Antifungal evaluation of extracts from selected medicinal plants against *Rhizoctonia solani*

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ABSTRACT

Maize production consumes about 40 million hectares of land in Sub-Saharan Africa (SSA). Approximately 50% of SSA nations cultivate maize as their primary cereal crop, and in about 75% of these nations, maize is one of the top two cereal crops. Over 100 grams of maize are consumed daily in more than half of the region's nations. Like other countries in sub-Saharan Africa, South Africa produces approximately 60% white maize serving as staple food for many South Africans and grows nearly 40% yellow maize used for animal feed. Maize is one of the cereal crops susceptible to various pathogens including *Rhizoctonia solani*, *Pythium* spp, *Sclerotium rolfsii* and *Macrophomina phaseolina.* Cultural, physical and chemical methods have been used in combating maize root rot caused by *Rhizoctonia solani*, However, the pathogen has developed resistance to existing treatment regiments, hence the need to introduce alternative environmentally friendly and sustainable treatment regiments such as medicinal plant extracts is inevitable. This study aimed to investigate the *in vitro* and *in vivo* antifungal potential of four selected medicinal plants against *R. solani* causing root rot in maize and to profile the phytochemistry of the selected medicinal plants.To attain the aim of this study, acetone and ethanol extracts of the medicinal plants were screened *in vitro* for their antifungal activity against *R. solani* using two methods; the agar disc diffusion assay and minimum inhibitory concentration (MIC) using a modified version of the micro titre plate methods. The phytochemical profiling was achieved through Ultra Performance Liquid Chromatography-MS/MS (UPLC-QToF/MS/MS). The medicinal plant extracts were further applied as seed treatment in the green house. Plugs containing the pathogen were inoculated into the vicinity of the seeds except in the control. Treatments and parameters including plant height, chlorophyll content, root length, number of root lesions, fresh and dry root weight, fresh and dry shoot weight were recorded weekly. All medicinal plant extracts, except *T. ametica* showed antifungal activity against the test pathogen. The mean inhibition of the plants against *R. solani* ranged from 3.9mm (*Cascabela thevetia* ethanol extracts) to 12.2mm *(A. arctotoides* acetone extracts). The highest inhibition was induced by both acetone and ethanol extracts of *A. arctotoides* (12.2 and 10.4 mm respectively), followed by acetone extracts of *V. amygdalina* (8.0mm) and acetone extracts of *C. thevetia* (7.5mm). Ethanol extract of *C. thevetia* exhibited the least antifungal activity with the mean inhibition of 3.8mm, while on the other hand both acetone and ethanol extracts of *Trichilia ametica* showed no activity against the pathogen. Acetone extracts had higher potency compared to ethanol, with an average of 71.77mm as

opposed to 55.99mm inhibition diameter. Based on the MIC results, acetone extracts from *A. arctotoides* exhibited the MIC value (0.2 mg/ml) among the tested medicinal plant extracts. *Arctotis arctotoides* ethanol *(AER1)*, *Vernonia amygdalina* acetone *(VAaR*1), *Vernonia amygdalina* ethanol *(VAER1), Cascabela thevetia* acetone *(CTaR1)* and *Cascabela thevetia* ethanol (*CTER1*) recorded the best five treatments with the highest plant height, chlorophyll content, root hairs and the lowest root lesions under greenhouse conditions. Both acetone and ethanolic extracts of *Trichilia ametica* (*TAER1 and TAaR1*) showed no activity against *R. solani*, which confirms the results obtained in the *in vitro* studies. The pathogen reduced plant height and chlorophyll content and had an increased number of root lesions compared to other treatments including the uninoculated control, which was expected as a confirmation that *R. solani* was pathogenic to maize. Acetone had high volatility, miscibility with polar and non-polar solvents, and minimal toxicity to test organisms, hence the results of the present study show high activity of acetone extracts in all measured parameters. *The A. arctotoides* and *V. amygdalina* plant extracts were regarded as the best treatments as they were among the top treatments in most of the measured parameters. The performance of these extracts indicates that they possess potential as biocontrol agents for the control of *Rhizoctonia* root rot pathogens of maize. The UPLC analysis identified numerous compounds archived through the comparison of the obtained mass spectra data to literature. Fifteen and eighteen bioactive compounds were identified in acetone and ethanolic extracts of T. *ametica* respectively, with terpenoid at 27.78% from the ethanolic extract. In *C. thevetia*, 17 compounds from acetone and 10 compounds from ethanolic extract were identified with the dominant one being terpenoid at 27.76%. A total of17 and 11 compounds were identified respectively from acetone and ethanolic extracts of *V. amygdalina* the dominant class being fatty acids from the ethanolic extract at 36,36%. In *A. arctotoides* 19 and 13 compounds were identified from acetone and ethanolic extracts respectively the dominant one being terpenoid at 53.85% from the ethanolic extract. The results of this study showed that medicinal plants harbour phytochemicals responsible for their reported potent antifungal activities that could be further explored against fungal pathogens.

Keywords: *Rhizoctonia solani*, Antifungal, Maize, Medicinal plant, Phytochemicals,

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APPROVAL SHEET

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DEDICATION

This work is dedicated to the man above, my Saviour Jesus Christ who tirelessly gave me strength and comfort. The greatest dedication goes to my late mother; Victoria Dorries Mkhonto, the generational curses are broken, I did it sesi!

DECLARATION

I am familiar with and understand the university's policy on plagiarism; thus, I certify that the thesis is my original work and that all other sources have been appropriately cited in accordance with the laws and regulations governing reference and citation in scientific writing. This thesis has been submitted in partial fulfilment of the Master of Science degree requirements at the University of Mpumalanga. I attest that the work has never been submitted for a degree at my institution or any other institution of higher education.

Christeldah Mkhonto

Signature: Date 06/03/2023

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CONFERENCE PRESENTATION

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PUBLICATIONS

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Chapter 1: Introduction

1.1 Background

After rice (Oryza sativa) and wheat (*Triticum* aestivum L), maize (*Zea mays* L)is the third most significant field crop grown worldwide (Ladha et al., 2016). Maize, wheat, and rice are the most dominant crops in the human diet (Ignaciuk and Mason-D'Croz, 2014) consumed directly or as second cycle products. Over 4.5 billion individuals in 94 developing nations get close to 30 percent of their entire daily caloric intake from these three crops (Melkie, 2021). In Sub-Saharan Africa, maize serves as a stable food source and a form of livelihood for more than 300 million people (Benjamin, 2018). South Africa is one of the top 10 maize producing countries globally, yielding between 10 and 12 million tons of maize grains on 3,1 million ha of land (Gravelet-Blondin, 2013; Plessis, 2003). However, South Africa is the number one maize producer (Mangani et al., 2019) and highest maize consuming country in Africa (Ranum et al., 2014a).

The maize industry plays a very significant role in the livelihoods and the economy of the country. South Africa produces both white and yellow maize concentrated in 4 provinces being Northwest, Free State, KwaZulu-Natal, and Mpumalanga. According to Gravelet-Blondin (2013), on a ten-year average, South Africa uses 4.1 million tons of maize production for direct human consumption, close to 3.9 million tons yellow maize including another 1 million ton of white maize used as animal feed, and lastly 650 000 tons of the annual produce is used for starch and glucose manufacturing. The report conducted by Gravelet-Blondin (2013) shows that the amount of maize produce used for animal feed is nearly equal to the amount of maize used for human consumption emphasizing the significance of the crop in the country's food security.

Root rot-inducing plant pathogens are a great threat to the production of horticultural and agricultural crops (Ketta and Hewedy, 2021) and maize is not an exception. The crop is also affected greatly by other diseases such as collar rot, wire stem, damping-off root rot induced by various plant pathogens such as *Fusarium verticillioides*-(Sacc.)and *Fusarium graminearum* Schwabe (Pechanova and Pechan, 2015). In many commercial crops, the soil-borne pathogenic fungus responsible for root rot is *Rhizoctonia solani* Kühn (Pane et al., 2013).

Due to its diversity in colony morphology, pathogenicity, aggressiveness, and molecular markers, *R. Solani* has been documented to be a very significant and damage inflicting species from the *Rhizoctonia* genus (Ajayi-Oyetunde and Bradley, 2018). The species is classified into

14 genetically isolated groups called Anastomosis groups based on the incompatibility of their hyphae and the specificity of their host (Pane et al., 2013). According to Ithurrart et al. (2004) the dominant and most aggressive anastomosis group of *R. solani* responsible for root rot in maize and sugar beet (*Beta vulgaris*) is Isolate AG 2-2-IIIB.

Rhizoctonia root rot is one of the four most studied stubborn fungal root rot due to the high proportions of crop loss they induce in agricultural crops (Bodah, 2017). Cultural, physical, biological, and chemical methods are used as management strategies for root rot (Sharma et al., 2022). These management strategies have been reported to be only partially successful in managing root rot due to the fungus' aggressive and host-specific nature (Kumari and Katoch, 2020).

The development of pathogenic fungi resistance towards chemical fungicides has resulted in a shift of attention towards the use of medicinal plants to treat plant pathogens. Increased fungal resistance to synthetic fungicides is also a big problem in agricultural crop production. This is due to their toxicity and disturbance to soil ecology from frequent use. Research by Mishra et al. (2020) shows that natural compounds from plants have traditional backing for their action and safety potential while modern chemicals possess more toxicity even though they have been validated for their actions. This, therefore, means medicinal plants can serve as an alternative treatment against fungal infestations in crops.

Medicinal plants are natural and provide pure phytocompounds which can also be in the form of standardized extracts with a matchless chemical diversity (Mishra et al., 2020) for treating a wide range of antimicrobial properties (Amadioha, 2000; Mahlo et al., 2010). Medicinal plants possess a wide range of active phytocompounds with antimalarial, antifungal, anti-inflammatory, antibacterial, and antinociceptive activity (Street and Prinsloo, 2013). The most common phytochemicals in medicinal plant extracts include alkaloids, flavonoids, triterpenes, and phenolics (Mojau, 2017). Phenolics are the largest category of plant phytochemicals with close to 10 000 identified cosmopolitan structures identified in the plant kingdom (Agostini-Costa et al., 2012). Although these medicinal plants possess limitless bioactive compounds that are both antifungal and antibacterial, no systematic research has been conducted to explore their potential in treating fungal plant pathogens affecting crops of economic importance.

In the present study, four medicinal plants *Arctortis Arctotoides, Trichilia Americana, Vernonia amygdalina and Cascabela Thevetia* were selected based on ethno pharmacological data indicating their traditional usage for the treatment of microbial diseases in South Africa

(Street and Prinsloo, 2013). In addition, the plants were selected because they are readily available and possess antimicrobial potency (Germano et al., 2005b; Oboh and Masodje, 2021; Otang et al., 2012; Sowjanya et al., 2013). Despite their extensive traditional usage, detailed chemical compositions, and bioactivity of these plants against *R. solani* responsible for root rot in maize are yet to be investigated.

1.2 Problem statement

More than 800 million people in developing countries lack access to adequate nutritious food and close to 10% of the food is lost due to plant diseases (Strange and Scott, 2005; Tapwal et al., 2011). Currently, the most dominant management of root rot disease in maize is the use of cultural, physical, and chemical methods. The cultural methods of managing the disease are not effective since the *Rhizoctonia Solani* is host-specific (Williamson-Benavides et al.,2021) 2021). The pathogen can infect crops grown in the same field (Gaulin et al., 2007) due to its ability to remain in the soil for 10 years (Williamson-Benavides et al.,2021) and therefore increasing the chances of re-infestation to the crops when crop rotated in the same rotation field.

The use of synthetic chemical fungicides is posing undesirable effects on beneficial soil micro-organisms (Tapwal et al., 2011). Negative effect on soil micro-organisms affect soil properties such as soil pH leading to plant death and adversely affecting agricultural productivity (Nuss and Tanumihardjo, 2010). The synthetic chemical fungicides affect soil biodiversity, including soil nutrients often leading to ill-balanced essential nutrients in maize crops. This is therefore detrimental to consumers who depend on maize as a major source of food (Nuss and Tanumihardjo, 2010).Aggressiveness of pathogens increase chances of malnutrition in both human and animals due to shortage in the world's maize protein and calorie production. This therefore means meeting the needs of the estimated 8 billion population increase by 2025 would be impossible (Surender et al., 2017). The commonly used chemical fungicides are synthetic and non-biodegradable and can accumulate in plants, water sources, and soils, consequently affecting humans and animal health through the food chain (Tapwal et al., 2011), hence the need for safer alternatives.

1.3 Justification

According to Tapwal et al. (2011), research on plant-based natural extracts for agricultural use has been declining for a long time. This decline can be linked to the daily increase in human diseases that have shifted the focus of research from the management of plant diseases to more

research on the management of human diseases. This, therefore, means there is a substantial need for research around natural plant products as a viable solution to environmental and food problems due to diseases and the use of synthetic chemical fungicides. The presence of antifungal compounds in plants is an important factor in disease prevention (Dixon, 2001). Plant based extracts are biodegradable, therefore, they pose a negligible threat to the environment. Plant based extracts appear to be a better alternative for root rot management since they exert minimal harmful effects on soil microbes, plants, humans, and animals in the food chain (Varma and Dubey, 1999).

Several studies have been conducted on the ability of the roots and shoots of *A. arctotoides* to inhibit the growth of a wide range of fungi such as *Aspergillus flavus* and *Penicillium notatum* (Afolayan, 2003b). Despite the well-documented reports on the chemical components and the pharmacological properties of the shoots and roots of this herb, no systematic research has been carried out on the antifungal activity of *A. arctotoides* against *R. solani.*

Like *A. arctotoides, T. ametica and C. thevetia* also possesses antifungal properties (Ojewole et al., 2010; Rahul et al., 2015). However, these antifungal properties are active against human pathogens. However, there has been no systematic study to document the ability of these plants to inhibit the growth of *R. solani* root rot of maize which informs the grounding of the current study.

1.5 Aims and objectives of the study.

The broad aim of the current study is to investigate the *in vitro* and *in vivo* antifungal activity of selected medicinal plant extracts against *Rhizoctonia* root rot of maize.

Specific objectives are:

1. To investigate the *in vitro* antifungal activity of *A. arctotoides, T. ametica and C. Thevetia* extract against *Rhizoctonia* root rot fungus.
2. To investigate the minimum inhibitory concentration (MIC) of the plant extracts.
3. To evaluate the potential of the plant extracts to control *Rhizoctonia* root rot of maize in greenhouse.
4. To investigate the phytochemicals of *A. arctotoides, T. ametica, and C. Thevetia* extracts.

1.6 Research questions

The main research question was: Do leaf extracts from *A. arctotoides, T. ametica, V. amygdalina and C. Thevetia* inhibit the growth of *Rhizoctonia solani*? Hence other questions had to be addressed to answer this main question.

1. What is the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration of the plant extracts?
2. Do leaf extracts from *A. arctotoides, T. ametica, and C. Thevetia* possess the potential to control the *Rhizoctonia* root rot of maize in a greenhouse?
3. Which phytochemicals are present in the extracts of these plants?
4. Which phytochemicals present in the extracts are responsible for inhibiting the growth of *Rhizoctonia solani*?

1.7 Overview of Chapter in dissertation

Chapter 1: Provides background on maize production and the impact *Rhizoctonia solani* on its production levels. In addition, the problem statement, significance of the study, aim and objectives are highlighted.

Chapter 2: provides an in-depth review of the literature on concepts relevant to maize root rot, medicinal plants and their uses, a summary of the active phytochemical classes found in medicinal plants, and a summary of the techniques for assessing antimicrobial activity.

Chapter 3: Focuses on evaluating the antifungal activity of selected medicinal plants against *Rhizoctonia solani in vitro* using the agar disc diffusion assay.

Chapter 4: Focuses on the potential of crude plant extracts to control *Rhizoctonia* root rot of maize under greenhouse conditions.

Chapter 5: Documents the phytochemicals identified from the crude extracts using UPLC Q-TOF/ MS/MS.

Chapter 6: Summarizes the study's key findings and draws recommendations based on those findings. This chapter also offers recommendations for increasing the availability of active medicinal plants for use by local farmers as biological fungicides.

Chapter 2: Literature Review

2.1 Introduction

Root rot diseases are the biggest threat to the annual productivity of maize and other commercial crops worldwide (Kumari and Katoch, 2020). The loss of maize produce can reach an economic threshold as influenced by factors such as the causal agent for the root rot, environmental conditions, and host susceptibility (Krupinsky et al., 2002). Oomycetes and fungi are reported to be the most dominant and common causes of the root of rot (Al-Jaradi et al., 2018) in commercial agricultural crops including maize, while bacteria have been reported to be dominant as a causal agent of root rot and damping off (Ortigosa et al., 2019). The most intensively studied and problematic fungal root rots in maize include Rhizoctonia root rot, Fusarium root rot, Panama root rot, and black root rot.

The susceptibility and prevalence of the fungal root rots in the crop are greatly influenced by soil compaction, high soil moisture, favourable pathogen growth temperature, mono-cropping systems, and poor drainage (Kumari and Katoch, 2020). Root rot symptoms usually go unnoticeable especially if the pathogen affects the germination of seeds (Bodah, 2017) and if symptoms appear aboveground, the crop fails to recover (Williamson-Benavides et al., 2021). Some of the noticeable symptoms associated with root rot in maize include stunning leaves, browning and softening of root tips, different lesion sizes, and colour, reduced yield, and most severe crop death (Bodah, 2017).

2.2 *Rhizoctonia solani* in commercial crops

Rhizoctonia Solani is one of the major and problematic soil-borne phytopathogenic fungi causing major crop losses in the entire world (Abbas et al., 2019). Due to its aggressive nature, the fungus can singly induce between 20-40% loss in crops annually throughout the world (Srivastava et al., 2016). Even though the fungus is host-specific, it has a wide range of hosts which it dominantly attacks. *R. solani* can survive in severe and critical conditions due to its ability to form defensive structures that are a source of sclerotic infection (Abbas et al., 2019). *R. solani* causes major diseases in many commercial crops including cereals, ornamental crops vegetables (Abbas et al., 2019), and plants in the nightshade family (*Solanum lycopersicum* , *Solanum melongena* L, and *Solanum tuberosum*.

In rice, *R. solani* causes sheath blight that potentially reduces rice yield by at least 25% under ideal conditions (Kumar et al., 2009). The fungal disease in rice presents itself as water-soaked lesions on the lower leaf sheath during its early stages of development (Kumar et al., 2009),

that expand till they appear bleached with a brownish border. In sugar beet, anastomosis group (AG) 2-2IIIB causes crown and root rot (Kluth and Varrelmann, 2010). Over 2% yield loss in sugar beet due to *R. solani* infection has been reported in Germany (Ayala et al., 2001), while over 50% of sugar beet yield losses have been documented in Europe due to *R. solani* infestation (Büttner et al., 2002), resulting in sugar beet quality reduction that led to low sales and hence economic losses.

2.3 *Rhizoctonia* root rot in maize

Rhizoctonia root rot is one of the most prevalent primary soil-borne fungal diseases in maize. The occurrence of *R. solani* is greatly influenced by environmental conditions (Buddemeyer et al., 2004) and its prevalence differs from field to field (Kluth and Varrelmann, 2010) while severity differs from cultivar to cultivar due to differences in environmental conditions such as high soil moisture, and temperature. In addition to maize, the pathogen also causes Root and Crown rot in sugar beet, and the main areas with incidents of the plant pathogen are identified by the rotation of the two crops (Maize and Sugar beet) within the same field (Buddemeyer et al., 2004). In a study conducted by Buddemeyer et al. (2004), maize cultivars inoculated with AG 2-2IIIB *R. solani* anastomosis developed round to elliptical, yellow to brown dry lesions, suppressed crown and roots. In some maize cultivars grown under German conditions, roots were rotten completely with some plant stalks broken below the ear (Buddemeyer et al., 2004). Both mature maize crops and maize seedlings are susceptible to *R. solani* infections from different anastomosis groups of the fungus (Buddemeyer et al., 2004).

2.3 Current management strategies for *Rhizoctonia* root

The most dominant management of the fungus is biological control which includes crop rotation practices. However, *R. solani* has a wide host range, the pathogen can inoculate crops grown in the same rotation field, hence crop rotation is reported as fully not an effective management strategy (Gaulin et al., 2007) and this is because the fungus can remain in the soil for 10 years (Williamson-Benavides, B.A and Dhingra, A, 2021) and therefore increasing the chances of re-infestation to the crops. The fungus is very aggressive and is, therefore, able to develop resistance to chemical fungicides as evident in maize and sugar beet rotation trials (Kluth and Varrelmann, 2010). The use of Synthetic chemical fungicides is ineffective because it affects beneficial soil microbes since the fungus is soil-borne. Therefore, there is a need to explore natural products such as medicinal plants as an alternative against fungal infestations in crops.

2.3.1. Biological control of R. solani

To archive quality production, farmers make use of chemical fungicides to manage and reduce damage by plant pathogens (Zheng et al., 2017). The attention has lately switched to biological control methods, even though chemical management of plant diseases has been practiced for years and has usually shown successful results. Chemicals are viewed as a hazard to both human health and natural biodiversity since they alter the rhizosphere and bulk soil microorganisms' structures and activities. Fungicide resistance is an inevitable risk when fungicides are employed (Hassan, 2017). Biological disease management techniques have been created in order to prevent the negative consequences linked to the usage of chemical control (Hassan, 2017). Other microorganisms are used in biological control measures to combat plant diseases. According to reports, microorganisms can defend common bean (Phaseolus vulgaris L.) against Pythium species by producing antifungal compounds, outcompeting hosts for resources, excluding the pathogen from its niche, parasitizing the pathogen, or by inducing plant resistance (Nzungize et al., 2012.

The biological management of soil-borne diseases is particularly difficult since they coexist with other microorganisms in a dynamic ecosystem at the rhizosphere interface (Nzungize et al., 2012). It is challenging to manage soil-borne diseases due to the rhizosphere's huge microbial activity, which includes a high population of microorganisms, fluctuations in pH, salt concentrations, osmotic potential, and water potential (Nzungize et al., 2012). Numerous microorganisms, including fungus, soil mycobacteria, and mycophage nematodes, can be employed to lessen disease severity and occurrence without the use of chemicals (Agrios, 2005). For instance, Pseudomonas putida bacteria or the 16 mycoparasite Verticillium lecanii treated cucumber seeds stimulate the host plant to produce phytoalexins and other defence mechanisms (Agrios, 2005).

2.3.2. Chemical control strategies for R. solani

The use of fungicides as seed treatment is the most common method of controlling root rot diseases. The treatment of maize seed with chemical fungicides is the most common treatment for controlling *R. solani* (Al-askar and Rashad, 2015). In order to lower the prevalence of diseases brought on by soil-borne infections, fungicides such as phenyl amides, mefenoxam and metalaxyl have been employed as seed treatments in the production of maize (Matthiesen et al., 2016). One of the most efficient ways to lessen damping-off, seedling blights, and root rots brought on by Rhizoctonia, mix systemic fungicides with broad-spectrum fungicides. It is known that seed treatments 15 followed by seedling spraying with the same or other fungicides

might lessen disease severity during the early phases of plant growth when soil infection levels are exceptionally high or soil wetness is extended (Agrios, 2005). Even though different R. solani AGs and subgroups have varying sensitivities to various fungicides and fumigants, systemic fungicides and soil fumigants are nevertheless used extensively to address Rhizoctonia root rot (Singh et al., 2019).

2.3.3. Cultural resistance for the control of *R. solani.*

Making sure that the root system of the host has access to enough water is one of the most crucial methods for minimizing root rots (Naseri and Moradi, 2015). The use of sprinkler irrigation as opposed to flood and furrow irrigation, which may cause water to accumulate, especially in low lying areas, is one way to successfully manage diseases brought on by Rhizoctonia. Through fostering environmental circumstances that are unfavourable for the development of the disease and hence support the growth of the host plant, Rhizoctonia root rots can also be reduced (Naseri and Moradi, 2015). Disease-free seeds should be used to combat both Rhizoctonia root rots (Yekelo, 2021). Raised seedbeds should be used by farmers in environments that encourage quick seedling development. Good soil surface and plant aeration is made possible by wide in-row spacing. Disease incidence is decreased by chemical soil sterilization and crop rotation (Yekelo, 2021). Pythium root rot incidence and severity have been decreased as a result of several cultural measures, such as efficient soil drainage, good air circulation, planting when environmental circumstances are favourable for rapid plant growth and providing suitable amounts of nitrate forms of nitrogen (Agrios, 2005).

2.4 Medicinal plants

Medicinal plants have in recent years gained popularity over allopathic medicine (Matyangaet al., 2020). They offer a wide range of chemical diversity which gives positive physiological changes to the human body (Zimudzi, 2014). Medicinal plants have been in use since the historical eras and continue to be predominantly used in Africa, Asia, and Latin America due to cultural beliefs (Tilburt and Kaptchuk, 2008). Over 80% of the African population still rely on indigenous medicinal plants for both cultural practices and healthcare needs (Ojewole, 2006). Southern Africa alone has been reported to be home to over 25% of higher plants in the entire world, making Africa the richest and most diverse continent in plant population (Van Wyk, 2008).

2.5 Use of medicinal plants in South Africa

The use of traditional knowledge and indigenous traditional medicine in South Africa dates back many centuries, especially the use of indigenous plants dating back to approximately 60 000 years ago (Fabricant and Farnsworth, 2001). The use of medicinal plants in South Africa remains evident in the history of many cultures and different ethnic groups (Monakisi, 2007). Through a long history of direct human dependence on the environment for needs like food, medicine, and even shelter, traditional knowledge about the use of medicinal plants has been accumulated. (Monakisi, 2007). Such Knowledge has been conserved through transfer between generations in the form of behaviour, beliefs, and social attributes (Monakisi, 2007; Springfield et al., 2005). Medicinal plants play a significant role in the livelihoods and health of many South Africans and play a vital role in the country's economic sector (Monakisi, 2007).

Many of the rural South African communities still rely on the use of indigenous remedies for the primary treatment of human and animal diseases. The use of traditional medicine in the country is attributed to the accessibility and limited affordability of modern medicine and health care systems (Monakisi, 2007). Positive results from the use of traditional herbs have increased trust in herbal medicines (Maregesi et al., 2008), hence their continuous use in the country. South Africa has a diverse population consisting of amongst others the Nguni, Bapedi, Basotho and bavenda tribes who depend largely on traditional medicine for the treatment of both human and domestic animal diseases (Afolayan et al., 2002). Similar to the population, the country's botanical heritage is diverse with over 30,000 plant species, close to 3000 of which are used for medicinal purposes (Van Vuuren, 2008), and nearly 147 used by the Zulu, Sotho, and Xhosa tribes for treatment and healing purposes (Louw et al., 2002). These plant species are used traditionally as anti-inflammatory, antiseptic, anti-peristaltic, antimitotic, diarrheic, antiviral, antimutagenic, and laxatives, sedative, haemostatic, and contraceptives (Khan and Yadava, 2010; Scarpa, 2004; Street and Prinsloo, 2013).

2.6 Safety evaluation of South African medicinal plants

Apart from contributing greatly to rural livelihoods, medicinal plants play a very significant role in the conservation of forest furrow land biodiversity (Gahukar, 2012). Most medicinal plants display several inhibitory and stimulatory biochemical reactions with other plants (Ushiki et al., 1996) which is an interaction commonly known as allelopathy. Allelopathic medicinal plants are often referred to as higher plants as they produce biological substances that prevent the growth and development of phytopathogens on several agricultural crops

including maize (Ushiki et al., 1996). Traditional medicinal plants are often assumed safe due to their recurring usage for the treatment of diseases, mental illnesses and knowledge passed down from one generation to the other over decades (Fennell et al., 2004). For example, some plants have been scientifically reported to possess toxic effects. Fennell et al. (2004) reported that the bark of *Rhamnus prinoides* exhibited anti-genotoxic effects. In the same study, these authors also indicated that the bark has low mutagen mitomycin effect which is a positive impact to human health.

In South Africa, a small percentage of the 4000 used ethnomedicinal taxa is regarded as toxic and harmful by traditional healthcare practitioners (Arnold et al., 2002; Fennell et al., 2004). According to Sorsdahl et al. (2010), close to 43% of reported deaths in South Africa between 1991 to 1995 were linked to poisoning from the use of traditional medicinal medicinal plants in Johannesburg. This is because medicinal plants are carriers of several mutagenic, carcinogenic and toxic compounds. Several medicinal plants such as *Aegle marmalos* (L.) Corrêa *have been reported to be hepato-toxic (Haq, 2004), making them extremely harmful to the human liver.*

Several popular and regularly used medicinal plants have also been reported to be toxic and serve as contributing factors to illnesses in humans and poor response of the human body to medication (Krishnaiah et al., 2007). *Allium sativum* L known as the popular garlic has for instance been reported to delay blood clotting time in humans (Borrelli et al., 2007), while some herbs such as the popular Aloe have been reported to potentially reduce body synthetic drug absorption due to the mucilage they contain (Haq, 2004). The poisonous and toxic effects of medicinal plants have been linked to inappropriate dosage and intake, misidentification due to lack of knowledge, and wrong preparatory procedures by traditionalists (Stewart et al., 1998).

Several scientific studies have been conducted to evaluate the safety of medicinal plants on the human body. A study by Fennell et al. (2004) tested close to 50 medicinal plant species for potential genotoxic effects using *in vitro* bacterial and mammalian cell assays. Results from this study indicated damage to the cell DNA by a majority of the plants which was detected using Comet assay (Fennell et al., 2004). Plants such as *Sclerocarrya birrea* (A.Rich.) Hochst) showed genotoxicity to human white blood cells under the micronucleus test. Multiple researchers suggest caution when using medicinal plants and extensive knowledge of the historical use and background of plants in use. Several researchers such as Biswas et al. (2002)

suggest the carrying out of thorough intensive research on toxicological and clinical attributes of medicinal plants to enhance their safety for use.

2.7 Choice of Medicinal plants used in this study.

Four medicinal plants were chosen in this study as follows: *Arctotis arctotoides* **(**L. f) O. Hoffm.*, Trichilia ametica* ***Vahl****, Vernonia amygdalina delile and Cascabela thevetia* **(L.), Feddes Repert. Álba'**. The choice of these plants was based on three criteria: Firstly, these plants have ethnopharmacological data indicating their traditional and medicinal use in the treatment of several microbial diseases such as tuberculosis (Afolayan et al., 2002; Mugovhani, 2009; Adinew, 2014). Secondly, these plants have been reported to have antifungal properties and lastly, they were chosen based on their availability.

2.7.1 Arctotis arctotoides (L. f) O. Hoffm.

Figure 2. 1: *Arctotis arctotoides* (Source: htpp ://www.plantzafrica.com)

Arctotis arctotoides is one of the low-lying plants possessing inhibitory properties over the growth of a wide range of bacteria (Afolayan et al., 2002). It belongs to the *Asteraceae* family and the genus *Arctotis*. The species is widely spread throughout the rainfall areas of South Africa and Lesotho. In South Africa, the species are found in eight provinces including Mpumalanga, Eastern Cape, Free State, Gauteng, Kwazulu-Natal, Limpopo, North West, and the Northern Cape (Afolayan, 2003a). The common names of *A. arctotoides* in South Africa include Botterblom (Afrikaans), Ubushwa (isiXhosa), and Putswa-pududu (seSotho). The shoots of the herb are traditionally used for the treatment of multiple health problems such as epilepsy, indigestion, and catarrh of the stomach (Afolayan, 2003a). The juice extracted from the plant is used as a topical paste to treat wounds (Afolayan, 2003a). Research conducted by

Afolayan (2003a) indicates that *A. arctotoides* displays a wide spectrum of biological and pharmacological activity and this has been supported by the work of Feltenstein et al. (2004) who showed the occurrence of sesquiterpenes in the roots of the herb with more than 5000 compounds.

2.7.2. Trichilia ametica Vahl

Figure 2. 2*: Trichilia ametica* (Source: htpp ://www.phytoimages.siu.edu)

Trichilia ametica is an evergreen plant growing up to 20-35 m in height. This tree is naturally and widely distributed in Sub-Saharan Africa. The plant grows in the KwaZulu Natal province of South Africa extending through Mpumalanga, Limpopo (Adinew, 2014). The plant grows inother countries such as Cameroon, Zimbabwe, and Swaziland (Adinew, 2014). *T. ametica* is one of the very coveted multifunctional trees from the Melaceace (Mahogany) family (Komane et al., 2011). The–plant has served as an integral part of the livelihoods of many African countries for ages. The bark of the plant has long been used by African households for implements, carving ornaments, and furniture. The Venda tribe in the Limpopo province uses the bark of the plant to make Mbilamutondo, a traditional musical instrument, while the seeds and of *T. ametica* are used as food for birds (Mugovhani, 2009). All the plant parts have traditional medicinal uses.

Traditionally, the bark of the plant is often soaked in water to create a decoction that relieves stomach pains and other ailments. The leaves are known to be used by the Zulu for rituals

associated with burials (Cunningham, 1993). The leaves are commonly used for healing bruises and wounds in many African countries. Several reports of the plant use indicate that the leaves are a major source medicine for the treatment of Syphilis in Nigeria and as a laxative in Zimbabwe (Cunningham, 1993). The traditional uses of *T. ametica* have been corroborated by several pharmacological studies. Several studies have assessed the antifungal, antibacterial, anti-inflammatory, and anti-cancer properties of the plant (Cao et al., 2021; García-Gómez et al., 2018; Opawale et al., 2015). A study by Germano et al. (2005a) reported the antibacterial activity of the *T. ametica against Staphylococcus aureus, Streptococcus pyogenes, Moraxella catarrhalis* and Haemophilus i*nfluenza* with Minimum inhibitory values <125mg/ml. Another study reported by Shai et al. (2008) reported the antifungal activity of *T. ametica* leaf extracts against *Cryptococcus neoformans, Aspergillus fumigatus, and Microccis canis* with an average minimum inhibitory value of 0.33mg/ml.

2.7.3 Cascabela thevetia (L.), Feddes Repert. Álba'

Figure 2. 3*: Cascabela thevetia* (Source: https://r.search.yahoo.com)

C. Thevetia is a well-known shrub cultivated as an ornamental plant in many tropical and subtropical parts of the world (Bandara et al., 2010) including India and several African countries (Basu and Tripura, 2021). The plant belongs to the Apocynaceae family and is popularly known as the Yellow Oleander in most of the areas where it is found. The plant possesses secondary metabolites such as terpenoids, steroids, glycosides, and flavonoids that promote its pharmacological activity (Gulati et al., 2000). Nearly all parts of the plant have documented medicinal properties (Bandara et al., 2010). Dried plant leaves have documented useful healing and therapeutic properties and are used as Kasisadi Taila formulation in India (Basu and Tripura, 2021).

In African countries, the plant leaves are soaked in water and used for the treatment of amenorrhea, used as eye drops for itchy and dry eyes (Maregesi et al., 2007). The plant's decoction is also commonly used for the treatment of flu, jaundice, and also as a laxative (Zade, 2015). Khan and Yadava (2010) reported the traditional uses of the plant in India. Oil extracted from the seeds of this plant is used for the treatment of several skin infections, while the dried bark is ground into fine powder for the treatment of diabetes (Khan and Yadava, 2010) and the dried leaf powder is used for the treatment of toothache (Basu and Tripura, 2021).

Several reports have documented the antifungal (Ambang et al., 2010), anti-diarrheal activity (Hassan et al., 2011), anti-inflammatory (Thilagavathi et al., 2010), and anti-spermatogenetic activities (Gupta et al., 2011). According to Tewtrakul et al. (2002), two glycosides from the leaves (Lavanone and Flavanol) possess active inhibitory properties against HIV-1 reverse and transcriptase and HIV-1integrase. Despite the documented usage, the plant has been reported to be toxic to cardiac muscles and the autonomic human nervous system (Boddupalli, 2021) due to the presence of cardiac glycosides (Basu and Tripura, 2021).

2.7.4 *Vernonia amygdalina* Delile

Figure 2. 4: *Vernonia amygdalina* (Source: https://www.google.com/url)

V. amygdalina is a woody shrub with vegetable leaves from the Asteraceae family (Uzoigwe and Agwa, 2011). The shrub has green petiolate leaves with an elliptic shape with a diameter of about 8mm (Uzoigwe and Agwa, 2011) characterised by a strong bitter flavour that has gained the plant the name African bitter leaf. The plant has a very common reputation in the east and west Africa as a multifunctional treatment for several ailments and diseases

(Atangwho et al., 2016). Traditionally all the plant parts have been used for disease treatment and control. The roots and bark are used as chew sticks for the treatment of stomach pains, fever and hiccups (Uzoigwe and Agwa, 2011). The plant has reported pharmacologically useful compounds that include phenolic acids, xanthones, anthraquinone (Tona et al., 2004) and have been reported active for the treatment of hypertension (Farombi and Owoeye, 2011), Gastro-intestinal disorders (Ifeoma and Chukwunonso, 2011), Amoebic Dysentery (Moundipa et al., 2005). According to Okolie et al. (2008) *V. amygdalina* leaf has been used traditionally for the treatment of blood clots and have shown a great decrease in blood sugar level at post-prandial time point.

2.8 Major groups of phytochemicals present in medicinal plants with antimicrobial properties

2.8.1 Phenolic compounds

Plant phenolics and polyphenols are natural secondary metabolites that are produced by plants. The malonate pathway, which can produce simple phenols, or the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or both biogenetically produce monomeric and polymeric phenols and polyphenols, which play a wide range of physiological roles in plants (Lattanzio, 2013). Phenolic compounds are the most extensively dispersed secondary metabolites, ubiquitously present in the plant kingdom (Lattanzio et al., 2009). Phenolics are not found in bacteria, fungus, or algae. Bryophytes are frequent makers of polyphenols, including flavonoids, but vascular plants contain the whole spectrum of polyphenols (Sadeek and Abdallah, 2019). Besides regulating the relationship between plants and their ecosystems, plant phenolics are involved both in resistance against pests and pathogens and in tolerance toward abiotic stresses (Boudet, 2007). Some of the major phenolic compounds include:

Phenolic acid

Gallic, p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids are examples of phenolic acids C6—C1(Lattanzio, 2013). Phenolic acids are commonly found in the bound soluble form conjugated with sugars or organic acids and are found in complex structures such as lignins and hydrolyzable tannins (Phane, 2009). Free and bounded phenolic acids are found in cereals. Hydroxybenzoic acid glycosides are also characteristic of some herbs and spices. Ellagic acid, aldehydes related to C6—C1 acids are also found in plants. Salicylaldehyde, p-hydroxybenzaldehyde (*Sorghum* spp.), p-anisaldehyde (Vanilla, Mimosa), and p-

protocatechualdehyde 1548 *V. Lattanzio* (*Cichorium intybus*) are also quite common in plants (Nyman and Julkunen-Tiitto, 2000).

Tannins

Plant tannins are a distinct class of phenolic compounds with relatively high molecular weight that may form strong complexes with carbohydrates and proteins. Tannins are water-soluble phenolic compounds with molecular weights ranging from 500 to 3000 (Boudet, 2007).In addition to the standard phenolic reactions, have unique features such as the capacity to precipitate alkaloids, gelatin, and other proteins (Phane, 2009)." Tannins are made up of two types of metabolites in higher plants: hydrolyzable tannins and condensed tannins (Phane, 2009). A third type of tannin, phlorotannins, has recently been identified in numerous algal taxa. Acids, bases, and, in certain situations. Hydrolytic enzymes (tannase) break down hydrolyzable tannins into sugars (typically D-glucose) or related polyols and phenolic acid. Gallic acid in the case of gallotannins, and vegetable tannins are some examples of tannins which are polygalloyl esters (Quideau et al., 2011).

Flavonoids

Flavonoids are naturally occurring chemicals with varied phenolic structures that may be found in fruits, vegetables, cereals, bark, roots, stems, flowers, tea plant leaves (Mojau, 2017). These natural compounds are widely known for their health benefits, and attempts are being undertaken to extract them. Flavonoids are now widely recognized as an essential component in a wide range of nutraceutical, pharmacological, therapeutic, and cosmetic uses (Cushnie and Lamb, 2005). This is because they have anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic qualities, as well as the ability to influence essential cellular enzyme performance (Cushnie and Lamb, 2005). Flavonoids have positive biological effects in disease treatment, hence drugs and treatments containing flavonoids have for centuries been used as principal physiologically active compounds to treat human diseases (Škerget et al., 2005). Flavonoids are classified into subgroups based on the carbon of the C ring to which the B ring is linked, as well as the degree of unsaturation and oxidation of the C ring. Almost all flavonoids can function as antioxidants. Flavones are reported appear to be the most potent flavonoids for protecting the body against reactive oxygen species (Nijveldt et al., 2001). Free radicals and reactive oxygen species, which are created during normal oxygen metabolism or

are triggered by external damage, are constantly threatening body cells and tissues. Some classes of flavonoids include Flavones which are are an important subclass of flavonoids. Flavones are found as glucosides in leaves, flowers, and fruits. Flavones are abundant in *Apium graveolens L, Petroselinum crispum, Capsicum annuum* and *Matricaria chamomilla L* (Škerget et al., 2005). Luteolin, apigenin, and tangeritin are examples of compounds found in this class (Panche et al., 2016). They have a double bond between positions 2 and 3 of the C ring and a ketone in position 4 (Panche et al., 2016). Most flavones of vegetables and fruits contain a hydroxyl group in position 5 of the A ring, but hydroxylation in other places, most notably in positions 7 of the A ring or 3′ and 4′ of the B ring, varies depending on the taxonomic classification of the specific vegetable or fruit (Manach et al., 2004).

Flavonols are flavonoids that include a ketone group. They are proanthocyanin building blocks. Flavonols are plentiful in a wide range of fruits and vegetables (Panche et al., 2016). The flavonols that have received the greatest attention include kaempferol, quercetin, myricetin, and fisetin (Iwashina, 2013). Flavonols, unlike flavones, have a hydroxyl group at position 3 of the C ring, which can also be glycosylated. Flavonols, like flavones, have a wide range of methylation and hydroxylation patterns, and when combined with the various glycosylation patterns, they are the most prevalent and biggest subclass of flavonoids found in fruits and vegetables (Iwashina, 2013). Flavonol consumption has been linked to a variety of health advantages, including antioxidant activity and a lower risk of cardiovascular disease (Manach et al., 2004).

Isoflavonoids are a diverse and separate subclass of flavonoids. Isoflavonoids have a restricted distribution in the plant kingdom, being found mostly in soyabeans and other leguminous plants. Some Isoflavonoids have also been discovered in microorganisms (Matthies et al., 2008). Szkudelska and Nogowski (2007) reported the effect of genistein on hormonal and metabolic alterations, which can influence many disease pathways.

2.8.2 Alkaloids

Alkaloids are plant secondary metabolites with potent pharmacological activities (Bribi, 2018b). With more than 12000 alkaloids extracted and studied, this group is regarded as one of the most diverse and rich phytochemical groups (Sadeek and Abdallah, 2019). Alkaloids are popular in the drug industry due to their limitless benefits to human health. They are good for muscle relaxation and their anti-cancer, narcotic analgesics and antimicrobial properties (Bribi, 2018b). This makes them one of the most used and studied phytochemical group in the drug

industry. Several drugs such as morphine, apomorphine, and codeine are derived from alkaloids.

2.8.3 Carotenoids

Carotenoids are secondary lipid phytochemical metabolites found in the tetraterpenoids class (Natividad and Rafael, 2014; Sadeek and Abdallah, 2019). Carotenoids are a very important phytochemical class with diverse bioactive properties (Sadeek and Abdallah, 2019). Close to 600 carotenoids have been identified, isolated, and studied to date (Sadeek and Abdallah, 2019). This class is responsible for pigmentation in plants, fruits, and vegetables. Several scientific studies report that they possess important roles associated with the management of aging-associated diseases in humans, cardiovascular diseases, and cancer (Kiokias et al., 2016). Limited studies have been conducted on the antimicrobial activity of carotenoids from plant extracts.

2.9 Overview of methods for evaluating antimicrobial activity

2.9.1 Diffusion methods

2.9.1.1 Agar disc diffusion method

This is a popular method used in many clinical microbiology laboratories for antimicrobial susceptibility testing of bacteria (Reller et al., 2009b). According to Espinel-Ingroff (2007), disc diffusion testing of antifungal agents has in the past been slow and therefore a need for a developed standardized agar disc diffusion method for fungi arose. The Clinical and Laboratory Standards Institute (CLSI) developed a disc diffusion method for antifungal susceptibility testing of *Candida spp.* This developed standardized method fulfilled the purpose of easy antifungal susceptibility testing and easy accessibility to clinical microbiology laboratories. Different methodological standards for agar disc diffusion are issued also by national and international institutions (Reller et al., 2009b). These standardized methods differ from the use of media to the use and inoculum preparation.

In this method, agar plates are inoculated with a standardized inoculum of the preferred study micro-organism in the test (Balouiri et al., 2016). About 6mm paper disc with test compound at known concentration are placed on the agar plate and the Petri dishes are sealed and incubated at a suitable temperature as per the standard method used (Balouiri et al., 2016). Antimicrobial agents from the paper disc diffuse into the agar plate preventing the growth of the microorganism under test. The zones of inhibitions are clear, observable, and measurable. This method has been used before its standardization to test Posaconazole against filamentous

fungi (López-Oviedo et al., 2006). This method is well-known and used after standardization due to the advantages it has over other methods. The disc diffusion method is simple to perform with low costs and is very effective in the antimicrobial screening of plant extracts, oils, and drugs (Konaté et al., 2012). Despite its popularity, this method cannot be used for the determination of minimum inhibitory concentrations due to its inability to quantify the number of microorganisms diffused into the agar plates (Balouiri et al., 2016)

2.9.1.2. Antimicrobial gradient test

This is a popular method used for the determination of the minimum inhibitory concentration values of antimycobacterial, antifungals, and antibiotics (Hausdorfer et al., 1998). The method integrates the principles of diffusion and dilution methods to produce the minimum inhibitory concentration value (Balouiri et al., 2016). Minimum inhibitory concentrations are established and can be calculated at the intersection of the strip and growth inhibition ellipse (Balouiri et al., 2016).

2.9.1.3. Agar well diffusion method

This method follows a similar procedure to the disc diffusion method (Balouiri et al., 2016) and is commonly used for the antimicrobial evaluation of plant extracts (Smaoui et al., 2010). The entire surface of the agar plate is inoculated with a volume of the microbial inoculum followed by a punctured hole of about 6 to 8 mm in diameter made out of a sterile cork borer (Gutef et al., 2020). An extract solution of the desired concentration is pipetted into the well and the agar plates are incubated under suitable conditions so the antimicrobial agent diffuses to inhibit the growth of the tested microbial strain (Holder and Boyce, 1994).

2.9.1.4. Agar plug diffusion method

This method is used to highlight microorganism competitiveness (Smaoui et al., 2010), following a procedure similar to that used in the disc diffusion method. An agar culture with the strain of study interest is prepared using the appropriate culture medium by tight streaks on the surface of the plate (Balouiri et al., 2016). Microbial cells release molecules that diffuse in the agar medium during their growth after incubation, a sterile borer is used to aseptically cut an agar plot (Balouiri et al., 2016) and is placed on another agar surface that has been previously inoculated with the study organism. Diffusion of the substance on the agar takes place leaving a visible zone of inhibition around the agar plug which is used to detect and measure antimicrobial activity (Hadacek and Greger, 2000).

2.9.1.5. Cross streak method

A rapidly used method for screening of microorganisms for antagonism (Lertcanawanichakul and Sawangnop, 2008). Seeding of the study strain is done through one streak at the centre of the agar plate (Mohseni et al., 2013). The inculcated plate is incubated based on the study strain and seeded with a microorganism that has been tested by a single steak perpendicular to the central streak (Velho-Pereira and Kamat, 2011) After more incubation period, antimicrobial interactions are analysed through observation of the zone of inhibition (Velho-Pereira and Kamat, 2011).

2.9.2. Bio autography Methods-Thin layer chromatography

2.9.2.1. Agar diffusion

This method is one of the least employed techniques popularly known as contact agar (Balouiri et al., 2016). In this method, the antimicrobial agent is transferred to a previously microbe inoculated agar plate through diffusion (Marston, 2011). The antimicrobial agent is left for some time till it diffuses then the chromatogram is taken out and the agar plate is incubated till zones of inhibition are visible and measurable (Balouiri et al., 2016). This method has been used for testing the activity of more than 22 medicinal plant extracts against gonorrhoea induced by gram-positive bacteria (Marston, 2011). A study by Fittler et al. (2010) was conducted to assess the optimization of the technique using two test organisms (*Candida albicans* and *Saccharomyces).* The study found clear zones of inhibition when using the Mueller-Hinton agar test supplemented with 2% and 0.5mg/ml of methyl blue inoculated with *Candida Albicans* (Fittler et al., 2010)

2.9.2.2. Direct bioautography

This is the most dominantly used TLC bioautography method (Balouiri et al., 2016). The TLC plates are sprayed or immersed in a microbial suspension (Bacterial or fungal) and the bioautogram is incubated for 48 hours under humid conditions at a temperature of 25 °C (Dewanjee et al., 2015). Meyer and Dilika (1996) suggest an absorbance of 0.84 at 560 nm for bacteria such as *Staphylococcus aureus*, while Schmourlo et al. (2005) suggest 106CFU/ mL suspension for both fungi and bacteria. Tetrazolium salts are frequently used for the visualization of microbial growth since they go through an intensely corresponding conversion of colour to formazan by the dehydrogenases of the microbe cells (Choma and Grzelak, 2011). The bioautogram is sprayed with salts and gets inoculated at 37°C for 3-4 hours (Runyoro et al., 2006). Clear white spots on a purple background on the TLC plate indicate antimicrobial activity (Das et al., 2010). Shahat et al. (2008) recommend supplementing Muller Hinton Broth

with agar for the maintenance of appropriate humidity and microbial adherence to the TLC plate because of enough medium fluid.

TLC direct bioautography is useful and effective in the screening of the chemical and biological composition of plant extracts. The sample can be analysed by liquid chromatography Mass spectrometry upon noticeable activity on the TLC plate to find out the involvement of new compounds in the antimicrobial activity (Dewanjee et al., 2015). Direct bioautography is useful for both fungal and microbial tests. This method gives consistent results when used for spore-producing fungi like *Cladosporium and* bacteria such as *Escherichia coli* (Horvath et al., 2010; Suleiman et al., 2010).

2.9.2.3. Agar Overlay assay

This is a mixture of both agar diffusion and direct bioautography popularly known also as immersion bio autography (Balouiri et al., 2016). This method applies to all microbes including moulds (Mehrabani et al., 2013). This bio autography is effective in providing clear zones of inhibition, simple to perform with low chances of contamination, and cheap to perform allowing its practice even in small laboratories that lack massive equipment (Marston, 2011). A study by (Galindo-Cuspinera and Rankin, 2005) characterized antimicrobial carotenoids of *Bixa Orellana* seed extracts using this method. Microbial activity was assessed by overlaying TLC plates with agar inoculated with *S. aureus* sprayed with tetrazolium salts (Galindo-Cuspinera and Rankin, 2005). In a different study, 8 antimicrobial peptides were characterized from *Galleria mellonella* larvae using tricine SDS-PAGE bio autography (Cytryńska et al., 2007).

2.9.3. Dilution methods

These are the most recommended methods for the determination of the minimum inhibitory concentration. They allow concentration estimation of the tested antimicrobial agent in the agar or broth medium which are the two used mediums for *in vitro* antimicrobial testing (Balouiri et al., 2016). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the growth of the microorganism under test (Balouiri et al., 2016). Dilution methods have been documented to be more quantitative and reproducible compared to agar disc diffusion, with limitations to certain antibiotics with the potential to produce inaccurate MIC values when tested in doubling dilutions (Hoffmann et al., 2013).

2.9.3.1. Broth dilution methods

This is the most basic antimicrobial susceptibility method divided into macro and micro. Macro broth dilution involves the preparation of two-fold dilutions for antimicrobial solution (1, 2,4,8,16, 32 and 64 μg/mL) in a liquid growth medium dispensed in test tubes with a minimum volume of 2mL (Bagul and Sivakumar, 2016; Balouiri et al., 2016). Micro broth dilution is regarded as the miniaturization and mechanization of the macro broth dilution test (Bagul and Sivakumar, 2016). Micro broth uses small disposable polystyrene panels containing 9 wells each with a volume of 100 mL (Bagul and Sivakumar, 2016). The major advantage of microdilution is the productivity and regent space that takes place as a result of the miniaturization of the test (Balouiri et al., 2016). The disadvantage therefore with macro broth dilution is its tedious and manual preparation nature (Balouiri et al., 2016). The microdilution broth method has an increased risk of error during antimicrobial solution preparations for each test and requires a large number of space and reagents (Reller et al., 2009a). According to Rodriguez-Tudela et al. (2003)), the method of inoculum preparation and preparation time, inoculum size (Gehrt et al., 1995), and the type of growth medium influence the minimum inhibitory concentration value.

2.9.3.2 Agar Dilution methods

This method involves the integration of different concentrations of the antimicrobial agent into the nutrient agar medium, followed by swapping off inoculation of standardized microbial cells using cotton swabs (Bagul and Sivakumar, 2016; Balouiri et al., 2016). The MIC is recorded at the lowest concentration of the antimicrobial agent that inhibited the growth of the agent under test (Balouiri et al., 2016), following conditions used in measuring the zone of inhibition (Bagul and Sivakumar, 2016). This method is useful for both antibacterial and antifungal tests. Since agar dilutions are performed in Petri dishes, multiple isolates can be tested in one petri dish, hence this method is the most preferred over broth dilution (Bagul and Sivakumar, 2016). Dilutions are made using small water volumes added to melt and cool agar (Bagul and Sivakumar, 2016). The effective and most preferred agar pH is between 7.2 and 7.4 at room temperature (Bagul and Sivakumar, 2016).

A review of the antimicrobial test method by Enriquez et al. (2016)) indicates a recommendation by the Clinical and Laboratory Standard Institute for use of agar dilution for fastidious organisms such as the helicobacter species. According to Baker et al. (1991), this method gives off a good correlation with Esther when used for gram-positive and gram-negative bacteria. Despite the effectiveness of this method over the broth method, it is time-consuming and requires intensive work to prepare the agar plates (Bagul and Sivakumar, 2016).

Chapter 3: Antifungal activity of selected medicinal plants against *Rhizoctonia solani,* casual root rot causing agent in *Zea mays* L.

Abstract

Medicinal plants possess antimicrobial activities against a wide range of pathogens. They are known to induce antagonistic effects to pathogens through the presence of bioactive compounds that induce cell wall disruption and inhibition of enzyme activities of the pathogen. In this study, four selected medicinal plants: *T. ametica, C. thevetia, A. arctotoides* and *V. amygdalina* were selected and screened for antifungal activity against *R. solani*. The antifungal agar disc diffusion assay was employed to test the crude extracts of four medicinal plants extracted in acetone and ethanol. The medicinal plant extracts, except *T. ametica* extracts exhibited antifungal activity against the pathogen. The mean inhibition diameter of the pathogen ranged from 3.9 (*C. thevetia* ethanol extracts) to 12.2mm (*A. arctotoides* acetone extracts). The extraction solvent that exhibited the highest inhibition is acetone with an average of 71.77mm compared to ethanol that exhibited 55.99 mm inhibition diameter. The minimum inhibitory concentration (MIC) for the crude extracts that showed antifungal activity was then determined using a modified version of a microplate method. Based on the MIC results, acetone extracts from *A. arctotoides* exhibited the lowest minimum inhibitory concentration (0.2 mg/ml) among the tested medicinal plant extracts, which supports the high antifungal activity of the plant observed in the agar diffusion assay of this study.

Keywords: Medicinal plants, Antimicrobial, Pathogens, Compounds, Inhibition, Antifungal

3.1 Introduction

Fungal diseases pose a significant threat to agricultural production. Pathogens such as *R. solani* are significant soil borne pathogens with adverse effects on crops by inducing pre- and post-emergence damping off, stem canker and root rot (Aslam et al., 2010; Khoury and Alcorn, 1973). *R. solani* causes root infections that result in heavy economic damage. The pathogen infects mostly seeds, roots and hypocotyls (Da Silva et al., 2017) and develops between crops on the plant rhizosphere (Sneh et al., 2013).

R. solani is cosmopolitan in occurrence from the *Alternaria* genus. The pathogen is necrotrophic and sustains itself through feeding from dead plant parts it destroys for nutritional sources (Zhang et al., 2019). Infected maize seedlings and mature maize plants develop reddish to brown lesions, lower stem canker and root brace. *R. solani* contributes 30% to maize yield loss due to its ability to induce root rot that causes plant logging in maize.

The control of fungal damages by *R. solani* includes the use of chemical fungicides, biological and cultural practices such as crop rotation and raised seed beds. The use of chemical fungicides such as Fludioxonil and azoxystrobin (Da Silva et al., 2017) to control *R. solani* remains the most popular method. Although these fungicides provide a quick effective control for farmers depending on them, the development of resistance by the pathogenic fungi to these fungicides and their toxic effects to non-target organisms, environmental and human health remains an issue of concern (Al-Askar and Rashad, 2010). The noticeable toxic effects of chemical fungicides have resulted in an increased demand for a safer sustainable and environmentally friendly alternative. So far, the use of natural products for the control of *R. solani* remains an interestingly effective alternative (Nguyen et al., 2009) as they contain environmentally safer active antifungal agents.

Several plant products including plant extracts have proven active antifungal biological activities, and are therefore used as active biological fungicidal agents (Mandal and Mandal, 2015). Medicinal plants have for long been used as diuretics, anti-inflammants and haemostatics (Burt, 2004), due to their active natural constituents such as saponins, fatty acids, alkaloids, flavones with reported antimicrobial properties (Rajput et al., 2018) that make them ideal alternatives for fungal crop disease management over chemical fungicides. Phytochemicals of plant origin such as carvone, pyrethroids and azadirachtin have reportedly been used as antifungal and anti-pesticidal agents in integrated disease management programs (Schmutterer, 1990). The use of plant extracts therefore can be a viable alternative to bridge

the gap on the management of fungal diseases over chemical fungicides due to their minimal adverse effects on plant physiological processes (Al-Askar and Rashad, 2010), less environmental toxicity (Aslam et al., 2010) and their biodegradable ability into soil nourishing organic material. *T. ametica, C. thevetia, A. arctotoides* and *V. amygdalina* have been reported to exhibit antimicrobial activities against several plants and human pathogens, hence the aim of this study was to evaluate the *in-vivo* antifungal activity of these selected plants against *R. solani.*

3.2 Materials and methods

3.2.1 Collection of plant materials

Vernonia amygdalina, Trichilia ametica, Cascabela thevetia and *Arctortis arctotoides (*MCK*001)* were selected to investigate their antifungal activity against *R. solani. Vernonia amygdalina (*MCK*002), Trichilia ametica (*MCK*003), Cascabela thevetia (*MCK*004)* were collected from the Nkomazi region of the Mpumalanga Province (25.5653° S, 30.5279° E), while A. *arctotoides* was collected from the Alice region of the Eastern Cape Province (32.2968° S, 26.4194° E). Voucher specimens for the collected medicinal plants were deposited at the South African National Biodiversity Institute (SANBI) herbarium for authentication.

3.2.2 Preparation of plant extracts

Plant leaves were washed, and oven dried at 40°C for three days. The dry plant leaves were powdered using a waring TBBK160K blender at low speed and stored in airtight containers on the laboratory bench to preserve the biomolecules present in the plant. The crude plant extracts were prepared using the Soxhlet extraction method (Redfern et al., 2014). Briefly, 50 g of each plant powdered material was extracted using 300 ml of ethanol and acetone separately for 24 hours. The extracts were filtered through Whatman no. 1 filter paper (**Figure 3.1**) and were later evaporated to dryness at a temperature of 40°C using a rotary evaporator (Otang-Mbeng, 2012). Thereafter, the extracts were dissolved in Dimethyl sulfoxide (DMSO) to yield 20 mg/ml stock solution.

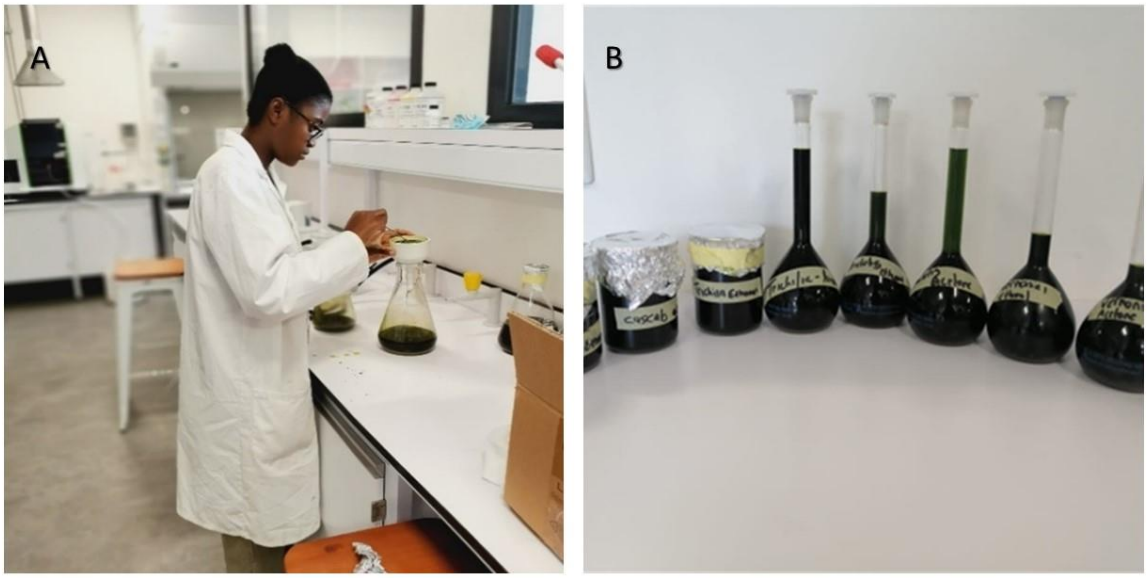

Figure 3. 1: Extract filtration process (a), Filtered plant extracts (b)

3.2.3 Microorganism and Media

The fungal pathogen used was chosen based on its aggressiveness and prevalence in causing root rot in maize. The strain used is from the anastomic group AG3-PT which was obtained from the Donhe Agricultural Institute plant pathology laboratory in the Eastern Cape. Pure cultures of the pathogen were prepared on Potato Dextrose Agar (PDA) plates five days prior to the assays. The PDA was used as a growth media and the PDA media plates were prepared as per the supplier's instruction (39 g for every 1L of distilled water). The PDA was mix by shaking and placed in the autoclave for 15 minutes at 200°C and left to cool in the laminar flow before into 8cm diameter petri dishes..

3.2.4 Antifungal Assay-Agar Disc diffusion

The extracts of four selected medicinal plants *A. arctotoides, T. ametica, V. amygdalina Vernonia.* and *Cascabela thevetia* were tested for antifungal activity against *Rhizoctonia solani* of the AG3-PT anastomosis group following the method described by Balouiri et al. (2016) and Afolayan et al. (2007). Since *R. solani* is a fast grower, pure fungal cultures were kept on PDA plates and recovered for sub culturing three days before the bioassay. Thereafter, 8cm diameter Petri dishes with PDA growth media were prepared and left to solidify for a few minutes. PDA plates were inoculated with 0.5 mm diameter disc of the *R. solani* test pathogen, 0.5 mm diameter Whatman no.1filter paper was prepared and sterilised in the autoclave for 5 minutes. Four 0.5mm diameter disc of the Whatman no.1 filter paper were dipped in 3g/ml

suspension of the plant extract and placed on the plate, two meters away from the inoculum disc. The Petri dishes were sealed with parafilm and incubated at 25±5°C till the pathogen in the control plates reached full growth and covered the entire plate. The experiment was repeated once. When the pathogen in the control had colonised the whole plate, mean inhibition diameters of the plant extracts were determined as the mean of the three replicates for each plant and pathogen interaction.

3.2.5 The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the plant extracts (*T. ametica, C. Thevetia, A. arctotoides)* against *Rhizoctonia solani.*

Minimum inhibitory concentrations of the four medicinal plants (*T. ametica, C. thevetia, Vernonia amygdalina* and *A. arctotoides*) that showed antifungal activity under the disc diffusion assay were evaluated further following the protocol described by Masoko et al. (2005) and a modified version of Eloff (1998) microplate method to test the antifungal activity of the plant extracts. In each experiment, plant extracts were evaluated in triplicate, and each assay was repeated in its entirety to validate the results. Residues of different extracts were dissolved in acetone to a concentration of 10mg/ml. The plant extracts (100 µl) were serially diluted 50% with water in 96 well microtitre plates (Eloff, 1998). To obtain a two-fold serial dilution, an aliquot of 100 µl from well A was mixed into well B and dilution continued downwards after which the excess 100 µl was discarded from the last well. This was followed by addition of 100 µl of fungal culture to each well. Untreated fungal culture and 100% acetone was used as the negative control. It was previously shown by Eloff et al. (2007) that the final concentration of acetone in the microplate well that the fungi are subjected to has no influence. As a growth indicator, 40 ml of p-iodonitrotetrazolium violet (INT) diluted in water at a concentration of 0.2 mg/ml was applied to each microplate well. To avoid fungal contamination in the laboratory, the microplates were sealed in a plastic bag and incubated for four days at 35°C and 100 percent relative humidity. The minimum inhibitory concentration (MIC) is the lowest concentration of the extract that inhibits fungal growth. The colourless tetrazolium salt works as an electron acceptor and is reduced by physiologically active organisms to a red-colored formazan product(Eloff, 1998). After incubation with INT, when fungal growth is suppressed, the solution in the well stays clear or displays a considerable drop in colour intensity on the growth of fungi.

Not only is the MIC value significant in determining which plants have the most potential for further development, but so is the quantity obtained from the plant material. Since the MIC

value is inversely proportional to the number of antifungal compounds present, the total activity was calculated by dividing the quantity extracted from 1 g of plant material in milligrams by the MIC concentration in mg per ml. The total activity is the maximum dilution of an extract from 1 g of plant material that still inhibits the development of the test organism (Eloff, 1998). It may also be used to assess losses during active compound separation and the existence of synergism (Eloff, 2004). The total activity can be calculated as:

$$\text{Total activity} = \frac{\text{Minimum inhibitory concentration (mg/ml)}}{\text{Quantity of material in mg extracted from1g of plant material}}$$

3.2.6 Statistical analysis

The mean inhibition values of the plant extracts towards *R. solani* were analysed using two-way analysis of variance (ANOVA) on GenStat 20th edition and reported as means of two replicates The Duncan multiple test (DMRT) at P=0.01 was used to separate the means.

3.3 Results

3.3.1 Agar disc diffusion

The inhibition zone diameters of tested medicinal plant extracts against *R. solani* are shown in Figure 3.3. The mean inhibition of the plants against *R. solani* ranged from 3.9mm (*Cascabela thevetia* ethanol extracts) to 12.2mm *(A. arctotoides* acetone extracts). The highest inhibition was induced by both acetone and ethanol extracts of *A. arctotoides* (12.2 and 10.4 mm respectively), followed by acetone extracts of *V. amygdalina* (8.0mm) and acetone extracts of *C. thevetia* (7.5mm). Ethanol extract of *C. thevetia* exhibited the least antifungal activity with the mean inhibition of 3.8mm, while on the other hand both acetone and ethanol extracts of *Trichilia ametica* showed no activity against the pathogen. The extraction solvent that exhibited the highest inhibition is acetone with an average of 71.77mm. Based on the inhibition diameters, the antifungal activity of the medicinal plants is in the order *Arctortis arctotoides*> *Vernonia amygdalina*> *Cascabela thevetia.* Based on the empirical values, *A. arctotoides* acetone extract was the most active with the highest inhibition zone diameter, the statistical analysis (One-way analysis of variance) shows that its antifungal activity was not significantly different (P>0.05) from that of the ethanol extracts of *A. arctotoides,* yet it differed significantly from both acetone and ethanol extracts of *V. amygdalina* and *C. thevetia*.

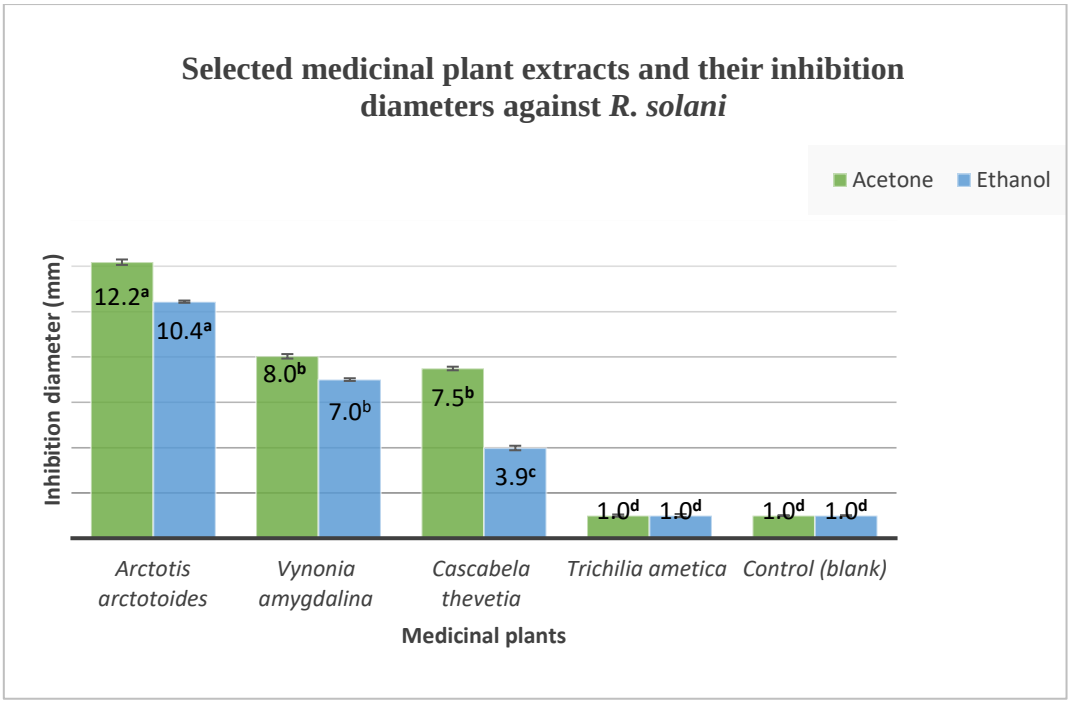

Figure 3. 2: Inhibition diameters of selected medicinal plant extracts; *A. arctotoides*, *C. thevetia* and *V. amygdalina against R.solani*

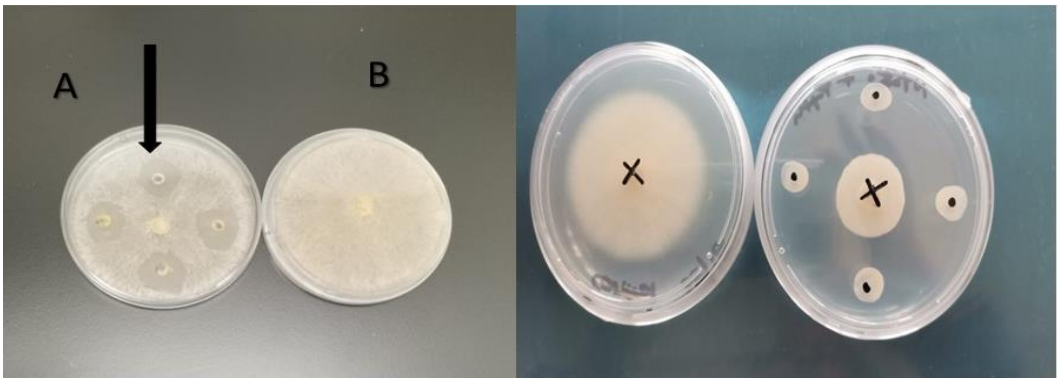

Figure 3. 3: In vitro assay of *V. amygdalina against R. solani (A)* with visible inhibition zones (Arrow). *(B) Is control plate with R. solani*

3.3.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of three medicinal plants that showed antifungal activity in the agar diffusion assay are shown in Table 3.1. The minimum inhibition concentration of mycelia growth ranged from 0.02 mg/ml concentration of acetone extracts of *A. arctotoides* acetone to 1.21mg/ml concentration of ethanol extracts of *Cascabela thevetia*. The most active plant based on the MIC is ethanol extracts of *A. arctotoides*, followed by ethanol extracts of *A. arctotoides* and acetone extracts of *V. amygdalina*. The extract that exhibited the least inhibition potential is ethanol extracts of *C. thevetia*, which exhibited a MIC value greater than 0.1mg/ml.

Table 3. 1: Antimicrobial activity (Minimum inhibitory concentrations = MIC) of 3 medicinal plant extracts against *R. solani*

Plant extract[1]	*MIC (mg/ml)*				
	Time[2] *(h)*	*Extractant*[3]			
		A	*E*	*Average*	*P-value*
V. a	*24*	***0.08***	***0.10***	*0.09*	*<0,02*
A. a	*48*	***0.02***	***0.03***	*0.02*	*<0,02*
C. t	*24*	***0.72***	*1.21*	*0.96*	*<0,02*
Average		*0.57*	*0.45*	*0.35*	

Means with the same superscript in the same column are not significantly different (P > 0.05)

* These are means of three replicates. *Values in bold are considered as noteworthy antimicrobial activity (MIC < 1 mg/ml)

*Values in bold are considered as noteworthy antimicrobial activity (MIC < 1 mg/ml).

[1]**Plant extract**: V. a.= *Vernonia amygdalina*, A. a. =*Arctotis arctotoides*, C. t. =*Cascabela thevetia*. [2]**Time**: MIC values after 24 h were at times not distinct, so they were kept incubating for another 24 h before reading the MIC. [3]**Extraction**: A= acetone, E= ethanol

3.3.3 Total Activity

MIC is inversely proportional to the plant total activity; therefore, noteworthy total antifungal activity was observed on extracts with low MIC values shown in Table 3.2 below. The total activity of the plant extracts ranged from 4.13 (*C. thevetia* ethanol extracts) to 170 (*A. arctotoides* acetone).

Table 3. 2: Total activity of acetone and ethanol extracts of the four medicinal plants under investigation

Plant extract[1]	**Total activity**			
	Time (h)[2]	**Extractant[3]**		
		A	E	Average
V. a	24	45	40	42.5
A. a	48	170	133.3	151.65
C. t	24	6.94	4.13	5.53
Average		110.97	59.14	66.56

[1]**Plant extract:** V. a.= *Vernonia amygdalina*, A. a. =*Arctotis arctotoides*, C. t. =*Cascabela thevetia*. [2]**Time**: MIC values after 24 h were at times not distinct, so they were kept incubating for another 24 h before reading the MIC. [3]**Extractio**n: A= acetone, E= ethanol.

3.4 Discussion

In this study, the antifungal activity of four medicinal plants was evaluated against *R. solani.* Based on the activity of the medicinal plant extracts in this study, it is noteworthy that acetone was a better extraction solvent than ethanol. The high antifungal activity of acetone extracts can be attributed to the ability of the solvent to extract a large spectrum of both polar and non-polar compounds. The results have shown that *A. arctotoides* exhibited the highest antifungal activity compared to the other three medicinal plants. It should be noted that there are no previous reports on the antifungal activity of *A. arctotoides* against plant pathogenic fungi in

literature. This therefore means little is known on the activity of this medicinal plant against plant fungal strains, more specifically *R. solani*, suggesting that this may be the first report of the antifungal activity of *A. arctotoides* against plant pathogen *R. solani*. Notwithstanding, the antifungal activity of the plant against various human pathogenic fungi has been reported by other authors. For example, the study of Afolayan et al. (2002) reported maximum antifungal activity (100% inhibition) by *A. arctotoides* against *Alternaria alternaria, Aspergillus niger, Mucorhiemalis* and *Schizophyllum commune.* Otang *et al*. (2012) reported the inhibition of *Cryptococcus neoformans* by *A. arctotoides*. The fungicidal and fungistatic activities of *A. arctotoides* may be attributed to the presence of various classes of bioactive compounds including quinines, phenols, tannins, and flavonoids which cause the disruption of cell wall/membrane integrity (Cho et al., 2013), inhibition of enzyme activities (Muhsin and Aubaid, 2001) and induction of oxidative stress (Lemar et al., 2005). Otang et al. (2011) attributed the mode of action of the acetone extracts of *A. arctotoides* to the induced cell wall impact, leading to alterations in fungal hyphae and the loss of cell wall strength. Unlike animal cells, fungal cells are sustained by an internal hydrostatic pressure that acts on the cell wall different to an internal proteinaceous cytoskeleton (Kaminskyj and Dahms, 2008), despite the fact that cell function requires a comparable cytoskeleton (Otang et al., 2011). This suggests that the cell wall may be one of the target sites for medicinal plants. The myriad reports on the antifungal activity of the plant underscore the antimicrobial relevance of *A. arctotoides* as a potent source for the development of antifungal agents for treating fungal infections.

In this study, the leaf extracts of *V. amygdalina* exhibited a higher antifungal activity against *R. solani* than the leave extracts of *T. ametica* and *C. thevetia*. This observation is consistent with Iwalokun et al. (2003) findings about the efficacy of *V. amygdalina* leaf extract which restored the effectiveness of chloroquine against strains of chloroquine-resistant *Plasmodium beiglei*. *V. amygdalina* has a high potential for treating disorders caused by fungal infections. For example, Okigbo and Mmeka (2008) reported high antifungal activity of *V. amygdalina* extracts against *Candida albicans* pathogen while Anibijuwon et al. (2012) reported activity of *V. amygdalina* against gram positive *Streptococcus mutans* and *Staphylococcus aureus*, using the agar diffusion method. Fungal strains can strive in a wide range of pH far from their optima due to the release of elements (Si, P, S, K, and Ca) that give them their virulence nature (Figueras and Guarro, 1997), however, radical variation in cytoplasmic pH can damage fungal strains through the disruption of plasma membrane and the inhibition of enzyme transport proteins (Nazzaro et al., 2013). Anibijuwon et al. (2012) reported effective antimicrobial

activity of *V. amygdalina* and attributed to the low pH value of the medicinal plant. This therefore suggest that medicinal plant acidity may be one of the influences behind the antifungal nature of medicinal plants through induced cytoplasmic leakage and alterations in cell wall permeability of the pathogen (Tolouee et al., 2010). Based on the results, *T. ametica* did not have inhibitory effects against *R. solani* in this study. It could be deduced from this study that the antifungal effect of these plant extracts is related to their chemical composition, therefore this disparity may be attributable to the fact that the concentration of active chemicals in the plant may change based on the time of harvest affecting the availability of antifungal compounds (Jainkittivong et al., 2009).

A crude extract must have a MIC value of 0.1 mg/ml or less to be deemed active (Aligiannis et al., 2001). Based on the MIC results, acetone extracts of *A. arctotoides* exhibited the lowest minimum inhibitory concentration value which supports the high antifungal activity of the plant observed in the agar diffusion assay of the study though it falls below the prescribes.

3.5 Concluding remarks

The results of this study showed that the highest antifungal activity was obtained for *A. Arctotoides.* The most active extraction solvent was acetone due to its ability to extract polar and non-polar compounds. *A. arctotoides* exhibited the lowest MIC value supporting its antifungal activity observed in the agar diffusion assay. *T. ametica* showed no activity against *R. solani*. The activity of these medicinal plants can further be explored against several more plant pathogens as they have the potential to be included in biocontrol programs against root rot.

CHAPTER 4: The potential of *T. ametica, C. thevetia, A. arctotoides and V. amygdalina* crude plant extracts to control *Rhizoctonia* root rot of maize *in vivo*

Abstract

Root rot infections are widely dispersed due to the huge production rates of maize. This wide distribution of root rot infections in maize results in lower yields. Even though biological control strategies are being pursued, the use of chemical fungicides remain the common root rot controlling strategy. The use of synthetic chemical fungicides remains unsustainable due to the pathogen resistance development. There is therefore a need for the development of alternatives that are more natural than synthetic. Medicinal plants possess antimicrobial activity against many human pathogens. However little information is available on their effect against plant pathogens. In this study, ethanol, and acetone extracts of four previously *in vitro* screened medicinal plants (*T. ametica, C. thevetia, A. arctotoides and V. amygdalina)* were assessed against *R. solani*. Plant extracts were assessed against the rhizoctonia root rot causing agent of maize under greenhouse conditions using rhizotrons. The crude plant extracts were applied as a seed treatment and the pathogen inoculated in the rhizosphere except in the control treatment. Four weeks after seed emergence, , plant height, leaf chlorophyll content, root length, number of root lesions, fresh and dry root weight, fresh and dry shoot weight were measured. *Arctotis arctotoides* ethanol *(AER1*), *Vernonia amygdalina* acetone (*VAaR1), Vernonia amygdalina* ethanol (*VAER1*), *Cascabela thevetia* acetone (*CTaR1)* and *Cascabela thevetia* ethanol (CTER1) were the best three treatments with the highest plant height, chlorophyll content, root hairs and the lowest root lesions. Both acetone and ethanolic extracts of *Trichilia ametica (TAER1 and TAaR1)* showed no activity against *R. solani*, which confirms the results obtained in the *in vitro* studies. The pathogen reduced plant height and chlorophyll content. The increased number of root lesions in treatments with *Trichilia ametica* compared to other treatments including the uninoculated control was expected as a confirmation that *R. solani* was pathogenic to maize. Acetone had high volatility, miscibility with polar and non-polar solvents, and minimal toxicity to test organisms, hence the results of this study show the high activity of acetone extracts in all measured parameters. The *A. arctotoides* and *V. amygdalina* plant extracts were regarded as the best treatments as they were among the top treatments in most of the measured parameters. The performance of these extracts indicates their potential as biocontrol agents for the control of *Rhizoctonia* root rot pathogens of maize. This study has provided a platform for further studies.

Keywords: Root rot, Fungicides, *Rhizoctonia*, Pathogen, Extracts

4.1 Introduction

Cereal grains give more nutrition to humans than any other food group and approximately half of their caloric needs (Ranum et al., 2014b). The three most significant field grains are corn, wheat, and rice, which account for around 94% of total cereal consumption (Awika, 2011). Maize is cultivated on more than 40 million hectares of land in sub-Saharan Africa (SSA). Approximately 50 percent of SSA nations cultivate maize as their principal cereal crop, and in almost three-quarters of these countries, maize is one of the top two cereal crops (Cairns et al., 2021). The average daily consumption of maize is more than 100 grams in more than half of the nations in the area. It is estimated that the population of SSA would quadruple in the next 30 years, and the demand for grains will grow by three-fold. This therefore means the output of maize has to expand by at least 2.2 percent annually in order to satisfy the requirements of future generations (Prasanna et al., 2021).

Root rot infections are widely dispersed due to the huge scale production of maize, resulting in lower yields (Galindo-Castañeda et al., 2019). Rhizoctonia species, together with Pythium spp. 48 and Fusarium species, are the most frequent root rot pathogens. *Phoma terrestis* is another prominent root rot pathogen. *Rhizoctonia* root rot, caused by *Rhizoctonia solani* Kühn, is a globally significant disease that causes significant crop yield losses (Al-Askar et al., 2016; Rashad et al., 2012). The pathogen is very resistant to synthetic fungicides and may persist on plant, soil and residue (Govaerts et al., 2006), making conventional agricultural practices ineffective and hence the use of chemical fungicides by farmers as a quick viable solution. Utilizing chemical fungicides, such as benomyl, carbendazim, and tolclofos-methyl, is the most prevalent method for combating disease (Rashad et al., 2018). The use of synthetic fungicides entails a great danger to human and animal health, environmental degradation, persistence, and probable carcinogenicity.

For the control of Rhizoctonia root rot of maize, medicinal plant extracts may offer an eco-friendly, effective, and economical strategy. The potential of plants to produce aromatic secondary metabolites is almost limitless; the vast majority of these are phenols or oxygen-modified derivatives of phenols (Hussain et al., 2012). This category of substances is comprised of several important subclasses, some of which include phenols, phenolic acids, quines, flavones, flavonoids, flavonols, tannins, and coumarins. They are reported to have antimicrobial properties. Plants use them as a defence mechanism against pathogens (Górniak et al., 2019). Both simple phenols and phenolic acid are examples of bioactive phytochemicals that include a single substituted phenolic ring in their structure. The site and quantity of

hydroxyl groups that are present in the phenolic compound are responsible for the toxicity of phenolic compounds to the pathogen (Al-Askar et al., 2016; Baka and Rashad, 2016; Rashad et al., 2012). In addition to their direct antifungal activity, medicinal plant extracts may work as "biotic elicitors" to promote plant resistance to invading pathogens and therefore plant extracts may be developed and used in plant disease management. This study aimed to evaluate the effectiveness *in vivo*, of selected medicinal plant extracts (*T. ametica, C. thevetia, A. arctotoides* and *V. amygdalina*) against *R. solani*, the root rot pathogen that affects maize, in a greenhouse setting.

4.2 Materials and methods

4.2.1 Experimental site

The experimental trial was conducted at the Donhe Agricultural Development Institute (-32° 31' 59.99" S, 27° 27' 59.99" E) in the Eastern Cape Province of South Africa. Experimental rhizotron trials were conducted in a greenhouse with a temperature of 30℃-day temperature, 25°C night temperature and 12 hrs photoperiod with daily irrigation.

4.2.2 Preparation of pathogen inoculum and pathogenity test

Pure cultures of the *Rhizoctonia solani* were prepared 5 days prior planting. Potato Dextrose Agar was prepared as per manufacturer's instructions and suspended into petri dishes and left to solidify for 30 minutes. Once the PDA had solidified, Rhizoctonia plugs from the previously prepared culture were inoculated into the plate and left to grow for 5 days (Till pathogen reached full growth) in an incubator with a temperature of 25±5°C.

To determine whether the *R. solani* isolate was pathogenic, an experiment was carried out in a greenhouse. Maize plant was planted in the greenhouse. One pot of the planted maize was inoculated with the pathogen and the second was planted without any inoculation as a control. The experiment was carried out over 4 weeks and the impact of the pathogen was observed as shown in figure 4.1.

4.2.3 Rhizotron experiment

The antifungal activity of the four medicinal plant extracts against Rhizoctonia root rot on maize was evaluated under greenhouse conditions using rhizotrons following the layout shown in **Table 4.1**.

4.2.4 Seed preparation

Maize seeds (P1788B, size F14) were first disinfected by washing in 70% ethanol for two minutes and rinsed twice thoroughly in sterilised distilled water. The maize seeds were bloated dry by paper towel and left to dry further for 2 hours on a laminar flow. About 16 maize seeds used for both positive and negative control were immersed in 20ml distilled water for 30 minutes before planting. For every 10 ml of each extract, eight seeds were soaked at 10% concentration for 30 minutes. The extracts in which seeds were soaked in are from *T.ametica*, *C. thevetia*, *A. arctotoides*, and *V. amygdalina* plants species. The extracts were extracted, separately using acetone and ethanol solvents.

4.2.5 Planting

Planting was carried out using the layout as shown in **Table 4.1.** A 10x15cm sized Rhizotrons were filled with filter sand supplemented with 5 grams of NPK (2:3:4(20) +0.5% Zn) fertilizer at planting**.** About two seeds from each extract were planted 1cm deep and test rhizotrons were inoculated with four mycelium plugs of 0,5mm diameter and placed 2 centimetres away from the maize seed. Each treatment had 4 replicates. The experiment had two controls comprising of untreated maize seed with no pathogen next to it and the other one was an untreated maize seed with a pathogen placed 2 centimetres away from the planted seed on either side. The rhizotrons were arranged in a randomised complete block design in the greenhouse with a 30 ℃-day temperature, 25°C night temperature and 12 hrs photoperiod with daily irrigation. In addition to the NPK application, 2.5 grams of LAN (28) was used as a topdressing supplement with nitrogen.

After surface sterilizing untreated maize seeds (cultivar P1788B, size F14) with distilled water, the seeds were left to air dry on the laboratory bench overnight. The seeds were obtained from the Dohne agricultural institution in the Eastern Cape region of the Republic of South Africa. One seed was placed in the centre of each of the four pots after they were each filled with topsoil to a capacity. Four agar plugs each measuring 4 millimetres (4x4 mm^2) squared and containing the mycelium of the pathogen were used to inoculate the soil with the pathogen roughly 2 centimetres distant from the seed. There was a total of four control pots, none of which were inoculated with the pathogen. During planting, each pot was given an additional 3g of NPK (2:3:4(20) +0.5% Zn) fertilizer attained from Omnia Nutriology, Bryanston, South Africa and 1g of LAN (28) from Sasol South Africa (PTY) LTD. After germination, the plants were allowed to grow for a total of four weeks while being irrigated on a regular basis. At the

end of the growth period, indications of root rot were evaluated. These included browning of the leaves and brown root lesions on the roots.

Table 4. 1: Layout of the Rhizotron treatments.

Treatment NO	**Treatment factors**					**Treatment name**	**Method of application**
	PT	**VAE**	**AAE**	**TAE**	**CTE**		
1	YES	-	-	-	-	Negative control	An agar cube with a pathogen mycelium was placed
2	-	YES	-	-	-	VAE	10ML of *Vernonia amygdalina* was drenched into the petri dish with seeds 30 minutes before panting.
3	-	-	YES	-	-	AAE	10ML of *Arctotis arctotoides* was drenched into the petri dish with seeds 30 minutes before panting.
4	-	-	-	YES	-	TAE	10ML of *Trichilia ametica* was drenched into the petri dish with seeds 30 minutes before panting.
5	-	-	-	-	YES	CTE	10ML of *Cascabela thevetia* was drenched into the Petri dish with seeds 30 minutes before panting.
6	YES	YES	-	-	-	VAE+PT	As per the application method of pathogen only and *Vernonia amygdalina* above
7	YES	-	YES	-	-	AAE+PT	As per the application method of pathogen only and *Arctotis arctotoides* above
8	YES	-	-	YES	-	TAE+PT	As per the application method of pathogen only and *Trichilia ametica* above
9	YES	-	-	-	YES	CTE+PT	As per the application method of pathogen only and *Cascabela thevetia* above
10	-	-	-	-	-	Positive control	No pathogen and No extract treatment applied, instead 10ml of water was applied

Key: VAE-*Vernonia amygdalina* extract AAE- *Arctotis arctotoides* extract
TAE-*Trichilia ametica* extract CTE- *Cascabela thevetia* extract.
PT-Pathogen (*R. solani*) C+P – Control and pathogen (*R. solani*)

4.2.6 Parameters Measured

The incidence of the disease was recorded following the method used by Al-Askar and Rashad (2010). Non germinated seeds percentage were recorded after 3 days of planting and the percentage of dead seedlings was recorded 21 days after planting and the percentage of live seedlings was recorded after 21 days of planting. Other parameters that were measured include

root volume, the number of root lesions, root dry weight, shoot dry weight, and shoot length. The trial was terminated after 4 weeks to allow the pathogen effect to be realized. Table 4.1 outlines the trial design treatment combination for the pot experiment including the Positive and negative controls used in the study.

4.2.7 Data collection and analysis

Parameters that were measured are the weekly plant height, root length, Chlorophyll Content Index (CCFI), number of root lesions, fresh & dry shoot weight, Fresh and dry root weight. Analysis of variance was done on all parameters and means were separated using Duncan's Multiple Range Test at 5% significance level on GenStat 20th edition.

Leaf parameters were also recorded. The measurement of all leaf parameters was on leaves which had 80% green leaf area, which included the number of leaves per plant: which were counted and recorded. Chlorophyll content index which was measured on the adaxial surface of the leaf using a CCM-200 Plus chlorophyll content meter weekly. The number of root lesions was visually counted at harvesting. Fresh and dry biomass weight (g) of the harvested material was measured after harvesting. The leaves and roots were harvested and put together per rhizotron in a well-labelled brown bag and taken to the laboratory for weighing using an electronic weighing scale. Thereafter, the harvested fresh rots and leaves were oven dried to a constant weight at 50˚C for 48 hours. The experiments were repeated once.

4.3 Results

After the planting period of the pathogenity test, the degree of root rot of both the experiment and control were assessed. The root length of the control was longer (**Figure 4.1 a**) as compared to the inoculated maize (**Figure 4.1 b**).

Figure 4. 1: Infection of maize seedlings by *R. solani. (A): control with no inoculation, (B)* maize seedling infected with *R. solani.*

4.3.1 Plant height

The mean weekly plant heights of maize treated with selected medicinal plants extracts against *R. solani* are shown in **Figure 4.2**. The results at harvest (week 4) show that all maize plants treated with medicinal plant extracts performed better than the inoculated control except the acetone extracts of *T. ametica* which showed the lowest plant height of 41.11cm. Based on the results at harvest, the overall performance of the medicinal plants extracts on maize plant height is in the order: *A. arctotoides* (acetone)>*V. amygdalina* (acetone) >*C. thevetia* (ethanol) with mean plant height of 68.73 cm, 67.72cm and 67.55cm respectively. The performance of the plant extracts as treatments was consistent in both experiments. In experiment 2, the results at harvest week 4 show that all maize plants treated with medicinal plant extracts performed better than the inoculated control except the acetone and ethanol extracts of *T. ametica* which showed the lowest plant height of 45.62 and 43.38cm respectively. Based on the results, the overall performance of the medicinal plants extracts on maize plant height is in the order: *V. amygdalina* (acetone) > *V. amygdalina* (ethanol) >*C. thevetia* (acetone) with mean plant height of 68.05cm, 66.51cm and 65.56cm respectively.

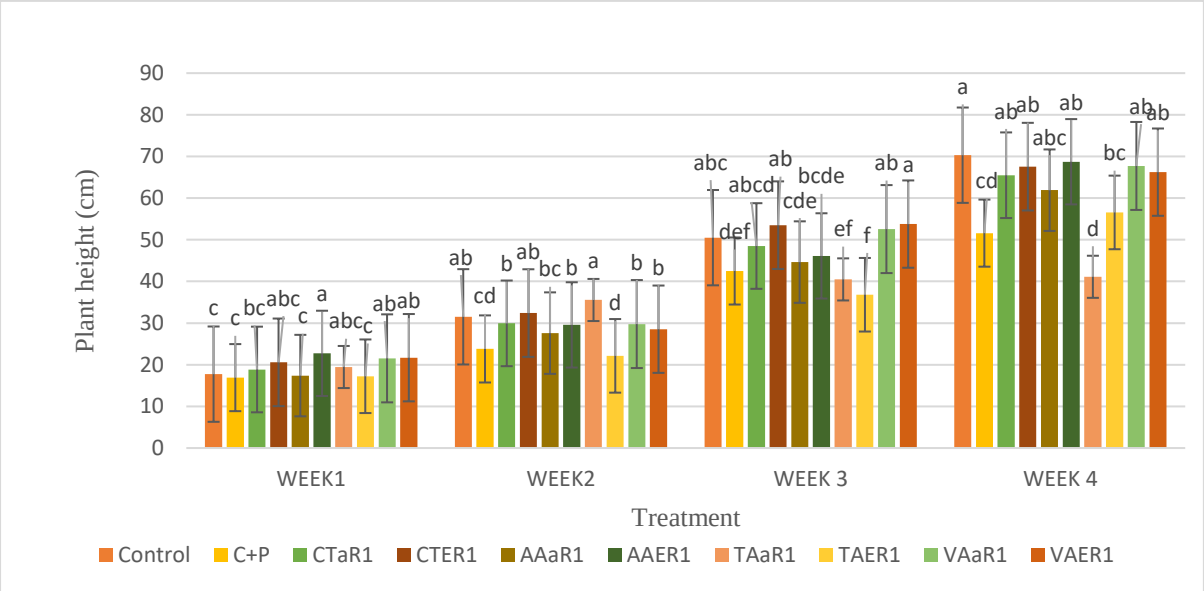

Figure 4. 2: The effect of medicinal plant extracts on the weekly plant height of maize inoculated with *R. solani* for Experiment 1. Mean plant height bars with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level

KEY: *CTaR1-Cascabela thevetia (acetone)* *CTER1- Cascabela thevetia (ethanol)*
AAaR1- Arctotis arctotoides (acetone) *AAER1- Arctotis arctotoides (ethanol)*
TAaR1-Trichilia ametica (acetone) *TAER1- Trichilia ametica (ethanol)*
VAaR1- Vernonia amygdalina (acetone) *VAER1-Vynonia amygdalina (ethanol)*

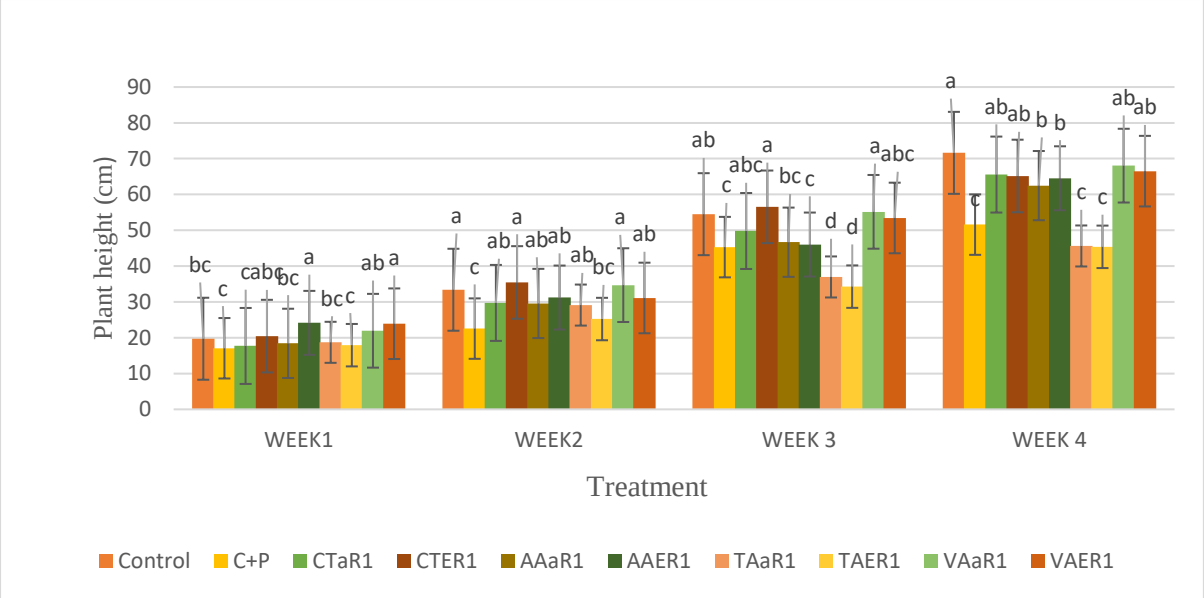

Figure 4. 3: The effect of medicinal plant extracts on the weekly plant height of maize inoculated with *R. solani* for Experiment 2. Mean plant height bars with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

KEY: *CTaR1-Cascabela thevetia* (acetone) *CTER1- Cascabela thevetia (ethanol)*
AAaR1- Arctotis arctotoides (acetone) *AAER1- Arctotis arctotoides (ethanol)*
TAaR1-Trichilia ametica (acetone) *TAER1- Trichilia ametica (ethanol)*
VAaR1- Vernonia amygdalina (acetone) *VAER1-Vynonia amygdalina* (ethanol)

4.3.2 Chlorophyll content

The weekly mean chlorophyll content of maize plants treated with selected medicinal plants against *R. solani* are shown in **Figure 4.3**. The mean chlorophyll content of the maize plant against *R. solani* ranged from 13.93 (*Trichilia ametica*) in week 1 to 51.03 (*Vernonia amygdalina*) in week 4. In Experiment 1, maize plants treated with C. *thevetia* (acetone), *V. amygdalina* (acetone) and *V. amygdalina* (ethanol) had a significantly higher chlorophyll content than other plant treatments during the first three weeks. At the end of the experiment (Week 4), *C. thevetia (*acetone), *V. amygdalina* (acetone) and V. amygdalina (ethanol) were the best three treatments with mean chlorophyll content of 48,18, 49,73 and 48,98 respectively. In Experiment 2, *V. amygdalina* (acetone) and *V. amygdalina* (ethanol) remained the best medicinal plant treatments throughout the experiment (Week 1-4). In this experiment the best three plant extract treatments were *V. amygdalina* (acetone), *V. amygdalina* (ethanol) and C.

thevetia (acetone) with mean plant chlorophyll contents of 49, 46,27 and 48,27 respectively in Week 4. In both experiments *T. ametica* (acetone) and *T. ametica* ethanol had the lowest chlorophyll content throughout the experiment and at harvest (week 4) and was lower than both controls in both experiments.

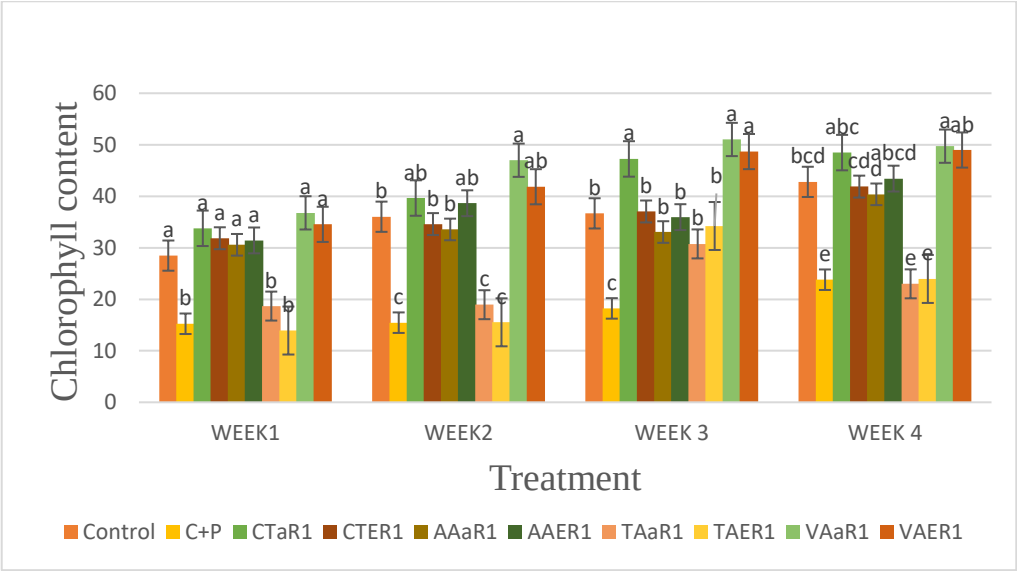

Figure 4. 4: The effect of medicinal plant extracts on the weekly plant chlorophyll content of maize inoculated with *R. solani* for Experiment 1. Mean plant chlorophyll bars with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

KEY: CTaR1-*Cascabela thevetia* (acetone)
AAaR1- *Arctotis arctotoides* (acetone)
TAaR1-*Trichilia ametica* (acetone)
VAaR1- *Vernonia amygdalina* (acetone)
CTER1- *Cascabela thevetia* (ethanol)
AAER1- *Arctotis arctotoides* (ethanol)
TAER1- *Trichilia ametica* (ethanol)
VAER1-*Vynonia amygdalina* (ethanol)

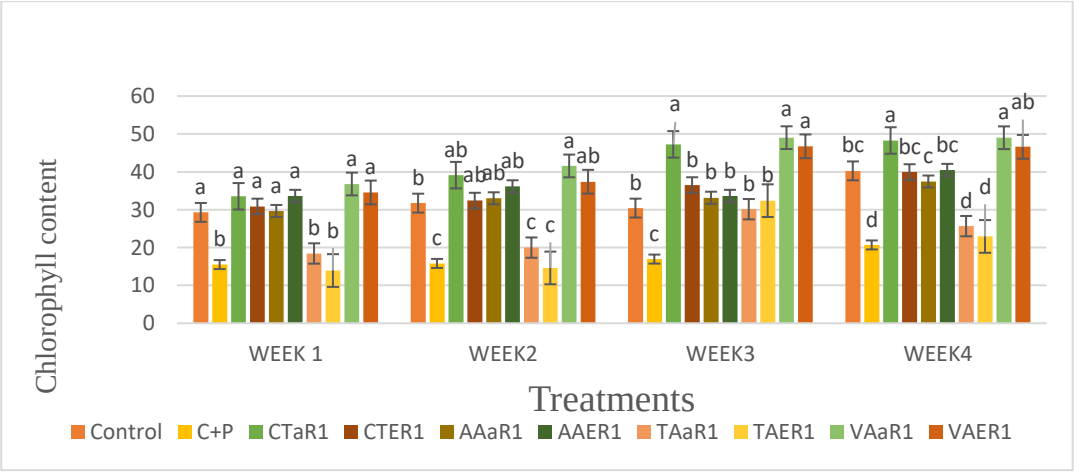

Figure 4. 5: The effect of medicinal plant extracts on the weekly plant chlorophyll content of maize inoculated with *R. solani* for Experiment 2. Mean plant chlorophyll bars with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level

KEY: CTaR1-*Cascabela thevetia* (acetone) CTER1- *Cascabela thevetia* (ethanol)
AAaR1- *Arctotis arctotoides* (acetone) AAER*1- Arctotis arctotoides* (ethanol)
TAaR1-*Trichilia ametica* (acetone) TAER1- *Trichilia ametica* (ethanol)
VAaR1- *Vernonia amygdalina* (acetone) VAER1-*Vynonia amygdalina* (ethanol

4.3.3 Root length

The root length of maize plant treated with medicinal plant extracts measured at harvest is shown in **Table 4.2**. Root length ranged from 14.23 to 27.88 cm in experiment 1. The mean root length of maize plants treated with acetone extracts of *V. amygdalina* (27.88cm) was higher than the maize plant treated with other medicinal plant extracts. The mean root length of this medicinal plant extract was also significantly different from the pathogen inoculated control (10.08cm). However, in Experiment 2, treatment of maize plants with acetone extracts of *A. arctotoides* showed the highest mean root length (27.88cm) and was significantly different from the rest of the treatments and the pathogen inoculated control (9.38cm). Based on the root length results, the extraction solvent that yielded a higher root length for both experiments is acetone with mean root length of 22.45 cm compared to ethanol with mean root inhibition of 21.75cm.

Table 4. 2: Mean root length (cm) of maize plants from experiment 1 and 2

Treatment	**Mean root length (Exp.1)**	**Mean root length (Exp.2)**
V. amygdalina (Acetone)	27.88^{a}	25.80^{b}
V. amygdalina (Ethanol)	27.03^{ab}	27.02^{ab}
A. arctotoides (Acetone)	25.85^{b}	27.88^{a}
Control (Uninoculated)	23.30^{c}	23.39^{c}
C. thevetia (Ethanol)	23.25^{c}	22.84^{c}
A. arctotoides (Ethanol	22.70^{cd}	22.70^{c}
C. thevetia (Acetone)	21.20^{d}	21.67^{c}
T. ametica (Acetone)	14.35^{e}	14.99^{d}
T. ametica (Ethanol)	14.23^{e}	14.21^{d}
Control (Inoculated)	10.08^{f}	9.38^{e}
P value	<0.01	<0.01
I.S.D	1.653	1.919
C.V%	5.4	6.3

*Mean root length of 4 treatment replicates. Mean root length with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

Figure 4. 6: Root system of plants from greenhouse experiment. Differences in root length and weight between maize plants pathogen inoculated control (C+P), uninoculated control (control) and maize treated with acetone extract of *Vernonia amygdalina (VAaR*).

4.3.4 Fresh and Dry root weight

The mean root fresh and dry weight of maize plants treated with medicinal plant extracts are shown **in Table 4.3.** There was a significant difference (P<0,05) between the treatments regarding the fresh and dry root weight. In Experiment 1, the mean fresh root weight of maize plants treated with acetone extracts of *A. arctotoides* (2.95g) was higher than other medicinal plant treatments while the highest mean root dry weight was noted in maize plants treated with acetone extracts of *A. arctotoides* (1.08g). The mean root fresh and dry weight of these two treated maize plants was also significantly different from the control maize and the pathogen inoculated control. However, in Experiment 2, treatment of maize plants with acetone extracts of *A. arctotoides* showed the highest mean root fresh weight (2.95g). *A. arctotoides* treated maize plants exhibited the highest mean root dry weight (1.15a) and was significantly different from the rest of the treatments and from the pathogen inoculated control.

Table 4. 3: Mean fresh and dry root weight (g) of maize plants from experiment 1 and 2.

Treatment	Mean root fresh weight (Exp.1)	Mean root dry weight (Exp. 1)	Mean root fresh weight (Exp. 2)	Mean root dry weight (Exp.2)
A.arctotoides (Acetone)	2.947^{a}	1.0603^{abc}	2.947^{a}	1.1846^{a}
A.arctotoides (Ethanol)	2.763^{ab}	1.0895^{abc}	2.763^{ab}	1.1504^{a}
Control (Uninoculated)	1.760^{de}	0.8826^{bcd}	1.760^{de}	0.8274^{cd}
V.amydalina (Acetone)	2.607^{ab}	1.1846^{a}	2.607^{ab}	1.0355^{ab}
V. amygdalina(Ethanol)	1.338^{e}	0.8355^{cd}	1.338^{e}	0.7326^{d}
C.thevetia (Acetone)	1.990^{cd}	0.9502^{abc}	1.990^{cd}	0.9361^{bc}
T.ametica (Ethanol)	0.488^{f}	0.4084^{f}	0.48^{f}	0.4335^{e}
T.ametica (Acetone)	0.698^{f}	0.5046^{ef}	0.698^{f}	0.4623^{e}
Control (Inoculated)	0.732^{f}	0.6897^{de}	0.732^{f}	0.4878^{e}
C.thevetia (Ethanol)	2.345^{bc}	1.1230^{ab}	2.345^{bc}	1.0902^{ab}
P value	<0.01	<0.01	<0.01	<0.01
I.S.D	0.5213	0.2312	0.5213	0.1917
C.V%	20.3	18.3	20.3	15.8

*Mean fresh and dry root weight of 4 treatment replicates. Mean number of fresh/dry root weight with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level

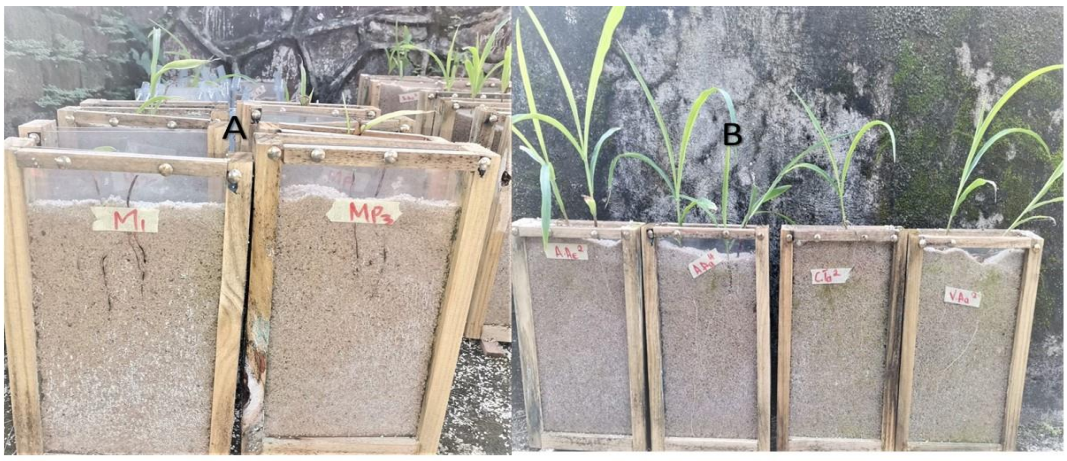

Figure 4. 7: Infection of maize roots by *R. solani.* (A): Control maize seedling infected with *R. solani*, (B) Inoculated maize seedling treated with plant extracts.

4.3.5 Root hairs

Mean root hairs of maize plants treated with selected medicinal plants are shown in **Table 4.4.** In both experiment 1 and 2, medicinal plant increased the number of root hairs in comparison to the inoculated control. The mean root hairs in experiment 1 ranged from 12.75 to 32.25. Maize plants treated with acetone extracts of *V. amygdalina* exhibited the highest mean root hairs, while maize plants treated with ethanol extracts of *T. ametica* exhibited the least number of mean root hair in experiment 1. In experiment 2, maize treated with ethanol extracts of *T. ametica* exhibited the lowest mean number of root hairs (13.50) while maize plants treated with acetone extracts of *A. arctotoides* exhibited the highest mean number of root hairs(32.25).

Table 4. 4: Mean root hair of maize plants from experiment 1 and 2

Treatment	**Mean root hair (Exp.1)**	**Mean root hair (Exp.2)**
A. arctotoides (Acetone)	27.75^{ab}	32.25^{a}
C. thevetia (Ethanol)	29.25^{ab}	29.50^{ab}
V. amygdalina (Ethanol)	29.50^{ab}	29.25^{ab}
V.amydalina (Acetone)	32.25^{a}	26.75^{b}
A. arctotoides (Ethanol)	29.50^{ab}	26.00^{b}
C. thevetia (Acetone)	25.00^{b}	25.00^{b}
Control (Uninoculated)	20.00^{c}	20.50^{c}
T. ametica (Acetone)	17.75^{c}	18.50^{c}
T. ametica (Ethanol	12.75^{d}	13.50^{d}
Control (Inoculated)	10.25^{d}	10.50^{d}
P value	<0.01	<0.01
I.S.D	4.235	4.491
C.V%	12.7	13.4

*Mean number of root hair of 4 treatment replicates. Mean number of root lesions with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level

4.3.6. Root lesions

Mean root lesions of maize plants treated with selected medicinal plants are shown in Table 4.5. In both experiment 1 and 2, medicinal plant treatments significantly ($P<0.05$) reduced the number of root lesions on maize roots. Dark brown root lesions were observed on the roots of the maize plants. The mean root lesions ranged in experiment 1 ranged from 3.500 from acetone treatment of *A. arctotoides* treatment to 10.500 in ethanol treatment of *T. ametica*. Maize plants treated with ethanolic extracts of *T. ametica* exhibited the highest mean root lesions while

maize plants treated with acetone extracts of *A. arctotoides* exhibited the least mean root lesions in experiment 1. In experiment 2, maize treated with acetone extracts of *T. ametica* exhibited the highest mean number of root lesions (9.750) while maize plants treated with acetone extracts of *A. arctotoides* exhibited the least mean number of root lesions (3.750). All medicinal plant treatments reduced the number of root lesions in maize significantly in comparison to the inoculated control. The reduction in root lesions ranged from 13.04 % to 68.48%. Acetone extracts of *A. arctotoides* exhibited the highest root lesion reduction effect (68.48%), while ethanol extracts of *T. ametica* exhibited the least root lesion reduction effect (13.04%). The root lesion reduction effect by the medicinal plant extracts was in the order: *A. arctotoides* >*V. amygdalina* >*C. thevetia*> *T. ametica*.

Table 4. 5: Mean and combined Mean root lesions of maize plants from experiment 1 and 2

Treatment	**Mean root lesions (Exp.1)**	**Mean root lesions (Exp.2)**
A. arctotoides (Acetone)	3.500^{e}	3.750^{c}
C. thevetia (Ethanol)	5.250^{d}	6.000^{c}
V. amygdalina (Ethanol)	4.750^{de}	4.000^{c}
V.amydalina (Acetone)	3.500^{e}	4.250^{c}
A. arctotoides (Ethanol)	4.000^{de}	4.250^{c}
C. thevetia (Acetone)	5.250^{d}	4.250^{c}
Control (Uninoculated)	1.000^{f}	1.000^{d}
T. ametica (Acetone)	8.750^{c}	9.750^{b}
T. ametica (Ethanol	10.500^{b}	9.500^{b}
Control (Inoculated)	12.500^{a}	10.50^{d}
P value	<0.01	<0.01
I.S.D	1.531	2.150
C.V%	17.7	24.8

Mean number of root lesions of 4 treatment replicates. Mean number of root lesions with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

4.4 Discussion

In this study, the antifungal activity of four medicinal plants was evaluated against *R. solani* under greenhouse conditions. The results show that acetone had a potentially interesting activity compared to ethanol extracts. Acetone extracts exhibited higher plant height and chlorophyll content and showed the least mean root lesions. Previous studies by Eloff et al. (2007) also reported acetone as a safe and effective extractant. This is due to its volatility, miscibility with polar and non-polar compounds, and minimal toxicity to test organisms (Eloff, 1999).

The results of the study show that seedlings treated with the extracts had an increased height for both experiment one and two in comparison to the maize inoculated control. The effective control of the *R. solani* pathogen by the selected medicinal plants in the study is similar to that reported by Rashad et al. (2018) on the effectiveness of khella extracts against *R. solani* pathogen in maize. The ability of the plant extracts to control the disease may be attributed to the antifungal action they directly exert against the fungi that cause disease and their indirect effects on the plants that are being affected. According to Akladious et al. (2015) and Abkhoo and Jahani (2017) medicinal plant extracts could indirectly increase the immunity of crops to pathogens, which would then activate the host's defensive mechanism against infections. Up-regulation of many genes involved in plant defences, activation of multiple pathogenesis-related (PR) proteins, accumulation of phytoalexins, deposition of lignin, and programmed cell death are all of plant response mechanisms induced by these medicinal plants extracts (Goel and Paul , 2015)

According to Argenta et al. (2004), maize chlorophyll values over 45.4 in the early stages are considered to be sufficient to achieve excellent maize grain production. In this study, maize chlorophyll content in this range was noted in maize plants treated with ethanol extracts of *C. thevetia* and *V. amygdalina* (Figure 3.3 and 3.4). This therefore suggests that these treated plants performed better, suppressed *R. solani* and can potentially give a better and improved maize grain yield.

The plant extract from *T. ametica* showed susceptibility to the pathogen and had the lowest mean root weight. In the event of Rhizoctonia root rot, differences in root mass may result in varying grain production. Trachsel et al (2013) demonstrated that the number of crown and brace roots with terminal decay appeared to influence grain yield and therefore, subsequent

research should be done focused on the impact of decreased root mass on shoot traits and grain yield, as well as variations in root mass regeneration among cultivars.

An increased amount of root hairs has been observed in maize plants treated with the selected plant extracts in previous studies as indicated above. The observed root dry weight in this study correlates well with the observed root hairs (Ma et al., 2001; Mommer et al., 2016). This observation therefore supports the increased root weight observed in maize plants treated with medicinal plants investigated in this study. The plant extracts that showed a significant increase in root dry weight as compared to the control were *A. arctotoides* (both ethanol and acetone extracts), *V. amygdalina* (Acetone) and *C. thevetia* (Ethanol). Moreover, the same extracts treatments showed a significantly higher number of root hairs on maize, which correlates positively with the root dry weights obtained. This means that these extracts protected the plant from the pathogen.

The use of plant extracts in this study resulted in a substantial decrease in the number of root lesions as compared to the control treatment inoculated with the pathogen. Root lesions are a common indication of root rot infections, which may results in the death of the entire plant (Jacobs et al., 2019). According to Chang et al. (2004) the increased number of root lesions in plants reduces the average daily growth rate, which could also be another factor linked to the low plant height in the inoculated control maize and that treated with *T. ametica* in figure 1 and 2. In this study not only did the plant extracts reduce the incidence of root lesions but, in the presence of *R. solani*, maize plants that had their seeds treated with medicinal plant extracts used in this research had a mean root length that was significantly greater than that of the control group that had been infected with the pathogen. Medicinal plant extracts were able to reduce the severity of root rot symptoms may create secondary metabolites and antimicrobial enzymes, both of which slow the progression of the disease caused by the pathogen (Solanki et al., 2015). Based on our results, *A. arctotoides* and *V. amygdalina* are the two most effective and strong plant species against *R. solani*. These results complement well the *in-vitro* antifungal results obtained on the performance of these plants in a subsequent chapter. This therefore means these plants are highly effective against *R. solani* both *in-vitro* and *in vivo*.

4.5 Conclusions

Arctotis arctotoides ethanol *(*AER1), *Vernonia amygdalina* acetone *(VA*aR1), *Vernonia amygdalina* ethanol *(VAER1), Cascabela thevetia* acetone *(CT*aR1) and *Cascabela thevetia* ethanol (CTER1) recorded the best three treatments with the highest plant height, chlorophyll content, root hairs and the lowest root lesions. Both acetone and ethanolic extracts of *Trichilia ametica* (*TAER1 and TAaR1*) showed no activity against to *R. solani*, which confirms the results obtained in the *in vitro* studies. The pathogen reduced plant height and chlorophyll content and had an increased number of root lesions compared to other treatments including the uninoculated control, which was expected as a confirmation that *R. solani* was pathogenic to maize. Acetone had high volatility, miscibility with polar and non-polar solvents, and minimal toxicity to test organisms, hence even the results of this study show high activity of acetone extracts in all measured parameters. *The A. arctotoides* and *V. amygdalina* plant extracts were regarded as the best treatments as they were among the top treatments in most of the measured parameters. The performance of these extracts indicate that they have potential as biocontrol agents for the control of *Rhizoctonia* root rot pathogens of maize.

Chapter 5: Phytochemical analysis of crude extracts of *T. ametica, C. thevetia, A. arctotoides* and *V. amygdalina* using UPLC Q-TOF/ MS/MS

Abstract

Medicinal plants have a large pool of bioactive compounds with active biological activities for the treatment of many diseases including microbial infestations. Understanding the bioactive constituents of these medicinal plants is essential for the application of new plant resources in ensuring food security and food health through healthy crop production. The current study aimed to profile the phytochemical composition of four medicinal plants using both acetone and ethanol extracts. The phytochemical screening was performed using Ultra Performance Liquid Chromatography-MS/MS. The phytochemical screening of ethanolic and acetone extracts of *T. ametica, C. thevetia, A. arctotoides and V. amygdalina* led to the identification of 121 compounds archived through the comparison of the obtained mass spectra data to literature. A total of 15 and 18 bioactive compounds were identified in acetone and ethanolic extracts of *T. ametica* respectively from 7 different classes. The dominant class of compounds was found to beterpenoid at 27,78% from the ethanolic extract. In *C. thevetia,* 17 compounds e from the acetone and 10 compounds from ethanolic extract were identified belonging to over 5 different classes. The dominant class of compounds was found to be terpenoid at 27.76% from the ethanolic extract. About, 17 and 11 compounds were identified from acetone and ethanolic extracts of *V. amygdalina* respectively belonging to over 5 classes, the dominant one being fatty acids from the ethanolic extract at 36,36%. In *A. arctotoides*, 19 and 13 compounds were identified from acetone and ethanolic extracts respectively belonging to over 6 classes, the dominant one being terpenoid at 53, 85% from the ethanolic extract. The results of this study showed that medicinal plants exhibit many phytochemical compounds with possible antifungal properties either singly or synergistically, validating their reported potent antifungal activities. There is need to carry out separation studies to determine which of the compounds have fungicidal properties that could be further explored against fungal pathogens.

Keywords: Medicinal plants, Bioactive compounds, Phytochemical, Classes

5.1 Introduction

Most medicinal plants possess secondary metabolites which are largely believed to be the drivers behind plant chemical defence activity in response to stress, diseases, and predators (Otang-Mbeng and Sagbo, 2019). With the evolution of plant defence mechanisms and activities, the emergence of exploring plant bioactive chemicals with valuable activities for use in medicine, pharmacy, and biotechnology (Otang-Mbeng and Sagbo, 2019) for the development of remedies against a variety of microbial illnesses is on the rise.

Fungal crop disease resistance remains of global agricultural concern (Westh et al., 2004) as they affect several crops. Due to the widespread use of chemical fungicides for the treatment of fungal pathogens, there has been an increase of numerous resistant pathogenic fungi in recent years. (Parekh and Chanda, 2007a). The emergence of aggressive and resistant pathogens like *Rhizoctonia solani* threatens the efficacy and value of many commercial fungicides. Despite the use of chemical fungicides with limited efficacy, there is a rise in serious adverse effects on the environment such as eliminating beneficial microbes, altering the soil pH, soil nutrient level and at worse contaminating water streams (Oruc, 2010).

Medicinal plant extracts have been used for decades for the treatment of fungal diseases (Ben-Shabat et al., 2020). The abundance of chemical components found in medicinal plants gives a large room for the discovery of new organic antifungal agents (Parekh and Chanda, 2007b). According to Basile et al. (2000), several studies have identified active compounds within medicinal plants with active metabolites against fungal strains. While effective compounds have been developed from traditional medicinal plant remedies with proven activity against antibiotic resistant strains of bacteria (Koné et al., 2004), there is still a need to identify new antimicrobial compounds with novel mechanisms of action against infectious plant fungal pathogens. The use of medicinal plant extracts may therefore be a better alternative for the discovery of active compounds for the treatment of fungal root rot diseases in maize and several agricultural crops.

The presence of these bioactive compounds gives plant medicinal properties and hence many of them have been studied and screened intensively for phytochemicals giving them antimicrobial, anti-oxidant, antibacterial, cathartic and anti-cancer properties (Cock, 2011). Despite the milestone, the level of profiling of medicinal plants bioactive compounds, the diversity of the compounds and their classes is still limited. This study, aimed at screening extracts from four medicinal plants; *T. ametica, C. thevetia, A. arctotoides and V. amygdalina*

for phytocehmicals using Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-QTOF-MS/MS) ran on positive and negative ionisation modes.

5.2 Materials and methods

The collection and extraction of plant extracts for the phytochemical screening using UPLC-Q-TOF-MS/MS analysis were done following the methods described in Chapter 3.

5.2.1 UPLC-Q-TOF-MS/MS analysis

Eight plant extracts (4 ethanol and 4 acetone of each plant) were analysed using ACQUITY Ultra Performance Liquid Chromatography mass spectrometry manufactured by Waters Corporation, **Milford Massachusetts**, United States. . An ACQUITY UPLC® BEH column (2.1 mm × 100 mm, ø 1.7 µm, Waters) was used for the UPLC analysis with a column temperature of 50 °C. The flow rate was 0.4 $mL \cdot min^{-1}$, and the mobile phase comprised of Water + 0.1 % formic acid (A) and MeOH + 0.1%$HC0_2H$ (B). The gradient program for the mobile phase was set as follows: 0 min (A: B = 95:5), 3 min (A: B = 75:25), 4 min (A: B = 35:65), and 10 min (A: B = 35:65). The concentration of each plant extract was 0.5 mg/mL in H_2O, and the injection volume was 10 µL. The Q-TOF/ MS/MS was operated in positive and negative electrospray ionization (ESI) modes. The operating parameters were set as follows: cone voltage of 40 V, capillary voltage of 2.6 kV, and source temperature of 120 °C. Data were recorded in the mass-to-charge (m/z) range of 50–1200 with a scan time of 1.05 s and an interscan time of 0.15 s for 20 minutes.

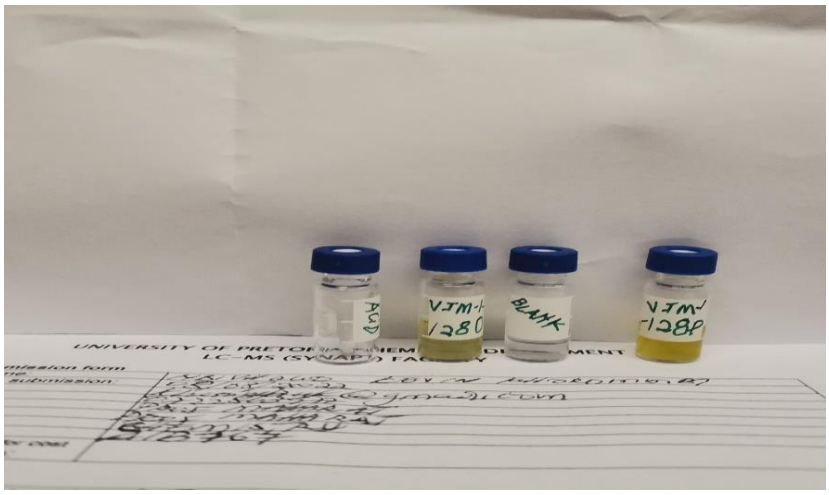

Figure 5. 1: Plant extracts prepared for mobile phase analysis in Water + 0.1 % formic acid (A) and MeOH + 0.1% $HC0_2H$

5.2.2. Data processing and alignment

A total of 16 chromatograms in positive and negative modes were obtained for the 8 plant extracts. Peak detection was performed using the MassLynx software (Waters) with the following parameters: peak intensity threshold of 50 counts, automatic determination of deisotoping, peak width, peak baseline threshold, and noise elimination level. The alignment of mass peaks across all chromatograms was performed using the mass range of m/z 60–1400, mass tolerance of 0.05 Da, retention time window of 0.25 min, and mass window of 0.1 Da. The results were output as a dataset containing 1340 and 661 ions represented as the retention time and mass-to-charge ratio (RT-m/z pair) in positive and negative modes, respectively.

5.2.3. Identification of *T. ametica, C. thevetia, A. arctotoides* and *V. amygdalina* extracts based on MS/MS spectra.

The MassLynx software program was used to identify and retrieve the chemical compounds in the AQUITY UPLC, and it suggested the molecular formula based on the elemental composition of the parent mass ion. A search of the major fragment ions in the MS and MS/MS spectra of potential chemical compounds was performed using Waters UNIFI Scientific Information System software and fragments were confirmed using the Dictionary of Natural Products, PubChem, chem draw. The obtained MS compound fragment data were compared to literature similar findings. The MS spectra results showed mass error values below 5 ppm signifying accuracy as mentioned by (Ismail et al., 2019).

5.3 Results

5.3.1 Compounds identified in acetone extracts of *T. ametica*

The bioactive compounds identified in acetone extracts of *T. ametica* are shown in Table 5.1. A total of 15 compounds were identified in ES+ and ES- ionization mode between 5.09 and 17.23 min. Based on the detector count, the three most abundant compounds are Tenacissoside K (100703), Kaempferol 3-Lathyroside (64059) and 19β-Glucosyl-14-deoxyandrographoside (62823) while the lease abundant is Linolenic acid (11120).

Table 5. 1: Phytochemical constituents identified in acetone extracts of *T. ametica* using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes

Peak no	RT (min)	Component name	Formula	MS/MS Fragments	ES Mode	Ontology/Class	Biological activity	References
1	5.09	Moxidectin	$C_{37}H_{53}NO_8$	528.2835,100,498.2694,39.62,496.20,575.45, 36.77,529.2886 32.96, 640.3805 26.47	+	Lactone	Antimicrobial Antiparasitic	(Anderson, 2020)
2	5,19	Kaempferol 3-Lathyroside	$C_{26}H_{28}O_{15}$	288.25,580.5	-	Flavonoid	Antibacterial	(Qin et al., 2019)
3	7,09	Evobioside	$C_{35}H_{54}O_{13}$		+	Steroid		(Khan and Wang, 2012)
4	8,5	Tenacissoside K	$C_{44}H_{62}O_{14}$	1098.5532,947.2794.9,957.1	-	Glycoside	Antimicrobial	(Tang et al., 2015)
5	9,31	Periplocin	$C_{36}H_{56}O_{13}$	-	+	Saponin	Antimicrobial	Zhao et al. (2019)
6	9,73	Cimidahuside D	$C_{37}H_{56}O_{12}$	-	+		Anticancer	(Alara et al., 2020)
7	9,74	Ginsenoside Rh4	$C_{36}H_{60}O_8$	620,4287	+	Saponin	Anti-inflammatory Antimicrobial	(Xue et al., 2020)
8	9,74	Periplocoside N	$C_{27}H_{44}O_6$	464,3157	+	Terpenoid	Antimicrobial Antioxidant	(Iqbal et al., 2012; Li et al., 2019)
9	9,75	(25R)-26-O-β-D-Glucopyranosyl-5β-furost-20(22)-en-3β,26-diol-3-O-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside	$C_{46}H_{76}O_{18}$	916.50300,276.14000	+	Glycoside	Anticancer	(Xu, 2018)
10	9,76	Trachelosperoside B-1	$C_{36}H_{58}O_{12}$	-	+	Steroid		(Monteiro et al., 2009)
11	12,64	Linolenic acid	$C_{18}H_{30}O_2$	93.0 100,95.0 85.79,91.0 71.97 108.0,66.37, 107.0 48.65	+	Fatty acid	Antifungal Anti-bacterial	(Chandrasekaran et al., 2011; Walters et al., 2004)
12	14,37	Stearidonic acid	$C_{18}H_{28}O_2$	231.3 100,177.2, 15.90,257.3 7.80 239.3130,93.1 0.60	-	Fatty acid	Antimicrobial	(Park et al., 2013)
13	15,62	Arvenin 1	$C_{38}H_{56}O_{13}$	691.23,30.77,322.01,400.01	-	Steroid	Antimicrobial	(Yasmeen et al., 2020)
14	16,07	Etiocholanolone	$C_{19}H_{30}O_2$	290.0 99.99,244.0 72.02, 272.0 61.23 257.0 58.97,216.0 44.01	-	Steroid	Antimicrobial Insecticidal	(Umar et al., 2020)
15	17,23	19β-Glucosyl-14-deoxyandrographoside	$C_{26}H_{40}O$	333.20727 191.05597 161.04514	-	Terpenoid	-	(Chia et al., 2020b)

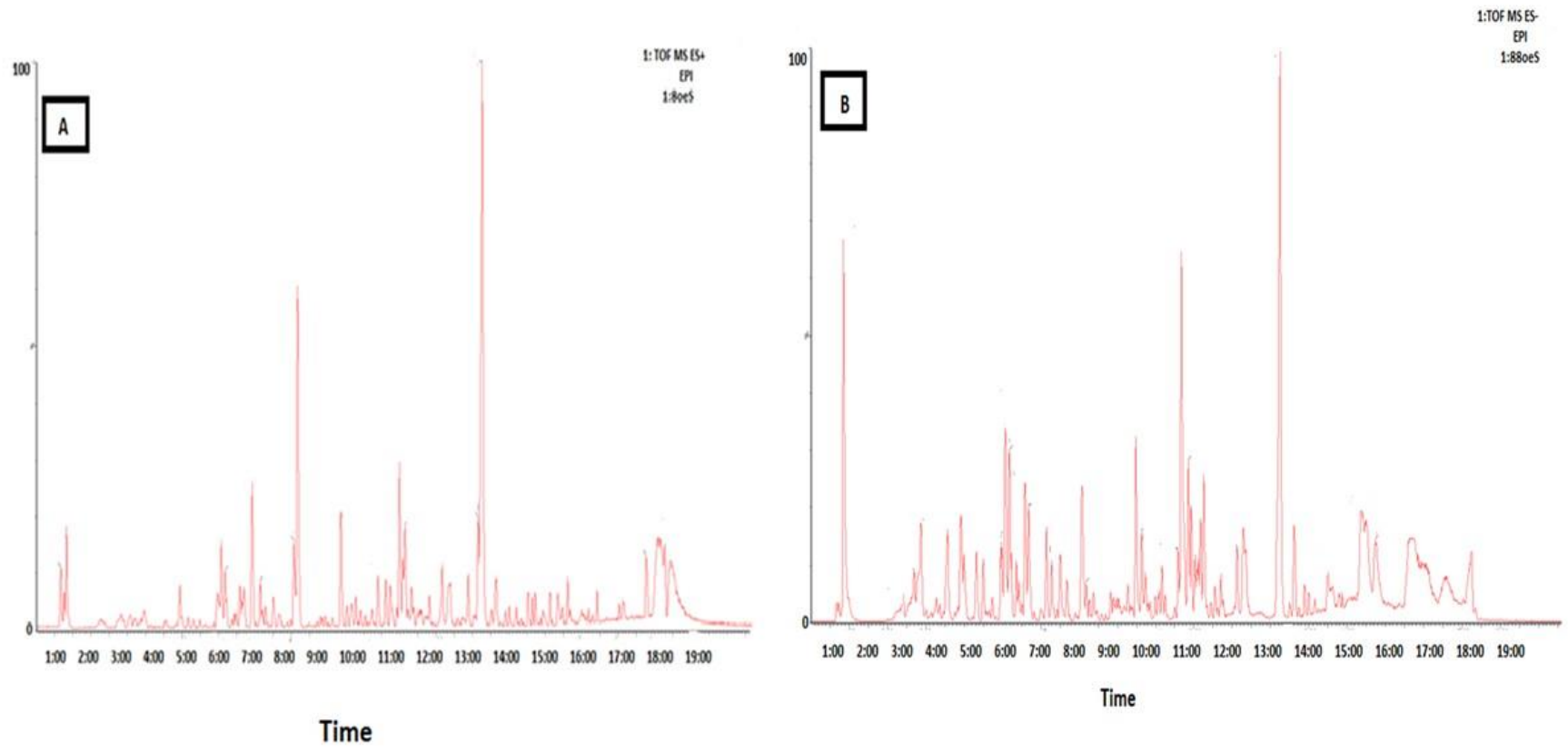

Figure 5. 2: The base peak chromatography of acetone extracts of *T. ametica* in ESI + (A) and ESI – (B)

Table 5. 2: Phytochemical constituents identified in ethanolic extracts of T. ametica using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes

Peak no	Observed RT (min)	Component name	Formula	MS/MS Fragments	ES Mode	Ontology/Class	Biological activity	References
1	5,23	Campesterol-β-D-glucoside	$C_{34}H_{58}O_6$	117.0 0.52, 93.234, 460.49	-	Steroid	Anticancer	(Maitani et al., 2005)
2	5,72	Aster saponin G	$C_{57}H_{92}O_{26}$	448,14	-	Saponin	Anti-inflammatory	(Su et al., 2019)
3	6,47	Cadambine	$C_{28}H_{30}O_{10}$	-	+	Alkaloid	Antifungal	(Dubey et al., 2011)
4	7,08	Daturametelin J	$C_{34}H_{48}O_{11}$	632.7,169.121	-	-	Antifungal	(Shah et al., 2014)
5	8,08	Lobetyolin	$C_{20}H_{28}O_8$	396.4187,396.4231,455.010, 100.365, 98,1234.	-	-	Antioxidant Antimicrobial	(Yang et al., 2019)
6	8,63	Cynanoside L	$C_{41}H_{62}O_{15}$	441.100,627.100, 673.100,771.100 297 100	-	Terpenoid	Antimicrobial Anti-inflammatory antioxidant	(Mamadalieva et al., 2011)
7	8,92	Picrasidine S	$C_{30}H_{29}N_4O_4$	449.5243,509.6001,313.4712 ,508.6341	-	Alkaloid	Antimicrobial	(Khatiwora et al., 2012)
8	8,99	Celosin C	$C_{35}H_{83}O_{25}$	295.5,292.4,285.309,175.0,160.6,1056	-	Terpenoid		(Sultan, 2018)
9	9,06	Allethrin	$C_{19}H_{26}O_3$	135.079,505.999,151.11,200. 406,169.121,338,123.1158 224.34,303.19,534.205	-	Terpenoid	Antimicrobial Anti-inflammatory	(Kulkarni et al., 2012; Pawar et al., 2021)
10	9,18	Arachidonic acid	$C_{20}H_{32}O_2$	79.023,91.0 0.75,80.059,117.052,93.0049	-	Fatty acid	Anti-inflammatory	(Ferrandiz and Alcaraz, 1991)
11	9,74	Darutigenol	C20H34O3	288.5,336.5,322.5,304.5	+	Terpenoid	Antimicrobial	(Reveglia et al., 2018)

12	9,92	Delbrusine	$C_{31}H_{44}O_9$	-	+	Alkaloid	Antifungal	(Alhilal et al., 2021)
13	10,05	Brucine	$C_{23}H_{26}N_2O_4$	395.197,403.999, 396.200,534.220,397.20,320.29	-	Alkaloid	Antimicrobial	(Thambi and Cherian, 2015)
14	10,21	Kushenol M	$C_{30}H_{36}O_7$	-	-	Flavonoid	Antimicrobial	(Oh et al., 2011)
15	10,56	Saucerneol	$C_{31}H_{38}O_8$	-	-	-	Anti-inflammatory Antimicrobial	(Saleem et al., 2005)
16	11,52	Burchellin	$C_{48}H_{74}O_{20}$	-	+	-	-	(Santos et al., 2012)
17	13,64	Bufalin	$C_{48}H_{72}O_{19}$	387.254,624.999,388.255,675.219, 389.2596 47	+	Glycoside	Antimicrobial	(Chang et al., 2022)
18	17,22	Oriediterpenol	$C_{20}H_{32}O_2$	-	-	Terpene	Antioxidant Antimicrobial	(Hossain and Yang, 2014)

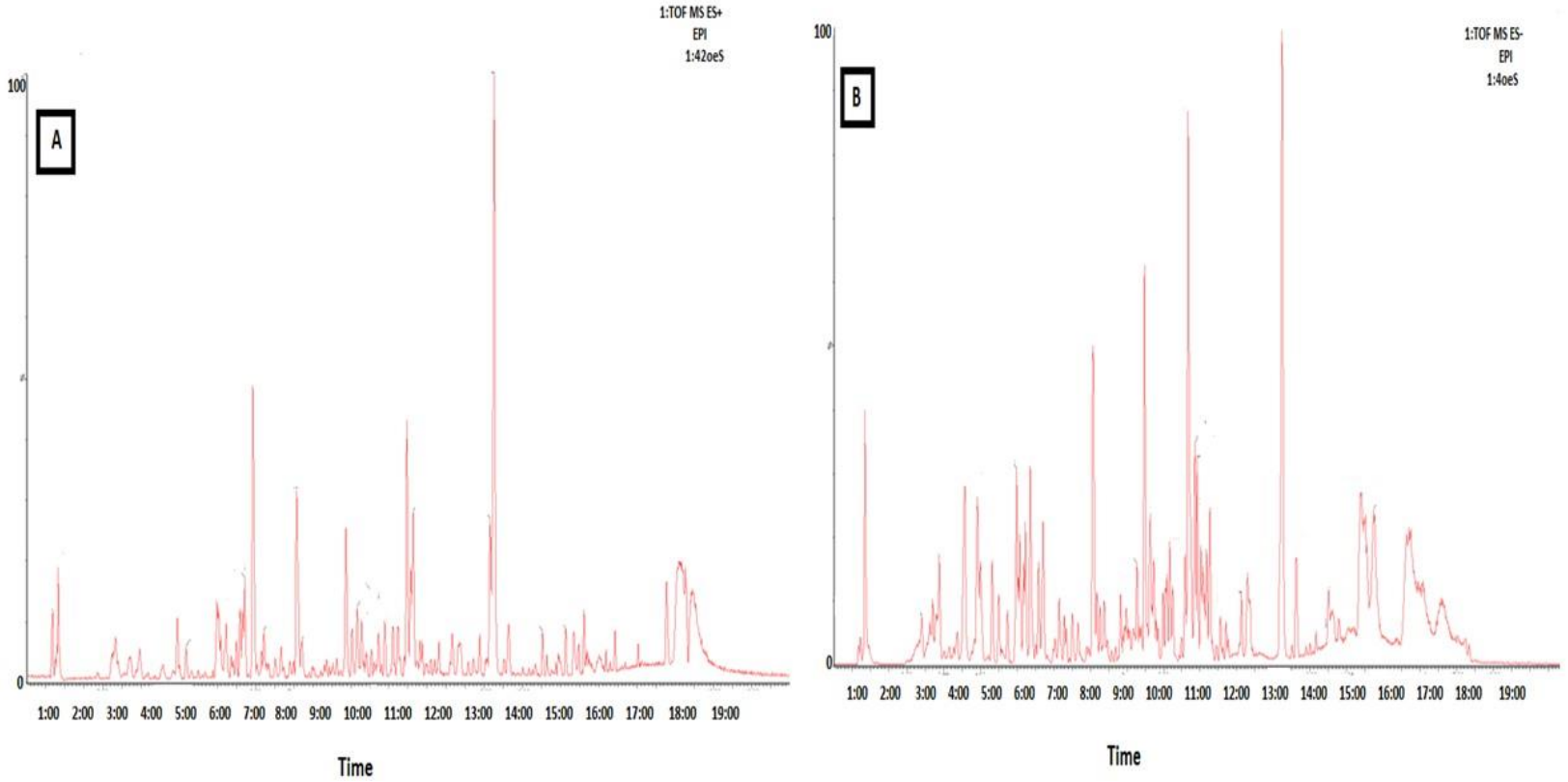

Figure 5. 3: The base peak chromatography of ethanolic and acetone extracts of T. ametica in ESI + and ESI –

Table 5. 3: Phytochemical constituents identified in acetone extracts of C. thevetia using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes

Peak no	RT (min)	Component name	Formula	MS/MS Fragments	ES Mode	Ontology/Class	Biological activity	References
1	2,18	Curzerenone	$C_{15}H_{18}O_2$	220.27,168.15,236.34,181.26	-	-	Antibacterial	(Joshi and Mathela, 2012; Zhu et al., 2020)
2	3,02	Flumioxazin (Pesticide)	$C_{19}H_{15}FN_2O_4$	355.1087,18.100,327.1139,11,78.307,107.499,204.045,329.09,311.034, 44.6571	-	-	Antimicrobial	(Retzlaff and Böger, 1996)
3	6,55	Cimicifugic acid A	$C_{21}H_{20}O_{11}$	181.051,331.100,253.03,364.663,241.978,191.0355,48.08,165.056503,29.21,134.036575 12.88	-	-	-	(Jahn and Petersen, 2021)
4	9,79	Glehlinoside B	$C_{35}H_{44}O_{15}$	137.2,150.3,122.6	-	-	Antifungal	(Cho et al., 2007; Yuan et al., 2002)
5	10,08	Pennogenin-3-O-α-L-rhamnopyranosyl(1→2)-[α-L-rhamno-pyranosyl(1→4)]-β-D-glucopyranoside	$C_{23}H_{24}O_{10}$	436.4,514.6,612.6	-	Flavonoid		(Nohara et al., 1982)
6	10,56	Matrine	$C_{15}H_{24}N_2O$	249.196,1.999	-	Alkaloid	Antimicrobial	(Yang and Zhao, 2006)
7	10,6	Daturametelin A	$C_{34}H_{48}O_9$	577.362,497.362,440.132	+	Terpenoid	Antifungal	(Shah et al., 2014)
8	10,6	Eclalbasaponin Ⅵ	$C_{42}H_{68}O_{17}S$	751.1,846.1234,661,831	+	Terpenoid	Antibacterial	(Ray et al., 2013)
9	10,94	Terrestrosin E	$C_{45}H_{76}O_{20}$	918.465,890.002,38.384	-	Steroid	Antimicrobial	(Farooq et al., 2012)
10	10,95	Picfeltarraenin Ⅳ	$C_{42}H_{64}O_{15}$	642.81876,658.8654,804.9923	-	Terpenoid		(Wang, H. et al., 2021)
11	10,97	Jesaconitine	$C_{35}H_{49}NO_{12}$	675.27,105.077,652.27,570.193	+	Alkaloid	-	(Yamashita et al., 2018)
12	11,24	Arachidonic acid	$C_{20}H_{32}O_2$	79.01,91.075,80.0059,117.0052,93.0 0.49 62.66,117.0,51.05,105.0 38.04,106.03353	+	Fatty acid	anti-inflammatory	(Sala et al., 2018)
13	11,4	Retinol	$C_{20}H_{30}O$	69.0699,218.100,43.054,213.87,83.08546.00,52.98,41.038,34.4670,201.12766,44.40,105.077,100.5678,91.079,83.55,43.311,79.189,64.47,69.142,63.16	+	Carotenoids	-	(Barua and Furr, 1998)
14	12,98	ar-Abietatriene	$C_{20}H_30$	180.24,202.33,160.25	+	Terpenoid	-	(Mothana et al., 2014)
15	13,77	Agroclavine		202.3	-	Alkaloid	Antimicrobial	(Wang et al., 2015)
16	14,11	Pimaradiene	$C_{20}H_{32}$	192.25,272.5,178.23,206.28	+	Terpenoid	anti-inflammatory	(Salleh et al., 2020)

Peak no	RT (min)	Component name	Formula	MS/MS Fragments	ES Mode	Ontology/Class	Biological activity	References
1	2,18	Curzerenone	$C_{15}H_{18}O_2$	220.27,168.15,236.34,181.26	-	-	Antibacterial	(Joshi and Mathela, 2012; Zhu et al., 2020)
17	15,07	12-O-Methylvolkensin	$C_{34}H_{46}O_9$	-	+	-	-	-

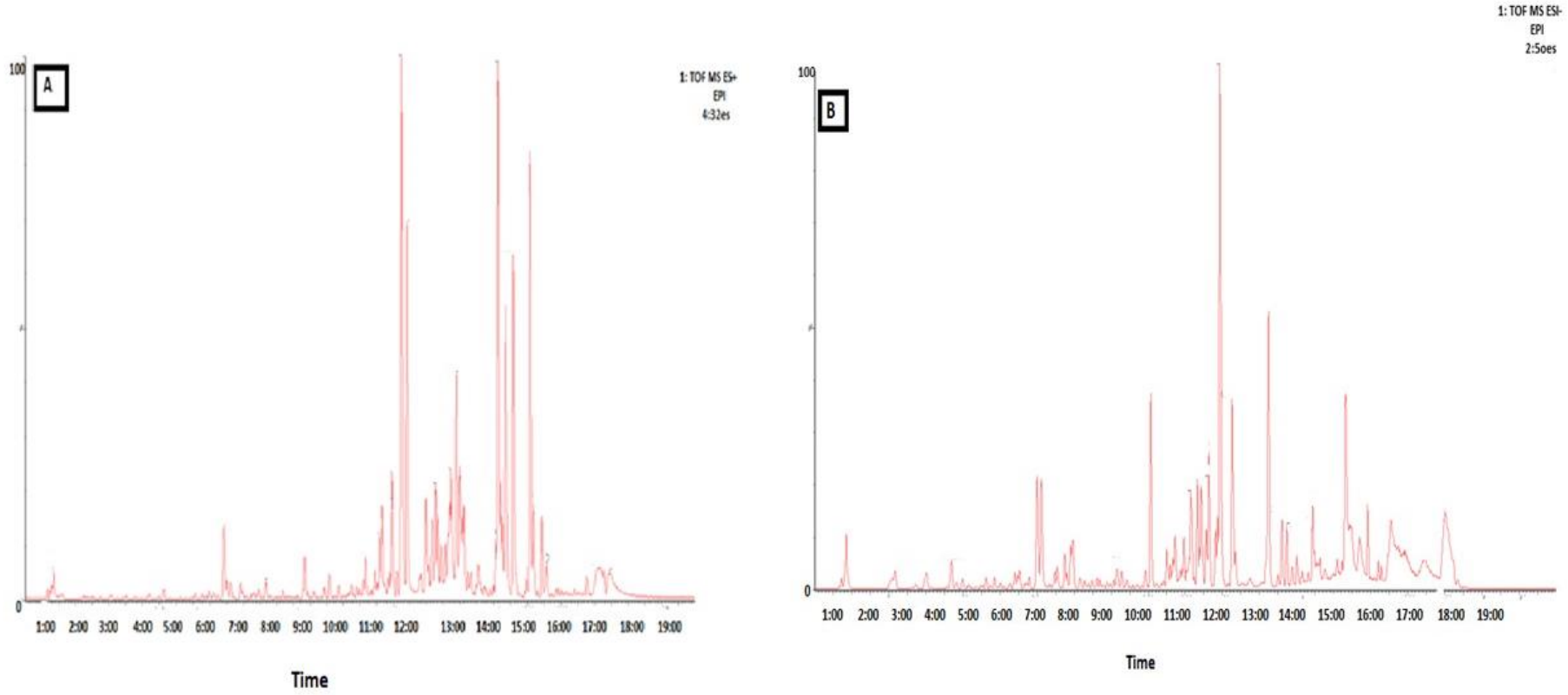

Figure 5. 4: The base peak chromatography of acetone extracts of C. thevetia in ESI + and ESI –

Table 5. 4: Phytochemical constituents identified in ethanolic extracts of *C. thevetia* using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes

Peak no	RT (min	Component name	Formula	MS/MS Fragments	ES Mode	Ontology/Class	Biological activity	References
1	2,3	Icariin	$C_{33}H_{40}O_{15}$	367.1206,317.999,368.1237,170,513.179,143.124, 366.1128 124,514.1827 40	-	Flavonoid	Antimicrobial	(Tan et al., 2016)
2	2,7	Bilirubin	$C_{33}H_{36}N_4O_6$	584,227,285.1100,539.212, 253.29,241.2 9,286.1 4	-	-	-	(McGeary et al., 2003)
3	3,5	Hookeroside C	$C_{41}H_{66}O_{12}$	751.461,17.283,546.999,455.351,38.708,438.3499,382.43,757.33499,340.3456,437.3513,554.275	-	Steroid	-	(Tang et al., 2018)
4	3,7	Hypaconitine	$C_{33}H_{45}NO_{10}$	366.1128,124.00,514.1827, 40.3645,241.2 9,286.14	+	Terpenoid	Antimicrobial	(Zhang et al., 2008)
5	4,2	Fritillebin D	$C_{40}H_{64}O_3$	-	+	Terpenoid	Anti-malarial Antimicrobial	(Lin et al., 2016)
6	3,9	Sevcoridinine	$C_{28}H_{47}NO_2$	-	+	Alkaloid	Antimicrobial	(Wang, Y. et al., 2021)
7	4	Protodiosgenin	$C_{33}H_{54}O_9$	-	+	-	-	(Hirai et al., 1984)
8	5.2	Ophiopogonin C'	$C_{39}H_{62}O_{12}$	-	+	Steroid	-	(Gao et al., 2018)
9	5,3	Oriediterpenol	$C_{20}H_{32}O_2$	168.15,236.34,181.26,304.2384	-	Terpenoid	-	(Guoping and Fengchang, 2002)
10	6,1	19β-Glucosyl-14-deoxyandrographoside	$C_{26}H_{40}O_9$	181.051331,253.033646,63.41,496.2706	-	Terpenoid	-	(Chia et al., 2020a)

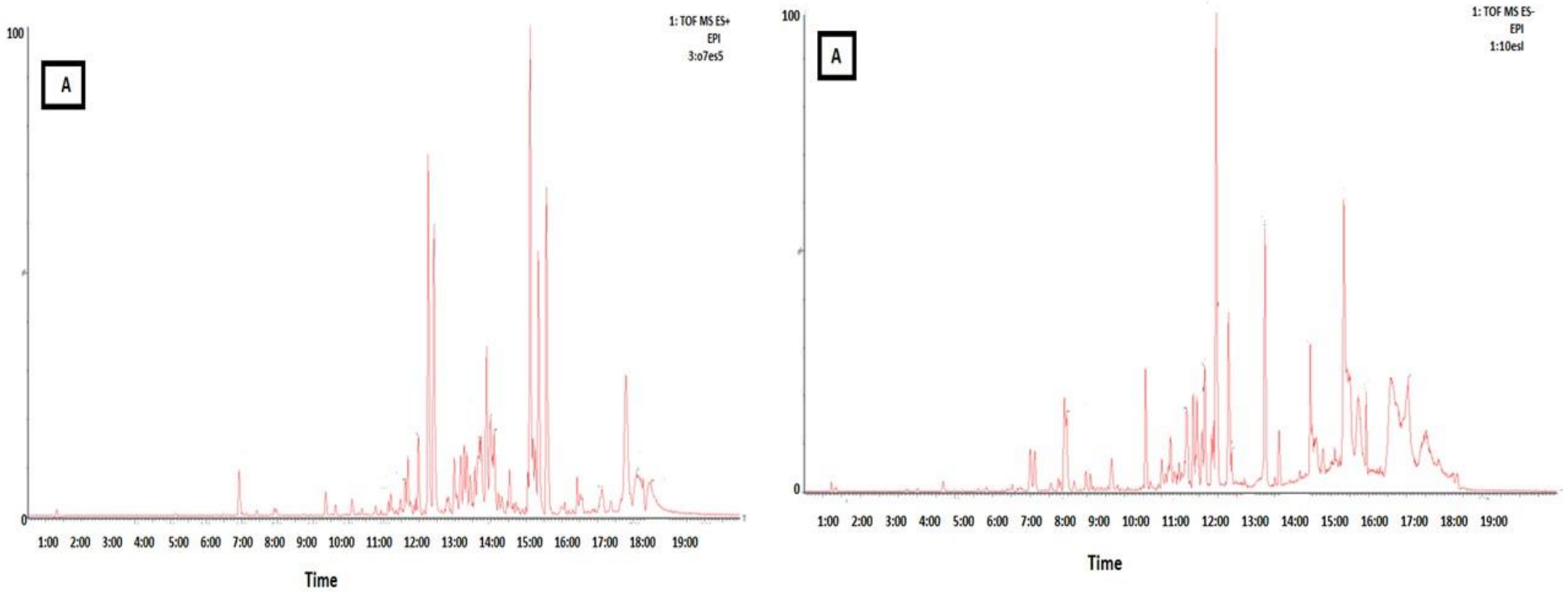

Figure 5. 5: The base peak chromatography of ethanolic extracts of C. thevetia in ESI + and ESI-

Table 5. 5: Compounds detected in acetone extracts of *V. amygdalina*.

Peak	RT (min)	Component name	Formula	MS/MS Fragments	ES mode	Ontology/Class	Biological activity	References
1	4.5	Agosterol A	$C_{33}H_{52}0_8$	-	+	Lipid	-	-
2	5.2	Vernolepin	$C_{15}H_{16}O_5$	-	+	Sequisterpene	-	Alara et al. (2017)
3	5.8	Vernodelol	$C_{20}H_{24}O_8$	-	+	Sesquiterpene	-	Erasto et al. (2006)
4	5.9	Kaempferol-3-O-glucuronoside	$C_{21}H_{18}O_{12}$	285.0405, 150, 461.072, 229.0511, 230.0, 113.0247, 329.23	-	-	-	Yuan et al. (2008) Panizzi et al. (2002)
5	5.99	Oleanolic acid	$C_{30}H_{48}O_3$	203.0, 250, 202.0, 249, 189.0260,320.0,130.234, 190.0266	-	Lipid	Antimicrobial	Corey and Lee (1993)
6	7.4	Apigenin	$C_{15}H_{10}O_5$	117.038, 150, 269.052, 151.008, 113,149.029	+	Flavonoid	Antibacterial	Alara et al. (2017)
7	8.1	Thiamine	$C_{12}H_{17}N_4OS+$	263.11, 2, 233.2, 0.28, 147.1, 115.2, 171.00, 90	+	Terpenoid	Antimicrobial	Alara et al. (2017)
8	8.53	Arctiin	$C_{17}H_{24}O_3$	202.454,234.100,206.055,262.14,14.4567,395.147.13,559.205,255.217,396.151917 1.19	+	Glycoside	Antimicrobial	Arslanyolu and Erdemgil (2006)
9	9.1	Vernomenim acetate	$C_{17}H_{18}0_6$	-	+	Sesquiterpene	-	Alara et al. (2017)
10	11.2	Ascorbic acid	$C_6H_8O_6$	176.0, 99.99, 72, 116.0, 59.2, 15.04, 159.0, 43.0, 130, 164.3, 10.5,85.0, 91, 164.3, 9.73,141.0, 33.5, 160.5, 9.46,147.0, 28.3, 100, 75.2, 117.0, 55.3, 118.3, 48.85,133.0, 32.5, 151.6, 22.62,1.0, 174.5, 146.35, 17.32	+	Terpenoid	Anti-inflammatory Antimicrobial	Alara et al. (2017)
11	12.24	Cholic Acid	$C_{24}H_{40}O_5$	372.09, 20, 271.0, 129, 310.5, 312, 90, 318, 86.21, 150, 253.0, 64.75, 324.0 350.2, 55.0, 323.6	+	-	Antimicrobial	Horai et al. (2010)
12	12.63	10-*O*-Methyl alismoxide	$C_{20}H_{28}O_3$	80.0, 252.2, 149.1	+	Terpenoid	-	Jin et al. (2012)
13	12.66	Aspersteroid A	$C_{28}H_{36}0_4$	220.0, 137.4,135.7, 77.5,70.1	+	Terpenoid		Jin et al. (2012)
14	14.21	Oxymatrine	$C_{15}H_{21}N_2O_2$	148,1	-	Alkaloid	Antimicrobial	Liu et al. (2014)
15	15.88	Notoginsenoside R2	$C_{41}H_{70}O_{13}$	609.4347, 162, 639.4401, 130,477.3912, 290, 457.7413, 309	-	-	-	Zhang et al. (2019)
16	15.63	6,9-Octadecanedioic acid methyl ester	$C_{19}H_{34}O_2$	-	-	-	-	-
17	16.01	Etiocholanolone	$C_{19}H_{30}O_2$	-	-	Steroid	-	-

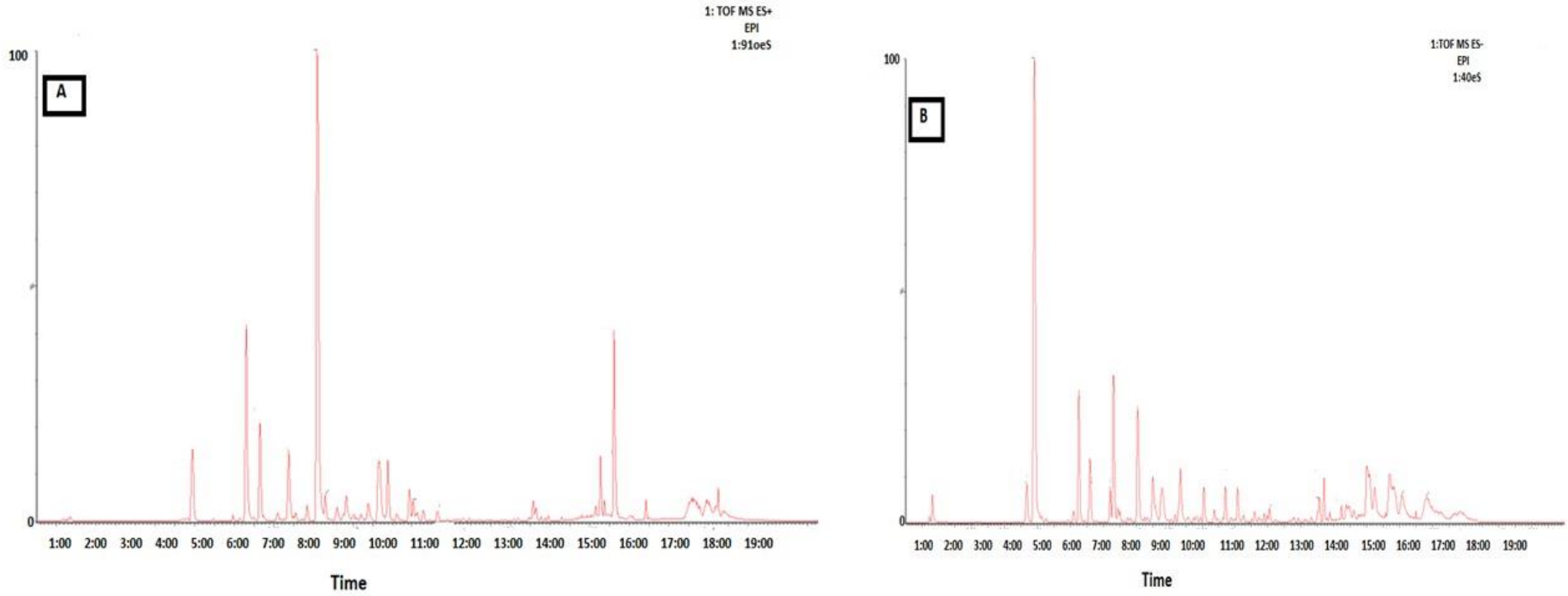

Figure 5. 6: The base peak chromatography of acetone extracts of V. amygdalina in ESI + and ESI-

Table 5. 5: Phytochemical constituents identified in ethanolic extracts of *V. amygdalina* using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes

Peak	RT (min)	Component name	Formula	MS/MS Fragments	ES mode	Ontology/Class	Biological activity	References
1	4.13	Quercetin deoxyhexoside	$C_{21}H_{19}O_{11}$	253,240.2, 10.9	+	Flavonoid	-	Tsamo et al. (2018)
2	4.8	Quercetin	$C_{16}H_{9}O_{7}$	179,151, 112.35	-	Flavonoid	Antimicrobial	Tsamo et al. (2018)
3	5.29	Mellein	$C_{10}H_{10}O_{3}$	161, 10.05, 178,133, 140.5,171, 6.35	+	-	-	Reveglia et al. (2020)
4	7.97	Luteolin	$C_{15}H_{10}O_{6}$	119.0, 1, 168.2, 200.0, 85.73, 161.0, 123.36, 258.0, 27.23, 262.0, 24.12	-	Flavonoid	Antifungal	Hasibuan et al. (2020)
5	7.97	2-Methoxycinnamic acid	$C_{10}H_{10}O_{3}$	172, 5.99, 147, 30.77, 91, 88.73, 118, 59.92, 77, 100.83	-	-	-	Hasibuan et al. (2020)
6	8.2	Linoleic acid	$C_{18}H_{29}O_{2}$	-	+	Fatty acid	Antimicrobial	Tsamo et al. (2018)
7	12.23	Silenoside A	$C_{18}H_{35}O_{12}$		-	Saponin	-	Mamarasulov et al. (2020)
8	13.19	oleonelic acid	$C_{20}H_{32}O_{3}$	92.9, 220, 40.7,265.78, 172.1, 302.5, 47.5, 257.1	-	Fatty acid	Antifungal	Zhanzhaxina et al. (2021)
9	13.41	Stearic acid	$C_{18}H_{35}O_{2}$	-	+	Fatty acid	Antifungal	Tsamo et al. (2018)
10	13.5	7α-hydroxy-8(17)-labden-15-oic acid (Salvic acid)	$C_{20}H_{34}0_{3}$	-	+	Terpenoid	Antimicrobial	-
11	13.65	Funkioside C	$C_{39}H_{62}O_{13}$	-	+	saponin	-	-
12	14.64	Glycerol monostearate	$C_{21}H_{42}O_{4}$	147.01, 210.2, 100, 255.36, 117.34, 205.8, 267,.85.6	+	Fatty acid	-	Kind et al. (2009)

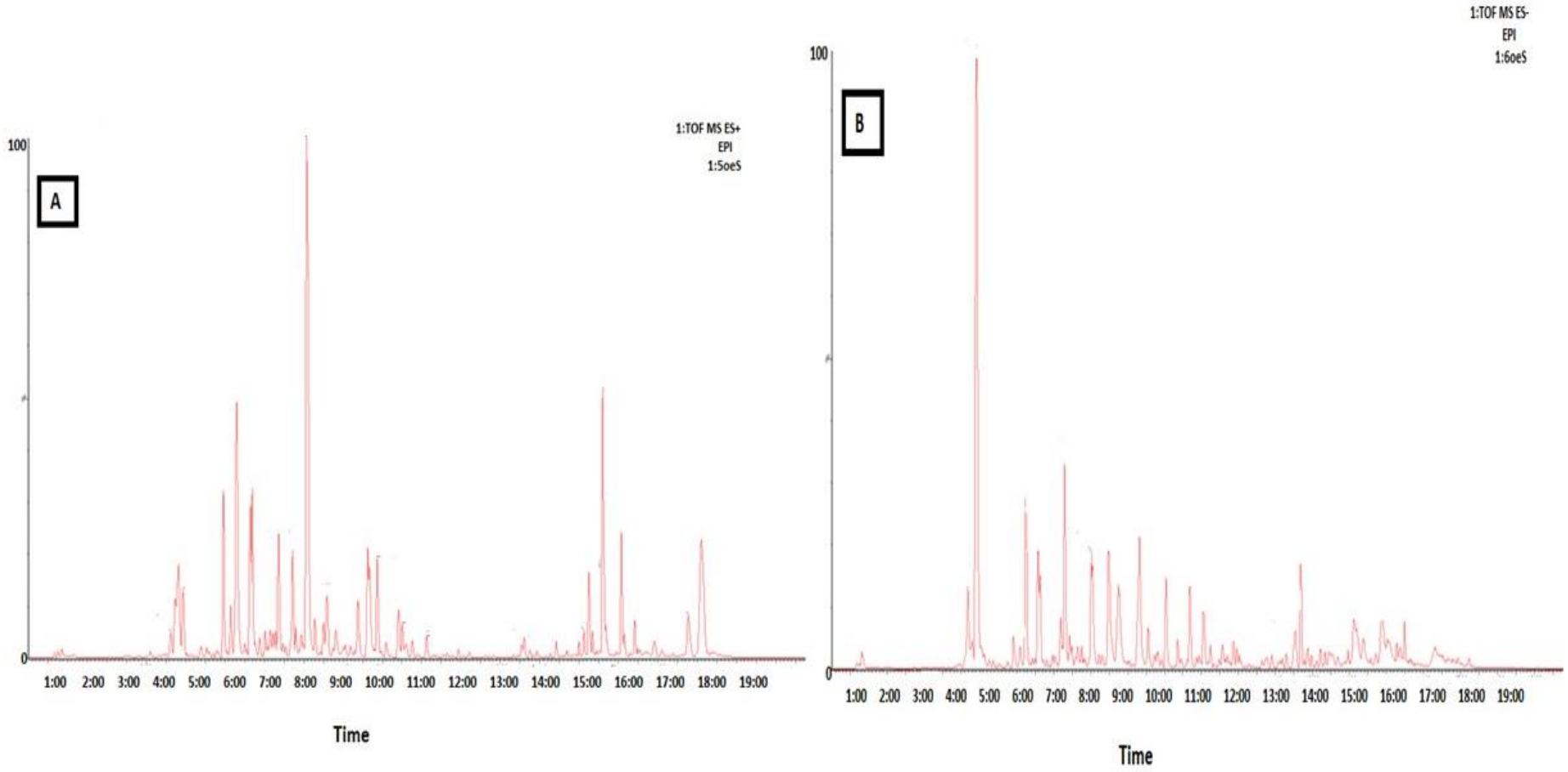

Figure 5. 7: The base peak chromatography of ethanolic extracts of V. amygdalina in ESI + and ESI –

Table 5. 6: Phytochemical constituents identified in acetone extracts of *A. arctotoides* using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes.

Peak no	RT (min)	Component name	Formula	MS/MS Fragments	ES mode	Ontology/Class	Biological activity	References
1	3,83	Etrimfos	$C_{10}H_{17}N_2O_4PS$	179.0982, 292.7811,181. 7789,153.001	-	Organic phosphate	Antimicrobial Insecticidal	(Fouad and Abotaleb, 2021)
2	4,81	Cichorioside C	$C_{21}H_{32}O_9$	424.4234,410.5008, 426.5001	-	Terpenoid	Antimicrobial	(Qadir et al., 2022)
3	6,49	Alisol A	$C_{26}H_{36}O_5$	549.3796, 100, 550.3825,36.71,603.3488, 15.23,551.3839 8.28,604.3552 7.10	+	Steroid	-	(Ma et al., 2016)
4	6,54	Phloyoside Ⅱ	$C_{17}H_{26}O_{13}$	-	-	Glycoside	Antimicrobial	(Li et al., 2017)
5	7,07	Arachidonicacid	$C_{33}H_{45}NO_{11}$	79.023, 630.75,80.0,552 0.59,117.0 0.52, 93.0, 521.49	+	Fatty acid	Antimicrobial	(Huang et al., 2010)
6	7,96	Stigmasterol-3-O-β-D-glucopyranoside	$C_{25}H_{24}O_6$	-	+	Terpenoid	Antifungal	Saleh-e-In and Van Staden, 2018)(Teles et al., 2014)
7	8,03	Allyl disulfide	$C_{15}H_{20}O_3$	41.0 99.99, 39.0 42.81, 45.0 20.31,81.012. 249, 146.09	+	Alkaloid	Antimicrobial	(Rattanachaikunsopon and Phumkhachorn, 2008)
8	8,33	Andrograpanin	$C_{30}H_{32}O_4$	456,2295	+	Terpenoid	Antibacterial	(Majumdar et al., 2020)
9	8,33	(-)-Borneyl ferulate	$C_{15}H_{16}O_2$	228,1148, 210,65, 20.176	-	Monocarboxylic acid	Antimicrobial	(Langat et al., 2018)
10	8,53	Arctiin	$C_{17}H_{24}O_3$	557.20, 245.4,100.558,206.055 272.14, 395.147,118.66, 559.205505 2.17, 396.151917 1.19	+	Glycoside	Antimicrobial	(Arslanyolu and Erdemgil, 2006)
11	8,79	Xanthosine	$C_{10}H_{12}N_4O_6$	147.100, 325.0, 65.87103.0 58.56 230.0 56.96,245.0, 48.65	-	Glycoside	Antifungal	(Wan et al., 2017)
12	8,81	Benzoylpaeoniflorin	$C_{42}H_{62}O_{16}$	480.5,496.5,496.5	+	Glycoside	Antimicrobial	(Yuan et al., 2020)
13	9,07	Angelol A	$C_{15}H_{16}O_3$	388.4,246.26	+	-	Antimicrobial	(Vancheva et al., 2015)

Peak no	RT (min)	Component name	Formula	MS/MS Fragments	ES mode	Ontology/Class	Biological activity	References
14	10,5	Arnicolide D	$C_{56}H_{88}O_{24}$	276.149,1261.03,100.245,295.12,277.240.05,313.135,345.38, 1591.05, 322.77 ,34.2,331.145538 30.71	+	Terpenoid	Antibacterial	(Huang et al., 2014)
15	11,21	Daucosterol	$C_{32}H_{48}O_8$	146.100, 72.74.07, 420,1587.128,02, 40.24, 167.27,173.765,206.22, 560.232,560,3333	+	Steroids	-	Saleh-e-In and Van Staden, 2018)
16	12,14	Myrigalone A	$C_{18}H_{20}O_4$	-	+	Flavanoid	-	(Khaled et al., 2019)
17	13,48	Isoproturon	$C_{12}H_{18}N_2O$	146.999,72.740,128.402,161 277, 206, 226	-	carbocyclic compound	Antifungal	(de Rodríguez et al., 2017)
18	15,07	Stearidonic acid	$C_{36}H_{61}N_7O_7$	-	+	Fatty acid	Antimicrobial	Saleh-e-In and Van Staden, 2018)
19	15,49	Xanthatin	$C_{36}H_{56}O_6$	285.4,234.33.24, 584.32	-	Terpenoid	Antimicrobial	(Ginesta-Peris et al., 1994)

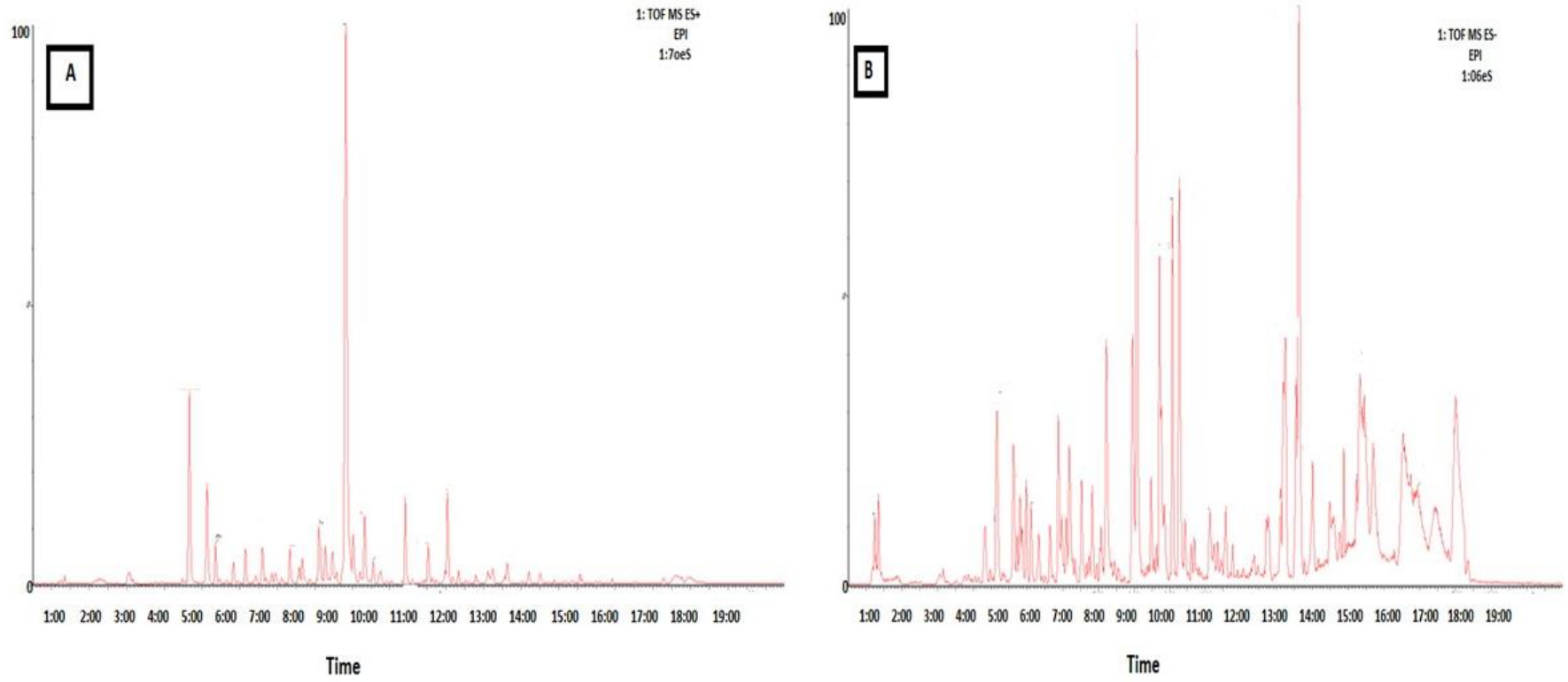

Figure 5. 8: The base peak chromatography of ethanolic extracts of A. arctotoides in ESI + and ESI –

Table 5. 7: Phytochemical constituents identified in ethanolic extracts of *A. arctotoides* using UPLC-Q-TOF-MS/MS analysis in ESI negative and positive modes**.**

Peak No	RT (min)	Component name	Formula	MS/MS Fragments	ES mode	Ontology/Class	Biological activity	References
1	6,17	Gerenic acid	$C_{10}H_{16}0_2$	69.99,100.99,414.0,246.40,100.678,168.10, 123.10.50,109 8.20		Terpenoid	Antifungal	(Yan et al., 2014)
2	8,33	Ganoderic acid MA	$C_{29}H_{38}O_7$	497.2915, 453.3003, 435.2869 301.1799, 285.187	+	Terpenoid	Antimicrobial	(Li et al., 2012)
3	8,56	Gedunin	$C_{28}H_{42}O_8$	-	+	Terpenoid	Antimicrobial Antimalarial Anticancer	(Ong et al., 2021; Patra and Mohanta, 2014)
4	8,73	Fargesone A	$C_{28}H_{38}O_8S$	-	+	-	Antimicrobial Anticancer Antioxidant	(Kelm and Nair, 2000)
5	8,831	Feselol	$C_{34}H_{34}O_{10}$	383.30, 218.809, 383.4 952,383.2670, 383, 68.1198, 383 ,565	+	Terpenoid	Antimicrobial	(Amin et al., 2016)
6	9,08	Lupanine	$C_{15}H_{24}N_2O$	136.101, 174, 172	-	Alkaloid	Antifungal	(Kwaśniewska-Sip et al., 2016)
7	9,26	Fludioxonil (Fungicide)	$C_{12}H_6F_2N_2O_2$	-	-	Alkaloid	Antifungal	(Shimshoni et al., 2020)
8	10,20	Genkwadaphnin	$C_{26}H_{42}O_4$	412.5,05.8018	+	Terpenoid	Antimicrobial	(Javidnia et al., 2003)
9	10,22	Cynanoside K	$C_{22}H_{30}O_6$	-	+	Glycoside	Antifungal	(Yan et al., 2014)
10	12,8	Lobetyolin	$C_{20}H_{28}O_8$	125.13, 158.80, 173.20, 180.40, 187.60, 182.73, 187.60, 191.27, 194.80, 212.87, 344.41, 359.41	-	-	Anticancer Antimalarial Antimicrobial	(Tadege et al., 2022)
11	10,56	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)- 6-methoxychromen-4-one (Jaceosidin)	$C_{33}H_{45}NO_{10}$	330.543,315,312,182	+	-	antioxidant anti-inflammatory antiallergic, antitumor	(Soromou et al., 2020)
12	12,09	Erythrodiol 3-palmitate	$C_{46}H_{80}O_3$	-	-	Terpenoid	Antimicrobial	(Ango et al., 2012)
13	14,71	Ganoderiol A	$C_{20}H_{28}O_3$	313.3102,265.2962,239.3218,211.3084,287.2020	+	Terpenoid	Antimicrobial	(Shi et al., 2021)

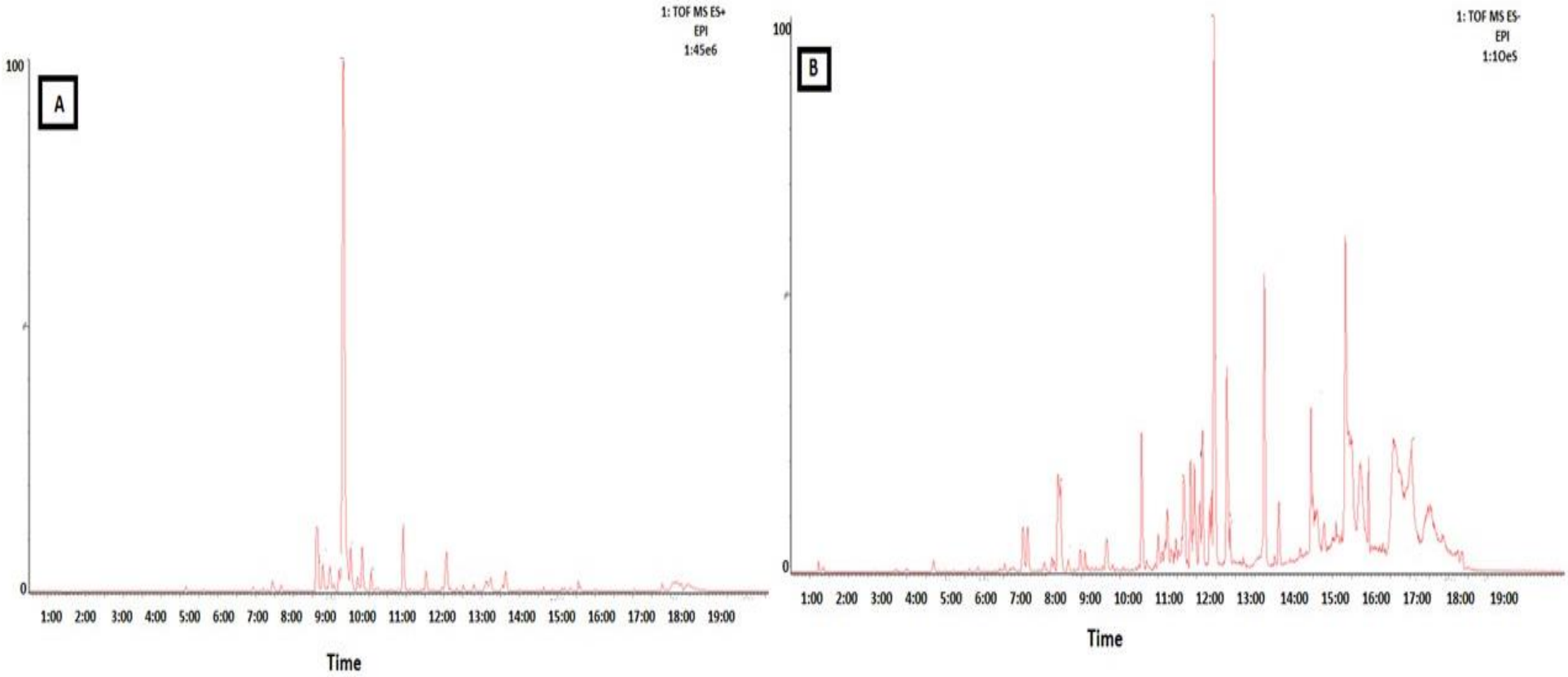

Figure 5. 9: The base peak chromatography of ethanolic extracts of A. arctotoides in ESI + (A) and ESI –(B)

5.3.3 Active chemical classes identified in the four selected medicinal plants

Table 5. 8: A summary of bioactive chemical classes identified in ethanolic and acetone medicinal plants of *T. ametica*, *C. thevetia*, *A. arctotoides* and *V. amygdalina*

Plant name	Class	Acetone	Percentage	Ethanol	Percentage
T. ametica	Terpenoid	2	13.33	5	27.78
	Steroids	4	26.67	1	5.56
	Saponins	2	13.33	1	5.56
	Fatty acids	2	13.33	1	5.56
	Glycosides	2	13.33	1	5.66
	Alkaloid.	0	0	4	22.22
	Flavonoids	1	6.67	1	5.56
	Other	2	13.33	4	22.22
	Total	**15**	**100**	**18**	**100**
C. thevetia	Terpenoid	5	27.78	4	40
	Alkaloids	3	16.67	1	10
	Steroids	1	15.88	2	20
	Flavonoids	1	15.88	1	20
	Fatty acids	1	5.88	0	0
	Carotenoids	1	15.88	0	0
	Other	5	29.41	2	20
	Total	**17**	**100**	**10**	**100**
V. amygdalina	Terpenes	4	23.53	1	9.09
	Fatty acids	0	0	4	36.36
	Alkaloid	1	5.88	0	0
	Flavonoids	1	5.88	3	27.27
	Sesquiterpenes	3	17.64	0	0
	Saponins	0	0	2	18.18
	Lipids	2	11.76	0	0
	Glycoside	1	5.88	0	0
	Steroid	1	5.88	0	0
	Other	4	23.53	1	9.09
	Total	**17**	**100**	**12**	**100**
A. arctotoides	Terpenoid	5	26.32	7	53.85
	Glycosides	4	21.05	1	7.69
	Steroids	2	10.53	1	7.69
	Fatty acids	2	10.53	0	0
	Flavonoid	1	5.26	0	0
	Alkaloids	1	5.26	1	7.69
	Other	4	21.05	3	23.08
	Total	**19**	100	**13**	**100**

5.4 Discussion

Several studies have been conducted to identify and characterise bioactive compounds in the leaves of medicinal plants, as a result, medicinal plants continue to provide inspiration for novel compound discovery as previously derived compounds continue to make important contributions towards the development of antifungal agents. The phytochemical screening of 4 ethanolic and acetone medicinal plant extracts using *UPLC* Q-TOF/ MS/MS in this study led to the identification of 121 compounds identified in negative mode and positive mode of ionization shown in Tables 5.3.1-5.3.8 above. Compounds were identified and presented based on their molecular formula, retention time, mass detected, detector count and their chemical classes which were therefore compared and confirmed through mass spectral data in the literature. The phytochemical constituents revealed in the plant extracts were terpenes (Highest in ethanol extracts of *A. arctotoides*), fatty acids (Highest in ethanolic extract of *V. amygdalina*), alkaloids (Highest in ethanolic extracts of *T. ametica*), flavonoids (Highest in ethanolic extracts of *V. amygdalina*), glycosides (Highest in acetone extracts of *A. arctotoides*), steroids, saponins, sequisterpenes, and lipids.

Terpenes are well known for their chemical defence properties in plants and their presence in the selected plants serves as a good potential tool for fungal disease control as recommended by over 44 revised papers (Zacchino et.al., 2017). Identified terpenes such as Geranic acid found in acetone extracts of *C. thevetia* (Table 5.3.3) possess strong antifungal activities against maize fungal pathogens *C. graminicola* and *F. graminicurum* (Yang at al., 2011). Terpenes change the permeability of a cell by getting in between the fatty acyl chains that make up the lipid bilayers of the membrane (Narishetty and Panchagnula, 2004). This disrupts the packing of the lipids and changes the pathogen fluid in the membrane (Dalleau et al., 2008). Similar to terpenes, plant glycosides show a remarkable degree of sensitivity against a wide variety of fungal species, and the effects are not restricted to one plant family or class of glycosides (Khan et al., 2017). Favel et al. (2005) reports the antifungal mechanism of glycosides is attributable to their capacity to form complexes with the sterols of fungal membranes, therefore producing spore-like structures that tear the membrane, resulting in the death of fungal cells. Although glycosides have fungal spore disruption potential, their antifungal activities are not as greatly exploited. Glycosides such as tenacissoside K in acetone extracts of *T. ametica* (Table 5.3.1) have been explored

against bacterial pathogens and not fungal pathogens and therefore there is a need to isolate these compounds and explore their antifungal activities.

There are over 10000 flavonoid compound detection counts in this study, proving flavonoids' dominance over other secondary plant metabolites and this is because flavonoids are widely distributed, found in all plant regions. Flavonoids remain an attraction to many researchers due to their wide range of reported pharmacological activities. This chemically diverse group is synthesized by plants in response to microbial infections (Górniak et al., 2019), hence a majority of them like Quercetin identified in ethanolic extract of *V. amygdalina* at ion mass 301,0352 m/z, Rt: 4,8 (Table 5.3.6) have been reported to be a potent antifungal agent against fungal strains such as *Cryptococcus neoformans* (Oliveira et al., 2016). In a different study by Tomita-yokotani et al. (2003), quercetin inhibited the growth of *Arabidopsis thaliana* and *Neurospora crassa*. According to Skadhauge et al. (1997), the fungal resistance role of plants such as barley is due to the flavonoid ability to cross link microbial enzymes, inhibition of microbial celluloses and pectinase, which could be used to support the antifungal activity of many more flavonoids and not just those from barley.

Sesquiterpenes are highly identified and documented in the Vernonia genus (Oladele et al., 2021). Identified Sequisterpenes such as Vernolepin from acetone extract of *V. amygdalina* (Table 5.3.5) are used largely in traditional medicine to treat fungal infections (Graham et al., 2000), while Vernodelol found at ion mas 392,41 m/z, Rt: 5,8 from acetone extract of *V. amygdalin* (Table 5.3.5) have reported higher antifungal properties against *Penicillum notatum* and *Aspergillus flavus* (Erastos et al., 2006), suggesting that they have a potential to can also be used for the development of antifungal agents.

In a TLC antifungal screening study by Kwaśniewska-Sip et al. (2016), a compound identified as lupanine in ethanolic extract of *A. arctotoides* (Table 5.3.8) was reported to be an antifungal alkaloid active against *Aspergillus Niger* Van Tiegen ATCC 6275(BAM4). Alkaloids are reported to exhibit the highest pharmacological actions (Bribi, 2018a). Several studies reported this group of compounds as the most active group with considerable biological activities including toxicity to other organisms (Jain et al., 2019). Although the group has reported to exhibit toxicity that even limits its use in clinical studies, they remain an important group in their role as active plant

defenders against plant pathogens (Jain et al., 2019). Compounds such as brucine identified from ethanolic extract of *T. ametica* (Table 5.2.2) have reported impressive preclinical pharmacological studies (Lu et al., 2020). Compound detected at ion mass of 534,3175 m/z, Rt: 10,05 has been identified as Brucine which has reported significant anti-inflammatory, analgesic effects and involved in the regulation of Wnt/b-catenin that signals a pathway for inhibition and growth of various cancer cells (Lu et al., 2020). Although highly significant, brucine has limited use due to its excessive toxicity to cells (Lu et al., 2020). The toxicity of the compound has however not been explored wide enough as it may be a potential antifungal agent against several plant pathogens affecting greatly the agricultural crop production. Alkaloids such as Matrine and Oxymatrine from acetone extracts of *C. thevetia* (Table 5.3.3) have been reported to have *in-vitro* antifungal properties against *Fusarium oxysporum, Valsa pini* and *Cladosporium oxysporum*. A study by Yang and Zhao (2006) found oxymatrine to have a strong inhibitory effect against *fusarium oxysporum* with EC50 values of 26 μg ·mL-1. Due to its adverse toxicological effects, prochloraz as a fungicidal agent for avocado fruits has now been restricted in other parts of the world including Europe (Shimshoni et al., 2020). Fludioxonil from ethanolic extract of *A. arctotoides* (Table 5.3.8) has been considered as a consequent replacement of prochloraz due to its safety and a broad-spectrum fungicidal value, is in postharvest application on many fruits in Europe and Israel (Shimshoni et al. 2020). This therefore emphasizes the need for the discovery and use of active plant compounds as viable antifungal agents to replace synthetic and chemical agents.

Stearidonic acid identified in acetone extract of *T. ametica* (Table 5.3.1), is a fatty acid that has been reported to have increased fungicidal activity against *Candida albicans* and *Candida dubliniensis* (Thibane et al., 2010), due to increase freedom of movement inside the fungal membrane (Pohl et al., 2011). Fatty acids have been recognised greatly for their antimicrobial activities for decades now (Fan et al., 2012). Majority of studies on fatty acids have involved their innate immunity function in humans and animals to protect against microbial enemies (Kanai and Kondo, 1979; Thormar and Hilmarsson, 2007). In animals and humans, fatty acids function as defences in plants against photogenic colonisers (Desbois and Smith, 2010; Zasloff, 2011) and are suggested as potential role players against microbial plant pathogens (Walters et al., 2004) Fatty acids employ inhibitory and antifungal mechanism based

on the target microbe and its microbial cell (Tsuchido et al., 1985). In addition to having potent inhibitory activities against fungal crop pathogens (Walters et al., 2004), fatty acids as antifungal agents are non-corrosive compared to other antifungal substances and disinfectants (Whittle and Basketter, 1993) that may otherwise halter the health of crops and cause toxicity to humans and the environment. Fatty acids such as linoleic acid identified in acetone extracts of *T. ametica* (Table 5.3.1) and oleonelic acid identified in acetone extracts of *C. thevetia* (Table 5.3.3) were found active against pathogenic fungi: *Alternaria solani, Colletotrichum lagenarium, Fusarium oxysporum f. sp. Cucumerinum, and Fusarium oxysporum f. sp* both *in-vivo* and *in-vitro* (Liu et al., 2008). These fatty acids were found to inhibit the growth of the mycelium and showed inhibition against fungal spore germination (Liu et al., 2008). The results obtained by Liu et al. (2008) therefore suggest the need to explore fatty acids as potent compounds effective against fungal pathogens in agricultural crops.

5.5 Conclusions

The results from this study showed that *T. ametica, C. thevetia, A. arctotoides and V. amygdalina* extracts exhibit several bioactive components, therefore, validating their ethnobotanical usage against fungal pathogens. As a result, isolating specific phytochemical components might lead to the discovery of new antifungal agents or a leading molecule.

Chapter 6: General Discussion, conclusion, and recommendations

6.1 General discussion

Maize crops are attacked continuously by various pathogens that lead to yield losses ranging from blemishes to complete crop losses. Pathogens such as *R. solani* are hard to control due to their aggressiveness. Pathogen resistance to fungicides has become one of the major concerns in crop production. This is due to their ability to evolve in time, space and genotype overcoming the action of the modern-day chemical fungicides. An increase in human population increases the pressure on food production, more especially agricultural crop production (Govoni et al., 2021). As the demand for food increases to feed the population, maintaining agro-ecosystem health remains a priority. Without reliable disease protection, agricultural crop production will be ineffective, failing to meet the nutritional needs of the population and the economy will suffer. Therefore, there is a large need to explore alternative antifungal and biological disease protection agents that will aid in maintaining an increased supply of food to maintain the growing population.

In the current study, 4 medicinal plants extracted in acetone and ethanol were tested *in vitro* and *in-vivo* for their antifungal activity against *Rhizoctonia solani*, a causative root rot agent in maize. 3 of the 4 medicinal plants showed activity against the pathogen while the other plant showed susceptibility to the pathogen. The activity of the selected medicinal plants in the study against the pathogen is reported for the first time. In both the *in vitro* and *in vivo* studies, *Arctortis arctotoides* exhibited the highest antifungal activity against *Rhizoctonia solani*. The plant inhibited the growth of the pathogen (*in-vitro*) and suppressed the pathogen and the pathogen symptoms on maize under greenhouse conditions. This, therefore, suggest that *A. arctotoides* has a high potential to be used as a botanical fungicide such as *Simmondsia chinensis* and *Laminaria digitate* whose isolates are used as ground fungicides. The presence of phytochemicals from different groups such as fatty acids, terpenoids and flavonoids identified in chapter 5 could have contributed to the biological activity of *A. arctotoides*.

A. arctotoides is distributed throughout the rainfall areas of South Africa and widely in the Eastern Cape province (Grierson et al., 2014). The plant grows in disturbed areas like road wedges and since it is a herbaceous plant, it requires minimal maintenance. This, therefore, means a larger scale propagation and cultivation of the plant is possible.

Due to the possible cultivation, the production of botanical fungicide from the plant can also be possible. This would therefore provide a much-cheaper and safe option for the control of fungal crop pathogens especially for rural farmers. Furthermore, the use of botanical fungicides derived from a traditional plant has been on the rise lately are used due to the culture of the people that depends greatly on the use of medicinal plants for the treatment of diseases. Because systemic fungicides do not reach the pathogen in roots or the basal regions of stems or trunks, foliar treatments against root or crown infections are ineffective. Plants can absorb a relatively tiny amount of a fungi toxic substance when fungicides are used as a soil treatment. Therefore, the use of the botanical fungicide would be a more efficient way of controlling root rot because seeds can be coated with the fungicide during planting without affecting the germination or causing any form of toxicity to the crop and the environment. Even when applied as foliar, the plant extract can reach the basal and root region of the crop leading to effective management of the fungal pathogen.

6.2. Limitations of the study

The researcher hailing from Mpumalanga, getting to the Eastern Cape and trying to establish where to find *A. arctotoides* was a bit challenging. Due to some unforeseeable factors, plant collection was done between June and August During this time getting more plants required much more extra effort as they were sterile due to the winter season. It is therefore advisable to collect plants during spring, summer or autumn when most plants are flowering to avoid sterile herbarium specimens. Despite the few challenges experienced in the journey, the success of this study greatly overshadowed the shortcomings experienced.

6.3. Conclusion

The study investigated the potential of medicinal plant crude extracts extracted in acetone and ethanol to control *R. solani*, which is a causative root rot agent in maize. Despite the large body of knowledge around the antifungal activity of medicinal plants, little remains unknown on the efficacy of medicinal plants against plant pathogens since more research is based on human pathogen. The study reports antifungal activity of medicinal plants against R. solani in chapter 3. The highest inhibition was induced by both acetone and ethanol extracts of *A. arctotoides* (12.2 and 10.4 mm respectively), followed by acetone extracts of *V. amygdalina* (8.0mm) and acetone extracts of *C. thevetia* (7.5mm). Ethanol extract of *C.*

thevetia exhibited the least antifungal activity with the mean inhibition of 3.8mm, while on the other hand both acetone and ethanol extracts of *Trichilia ametica* showed no activity against the pathogen. Acetone extracts had higher potency compared to ethanol, with an average of 71.77mm as opposed to 55.99mm inhibition diameter. Based on the MIC results, acetone extracts from *A. arctotoides* exhibited the MIC value (0.2 mg/ml) among the tested medicinal plant extracts. In chapter 4, *Arctortis arctotoides* ethanol *(A*ER1), *Vernonia amygdalina* acetone *(VA*aR1), *Vernonia amygdalina* ethanol *(VAER1), Cascabela thevetia* acetone *(CT*aR1) and *Cascabela thevetia* ethanol (*CT*ER1) recorded the best five treatments with the highest plant height, chlorophyll content, root hairs and the lowest root lesions under greenhouse conditions. Both acetone and ethanolic extracts of *Trichilia ametica* (TAER1 and TAaR1) showed no activity against *R. solani*, which confirms the results obtained in the *in vitro* studies. The pathogen reduced plant height and chlorophyll content and had an increased number of root lesions compared to other treatments including the uninoculated control, which was expected as a confirmation that *R. solani* was pathogenic to maize. Acetone had high volatility, miscibility with polar and non-polar solvents, and minimal toxicity to test organisms, hence the results of the present study show high activity of acetone extracts in all measured parameters. *The A. arctotoides* and *V. amygdalina* plant extracts were regarded as the best treatments as they were among the top treatments in most of the measured parameters. The performance of these extracts indicates that they possess potential as biocontrol agents for the control of *Rhizoctonia* root rot pathogens of maize. The UPLC analysis in chapter 5 identified numerous compounds archived through the comparison of the obtained mass spectra data to literature. Fifteen and eighteen bioactive compounds were identified in acetone and ethanolic extracts of T. *ametica* respectively, with terpenoid at 27.78% from the ethanolic extract. In *C. thevetia*, 17 compounds from acetone and 10 compounds from ethanolic extract were identified with the dominant one being terpenoid at 27.76%. A total of17 and 11 compounds were identified respectively from acetone and ethanolic extracts of *V. amygdalina* and the dominant one being fatty acids from the ethanolic extract at 36,36%. In *A. arctotoides* 19 and 13 compounds were identified from acetone and ethanolic extracts respectively the dominant one being terpenoid at 53.85% from the ethanolic extract. The results of this study showed that medicinal plants harbour phytochemicals responsible for their reported potent antifungal activities that could be further explored against fungal pathogens. Therefore, this study reports for the first time the activity of the selected medicinal plant extracts against *R. solani* which is a plant pathogen.

6.4. Recommendations

Based on the findings of this study, the following recommendations can be advanced:

1. The study strongly recommends further research on these medicinal plants in the field of natural products. Research that will focus on isolation, purification and characterisation of antifungal compounds using advanced performance chromatograms such as NMR.
2. In addition, the study also recommends that isolated compounds be studied against fungal pathogens such as *R. solani* to know which of the compounds specifically inhibit the growth and development of the pathogen *in-vitro.*
3. The study recommends that the plants be further studied for their antimicrobial activity against other plant pathogens to increase the body of knowledge that exists on the antimicrobial activity of medicinal plants against plant pathogens
4. The study further recommends the exploration of propagation methods to extend the availability of the active medicinal plants especially *A. arctotoides* so that it can be cheaply made available to small-scale farmers in other provinces outside the Eastern Cape.

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Appendix A: Certificate of study approval.

UNIVERSITY OF MPUMALANGA

FACULTY OF AGRICULTURE AND NATURAL SCIENCES

Postgraduate Studies Committee

Certificate of Approval – Research Proposal

Date of this Approval:	**26 July 2021**

Student Details

1	Student Name:	Christeldah Mkhonto
2	Student Number:	201707659
3	School	School of Biology and Environmental Sciences
4	Degree Registered for:	Master of Science
5	Date of First Registration:	March 2021
6	Supervisor(s):	Prof W. Otang Mbeng Co-Supervisor(s): Dr I. J. Sagbo and Dr B. P. Kubheka

The research proposal entitled '**Antifungal evaluation of extracts from selected medicinal plants against *Rhizoctonia solani***' has been evaluated and approved by the Postgraduate Studies Committee of the Faculty of Agriculture and Natural Sciences.

UNIVERSITY OF MPUMALANGA
2[illegible]-07-2021
FACULTY OF AGRICULTURE AND NATURAL SCIENCES

Chairperson: Prof. Victor Mlambo

Signature: [signature]

Date & Official Stamp:

Appendix B: Ethical approval of the study.

Research Ethics Clearance Letter *UMP*

Office 206. Building 4. C/o R40 & D725. Private Bag X11283. Riverside Mbombela South Africa 1200.
Website: www.ump.ac.za | *Tel*: (013) 002 0196 | **E***mail***:** Estelle.Boshoff@ump.ac.za

RESEARCH ETHICS CLEARANCE LETTER

Ref: UMP/Mbeng/2/2021

Date: 29 August 2021

Name of Researcher: Prof. Wilfred Otang-Mbeng

Address of Researchers:

Prof. Wilfred Otang-Mbeng

School of Biology and Environmental Sciences

Faculty of Agriculture and Natural Sciences

University of Mpumalanga

RE: APPROVAL FOR ETHICAL CLEARANCE FOR THE STUDY:

Sustainable management of Rhizoctonia and Pythium root rot of maize, using extracts from Arctortis arctotoides.

Reference is made to the above application.

I am pleased to inform you that the Chairperson has on behalf of the University of Mpumalanga's Research Ethics Committee, **approved ethical clearance** of the above mentioned study.

Please Note

Any alteration/s to the approved research protocol i.e. Questionnaire/Interviews Schedule, Informed Consent form, Title of the project, Location of the study, Research Approach and methods must be reviewed and approved through the amendment/ modification prior to its implementation.

Regards

Prof Estelle Boshoff
Chairperson: University of Mpumalanga's Research Ethics Committee.

Appendix C: South African National Biodiversity Institute field label for plant collection.

Collector: ..... No.: ..... Date: .....

Provisional name:

Region:	Grid:	Alt: m
GPS ° ' S	° ' E	
Locality		

Biome	Fynbos Grassland Savanna Nama Karoo Succulent karoo Forest Indian Ocean Coastal Belt Albany Thicket Desert Azonal
Vegetation type	
Habitat	mountain peak mountain slope hilltop hill slope ridge cliff face ravine/kloof/gorge talus/scree plateau valley floodplain waterfall river/stream bank river/stream dry streambed donga/gulley/ditch pan depression marsh swamp wetland seepage dune (desert) dune (coastal) estuary littoral lagoon sea lake dam pond plain other:
Substrate	soil stony soil rocky soil gravel bare rock in water termite mound bark leaf leaf litter roots other:
Soil type	gravel sand loam black turf humus clay salt/brack baserock
Lithology	sandstone shale granite quartzite calcrete dolomite dolerite
Moisture regime	well-drained seasonally waterlogged free standing water tidal mist/fog moist/damp permanently waterlogged running water other:
Exposure	shade partial shade full sun
Slope	none gentle moderate steep
Aspect	N S W E NE NW SE SW
Biotic effect	abandoned land cultivated land pasture recently burned garden roadside plantation grazed disturbed none seen other:
Life form	tree shrub dwarf shrub herb graminoid geophyte epiphyte climber parasite succulent hydrophyte bryophyte lichen scrambler saprophyte lithophyte other:

Plant features (underground parts, bark, leaves, flowers, fruit, seeds, aroma)

Flowers: present absent	**Fruit:** present absent	**Plant height:** m

Notes (local abundance, phenology, pollinators, herbivory, economic & ethnobotanical factors, voucher specimen)

Permit Number:	**Issuing authority:**

Voucher: photo ecology cytology anatomy seed spirit

Plant name: .....

.....

Genspec: ..... / ..... Det.: ..... Date: ..... No. of labels: .....