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Ethnobotanical uses, anatomical features, phytochemical properties, antimicrobial activity, and cytotoxicity of the Sotho medicinal plant *Searsia erosa* (Anacardiaceae)

BY

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Dedication

I dedicate this dissertation to my mothers, Mrs T. A Mashimbye and Mrs Maluleke for raising my child while I continued with my research project, my daughter R. T Maluleke for putting up with my absence, and God, without whom this would not have been possible.

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Abstract

The species *Searsia erosa* (Thunb.) Moffett is one of the 111 species of *Searsia* occurring in southern Africa, where it occurs in South Africa (Free State and Northern Cape Provinces) and Lesotho. The species is used traditionally by the Basotho people for tobacco flavouring, for the treatment of gastro-intestinal problems in both humans and animals, respiratory conditions, and cancer, as well as for tanning of animal skin. However, the plant has not been tested for its pharmacological activity nor its chemical properties. The leaves emit a strong turpentine or resinous odour when crushed, which may explain the utilisation for tobacco flavouring. The first aim of the project was therefore to evaluate the leaves for the presence of essential oils, to determine whether there is variation in the composition of essential oils between and within populations, and whether the variation is affected by seasonal changes. Essential oils are often located in glandular trichomes and secretary canals in different plant parts. The second aim of the study was to analyse the anatomical features of the petioles, leaves, and young stems for the presence of glandular trichomes and secretary structures. The oil is variable both in yield and composition, but α pinene, 3-Cyclohexen-1-ol, 4-methyl-1(1-methylethyl)-, Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1methylethylidene)-, Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-, Bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene- and 1,4-Cyclohexadiene, 1-methyl-4-(1methylethyl)- appear to be characteristic of *S. erosa's* oil from the plant samples collected in Lesotho, while α phellandrene, β Ocimene, α terpineol, caryophyllene and α pinene appear to be characteristic of the oil from plant samples collected in the Free State Province. The composition was found to vary both within and between populations. Furthermore, histochemical techniques were used to observe the specific location of tannins, alkaloids, and essential oil accumulation in the internal (canals) and external structures (trichomes). The results of histochemical test confirmed that secretory trichomes and canals produce essential oils (terpenoids), lipids and phenolic compounds. Alkaloids and tannins were present in the epidermal cells, subepidermal collenchyma, inner cells of parenchyma surrounding the bundles and inner cell layers of cortex in petioles, leaf laminar and stem. The HPLC analysis showed that gallic acid, p-oH- benzoic acid, protocatechuic acid and tannic acid were the major phenolics. The third aim was to determine whether the extracts (organic

and aqueous) and essential oils of *S. erosa* show any activity against some of the prevalent human pathogenic bacterial strains associated with gastrointestinal (i.e. *Bacillus cereus* and *Escherichia coli*), respiratory tract infections (RTIs) (*Cryptococcus neoformans, Klebsiella pneumoniae,* and *Staphylococcus aureus*) and one general pathogen (*Streptococcus agalactisae)*. The minimal inhibitory concentration (MIC) assay was undertaken on dichloromethane-methanol (CH2CI2: MeOH) extracts, aqueous crude extracts and essential oils using a microtiter plate in duplicates. The highest antimicrobial activity of the organic extracts against gastro-intestinal pathogens was exhibited against *Bacillus cereus* with a mean MIC value of 0.05 mg/ml, while the most susceptible respiratory tract pathogens were *Cryptococcus neoformans* (0.125 mg/ml) and *Staphylococcus aureus* (0.25 mg/ml). The aqueous extracts were only active against *Cryptococcus neoformans* with a noteworthy activity of 0.125 mg/ml and *Staphylococcus aureus* with a moderate activity of 0.25 mg/ml. The aqueous extracts were only moderately active against *Cryptococcus neoformans* (0.25 mg/ml). The essential oil from three samples from the Free State Province (FS1, FS2, and FS3) were found to have strong activity against Streptococcus *agalactiae* with values of 0.375 mg/ml, 0.5 mg/ml, and of 0.75 mg/ml respectively. Of the RTI pathogens, the essential oils displayed the highest activity against *Cryptococcus neoformans* with values of 0.125 mg/ml (FS3) and 0.25 mg/ml (in FS1 and FS2), while moderate activity was observed against *Klebsiella pneumoniae* with a value of 1.5 mg/ml (in FS2 and FS3). The cytotoxicological evaluation of *S. erosa* using the brine shrimp lethality assay revealed that both organic and aquoues extracts are non-cytotoxic. The aqueous extracts exhibited 0.813% mortality after 24 hrs of exposure, while the organic extract exhibited 26.37% mortality.

Keywords: anatomy, antimicrobial, essential oils, ethnobotany, histochemistry

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Chapter 1 General introduction

1.1 Background

Throughout the history of mankind, human beings have relied on nature for the production of their fundamental needs, such as food, fertilizers, shelter, medicines, and clothing (Karunamoorthi et al., 2012). Since time immemorial, plants have been a major source of medicine, traditionally for raw material and finished herbal drugs (Manoharachary and Nagaraju, 2016). Several infectious and non-infectious diseases have been treated using plant species with medicinal value for many centuries (Buwa and Van Staden, 2006). The term 'medicinal plant' is defined differently by different authors, for example, according to Fellows (1991), as cited by Iroka et al. (2016), "the term 'medicinal' as applied to a plant indicates that it contains matter that regulates beneficially the physiology of a sick mammal". Simply put, medicinal plants are plants that are claimed to have therapeutic properties (Farnsworth and Soejarto, 1991). In many developing countries across the globe, a large portion of inhabitants rely on medicinal plants and traditional practitioners in order to meet their health care requirements. In fact, today more than 50% of all the medicine in clinical use are made from natural products and their derivatives (Buwa and Van Staden, 2006). According to Maridass and Debritto (2008), 25% of modern medicine is made from products derived from higher plants which were initially used in traditional medicine, as pure active products. Although modern medicine exists, the traditional practice of using herbal medicine continues to maintain its popularity mainly due to historical and cultural reasons, but also due to the fact that the western drugs are considered to be expensive. Furthermore, according to Wachtel-Galor and Benzie (2011), *Ginkgo biloba* L.*, Allium sativum* L., and *Panax ginseng* C.A. Mey., which produce the highest plant products, can be traced back to origins of traditional Chinese medicine, but are still being used today used for the treatment of various diseases. Interestingly, a recent study (Oyebode et al., 2016) investigated the use of traditional medicine in different countries, namely China, Ghana, India, Mexico, Russia, and South Africa, over a three-year period, and concluded that it is in fact on the decline.

1.1.1 African traditional medicine

Africa is the world's second largest and most populous continent, known to have rich sources of indigenous plant species which play an important role in traditional medicine for the treatment of contagious and non-contagious diseases (Buwa and Van Staden, 2006). These natural drugs were taken in various dosage forms such as ointments, powders, tinctures, teas, fresh extracts, and other herbal formulations (Kumadott and Ofori-Kwakye, 2017). Despite their popularity in Africa, there is still lack of scientific information on the efficacy and side effects for many of these plants (Ozioma and Chinwe, 2019). This reliance is mainly due to the lack of adequate healthcare facilities, for example, in countries such as Mozambique, Lesotho, and South Africa currently, only 40% of inhabitants have access to the public health system (Bruschi et al, 2011). The problem is exacerbated by the lack of funds and adequate transportation in the rural areas, which make the population more dependent on their natural resources (Buwa and Van Staden, 2006). Although humans did not understand the science behind traditional medicines, to some extent, they knew that some medicinal plants are highly effective only when used at corrective doses (Karunamoorthi et al., 2013). However, this lack of understanding medicinal plants are sometimes considered to be a form of witchcraft, which is often coupled with superstition (Karunamoorthi et al., 2013; Boehme, 1982). On the other hand, both traditional and modern health systems exist in African societies (Mathibela, 2013), whereby people consult both systems, for different causes and occasions, besides the disease encountered. In addition, the traditional African healing systems conceptualizes illness differently from the western healing system. Modern medicine focuses only on physiological symptomatology while the traditional healing model goes beyond the physiology by tackling the mind, body, and spirit (Iwu, 1993).

1.1.2 Basotho traditional medicine

The Basotho people in southern Africa are found mostly in Lesotho and the Free State Province of South Africa. Lesotho is completely landlocked by South Africa where the majority of the population are Sesotho speaking and traditional medicine plays an important role in the treatment and well-being of the rural population

(Mugomeri et al, 2014; Shale et al, 1999). The Basotho people in the Free State Province of South Africa are bound to the people across the border by more than just a common linguistic and ethnic lineage (Steinberg, 2005), they also share a number of cultural practices (Moteetee et al., 2019). South Africa equally has a strong history with regards to the utilisation of medicinal plants for traditional healing. Furthermore, the country has a huge diversity of tribes which is reflected in the systems of medicinal preparations (Buwa and Van Staden, 2006). As eloquently stated by Masupha et al. (2013), “Basotho, like other communities have their own unique traditional knowledge, beliefs and culture, that helps them protect themselves and raise their children”. They acquire valuable indigenous knowledge from their elders to harvest the medicinal plants that they use, however, this knowledge is often hidden and undocumented (Ahmed et al., 2014). This knowledge is often transferred through visual memory and unfortunately most of the knowledge is getting lost as elderly people with this knowledge are dying. Furthermore, the communities are becoming westernized, as they adapt to western lifestyles (Shale et al., 2013). As with other cultures, this dependence on nature is similarly guided by history, experience, instinct, and taste, and is then proceeded from one generation to the next (Niazi, 2006; York et al., 2011). Some remedies are carefully prepared and preserved as family secrets, thus making the training of new healers difficult as a cross-exchange of ideas is prevented (Iwu, 1993).

Like many other cultures in southern Africa, the Basotho people utilise numerous plants for a variety of purposes including arts, crafts, food, mats, magic and medicine. The use of medicinal plants for the treatment of different ailments includes the use of leaves, flowers, fruits, and roots for their therapeutic and medicinal value, either as self-medication or consultation of traditional healers (Moteetee and Van Wyk, 2011). In addition, it is common knowledge that simple ailments such as colds, coughs, and headaches, can be treated by drinking teas and juices of plant extracts, inhaling smoke from burning sticks, chewing roots, or applying pastes on infected skin (Moteetee and Van Wyk, 2011). In an earlier review on the use of plants in Lesotho, Moteetee and Van Wyk (2011) recorded a total of 303 plant species used medicinally. The review highlighted *Artemisia afra* Jacq. and *Dicoma anomala* Sond. as the two most important medicinal plants used by the Basotho people for the two

most common everyday ailments, namely respiratory and digestive problems respectively. In a later review encompassing the Basotho living in both Lesotho and the Free State Province, Moteetee et al. (2019) revealed that 355 plants are used for medicinal purposes. Both lists included *Searsia erosa* (Thunb.) Moffett, which is the focus of this study.

1.2 The genus *Searsia* F.A. Barkley

Searsia is one of the genera in the sumac family Anacardaceae, with widely distributed species (Moffett, 1994). The family comprises approximately 82 genera and 800 species, which are distributed primarily in tropical and subtropical areas of the world (Phongkrathung et al., 2016). Members of the family Anacardiaceae are resinous trees and shrubs with leaves that are trifoliolate or pinnately compound with an alternate pattern (though some species bear simple leaves). *Searsia* is one of the most widespread genera, distributed mostly in southern Africa with a few species in Eastern Asia (Yang et al., 2016). The genus *Searsia* is named after Paul B Sears who was the head of Yale School of Botany (Moffett, 1994). It was formally part of the *Rhus* complex but was segregated by Barkley (1943) after he proposed the correct name for this genus (Yang et al., 2016). The genus is easily recognised by its trifoliate leaves which have a resinous smell when crushed. According to Moffett (2007), *Searsia* comprises approximately 120 species (and 28 infraspecific taxa), with 111 of them occurring in southern Africa (Yang et al., 2016).

Searsia erosa (Thunb) Moffett is one of the southern African species of the genus and its specific name '*erosa'* is Latin for the toothed leaf margins (Moffett, 1994). The species is further characterised by its conspicuous, trifoliate leaves which comprise long and narrow leaflets. These leaflets appear leathery in texture, sticky and fine, while the midrib of the leaflets appears prominent. The leaves have a strong turpentine resinous aroma when crushed, suggesting the presence of essential oils which might be secreted from glandular trichomes. The leaves are either yellowish green or olive-green in colour and attached to the stem by petioles (Figure 1.1). *S. erosa* is an evergreen branched sprawling shrub or small tree with brown bark and prominent lenticels of 3-4 m in length as shown in Figure 1.2. The plant grows fairly fast and occurs on rocky (or stony) hills or gravel soil (Drummond and Moll, 1977).

Figure 1.1: *S. erosa's* yellowish green leaves attached to stem by long petioles.

Figure 1.2: *S. erosa* bark showing abundant lenticels

1.2.1 Ethnobotanical uses of *Searsia* species

The Southern African species of *Searsia* such as *S. lancea* (L.f.) Barkely (karree), *S. leptodictya (*Diels) T.S.YI, A.J. Mill and J.Wen (mountain karree), *S. pendulina* (Jacq) Moffett (white karree), *S. erosa* (broom karree) and *S. pyroides* (Burch) Moffett var. *gacillis* (Engl) Moffett are known for their importance in providing shelter, food, brooms, fencing poles, fuel and as garden ornamentals (Moffett, 1994). The bark of some species such *Searsia lancea* contains high amounts of tannins and were therefore used for tanning in the past (Edris, 2007). Furthermore, species such *as S. burchellii* (Sond. ex. Engl.) Moffett, *S. divaricata* (Eckl. & Zeyh.) Moffett, *S. lancea*, *S. pyroides*, *S. undulata* (Jacq.) T.S. Yi, A.J.Mill. & J.We, are used for traditional medicine in southern Africa for the treatment of a wide range of illnesses. For example, *S. chirindensis* (Baker f.) Moffett*, S. gueinzii* (Sond.) F.A.Barkley, *S. incisa* (L.f.) F.A.Barkley*, S. lancea, S. natalensis* (Bernh. ex C.Krauss) F.A.Barkley*, S. pendulina*, *S. pentheri* (Zahlbr.) Moffett*,* and *S. rogersii* Schonl, are all used traditionally for treating intestinal disorders (Madikizela et al., 2013; Ahmed et al., 2014), while *Searsia pyroides* root extract are drunk as cough medicines (Maroyi, 2013). *Searsia erosa* grows in the Northern Cape and the Free State Provinces of South Africa, as well as in Lesotho (Moteetee and Van Wyk, 2011). Due to its confined geographical distribution pattern, the species is used only by the Basotho people and therefore its therapeutic properties are not widely-known. It is used traditionally by the Basotho people for the treatment of diarrhoea in both humans and cattle, colds, and uterine cancer, and for tobacco flavouring, in rainmaking rituals, and for tanning of animal hide (Moteetee and Van Wyk, 2011). The species is also used for making rough brooms, thatching outbuildings (the branches and leaves) and grown in gardens (as a hedge) (Moffett, 2010).

1.2.2. Anatomical features of *Searsia*

1.2.2.1 *Trichomes*

Trichomes are ‘hair-like’ appendages present on the surfaces of leaves, stems, roots, and floral structures of plants (Evert, 2006). These structures have the

capability to secrete and function, and differ in size, shape, and location. Trichomes can be classified into two groups, namely; non-glandular and glandular (secretory).

Non-glandular trichomes can be unicellular, multicellular or branched while glandular trichomes can be peltate, capitate, and sub-sessile or branched (Evert, 2006). Glandular trichomes consist of a small basal cell, short uniseriate stalk and a unicellular or multicellular spherical head (Jordaan and Kruger, 1992; Madani and Farouk, 2019). Trichomes may be randomly distributed on the leaves, but they are denser on the adaxial surface than on the abaxial surface in most cases. On the other hand, essential oils that confer therapeutic properties are synthesized and stored in glandular trichomes (Garcia et al., 2014).

Trichome type and distribution have a significance in taxonomy, where they have shown unique morphological and distribution patterns among taxa in comparative studies (Jia et al, 2013). Furthermore, trichomes play an important role in plant defence mechanisms as they may complement the chemical defense of the plant by possessing oils and other structures such as druse crystals and secretary canals which exude metabolites associated with the sense of smell or taste for repellent (Levin, 1973). Simple trichome types might serve to prevent water loss, influence pollination, or act as a mechanical barrier to herbivores, while glandular trichomes might be involved in the production and secretion of chemicals for protection against pests or attraction of pollinators (Levin, 1973). According to Metcalfe and Chalk (1979), plant protection by glandular trichomes could occur in four ways: “by capturing pests so that overall movement across/within leaves is prohibited; by obstructing the movement of pests into leaf tissue; by producing or secreting volatile or non-volatile secondary metabolites; and by producing proteins that directly poison or actively deter pests”.

According to Jordaan and Kruger (1992) and Andrés-Hernández and Terrazas (2009), the genus *Rhus* has simple acicular trichomes (non-glandular trichome) and capitate glandular trichomes. Species such as *Rhus capallira* L. has the two types of trichomes, commonly small capitate but also larger ovate trichomes (AndrésHernández and Terrazas, 2009). According to Hardin and Phillips (1985), *Rhus* subgenus *Rhus* have bulbous glandular trichomes types and acicular trichomes. *Searsia burchellii* does not have acicular trichomes but has peltate

trichomes with small basal cells, unicellular stalks and multicellular heads consisting of 4 - 8 cells (Jordaan and Kruger, 1992). On the same hand, *Searsia chinesis* was reported to have capitate glandular trichomes and non-glandular trichomes (Eminagaoglu and Ozcan, 2018).

1.2.2.2 Secretary canals

Secretory cavities and canals (ducts) are two types of secretory spaces which differ from each other by their length: the canals are long secretory spaces whereas the cavities are short secretory spaces (Ellis, 1974; Evert, 2006). In plants, three growth types of secretory cavities and canals are found, namely; schizogenous, lysigenous, and schizolysigenous (Evert, 2006). These canals and ducts have been described as follows by Evert (2006): the schizogenous type forms by separation of cells, which results in a space lined with secretory epithelial cells while, lysigenous cavities and ducts result from a dissolution (autolysis) of glandular cells. In the latter type, the secretory product is formed in the cells that eventually breakdown and release the product into the resultant space. In addition, partly disintegrated cells occur along the periphery of the space. Furthermore, the development of schizolysigenous cavities and canals initially is schizogenous, but lysigeny occurs in later stages as the epithelial cells lining the space undergo autolysis, further enlarging the space. The contents of the cavities and canals may consist of terpenoids, carbohydrates and other substances. According to Sant'Anna-Santo et al. (2006), *Spondia dulcis* (Anacardiaceae) canals contain essential oils, polysaccharides and phenolic compounds.

Secretory canals are present in primary and secondary phloem in members of Anacardiaceae such as *Rhus diversiloba* (McNair, 1918). The secretory canals of *Searsia burchelli* are not only situated in the primary and secondary phloem but also on the secondary vein of its leaves (Jordaan and Kruger 1992). According to Ellis (1974), Sieck stated that the canals of the Anacardiaceae are of schizolysigenous origin, but Fahn (1969) states that they are schizogenous and develop between resin-producing parenchyma cells which form the duct epithelium.

1.3 Secondary metabolites in Anacardiaceae

Plants are complex chemical factories that continuously synthesize biologically active compounds that are referred as phytochemicals. These diverse compounds can be classified as primary or secondary metabolites depending on their role in the plant metabolism (Saxena et al., 2013). These plant metabolites can be extracted from plants to treat different ailments whether chronic or infectious (Madikizela et al., 2013). Primary metabolites are compounds which perform essential metabolic activities in plants because they are involved in normal metabolic processes of growth and development. Examples include chlorophyll, amino acids, lactic acid, nucleotides, simple carbohydrates and membrane lipids. On the other hand, secondary metabolites are produced by the plant naturally with no direct primary role in growth and development, but rather as a defense mechanism against predation by micro-organisms, insects and herbivores (Tariq and Reyaz, 2013). Furthermore, secondary metabolites differ from primary metabolites in having a confined distribution in the plant kingdom (Mazid et al., 2011). In addition, secondary metabolites are mostly found in taxonomically related species, whereas primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 2006).

In plants, a diverse array of organic compounds (or metabolites) are produced as a result of metabolic processes (Mazid et al., 2011). Most of these natural products are phenols or their oxygen substituted substances, which are non-nutritive (Naidoo, 2007). Based on literature, phenolics are said to be the most numerous and structurally diverse of all phytochemicals (Saxena et al., 2013). Secondary metabolites are commonly found in the family Anacardiaceae. According to Suzimone et al. (2006), phytochemical studies up to today have shown that species of the family Anacardiaceae contain mixtures of polyphenols, terpenoids, fatty acids and steroids, with polyphenols being the major phytochemicals in the family (Kabir et al, 2017). The genus *Rhus* is rich in phenolics such as flavonoids, mainly bioflavonoids (Suzimone et al., 2006). Furthermore, leaves of *Anacardium humile* A.St.-Hil., are reported to possess flavonoids and tannins (Ferreira et al., 2012), while *A. occidentale* L. was found to contain flavonoids, glycosides, tannins, and terpenoids, which confer medicinal properties (Mustapha et al., 2015). In *Mangifera indica* L. polyphenols such as catechins, quercetin, gallic acid, ellagic acid,

kaempferol and benzoic acid were found to be the major polyphenols present (Kabir et al, 2017). The following subsection will be dedicated to the different classes of terpenoids, essential oils (especially monoterpenoids and sesquiterpenoids as economically important groups) saponins, phenolics as well as alkaloids as compounds occurring in Anacardiaceae.

1.3.1 Terpenes or terpenoids

These are the most diverse and largest group of secondary metabolites, which are characterised by isoprene units which are usually jointed in a head to tail fashion (Wink, 1999). Terpenoids are commercially interesting because of the fragrances and flavours in food and have useful applications in industrial compounds, cosmetics production, and pharmaceuticals (Naidoo, 2007). Major classes of terpenoids include mono-, sesqui-, and diterpenes, which are secondary metabolites, as well as tri- and tetraterpenes, which are commonly primary metabolites (Naidoo, 2007). However, the vast majority are secondary metabolites, such as the volatile constituents of essential oils, in particular monoterpenes and sesquiterpenoids. Terpenoids are classified on the basis of the number of isoprene units in the basic skeleton units with C-5, C-10, C-15, C-20, C-30 and C-40 skeletons (Zwenger and Basu, 2008), as shown in Table 1.1. Examples of monoterpenes are: α- pinene, βpinene, Linalool, Menthol, Borneol and 1,8-cineol (Naidoo, 2007). Iridoids and pyrethrins are included in this group but not commonly found. Sesquiterpenes such as bisabolol, humulene and caryophyllene are also chemical constituents of essential oils of many plants. The Anacardiaceae family commonly have terpenoids but are particularly rich in monoterpenoids (Kostermans and Bompard, 2012). For example, in *Rhus cotinus* L. monoterpenes such as α pinene, β-pinene and limonene are the predominant ones (Joshi and Mathela, 2014).

Table 1.1: Different classes of terpenoids

Class of terpenoids	No of carbons	No of isoprene units	example
Hemiterpenoid	5	1	Isovalenic acid
Monoterpenoid	10	2	α- Pinene, Limonene
Sesquiterpenoid	15	3	Spathulenol, caryophyllene
Diterpenoids	20	4	phytol
Triterpenoids	30	6	Oleanolic acid
Tetraterpenoids	40	8	Carotene

1.3.1.1 *Essential oils*

These are aromatic oily liquids that are produced naturally by plants (Edris, 2007). Oils are complex mixtures of components that can comprise up to 80 or more constituents. They are usually characterised by three or four major components which occur at fairly high concentrations (> 10%) or area percentages. These major components may determine their biological activity. The production of essential oils and resins in plants is generally associated with the presence of specialized secretory structures such as glandular trichomes and oil or resin canals and cavities. Essential oils can be present or absent in a plant and are distinguished from fatty vegetable oils, as they volatise within the air (Naidoo, 2007). In addition, essential oils can only be distilled by using water and heat and are subjected to both quantitative and qualitative tests in order to determine their chemical characteristics and biological activity (Edris, 2007). According to Naidoo, (2007) minimum chemical change can be done but, it is also necessary to distil or to isolate the essential oil as completely as possible from the mass of inert cellular matter with the minimum amount of chemical change. On the other hand, the oil yield can vary between batches from the same trees and between sites of batches because oil yield is affected by environmental conditions and the type of distillation process (Rai, 2013). The family Anacardiaceae is a rich source of volatile oils, which are mostly distilled from fruits and leaves (Montanari et al., 2012). According to Kostermans and Bompard (2012) Species of *Pistacia* and *Mangifera* in the family Anacardiaceae produce essential oils that are of monoterpenes than sesquiterpenes. Furthermore, plant essential oils may naturally have a defensive role against herbivore, as well as having the potential of being antibacterial, antifungal, anthelmintic, antimalarial and

molluscicidal. In addition, the volatile oils from the family Anacardiaceae are known to have antibacterial activities (Montanari et al., 2012). For example, essential oils from *Pistacia* species showed significant inhibition of fungal growth with α-pinene as the main chemical compound, while the essential oils from *Schinus terebinthifolius* Raddi leaves were found to exhibit antimicrobial activity against both bacteria and fungi (Ismial et al., 2013; Montanari et al., 2012).

1.3.1.2 *Saponins*

These represent a group of 30-carbon compounds with sugar molecules which are referred to as triterpenes. They have a soap like property and often have an important biological activity. Their structures are made from two parts: the aglycone (sugar free part) and the glycoside (the attached sugar residue). They are distributed in the plant kingdom in plant portions such as leaves, roots, seeds, stems, and flowers (Wink, 1999). According to Umadevi et al. (1988), saponins are rarely found in members of the family Anacardiaceae but some species contain large amounts of saponins accumulating mostly in leaves and stem bark. For example, species such as *Searsia tenuinervis* (Engle) Moffett, contain saponins in root and bark (Dushimemaria et al. 2012). Saponins help humans to fight fungal infections, viruses, and combat other microbes as they have been found to possess a range of biological activities including immune-stimulation and anticancer properties (Okwu and Nnamdi, 2008).

1.3.2 Phenolics

Phenols are the most widely distributed phytochemicals in the plant kingdom (Saxena et al., 2013). They are characterized by an aromatic ring attached to various substituent groups such as hydroxyl and methoxyl (-O-CH3) groups, as well as other non-aromatic ring structures (Dai and Mumper, 2010). Classes of phenolic compounds include tannins, coumarins, quinones, and flavonoids such as flavones and flavonols, and serve as an effective defence against herbivores (Wink, 1999; GuribFakim, 2006). Phenolic compounds are accumulated and distributed in the plant kingdom, mainly for colour and flavour in different plants (Dai and Mumper, 2010). Most phenolic compounds such as flavonoids and tannins are widespread in the family Anacardiaceae. According to Umadevi et al. (1988), tannins are the most common, which explains why *Searsia* species in the family are valued in the timber

and tannin industries. According to Abu-Reidah et al. (2014), galliotannins are a characteristic property of the *Rhus* species. Tannins and flavonoids have an antibacterial and antiviral effects that can inhibit insect growth and disrupts digestive events in animals, while alkaloids have a mode of antidiarrheal action, due to their effects in small intestines (Ukoha, 2011; Farjana et al., 2014). For example, *Searsia dentata* whose leaf sap is used as a remedy for diarrhoea, stomach, and ulcer problems contains bioflavonoids, which are known to be biologically active (Maroyi, 2013). On the other hand, coumarins have been found to stimulate macrophages, which have indirect effects in infections (Cowan, 1999). It has been reported that tannins, flavonoids, and coumarins have a wide range of pharmaceutical activities such as anti-inflammatory, analgesic, antitumour, anti-HIV, anti-infective (anti-diarrhoeal, antifungal), anti-hepatotoxic, antilipolytic, antioxidant, vasodilatory, immunestimulant, and anti-ulcerogenic (Wink, 1999 and Gurib-Fakim, 2006). Therefore, due to the structural diversity of plant metabolites and the pharmacological properties that they possess, medicinal plants serve as a starting point for drug and drug discovery.

1.3.3 Alkaloids

These are secondary metabolites that contain heterocyclic nitrogen atoms. These organic nitrogenous bases have medicinal properties such as analgesic and antimicrobial activities found primarily in plants and to a lesser extent in animals and micro-organisms (Wink, 2000). Alkaloids can be grouped according to the heterocyclic ring system they contain. According to Croteau et al. (2000), alkaloids are distributed in about 20 % of all flowering plants. Therefore, they are significant in chemotaxonomy as markers because plant species accumulate alkaloids in unique patterns (Croteau et al., 2000). Umadevi et al. (1988) reported that alkaloids were absent in most members of the family. However, according to Pallardy (2008), “alkaloids also sometimes occur in wood, and the wood of some species of the families Anacardiaceae, Apocynaceae, Euphorbiaceae, the legume families,

Rutaceae, and Rubiaceae contains so much alkaloid that it produces dermatitis”. Alkaloids have many pharmacological activities such as antimalarial activities (like quinine), antiarrhythmic effect (quinidine, spareien), antifungal and antibacterial (by ensuring plant survival). However, due to their cytotoxicity and addictive nature, their

use is regulated (Roy, 2017; Saxena, 2013). In addition, most alkaloids are rarely used in their pure form but rather as semi-synthetic analogues (Croteau et al., 2000).

1.4 Antimicrobial properties of *Searsia* species

Plants are the largest drug stores, producing endless bioactive chemical compounds which have direct effects on animal and human health (Abdalla and Abdalla, 2016). The interest in plants with antimicrobial properties has increased due to the problems associated with the use of antibiotics (Mativandlela, 2005). Bacterial species have the genetic ability to transmit and acquire resistance to drugs, which are used as therapeutic agents (Girish and Satish, 2008; Khatri et al, 2016). According to Eloff (1999), antimicrobial compounds from plants may inhibit bacteria by a differently than the presently used antibiotics. In addition, bacterial resistance has propelled research in the direction of combination therapy to enhanced efficacy (Hübsch, 2014). Many species of *Searsia* such as *S. dentata, S. pyroides,* and *S. tenvinervis* (Engel) Moffett have been shown to display important antimicrobial activity (Maroyi, 2013). Furthermore, *S. lancea* has shown significant activity against *Escherichia coli*, *Aspergilius spp*, and *Clostridium perifringens* (Swanepoel, 2016). According to Walker et al. (2013) diarrhoea and respiratory tract infections, together account for the 30% of all child deaths worlwide.

1.4.1 Gastrointestinal problems

Searsia erosa is of particular interest for treatment of diarrhoea (in humans and cattle), which is a major problem in many African countries due to lack of safe drinking water. In addition, diarrhoea causing pathogens are becoming resistant to antimicrobial medications (Madikizela et al., 2013), making it important to look for possible alternative treatments from plants. According to De la Cruz-Jimenez et al. (2014), the most common gastrointestinal disorders are indigestion, ulcers, diarrhoea, stomach pain, and dysentery, which have common causes as contaminated food, nutritional factors, and pathogens like bacteria, viruses, parasites and helminthes. Diarrhoea is regarded as one of the major causes of death worldwide and it continues to kill more children under the age of five years than AIDS (Acquired Immunodeficiency Syndrome), measles and malaria combined (Semenya

and Maroyi, 2012; Kirk et al. 2017). Furthermore, diarrhoea often results from food and water contaminated by *Salmonella typhmurium*, *Campylobacter jejuni*, and Shiga toxin-producing *Escherichia coli*, as well as water sources contaminated by parasites such as *Giardia intestinalis* and *Cryptosporidium parvum* which cause foofborne disease (Kirk et al. 2017). Diarrhoea is also a leading cause of morbidity and mortality in HIV-infected children. HIV-infected children admitted with diarrhoea are more likely to have prolonged diarrhoea which causes recurrent hospital admissions due to the fact that HIV infected persons experience a major burden of infection from contaminated food. They have a higher frequency of recurrent diarrhoea and require a longer hospital stay than an adult (Kirk et al. 2017). Treatment of diarrhoea by antibiotics often results in drug resistance when caused by bacterial species. Within the family Anacardiaceae, *Rhus coriaria* L. was found to have noteworthy activity against different bacteria such as *B. cereus* and *E. coli* (Abu-reidah et al., 2014). Other species of *Searsia* such as *S. chirindensis, S. gueinzii* (Sond.) F.A. Barkley, *S. incisa* (L.f.) F.A. Barkley, *S. lancea, S. natalensis* (Bernh. ex C. Krauss) F.A. Barkley, *S. pendulina*, *S. pentheri* (Zahlbr.) Moffett, and *S. rogersii* Schonl, are used traditionally for treating intestinal disorders (Madikizela et al., 2013; Ahmed et al., 2014). Extracts from many of these plants have been found to exhibit noteworthy antibacterial, antimycobacterial, and antifungal activities (Ahmed et al. 2014).

1.4.2 Respiratory tract infections

The increasing global prevalence of resistant respiratory tract pathogens is a serious public health problem worldwide (Morobe et al, 2012). Infectious diseases have been and still continue to be an ever-present threat to mankind (Kolodziey, 2011). In both developing and developed countries infectious diseases that cause respiratory infections are a major cause of death. According to Moteetee et al. (2019) respiratory conditions are one of the most frequent ailments treated through traditional healing in Lesotho and the Free State Province in South Africa. This is because of limited access to health care facilities, costly antibiotics, and low vaccination coverage. Lack of access to antimicrobials and resistance to those available may increase the morbidity of vaccines (Gibson et al, 2011). According to Bates et al. (2017), respiratory tract infections are the most prevalent causes of death in Africa. They

consist of lower respiratory tract infections (such as bronchitis, pneumonia, and acute bronchitis) and upper respiratory tract infections (which include tonsillitis, laryngitis and common colds). In South Africa, lower respiratory infections are ranked third in the top 20 leading causes of death (Suliman, 2010). On the other hand, investigations of alternative therapies from medicinal plants and herbal medicines that are used traditionally for their effectiveness in treating respiratory infections, could prove to be a valuable source, especially in developing countries where acute respiratory infections are very prevalent (Suliman, 2010). The ever-increasing emergence of resistant micro-organisms to regular antimicrobial therapy, specifically related to respiratory tract infections is a serious problem. Acute respiratory tract infections have a vast impact on the deaths of children. This is evident in developing countries, where 50% of deaths occur in children who are younger than five years and 25-33% of these deaths are caused by respiratory infections (Suliman, 2010). According to Maroyi, (2013), *Searsia pyroides* has pharmacological activities with bioflavonoids that cure respiratory conditions. Furthermore, species such as *Searsia tenuinervis* have been found to have noteworthy activity against *S. aureus* (Dushimemaria et al., 2012). This suggests that the genus *Searsia* has potential in the treatment of different ailments as many other species of the genus have noteworthy properties.

1.5 Cytotoxicity of *Searsia* species

Plants used in traditional medicine are assumed to be safe and non-toxic due to their natural origin and the long use in traditional medicine to treat various ailments that humans encounter (Fennell et al., 2004). However, the safety and effectiveness of medicinal plants used in traditional medicine needs to be evaluated. According to Ahmed (2014), medicinal plants used in traditional medicine are taken several times a day, until the symptoms are resolved, but the dosage is not controlled in most cases. Furthermore, scientific studies of some medicinal plants have indicated that there are phytochemicals that have cytotoxic effects when used for prolonged periods. In addition, Hübsch et al. (2014) stated that toxicity is usually only evident when the medication is consumed in large quantities or for an extended period. Previous toxicity studies on some species of *Searsia,* for example *S. pendulina*, *S. undulata* and *S. leptodictya* demonstrated

that these species have low cytotoxicity levels (Amed, 2014; Kabonga-Kayoka et al. 2016). According to Mbuyi and Chacha (2019), *Searsia longipes* demonstrated concentration dependent mortality induction. Therefore, the use of *Searsia* species to treat ailments needs be monitored as toxicity limits can be exceeded.

1.6 Aims and objectives of the study

The aim of this study is to investigate the ethnobotanical uses, anatomical features, chemical constituents (including essential oils), and antimicrobial activity of *S. erosa* against some of the prevalent pathogens.

The objectives of the study were to find a scientific rationale for the use of the Sotho medicinal plants in order to validate its traditional use.

Specific objectives:

- To detect the phytochemicals, present in the plant (particularly essential oils and tannins).
- To conduct a population analysis of essential oils in order to detect whether there is variation in the composition of essential oils between and within populations.
- To investigate whether the variation between and within populations is affected by seasonal changes.
- To evaluate the antimicrobial activity of both plant extracts and essential oils.
- To analyse the anatomical features of the leaves for the presence of glandular trichomes.

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Chapter 2

Materials and methods

2.1 Plant collection

Fresh material of *Searsia erosa* leaves was collected in various areas of Cholotsa Hill, Lekokoaneng and Ha Seeiso village, both in the Berea District (but 35 km apart) in Lesotho and the Free State Province of South Africa. The material was identified by Prof A. Moteetee at the University of Johannesburg Herbarium (JRAU) where voucher specimens were also deposited.

2.2 Anatomical procedures

Fresh leaf, petiole and young stems were placed into a small clear plastic jar (120 mm) with 70% ethanol for three days. The plant materials were then dehydrated with 100% ethanol twice for 4 hrs. Each plant material was then cut into small pieces and further treated according to the glycol methacrylate (GMA) method of Feder and O'Brien (1968). This involves further dehydration of the small pieces of plant material through alcohol series before infiltrating with GMA and embedding in capsules containing GMA. The capsules were then placed in a wooden rack in an oven at 60℃ for 24 hrs. Sections of 5 µm thick were made using a Porter-Blum ultramicrotome. Staining was done with Periodic acid- Schiffs (PAS) and Toluidine blue. The microscope slides were perceived under a light microscope equipped with digital and a computerized data capturing system.

Radial, tangential and cross sections of bark and wood as well as cross sections of stems, were made using a freezing microtome (Ernst Leiz GMBH, Wetzlar, Germany). Sections from fresh unstained material were mounted in glycerol and immediately studied under a light microscope. Sections from fixed material were stained with 1.1 safranin/ alcian blue mixture (Jansen et al., 2004) together with series of alcohol and mounted on Euparal.

Maceration of secondary xylem was immersed in Jeffrey's liquid (consisting of equal volumes of 10% nitric acid and 10% chromic acid of potassium bichromate) for 48 hrs. The length of vessel elements and length of fibre was determined from macerated material mounted in glycerol. The standardized descriptive terminologies

for wood structure proposed by the International Association of Wood Anatomists (IAWA) list of microscopic features for hardwood identification (Wheeler et al., 1989) were followed throughout and the terminology used to describe the bark structure follows Trocknbrodt (1990).

2.3 Histochemistry

Histochemistry aims to describe the organisation of cells and tissues in terms of their structure, composition, and function (Casselman, 1959). The procedure and methods were described by Mazzoni-Viveros and DeMaraes Castro, (2016). All histochemical tests were performed on fresh hand-made sections.

2.3.1 Tannins test

Vanillin-hydrochloric acid

Fresh sections were exposed to a drop of saturated alcoholic vanillin, followed by several drops of concentrated hydrochloric acid (HCL). Observation: The presence of condensed tannin in the tissue was indicated by the development of bright red colour.

2.3.2 Phenolic test

Ferric chloride

Preparation of solution: 2 g of ferric chloride was added into 100 ml of 95% ethanol. Fresh sections were immersed into ferric chloride solution for 5 min; rinsed in 95% ethanol and then examined. Observation: Presence of a green colour indicates positive results for polyphenols while a blue-black colour indicates presence of tannins.

2.3.3 Terpenoid tests

Nadi reagent

Preparation of solutions: 0.5ml of alpha-naphthol 0.1% in 40% ethanol (solution 1) and 1% NN- dimethyl-p-phenylenediamine chlorohydrate in water (solution 2). Equal amounts were only mixed prior to use. Fresh sections were submerged in Nadi reagent for 1 hour at room temperature, then washed for 2 min in potassium

phosphate buffer 0.1M at pH 7.2 and mounted in some potassium phosphate buffer. Observation: Presence of a blue colour indicates positive results for essential oils while a violet colour indicates presence of oleoresin, and a red colour indicates presence for resiniferous acids.

2.3.4 Mucilage

Ruthenium red

The sections were submerged for ± 10 min in a solution of 5% Ruthenium red in 1% calcium chloride, washed in distilled water and mounted in distilled water.
Observation: Presence of a bright red colour indicates positive results for mucilage.

2.3.5 Lipid test

Sudan IV

The sections were exposed to a saturated solution of Sudan IV in 70% ethanol, for 15 min, at room temperature, rinsed in 70% ethanol, washed in distilled water and then mounted in distilled water. Observations: The presence of a blue, black or brownish-black colour indicates positive results for total lipids.

2.3.6 Alkaloids

Ellram's reagent

Preparation of reagent: 1 g of potassium iodide was added in 100 ml of 40% sulphuric acid. Observation: Presence of reddish pink colour indicates positive results for alkaloids.

2.4 Distillation of essential oils

Fresh leaves from 26 samples [19 for seasonal variation, 7 for geographical distribution (four from Lesotho and three from the Free State Province of South Africa)] were firstly weighed and then subjected to hydro-distillation for 5 hr using a Clevenger-type apparatus. The oils were then weighed and stored in sealed vials before analysis.

2.4.1 Percentage yields of essential oil samples

The percentage yield was calculated by dividing the total weight of essential oil obtained (after hydro-distillation) by total weight of plant material used in the preparation of the essential oil. The obtained value was then multiplied by hundred to obtain a percentage, weight per weight (w/w).

2.4.2 Gas Chromatography- Mass Spectrometry analysis of essential oils.

Gas Chromatography- Mass Spectrometry (GCMS) is a powerful, universal and useful tool used for quantitative and qualitative analysis for volatile compounds (Clement and Taguchi, 1989). The GCMS analysis technique was used to compare chemical components present in the essential oil to investigate whether there is variation in the composition between and within populations, as well as to investigate whether the variation is affected by seasonal changes.

2.4.3 GCMS instrument and operating settings for analysis of *S. erosa* essential oils.

The oils (20-30%) were diluted in dichloromethane (DCM) and analysed using a Shimadzu QP2010 gas chromatography-mass spectrometry (GC-MS) equipped with GC-MS solution software which was fitted with a Rxi-5ms column (30 m x 250 µm i.d x 0.25 µm film thickness). The column oven temperature program was 60°C for the first 10 min, rising to 250 °C to 280 °C at a rate of 5 °C/min and held for 15 min yielding a total run of 59 min. Helium (Afrox, South Africa) was used as carrier gas at a constant flow of 1.61 ml/min. The injector and transfer line were maintained at 250 °C. A volume of 1µl was injected in the split mode (using a split ratio of 20:1) and an inlet temperature of 200°C. Often in GC-MS, an internal standard is added to all samples but since the chemical constituents of this plant from its oils have not been studied before, no standards were used. The mass spectra were obtained on a detector voltage 1kV with 1000 threshold, scanning from 5 to 500 m/z. Library searches were carried out using NIST® and Wiley libraries.

2.4.4 Identification of components.

N-alkanes were used as reference points in the calculation of retention indices (RI). Component identifications were made by comparing mass spectra and retention indices, using the database of National Institute Standard and Technology (NIST).

The spectrum of an unknown component was compared with that of a known component stored in the NIST library.

The retention indices were calculated using the non-isothermal indices formula described by Babushok et al. (2011).

$Ix = 100n + 100(tx - tn) / (tn+1 - tn)$

2.5 Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analysis of *S. erosa* extract.

Chloroform and water leaf extracts were analysed in HPLC to investigate the quality and qualitative amount of tannins and other phytochemicals present within the plant. The procedure and methods were described by Durgwale et al. (2016) and additions by Dr Ambushe (Department of Chemistry, University of Johannesburg). The RPHPLC technique is a type of liquid chromatography which involves the separation of molecules based on hydrophobicity in which the stationary phase is less polar than the mobile phase (Lukhele, 2012). The RP-HPLC is coupled with UV photodiode array (PDA-UV) detector.

2.5.1 Preparation of extract:

2.5.1.1 Aqueous extract

Ten g of ground material was immersed in a round bottom flask (500 ml) and soaked with 50 ml distilled water and 10 ml of chloroform as preservative for 24 hrs with occasional shaking; then the pellet was removed by filtering the extract through a 0.45 µm filter, using vacuum pump, and was concentrated on a water bath at 50°C. It was then kept in the refrigerator below 4°C till experimental study.

2.5.1.2 Preparation of mobile phase

The mobile phase was prepared by mixing methanol and water in 1 to 1 dilution of 700 ml: 700 ml and filtered through a 0.45 µm nylon filter membrane, using vacuum pump and degassed by sonication for 30 min before use.

2.5.1.3 Preparation of calibration curve of tannic acid (internal standard)

Tannic acid (0.014 g) was dissolved in 10 ml of mobile phase to prepare a stock solution with a concentration of 1000 µg/ml. A series of dilutions with concentrations of 10, 20, 30, 40 and 50 µg/ml were prepared by taking aliquots of 0.1, 0.2, 0.3, 0.4 and 0.5 ml of stock solution (1000 µg/ml) and diluting up to 10 ml with mobile phase. All concentration dilutions were filtered through a 0.45 µm nylon syringe filter membrane and transferred into sampler vials. Each dilution (20 µl) was injected in triplicate. Ideally tannic acid (an internal standard) is a solute with a retention time close to that of the analyte.

2.5.1.4 Preparation of sample (analyte)

Sample (4 ml) was dissolved in 10 ml of mobile phase and allowed to stand for 4 hr with occasional stirring and there after filtered through a 0.45 µm nylon syringe filter membrane and transferred into sampler vials and degassed by sonication for 30 min. Quantification was carried out using an absolute calibration curve method with standard solutions of tannic acid. The chromatographic conditions for investigation were as mentioned in Table 2.1. The mobile phase was run before, to monitor the system pressure and avoid sample carry over.

Table 2.1: Chromatographic conditions for quantitative estimation of tannins present within the plant.

Parameter	**Chromatographic conditions**
HPLC system	600E HPLC system (Milipore)
Pump	Fx-10 UHPLC Pump
Detector	UV photodiode array (PDA-UV) detection
Column	C18 (250 mm length x4.6 mm I.D x 5 µm particle size)
Column temperature	22 °C
Mobile phase	Methanol: water (50:50)
Wavelength of detection	270
Flow rate	1 ml/min
Sample volume	20 µl
Run time	12 min
Retention time	3.1

2.5.2 Identification and confirmation

Identification and confirmation of the fifteen compounds were achieved by comparing the retention times with that of calibration solution and standard chromatograms (Gilala, 2010; Karpagasundan and Kulothungan, 2014).

2.6 Antimicrobial activity

Organic and aqueous extracts as well as oil samples were investigated for antimicrobial activity using the minimum inhibitory concentration (MIC) microtitre plate method described by Eloff (1998). The MIC is a quantitative measure of antimicrobial activity that can be defined as the lowest concentration at which the bacterial growth (or pathogen growth) is inhibited after incubation in a specified growth medium (Eloff, 1998; Van Vuuren, 2008).

2.6.1 Preparation and extraction of plant materials

Ground plant material was extracted using both aqueous and organic solvents. In the case of organic extracts, the plant was immersed in a 1:1 mixture of methanol and dichloromethane (DCM) for 24 hr in a shaker incubator at 37°C. After 24 hrs, the supernatant was separated from the pellet by transferring the supernatant into a beaker. In addition, the pellet was topped with mixed solvent (equal quantity as the pellet) and incubated for a further 24 hrs at 37°C. Thereafter, the supernatant was removed and combined with previous supernatant. This was left to dry in a fume cupboard. The residues were re-suspended in acetone to give a starting concentration of 32 mg/ml.

The aqueous extracts were prepared by adding the ground material to sterile distilled water at 30°C for 24 hrs, in a shaker incubator. The samples were then filtered and placed in -80°C fridge. Thereafter, the extracts were then lyophilized (BenchTop Pro with Omnitronics, SP Scientific) for 24 hrs at University of the Witwatersrand. The lyophilized material was then re-suspended in sterile water to give a starting concentration of 32 mg/ml.

The essential oil samples as prepared in Section 2.2 were also included for antimicrobial studies and also prepared to a concentration of 32 mg/ml. The data on *S. erosa* plant samples that were used for antimicrobial activity is presented in Table 2.2 with their voucher numbers and localities in southern Africa

Table 2.2: Voucher number of all leaf *S. erosa's* samples investigated with their different localities.

Voucher number	**Locality**	**Type of plant extract used for analysis**
A. Moteetee & L. Moteetee 29 (1A*)	Cholotsa Hill, Lekokoaneng, Berea District	Essential oil
A. Moteetee & L. Moteetee 30 (2B*)	Cholotsa Hill, Lekokoaneng, Berea District	Essential oil
A. Moteetee & L. Moteetee 31(3C*)	Cholotsa Hill, Lekokoaneng, Berea District	Essential oil
A. Moteetee & L. Moteetee 32 (4D*)	Cholotsa Hill, Lekokoaneng, Berea District	Essential oil
A. Moteetee & L. Moteetee 33 (5E*)	Cholotsa Hill, Lekokoaneng, Berea District	Essential oil
A. Moteetee & L. Moteetee 35	Ha Seeiso village, Berea District, Lesotho	Essential oil, organic and aqueous extract
A. Moteetee & L. Moteetee 36	Ha Seeiso village, Berea District, Lesotho	Essential oil
A. Moteetee & L. Moteetee 37	Ha Seeiso village, Berea District, Lesotho	Essential oil
A. Moteetee & L. Moteetee 38	Ha Seeiso village, Berea District, Lesotho	Essential oil
A. Moteetee 39 (FS1[$])	25 km past Bots'abelo on way to Bloemfontein	Essential oil
A. Moteetee 40 (FS2[$])	15 km before Thaba-Nchu on the road to Bloemfontein	Essential oil, organic and aqueous extract
A. Moteetee 41 (FS3[$])	20 km after Thaba-Nchu on road to Bloemfontein	Essential oil

*1A, 2B, 3C, 4D and 5E = Seasonal and geographical variation study (five populations from five different localities)

[$]FS1, FS2 and FS3 = Free State collection used for the geographical variation study.

2.6.2 Test micro-organisms and incubation conditions

The samples were screened against six bacterial strains (two pathogens commonly associated with gastro-intestinal complaints, three pathogens commonly associated with respiratory tract infections and one systematic infection). The microorganisms used for tests were obtained from the Department of Pharmacy and Pharmacology, University of the Witwatersrand. The pathogens were selected based on the traditional use of the plant. These are shown in Table 2.3 with their reference strain numbers and ailments they are commonly associated with.

Table 2.3: Micro-organisms used for antimicrobial activity tests for *S. erosa* extracts.

Test microorganism	Reference strain number	Gram stain	Primary infection related to:
Bacillus cereus	ATCC* 11778	Gram-positive	Gastrointestinal tract
Cryptococcus neoformans	ATCC* 14116	yeast	Respiratory tract
Escherichia coli	ATCC* 85900	Gram -negative	Gastrointestinal tract
Klebsiella pneumoniae	ATCC* 13887	Gram-negative	Respiratory tract
Staphylococcus aureus	ATCC* 25923	Gram-positive	Respiratory tract
Streptococcus agalactiae	ATCC* 55618	Gram- positive	Systemic infections

* ATCC = American type culture collection.

2.6.2.1 Gastrointestinal micro-organisms

B. cereus and *E. coli* were sub-cultured into Tryptone Soya Broth (TSB), (Oxoid) at 37°C for 24 hrs.

2.6.2.2 Respiratory tract infection (RTI) micro-organisms

B. neoformans was sub-cultured in TSB (Oxoid) at 37°C for 48 hrs, whereas *K. pneumonia* and *S. aureus* were sub-cultured in TSB (Oxoid) at 37°C for 24 hrs.

2.6.2.3 General pathogen responsible for systematic infections

Streptococcus agalactiae was sub-cultured in Haemopholius media, (Oxoid) at 37°C for 24 hrs.

2.6.3 Minimum inhibition concentration assay

Each 96 well micro-titre plate was filled with 100 µl of sterile broth. Extracts and oils diluted in acetone were applied (100 µl) to the first row of the microtitre plate at starting concentrations of 32 mg/ml. Serial doubling dilutions were performed to yield concentrations varying from 32 mg/ml to 0.5 mg/ml. The cultures were diluted to an approximate inoculum size 1 x 10^8 colony forming units (CFU)/ml and then introduced to all wells of the microtitre plate. Ciprofloxacin at starting stock concentrations of 0.01 mg/ml was used as the positive control. The microtiter plates were sealed with sterile adhesive and incubated for 24 hrs and 48 hrs for *Cryptococcus neoformans* (14116) at 37 °C. The colour reagent *p*-iodonitrotetrazolium violet (INT; Sigma-Aldrich) was prepared (0.4 mg/ml) and 40 µl was transferred to all the inoculated wells after incubation. The microtiter plates were examined for colour changes (indicating microbial growth) after 6 hrs while *Cryptococcus* neoformans was examined after 24 hrs. The MIC value was read as the lowest dilution having no evidence of bacterial growth. A summary of classification for antimicrobial activity seen in the recent review of Kuete and Efferth, (2010) and Van Vuuren and Holl, (2017), (Table 2.4) was used to determine a suitable scheme for classification of activity.

Table 2.4: Classification for antimicrobial activity according to MIC values.

Plant sample	MIC (mg/ml)	Classification of antimicrobial activity	Reference
Plant extract	> 0.625	low antimicrobial activity	Kuete and Efferth, (2010)
	0.1- 0.625	Moderate antimicrobial activity	Kuete and Efferth, (2010)
	<0.1	Noteworthy antimicrobial activity	Van Vuuren and Holl, (2017)
	≤ 0.16	Interesting with potentially useful activity	Van Vuuren and Holl, (2017)
Essential oils	0.10	Very Strong activity	Van Vuuren and Holl, (2017)
	0.1-0.5	Strong activity	Van Vuuren and Holl, (2017)
	0.5-1	Strong activity	Van Vuuren and Holl, (2017)
	1	Noteworthy antimicrobial activity	Van Vuuren and Holl, (2017)

Table 2.5: Classification for antimicrobial activity for the current study.

Plant	MIC (mg/ml)	Classification
Plant extract	0.1	Noteworthy/ significant activity
	≥0.1- 0.625	moderate activity
	≥0.625	low activity
Essential oils	≤ 0.1	Very strong activity
	0.5 - 1	strong activity
	1	Noteworthy activity

2.7 Cytotoxicity

The plant extracts were screened in the current study using the brine shrimp lethality assay as described by Hübsch et al. (2014) and Seleteng-kose et al. (2019). Brine shrimp (*Artemia franciscana*) eggs (Ocean Nutrition™) were added to the artificial salt water and a constant source of light was provided with a lamp (220–240 V) (Kiho). The eggs were incubated for 18-24 hrs (at 25 °C) to allow hatching. The study

was conducted in a micro-titre plate, where 400 µl salt water containing 40-60 live brine shrimp which was added to each well. Thereafter, 400 µl plant sample diluted in distilled water (aqueous extracts) or 1% DMSO for organic extracts were added in triplicate to wells. All samples were tested for cytotoxicity at a concentration of 1 mg/ml (Bussmann et al. 2011). The the positive control consisted of 1.6 mg/ml potassium dichromate and negative control consisted of 32 g/l salt water (Sigma–Aldrich). The plates were observed under a light microscope (Olympus) (40× magnification) immediately after sample addition at T0 (at time 0) to note any dead brine shrimp, which would be excluded from the percentage mortality calculations. Dead brine shrimp were then counted after 24 h. Thereafter, a lethal dose of 50 µl of glacial acetic acid (100% v/v) (Sigma–Aldrich) was added to each well and percentage mortality calculated. Plant species demonstrating percentage mortality greater than 50% were considered cytotoxic (Bussmann et al. 2011). All tests were undertaken at least in triplicate.

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Chapter 3

Results and Discussions

3.1 Anatomical studies

3.1.1 Anatomical description of *Searsia erosa* bark

Sample examined*:* A. Moteetee and L. Moteetee 34 (MM34) [Cholotsa Hill, Lekokoaneng, Berea District, Lesotho].

The epidermis on young stems is composed of a single layer of more or less isodiametric cells with thin inner walls and thick outer walls covered by thin cuticle. Peltate and capitate trichomes were found as shown in Figure 3.1.

The cortex is very narrow (up to 3-5(7) cells in width), composed of only collenchyma. Cortical collenchyma is lamellar. Collenchyma cells are 10-20 µm in tangential diameter, with outer collenchyma cells occasionally with brown deposits. There were no crystals found in the cells of the cortical collenchyma. Pericyclic fibres are in continuous band of fibres near the secretory canals (of 5-7 cells in width), separated by narrow (2-4 seriate) medullary rays. Secretory canals are in the phloem parts of conductive bundles. The lumina of the canals are commonly 40-65 µm in tangential diameter. Dilation of the cortical tissue is not observed. Chloroplast were observed in cells of cortical collenchyma.

Mature bark non-peeling, brittle, with shallow fissured surface. The initiation of firstformed periderm is in the sub-epidermal layer of cells. The phellem is composed of 4-7 layers of isodiametric to somewhat radially flattened or radially elongated cells with thin cell walls (0.3-0.9 µm thick). The phelloderm comprises of 8-12 layers of radially flattened, thin-walled cells. No crystalliferous cells were found in periderm.

Sieve tubes members are 11-19 µm wide, their mean length is 424.5 µm and vary from 277-606 µm. Sieve tube are in radial groups or clusters of 4-9, while sieve plates are composed of 2-4 sieve areas that are located on vertical or slightly oblique cross wall. Axial parenchyma cells associated with conductive elements and in 4-10 cells. Prismatic crystals occur in axial cells. Axial secretary canals are scattered throughout the secondary phloem. They are lined by a single layer of 20-60 µm in

tangential diameter. 2-4 seriate parenchyma sheaths near the secretary canals were found. The transition from non-collapsed to collapsed secondary phloem is sharp. Axial parenchyma cells in collapsed secondary phloem occur as thin-walled strands and as tangential stretched cells and in tangential- strands of 2-4 cells crystalliferous cells containing prismatic crystals. Large clusters of sclereids occur in 40-65 µm in tangential diameter.

Secondary phloem rays are unseriate and 2-3 seriate. Uniseriate rays are composed of square and upright cells while, the 2- and 3- seriate rays have procumbent cells or also upright and square cells (mostly in uniseriate portions). No dilated rays found.

Prismatic crystals occur in ray cells. Radial secretory canals present.

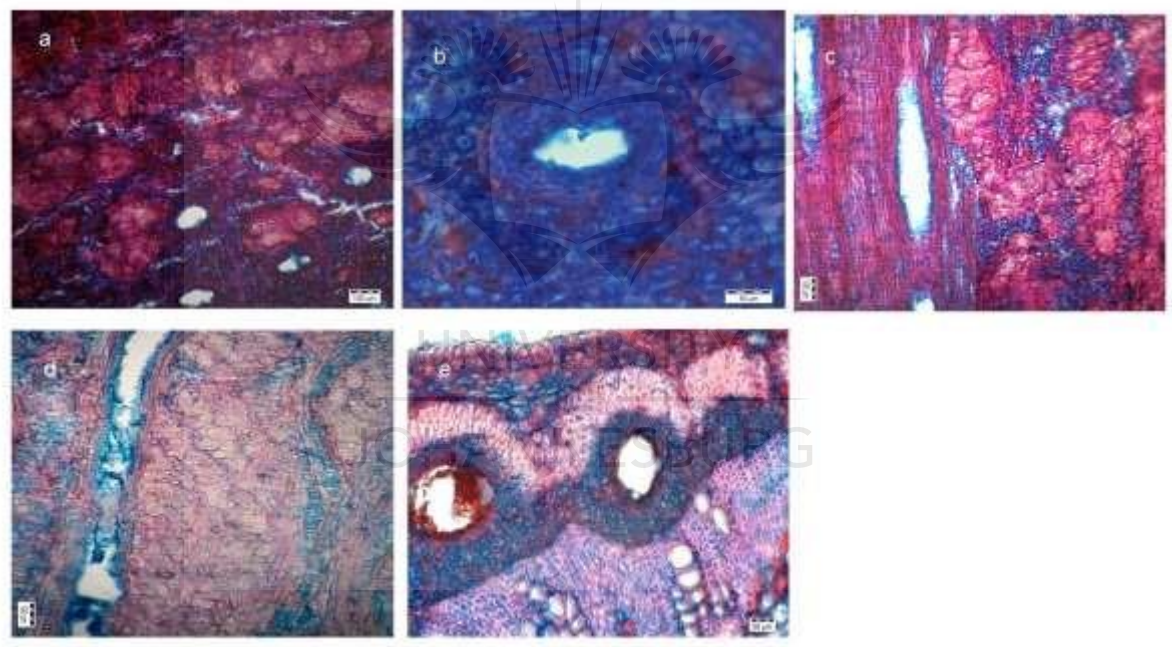

Figure 3.1: (a). Cross section of bark with sieve elements and axial secretory canals. (b) Secretory canal in secondary phloem of *S. erosa* (40x magnification). (c). Radial section of non-collapsed and collapsed secondary phloem showing axial secondary canals, phloem rays and sclereids. (b). Radial section of collapsed secondary phloem showing sclereids, parenchyma cells with prismic crystals, and axial secretory canal. (e). Cross section of young stem showing epidermis, cortex, continuous ring of pericyclic fibres and cortical secretory canals.

The bark anatomical features (sieve tubes of intermediate length, oblique composed sieve plates, axial parenchyma in conspicuous tangential bands, heterocellular phloem rays, prismic crystals, sclerenchyma cells in both phloem and cortex, presence of secretory canals in cortex and secondary phloem) examined in the present study are in accordance with those previously described for the genus *Searsia* (*S. chirindesis* Bak. F, *S. gueinzii* Sond, *S.* lancea L.f, *S. leptodictya* Diels, *S. natalensis* Berhn, *S. pendulina* Jacq and *S. pyroides* Burch) (Meltcalfe and Chalk, 1957; Ramovha, 1997). Among other related taxa, *Rhus coriaria* has druse crystals and not prismic crystals like other Rhoeae genera. *Pistacia lentiscus* L. lacks sclerenchyma cells in both phloem and cortex (Crivellaro, 2012).

Searsia erosa differs from the southern Africa species of *Searsia* and the genus *Rhus* by not having any dilatation tissue. Dilatation tissue is only well developed in *S. chirindesis*, *S. leptodictya*, *S. natalensis*, *S. pendulina* and *S. pyroides*, while some rays become dilated in *Rhus coriaria* L (Crivellaro, 2012). *Searsia erosa* also differs to the genus *Lanneae* (tribe Spondiea) and genus *Mangifera* (in the tribe Anacardium). Dilatation tissue is well developed, derived from phloem parenchyma and rays by the presence of dilatation tissue derived from phloem parenchyma and rays, which is present in *Lannea antiscorbutica* (Hiern) Engl., *Lannea discolour* (Sond.) Engl. and *Magifera indica* L (Ramovha, 1997).

3.1.2 Anatomical description of *Searsia erosa* wood

Sample examined: A. Moteetee and L. Moteetee 34 (MM34) [Cholotsa Hill, Lekokoaneng, Berea District, Lesotho].

Growth rings are distinct, marked by 3-6 rows of radially flattened fibres. Wood diffuse-porous, occasionally semi–ring-porous. The vessels are rounded in outline, narrow (tangential diameter up to 15- 125 µm) and few (vessels frequency 38.5 per mm^2 Vessels are solitary and in radial multiples or in small clusters of 2-7. Vessel elements are 260μm (143-355 µm) long. Perforation plates are simple. Intervessel pits are alternate, small, 2.0- 4 µm in vertical size, circular to oval in shape, with slit-like apertures and rounded borders. Vessel ray pits are larger than intervessel pits, which are outlined oval and horizontally elongated (scalariform), simple with reduced

borders. Helical thickenings occur on the wall of some vessel elements. No tyloses found.

Fibres libriform, septate, thin to thick walled, with minute small simple pits on radial walls. Fibre length of 545 µm (356-778 µm) long.

Axial parenchyma is scanty paratracheal, in solitary or incomplete sheet around the vessels, with 3-6 cells per strand. Rays 4.9 – 7.3 per mm, Uni- and 2-3 seriate, occasionally 4-5-seriate with radial canals. Ray height up to 0.65 mm. All rays are heterogeneous, composed of procumbent, square and upright cells mixed throughout. Radial canals in few wide rays of 5 seriate. Prismatic crystals in nonchambered upright and square ray cells.

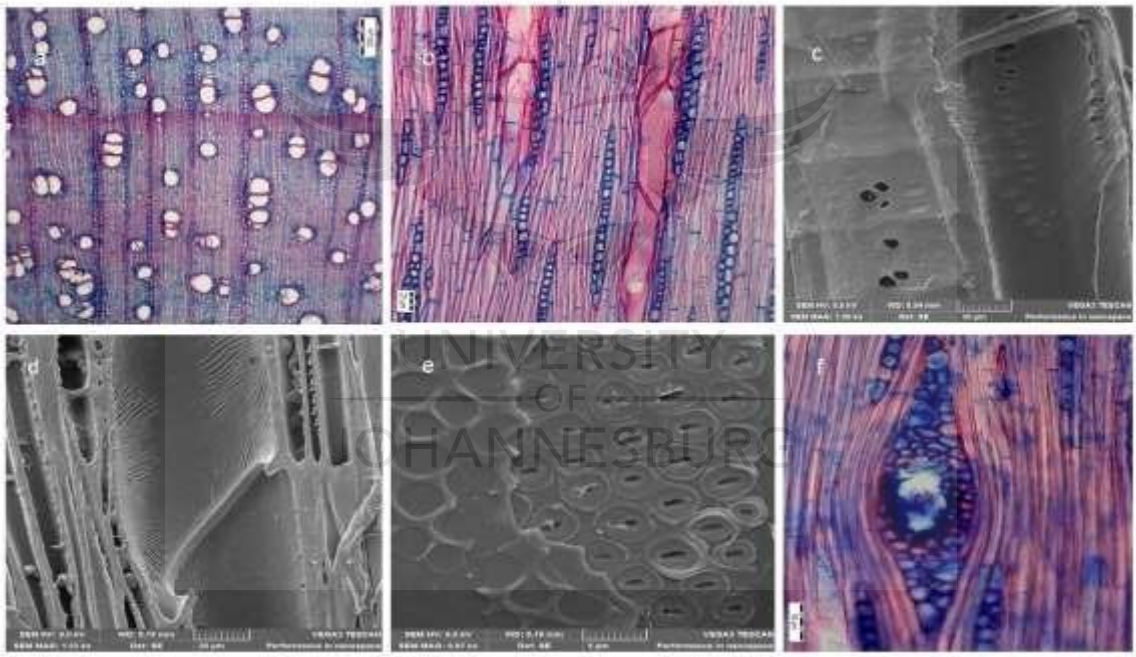

Figure 3.2: (a). Cross section of wood showing vessels. (b). Tangential section of wood showing simple perforation plates, intervessel pits, septate fibers, 1-3-seriate rays. (c). Radial section of wood showing ray-vessel pits (SEM). (d). SEM section showing helical thickenings on wood vessel walls. (e). SEM section showing intervessel pits. (f). Tangential section of wood showing radial secretary canal (40 x magnification).

The genera of Anacardiaceae show exclusively simple perforation plates, wood diffuse-porous, occasionally ring porous, axial parenchyma paratracheal: scanty, vasicentric or aliform, rays heterogeneous, uni or 2-3 seriate but larger in some species, fibres with simple pits (libriform) and septate, the presence of radial secretory canal. This set of anatomical characters, found in *S.erosa*, is typical for a large majority of wood in the family Anacardiaceae within the tribes Rhoeae, Spondieae and Anacardieae (Meltcalfe and Chalk, 1957; Mitchell and Daly, 2015; Dong and Baas, 1993). Only *Dracondtomelon* Blume, *Cotinus* (Tourn.) Mill, *Toxicondendron* (Tourn.) Mill, *Anacardium* L and Magnifera L lack radial canals.

Despite similarities*, Searsia erosa* is distinct from other members of Anacardiaceae by its vessel-ray pits that are larger than the intervessel pits, which are outlined oval and horizontally elongated (scalariform), simple with reduced borders. Vessel-ray and vessel parenchyma pits are similar to other members of the family (Dong and Baas, 1993). Tyloses and silica bodies are present in the, *Anacardium* (L), *Buchanania* and *Mangifera*, *Cotinus* (Tourn.) Mill, *Pistacia* L. and *Toxicondendron* (Tourn.) Mill, which are absent in *Searsia erosa* (Dong and Baas, 1993).

Searsia erosa differs from the tribe Rhoeae in having helical thickenings on the wall of some vessel elements and not having spiral thickenings (in genera such as *Cotinus* and *Toxicondendron*) with oblique vessel pattern and marginal parenchyma (Dong and Baas, 1993). According to Crivellaro, (2012), *Rhus coriaria* L and the genus *Pistacia* (*Pistacia atlantica* L and *Pistacia lentiscus* L) has helical thickenings in vessel elements, mostly in narrower vessel elements.

Searsia erosa is similar to the outgroup genera *Cotinus*, *Rhus, Pistacia* and *Toxicondendron* (in the tribe Rhoeae) by having distinct growth rings (Dong and Baas, 1993; Crivellaro, 2012). However, the genus *Searsia* and the genera within the tribe Rhoeae differ from tribe Anacardieae in not having growth rings (absent or faint). According to Dong and Baas (1993), growth rings in *Anacardium*, *Buchanania* and *Mangifera* are faint, sometimes marked by denser bands of fibres and sometimes indicated by narrow to moderately wide bands of parenchyma

3.1.3 Anatomical description of *Searsia erosa* leaf

Sample examined: A Moteetee and L Moteetee 34 (MM34) [Cholotsa Hill, Lekokoaneng, Berea District, Lesotho].

The leaves of *Searsia erosa* are hairy mostly on the adaxial surface than the abaxial surface. Capitate and peltate glandular trichomes which are more diffusely distributed on the Adaxial surface than the abaxial surface (Figure 3.3 c and d). Capitate glandular trichomes have a unicellular base, 1-2 celled stalk and a unicellular head. Furthermore, the peltate glandular trichomes have unicellular base, 1-2 celled stalk and 4-12 peripheral celled head (Jia et al., 2013). The leaf blade is dorsiventral, 231.6-315 µm. thick, with palisade mesophyll beneath the upper epidermis. The adaxial epidermal cells differ from the abaxial ones in their shape and size; the abaxial cells are polygonal, square to rectangular in transection and in surface view while the adaxial cells are circular to oval in shape. The epidermal cells have curved anticlinal walls with slightly thickened outer and inner cell walls of 6.713 µm (abaxial surface) and 12-15.8 µm (adaxial surface). The outer walls of the epidermis are covered by cuticle of ca. 2-3 µm thick. Hypodermis is absent. The midrib of *S. erosa* has five bundles (in the secondary phloem) arranged in an arc (a part of a curve) as shown in figure 3.3 a. The palisade tissue consists of two to three cell layers. The palisade cells are upright to long upright (height/width ratio1.5 to 4). The intercellular spaces in the spongy parenchyma are large. Some of the large parenchyma cell, contain a druse crystal and are scattered in the photosynthetic tissue (Figure 3.3 b). Some canals end up in the mesophyll tissue blindly. The vascular bundles are sheathed by 1 or 2 layers of parenchymatous to slightly collenchymatous cells. In addition, the abaxial and adaxial extensions are associated with the sheath of larger vascular bundles. The vascular bundles are round, collateral with tangentially extended phloem and xylem zones, usually accompanied with a secretory canal. Most mesophyll cells are completely filled with brownish and black substances which may represent oxidized tanniniferous substances which may be different from those in the epidermal layer. Stomata are anomocytic, scattered on

the abaxial and adaxial surfaces (amphistomatous), situated mostly at the level of the outer epidermal cell wall.

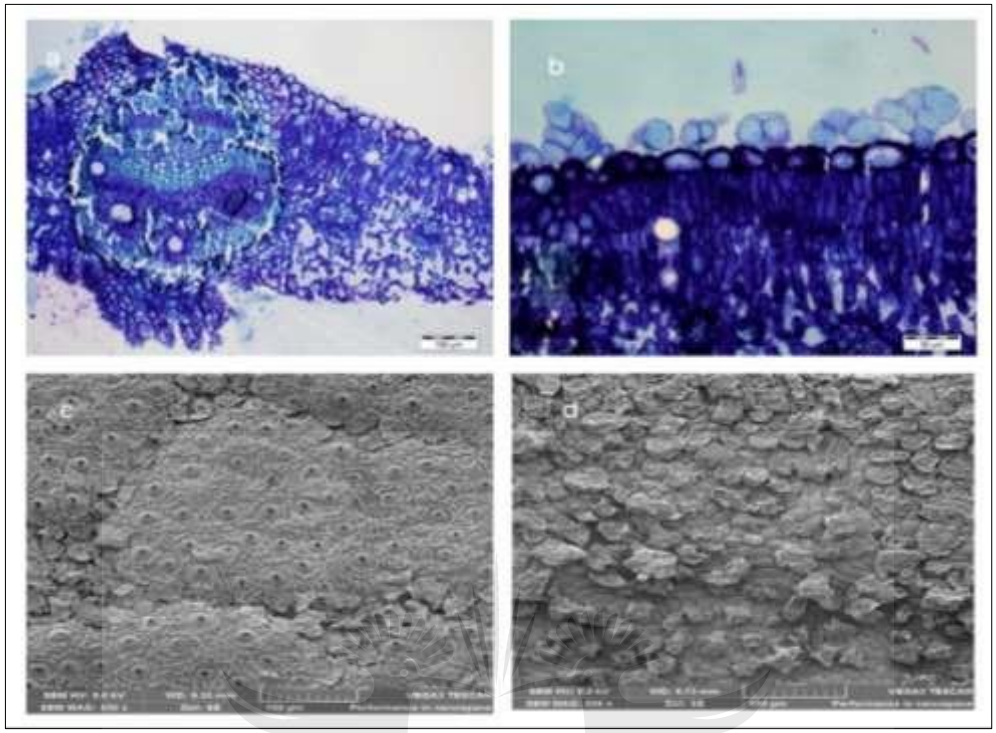

Figure 3.3: (a). Light micrographs of *S. erosa* leaf in transverse section. (b). Light micrographs of *S. erosa* leaf laminar (c). Abaxial surface of *S. erosa* leaf with fewer trichomes but more numerous stomata than there are on adaxial surface (Fig. 3.3 d). (d). Adaxial section of *S. erosa* leaf with numerous trichomes.

The leaf of *Searsia erosa* shows such characteristic traits of the majority of Anacardiaceae genera as dorsiventral structure of blade, non-glandular and glandular trichomes, amphistomatous uniseriate epidermis, circular band (arc) of vascular bundles in the midrib, presence of secretory canals in the midrib and mesophyll tissue, and the occurrence of druse crystals in mesophyll cells (Meltcalf and Chalk, 1957; Jordaan and Kruger, 1992; Einagaoglu and Ozcan, 2018). Only *Pistacia vera* has isobilateral appearence (Al-saghir et al, 2006).

Searsia erosa has peltate and capitate trichomes on its leaves. The capitate glandular trichomes found also in other *Searsia* species, i.e. in *S. chinensis* (Einagaoglu and Ozcan, 2018), and *S. burchellii* (Jordaan and Kruger, 1992). Apart from *Searsia*, capitate glandular trichomes occur in *Rhus* and *Camonosperma*

(Meltcalfe and Chalk, 1957; Andrés-Hernandez and Terrazas, 2009); this feature is seemingly synapomorphic for the tribe Rhoeae.

According to Jordaan and Kruger (1992), the epidermal cells in *S. burchellii* are partially or completely filled with tanniferous substances. This agrees with the results of the histochemical tests of *Searsia erosa* from both tests (Vanillin- HCL and Ferric chloride) for the presence of tanniferious substances in the epidermis. *Searsia erosa* differs from *S. chinensis* in having a single palisade layer and 3-4 spongy layers, while *S. burchellii* and other genera of Anacardiacese consist of two to three cell layers. Druse crystals in *S. chinensis* are present in collenchymatous cells of midrib and palisade cells of lamina, while in *S. burchellii* and *S. erosa* druse crystals are present in large parenchyma cells which are scattered in photosynthetic tissues (Einagaoglu and Ozcan, 2018).

The secretory canal arrangement of *S. erosa* is similar to *that of S. diversiloba* while arrangement in *Smodignium argutum* is similar to *Toxicodendron radicans* and *S. diversiloba*. However, *S. diversiloba* canal arrangement differs from *Smodignium argutum* and *Toxicodendron radicas* by a single bundle that is in a dorsal arrangement (Ellis, 1974). According to Ellis (1974), all the lateral veins of the leaves of the poison ivy and *Smodignium argutum* contain at least one secretary canal on their dorsal sides in the phloem of the vascular bundle.

3.2 Histochemistry

The histochemical results show that the secretory trichomes on the leaf surface as well as secretory canals in the stem cortex, secondary phloem, pith, petioles and leaves produce essential oils and lipids.

The results from Table 3.1 show that the secretory canals in the stem cortex, secondary phloem, pith, petioles and leaf midrib produce essential oils and oleoresin (terpenoids). These products were not seen in the sub-epidermal collenchyma and sclerenchyma caps of the stem and petioles, while in the midrib terpenoids were seen in the trichomes, epidermis and epithelial cells of secretory canals as shown in figure 3.4. Lipids were absent in trichomes (in the stem and petioles), sub-epidermal collenchyma (petioles and midrib) and sclerenchyma cap (leaf midrib). On the other hand, alkaloids were not present in the trichomes, epidermis and epithelial cells of

secretory canals of the stem, while in the petiole alkaloids were only present in the sclerenchyma cap, xylem/phloem parenchyma and pith parenchyma which is shown in Figure 3.5 (a and b). On the other hand, alkaloids were absent in the leaf midrib.

Furthermore, tannins were present in the epidermis, sub-epidermal collenchyma and pith parenchyma, while in the xylem /phloem parenchyma tannins were only seen in ferric chloride test (Figure 3.6 a, b and c). In the petiole's tannins were absent in the trichomes, sclerenchyma caps and pith parenchyma, while in the cortex parenchyma and xylem/phloem parenchyma tannins were present based on vanillin HCL test. Furthermore, tannins were present in the epidermis and sclerenchyma cap, while in the trichomes and sub-epidermal collenchyma, tannins were present in ferric chloride test. In the vanillin- HCL test xylem/phloem parenchyma tested positive for the presence of tannins (Figure 3.5, c, d and e). On the other hand, mucilage was present in the epidermal cells of stems, petioles and leaf midribs, while in the petiole mucilage was also present in the epithelial cells of secretary canals and pith parenchyma cells as shown in Figure 3.7(a, b and c). Furthermore, in the leaf midrib mucilage was present in the sub-epidermal collenchyma.

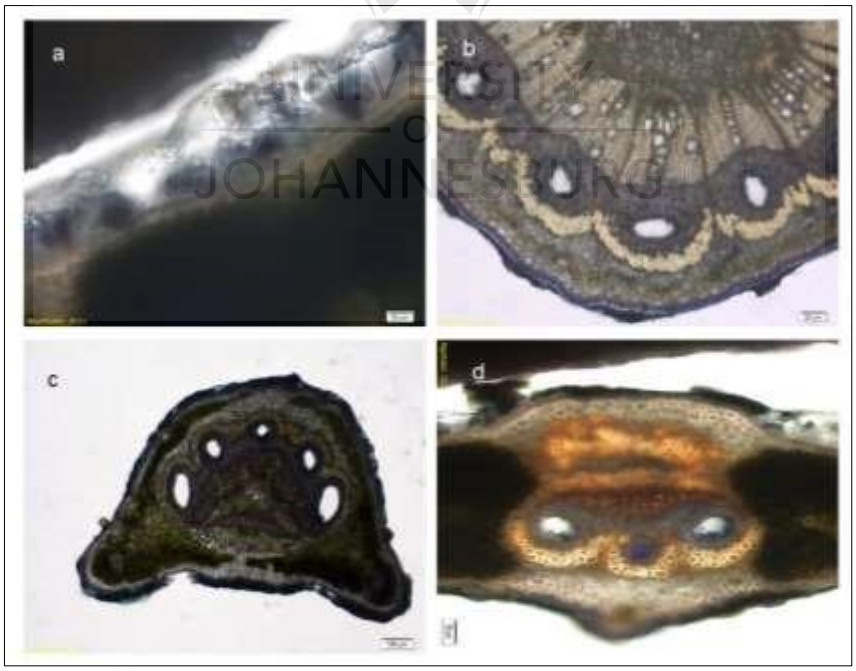

Figure 3.4: (a). Cross section of *S. erosa* trichomes showing positive reaction to Nadi reagent (blue essential oils and violet for oleoresin). (b). Cross section of *S. erosa* stem showing positive reaction to Nadi reagent (blue essential oils and violet for

oleoresin). (c). Cross section of *Searsia erosa* petiole showing positive reaction to Nadi reagent (blue essential oils and violet for oleoresin). (d). Cross section of *S. erosa* leaf (midrib and lamina) showing positive reaction to Nadi reagent (blue essential oils, violet for oleoresin, and- red colour for resiniferous acids).

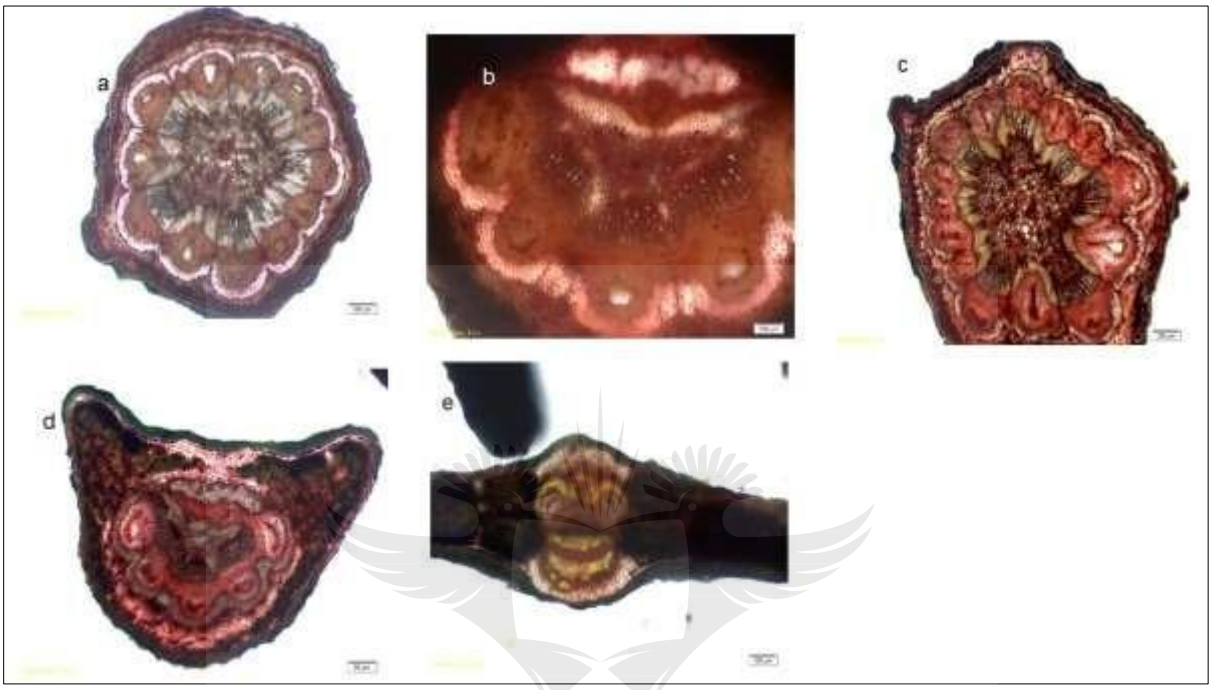

Figure 3.5: (a). Cross section of *S. erosa* stem showing positive reaction to Ellram's red (reddish pink) for presence of alkaloids on sclerenchyma cap and some cells of pith. (b). Cross section of *S. erosa* petiole showing positive reaction to Ellram's red (reddish pink) for presence of alkaloids on sclerenchyma cap and some cells of pith. (c). Cross section of *S. erosa* stem showing positive reaction to Vanillin- HCL (bright red colour) within different cells and tissues of *S. erosa* for presence of tannins. (d). Cross section of *S. erosa* of petiole showing positive reaction to Vanillin- HCL (bright red colour) within different cells and tissues of *S. erosa* for presence of tannins. (e). Cross section of *S. erosa* leaf (midrib and laminar) showing positive reaction to Vanillin- HCL (bright red colour) within different cells and tissues of *S. erosa* for presence of tannins.

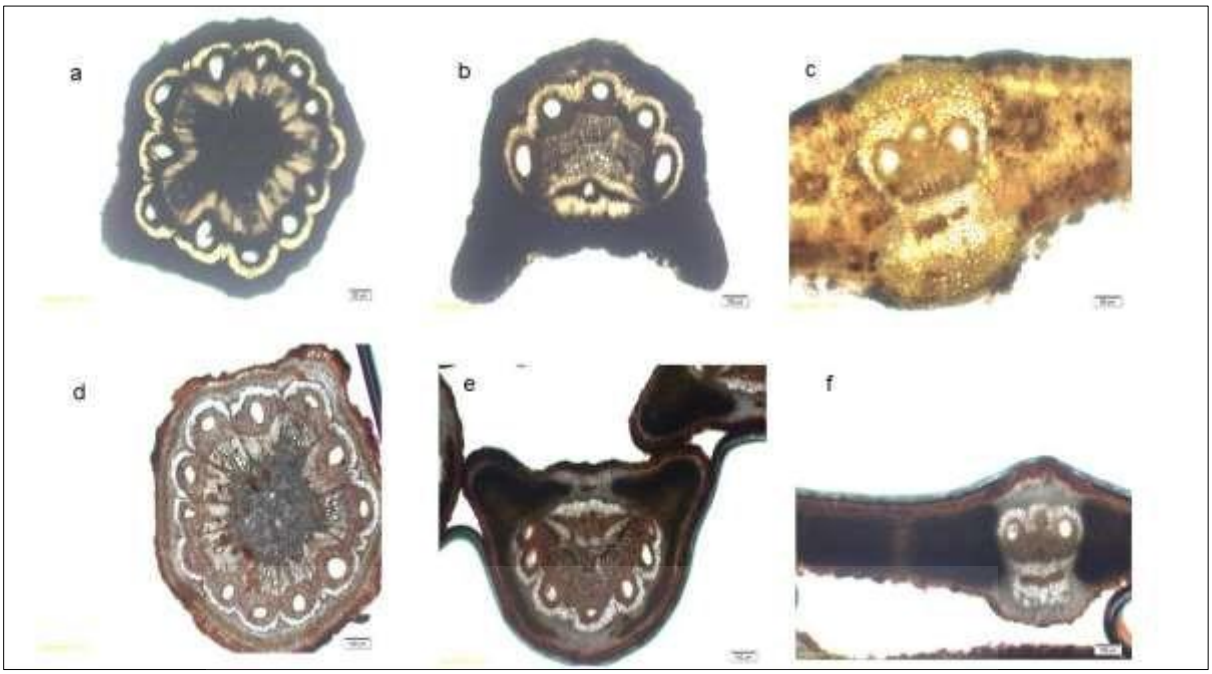

Figure 3.6: (a). Cross section of *S. erosa* stem showing positive reaction to Ferric chloride (green for polyphenols and black for tannins) for presence of tannins and polyphenols. (b). Cross section of *S. erosa* petiole showing positive reaction to Ferric chloride (green for polyphenols and black for tannins) for presence of tannins and polyphenols. (c). Cross section of *S. erosa* leaf (midrib and laminar) showing positive reaction to Ferric chloride (green for polyphenols and black for tannins) for presence of tannins and polyphenols. (d). Cross section of *S. erosa* stem showing positive reaction to Sudan (blue, black or brownish) for presence of lipids. (e). Cross section of *Searsia erosa* petiole showing positive reaction to Sudan (blue, black or brownish) for presence of lipids. (f). Cross section of *S. erosa* (midrib and lamina) showing positive reaction to Sudan (blue, black or brownish) for presence of lipids.

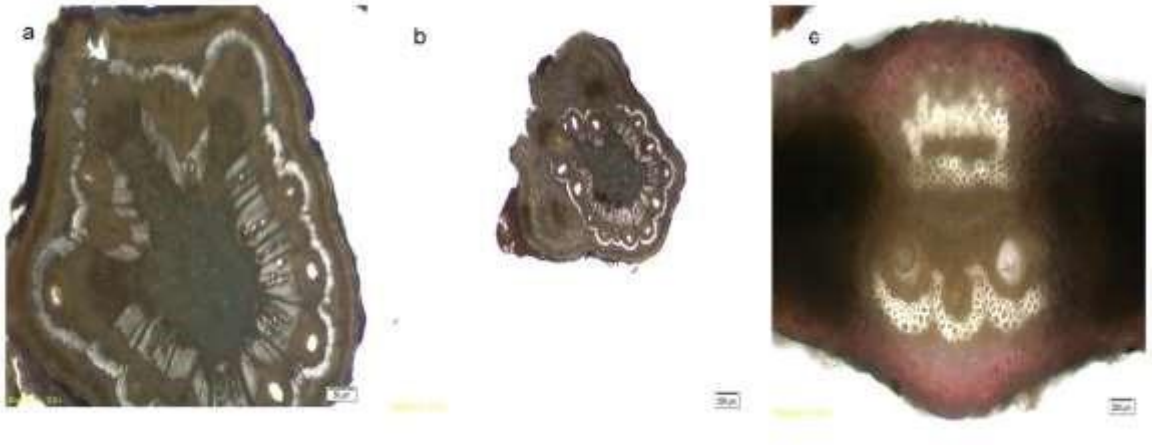

Figure 3.7: (a). Cross section of *S. erosa* stem showing positive reaction to Ruthenium red (bright red colour) for presence of mucilage. (b). Cross section of *S. erosa* petiole showing positive reaction to Ruthenium red (bright red colour) for presence of mucilage. (c). Cross section of *S. erosa* leaf (midrib and lamina) showing positive reaction to Ruthenium red (bright red colour) for presence of mucilage.

The histochemical results shown in Table 3.2 show that secretory trichomes and canals on the leaf laminar produce essential oils, oleoresins and lipids. Terpenoids were only absent in the palisade mesophyll cells. Furthermore, tannins were present in the epidermal cells and parenchyma cells (palisade and mesophyll parenchyma) while no alkaloids were present in the leaf laminar.

Table 3.1: Histochemical tests for fresh sections of stems, petioles and leaf midribs.

Stem										
Metabolite group		**tests**	**localization**							
			trichomes	**epidermis**	**cortex parenchyma**	**subepidermal collenchyma**	**sclerenchyma cap**	**xylem/phloem parenchyma**	**epithelial cells of secretory canal**	**pith parenchyma**
Alkaloids		Ellram's	-	-	+	+	+	+	-	+
Phenolic compound	tannins	Vanillin-HCL	-	+	+	-	-	-	-	+
		Ferric chloride	-	+	+	-	-	+	-	+
Lipids	total	Sudan IV	-	+	+	+	+	+	+	+
Terpenoids	essential oils	Nadi reagent	-	+	+	-	-	+	+	+
	oleoresin		-	+	+	-	-	+	-	+
Mucilage		Ruthenium red	-	+	-	-	-	-	-	-
Petiole										
Akaloids		Ellram's red	-	-	-	-	+	+	-	+
Phenolic compounds	tannins	Vanillin-HCL	-	+	+	+	-	+	+	-
		Ferric chloride	-	+	-	+	-	-	+	-
Lipids	total	Sudan IV	-	+	+	-	+	+	+	+
Terpenoids	essential oils	Nadi reagent	+	+	+	-	-	+	+	+
	oleoresin		+	+	+	-	-	+	-	+
Mucilage		Ruthenium red	-	+	-	-	-	-	+	+
Leaf midrib										
Alkaloids		Ellram's red	-	-	-	-	-	-	-	-
Phenolic compounds	tannins	Vanillin-HCL	-	+	-	-	-	+	+	-
		Ferric chloride	+	+	-	+	-	+	-	-
Lipids	total	Sudan IV	+	+	+	-	-	+	-	-
Terpenoids	essential oils	Nadi reagent	+	+	-	-	-	-	+	-
	oleoresin		+	+	-	-	-	+	-	-
Mucillage		Ruthenium red	-	+	-	+	-	-	-	-

+ = Present; - = Absent

Table 3.2: Histochemical tests for fresh leaf lamina sections of *Searsia erosa.*

Metabolite group		**tests**	**localization**			
			trichomes	**epidermis**	**Palisade parenchyma**	**Spongy parenchyma**
Alkaloids		Ellram's red	-	-	-	-
Phenolic compounds	tannins	Vanillin- HCL	-	+	+	+
		Ferric chloride	_	+	+	+
Lipids	total	Sudan IV	+	+	+	+
Terpenoids	essential oils	Nadi reagent	+	+	-	-
	oleoresins		+	+	-	-
Mucillage		Ruthenium red	-	+	-	-

+ = Present

- = Absent

3.3 Essential oils

The data of the percentage yield of essential oils is presented in Tables 3.3 and 3.4. The oil is variable in yield between the individual plants in the same season and in each individual during different seasons. However, in three of the five individuals, the yield was found to be the highest in summer. Although not related, in the species *Mentha longifolia* the highest essential oil yield was obtained during winter, however this corresponded with the flowering stage of this species (Zouari-Bouassida et al., 2018). It is worth noting that *Searsia erosa* produces flowers from late October to December (late spring- early summer. On the other hand, the remaining two individuals in this population produced the highest yields in autumn, during the vegetative stage of this species.

Table 3.3: Percentage yield values of essential oil samples investigated throughout the year.

Voucher number	Locality	Percentage yield (w/w)			
		Summer	Autumn	Winter	Spring
A. Moteetee & L. Moteetee 29 (1A*)	Cholotsa Hill, Lekokoaneng, Berea District	0.25	0.44	0.326	N/A
A. Moteetee & L. Moteetee 30 (2B*)	Cholotsa Hill, Lekokoaneng, Berea District	0.2	0.302	0.231	0.128
A. Moteetee & L. Moteetee 31 (3C*)	Cholotsa Hill, Lekokoaneng, Berea District	0.34	0.298	0.28	0.102
A. Moteetee & L. Moteetee 34 (4D*)	Cholotsa Hill, Lekokoaneng, Berea District	0.34	0.190	0.29	0.23
A. Moteetee & L. Moteetee 33 (5E*)	Cholotsa Hill, Lekokoaneng, Berea District	0.32	0.230	0.206	0.247

*1A, 2B, 3C, 4D and 5E = Seasonal variation study (five individuals from five different population); N/A = Not available (dried due to drought)

For the geographical variation study, the Lesotho collection had variable yield while the Free State collection had similar oil yields.

Table 3.4: Percentage yield values for geographical variation of essential oil samples investigated.

Voucher number	Locality	Percentage yield (w/w)
A. Moteetee & L. Moteetee 35 (LS1^)	Ha Seeiso village, Berea District, Lesotho	0.16
A. Moteetee & L. Moteetee 36 (LS2^)	Ha Seeiso village, Berea District, Lesotho	0.21
A. Moteetee & L. Moteetee 37 (LS3^)	Ha Seeiso village, Berea District, Lesotho	0.25
A. Moteetee & L. Moteetee 38 (LS4)	Ha Seeiso village, Berea District, Lesotho	0.2
A. Moteetee 39 (FS1$^{\$}$)	25km past Bots'abelo on way to Bloemfontein	0.35
A. Moteetee 39 (FS2$^{\$}$)	15km before Thaba-nchu on the road to Bloemfontein	0.36
A. Moteetee 39 (FS3$^{\$}$)	20km after Thaba-nchu on the road to Bloemfontein	0.30

^LS1, LS2 and LS3 (LS - Lesotho) = Lesotho collection for geographical variation study. $FS1, FS2 and FS3 (FS – Free State) = Free State collection for geographical variation study.

3.3.1 Essential oil composition

From the current study a total of 34 volatile compounds were identified (as major compounds) in twenty-six samples studied. The major compounds include several monoterpenes and sequiterpenes. From the Lesotho samples (23 in total samples), the main compounds identified were: α pinene, 3-Cyclohexen-1-ol, 4-methyl-1-(1methylethyl)-, Cyclohexane,1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1methylethylidene)-, Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-, Bicyclo [3.1.0] hex-2-ene,2-methyl-5-(1-methylethenyl) and 1,4-Cyclohexadiene, 1-methyl-4(1-methylethyl)- which were present in all or most of the samples (table 3.5). From the Free State Province (three samples), main compounds identified were: α phellandrene, β Ocimene, α terpineol, caryophyllene and α pinene in most of the samples. All the main compounds (from the samples collected from Lesotho and Free State Province) demonstrate some variability and none of the constituents is present as a major compound in all the samples, except Alpha-phellandrene which is the main constituent in the Free State populations FS1 (69.4%) and FS2 (57.27), as well as Dlimonene, which is a major compound in the Ha Seeiso village (Lesotho) individuals MM35 (49.3%) and MM37 (38.2%). It is not surprising that α-pinene is found in all samples studied as it is the most abundant terpene in nature (Noma and Asakawa, 2010). This constituent was also found to occur in *Rhus* (*Searsia*) *lancea*, together with δ-3-carene (Gundidza et al., 2010), which occurs sporadically in the studied samples. Bulgarian species of the closely related genus *Rhus,* were found to possess α-pinene, β-pinene, and limonene as the major constituents (Tsankova et al., 1993), while *R. continus* L. (now *Cotinus coggygria* Scop.) from India had βpinene, camphene and limonene as the main components of the oil (Joshi and Mathela, 2014).

Table 3.5: Summary of the main compounds found in five populations of S. erosa essential oils.

Location			Cholotsa Hill, Lokokoaneng, Berea district Lesotho (Population1)																		Ha seeiso village Lesotho (Population 2)				Free State Province (Population 3,4,5)		
Seasonal / geographical distribution			Winter					Autumn					Spring				Summer					Geographical distribution				Geographical distribution		
Voucher specimen			1A	2B	3C	4D	5E	1A	2B	3C	4D	5E	2B	3C	4D	5E	1A	2B	3C	4D	5E	MM35	MM36	MM37	MM38	FS1	FS2	FS3
NO	RI	Major compounds																										
1	1017	**α-Pinene**	**5.9**	**37.2**	**14.1**	**4.5**	**10.7**	**27,6**	**8**	**10.2**	**17.3**	**37.8**	**10.2**	**18.7**	**23.8**	**36.3**	**42**	**39.1**	**7**	**-**	**12.4**	**6.7**	**24.2**	-	-	**23.7**	**0.02**	**0.45**
2	1062	**α-phellandrene**	4	-	0.4	0.4	1.4	-	-	0.2	0.014	0.4	-	-	-	0.2	-	-	-	-	-	-	1.9	0.3	0.4	**69.4**	**57.27**	-
3	1091	**Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-**	**3.6**	**25.9**	**5.9**	**2.5**	**2.1**	**35.3**	**34.3**	**36.6**	**28.7**	**26.6**	**28.1**	**2.6**	**19.1**	**24.6**	**-**	**-**	**42.1**	**-**	**-**	**13.8**	**21.7**	**7.9**	**12.6**	**-**	**-**	**-**
4	1088	**Bicyclo[3.1.0]hex-2-ene,2-methyl-5-(1-methylethyl)**	**1.6**	**14.1**	**2.3**	**3.6**	**1.7**	**-**	**1.1**	**1.9**	**1.3**	**0.09**	**1.1**	**1.7**	**0.04**	**-**	**2.3**	**-**	**1.2**	**-**	**1.3**	**-**	**1.5**	**-**	**1.2**	**0.12**	**-**	**8.2**
5	1052	**β-ocimene**	2.7	-	1	2.2	1.6	1.1	3	0.4	0.9	0.98	3.9	3.4	0.9	0.9	-	2.6	0.8	-	1.2	0.53	2.5	0.4	1.3	**0.46**	**8.04**	**6.7**
6	1033	**1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-**	**8.4**	**-**	**18.6**	**8.9**	**9**	**3.9**	**6.9**	**8.1**	**6**	**5.1**	**7.3**	**9.5**	**-**	**3.2**	**-**	**6.8**	**0.02**	**0.8**	**5.2**	**5.3**	**5.1**	**1.4**	**4.6**	**-**	**-**	**-**
7	1075	**3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-**	**12.6**	**-**	**2.9**	**16.4**	**14.3**	**7.8**	**7.9**	**11.6**	**7.6**	**9**	**11.9**	**23.9**	**9.7**	**4.1**	**-**	**12.6**	**8.4**	**6.4**	**8.4**	**9**	**10.6**	**2.9**	**9.5**	**-**	**-**	**4**
8	1540	**Caryophyllene**	**1**	**-**	**1.3**	**1**	**3.2**	**-**	**1**	**0.2**	**-**	**0.4**	**0.3**	**0.4**	**0.4**	**0.8**	**-**	**0.38**	**0.4**	**1.6**	**1.2**	**0.09**	**0.2**	**0.9**	**0.4**	**1.69**	**7.62**	**3.8**
9	1057	**α - Terpineol**	**-**	**-**	**0.14**	**0.45**	-	-	-	-	-	-	**0.4**	**0.6**	**0.4**	**0.2**	**-**	-	**0.3**	-	**0.4**	**0.4**	**0.6**	**0.29**	**6.4**	**0.55**	**1.79**	**18.4**

3.3.2 Seasonal variation

The essential oils from all the 19 samples of population 1 have shown variability with less similar GC profiles. However, 17 compounds (from the total 34 volatile compounds), namely; α-phellandrene, β phellandrene, 2-hexanal, 3-hexen-ol-1, Bicyclo[3.1.1]heptane,6,6-dimethyl-2-methylene-,1R,3Z,9S2,6,10,10Tetramethylbicyclo[7.2.0]undeca-2,6-diene, 1,5-Cyclodecadiene, 1,5-dimethyl-8-(1methylethenyl), 8 -Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene, Cyclohexene,4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-, cyclohexane,1-ethenyl-1methyl-2-(1-methylethenyl)-4-(1-methylethylidene)- ,1,6-Cyclodecadiene, 1-methyl-

5-methylene-8-(1-methylethyl)-, 1R α-pinene, 3-carene, α–Terpineol and (-) Spathulenol, showed much variation as they were absent in most of the individual plants of population1 (i.e. Cholotsa hill, Lokokoaneng Berea District). On the other hand, Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, 2-Cyclohexen-1ol,3-methyl-6-(1-methylethyl) trans- and 1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha., 7.alpha.,7a.beta.,7b.alpha.)]- were the three compounds that showed lesser viability within the four seasons.

In the samples collected in autumn, α pinene, Bicyclo [3.1.0] hexane, 4-methylene-1(1-methylethyl)-, 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, were the main compounds present in all the five individuals. However, Bicyclo [3.1.1] heptane, 6,6dimethyl-2-methylen- and 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- were present in some individuals. Samples collected during the winter season had α pinene, 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- as the main compounds present in all the individuals. 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- was present in some individuals. Furthermore, α pinene, Bicyclo [3.1.0] hexane, 4methylene-1-(1-methylethyl)- were the main components in the spring collected samples present in all the individuals. However, 3-Cyclohexen-1-ol, 4-methyl-1-(1methylethyl)- and Cyclohexane,1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1methylethylidene)- were present in some individuals. In addition, α pinene, 3Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- and Cyclohexane,1-ethenyl-1-methyl2-(1-methylethenyl)-4-(1-methylethylidene)- were present in most of the individuals of the summer season (table 3.7).

It was determined that α-pinene was consistently present throughout the different seasons in almost all the studied individuals (except in 4D of the summer season). The shrub (4D) did not survive the previous season's drought and was completely dry. More interestingly however, was the presence of (+) 4-carene in all the individuals of spring and summer season (population1), while it was absent together with 3-carene in almost all the individuals of autumn and winter season (Table 3.6). The absence of these constituents in during the autumn and winter months could be

due to ecological factors such as precipitation, pH of the soil, temperature, etc. In a study by Liu et al. (2015), annual average rainfall was found to have an inverse relationship with the active ingredients in the Chinese medicinal plant *Sinopodophyllum hexandrum* (Royle) T.S. Ying.

At all stages during the different seasons, monoterpenes were dominant in the composition of *S. erosa* essential oils (Table 3.7). Samples collected during the autumn season had high α-pinene (8% - 37.8%) than in the winter collected samples (5.9% - 37.2%). In the summer samples not all the individuals had α-pinene present (4D in summer had no α pinene). 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl) and 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- had few variations within the individuals of population 1. Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-, Bicyclo [3.1.0] hex-2-ene,2-methyl-5-(1-methylethenyl) had much variation within the summer season. Cyclohexane,1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1methylethylidene) had variation within and between populations (Table 3.7).

Table 3.6: Seasonal variation of the main compounds found in five populations of S. erosa essential oils.

Location			Cholotsa Hill, Lokokoaneng, Berea district Lesotho (Population1)																	
Seasonal / geographical distribution			Winter					Autumn					Spring				Summer			
Voucher specimen			1A	2B	3C	4D	5E	1A	2B	3C	4D	5E	2B	3C	4D	5E	1A	2B	3C	4D	5E
NO	RI	Major compounds																			
1	1062	**Alpha phellandrene**	**4**	**-**	**0.4**	**0.4**	**1.4**	**-**	**-**	**0.2**	**0.014**	**0.4**	**-**	**-**	**-**	**0.2**	**-**	**-**	**-**	**-**	**-**
2	1072	Beta phellandrene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16.3	-	-	33
3	1091	**Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-**	**3.6**	**25.9**	**5.9**	**2.5**	**2.1**	**35.3**	**34.3**	**36.6**	**28.7**	**26.6**	**28.1**	**2.6**	**19.1**	**24.6**	**-**	**-**	**42.1**	**-**	**-**
4	1035	**Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-**	**0.04**	**-**	**-**	**-**	**-**	**-**	**-**	**11.3**	**11**	**0.01**	**-**	**-**	**9.6**	**-**	**10**	**-**	**-**	**-**	**-**
5	1060	(+)-4-Carene	1.1	-	-	-	-	-	0.04	-	-	-	5.3	5.2	2.4	1.9	2.8	2.84	0.03	0.5	0.5
6	1012	3 Carene	-	-	-	-	-	-	-	-	-	-	10.9	9.2	3.6	-	8.4	-	5.5	-	-
7	1524	1R,3Z,9S-2,6,10,10-Tetramethylbicyclo[7.2.0]undeca-2,6-diene	12.6	-	-	-	13	-	-	-	-	-	-	-	-	-	0.2	8.2	-	-	-
8	1514	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethenyl)-	0.01	-	-	14.8	-	8.9	-	-	-	3.3	0.02	-	-	4.6	-	-	0.08	-	6.5
9	1064	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl) trans	0.12	-	0.4	0.25	0.2	-	0.05	0.13	0.1	0.1	0.1	0.2	0.07	0.04	-	-	0.09	-	0.09
10	1553	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-	2.5	-	3.4	1.9	3.5	0.46	-	-	-	-	0.03	0.02	-	-	1.2	-	-	-	6.5

3.3.3 Geographical variation

Two individuals of population 2, i.e. Ha Seeiso village (MM35 and MM36) differ from the other two plants (MM37 and MM38) within the same population in the presence and absence of isomer 1R α pinene respectively. However, Bicyclo [3.1.0] hexane, 4methylene-1-(1-methylethyl)- was present in all the four individuals of population 2. Bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene-1,4-Cyclohexadiene, 1-methyl-4(1-methylethyl)-**,** bicycle [3.1.0]hex-2-ene,2-methyl-5-(1-methylethyl) and

3Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- were present in some individuals of population 2. Furthermore, bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-, bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene-1,4-Cyclohexadiene,1-methyl-4-(1methylethyl)-, Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis(1-methylethenyl)-, 2Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-, 1,3-Cyclohexadiene, 1-methyl4-(1-methylethyl)-, 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- were absent in the in population 3, 4 and 5 i.e. Free State Province. Interestingly, bicyclo[3.1.0]hex-2ene,2-methyl-5-(1-methylethyl) was only not present in population4 (individual FS2) but present in populations 3 and 5.

The isomer 1R α pinene was only found in samples of population 2 but not in populations 1, 3, 4 and 5. D limonene was found to be the only main compounds occurring in individuals of population 2 that is not present in population 1 and 3 due to its high percentage area in individual MM35 and MM37. Furthermore, (+)- 4 carene was present in all the individuals of population 2 while only population 5 (individual FS3) in the Free State Province collection had no (+)- 4 carene present. 3 carene was not present in all the individuals of population 2 and population 3, 4 and 5. 1R,3Z,9S-2,6,10,10-Tetramethylbicyclo[7.2.0]undeca-2,6-diene, and 8 Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene, were not present in all the individuals of population 2 while they were only present few of the individuals of population 1. 8 Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene, was present in all the individuals of population three, while 1R,3Z,9S-2,6,10,10-Tetramethylbicyclo[7.2.0]undeca-2,6diene, was absent in all the individuals of population 3 similar to population 2.

According to Kabuba (2009), oil from the same species is generally constant in its constituents. The results from the current study however, indicate that the composition of the oil from *Searsia erosa* is extremely variable even within the same population. The results concur with Lakušića et al.'s (2013) conviction that the same genotype can produce oils that are so different, during different growing seasons that they can be classified as different chemotypes. The study further confirms that the chemical composition of essential oils depends on the time of collection, climate, population, and geography. Essential oil studies are complex due

to the heterogeneous nature of the oil, harvest season, environmental and climate differences (Van Vuuren, 2009). The complexity of the oil, may cause multiple overlapping peaks. According to Makgwana, (2006) the GC must always be coupled with the mass spectroscopy to recognise the overlap. The availability of accurate mass spectra (MS) and retention times are important for correct identifications and calculations of retention indices for comparison with libraries such as NIST library or literature that has retention indices for frequently reported compounds of pant essential oils. According to Makgwana, (2006) accurate retention times are often of greater importance due to the mass spectral similarities of the isomers.

Table 3.7: The major (percentage area) of essential oil samples from of *S. erosa* collected at two localities, as identified by GC-MS.

Location			Cholotsa Hill, Lokokoaneng, Berea district Lesotho (Population1)																		Ha seeiso village Lesotho (Population 2)				Free State Province (Population 3,4,5)		
Seasonal / geographical distribution			Winter					Autumn					Spring				Summer					Geographical distribution				Geographical distribution		
Voucher specimen			1A	2B	3C	4D	5E	1A	2B	3C	4D	5E	2B	3C	4D	5E	1A	2B	3C	4D	5E	MM35	MM36	MM37	MM38	FS1	FS2	FS3
NO	RI	Major compounds																										
1	1017	**α-Pinene**	**5.9**	**37.2**	**14.1**	**4.5**	**10.7**	**27,6**	**8**	**10.2**	**17.3**	**37.8**	**10.2**	**18.7**	**23.8**	**36.3**	**42**	**39.1**	**7**	**-**	**12.4**	**6.7**	**24.2**	-	-	**23.7**	**0.02**	**0.45**
2	1017	**1R- α.-Pinene**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**24.2**	**30.7**	-	**-**	**-**
3	1062	**Alpha phellandrene**	4	-	0.4	0.4	1.4	-	-	0.2	0.014	0.4	-	-	-	0.2	-	-	-	-	-	-	1.9	0.3	0.4	**69.4**	**57.27**	-
4	1072	Beta phellandrene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16.3	-	-	33	-	-	-	-	0.43	7.89	
5	853	2-Hexanal	-	-	0.4	0.17	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-	-
6	850	3-Hexen-1-ol	0.08	-	-	-	-	-	-	-	-	-	-	0.14	0.1	-	-	-	-	-	-	-	-		--	0.02	-	0.06
7	1091	**Bicyclo[3.1.0]hexane, 4-methylene-1(1-methylethyl)-**	**3.6**	**25.9**	**5.9**	**2.5**	**2.1**	**35.3**	**34.3**	**36.6**	**28.7**	**26.6**	**28.1**	**2.6**	**19.1**	**24.6**	**-**	**-**	**42.1**	**-**	**-**	**13.8**	**21.7**	**7.9**	**12.6**	**-**	**-**	**-**
8	1088	**Bicyclo[3.1.0]hex-2-ene,2-methyl-5-(1-methylethyl)**	**1.6**	**14.1**	**2.3**	**3.6**	**1.7**	**-**	**1.1**	**1.9**	**1.3**	**0.09**	**1.1**	**1.7**	**0.04**	**-**	**2.3**	**-**	**1.2**	**-**	**1.3**	**-**	**1.5**	**-**	**1.2**	**0.12**	**-**	**8.2**
9	1035	**Bicyclo[3.1.1]heptane, 6,6-dimethyl2-methylene-**	**0.04**	**-**	**-**	**-**	**-**	**-**	**-**	**11.3**	**11**	**0.01**	**-**	**-**	**9.6**	**-**	**10**	**-**	**-**	**-**	**-**	**-**	**15.9**	**10.9**	**18.6**	**-**	**-**	**-**
10	1028	**Beta.-Myrcene**	3.1	22.3	3.7	4.3	7.8	-	1	1.7	2.7	3.8	4	6.2	3.6	3.1	1.7	-	0.9	-	1.6	2.7	3	2.1	3	0.02	-	**39.6**
11	1060	Benzene, 1-methyl3-(1-methylethyl)- (Z)	1.2		5.4	1.4	1.8	-	0.7	1	0.4	0.5	1.6	2.8	0.6	0.3	-	2.6	1.4	-	0.9	0.9	4.2	1.2	1.2	-	-	0.39
12	1024	**D limonene**	3	-	3.5	4.4	4.2	-	2	1,7	2.1	0.5	2.2	2.6	2	1.7	2.3	2.14	1.5	-	2.4	**49.3**	-	**38.2**	-	-	0.48	0.01
13	1052	**β-ocimene**	2.7	-	1	2.2	1.6	1.1	3	0.4	0.9	0.98	3.9	3.4	0.9	0.9	-	2.6	0.8	-	1.2	0.53	2.5	0.4	1.3	**0.46**	**8.04**	**6.7**
14	1033	**1,4-Cyclohexadiene, 1-methyl-4-(1methylethyl)-**	**8.4**	**-**	**18.6**	**8.9**	**9**	**3.9**	**6.9**	**8.1**	**6**	**5.1**	**7.3**	**9.5**	**-**	**3.2**	**-**	**6.8**	**0.02**	**0.8**	**5.2**	**5.3**	**5.1**	**1.4**	**4.6**	**-**	**-**	**-**

Table 3.7 continued.

Location			Cholotsa Hill, Lokokoaneng, Berea district Lesotho (Population 1)																		Ha seeiso village Lesotho (Population 2)				Free State Province (Population 3,4,5)		
Seasonal / geographical distribution			Winter				Autumn					Spring					Summer					Geographical distribution				Geographical distribution		
Voucher specimen			1A	2 B	3 C	4 D	5E	1 A	2B	3 C	4 D	5 E	2B	3 C	4 D	5E	1 A	2B	3 C	4 D	5E	MM 35	MM 36	MM 37	MM 38	FS1	FS2	FS3
NO	RI	Major compounds																									
15	1060	(+)-4-Carene	1.1	-	-	-	-	-	0.04	-	-	-	5.3	5.2	2.4	1.9	2.8	2.84	0.03	0.5	0.5	0.73	0.7	0.25	0.7	0.38	9	-
16	1012	3 Carene	-	-	--	-	-	-	-	-	-	-	10.9	9.2	3.6	-	8.4	-	5.5	-	-	-	-	-	-	-	-	-
17	1057	1,3-Cyclohexadiene, 1-methyl-4-(1methylethyl)-	-	-	8	0.1	4.5	2.6	-	1.3	3.3	3	4.2	-	3.4	-	-	-	3.1		2.7	2.4	2	-	2.3	-	-	-
18	1543	Cyclohexane, 1ethenyl-1-methyl-2,4bis(1-methylethenyl)-	20.6	-	0.15	0.08	0.7	8.4	0.3	0.09	0.12	2.3	-	0.02	1.2	10.1	10	-	0.04	-	0.23	-	-	-	0.1	-	-	-
19	1524	1R,3Z,9S-2,6,10,10Tetramethylbicyclo[7.2.0]undeca-2,6-diene	12.6	-	-	-	13	-	-	-	-	-	-	-	-	0.2	8.2	-	-		0.01	-	-	-	-	-	-	-
20	1514	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1methylethenyl)-	0.01	-	-	14.8	-	8.9	-	-		3.3	0.02	-	-	4.6	-	-	0.08	-	6.5	-	0.02	2.7	3.8	0.16	1.04	1.13
21	1064	2-Cyclohexen-1-ol, 1methyl-4-(1methylethyl) trans	0.12	-	0.4	0.25	0.2	-	0.05	0.13	0.1	0.1	0.1	0.2	0.07	0.04		-	0.09		0.09	0.07	0.06	0.03	0.07	-	0.95	9.3
22	1075	**3-Cyclohexen-1-ol, 4-methyl-1-(1methylethyl)-**	**12.6**	**-**	**2.9**	**16.4**	**14.3**	**7.8**	**7.9**	**11.6**	**7.6**	**9**	**11.9**	**23.9**	**9.7**	**4.1**	**-**	**12.6**	**8.4**	**6.4**	**8.4**	**9**	**10.6**	**2.9**	**9.5**	**-**	**-**	**4**
23	1057	α - Terpineol	-	-	0.14	0.45	-	-	-	-	-	-	0.4	0.6	0.4	0.2	-	-	0.3		0.4	0.4	0.6	0.29	6.4	0.55	1.79	18.4
24	1025	2-Cyclohexen-1-ol, 3methyl-6-(1-methylethyl)-, trans-	0.3	-	0.8	0.45	0.3	-	-	0.17	0.07	0.2	0.3	0.5	0.2	0.1	-	-	0.1		0.18	0.12	0.2	0.05	0.2	-	-	-
25	1540	**Caryophyllene**	1	-	1.3	1	3.2	-	1	0.2	-	0.4	0.3	0.4	0.4	0.8	-	0.38	0.4	1.6	1.2	0.09	0.2	0.9	0.4	**1.69**	**7.62**	**3.8**

Table 3.7 continued.

Location			Cholotsa Hill, Lokokoaneng, Berea district Lesotho (Population 1)																		Ha seeiso village Lesotho (Population 2)				Free State Province (Population 3,4,5)		
Seasonal / geographical distribution			Winter					Autumn					Spring				Summer					Geographical distribution				Geographical distribution		
Voucher specimen			1A	2B	3C	4D	5E	1A	2B	3C	4D	5E	2B	3C	4D	5E	1A	2B	3C	4D	5E	MM35	MM36	MM37	MM38	FS1	FS2	FS3
NO	RI	Major compounds																										
26	1553	1,6-Cyclodecadiene, 1methyl-5-methylene-8(1-methylethyl)-, [s(E,E)]-	2.5	-	3.4	1.9	3.5	0.46	-	-	-	-	0.03	0.02	-	-	1.2	-	-		6.5	0.36	0.06	1.6	0.13	0.67	0.12	1.16
27	1529	Cyclohexane, 1-ethenyl-1-methyl-2-(1methylethenyl)-4-(1methylethylidene)-	0.03	-	11.8	-	-	-	14.5	-	11.6	-	5.7	6.1	7	-	-	6.7	14.5	66.5	-	1	1.1	-	-	1.86	0.39	1
28	1519	Cyclohexene, 4-(1,5dimethyl-1,4hexadienyl)-1-methyl-	-	-	-	8.9	-	-	-	-	-	-	-	-	0.9	0.06	-	-	-	-	-	1.8	0.8	-	-	-	0.21	-
29	1568	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7boctahydro-1,1,4,7tetramethyl-, [1aR(1a.alpha.,7.alpha.,7a.beta.,7b.alpha.)]-	0.04	-	0.04	0.01	0.05	-	0.05	0.03	0.06	0.03	0.03	0.03	0.03	-	-	0.3	-	11	T	0.02	0.04	0.02	0.01	-	-	-
30	1575	8-Isopropenyl-1,5dimethyl-cyclodeca-1,5diene	5.6	-	0.1	0.4	-	-	T	0.06	-	2.6	-	-	-	2	7.2	-	-	-	4.2	-	-	-	-	0.2	2	1.9
31	1580	(-)-Spathulenol	0.13	-	-	-	0.05	0.06	0.02	-	-	-	-	-	-	0.02	-	-	-	1.7	-	0.07	0.06	0.06	0.1	0.29	-	3.5
32	1559	Veridiflorol	0.16	-	0.3	0.015	0.2	-	0.14	0.05	0.2	0.08	0.09	0.1	0.13	0.07	0.01	-	0.08	1.2	0.05	0.07	0.03	0.06	0.06	-	-	-
33	919	Nonanol	0.11	-	-	0.25	0.2	-	-		-	0.04	-	-	0.03	0.04	-	-	-	-	-	-	-	-	0.02	-	-	0.26
34	1553	Globulol	0.03	-	0.05	0.04	0.2	-	0.03	0.02	0.03	0.002	0.03	-	-	-	-	-	0.01	1.7	T	0.085	0.07	0.12	0.12	-	-	-
		Total Area %	90.4	99.5	84.3	79.4	99.7	89.3	81	88.5	93.5	96.9	97.7	94.7	88.2	92.5	96	85.6	87.6	91.5	89.2	95.2	96.5	95.6	97.4	99.95	97.65	99.86

In Bold = the yield of important compounds (main compounds)

T= insufficient/ trace amount (less than 0.01)

RI = Retention Indices relative to C_9 -C_{15} n-alkenes

3.4 RP-HPLC analysis

The leaf extracts of *S. erosa* were analysed by HPLC to screen for the occurrence of phytochemicals present within the plant. The HPLC analysis technique is a method of choice for the separation and authentication of plant extracts. According to Beelders, (2011), HPLC analyses of phenolic compounds are usually carried out in the RP mode (RP-HPLC) employing octyl C8 or octadecyl C18 – bonded silica columns because phenolic compounds are fragile acids that can be separated as neutral, relatively hydrophobic compounds in a fragile acid matrix. HPLC chromatograms gives substantial information and fingerprint on the chemical composition of an extract. Fifteen compounds are observed on the representative chromatograph (Figure 3.8) and the area percentage of the phenolic extracts were determined as presented in Table 3.8. The Chromatograms for tannic acid (standards) are shown in appendix 1.

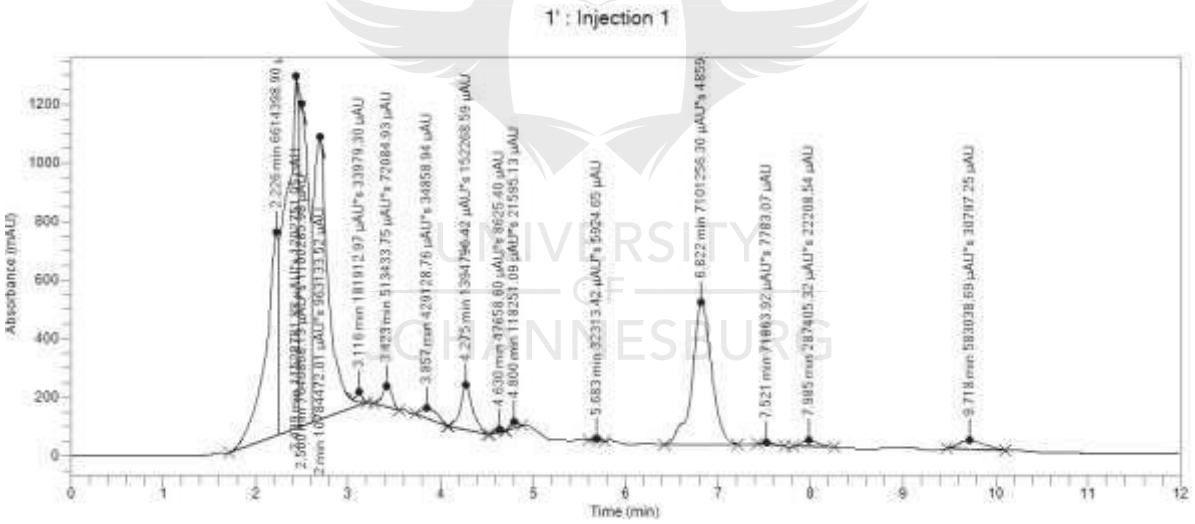

Figure 3.8: Representative chromatograph of sample extract of *S. erosa* leaves.

Table 3.8: Retention times of *S. erosa* phenolic peaks from RP-HPLC profile

Peak #	RT (min)	Literature (RT)	Component Name	Area	Area percentage %	Height
1	2.226	2.477	Tannic acid	6614398.9	14	695930.3
2	2.439	2.54	Gallic acid	11528781.9	24.4	1202751.1
3	2.500	2.67	Protocatechuic acid	7646868.1	16.2	1100286.0
4	2.702	2.94	p-hydroxybenzoic acid	10784472.0	22.8	963133.5
5	3.116	3.14	Vanillic acid	181913.0	0.4	33979.3
6	3.423	3.6	Catechol	513433.7	1.08	72084.9
7	3.857	3.90	Ellagic acid	429128.8	0.91	34858.9
8	4.275	4.28	Mycertin	1394796.4	2.9	152268.6
9	4.630	4.737	Acetyl salicylic acid	47658.6	0.1	8625.4
10	4.800	4.76	Quercetin	118251.1	0.24	21595.1
11	5.683	6.06	Kaempferide	32313.4	0.07	5924.6
12	6.822	7.00	Catechin	7101256.3	15	485993.4
13	7.521	7.44	Galloylhexose I	71863.9	0.15	7783.1
14	7.985	7.87	Epicatechin	287405.3	0.6	22208.5
15	9.718	9.86	Galloylhexose III	583038.7	1.2	30797.3
Total				47335580.2	100	

The family Anacardiaceae is known as polyphenol rich and high economic importance (Schulze-Kaysers et al., 2015). The results of *S. erosa* showed that the major identified compounds were; gallic acid, p-oH- benzoic acid, protocatechuic

acid and tannic acid (Figure 3.8). According to Sebothoma (2009), four phenolic compounds are present in the sumac extracts as determined by HPLC analysis; gallic acid (the main component), protocatechuic acid, p-oH- benzoic acid and vanillic acidic. Furthermore, gallic acid was found to be the main phenolic acid in *Rhus coriaria* (Kosar et al. 2007). This supports the HPLC results of *Searsia erosa*, as all the four phenolic compounds are present but only vanillic acid is not part of the four major phenolic compounds identified. According to Cai, et al. (2004) *Searsia chinesis* has hydrolyzable tannins (gallotannins) and phenolic acids (gallic acid). Furthermore, Ahmed et al. (2014) stated that the tannin constituents found in *Searsia* species (*S. leptodictya*, *S. pendulina* and *S. panther*) are mostly of the proanthocyanidin type (condensed tannins, such as epicatechin and catechin). Interestingly, Ahmed et al. (2014) also stated that *Ozaroa* species (*O. paniculosa* and *O. mucronata*) contained relatively high quantities of gallotannins (hydrolysable tannins), while the *Searsia* species, *S. leptodictya*, *S. pendulina* and *S. panther* had low gallotannins. This does support the results as condensed tannins are present in *S. erosa* and only two gallotannins were present (galloylhexose I and galloylhexose III). Gallotannins are not gallic acids but are polymers formed when galic acid esterifies and binds with a hydroxyl group of a polyol carbohydrate such as glucose [derivatives of galic acids] (Khanbabaee and Van Ree, 2001). Furthermore, the genera *Ozaroa* and *Searsia* are both used in South African traditional medicine treating gastrointestinal disorders (especially diarrhoea) and microbial infections (Ahmed et al. 2014). Phytochemicals present in the plant (particularly tannins) were analysed because *S. erosa* was used traditionally for tanning purposes such as leather processing.

According to Karpagasundan and Kulothungan (2014) the qualitative and quantitative RP-HPLC analysis of the actual phenolic compounds present in a plant sample are facilitated by means of comparison with standard chromatograms, which enables identification and confirmation of the presence of any phenolic compounds in the research sample. Using varying ratios of the mobile phase solvents to determine unknown compounds present in the plant sample by using standard HPLC phenolic compounds, can result to changes of known retention times of reference

standards (Deepa and Murugesh, 2014; Mradu et al, 2012). According Gilala (2010), changes of retention times should not exceed ± 10% within a batch.

The compounds identified from the chromatographs of extracts were confirmed by retention times, type of methodology, mobile phase and the eluting gradient solvents (Gilala, 2010; Karpagasundan and Kulothungan, 2014). The mobile phase used in reverse phase HPLC is more polar than the stationary phase (Lukhele, 2012). Therefore, the first four identified compounds (i.e. tannic acid, gallic acid, Protocatechuic acid, and p-hydroxybenzoic acid) are very polar and hydrophilic but their peaks are not well resolved. This could mean that they are not well separable under the analysis conditions.

3.5 Antimicrobial activity

The minimum inhibitory concentration MIC is a quantitative measure used to determine the *in vitro* antibacterial activity of plant extracts (Eloff, 1998). Antimicrobial activity was determined after 24 hours and 48 hours for some microorganisms [such as *Cryptococcus neoformans* (14116)].

3.5.1 Plant extracts

3.5.1.1 Gastro-intestinal microorganisms

Plant extracts were considered to exhibit noteworthy activity if their MIC values were ≤ 0.16 mg/ml (Kuete and Efferth, 2010; Van Vuuren and Holl, 2017). From the screening of the Lesotho samples, *S. erosa* was found to inhibit all gastrointestinal pathogens with strong antimicrobial activity: the highest antimicrobial activity was observed for the organic extracts against *Bacillus cereus* with a value of 0.05 mg/ml and *Escherichia coli* with a value of 0.125 mg/ml. The aqueous extracts displayed low activity against the gastrointestinal microorganisms with the highest being *Bacillus cereus* with a value of 1 mg/ml. As expected, the aqueous extracts of the material from the Free State Province did not possess noteworthy activity against the gastrointestinal microorganisms (except for the general pathogen *S. agalactiae* where it exhibited a low activity of 1 mg/ml). This may be due to better solubility of components in organic solvents than water. The organic extracts were active against *Bacillus cereus* (0.188 mg/ml). The results validate to some degree the traditional use of *S. erosa* for the treatment of gastrointestinal conditions.

3.5.1.2 Respiratory tract micro-organisms

Both organic and aqueous extracts of *S. erosa* material from Lesotho were found to display moderate activity against the three respiratory tract pathogens tested, while organic extracts of the Free State material showed noteworthy activity against two micro-organisms, namely, *C. neoformans* (0.125 mg/ml) and *S. aureus* (0.25 mg/ml) and the aqueous extracts were only active against *C. neoformans* (0.25 mg/ml). These findings support the medicinal use of *S. erosa* in the treatment of RTIs to some extent. On the other hand, *S. erosa's* organic extract from the Free State Province had strong activity against the general pathogen *S. agalactiae* (0.125 mg/ml), as well as the organic extract from Lesotho collection had strong activity with a value of 0.0625 mg/ml. Therefore, *S. erosa* is able to treat other ailments other than the two primary infections.

3.5.1.3 The effects of the chemical composition to the biological activity of the plant

In order to explain the activity of the plant extracts, HPLC was used to investigate the phenolic compound. According to Khanbabaee and van Ree, (2001) gallic acid is one of the most biological- active phenolic compounds of plant origin. It has been reported to be active against *S. aureus* (Afarraj et al. 2018; Khanbabaee and van Ree, 2001). According to Khanbabaee and Van Ree, (2001) tannin-containing plant extracts are used as astrigents against diarrhoea. Furthermore, Colak et al. (2009) stated that tannic acid exhibited activity against *S. aureus* and *E. coli*. Gallic acid in combination with tannic acid was found to impair bacterial growth levels (Afarraj et al. 2018). The compound Protocatechuic acid (PCA) lysis the bacterial membrane by decreasing murine cytochrome P450 and phase II enzyme leading to diminishing of lipid oxidation level (Khan et al. 2015).

3.5.2 Essential oils

Africa is a home to a number of aromatic plants. Screening studies are mostly focused on specific genus and species with antimicrobial activity that has been reported together with its chemical composition (Van Vuuren, 2008). Essential oils are considered to exhibit noteworthy activity if their MIC values are ≤ 1 mg/ml (Kuete and Efferth, 2010; Van Vuuren and Holl, 2017). In the present study, essential oils

from Lesotho material (1A, 2B, 3C, 4D, 5E, MM35, MM36, MM37 and MM38) did not show any noteworthy activity as the values were > 1 mg/ml. According to Kakad et al. (2015), poor activity does not mean absence of bioactive constituents nor that the plant is inactive, but active compounds may be present in insufficient quantities in the extract to show much expected activity with the dose level. Furthermore, the oils maybe active in other bacterial strains within the two primary infections.

Searsia erosa material from the Free State Province was found to have strong activity, the extracts inhibited the pathogens *S. agalactia* with values of 0.375 mg/ml (FS3), 0.5 mg/ml (FS2) and of 0.75 mg/ml (FS1) respectively. Furthermore *S. erosa* had noteworthy activity against *B. cereus* with a value of 1mg/ml (FS2). Therefore, *S. erosa's* ethnobotanical use to treat diarrhoea is validated by the antimicrobial activity of the essential oils *Bacillus cereus*. The essential oils were found to inhibit the RTI pathogen *C. neoformans* with a value of and 0.125 mg/ml (FS3) and 0.25 mg/ml (in FS1 and FS2) respectively, while moderate activity was observed against *K. pneumoniae* with a value of 1.5 mg/ml (in FS2 and FS3). These pathogens are most common microbes responsible for respiratory ailments and are associated with infection in immune-compromised patients (Viljoen, (1995); Viljoen et al. 2005)

According to Montanari et al. (2012), the essential oils distilled from *Schinus terebinthifolus* in July were more active against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* as opposed to the samples collected in March. Furthermore, the essential oils from *S. lancea* had noteworthy activity against *E. coli* (Gundidza et al., 2008). In the current study, the essential oils of *S. erosa* displayed moderate antimicrobial activity against gastrointestinal pathogens and respiratory tract pathogens. According to Pavani (2014), *B. cereus* is not only a gastrointestinal pathogen but also affects the respiratory tract and causes pneumonia. These findings justify *S. erosa's* traditional use in African traditional medicine to treat symptoms associated with diarrhoea, colds, and flu. Furthermore, the findings indicate that the composition of *S. erosa* essential oils display geographical and seasonal variation, as the Free State Province samples had strong activity compared to the Lesotho samples.

3.5.3 Effects of the chemical composition of the essential oil on the geographical variation study.

For a fact, chemical composition of the essential oil potentially affects the biological activity of the plant (Lakušića et al. 2013). The antimicrobial activity of the essential oils may be associated with the presence of the monoterpene α-pinene which occurred as the main constituent (in almost all the 26 individual samples). Similar findings were reported for the species *S. lancea* by Gundidza et al. (2008). According to Gundidza et al. (2008) α-pinene is known to have antibacterial activity. The Free State Province material indicated major variability between localities being distinctly different both chemically and microbiologically as essential oils from FS1 (population 3), FS2 (population 4), and FS3 (population 5) had strong antimicrobial activity but FS3 had the highest activity against both gastrointestinal pathogens and respiratory tract pathogens. β-myrcene was the compound that had the highest percentage area (39,6%) in population FS3, which was not present in populations FS2 and FS1.

According to O'Bryan et al. (2008), β-myrcene exhibited antimicrobial activity against *Salmonella* spp. Interestingly, FS1 and FS2 had high percentage areas of α phellandrene (69.4% in FS1 and 57.3% in FS2). According to Iscan et al. (2012), antimicrobial, antifungal, and anti-inflammatory activities against *Candida* species.

Table 3.9: Antimicrobial activity of *S. erosa* plant extracts from Lesotho and Free State Province South Africa (values indicated in bold show noteworthy activity, while those in italics show moderate activity).

Extracts	Code	MIC mean values (of two experiments) for one general pathogen, two gastrointestinal and three respiratory pathogens from Lesotho						Code	MIC mean values (of two experiments) for one general pathogen, two gastrointestinal and three respiratory pathogens from Free State Province					
		B cereus # 11778	*E coli* # *85900*	*S agalactiae* # 55618	*K pneumoniae* #13887	*S aureus #25923*	*C neoformans* #14116		*B cereus* #11778	*E coli* #85900	*S agalactiae* # 55618	*K pneumoniae* #13887	*S aureus* #25923	*C neoformans* #14116
Essential oils	1A	4	8	4	>8	>8	4	FS1	2	>8	**0.75**	3	>8	**0.25**
Essential oils	2B	8	8	2	>8	>8	4	FS2	1	>8	**0.5**	1.5	>8	**0.25**
Essential oils	3C	6	>8	2	>8	8	3	FS3	1.5	>8	**0.375**	1.5	>8	**0.125**
Essential oils	4D	4	6	8	>8	8	4							
Essential oils	5E	6	8	8	>8	>8	4							
Essential oils	MM35	4	>8	8	>8	8	8							
Essential oils	MM36	6	>8	6	>8	8	8							
Essential oils	MM37	8	>8	>8	8	>8	4							
Essential oils	MM38	>8	>8	>8	8	>8	3							
aqueous extract	NN1	1	2	4	1	1	8	NN3	*0.188*	2	**0.125**	*1*	*0.25*	**0.125**
organic extracts	NN2	**0.05**	**0.125**	**0.0625**	1	4	1	NN4	4	4	*1*	2	4	*0.25*
	+ c	0.00625	0.00125	0.000625	0.00125	0.0025	0.000625	+ c	0.000078	0.0025	0.000625	0.000078	0.000625	0.000156
	- c	>8	>8	>8	>8	>8	>8	- c	>8	>8	>8	>8	>8	>8

^MM35 = Used for essential oils and extracts (aqueous extracts, and organic extracts) but given code NN1 (for aqueous extract) and NN2 (for organic extract.

~FS2 = Used for essential oils and extracts (aqueous extracts, and organic extracts) but given code NN3 (for aqueous extract) and NN2 (for organic extract) as shown in Table 2.2.

3.6 Cytotoxicity

The cytotoxicity results using brine shrimp lethality assay using aqueous and organic extracts are present in Table 3.10. From the resuts, the aqueous extracts are exhibited to be the safest due to low cytotoxicity (% mortality <50). According to Lourens et al. (2011), cytotoxic componets are present in higher concentrations in organic extracts. Therefore, the cytotoxic components are not a problem when the plant is used in traditional preparations. Furthermore, the cytotoxicity of these extracts are also low when compared with the potassium dichromate which was used as positive control, indicating that the plant has some level of safety but their use needs to be monitored to avoid any toxicity limits as dosage is sometimes not considered in traditional medicine. According to Amed (2014), species of genus *Ozoroa* such as *O. paniculosa* was found to be toxic with L_C= 16.58 µg/ml (their toxicity threshold level for extract is less than L_C= 20 µg/ml) while *O. mucronate* was non-cytotoxic. Species of *Searsia* such as *S. pentheri* were found to be non-cytotoxic but *S. leptodictya* (L_C= 25.09 µg/ml) and S. pendulina (L_C= 20.30 µg/ml) had some level of toxicity. The two species of *Ozoroa* had different toxicity levels though they are from the same genus. The two species may have quite different chemical composition to account for the difference. According to Amed (2014), *O. mucronate* had less flavonol, total flavonoid, gallotannins, condensed tannins and proanthocyanidin than *O. paniculosa*. Therefore, cytotoxicity is dependent on the presence of potent bioactive compounds, as desired biological response is not due to one component.

Table 3.10: Cytotoxicity using the brine shimp assay in *Searsia erosa* extracts.

Plant part	% mortality after 24 hrs when exposed to organic extract	% mortality after 24 hrs when exposed to aqueous extract
Leaves	26.37	0.813
Positive control	100	100
Negative control	0.56	0.57

3.7 References

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Chapter four

Dissertation summary and conclusions

4.1 Anatomical and histochemical characteristics of *S. erosa*

The anatomical structure of this species is reported here for the first time. *Searsia erosa* shares a number of anatomical features with other species of the genus these include the presence of sieve tubes of intermediate length, axial phloem parenchyma, heterocellular phloem rays, mature periderm, prismic crystals and sclerenchyma that is not affected by dilatation. However, there are some differences, for example *S. erosa* lacks dilatation tissue, gelatinous fibres and sheaths surrounding the secretory canals which are present in other southern Africa species of the genus. The current studies revealed the presence of secretory canals below the midrib, in some vascular bundles of the leaves in the leaf laminar, and petioles, in addition to the glandular trichomes. The histochemical results allowed characterizing anatomically the leaf, petiole and stem, outlining a detailed profile of its constituents and allowed determination of which anatomical structures these constituents are produced by the plant.

4.2 Essential oil composition

The results have confirmed the presence of essential oils in the leaves of *S. erosa*, this thus explains the strong turpentine aroma emitted from the crushed leaves, in particular the terpene α-pinene, which is found in almost all the individuals. The oil was found to be extremely variable in both yield and more so in composition, with the composition varying between the Lesotho and the Free State populations, within these populations, and within individuals during different seasons. This implies that no two plants of *Searsia erosa* are chemically alike, i.e. each plant could potentially be described as a chemotype. The best yield is during the flowering/fruiting stage, which is in summer.

The highest percentage area was exhibited by Alpha phellandrene; however, it is a major compound in only two individuals. The fact that this plant contains essential oils may support the traditional use of this plant in the flavouring of tobacco snuff. The chemical composition of the oil gives indications on geographical factors which

influence phytochemicals and biological activity. Furthermore, the essential oil variation of the constituents gave insight into the antimicrobial activity of the plant. The individuals from Free State Province had strong antimicrobial activity, with the third population (FS3) having the highest activity against both gastrointestinal and respiratory tract pathogens.

4.3 Phenolic compounds

The leaves were found to be rich in phenolic compounds, which could explain their significant biological activity. The HPLC analysis showed that gallic acid, p-oH-benzoic acid, protocatechuic acid and tannic acid were the major phenolics. In a previous study by Kosar et al. (2007), gallic acid was also found to be the main phenolic acid in *Rhus coriaria*. These findings agreed with the findings of Sebothoma (2009), as gallic acid was found to be the main component among the four major phenolic compounds known to be present in *Rhus leptoditya*.

4.4 Antimicrobial activity

4.4.1 Organic and aqueous extracts

From the results of the antimicrobial screening of the organic and aqueous extracts of *S. erosa* leaves, it can be concluded that the traditional use of this plant to treat gastrointestinal and respiratory tract ailments has been validated to some degree. This is evidenced by the noteworthy activity displayed by organic extracts against all gastro-intestinal pathogens, i.e. *B. cereus* (0.05 mg/ml), and *E. coli* (0.125 mg/ml), as well as the RTI pathogen *C. neoformans* (0.125 mg/ml). Of particular importance is the good activity of the aqueous extracts against *C. neoformans* and *S. agalactiae* since this is the dosage form in which the medicines are ingested. According to Pavani (2014), *B. cereus* is not only a gastrointestinal pathogen but also a respiratory tract pathogen that causes pneumonia. The antimicrobial screening against the generalist pathogen *S. agalactiae,* showed noteworthy activity in the organic extract (0.065 mg/ml), as well as aqueous extract against (0.125 mg/ml). This suggests that *S. erosa* could also be used for the treatment of other ailments than its documented traditional use, since S*. agalactiae* is responsible for systemic infections. *Streptococcus agalactiae* causes serious infections in new-born babies in humans,

including meningitis (Nagao, 2015), as well as other complications such as urinary tract infections in the non-pregnant adults (Low, 2012) and invasive infections in immunocompromised, chronically ill, or elderly patients (Batista and Ferreira, 2015). *Klebsiella pneumoniae* is one of the common microbes responsible for respiratory tract ailments and is similarly associated with infections in immunecompromised patients (Viljoen et al., 2005). In this study, this microbe was found to be resistant to *S. erosa* extracts.

4.4.2 Essential oils

Although essential oils are not necessarily traditionally used for medicinal purposes per se, it is common knowledge that they have several medicinal properties. It is on this basis that the *S. erosa* essential oils were screened for their antimicrobial potential. The essential oils displayed noteworthy activity against S*. agalactiae* (0.375-0.75 mg/ml), *B. cereus* (1 mg/ml) and all the three respiratory tract pathogens tested in this study, namely; *C. neoformans* (0.125-0.5 mg/ml), *S. aureus* (0.25 mg/ml), and *K. pnemoniae* (1 mg/ml) respectively. The ethnobotanical use of *S. erosa* to treat respiratory ailments correlates with the high antimicrobial activities found against these pathogens*.* Furthermore, *S. erosa's* ethnobotanical use to treat diarrhoea is validated by the antimicrobial activity of the essential oils against *B. cereus.* These findings justify *S. erosa's* traditional use in African traditional medicine to treat symptoms associated with diarrhoea, colds, flu and other ailments. Individuals from the Free State Province displayed major variability between localities both chemically and microbiologically. For example, the essential oils from the individuals FS1, FS2, FS3 had strong antimicrobial activity, but FS3 had the highest activity against both gastrointestinal and respiratory tract pathogens. Interestingly, the compound β myrcene had the highest percentage area (39.6%) in FS3, which was not present in FS2 and FS1. Whereas FS1 and FS2 had the highest percentage areas of α phellandrene (69.4% in FS1 and 57.3% in FS2) than FS3.

4.5 General conclusions

The phytochemical content of the plant correlates with its antimicrobial activity against RTI's and gastrointestinal pathogens studied, particularly the oils collected Free State Province were found to display strong activity against *S. agalactiae* and *C. neoformans* (which may be associated with the presence the monoterpene α-pinene which occurred as the dominant constituent in almost all the 26 individuals). It can further be concluded that *Searsia erosa* is safe to use as the toxicity is low, particularly the form in which it is traditionally used, i.e. aqueous. The traditional use of this plant for tanning of animal hide is corroborated by the presence of high quantities of tannic acid, which suggests that the plant can be a good source of tannins.

4.6 Recommendations for future research

The current study screened *S. erosa* against RTI's and gastrointestinal pathogens in order to scientifically validate its antimicrobial potential, both organic and aqueous extracts, as well essential oils, showed good antimicrobial activity. The plant is also used traditionally for the treatment of cancer, it should therefore be assessed for its anti-cancer properties. This species should also be tested for their pharmacological properties in the treatment of other illnesses such as diabetes and heart problems as species of *Searsia* such as *S. lancea* are traditionally used to treat these ailments (Moteetee and Van Wyk, 2011). Furthermore, the therapeutic potential of the plant against other infections should also be assessed since *S. agalactisae* is a pathogen that causes systemic infections (Ya'qoub et al, 2018). The study also evaluated the plant for the presence of essential oils to corroborate its traditional use for tobacco flavouring. The oil was found to be very variable within and between populations, both seasonally and geographically, this variation should be further explored. Pharmacological activities such as antioxidants, anti-asthma activities, anti-mycobacterium and anti-inflammatory should be studied more on the species as other *Searsia* species such as *S. chiridensis* and *S. undulata* have been reported to have good anti-inflammatory activity (Kabonga-Kayoka, 2016).

4.7 References

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Appendix

Chromatograms of tannic acid (standards)

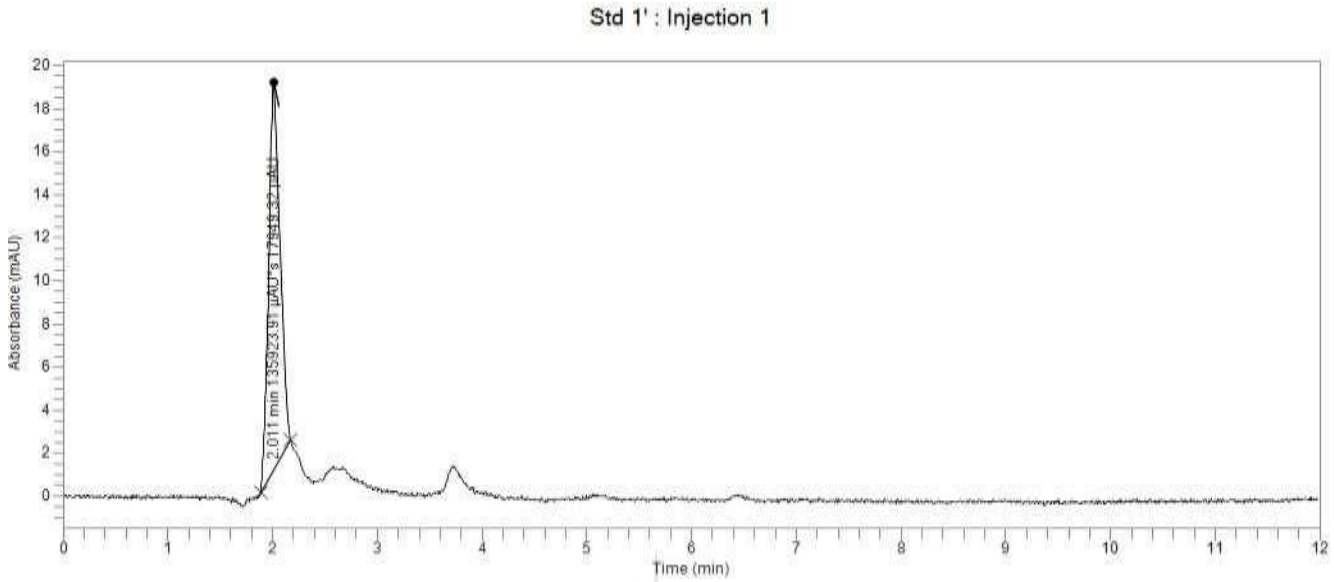

Figure A1: Tannic acid standard chromatogram analysed by RP-HPLC

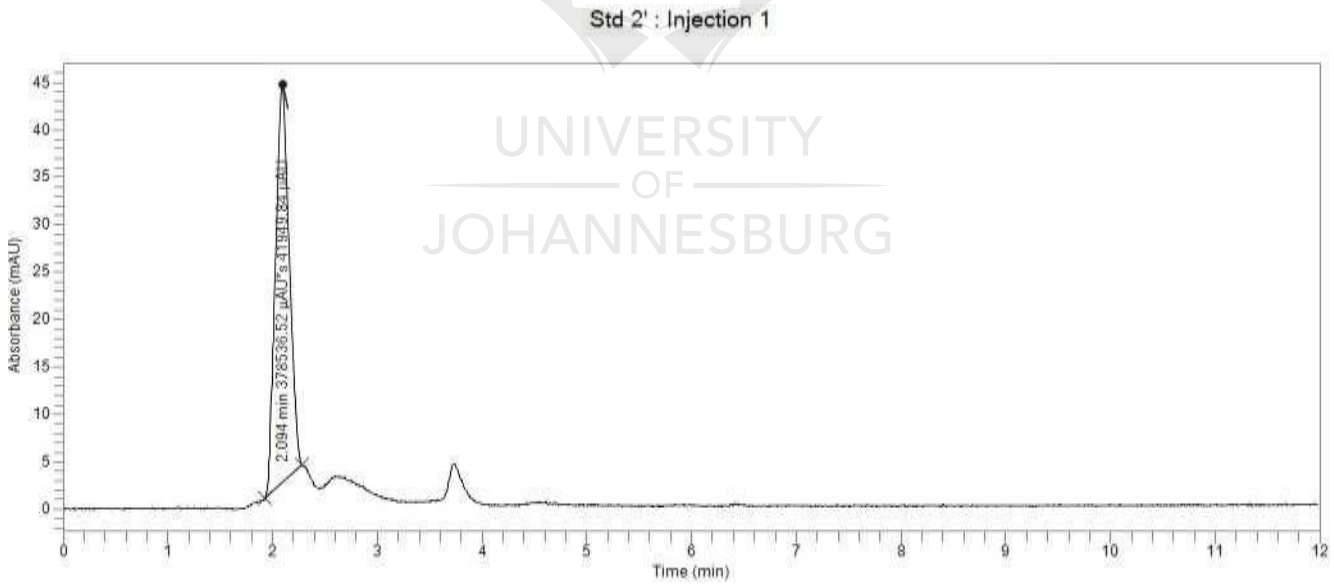

Figure A2: Tannic acid standard chromatogram analysed by RP-HPLC

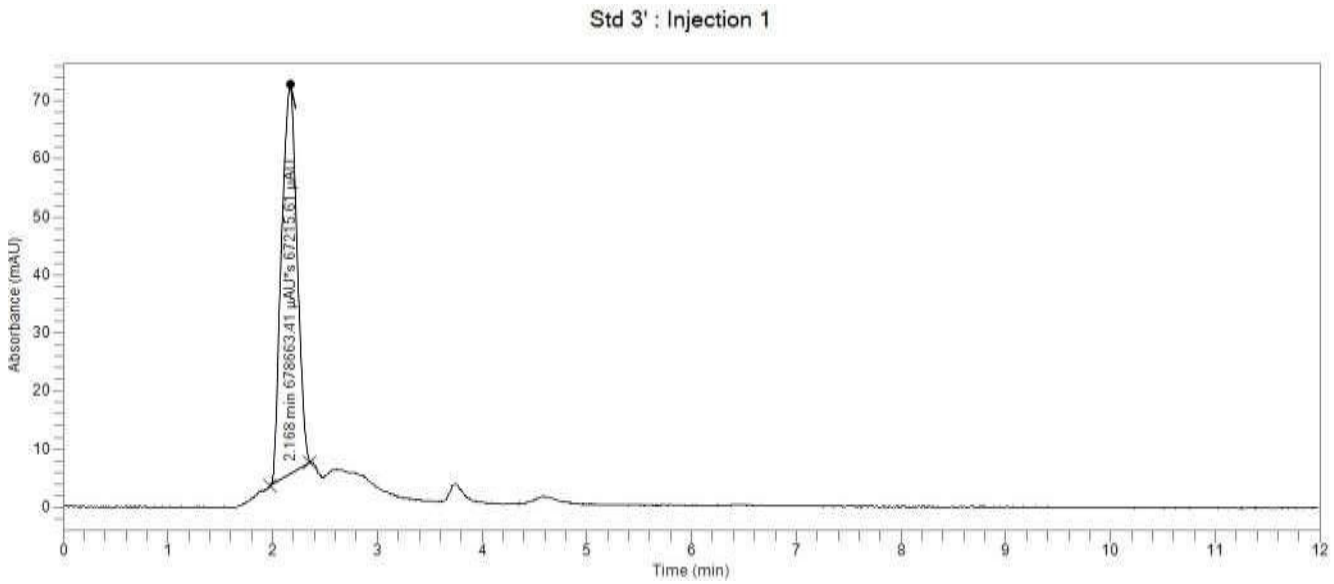

Figure A3: Tannic acid standard chromatogram analysed by RP-HPLC

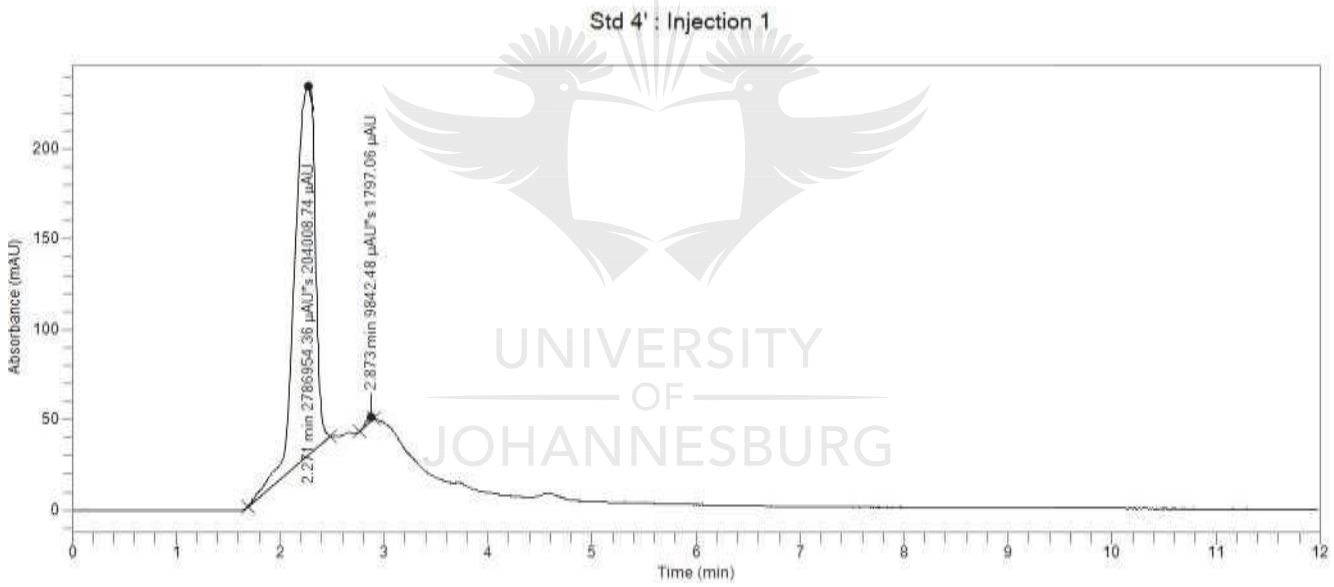

Figure A4: Tannic acid standard chromatogram analysed by RP-HPLC

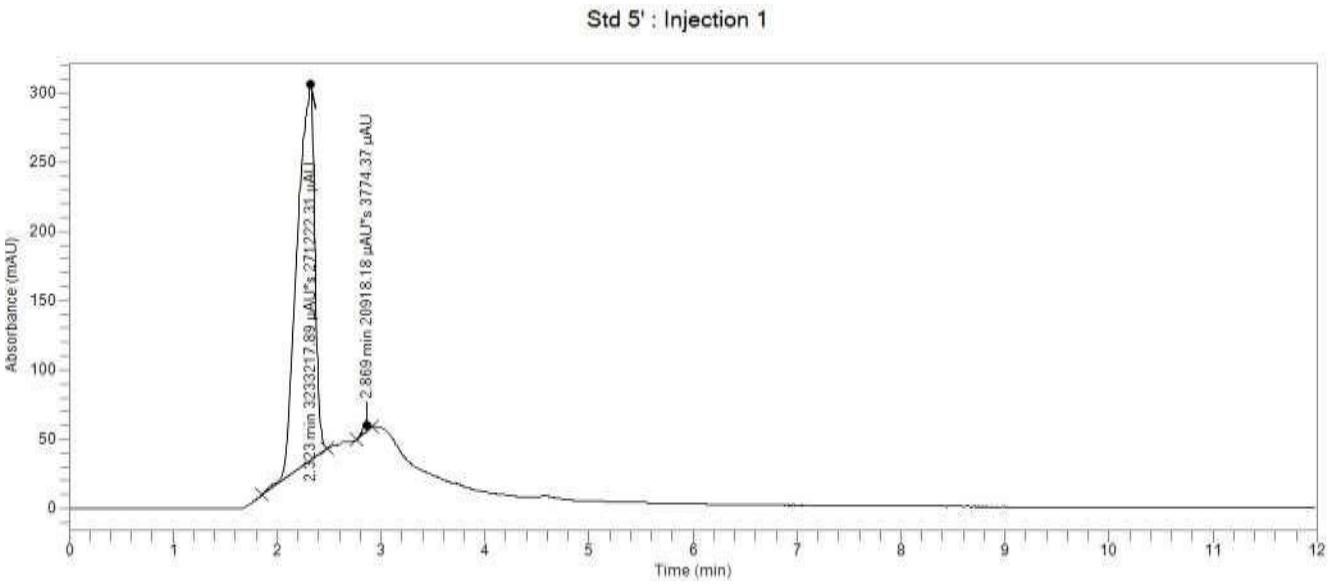

Figure A5: Tannic acid standard chromatogram analysed by RP-HPLC

Chromatograms of GC-MS analysis

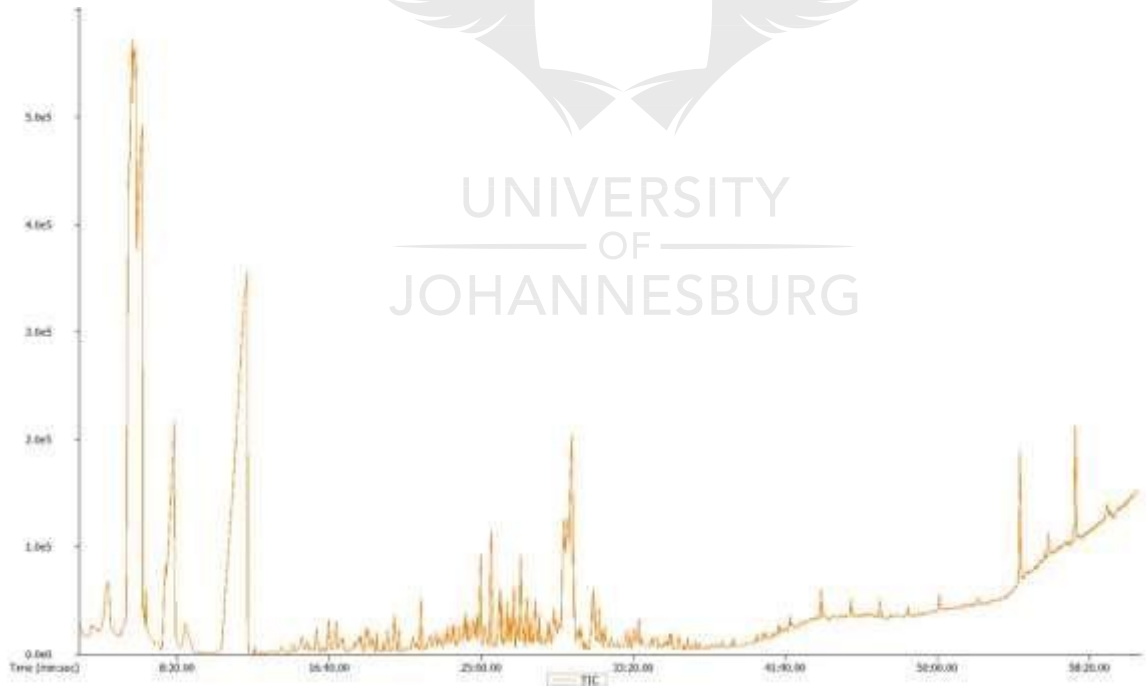

Figure A6: FS1 essential oil chromatogram

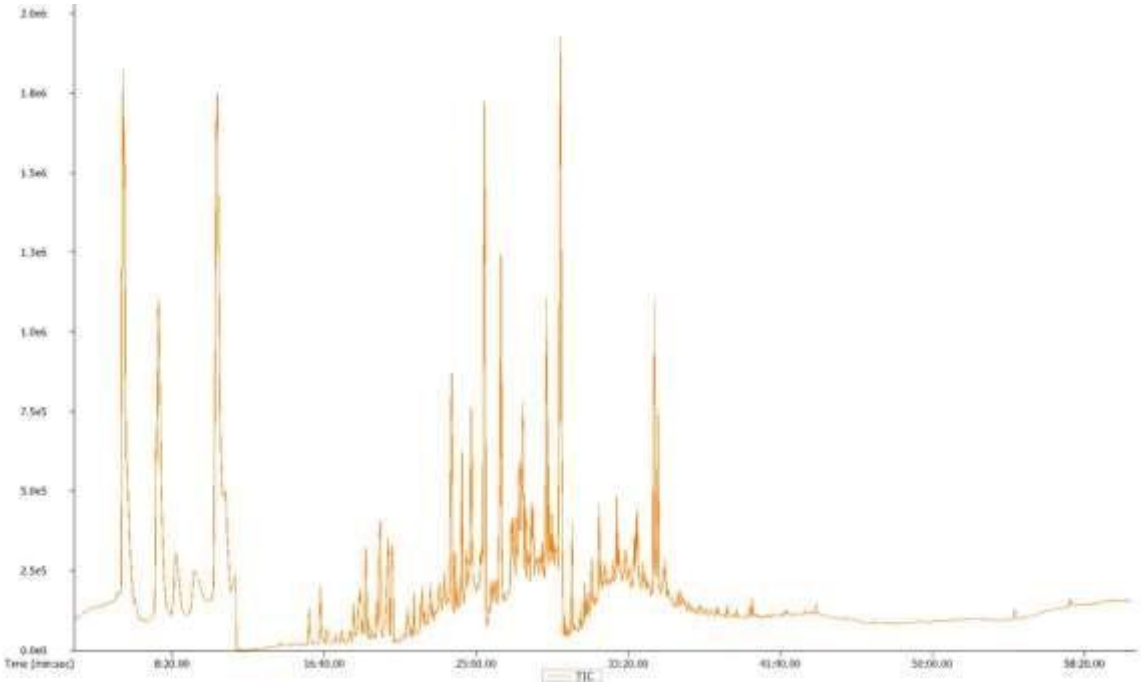

Figure A7: FS2 essential oil chromatogram

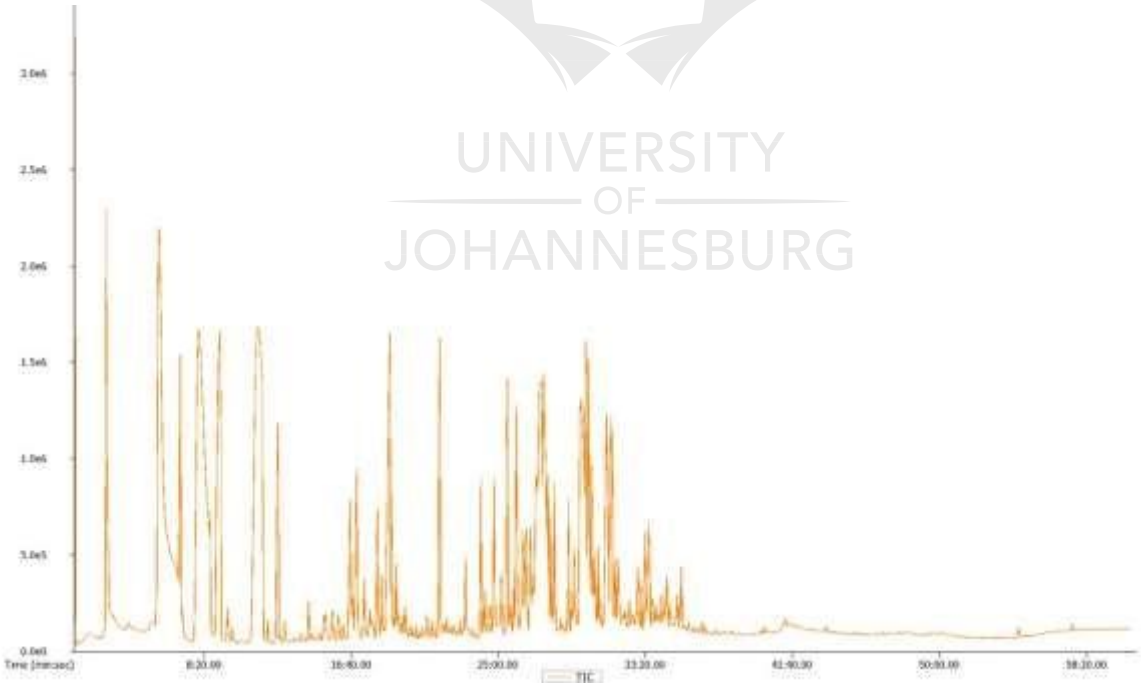

Figure A8: FS3 essential oil chromatogram