PHYTOCHEMICAL INVESTIGATION OF THE AERIAL PARTS OF *KLEINIA LONGIFLORA*

Department of Chemical and Forensic Sciences

Faculty of Sciences

Keabetswe Sewawa

Keabetswe.sewawa@studentmail.biust.ac.bw, +267 77417434

A dissertation submitted to the Faculty of Sciences in partial fulfilment for the award of the Degree of Master of Science in Chemistry of Botswana International University of Science and Technology.

Supervisor:

Dr Tshepo Pheko-Ofitlhile

Department of Chemical and Forensic Sciences

College of Science

phekot@biust.ac.bw, +267 76156455

Signature:  Date: 11/01/2021

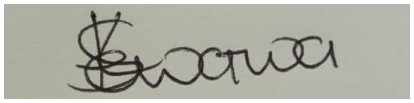
ABSTRACT

Kleinia longiflora belongs to the family Asteraceae and genus *Kleinia* (synonym *Senecio*) which comprises of about 40 species. Preliminary phytochemical investigation done on this plant showed the presence of terpenoids, tannins, alkaloids and flavonoids. The phytochemical investigation on the non-volatile compounds from the plant led to the isolation of a triterpenoid lupeol **1** (0.403 g), a flavonoid named isorhamnetin-5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranose **11** (0.658 g), a steroid stigmasterol **12** (0.248 g).

Hexane extract and *Kleinia longiflora* essential oil analysis was done using GC-MS. 21 compounds were found to be present in the hexane extract and the main constituents were found to be β-eudesmol (80.6%), lupeol (53.1%), ambrosin (48.4%) and (-)-α-panasinsen (43.6%). 20 compounds were identified from *Kleinia longiflora* essential oil and the main constituents of the oil were hexanedioic acid, bis (2-ethylhexyl) ester (71.5 %) which is a diester, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (63.2%) which is a fatty acid glycerol ester, *cis*-carveol (61.0%) which is a monoterpenoid alcohol and octadecanoic acid, 2,3-dihydroxypropyl ester (50.7%) which is a fatty acid ester.

DECLARATION AND COPYRIGHT

I, Keabetswe Sewawa, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other university for a similar or any other degree award.

Signature**:** 

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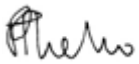
CERTIFICATION

The undersigned certifies that she has read and hereby recommends for acceptance by the Faculty of Sciences a dissertation titled "Phytochemical investigation of aerial parts of *Kleinia longiflora*" in fulfilment of the requirements for the degree of Master of Science in Chemistry of the BIUST.

Signature: 

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CHAPTER 1: INTRODUCTION

1.1 Medicinal Plants

The term medicinal plants refer to a variety of plants that have medicinal properties and are a rich source of phytochemicals that can be used as drugs (Jamshidi-Kia et al., 2018). Medicinal plants can be extracted and processed for direct consumption as herbal medicine or prepared for experimental purposes (Abubakar & Haque, 2020). Different parts of the plant like stems, roots, leaves, fruits and flowers can be used and are usually air dried and cut into smaller pieces or powder to increase the surface area for easy infusion in solvents (Jamshidi-Kia et al., 2018). The phytochemicals in medicinal plants can be used to synthesize useful therapeutic agents, therefore they are of great importance to the health of individuals and communities in general (Yusuf et al., 2014).

The World Health Organization (WHO) stated that between 70-80% of the people in developing countries still depend on medicinal plants to meet their primary health care needs. The plants are a source of income which positively impacts on the livelihood of communities while some just sell the herbs to treat ailments or for use as supplements (Maroyi & Mosina, 2014).The phytochemicals found in medicinal plants include alkaloids, tannins, essential oils, phenolics and flavonoids. The abundance of scientific evidence indicates that phytochemicals have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelets aggregation, modulation of hormone metabolism and anticancer properties (Gujjeti & Mamidala, 2013; Kumar et al., 2015; Nyamai et al., 2016). Due to these properties traditional healers belief that some medicinal plants are more effective in treating infectious diseases than synthetic antibiotics (Das et al., 2010). This could be due to the

fact that medicinal plants are used as a whole which gives a synergic effect compared to when the single pure bio-active component is used, therefore the crude plant extract often has greater *in vitro* or /and *in vivo* activity than the isolated constituents at an equivalent dose (Rasoanaivo et al., 2011). Pure synthetic drugs produced industrially or isolated from plants may be chosen for their high activity against human diseases, but they have disadvantages (Rasoanaivo et al., 2011) like side effects and drug resistance which can lead to death. Approximately 8% of hospital admissions in the United States of America are due to different side effects of synthetic drugs. About 100 000 people die each year due to these toxicities (Abubakar & Haque, 2020; Arnold, 2013; Karimi et al., 2015).

Antibiotic resistance has become a serious and widespread problem in the world. It results in reduced efficacy of anti-bacterial drugs making the treatment of patients difficult, expensive or even impossible (Wikaningtyas & Sukandar, 2016). The effect on vulnerable patients results in prolonged illness and they can end up losing their lives. One strategy to avoid this is by using alternative therapeutic agents from plants that are effective against antibiotic resistant bacteria. (Raghunath, 2008; Wikaningtyas & Sukandar, 2016).

1.2 Preliminary phytochemical screening of medicinal plants

Preliminary screening of phytochemicals is a valuable step in the detection of the bioactive compounds present in medicinal plants, it may subsequently lead to drug discovery and development (Visweswari et al., 2013). Different qualitative tests are performed to establish the profile of given extracts and to detect different phytochemicals present in them like tannins, flavonoids, terpenoids, saponins, steroids, carbohydrates, glycosides, coumarins, alkaloids, proteins, emodins, anthraquinones, anthocyanins and leucoanthocyanins. The summary of the

methods used as outlined by (Visweswari et al., 2013) are in Table 1. This screening is imperative in order to discover and develop new therapeutic agents with improved efficacy (Yadav et al., 2014). Preliminary phytochemical screening helps to determine whether the plant is worth studying or not. If there are few phytochemicals present, or the phytochemicals of interest to the researcher are not shown by preliminary tests, then there will be no point to continue with the study of the plant.

Table 1: Standard Procedures for Qualitative Detection of Phytochemical constituents

Phytochemical	**Test/ Reagent used**	**Description**	**Expected colour change**
Alkaloids	Mayer's reagent	Filtrates are treated with the reagent	Yellow coloured precipitate
	Wagner's reagent	Filtrates are treated with the reagent	Brown/reddish precipitate
Flavonoids	Sodium hydroxide and hydrochloric acid	Small amount of extract treated with NaOH and HCl	Yellow-orange colour
	Concentrated sulphuric acids	A fraction of the extract treated with concentrated sulphuric acid	Orange colour
Terpenoids	Liebermann-Burchard test	4 mg extract treated with 0.5 ml of acetic anhydride and 0.5 ml acetic acid. Then concentrated sulphuric acid added slowly.	Blue-green colour for terpenoids Reddish-brown colour for steroids
Saponins	Foam test	About 2 g of extract mixed with 10 ml distilled water and shaken vigorously.	Appearance of froth
Tannins	Ferric chloride test	0.5 g of the dried powdered sample boiled in 20 ml of water in a test tube and filtered.	Brownish green-black or blue-black
	Lead acetate test	3 ml of plant extract is combined with 2 ml of distilled water. 0.01 g lead acetate added and shaken well	Development of white turbidity and precipitate
Phenols	Ferric chloride test	2 ml plant extract taken to water and warmed at 45-50 ℃. Then 2 ml of 0.3% ferric chloride added.	Green or blue colour
Glycosides	Fehling's test	Fehlings' solution A and B diluted with distilled water and boiled for 1 minute. To this 8 drops of plant extracts added and then mixed with 1 ml of Fehlings' solution and boiled in water bath for 5 minutes.	Brick-red precipitate

1.3 Essential oils

The term essential oil dates back to the 16th century and it is derived from the drug *Quinta essentia* named by Paracelsus von Hohenheim of Switzerland (Dhifi et al., 2016). Essential oils are concentrated and complex substances which have the form of oily drops present in the organs of the aromatic plant like flowers (jasmine), leaves (sage), fruits (orange), seeds (fennel), bark (cinnamon) and roots (angelica) (Mejri et al., 2018). They are a complex mixture of several chemical compounds such as terpenes, terpenoids and phenylpropenes and can be produced by more than 17 000 aromatic plant species commonly belonging to angiosperm families such as Lamiaceae, Zingiberaceae and Asteraceae (Mejri et al., 2018). Essential oils have been used to prevent and treat various diseases because they exhibit a wide range of bioactivities especially antimicrobial activity. Each plant has unique essential oil composition due to the fact that they have to protect themselves from particular predators (Blowman et al., 2018).

Essential oils are broadly categorized into oxygenated compounds and hydrocarbons based on their chemical composition. Oxygenated compounds include esters, aldehydes, ketones, alcohols, phenols and oxides. Other active groups include aromatic and sulphur containing components. Terpenes are composed of varying isoprene units (C5), monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20). Monoterpenes contribute 90% of essential oil overall constituents. Both monoterpenes and sesquiterpenes offer a large variety of structures through adjoining with other functional groups and addition of oxygenated groups (monoterpenoids and sesquiterpenoids) (Blowman et al., 2018). Terpenes can be acyclic, monocyclic or bicyclic and may contain an aromatic group. The chemical variations possible depends on the length of the isoprene chain (Blowman et al., 2018).

1.4 Justification of the study

Kleinia longiflora is a commonly used medicinal plant in Southern Africa but the most well-known use in Botswana is for ritual purposes and the plant have not been studied in Botswana. This study intends to explore the *Kleinia longiflora* growing in Botswana at Lecheng village and study its chemical composition in order to figure out if it can be a potential medicinal plant.

1.5 Aims and objectives of the study

1.5.1 Aim

The aim of the study was to extract and identify the phytochemicals present in *Kleinia longiflora.*

1.5.2 Objectives of the study

The study objectives are to:

- To establish the chemical profiles and structures of the constituents of the aerial parts extract of *Kleinia longiflora*
- To establish the identities of the chemical constituents of the aerial parts of *Kleinia longiflora*
- Compare GC/MS results of the hexane extract with that of the essential oil

CHAPTER 2: LITERATURE REVIEW

2.1 The genus *Kleinia* (synonym S*enecio*)

2.1.1 *Kleinia* botanical descriptions and distribution

The genus *Kleinia*, with some 40 species in the Canary Islands, North Africa, Tropical and Southern Africa, Madagascar, Arabia, India and Sri Lanka, has a strong concentration of species in the Horn of Africa from where about 20 species are known (Thulin, 2014). *Kleinia* genus was first proposed by Linnaeus and included 4 species which he later re-classified under *Cacalia L* but most modern workers now accept the genus *Kleinia* as including *Notonia* (Halliday, 1986). *Kleinia* belongs to the Gunuroid group of subtribe senecioninae, which include *Kleinia*, *Gynura* and *Solanecio*. It is characterized by the presence of prominent drusiform crystals in the ovary wall, always *discoid capitula* and mostly a sub-succulent to succulent habit (Vanijajiva et al., 2014).

2.1.2 Traditional uses of *Kleinia*

The genus have been used in traditional medicine for the treatment of wounds, asthma, coughs, bronchitis, stomach-ache, blood purifiers for skin eruptions and burns (Joshi et al., 2019). *Kleinia pendula* has been used by the Saudi and Ethiopian traditional healers to treat inflammation of the ear (otitis) and swollen body parts respectively(Alfaifi et al., 2020). *Kleinia grandiflora* has been used to treat ear aches and skin infections, studies have shown it to possess anti-microbial and anti-inflammatory activity (Pendyala et al., 2018). *Kleinia squarrosa* is known to have repellent activity

against mosquitoes, they use the smoke obtained from burning *K. squarrosa* as a fumigant to kill mosquitoes (Guta et al., 2018).

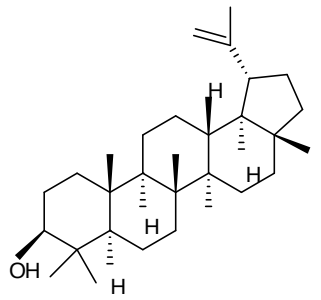
2.1.3 Reported *Kleinia* phytochemicals

2.1.3.1 Terpenoids reported from *Kleinia*

From the South African *Kleinia* group, several species have been investigated and triterpenes mainly lupine derivatives seem to be widespread in this group as shown in Table 2. The Table shows different classes of terpenes which include germacranes, oplopanes,triterpenes and acyl pyrroles found in *Kleinia* groups. These groups are rich in terpenes since they all have triterpenes which is a clear indication that triterpenes are widespread in *Kleinia* (Bohlmann et al., 1981). Other groups of the succulent species in addition to triterpenes posess germacranes while oplopane and acyl pyrroles was present in only group one and group two respectively (Bohlmann et al., 1981).

Abrotanifolone derivatives were reported in *Kleinia tomentosa* and two new acyl pyrrole derivatives were identified in *Kleinia kleinioides* while three triterpenoids namely lupeol **1**, lupenone **2** and lupeol acetate **3** were isolated from *Kleinia odora* (Al-Taweel et al., 2004). Previous chemical investigation of *Kleinia odora* growing in Saudi Arabia resulted in the isolation and characterization of lupeol **1**, lupenone **2**, epilupeol, lupeol acetate **3**, ursolic acid, β-11-α-dihydroxy- urs-12-ene, brein and ursolic acid lactone (Al Musayeib et al., 2013). *Kleinia articulata* was reported to have lupenone **2**, lupeol **1**, lupeol acetate **3**, 28-hydroxylupeol, α-zingibenene **4**, α-curcumene and bicyclogermarene while *Kleinia mandraliscae* composed of germacrene D, lupenone **2** and lupeol acetate **3** (Bohlmann et al., 1981)**.** Re-investigation of the ethanol extract of the aerial parts of *Kleinia odora* led to the isolation of two new triterpenes

klodorone A and klodorol A together with two known compounds β-amyrin and germanicol (Al Musayeib et al., 2014). The reported terpenoids examples are shown in Figure 1.



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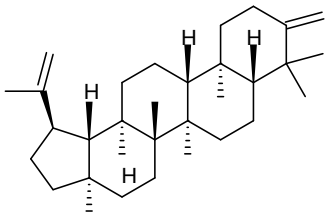

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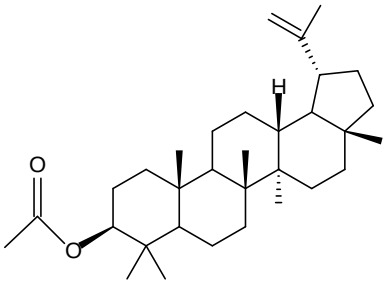

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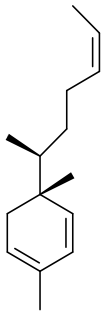

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Figure 1: Terpenoids reported from the genus *Kleinia*

Table 2: Distribution of the main terpene types in the proposed *Kleinia* groups (Bohlmann et al., 1981)

Groups	**Germacranes**	**Oplopanes**	**Triterpenes**	**Acylpyrroles**
Group 1				
K. tormentosa	-	+	+	
Group 2				
K. acaulis	+	-	+	
K. archeri	+	-	+	
K. articulates	+	-	+	
K. crassfolius	+	-	+	
K. cylindricus	+	-	+	
K. ficoides	+	-	+	
K. mandraliscae	+	-	+	
K. serpens	+		+	
Group 3				
K. barbertonicus	-	-	+	+
K. phonolishicus	+	-	+	
K. rimincilis	-	-	+	
Group 4				
K. anteuphorbium	-	-	+	
J. coccineiflorus	-	-	+	
K. coccinea	-	-	+	
K. fulgens	+	-	+	
K. kleinioides	-	-	+	+
K. longiflora	-	-	+	+
K. neriifola	-	-	+	
K. perraeus	+	-	+	

Key; + presence of the terpene type, - absence of the terpene type

2.1.3.2 Flavonoids from *Kleinia*

Flavonoids were surveyed in leaves of 44 clones from 25 *Kleinia* species, mainly those belonging to the succulent *Senecio radicans* complex. The common flavonoids identified were 3-glucosides and 3-rutinosides of kaempferol, quercetin **5** and apigenin-7-glucoside. Rarer constituents present were 3′-methylquercetin **6**, glycosylflavone mangiferin and isomangiferin **7** (Gleenie et al., 1971).

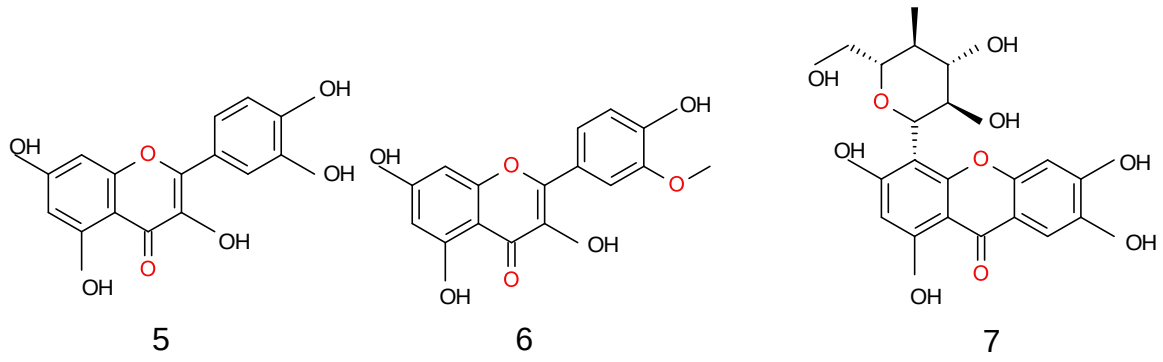

Figure 2: Flavonoids reported from the genus *Kleinia*

2.1.3.3 Alkaloids from *Kleinia*

A phytochemical investigation of *Senecio glabellus* resulted in the isolation and identification of five pyrrolizidine alkaloids florosenine (acetylotosenine), senecionine **8**, integerrimine, otosenine and senkirkine (Kapadia et al., 2008). The alkaloid content of *Senecio madagascariensis* collected from Australia and Hawaii was examined and the alkaloids identified from the aerial parts by GC-MS analysis included senecivernine, otosenine, acetylsenkirkine, descicetyldoronine, florosenine and doronine (Gardener et al., 2006) The

distribution of toxic pyrrolizidine alkaloids in the Icelandic flora constitutes senecionine **8**, seneciphylline **9,** retrorsine**,** riddelliine **10,** senecionine oxide and seneciphylline oxide isolated from *Senecio vulgaris* L. The presence of riddelliine **10** was confirmed by high resolution NMR Spectroscopy while senecionine oxide and seneciphylline oxide were isolated by preparative centrifugal chromatography (Gardener et al., 2006). Examples of some of the reported alkaloids are shown in Figure 3.

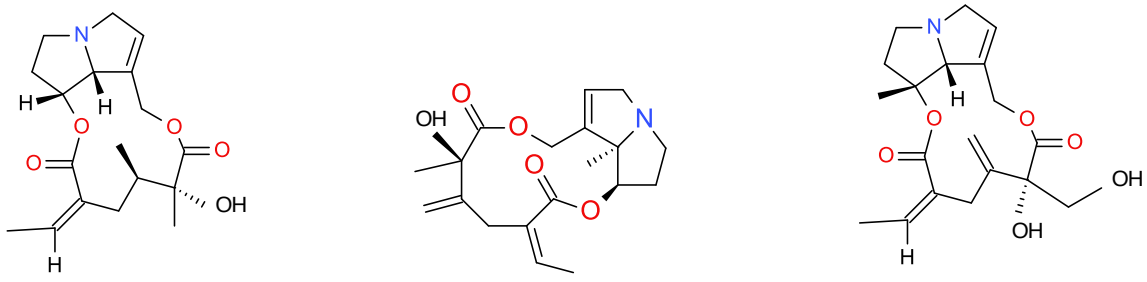

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Figure 3: Flavonoids reported from the genus *Kleinia*

2.1.3.4 Essential oil composition and biological activity of some *Kleinia* (syn *Senecio*) species

The composition of essential oil obtained from *Kleinia pendula* grown in Somalia was found to consist of myrcene, *alpha* –humulene, *beta*-elemene, T-cardinol and 4-α(H)-eudesm-5-α-ol which are the main constituents (Al-Taweel et al., 2004). It showed analgesic, cytotoxic and anti-inflammatory activities (Alfaifi et al., 2020). *Kleinia odora* GC-MS analysis resulted in the identification of (+)-epi-bicyclosesquiphellandrene, caryophyllene and α-pinene as the main components, the oil showed a moderate antimicrobial activity against *E-coli* and

antifungal activity against *Candida albicans* (Al-Taweel et al., 2004). *Kleinia squarrosa* oil gas chromatogram resulted in the identification of (E)-iso-γ-bisabolene, α-pinene, caryophyllene and sabinene as the main constituents. The presence of the monoterpenoids and sesquiterpenoids in essential oils is mostly associated with mosquito repellent activity (Guta et al., 2018)

The oil from *Kleinia grandiflora* showed a moderate antimicrobial activity, against *Escherichia coli* with a zone of inhibition (ZI) of 9 mm while the control tetracycline had a ZI of 19 mm and anti-fungal activity against *Candida albicans* with a ZI of 9 mm and the control amphotericin was at 20 mm (Al-Taweel et al., 2004). *Kleinia grandiflora* leaf extract was also found to be active against *Escherichia coli*, *Staphylococcus epidermidis* and *Bacillus pumilus* with ZI at 200 mg/ml of 0.32, 0.34 and 0.28 cm respectively. (Pendyala et al., 2018). *Senecio nudicaulis* essential oil was tested for antioxidant activity using DPPH scavenging assay. The IC_{50} values of *Senecio nudicaulis* essential oil and ascorbic acid were 10.61 ± 0.14 μgmL^{-1} and 12.08 ± 0.42 μgmL^{-1} respectively and the ABTS radical scavenging activity of the oil was found to be 11.85 ± 0.28 μgmL^{-1} while that of ascorbic acid was 13.51 ± 0.16 μgmL^{-1}. In the nitric oxide scavenging assay IC_{50} was found to be 11.29 ± 0.42 μgmL^{-1} for the essential oil and 12.26 ± 0.78 μgmL^{-1} for ascorbic acid. This reflects the ability of the *Senecio nudicaulis* oil to donate electrons or hydrogen atoms to inactive free radial or cation (Sharma & Shah, 2015). The flower and leaf volatile oils of *Senecio aegyptius* showed significant level of anti-

fungal activity against *Candida albicans* and moderate effect against Gram positive bacteria, however, it has weak activity against Gram negative bacteria (El-Shazly et al., 2002).

2.2 *Kleinia longiflora* species (synonym *Senecio longiflorus*)

2.2.1 Botanical description of *Kleinia longiflora*

Kleinia longiflora (synonym *Senecio longiflorus)* belong to the genus *Kleinia* (synonym *Senecio*) and family Asteraceae or Compositae. The plant photograph is shown in Figure 4. It is a perennial much branched shrub and has very succulent blue- green stems, it can grow up to a height of 30-70 cm in an erect upright manner. It grows in dry areas among rocks or under bushes and on rocky ridges. It is found predominantly in South West Africa/ Namibia, Botswana, the northern, north-eastern, western and central tropical Africa and Madagascar. In South Africa it can be found in the Eastern Cape, Free State, Gauteng, Limpopo, Mpumalanga, Northern Cape, North West and Western Cape (Oliver & Hiern, 1877).

Figure 4: Photograph of *Kleinia longiflora* plant

2.2.2 Ethnomedicinal use of *Kleinia longiflora*

Kleinia longiflora is a commonly used medicinal plant by traditional healers in southern Africa. In many tribes in Botswana, when people return from the burial, they wash hands with water mixed with *Kleinia longiflora* which is believed that through such washing, mourners are washing death from their midst and to let go of the loved one who died (Larson, 1986). The decoction of the plant is used to induce vomiting (Damme et al., 1922)

In case of poisoning, Vhavenda in the Vhende district of Limpopo province chew the green and fresh branches of *Kleinia longiflora* and swallow the juice to act as an emetic (Magwede et al., 2019). The Vhavenda also belief that chewing the soft branches may induce love in women whom a man may meet, the stems can also be used as an ingredient of snuff (Mabogo, 1990).

Bapedi traditional healers use *Kleinia longiflora* in the treatment of female reproductive ailments in the Limpopo area, mainly female infertility (Semenya et al., 2013). *Kleinia longiflora* and other herbs are also used to a lesser extent in initiating abortion, and to manage breast cancer, menstrual disorders, period pains, vaginal candida and womb problems (Potroz & Cho, 2015). In the Sekhukhune district and Peri-urban domestic gardens of Limpopo province, traditional healers use the root of *Kleinia longiflora* to treat bacterial STIs specifically Chlamydia (Maroyi & Mosina, 2014; Potroz & Cho, 2015; Semenya et al., 2013).

Kleinia longiflora is mixed with fresh bulb of *Drimia sanguinea* and dried whole plant of *Enicostemma axillare* by the traditional health practitioners in the Limpopo Province to treat pneumonia, tuberculosis and its opportunistic infections, they are boiled for three minutes and the steam is inhaled nasally under the blankets three times a day (Maroyi & Mosina, 2014; Semenya & Maroyi, 2019).The stem infusion is also used by traditional healers in the Municipality of Waterberg district in the Limpopo province, the Topnaar of the Kuiseb in the Namib dessert and the in Northern India to treat ear ache (ear drops), sore eyes (wash the eye), tooth ache and headache (drunk) (Damme et al., 1922; Gupta et al., 2015; Potgieter & Maema, 2016). The Batswana traditional healers in the Ngaka Modiri Malema district Municipality use it to treat skin diseases (Asong et al., 2019). According to Cheikhyoussef et al., 2011, *Kleinia longiflora* is used in the treatment of mental illness, colloid and for fertility in the Oshiko region in Namibia.

2.2.3 Phytochemicals discovered from *Kleinia longiflora*

Kleinia longiflora have been reported to consist of two pyrrolizidine alkaloids being senecionine **8** and seneciphylline **9** (Figure 3). Pyrrolizidine alkaloids cause serious diseases in domestic animals and humans (Hartmann & Witte, 1995). *Kleinia longiflora* also has quercetin **5** (Figure 2), which belongs to a group of plant pigments called flavonoids that give many fruits, flowers and vegetables their colour. Flavonoids such as quercetin **5** are antioxidants, they scavenge damaging particles in the body known as free radicals that damage cell membranes, tamper DNA and even cause cell death. It acts as an antihistamine and an anti-inflammatory and may help protect against heart disease and cancer (Bohm & Stuessy, 2001). *Kleinia longiflora* was also reported to be rich in triterpenes and acylpyrroles (Bohlmann et al., 1981).

2.2.4 Biological activity of *Kleinia longiflora*

Asong et al., 2019 conducted a study on the antimicrobial activity of *Kleinia longiflora* and showed MIC values of 0.9 mg/ml (*Bacillus cereus*), 56.2 mg/ml (*Shigella flexneri*), 1.8mg/ml (*Candida glabrata*), 1.8 mg/ml (*Trichophyton rubrum*) and 1.8 mg/ml (*Trichophyton tonsurans*).

Trichophyton tonsurans is an occasional cause of scalp ringworm in adults and *tinea capitis* in children. It was found to have become the predominant cause of *tinea capitis* in Birmingham, UK, accounting for 72% of infections investigated in 1993 (Hay, 2017). *Trichophyton rubrum* is a dermatophytic fungus that parasitizes keratinised tissues (stratum, corneum and nails) of humans, the organism produces exocellular proteinases to utilise host proteins as a nutrient source, the fungi cause a variety of skin infections including athlete's foot (*Tinea pedis*), jock itch *(Tinea cruris*) and ringworm (*Tinea capitis*) (Apodaca & McKerrow, 1989; Martinez et al., 2012).The observed antimicrobial activity provided the scientific rationale for the ethnomedicinal use of *Kleinia longiflora* for treatment of skin diseases.

CHAPTER 3: MATERIALS AND METHODS

3.1 Collection of the plant material

Kleinia longiflora plant was collected from Lecheng village, Botswana in the month of May 2015. The stems were cut into small pieces, dried under shade and crushed into powder. Dr Mbaki Muzila, senior lecturer at University of Botswana and Herbarium Curator for University College Botswana Gaborone (UCBG), did the verification of the plant.

3.2 Preliminary phytochemical screening of the extracts

In preparation for preliminary phytochemical screening, the dried plant powder was extracted with hexane, methanol, ethanol, dichloromethane and acetone to make 5 crude extracts.1 g of *Kleinia longiflora* powder was blended with 50 ml of each solvent with agitation at room temperature for 24 hours. The extracts were concentrated using a rota vapour at 37°C under reduced pressure. The extracts were then weighed, stored at -20°C and percentage yields were calculated.

3.2.1 Test for Flavonoids

Ammonia solution (5 ml) was diluted with 0.5g of the aqueous filtrate of each plant extract followed by addition of concentrated sulphuric acid. A yellow coloration observed in each

extract indicated the presence of flavonoids. The yellow coloration would disappear on standing (Edeoga et al., 2005).

3.2.2 Test for steroids

Acetic anhydride (2 ml) was added to 0.5 g ethanolic extract then 2ml sulphuric acid was added. The colour change from violet to blue/green indicated the presence of steroids (Edeoga et al., 2005).

3.2.3 Test for terpenoids

Each solvent extract (5 ml) was mixed with 2 ml of chloroform and concentrated sulphuric acid (3 ml) was added to form a layer. A reddish- brown coloration of the interface was formed to show positive results for the presence of terpenoids **(**Edeoga et al., 2005).

3.2.4 Saponin test

The five solvent extracts were diluted with distilled water and made up to 20 ml. The suspensions were shaken in a graduated cylinder for 15 minutes. A thick 2 cm layer of foam indicated the presence of saponins (Gujjeti & Mamidala, 2013).

3.2.5 Test for phenolic compounds

The solvent extracts were diluted to 5 ml with distilled water. To this a few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated presence of phenolic compounds (Gujjeti & Mamidala, 2013)

3.2.6 Test for tannins

Plant extract solution (3 ml) was taken in a test tube and diluted with chloroform (2 ml) and acetic anhydride (1 ml). Finally, sulphuric acid (1 ml) was added carefully to the solution. A green colour was formed which showed the presence of tannins (Hossain et al., 2013).

3.2.7 Test for alkaloids

To 1 ml of each solvent extract, 2 ml of Wagner's reagent was added. A reddish-brown colour precipitate indicated the presence of alkaloids (Yusuf et al., 2014)**.**

3.3 Chromatographic Separation Methods

Column chromatography was used to separate the crude into simpler fractions. This was done using different sized columns packed with Merck silica gel, 60-80 mesh and particle size of 0.0400-0.0630 mm. The column eluents were monitored using thin layer chromatography pre-

coated Merck silica gel $60HF_{254}$ aluminium sheets. All the solvents used were analytical reagent (AR) grade. Sephadex LH-20 was used to do gel filtration in order to separate compounds with similar polarities but with different molecular masses.

Preparative TLC plates were prepared using 0.5 mm thick layer Merk silica gel $60HF_{254+366}$ coated on a 20x20 cm glass plates**.** Compounds separated using thin layer chromatography and preparative thin layer chromatography were observed under UV lamp of wavelength 254 and 366 nm.

3.4 Physical and Spectroscopic Measurements

3.4.1 UV Analysis

UV analysis was done using Shimadzu (UV 2101 PC) UV-VIS Spectrometer. The extracts were first dissolved in methanol and then the UV spectra recorded. Freshly prepared shift reagents were then used which were the following; Aluminium chloride (5 g of fresh anhydrous $AlCl_3$ was dissolved in 100 ml of dry methanol), Hydrochloric acid (HCl) (50 ml of concentrated HCl in 100 ml water) and Sodium acetate (NaOAc) (Anhydrous powdered analytical reagent was used)

3.4.2 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance **(**NMR) spectra were run using Bruker Avance DPX operating at frequency of 300 MHz. The solvents used were chloroform-d (δ_H 7.27, δc 77.2), acetone-d (δ_H 2.05, δc 29.9), methanol-d (δ_H 3.31, 4.81, δc 49.1)

3.4.3 Gas Chromatography-Mass Spectrometry (GC-MS)

In order to determine the phytochemical profile of the plant, GC-MS spectroscopy was done as detailed by Asong et al., 2019 with a few alterations. GC: 7890A, manufactured by Agilent Technologies was used, auto injector-GC ALS. Capillary column of fused silica (30 m × 250 µm × 0.25 µm) coated with 5% Phenylmethylsilox (HP-5MS). Helium ultra-pure was used as carrier gas at a flow rate of 1 ml and a speed of 37 cm/s. For the analysis, the dilute sample (1µL) was injected into the column with an inlet temperature of 250 ^{O}C and a splitless mode of 30 s. The initial temperature of the oven was 55 ^{O}C with a steady (at the rate of 10 ^{O}C per minute with a holding time of 3 minutes at each increment) increment until 280 ^{O}C. The total run time was 46.33 minutes and the injection mode were split less. MS detection was done using Mass Spec: 5975C, manufactured by Agilent Technologies. The scan of *m/z* was from 50-500 and the start-stop masses were 50-500. For the identification of the compounds,

reference was made to the library in the National Institute of Standards and Technology (NIST). Compounds with match factor of above 700 were considered acceptable.

3.5 Extraction of non-volatile compounds from *Kleinia longiflora*

The powdered stem of *Kleinia longiflora* (1,292 g) was extracted exhaustively using 70% ethanol. The solvent was then evaporated using a rotary evaporator. The total mass of the crude extract got was 164.07 g.

The extract was dried, adsorbed in coarse silica gel and then subjected to column chromatography in a column packed with silica gel. Hexane was used to pack the column. Column elution was carried out using gradient elution; hexane, hexane/Ethyl acetate, Ethyl acetate, Ethyl acetate/ Methanol and finally methanol. This was done by doing a 5 % increment of polarity. TLC was carried out and then the similar fractions combined which gave a total of eight major fractions.

Fraction 1 (79.24 g) was subjected to column chromatography using gradient elution (hexane, Ethyl acetate and methanol) and got fractions 1a (0.021 g), 1b (0.017 g) and1c (0.0264 g). Fraction 1a which was the hexane extract was submitted for GC-MS, while TLC was carried out on fractions 1b and 1c and found to be similar and were combined to form fraction 1bc (0.038 g). It was then subjected to preparative TLC (Toluene: Ethyl acetate: Acetic acid 44:5:1) before taken for 1H NMR and their proton spectra showed that it was a mixture of terpenoids.

Fraction 2 (19,09 g) was eluted with Hexane: ethyl acetate (9:1) in a small column and got fractions 2a (0.0036 g), 2b (0.0015g), 2c (0.0053 g). The fractions were not clean and in small quantities, so no further experiments were done on them, but the ^{1}H NMR spectra revealed that they were a mixture of terpenoids.

Fraction 3 (0.04 g) was subjected to preparative TLC (Hexane: Ethyl acetate 9:1) and got fractions 3a, 3b and 3c. Fractions 3a and 3b were combined to form fraction 3ab (0.0044 g). This fraction was also in small quantities and not very clean therefore did not undergo further analysis. The ^{1}H NMR spectra revealed that it was a mixture of terpenoids.

Fractions 4 (0.101 g), 5 (0.403 g) and 6 (0.025 g), formed some crystals. The crystals were harvested and subjected to analysis by TLC. TLC was done for the three fractions. fraction 6 was found to be pure and further analysis was done on it to determine the structure of the compound. ^{1}H and ^{13}C NMR analysis were done and compared with literature and no further analysis was performed and was named compound **12.** TLC for fraction 5 revealed that the fraction was pure and GC-MS was done to characterise the fraction, it was named compound **1**. Fraction 4, was impure and too small to be purified for further analysis but the ^{1}H NMR revealed it to be a Lupeol derivative.

Fraction 7 (32.43 g) was subjected to column chromatography using Acetone: methanol 8:2 from which fraction 7a was obtained. This extract was then run through Sephadex and got

fractions 7aa, 7ab and 7ac. These fractions were too small for further analysis, but the ^{1}H NMR showed that they were also a mixture of terpenoids.

Fraction 8 (32.7 g) was subjected to a small silica column and total number of fractions got were seventeen. On TLC fractions first five fractions showed no spots while the remaining twelve fractions showed one yellow spot and they were combined and named fraction 8A. Preparative TLC was done on fraction 8A and only one band appeared on the plate weighing 0.248 g. The fraction was taken for further analysis and different experiments like ^{13}C NMR, HMBC, COSY, and HMQC were done to determine the structure and finally UV analysis using shift reagents was done to confirm the positions of the other groups This was named compound **11**.

3.6 Extraction of *Kleinia longiflora* essential oil

The dried powdered stem and leaves of *Kleinia longiflora* (50 g) was subjected to hydro distillation (with 1000 ml distilled water) for three hours using a Clevenger-type apparatus. The oil was collected and dried over anhydrous sodium sulphate. The mass of the oil obtained was 0.048 g and was kept in a brown glass vial at 40 ^{O}C for further analysis using GC-MS.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Preliminary phytochemical screening of *Kleinia longiflora*

Preliminary phytochemical screening was the first step as it helps to figure out if the plant is worth studying. This was done by extracting using five different solvents to get five extracts.

The yield of the extracts from different solvents was calculated as a percentage mass obtained from 1 g of the crude. The methanol and ethanol extracts were green in colour and had high yields of 60.7% and 66.6% respectively. Dichloromethane extract was yellowish green with a yield of 55.1%. The hexane and acetone extracts which were yellow and green had low yields of 32.4% and 30.0% respectively. The percentage yield of the extracts is shown in Table 3.

Table 3: Percentage yield of the extracts

Sample no.	**solvent**	**Colour of extract**	**Yield of the extract (g)**	**Percentage yield (%)**
1	Hexane	yellow	0.324	32.4
2	Methanol	green	0.607	60.7
3	Ethanol	green	0.666	66.6
4	Dichloromethane	Yellowish-green	0.551	55.1
5	Acetone	green	0.300	30.0

Preliminary phytochemical analysis of the five extracts indicated the presence of alkaloids, saponins, tannins, flavonoids, phenolic compounds and terpenoids as shown in Table 4. All the five extracts showed a positive test for terpenoids and flavonoids, ethanol and acetone extracts showed positive results for tannins. Dichloromethane, ethanol and acetone extracts indicated the presence of alkaloids. Methanol, ethanol and acetone extracts showed positive results for

phenolic compounds, methanol and ethanol extracts indicated the presence of saponins. None of the extracts showed a positive test for steroids.

Table 4: Preliminary phytochemical screening of *Kleinia longiflora* extracts.

Test	**Hexane**	**Methanol**	**Ethanol**	**Dichloromethane**	**Acetone**
Terpenoids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannins	-	-	+	-	+
Steroids	-	-	-	-	-
Alkaloids	-	-	+	+	+
Phenolic compounds	-	+	+	-	+
Saponins	-	+	-	-	-

Key: -Results negative, +Results positive

From these results, acetone and ethanol extracted more compounds followed by methanol and dichloromethane and lastly hexane. This indicates that most of the compounds present in *Kleinia longiflora* are medium polar to polar and only a few of them are non-polar. Since all the five extracts showed positive results for terpenoids and flavonoids, it is a clear indication that *Kleinia longiflora* is rich in terpenoids and flavonoids. The results also reveal that alkaloids and phenolic compounds are in moderate quantities followed by tannins, therefore *Kleinia longiflora* is worth studying.

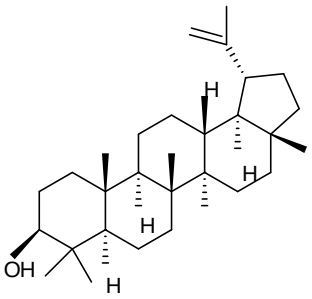
4.2 Isolated compounds from *Kleinia longiflora* and their characterization

The phytochemical investigation of *Kleinia longiflora* resulted in the isolation of three secondary metabolites which were lupeol **1,** isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-

glucopyranoside **11** and stigmasterol **12**. The structures of compounds **11** and **12** were deduced from ^{1}H NMR, ^{13}C NMR, HMBC, HMQC, COSY and UV while compound **1** was identified using GC-MS. The other five extracts were difficult to clean up but ^{1}H NMR revealed that they were a mixture of terpenoids.

4.2.1 Structure elucidation of lupeol 1

Compound **1** was isolated as an impure product as its TLC profile showed two spots that were very close to each other after preparative TLC. It was then analysed using GC-MS. The identification of the components was performed by aid of the NIST library search and revealed that the compound was lupeol **1.** The structure is shown in Figure 5.



1

Figure 5: Structure of lupeol **1**

4.2.2 Structure elucidation of Isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranoside 11

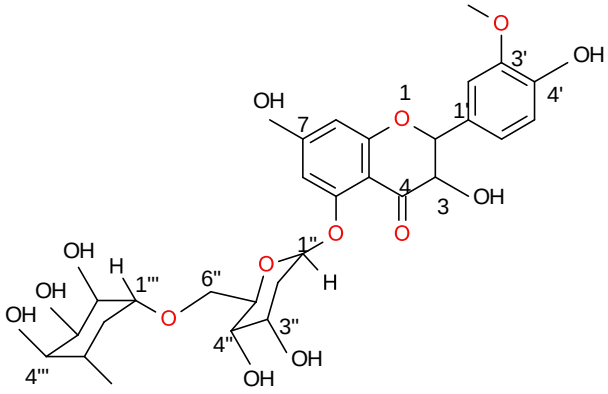
Isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranoside **11** (Figure 5) was obtained as a yellow powder of 0.248g. The ^{13}C- NMR data gave 28 signals. The UV spectra for this compound gave a typical methanol spectrum of a quercetin derivative (Guvenalp et al., 2005).

The ^{1}H and ^{13}C NMR spectra for this compound showed the signals in the aromatic region for the isorhamnetin aglycone. The proton NMR spectra for ring B of the quercetin derivative showed an ABX system consisting three protons which are proton 6', δ_H 7.59, dd, J [2.1, 8.4] which is *ortho* to proton 5', δ_H 6.91, d, J [8.4], and *meta* to proton 2', δ_H 8.09, d, J [2.1]. Ring A had two protons which are *meta* to each other namely proton 6, δ_H 6.20, d, J [2.05] and proton 8, δ_H 6.42, d, J [2.17]. On the proton spectra, there were two doublets at δ_H 5.24 (1H, d, J=8) and δ_H 4.56 (1H, d, J=1.7) which indicated two anomeric protons of a sugar moiety. These anomeric proton signals showed the presence of a β-D configuration of a glucose, and α-L configuration of a rhamnose respectively. From HMBC spectra rhamnose showed correlation with sugar carbons only therefore the rhamnose is not attached to the flavonoid. There is a shift on Carbon 5 from the usual δc 163 to δc 167.8 ppm that revealed that there is a group attached to carbon 5. The HMBC spectra also revealed that there is a correlation between proton δ_H 5.24 from glucose with carbon δc 167.8 ppm which proved that the glucose is attached to the flavonoid.

The UV spectral analysis also revealed that there was no complex formed in band II when $AlCl_3$ shift reagent was added which proved that position 5 in the benzoyl part of the quercetin derivative was occupied. The sodium acetate spectrum also proved this since there was a bathochromic and hyperchromic shift after sodium acetate powder was added to the sample solution (Guvenlp et al., 2005). The signal at δ_H 3.98 (3H, s) and δ_C 56.7 confirmed the presence of a methoxy group.

To deduce the positions of the methoxy group, HMBC was used which showed cross peaks at carbon 3' / OMe 3'. This was also confirmed by the UV spectral analysis which revealed that there was no complex formed in band I when $AlCl_3$ shift reagent was added which proved that position 3' in the cinnamoyl part of the quercetin derivative was occupied (Guvenalp et

al.,2005). This compound was characterized as isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranoside **11**. The assignment was consistent with literature since Guvenalp et al, 2005 extracted the same compound. Table 5 shows the protons and carbons and Figure 6 shows the structure for this compound.



11

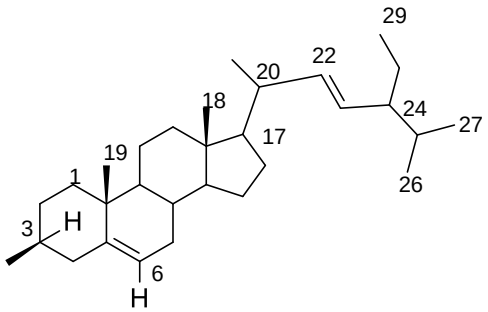
Figure 6: Structure of isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranoside **11**

Table 5: ^{1}H-NMR (3000 MHz) and ^{13}C NMR (75 MHz) chemical shift for isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranoside **11** in CD_3OD (J values in Hz)

Position	**^{1}H NMR (CD_3OD, 300 MHz)**	**^{13}C NMR (CD_3OD, 75 MHz)**	**^{13}C NMR (Literature value, CD_3OD, 75MHz) (Guvenalp et al, 2005)**
2		157.4	156.5
3		134.1	133.8
4		178.0	170.0
5		164.8	159.5
6	6.20 (1H, d, *J*=2.05)	103.6	102.5
7		164.7	163.8
8	6.42 (1H, d, *J*=2.2)	93.5	95.8
9		157.4	158.3
10		104.2	104.8
1'		121.5	128.6
2'	8.09 (1H, d, *J*=2.1)	114.6	114.5
3'		147.0	148.5
4'		149.5	146.7
5'	6.91 (1H, d, *J*=8.4)	113.3	116.2
6'	7.59 (1H, dd, *J*=2.1, 8.4)	122.4	124.0
3'- OCH_3	3.98 (3H, s)	57.7	56.7
Glucose 1"	5.24 (1H, d, *J*=8.0)	105.6	102.5
2"		72.4	75.9
3"		75.4	77.4
4"		68.8	71.6
5"		76.9	78.2
6"		65.2	69.8
Rhamnose 1'''	4.56 (1H, d, *J*=1.8)	99.9	102.0
2'''		71.5	72.1
3'''		69.8	72.3
4'''		74.4	73.9
5'''		67.3	71.6
6'''	1.20 (3H, d, *J*=2.6)	15.4	18.1

4.2.3 Structure elucidation for stigmasterol 12

Stigmasterol **12** was isolated as colourless crystals of mass 0.248 g. The proton NMR spectrum showed a downfield ^{1}H intensity at δ_H 5.40 ppm indicative of an oefinic proton (H-6), it also showed oefinic protons at δ_H 5.02 ppm and at δ_H 5.45 ppm (H-22 and H-23). The spectrum had a multiplet at δ_H 3.57 ppm indicative of an oxymethine proton (H-3). The spectrum showed the presence of six methyl protons at δ_H 0.690 (H-18), 1.00 (H-19), 0.82 (H-27), 0.84 (H-26), 0.86 (H-21) and 0.82 (H-29) respectively. ^{13}C NMR spectrum revealed the presence of 29 carbons suggestive of a steroidal compound. The signals at δ_C 140.08 (C-5), 42.41 (C-13) and 36.05 (C-10) were assigned to three quaternary carbons. The signal at δ_C 71.85 ppm was for oxymethine carbon (C-3). Two olefinic carbons signals at δ_C 140.08 and 121.73 ppm were for (C-5 and C-6) and signals at δ_C 138.00 and 129.03 ppm for (C-22 and C-23). According to this, the compound was named stigmasterol **12**. Table 6 shows the NMR data and Figure 7 shows the structure for stigmasterol **12.** The proton and carbon spectra were similar to the stigmasterol reported by (Khatun et al., 2012).



12

Figure 7: Structure of stigmasterol **12**

Table 6: ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) chemical shift for compound stigmasterol **12** in CD_3OD (J values are in H)

Position	**^{1}H NMR**	**^{13}C NMR**	**^{13}C NMR Literature (Khatun et al., 2012)**
1		37.3	37.3
2		31.7	31.7
3	3.57 (m)	71.8	71.8
4		42.4	42.3
5		140.8	141.0
6	5.40 (1H, d, *J*=5.3)	121.7	121.7
7		31.7	31.7
8		31.8	31.8
9		51.2	50.2
10		36.5	36.2
11		21.1	21.1
12		39.7	40.1
13		42.4	42.4
14		56.0	56.1
15		24.4	23.1
16		29.7	29.7
17		56.0	56.1
18		12.1	12.0
19		19.4	19.4
20		40.5	41.1
21		21.2	21.1
22	5.02(1H, dd, *J*=8.5,15.1)	138.3	138.0
23	5.45(1H, dd,*J*=8.5,15.1)	129.3	128.9
24		50.2	50.2
25		31.9	31.7
26		21.1	21.3
27		19.0	19.1
28		24.4	25.4
29		12.1	12.3

4.2.4 The biology of isolated compounds from *Kleinia longiflora*

Isorhamnetin 5-O-α-rhamnopyranosyl (1'''-6'')-β-glucopyranoside **11** is a flavonoid and flavonoids are well known to be antioxidants and free radical scavengers which prevent oxidative cell damage. They are also known to have strong anti-cancer activity and protect the cell against all stages of carcinogenesis (Tyagi & Agarwal, 2017). Stigmasterol is known to exhibit antimicrobial, anticancer, anti-arthritic, anti-asthma, diuretic and anti-inflammatory activities (Lalitha et al., 2015). It also acts as a precursor in the manufacture of semi synthetic progesterone, which is a valuable hormone that plays an important physiological role in the regulatory and tissue-rebuilding mechanisms related to oestrogen effects. Stigmasterol acts as an intermediate in the biosynthesis of androgens, oestrogens and corticoids (Lalitha et al., 2015; Tyagi & Agarwal, 2017).

Lupeol has been reported to show a broad spectrum of biological activities including anti protozoal, anti-inflammatory, anti-microbial, antioxidant, antiviral, anti-flue, anti-hyperglycaemic, anti-tumour and a chemo preventive agent (Sudha et al., 2013). Stigmasterol is a major phytosterol in various herbal plants and have been proposed as a candidate for anti-cancer agents (Kangsamaksin et al., 2017).

Beneficial effect of lupeol was investigated on the i*n vitro* development of bovine embryos.it was discovered that apoptosis negatively affects bovine embryo development, implantation and the pregnancy rate, treatment with 2.0 µM lupeol significantly improved blastocysts quality by reducing the number of apoptotic cells and increasing the total cell number (Khan et al., 2018; Siddique & Saleem, 2011). The above studies confirm the ethnomedicinal use of *Kleinia longiflora* by Bapedi traditional healers to manage breast cancer and in the treatment of female reproductive ailments, mainly infertility as stated by Potroz & Cho, 2015.

4.3 The GC-MS Analysis of *Kleinia longiflora*

4.3.1 *Kleinia longiflora* hexane extract

Kleinia longiflora hexane extract was found to contain 21 compounds as shown in Table 7. From the 21 compounds, eight of them are sesquiterpenes, two are sesquiterpene alcohols, the other two are monoterpenes, one is a sesquiterpene lactone and another one is a triterpene while the rest are hydrocarbons. The main constituents of the extract were found to be; β-eudesmol (80.6%) which is a sesquiterpene alcohol, lupeol (53.1%) which is a triterpene, ambrosin (48.4%) which is a sesquiterpene lactone and (-)-α-panasinsen (43.6%) which is a sesquiterpene. Compounds reported in Table 7 had a match factor of above 700. Studies by Asong et al, 2019 on non-polar extracts of *Kleinia longiflora* have shown that the extracts are rich in terpenoids. The main constituents of non-polar extracts of *Kleinia longiflora* studied by Asong et al., 2019 include dotriacontane, 6β-bicyclo [4.3.0] nonane, 5β-iodomethyl-1β-41) isopropenyl-4α, 5α-dimethyl-, lupeol,1,3,6,10-cyclotetradecatetraene, 14-isopropyl-3,7,11-trimethyl-, 9,19-cyclolanostan-3-ol, acetate, (3β), heptacosane, lup-20 (29)-ene-3-one, lupeol and 1,3,6,10-cyclotetradecatetraene, 14- isopropyl-3,7,11-trimethyl-. These results are consistent with the constituents that were found in the *Kleinia longiflora* hexane extract. The differences may be because chemical constituents may vary due to location and season of harvesting.

β-eudesmol which is one of the major constituents of *Kleinia longiflora* hexane extract, has been reported to inhibit the proliferation of some tumour *in vitro* growth of mouse H_{22} and S_{180} tumour, and formation of new blood vessels in tumour tissues *in vivo* (Ma et al., 2008). It was also shown to have unique effects on the nervous system including blocking the nerve-evoked contraction and markedly alleviating muscle fasciculation, tremor and convulsion (Yu et al.,

2008). D-limonene which is the natural occurring monoterpene was found to have chemo preventive and chemo therapeutic activity against many rodent solid tumour types (Crowell & Gould, 1994). Ambrosin was also reported to have a very potent anti-cancer activity, it was evidenced to be a potential NF-Kβ inhibitor in *in vitro* assays (Khalil et al., 2019). These results show the potential for *Kleinia longiflora* to manage breast cancer as well as to treat mental illness and colloid as observed also in its ethno medicinal use (Cheikhyoussef et al., 2011; Potroz & Cho, 2015)

Table 7: Composition of *Kleinia longiflora* hexane extract

	Compound	**Chemical formula**	**Retention time (minutes)**	**Retention Indices**	**Peak area %**
1	4-methyl-1-(1-methylethyl)-Cyclohexene (terpenene)	$C_{10}H_{16}$	4.414	894.4	17
2	Triacontane	$C_{30}H_{62}$	26.642		13.2
3	Cubenol	$C_{15}H_{26}O$	10.282	1237.6	10.6
4	Ambrosin	$C_{15}H_{18}O_3$	24.706	1301	48.4
5	Cubenol	$C_{15}H_{26}O$	10.552	1238.8	9.51
6	Veridiflorol	$C_{15}H_{26}O$	10.365	1238	17.4
7	β-eudesmol	$C1_5H_{26}O$	10.837	1240	80.6
8	(*E*, *E*)-12-acetoxy-6-hydroxymethyl-2,10-dimethyl-2,6,10-dodecatriene	$C_{17}H_{28}O_3$	12.383	1244.5	17.1
9	Tetratricontane	$C_{34}H_{70}$	31.633		16.5
10	Lupeol	$C_{30}H_{50}O$	32.853		53.1
11	(-)-α-panasinsen	$C_{15}H_{24}$	9.742	1235.2	43.6
12	α-amorphene	$C_{15}H_{24}$	9.540	1234.3	4.59
13	Decahydro-4a-methyl-1-methylene-7(1-methylethenyl)-[4aR-(4aα,7α,8aβ)] naphthalene (β-eudesmene or β-selinene)	$C_{15}H_{24}$	9.483	1234.1	22.7
14	β- cubebene	$C_{15}H_{24}$	9.421	1233.8	37.7
15	[1S-(1α,4α,7α)],1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-Azulene(α-Guaiene)	$C_{15}H_{24}$	9.364	1233.6	15.7
16	Seychellene	$C_{15}H_{24}$	9.265	1233.1	6.41
17	Ylangene	$C_{15}H_{24}$	9.058	1232.2	20.1
18	Caryophyllene	$C_{15}H_{24}$	8.902	1231.5	6.83
19	Dodecane	$C_{12}H_{26}$	6.697	1055.2	30.3
20	D-limonene	$C_{10}H_{16}$	5.005	899.9	22.2
21	Copaene	$C_{15}H_{24}$	8.492	1229.7	33.3

4.3.2 *Kleinia longiflora* essential oil

Kleinia longiflora essential oil was found to contain 20 compounds with match factor of over 700 among these five were monoterpenes, twelve were sesquiterpenes and three were esters. The main constituents of the oil were hexanedioic acid, *bis* (2-ethylhexyl) ester (71.5 %) which is a diester, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (63.2%) which is a fatty acid glycerol ester, *cis*-carveol (61.0%) which is a monoterpenoid alcohol and octadecanoic acid,2,3-dihydroxypropyl ester (50.7%) which is a fatty acid ester. The oil is rich in terpenes and this was confirmed by Bohlmann et al 1981 as well as in esters.

Hexadecanoic acid,2-hydroxy (hydroxymethyl) ethyl ester which is one of the main constituents was reported to be haemolytic, antioxidant and anti-androgenic (Tyagi & Agarwal, 2017), α-cadinol was reported to possess anti-inflammatory and anti-carcinogenic activities while caryophyllene oxide was shown to be a common sesquiterpene which possesses anti-inflammatory and anti-carcinogenic activities (Nadarajan & Pujari, 2014).Fatty acid esters play an important role in regulating human body health in terms of anti-inflammatory function, enhancing immunity, promoting infant development and boosting memory (Xiao et al., 2019).

Kleinia longiflora essential oil has not been studied before, Asong et al., 2019 did the GC-MS analysis on the non-polar and polar extracts of the aerial parts and 4-hydroxy-4-methylhex-5-enoic acid, tert-butyl ester was part of the main constituents, this shows that *Kleinia longiflora* has esters and this explains why it has such an outstanding aroma.

Table 8: Composition of *Kleinia longiflora* essential oil

Name of compound	**Retention time**	**Retention indices**	**Peak area %**
cis-carveol	10.90	955.4	61
β-eudesmol	19.54	1278.3	47.8
Caryophyllene oxide	20.12	1280.9	43.6
α-epi-muurolol	21.43	1286.6	48
Hexanedioic acid, bis (2-ethylhexyl) ester	35.59	1340.31	71.5
Hexadecanoic acid, 2-Hexadecanoyl-1-(hydroxymethyl) ethyl ester	37.30	1359.4	63.2
Octadecanoic acid, 2,3-dihydroxypropyl ester	40.39	1388.1	50.7
Caryophyllene	16.09	1263.1	30.9
δ-cadinene	18.59	1274	33.2
6-(1-hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	31.27	1329.9	32.9
Verbinol	9.60	943.1	27.8
Trans-p-mentha-1 (7), 8-dien-2-ol	10.12	948	30.2
(-)-Carvone	11.55	961.5	39.8
α-copaene	14.98	1258.2	35.8
1H-cyclopron (e) azulen-4-ol, decahydro-1,1,4,7-tetramethyl- [1aR-(1aα ,4β ,4aβ ,7α ,7aβ ,7bα)]	20.36	1281.9	29.8
α –cadinol	21.75	1288	25.1
2,4-decadienal, (E, E)-	13.36	821.5	21.2
γ-gurjunenepoxide-(2)	23.26	1294.7	24.8
2-(4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydro-napthalen-2-yl)-prop-2-en-1-ol	24.62	1300.7	23.2
Aromadendrene, dehydro-	24.86	1301.7	20.8

4.3.3 Comparison of the constituents of *Kleinia longiflora* essential oil and the hexane extract

This study has shown that both the *Kleinia longiflora* hexane extract and the essential oil are rich in terpenes. The monoterpenes and sesquiterpenes found in this plant are known to possess anti-inflammatory and anti-carcinogenic activities (Nadarajan & Pujari, 2014). *Kleinia longiflora* essential oil has esters that form part of the main constituents of the oil while hexane

extract does not have esters as part of the main constituents. This may be due to several factors like time and season of harvest.

4.3.4 Comparison of *Kleinia longiflora* essential oil main constituents with that of other *Kleinia* species

When comparing *Kleinia longiflora* essential oil with essential oils from other *Kleinia* species there is a clear indication that *Kleinia* species are rich in triterpenes as outlined in Table 9. However, *Kleinia longiflora* essential oil has shown a marked difference from essential oils from other *Kleinia* species by having esters as the main constituents. For instance, when comparing *Kleinia longiflora* with other *Kleinia* species like *Kleinia pendula*, *Kleinia odora* and *Kleinia squarrosa* as shown in Table 9, it has a diester, fatty acid glycerol ester and a fatty acid ester. This difference may be due to geographical location and the soil composition since the other *Kleinia* species are not from Botswana.

Table 9: Main constituents of other *Kleinia* essential oils

K.longiflora	*K.odora*	*K.pendula*	*K.squarrosa*
Hexanedioic acid, bis (2-ethylhexyl) ester Hexadecanoic acid, 2-hydroxy-1-(hydroxy methyl) ethyl ester . *Cis*-carveol Octadecanoic acid,2,3-dihydroxypropyl ester	(+)-epi-bicyclosesquiphellandrene Caryophyllene α-caryophyllene α-pinene	Myrcene α-humulene β-elemere T-cardinal 4-α-H-eudesm-5α-ol	(E)-iso-γ-bisabolene α-pinene Caryophyllene Sabinene

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1 Conclusion

Kleinia longiflora belongs to the family Asteraceae and genus *Kleinia* (synonym *Senecio*) which comprises of about 40 species. Preliminary phytochemical investigation was done on this plant using standard methods and showed the presence of terpenoids, tannins, alkaloids and flavonoids. From the results, acetone and ethanol have shown to be better solvents for extraction of phytochemicals since they extracted more compounds. *Kleinia longiflora* was found to be rich in terpenoids and flavonoids since the extracts from the solvents used showed positive results for terpenoids and flavonoids. The non-volatile compounds isolated from the plant included a triterpene named lupeol **1,** a flavonoid named isorhamnetin 5-O-α-rhamnopyranosyl (1'"-6")-β-glucopyranose **11** and a sterol named stigmasterol **12.** These are well known to have anti-cancer effects. *Kleinia longiflora* plant is rich in terpenoids, and this is a confirmation of what is shown in Table 2 on the distribution of the main terpenoid types in the proposed *Kleinia* groups by Bohlmann et al., 1981.

GC-MS analysis of *Kleinia longiflora* hexane extract revealed the presence of lupeol, β-eudesmol, α-cadinol, caryophyllene oxide, ambrosine, d-limonene. *Kleinia longiflora* essential oil showed the presence of Hexanedioic acid, bis (2-ethylhexyl) ester, hexadecanoic acid, 2-hydroxy-1-(hydroxy methyl) ethyl ester, cis-carveol and octadecanoic acid,2,3-dihydroxypropyl ester. Majority of the main constituents are esters, which indicate that *Kleinia longiflora* is also rich in esters.

5.2 Future work

Further work should be done on isolation of more non-volatile compounds by coming up with ways which will enable ease isolation of terpenoids since from the work done so far there is a clear indication that the plant is rich in terpenoids. Previous studies on the chemistry of the plant by Bohm et al 2001 revealed that *Kleinia longiflora (*syn *Senecio longiflorus*) has quercetin. Hartmann & Witte, 1995 also stated that *Kleinia longiflora* has alkaloids seneciphylline and senecionine. There is need for further work to investigate whether the *Kleinia longiflora* found in Botswana contain these secondary metabolites. Research must be done on the best method to be used for *in vitro* and *in vivo* antimicrobial activity studies on bacteria associated with skin infections, cancer and infertility since all the experiments conducted failed and the problem may be due to the bacteria used as the results were inconclusive. Cytotoxicity should be done as it will help to determine whether the plant is safe to taken orally and the quantity to take.

REFERENCES

Abubakar, A. R., & Haque, M. (2020). Preparation of medicinal plants Basic extraction and fractionation procedures for experimental purposes. *Journal of Pharmacy & BioAllied Sciences*, *12*(1), 1–10.

Al-Taweel, A. M., El-Deeb, K. S., & Al-Muhtadi, F. J. (2004). Chemical composition and antimicrobial activity of the essential oil of *Kleinia odora. Saudi Pharmaceutical Journal*, *12*(1), 47–50.

Al Musayeib, N. M., Mothana, R. A., Ibrahim, S. R. M., El Gamal, A. A., & Al-Massarani, S. M. (2014). Klodorone A and klodorol A: New triterpenes from *Kleinia odora*. *Natural Product Research*, *28*(15), 1142–1146.

Alfaifi, M., Alsayari, A., & Annadurai, S. (2020). Analgesic, anti-inflammatory, cytotoxic activity screening and UPLC-PDA-ESI-MS metabolites determination of bioactive fractions of *Kleinia pendula*. *Molecules*, *25*(418), 1–14.

Apodaca, G., & McKerrow, J. H. (1989). Regulation of *Trichophyton rubrum* proteolytic activity. *Infection and Immunity*, *57*(10), 3081–3090.

Arnold, C. (2013). The new danger of synthetic drugs. *World Report*, *382*(9886), 15–16.

Asong, J. A., Ndhlovu, P. T., Khosana, N. S., Aremu, A. O., & Otang-Mbeng, W. (2019). Medicinal plants used for skin-related diseases among the Batswanas in Ngaka Modiri Molema District Municipality, South Africa. *South African Journal of Botany*, *126*, 11–20.

Blowman, K., Magalhães, M., Lemos, M. F. L., Cabral, C., & Pires, I. M. (2018). Anticancer properties of essential oils and other natural products. *Evidence-Based Complementary and Alternative Medicine*, *2018*.

Bohlmann, F., Ahmed, M., Jakupovic, J., & Jeffery, C. (1981). Sesquiterpenes from *Kleinia* species. *Phytochemistry*, *20*(2), 251–256.

Bohm, B. ., & Stuessy, T. (2001). Flavonoids of the sunflower family (Asteraceae), Springer-Verlog, Australia, 349.

Cheikhyoussef, A., Shapi, M., Matengu, K., & Mu Ashekele, H. (2011). Ethnobotanical study of indigenous knowledge on medicinal plant use by traditional healers in Oshikoto region, Namibia. *Journal of Ethnobiology and Ethnomedicine*, *7*, 1–11.

Crowell, P. ., & Gould, M. . (1994). Chemoprevention and therapy of cancer by d-limonene. *Critical Reviews in Oncogenesis*, *5*(1), 1–22.

Damme, P., Eynden, V., & Vernemmen, P. (1922). Plant uses by the Topnaar of the Kuiseb valley Namib dessert. *Afrika Focus*, *8*(3), 223–252.

Das, K., Tiwari, R. K. S., & Shrivastava, D. K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*, *4*(2), 104–111.

Dhifi, W., Bellili, S., Jazi, S., Bahloul, N., & Mnif, W. (2016). Essential oils' chemical characterization and investigation of some biological activities: A critical review. *Medicines*, *3*(4), 25.

Edeoga, H. O., Okwu, D. E., & Mbaebie, B. O. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*, *4*(7), 685–688.

El-Shazly, A., Doral, G., & Wink, M. (2002). Chemical composition and biological activity of the essential oils of *Senecio aegyptius* var. discoideus Boiss. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences*, *57*(5–6), 434–439.

Gardener, D. ., Thorne, M. ., Molyneux, R. ., Pfister, J. ., & Seawright, A. . (2006). Pyrrolizidine alkaloids in *Senecio Madagascariensis* from Australia and Hawaii and assessment of possible livestock poisoning. *Biochemical Systematics and Ecology*, *34*(10), 736–744.

Gleenie, C. ., Harbone, J. ., & Rowley, G. . (1971). Correlations between flavonoid chemistry and plant geography in the *Senecio radicans* complex. *Phytochemistry*, *10*(10), 2413–2417.

Gujjeti, R. P., & Mamidala, E. (2013). Phytochemical screening and thin layer chromatographic studies of *Aerva lanata* root extract. *International Journal of Innovative Research in Science, Engineering and Technology (An ISO Certified Organization)*, *3297*(10), 5725–5730.

Gupta, V., Bansal, P., Bansal, R., & Mittal, P. (2015). Folklore herbal remedies used in dental care in Northern India and their pharmacological potential. *American Journal of Ethnomedicine*, 2(6), 365–372.

Guta, M., Teka, F., Bisrat, D., & Lindemann, P. (2018). Repellent activity of essential oil of the stem of *Kleinia squarrosa* against mosquitoes. *Ethiopean Pharmaceutical Journal*, *33*(July), 75–82.

Halliday, P. (1986). The genus *Kleinia* (compositae) in North Africa and the Canary Islands. *Kew Bulletin*, *41*(2), 279–285.

Hartmann, T., & Witte, L. (1995). Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In *Alkaloids: Chemical and Biological Perspectives* (pp. 155–233).

Hay, R. J. (2017). *Tinea Capiti*s: Current Status. *Mycopathologia*, *182*(1–2), 87–93.

Hossain, M. A., Weli, A. M., & Al-riyami, Q. (2013). Study of total phenol , flavonoids content and phytochemical screening of various leaves crude extracts of locally grown

Thymus vulgaris. *Asian Pacific Journal of Tropical Biomedicine*, *3*(9), 705–710.

Jamshidi-Kia, F., Lorigooini, Z., & Amini-Khoei, H. (2018). Medicinal plants: Past history and future perspective. *Journal of HerbMed Pharmacology*, *7*(1), 1–7.

Joshi, B. C., Kumar, V., Chandra, B., & Kandpal, N. D. (2019). Chemical composition and antibacterial activity of essential oil of *Senecio graciliflorus*. *Journal of Drug Delivery and Therapeutics*, *9*(1), 98–100.

Kangsamaksin, T., Chaithongyot, S., Wootthichairangsan, C., Hanchaina, R., Tangshewinsirikul, C., & Svasti, J. (2017). Lupeol and stigmasterol suppress tumor angiogenesis and inhibit cholangiocarcinoma growth in mice via downregulation of tumor necrosis factor-a. *PLoS ONE*, *12*(12), 1–16.

Kapadia, G. ., Ramdass, A., & Bada, F. (2008). Pyrrolizidine alkaloids of *Senecio Glabellus*. *International Journal of Crude Drug Research*, *28*(1), 67–71.

Karimi, A., Majlesi, M., & Rafieian-Kopaei, M. (2015). Herbal versus synthetic drugs; beliefs and facts. *Journal of Nephropharmacology*, *4*(1), 27–30.

Khalil, M. N. A., Choucry, M. A., Senousy, A. S. El, Hassan, A., El-marasy, S. A., Awdan, S. A. El, & Omar, F. A. (2019). Ambrosin , a potent NF- κβ inhibitor , ameliorates lipopolysaccharide induced memory impairment , comparison to curcumin. *PLoS ONE*, *14*(7), 1–23.

Khan, I., Chowdhury, M. M. R., Song, S. H., Mesalam, A., Zhang, S., Khan Khalil, A. A., Jung, E. H., Kim, J. B., Jafri, L., Mirza, B., & Kong, I. K. (2018). Lupeol supplementation improves the developmental competence of bovine embryos *in vitro*. *Theriogenology*, *107*, 203–210.

Khatun, M., Billah, M., & Quader, M. A. (2012). Sterols and Sterol Glucoside from

Phyllanthus Species. *Dhaka University Journal of Science*, *60*(1), 5–10.

Kumar, A., Lather, A., Kumar, V., Sherawat, V., & Tyagi, V. (2015). Pharmacological potential of plant used in dental care : A Review. *Journal of Herbal Drug*, *5*(4), 179–186.

Lalitha, S., Parthipan, B., & Mohan, V. R. (2015). Determination of Bioactive Components of *Psychotria nilgiriensis* Deb & Gang (Rubiaceae) by GC-MS Analysis. *International Journal of Pharmacognosy and Phytochemical Research*, *7*(4), 802–809.

Larson, T. . (1986). The ethnomedicine of the Hambukushu in 1950. *Botswana Notes and Records*, *18*, 39–47.

Ma, E. L., Li, Y. C., Tsuneki, H., Xiao, J. F., Xia, M. Y., Wang, M. W., & Kimura, I. (2008). β-Eudesmol suppresses tumour growth through inhibition of tumour neovascularisation and tumour cell proliferation. *Journal of Asian Natural Products Research*, *10*(2), 159–167.

Mabogo, D. (1990). The ethnobotany of the Vhavenda, MSc thesis, University of Pretoria.

Magwede, K., Wyk, B. Van, & Wyk, A. E. Van. (2019). An inventory of Vhavenḓa useful plants: An inventory of Vhavenḓa useful plants. *South African Journal of Botany*, *122*, 57–89.

Maroyi, A., & Mosina, G. K. E. (2014). Medicinal plants and traditional practices in peri-urban domestic gardens of the Limpopo province, South Africa. *Indian Journal of Traditional Knowledge*, *13*(4), 665–672.

Martinez, D. A., Oliver, B. G., Gräser, Y., Goldberg, J. M., Li, W., Martinez-rossi, N. M., Monod, M., Shelest, E., Barton, R. C., Birch, E., Brakhage, A. A., Chen, Z., Gurr, S. J., Heiman, D., Heitman, J., Kosti, I., Rossi, A., Saif, S., Samalova, M., White, T. C. (2012). Comparative genome analysis of *Trichophyton rubrum* and related dermatophytes reveals candidate genes involved in infection. *MBio*, *3*(5), 1–14.

Mejri, J., Aydi, A., Abderpabba, M., & Mejri, M. (2018). Emerging extraction processes of essential oils: A review. *Asian Journal of Green Chemistry*, *2*(3), 246–267.

Nadarajan, S., & Pujari, S. S. (2014). Leaf essential oil composition and biochemical activity of an endangered medicinal tree *Syzygium caryophyllatum* (L) Alston , (Wild black plum). *Journal of Essential Oil Bearing Plants*, *17*(3), 371–379.

Nyamai, D. W., Arika, W., Ogola, P., & Njagi, E. N. M. (2016). Medicinally important phytochemicals : An untapped research avenue. *Journal of Pharmacognosy and Phytochemistry*, *4*(1), 35–49.

Oliver, & Hiern, W. . (1877). Flora of tropical Africa. L. Reef, London, 1, 1868-1902.

Pendyala, V., Suryadevara, V., Tokala, D., & Nelluri, N. (2018). Formulation and evaluation of a polyherbal ointment for treatment of acne. *Asian Journal of Pharmacy and Pharmacology*, *5*(1), 143–148.

Potgieter, M. J., & Maema, L. (2016). Ethnomedicinal uses of indigenous plant species in Mogalakwena Municipality of Waterberg District , Limpopo Province South Africa. *International Journal of Traditional and Complementary Medicine*, *1*(4), 0028–0044.

Potroz, M. G., & Cho, N. (2015). Natural Products for the Treatment of Trachoma and *Chlamydia trachomatis*. *Molecules*, *20*, 4180–4203.

Raghunath, D. (2008). Emerging Antibiotic resistance in bacteria with special reference to India. *Journal of Bioscience*, *33*(4), 593–603.

Rasoanaivo, P., Wright, C. W., Willcox, M. L., & Gilbert, B. (2011). Whole plant extracts versus single compounds for the treatment of malaria : synergy and positive interactions. *Malaria Journal*, 10(Suppl 1), 1–12.

Semenya, S. ., Maroyi, A., Potgieter, M. J., & Erasmus, L. J. . (2013). Herbal Medicines Used

by Bapedi Traditional Healers to Treat Reproductive Ailments in the Limpopo Province , South Africa. *African Journal of Traditional, Complementary and Alternative Medicine*, *10*(2), 331–339.

Semenya, S. S., & Maroyi, A. (2019). Ethnobotanical survey of plants used by Bapedi traditional healers to treat tuberculosis and its opportunistic infections in the Limpopo Province, South Africa. *South African Journal of Botany*, *122*, 401–421.

Sharma, P., & Shah, G. . (2015). Composition and antioxidant activity of *Senecio nudicaulis* Wall . ex DC . (Asteraceae): A medicinal plant growing wild in Himachal Pradesh , India. *Natural Product Research*, *29*(9), 883–886.

Siddique, H. R., & Saleem, M. (2011). Beneficial health effects of lupeol triterpene: A review of preclinical studies. *Life Sciences*, *88*(7–8), 285–293.

Sudha, T., Chidambarampillai, S., & Mohan, V. . (2013). GC-MS Analysis of Bioactive Components of Aerial parts of *Fluggea leucopyrus* willd.(Euphorbiaceae). *Journal of Applied Pharmaceutical Science*, *3*(05), 126–130.

Thulin, M. (2014). New species and combination in *Kleinia* (Asteraceae) from the horn of Africa. *Nort.J.Bot*, 419–426.

Tyagi, T., & Agarwal, M. (2017). Phytochemical screening and GC-MS analysis of bioactive constituents in the ethanolic extract of *Pistia stratiotes L* . and *Eichhornia crassipes* (Mart .) solms. *Journal of Pharmacognosy and Phytochemistry*, *6*(1), 195–206.

Vanijajiva, O., Pornpongrungrueng, P., & Pongamornkul, W. (2014). *Kleinia grandiflora* (Asteraceae: Senecioneae), a species and genus newly discovered in Thailand. *Phytotaxa*, *159*(1), 017–022.

Visweswari, G., Christopher, R., & Rajendra, W. (2013). Phytochemical screening of active

secondary metabolites present in *Withania somnifera* root. *International Journal of Pharmaceutical Sciences and Research*, *4*(7), 2770–2776.

Wikaningtyas, P., & Sukandar, E. Y. (2016). The antibacterial activity of selected plants towards resistant bacteria isolated from clinical specimens. *Asian Pacific Journal of Tropical Biomedicine*, *6*(1), 16–19.

Xiao, Y., Li, M., Mao, P., Yang, L., & Qu, L. (2019). Grain & Oil Science and Technology Enzymatic synthesis , antioxidant ability and oil-water distribution coefficient of troxerutin fatty acid esters. *Grain & Oil Science and Technology*, *2*(3), 78–84.

Yadav, M., Chatterji, S., Gupta, S. K., & Watal, G. (2014). Preliminary phytochemical screening of six medicinal plants used in traditional medicine. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(5), 539–542.

Yu, F., Harada, H., Yamasaki, K., Okamoto, S., Hirase, S., Tanaka, Y., Misawa, N., & Utsumi, R. (2008). Isolation and functional characterization of a β-eudesmol synthase , a new sesquiterpene synthase from Zingiber zerumbet Smith. *FEBS Letters*, *582*, 565–572.

Yusuf, A. Z., Zakir, A., Shemau, Z., Abdullahi, M., & Halima, S. A. (2014). Phytochemical analysis of the methanol leaves extract of *Paullinia pinnata linn. Journal of Pharmacognosy and Phytotherapy*, *6*(2), 10–16.