Chapter 1

General Introduction

1.1 Introduction

Mankind has always relied on plants not only because of their nutritive value but for disease cure and control. The practice of traditional medicines through herbal practitioners has been shrouded in secrecy and appears mystic to the extent that some traditional healers use religious operations to determine nature of illness and the right prescription [1]. Traditional medical practice is a health practise that involves all beliefs, approaches and knowledge pertaining to the use of plants to cure disease, relieve pain and perform ritual practises [1]. An herbal medicine is any remedy obtained exclusively from plant sources either in the raw state or as pharmaceutical combinations [2].

World Health Organisation (WHO) describes a medicinal plant as a plant whose parts can be used in the management of disease [3-4]. It also estimates that 80% of the world’s population regard plant medicine as a primary source of health care and as such many countries recognise the use of plant-based traditional medicine in their health care delivery systems [5]. However, the struggle has been to document traditional medicinal plants, establish effective dosages and contraindications for the safe and efficient use of these herbal remedies [5]. Herbal remedies form part of their folklore and this knowledge has been passed on for thousands of years through oral tradition [6]. Traditional herbal remedies are often multi drug formulations and they are viewed to provide leads to drug discovery. Even though the therapeutic value of certain plants has been proven wrong, many plant based remedies are based on first hand practical experience and empirical findings of lengthy periods of time [7].

The increasing cost of pharmaceutical drugs and modern cost of health care has led many people to turn to plant medicine. This has also been compounded by the introduction of multi drug resistant pathogens to pharmaceutical drugs and the side effects associated with them.
Multi drug formulations and enhancement of the potency of the herbal drugs may provide a panacea to drug resistance. This has resulted in combinations between herbal and synthetic drugs and involvement novel forms of disease cure like nanoparticles [8]. Over reliance on herbal remedies has led to over harvesting of medicinal plants to meet increasing demand. Cultivation has been viewed as a means to counter resource depletion and also to meet demand for mass production of plant medicinal drugs. This has been found to relieve harvest pressure which has a large potential to upset ecological systems. Pressure has often been felt for those slow growing plants that are prone to destructive harvesting due to the use of the whole parts of the plant such as roots, stem and the aerial parts of the plant [9]. However cultivation or replenishment of harvested plants is not the only means to the end of destructive harvesting since some plants take too long to grow. Alternative sources of medicine need to be sought.

The major drawback with traditional herbal remedies is lack of scientific validation to confirm their therapeutic values and toxicity levels. This has been primarily due to lack of documented scientific evidence on the negative effects and contraindications of these herbal remedies. The lack of scientific knowledge or lack of clinical data on the components of these herbs has lead to many herbal practitioners to ignorantly prescribe these herbs. However knowledge of the desired outcome and potential health benefits which have been acquired through experience often results in success [10-11]. Among many applications of plant remedies healing of pain and inflammation always feature prominently. Treatment of pain due to inflammation brings instant relief and reduces discomfort [12]. Inflammation is a word derived from the Latin word *inflammatio* referring to fire. It is a response by vascular tissues of organisms to stimuli or irritants in their effort to remove foreign bodies or repair damaged tissue [12]. Injury to cell membranes results in the release of the fatty acid arachidonic acid. The catalysis of arachidonic acid by cyclooxygenase enzymes results in the production of
inflammation mediators such as prostaglandins [13-14]. Prostaglandins and other products of the cyclooxygenase pathway enhance vascular permeability of the cell walls leading to a large inflow of substances such as proteins, thromboxanes, phagocytes and other inflammation mediators which cause pain and inflammation [15-16]. Macrophages get rid of the offending cells and damaged cells by engulfing and digesting them. The white blood cells form a puss filled barrier that promotes healing. Inflammation can be referred to as acute or chronic depending on the duration of response to the stimulus. Acute inflammation is the body’s immune system response to injury and is short-lived since the swelling is quickly resolved and the tissue hydrostatic pressure returns to normal [17]. Pain can be described as acute and chronic. Acute pain is sharp, stabbing and is immediately observed. It is as a result of stimulation of nociceptive receptors in the nervous system [18-20]. Chronic pain is associated with harm or injury to body organs and disease and is long lasting. Chronic pain and inflammation are long term and reflect the development of disease. Pain may not be exclusively a sign and symptom of an inflammatory disease. Symptoms of inflammation include redness of the skin, swollen joints, joint stiffness and loss of joint function [21]. Inflammation can also be associated with muscle stiffness, fatigue and fever. Diseases associated with chronic pain and inflammations include rheumatoid arthritis, nephritis and gouty arthritis among others [21]. Inflammation and pain can be treated by drugs such as non-steroidal anti-inflammatory drugs (aspirin, meloxicam and ibuprofen) [22]. However, due to the prevalence of side effects both short term and long term to pharmaceutical drugs, focus has turned on plant medicine [23]. In contrast to synthetic pharmaceuticals which are based on single chemicals, many plant remedies cure a wide array of diseases due to the synergistic actions of several metabolites inherent in the plants [23]. In many rural communities traditional plant medicines are more readily available compared to pharmaceutical drugs [24].
such as buchu (A betulina) and meadowsweet (F ulmaria) for therapeutic purposes has been passed from one generation to the next through oral tradition and through traditional doctors. However the majority of these plant medicines have not been evaluated through scientific analysis to establish their toxicity profiles, their effective dosages and exploration of other potential uses [24].

1.2 Research gap

Despite the long-time use of A betulina and F ulmaria as traditional herbs in treating pain and inflammation in South Africa, there has been little pharmacological research to back these claims and the establish effective dosages. Traditional medical practitioners often rely on the infusion extracts and use of the essential oil has been relegated to aromatherapy. This calls for research on comparative efficacies between the volatile and non-volatile extracts. Metabolite composition differs with geographical location of the plant, climatic conditions, and genetic composition among others. We therefore cannot generalise herbal applications of medicinal plants located in different geographical locations. This leaves scope for determination and comparison of metabolites composition with literature reports and value addition to the non-volatile crudes extracts. Value addition by synthesis of AgNPs may enhance their bioactive properties of the secondary metabolites.
1.3 Aims and objectives

Aims

- Determine volatile oil composition of *A betulina* and *F ulmaria* extract the non-volatile crude using ethanol from the two plants and run qualitative tests to determine metabolite composition.
- Determine the anti-inflammatory and analgesic properties of the volatile components, non-volatile components and the synthesised silver nanoparticles.

Objectives

- Isolate the volatile and non-volatile compounds using chromatography.
- Full structural elucidation of the isolates using spectroscopic methods.
- Plant mediated silver nanoparticles synthesis using the ethanolic crude extract of *A betulina* and *F ulmaria* using the green synthesis approach.
- Full characterisation of the synthesised silver nanoparticles using spectroscopic methods.
1.4 References


extract of *Uapaca guenensis* (Euphorbiaceae). *Journal of Medicinal Plants Research, 3*(9), 635-640.


2.1 Secondary metabolites

Medicinal plants have inherent active ingredients that have curative properties. These ingredients are termed secondary metabolites. Secondary metabolites are bioactive compounds with intrinsic non-nutrition components that are beneficial to the health of consumers of these foods [1]. Although medicinal herbal therapy has been practiced for thousands of years, the identification and determination of the synergistic function of phytochemicals remains a continuously ongoing and improving process. Plant secondary metabolites represent a diverse group of molecules that broadly differ from plant to plant even of the same species. They do not form part of the primary biochemical pathways of the plant which are necessary for plant growth and reproduction [2]. Various factors have been observed to influence metabolite composition among plant species which include geographical location, climatic conditions, and genetic composition of the plants among others [3]. Secondary metabolite composition and study has risen dramatically in recent years due to the diverse bioactive properties that they offer. These include among antioxidant, antiviral, antibacterial, antifungal and anticancer properties. Some of these metabolites are involved in giving characteristic flavours and odours to plants and others are used for the seasoning of food [3]. Despite the positive health contributions of phytochemicals some have been reported to have toxic effects such that their effective dosages need to be established and be used under the guidance of health practitioners. Such information will give guidelines to traditional medical practitioners in dispensing herbal medicines. Toxic metabolites have been estimated to constitute less than 2% of the dry matter and can have negative health effects such as kidney failure, reproductive problems, neurological problems and even death.
2.2 Classification of phytochemicals

Plant secondary metabolites can be described as volatile and non-volatile. The volatile components are also referred to as essential oils.

2.2.1 Non-volatile secondary metabolites

2.2.1.1 Tannins

Tannins are water-soluble polyphenols. They naturally bind and precipitate proteins. They are anticarcinogenic and antimutagenic. Their antimicrobial properties are associated with the hydrolysis of ester linkage between Gallic acid (2.1). Tannins in fruits serve as a natural defence mechanism against microbial infections [4]. Tannins have also been reported to exert their antimicrobial properties through hydrogen bonding and interactions with proteins found in microorganisms and viruses [5].

![Gallic acid](image)

2.2.1.2 Alkaloids

Alkaloids are compounds that mostly contain nitrogen, carbon and hydrogen and rarely contain elements such as halogens, oxygen and sulphur. Many alkaloids have nitrogen in a hetero cycle. Examples include nicotine (2.2).
Many alkaloids even though have been found to be bitter and poisonous; they are also useful as plant medicines. For example, curarine, found in the deadly extract curare, is a powerful muscle relaxant and physostigmine is a specific for certain muscular diseases. Alkaloids such as morphine and codeine have been used in the management of pain. Alkaloids cause lysis and morphological changes of bacteria [6-7].

2.2.1.3 Flavonoids

Flavonoids are phytochemicals that contain phenyl benzyl pyran functionality. They are large class of polyphenolic compounds prevalent in the plant kingdom. Their basic nucleus contains 15 carbons arranged in a C6-C3-C6 configuration. It can be visualised as two benzene rings joined to a 3 carbon chain. [8]. Variations in the heterocyclic ring give rise to flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids [9]. Example Isoflavan (2.3)

Flavonoids have been found to have antimicrobial, antiviral, antibacterial, antiallergic and anti-inflammatory activities [10-12]. The anti-inflammatory action of the flavonols is partly explained by their capacity to inhibit the production of cytokines and prostaglandin at the
inflammatory sites [13-14]. Flavonoids can be tolerated by the human body since they can be absorbed by the human body for a long time with relatively less adverse effects. The presence of hydroxyl group or oxygen makes flavonoids strong scavengers of free radicals which also make them good antioxidants [15].

2.2.1.4 Terpenes

Terpenes are biosynthetically obtained from the isoprene unit. Their formula comprises of units of the isoprene molecule. Monoterpenes contain two isoprene units, sesquiterpenes contain 3 isoprene unites, diterpenes contain 4 isoprene units (2.4) and triterpenes contain 6 isoprene units such as Betulinic acid (2.5)

\[
\text{C}_15\text{H}_{24}\text{O}_3\
\]

Monoterpenes are found in essential oils. They possess a wide variety of health benefits. Limonene, menthol, eugenol and menthone have analgesic and anti-inflammatory properties [16]. Terpeneol and linalool possess free radical scavenging ability making them good antioxidants [17].

2.2.1.5 Phenols

Phenols are aromatic compounds that contain a benzene ring with the molecular formula \(\text{C}_6\text{H}_5\text{OH} (2.6)\). It is a volatile compound that can also be found as a white crystalline
Plant phenols have antimicrobial, antioxidant and antiviral properties [18-19]. They have also been reported to interfere in the cancer formation process resulting in reduced cancer risk as a health benefit. They reduce potential harm by direct scavenging of the carcinogen [20].

2.2.1.6 Glycosides

A glycoside has a sugar molecule bonded through its anomic carbon to another functional group by a glycosidic bond. The sugar bonded group is referred to as the glycone part while the non sugar group is referred to as the aglycone part. The glycone part can contain one sugar group known as a monosaccharide or it can contain several sugar molecules (polysaccharide) joined by the glycoside bond [21]. E.g. salicylin (2.7)

2.2.1.7 Saponins

Saponins are a class of chemical compounds found in particular abundance in various plant species. They have one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. The aglycone portions of the saponins are termed sapogenins. Saponins
occur in many plant foods and get their name from their soap-like qualities. Saponins like Solanin (2.8) have been reported to lower cholesterol levels and reduce the risk of heart disease and reduce risk of developing tumours and cancer [22].

2.8

2.2.2 Volatile secondary metabolites

Essential oils are volatile compounds obtained in plants and they readily evaporate at room temperature. They derive their name from the Latin word *volare* which means to fly because they readily escape into the atmosphere [23]. Base oils are heavier hence they evaporate slowly whereas middle or top note oils quickly evaporate [23]. Essential oils exude beautiful aroma and are only found in about 10% of the plant kingdom. Conventionally they are stored in specialised secretory cells such as gland and secretory cavities and they readily oxidise in air [23]. Essential oils are termed essential not because of their important therapeutic properties but because the main component responsible for the fragrance of the plant is referred to as the “essence” of the plant [24]. It is widely accepted that the beautiful aroma
and taste of the essential oils emanate mostly from the oxygenated components. Presence of oxygen also has a significant contribution towards the solubility of the essential oils in alcohols and variably in water [24]. About 3000 essential oils are known, yet only a small portion plays a significant role in the fragrance market [24]. It is not uncommon for an essential oil to contain over 200 two hundred components and yet often the trace substances (in ppm) are responsible for the odour and flavour. Although a specific action of a plant may appear to depend on a single chemical constituent, isolating it may not enhance its physical characteristics or its bio-potential. The absence of even one component may change the aroma. Plant phytoconstituents have their own synergistic action that is irreplaceable. [25].

Composition of essential oils can vary according to the geographic and climatic conditions, location, growing season and time of day when harvesting is achieved [23-25]. Genetic composition has also been found to have a bearing on the composition of the essential oils. Plants of the same species can therefore produce oils of different composition, fragrance and different therapeutic properties. Variations of plants of the same species have led to the introduction of chemotypes. A chemotype is defined as a plant of the same species that has a different chemical profile of secondary metabolites [25]. The slight variation in chemical composition of chemotypes can produce large variations in bioactive potential.

Essential oils generally comprise of hydrocarbons and oxygenated compounds. The hydrocarbon compounds contain the monoterpenes and sesquiterpenes where-as the oxygenated non-terpene compounds comprise of ketones, alcohols, aldehydes, lactones, coumarins, esters, etc.

Terpenes are composed of hydrogen and carbon atoms only. All terpenes are based on the isoprene unit, an essential building block in plant biochemistry [26]. They make up the largest group of secondary metabolites with some 1000 monoterpenes and 3000
sesquiterpenes known [26]. Terpenes make up the largest single class of compounds, although phenylpropenes tend to have the largest impact on the aroma [27]. Terpenes arise from the mevalonic pathway.

2.2.2.1 Mevalonic Pathway

Melavonic acid, a six carbon compound forms the precursor for the biosynthesis of intermediate compounds in this pathway. This acid plays a prominent role towards the survival of the plant since it is involved in the synthesis of the bulk of essential terpenes in the plant [28]. Melavonic acid is converted to a 5 carbon structure which resembles the isoprene arrangement. This intermediate is then further converted to Geranyl pyrophosphate (GPP). This represents the first ten carbon molecule from this pathway. Enzymatic catalysis can then pursue rearrangement of these carbon atoms to form a sesquiterpene farnesyl pyrophosphate (FPP). Oxygenation of the first terpene geranyl pyrophosphate can further take place to add other functional group such as alcohols or aldehydes.

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Acetyl-CoA

\[CH_3CO\]

Acetoacetyl-CoA

\[CH_3\-C\equiv-CO\-CH\equiv-C\equiv-CO\-CH_3\]

HMG-CoA

\[\text{Mevalonate}\]
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Monoterpenes contain ten carbon atoms. This comprises of two isoprene units making the monoterpenes the basic building unit as found in nature. Monoterpenes make up the largest number of terpenes and can be subdivided into groups that indicate their structure. They are light molecules and evaporate quickly. They oxidize easily and combine with oxygen over time to become alcohols. In aromatherapy, the term terpenoid is given to terpene molecules that include oxygen [25]. These terpenes can also have several functional groups such as...
ketones, aldehydes, alcohols, etc [29]. Examples of monoterpenes include: myrcene, ocimene, β-Pinene, α-pinene and p-cymene (2.9).

2.9

2.2.2.3 Sesquiterpenes

Sesquiterpenes are composed of three isoprene units and therefore have 15 carbon atoms. Example of sesquiterpenes characteristic of essential oils: caryophyllene and β-bisabolene (2.10).

2.10

2.2.2.4 Esters

Esters are a combination of an acid and an alcohol and they take their name from the acid and the alcohol. Esters are sweet smelling and give a pleasant smell to the oils [30]. e.g. ethyl cinnamate (2.11)

2.11
2.2.2.5 Oxides

Oxides have an oxygen bridge between two neighbouring carbon atoms. An oxide has an oxygen atom in a chain of carbons, which forms a ring (but not a benzene ring). They contribute strongly to the odour of the plant [31]. E.g. scareol oxide (2.12)

![2.12]

2.2.2.6 Lactones

Lactones like coumarins have an ester group integrated into a carbon ring system. Lactones have an oxygen atom double bonded to a carbon atom. The carbon atom is often bonded to another oxygen in a ring formation. They are very high molecular weight compounds [32]. E.g. Citroptene (2.13)

![2.13]

2.2.2.7 Alcohols

Alcohols are found in many essential oils. Alcohols have a hydroxyl group attached to one of their carbon atoms. In addition to their pleasant fragrance, alcohols have several therapeutic benefits and fewer reported contra-indications [33]. E.g. nonanol (2.14)
2.2.2.9 Aldehydes

An aldehyde has an oxygen atom double bonded to a carbon atom at the end of a carbon chain. The fourth bond is always a hydrogen atom [35]. Aldehydes are unstable and oxidize easily. Example of an aldehyde is nonanal (2.15)

2.2.2.11 Ketones

A ketone is derived from an alcohol by oxygenation and has an oxygen atom double bonded to a carbon atom, which is also bonded to two other carbon atoms [36]. Example of a ketone is jasmone (2.16) [26].
2.3 Essential oil extraction methods

The major methods of extracting oils currently in use are Cold expression, distillation, supercritical fluid extraction and solvent extraction.

2.3.1 Solvent-Extraction:

Essential oils contain many thermo-labile compounds which are highly unstable to excess heat. This makes choice of temperature of extraction a very important factor that influences yield and collective quality of the oil. Solvent extraction is a method of choice for those essential oils that cannot withstand high temperatures which are used in steam distillation. Solvent extraction uses solvents such as hexane, petroleum ether, methanol, ethanol among others. End users in aromatherapy advocate against the use of these solvents for fear of contamination of the end product which may make them unusable if the solvent remains in the oil. Solvents to be used dissolve all extractable material which includes the essential oil and the non essential oil such as waxes and pigments. Filtration of the mixture precedes low pressure distillation to recover the solvent leaving out the concrete. This concrete contains the volatile component. The concrete is then washed with ethanol and slight heating and stirring. The essential oil dissolves more readily into the alcohol leaving out the wax. The little wax that dissolves in the alcohol can then be removed by exposing it to very low temperatures to allow it to freeze out leaving the wax free volatile oil referred to as the absolute [37-39]

2.3.2 Super Critical CO₂ Extraction:

Supercritical carbon dioxide extraction (SCO₂) involves the use of carbon dioxide as a carrier solvent for extracting essential oils. Carbon dioxide when exposed to very high pressure it turns into a liquid. Under these conditions CO₂ looks like a dense fog. This inert liquid can then be used as a liquid solvent for extraction of essential. The essential oil is dissolved and
trapped into the liquid gas. When the pressure is released carbon dioxide expands and reverts back to its gaseous state allowing it to escape leaving the essential oil behind [37-39].

2.3.3 Steam distillation Method:

There are varieties of distillation procedures yet in all of them water is heated to produce steam which carries the volatile oil with it. As the steam passes through a condenser the distillate is collected into a receiving container. The essential oil floats on the hydrosol which is all the remaining aqueous content dissolved in the water. Steam distillation is suitable for those essential oils that are not heat sensitive and therefore will not deteriorate or decompose upon application of heat through the steam.

Fresh or dry material can be distilled by placing them in a Clevenger apparatus filled with hot water to soften the oil secreting cells so that the essential oil can vaporise and escape with the steam. Temperature should however be controlled so as not to destroy the plant volatile component. A little organic solvent is normally added so that as the condensate passes through the solvent the oil is trapped it leaving the condensed vapour [37-39].

2.4 Bioactive properties and therapeutic benefits of essential oils

2.4.1 Aromatherapy

Essential oils have found considerable use in aromatherapy. It has been observed that essential oils are readily absorbed into the blood stream when massaged onto the body or when taken through oral ingestion or inhalation [37-39]. When essential oils are inhaled through the nose they get in contact with the olfactory nerve thereby affecting the brain’s limbic system. This subsequently produces an emotional response which lowers stress levels and has a positive effect on improved hormonal control. Aromatic components of essential oils also positively affects some functions controlled by the hypothalamus such blood
pressure, breathing rate and heart rate [40-41]. Lavender essential oil has been observed to reduce pain significantly and is recommended for long term use in aromatherapy [42].

2.4.3 Anti-inflammatory properties

Essential oils from *Boswellia carteri* and *Boswellia serrata* have been used for the treatment of arthritis and other inflammation elated or linked diseases in traditional medicine for several years. Studies showed that frankincense oil derived from *Boswellia* species possess anti-inflammatory activity through inhibition of immune cytokines production and leukocyte infiltration [43]. The essential oil of *Cordia verbenaceae* contains 4.64 % α-humulene, which accounts for the anti-inflammatory activity [44]. Thymol has been reported to contribute extensively to the anti-inflammatory properties of plants bearing the essential oil component. It is believed that it reduces inflammation possibly by reducing the production of proinflammatory mediators in the acute inflammation models [45].

2.4.4 Analgesic properties

Pain is an unpleasant emotional experience which is often accompanied by tissue damage and has a potential to cause harm to living cells [46-48]. Most pain models have reported the analgesic properties of essential oils. Some components such as β-pinene and 1, 8-cineole have been reported to be very potent even at low doses [49]. Chavan et al reported that santalol a sesquiterpenoid causes analgesia in mice because of an inhibitory binding effect on the δ2-opioid receptor [50-51].

2.4.5 Toxicity of essential oils

Many substances essential oil included become toxic if their use or consumption is not regulated. Essential oils despite their ecofriendliness and less hazardous nature do have their
consumption limits and safety exposure levels. Safe, regulated and correct use according to professional recommendations helps to avert potential hazards to consumers of these essential oils for their therapeutic value [53].

Reports on toxicities of essential oil have largely pointed monoketones as chief culprits for many of the adverse reactions to essential oils. Monoterpene ketones such as camphor, pulegone and pinocamphone have been found to be toxic in high concentrations [54-55]. The presence of one of these compounds or other chemically related components as major constituents tends to shift these essential oils towards the toxic class resulting in human health risks. Convulsion reports have been received on adults and children who use essential oils for therapeutic purposes [56-57]. Evidence has also been produced showing the dermatitis problems such as skin irritation by those that use essential oils in topical applications. Oil constituents such as furanocoumarins produce skin irritation when ingested or applied to the skin [58]. Skin irritation has been found to be more on those with fair skin and the elderly.

Essential oils such as Garlic, Tagetes, Geranium and True Lavender have been shown to have hypotensive effects in laboratory animals. Both eucalyptus and pennyroyal oil were shown to produce a slight increase in both systolic and diastolic blood pressure in animal models [58].

Some oils have carcinogenic properties when tested in animal models. Carcinogens give rise to tumour production, which is an unrestrained malignant proliferation of cells, resulting in a progressively growing mass of abnormal tissue. Eucalyptus oil, Acetaldehyde and phellandrene or one of its constituents, have also been tested as promoting agents on mouse skin [59].
2.5 References


Chapter 3

Phytochemical analyses and bioactivity testing of the volatile and non-volatile components of *Filipendula ulmaria* (L.) Maxin

3.1 Introduction

*Filipendula ulmaria* is mostly referred to as meadowsweet. It belongs to the genus *filipendula* and family Rosacea. It is known by other common names such as Meadow-wort, lady of the meadow, queen of the meadow, and ulmaria. It is a perennial plant that grows in damp meadows or wet swamps. It grows to about two meters in height with cream white flowers that exude a strong characteristic scent (Figure 3.1). It is native to Europe and North America [1].

![Figure 3.1: *F. Ulmaria* (Meadowsweet)](image)

3.2 Phytochemical composition and therapeutic value

*F. ulmaria* contains tannins, which might decrease inflammation (swelling) and decrease mucus (phlegm). It contains variably small to large amounts of salicylates. Salicylates are similar to aspirin [2-3]. The phytochemical constituents include salicylates, flavonoids, vitamin C, volatile oils, mucilage, coumarin and carbohydrates [4]. The essential oil of *F. ulmaria* has been found to constitute mostly of Salicylic acid, salicylates, limonene, and
menthone [4-5]. The therapeutic benefits of meadowsweet are resemble those of aspirin yet it has been found to have less side effects than aspirin [6]. The significantly large quantity of tannins and mucilage are particularly valuable in the protection of the gastric lining. This makes it very useful in the treatment of gastritis and other gastric related problems. The high presence of salicylates derivatives both as volatile and non-volatile components have been reported to be responsible for the action of the plant as an anti-inflammatory and painkilling agent in treating ailments associated with swelling such as arthritis [7-8]. This medicinal herb has been renowned as a very powerful medicinal herbal remedy in traditional medicine. It is quite effective in treating diarrhoea, pneumonia, dysentery and diphtheria in children [9]. *F. ulmaria* herbal remedies have been used in Austrian traditional medicine internally as tea for treatment swelling infections, and fever [10]. The whole plant is often used in infusion extracts but commonly used parts of the plant are fresh and dry leaves, roots and stem. *F. ulmaria* has however been contra indicated against people with asthma and breathing problems as it has been found to cause bronchospasm. Most often it causes gastric ulcerations for those allergic to aspirin and related drugs [11-12].

### 3.4 Experimental investigation

#### 3.4.1 Hydrodistillation

Dried stem, flowers and leaves of *F. ulmaria* (Figure 3.2) were supplied by Mr Reuben Matewu, an herbalist from King William’s town in the Eastern Cape Province of South Africa in the month of August 2013. The plant material was kept under room temperature and pressure and allowed to dry naturally before being taken for hydrodistillation.
Plant leaves were placed in a round bottomed flask with excess distilled water (Figure 3.2). 300g of dry plant material (leaves, stem and flowers) were hydrodistilled using a modified Clevenger apparatus for 3 hours.

Figure 3.2: *F. ulmaria* dried plant material

### 3.4.2 Gas Chromatography: GC/MS analysis

GC/MS analyses of the oils were performed on an Agilent 5973N Gas Chromatography- mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS fused silica capillary system with a 5% phenylmethylsiloxane stationary phase. Capillary column parameter was 30m by 0.25mm, film thickness 0.25µm. The initial temperature of the column was 70°C and was heated to 250°C at a rate of 5°C/min. The final temperature was kept for a run time of 77.25 minutes. Helium was used as the carrier gas at a flow rate of 1 ml/min. The split ratio was 100:1. Scan time was 78 minutes with a scanning range of 35 to 450 amu. One micro litre (1µl) of the diluted oil (in hexane) was injected for analysis. *n*-alkane of C<sub>8</sub> to C<sub>30</sub> were run under the same condition for Kovat Indices determination.

### 3.4.3 Identification of compounds

The essential compounds were identified by gas chromatography using retention indices. A homologous series of alkanes under the same operating conditions was run in order to determine the retention indices. The components of the oils were identified by matching their
spectra and retention indices (Kovat Index) using the NIST spectrometer data bank. Identification of the main components was also carried out by the comparison of both the GC retention times and the MS data against those of the reference standards, Kovats retention indices (KI) and comparison with previous literature. The homologous series of n-alkanes C\textsubscript{8}-C\textsubscript{30} was used as standards [13-14].

3.4.4 Pharmacological studies

The essential oil and the ethanolic extract were evaluated for analgesic and antiinflammatory activity. The formalin test model and the egg albumin test model were used for evaluating the secondary metabolites ability to reduce pain and inflammation respectively.

3.4.4.1 Animals

Twenty four Sprague Dawley rats weighing 180-250 g and twenty four Swiss albino mice weighing 18-35 g were obtained from the South African Vaccine program. The animals were housed during the experimental period in the Department of Biology and Environmental Sciences animal holding facility at Walter Sisulu University and were used according to the Walter Sisulu Ethical Clearance Committee (N0 0009/07) ethics. The animals were allowed to acclimatize in the new environment for 2 weeks. Room temperature was maintained at 24°C while lighting was provided exclusively by daylight. Wood shavings were used as bedding. During this period of acclimatization, animals had free access to pellets (EPOL SA: protein-180 g/kg, Moisture-120 g/kg, Fat-25 g/kg, Fibre-60g/kg, Calcium-18 g/kg and Phosphorus-7 g/kg) and water \textit{ad libitum}. Cages were cleaned and bedding replaced 2 times per week.
3.4.4.2 Drugs used in the study

Aspirin (Sigma Aldrich, St. Louis, USA) and meloxicam (Sigma Aldrich, St. Louis, USA) used in this study were of analytical grade.

3.4.4.3 Analgesic test on the essential oil and ethanolic extract of *F. ulmaria*

The formalin test was carried out to determine analgesic activity as described by Prabhu et al. (2011) with some modifications [15]. Four groups of six mice each were selected for each investigation in the study. Analgesic activity studies were undertaken on the treated groups namely *F. ulmaria* plant extracts (essential oil and ethanolic extract)

The animals were randomly assigned to treatment groups as follows:

- Group I – 1 ml normal saline the control group.
- Group II – 100 mg/kg Aspirin the standard group
- Group III – 200 mg/kg essential oil.
- Group IV – 200 mg/kg ethanolic extract.

One hour after treatment with the oil and extract, animals were injected sub-plantarly with a 100 μl of 2.5% v/v formalin solution in saline. Animals responded to formalin injection by licking or biting the injected paw. The number of times the animal licks/bites the paw was recorded during the first 5 min (neurogenic phase) and then 20-30 min (inflammatory phase) after formalin injection.

- The percentage inhibition was then calculated using the following formula:
  
  \[(1 - \frac{\text{T}}{\text{C}}) \times 100\]  

  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
3.4.4.4 Anti-inflammatory evaluation of essential oil and ethanolic extracts of *F. ulmaria*

Acute inflammation study was carried out using 24 male Wistar rats per experiment. Animals were divided into an experimental group of six rats each per study. Anti-inflammatory activity studies were undertaken on the *F. ulmaria* essential oil and ethanolic extract.

The animals were randomly assigned to treatment groups as follows:

- Group I – 1 ml normal saline control group.
- Group II - 15 mg/kg Meloxicam the standard group.
- Group III – 200 mg/kg essential oil.
- Group IV – 200 mg/kg *F. ulmaria* plant extract.

The animals were placed in transparent cages for observation.

Acute inflammation study was carried out using the induced rat hind paw edema model as described by Ojewole et al (2006) with slight modifications (Paw oedema test). 24 rats of both sexes were used with fresh egg albumin as the phlogistic agent injected into the sub-plantar surface of the left hind paw [16]. Thirty minutes post treatment, inflammation of the left hind paw was induced by injecting 1ml of fresh egg albumin into the sub-plantar surface of the left hind paw. The control group was treated with the vehicle while standard group received meloxicam. The other two groups were treated with essential oil and ethanolic crude extract (200 mg/kg). The paw diameters of the animals were measured with a digital caliper. Baseline paw diameters were recorded. Then, the hourly reading was taken after the injection of 50 % v/v fresh egg albumin until the 5th hour.

Percentage inhibition = \((1-D/C \times 100\%)\)

Where D is difference in paw volume after administration of test drugs and C is the difference on paw volume in control groups.
3.4.4.5 Statistical Analysis

Graph Pad Prism (5) software was used for statistical analysis. Comparison of the data was made between the control group and the experimental group by one way analysis of variance (Anova) followed by the Dunnet post hoc test. The significance level was set at P<0.05. Results were presented as Mean±SEM (standard error of mean).

3.5 Results and discussion

3.5.1 Hydrodistillation of *F. ulmaria* plant material

Light yellow oil was obtained (Figure 3.3). The quantity of the oil was 2.3g which represented a yield of 0.8% (w/w) (Table 3.1).

![Figure 3.3: *F. ulmaria* essential oil](image)

**Table 3.1: Physicochemical analysis of the essential oil of *F. ulmaria***

<table>
<thead>
<tr>
<th>Colour of oil</th>
<th>Quantity of oil Obtained (g)</th>
<th>Yield (w/w %)</th>
<th>Odour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried leaves</td>
<td>Tint yellow</td>
<td>2.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>
The essential oil of *F. ulmaria* was analysed by GC-MS analysis (Appendix 1). The results obtained were presented in Table 3.2 below.

**Table 3.2: Chemical composition of the essential oil of *F. ulmaria***

<table>
<thead>
<tr>
<th>No</th>
<th>Compound name</th>
<th>RI</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexanal</td>
<td>806</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>Hexenal</td>
<td>814</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>Benzaldehyde</td>
<td>982</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>2-Pentylfuran</td>
<td>1040</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>p-Cymene</td>
<td>1042</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>1,8 Cineol</td>
<td>1059</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>Salicylaldehyde</td>
<td>1064</td>
<td>10.6</td>
</tr>
<tr>
<td>8</td>
<td>Linalool oxide</td>
<td>1071</td>
<td>0.2</td>
</tr>
<tr>
<td>9</td>
<td>Linalool</td>
<td>1082</td>
<td>2.7</td>
</tr>
<tr>
<td>10</td>
<td>Nonanal</td>
<td>1104</td>
<td>1.1</td>
</tr>
<tr>
<td>11</td>
<td>β-Thujone</td>
<td>1114</td>
<td>0.8</td>
</tr>
<tr>
<td>12</td>
<td>Camphor</td>
<td>1121</td>
<td>2.4</td>
</tr>
<tr>
<td>13</td>
<td>Menthone</td>
<td>1148</td>
<td>8.1</td>
</tr>
<tr>
<td>14</td>
<td>Isomenthone</td>
<td>1148</td>
<td>6.3</td>
</tr>
<tr>
<td>15</td>
<td>Menthol</td>
<td>1164</td>
<td>8.6</td>
</tr>
<tr>
<td>16</td>
<td>Methyl salicylate</td>
<td>1172</td>
<td>17.3</td>
</tr>
<tr>
<td>17</td>
<td>Pulegone</td>
<td>1212</td>
<td>3.0</td>
</tr>
<tr>
<td>18</td>
<td>Carvone</td>
<td>1236</td>
<td>1.0</td>
</tr>
<tr>
<td>19</td>
<td>Ethyl salicylate</td>
<td>1249</td>
<td>5.8</td>
</tr>
<tr>
<td>20</td>
<td>Anethole</td>
<td>1254</td>
<td>1.0</td>
</tr>
</tbody>
</table>
The GC/MS analysis of the oil gave 41 constituents in the chromatogram and 34 constituents were identified representing 92.1% of the oil [Appendix 1]. The major components in the oil were found to be methyl salicylate (3.1) (17.3 %), salicylaldehyde (3.2) (10.6 %), menthol (3.3) (8.6 %), anisaldehyde (8.2 %), menthone (3.4) (8.1 %) [Table 3.2]. The oil comprised of ketones (1.3 %), aldehydes (21.5 %), esters (26.1 %), sesquiterpenes (2.3 %) and hydrocarbons (0.3 %). Oxygenated mono terpenes constituted a major component in the essential oil (39.1 %) (Table 3.3). There were qualitative differences observed between the oil obtained from this plant and other plants of the same species in different regions. A high
content of salicylaldehyde (36.0%) and methyl salicylate (19.0 %) was reported from the aerial parts of *F. ulmaria* of Russian origin [16].

Table 3.3: Summary of the chemical profile of the essential oil of *F. ulmaria*

<table>
<thead>
<tr>
<th>Class of compounds</th>
<th><em>F. ulmaria</em> essential oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of compounds</td>
</tr>
<tr>
<td>Monoterpene</td>
<td>1</td>
</tr>
<tr>
<td>Oxygenated monoterpenes</td>
<td>15</td>
</tr>
<tr>
<td>Sesquiterpenes</td>
<td>4</td>
</tr>
<tr>
<td>Oxygenated sesquiterpenes</td>
<td>2</td>
</tr>
<tr>
<td>Ester</td>
<td>3</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>6</td>
</tr>
<tr>
<td>Ketones</td>
<td>1</td>
</tr>
<tr>
<td>Ethers</td>
<td>1</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>1</td>
</tr>
</tbody>
</table>

Further comparison with oils obtained from the same genus but different species showed that the essential oil from the leaves of *F. vulgaris* of Serbia origin had a characteristically higher content of salicylaldehyde (68.6 %) with lower values for benzaldehyde 2.3 % methyl salicylate 2.4 % and linalool 1.8 % than the plant species used in this study [17]. The marked qualitative differences could be due to differences in the parts of the plant used, genetic composition and geographical location of the plants. Temperature used in the drying process of the plant material has been found to influence the quality and composition of essential oils. As the oil components are brought to the surface during evaporation some compounds are lost or have their concentration reduced beyond recognition [18-19].

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3.5.2 Non-volatile components extraction

The plant materials from the individual plants were crushed into thin material to make 500 g each of meadowsweet and buchu to make them ready for solvent extraction. The plant materials were separately soaked in 2L of 95 % (v/v) ethanol solvent on a shaking vibrator for one week to allow for optimum contact between solvent and plant material and obtain optimum yield [20]. The crude was then filtered with cotton wool and filter paper and later removed under vacuum at 40°C.

3.5.3 Qualitative phytochemical analysis

Chemical test were carried out using aqueous crude extracts to identify various metabolites using standard methods [21-22]. The results were collated in Table 3.4

**Test for Alkaloid:** 3 ml aqueous extract was mixed with 3 ml of 1 % HCl and stirred in a warm water bath. After stirring the mixture Mayer and Wagner’s reagent was then gradually added. No turbid precipitate was produced. This was taken as confirmation of the unavailability of alkaloids.

**Test for Tannins:** 2 ml of the ethanolic extract was carefully mixed with 2 ml of distilled water under continuous stirring. A few drops of FeCl₃ Solution were later added. A green precipitate was obtained. This was taken as confirmation for the availability tannins.
**Test for Saponins:** 5 ml of the ethanolic extract were gradually mixed with 5 ml of distilled water under constant and vigorous stirring in a warm water bath. Stable foam was produced which was taken as confirmation of the availability of saponins.

**Test for Flavonoids:** To 1 ml of ethanolic extract, 1 ml of 10% lead acetate solution gradually added while stirring. A yellow precipitate was obtained indicating the availability of flavonoids.

**Test for Terpenoids:** 2 ml of the ethanolic extract were gradually dissolved in 2 ml of chloroform and allowed to evaporate to dryness. 2 ml of concentrated sulphuric acid was then added and heated for about 2 min. A greyish colour was obtained which confirmed the availability of terpenoids.

**Tests for glycosides (Liebermann’s test):** 2 ml of the ethanolic extract were dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added in it. The mixture was allowed to cool in an ice bath. Sulphuric acid was then gradually added. A colour change from violet to blue was obtained. This was taken as confirmation for the presence of a steroidal nucleus (aglycone portion of glycoside).

**Tests for steroids:** 2 ml of chloroform were mixed with 2 ml of the ethanolic extract. After uniform mixing 2 ml of concentrated sulphuric acid were then added to the mixture. The red colour obtained was taken as an indicator for the presence of steroids.
3.5.4 Isolation

3.5.4.1 Pre-column preparation

TLC of each of the crude extracts was carried out to ensure that the right choice of eluting solvent mixture was used and also to understand the chemical profile of the crude extracts. Various mobile phases of varying polarities ranging from hexane to ethyl acetate were used. The spots were identified using vanillin-sulphuric acid spray reagent.
3.5.4.2 Isolation of compound MSV8 using Vacuum Liquid Chromatography

The ethanol extract was partitioned into a hexane fraction and a remaining ethanol fraction. A VLC column was prepared with TLC grade silica gel. A short column having 10 cm length, 6 cm diameter and 5 cm height was packed under reduced pressure. The column was washed with hexane to enable compact packing. 2.35 g of the hexane fraction was mixed with hexane and allowed to dry and then applied onto the top of the adsorbent. The VLC column was
eluted with hexane and the polarity gradient gradually increased by addition of small calculated percentage volumes of ethyl acetate. Results from thin layer chromatography were used to combine fractions with similar R_f values. These isolates were obtained at 80:20 hexane: ethyl acetate mixture. The isolates was further purified by short column chromatography eluting with hexane gradually increasing the polarity with silica gel as the stationary phase. A column of diameter 1.5 cm and 6.8 g silica gel 60 F_{254}, mesh was used as adsorbent. The rechromatographed isolate was obtained at 80:20 hexane: ethyl acetate mixture.

3.5.4.3 Isolation of compounds M5

Column chromatography was used to isolate the compound from the ethanol fraction. 14.5 g of the hexane crude extract was subjected to column chromatography over silica gel 60 F_{254}, 70-230 mesh as adsorbent, eluting with 100 % hexane. Hexane was used as the eluting solvent gradually increasing the polarity `with ethyl acetate. The isolate was obtained from the 60:40 hexane: ethyl acetate ratio. The isolate was further purified through a series of recrystallisation procedures.

3.5.4.4 Purification of isolates by recrystallisation

Recrystallisation is a slow, selective formation of the crystal framework due to molecular recognition of similar pure compounds [23]. The impure isolates were individually dissolved in a minimum volume of hot methanol solvent. Insoluble coloured impurities were removed by filtration. The dissolved impure solid was placed in an Erlenmeyer flask. The solution in the Erlenmeyer flask heated in a water bath, and hot methanol solvent was gradually added to the flask until the isolates dissolved. The solution was shaken using a stirring rod between additions of methanol solvent. The solution was then filtered to remove coloured impurities.
The flask containing the hot filtrate was cooled using ice water. As the solution cooled, the solubility of the dissolved compound decreased and the solid isolates were formed from the solution. The TLC results and the yield after the purification were presented in Table 3.5 and Table 3.6.

**Table 3.5: TLC Results of Isolates after purification**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MSV8</th>
<th>M(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R&lt;sub&gt;F&lt;/sub&gt; value</strong></td>
<td>7.8</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>Mobile phase (Hex:EtOAc)</strong></td>
<td>80:20</td>
<td>60:40</td>
</tr>
</tbody>
</table>

**Table 3.6: Yields from the purification process**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MSV8</th>
<th>M(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yield %</strong></td>
<td>0.057</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Weight(g)</strong></td>
<td>0.0082</td>
<td>0.0039</td>
</tr>
</tbody>
</table>

3.5.4.5 Melting Point Determination

Melting points were determined on a Gallenkamp melting point apparatus MFB-595 010M. The melting point was done in triplicate and the average was determined to improve precision and validity of the results (Table 3.7).

**Table 3.7: Melting points results of isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MSV8</th>
<th>M(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Melting point °C</strong></td>
<td>211-216</td>
<td>243-245</td>
</tr>
</tbody>
</table>
3.5.4.6 Structural elucidation of isolate MSV8

The isolate MSV8 eluted from the solvent ratio of 80:20 (Hexane:Ethyl acetate) was elucidated with the aid of 1D and 2D NMR spectra at 600MHz using DMSO as the carrier solvent [Appendix 2-9]. The results are summarised in Table 3.8.

Table 3.8: $^1$HNMR and $^{13}$CNMR of MSV8 (600MHz, DMSO)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.7</td>
<td>38.1</td>
<td>37.2</td>
<td>38.7</td>
<td>CH$_2$</td>
<td>0.9, 1.6 (m)</td>
</tr>
<tr>
<td>2</td>
<td>27.4</td>
<td>25.1</td>
<td>29.9</td>
<td>27.4</td>
<td>CH$_2$</td>
<td>1.5 (m)</td>
</tr>
<tr>
<td>3</td>
<td>78.9</td>
<td>79.0</td>
<td>79.0</td>
<td>78.9</td>
<td>CH</td>
<td>3.2dd</td>
</tr>
<tr>
<td>4</td>
<td>38.8</td>
<td>38.7</td>
<td>38.7</td>
<td>38.9</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>55.3</td>
<td>55.3</td>
<td>55.2</td>
<td>55.3</td>
<td>CH</td>
<td>0.8(m)</td>
</tr>
<tr>
<td>6</td>
<td>18.3</td>
<td>18.3</td>
<td>18.1</td>
<td>17.1</td>
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<td></td>
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<tr>
<td>7</td>
<td>34.2</td>
<td>34.3</td>
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<td>40.8</td>
<td>40.0</td>
<td>40.7</td>
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<td></td>
</tr>
<tr>
<td>9</td>
<td>50.4</td>
<td>50.4</td>
<td>50.4</td>
<td>50.5</td>
<td>CH</td>
<td>1.3 (m)</td>
</tr>
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<td>10</td>
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<td>20.9</td>
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<td>25.1</td>
<td>25.5</td>
<td>CH$_2$</td>
<td>1.6(m)</td>
</tr>
<tr>
<td>13</td>
<td>38</td>
<td>38.9</td>
<td>38.7</td>
<td>38.2</td>
<td>CH</td>
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</tr>
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<td>42.4</td>
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<td>27.4</td>
<td>30.6</td>
<td>CH$_2$</td>
<td>1.9(m)</td>
</tr>
<tr>
<td>16</td>
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<td>35.6</td>
<td>35.6</td>
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<td>42</td>
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<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
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</tr>
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<td>48.2</td>
<td>48.3</td>
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<td>49.2</td>
<td>CH</td>
<td>1.5(m)</td>
</tr>
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<td>48.0</td>
<td>48.1</td>
<td>47.6</td>
<td>CH</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>150.9</td>
<td>151.0</td>
<td>151.0</td>
<td>150.8</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>29.8</td>
<td>29.8</td>
<td>28.0</td>
<td>29.7</td>
<td>CH₂</td>
<td>1.9 (m)</td>
</tr>
<tr>
<td>22</td>
<td>40.0</td>
<td>40.0</td>
<td>35.9</td>
<td>40.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>28</td>
<td>28.0</td>
<td>27.4</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>15.4</td>
<td>15.4</td>
<td>14.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>16.1</td>
<td>16.1</td>
<td>16.2</td>
<td>16.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>15.9</td>
<td>16.0</td>
<td>15.7</td>
<td>16</td>
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<tr>
<td>27</td>
<td>14.5</td>
<td>14.6</td>
<td>14.2</td>
<td></td>
<td></td>
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<tr>
<td>28</td>
<td>109.3</td>
<td>109.3</td>
<td>109.3</td>
<td>109.4</td>
<td>CH₂</td>
<td>4.5; 4.7, 5.2(s,s)</td>
</tr>
<tr>
<td>29</td>
<td>18</td>
<td>18.0</td>
<td>18.1</td>
<td>18.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>19.3</td>
<td>19.3</td>
<td>18.3</td>
<td>19.3</td>
<td></td>
<td>1.7 (s)</td>
</tr>
</tbody>
</table>

**FTIR:** The FTIR spectrum (Appendix 9) showed a hydroxyl group at 3450 cm⁻¹ while the band at 2990 cm⁻¹ and 1450 cm⁻¹ (aliphatic C-H) represented the bending vibrations of methyl groups and 2380 cm⁻¹ (vinyl stretching), 1480 cm⁻¹ medium band vibration for a methylenic signal and 1150 cm⁻¹, 1090 cm⁻¹ and 950 cm⁻¹ (methyl)

**¹H NMR:** 0.75, 0.80, 0.85, 0.90, 1.0, 1.85, 3.70, 2.50, 4.50, 4.7, 5.20. ¹HNMR showed a pair of two singlets at δ 4.5(brs) H29a and 4.7(brs) H29b. The presence of a doublet at δ 3.2(dd) was due to the proton attached to the carbon bearing hydroxyl group at H3; δ 78.9 C-3; δ 150.8 C-20 and δ 109.4, C-28(34; 35; 36) [Appendix 2].
\[\text{\textsuperscript{13}C NMR: } 38.7(\text{C1}); 27.4(\text{C2}); 78.9(\text{C3}); 38.9(\text{C4}); 55.3(\text{C5}); 17.1(\text{C6}); 34.3(\text{C7}); 40.7(\text{C8}); 50.5(\text{C9}); 37.1(\text{C10}); 20.8(\text{C11}); 25.5(\text{C12}); 38.2(\text{C13}); 42.4(\text{C14}); 30.6(\text{C15}); 37(\text{C16}); 42(\text{C17}); 49.2(\text{C18}); 47.6(\text{C19}); 150.8(\text{C20}); 29.7(\text{C21}); 40.0(\text{C22}); 28(\text{C23}); 16.1(\text{C25}); 16(\text{C26}); 109.4(\text{C28}); 18.3(\text{C29}); 19.3(\text{C30}) \]  

Compound MSV8 was isolated as a white amorphous powder and its NMR spectral data suggested a pentacyclic triterpene with a lupane skeletal back bone (C30). The \textsuperscript{13}C-NMR spectral assignments of various carbons of the compound were substantiated by DEPT 135 [Appendix 4] experiments which revealed 11 methylene carbons, 7 methyl groups, 6 quaternary carbons. Within these carbon assignments were an exomethyelene carbon at \(\delta\) 109.3, quaternary carbon attached to the exomethyelene at \(\delta_{\text{C}}\) 150.8 and the oxygenated methyne at \(\delta_{\text{C}}\) 78.9 which were assigned to C20, C3 and C28 respectively. These values were also supported by the presence of olefinic carbon (C20 and C28) absorptions at 1600 cm\(^{-1}\) on the FTIR spectrum and the broad absorption spectrum at 3400cm\(^{-1}\) which is typical of a hydroxyl group attached to a carbon ring system on C3 [24].

The \textsuperscript{1}H NMR revealed tertiary methyl protons at 0.80, 0.75, 0.85, 1.00 and 1.90. A multiplet at 3.20 coupled with a broad pair of singlets at 4.50 and 5.50 signified the presence of olefin protons which were assigned to C20 and C28. The assignments are in agreement and indicative of a Lupeol structure [24-26]. HSQC and HMBC [Appendix 5 and 6] were also used to demonstrate the correlations between the carbon atoms and to assign the carbon groups. The methine proton (H3) at \(\delta_{\text{H}}\) value of 3.3 showed correlation with the methyl carbon resonance at \(\delta_{\text{C}}\) value of 18.5 (C6). The methyl resonance at \(\delta_{\text{H}}\) value of 2.4 (H19) with two methylene signals at \(\delta_{\text{C}}\) value of 29.8 (C21), \(\delta_{\text{C}}\) value of 48 (C19) and the quaternary carbon at 150.8 (C20). The broad singlets at 4.5 and 4.7 showed correlation with the methylene carbon resonance at \(\delta_{\text{C}}\) value 48(C19) and \(\delta_{\text{C}}\) value of 19.3 [Appendix 5].
chemical shifts assigned were in agreement with the structure for Lupeol. We therefore proposed the structure isolated to be Lupeol (3.5)

![Chemical structure of Lupeol](image)

3.5 (3β-Lup-20(29)-en-3-ol)

3.5.4.7 Structural elucidation of isolate M5

The isolate M5 eluted from the solvent ratio of 60:40 (hexane:ethyl acetate) were elucidated with the aid of 1D and 2D NMR spectra (600MHz) with DMSO used as the carrier solvent [Appendix 10-16]. The results obtained were summarised in Table 3.9.

**Table 3.9: \(^1\)H, \(^{13}\)C and DEPT NMR data for M5 (600MHz, DMSO)**

<table>
<thead>
<tr>
<th>Carbon Number</th>
<th>Ref [27]</th>
<th>Ref [28]</th>
<th>Ref [29]</th>
<th>M 5</th>
<th>Dept</th>
<th>(^1)HNMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.7</td>
<td>38.8</td>
<td>38.8</td>
<td><strong>38.9</strong></td>
<td>CH2</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>27.4</td>
<td>27.3</td>
<td><strong>27.4</strong></td>
<td>CH2</td>
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<tr>
<td>3</td>
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<td>77.3</td>
<td>78.8</td>
<td><strong>77.4</strong></td>
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<td>38.8</td>
<td><strong>38.7</strong></td>
<td>C</td>
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</tr>
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<td>5</td>
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<td>55.2</td>
<td>55.4</td>
<td><strong>55.3</strong></td>
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<td>47.3</td>
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<td>C</td>
<td></td>
</tr>
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<td><strong>52.9</strong></td>
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</tr>
<tr>
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<td>39.5</td>
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<td>39.1</td>
<td><strong>39.3</strong></td>
<td>CH</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>38.6</td>
<td>38.8</td>
<td>38.8</td>
<td><strong>38.9</strong></td>
<td>CH</td>
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<td><strong>30.7</strong></td>
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<td>28.7</td>
<td>28.2</td>
<td><strong>28.0</strong></td>
<td>CH3</td>
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</tr>
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<td>15.5</td>
<td><strong>17.5</strong></td>
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<tr>
<td>25</td>
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<td>15.6</td>
<td>15.7</td>
<td><strong>15.7</strong></td>
<td>CH3</td>
<td>0.90(m)</td>
</tr>
<tr>
<td>26</td>
<td>17.7</td>
<td>17.3</td>
<td>16.9</td>
<td><strong>16.5</strong></td>
<td>CH3</td>
<td>0.70(m)</td>
</tr>
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<td>23.7</td>
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<td><strong>23.8</strong></td>
<td>CH3</td>
<td>1.00(m)</td>
</tr>
<tr>
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<td>172.8</td>
<td>178.7</td>
<td>177.7</td>
<td><strong>178.8</strong></td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>15.4</td>
<td>17.4</td>
<td>16.9</td>
<td><strong>16.5</strong></td>
<td>CH3</td>
<td>0.80(m)</td>
</tr>
</tbody>
</table>
\[1H \text{ NMR: } 0.4, 0.5, 0.70, 0.80, 0.90, 1.00, 2.98(s), 5.2(s), 2.10(d), 4.5(brs) \] [Appendix 10].

\[13C \text{ NMR: } 38.9(C1), 27.4(C2), 77.4(C3), 38.7(C4), 55.3(C5), 18.5(C6), 33.3(C7), 39.9(C8), 47.5(C9), 37.0(C10), 23.3(C11), 125.1(C12, (C13), 42.1(C14), 28.7(C15), 24.3(C16), 47.3(C17), 52.9(C18), 39.3(C19), 38.9(C20), 30.7(C21), 36.7(C22), 28.0(C23), (C24), 15.7(C25), 16.5(C26), 23.8(C27), 178.8(C28), 16.5(C29), 21.5(C30) \] [Appendix 11].

The \[13C\text{NMR} \] showed resonances that matched with known values in literature (Table 3.9).

Analysis from the DEPT showed 9 CH\(_2\) groups, 7 CH groups and 7C groups [Appendix 12].

The resonance at \(\delta_c 177.7\) is a characteristic acetyl group of an ursolate ester commonly occurring at C28. The \(\delta_c\) values at 138.7 and 125.5 are typical Ursane resonance carbons commonly occurring at C12 and C13 respectively [29]. The carbon at \(\delta_c\) value of 78.9 showed HMBC correlations with methyl resonance protons \(\delta_h\) values of 0.60 and 0.85 \(1H\).

These methyl resonances also had HMBC correlations with the methine carbon at \(\delta_c 55.2\) [Appendix 14]. Upon comparison with literature values these methyl groups were assigned to C23 and C24. These methyl groups also correlated with the carbon with \(\delta_c\) value of 38.7 and was thus assigned to C3. The methyl resonance at \(\delta_h\) value of 0.8 correlated with a methine carbons at \(\delta_c\) values of 52.9 and 38.9. This methyl resonance was assigned to C29 (\(\delta_h 0.8\)).

The carbon resonance at \(\delta_c\) value of 42.1 was found to correlate with a methyl resonance at \(\delta_h 1.00\) and it showed a methyl correlation with the carbon at \(\delta_c 28.3\). These were assigned as C27 (\(\delta_h 1.00\) and C14 (\(\delta_c 42.1\)). All the methyl resonances were assigned using DEPT values, HSQC, HMBC correlations and comparing with literature values [27-29]. From the
spectra data (Appendix 11-17) it can be observed that the isolate resembles a methyl ursolate (3.6) which is a known compound.

3.6 (Methyl-3β)-3-hydroxy-urs-12-en-28-oate)
3.6 Bioactivity testing

The results obtained from the analgesic test (Table 3.11) showed that the ethanolic extract and oil of *F. ulmaria* at 200 mg/kg significantly (p<0.05) inhibited paw licking in neurogenic phase by 78 and 71 % respectively followed by a reduction of 72 and 81 % respectively in the inflammatory phase (Table 3.11). The standard drug (Aspirin) significantly inhibited pain (p<0.05) in the neurogenic phase by causing a reduction in licking of the hind paw by 84.7 % in the neurogenic phase and 74 % in the anti-inflammatory phase.

Table 3.11: Analgesic effect of *F. ulmaria* ethanolic extract and essential oil on formalin induced pain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>neurogenic phase</th>
<th>Anti-inflammatory phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no of licks Mean± sem</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Vehicle</td>
<td>saline</td>
<td>36±1.4</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>100 mg/kg (aspirin)</td>
<td>5.5±1.05</td>
<td>84.7 **</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>200 mg/kg</td>
<td>16.3±1.60</td>
<td>78</td>
</tr>
<tr>
<td>Essential oil</td>
<td>200 mg/kg</td>
<td>10.2±1.17</td>
<td>71</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. P value *P<0.05, **P<0.01

The oil and extract at a dosage of 200 mg/kg *F. ulmaria* significantly (p<0.01) reduced the paw inflammation induced by the egg albumin in the rats (Table 3.11). Results show a significant reduction of inflammation with time by both the crude extract and the essential oil. More reduction is observed from the administration of the oil than the ethanolic crude extract. Values for inhibition of inflammation peaked up to 47 % for the crude extract.
Table 3.12 shows the results obtained from the antiinflammatory test. The oil and extract at a dosage of 200 mg/kg *F. ulmaria* significantly (p<0.01) reduced the paw inflammation induced by the egg albumin in the rats (Table 3.12). Results show a significant reduction of inflammation with time by both the crude extract and the essential oil. More reduction is observed from the administration of the oil than the ethanolic crude extract. Values for inhibition of inflammation peaked up to 47 % for the crude extract and 63 % for the essential oil. There is significant reduction in inflammation of the group treated with the essential oil between 3rd -5th hour. Administration of the essential oil results in higher inhibition of inflammation than the crude extract as shown in Table 3.12.

Table 3.12: Effect of *F. ulmaria* oil and ethanolic extract on egg albumin-induced paw oedema test.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control Mean±SEM</th>
<th>Meloxicam Mean±SEM</th>
<th>% inhibition</th>
<th>Essential oil Mean±SEM</th>
<th>% inhibition</th>
<th>Ethanol extract Mean±SEM</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1hr</td>
<td>3.68±0.3</td>
<td>2.810±0.0518</td>
<td>31</td>
<td>2.25±0.0548</td>
<td>**</td>
<td>2.22±0.0867</td>
<td>**</td>
</tr>
<tr>
<td>2hr</td>
<td>2.66±0.215</td>
<td>2.346±0.0388</td>
<td>34</td>
<td>1.59±0.209</td>
<td>**</td>
<td>1.80±0.0623</td>
<td>**</td>
</tr>
<tr>
<td>3hr</td>
<td>2.39±0.155</td>
<td>2.146±0.0231</td>
<td>31</td>
<td>1.71±0.186</td>
<td>**</td>
<td>1.98±0.0795</td>
<td>**</td>
</tr>
<tr>
<td>4hr</td>
<td>2.18±0.211</td>
<td>1.614±0.0873</td>
<td>42</td>
<td>1.38±0.0536</td>
<td>**</td>
<td>1.44±0.0938</td>
<td>**</td>
</tr>
<tr>
<td>5hr</td>
<td>1.66±0.273</td>
<td>1.150±0.0420</td>
<td>37</td>
<td>0.79±0.111*</td>
<td>47</td>
<td>0.874±0.169</td>
<td>**</td>
</tr>
</tbody>
</table>

The formalin test is a chemical model for stimulation of pain. It is a bi-phase model comprising of neurogenic and inflammatory mechanisms of nociception [30-31]. The primary phase lasts between 5-10 minutes and measures acute pin emanating from stimulation of nociceptive receptors due to injury of the hind paw when injecting formalin. The secondary phase measures pain due to inflammation. It is referred to as the anti-inflammatory phase. It lasts between 20-30 minutes and is a result of the action of pain mediators such as prostaglandins and histamines. Drugs which inhibit pain in both phases of this pain model are
referred to as centrally acting while those inhibiting the secondary phase are referred to as peripherally acting [32-34]. *F. ulmaria* oil and ethanolic extract significantly inhibited both neurogenic and anti-inflammatory pain P<0.05-P<0.01 as shown in Table 3.12. The current results showed that both the essential oil and the ethanolic extract are able to inhibit the action of mediators of inflammation in both phases of the pain model. Essential oil components such as menthone, isomenthone, salicylic acid and its derivatives which have been reported to have both analgesic and anti-inflammatory properties in earlier reports using the same test method [35]. Salicylates are a class of compounds that are widely valued for their pain killing, antipyretic and anti-inflammatory properties [36-37]. Their mode of action is the inhibition of the synthesis of prostaglandin and its derivatives that cause inflammation [38-39].

Egg albumin has been widely used as a phlogistic agent [40]. The early phase of the albumin inflammation occurs within one hour after sub-plantar injection of the phlogistic agent and is due to the action of inflammatory mediators such as histamines and serotonin [41]. These are released from mast cells and increase vascular permeability thus favouring formation of oedema. Our results showed a gradual inhibition of inflammation by the oil and extract of *F. ulmaria* indicating that this plant oil and extract may exert their action by inhibiting the release/effect of histamine and serotonin [42]. The anti-inflammatory effects of *F. ulmaria* can be attributed to the presence of essential oil components such as salicylates, eugenol, menthol and glycosides, flavonoids and steroids from the ethanolic crude extract. Salicylates are prominent [43] while menthol and eugenol are used for topical pain relief [44-45]. Studies suggest that eugenol exerts its analgesic effects via the capsaicin receptor and high voltage activated calcium channel inhibition [46].
3.7 Conclusion

The present study showed that *F. ulmaria* oil and ethanolic extract have significant anti-inflammatory properties thus confirming their use for the treatment of inflammation and management of pain in traditional medical practice.
3.8 References


Chapter 4

Determination of the essential oil and ethanolic extract composition of *Agathosma betulina* (Berg.) Pillans and their bioassays

4.1 Introduction

Buchu belongs to the genus *Agathosma* and the family Rutaceae. It is native to South Africa and the southern parts of Africa. However two plant species are often referred to as buchu, namely *Agathosma betulina* and *Agathosma crenulata*. They possess small shrubs with woody stems (Figure 4.1). Buchu is a highly aromatic plant well known for its production of essential oil [1-2]. Most commonly used in trade and commerce is the *Agathosma betulina* type. Buchu (*Agathosma betulina*) is a protected plant species in South Africa under the Kingdom flora of the Cape Floristic Region. Harvesting of the plant is strictly controlled by the Cape Nature Conservation [3]. Buchu has been in use in the Western Cape region of South Africa for centuries finding prominent use by the Khoisan people around the 1650s [4]. It has been reported that the Khoi and the San people prepared brandy by distilling the leaves with wine. This produced efficient remedies for the bladder and stomach ailments [5]. Significant revenue has been obtained through the extensive export of buchu oil and dried leaves to European markets and the USA. Simpson reported that over 180000kg of dry buchu was exported from the cape colony to Reece & Company in 1873 [6].

![Figure 4.1: Agathosma betulina (Berg.) Pillans (Buchu)](image-url)
4.2 Phytochemical composition and ethno medicinal value

The essential oil composition of buchu from the Western Cape region of South Africa 8-mercapto-p-menthan-3-one, a sulphur containing terpene has been isolated and found to strongly contribute to the flavour and aroma of buchu [7]. The essential oil is used in the manufacture of flavourings and perfume [7]. In traditional practice, A. betulina is usually taken orally in an aqueous infusion extracts sometimes sweetened with brown sugar. Other dosage forms include a vinegar infusion, for external application as an antiseptic wash, urinary tract infections, cleansing of wounds, colds and coughs, rheumatism and diuretic [7]. An infusion of the leaves in brandy known as Buchu brandy is used in the Western Cape Province of South Africa as a stimulant tonic and as a remedy for indigestion and stomach complaints [4]. Buchu vinegar is also highly regarded in the washing and cleaning of wounds. Over the years buchu has been used in the management of inflammation, and kidney and urinary tract infections [4]. Traditional medical practitioners report gastrointestinal irritation as a possible side effect of oral use. Patients suffering from kidney infections are normally warned to use buchu remedies with caution. Essential oil components diosmin and pulegone are also suspected to cause renal irritation. Pulegone is a known abortion inducing agent and has also been reported to prolong and increase the menstrual flow hence it has contraindicated against pregnant women [8].

4.3 Experimental design

4.3.1 Preparation of plant material

Dried stem, flowers and leaves of A. betulina (Figure 4.2) were supplied by Mr Reuben Matewu, an herbalist from King William’s town in the Eastern Cape Province of South Africa in the month of August 2013. The plant material was kept under room temperature and pressure and allowed to dry naturally before being taken for hydrodistillation.
4.3.2 Hydrodistillation of the essential oil

Dry plant leaves (Figure 4.2) were placed in a round bottomed flask with excess distilled water. The dry leaves were hydrodistilled using a modified Clevenger apparatus for 3 hours. After the extraction process the oil was placed in a vial for storage at 4°C.

![Figure 4.2: A. betulina dried leaves](image)

4.3.3 GC/MS analysis

GC/MS analyses of the oils were performed on an Agilent 5973N Gas Chromatography-mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS fused silica capillary system with a 5% phenylmethylsiloxane stationary phase. Capillary column parameter was 30m by 0.25mm, film thickness 0.25µm. The initial temperature of the column was 70°C and was heated to 250°C at a rate of 5°C/min. The final temperature was kept at 450°C and run time of 77.25 minutes. Helium was used as the carrier gas at a flow rate of 1ml/min. The split ratio was 100:1. Scan time was 78 minutes with a scanning range of 35 to 450 amu. One micro litre (1µl) of the diluted oil (in hexane) was injected for analysis.

4.3.4 Identification of compounds

The essential compounds were identified by gas chromatography using retention indices. A homologous series of alkanes under the same operating conditions was run in order to
determine the retention indices. The components of the oils were identified by matching their spectra and retention indices (Kovat Index) using the NIST spectrometer data bank. Identification of the main components was also carried out by the comparison of both the GC retention times and the MS data against those of the reference standards, Kovats retention indices (KI) and comparison with previous literature [9]. The homologous series of n-alkanes (C8-C30) was used as standards [10].

4.3.5 Pharmacological studies

4.3.5.1 Animals

Twenty four Sprague Dawley rats weighing 180-250 g and 24 Swiss albino mice weighing 18-32 g of both sexes were obtained from the South African Vaccine program October 2013. The rats were allowed to acclimatize in the new environment for 2 weeks at Walter Sisulu University animal holding facility and were used according to the Walter Sisulu University Ethical Clearance Committee (N0 0009/07) ethics. Room temperature was maintained at 24°C while lighting was provided exclusively by daylight.

Wood shavings were used as bedding. During this period of acclimatization, animals had free access to pellets (EPOL SA: protein-180 g/kg, Moisture-120 g/kg, Fat-25 g/kg, Fibre-60g/kg, Calcium-18 g/kg and Phosphorus-7 g/kg) and water ad libitum. Cages were cleaned and bedding replaced 2 times per week.

4.3.5.2 Drugs used in the study

Aspirin (Sigma Aldrich, St. Louis, USA) and meloxicam (Sigma Aldrich, St. Louis, USA) used in this study were of analytical grade.
4.3.5.3 Analgesic evaluation of essential oil and ethanolic extracts

The formalin test was carried out to determine analgesic activity as described by Prabhu et al. (2011) with some modifications [11]. 4 groups of 6 mice each were selected for each investigation in the study. Three series of analgesic activity studies were undertaken on the treated groups namely *A. betulina* plant extracts (essential oil and ethanolic extract). The animals were randomly assigned to treatment groups as follows:

- Group I – 1 ml normal saline the control group.
- Group II – 100 mg/kg Aspirin the standard group
- Group III – 200 mg/kg essential oil
- Group IV – 200 mg/kg ethanolic extract.

Animals were placed in transparent observation cages.

One hour after treatment with the oil and extract, animals were injected sub-plantarly with a 100 μl of 2.5% v/v formalin solution in saline. Animals responded to formalin injection by licking or biting the injected paw. The number of times the animal licks/bites the paw was recorded during the first 5 min (neurogenic phase) and then 20 to 30 min (inflammatory phase) after formalin injection. The percentage inhibition was 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

4.3.5.4 Anti-inflammatory evaluation of essential oil and ethanolic extract

Acute inflammation study was carried out using 24 male Wistar rats per experiment. Animals were divided into groups of six rats each per study. Anti-inflammatory activity studies were
undertaken on each group for *A. betulina* plant material essential oil and ethanolic extract).

The animals were randomly assigned to treatment groups as follows:

- **Group I** - The control group (which was administered (0.09 %) normal saline).
- **Group II** - 15 mg/kg Meloxicam
- **Group III** – 200 mg/kg essential oil.
- **Group IV** – 200 mg/kg ethanolic extract.

The animals were placed in transparent cages for observation.

Acute inflammation study was carried out using the induced rat hind paw edema model as described by Ojewole et al (2006) with slight modifications (Paw oedema test). 24 rats of both sexes were used with fresh egg albumin as the phlogistic agent injected into the sub-plantar surface of the left hind paw [12]. Thirty minutes post treatment, inflammation of the left hind paw was induced by injecting 1ml of fresh egg albumin into the sub-plantar surface of the left hind paw. The control group was treated with the vehicle while standard group received meloxicam. The other two groups were treated with essential oil and ethanolic crude extract (200 mg/kg). The paw diameters of the animals were measured with a digital caliper. Baseline paw diameters were recorded. Then, the hourly reading was taken after the injection of 50% v/v fresh egg albumin until the 5th hour.

\[
\text{Percentage inhibition} = (1 - \frac{D}{C} \times 100\%)
\]

Where D is difference in paw volume after administration of test drugs and C is the difference on paw volume in control groups.

### 4.3.5.5 Statistical analysis

The data were subjected to statistical analysis and the results presented as mean ± standard error of mean (SEM). Comparison between the experimental group and the control group
were made by one way analysis of variance (ANOVA) followed by the Dunnet post hoc test. P<0.05 was taken to be statistically significant.

4.4 Results and discussion

4.4.1 Volatile extractive analysis

Hydrodistillation of the dried leaves produced 0.73 g (Table 4.1) of light yellow oil (Figure 4.3).

![Figure 4.3: A. betulina essential oil](image)

**Table 4.1: Physicochemical analysis of the essential oil of A. betulina**

<table>
<thead>
<tr>
<th></th>
<th>Colour of oil</th>
<th>Quantity of oil Obtained (g)</th>
<th>Percentage Yield (w/w %)</th>
<th>Odour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried leaves</td>
<td>Tint yellow</td>
<td>0.73</td>
<td>0.37</td>
<td>Minty black current</td>
</tr>
</tbody>
</table>

The essential oil obtained from hydrodistillation of the leaves of *A. betulina* was analysed by GC-MS (Appendix 17) and the results were collated in Table 4.2. The essential oil constituents were further classified into their essential oil categories as shown in Table 4.3.
Table 4.2: Chemical composition of the essential oil of *A. betulina*

<table>
<thead>
<tr>
<th>№</th>
<th>Retention time</th>
<th>KI</th>
<th>Compound name</th>
<th>%Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.1388</td>
<td>1018</td>
<td>Limonene</td>
<td>9.1</td>
</tr>
<tr>
<td>2</td>
<td>8.736</td>
<td>1082</td>
<td>Linalool</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>10.529</td>
<td>1148</td>
<td>Isomenthone</td>
<td>26.1</td>
</tr>
<tr>
<td>4</td>
<td>10.726</td>
<td>1148</td>
<td>menthone</td>
<td>12.1</td>
</tr>
<tr>
<td>5</td>
<td>10.94</td>
<td>1182</td>
<td>Terpene-4-ol</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>12.489</td>
<td>1256</td>
<td>Pulegone</td>
<td>10.5</td>
</tr>
<tr>
<td>7</td>
<td>13.183</td>
<td>1273</td>
<td>p-Diosphenol</td>
<td>12.8</td>
</tr>
<tr>
<td>8</td>
<td>14.063</td>
<td>1296</td>
<td>Diosphenol</td>
<td>17.9</td>
</tr>
<tr>
<td>9</td>
<td>16.667</td>
<td>1361</td>
<td>8-mercapto-p-menthane-3-one</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>21.154</td>
<td>1444</td>
<td>acetylthiomenthane-3-one</td>
<td>2.9</td>
</tr>
<tr>
<td>11</td>
<td>25.43</td>
<td>1733</td>
<td>Ascabiol</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 4.3: Summary of the chemical profiles of the essential oil of *A. betulina*

<table>
<thead>
<tr>
<th>Class of compounds</th>
<th>Number of compounds</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-terpenes</td>
<td>1</td>
<td>9.1</td>
</tr>
<tr>
<td>Oxygenated monoterpenes</td>
<td>7</td>
<td>84.2</td>
</tr>
<tr>
<td>Esters</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Sulfonated (sulphur containing) monoterpenes</td>
<td>2</td>
<td>5.4</td>
</tr>
</tbody>
</table>
Light yellow oil with a strong blackcurrant smell was obtained from hydrodistillation of the dried leaf of *A. betulina*. The major oil components were found to be isomenthone (4.1) (26.1 \%) and diosphenol (4.2) (17.9 \%), menthone (4.3) (12.1 \%) and pulegone (4.4) (10.5 \%). Further analysis of the chemical profile of the essential oil revealed that the oil had more oxygenated monoterpenes (84.2 \%) in higher proportion than any other group followed by monoterpenes (9.1 \%) and sulphur containing monoterpenes (5.4 \%). The results from this experimental investigation concur with previous reports that buchu oil comprises mostly of oxygenated monoterpenes [13-14]. Collins *et al* (1996) reported that there are two main chemotypes of *A. betulina* [7]. There is the Isomenthone type and the Diosphenol type. The Diosphenol chemotype has a relatively high content of Diosphenol content hovering around 12 \% and a lower content of Isomenthone and menthone of less than 29.5 \% and 9.6 \% respectively whereas the other (Isomenthone) chemotype has a lower Diosphenol content below 0.14 \% and a very high content of Isomenthone and menthone with compositions of greater than 31 \% and 27 \% respectively [15-16]. Based on that classification the plant material used in this study was classified as the Diosphenol chemotype due to a relatively high Diosphenol (17.9 \%) and p-Diosphenol (12.1 \%) content and correspondingly lower Isomenthone (26.1 \%) and menthone (12.1 \%) content. The essential oil of *A. betulina* can also be identified by the Pulegone (4.4) content which normally ranges from 2.5-4.5 \% even though there are hybrids which have been found to range from 7-28 \% [13-14] High pulegone content has been found to be toxic [17]. In this investigation the Pulegone content was found to be 10.5 \% which did not result in any deaths of mice at the administered dose.
4.4.2 Non-volatile extractive analysis

Phytochemical analyses was carried out to determine the metabolite composition of the crude using standard methods [18-19]. Chemical analyses were carried out using aqueous crude extracts to identify various metabolites using standard methods [21-22]. The results obtained were collated in Table 4.4.

**Test for Alkaloids:** 3 ml aqueous extract was shaken with 3 ml of 1% HCl in a warm water bath. After stirring the mixture Mayer and Wagner’s reagent was then gradually added. A turbid precipitate was produced. This was taken as confirmation of the availability of alkaloids.

**Test for Tannins:** 2 ml of the ethanolic extract was carefully mixed with 2 ml of distilled water under continuous stirring. A few drops of FeCl₃ solution were later added. A green precipitate was obtained. This was taken as confirmation for the availability tannins.

**Test for Saponins:** 5 ml of the ethanolic extract were gradually mixed 5 ml of distilled water under constant and vigorous stirring in a warm water bath. Stable foam was produced which was taken as confirmation of the availability of saponins.
Test for Flavonoids: To 1 ml of ethanolic extract, 1 ml of 10 % lead acetate solution gradually added while stirring. A yellow precipitate was obtained indicating the availability of flavonoids.

Test for Terpenoids: 2 ml of the ethanolic extract were gradually dissolved in 2 ml of chloroform and allowed to evaporate to dryness. 2 ml of concentrated sulphuric acid was then added and heated for about 2 min. A greyish colour was obtained which confirmed the availability terpenoids.

Tests for glycosides: Liebermann’s test: 2 ml of the ethanolic extract were dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added in it. The mixture was allowed to cool in an ice bath. Sulphuric acid was then gradually added. A colour change from violet to blue was obtained. This was taken as confirmation for the presence of a steroidal nucleus (aglycone portion of glycoside).

Tests for steroids: 2 ml of chloroform were mixed with 2 ml of the ethanolic extract. After uniform mixing 2 ml of concentrated sulphuric acid were then added to the mixture. The red colour obtained was taken as an indicator for the presence of steroids.

Table 4.4: Phytochemical screening results

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Terpenes</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
</tr>
</tbody>
</table>

+present and -absent
4.4.3. Bioactive analysis

4.4.3.1 Analgesic activity of the oil and extract

The results of the analgesic test (Figure 4.4) on the formalin test showed that the oil and extract at 200 mg/kg significantly (p<0.01) reduced paw licking by 59 and 54 % respectively in the neurogenic phase; also caused significant (p<0.01) reduction in paw licking by 61 and 45 % respectively in the inflammatory phase. Aspirin caused significant (p<0.01) reduction in paw licking in the neurogenic (84.7 %) and inflammatory (81 %) phases respectively. Comparison between the oil and the crude extract show that more inhibition is obtained from administration of the oil than the ethanolic crude extract.

![Figure 4.4: Effects of oil and extract on formalin-induced paw licking in rats](image)

VEH is vehicle (normal saline). Each bar represents Mean±SEM. **P<0.01 Statistically different from vehicle (ANOVA, Dunnett’s test)
4.4.3.2 Anti-inflammatory activity of the oil and extract

Results obtained (Figure 4.5) show that both the oil and the extract caused significant (p<0.01) reduction in the rat paw oedema with time compared to the negative control group administered with normal saline where there was little reduction in the oedema size. The oil showed better anti-inflammatory effects compared to the extract. Values for inhibition of inflammation reached a peak effect of 52 % for the crude extract 67 % for the essential oil. More inhibition of inflammation is observed between 3-5 hours for the essential oil and during the 5th hour for the crude extract after administration of oil and extract respectively.

![Graph showing the effect of oil and extract on egg albumin-induced paw oedema in rats](image)

**Figure 4.5: Effect of oil and extract on egg albumin-induced paw oedema in rats**

Each bar represents Mean±SEM. **p<0.01 statistically lower than vehicle (ANOVA, Dunnett’s)**

Formalin test is a tonic model of pain resulting from formalin-induced tissue injury and irritation. It encompasses inflammatory and neurogenic mechanisms of nociception [20-21]. It is considered a more valid model for clinical pain than tests involving mechanical or thermal stimulation [22]. The neurogenic phase lasts between five and ten minutes and results
from the acute response to injection of formalin due to the stimulation of nociceptors while the anti-inflammatory phase lasts for about 20-30 minutes after the injection of formalin [23-24]. The second phase results from the action of inflammatory mediators released locally, such as prostaglandins, serotonin, histamine, and bradykinin [25]. The results showed that both the crude extract and the oil significantly inhibited pain in the neurogenic and anti-inflammatory phase. This shows that the crude extract and the oil have constituents that inhibit pain mediators in both phases. Essential oil components present in the oil such as menthone and Isomenthone have been reported to have analgesic properties and flavonoids, steroids and glycosides found in the non-volatile crude extracts have been found to contribute to the analgesic properties of plants [26]. The presence of these metabolites in both the crude extract and the oil could be responsible for the analgesic properties observed in this investigation.

Inflammation is a biological response by vascular tissues to harmful stimuli or foreign irritants. It can be classified as acute or chronic depending on the duration of response to the stimulus. Acute inflammation can be caused by an irritant or a phlogistic agent such as egg albumin. Egg albumin induced inflammation has been used in several studies to test for anti-inflammatory activity [27-28]. The early phase of the inflammation occurring within one hour after sub-plantar injection of the phlogistic agent results due to the action of inflammation mediators such as histamines and serotonin [29-31]. The late phase can occur up to 12 hours due to the release of inflammation mediators such as prostaglandins [30-31]. There was significant reduction in inflammation consistently within all the treated groups from the 1st-5th hr (Figure 4.5) with higher inhibition values obtained after 3h in both the crude extract and the oil. The higher inhibition values observed in the later phase of inflammation could be due to the inhibition of cyclooxygenase (COX) enzyme which catalyses the rate determining phase for the synthesis of prostaglandins [30-31]. However,
comparisons on the percentage inhibition of inflammation showed that the oil had inhibited inflammation more than the crude extract along entire length of the treatment period. The results show that Buchu oil and the crude extract were as effective as meloxicam, a known specific COX-2 inhibitor [32]. Limonene present in the essential oil has been reported to be effective in reducing the synthesis of cyclo-oxygenase 1 and 2 and pro-inflammatory prostaglandins which result in reducing inflammation [33]. Anti-inflammatory activity of buchu can therefore also be attributed to the significantly high amounts of limonene and other components such as menthone and Isomenthone. Phytochemical screening of Buchu ethanolic crude extract showed the presence of phenolics, steroids and flavonoids. Flavonoids and steroids have been reported to have the ability to inhibit the release of inflammatory mediators. These could among other phytochemicals be responsible for the reduction of inflammation and inhibition of pain shown by the buchu crude extract in the investigation [34-35].

4.5 Conclusion

From the results obtained in this investigation we concluded that A. betulina ethanolic extract and the essential oil possess analgesic and anti-inflammatory properties which can be used to validate the use of A. betulina plant in the management of pain and inflammation in traditional medicinal practice.
4.6 References


Chapter 5

Mediated synthesis of silver nanoparticles from *Filipendula ulmaria* (L.) Maxin plant

5.1 Introduction

Biosynthesis of nanoparticles using plant extracts is an attractive method of ecofriendly production of nanoparticles. It has been exploited to a vast extent because plants are widely distributed, easily available, safe to handle and have a diverse range of metabolites [1]. Plant mediated biological synthesis of nanoparticles, hence is gaining importance due to its simplicity and fast rate of reaction. Metabolites are bioactive compounds with intrinsic non-nutrition food components that are beneficial to the health of consumers of these foods. Plant phytochemicals can act both as electron shuttles for the reduction of the noble metals and as capping agents to prevent aggregation and post surface modification of the nano particles in a single pot synthesis [2]. Since the synthesis of silver nanoparticles using plant extracts is a benign method to the environment it can therefore be classified as a green method. Synthesis methods of nanoparticles can be described as green or non-green depending on how eco-friendly the starting materials are. Many of the nanoparticle synthesis methods employed involve toxic and hazardous chemicals which are characterised by low material conversions, high energy requirements, difficult and wasteful purification [3]. Among some of the main principles of green chemistry are waste prevention, energy conservation; maximisation of atom economy, use of safer solvents, and use of catalyst [4]. For a synthetic method to be classified as green, all factors including capping agent, reducing agent and the solvent should be less toxic to the environment [3]. Biological methods of synthesis offer more distinct advantages than their physical and chemical counterparts because the inherent components such as enzymes and phytochemicals can act as reducing and capping agents. As mentioned earlier, reactions involving plant extracts are reproducible and have very low energy
requirements. The nature of the nanoparticles such as particle shape and size are not affected by large scale production [4]. Chemical and physical methods unlike biological methods depend on severe reaction conditions that involve the use of aggressive agents such as sodium borohydride and hydrazinium hydroxide which are toxic to environment and ecology. Among the solvents used for the green synthesis of silver nanoparticles, water is the most environmentally benign solvent. Ecofriendly capping agents such as polysaccharides like glucose, maltose and cellulose have always been used. The binding interactions between these polysaccharides and silver nanoparticle are weak and hence allow their ease of separation. The hydrogen bond network provides stability to the nanoparticle system and controls crystal growth [5]. Gelatin has been used as a green agent for the synthesis of silver nanoparticles. Particles sizes were reported to get smaller with increase in temperature [6-7]. Disaccharides such as maltose and lactose have reducing ability, since at least one of their monomers can assume an open chain form. In a related study, Oluwafemi et al. synthesised silver nanoparticles as small as 3.76 nm using gelatin and maltose as capping and reducing agent respectively. The use of such biocompatible and biodegradable capping and reducing agents marks the beauty of the green approach [8]. No hazards are posed to the environment.

5.2 Experimental design

5.2.1 Synthesis of silver nanoparticles using F. ulmaria extracts (ethanolic extract and hydrosol)

Aqueous solutions (1 mM and 0.1 mM) of silver nitrate (AgNO₃) were used in this investigation. For the ethanolic crude extract, the concentration of the meadowsweet plant material was varied by changing the quantity of crude extract dissolved in distilled water. Crude extract between 1-5 g were dissolved in distilled water to make up 50 g (w/w) of
solution. In studies using the hydrosol, the concentration of the hydrosol was varied by varying the quantity of the hydrosol used while temperature was maintained at 40°C. Volumes of the hydrosol ranging from 1-5 ml were used and topped up with distilled water to make 50 ml (v/v) hydrosol solution. The respective solutions were allowed to stand for two hours to allow the powder and the hydrosol to completely dissolve and for the solution to settle before adding silver nitrate solution.

5.3 Results and discussion

The *F. ulmaria* ethanolic extract and hydrosol solution was dark brown in colour. Immediately after adding all the reactants the colour changed from brown to silver grey indicating that there was a reaction between the meadowsweet material and the silver nitrate solution. The absorption spectra were recorded at different reaction times of 15 min, 30 min, 45 min, 60 min, 1 h, 2 h, 3 h and 24 h. The absorption spectra of the synthesised Ag-NPs at different reaction times showed SPR peak positions between 300 to 500 nm. SPR peaks below 400 nm were observed when 5 ml *F. ulmaria* hydrosol and 5 ml of 0.1M AgNO₃ were used. There was immediate aggregation and precipitation of particles from the solution. These results show that rapid reduction of silver ions to nanosilver took place but the synthesised silver nanoparticles were not stable.

5.3.1 UV-Vis analysis of *F. ulmaria* mediated silver nanoparticles

Figure 5.1 shows the colour of the colloidal solutions at different reaction times. The colour changed from green to silver brown. This was taken as evidence for the reduction of silver ions. This was however followed by immediate precipitation and aggregation of these particles.
Figure 5.1: Colloidal solutions of Ag-NPs synthesised using 5 g ethanolic extract /1ml, 1M AgNO₃

Figure 5.2 shows the absorption spectra of the as-synthesised Ag-NPs when 5 ml of hydrosol and 5 ml of 1M AgNO₃ were used. At the employed concentration of silver nitrate and hydrosol there was no observed absorption in the UV-Vis region for the silver nanoparticles (Figure 5.2). There was evidence of aggregation and precipitation of particles from the solution. This showed that at these reaction conditions there was not enough passivation agent to prevent aggregation of the nanoparticles.

Figure 5.2: Absorption spectra of Ag-NPs synthesised using 5 ml of hydrosol and 5 ml, 1M AgNO₃
Figure 5.3 shows the colour change of the colloidal solution upon the addition of silver nitrate solution. There was a colour change from green to brown followed by a silver grey colour. This showed the reduction of the silver ions but however this was immediately followed by precipitation and aggregation of particles from the solution.

Figure 5.3: Colloidal solutions of Ag-NPs synthesised using 5 ml hydrosol and 5 ml 0.1 MAgNO₃

Results from Figure 5.4 showed that from the beginning of the reaction up to 1h there was no observable difference between the crude absorption and the sampled solutions under UV-Vis. However, at 1h a red shift was observed from the crude UV-Vis absorption indicating the formation of silver nanoparticles [9]. However, the particles quickly aggregated and precipitated out of the solution. The UV spectra suggests that the reduction of silver ions to silver nanoparticles optimally took place at 1 h due to the observed peak between 400 and 500 nm which is a typical silver nanoparticle surface plasmon resonance (SPR) absorption range.
Figure 5.4: Absorption spectra of Ag-NPs synthesised using 5 ml hydrosol and 5 ml 0.1M AgNO₃

Figure 5.5 shows the colloidal solution of the reaction upon addition of aqueous silver nitrate to the extract solution. The colour changed immediately changed from green to brown and silver grey. There was rapid precipitation of particles from the aqueous solution. This showed aggregation of particles.

Figure 5.5: Colloidal solutions of Ag-NPs synthesised using 1 g ethanolic extract and 5 ml of 0.1M AgNO₃

The results from Figure 5.6 show that from 15 min to 60 min there is no significant observable change between the crude UV-Vis absorption and the sampled solutions. However, there is notable change observed between 1-3h. The absorption position shifts to smaller wavelength below 400 nm. All the particles rapidly aggregated and precipitated in the solution. This probably explains that even though the reduction took place, the extract failed to control particle growth and stability of the Ag-NPs under these reaction conditions.

![Absorption Spectra](image)

**Figure 5.6: Absorption spectra of Ag-NPs synthesised using 1 g ethanolic extract and 5 ml of 0.1M, AgNO₃.**

The immediate precipitation and aggregation of particles showed that the synthesis of Ag-NPs mediated by *F. ulmaria* extracts was not successful. However, the immediate change in colour ranging from brown to silver grey showed that there was reduction of silver ions. The passivation agent was not effective at the employed reaction conditions.

**5.4 Future work**

There is more work to be done on plant mediated synthesis of Ag-NPs using this plant. More reaction parameters need to be varied upon availability of plant material in order to make conclusive arguments.
6.5 References


Chapter 6

Mediated synthesis of silver nanoparticles from *Agathosma betulina* (Berg.) Pillans plant and their biological studies

6.1 Introduction

Nanotechnology represents a technological frontier that is able to assemble and reproduce small particles [1]. Nanomaterials have special physicochemical properties as a result of their small size [2]. Polak 2008 described nanoscience as the practical ability to manipulate and make predictions for reactions at a nano-scale (1-100 nm) [3]. It delves into previously unimaginable dimensional realms. Target range of particle size is normally between 1-20 nm. At such nano-scale sizes, nano-particles exhibit very high surface area to volume ratio and increased bioactivity. Completely new properties have been found that are dependent on particle size, morphology and size distribution [2].

Nanotechnology takes advantage of the fact that a decrease in the size or volume of a solid material is accompanied by an increase in the specific surface area. This results in a phenomenal increase in surface reactivity and other quantum-related effects [1-3]. The properties of bulk noble metals change enormously when converted to nanoparticles. Nanoparticles are classified solely based on their size and may exhibit size-related properties that differ significantly from those observed in bulk materials.

6.2 Optical properties of silver

Plasmon resonance refers to the collective excitation of the electrons in the conduction band near the surface of the nanoparticles when excited by electromagnetic (EM) radiation. Electrons are limited to specific vibrations modes by the particle’s size and shape. Therefore,
metallic nanoparticles have characteristic optical absorption spectrums in the UV-Vis region [16].

Silver nano-particles exhibit optical properties which are observed neither in molecules nor in bulk metals. Silver has an absorption band within the light region at around 450 nm [17]. Plasmon absorbance at 400 nm has been observed as a characteristic for spherical or roughly spherical faceted silver nano-particles [18]. The absorption band encroaches into the red wavelength region as the silver nano-particles aggregate [19]. Due to plasmon resonance, different particle shapes and size absorb different wavelengths of light. This explains why certain particle sizes are synonymous with certain colours when illuminated with white light.

6.3 Synthesis of silver nanoparticles

6.3.1 Bottom up and Top down approaches

Nano synthesis methods can be classified generally into two categories which are bottom up and top up approaches. In the bottom-up approach, the structure of nanoparticles is constructed by atoms, molecules or clusters with a targeted precursor particle which is allowed to grow in size. In top-down approach, a bulk piece of a required material is reduced to nanosized dimensions using cutting, grinding and etching techniques. In both approaches choice of synthesis method is heavily dependent on the ability to control particle size, shape, distribution, composition and prevent particle aggregation. Nanomaterials prepared from larger entities have less atomic-level control [20-21]. Although small particle sizes ranging from 10 to 100 nm can be obtained when using bottom up technique however, defects in the surface structure are likely to be present [22]. Top-down techniques are the methods of choice for the synthesis of highly complex structures. The bottom-up approaches involve wet chemistry techniques. The major bottom up synthetic method for silver nanosynthesis
involves silver salt precursor in an aqueous medium. The silver ion is reduced and stabilised via a chemical reaction through nucleation and growth [23]. Capping agents are often added to control crystal growth, prevent aggregation, provide particle stability and conjugation for different applications [24-25].

Synthesis of nanoparticles can be broadly classified into three methods namely chemical, physical and biological. Silver nanoparticles are synthesized in various shapes. These shapes of silver nanoparticles have often been grouped into 1, 2 and 3 dimensional. The one dimensional shapes are represented by thin films, the two dimensional shapes by nanorods and wires while the three dimensional shapes are represented by spherical. Various shapes of silver nanoparticles have been reported or synthesised. Spherical particles are the most prevalent among silver nanoparticles [26]. The shape of silver nanomaterials might have an impact on some of its properties. These shapes have been reported to exert varying biological activities on microorganisms. Pal et al in their study reported that triangular silver nanoparticles were more biocidal against Gram-negative bacterium *Escherichia coli* than the spherical and rod shaped silver nanoparticles [27].

### 6.3.2 Stabilisation mechanism of a nanoparticle system

Nanoparticles have high surface area to volume ratio which gives them a large tendency to aggregate or agglomerate to larger particles to minimise interfacial energy of their system. In order to avoid aggregation nanoparticles have to be kinetically stabilized [28]. Forces of attraction such as Van der Waals are majorly responsible for the aggregation of the nanoparticles. Forces of repulsion are therefore necessary to counter the effects of these forces of attraction. Capping agents maintain the stability of the colloidal system preventing particle agglomeration at the solid liquid interphase obtainable on colloidal nanocrystals systems. Stability of the system involving capping agents goes through steric, electrostatic or
ionic repulsion [29]. Several factors which include temperature, pH of the system, electrolyte composition, ionic strength of reducing agent, capping agent among others have a strong influence on colloidal stability [28-34]. This therefore calls for optimisation of these parameters in order to get the best for stability of a nanoparticle system.

### 6.3.3 Chemical reduction methods

This technique involves chemical reduction of a silver salt with strong reducing agents such as sodium borohydride (NaBH$_4$), hydrazine (N$_2$H$_4$), sodium citrate, etc. Stability of the resultant nanoparticle solution is the challenge that is usually encountered in this method. Hence, capping agents are required to stabilise the system, control crystal growth and prevent particle aggregation or agglomeration. Some of these reactions can take place at room temperature and some require elevated temperature. It has been experimentally proven that stronger reducing agents promote the formation of nuclei with smaller diameters, which then continue to grow [36]. Other than temperature, reaction kinetics is dependent on several factors such as pH of the system, electrolyte composition, ionic strength of redox agent, capping agent among others. Guzmán et al., synthesised silver nanoparticles (Ag-NPs) using silver nitrate solution and sodium dodecyl sulphate as a metal salt precursor and stabilizing agent respectively. Hydrazine hydrate was also used as a reducing agent coupled with sodium citrate as stabilizing agent [37]. The bulkiness of sodium citrate is used to control particle agglomeration and stabilise the system through both steric hindrance and electrochemical stabilisation [38-39]. Silver nanoparticles can also be synthesised by reduction with an organic base without the use of any capping agents [38].
6.3.4 Plants mediated synthesis method

This technique involves the use of plant extracts for reduction of silver salts to silver nanoparticles. The mechanism of metal nanoparticle synthesis using plant extracts includes 4 main phases namely:

- the activation phase which is accompanied by the reduction of metal ions and nucleation of the reduced metal atoms
- the growth phase where-by the small adjacent nanoparticles spontaneously coalesce into particles of a larger size and direct formation of nanoparticles by means of heterogeneous nucleation
- growth, and further metal ion reduction, which is accompanied by an increase in the thermodynamic stability of nanoparticles
- Termination phase which determines the final shape of the nanoparticles. The presence of plant phytochemicals plays a significant role in the stabilisation of the nanoparticles [39-41].

Plant extracts reduce the metal ions in a shorter time compared to microorganisms. Synthesis of nanoparticles using plant extracts is highly dependent on phytochemical composition of the plant. The time of synthesis ranges from few minutes to hours [42]. Different phytochemical (metabolites) composition also results in different mechanisms for the reduction and capping of the nanoparticles since these metabolites come with different functional groups. These factors could influence the level of metal deposition around already existing nanoparticles, and also the prospect of new nucleation events. The nature of phytochemicals and their composition also have a bearing on factors such as particle shape, size and morphology distribution [43]. The silver nanoparticles synthesised using leaves of *O Sunctum* were found to be surrounded by proteins and various metabolites such as terpenoids, alcohols and carboxylic acids. These demonstrated the involvement of plant metabolites in
the reduction and stabilisation of silver nanoparticles [44-45]. In another report, well-dispersed, single, flower like silver nanoparticles were synthesised using red apple extract in a single pot. The rate of reaction was found to be dependent on the concentration of the plant extract. The particles obtained were poly-disperse mixtures in the range 50 to 300 nm. The stability of the nanoparticles at room temperature was tested using the zeta potential and this stability was largely attributed to the presence of metabolites such as flavonoids, proteins and reducing sugars present in the extract [46]. The synthesis of silver nanoparticles using plant extracts has the ability to produce nanoparticles without toxic chemicals. This also gives it an advantage over the use of microorganisms which require difficult and laborious culturing and purification methods [47]. Plants use has an added advantage in that it can be suitably scaled up for large-scale synthesis of nanoparticles [48-49].

6.4 Anti-inflammatory properties of Ag-NPs

Prostaglandins are chemicals secreted by the body’s immune system to fight off bacteria and other infection causing organisms. Located around wounds, these chemicals cause pain and inflammation. Following bacterial infection, prostaglandins are released by the body which is characterised by a rise in temperature. In their capacities to cause pain, inflammation, and fever, prostaglandins are a nuisance. Anti-inflammatory drugs and silver nanoparticles work on inhibiting their production and consequently reducing pain, inflammation, and fever. Nanosilver dressings as well as nanosilver-derived solutions have been reported to show anti-inflammatory activity [50]. In animal models, nanosilver alters the expression of matrix metallo-proteinases enzymes that are important in various inflammatory and repair processes [51] and induces apoptosis of inflammatory cells [52-53].
6.5 Experimental design

6.5.1 Synthesis of silver nanoparticles using *A. betulina* plant extract

Thirty grams of *A. betulina* ethanolic extract were dissolved in 100 ml of water and filtered. From this solution 10ml was taken and mixed with 10ml of 0.1M AgNO₃. Aliquots were taken at different reaction times for optical analysis. The experiment was repeated at different temperatures of 60⁰C and 75⁰C.

6.5.2 Pharmacological studies

6.5.2.1 Animals

Forty two Swiss albino mice weighing between 18-35 g were obtained from the South African Vaccine program. The animals were housed during the experimental period in the Department of Biology and Environmental Sciences animal holding facility at Walter Sisulu University and were used according to the Walter Sisulu Ethical Clearance Committee (N0 0009/07) ethics. The animals were allowed to acclimatize in the new environment for 2 weeks. Room temperature was maintained at 24°C while lighting was provided exclusively by daylight. Wood shavings were used as bedding. During this period of acclimatization, animals had free access to pellets (EPOL SA: protein-180 g/kg, Moisture-120 g/kg, Fat-25 g/kg, Fibre-60 g/kg, Calcium-18 g/kg and Phosphorus-7 g/kg) and water *ad libitum*. Cages were cleaned and bedding replaced 2 times per week.

6.5.2.2 Drugs used in the study

Aspirin (Sigma Aldrich, St. Louis, USA) used in this study was of analytical grade.
6.5.2.3 Analgesic evaluation of ethanolic extracts and silver nanoparticles

The formalin test was carried out to determine analgesic activity as described by Prabhu et al., (2011) with some modifications [54]. Seven groups of six mice each were selected for each investigation in the study (n=6).

The animals were randomly assigned to treatment groups as follows:

- Group I – 1ml normal saline, the control group.
- Group II -100 mg/kg Aspirin, the standard group
- Group III - 200 mg/kg ethanolic extract.
- Group IV - 200 mg/kg of Ag-NPs produced at 40°C.
- Group V - 200 mg/kg of Ag-NPs produced at 60°C.
- Group VI - 200 mg/kg of Ag-NPs at 75°C harvested after 15 mins.
- Group VII - 200 mg/kg Ag-NPs produced at 75°C harvested after 24h.

Animals were placed in transparent observation cages.

One hour after treatment with the oil and extract, animals were injected sub-plantarly with a 100 μl of 2.5% v/v formalin solution in saline. Animals responded to formalin injection by licking or biting the injected paw. The number of times the animal licks/bites the paw was recorded during the first 5 min (neurogenic phase) and then 20 to 30 min (inflammatory phase) after formalin injection.

The percentage inhibition was 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.
6.5.2.4 Statistical analysis

The data were subjected to statistical analysis and the results presented as mean ± standard error of mean (SEM). Comparison between the experimental group and the control group were made by one way analysis of variance (ANOVA) followed by the Dunnet post hoc test. P<0.05 was taken to be statistically significant.

6.6 Results and discussion

6.6.1 UV-Vis analysis of A. betulina mediated silver nanoparticles

Figure 6.1 shows the colour of the colloidal solution after addition of aqueous silver nitrate to the extract solution at 40°C. The yellow brown colour formed signified the production of Ag-NPs [43-45]. The intensity of the colour increased as the reaction time increased indicating increase in the concentration of the Ag-NPs present in the solution.

Figure 6.1 Colloidal solutions of Ag-NPs synthesised using ethanolic extract at 40°C at different reaction time.
Figure 6.2 shows the absorption spectra of the Ag-NPs synthesised using *A. betulina* ethanolic extract at 40ºC. The reaction progressed slowly and the intensity of the colour of the samples took time to change. Weak SPR absorptions were observed from 15mins to 5h as shown in Figure 6.2. Absorptions in the UV-Vis region were observed between 430-500 nm. These typical Ag-NPs SPR absorptions indicated the reduction of silver ions to Ag-NPs. After 5h there was a gradual shift in the SPR absorptions to around 550 nm.

![Absorption spectra of Ag-NPs](image)

**Figure 6.2 Absorption spectra of Ag-NPs synthesised using ethanolic extract at 40ºCat different reaction time.**

Figure 6.3 shows the change in the colour of the extract solution upon addition of the aqueous silver nitrate at 60 ºC. The change in the colour of the extract to brown signified the reduction of silver ions to Ag-NPs [43-45]. There was no precipitation of material from the extract solution.
Figure 6.3: Colloidal solutions of Ag-NPs synthesised using ethanolic extract at 60ºC at different reaction time.

Figure 6.4 shows the absorption spectra of Ag-NPs synthesised using A. betulina ethanolic extract at 60ºC. Typical Ag-NPs SPR absorptions were obtained around 480 nm between 15mins and 3 h indicating the production of Ag-NPs. These SPR absorptions shifted to shorter wavelengths (around 430 nm) at longer reaction times (20-24 h) indicating decrease in particle size.

Figure 6.4: Absorption spectra of Ag-NPs synthesised using ethanolic extract at 60 ºC at different reaction time.
Figure 6.5 shows the colour of the colloidal solution after addition of silver nitrate solution to the plant extract solution at 75 °C. The colour changed from light yellow to brown as the reaction time increased. The brown colour indicated the reduction of silver ions to Ag-NPs by the phytochemicals present in the ethanolic extract [43-45].

Figure 6.5: Colloidal solutions of Ag-NPs synthesised using ethanolic extract at 75⁰C at different reaction time.

Figure 6.6 shows the absorption spectra of Ag-NPs synthesised using A. betulina ethanolic extract at 75⁰C. Plasmon absorption peak was observed at around 480 nm between 15min and 3h indicating the formation of Ag-NPs. The intensity of the golden brown colour gradually increased with time until 3 h showing an increase concentration of silver nanoparticles in the solution. From 4-20 h, there was a red shift in the position of the SPR to longer wavelengths at around 600 nm.
The silver nanoparticles exhibit yellow brownish colour in aqueous solution due to the excitation of surface plasmon vibrations in silver nanoparticles [44-47]. In order to determine the formation of the nanoparticles in the colloidal solution, UV-Vis spectroscopy was used [48]. Comparing the absorption spectra in Figure 6.2, 6.4 and 6.6 it can be suggested that the plant mediated synthesis of silver nanoparticles using A. betulina is dependent on both temperature and time. At temperatures around 40°C surface plasmon resonance bands in the UV-Vis region (SPR) around 400 nm were observed. These are typical Ag-NPs SPR absorptions [49]. The broadening of the SPR absorption band reflected the presence of particles of various sizes and morphologies [49]. Increasing the temperature to 60°C initially produced broad SPR bands at around 550 nm. However, at longer incubation time a blue shift in the SPR peak was observed to around 430 nm. The SPR bands were sharp and narrow which indicated the production of smaller Ag-NPs with a narrow and distinct particle size.

Figure 6.6: Absorption spectra of Ag-NPs synthesised using ethanolic extract at 75°C at different reaction time.
distribution [45]. A further increase in temperature to 75°C initially produced a sharper and focused SPR absorption bands between 430-480 nm at shorter reaction time. As the reaction progressed a red shift in the position of the SPR peak to around 580 -680 nm was observed. This indicates the formation of large particles with broad size distribution [45]. The broadening and increase in bandwidth showed that at 75°C, the plant phytochemicals had their ability to act as passivating agents reduced drastically after 20 h reaction time. The prolonged high temperature may have caused degradation of some functional groups that are actively involved in the reduction and passivation of the silver nanoparticles [47]. This might result in the formation of anisotropic particles [47-48].

6.6.2 FTIR Analyses of *A. betulina* ethanolic extract and *A. betulina* mediated silver nanoparticles

Figure 6.7 shows the FTIR spectra of *A. betulina* extract and as-synthesised Ag-NPs. The prominent peaks in the silver nanoparticles were 396, 460, 600, 1100, 1387, 1615, 2900 and 3400 cm⁻¹. The FTIR study indicates the presence of secondary metabolites surrounding the silver nanoparticles hereby confirming their involvement in the reduction and stabilisation of the Ag-NPs.
A closer look at the peaks therefore shows that the main peaks that were involved in the reaction are the new ones and those that changed in shape and intensity in the crude extract spectra. The bands in the nanoparticles spectrum at 396 cm\(^{-1}\), 460 cm\(^{-1}\) and 600 cm\(^{-1}\) originate from Ag-NPs-ligand stretching vibrations that appear due to interaction of bio-molecules with the nanoparticles [56]. From the three FTIR spectra, it is quite evident that the Ag-NPs synthesised at 60\(^{0}\)C and 75\(^{0}\)C absorb more strongly at 396 cm\(^{-1}\) and 460 cm\(^{-1}\) than the Ag-NPs at 40\(^{0}\)C. The absorption at 396 cm\(^{-1}\) was stronger than the one at 460 cm\(^{-1}\) for both the nanoparticles at 60\(^{0}\)C and 75\(^{0}\)C. This could mean a higher concentration of silver nanoparticles of a particular size that absorb at that wavelength. However, even though the nanoparticles at 40\(^{0}\)C absorb at the two peaks of 396 cm\(^{-1}\) and 460 cm\(^{-1}\) the absorption at 460 cm\(^{-1}\) is stronger than the absorption at 396 cm\(^{-1}\). This is reflective of the high concentration of nanoparticles with absorption at 460 cm\(^{-1}\). The absorption band at 3400 cm\(^{-1}\) which is a broad peak could be indicative of NH groups or OH bonded to a carbohydrate which shifts from a broad peak to a narrow stretching peak at the same value in the nanoparticles. This shows that

**Figure 6.7: FTIR spectra of ethanolic extract and synthesised Ag-NPs at different temperature.**
the NH and OH groups among a host of other functional group could be broadly responsible for the reduction of ionic silver to zero valent silver nanoparticles [56]. The peak at 2900 cm$^{-1}$ is attributed to the alkane stretching band. This is quite prominent in the crude but decreases in intensity in the nanoparticles. It could indicate a realignment of C-H bonds of the organic compounds in the crude during the nanoparticle synthesis. [57-58]. The crude extract band at 1490 cm$^{-1}$ represents amide bond arising from C=O and NH groups stretching vibrations [59] while the band at 1615 cm$^{-1}$ is the fingerprint region for C=O and C-O functional groups. It corresponds to the $-\text{C}=\text{O}$– stretching vibration modes of tertiary amides coupled with C-N stretch. This band totally disappears in the Ag-NPs showing active participation of amides in the nanoparticle synthesis. The bands between 950 cm$^{-1}$ and 1200 cm$^{-1}$ in the crude extract represents carbohydrates (C-OH) stretching band [59]. The peaks observed from FTIR analysis confirm that the nanoparticles are surrounded by proteins, carbohydrates and flavonoids.

Phytochemical screening of the crude confirmed the presence of proteins, carbohydrates and flavonoids. These contain functional groups observed from FTIR analysis such as C=O, C-O, OH and amide groups (CO-NH). The alcohol reduction process of silver ions has been reported to be a very general process for the production of metal nanoparticles, often stabilized by organic polymers. Alcohols are oxidised to carbonyl compounds during the reduction of silver ions to silver nanoparticles. Flavonoids, a large group of polyphenolic compounds contain functional groups capable of nanoparticle formation. It has been reported that the tautomeric transformations of flavonoids from the enol-form to the keto-form may release reactive hydrogen that can be used in the reduction of silver ions [61]. Reduction of silver ions therefore serve to support the notion that plant phytochemicals can act both as electron shuttles in reduction of the noble metals and as capping agents to prevent aggregation and post surface modification of the nanoparticles [62]. These findings is in
accordance with the Gole et al., observation who reported that proteins can bind to nanoparticles through free amine groups by the electrostatic attraction of polar and negatively charged ionic functional groups for stabilisation of silver nanoparticles [63].

6.6.3 TEM Analysis

The TEM images of *A. betulina* ethanolic extracts synthesized silver nanoparticles are shown in Figures 6.8. TEM analysis was performed in order to have a better insight of the morphology and size distribution of the Ag-NPs. The images confirmed that the synthesized silver nanoparticles are polydispersed. The synthesized silver nanoparticles are spherical and oblong and some have dispersed undefined shapes. The *A. betulina* ethanolic extract synthesized silver nanoparticles diameter varied from 5-60 nm in size with an average particle diameter between 30 nm (Figure 6.9).

![Figure 6.8: Typical TEM images of Ag-NPs synthesised at 60ºC](image-url)
6.6.4 Evaluation of Analgesic activity

Results in Table 6.1 show that all the silver nanoparticles significantly reduced the number of paw licks at a dosage of 200 mg/kg. Inhibition values ranged from 73 to 98 % (P< 0.05) for all the nanoparticles for the neurogenic phase while inhibition values ranged from 55-80 % for the anti-inflammatory phase. The inhibition values for *A. betulina* ethanolic extract were 55 % and 45 % for the neurogenic phase and the anti-inflammatory phase respectively. The inhibition values for the positive control (Aspirin) were 84 % and 81 % for the neurogenic and the anti-inflammatory phase respectively.
Table 6.1: Effect of nanoparticles on paw licking in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/Kg)</th>
<th>neurogenic phase</th>
<th>Anti-inflammatory phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no of lick Mean±SEM</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Control</td>
<td>Normal saline</td>
<td>42.3 ± 1.5</td>
<td>30.5 ± 1.7</td>
</tr>
<tr>
<td>Standard (Aspirin)</td>
<td>100 mg/Kg</td>
<td>7 ± 1.3</td>
<td>83</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>200 mg/Kg</td>
<td>19.7 ± 0.7</td>
<td>54</td>
</tr>
<tr>
<td>NP 40°C</td>
<td>200 mg/Kg</td>
<td>6.7 ± 2.3</td>
<td>84</td>
</tr>
<tr>
<td>NP 60°C</td>
<td>200 mg/Kg</td>
<td>0.8 ± 0.5</td>
<td>98</td>
</tr>
<tr>
<td>NP 75°C @ 15 mins</td>
<td>200 mg/Kg</td>
<td>11.3 ± 1.3</td>
<td>73</td>
</tr>
<tr>
<td>NP 75°C @ 24 h</td>
<td>200 mg/Kg</td>
<td>6.3 ± 0.6</td>
<td>85</td>
</tr>
</tbody>
</table>

P<0.05-P<0.01 statistically lower than the vehicle. ##P<0.01 statistically higher compared to A. betulina ethanolic extract.

The Ag-NPs significantly reduced the number of paw licks showing that silver nanoparticles have the ability to inhibit the action of pain mediators (Table 6.1). Analysis of variance between the experimental groups showed that the inhibition values of the nanoparticles were statistically higher than those administered with A. betulina ethanolic extract alone. The secondary metabolites revealed during qualitative analysis of the ethanolic extract surrounding the silver nanoparticles could provide a synergistic increase in the analgesic activity of the silver nanoparticles. Our results show that the Ag-NPs significantly inhibit the effect of inflammation mediators such as prostaglandins and its derivatives that cause pain and inflammation more than the ethanolic extract [66-67]. The nanoparticles produced at
75°C at 15 mins had smaller inhibition values of pain for both the neurogenic and anti-inflammatory phase. This could be due to the lower concentration of silver nanoparticles during the early course of the reaction. The low concentration of silver nanoparticles however, comparably reduced paw licking in mice. This shows that silver nanoparticles are bioactive even at low concentrations [68]. The silver nanoparticles inhibit pain as effectively as aspirin. The results obtained show that silver nanoparticles are more effective than buchu extracts in the management of pain.

6.6 Conclusion

Green synthesis of silver nanoparticles using *A. betulina* ethanolic extract is reported for the first time. Stable silver nanoparticles were synthesised and characterised by UV-Vis, FTIR and TEM. The synthesis of Ag-NPs mediated by *A. betulina* ethanolic extract was time and temperature dependent. The reaction was favoured by moderate temperatures at 60°C and longer reaction times. Spherical and oblong Ag-NPs were obtained with particles size ranging from 5-60 nm for those produced at 60°C. FTIR analysis showed that the Ag-NPs were surrounded by secondary metabolites. The high inhibition values obtained in the analgesic test for the silver nanoparticles compared to the buchu ethanolic extract show that there was significant enrichment to the buchu crude extract. We therefore conclude that green synthesised silver nanoparticles from *A. betulina* ethanolic extract can be used in the management of pain with significant success.
5.7 References


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dysfunction to bis function: On the design and application of functionalised ionic liquids. *Chemistry-a European Journal*, **12**(8), 2122-2130.


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Approaches. *Advances in Microbial Physiology*, 38, 177.


Appendix 1 - GC/MS chromatogram of the essential oil of *Filipendula ulmaria*
Appendix 2: $^1$H-NMR (600 MHz in DMSO) for MSV8 from *F. ulmaria*
Appendix 3: $^{13}$C NMR (600 MHz in DMSO) for MSV8 from *F. ulmaria*
Appendix 4: $^{13}$C DEPT NMR (600 MHz in DMSO) for MSV8 from *F. ulmaria*
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