COMPARISON OF PHARMALOGICAL ACTIVITY OF *RHOICISSUS TOMENTOSA* AND *RHOICISSUS TRIDENTATA* FOR THE TREATMENT OF ELEPHANTIASIS IN SOUTH AFRICA

BY

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DECLARATION

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DEDICATION

This work is dedicated to my supervisor, my family and friends, for supporting me all the way up to so far.

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It is through the grace and guidance of the Almighty that I have made it this far in my studies.

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ABSTRACT

Elephantiasis is a symptom of a variety of diseases that is typically characterised by thickening of the skin and subcutaneous tissue, resulting in grossly enlarged and very swollen body parts. The swelling is associated with the accumulation of lymph. The disease is mostly noticeable in the lower limbs, but also commonly affects the scrotum in males, and may also affect breasts and arms. Elephantiasis could either be filarial or non-filarial. Filarial elephantiasis is caused by parasitic nematodes whereas non-filarial elephantiasis is due to a number of cases such as tuberculosis, cancer treatment, leishmaniasis, leprosy, sexually transmitted diseases like lymphogranuloma venereum and repeated streptococcal infections.

Elephantiasis is a worldwide problem that could be treated and managed if correct measures are taken. Elephantiasis is treated with a combination of albendazole and diethylcarbamazine or ivermectin and albendazole. Since secondary bacterial infections play an important role in advancing acute symptoms and progression of lymphoedema, antibiotics are also used in the management of the disease. Medicinal plants are also used to treat and manage elephantiasis and its symptoms.

South African traditional healers uses various plants to treat a wide number of health conditions. Research into the scientific validation of South African medicinal plants showed that medicinal plants are used in the treatment of pain, inflammation, hypertension, parasitic diseases and a number of bacterial infections. *Rhoicissus tomentosa* and *R. tridentata* are used by South African traditional healers to treat elephantiasis, inflammation, parasitic worms and to ensure safe delivery during pregnancy.

Rhoicissus tridentata and *R. tomentosa* were collected with the help of traditional healers, extracted and tested for antibacterial, antifungal, antimycobacterial, anthelmintic and anti-inflammatory activities. In antibacterial activity, the *R. tridentata* and *R. tomentosa* ethanol and acetone extracts inhibited all test microorganisms with minimum inhibitory concentration (MIC) values ranging between 0.098 and 0.39

mg/ml. The acetone extract of *R. tridentata* displayed the best antifungal activity by inhibiting all fungal strains at 0.195 mg/ml. Concerning antimycobacterial activity, the organic solvent extracts prepared from both plants inhibited *Mycobacterium tuberculosis* at 0.195 mg/ml. A moderate activity of1.56 mg/ml was observed with water extracts from both *R. tomentosa* and *R. tridentata*. The ethanol extract of *R. tridentata* displayed the best anthelmintic activity against *Haemonchotus contortus* with 100% larval mortality at 2 mg/ml. Fifty percent larval mortality was observed with water extract. The *R. tomentosa* and *R. tridentata* displayed the best anti-inflammatory activity by inhibiting 5- lipoxygenase (5-LOX) with IC_{50} values ranging between 0.10± 72.50 and 0.13± 6.650 µg/ml. The extracts from *R. tomentosa* and *R. tridentata* displayed 82% to 99% inhibitory effects against 5-LOX in the anti-inflammatory activity. Concerning the evaluation of chemical profile using thin layer chromatography, the extracts from both plants displayed the presence of band A, B, C, E and F. In bioautography bioassay, band E and F inhibited the growth of *S. aureus*.

These results have validated the claims made by traditional healers on the use of *R. tridentata* and *R. tomentosa* in the treatment and management of elephantiasis and related sysmptoms.

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LIST OF ABBREVIATIONS

5-LOX - 5- Lipoxygenase

AD - Anno Domini

ADLA - Adenolymphangitis

AIDS - Acquired Immune Deficiency Sndrome

BC - Before Christ

Ca - Latin word' around' about'

CDC -Centre for Disease Control

CDCP- Centres for Disease Control and Prevention

DMSO - Dimethylsulfoxide

GPELF - Global Programme for the Elimination of Lymphatic Filariasis

IC - Inhibitory concentrations

INT - p-iodonitrotetrazolium violet

MDA - Mass Drug Administration

MH - Mueller Hinton Broth

MIC - Minimum inhibitory concentrations

NCCLS - National Committee for Clinical Laboratory Standard

NDGA - Nordihydroguaiaretic acid

OADC - Oleic acid+ albumin+ dextrose+ catalase

OD - Optical Density

R_f - Retardation factor

TB - Tuberculosis

TLC - Thin Layer Chromatography

UV - Ultra violet

WHO - World Health Organization

LIST OF SI UNITS

°C - Degrees Celsius

µl - Microlitre(s)

cm - Centimetre(s)

g - Grams

H - hours

nm - nanometre(s)

mg – milligram (s)

ml – millilitre (s)

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Elephantiasis is a syndrome of a variety of diseases where parts of a person's body swell to enormous proportions (Addiss, 1998). It is typically characterised by thickening of the skin and subcutaneous tissue that results in grossly enlarged and very swollen limbs (Mandal, 2014; Komoreng *et al*., 2017). The swelling is associated with accumulation of lymph fluid of (lymphoedema). The disease is mostly noticeable in the lower limbs, but also commonly affects the scrotum in males, and may also affect breasts and arms. In fact, it is one of the most painful and intensely disfiguring diseases ever known. Elephantiasis is known to be common in the tropical and subtropical regions of Asia, Africa, South America and Pacific Island affecting millions of men and women (Cano *et al*., 2014; Maurya *et al*., 2015).

Elephantiasis is a worldwide problem that could be treated and managed if correct measures are taken. The Global Programme for the Elimination of Lymphatic Filariais (GPELF) was established in order to stop the spread of the infection and alleviate suffering among patients (WHO, 2016). The current mass treatment drugs used to treat elephantiasis are diethylcarbamazine, ivermectin and albendazole. These drugs result in 90% microfilariae reduction in the blood (Taylor *et al*., 2010). Since secondary bacterial infections play an important role in advancing acute symptoms and progression of lymphoedema, antibiotics together with hygiene measures are also used in the management of the disease (WHO, 2018). However, these antibiotics are ineffective against the adult worms, which are the cause of the disease (Debrah *et a*l., 2007; Komoreng *et al*., 2017).Monotherapy is no-longer advisable for the treatment of elephantiasis, which means combinations are used for therapy purposes. Combinations are quite expensive thereby significantly affecting the income of the infected members. Furthermore, the treatment requires an extended period for the curative purposes of elephantiasis. Many chronically ill patients are non-productive for the rest of their lives and become a burden to their families and society (Ramaiah *et al*., 1998 & 2000; WHO, 2009; Fernando *et al*., 2011).

1.2 History of elephantiasis

Elephantiasis is one of the oldest diseases that have a major effect on man. It has a long history of worldwide distribution from Far East to the Western horizon (Routh & Bhowmik, 1993). According to a study undertaken by Rajan (2000), elephantiasis has existed as a recognisable disorder from the very beginning of recorded human history. Both ancient Chinese and Indian writings describe swellings of extremities and the genitalia that are highly reminiscent of filarial lesions (Rajan, 2000). The Indian physician compiled *Sushruta Samhita* the extant version which was probably in its final form by 70 AD (Demaitre, 2007), he refered to it as a disease called *slipada* (*sli*=elephant; *pada*=leg) (Rajan, 2000). The description of this condition included diseases other than elephantiasis. It is nonetheless clear that at least some of the patients exhibiting *slipada* are cases of elephantiasis. The physician also noted that the disease is particularly present in individuals who live close to stagnant water.

The disease was clearly known by the Arab physicians as early as around the 10th century as well (Rajan, 2000). In the 13th century, as the Greek physicians began to translate Arabic literature and encountered descriptions of the disease, the Greeks incorrectly attributed the lesions to leprosy (Routh & Bhowmik, 1993; Rajan, 2000). To the Greeks, including Hippocrates (ca 460–370 BC), Galen (129–200 AD) and Paulus Aegineta (625–690 AD), the term elephantiasis was associated with ambiguity, implying both filariasis and leprosy (Demaitre, 2007; Golzari *et al*., 2012). It was not until 807–870 AD that Ali ibn Sahl Rabban al-Tabbari first described elephantiasis as Daa al-Fil (daa=disease, fil=elephant) and denoted treatments in Firdows Al-Hikmat (Paradise of Wisdom), the first existent medical book in medieval Persia (Madani & Borujerdi, 2009; Golzari *et al*., 2012).

In the half of the 19th century, Patrick Manson attributed the profound, deforming swelling of extremities to infestation with filarial parasites (Rajan, 2000). However, early records of this disease date back before the biblical time (Hajdu, 2002; Golzari *et al*., 2012).

1.3 Causes of elephantiasis

Elephantiasis can be categorised into filarial and non-filarial disease. Filarial elephantiasis is the gross abnormality caused lymphoedema, often as a result of infection in the lymphatic system. It is the type of elephantiasis resulting from infection with parasitic nematodes. It can also be confusing as it is caused by a variety of factors including exposure to certain types of soils (e.g. irritant alkaline clay soil) even without infection (Korevaar & Visser, 2012). Filarial elephantiasis is a parasitic disease caused by microscopic, thread-like worms (nematodes) of the family Filarioridea (Kagai, 2013; WHO, 2017). The three types of these thread-like worms are *Wuchereria bancrofti* which is responsible for 90% of all the cases, *Brugia malayi* which is responsible for less of the cases and *Brugia timori* (which occurs in individuals of all ages and does not choose the sex of a person, but also advances more in areas with low social and economic levels (De Souza, 2010). These three parasitic filarial worm species are commonly known for causing elephantiasis and are located mainly in the lymphatic system (Yimer *et al*., 2015). Infection due to *W. bancrofti* involves the entire affected limb, breast or genitals, whereas infection due to *B. malayi* only involves swelling below the knee or elbow (Shenoy, 2008).

Non-filarial elephantiasis is caused by minority of cases like tuberculosis, leishmaniasis, leprosy, sexually transmitted diseases like lymphogranuloma venereum and repeated streptococcal infections. These infections result in severe lymphatic damage which allows persistent lymphoedema and successive development of elephantiasis (Korevaar & Visser, 2012). The common etiological agents for the disease include, but not limited to, *Mycobacterium tuberculosis*, *M. leprae*, *Chlamidia trachomatis*, *Streptococcus pyogenes*, *S. pneumoniae*, *Staphylococcus aureus*, *Klebsiella granulomatosis*, and protozoan parasites. According to Carter *et al*. (1999) and O'Farrell (2002) *K. granulomatosis* is 99% similar to *K. pneumoniae*. Bacteria may infect the skin causing secondary infections which further complicates the condition (Chris, 2015). Elephantiasis can also be caused by cancer treatment and its reported frequency varies between 1% and 47%. Pelvic irradiation increases the frequency of leg lymphedema after cancer surgery for conditions such as malignant melanoma, prostate cancer, and gynaecological malignancies (Cormier *et al*., 2010).

1.4 Transmission of filarial elephantiasis

Filarial elephantiasis is a zoonotic whereby the nematode is transmitted to human through mosquito bite. Mosquitoes of the genera *Aedes, Anopheles, Culex* and *Mansonia* are the biological vectors of the disease (Chandy *et al*., 2011). According to Maurya *et al*. (2015), the filarial worms need human or other mammalian hosts and mosquito vector to complete life cycle. A successful lymphatic filarial infection is due to dynamic interaction between the environment, host and the parasite. It is known that the interaction between a host and a parasite has a profound effect on the outcome of the infection. Like most helminthic infections, filariasis usually presents a relatively chronic infection and this dynamic host parasitic interaction in human elephantiasis may results in chronic infection associated with a wide clinical spectrum (Mak, 2012). The efficiency of parasitic transmission to humans differs according to the genera of mosquito. For example, *Culex* mosquito is widespread across urban and semi-urban areas, *Anopheles* mainly in rural areas, and *Aedes* mainly in the Pacific Islands and parts of the Philippines (WHO, 2014).

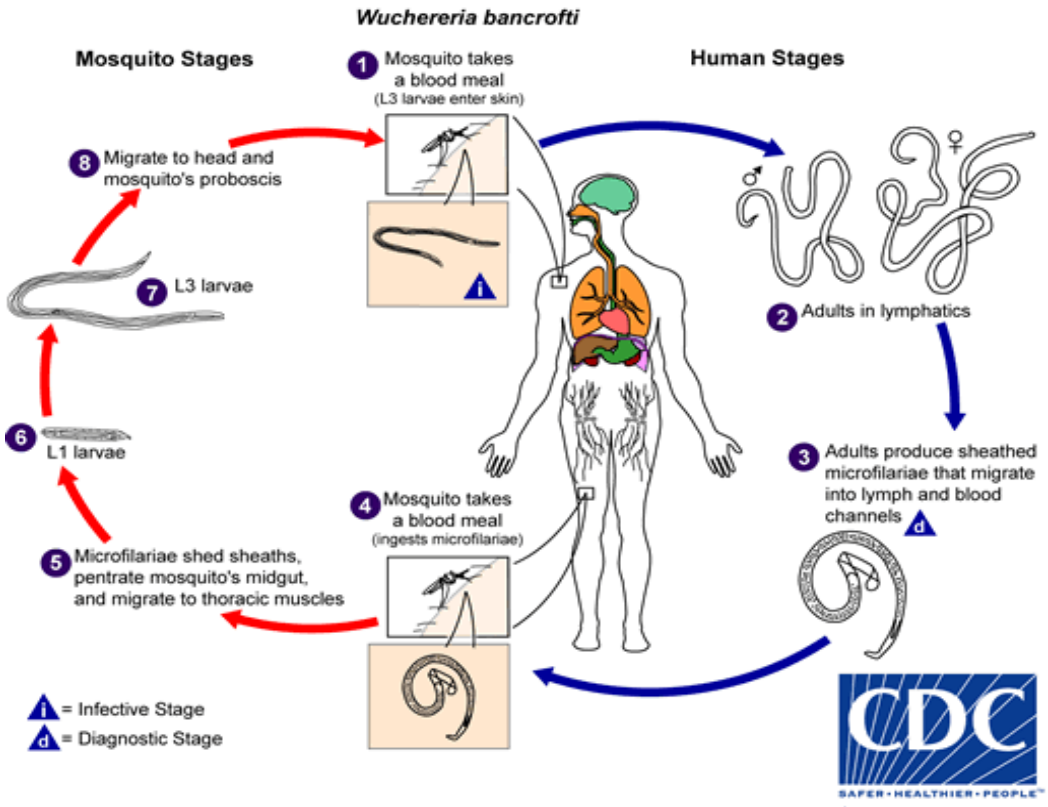

Figure 1.1 A life cycle of elephantiasis (CDC, 2017)

The adult worm lives in the human lymph vessels, mates and produces millions of microscopic worms (CDC, 2017). When a mosquito bites a person who has elephantiasis, it ingests the microfilaria and becomes infected. While inside a mosquito, the microfilaria grows and develops over a period of a week into infectious larvae. Subsequently, when the mosquito bites another person, the larval worms pass from the mosquito and migrate to the human lymphatic system. They grow into adult worms which is a process that takes at least 6 months. Adult worms live for about 5 to 7 years, they mate and release millions of microfilariae into blood stream (Taylor, 2010).

1.5 Pathogenesis of elephantiasis

The pathogenesis of elephantiasis is linked to host inflammation invoked by the death of the parasite, causing lymphoedema and elephantiasis (Taylor *et al*., 2010). The infection involves asymptomatic, acute and chronic conditions (WHO, 2014 & 2017). According to Molyneux (2003), the infection is usually acquired in

childhood where it is asymptomatic and then progresses till it reaches adult stage where it is now chronic.

The asymptomatic stage of filarial infection is characterized by the presence of live adult parasites in the lymphatic system and microfilariae in the blood without any outward evidence of the disease. Although the parasite damages the lymphatic system, most individuals can remain asymptomatic for years and will never develop clinical symptoms. Hence, most people who are infected by the disease are completely unaware of their condition especially in the early stage of the infection but only realise when the disease has advanced and certain symptoms are being manifested as presented in Figure 1.1 (Bockarie *et al.*, 2009; Maurya *et al*., 2015).

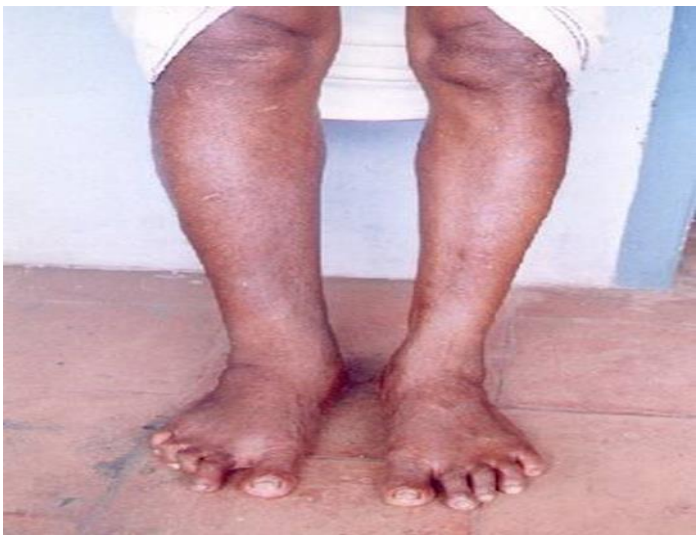

Figure 1.2 Asymptomatic stage of elephantiasis (Oscar *et al*., 2014)

Acute manifestations of elephantiasis are characterised by symptoms of adenolymphangitis (ADLA) with high fever, swollen and tender lymph glands, and inflammation of lymph vessels (Dreyer & Piessens, 2000). In addition, according to Shenoy (2008), the most widely recognized acute clinical signs in elephantiasis include fever, chills, vomiting, toxemia, changed sensorium and urinary incontinence. These scenes are more continuous in higher grades of lymphoedema. The most infected region become painful, warm, red, swollen and tender. Red streaks might be visible along the swollen lymphatic vessels (Figure 1.2). The draining lymph nodes in the groin or axilla may become swollen and

tender. The presentation may be with lymphangitis, lymphadenitis, cellulitis, or abscess formation (Shenoy, 2008).

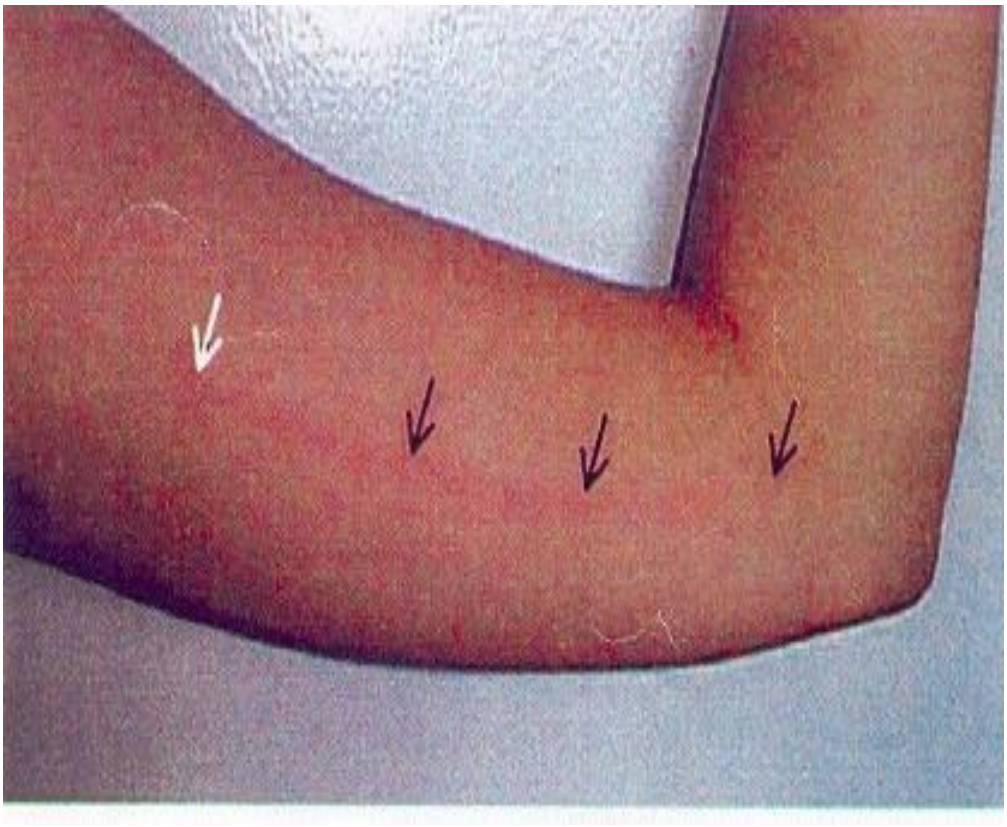

Figure 1.3 Acute stage of elephantiasis (Oscar *et al*., 2014)

Lymphoedema is a chronic clinical manifestation of elephantiasis that evolves slowly over the years (Shenoy, 2008). The lower limbs are usually affected, either unilaterally or sometimes bilaterally in which case the swelling tends to be asymmetrical. The upper limbs, male genitalia (Figure 1.3) and rarely breasts in females may also be affected. According to Kumaraswani (2000) and Shenoy (2008), the lymphoedema of limbs is commonly categorized into four grades: pitting oedema which is reversible on elevation of the affected limb (Grade I); pitting or non-pitting oedema which does not reverse on elevation of the affected limb and there are no skin changes (Grade II); non-pitting oedema that is not reversible, with thickening of the skin (Grade III) and non-pitting oedema that is not reversible, with thickening of the skin along with nodular or warty excrescences which is the last stage of elephantiasis (Grade IV).

In advanced stages of lymphoedema, the skin is thickened with deep folds, often with hypertrichosis, black pigmentation, nodules, warty growth, intertrigo in the webs of toes or chronic non-healing ulcers (Burri *et al*., 1996; Shenoy, 2008). The deep folds are often susceptible to fungal and bacterial infections (Shenoy, 2008). According to Shenoy (2008), inactivity of the lymph encourages bacterial growth. These bacteria, particularly streptococci and other pathogens resulted by the acute attack of ADLA (Suma *et al*., 1997; Vincent *et al*., 1998; Shenoy, 2008). The patient suffering from advanced stages usually are incapacitated.

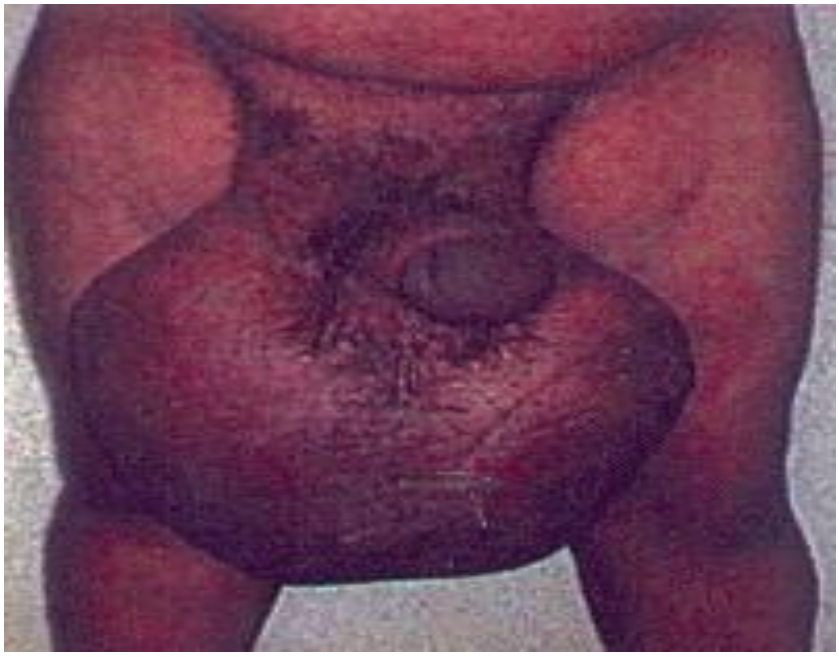

Figure 1.4 Chronic stage of elephantiasis (Oscar *et al*., 2014)

1.6 Prevalence of elephantiasis

Elephantiasis tends to receive less attention than other diseases such as Acquired Immune Deficiency Syndrome (AIDS) and tuberculosis (TB) since it is not a fatal disease. However, its most obvious manifestations disfigure and socially isolate people (Ottesen *et al*., 1997). The disease is known to occur in 73 countries, in which 38 are in the African region, 7 in the American regions, 4 in the Eastern Mediterranean region, 8 in the South-East region and 16 in the Western Pacific region (Mandal, 2014). Elephantiasis is a long-standing persistent disease which is widespread in many parts of the tropics and sub-tropics on the planet affecting millions of men and women (Maurya *et al*., 2015). It is estimated to be transmitted in 37 out of the 49 sub-Saharan African countries with an estimated at-risk population of 432 million people (Sodahlon *et al*., 2016). In the 20th century, more

than 120 million people were reported to be affected by the disease with 40 million people seriously incapacitated and disfigured by the disease (Maurya *et al*., 2015; WHO, 2017).

Currently, the global baseline estimate of persons affected by elephantiasis is 25 million men with hydrocele and over 15 million people with lymphoedema (WHO, 2017). Globally, about 40% of men and women have filarial burdens (Maurya *et al*., 2015), and almost 25 million men within this population suffer penile filariasis while another 25 million people are enduring more as they are starting to become dysphemistic due to the disease (WHO, 2006). Frequently, people from poor background are the ones suffering from elephantiasis and about 1. 4 billion people are at risk of infection in 72 countries where filaria is endemic (WHO, 2009; Maurya *et al*., 2015). A few reports have shown that youngsters in endemic zones experience the ill effects of lymphoedema of the appendages, hydrocele and ADLA assaults (Ramaiah, 2000; Shenoy, 2008). The presence of live adult filarial worms were observed on Doppler sonography and lymphatic dilatation by lymphoscintigraphy in children between the ages of 3 and 15 years (Shenoy, 2008). In an investigation on counteractive action of ADLA assaults in adult patients with filarial lymphoedema, 32% of the subjects interviewed recalled that the malady initially manifested before they were15 years old (Suma *et al*., 2002; Shenoy, 2008).

Within the African continent, elephantiasis is endemic in 39 countries and the population at risk is estimated at 390 million (Zagaria & Savioli, 2002; Simonsen *et al*., 2008). According to WHO (2006), people afflicted by this disease in the African continent rate up to 34%. Approximately 11 African countries reported 45 463 lymphoedema and 72 548 hydrocele cases (WHO, 2016). Nigeria has the heaviest elephantiasis burden with an estimated 120 million people at risk (Brant *et al*., 2018). The country has the highest number of cases of elephantiasis and ranks third globally (Hotez *et al*., 2012; Okorie *et al*., 2015). The main vectors of elephantiasis in Nigeria are mosquitoes of the *Anopheles gambiae* (principally *A. gambiae senso stricto* and *A. arabiensis*) and *A. funestus* complexes (Lenhart *et al*., 2007; Okorie *et al*., 2011 & 2015). Elephantiasis has been reported in the coastal and rain-forest zones of Nigeria (Ufomadu *et al*., 1992; Mbah & Njoku,

2000; Anosike *et al*., 2005; Christiana *et al*., 2014). Many of the studies carried out in Nigeria have reported the infection rates which are far beyond, WHO recommended 1% threshold necessary for the initiation of mass treatment (Udoidung *et al*., 2008; Christiana *et al*., 2014).

Similarly, it has been well established that elephantiasis is endemic in rural areas of Ghana (Gbakima *et al*., 2005; Simonsen & Mwakitalu, 2013; Yirenkyi, 2015). In Ghana, elephantiasis is prevalent in 61 out of 128 districts and in 9 out of 10 regions (Yirenkyi, 2015). The disease is distributed along the coastal belt and the northern region of the country (De Souza et al., 2010; Aboagye-Antwi *et al*., 2015; Yirenkyi, 2015). Stanton *et al*. (2015) identified 92 cases, 58 of which reported hydrocele, 33 reported lymphoedema and one reported both conditions.

Kenya showed a high prevalence of bancroftian filariasis (Njenga *et al*., 2011). The overall microfilarial density was 223 microfilariae/ml blood and significantly higher in males (336 microfilariae/ml) than in females (212 microfilariae/ml) (Estambale *et al*., 1994). The prevalence of clinical signs was age-dependant with infection being higher in males (16.5%) than in females (Estambale *et al*., 1994), with hydrocele being the major clinical sign (Estambale *et al*., 1994; Njenga *et al*., 2011). In Malawi, elephantiasis is highly endemic and is transmitted by a combination of both *Anopheline* and *Culicine* mosquito vectors (Bøgh *et al*., 1998). Approximately 12.8 million people in Malawi are at risk of infection with *W. bancrofti* (Smith *et al*., 2014; WHO, 2014). The Gambia is among 73 countries considered endemic for elephantiasis (WHO, 2012; Rebollo *et al*., 2015). Data from the Ministry of Gender, Sports, Culture and Social Services reveals that the number of traditional medical practitioners registering their commercial enterprises/herbal clinics (usually in the urban areas) is on the increase. Further, the number of patients being treated in these herbal clinics is on the increase, sometimes reaching well over 500 patients per month (Njoroge, 2012).

In South Africa, elephantiasis is known as *umeqo* (isiZulu), *inyawo lendlovu* (isiXhosa) and *leoto la tlou* (Sesotho). According to a study undertaken by Berger (2014), approximately 31% of the South African population is at risk of being infected with elephantiasis. Of the total population at risk, 530 000 - 1.06 million

people are suffering from lymphoedema (Davey, 2014; Komoreng *et al*., 2017). In reports published by Dlamini (2011), News24 (2012) and eNCA (2013), patients are often turned away from some of the public hospitals in South Africa, reasons being that there is no treatment in the country or that the disease needs specialist care. People who are affected by this disease suffer from disability, as well as social and financial losses contributing to stigma and poverty (Evans *et al*., 1993, Ramaiah & Ottesen, 2014, Maurya *et al*., 2015; Komoreng et al., 2017). Treating elephantiasis remains a big problem in South Africa (eNCA, 2013).

1.7 Treatment of elephantiasis

Elephantiasis is not just treatable or controllable but it can be eliminated and cured if correct measures are taken (Ottessen, 2006). According to WHO (2016), the GPELF was launched in 2000 with mass drug administration (MDA) as the main strategy to stop the transmission of the infection and to eliminate the disease as a public health problem. Mass Drug Administration involves treatment with albendazole (400 mg) together with diethylcarbamazine (6 mg/kg) or albendazole (400 mg) plus ivermectin (150-200 µg/kg) (WHO, 2016). The MDA reduces the development or progression of microfilariae in the blood (Taylor *et al*., 2010). Antibiotics may also be used in treating bacterial cellulitis which arises secondary to lymphoedema and elephantiasis (AL-Niaimi, 2009; WHO, 2013, Asdourian *et al*., 2016). The antibiotic doxycycline is reported to be effective against infection caused by *W. bancrofti* (Asdourian *et al*., 2016). However, the mass treatment drugs of choice used to treat elephantiasis have side effects such as dizziness, nausea, fever, headache, or pain in muscles or joints (CDC, 2017; Maurya *et al*., 2015). There is, therefore, a need for an alternative treatment that is cheaper and readily available with fewer or no side effects. Some leads have been made by researchers to investigate the effects of several medicinal plants on filarial worm and many of them have been reported to have anti-filarial activity (Lin *et al*., 1999; Sahare & Singh, 2013; Maurya *et al*., 2015; Rana *et al*., 2016).

A large proportion of the population of developing countries uses traditional medicine alone but in some cases, they use it in combination with the western drugs to treat a wide variety of ailments. Herbal medicines have been used by mankind since time immemorial (Gurib-Fakim, 2006). Also in recent years, studies

on the pharmacological significance of medicinal plants have received tremendous attention of the pharmaceutical and scientific communities (Van Wyk, 2011). Treating elephantiasis using medicinal plants is a common practice in some parts of the world (Komoreng *et al*., 2017). For example, there are several herbs that have been prescribed by Ayuverdic medicine to treat elephantiasis (Agrawal, 1997; Ravishankar & Shukla, 2007). *Piper guineense* has been used in Ghana (Erhenri & Obadoni, 2015) while *Strychnos acudeata* is used in Madagascar (Heinrich, 2013) to treat elephantiasis.

Medicinal plants are also used in South Africa to treat elephantiasis. Research into the scientific validation of South African medicinal plants showed that they are used in the treatment of pain, inflammation, hypertension and parasitic diseases including those with anthelmintic and anti-bacterial activity (Taylor *et al*., 2000). Among the plant species that are used in traditional medicine are two plants of the genus *Rhoicissus* in the grape family Vitaceae with 15 accepted species (The Plant Africa, 2018).

The grape family (Vitaceae) is vast, with around 1000 species spread all through the warm nations of the world and is well known for its most commended part, the grapevine, *Vitis vinifera* (PlantzAfrica, 2018). In Southern Africa, this family is represented by 5 genera (*Ampelocisssus*, *Cayratia*, *Cissus*, *Cypostemma* and *Rhoicissus*) and 53 species (PlantzAfrica, 2018). The genus *Rhoicissus* has 10 species that are found in every territory of South Africa, with the exception of the Northern Cape, and all other southern African countries except from Namibia and Botswana (PlantzAfrica, 2018). The name *Rhoicissus* is derived from the Greek *rhoia*, which means pomegranate and ivy (PlantzAfrica, 2018). It has a great number of common names, for the most part in regards to its similarity to the developed grapevine, or its rope-like stems.

1.7.1 *Rhoicissus tomentosa* (Lam.) Wild & R.B. Drumm.

Rhoicissus tomentosa (Figure 1.4) is commonly known as '*Isinwazi*' in isiZulu. It is an evergreen tendril climber with ornamental, vine-like leaves with purple grape like fruits. *Rhoicissus tomentosa* is generally simple to distinguish since it has straightforward or shallowly lobed leaves and it is the main shelter climber

(PlantzAfrica, 2018). The Latin species name *R*. *tomentosa* implies felt-like, with a thick woolly covering, insinuates the rust-hued hairs that cover the youthful development, the underside of the leaves, buds and rings (PlantzAfrica, 2018). It grows from Cape Peninsula, all the way through the Eastern Cape up to northern KwaZulu-Natal, Mpumalanga, Limpopo, Zimbabwe and Malawi (Gumede, 1978; Hutchings, 1996). It is used to treat elephantiasis, inflammation, to expel worms and to ensure safe delivery during pregnancy (Hutchings, 1996; Nqolo, 2008).

Figure 1.5 *Rhoicissus tomentosa* (PlantzAfrica, 2018)

1.7.2 *Rhoicissus tridentata* (L.f.) Wild & R.B.Drumm.

Rhoicissus tridentata (Figure 1.5) is commonly known as '*Imboziso*' in isiZulu. It is a shrub that grows in grassy hillsides of Western Cape, KwaZulu-Natal, Free State, Lesotho, Mpumalanga, Swaziland, Limpopo, North-West, Gauteng, Mozambique and Yemen. The leaves are leathery, shiny dark green above and pale underneath (PlantzAfrica, 2018). The fruits are roundish, about 18 mm in diameter and ripen in late summer-winter. Chopped roots are used to treat inflammation; it is also used as an anthelmintic and to ensure safe delivery during pregnancy (Hutchings, 1996; Nqolo, 2008). According to a study undertaken by Katsoulis *et al*. (2000), the tuberous rootstock is utilized therapeutically although it is poisonous. The roots boiled in milk are given to calves to expel intestinal worms (PlantzAfrica, 2018). The South African traditional healers use roots of *R. tridentata* as an anthelmintic

and anti-inflammatory (Komoreng *et al*., 2017). It is also used to treat babesiosis, a tick-borne disease of cattle (Naidoo *et al*., 2006).

Figure 1.6 *Rhoicissus tridentata* (PlantzAfrica, 2018)

1.7.3 Ecology of *R. tomentosa* and *R. tridentata*

According to a report by PlantzAfrica (2018), the organic products of *R. tomentosa* and *R. tridentata* are eaten by feathered creatures like birds and warm blooded creatures (Human beings) and the leaves are perused by diversion. The natural products are said to be especially well known with Knysna and Purple-peaked louries. The swollen tubers, albeit dangerous, are eaten by bushpigs and porcupines. Silver Striped Hawkmoth caterpillars (Hippotion celerio) have been recorded eating the leaves of *R*. *tomentosa* and *R. tridentata* (PlantzAfrica, 2018).

The leaves display the wonder of guttation exceptionally well, with drops of clear, watery liquid on the edges. These drops of water are not dew but drops of fluid constrained out of the plant. When soil moisture is high and climatic conditions are bad for transpiration, the root weight develops and water is pushed out of the tips of veins (PlantzAfrica, 2018).

1.8 Justification of the study

Rhoicissus tomentosa and *R. tridentata*, species of the family vitaceae, are indigenous to the Afromontane forests of southern Africa (PlantzAfrica, 2018; Van Nieukerken & Geertsema, 2015). Both species are known for their medicinal properties, especially for their use in ensuring safe delivery, the treatment of inflammation and to expel worms (Hutchings, 1996; Varga & Veale 1997; Nqolo, 2008). The *R. tridentata* is known to possess good antioxidant properties while *R. tomentosa* only possess these properties at very high concentrations (Atawodi, 2005). The *R. tridentata* is also reported to possess antimicrobial and anti-inflammatory properties (Steenkamp *et al.,* 2007; Adebayo *et al*., 2015). Decoction is prepared from the tuber and taken as an uterotonic (Katsoulis *et al*., 2002). Leaves of *R. tridentata* were also reported to contain anthocyanins, coumarins, flavonoids, saponins, sterols and triterpenes (Chhabra *et al*., 1984; Katsoulis, 2000). Rhizomes of *R. tomentosa* were reported to contain tannins, flavonoids, steroids, reducing sugars, alkaloids and saponins (Nqolo, 2008). According to studies undertaken by Lin *et al*. (1999), Nqolo (2008) and Maphosa *et al*. (2010), *R. tomentosa* possess antimicrobial and anti-inflammatory properties.

The presence of these reported phytochemicals, antimicrobial, anti-inflammatory and antioxidant properties justifies the usage of these two plant species in the treatment of elephantiasis and its symptoms. Moreover, since the two plant species are reportedly used by South African traditional healers to treat and manage elephantiasis, it was imperative to conduct a study comparing their biological and anti-inflammatory activities as well as their chemical profiles.

1.9 Aim

The aim of this study was to conduct a comparative study on pharmacological activities of *R. tridentata* and *R. tomentosa*, which are reportedly commonly used in the treatment of elephantiasis in some parts of South Africa.

1.10 Objectives

The specific objectives were:

- To assess antibacterial activity of *R. tomentosa* and *R. tridentata*.

- To assess antifungal activity of *R. tomentosa* and *R. tridentata*.
- To assess antimycobacterial activity of *R. tomentosa* and *R. tridentata*.
- To assess anti-inflammatory activity of *R. tomentosa* and *R. tridentata*.
- To assess anthelmintic activity of *R. tomentosa* and *R. tridentata*.
- To compare the chemical profiles of *R. tomentosa* and *R. tridentata*.

CHAPTER 2
MATERIALS AND METHODS

2.1 Study area

The study was based in Vryheid in the eastern part of KwaZulu-Natal, in Abaqulusi municipal area (Figure 2.1), which is a rural settlement. Vryheid lies within the coordinates latitude 27°46.1712′ S and longitude: 30°47.499′ E (Dateandtime.info, 2018). This area is surrounded by farms and dominated by a grassland biome. The area normally receives about 688 mm of rain per year, with most rainfall occurring during summer. It receives the lowest rainfall (3 mm) in June and the highest (122 mm) in December (Map data, 2017). The monthly distribution of average daily maximum temperatures shows that the average midday temperatures for Vryheid range from 19.6°C in June to 26.4°C in January. The region is the coldest during July when the mercury drops to 3.5°C on average during the night (Map data, 2017).

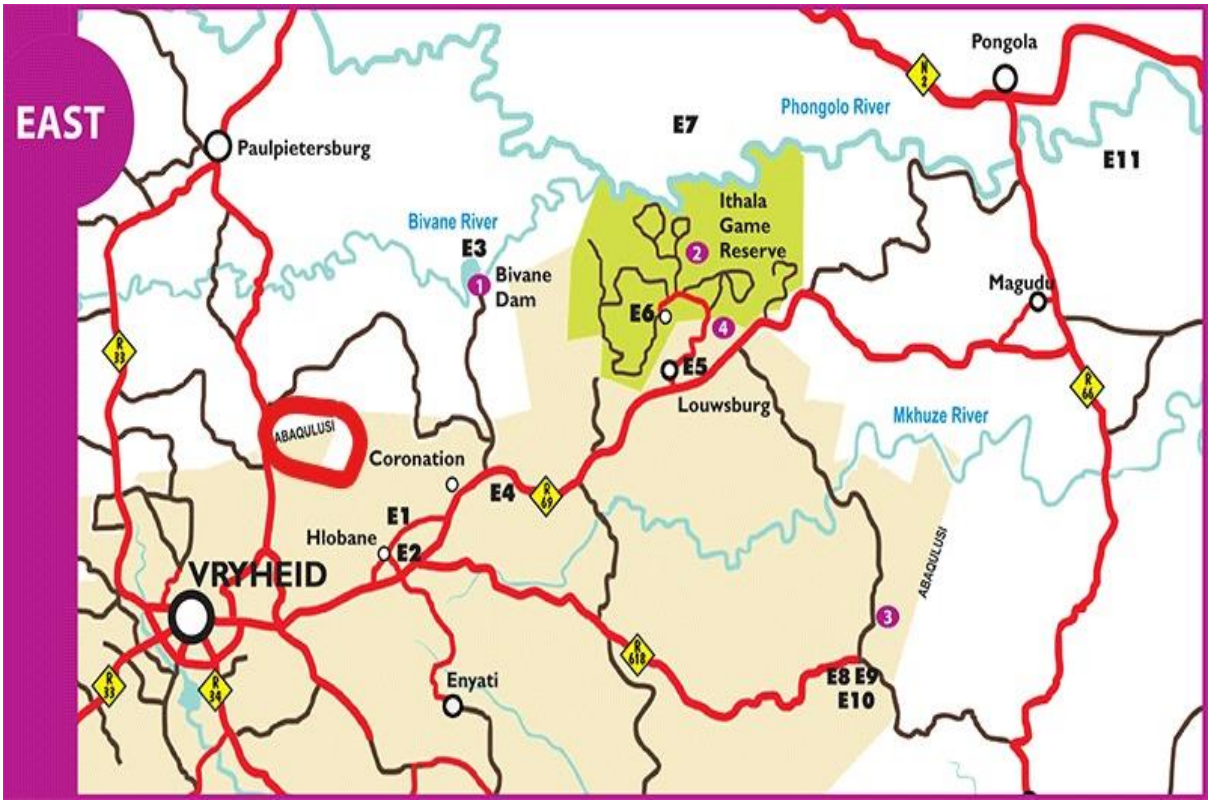

Figure 2.1: A map showing the study area, Vryheid within Abaqulusi Municipality (Map data, 2017)

2.2. Plant Collection

Rhoicissus tomentosa and *R. tridentata* were collected through consultations with traditional healers and herbalists from Vryheid in the Abaqulusi Municipal area in KwaZulu-Natal Province of South Africa. Voucher specimen (MDLE 01 and MDLE 02) for each plant was prepared and deposited at the herbarium of the University of the Free State (QwaQwa Campus).

2.3. Intellectual property agreement statement

All the traditional healers and herbalists who contributed information to this project were adequately financially rewarded with further verbal agreement that this research shall not be for commercial purposes, but to serve as an enlightenment to the community and the entire country on the two plant species that are used to treat elephantiasis.

2.4. Plant preparation

The collected plants were oven dried at 40°C and ground to fine powders using a blender (Model KM 1500, manufactured by RADWAG Wagi elektroniczne in Poland (EU). Subsequently, they were stored in sealed clear-plastic honey jars in the dark at room temperature until further processing.

2.5. Plant extraction

Three separate samples of 5 g each were extracted with 50 ml of distilled water, ethanol and acetone, respectively. Plant extraction was performed by leaving powdered plant material with solvents (water, acetone and methanol) in a shaker overnight. The plant extracts were then filtered through Whatman No. 1 filter papers. The filtrates were dried with a fan. Plant extraction was done by weighing plant extracts until a constant weight was obtained. The residues were stored at 10°C in the fridge until further use.

2.6. Antibacterial activity

Pseudomonas aeruginosa, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Enterococcus feacalis*, *Proteus vulgaris*, *Shigella flexneri* were obtained from the University of Fort Hare Department of Biochemistry and Microbiology. They were then maintained on Mueller Hinton (MH) agar plates and invigorated for bioassay by culturing a single colony in 2 ml MH broth for 24 h, after which the optical density (OD) at 600 nm for each liquid culture was determined. The saturated bacterial cultures were then diluted with MH broth (1 ml bacteria: 99 ml broth), to ascertain the log phase of bacteria when the test started.

The microplate method of Eloff (1998) was used to determine the minimal inhibitory concentration (MIC) values for plant extracts with antibacterial activity. Residues of plant extracts were dissolved at 50 mg/ml with the extracting solvents. All extracts were initially tested at 12.5 mg/ml 96-well microplates and serially diluted two-fold to 0.098 mg/ml, after which 100 µl bacterial cultures were added to each well. The antibiotic neomycin was included as standard in each essay. Extract-free solution was used as a blank control. The microplates were incubated overnight at 37°C. As an indicator of bacterial growth, 40 µl *p*-iodonitrotetrazolium (INT) violet dissolved in water was added to the wells and incubated at 37°C for 30 minutes. MIC values were recorded as the lowest concentration of the extract that completely inhibited bacterial growth, for example a clear well. When colorless tetrazolium salt accepts an electron, it changes to a red formazan present in organisms while bacterial growth is inhibited, the solution in the well remain clear after incubation with INT (Eloff, 1998). All extracts were tested in triplicates.

2.7. Antifungal screening

A modification of the National Committee for Clinical Laboratory Standards proposed method (M27-P), the broth microdilution test was performed as described previously (Espinel-Ingroff *et al*., 1995; Buwa & van Staden, 2006). Four millilitres of sterile saline were added to approximately 400 µl of 24-h-old *Candida albicans*, *C. vulgaris* and *Trichophyton mucoides* which were used as test microorganisms. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 McFarland standard solution. From the prepared stock fungal cultures, a

1:1000 dilution with nutrient broth (e.g. 10 µl stock yeast culture: 10 ml broth) was prepared.

Water extracts were dissolved in distilled water whereas organic solvent extracts were dissolved in dimethyl sulfoxide (DMSO). All extracts were dissolved at 100 mg/ml. One hundred microliters of nutrient broth was added to each well of a 96-well microplate. One hundred microliters of the water extract was added to well (A) and serially diluted from (A) by taking 100 µl into (B). This two-fold dilution was continued down the plate and 100 µl from the last well (H) was discarded. In the case of organic solvent extracts, 25 µl of the extracts was added to 175 µl broth and serially diluted. Three replicates were prepared for each extract. All the wells were then filled with 100 µl of stock fungal culture. Amphotericin B was used as a standard for this experiment and the following controls were prepared: wells containing broth only, fungal strain with no extract, and serial dilutions of DMSO with the fungi. The plates were covered and incubated at 33°C overnight. After incubation, 40 µl of INT was added to the wells and incubated for 30 minutes. MIC values were recorded.

2.8 Antimycobacterial activity

Preparation of Mycobacterium stocks

Mycobacterium tuberculosis (ATCC 25177) was maintained in Middlebrook 7H9 broth containing 10% oleic acid + albumin + dextrose + catalase (OADC). Inoculum was prepared by transferring the stock bacterial culture to two 5 ml supplemented 7H9 broth (Middlebrook 7H9 + 10% OADC) and grown for 72 h on a shaking water bath at 37°C. Twenty percent sterile glycerol was added to each culture and 500 µl aliquots were made into sterile Eppendorf tubes. These stocks were named G1 stocks and were stored at -30°C. A single G1 stock was used to inoculate supplemented Middlebrook 7H10 agar (7H10 + 10% OADC) plates and incubated at 37°C for four days or until growth was observed. From this culture, a single colony was used to inoculate 5 ml supplemented 7H9 broth. This was grown on a shaking water bath at 37°C for 72 h and used for the experiment.

The broth microdilution method (Swenson *et al*., 1982) was used to determine the MIC values for plant extracts with antimycobacterial activity. The aqueous extract

residues were dissolved in distilled water and other extract residues were dissolved in DMSO. All extracts were dissolved to a concentration of 100 mg/ml. One hundred microliter of the supplemented 7H9 broth was added to all the wells of microtitre plates. All extracts were tested at the highest concentration of 25 mg/ml and serially diluted to 0.195 mg/ml. The optical density of the 72-h-old broth culture was determined and adjusted at 550 nm. One hundred microliter of the diluted culture was added to every well of the microtitre plate. The controls included the solvent used to dissolve plant extracts, Middlebrook 7H9 broth alone, and the antibiotic streptomycin (1.56 mg/ml) as a positive control. The plates were covered and incubated at 37°C for 72 h. After incubation, 40 µl of 0.4 mg/ml solution of INT was added to each well of the plate. The plates were covered and incubated for 24 h at 37°C. All extracts were tested in triplicates.

2.9 Anthelminthic activity

The method of Rasoanaivo & Ratsimamanga-Urverg (1993), modified by McGaw *et al*. (2007), was used. Sheep faecal samples were collected from Golden Gate Highlands National Park, in Eerstegulk farm in the Free State Province. About 10 g each of faecal sample was weighed and incubated with 10 grams of vermiculite at 26ºC for 7- 10 days. The L_3 larvae were harvested from the *in vitro* cultures prepared and transferred into a single petri dish. One hundred and fifty microlitres of the solution containing about 10 - 15 nematodes was incubated with 0.5, 1 and 2 mg/ml of plant extracts respectively for 2 h at 25 °C in the dark. The anthelmintic Levamisole was used as a positive control, and solvent blanks were included. The percentage of living nematodes was estimated using a stereomicroscope.

All live and motile L_3 stage larvae in each petri dish were counted and a percentage inhibition of larval development was calculated using the formula described by Coles *et al.* (1992) & Bizimenyera *et al*. (2006) with slight changes:

Inhibition percentage (%) = $100(1-X_1/X_2)$

Where X_1 is the initial number of larvae in test extracts pre-treatment, and X_2 is the number of larvae obtained post-treatment.

2.10 Anti-inflammatory assay

The 5-lipoxygenase (5-LOX) enzyme activity was determined using the method reported by Evans (1987) and modified by Baylac & Racine (2003) & Trouillas *et al*. (2003). Linoleic acid was used as the substrate. A stock solution containing 10 µl of plant extract dissolved in a 1:29 w/w solution of Tween 20 in DMSO, 2.95 ml pre-warmed 0.1 M potassium phosphate buffer (pH 6.3) prepared with analytical grade reagents and 50 µl linoleic acid solution (100 µM final concentration) was prepared. Twelve microliters of 5-LOX and 12 µl phosphate buffer were added. All extracts were initially tested at a concentration of 0.4 mg/ml and serially diluted to 0.02 mg/ml. The mixture was then transferred to a cuvette and the contents of the cuvette were shaken and read in the spectrophotometer (Model 7305, manufactured by Cole-Parmer Ltd in UK). Absorbance was measured at 234 nm over a period of 10 min. Nordihydroguaiaretic acid (NDGA) was used as a positive control (enzyme inhibition). The IC_{50} value, i.e. the concentration at which 50% of the enzyme was inhibited for each test sample was determined using Graphpad prism. The percentage inhibition of the enzyme activity was calculated by comparing with the negative control (Tween® 20 and DMSO). The experiment was conducted in duplicates.

2.11 Analysis of chemical composition

Approximately 0.5 mg of *R. tomentosa* and *R. tridentata* ethanol and acetone extracts were applied onto a plastic-backed Thin Layer Chromatography (TLC) plates (Merck Silica gel 60 $F_{254\ nm}$) as 1 cm bands. The plates were developed over 14.5 cm using ethyl acetate hexane (1:1). The chromatographs of plates were evaluated directly under UV $_{254\ nm}$ and UV $_{365\ nm,}$ after which they were stained with anisaldehyde/ sulphuric acid spray reagent, heated for 5 - 10 min at 100°C and observed for colour development.

Data was recorded by scanning anisaldehyde and bioautography and also by taking photographs of different fingerprints of the various extracts under UV light at 366 nm (long wavelength exposing blue light) and 254 nm (short wavelength exposing green light). UV light detection is non-destructive (Gibbons & Grey,

1998), making it a favourable technique for compound detection. Retardation Factor (Rf) values determine the locality of various compounds. These values were calculated by determining the ratio of the distance travelled by the band maximum to the distance travelled by the leading edge of the mobile phase.

2.11.1 Bioautography assay

Organic solvent extracts made from both *R. tomentosa* and *R. tridentata* (0.125 mg) were spotted onto a large TLC plate. The plate was developed in a solvent system over 17 cm. The solvent front was marked and the plate allowed to dry before visualisation under UV_{254nm} and $UV_{366nm.}$ For the bioautographic assay, *S. aureus* was used as the test organism (Martin & Eloff, 1998). An overnight culture of *S. aureus* was prepared in MH broth medium. The culture was centrifuged at 3000 g for 10 min and the supernatant medium discarded. The pellets of bacteria cells were combined and re-suspended in 10 ml of fresh MH broth. This broth culture was then sprayed onto a TLC plate and incubated overnight at 37°C in 100% humidity. Subsequently, the plate was allowed to dry slightly before spraying with a 2 mg/ml solution of INT. The plate was then re-incubated for a further 30 - 60 min to allow for colour development. Zones of inhibition appeared as white spots against a pink coloured background (Begue & Kline, 1972).

CHAPTER 3
RESULTS

3.1 Antibacterial activity

The results for antibacterial activity of extracts prepared from *R. tomentosa* and *R. tridentata* are presented in Table 3.1 below. In this study, extracts were considered highly active when the MIC values were between 0.098 and 0.78 mg/ml, moderately active at 1.56 mg/ml and poor activity between 3.125 and 12.5 mg/ml. The extracts prepared from both plants were highly active and showed the best activities against all the tested bacteria. Concerning *R. tridentata*, the ethanol and acetone extracts displayed the best antibacterial activity by inhibiting all test micro-organisms with MIC values ranging between 0.098 and 0.39 mg/ml. The water extract displayed the best activity against *E. coli* and *S. flexneri* at 0.39 mg/ml, with moderate activity (1.56 mg/ml) against the rest of the test microorganisms. The *R. tomentosa* extracts displayed the best antibacterial activity against the majority of the test bacteria with MIC values ranging from 0.098 to 0.39 mg/ml. Moderate activity was observed with aqueous extract against *K. pneumoniae* and *S. epidermidis* at 1.56 mg/ml.

Table 3.1: Antibacterial activity of *R. tomentosa* and *R. tridentata* extracts (MIC recorded in mg/ml)

Plant name	Part used	Extract yield (g)	Extracting solvents	Bacterial strains tested								
				E. faecalis (+)	*E. coli* (-)	*P. aeruginosa* (-)	*S. aureus* (+)	*P. vulgaris* (-)	*S. flexneri* (-)	*B. cereus* (+)	*K. pneumoniae* (+)	*S. epidermidis* (+)
R. tridentata	Shoot	110.9905	Ethanol	0.098	0.098	0.098	0.39	0.098	0.098	0.098	0.39	0.098
		110.3524	Acetone	0.098	0.098	0.098	0.098	0.098	0.39	0.098	0.39	0.098
		112.9155	Water	1.56	0.39	1.56	1.56	1.56	0.39	1.56	1.56	1.56
R. tomentosa	Bulb	112.4151	Ethanol	0.098	0.098	0.098	0.098	0.195	0.098	0.098	0.098	0.098
		113.9974	Acetone	0.39	0.195	0.098	0.098	0.098	0.098	0.195	0.39	0.195
		114.3987	Water	0.098	0.39	0.195	0.098	0.39	0.195	0.098	1.56	1.56
Neomycin (µg/ml)				0.39	0.78	0.39	0.78	0.39	0.78	0.39	0.78	0.39

(-) = Gram-negative; (+) = Gram-positive

3.2 Antifungal Activity

The results for antifungal activity of extracts prepared from *R. tomentosa* and *R. tridentata* are presented in Table 3.2 below. Plant extracts were considered highly active if MIC values ranged between 0.195 and 0.78 mg/ml, moderately active at 1.56 mg/ml and poor activity between 3.125 and 25 mg/ml. The acetone extract of *R. tridentata* displayed the best antifungal activity by inhibiting all three fungal strains at 0.195 mg/ml. The rest of the extracts displayed poor activity with MIC values ranging between 3.125 and 25 mg/ml.

Table 3.2: Antifungal activity of *R. tomentosa* and *R. tridentata* extracts (MIC recorded in mg/ml)

Plant name	**Part used**	**Extract yield (g)**	**Extracting solvent**	**Fungal strains tested**		
				T. mucoides	*C. albicans*	*C. vulgaris*
R. tridentata	Shoot	4.9905	Ethanol	12.5	12.5	12.5
		4.3524	Acetone	0.195	0.195	0.195
		4.9155	Water	25	3.125	25
R. tomentosa	Bulb	4.4151	Ethanol	3.125	6.25	3.125
		4.9974	Acetone	3.125	3.125	6.25
		4.3987	Water	6.25	12.5	12.5
Amphotericin B (µg/ml)				0.012	0.012	0.012

3.3 Antimycobacterial activity

The results for antimycobacterial activity are presented in Table 3.3. The organic solvent extracts displayed the best activity compared to water extracts. The organic solvent extracts prepared from both plants inhibited *M. tuberculosis* at 0.195 mg/ml. Moderate activity was observed with water extracts from both *R. tomentosa* and *R. tridentata* at 1.56 mg/ml.

Table 3.3: Antimycobacterial activity of *R. tomentosa* and *R. tridentata* (MIC recorded in mg/ml)

Plant name	**Part used**	**Extract yield (g)**	**Extracting solvent**	***M. tuberculosis***
R. tridentata	Whole plant	4.9905	Ethanol	0.195
		4.3524	Acetone	0.195
		4.9155	Water	1.56
R. tomentosa	Bulb	4.4151	Ethanol	0.195
		4.9974	Acetone	0.195
		4.3987	Water	1.56
Streptomycin (µg/ml)				0.012

3.4 Anthelmintic activity

The results for anthelmintic activity of plant extracts are presented in Table 3.4 and Figure 3.1 - 3.4 below. *Haemonchus contortus* was used as a model nematode due its anatomical resemblance to *W. bancrofti*. The water extract of *R. tridentata* displayed good anthelmintic activity against *H. contortus* with 50% larval mortality at a concentration of 2 mg/ml. The ethanol extract of *R. tridentata* displayed the best anthelmintic activity with 100% mortality at a concentration of 2 mg/ ml. At 1 mg/ml, 40% nematode mortality was observed.

Concerning *R. tomentosa*, 60% nematode mortality was observed at a concentration of 2 mg/ml, with 40% mortality at 1 mg/ml. The water extract displayed poor anthelmintic activity as compared to the control, levamisole.

Table 3.4: Anthelmintic activity of *Rhoicissus tomentosa* and *Rhoicissus tridentata* observed for a period of two hours

Plant name	Extracting solvent	Concentration (mg/ml)	Larval mortality (%)	Levamisole (positive control)	Extracting solvent (negative control)
R. tridentata	Water	0.5	10	100	00.0
		1	20	100	00.0
		2	50	100	00.0
	Ethanol	0.5	20	100	00.0
		1	40	100	00.0
		2	100	100	00.0
R. tomentosa	Water	0.5	10	100	00.0
		1	20	100	00.0
		2	30	100	00.0
	Ethanol	0.5	20	100	00.0
		1	40	100	00.0
		2	60	100	00.0

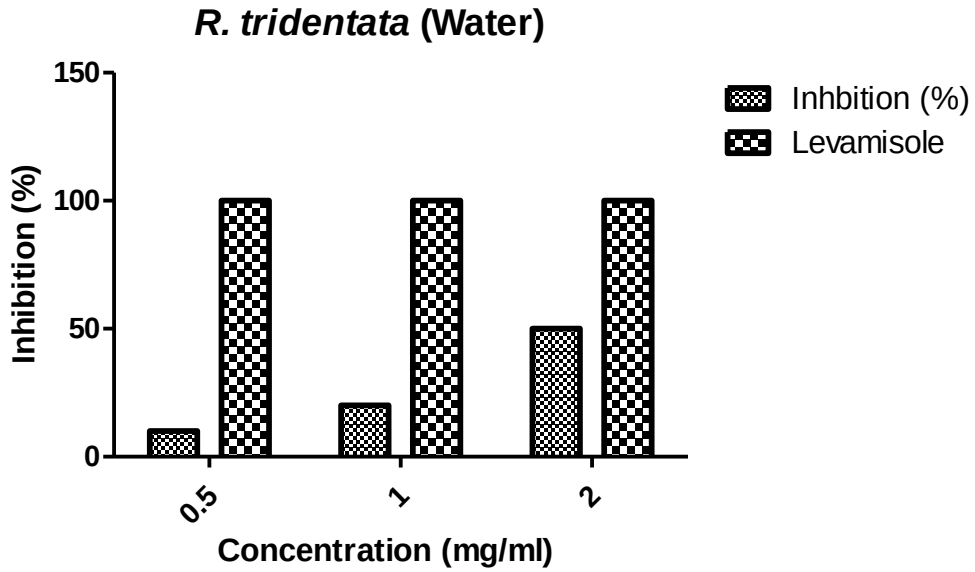

Figure 3.1: Anthelmintic activity of *R. tridentata* water extract

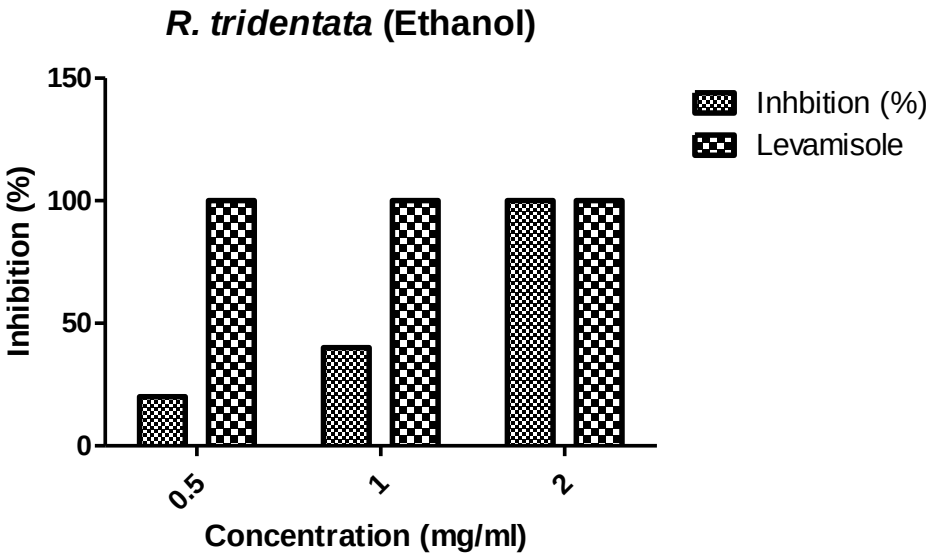

Figure 3.2: Anthelmintic activity of *R. tridentata* ethanol extract

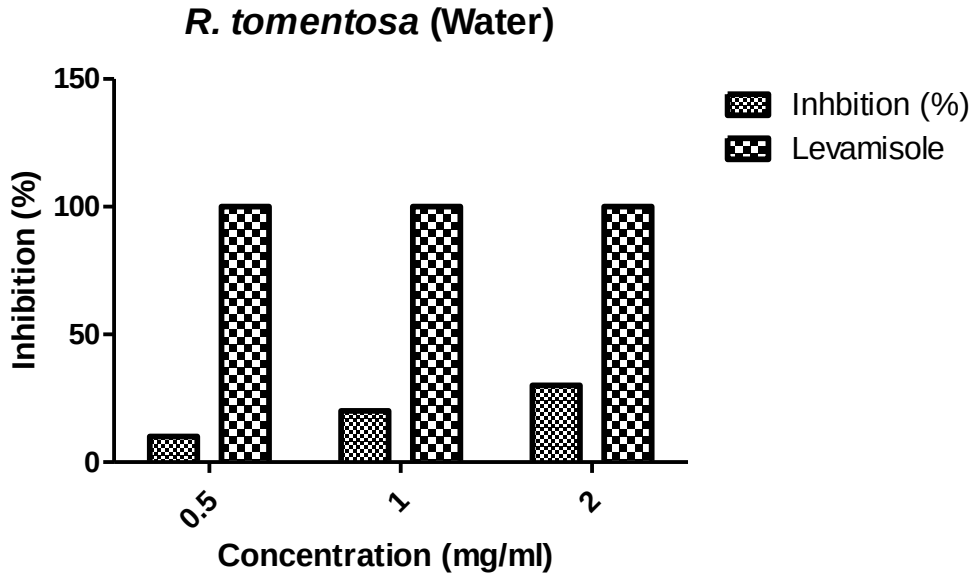

Figure 3.3: Anthelmintic activity of *R. tomentosa* water extract

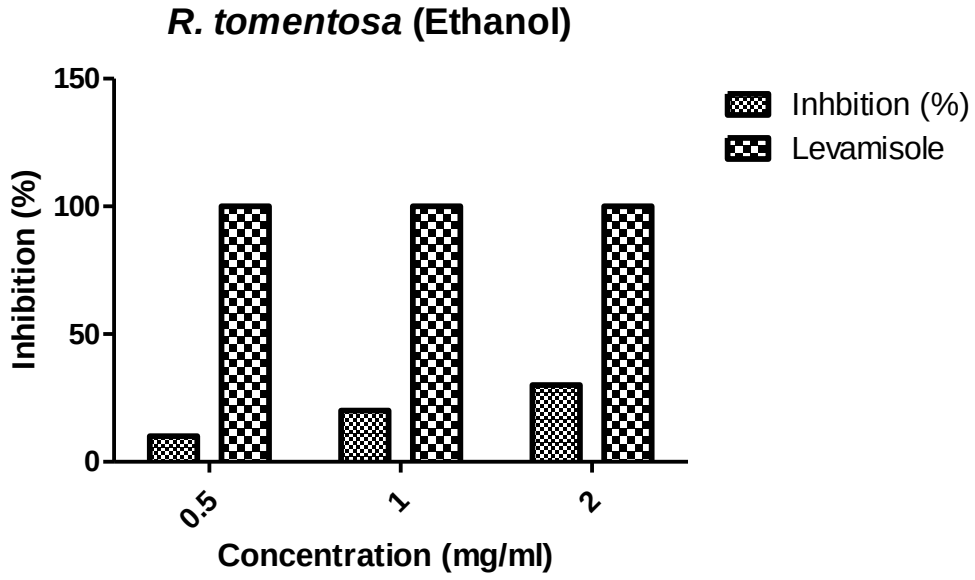

Figure 3.4: Anthelmintic activity of *R. tomentosa* ethanol extract

3.5 Anti-inflammatory activity

The results for anti-inflammatory activity of plant extracts are shown in Table 3.5 and Figure 3.5 to 3.10. The percentage of inhibition of ethanol, acetone and methanol extracts of two different plants at different concentrations is in comparison with NDGA. A higher inhibitory activity was associated with a lower or reduced IC_{50} value. Extracts from both *R. tomentosa* and *R. tridentata* displayed the best anti-inflammatory activity by inhibiting 5-LOX with IC_{50} values ranging between 0.10± 72.50 and 0.13± 6.650 µg/ml (Table 3.5). The *R. tomentosa* acetone, ethanol and water extracts displayed 92-99% inhibition as the concentration increased from the lowest (0.02 mg/ml) to the highest (0.40 mg/ml) (Figure 3.8 to Figure 3.10). Concerning *R. tridentata*, acetone and water extracts displayed 98 - 99% inhibition from the lowest (0.02 mg/ml) to the highest concentration (0.40 mg/ml) (Figure 3.5 and Figure 3.6). The *R. tridentata* ethanol extract displayed 83-92% inhibition, as the concentration increased from the lowest (0.02 mg/ml) to the highest concentration (0.40 mg/ml) (Figure 3.7).

Table 3.5: Anti-inflammatory activity of *R. tomentosa* and *R. tridentata* (IC_{50} values in µg/ml)

Plant name	**Extracting solvent**	**IC_{50}(µg/ml)**
R. tridentata	Water	0.10 ± 72.50
	Acetone	0.12.0 ± 5.300
	Ethanol	0.13 ± 29.30
R. tomentosa	Water	0.12 ± 145.0
	Acetone	0.13 ± 6.650
	Ethanol	0.11 ± 0.8700
NDGA	Control	0.14 ± 0.043

IC_{50} (mg/ml) ± Standard Deviation

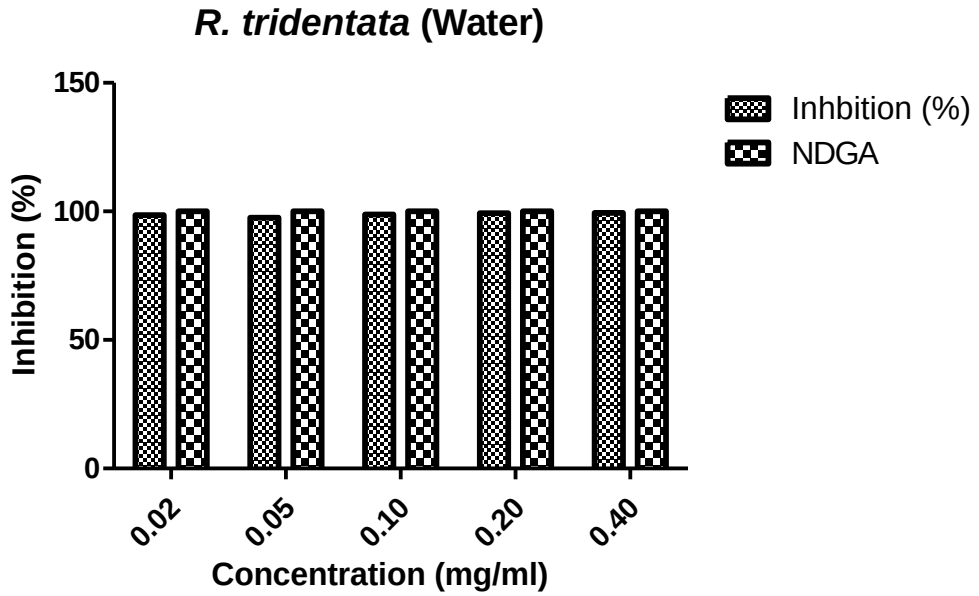

Figure 3.5: Anti-inflammatory activity of *R. tridentata* water extract

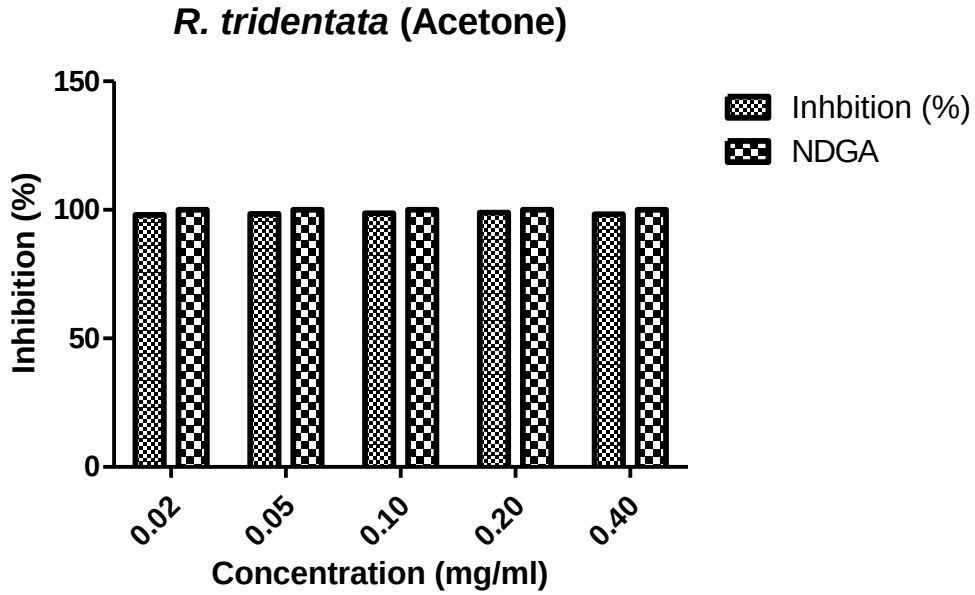

Figure 3.6: Anti-inflammatory activity of *R. tridentata* acetone extract

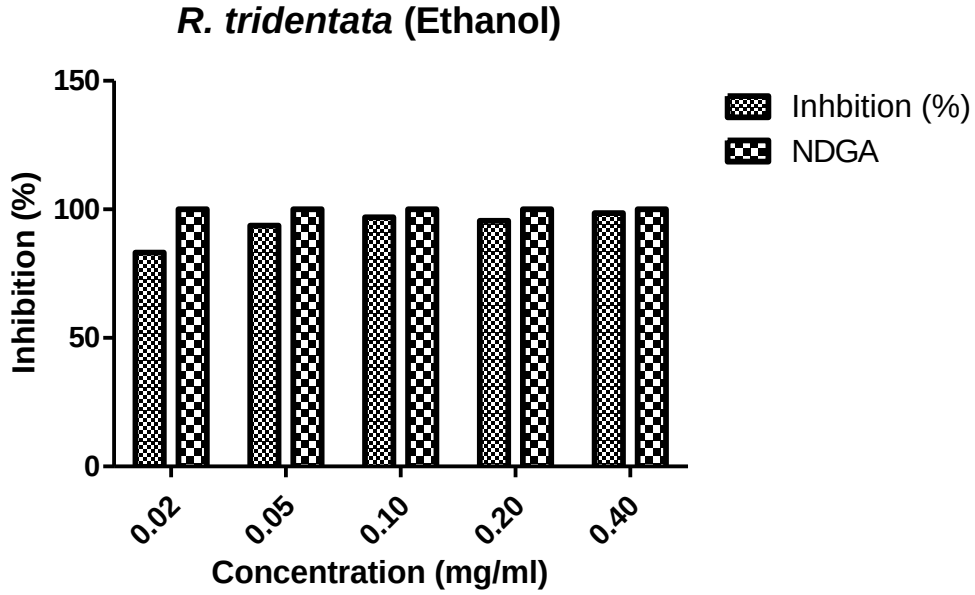

Figure 3.7: Anti-inflammatory activity of *R. tridentata* ethanol extract

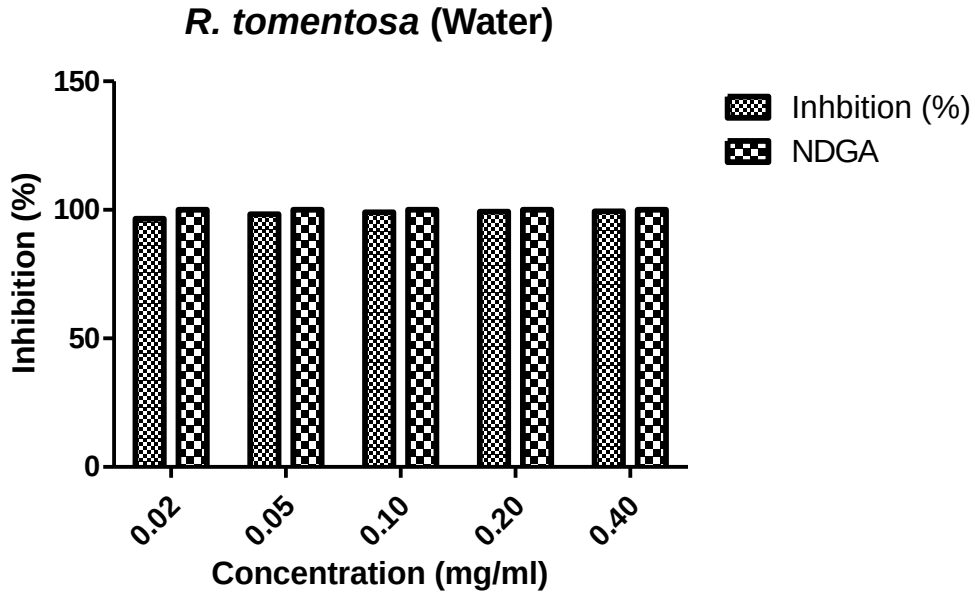

Figure 3.8: Anti-inflammatory activity of *R. tomentosa* water extract

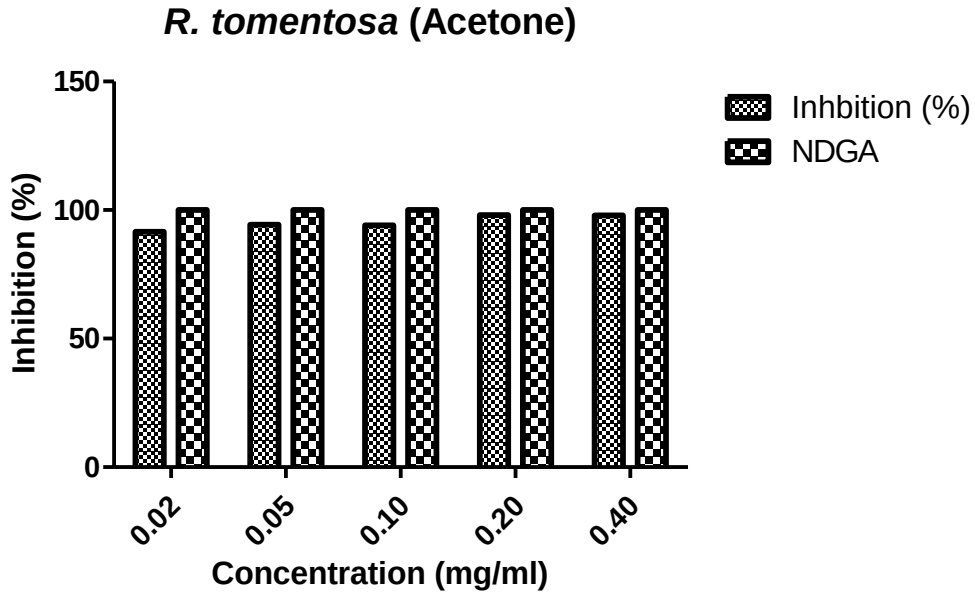

Figure 3.9: Anti-inflammatory activity of *R. tomentosa* acetone extract

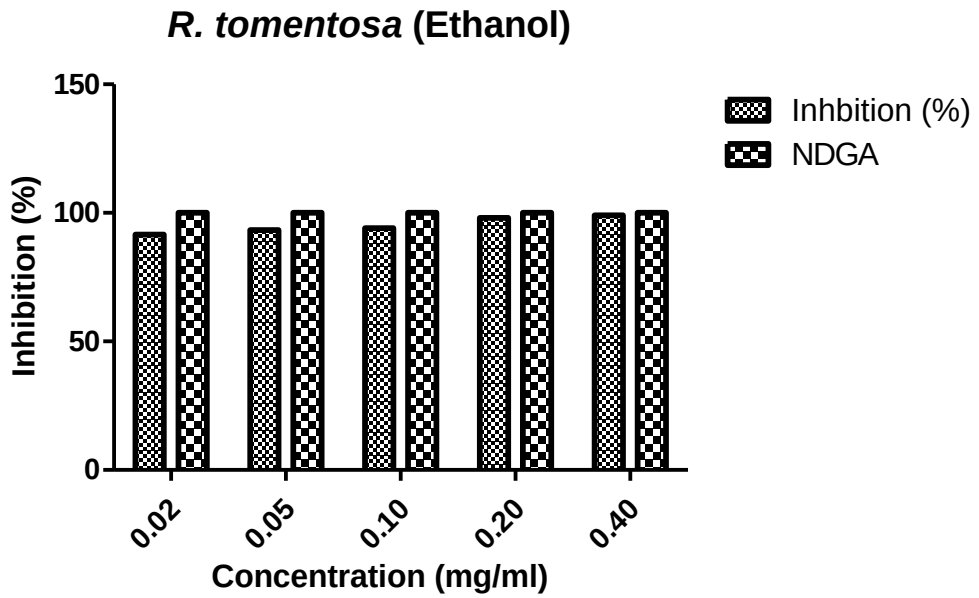

Figure 3.10: Anti-inflammatory activity of *R. tomentosa* ethanol extract

3.6 Analysis of chemical profile

The results for the analysis of chemical profile of *R. tomentosa* and *R. tridentata* are shown in Figures 3.11 to 3.13. In this study, the same R_f values and colours of various components indicated the same substance. Components were clear when viewed under UV 366 nm (Figure 3.12) whereas they were undetected at UV 254 nm. The TLC profile of both *R. tomentosa* and *R. tridentata* displayed the presence of bands/spots A and D with R_f values of 0.93 and 0.83 (band D), respectively. The *R. tomentosa* acetone (RTO_A) and ethanol (RTO_E) extracts displayed a red fluorescing compound (band B) with an R_f value of 0.89 (Figure 3.12). Furthermore, the RTO_A and RTO_E showed a green fluorescing compound (band C) with an R_f value of 0.85. A black compound (band E) with an R_f value of 0.79 was detected in *R. tridentata* acetone (RTR_A) and ethanol (RTR_E) extracts and *R. tomentosa* acetone extract (RTO_A).

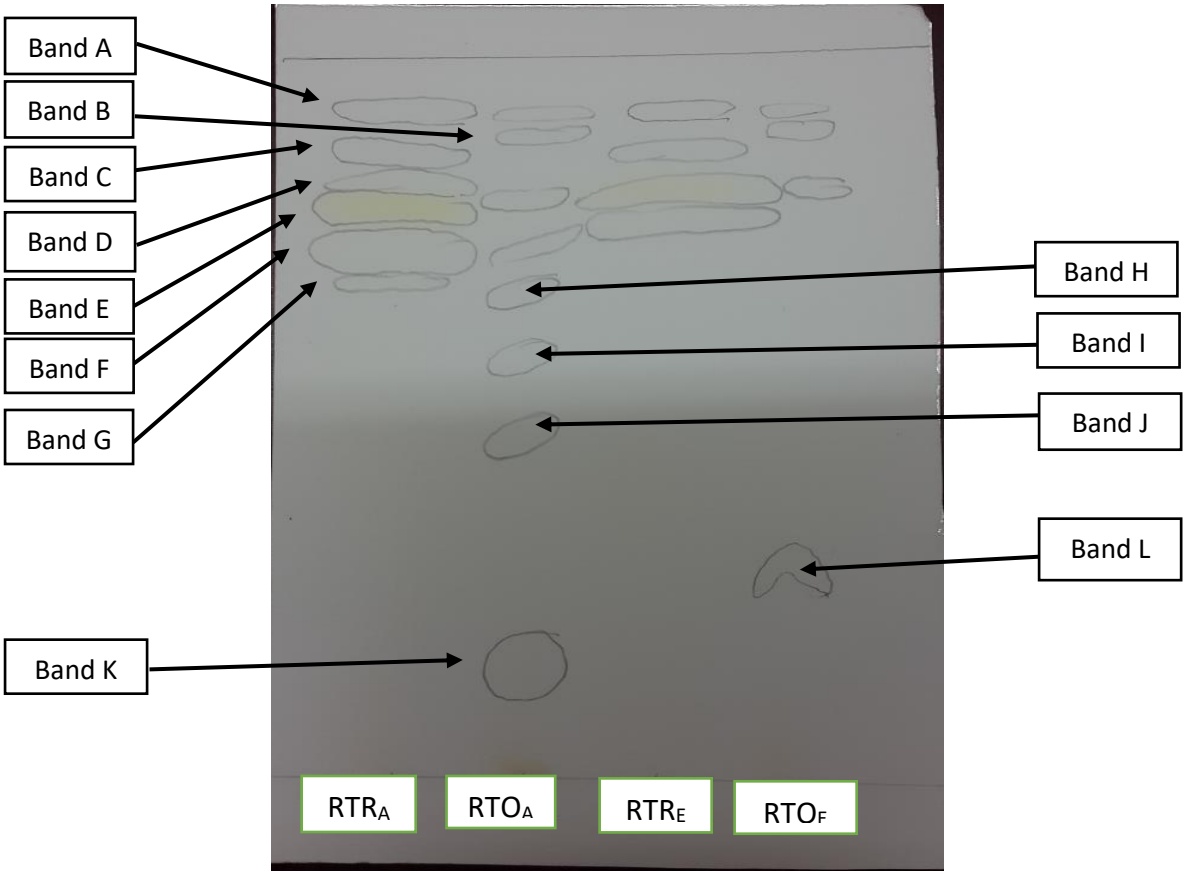

Figure 3.11: The TLC profiles of *R. tridentata* (RTR) and *R. tomentosa* (RTO) showing chemical components under visible light. Key: RTR_A: *R. tridentata* acetone; RTR_E: *R. tridentata* ethanol; RTO_A: *R. tomentosa* acetone; RTO_E: *R. tomentosa* ethanol. The TLC plate was developed in ethyl acetate: hexane (1:1).

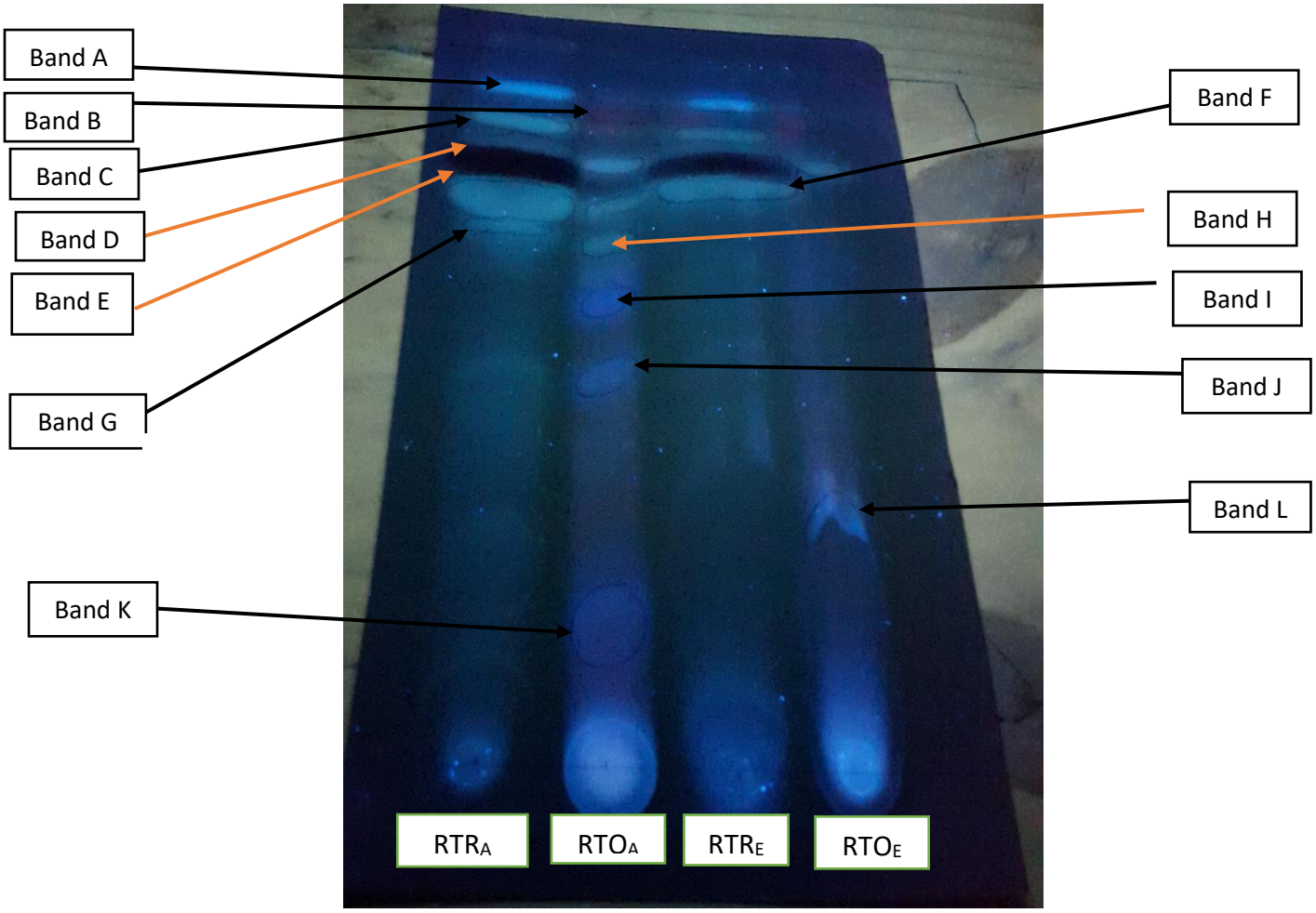

Figure 3.12: The TLC profiles of *R. tridentata* (RTR) and *R. tomentosa* (RTO) showing chemical components under UV light (366 nm). Key: RTR_A: *R. tridentata* acetone; RTR_E: *R. tridentata* ethanol; RTO_A: *R. tomentosa* acetone; RTO_E: *R. tomentosa* ethanol. The TLC plate was developed in ethyl acetate: hexane (1:1).

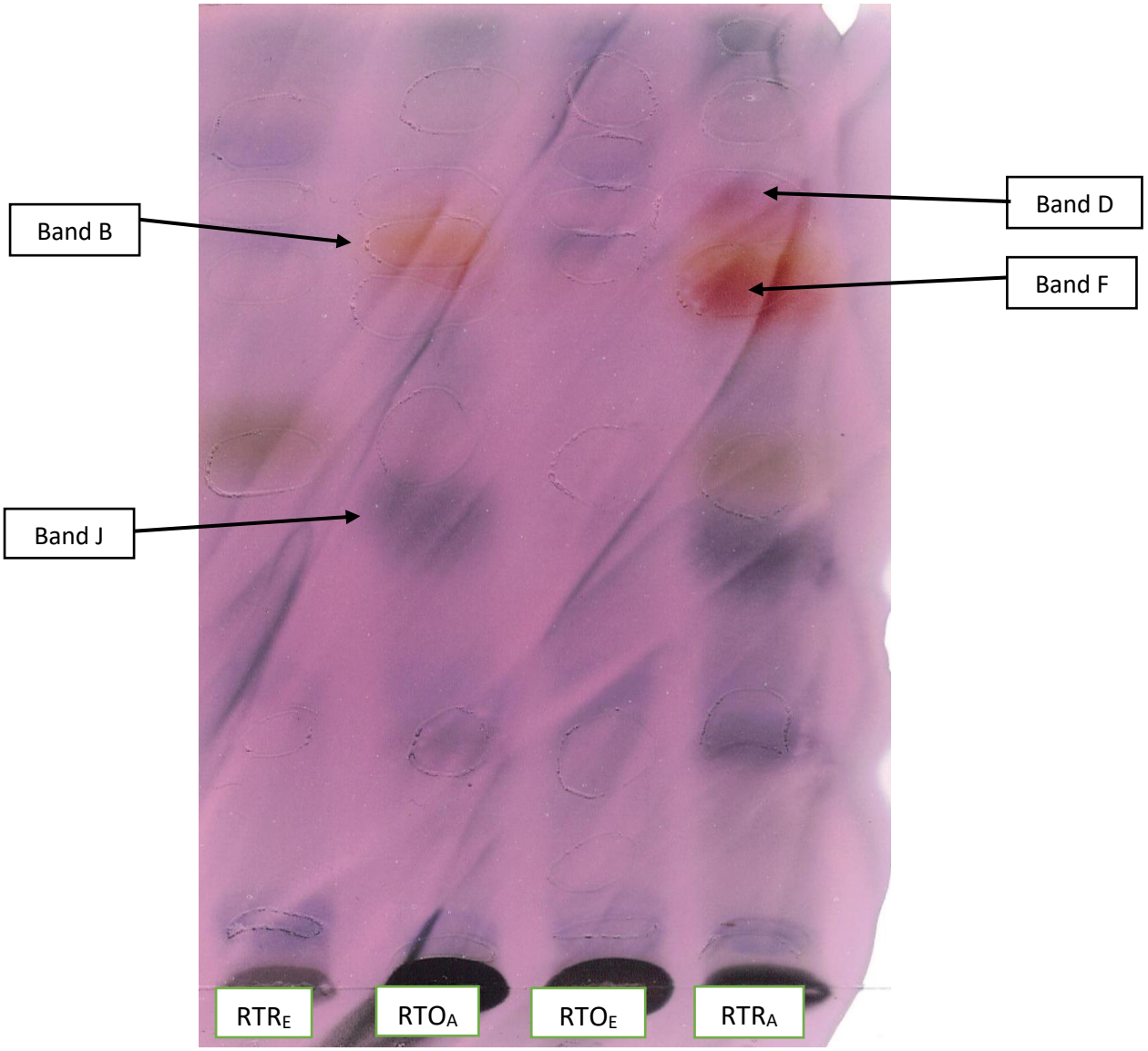

Figure 3.13: The TLC profiles of *R. tridentata* (RTR) and *R. tomentosa* (RTO) showing chemical components stained with anisaldehyde. Key: RTR$_A$: *R. tridentata* acetone; RTR$_E$: *R. tridentata* ethanol; RTO$_A$: *R. tomentosa* acetone; RTO$_E$: *R. tomentosa* ethanol. The TLC plate was developed in ethyl acetate: hexane (1:1).

3.6.1 Bioautography assay

The bioautography results are shown in Figure 3.14. The white spots displayed inhibition of *S. aureus*. The ethanol extract from both *R. tomentosa* and *R. tridentata* displayed inhibition zones in band E (R_f =0.79) and band D (R_f =0.83). Ethanol and acetone extracts from *R. tridentata* and *R. tomentosa* also inhibited *S. aureus* by displaying clear spots at an R_f value of 0.74. Lastly, the acetone extract from *R. tridentata* inhibited *S. aureus* at an R_f of 0.74.

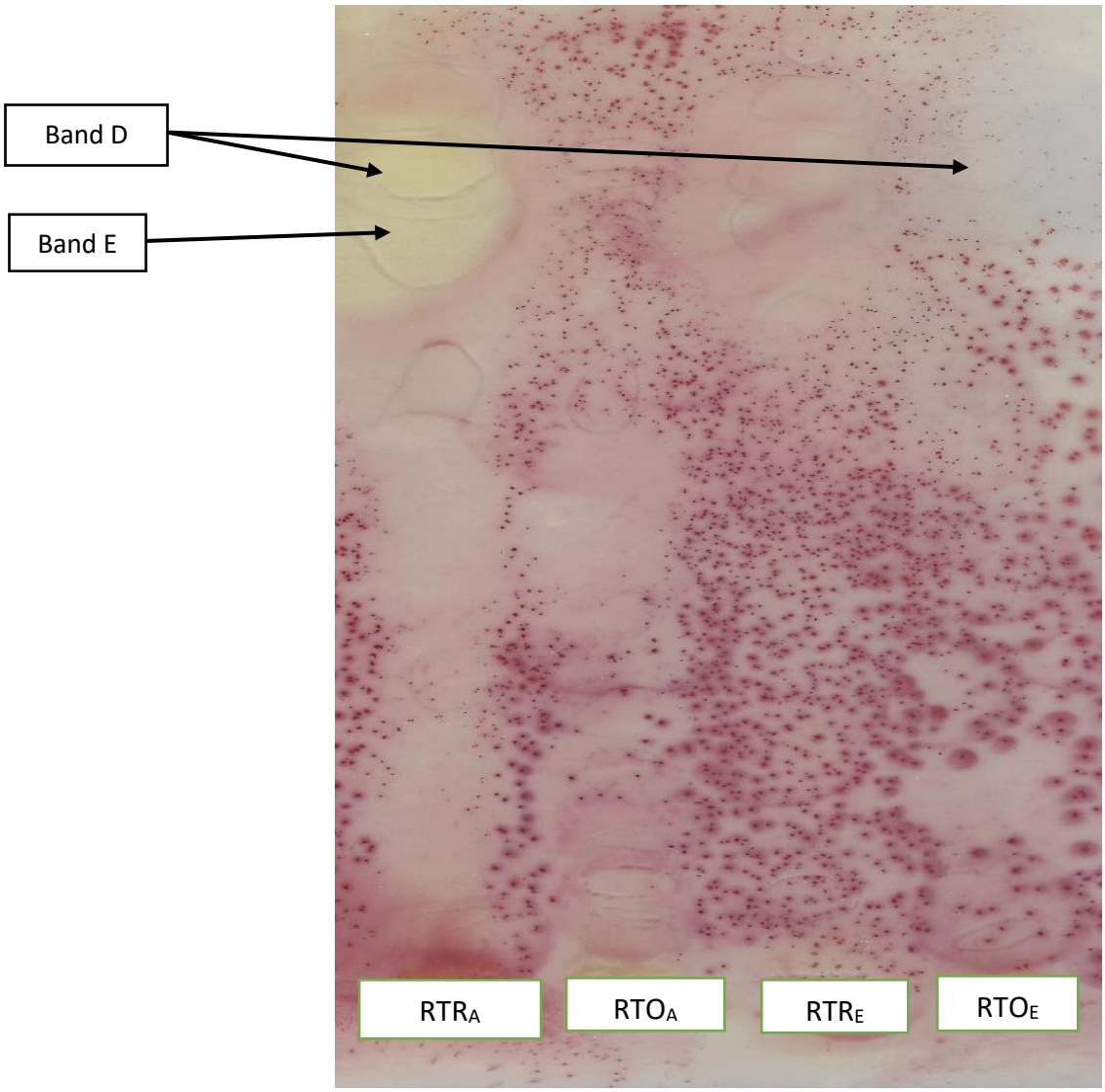

Figure 3.14: The TLC profiles of *R. tridentata* (RTR) and *R. tomentosa* (RTO) showing chemical components stained with *S. aures*. Key: RTR$_A$-*R. tridentata* acetone; RTR$_E$-*R. tridentata* ethanol; RTO$_A$- *R. tomentosa* acetone; RTO$_E$- *R. tomentosa* ethanol. The TLC plate was developed in ethyl acetate: hexane (1:1).

CHAPTER 4
DISCUSSION

4.1 Antibacterial activity

The extracts prepared from both plants were highly active and showed the best activities against all the tested strains of bacteria. The *R. tomentosa* extracts exhibited high antibacterial activity by inhibiting the majority of test bacterial strains. In a study undertaken by Kapoor *et al*. (2015), *R. tomentosa* extracts showed different levels of activity against the majority of test microorganisms. The methanolic extracts of *R. tomentosa* leaf also showed different levels of anti-microbial activities against almost all tested microorganisms in a study reported by Lin *et al*. (1999). Antimicrobial activities exhibited by plants could be due to the highly potent presence of alkaloids, flavonoids and carbohydrates (Meta and Parahydroxyl compounds) (Fennel *et al*., 2004). Concerning *R. tridentata*, the ethanol and acetone extracts displayed the best antibacterial activity by inhibiting all test microorganisms between 0.098 and 0.39 mg/ml. In a study by Samie *et al*. (2005), *R. tridentata* methanol and acetone extracts were found to be the most active with Gram-positive bacteria being the most sensitive to the extract as compared to Gram-negative bacteria. Furthermore, reports by Lin *et al*. (1999), Opoku *et al*. (2007) & (Mamba *et al*., 2016) indicated that *R. tridentata* crude extracts possessed good antimicrobial activity. In this study, the aqueous extract of *R. tridentata* displayed good activity against two Gram-negative bacterial strains (*E. coli* and *S. flexneri*) at 0.39 mg/ml. Generally, Gram-negative bacteria are more resistant to antimicrobial agents than Gram-positive bacteria (Brantner *et al*., 1996; Nostro *et al*., 2000; Ojala *et al*., 2000). The resistance of Gram-negative bacteria is due to lipopolysaccharides that are in their outer cell membrane (Gao *et al*., 1999; Takeuchi *et al*., 1999). This is controversial with the results obtained from this study where the *R. tridentata* water extract displayed prominent activity against Gram-negative bacteria compared to Gram-positive bacteria.

4.2 Antifungal Activity

The acetone extract of *R. tridentata* was the only extract that displayed the high antifungal activity by inhibiting all three tested fungal strains (*C. albicans*, *C. vulgaris* and *T*. *mucoides*) at 0.195 mg/ml. In a study undertaken by Brookes & Katsoulis (2006), the acetone extract of *R. tridentata* tubers was active against all *Fusarium* spp. tested with the MIC values varying from 0.95 to 3.75 mg/ml. This is in agreement with the present study where the acetone extract of *R. tridentata* also showed good antifungal activity. The type of solvent used, thus, determined the success of isolation process (Masoko *et al*., 2007). According to Eloff (1998b), acetone was the preferable solvent compared to methanol, ethanol and water when screening plants. This is because, acetone is volatile, can extract highly polar components, and it relatively has low toxicity to the test micro-organisms. The rest of plant extracts displayed poor activity. The observations made by other investigators state that pathogenic fungi are generally more resistant to plant extracts than pathogenic bacteria (Leven *et al*., 1979; Naqvi *et al*., 1991; Heisey & Gorhan, 1992; Buwa & Van Staden, 2006). Negative results do not mean absence of bioactive constituents nor that the plant is inactive, plant extracts may act in other way by stimulating the immune system of the patient, or by creating internal conditions that are unfavourable for the multiplication of the microorganisms (Buwa & Afolayan, 2009). The factors that seemed to affect the anti-microbial activity of the plant extracts were found to be the location and/ or the season in which the plants were collected (Lin *et al*., 1999*).*

4.3 Antimycobacterial Activity

The organic solvent extracts prepared from both *R. tomentosa* and *R. tridentata* inhibited *M. tuberculosis* at 0.195 mg/ml, whereas a moderate activity was observed with water extracts (1.56 mg/ml). The moderate activity might be due to that aqueous solvent does not extract all the active compounds that might be present in the plant as compared to the lipophilic solvents (Buwa & Van staden, 2006). Water, is one of the solvents used by traditional healers to prepare the traditional remedies. Hence, the medication is applied at high volumes, whereas applying the same dosage from lipophilic solvent may be dangerous. Several studies have shown lipophilic solvents, particularly acetone, to be efficient extractants of diverse bioactive compounds that

may act in synergy and produce a greater antimycobacterial activity, producing susceptibility of patterns (Eloff, 1998b & Martin, 1998; Kotze *et al*., 2002; Madamombe & Afolayan, 2003; Asres *et al*., 2006). Studies undertaken by Mukundi *et al*. (2015) & Uche-Okereafor *et al*. (2016) showed that *R. tomentosa* and *R. tridentata* possessed alkaloids, flavonoids, saponins and tannins. Flavonoids have been shown to be active against *M. tuberculosis* (Okunade *et al*., 2004).

4.4 Anthelmintic Activity

The *R. tridentata* ethanol extract was generally more potent than water extract. It displayed the best anthelmintic activity with 100% larval mortality at a concentration of 2 mg/ml. This could probably be attributed to the kind of bioactive substances extracted by the two solvents; agreeing with reports by Harbone (1973a) that different solvents extract different compounds depending on type of substances and polarity. Harbone (1973b) recommended alcohol as a "good for all-purpose" solvent for preliminary extraction. Brookes & Katsoulis (2006) reported that *R. tridentata* has twenty novel compounds with health-promoting properties. A study undertaken by Naidoo *et al.* (2005) reported tannins as the major chemical constituent which probably accounts for the health properties although synergy by other compounds could have enhanced the activity. The role of condensed tannins in helminths control has been demonstrated by Athnasiadou *et al.* (2001), Cenci *et al.* (2007) and Brunet *et al.* (2008). Chemically tannins are polyphenolic compounds (Muchuweti *et al*., 2006) and synthetic phenolic anthelmintics like niclosamide and oxyclozanide are said to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation (Martin *et al.*, 2005). Concerning *R. tomentosa*, 60% nematode mortality was observed with ethanol extract at a concentration of 2 mg/ml, with 40% mortality at 1 mg/ml.

4.5 Anti-inflammatory activity

The anti-inflammatory activity of plant extracts was compared with NDGA. The NDGA is used as a reference compound for studies of 5-lipoxygenase inhibition due to its widely reported strong inhibitory activity on this enzyme (Safayhi *et al*., 1992; Abad *et al*., 1995). Extracts from both *R. tomentosa* and *R. tridentata* displayed the high anti-inflammatory activity by inhibiting 5-LOX with IC_{50} values ranging between 0.10± 72.50

and 0.13± 6.650 µg/ml. *R. tridentata* has cholesterol-lowering properties, and triterpenoids such as oleanolic acid, lupenone and taraxastananol are known for their anti-inflammatory action (Brookes & Katsouluis, 2006). Both *R. tridentata* and *R. tomentosa* possessed anti-inflammatory properties and this is in agreement with a study conducted by Lin (1999). Furthermore, in a study undertaken by Afolayan & Masika (2003), *Rhoicissus* species was found to possess anti-inflammatory properties. In a study by Mukundi *et al*. (2015), *R*. *tridentata* was found to possess phytochemical constituents (flavonoids, saponins, alkaloids and tannins) that possess anti-inflammatory properties. The *R. tridentata* ethanol extract displayed 83-92% inhibition, as the concentration increased from the lowest (0.02 mg/ml) to the highest concentration (0.40 mg/ml).

4.6 Chemical profile analysis

The same colour of spots from both plants with the same R_f values were displayed, as shown in Figure 3.11 to 3.13 of Chapter 3. The spots that displayed high R_f values are less polar and more attractive to the mobile phase compared to those that are less polar which are attracted to the stationery phase (Braithwaite & Smith, 2012). Extracts from both plants displayed the presence of chemical profile in band A, B, C, E and F (Figures 3.11-3.13). In the bioautography bioassay, band D and E inhibited the growth of *S. aureus*. According to these results, the *R. tomentosa* bulb and *R. tridentata* shoot system possessed the same constituents. A similar chemical composition between the two species suggest that traditional healers could substitute the plant parts they use in their traditional medicines. According to Zschocke *et al*. (2000), traditional healers could substitute plant parts from the same plant species.

In terms of conservation, it is advised to use the shoot system rather than an underground plant part as this will counter extinction and accelerate the usefulness of important species. Herbalists normally use underground plant parts most frequently, believing that they contain the highest concentration of potent healing agents (Shale *et al*., 1999), with leaves or flowers used less frequently in traditional practice (Bangani *et al*., 1999; Crouch *et al*., 1999; Zschocke *et al*., 2000; Kelmanson *et al*., 2000; Louw, 2002). This study has shown that leaves from *R. tridentata* could be used in the place of *R. tomentosa* bulb to prevent over-harvesting of the plant species. Medicinal plants

are often at risk from habitat destruction, bioprospecting for new drugs and over harvesting of known medicinal species (Sulaiman & Balachandran, 2013).

Chapter 5
Conclusion and Recommendations

5.1 Conclusion

The screening of plant extracts as described in Chapter 3 showed the potential activities of *R. tomentosa* and *R. tridentata* against the majority of the tested microorganisms. The *R. tomentosa* extracts displayed high antibacterial activity against the majority of test bacteria with MIC values ranging from 0.098 to 0.39 mg/ml. Moderate activity was observed with aqueous extract against *B. cereus* and *S. epidermidis*. The water extract displayed the high activity against *E. coli* and *S. flexneri* at 0.39 mg/ml, with moderate activity (1.56 mg/ml) against the rest of the test microorganisms. According to these finding, water is an effective solvent that could be used in the extraction of antibacterial components. Concerning *R. tridentata*, the ethanol and acetone extracts displayed the best antibacterial activity by inhibiting all test microorganisms with MIC values ranging between 0.098 and 0.39 mg/ml. The extracts prepared from both plants were highly active and showed the best activities against all the tested bacteria.

Concerning antifungal activity, the acetone extract of *R. tridentata* displayed the best activity by inhibiting all three fungal strains at 0.195 mg/ml. The rest of the extracts displayed poor activity. Not all plants exhibited the best activity but even so, the absence does not mean that neither the bioactive constituents nor the plant is inactive.

The organic solvent extracts prepared from both plants inhibited *M. tuberculosis* at 0.195 mg/ml whereas moderate activity was observed with water extracts.

The water extract of *R. tridentata* displayed good anthelmintic activity against *H. contortus* with 50% larval mortality at 2 mg/ml. The ethanol extract of *R. tridentata* displayed the anthelmintic activity with 100% mortality at 2 mg/ ml. Concerning *R. tomentosa*, 60% nematode mortality was observed at 2 mg/ml. The water extract displayed poor anthelmintic activity compared to levimasole. This makes water as an ineffective solvent in extracting compounds with anthelmintic properties.

The *R. tomentosa* and *R. tridentata* had an effective anti-inflammatory potential or better inhibition of the 5-LOX enzyme as demonstrated by reduced IC_{50} values which were lesser than NDGA. With IC_{50} values ranging between 0.10± 72.50 and 0.13± 6.650 µg/ml (Table 3.5. All the plant extracts showed moderate and promising inhibition of the 5- LOX enzyme.

The TLC chemical profile of both *R. tomentosa* and *R. tridentata* extracts displayed the presence of common bands/spots which shows the presence of the same chemical. Both *R. tomentosa* and *R. tridentata* displayed the presence of band A, B, C, E and F. In bioautography bioassay, band D and E inhibited the growth of *S. aureus*. A similar chemical composition between the two species suggest that traditional healers could substitute the plant parts they use in their traditional medicines.

The extracts of *R. tomentosa* and *R. tridentata* have demonstrated some useful activities that support their traditional use against elephantiasis and related ailments. Our results confirmed that *R. tomentosa* and *R. tridentata* had an effective or better inhibition of antimicrobials that cause secondary infections in people suffering from elephantiasis. Therefore, this study has showed that extracts of these plants have potential to act as therapeutic/curative agents against pathogens causing elephantiasis.

5.2 Recommendations

Further investigation on isolation and purification of active compound responsible for the antimicrobial, antioxidant, anticancer and anti-inflammatory activity is recommended.

Studies on toxicity of the plant extracts are also required.

Compounds with potential anti-inflammatory properties need to be tested *in vivo* or against biochemical models of inflammation.

Concerning anti-inflammatory assay, it is recommended that other anti-inflammatory routes are used in order to determine whether the solvent extracts do exert anti-inflammatory activity in other steps of the complex anti-inflammatory cascade.

Since the root and the shoot system possessed the same chemical profile it is more advisable to use leaves other than roots to prevent extinction of plant species.

Municipalities and institutions (e.g. Universities, National Parks) must also play a role of educating the communities about the conservation of these valuable medicinal plants and also establish botanical gardens for these plants.

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