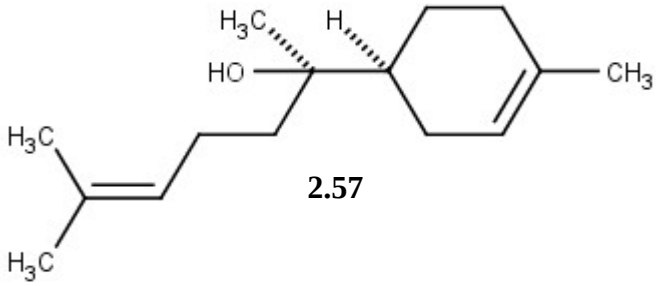

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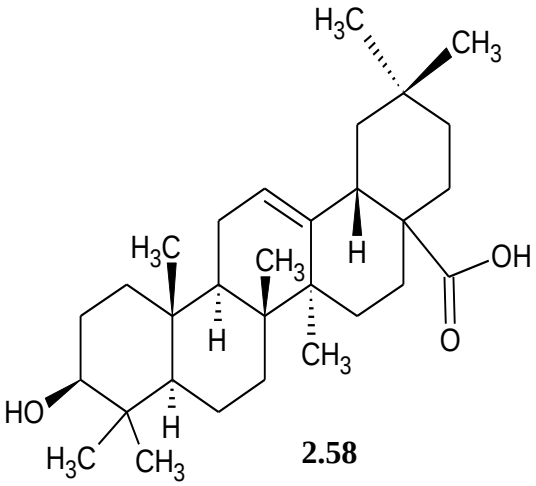
2.6.4 Triterpenoids role as anti-inflammation inhibition

Oleanolic acid (**2.58**) and ursolic acid (**2.59**) are common triterpenoids isolated especially in most spices and fruits. They function by the suppressing of COX-1, LTB4, COX-2, and NO. High activities from their derivatives had also been investigated; Honda et al, reported high activity of all the derivatives synthesized with about 10,000 times activity when compared to the lead compounds[71]. Natural products inhibiting inflammation from marine organisms, and fatty acids sources as well had been reported[72].



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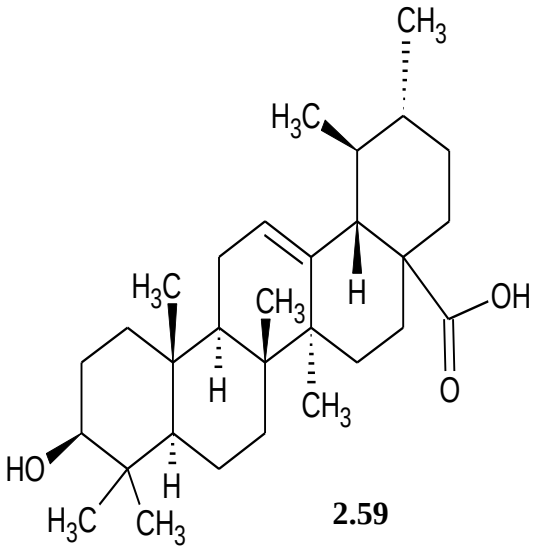

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2.7 Review on *Acacia karroo* Hayne and *Acacia mearnsii* De Wild

2.7.1 *Acacia species*

Acacia is a generic name for species of Mimosaceae of the family Fabaceae, Leguminosae. It ranked as second most abundant genus in the Leguminosae family. It was first classified in 1773 by Carl Linnaeus, a Swedish botanist [73]. Approximately about 1350 *Acacia* species had been collected and identified worldwide with 960 species domicile in Australia, 144 in Africa, 89 species in Asia and about 185 species in the Americas [74]. *Acacia* genus comprises of tall trees, herbs and possess high timber content. *Acacia's common names includes* whistling thorns, black locust, wattles or thorn trees.

2.7.1.1 Description

The leaves of *Acacias* are arranged on either side (pinnate) generally. However, in some Australian and temperate Pacific Island species, the leaf-lets is short and flattened, thereby serving as leaves. The vertical orientation of the petiole shield them from the extreme degree of the sunlight with enough exposure to accommodate photosynthesis. Some species like *A. glaucoptera* and *A. alata* are without leaves but instead uses its cladodes (modified stems) for photosynthetic activities [75]. As trees, they can grow up to 6-20 m tall, unarmed gray or brown while as shrubs, armed or unarmed growing up to 3-8 m tall. The flowers are arranged in panicles, yellowish to whitish in colour while the seeds are black, shiny and ovoid in shape[76].

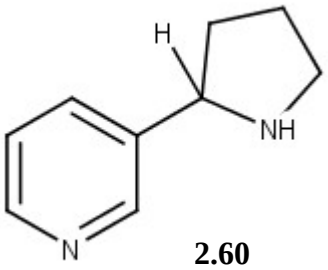
2.7.1.2 Uses

The uses of *Acacia* plants ranges from its use as food (*A. pennata*), gum (commonly known as gum Arabic), ornamental uses (*A. dealbata*), paints and perfume (*A. farnesiana*), symbolism and ritual, tannin (*A. mearnsii*,), wood and timber (*A. karroo*) and land

reclamation[77], wound dressing for burns (*A. tortilis*) and as seed for human consumption (*A. colei*)[78].

2.7.1.3 Phytochemistry of *Acacia* plants

Acacia produce a large number of secondary metabolites with potential for industrial application and as food additives. Few examples of these important compounds with pharmacological importance includes; Nicotine from *Acacia concinna (***2.60***)*[79], *Hordenine (***2.61***) and nornicotine (***2.62***) from A. rigiduda and A. berlandieri*[80, 81]. Mutai and his co-worker isolated 3 new pentacyclic triterpenoids from the stem bark of *Acacia mellifera*, these triterpenoids ((20*R*)-3-oxolupan-30-al (**2.63**), ((20*R*)-28-hydroxylupen-30-al-3-one (**2.64**) and ((20S)-3β-hydroxylupan-30-al (**2.65**) exhibited significant cytotoxic activity against the NSCLC-N6 cell line[82].



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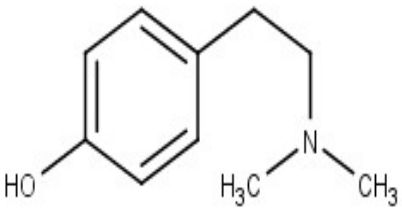

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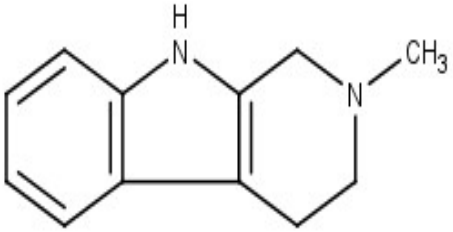

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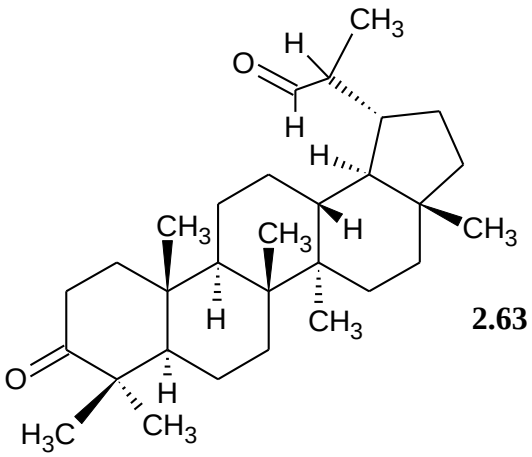

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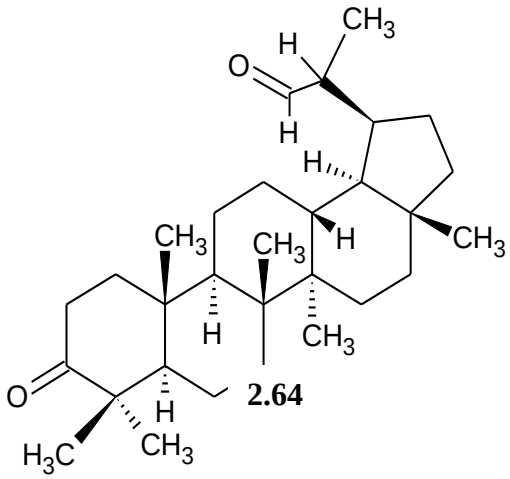

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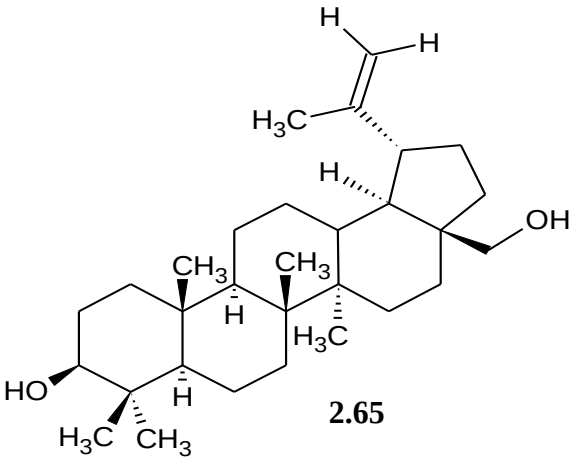

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2.7.1.4 Volatile terpenoids

Chemical studies of the essential oil of some species of this genus have been reported previously. Two *Acacia species, A. nilotica* Linn. and *A. albida* Delile (Leguminosae) from Western Nigeria, West Africa had been shown to be highly rich in menthol (34.9%) and α-pinene (18.6%)[9], *Acacia tortilis* from West Africa also contains α-humulene (12.0%), and α-cadinol (10.6%) as the major components[83,84]. *Acacia aroma* and *Acacia caven* Moll collected in Argentina shows a relatively high amount of methyl salicylate (46.0%) and *p*-anisaldehyde (14.4%) respectively[85]. *A. cyclops* analysed in South Africa shows (Z)-*β*-Ocimene (70%) as the most abundant constituents[86]. The constituents in the different species so far analysed shows no distinctive pattern in the components as some are hydrocarbon based[87] while others are mostly monoterpenoids[84].

2.7.2 Plant of study

2.7.2.1 *Acacia karoo* Hayne

Acacia karroo Hayne or sweetly thorn is a remarkably beautiful and important flora of the Southern Africa. It is distributed within the fringes of Western Cape through the Eastern Cape and spreads down to the Zambian and the Angola. It is commonly known as Cape thorn tree, karro-thorn, mimosa thorn, sweet thorn or white thorn [88].

Among the Afrikaans it's known as doringboom, karoodoring while among the Xhosa's and the Zulu's its known as umunga[89]. *Acacia karroo* is a household material among the South African's. Among the Zulu's for instance, it can be weaved into raft to fence royal houses of the Zulu's and also the thorns were used by earlier ancestral to pin insects. *A. karroo* can thrive in virtually tough regions, including the deserts (a height of 3-4 metres, with big thorns and high content of leave tannins) and coastal woodlands (40 m tall, with small thorns, low tannin content with low chemical defence mechanisms).

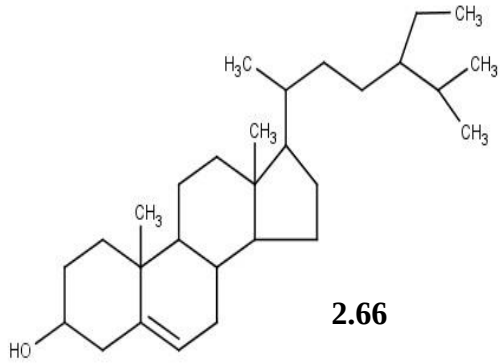
Figure 2-7 *Acacia karoo* (a) flowers and leaves

Figure 2-8 *Acacia karoo* full plant with stem bark

A. karroo is characterised with red-brown to dark thorn less trunk, having colour changes based on the age of plant. The leaves ranges from small to large sizes, dark green in colour and sometimes are shed during the winter season. Their flowers are golden yellow in colour as shown in Fig 2-7, blossoms during summer in a crowded yellow pompons shape. The seed is flat, oval shaped and 4.5-8 mm long arranged on both sides of the pods. Pods are slender, elliptical and brownish colour when dried. The thorns as shown in Fig. 2-8, are arranged on both sides of the stem bark, sharp and firm when plant matures [90].

Medicinal activities such as anti-fever, antiseptic, anti-diarrhoea and anti-dysenteric had been reported from the Sweet thorn trees. A decoction of some species of *A. karroo* is administrated as a remedy for malaria[91], stomach ailments and pneumonia. In the tropics, A. karroo is an outstanding forage for goats, cattle's, horses and herbivorous animal [92]. The wood burns with less smoke, gums can be eaten, the root bark can be used as twine, and for treating plant poisons in cattle [93].

Acacia karroo aqueous extract exhibited high anti-inflammatory and analgesic property on an animal model[94] and also inhibited the HIV-1 reverse transcriptase with IC_{50} of about 70%[95]. Bioassay guided fractionation of *Acacia karroo* ethyl acetate extract led to the isolation of 3 compounds which are β-sistosterol (**2.66**), epicatechin (**2.67**) and epigallocatechin (**2.68**)[96]The analysis on the essential oils had not yet been reported in the literature.



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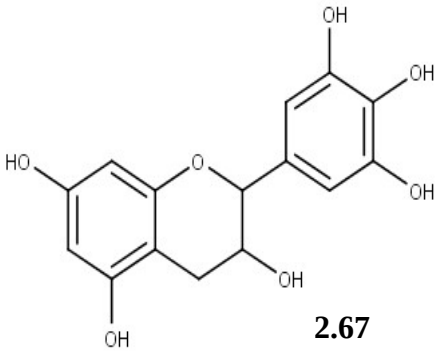

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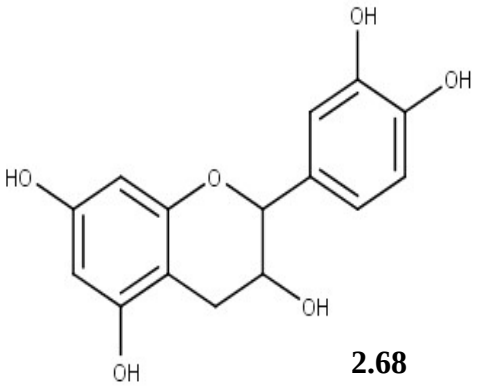

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2.7.2.2 *Acacia mearnsii* De Wild

Acacia mearnsii De Wild is a genus of Mimosoideae family. It is commonly known as black wattle, blue passionflower, green wattle, tan wattle and Australia *acacia*. Among the Swahili's, its commonly called *acacia* negra or ***Uwatela*** (Zulu) [97]. It is a rapid growing Leguminosae tree and contains a large deposit of tannins and also serves as a good source of

fuel (wood) employed by rural dwellers [98]. *Acacia mearnsii* seeds were dispersed during the voyage and slavery period from Australia to South Africa and has since became one of the most invasive species in the continent of Africa [99]. It was mostly used for firewood, fences for buildings and as livestock shades. About 1888, the first tannin extraction were done from the bark and had since become a leading source of tannins for leathers and pulp making [100].

Figure 2-9 *Acacia mearnsii*, leaves and flowers

Figure 2-10 *Acacia mearnsii* stem bark.

Acacia mearnsii occupies about 8.9% of the South African agricultural reserved land, totalling to 104,055 hectare. It is a tall woodland tree common in subtropical and warm temperate regions. They grow naturally along the river beds and the trees are like canopies, erect, slender or fleshy depending on the age of plant, 5-10 m tall with brownish-grey bark colour. *Acacia mearnsii* leaves as shown in Fig. 2-9 are greenish, evergreen, and alternate with very soft hair, 8-15 cm long. The flowering season spreads from August to October and flowers at 2 years after cultivation [101].

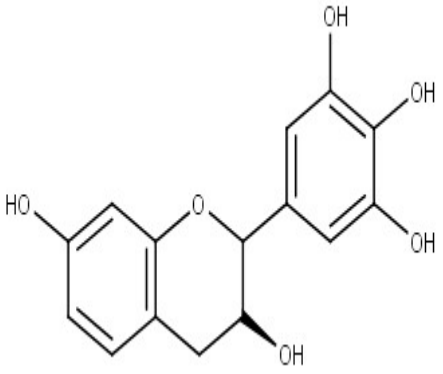
The uses of *Acacia mearnsii* include the use of its fodder as sheep milled leaves to feed cattle. It is commonly used as a source of fuel wood and the charcoal is used widely in Southern America and Southern Africa. *Acacia mearnsii* is about 320 kg/cubic rich in pulp and had been applicable as paper wrapping and hardboard[102].

In the Java and in Kenya region, the leaves stock serves as a source of manure for cultivation in order to increase harvest. Serving as a large source of wood, the sawdust is used in China for growing domestic mushrooms for consumption[103]. The wood serves as electricity poles, furniture, flooring and tool handles. The name black tan is derived mainly because of its wide use as tans as a result of high content of tannins. *Acacia mearnsii* contains tannins or dyestuff and its tannins is a major exporting commodities in Tanzania, Kenya, Brazil and South Africa. About 36-44% of tannins are recovered in the bark of *Acacia mearnsii* alone with variation in this yield as a result of abiotic conditions (plant age, genetic code and climatic conditions) [104].

Tannins isolated form *Acacia mearnsii* includes the hydrolysable and condensed tannins[105]. In addition, South Africa became the number one supplier of tannins in 1997, while Australia's leather and furniture industries were the highest users[106]. Methanolic crude extract

of *A. mearnsii* stem bark exhibits an extremely high inhibition against Shigellosis causing bacteria, thus confirming the antimicrobial potential of *Acacia mearnsii*[107].

The water extract of *Acacia mearnsii* bark led to isolation of some proanthocyanidins with high amylase and lipase inhibitory activity; they include robinetinidol (**2.69**), syringic acid (**2.70**), taxifolin (**2.71**), robinetinidol-(4α,8)-gallocatechin (**2.72**), robinetinidol-(4α,8)-catechin (**2.73**), fisetinidol-(4α,8)-catechin (**2.74**) and 3,5-dimethoxy-4-hydroxybenzyl alcohol 4-O-β-$_D$-glucopyranoside (**2.75**)[108]. The terpenoids content of this plant species is yet to be investigated and reported.



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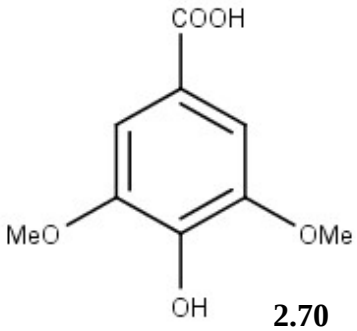

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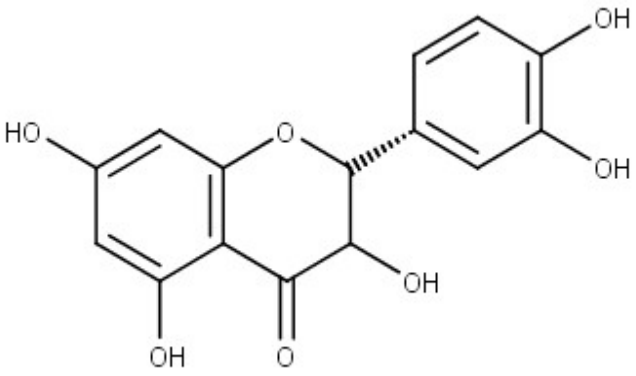

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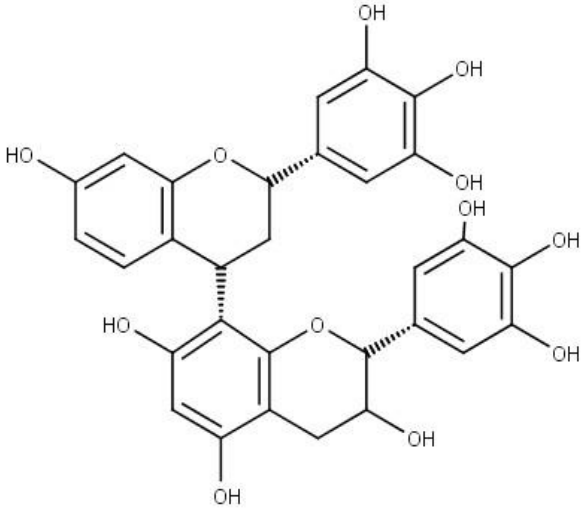
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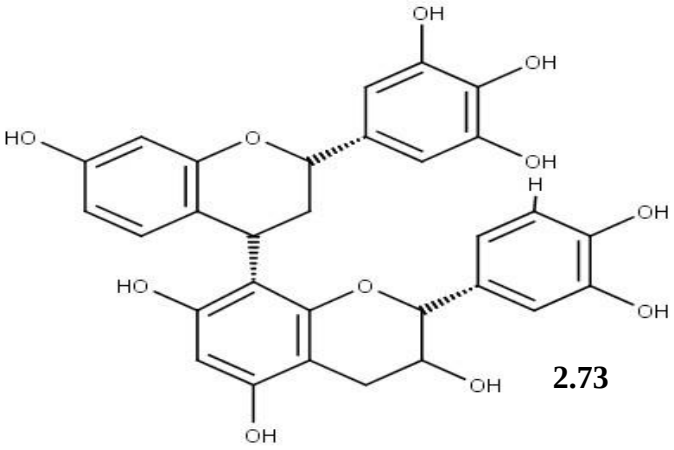
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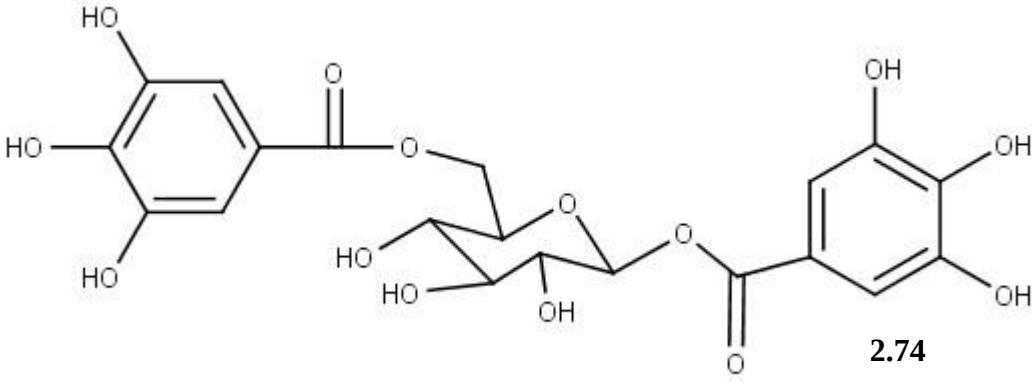
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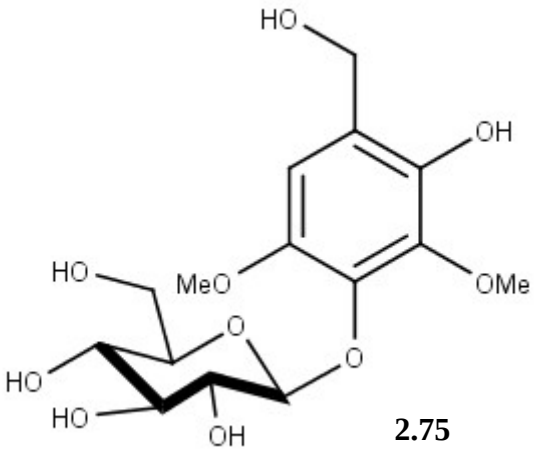

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CHAPTER 3

Chemical composition and Anti-inflammatory activities of the Essential oils from *Acacia mearnsii* De Wild

3.1 Introduction

Acacia mearnsii de Wild (Fabaceae) commonly referred to as ‘’black wattle’’ is a fast-growing leguminous tree that is native to Australia. It was introduced to South Africa about 150 years ago primarily for the tanning industry[1]. The tree grows to 5-20 m high, unarmed smooth bark with dark olive-green colour. The leaves are bi-pinnate and crowded and the flower is pale yellow or cream in colour while the fruit pod is more or less straight. In folklore medicine, it had been applied in the treatment of dysentery and inflammations in rural areas in South Africa [2]. The medicinal application of prothocyanidin’s from *A. mearnsii* includes anti-obesity, anti-diabetes [3], α-Amylase and lipase inhibitors and itching inhibition [4].The anti-inflammatory of most of the extracts reveals a high activity and potency even at very low concentrations [5–7].

Volatile terpenoids from *Acacia* species shows that the species consist of varying constituents of components; eugenol (oil of *A. caven*), methyl salicylate, eugenol (oil of *A. aroma*) [8], while (Z)-β-ocimene, is dominant in *A. cyclops* from South Africa [9]. The essential oils of *A. tortilis* are characterized by α-humulene, α-cadinol, nerolidol [10], eugenol (*A. praecox*) [11]. This chapter presents the chemical constituents of the volatiles of the leaves and stem (dry and fresh) of *A. mearnsii* and its anti-inflammatory activity in a male wistar rat paw edema which had not been formerly reported.

3.2 Experimental Section

3.2.1 Plant materials:

The leaves and stem bark of *A. mearnsii* were collected in the Walter Sisulu University, Mthatha, Eastern Cape, South Africa (31°36'08.35"S 28°45'02.48"E) botanical garden on the 5th of November, 2012 within the hours of 1400 h and 1800 h and was taxonomically identified at Selmar Schonland Herbarium, Grahamstown (GRA), South Africa by Mr. T. Dold; A voucher specimen (AOM001) of the collected sample was deposited in the herbarium for future reference.

3.2.2 Essential oil isolation:

The stem bark and leaves (fresh and air-dried) of the plant (300 g) were subjected to hydro-distillation for 3 h using Clevenger-type apparatus. The oil was collected over hexane and kept in a tight screw-capped bottle and kept till further analysis.

3.2.3 Gas Chromatography- Mass Spectrometry analysis:

3.2.3.1 GC-MS Analysis.

GC-MS analysis were performed with an Agilent 7890A gas chromatography equipped with a HP-5 capillary column (30 m x 250 µm; film thickness 0.25 µm) and a 5975C VL MSD (Mass Selective Detector) detector. EM Voltage: 1835.294, Gain Factor 1.01 (Actual/Resulting EMV: 2035.29) MS Source Temp.: 250 °C MS Quad. Temp.: 200 °C. Analytical conditions: injector temperatures were 250°C, oven temperature programmed from 45°C, hold up time was 5 min and the final temperature was 310°C at 4°C/min; carrier gas was hydrogen at a flow rate of 1 mL/min; injection of 0.5 µL; split ratio 1:30. Mass spectra were recorded at 70 eV. The acquisition scanning range was 30-300 m/z at a scan rate of 1 scan/s.

3.2.4 Identification of compounds

Essential oils components were ascertained by matching their spectra and retention index (Kovat Index) alongside those of the original/pure samples operated under the same condition and literature values [12–14].

3.2.5 Anti-inflammatory activity

3.2.5.1 Study Animals

Wistar male rats weighing 200–240 g were provided by South African Vaccine Producers (SAVP). The animals were housed in the Zoology Department of Walter Sisulu University animal holding facility of Walter Sisulu where they had free access to standard pellet food and water, acclimatized for two weeks before the experiments started. They were later placed randomly to one of 6(six) groups consisting of 5 wistar rats each as follows:

Group 1- control group (0.9% NaCl),

Group 2- Ibuprofen treated group (standard),

Group 3- treated with fresh leaves essential oils of *A. mearnsii,*

Group 4- treated with essential oils from *A. mearnsii* dry leaves

Group 5- treated with essential oils from fresh stem bark of *A. mearnsii* and

Group 6- treated with essential oils of the dry stem bark of *A. mearnsii.*

The bioassay were performed according to Walter Sisulu University Ethical Clearance Committee (No: 0009/07) ethics.

3.2.5.2 Drug

Ibuprofen (Sigma Aldrich, 2014).

3.2.5.3 Fresh egg albumin-induced edema

On day 1 of experimentation, baseline paw diameters (mm) of all rats were determined using a pair of digital calipers (Yato). Rats in the essential oils treatment groups received 1 ml of 2% essential oils as per assigned group. On day 2, rats were induced with 1 mL NaCl (1st group), 100 mg/kg ibuprofen (group 2) or 1 ml of 2% essential oils as per assigned group on day 1 of tests. 30 minutes later, 0.5 ml of 50% (v/v) of fresh egg albumin was injected subplantar on the right hind paw. Paw sizes were again measured at hourly interval till the 4th after drug treatment. Inflammation was determined as change in paw size = paw size at any given time – paw size at start of experiment[15,16].

3.2.5.4 Statistical analysis

Analysis of data was done with the aid of GrapPad Instat®. ANOVA with post hoc test was performed to establish the difference between treatment groups, control group and the standard drugs; difference were regarded to be statistically significant when $p < 0.05$. Results were expressed as the mean ± SEM.

3.3 Results and Discussion

3.3.1 Essential oils

Table 3-1 presents the volatile constituents from the fresh, dry leaves and stem bark of *Acacia mearnsii* and their percentages according to elution order on a DB-5 column. The oil yields were 0.25%, v/w, fresh leaves (**A**); 0.50%, v/w, dry leaves (**B**); 0.78%, v/w, fresh stem (**C**); and 0.11%, v/w, dry stem (**D**) of the pale yellow oils obtained. A total of 132 compounds in all were identified (Table S1): 20 components in **A** (**91.2%** of the total oil), 37 in **B** (**92.1%**

of the oil), 27 in **C (78.6%)** and 49 components for the dry stem bark (**94.3%)**, **D**. The spectra of the chromatogram are shown in **Appendix A.**

The amount of terpenoids present in different parts varied as we move from fresh parts to dry parts. High amount of monoterpenoids (55.45%) were present in the dry leaves. The main components were Verbenol (29.9%), β-cubebene (6.1%) and β-Carryophyllene (4.2%). Alcohol based compounds (53.2%) were predominant in the fresh leaves, showing stearyl alcohol or octadecyl alcohol (25.5%), phytol (10.5%) and 2-Hexenal (E) (8.60%) as the main components.

In addition, oils from the fresh stem bark reveals high percentage of alcohol based compounds accounting for 28.9% with phytol (23.4%), and 3-Hexen-1-ol (10.6%) being the major constituents. A considerable amount of monoterpenoids and sesquiterpene were confirmed in the dry stem oils. Cis-verbenol (10.0%), β-buorbonene (5.1%) and phytol (10.1%) were some of the major components. Same constituents were observed for the oils of the dry stem bark with and phytol (10.1%) accounting for the major content of the oil.

2-Hexenal (E), which is a common leaf aldehyde [9], occurs as a characteristic constituent in all the parts of the plants analyzed. The occurrence of sesquiterpenoids and monoterpenoids in the oils of *A. mearnsii* is of keen interest, as observed in the oils of *A. albida*, *A. nilotica* and *A. nuperimma* which are majorly monoterpenoids and sesquiterpenoids (52.2%) [10,11,17]. The constituents of both the fresh and dry stem bark are similar except in the quantity. Furthermore, the carbonyl and hydrocarbons in the fresh leaves and the dried stem occurs moderately, while the other major constituents; phytol, 2-hexenal (E) and 3-hexen-1-ol had also been reported in the oils of *A. howitti* (phytol= 1.5%) [18] . In this report, hydrocarbons constituents accounted for 12.37%, 12.28% and 14.21% from the oils of the fresh leaves and stem (fresh and dry) respectively, which resembles the oil of *A. praecox* (hydrocarbons about 20 %) [11] and *A. Cyclops* [9].

Table 3-1: The essential oils constituents of the Fresh leaves (**A**), Dry leaves (**B**) and fresh stem bark (**C**), dry stem bark(**D**) of *Acacia mearnsii* in series of elution from DB-5 column

RI[a]	RI[b]	Compounds	A	B	C	D
Monoterpenes						
988	991	Myrcene	-	4.9	-	-
1002	1001	α-Phellandrene	-	3.9	-	1.3
1020	1021	ρ-Cymene	1.5	1.8	1.2	1.5
1022	1023	o-Cymene	-	0.7	-	1.1
1025	1033	β-Phellandrene	-	1.9	-	-
			1.50	**12.67**	**1.20**	**2.32**
Monoterpenoids						
1096	1101	Linalool	-	4.0	0.7	0.3
1139	1141	(S)-cis-Verbeneol	-	29.9	-	10.0
1165	1168	Borneol	-	0.1	-	0.1
1189	1195	α-Terpineol	-	0.3	-	1.3
1205	1204	Verbenone	-	7.9	-	0.1
1249	1282	Piperitone	-	0.3	-	2.0
1356	1356	Eugenol	-	0.3	-	1.1
				42.78	**0.73**	**14.76**
Sesquiterpenes						
1376	1378	α-Copaene	-	0.3	-	0.1
1387	1384	β-Bourbonene	-	0.2	-	5.1
1388	1390	β-Cubebene	-	6.1	-	3.2
1418	1428	β-Caryophyllene	-	4.2	-	0.1
1441	1449	Aromandedrene	-	0.3	-	-
1478	1479	γ-muurolene	-	0.3	-	-
1483	1485	α-Amorphene	-	1.1	-	-
1498	1492	γ-Elemene	-	0.5	-	-
1500	1501	α-muurolene	-	0.2	-	-
1523	1526	δ-cadinene	-	0.4	-	4.2
				13.43	**-**	**12.69**
Sesquiterpenoids						
1561	1572	Nerolidol (cis)	-	0.1	-	1.1
1578	1574	Spathunelol	-	0.4	-	-
1582	1581	Carryophyllene oxide	-	0.9	-	0.1
1626	1645	τ-Cadinol	-	0.1	-	0.1
1649	1649	β-Eudesmol	-	3.8	-	-
1685	1686	α-Bisabolol	-	3.4	-	-
1690	1693	Bergamotol, - (Z)-α-trans	0.10	-	-	-
1742	1741	E,Z-Farnesol	-	1.4	-	2.3
1748	1746	α-Bisabolol oxide	-	2.9	-	-
			0.10	**13.03**	**-**	**3.54**
Hydrocarbons						
510	500	α-Pentene	-	0.3	-	0.1
600	600	Hexane	0.5	-	0.3	0.3
700	700	Heptane	0.7	-	0.5	0.1
800	800	Octane	-	-	0.4	-

883	883	1,2,3-trimethyl	-	0.3	-	0.1
900	900	Nonane	0.5	-	0.5	0.5
897	897	1-ethyl-1,4-cyclohexane	-	0.7	-	-
1700	1700	Heptadecane	-	-	0.7	-
1800	1800	Octadecane	-	-	1.3	-
2000	2000	Eicosane	3.9	-	3.2	1.1
2100	2100	Heneicosane	-	-	1.7	6.6
2300	2300	Tricosane	0.8	-	-	1.0
2400	2400	Tetracosane	4.8	-	1.1	-
2800	2800	Octacosane	1.7	-	1.0	0.1
2900	2900	Nonacosane	-	-	1.2	1.1
4400	4400	Tetratetracosane	0.9	-	2.1	3.5
			13.51	**1.32**	**14.05**	**14.36**
Carbonyl Compounds						
846	862	2-Hexenal (E)	2.5	-	3.0	1.1
964	964	2-Heptenal, (Z)	-	-	0.5	1.0
984	992	2-Pentyl furan	-	-	0.4	1.5
1004	1008	3-Hexenyl Acetate (Z)	6.7	-	3.0	1.2
1100	1104	Nonanal	-	-	7.2	5.2
1157	1162	(E)-2- Nonenal	8.2	-	-	8.6
1201	1205	Decanal	4.2	-	1.7	-
1235	1245	Cis-3-Hexenyl	1.3	-	1.7	-
1565	1570	3-Hexenyl benzoate	-	-	0.5	-
			22.97		**17.98**	**18.65**
Alcohol based Compounds						
861	862	2-Hexen-1-ol (E)	-	-	3.5	2.5
851	866	3-Hexen-1-ol	8.6	-	10.6	2.0
869	868	1-Hexanol	8.6	-	6.5	1.2
977	978	1-Octen-3-ol	-	0.2	-	1.3
1070	1087	1-Octanol	-	0.2	0.6	0.5
1712	1727	5-Azulenemethanol	-	6.0	-	1.1
2232	2080	Stearyl alcohol	25.5	-	-	8.1
2138	2122	Phytol	10.5	-	23.4	10.1
			53.2	**6.35**	**44.6**	**26.7**
Unknown						
	1574		-	2.08	-	-
			91.24	**92.1**	**78.56**	**94.31**

–: Not detected; RI[a] Retention indices on HP-5MS capillary column; Literature retention indices, ESO 2000 and its lower versions). RI[b]: Kovats Index in DB-5 column

3.3.2 Anti-inflammatory properties of *A. mearnsii*

The anti-inflammatory effects of the essential oils of *A. mearnsii* which was established by the difference in paw size using albumin as the phlogistic agent. As illustrated in Figure 1, albumin induced inflammation was highest 1 hour after treatment. Like ibuprofen, all the essential oils inhibited albumin induced inflammation significantly (0.01) from 1-3 h after administration of drugs. Beyond 3 h, only oils from the fresh leaves and stem bark of *Acacia mearnsii* showed significant ($p < 0.01$) anti-inflammatory effects.

3.3.2.1 Anti-inflammatory: Albumin induced

The ability of plant metabolites to inhibit inflammation has been widely reported [19,20]. Basically, the inflammatory process involves a series of events that can be triggered by numerous stimuli such as histamine, leukotrienes and prostaglandins. *Acacia* species

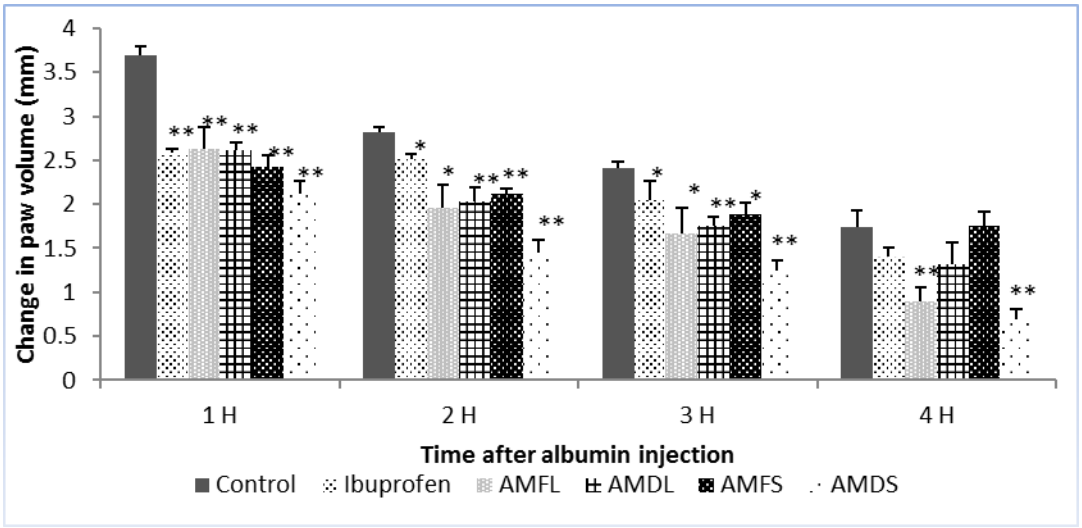


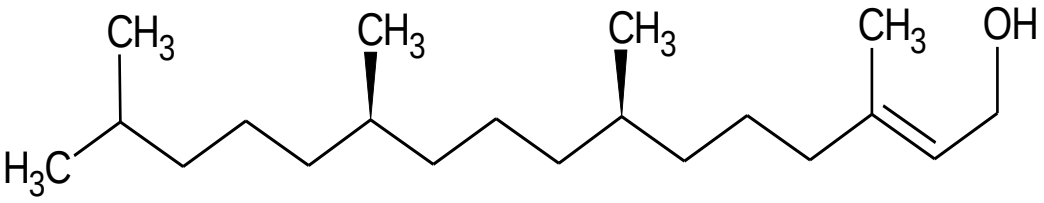
Figure 3-1: Anti-inflammatory effects of the essential oil of *Acacia mearnsii* (AM): AMFL = essential oils from fresh leaves; AMDL = essential oils from dry leaves; AMFS = essential oils from fresh stem; AMDS = essential oils from dry stems. Each bar signifies mean ± S.E.M for 5 rats. *=$p < 0.05$, **=$p < 0.01$, compared to control.

such as *A. modesta*, *A. farnesiana*, *A. nilotica* and *A. karroo* have been shown to have anti-inflammatory properties [5,21,22]. The presence of large amounts of phytol (**3.1**) and cis-

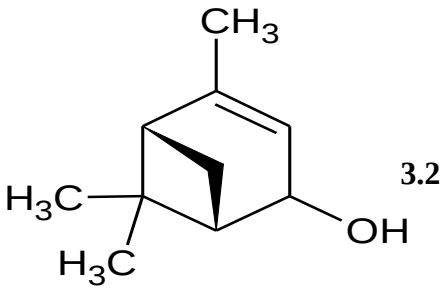
verbeneol (**3.2**) in most of the essential oils might be the factor responsible for the anti-inflammatory activity. These terpenes have strong anti-inflammatory effect by inhibiting the COX1 and the leukotriene[23]. Edema growth is a tri-phasic process – the liberation of histamine and serotonin in the first phase (0–2 h), cytokines, at the 2nd phase (3rd h) and prostaglandin in the 3rd phase (>4 h) [24]. The anti-inflammatory effects of *A. mearnsii* show high potency from the 1st to 4th h. Thus, it inhibits at all stages of edema development.

Essential oils from different parts of *A. mearnsii* show varying inhibition at different hours of analysis. Essential oils from the fresh leaves and dry stems showed greatest inhibition and which was of longer duration. The essential oils of *A. mearnsii* may therefore possess compounds inhibiting at different phases of inflammation thus making it a potent anti-inflammatory agent as shown our results.

3.4 Structural presentation of major compounds



3.1



3.2

3.5 Conclusion

This report shows the effect of drying on the chemical composition, quantity and anti-inflammatory activity of the essential oils of *A. mearnsii*. The composition and quantity of the oils increases in moving from fresh plant parts to dry parts and this also shows in the anti-inflammatory studies. The high contents of monoterpenoids and sesquiterpenoids observed in the dry parts could had been the major reason for their outstanding anti-inflammation activity. This marked difference may be due to the effect of moisture on quality, quantity and activity. Although the anti-inflammatory mechanism is not yet understood, the activity of essential oils may be influenced by drying. The marked differences in the quantity and components in the oils may be attributed to large tannins that are present in the plant and its geographical distribution.

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CHAPTER 4

Volatile components of leaves and Stem bark of *Acacia karroo* Hayne and their Anti-Inflammatory Properties

4.1 Introduction

The genus *Acacia* comprises about 1200 species[1,2] indigenous to humid climate and subtropical grasslands, mostly in Australia and southern Africa[3]. It belongs to the Mimosaceae of the Fabaceae (Leguminosae) family. *A. karroo* can thrive in virtually tough regions, including the deserts (a height of 3-4 metres, with big thorns and high content of leave tannins) and coastal woodlands (40 m tall, with small thorns, low tannin content with low chemical defence mechanisms)[4]. It is widely distributed in all the nine provinces in South Africa[5]. *Acacia karroo* can be a short shrub or grow to a tall tree with rounded top with minimal branches on the trunk. The colour changes from red to dark-brown as the age increases. The leaves are small in size and easily falls off during the winter season. The yellow plume nature of the flowers makes it an ornamental plant and emerge in early part of summer [6].

Ethno pharmacological applications of *A. karroo* reveals its application as a remedy for malaria, stomach ailments and boiled in water to treat diarrhoea in goats[7,8] as a feed forage for herbivores [9]; its gum has been used to treat mouth ulcers and applied medically as emulsifiers, stabilizers of suspensions and additives for solid formulations[5,10]. An herbal mixture of some species of *Acacia* is dispensed as a cure for malaria, stomach ailments and pneumonia[11]. Its use as analgesic and anti-inflammatory potential had also been investigated [12].

A. karroo had been reported to be a good source of flavonoids and prothocyanidins which had been shown to exhibit membrane-stabilizing activities and enhances metabolic intermediate pathway processes[10,13]. Report had shown that *A. karroo* from Southern Africa contain high proportions of prothocyanidins[4]. The volatile constituents from other *Acacia* species had been reported showing variation in the quality and quantity of its component. α-humulene (12.0%) and α-cadinol (10.6%), are dominant in (oil of *A. tortilis*) [14], menthol (34.90%) and limonene (15.3 %) (*A. nilotica*) [15], ρ-anisaldehyde (14.4%) and eugenol (11.2%) (*A. caven*), methyl salicylate (42.0%) and eugenol (15.5%) (*A. aroma*) [16]. Furthermore, linalool (13.5%) and eugenol (10.5%) (*A. praecox*) [17], terpine-4-ol (*A. farnesiana*) [18]. Hex-1-en-3-one (7.5%) and oct-1-en-3-one (44.5%) were the main constituents in the oils of *A. howittii* [19], while *A. nuperrima* had been shown to be abundant in Kessane sesquiterpenoids and monoterpenoids, α-pinene [20]. This represents the first literature discussion on the volatile constituents of *A. karroo* and its anti-inflammatory properties.

4.2 Experimental Section

4.2.1 Plant Collection and identification:

A. karroo leaves and stems bark were collected in Walter Sisulu University, Mthatha, Eastern Cape, South Africa (31º36'08.35"S 28º45'02.48"E) botanical garden on the 5th of November, 2012 within the hours of 1400 h and 1800 h and was taxonomically identified at Selmar Schonland Herbarium, Grahamstown (GRA), South Africa by Mr. T. Dold; Voucher sample was deposited and Voucher number (AOK001) was collected at the herbarium.

4.2.2 Essential oil isolation:

The fresh and dried (leaves and stem bark) of the plant (300 g) were hydro-distilled with Clevenger-type apparatus for 3 h. Extracted essential oil with pale yellow colour was kept in a tight screw-capped bottle and kept till further analysis.

4.2.3 Gas Chromatography-Mass Spectrometry Analysis.

GC-MS was operated on an Agilent 7890A gas chromatography equipped with a HP-5 capillary column (30 m x 250 μm; film thickness 0.25 μm) and a 5975C VL MSD (Mass Selective Detector) detector. EM Voltage: 1835.294, Gain Factor 1.01 (Actual/Resulting EMV: 2035.29) MS Source Temp.: 250 °C MS Quad. Temp.: 200 °C. Analytical conditions: injector temperatures were 250^{o}C, oven temperature programmed from 45^{o}C, hold up time was 5 min and the final temperature was 310^{o}C at 4^{o}C/min; carrier gas was hydrogen at a flow rate of 1 mL/min; injection of 0.5 μL; split ratio 1:30. Mass spectra were recorded at 70 eV. The acquisition scanning range was 30-300 m/z at a scan rate of 1 scan/s.

4.2.4 Identification of compounds

The essential oils components were identified on the basis of comparism of their spectra and retention indices (Kovat Index) alongside those of pure samples and literature values [21–23]

4.2.5 Anti-inflammatory activity

4.2.5.1 Study Animals

200-240 g Male Wistar rats was provided by South African Vaccine Producers (SAVP). The rats were accommodated in the Zoology Department animal facility of Walter Sisulu University, Mthatha. The animals were kept in a metal steel cages, where they had unrestricted supply to water and standard pellet food. They were acclimatized for two weeks before commencement of experiment. The animals were assigned at random to a group of 6 consisting of 5 animals each as detailed below:

Group 1- control group (0.9% NaCl),

Group 2- Ibuprofen treated group (standard),

Group 3- treated with fresh leaves oil of *A. karroo*,

Group 4- treated with dry leaves oil of *A. karroo,*

Group 5- treated with fresh stem bark essential oil of *A. karroo* and

Group 6- treated with dry stem bark of oil *A. karroo.*

The bioassay were performed according to Walter Sisulu University Ethical Clearance Committee (No: 0009/07) conditions.

4.2.5.2 Drug

Ibuprofen (Sigma Aldrich, 2014).

4.2.5.3 Evaluation of Anti-inflammatory activity

4.2.5.4 Fresh egg albumin-induced edema

Modified method for rat paw edema as reported by Xiao-Shun et al.[24] was used. Baseline paw diameters were measured on day 1 of experimentation, using a pair of digital calipers (Yato). Rats in the essential oils treatment groups received 1 ml of 2% essential oils orally as per assigned group. On day 2, animals were fed with 1 ml NaCl (1st group), 100 mg/kg ibuprofen (group 2) or 1 ml of 2% essential oils as per assigned group on day 1 of tests. 30 minutes later, 0.5 mL, 50% (^{v}/v) of fresh egg albumin (phlogistic agent) was injected subplantar to the right hind paw of animals. Paw sizes were again measured hourly till the 4th hour after drug (essential oils) administration.

Inflammation was determined as change in paw size = paw size at any given time – paw size at start of experiment[25,26] and the percentage inhibition were deduced from the formula,

Percentage inhibition of edema= [(mean of control-mean of test) / mean of control] x 100.

4.2.5.5 Statistical analysis

Analysis of data was done with the aid of GrapPad Instat®. ANOVA with post hoc test was performed to establish the difference between treatment groups, control group and the standard drugs; difference were regarded to be statistically significant when $p < 0.05$. Results were expressed as the mean ± SEM.

4.3 Results and Discussion

4.3.1 Essential Oils Components of *A. karroo*

Pale yellowish oils obtained by hydro-distillation yield 0.24% (v/w), 0.55% (v/w) and 0.35% (v/w), 0.05% (v/w) of leaves (fresh and dry) and stem bark (fresh and dry) of *A. karroo* respectively, were characterized chemically using GC-MS. Table 4-1 shows the oil components, their amounts, Kovats index and reference indices. The spectra of the chromatogram are shown in **Appendix B.**

147 compounds in all were identified, 36 for dry leaves, accounting for 97.01% of the total oil and 16 for fresh leaves accounting for 86.10% while 30 for 90.38% (fresh stem bark), and 65 components for the dry stem bark accounting for 97.76%.

The oil of the fresh leaves is characterized largely by β-pinene (14.30%) and (Z)-2-Hexen-1-ol (10.21%). The dry leaves are made of Hexanal (10.67%), ß-ionone (9.74%), phytone (7.44%), tetracosane (6.92%), α-cadinol (5.96%) and nonanal (4.07%). In addition, the fresh stem bark gives array of constituents with phytol (23.38%), 2-Hexenal-(E) (10.56%) and nonanal (7.19%) occurring in high percentage. Octacosane (10.59%) and geranyl acetone (5.33%) are the dominant constituents of the dry stem bark of *A. karroo*.

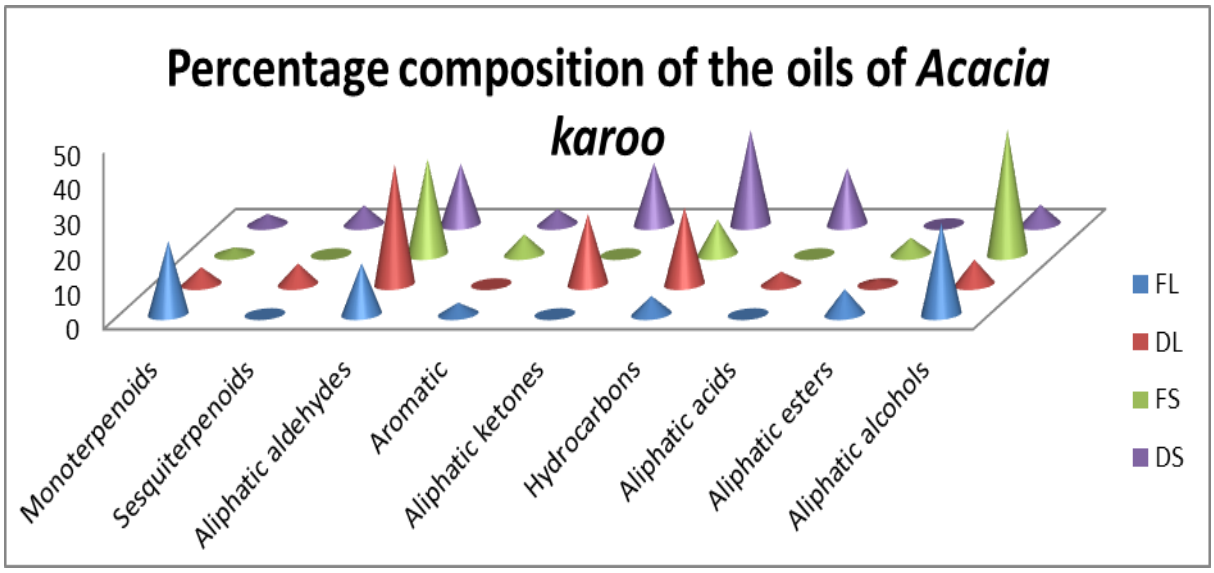


Figure 4-1: Graphical representation of the essential oils components of *A. karroo*

Reports obtained from this analysis, show that as the plants parts are dried, the constituent increases and resembles each other as observed for the dry samples (leaves and stem bark). However, the hydrocarbons components in the dry leaves and stem bark of *A. karroo* is similar to those of *A. praecox* with hydrocarbons percentage of >20 [17] and of *A. leucophlae* [27]. β-pinene which is the major component from the fresh leaves of *A. karroo* had also been reported in the oils of *A. albida* [15]. The significant amounts of α-cadinol (5.96%) present in the dry leaves of *A. karroo* is in accordance with the oils of *A. tortilis* [14]. The presence of eugenol in the dry leaves and stem bark shows similarities with the oil from *A. aroma*, *A. praecox*, *A. caven* and *A. tortilis* previously reported.

The constituent from the fresh and dry leaves differs considerably in yield and composition. Difference in generic, climate, time of collection, age of plant could have been the major reasons responsible for the marked alterations in the yield, and the oils constituents[28].

Table 4-1: Volatiles identified in the leaves (fresh and dry) and Stem (fresh and dry) of *Acacia karroo*

Compound	KI[a]	KI[b]	Fresh leaves	Dry leaves	Fresh Stem bark	Dry Stem bark
	Monoterpenoids					
β-pinene	930	**974**	**14.30**	-	-	-
δ-3-carene	1012	1008	-	1.68	-	-
(Z)-β-Ocimene	*1032*	1044	6.61	-	-	-
(E)-β-Ocimene	1045	1047	-	-	1.20	-
Linalool	1100	1095	-	-	0.73	0.25
Isophrone	1117	1119	-	-	-	0.51
Safranal	1201	1196	-	3.92	-	0.24
β-cyclocitral	1218	1217	-	2.41	-	1.31
β-homocyclocitral	1254	1256	-	-	-	0.38
Eugenol	1365	1356	-	0.45	-	0.24
			20.91	**8.46**	**1.93**	**2.93**
Sesquiterpenoids						
Valencene	1491	1496	-	-	-	0.37
α-bulnesene	1505	1509	-	-	-	3.76
(-)-Spathunelol	1577	1577	-	-	-	0.45
α-Cadinol	1654	1652	-	5.96	-	0.45
Bulnesol	1672	1670	-	-	-	0.80
			-	**5.96**	-	**5.38**
Aliphatic aldehydes						
Hexanal	801	801	5.99	**10.67**	3.55	4.55
2-Hexenal (E)-	863	846	5.07	3.36	**10.56**	0.19
Heptanal	902	901	-	0.88	0.54	1.11
2-Heptenal, (E)-	964	964	-	1.02	-	-
2-pentylfuran	995	984	5.53	3.84	0.36	0.16
trans-2-(2-Pentenyl) furan	1003	1003	-	1.60	-	-
Octanal	1006	1004	-	-	0.39	1.16
2-Heptenal, (Z)	1041	1011	-	1.02	0.51	-
(2-Octenal, (E)-	1056	1056	-	-	-	0.44
Nonanal	1102	1100	-	4.07	7.19	4.53
(2E,6Z) -Nonadienal	1154	1150	6.84	0.52	-	-
2E-Nonenal	1155	1157	-	-	-	1.29
Decanal	1207	1201	-	-	3.68	0.95
2(E)-Decenal	1261	1260	-	-	-	0.53
(2E, 4E)-Decadienal	1314	1315	-	-	-	0.94
2E-Undecenal	1365	1357	-	-	-	0.36
Pentadecanal	1711	1713	-	0.90	-	-

9,17-Octadecadienal, (Z)-	1997	1999	-	-	-	0.28
Octadecanal	2021	2077	-	-	-	0.36
			14.59	**34.20**	**27.30**	**17.30**
	Aromatic Compounds					
Toluene	773	775	-	-	0.28	0.21
Benzaldehyde	961	952	-	-	-	0.23
Benzene, 1,4-bis(1-methyletheyl)	1168	1170	-	-	-	0.24
Cis-3-hexenyl benzoate	1570	1565	3.34	-	-	-
Heptyl phthalate	2497	2500	-	-	-	3.61
Fleximel	2507	2510	-	-	5.39	-
Diisobutylphthalate	2526	2527	-	-	-	0.27
			3.34	**-**	**5.67**	**4.32**
Aliphatic ketones						
3-Penten-2-one, (E)-	844	846	-	-	-	0.23
2-Heptanone	895	889	-	-	-	0.12
Sulcatone	981	986	-	0.73	-	1.41
3,5-Octadien-2-one	985	1000	-	1.38	-	-
2,2,6 trimethylcyclohexane	1036	1038	-	-	-	0.37
Octan-3,5-dione	1094	1098	-	0.66	-	0.53
Carvenone	1252	1255	-	0.75	-	-
α-ionone (E)	1426	1428	-	-	-	3.57
Geranyl acetone	1453	1453	-	-	-	5.33
β-ionone (E)	1485	1487	-	9.74	-	3.52
Phytone	1849	1851	-	7.44	-	0.28
5E,9E-farnesyl acetone	1919	1913	-	-	-	1.51
Isopropyl hexadecanoate	2027	2024	-	-	-	0.29
			-	**20.07**	-	**17.52**
	Hydrocarbons					
Nonane	900	900	-	0.69	0.54	-
Hexadecane	1600	1600	-	-	-	0.26
Heptadecane	1700	1700	3.20	3.29	0.67	2.10
Octadecane	1800	1800	-	0.55	1.30	-
Eicosane	2000	2000	-	-	3.15	4.17
Heneicosane	2100	2100	2.02	3.02	0.98	-
Docosane	2200	2200	-	1.33	-	0.44
Tricosane	2300	2300	-	1.32	-	1.07
Tetracosane	2400	2400	-	6.92	1.13	1.93
11-butyldocosane	2462	2467	-	-	-	0.52
Octacosane	2800	2800	-	1.85	1.04	10.59
Nonacosane	2900	2900	-	-	1.15	1.08
Triacontane	3000	3000	-	-	-	2.71

Hentriacontane	3100	3100	-	-	-	1.98
Tritetracontane	4300	4300	-	3.07	-	-
Tetratetracontane	4400	4400	-	-	2.12	-
			5.22	**22.04**	**9.91**	**26.85**
	Aliphatic acids					
n-Decanoic acid	1373	1364	-	-	-	0.61
Lauric acid	1568	1570	-	-	-	2.56
Myristic acid	1768	1800	-	-	-	4.22
Pentadecanoic acid	1866	1868	-	-	-	0.34
Palmitic acid	1984	1988	-	3.59	-	7.72
Linoleic acid	2173	2132	-	-	-	0.59
			-	**3.59**	**-**	**16.04**
	Aliphatic esters					
(Z)-3-hexenyl-2-methylbutanoate	1191	1194	4.05	-	-	-
Geranyl acetate	1453	1379	-	-	-	-
3-hexenyl acetate, (Z)-	1509	1004	-	1.37	-	-
Cis-3-hexenyl isovalerate	1570	1243	-	-	2.97	-
Terpinyl pentanoate	1626	1628	3.14	-	1.71	-
			7.19	**1.37**	**4.68**	**-**
	Aliphatic alcohols					
n-Hexanol	851	863	7.49	-	2.51	0.44
3-Hexen-1-ol	858	844	4.66	-	6.15	-
2-Hexen-1-ol	861	854	-	-	3.01	-
(Z)-2-Hexen-1-ol	880	**859**	**10.21**	-	-	-
2-Cyclohexen-1-ol, 2,4,4-trimethyl	896	900	-	-	-	0.53
1-Octen-3-ol	979	974	-	2.76	-	-
Bicyclo [3.1.0] hexan-2-ol	1051	1053	-	1.26	-	0.27
Capyrilic alcohol	1071	1075	-	-	0.60	-
(E)-2-Octen-1-ol	1104	1060	3.65	1.72	-	0.74
Cis-11-Tetradecen-1-ol	1678	1701	-	-	-	-
Octadecyl alcohol	2080	2082	-	-	3.34	3.63
			26.01	5.44	12.27	5.61
Diterpenoids						
Phytol	2128	1942	-	1.57	**23.38**	-
Total			**86.10**	**97.01**	**90.38**	**97.76**

[a] = Compounds arranged according to elution from a DB-5 column;
- = not detected
KI[a] = Programmed temperature retention indices determined on apolar DB-5 column,
KI[b] - Retention indices from literature references.

4.3.2 Anti-inflammatory properties of *A. karroo*

The anti-inflammatory effects of the essential oils of *A. karroo* which was established by the difference in paw size using albumin as the phlogistic agent is summarized in Fig. 4-2.The essential oils of *A. karroo* from all parts at a 2% dose considerably inhibited the inflammation ($p< 0.01$) for 1st h after treatment. As shown in the figure, the rate of inhibition reduces significantly for most of the extract as the reaction time increases. The essential oils of the fresh leaves (AKFL) only shows significant inhibition of the order ($p< 0.01$) and ($p< 0.05$) for the 2nd and 3rd h respectively.

4.3.2.1 Anti-inflammatory: Albumin induced

Acacia karroo is a traditional herbal concoction for treatment of ulcers, malaria, inflammation and other pain related conditions in the Eastern Cape province of South Africa. The significant ability of plant metabolites to inhibit inflammation are due to the synergistic role they play in the multiple steps of the anti-inflammatory cascade compared to a single

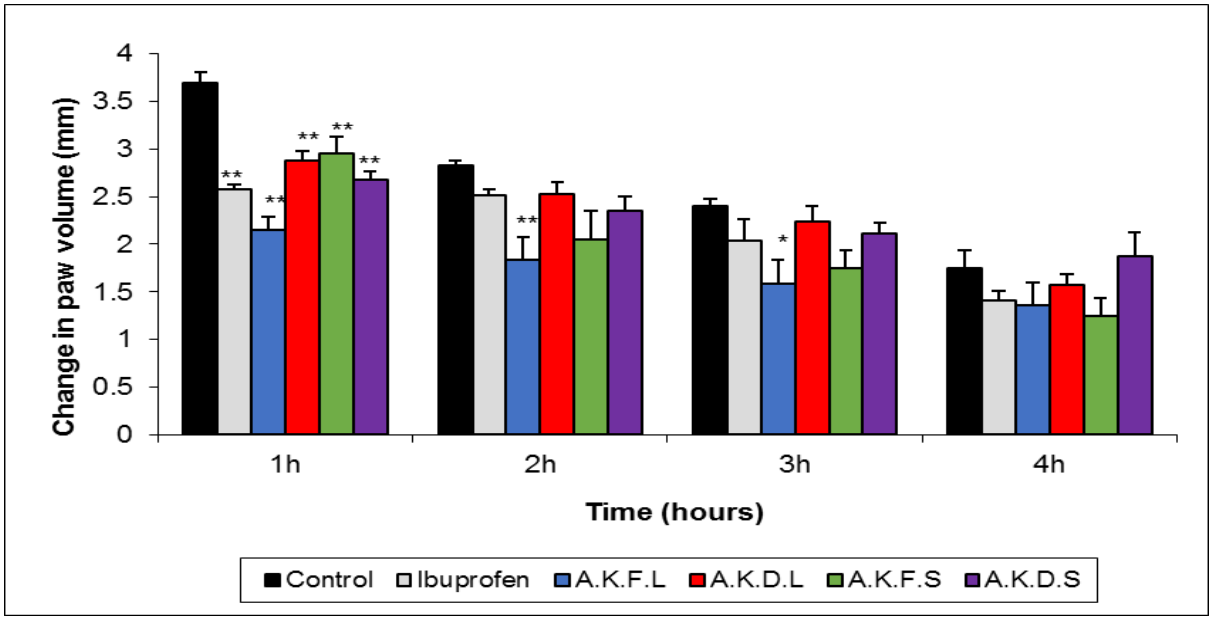


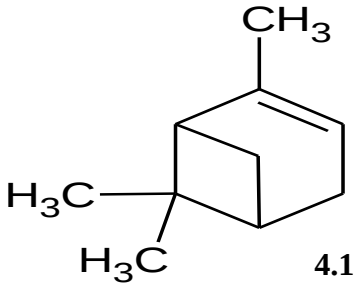
Figure 4-2: Anti-inflammatory effects of the essential oil of *Acacia karroo* (AK): AKFL = essential oils from fresh leaves; AKDL = essential oils from dry leaves; AKFS = essential

oils from fresh stem; AKDS = essential oils from dry stems. Each bar signifies mean ± S.E.M for 5 rats. *=p <0.05, **=p <0.01, compared to control.

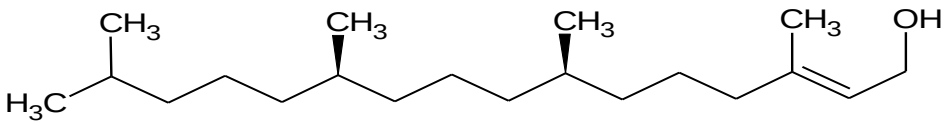
step of a synthesized drug[29]. For instance, some essential oils had been reported to act as 5-lipoxygenase, leukotriene, NO production, COX-I and COX-II enzymes inhibition [30–32]. However, the actual release of the mediators are time-dependent in an animal model; Edema growth is a tri-phasic process – the liberation of histamine and serotonin in the first phase (0–2 h), cytokines, at the 2nd phase (3rd h) and prostaglandin in the 3rd phase (>4 h) [33].

The presence of some major constituents in these essential oils could have been responsible for synergistic anti-inflammatory effect. As seen from Fig. 4-2; the inhibition of the release of histamine and serotonin are observed for all the essential oils at a significance level of ($p<0.01$). However, suppression of the cytokines is only observed for the *Acacia karroo* fresh leaves (AKFL).

The effect of β-pinene (**4.1**) and phytol (**4.2**), in this oil had also been reported in other essential oils to exhibit high anti-inflammatory inhibitors[34]. Avoseh et al. observed that the presence of phytol and cis-Verbenol constituents in the oil of *A. mearnsii* led to a high anti-inflammatory effect in Swiss rat to the order of ($p<0.01$) for the entire duration of analysis[35]. Significant differences exists in the action of different plant parts essential oils of *A. karroo.* The actions of the fresh leaves lasted longer with significant inhibition up to the 3rd, observed as well for the dry stem. The anti-inflammatory behavior of the extracts acts as a research based proof of the local use of *A. karroo* for anti-inflammation purposes.



4.1



4.2

4.3.3 Conclusion

Volatile terpenoids from *Acacia karroo* Hayne isolated reveals a low content essential oils affected by drying. The essential oils is composed of different class of components (monoterpenoids, sesquiterpenoids, alcohols, carbonyls, etc) which are strong pharmacological metabolites responsible for diverse healing effect. The anti-inflammatory ability of the essential oils suggests that it is a rapid metabolic absorbing components showing activity at an early stage of reaction. However, future work will involve understanding the mechanism of metabolism and isolation of the main component responsible for this activity.

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CHAPTER 5

Biosynthesis of Silver nanoparticles from *Acacia mearnsii* De wild stem bark and its Analgesic and Inflammatory properties.

5.1 Introduction

Nanotechnology has become an interdisciplinary field of research that is growing rapidly and becoming integrated into other areas of science and technology[1]. This technology involves the study of the physical properties of materials with a dimension ≤ 0.1 μm or 100 nm. It also involves scientific control of matter at the molecular level [2,3].

Nanoparticles are extremely important because of their exceptionally small size and large surface area to volume ratio known as quantum confinement. This determines their mechanical, physical, chemical, electrical, optical, solubility and stability properties[4,5]. Extensive plasmon excitation, large surface energy and specific electron structure brought about by the effective transition between the molecular and metallic states are some reasons for the application of this material for sensors devices, water purification, drug delivery, biomedical application, cosmetics, processed food, electronics, optics, energy production, and metrology[6–9]. Several drug-loaded nanoparticles are currently at the last stage of clinical trial/ administration,; they include liposomal amphotericin B for treatment of fungal infections, Liposomal verteporfin and micellular estradiol[10] for age related diseases and menopausal therapy respectively just to mention a few

Among the various metal nanoparticle (NPs) like copper, titanium, zinc, iron and gold, silver nanoparticles (Ag-NPs) have become desirable as a result of its outstanding surface plasmonic resonance (SPR) and its potent biological activities such as antimicrobial, antibacterial, anti-inflammatory, anticancer, antitumor and antimalarial[11–16]. Research had

shown that, the smaller the sizes of the silver nanoparticles, the higher the inhibition potential. This high inhibition potential has been attributed to the large surface area of the smaller particle [17–19]; Morones *et al*., in their report showed that the bactericidal activity of Ag-NPs were size dependent. Ag-NPs with diameter between 1-10 nm showed high activity against gram negative bacteria than those with larger sizes [20].

Several methodology have been employed for synthesising nanoparticles; these include reverse micelles, chemical reduction, electrochemical, sonoelectrochemical and laser ablation methods among others. Chemical reduction offers a simple method of synthesis which is achieved by reduction of metal ion salt solution. This simple method employs several organic reagents such as sodium borohydride ($NaBH_4$), hexadecylamine (HDA), tri-n-octylphosphine (TOP), ethylene glycol, poly(vinyl pyrrolidine) (PVP) as the reaction solvent, reducing agent and capping agent[21–25]. However, these reagents/solvents are toxic, expensive, and environmentally unfriendly and produce bye-products that are toxic. To circumvent this effect, greener methods involving the use of plants, micro-organisms and marine organisms have been reported.

This method had proven to be effective, simple, inexpensive, and environmentally friendly and yields stable size-controlled silver nanoparticles. The ability of plant and micro-organisms to function in phytomining and phytoremediation of heavy metals further enhances their potential for the biosynthesis of Ag-NPs[2]. It has been reported that certain magnetostatic bacteria can synthesize magnetic nanoparticles[26]. Gardea-Torresdey J. L. *et al*; reported the first reduction of metallic solution using plant. In their work, Au and Ag nanoparticles with particle size between 6-10 nm and with face centered cubic (fcc) crystalline structure were produced.[27,28]. In another development, Huang *et al*., successfully prepared triangular and spherical silver nanoparticles from the biomass of *Cinnamomum camphora* at ambient temperature by controlling the amount of biomass. Thus, biosynthesis of nanoparticle using

plant has been used in controlling the monodispersity in terms of size and shape [29]. In addition to acting as reducing agent, plant materials also act as capping agent for stabilisation of Ag-NPs. The latex, *Jatropa curcas* has also been exploited for synthesising Ag-NPs. The stability of the material was as a result of cyclic peptides present in the latex [30]. Palanivel et al., reported the synthesis of 10-20 nm Ag-NPs from the root extract of *Zingiber officinale*. The as-synthesised Ag-NPs showed high antibacterial efficacy against *Staphylococcus spp.* and *Listeria spp*[31]. Silver nanoparticle of 5.7 ± 0.2 nm in size synthesized from *Anogeissus latifolia* were reported to exhibit high antibacterial activity against gram $^{+}$/- bacteria[31]. *Acacia leucophlae,* a specie from the *Acacia* family had also been reported to produce sphere-shaped Ag-NP with an average diameter of 17-29 nm and exhibits high antibacterial activity[32] . Velmurugan *et al*., reported the synthesis of Ag-NPs using *bacillus subtilis* EWP-46 cell, a micro-organism extract acting as reducing and capping agents. The particles size were within 10-20 nm with minimum inhibition concentration of 129 ± 10.8 µg/ml and 116 ± 12.6 µg/ml when tested against *P. fluorescens* and *S. aureus* respectively[33].

Acacia mearnsii De Wild is a genus of Mimosoideae family, commonly known as black wattle, blue passionflower, green wattle and tan wattle. It originates from the Australia and had been spread to other continents including the America, Asia and Africa. The economic importance of *A. mearnsii* as source of tannins led to its cultivation in South Africa [34]. The therapeutic use of prothocyanidin's from *A. mearnsii* are anti-obesity, anti-diabetes [35], α-Amylase and lipase inhibitors and itching control [36].The anti-inflammatory study discloses that their extracts are active at considerable concentrations [37–39]. The phytochemicals analysis of the plant shows a high content of tannins as reported by Kusano R. *et al*., who had isolated some proanthocyanidins oligomers such as 5-deoxyflavan-3-ols, 4'-O-methylrobinetididol 3'-O-β-D-glucopyranoside, syringic acid, butin, gallocatechin and catechin from the plant [40]. The essential oils from this plant were shown to contain majorly alcohol based compounds of

phytol and cis-verbeneol, with high anti-inflammatory activity [41]. In this report, we explore the synthesis of Ag-NPs using essential oils hydrosols of the *Acacia mearnsii*, as both reducing and capping agents. Furthermore, the sequential evolution of extract volume and precursor concentrations on the optical and morphological properties was investigated.

5.2 Experimental section

5.2.1 Plant material:

The stem bark of *A. mearnsii* (**Fig 5-1; inset (stem bark)**) was collected at the Walter Sisulu University, Mthatha, Eastern Cape, South Africa (31°36'08.35"S 28°45'02.48"E) botanical garden on the 5th of November, 2012 within the hours of 1400 h and 1800 h and was taxonomically identified at Selmar Schonland Herbarium, Grahamstown (GRA), South Africa by Mr. T. Dold; A voucher number AOM001 for *A. mearnsii* was collected and voucher specimen deposited in the herbarium for future reference.

Figure 5-1: *Acacia mearnsii* whole plant; Inset (a) *Acacia mearnsii* stem bark

5.2.2 Chemicals.

Silver nitrate was purchased from Sigma-Aldrich, U.S.A. unless stated; all chemicals are used as purchased.

5.2.3 Preparation of Acacia hydrosol of *Acacia mearnsii*

300 g of the dried stem bark of each sample were hydro-distilled in a Clevenger-type apparatus for 3 h at 100 ^{o}C. Upon completion of the hydro-distillation, the plant residue in the flask was allowed to cool, followed by filtration using 250 µm mesh size. The extract was kept in an amber bottle and stored at 4°C for further analysis. To avoid growth of mucus, extract prepared were analysed at an interval of one week.

5.2.4 Analgesic and Inflammation analysis

5.2.4.1 Methods for Formalin Test

With slight modifications to the Prabhu *et al*., methodology, the formalin test was investigated [42]. Six groups of mice were selected for the present study.

Group 1- Control group received 0.09 % NaCl,

Group 2- Standard drug (Aspirin drug (100 mg/kg)

Group 3- *Acacia mearnsii* dry stem extract (AMDS) (200 mg/kg),

Group 4- as-synthesized silver nanoparticle (AMDS-Ag-NPs) (200 mg/kg), and

One hour (1 h) after treatment with the various drugs, animals were injected sub-plantaly with a 100 µl of 2.5% formalin solution (diluted in saline). Swiss rat were used because of their high sensitivity to pain. Nociceptive response in Swiss rat is characterised by an intense period of biting and licking of the rat paw for 5 mins after injection of diluted formalin to the hind paw. The biting subsides for 15 mins (rest period) and later commences with periodic bites and licks of the hind paw for 20-30 mins. Concurrent counts of bites and licks was recorded for the neurogenic phase (phase 1) and anti-inflammatory phases (phase 2). The percentage inhibition is then calculated using the following formula: $[1-(T/C)] \times 100$

Where, T is the number of times treated mice licked/bit the injected paw; C is the number of times control mice licked/bit the treated paw.

5.2.5 Synthesis of AMDS-Ag-NPs

An aliquot (5-50 mL) of the AM extract was added to a three necked flask, already equipped with temperature sensor and placed in a jacketed heating mantle. 10 mL of 0.1 M $AgNO_3$ solution (aqueous) was transferred into the stabilised plant extract with constant stirring at 200 rpm at room temperature, 40^oC and 60^oC. The reaction vessel was covered with an aluminium foil and placed in a dark cupboard to avoid auto-reduction of the $AgNO_3$ because of its photosensitivity [43]. The appearance of milky colour after few reaction minutes shows the formation of silver nanoparticles as shown in **Fig. 5-2**. Aliquots sample were withdrawn at different time interval to observe the growth of the Ag-NPs.

5.2.6 Optimization of Ag-NPs production

The optimization process was carried out with the aim of obtaining the final optimal reaction parameters. The reaction parameters involved in the optimization process were reaction temperature (room temperature, 40, and 60 ^oC, reaction time (15, 30, 60 and 180 min), extract volume (5, 10, 30 and 50 mL) and metal ion concentration (0.1 and 1.0 M).

5.3 Characterisation

The synthesized Ag-NPs/*Acacia mearnsii* were characterized by ultraviolet-visible spectroscopy (UV-Vis), X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy dispersive X-ray fluorescence spectrometry (EDXRF) and Fourier transform infrared (FT-IR) spectroscopy. The UV-visible spectra were recorded over the 200–800 nm range with a UV 1650 PC-Shimadzu B UV-visible spectrophotometer (Shimadzu. Osaka, Japan). Size, shape and morphology of as-synthesized Ag-NPs were observe using the Transmission electron microscope (TEM). TEM analysis

was carried out using a JEOL JEM 2100 (TEM) operated at 200 KV. Samples were mounted on a carbon coated copper grid, and then dried on a filter paper.

The crystallinity of the Ag-NPs produced were examined by X-ray diffraction (Bruker aXS D8 advanced diffractometer). The XRD patterns were recorded at a scan speed of 4°/min with Cu Kα radiation (λ=1.5406Å) operated at 40 kV and 40 mA. SEM was performed using a Philips XL-30 instrument (Philips, Eindhoven, while EDXRF was carried out on a DX-700HS spectrometer (Shimadzu). The FT-IR spectra were recorded over the range of 370 – 4000 cm^{-1} using a FT-IR Series 100, 1650 Perkin Elmer spectrophotometer (Los Angeles, California, USA).

5.4 Results and Discussion

5.4.1 Optical characterization

5.4.1.1 UV-vis spectroscopic analysis

a. Effect of temperature

In this reaction, the hydrosol extract of *A. mearnsii* acted as both reducing and stabilisation (capping) agents. The preliminary observation of colour change from deep brown to milky colour within 15 mins of reaction confirmed the reduction of $AgNO_3$ to Ag-NPs (**Fig.5-2**). The strong resonance centred at 480 nm was clearly observed, which corresponds to the excitation of longitudinal plasmon vibrations of silver nanoparticles in solution. **Fig. 5.3-5.5** depicts the surface plasmon resonance (SPR) spectra of the as-synthesized AM-AgNPs at different temperature of 60 ^oC, 40 ^oC and room temperature respectively. At temperature of 60 ^{o} C and 40 ^{o} C, the maximum SPR peak position at 15 min, 30 min and 60 mins are very close to each other indicating particle with same size and size distribution [44]. As the

temperature decreases, the broadness of the SPR peak increased indicating particle with broad size distribution. The narrow size distribution at elevated temperature has been ascribed to the greater reduction rate. This resulted in smaller particle size and hence narrow size distribution. At room temperature, the maximum SPR peak of the as-synthesised NPs disappeared and shows a resemblance with that of the plant extract. These results show that the optimum condition for the synthesis of *Acacia mearnsii*-Ag stabilised NPs is high temperature. *Acacia mearnsii* had been shown to contain tannins embedded in the cell cavity of the plant, which can be released at high temperatures thereby resulting in better reduction and stabilisation process.

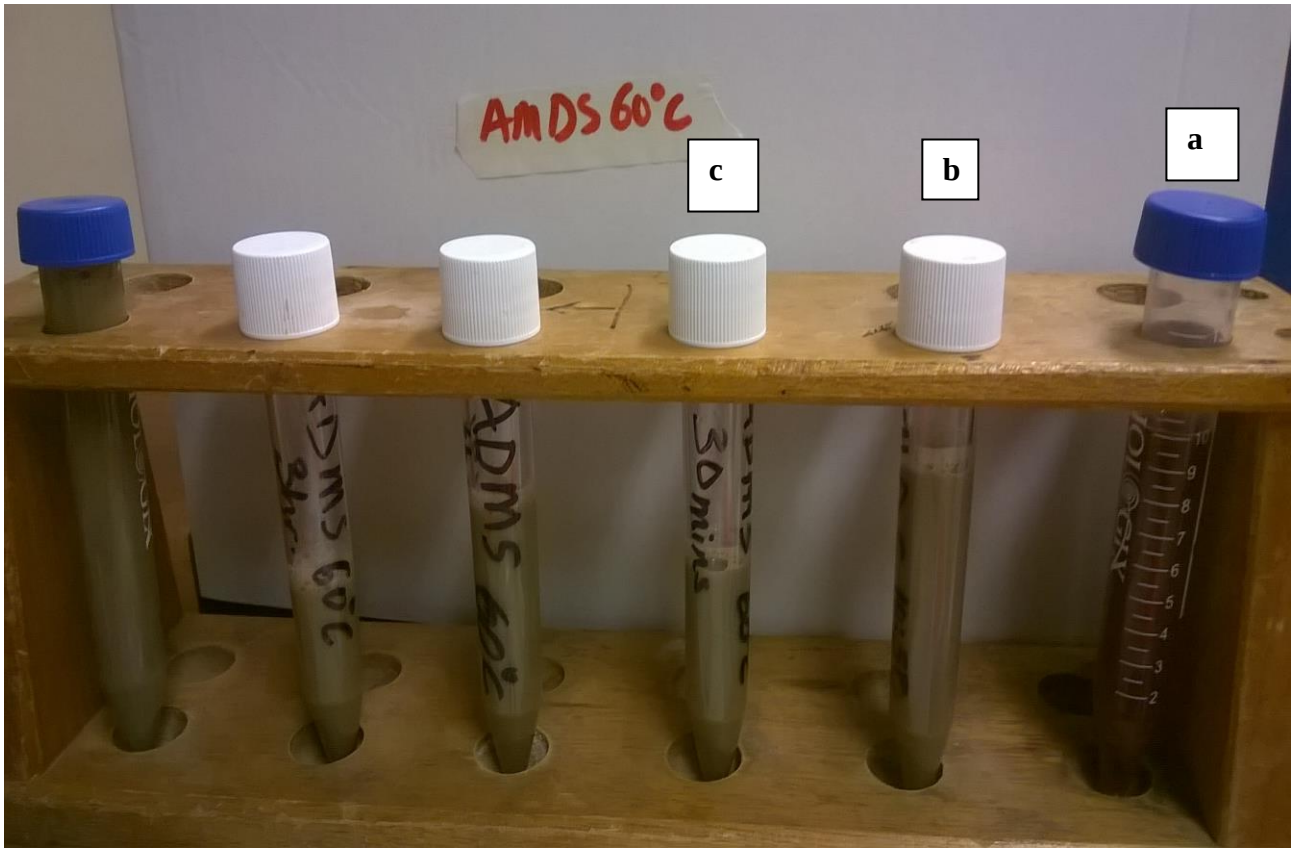

Figure 5-2: Colour of the *Acacia mearnsii* extract (a) colour change at (b) 15 m (c) 30 m of the solution after the formation of AgNPs.

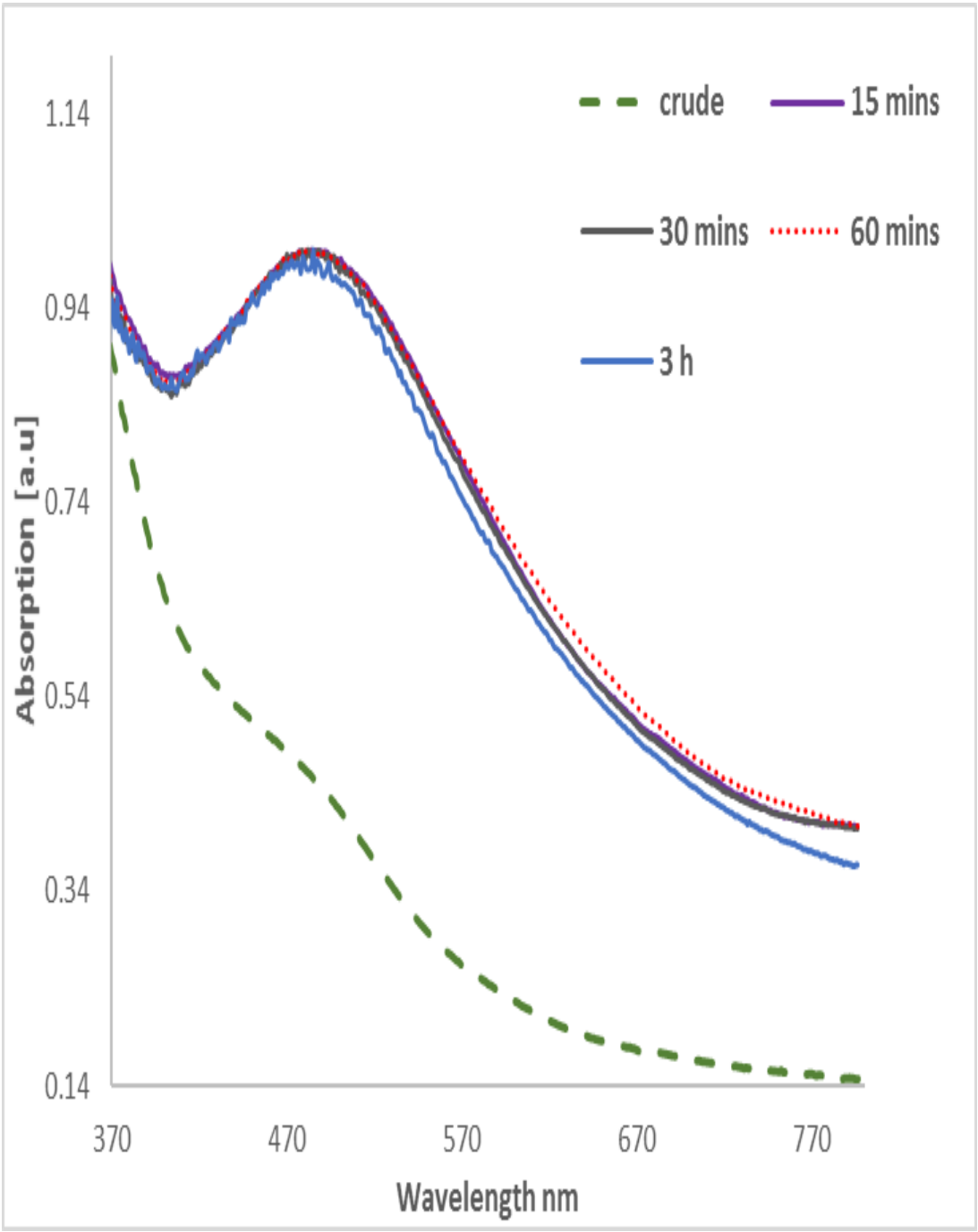

Figure 5-3: Absorption spectra of AM-AgNPs synthesized using 0.1 M $AgNO_3$ at 60 ^oC

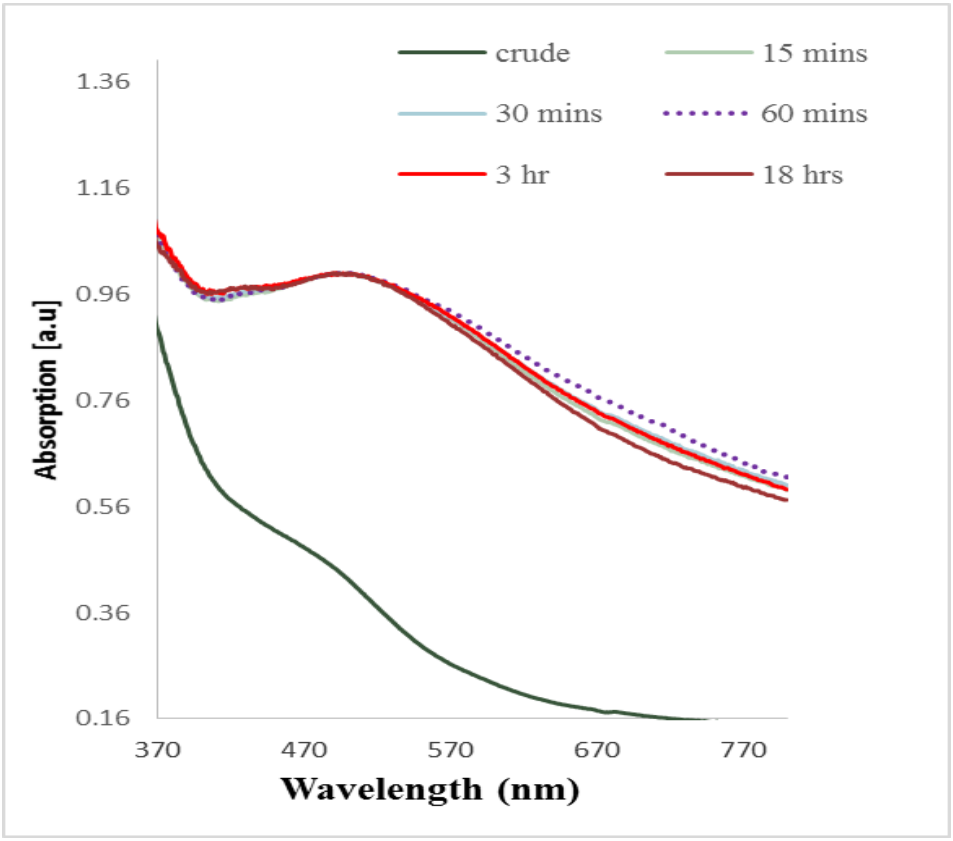

Figure 5-4: Absorption spectra of AM-AgNPs synthesized using 0.1 M $AgNO_3$ at 40 ^oC

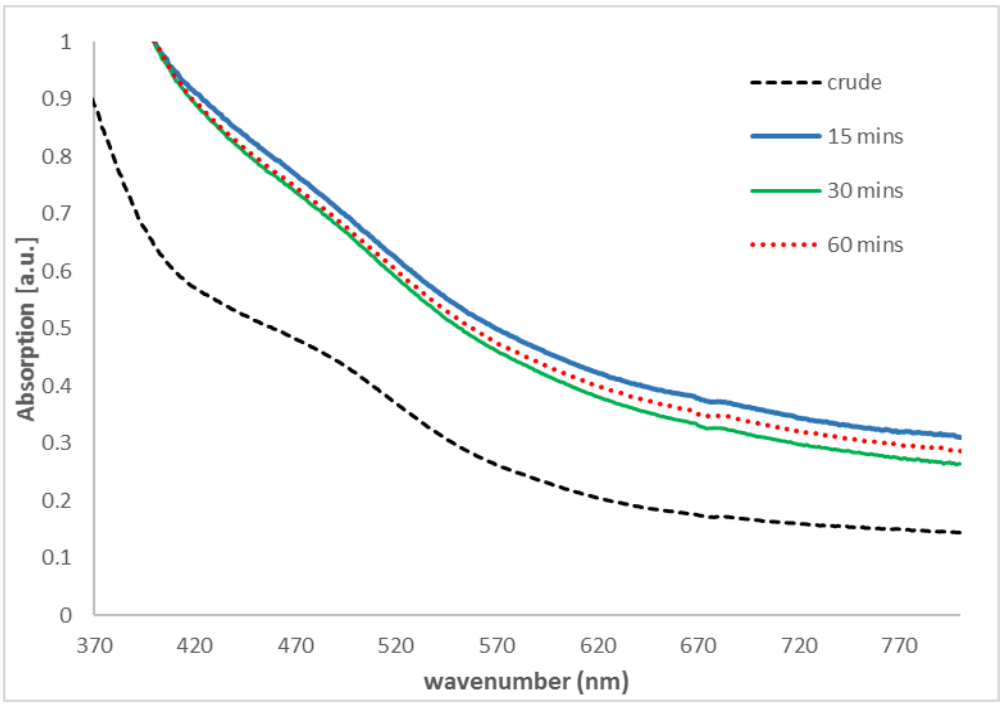

Figure 5-5: Absorption spectra of AM-AgNPs synthesized using 0.1 M $AgNO_3$ at room temperature.

b. ***Effect of volume***

The relative influence of different amounts of *A. mearnsii* Stem bark extract (hydrosol) on the reduction, size and stability of the synthesized AM-AgNPs was investigated by maintaining the Ag precursor solution while varying the volume of the extract. Studies have shown that at larger concentration of plant extracts, fast nucleation occurs followed by slower growth of the nanoparticles due to stronger interaction between the protective bio-molecules and the growing nanoparticles[45]. **Figure 5-6** shows the absorption spectra for the Ag-NPs synthesised using 1.0 M $AgNO_3$ and 5 ml of the extract. As the reaction time increased, the SPR peak position became gradually red-shifted signifying increase in particle size. This shift is also accompanied by sharpness of the SPR peak position at 470 nm. However, as the reaction time exceeded 1 h, broadness of the peak occurred. This might be attributed to the formation of larger particles i.e smaller NPs dissolves in the solution as the reaction continues to produce larger particles via the process of Ostwald ripening. The formation of larger particles resulted in the broadness of the peak.

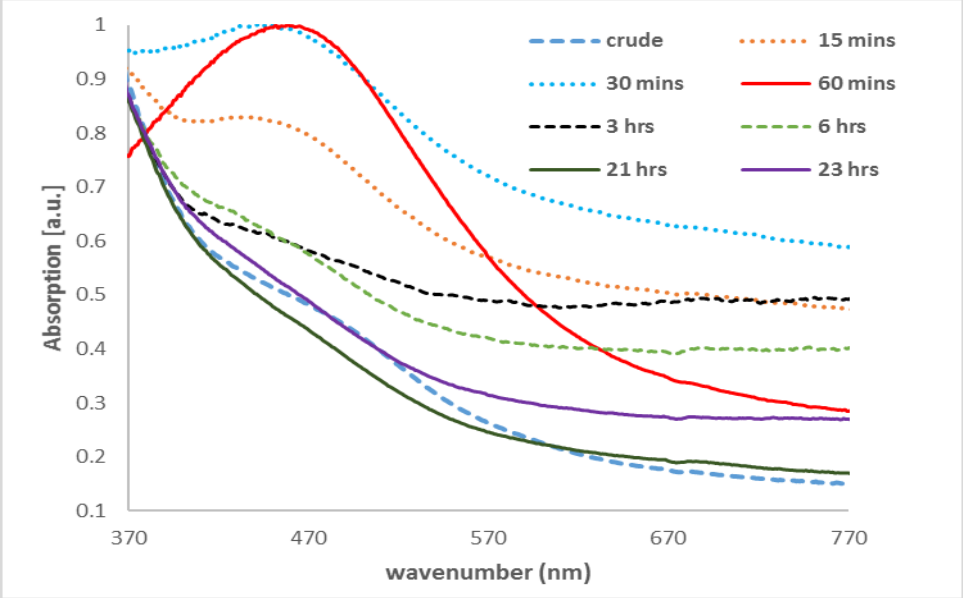

Figure 5-6: Absorption spectra of AM-AgNPs synthesised using; 45 mL 1.0 M $AgNO_3$ vs 5 ml of extract.

c. Effect of Reaction time

The absorbance spectra of the as-synthesized Ag-NPs at different reaction time shows no specific sharp peaks except for the colour change to light-brownish colour observed. The absorption spectra of the Ag-NPs produced by using 40 ml of the silver precursor and 10 ml of the extract are shown in **Fig.5-7** .The absorption spectra followed the same pattern as those reported in **Fig. 5-6** i.e narrow absorption spectra as the reaction time increased up to one hour followed by broadness of the spectra. However, the maximum SPR peak position under this reaction condition occurs at lower wavelength indicating decrease in particle size. This decrease in particle size could be attributed to the increase in the amount of plant extract i.e increase in the amount of reducing agent.

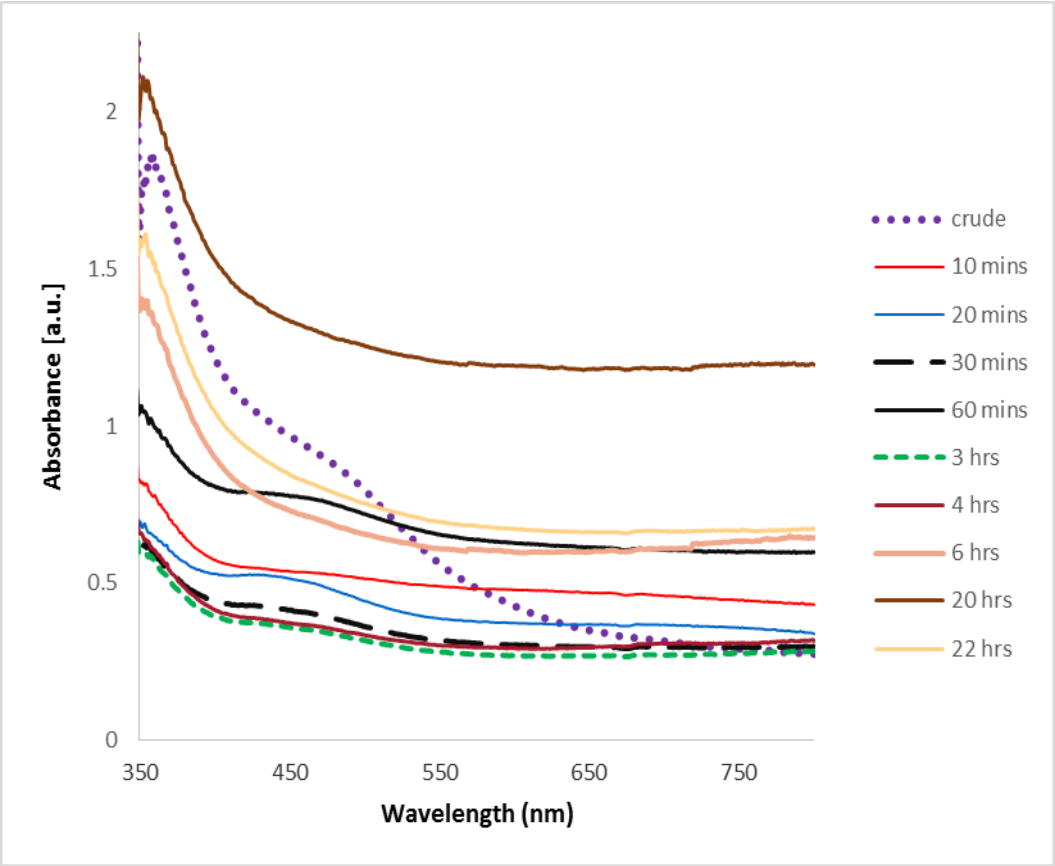

Figure 5-7: UV spectra of AM-Ag-NPs synthesized using 40 mL of 1.0 M $AgNO_3$ and 10 mL of the extract.

d. Effect of Concentration

The absorption spectra of the Ag-NPs formed by using 30 ml of the silver precursor and 20 ml of the extract are shown in **Fig.5-7**. The spectra is characterise by a narrow absorption peak up to 60 mins proceeded by peak broadness. The SPR peak occurs at a lower wavelength indicating decrease in particle size. As observed, the decrease in particle size could be as a result of large amount of extract available for reduction of the silver salt.

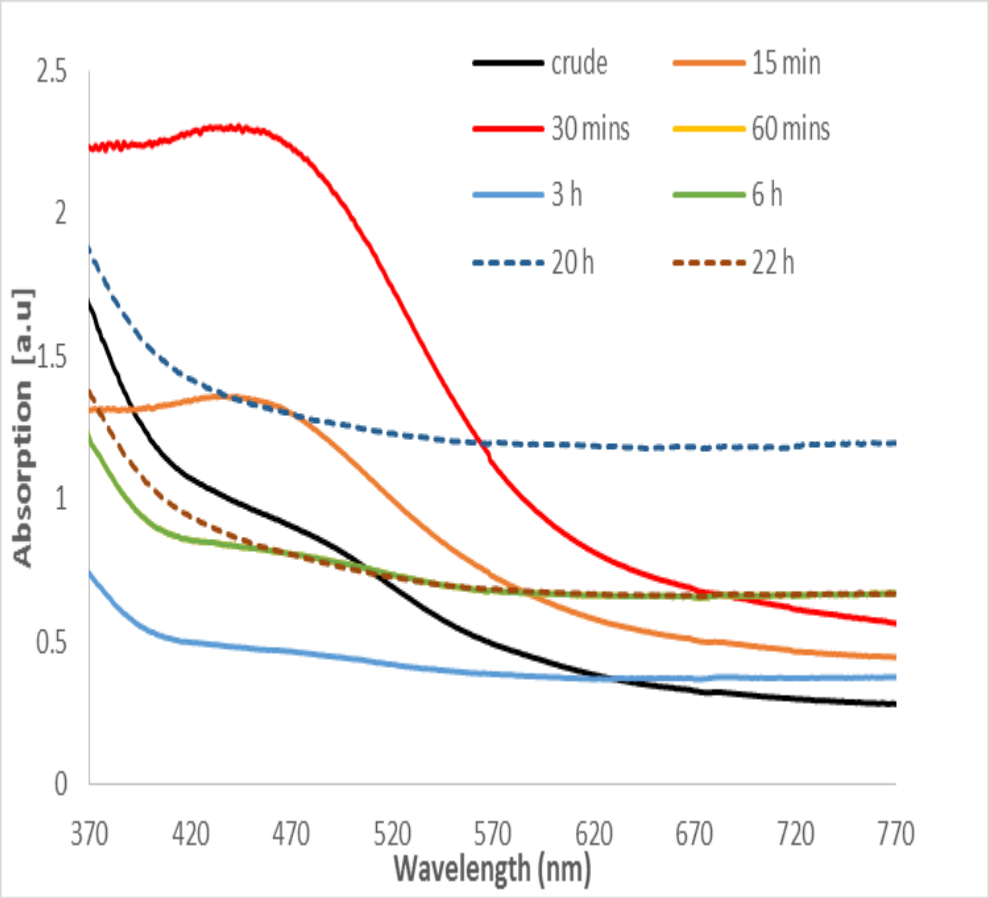

Figure 5-8 UV spectra of AM-Ag-NPs synthesized from 30 mL of 1.0 M $AgNO_3$ vs 20 mL extract

5.4.1.2 Fourier transform infrared spectroscopy

To determine of probable bio-reducing functional groups liable for the reduction and passivation of the Ag-NPs surface under this synthetic method, FTIR analysis was performed. **Fig 5-9** shows the spectral of the crude material and that of the as-synthesised AM-AgNPs. Characteristics changes in the position, emergence of new peaks and change in the peak intensities are some confirmatory parameters for the biomolecules responsible for the reduction and successful capping of the metal nanoparticles. The change and assignments of the peaks are outlined in Table 5-1. Representative functional groups of some biomolecules

such as protein, flavonoids, alkaloids, and terpenoids are observed from the crude extract. The free O-H of alcohol, phenols, C=O of the amide group (protein), C-N of the amines, alkaloids, N-O of nitro containing terpenoids, $–OCH_3$ of flavonols and halogens peaks as revealed by the EDX (**Fig.5-12**) were identified.

In the as-synthesized AM-Ag-NPs, the broadness of the peak increases and the peaks assigned for the free O-H stretching vibration shifted from 3715.8 to 3855.5 cm^{-1}. In addition, the C=O of the amide which appeared at 1619.3 in the crude extract was shifted to 1608.4 cm^{-1}. Furthermore, the peaks at 1452.7, 1320.1, 1204.0 and 1154.9 cm^{-1} assigned to the C=C of aromatic, C-N of amide, C-N bond of amide and C-O of ester stretching vibrations in the AM extract shifted to 1440.06, 1396.95, 1278.89, 1192.51, 1104.2, 1030.1 and 523.1 cm^{-1} respectively. Previously observed peak at 2124.6 cm-1 earlier attributed to C≡C triple bond stretching vibrations in AM extract disappeared, with emergence of a new peak at 2856.7 cm^{-1}. This peak is designated to the alkane C-H stretching vibration in the as- synthesised AM-AgNPs.

Table 5-1: Peak assignment for the FT-IR of the crude extract and the AMDS-Ag-NPs

λ cm-1 of crude extract	λ cm-1 of AMDS-Ag-NPs	Assignment	Reference
3751.8	3855.5	Free O-H of the alcohol	[46]
3413.7	Disappear	C-H-stretch of terminal alkynes	[47]
3200	3150.4	N-H bond of the amide	[48]
2923.8	2910.3	C-H stretch of CH_3	[49]
	2856.7	C-H stretching for alkanes	[50]
2124.6	Disappear	C≡C bond of terminal alkynes (mono substituted)	[47]
1619.3	1608.4	C=O bond of amide	[49]
1516.2	1518.2	N-O bond of nitro compounds	[51]
1452.7	1440.1	(C=C-C of aromatic chromones	[52]
1320.1	1397.0	C-N bond of amide	[48]
Absent	1318.35	Acyl and phenyl C-O, Ag/A.M vibrations	[53]
1204.0	1278.89	C-N bond of the amines	[54]
1154.9	1192.51	C-O-C bond of ester	[55]
1030.7	1030.1	-OCH_3 of ether linkage	[56]
842.6-662.2	850-600	C-halogen bonds, C-H bends of the alkynes, Ag-O (deformation) bond of the silver metal	[57]

The bioreduction and stabilization of the as-synthesized Ag-NPs could be credited to the presence of –OH, C≡C and the –C=O functional group of the amide and synergistic effect of other functional groups earlier highlighted. Earlier reports had indicated the ability of protein

moiety (amines, amides and peptides) to bind themselves to nanoparticles surfaces thereby acting as capping agents to prevent aggregation [58,59,60].

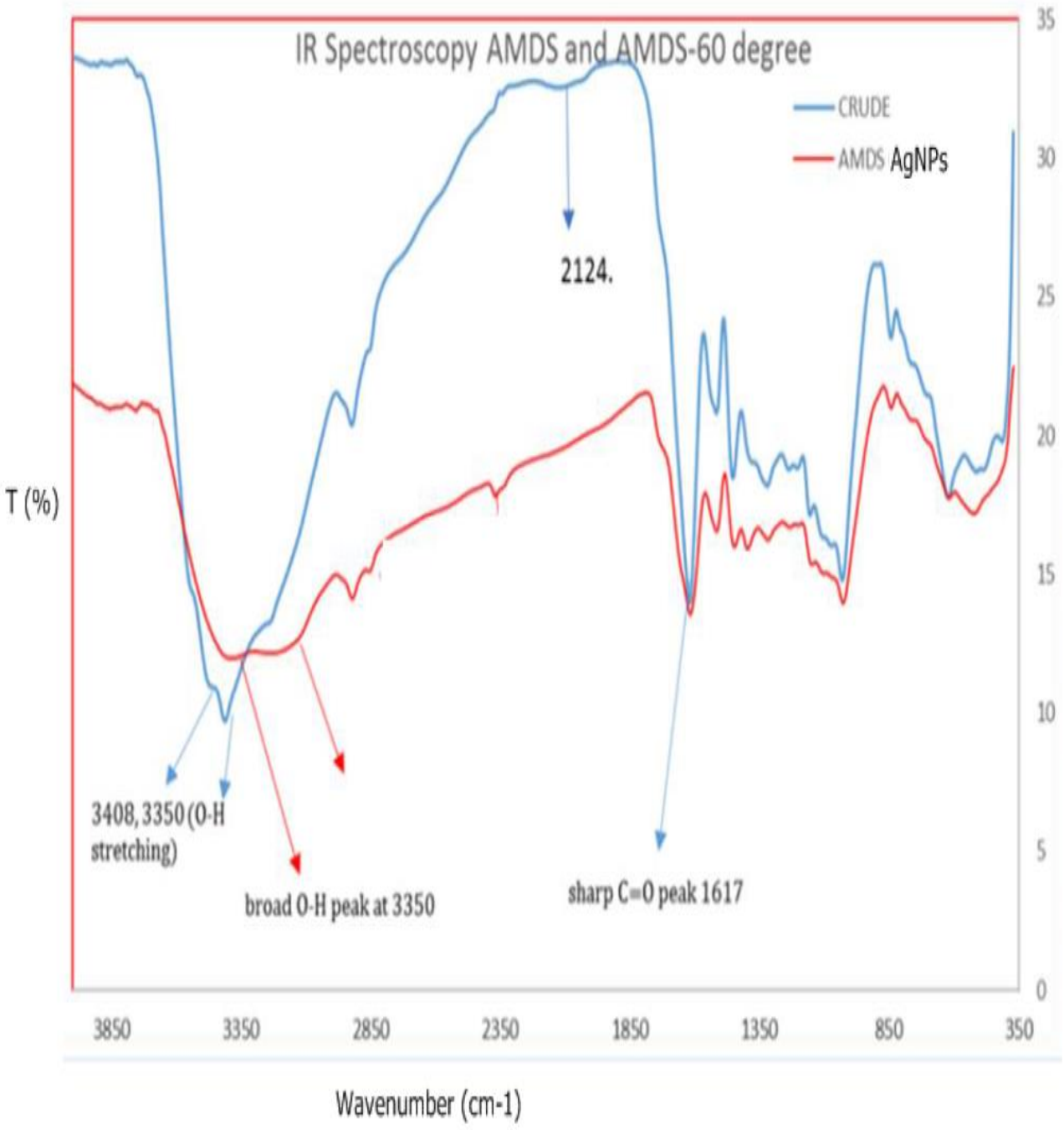

Figure 5-9: FT-IR of crude sample and synthesized AM-AgNPs.

5.4.2 Morphological studies

5.4.2.1 SEM AND EDX analysis

The morphology of the as-synthesised Ag-NPs investigated using scanning electron microscope (SEM) is shown in **Fig.5-10**. The micrograph shows that surface of the crude is coarse while the synthesized Ag-NPs shows little follicles. The micrograph for other synthesis are shown in Appendix **C.** The formation of Ag-NPs and its purity was further confirmed using EDX (**Fig.5.11 & 5.12**). The EDS spectrum of the crude extracts (**Fig.5-11**) show C and O as the major peaks with traces of Cl, N and S. However, the Ag-peak is conspicuously absent. On the other hand, the EDS spectrum of the as-synthesised Ag-NPs (**Fig.5-12**) show Ag peak as the major peak with traces of Cl, C, O, N and S.

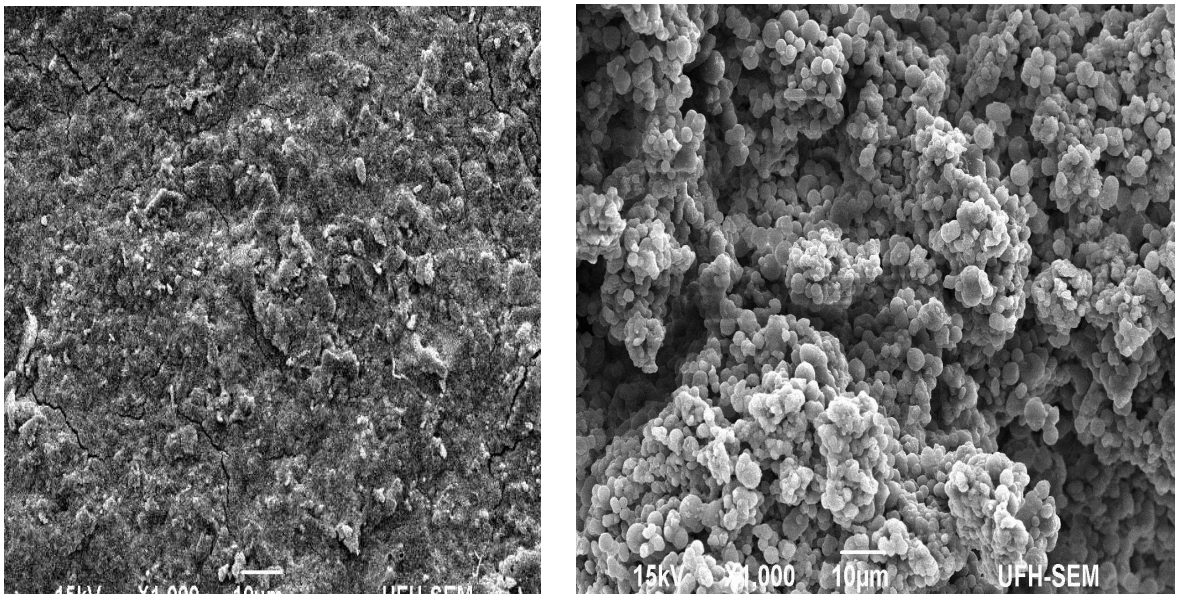

Figure 5-10: SEM micrograph of (a) Crude *Acacia Mearnsii* (b) Synthesized AM-AgNPs

The weight percentage of the Ag, Cl, C, O, N and S are 75.4%, 11.1%, 0.9%, 12.5%, 1.0% and 0.1% respectively. This further confirms the formation and purity of the as-synthesised Ag-NPs.

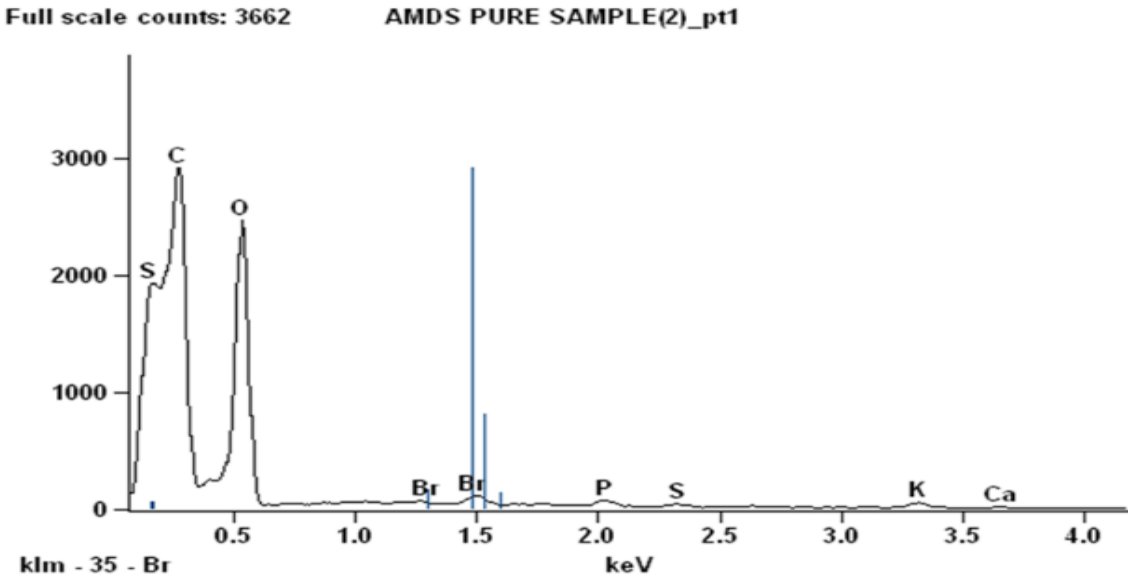

Figure 5-11: EDS spectrum of AMDS pure sample

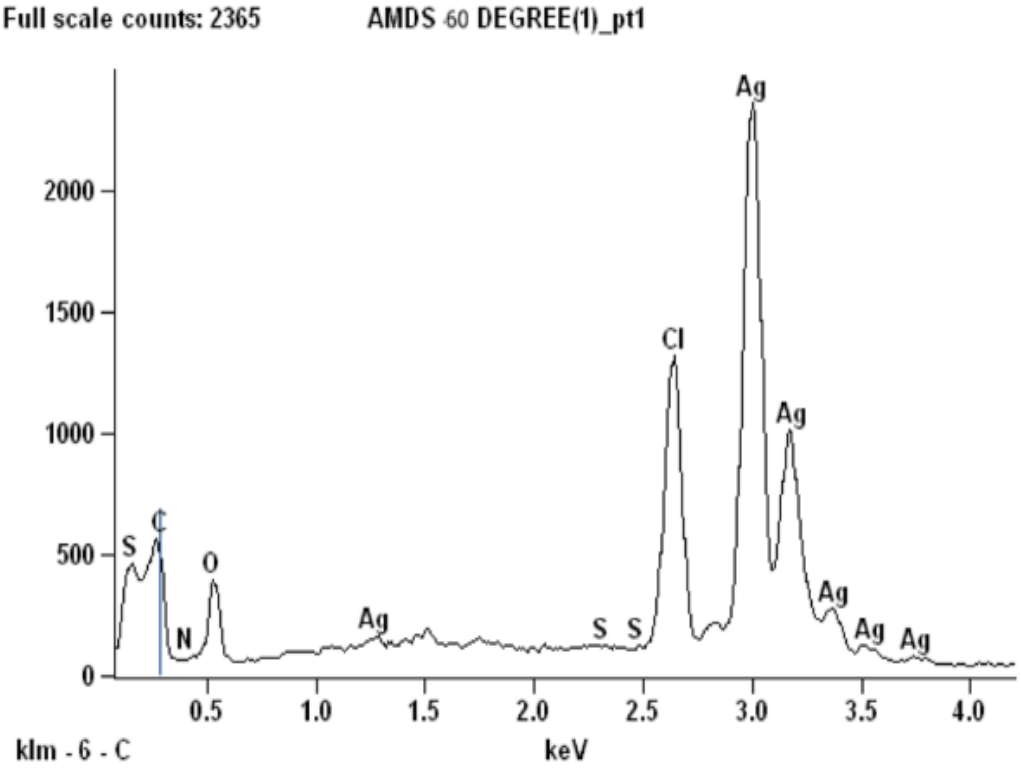

Figure 5-12: EDX Spectrum for synthesized AM-AgNPs.

5.4.2.2 XRD analysis

The crystallinity of the as-synthesised Ag-NPs were confirmed using XRD analysis. **Figure 5-13** shows the typical XRD pattern of the Ag-NPs synthesised at different temperatures while **Fig. 5-14** was the XRD pattern of the Ag-NPs synthesised at different extract volume. All the XRD pattern show diffraction peaks at 2Θ values of 38.63, 46.54, 64.97 , 77.42 and 81.99 corresponding to the (111), (200), (220), (311) and (222) crystallographic planes of face centred cubic crystalline structure of metallic Ag respectively (JCPDS no 04-0783). The broad nature of the XRD peaks is attributed to the nanocrytalline nature of the Ag-NPs. The unassigned peaks may result from the bio crystalline particles of the plant[49]. **Table 5-2** and **5-3** summarises the calculated particle size using the Debye-Sherrer,s formula shows an

average particle size of 20.77 nm and 24.91 nm for Ag-NPs synthesized at 60^{o}C and 40^{o}C respectively . This indicate that increase in temperature favours the formation of smaller particle sizes.

Table 5-2: Particle sizes of nanoparticles calculated using the Sherrer's equation at 60^{o}C.

2θ position	Particle size (nm)
38.41	19.61
44.57	13.77
64.79	19.37
77.68	26.00
82.03	30.10
Average	**20.77**

Table 5-3: Particle sizes of nanoparticles calculated using the Sherrer's

2θ position	Particle size (nm)
38.37	23.76
44.54	16.45
64.72	22.13
77.63	30.14
81.82	32.05
Average	**24.91**

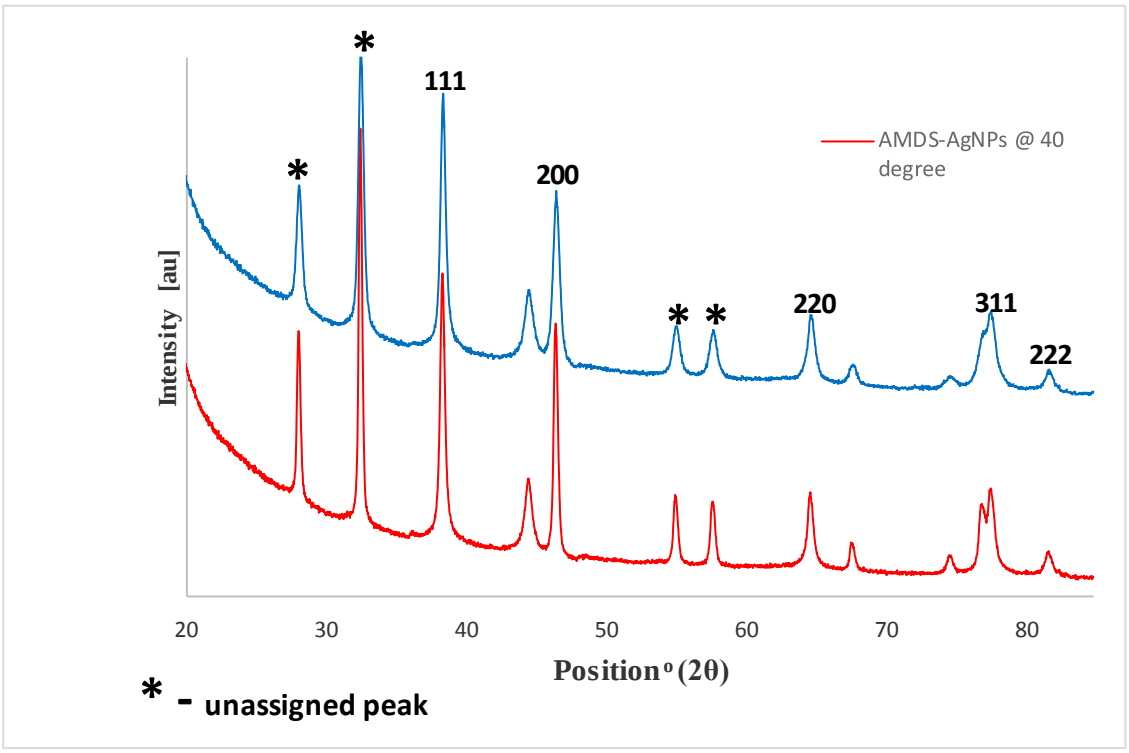

Figure 5-13: A typical XRD pattern of Ag-NPs synthesized at different temperatures.

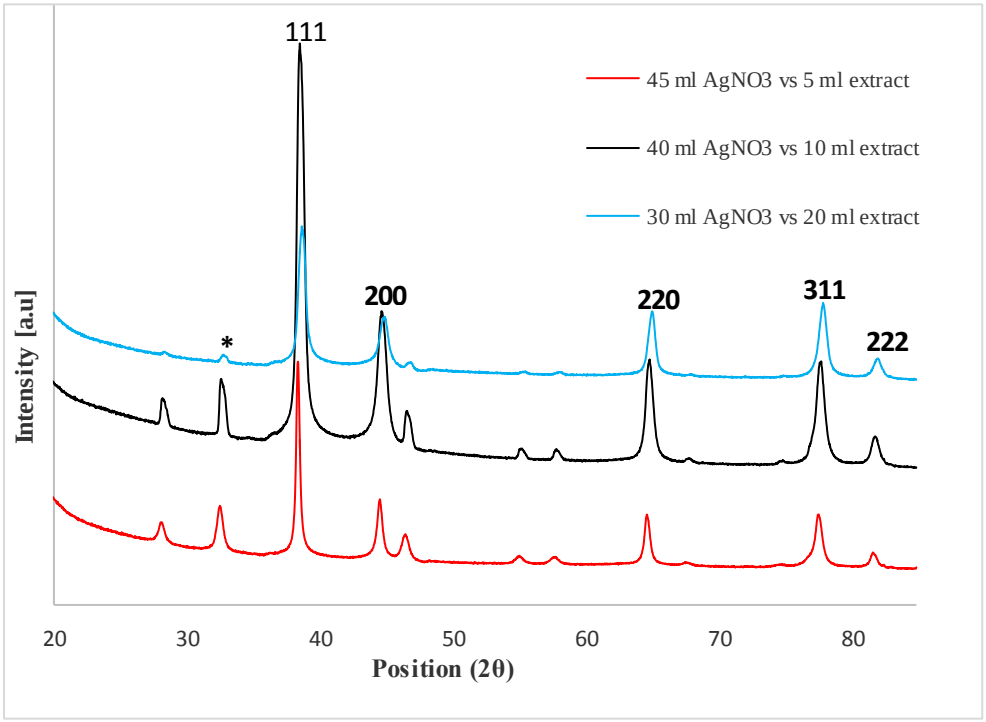

Figure 5-14: A typical XRD pattern of Ag-NPs synthesized with different extract volume.

The XRD patterns of the as-synthesized Ag-NPs at different extract volume (Fig. 5-14) shows a well resolved peak with minimum bio-crystalline peaks (unassigned peaks). The diffraction peaks patterns follows the same as those synthesized with increased temperature and lower concentration (i.e. 0.1 M $AgNO_3$). Mean particle size of the as-synthesized Ag-NPs computed with the Scherrer's equation from variation of extract volume shows a slight decrease in particle size as the extract volume increases as shown in Table 5-4, 5- 5 and 5-6. This shows that as extract volume increases, further bio-reduction occurs resulting in the breaking down of the metal salt to form Ag-NPs.

Table 5-4: Particle sizes of nanoparticles synthesized using 45 ml 1.0 M $AgNO_3$ and 5 ml of extract.

Position 2θ	Particle sizes (nm)
38.34	29.48
44.52	21.07
64.68	22.38
77.63	19.05
81.84	16.10
Average	**21.62**

Table 5-5: Particle sizes of nanoparticles synthesized using 40 ml of 1.0 M $AgNO_3$ and 10 ml of extract.

Position 2θ	Particle sizes
38.52	14.71
44.68	12.76
64.85	16.16
77.76	15.94
81.12	16.14
Average	**15.14**

Table 5-6: Particle sizes of nanoparticles synthesized using 30 ml of 1.0 M $AgNO_3$ and 20 ml of extract.

Position 2θ	Particle sizes
38.66	15.06
44.84	13.13
65.06	17.16
77.94	16.85
82.27	18.10
Average	**16.06**

5.4.2.3 TEM Analysis

The TEM micrograph of AM-Ag-NPs are illustrated in Fig 5-15. The shape of nanoparticles formed are spherical in shape with sizes ranging from 20 and 50 nm. The dark patches/ shades on the surface of the nanoparticles could be attributed to the presence of biomaterials acting as capping agent for the Ag-NPs. The nanoparticles are poly-dispersed and form agglomerations.

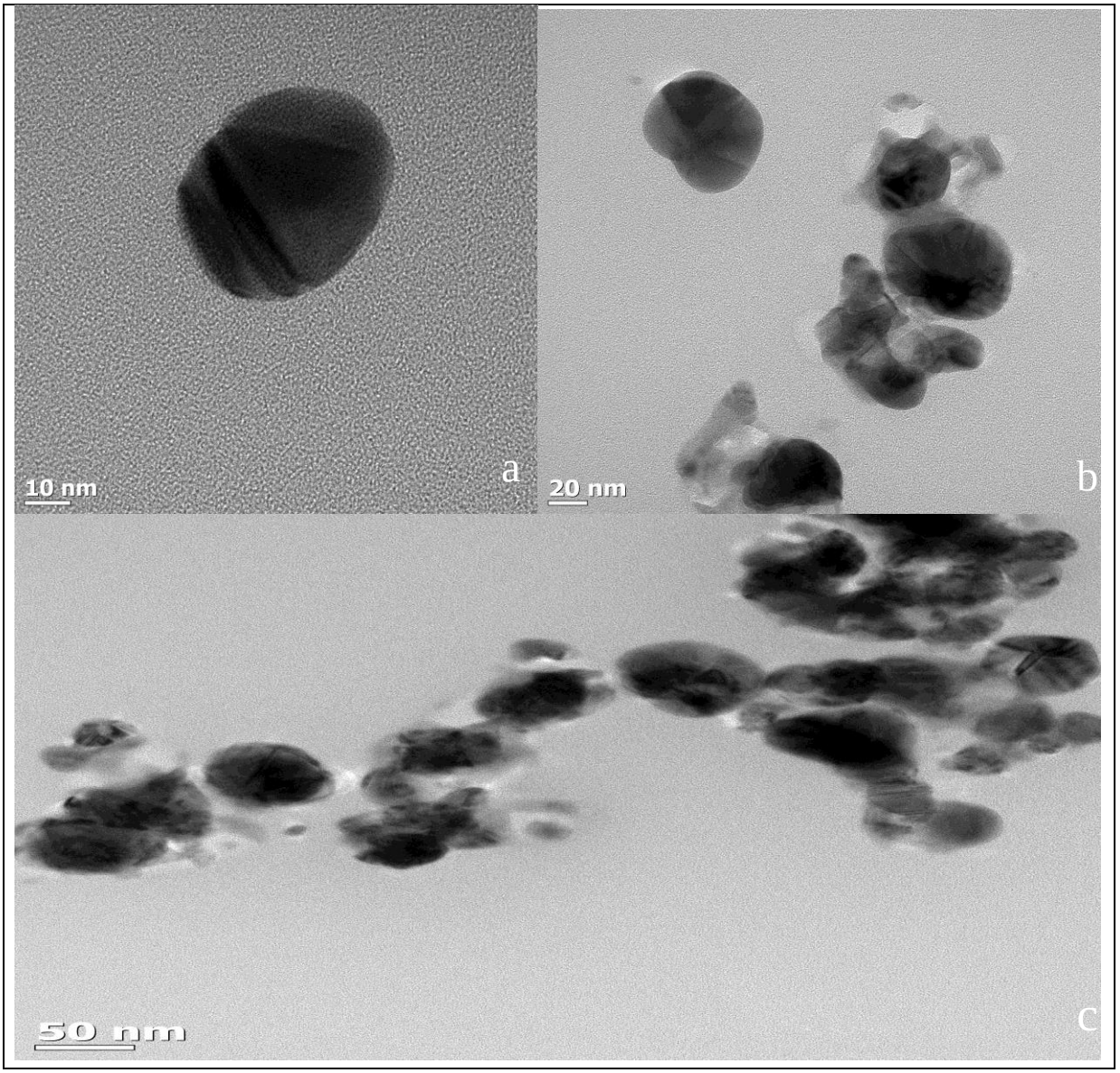

Figure 5-15: TEM image of AM-AgNPs synthesized (a) 10 nm (b) 20 nm and (c) 50 nm resolutions.

5.5 Antinoniceptive and Inflammation Activity

Formalin induced test is a pain model used to analyse the both the analgesic and the anti-inflammatory properties simultaneously. Formalin induces pain of moderate intensity characterised by irritation, tissue damage and oedema formation as a result of the release of inflammatory mediators[61]. Typically, formalin-induced pain involves two phases. The initial phase is linked to the stimulation of nociceptors and principal afferent fibres by the formalin,

triggering the liberation of bradykinin and tachykinins which occurs for 5 mins[62]. The latter phase which lasts for 20 to 30 mins after injection is accompanied by the discharge of inflammation facilitators such as prostaglandins, cytokines, histamine, nitric oxide (NO), and serotonin. In addition, these phases are inhibited by different classes of drugs as opioid drugs inhibits the early phase and non-steroidal, anti-inflammatory drugs (NSAIDs) and opioid drugs for the latter phase.

Table 5-7: Antinonicepive effect of oral treatment with AMDS-AgNPs on formalin-induced pain.

		Neurogenic phase (0-5mins)		Inflammatory phase (10-30mins)	
Treatment	Dose (mg/kg)	No of licks	Inhibition (%)	No of licks	Inhibition (%)
Control	Normal saline	42.3 ± 1.5		30.5 ± 1.7	
Standard (Aspirin)	100 mg/Kg	7 ± 1.3**	83	6.8 ± 1.4**	78
AMDS crude	200 mg/Kg	17.5 ± 0.9**	59	9.0 ± 2.0 **	70
AMDS NP 60^{o}C	200 mg/kg	20.8 ± 3.2**	51	7.3 ± 1.9 **	76

In this model, a lower number of licks is an indication of high inhibition. As expected, aspirin used as the standard drug significantly inhibited the 1st phase (83%) and 2nd (73%) phases of the test which is expected for the non-steroidal drug. The crude sample (AMDS) and the AMDS-AgNPs exhibited a high activity at the inflammatory phase with 70% and 76% inhibition respectively at dose level of 200 mg/kg. Silver nanoparticles had been shown to possess a very high wound healing and anti-inflammation effects due to their high rate of absorption into cell membranes and ability to modulate cytokine production[63–65]. The AgNPs synthesized were significant to an order of $P > 0.01$ at both phases and the result shows that

the nanoparticle possess better activity than the crude plant at the second phase only. In addition, the anti-inflammatory activity of the AgNPs at a dosage of 200 mg/kg AgNPs is nearly similar to 100 mg/kg dose level of the standard drug (Aspirin).

5.6 Conclusion

Silver nanoparticles (Ag-NPs) were successfully synthesized from bio-reduction of silver nitrate solutions using *Acacia mearnsii* stem bark hydrosol. The plant extract acted as both reducing agent and capping agents. Optimization of synthetic parameters reveals a simple, efficient method favoured by an elevation in temperature and short reaction time. The as-synthesized Ag-NPs were characterised using UV-vis spectroscopy, scanning electron microscopy (SEM-EDX), Fourier Transform Infra-Red spectroscopy (FTIR), X-ray-Diffraction (XRD) and Transmission Electron Microscope (TEM). At 0.1 M $AgNO_3$ against 50 ml of extract at 40 ^oC and 60^oC temperature, a uniformed sized nanoparticles were observed. The Ag-NPs are spherical in shape with face centred cubic (fcc) crystalline structure. The average particle sizes as-calculated using the Scherer's equation are between 16-30 nm. The FTIR analyses showed that the alkyne, carboxyl and amide groups from *Acacia mearnsii* extract were liable for the bio-reduction of the Silver ions to nanoparticles and the consequent passivation of the surface.

The anti-inflammation efficacy analysed by the formalin model, reveals that the as-synthesized Ag-NPs was more effective than the crude extract at the second phase (inflammation phase) and with an inhibition that is close to the standard drug, Aspirin. This investigation shows that AgNPs synthesized from the stem bark of *Acacia mearnsii* can be used as drug to modulate various inflammatory mediators such as cytokines, prostaglandins,

NO, histamine and serotonin. Further studies on the inflammation mechanism of the AMDS-AgNPs and its action at the molecular level will still be investigated coupled with the cytotoxicity of the synthesized Ag-NPs.

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CHAPTER 6

Synthesis, characterisation, Analgesic effect and Anti-inflammatory activities of *Acacia karroo* Hayne mediated-Silver nanoparticles.

6.1 Introduction

Metal nanoparticles are unique in their characteristics behaviour (optical, electronic and catalytic) as a result of small size and their high surface to volume ratio. This unique ability render them useful in many applications such as biosensors, catalysts, data storage, optics, drug delivery and packaging[1–7]. Several drug-loaded nanoparticles are currently at the last stage of clinical trial/ administered,; they include Liposomal amphotericin B for treatment of fungal infections, Liposomal verteporfin and Micellular estradiol[4] for age related diseases and menopausal therapy respectively just to mention a few.

Silver nanoparticles (Ag-NPs) had generated tremendous interest due to their vast antimicrobial, exceeding fascinating catalytic, optical and electrical applications. Ag-NPs had been shown to supress microbes intermingling with enzymes, proteins or DNA of the cancer cells to inhibit cell proliferation. Rogers et al, investigated the activity of Ag-NPs on Monkey pox virus (MPV), the findings showed that nanoparticles size of 10 nm inhibited the virus in vitro[8]. Other biological activities of silver nanoparticles include antifungal[9–11], anti-cancer[12,13], anti-malarial[14,15], anti-diabetes and anti-inflammatory etc.

Several physical, chemical and thermal methods has been in application for synthesising silver nanoparticles; this includes laser ablation, photochemical reduction, lithography, pyrolysis, spark discharge and electrochemical synthesis. Chemical reductions results in the

formation of colloidal silver particles which can further results in larger particles due to agglomeration to form oligomeric clusters. To circumvent these limitations, most methods incorporate the use of particles stabilisers or capping agent which could be organic/inorganic solvents. Hydrazine, hydroxylamine, sodium citrate, sodium borohydride ($NaBH_4$), ethylene glycol, poly (vinyl pyrrolidone) (PVP)[16], oleylamine[17], n-dodecyl sulfide[18] and carboxymethylated chitosan (CMCTS) [19] are few examples of capping and reducing agents commonly used in the synthesis of nanomaterials. It had been reported that majority of these reagents are toxic, environmentally unfriendly, expensive and produces a large amount of toxic waste[19].

Conversely, a biosynthetic route offers one of the safest, simple and direct methods for silver nanoparticles synthesis. Several organisms including bacteria[20], fungi[21], actinomycetes[22] to the higher organism such as plants[23–26] have been used to produce nanoparticles of controlled sizes and shapes. Stabilized and size controlled silver nanoparticles have been synthesized from plant such as *Cinnamomum camphora*[27], *Bixa orellana*[28], *Potamogeton pectinatus* L[29]., *Callicarpa maingayi*[30], *Anacardium occidentale* L.[31], water hyacinth[32], *Ocimum basil*[33], clove[34], *Delonix elata*[35], *Jatropa curcas*[23] and *Camellia sinesis*[25]. Silver nanoparticles synthesized from living organisms such as plant had been shown to exhibit biological and pharmaceutical properties. Palanivel et al., reported the synthesis of 10-20 nm Ag-NPs from the root extract of *Zingiber officinale*. The as-synthesised Ag-NPs showed high antibacterial efficacy against *Staphylococcus spp.* and *Listeria spp* [36]. Silver nanoparticle of 5.7 ± 0.2 nm in size were synthesized from *Anogeissus latifolia* and were reported to exhibit high antibacterial activity against gram $^{+}$/- bacteria[37].

Acacia karroo Hayne otherwise called sweet thorns can thrive in virtually all tough regions, including the deserts (a height of 3-4 metres, with big thorns and high content of leave tannins) and coastal woodlands (40 m tall, with small thorns, low tannin content with low

chemical defence mechanisms) [38]. It is widely distributed in all the nine provinces in South Africa[39]. Ethno pharmacological applications of *A. karroo* reveals its application as a remedy for malaria, stomach ailments and boiled in water to treat diarrhoea in goats[40,41] as a feed forage for herbivores [42]. *A. karroo* had been reported to be a good source of flavonoids and prothocyanidins which had been shown to exhibit a high antioxidant properties. Aqueous extract from the stem bark had been shown to significantly reduce the oedema in mice by 90.8% at a dose of 200 mg/kg[43–45].

In this report, we explore the ability of *Acacia karroo* stem bark to act as promising material for the synthesis of silver nanoparticles, and thereby test the analgesic and the anti-inflammatory capacity of the synthesized silver nanoparticles.

6.2 Experimental Procedure

6.2.1 Preparation of *Acacia karroo* hydrosols (extracts).

Acacia karroo stem bark (**Fig. 6.1**) were collected at Walter Sisulu University, Mthatha, Eastern Cape, South Africa (31°36’08.35”S 28°45’02.48”E) botanical garden on the 5th of November, 2012 within the hours of 1400 h and 1800 h and was taxonomically identified at Selmar Schonland Herbarium, Grahamstown (GRA), South Africa by Mr. T. Dold; Voucher sample was deposited and Voucher number (AOK001) was collected at the herbarium.

The hydrosol was prepared by subjecting about 300 g of dried stem bark to hydro-distillation using a Clevenger-type apparatus. Upon completion of essential oils extraction, the extract in the flask were allowed to cool, filtered and kept for analysis. The extract was used after 3 days of preparation to prevent photo-oxidation and mucus growth on the extract.

Figure 6-1: *Acacia karroo* full plant

6.2.2 Synthesis of silver nanoparticles

Silver nitrate ($AgNO_3$), were purchased from Shalom chemicals Ltd, South Africa and were used as purchased. During the synthesis, 50 mL of the extract was added to a three-necked flask with continuous stirring at 200 rpm and heated at 60^{o}C and 80^{o}C respectively to stabilise the solution. 10 ml of 0.1 M aqueous $AgNO_3$ was added to the extract in the dark under continuous stirring. Aliquots were withdrawn at different intervals for optical characterization.

6.2.3 Characterisation:

6.2.3.1 UV-analysis

The bio-reduction and stability of Ag-NPs in colloidal solution was examined periodically by sampling of 2.0 mL aliquots of the solution. The UV-vis spectroscopy studies of the

nanoparticles synthesised was monitored on a UV−visible (UV−vis) spectrophotometer (SHIMADZU model UV-1650 PC, Japan) in the range of 200-800 nm.

6.2.3.2 Fourier Transform Infra-Red Spectrophotometer analysis:

To analyse the biomolecules responsible for the reduction and the capping, the synthesized nanoparticles was subjected to FTIR. A Perkin Elmer 2000 FTIR spectrophotometer was used by preparing the solid sample with KBr pellet.

6.2.3.3 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray Spectrometer (EDX) Analysis.

Ag-NPs were obtained from the synthesized suspension by centrifuging 5mL of solution at 40,000 rpm (Eppendorf Centrifuge 5702 R). The fine particles obtained was dehydrated in an oven at 50 ^{o}C to obtain a powdery form which was used further for EDX investigation. Powdered sample of the crude and synthesized nanoparticles were spread evenly on a copper block. For SEM analysis, a JEOL JSM 6390 LV SEM at 15Kv (Japanese Electron Optical Lab.). The specimens were coated with a thin layer of gold in order to avoid charging effects.

6.2.3.4 XRD measurement

The powdered AK-Ag-NPs were subjected to XRD analysis. The XRD studies was performed on the powdered samples using a Bruker D8 Advanced X-ray diffractometer (Bruker Optics GmbH, Ettlingen, Germany) subjected to $Cu_{K\alpha}$ radiation (λ = 1.54 Å, rated as 1.6 kW) in the 2θ range of 10-85 operate data voltage of 40 kV and a current of 30 mA.

$$D = \frac{0.89\lambda}{\beta \cos\theta} \quad (1)$$

D is the crystallite size (nm), λ is the wavelength of incident X-ray (nm), β is the full width at half maximum in radian, and θ is the diffraction angle.).

6.2.3.5 TEM analysis

Size, shape and morphology of as-synthesized Ag-NPs were observe using the Transmission electron microscope (TEM). TEM analysis was carried out using a JEOL JEM 2100 (TEM) operated at 200 KV. Samples were mounted on a carbon coated copper grid, and then dried on a filter paper.

6.2.4 Analysis of the analgesic

6.2.4.1 Methods for Formalin Test

The formalin test was carried out using the Prabhu et al., method with some modifications[46]. Four groups of mice were selected for the present study and grouped as follows;

Group I: Vehicle treated Control Group **0.09%**

Group II: Aspirin Group (standard) **100 mg/kg**

Group III: AKS extract (*Acacia Karroo* Stem) **200 mg/kg**

Group IV: AKS-AgNPs (*Acaci a Karroo*-Silver nanoparticles) **200 mg/kg**

One hour (1 h) after treatment with the various drugs, animals were injected sub-plantarly with a 100 µl of 2.5% formalin solution (diluted in saline). Swiss rat were used because of their high sensitivity to pain. Nociceptive response in Swiss rat is characterised by an intense period of biting and licking of the rat paw for 5 mins after injection of diluted formalin to the hind paw. The biting subsides for 15 mins (rest period) and later commences with periodic bites and licks of the hind paw for 20-30 mins. Concurrent counts of bites and licks was recorded for the neurogenic phase (phase 1) and anti-inflammatory phases (phase 2). The percentage inhibition is then calculated using the following formula: $[1-(T/C)] \times 100$

Where, T is the number of times treated mice licked/bit the injected paw; C is the number of times control mice licked/bit the treated paw.

6.2.4.2 Statistical Analysis

GraphPad Instat® was used to perform ANOVA followed by Dunnet's test. Comparisons of the results for each treatment were done with those of the control group. Results were expresed as ± SEM (Standard Error of Mean). Statistical significant value, $p < 0.05$ was used.

6.3 Results and Discussion

6.3.1 Ultra-Violet Spectroscopy

Certain noble metallic nanoparticles especially Ag and Au, exhibit intense interactions with visible light resulting in conduction of electrons on metal surface leading to their collective oscillation. This phenomenon called Surface Plasmon Resonance (SPR), leads to remarkably strong scattering and absorption characteristics. Specifically, silver nanoparticles can display an effective extinction (scattering + absorption) cross sections ten times larger compared to their physical cross section. This collective oscillation leads to the observed UV spectrum of a typical silver nanoparticle. Furthermore, metal nanoparticle's resonance frequency is shape, size, type of metal and surrounding medium dependent [47–52].

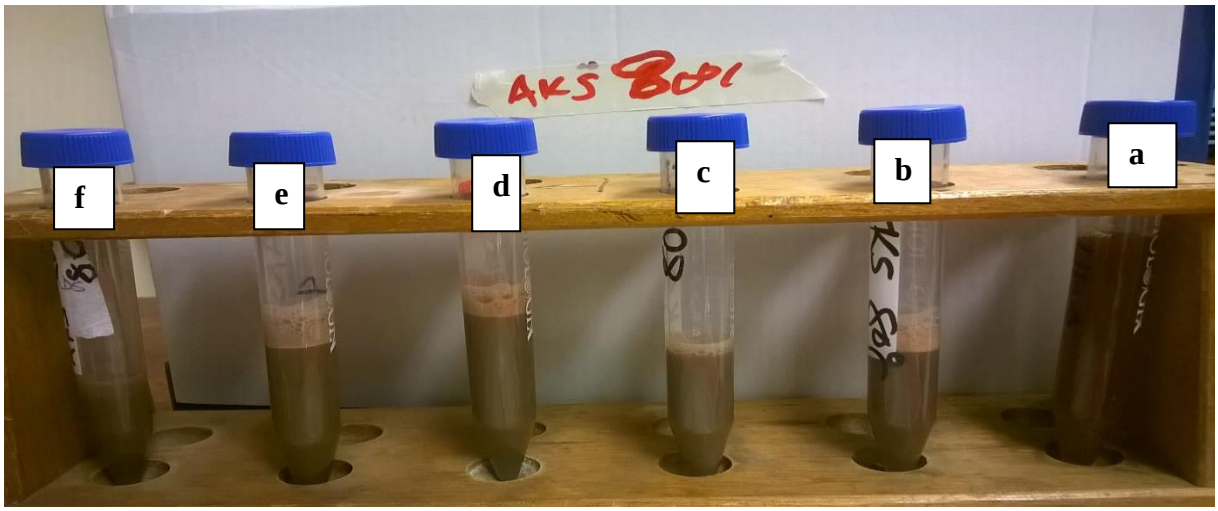

Figure 6-2; Colour change of extract before synthesis (a) after synthesis at different time interval (b) 15 mins (c) 30 mins (d) 3 h (e) 4 h and (f) 21 h.

By keeping the volume and concentration of the $AgNO_3$ precursor solution constant (10 ml, 0.1 M), the effect of temperature on bio-reduction and stability of nanoparticles was investigated. As the reaction time increased, the colour of the solution shifted from dark brown to grey-brownish colour indicating the formation of Ag-NPs (**Fig. 6-2)**. Fig. **6-3** and **6-4** show the UV spectra of the Ag-NPs synthesized at 60 ^{o}C and 80^{o}C respectively. At 60^{o}C reaction temperature, narrowness of peak centred at 470 nm was observed which could be attributed to uniform size distribution for the 15 mins to 60 mins. However, at the 3rd h, the SPR peak position gradually red-shifted to 474 nm indicating increase in particle size distribution. On the other hand, increase in reaction temperature to 80^{o}C, led to a uniform spectra for all reaction time with the SPR peak position centered at 484 nm. This could result from the stability of the AK-Ag-NPs over the reaction time. The peak broadness can be attributed to formation of particles with different sizes as shown in Fig. **6-4**. AK-Ag-NPs synthesized in this reaction condition shows that stability of the synthesized Ag-NPs is favoured by increase in temperature as observed for Ag nanoparticles synthesized from *Acacia leucophlae* [53] .

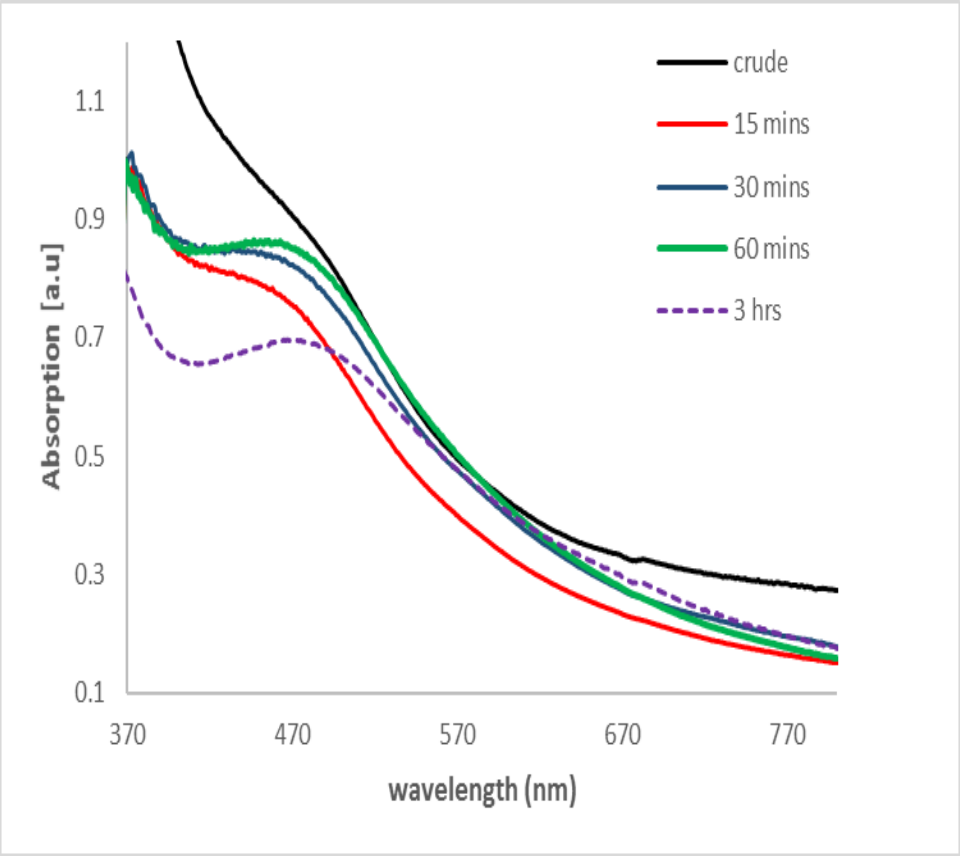

Figure 6-3: UV Spectra of AK-Ag-NPs synthesized at 60^{o}C

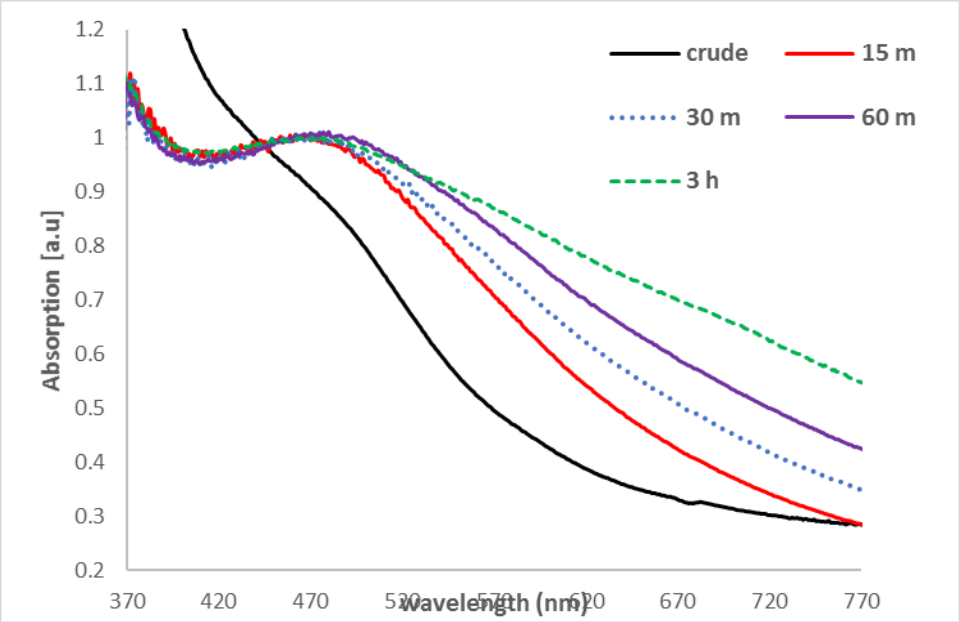

Figure 6-4; UV Spectra of AK-AgNPs synthesized at 80^{o}C

6.3.2 FT-IR analysis.

FT-IR spectra of the crude and as-synthesized Ag nanoparticles are indicated in **Fig. 6-5** and **6.6** above, with further elucidation in Table 6-1. Change in peak intensity, band shifting, appearance and disappearance of peaks are some characteristics feature of nanoparticles reduction and stabilisation. The crude sample manifests a broad absorption peak at 3409.8 which are characteristics of the stretching vibrations of the alcoholic group present on the flavonoids and the terpenoids of *A. karroo*. Other prominent peaks includes those of the C-H stretching of sp^3 which appeared at 2948 cm^{-1}, C=O of the amides at 1615.8 cm^{-1}, and C-N stretching peaks of the amine/amide family which appeared at 1400 cm^{-1}. The presence of these peaks can be attributed to the proteins and the alkaloids present in the stem bark. Some characteristics peaks observed after the synthesis include; 3754.0 and 3136.2 cm^{-1} which are peaks within the region of –OH and the N-H's stretching vibrations.

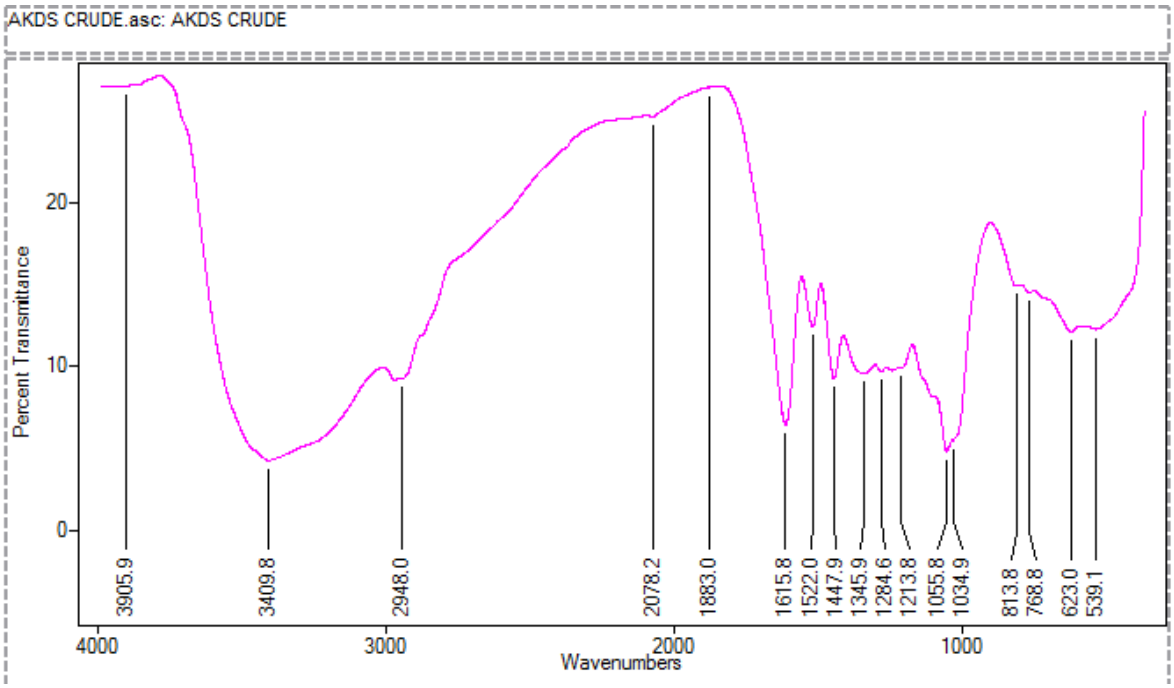

Figure 6-5: FT-IR spectra of *Acacia karroo* hydrosols (extract)

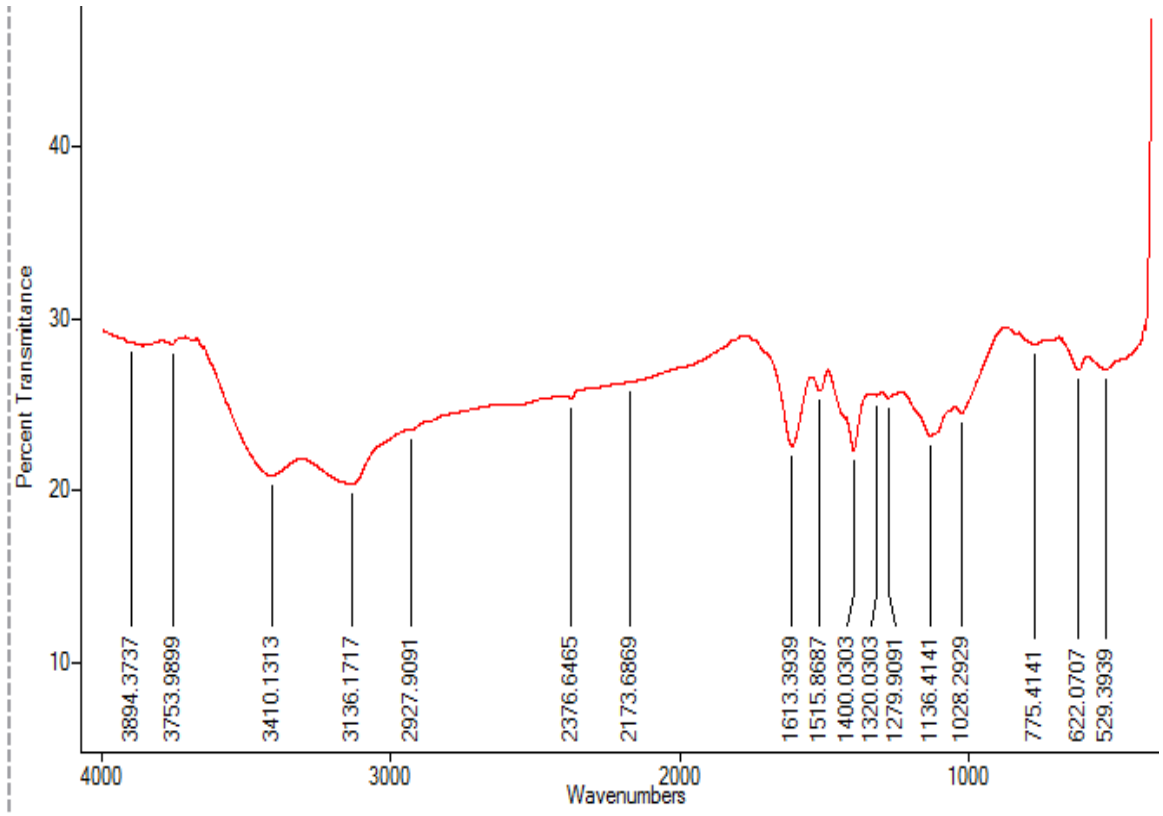

Figure 6-6: FT-IR spectra of *Acacia karroo-AgNPs synthesized at 80^{o}C*

The conversion of the broad –OH on the crude extract to two significant peaks, shows the effect of reduction/ capping ability of the extract.[54]. Absorption at 2078.2 cm^{-1} corresponding to the - Diazo (RCH=N-N, stretching) peak was shifted from 2078.2 cm^{-1} to 2176.6 cm^{-1}. This could be attributed to the effect of resonance or conjugation of the diazo functional group. Shift were also observed for the C-N bond (1284.6 to 1279.9 cm^{-1}), C-O-C bond (1213.8 to 1136.4cm^{-1}) and the N-O symmetric stretch (1345.9 to 1341.6 cm^{-1}). Other peaks include O-H bend (950-910), 910-665 N-H wag, and the 900-675 C-H ("oops") bending vibrations [28]. The synergistic effect of these functional groups could had resulted into the reduction of the metallic salt as well as subsequent capping of the nanoparticles. The change in the spectra shows the bio-reduction and capping ability of *Acacia karroo* in the synthesis of silver nanoparticles.

Table 6-1: Summary of the bands and their respective functional groups transformations of *A. karroo* and AK- Ag-NPs.

λ (cm^{-1}); *Acacia karroo* crude	**AK-Ag-NPs**	**Functional group**	**References**
3409.8	3410.1	-OH or NH of tertiary amines	[55]
Absent	3136.2	-OH or NH vibrations of 1^o, 2^o amines, amides or alcohol	[56]
2948.0	2927.9	C-H asymmetric stretch of CH_3	[57]
2078.2	2173.7	RCH=N=N stretching of the diazo compounds	[58]
1883.0	Disappear	5-membered pyran resulting from pyranose group/ Ag-O bond	[30,55]
1615.8	1613.4	C=O of the amides from the proteins	[30]
1522.0	1515.9	C=C-C aromatic ring stretch common to chromones moieties of the flavonoids	[30]
1447.9	1400.0	C-N bonds of the amino groups/ symmetric vibrations of the carboxylate group (-COO-)	[59]
1345.9	1341.6	N-O Aromatic nitro groups	[55]
1284.6	1279.9	C-N bonds of aliphatic amines	[56]
1213.8	1136.4	C-O stretch of phenol	[55]
1055.8	Disappear	-C-O stretch of alcohols	[59]
1034.9	1028.3	$-OCH_3$ of ether linkage in the chromones	[60]
900-540	Below 800	C-H bending of the Vinyl group, C-halogen bonds stretch, C-H "oops" of aromatic, N-H wag of 1^o and 2^o amines	[55]

6.3.3 X-ray diffraction (XRD) studies

The crystalline nature of the dried powder of the as-synthesized Ag-NPs was confirmed by X-ray diffraction analysis. The XRD pattern shows numbers of Bragg's reflections that can be indexed on the face centred cubic (fcc) of silver. The peaks at the 2θ degree values of 38.37°, 44.35°, 64.60°, 77.06°, and 81.39° corresponds to (111), (200), (220), (311) and (222) respectively when compared to the standard silver nanocrystals, JCPDS card No: 89- 3722).

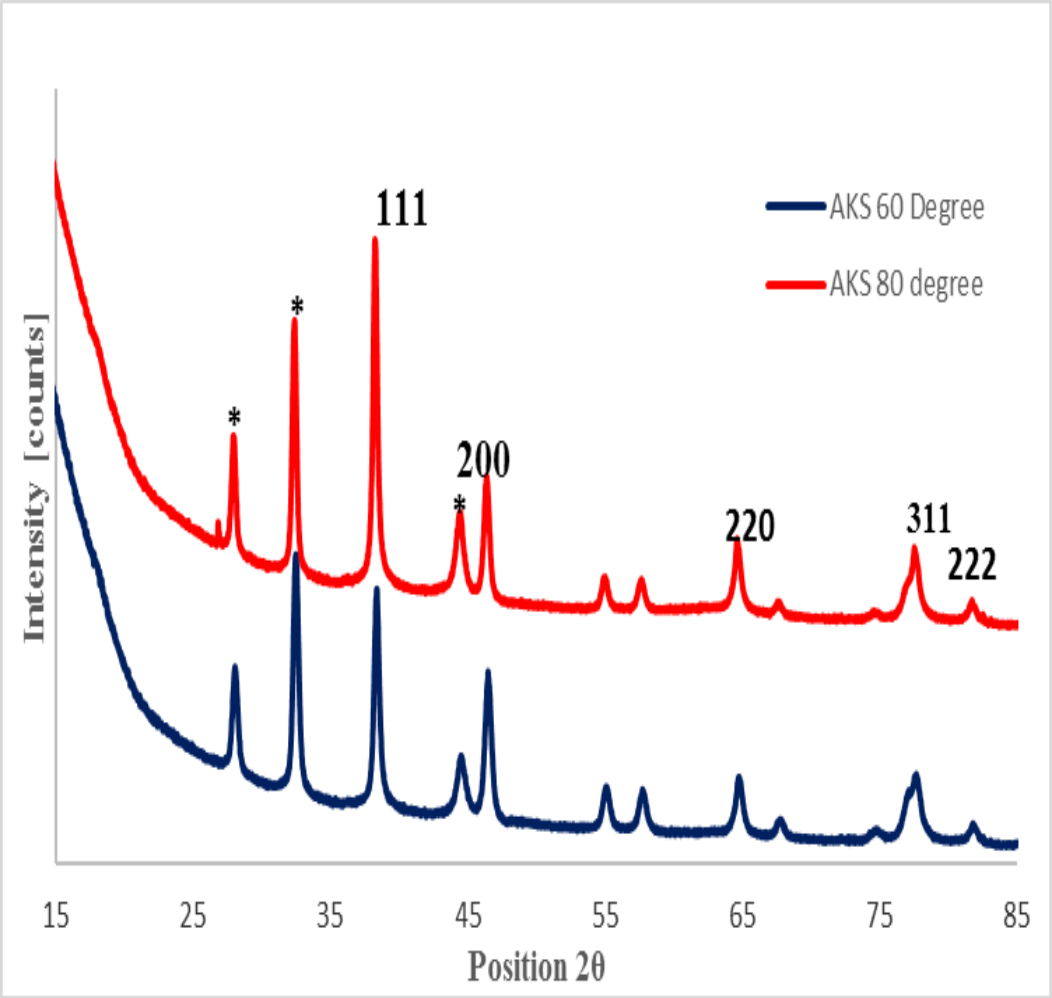

Figure 6-7; X-ray diffraction pattern of the AK-Ag-NPs. Labelled peaks correspond to the characteristic diffraction peaks of elemental Ag°.

The unassigned peaks at 28.03°, 32.50° and 46.47° may be ascribed to the bio-crystalline components or the amorphous organic phase. The lack of any peaks resembling metals apart

from that of pure silver in the spectra confirmed the purity of the synthesized Ag-NPs. In addition, peak broadening indicated the monocrystalline nature of the Ag-NPs. The calculated average crystallite size shows that increasing the reaction temperature from 60^{o}C to 80^{o}C led to an increase in size from 17.82 nm to 20.34 nm, as shown in Table 6-2. This could be attributed to the fact that as smaller particles are formed in this temperature, they easily agglomerate to form larger nanoparticles through the Ostwald ripening.

Table 6-2: Average particle diameter of silver nanoparticles synthesized using 50 ml of extract and 10 ml of 0.1 M $AgNO_3$ at 60^{0}C.

Peak angle	**Diameter (nm); Sherrer's Equation**
32.57	20.51
46.53	18.42
64.78	18.24
77.64	9.83
81.39	22.12
Average	**17.82**

Table 6-3: Average particle diameter of silver nanoparticles synthesized using 50 ml of extract and 10 ml of 0.1 M $AgNO_3$ at 80^{0}C

Peak angle	**Diameter (nm); Sherrer's Equation**
38.31	26.51
44.46	15.74
64.67	21.02
77.58	14.43
81.97	24.02
Average	**20.34**

6.3.4 SEM and EDS Analysis

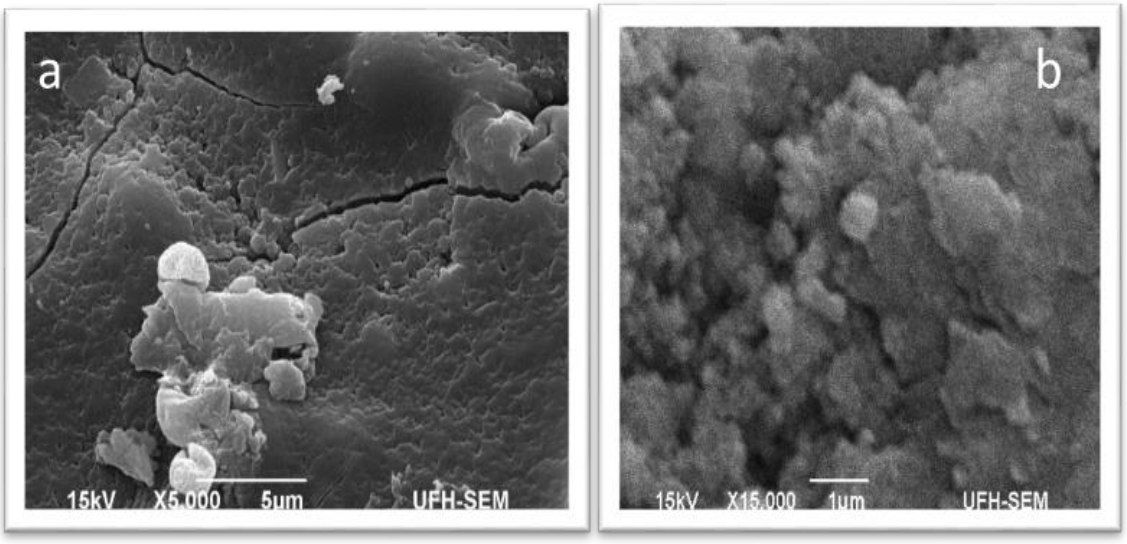

Figure 6-8; SEM analysis of (a) *Acacia karroo* crude extract (b) *Acacia karroo*- AgNPs

The morphology of the synthesized nanoparticles were further characterised by scanning electron microscope. **Figure 6-8a** and **6.8b** show the micrograph of the crude sample and the synthesized nanoparticles. The micrographs at other magnifications are shown in Appendix **D**. The micrograph showed that the silver nanoparticles are spherical and poly dispersed with large agglomeration. Similar observation was reported using the extract of *Ocimum sanctum*[61] and *Ipomoea indica* flowers[62] .

Fig. 6-9 shows the energy dispersive spectrum of the AK-Ag-NPs which confirmed the presence of silver. The relative weights of the respective metals are shown as well.

Metallic nanocrystals mostly display a distinctive optical absorption peak at 3 KeV as a result of SPR and thus can be confirmed for the silver formation in the nanoparticles.

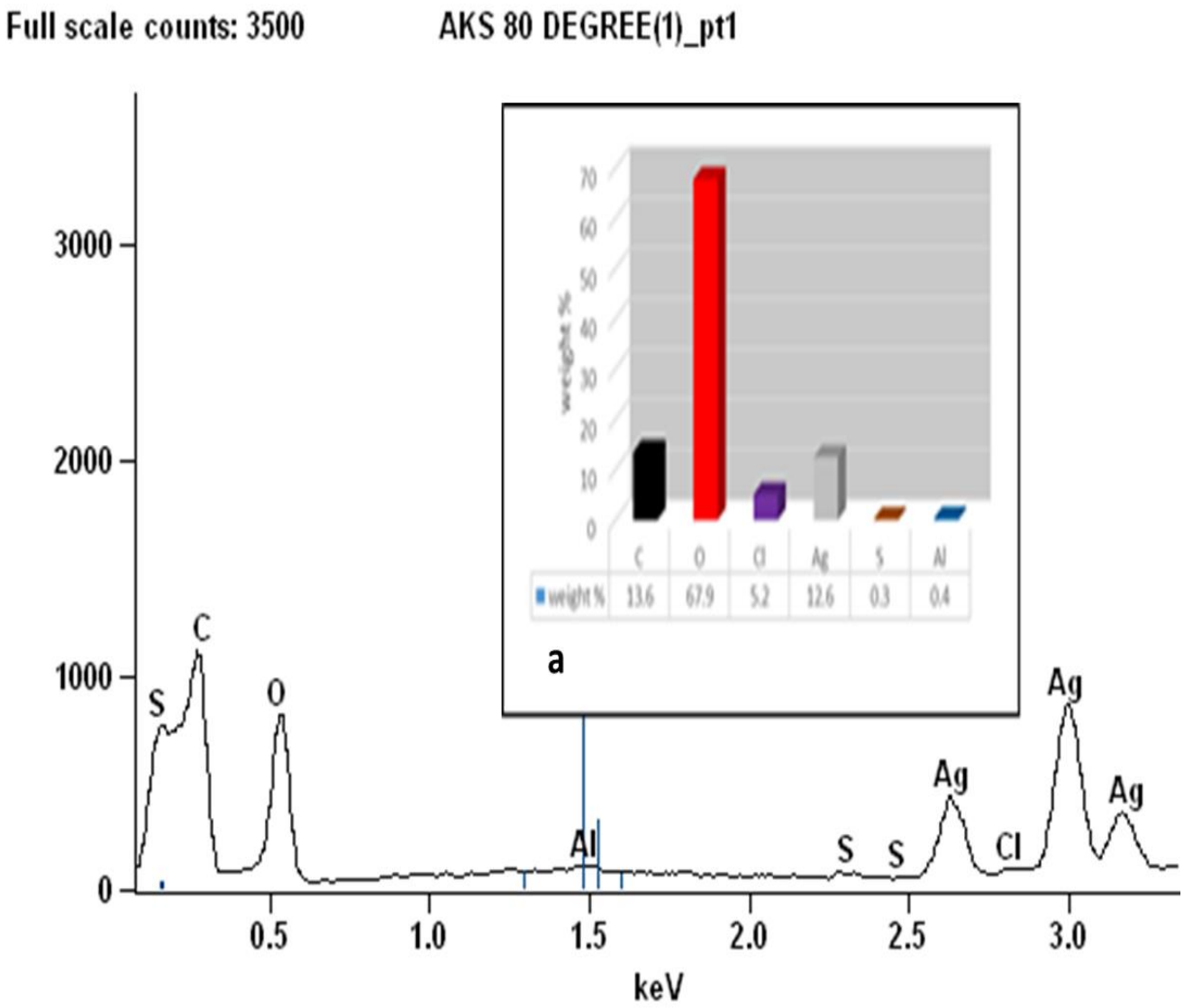

Figure 6-9: EDS spectra of the synthesized AK-Ag-NPs at 80^{o}C Inset (a) Graphical description of the weight % of elements

6.3.5 Transmission Electron Microscopy (TEM)

Fig. 6-10 and 6-11 shows the TEM images of the silver nanoparticles synthesized from *Acacia karroo* dry stem. The nanoparticles are spherical, polydisperse and some irregular shapes at 100 nm. However, images at 20 nm shows a well dispersed, spherical nanoparticles. The coated black spots on the surface of the nanoparticles could be ascribed to the capping influence of the metabolites in the plant material.

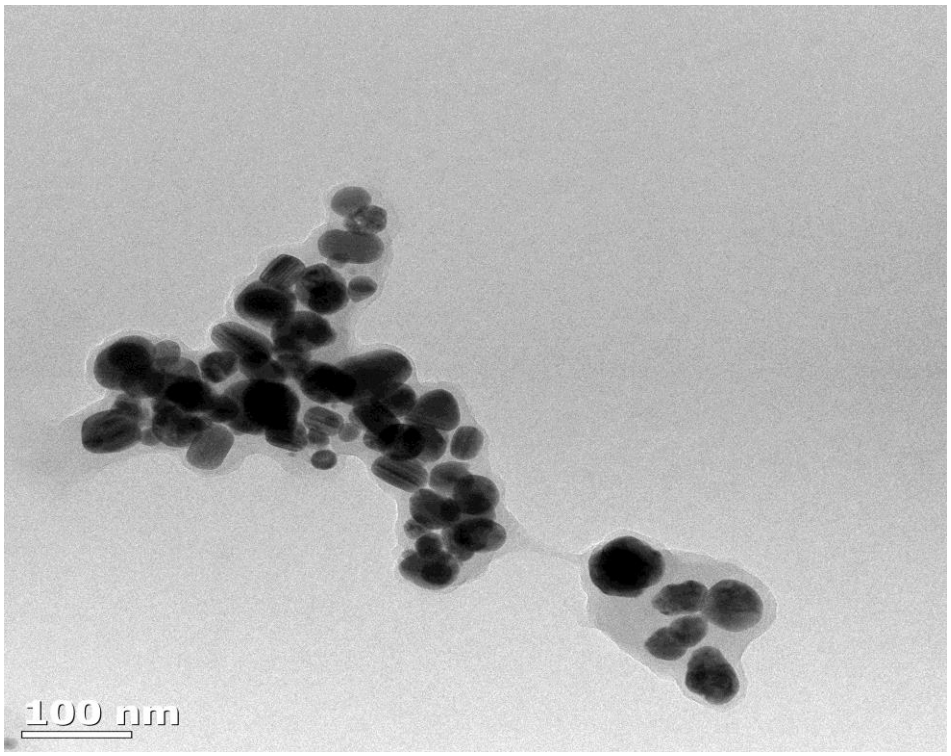

Figure 6-10: TEM images of Ag-NPs synthesised with 50 ml extract of *A. karroo* and 0.1M $AgNO_3$ at 100 nm.

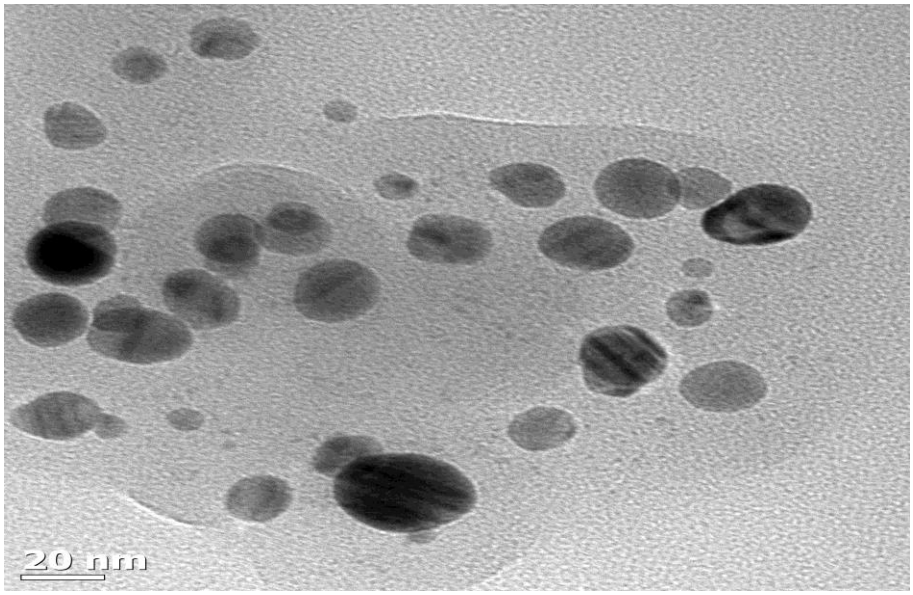

Figure 6-11: TEM images of Ag-NPs synthesised with 50 ml extract of *A. karroo* and 0.1M $AgNO_3$ at 20 nm.

6.3.6 Formalin-induced nociception:

The effect of silver nanoparticles as potent anti-inflammatory agent had not been fully investigated. However, the wound healing effect of silver nanoparticles had been widely reported[63–65]. The mechanisms of wound healing and inflammation are closely related as they involves the formation and oedema, followed by mediation of inflammation mediators, such as cytokines, prostaglandins, NO and the histamines[66]. A typical Formalin inducement produces a kind of flinching and licking characteristics on the mice. This test is a universally accepted model for explaining pain and analgesia pathways when compared to other mechanical/ physical test like tail flick[67]. As shown in **Table 6-4** , the model constitutes of two separate phases. The early phase normally peaked 0-5 min (neurogenic phase), results in interaction of the drug with the opioid system, accompanied by the release of substance P via the central mechanism of inflammation followed by a period of remission. At the expiry of the relapse time, the phase 2 begins and last for about 10-60 mins. These phase is

characterised by the release of several inflammatory mediators inhibited by non-steroidal anti-inflammatory drugs (NSAID)[68].

Table 6-3 shows the analgesic effect of the synthesized AK-Ag-NPs on mice administered with formalin. As expected, aspirin used as the standard drug significantly inhibited the 1st phase (83%) and 2nd (73%) phases of the test. The crude sample and the as-synthesised AK-AgNPs reduced both phases of the formalin test and the results were significant which is in agreement with previous reports. The plant extract exhibited moderate inhibition at the neurogenic phase (39%) as compared to the synthesized AK-Ag-NPs which shows higher rate of inhibition (51%) with significance of $p<0.01$.

Table 6-4: Antinonicepive effects of AKDS-Ag-NPs on formalin-induced pain

		Neurogenic phase (0-5mins)		Inflammatory phase (10-30mins)	
Treatment	Dose (mg/kg)	No of licks	Inhibition (%)	No of licks	Inhibition (%)
Control	Normal saline	42.3 ± 1.5		30.5 ± 1.7	
Standard (Aspirin)	100 mg/Kg	7 ± 1.3**	83	6.8 ± 1.4**	78
AKDS crude	200 mg/Kg	26 ± 2.1**	39	4.7 ± 1.3**	85
AK-AgNPs	200 mg/kg	20.8 ± 0.8**	51	20± 2.9**	39

Values expressed as mean ± SEM. (n=5), *p<0.05, **p<0.01

The analgesic properties of the AK-Ag-NPs reduce in the inflammatory stage when matched to the neurogenic phase. Rapid activity of the nanoparticles synthesized is an indication of the high absorption of AKDS-Ag-NPs at the early stage of the analysis, which indicates that it can be used as an opioid regulatory drug. Other suggestive factors for the low inhibition at

the inflammatory phase could be the dose, solubility and the rate of inhibition. Previous report had shown the high anti-inflammatory potential of *Acacia karroo* extracts as confirmed by this analysis[69]. Conclusively, the synthesized Ag-NPs block both phases although the effect was more evident at the neurogenic phase.

6.4 Conclusion

We have demonstrated the reductive and stabilising ability of the hydrosol extract of *Acacia karoo* on silver nitrate to yield a nanoparticle having a mean diameter of 10-20 nm at 80^{o}C. This synthetic pathway is simple, economical and void of hazardous chemicals. The analgesic potential of the synthesized Ag-NPs is significant as observed from the formalin-induced pain on mice.

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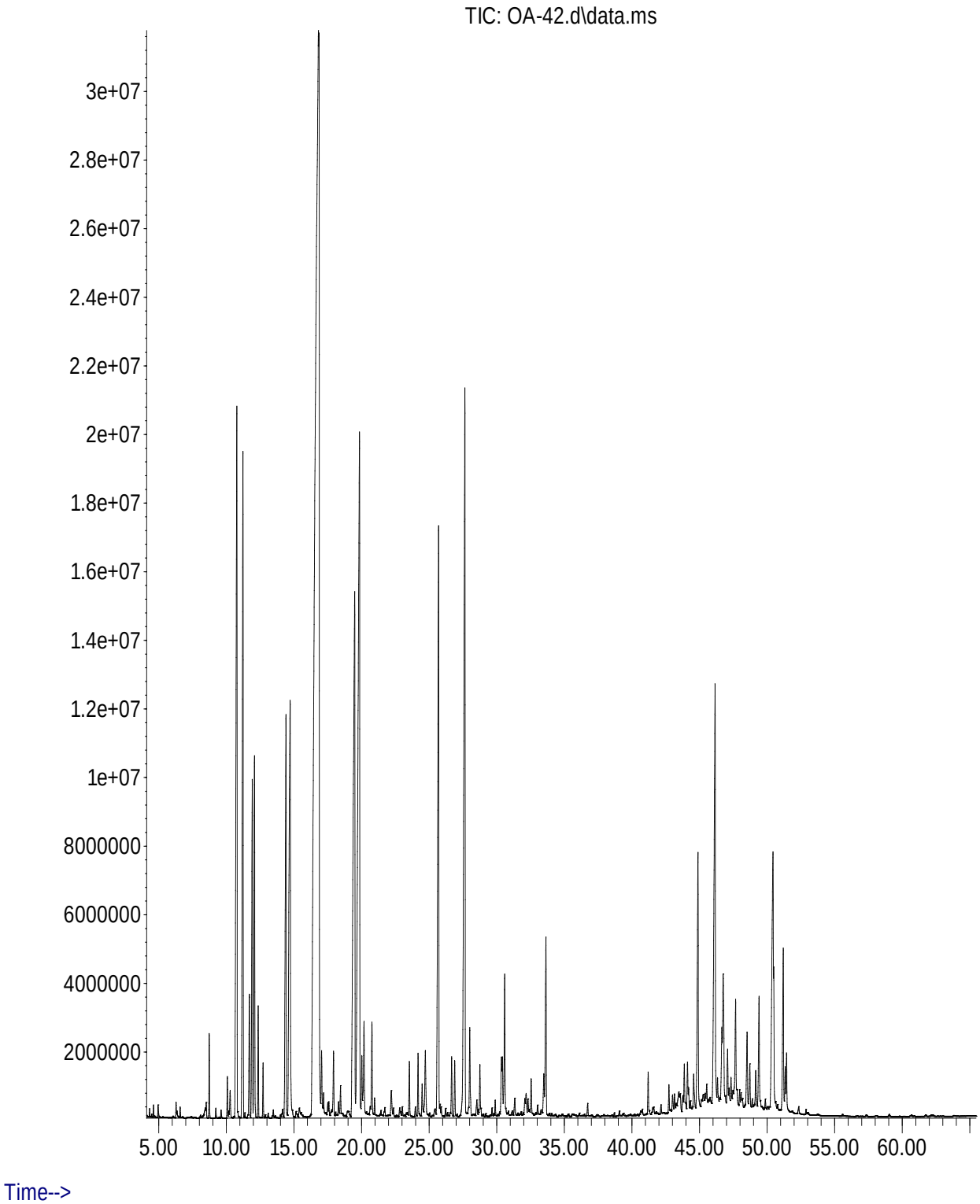
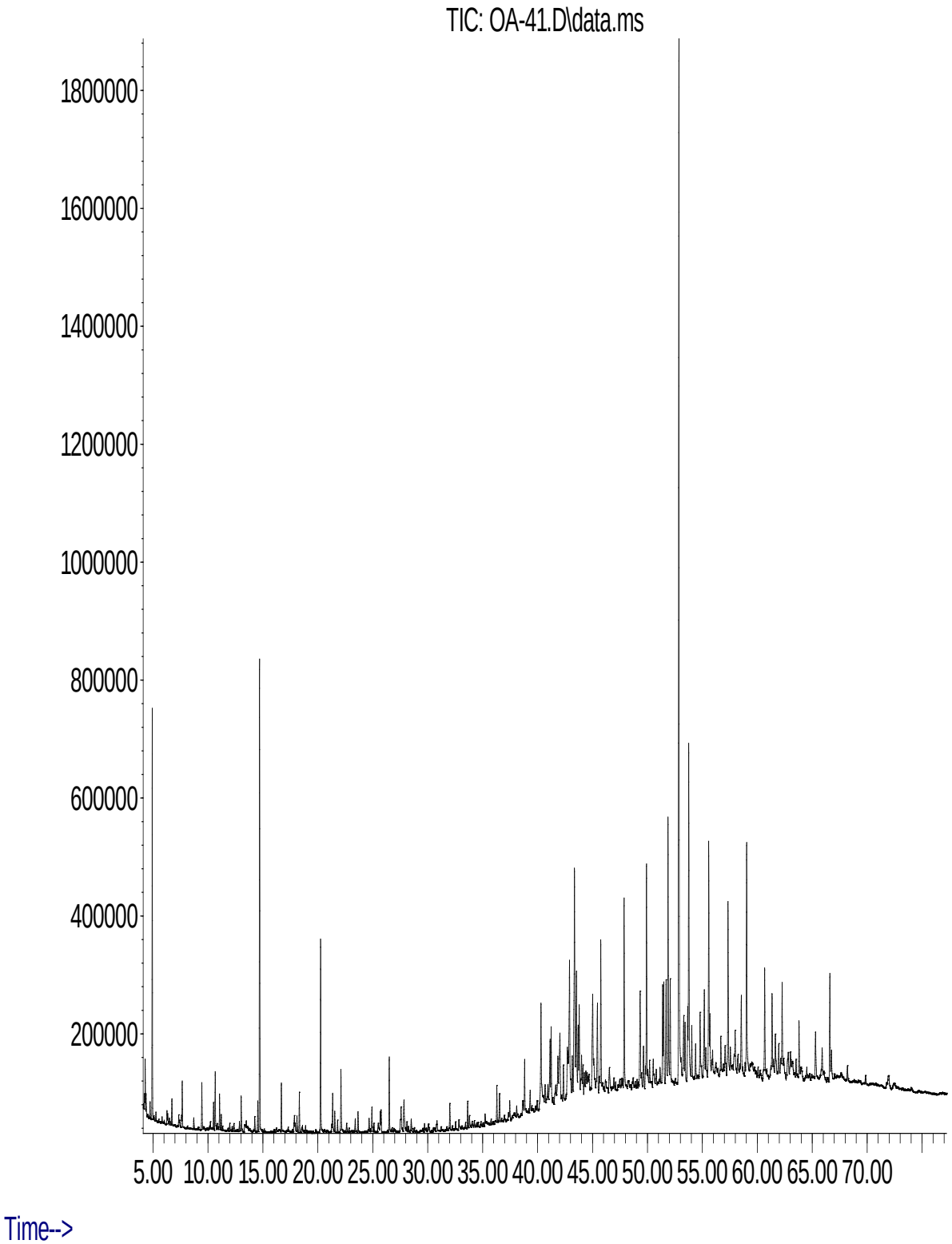
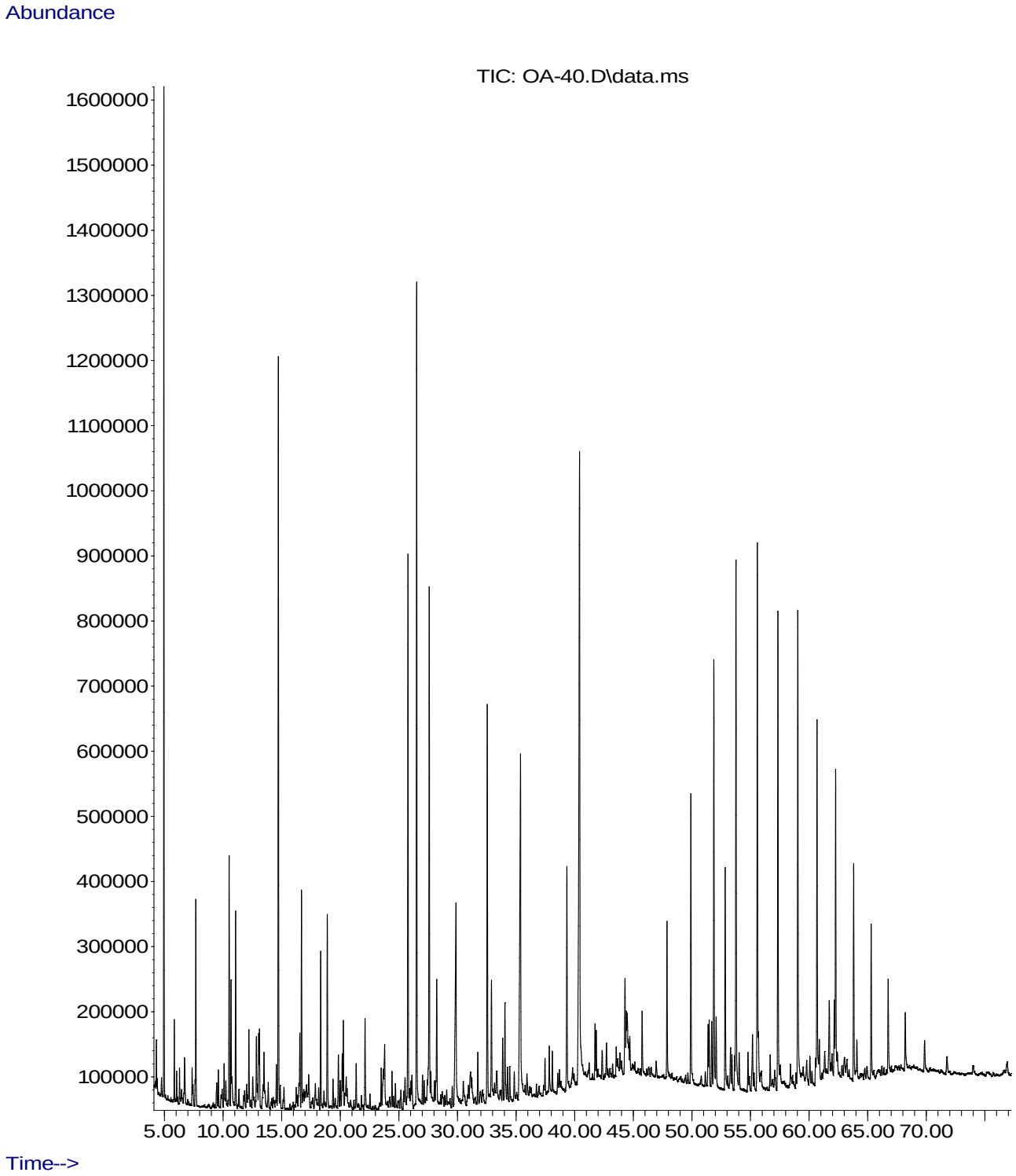
APPENDIX A- 1: GC-MS of *Acacia mearnsii* dry leaves



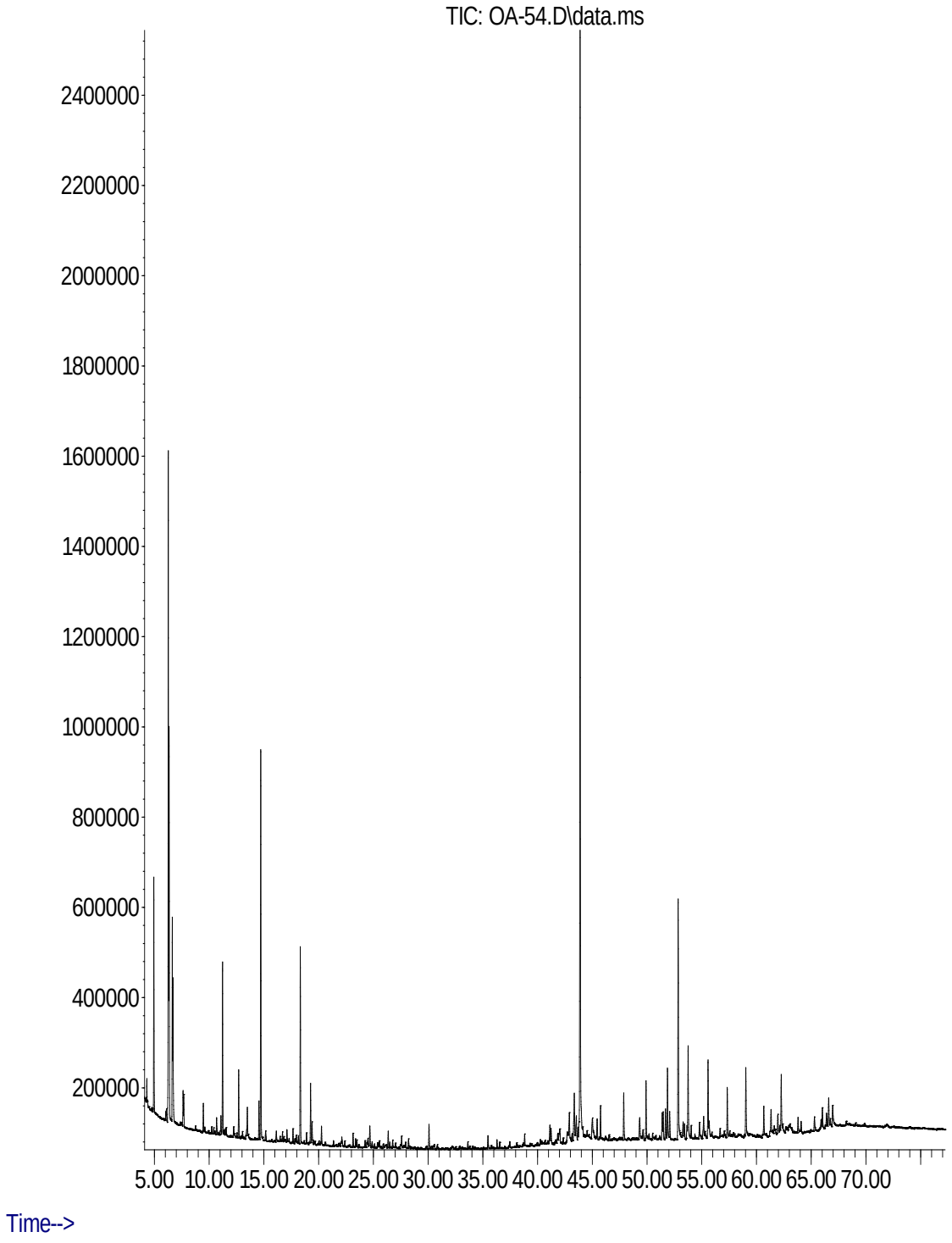
APPENDIX A- 2: GC-MS of *Acacia mearnsii* fresh leaves



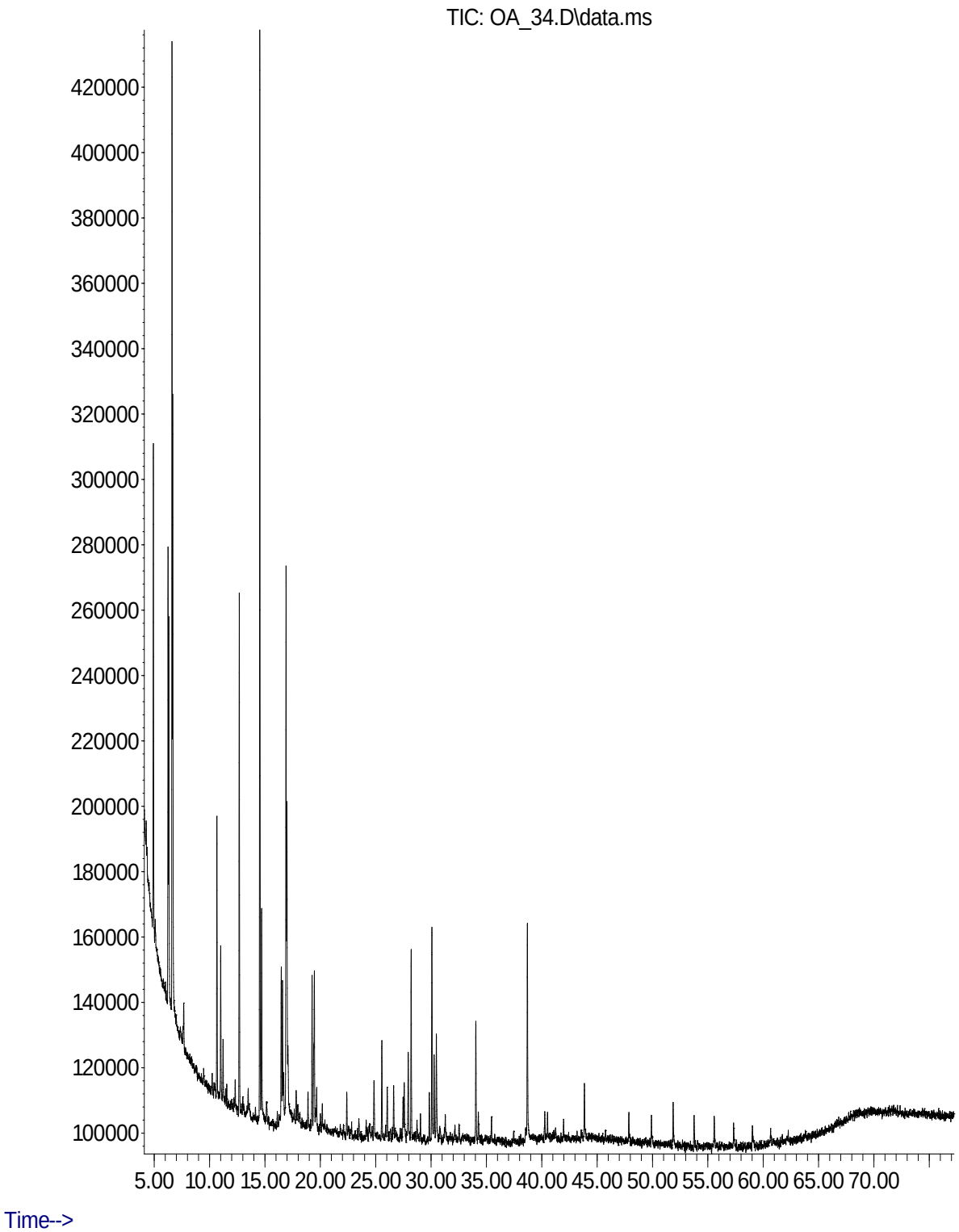
APPENDIX A- 3: GC-MS of *Acacia mearnsii* dry stem



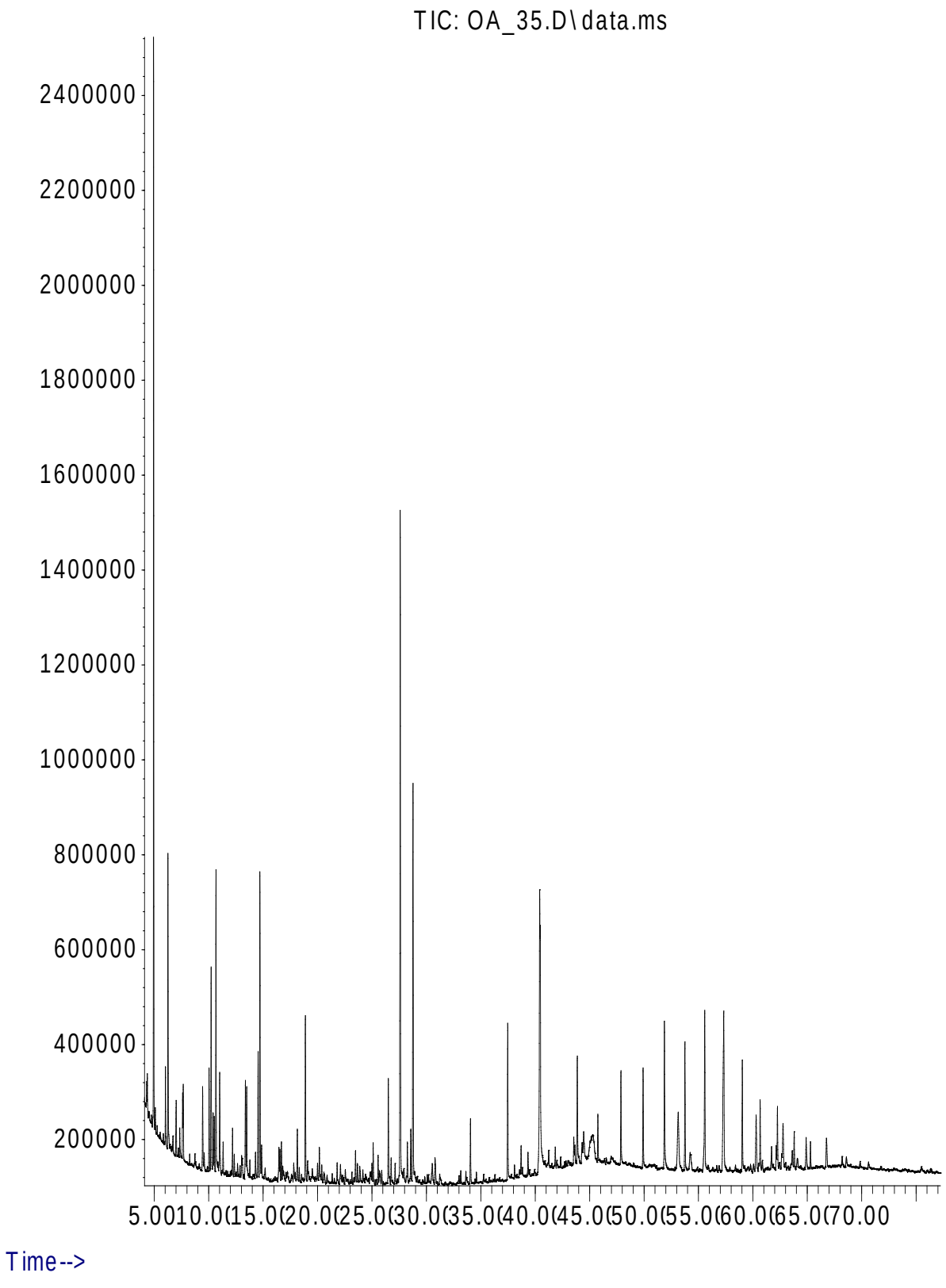
APPENDIX A- 4: *Acacia mearnsii* fresh stems



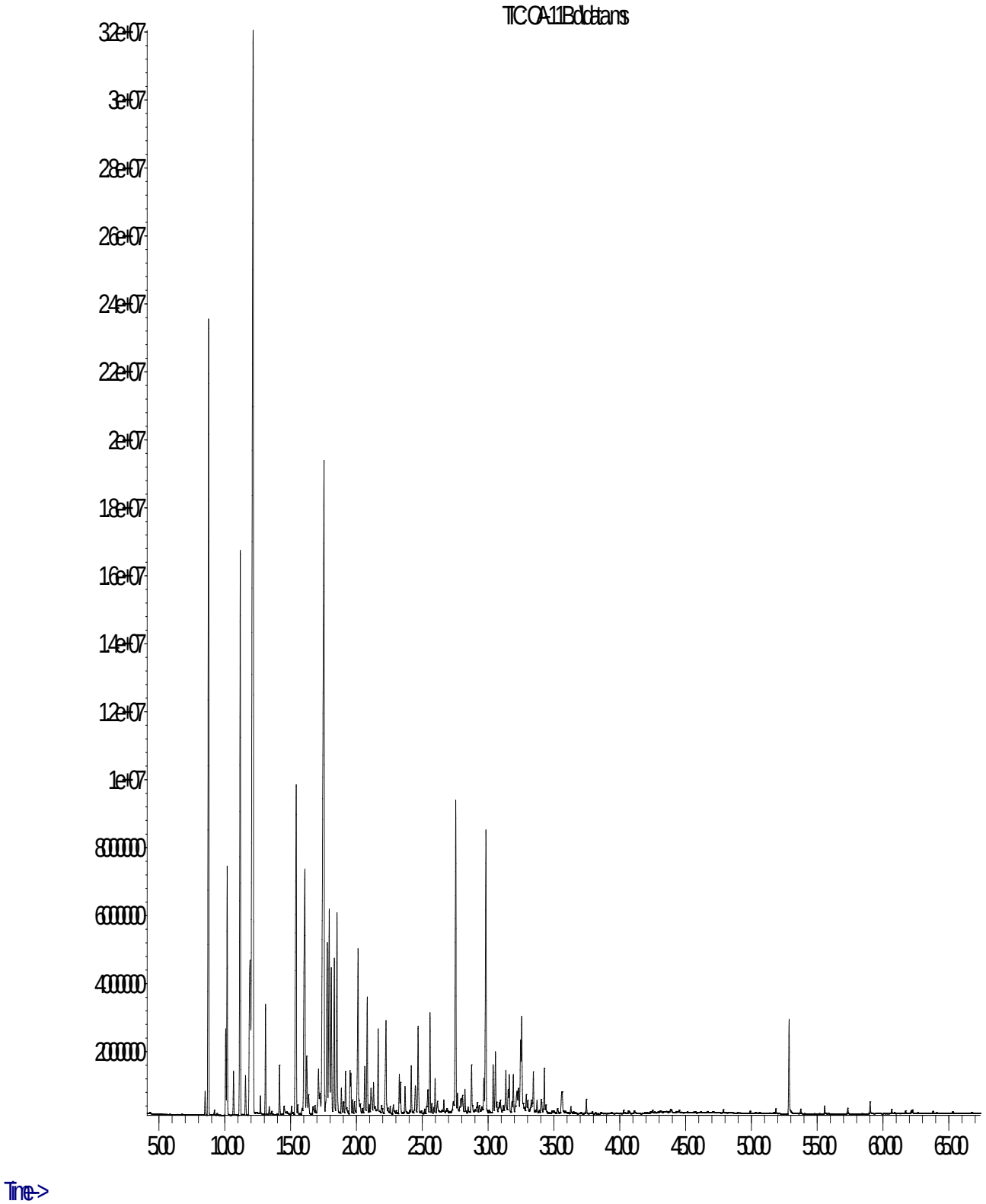
APPENDIX B- 1: *Acacia karroo* fresh leaves



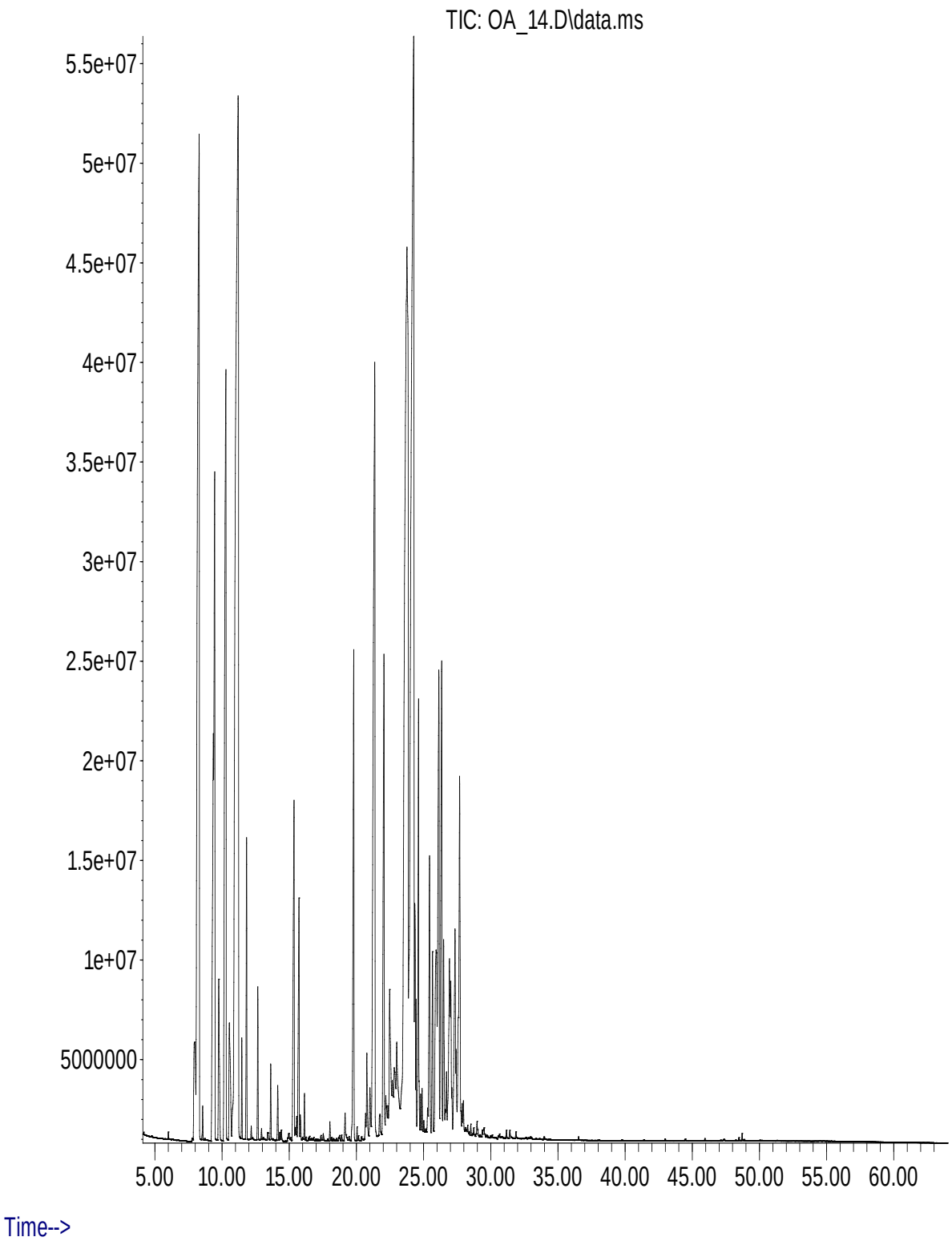
APPENDIX B- 2: *Acacia karroo* dry leaves.



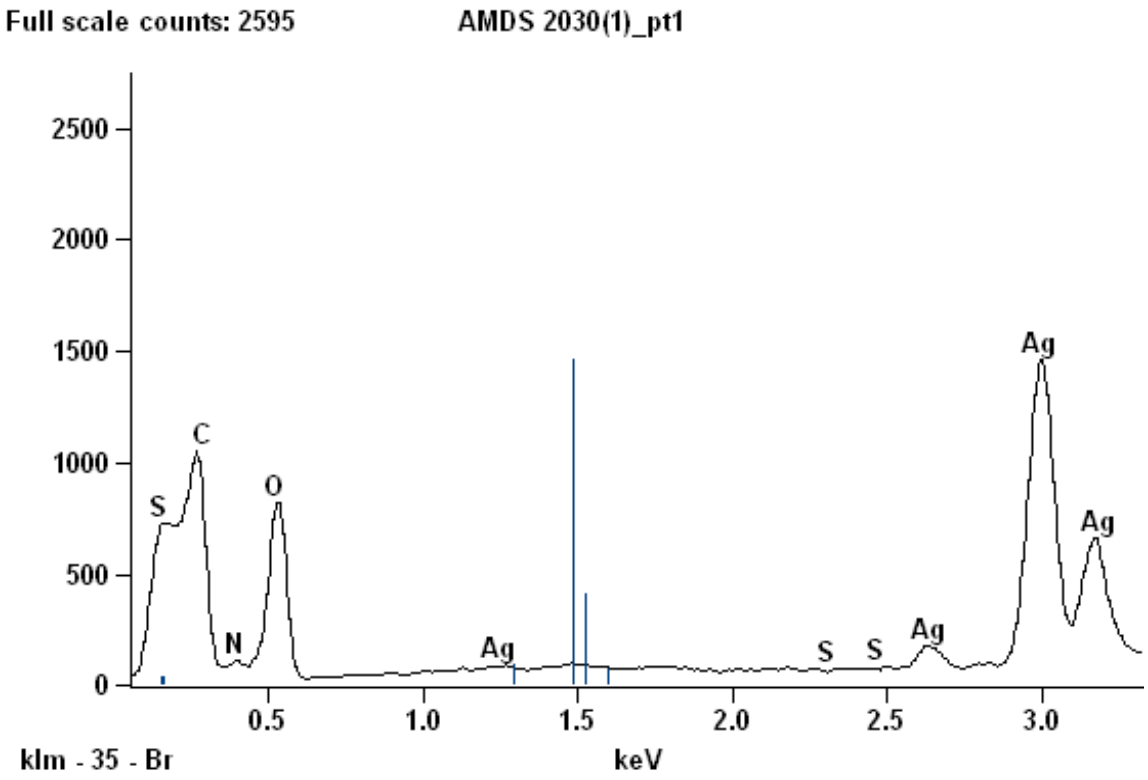
APPENDIX B- 3: GC-MS of *Acacia karroo* fresh stem bark



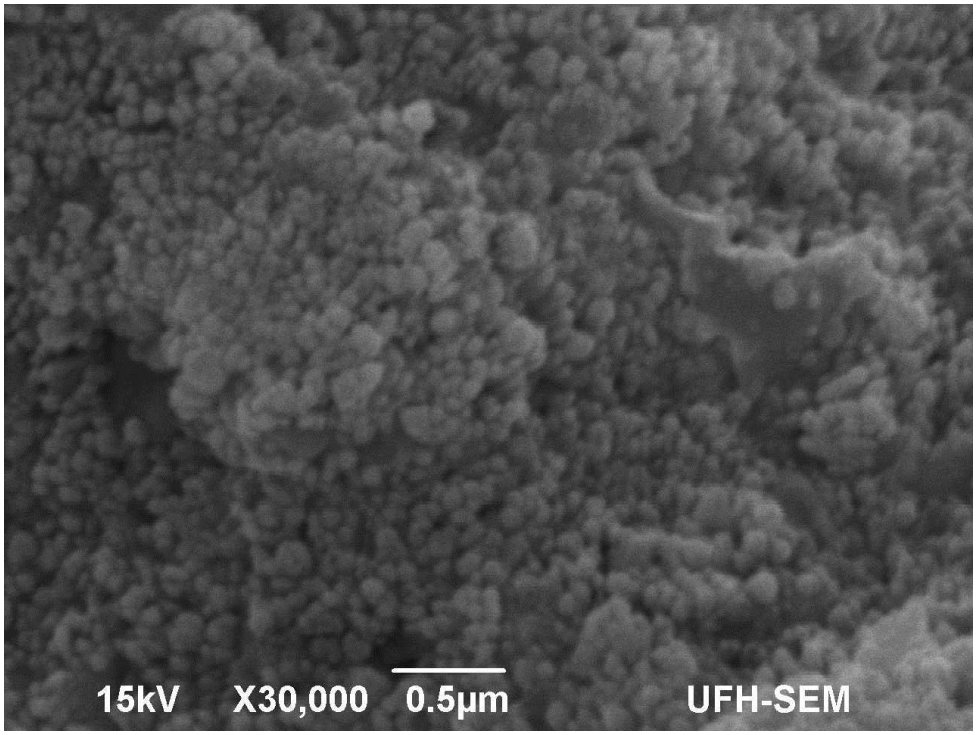
APPENDIX B- 4: GC-MS of *Acacia karroo* dry stem bark



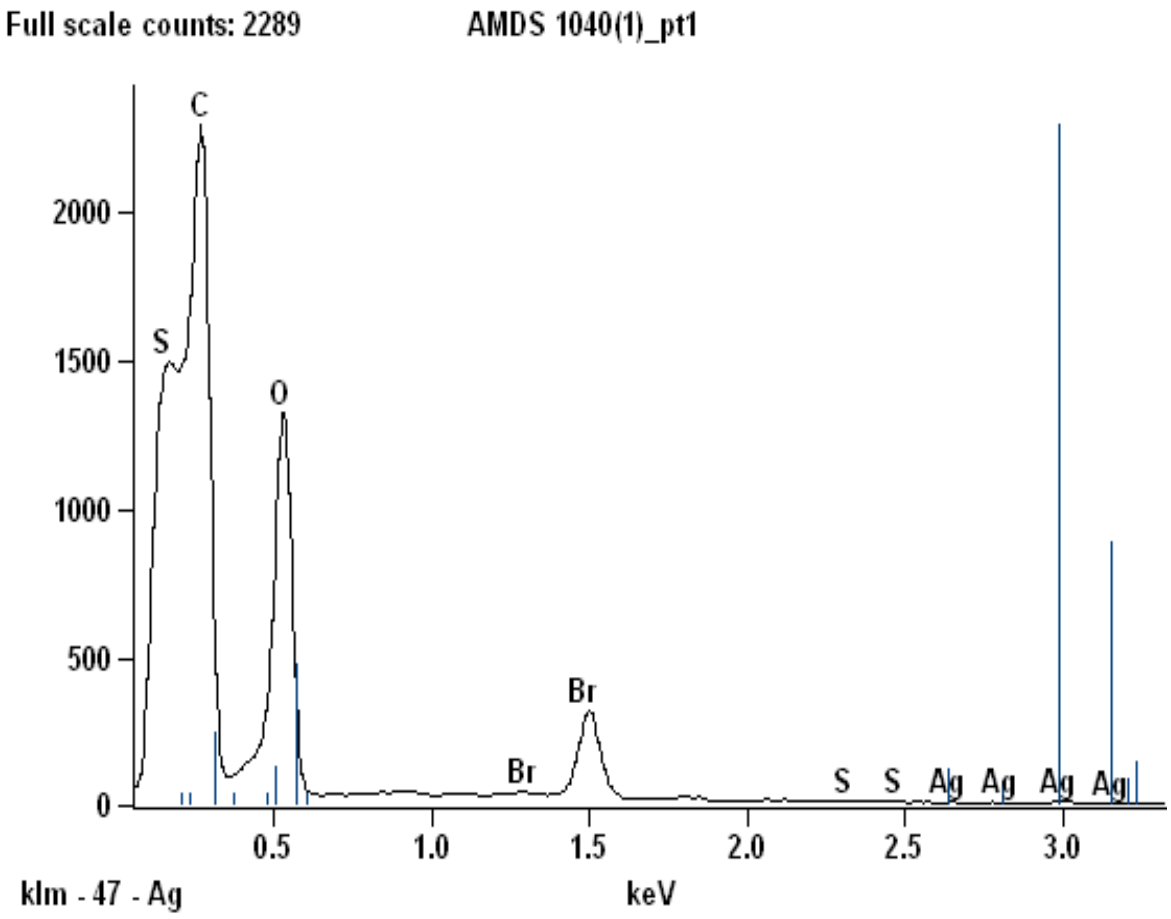
APPENDIX C- 1: EDX spectrum of AMDS-Ag-NPs synthesised at 20:30 (extract: $AgNO_3$)



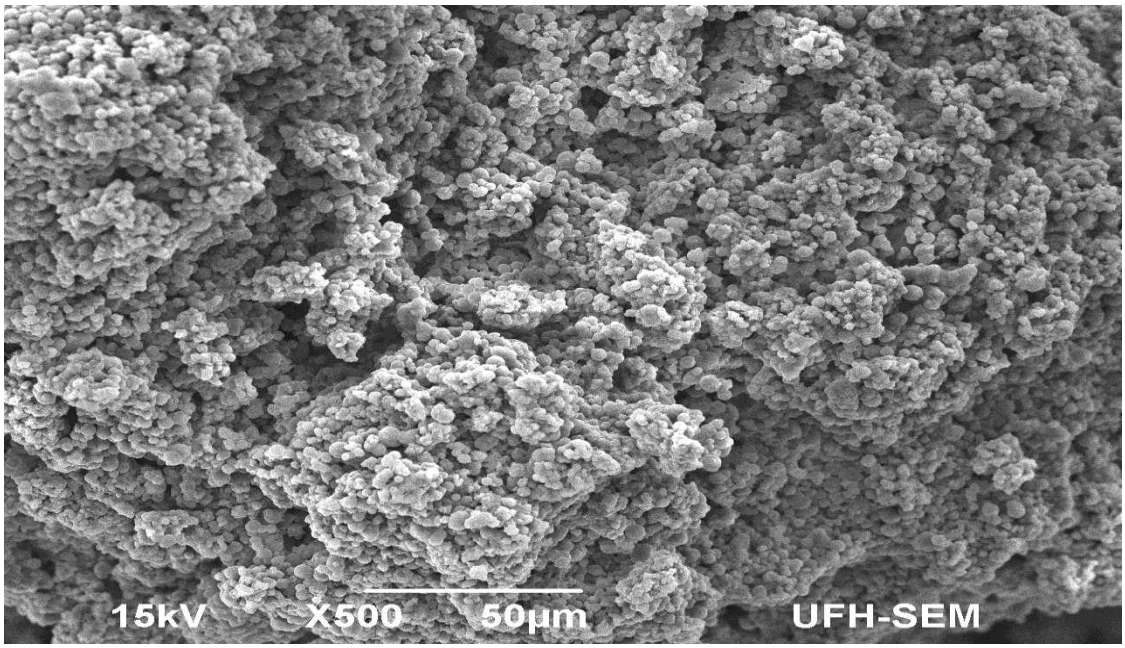
APPENDIX C- 2: SEM micrograph of AMDS-Ag-NPs 20:30 at mag. X 30,000



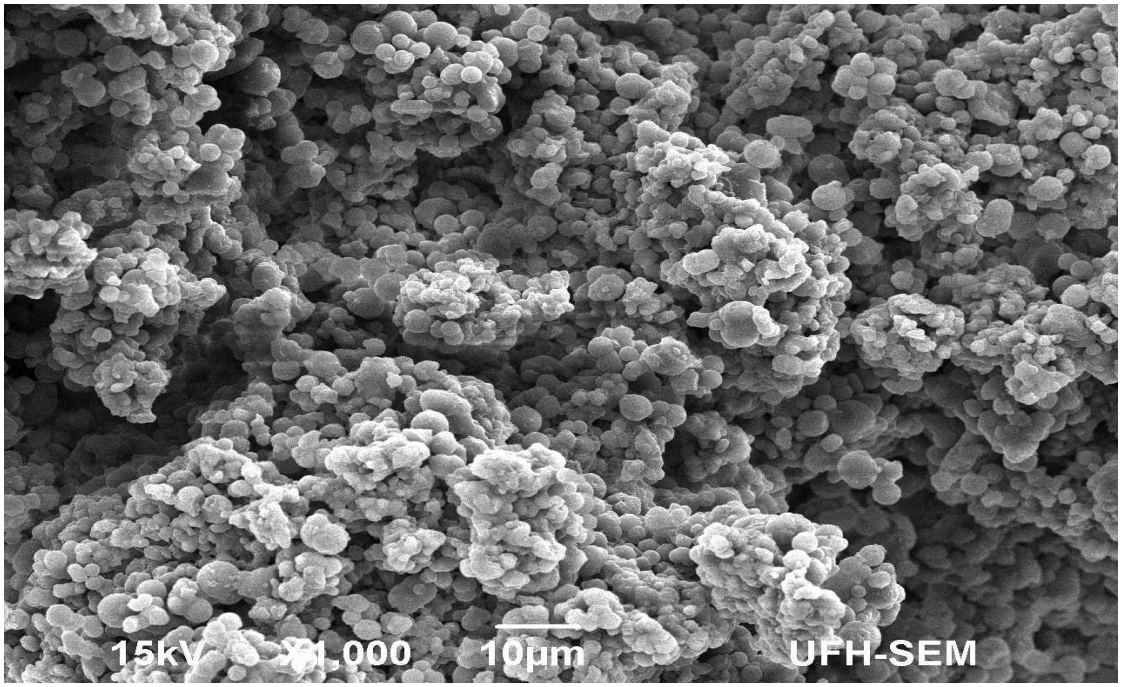
APPENDIX D- 1: EDX of AMDS-Ag-NPs at 10: 40



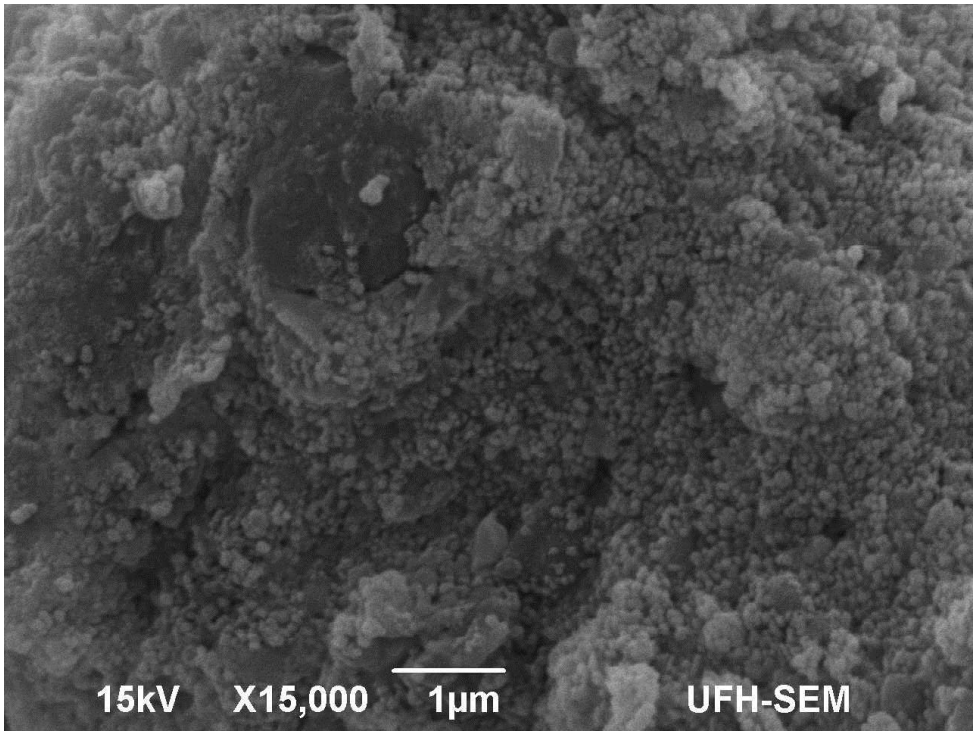
APPENDIX D- 2: SEM autogram of AMDS-Ag-NPs at 60 degree



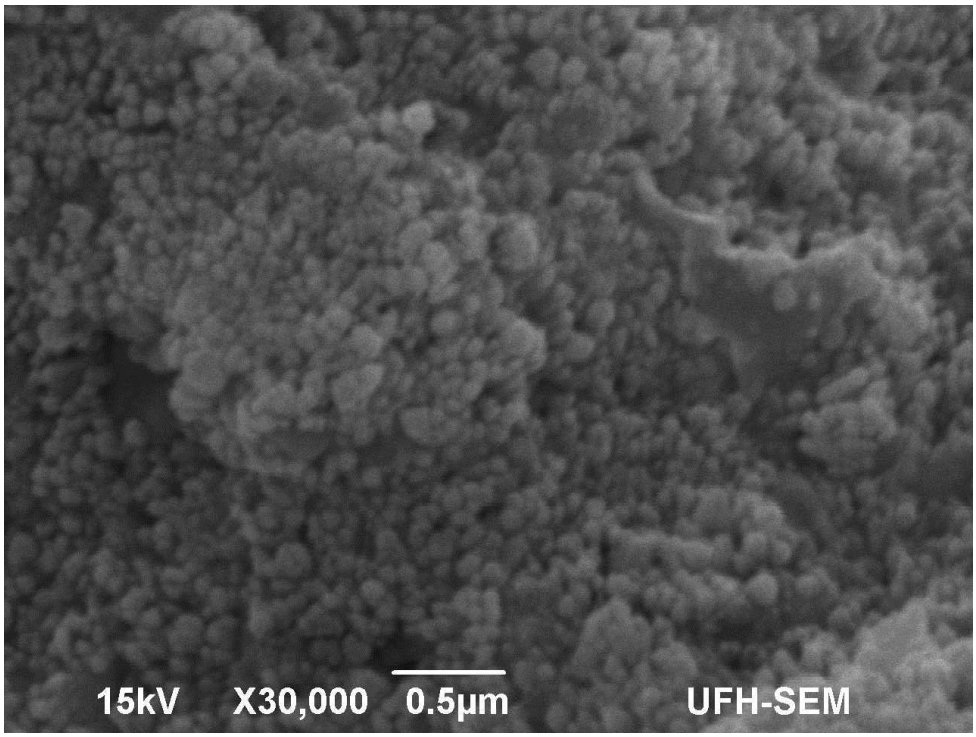
APPENDIX D- 3: SEM autogram of AMDS-Ag-NPs at 60 degree X1, 000



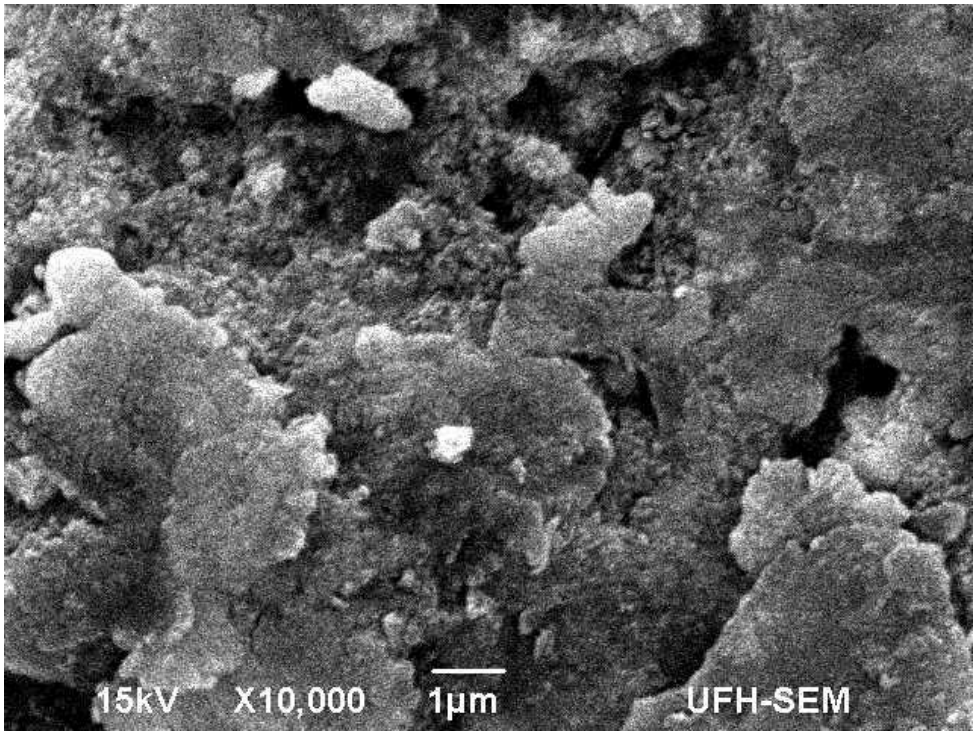
APPENDIX D- 4: SEM autogram of AMDS-Ag-NPs at 60 degree X15, 000



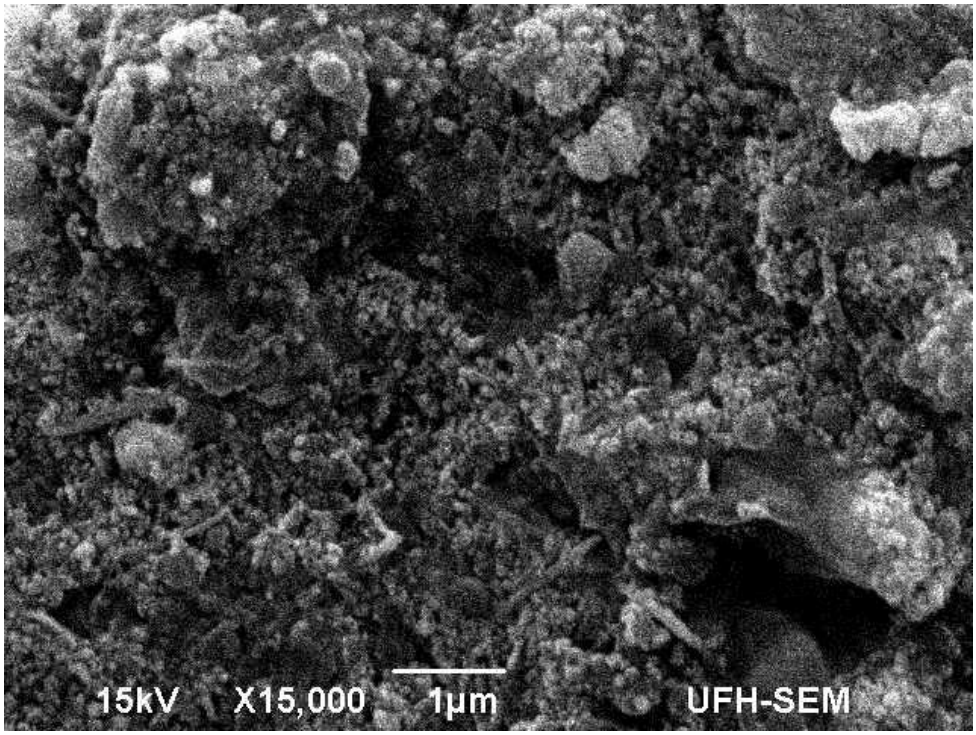
APPENDIX D- 5: SEM autogram of AMDS-Ag-NPs at 60 degree X30, 000



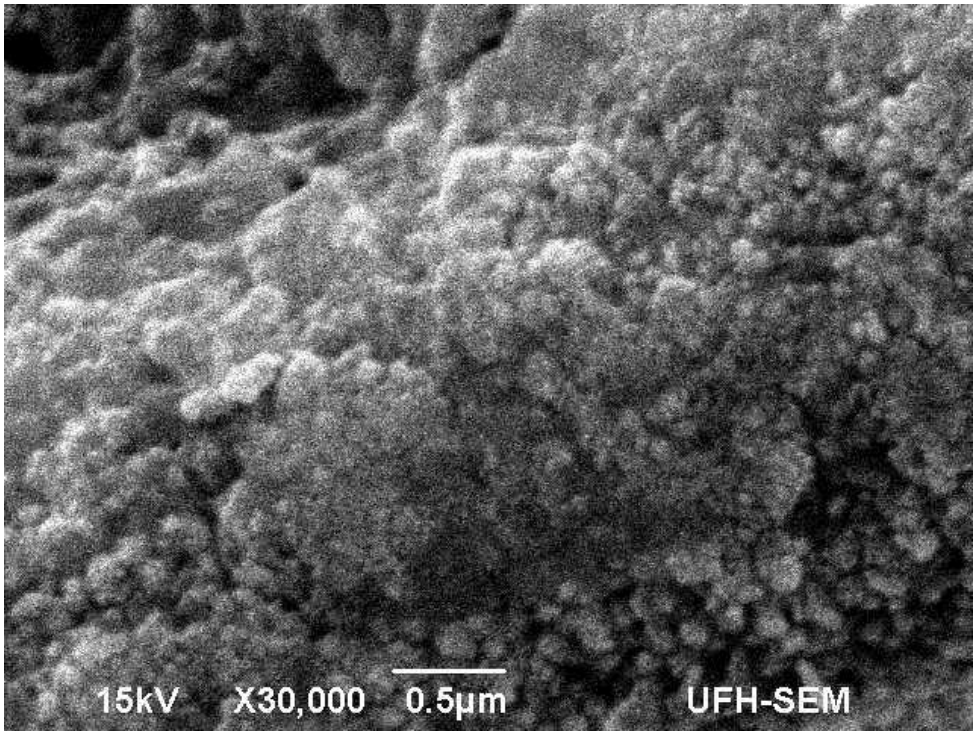
APPENDIX E- 1: SEM autogram of AMDS-Ag-NPs at 40 degree X 10,000



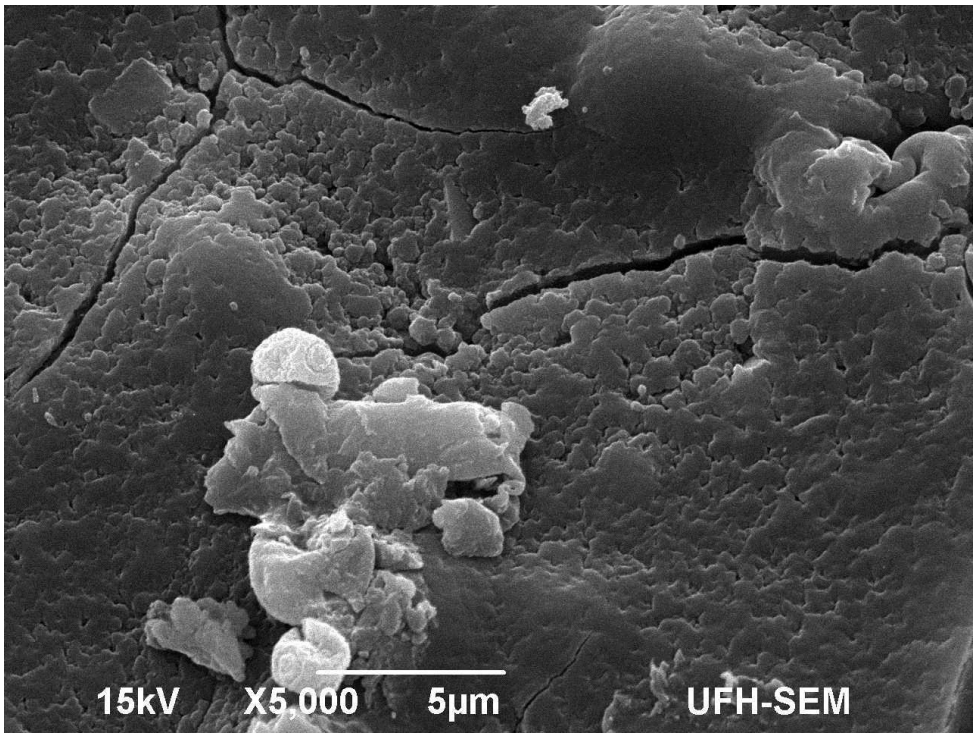
APPENDIX E- 2: SEM autogram of AMDS-Ag-NPs at 40 degree X 15,000



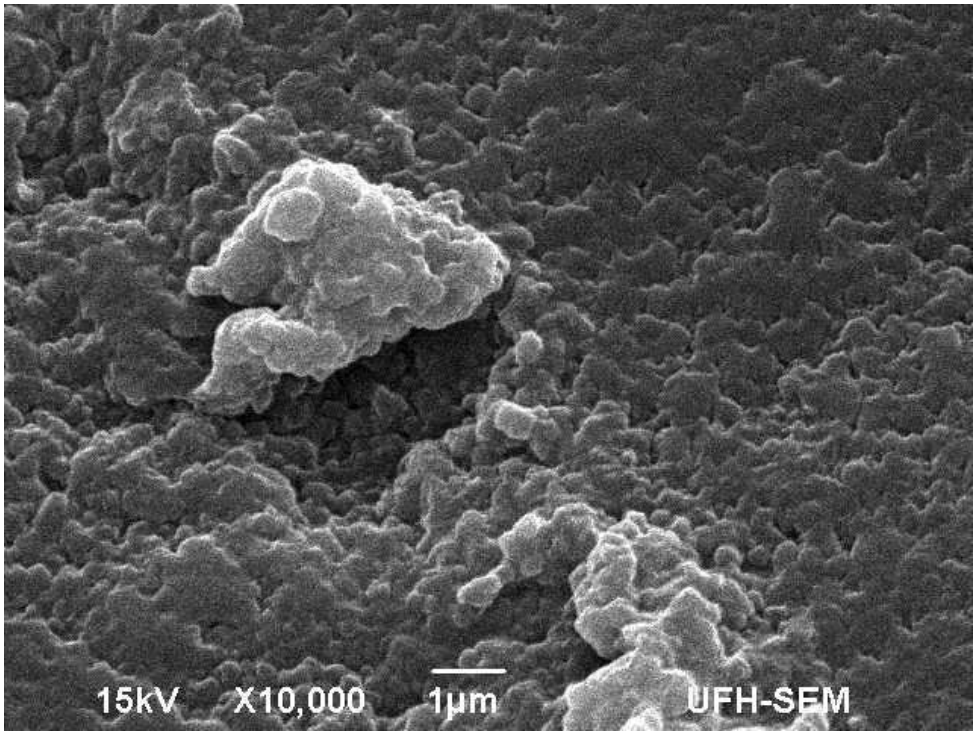
APPENDIX E- 3: SEM autogram of AMDS-Ag-NPs at 40 degree X30, 000



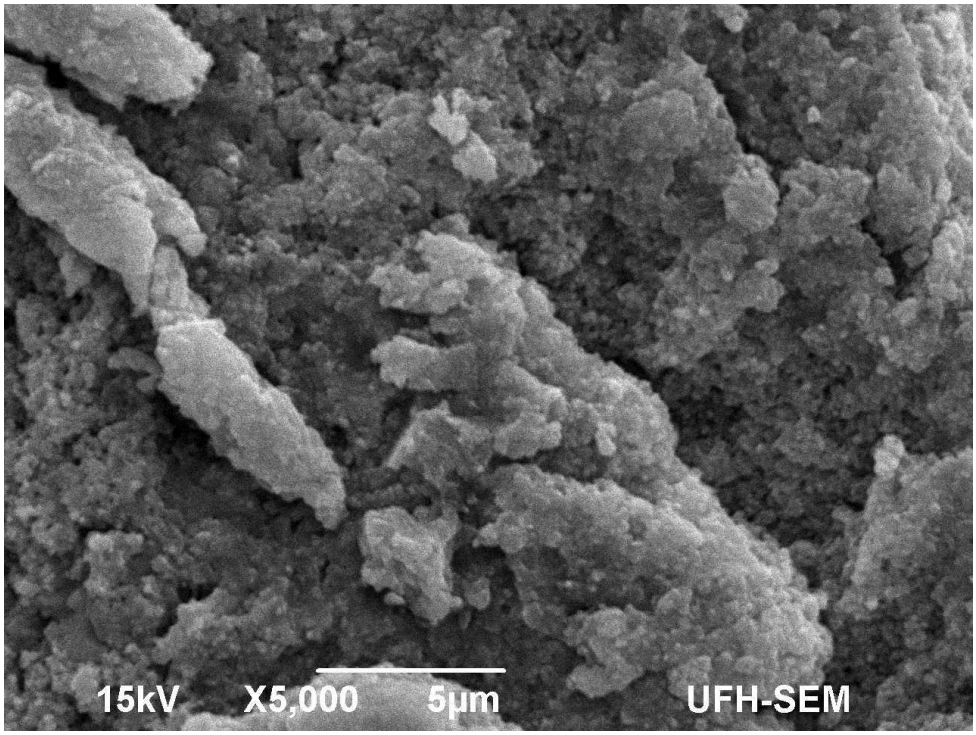
APPENDIX F- 1: SEM autogram of AKDS crude X5, 000



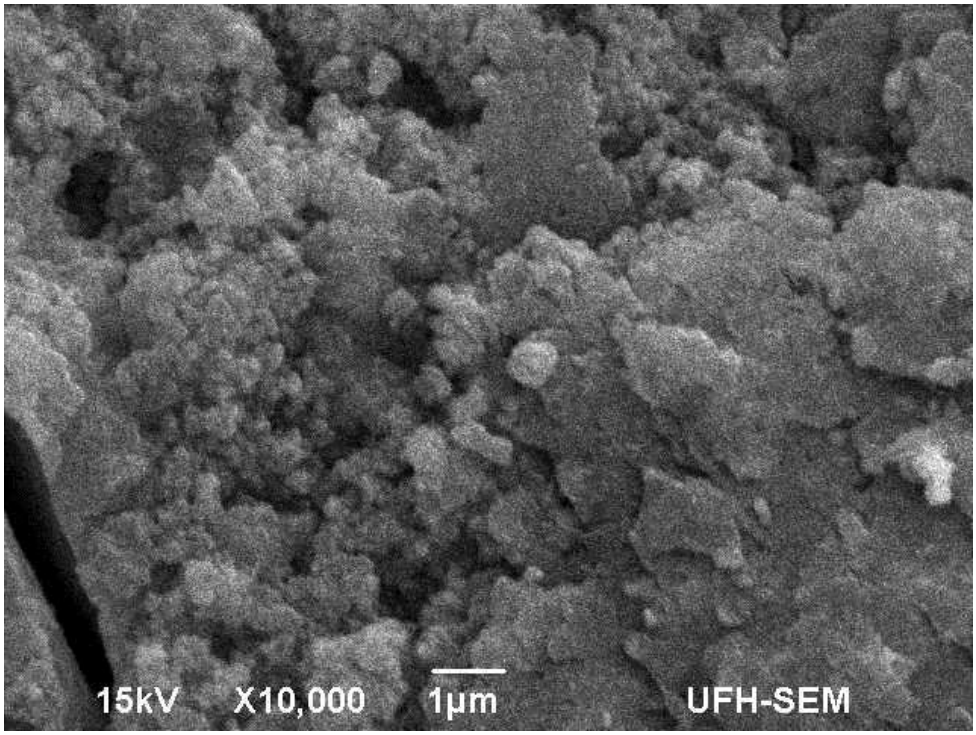
APPENDIX F- 2: SEM autogram of AKDS crude X 10, 000



APPENDIX G- 1: SEM autogram of AKDS-Ag-NPs at 80 degree X 5, 000



APPENDIX G- 2: SEM autogram of AKDS-Ag-NPs at 80 degree X 10, 000



APPENDIX G- 3: SEM autogram of AKDS-Ag-NPs at 80 degree X 15, 000