A comparative evaluation of the biological activities and phytochemical properties in *Ehretia obtusifolia* and *Ehretia rigida*

By

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Contents

College of Agriculture, Engineering and Science

Declaration 1 – Plagiarism

I, Mzamo Mpendulo Ntethelelo Mnikathi, student number: 214533521, declare that

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A comparative evaluation of the biological activities and phytochemical properties in Ehretia obtusifolia and Ehretia rigida

I, Mzamo Mpendulo Ntethelelo Mnikathi, student number:214533521 declare that:

(i) The research reported in this dissertation is the result of my own independent work at the Research Centre for Plant Growth and Development (RCPGD), School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

(ii) This dissertation will not be submitted for any other degree or examination at any other University.

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Signed at UKZN, Pietermaritzburg Campus on the 28th day of November 2021

Declaration by Supervisors

We hereby declare that we acted as supervisors for this MSc student:

Students full names: Mzamo Mpendulo Ntethelelo Mnikathi

Student number: 214533521

Title of dissertation: A comparative evaluation of the biological activities and phytochemical properties in *Ehretia obtusifolia* and *Ehretia rigida*

Consultation between the student and we took place recurrently in the duration of the project. The student was advised to the best of our ability, and we find the dissertation acceptable to be submitted to the School of Life Sciences Higher degrees office, for examination by the university appointed examiners.

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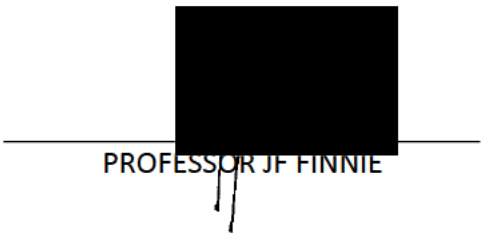

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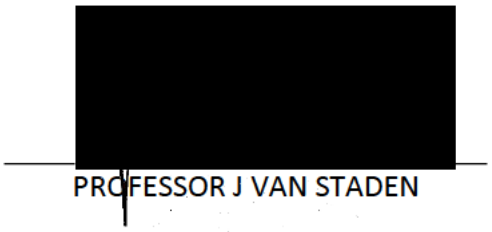

PROFESSOR J VAN STADEN

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List of Figures

List of Tables

List of Abbreviations

DCM:

Dichloromethan

e DMSO:

Dimethylsulphox

ide

DPPH: 2,2-diphenyl-1-

picrylhydrazylEA: Ethyl-

acetate

EO: *Ehretia obtusifolia*

ER: *Ehretia rigida*

MIC: Minimum Inhibitory

Concentration MFC: Minimum

Fungicidal Concentration

$Na_2HPO_4 \cdot 7H_2O$: Sodium monohydrogen phosphate

heptahydrate$Na_2H_2PO_4H_2O$: Sodium dihydrogen

phosphate monohydrate $NaCO_3$: Sodium carbonate

NaCl: Sodium

chloride

NaOH: Sodium

hydroxide

$NaCl_3$**:** Sodium

nitrite

pNPG: p-Nitrophenol-α-

glucopyronosideTFC: Total

flavonoid content

TPC: Total phenolic content

Abstract

Ehretia from the Boraginaceae family is predominantly found in parts of Asia and North America, with fewer species found in Africa, Europe, and Australia. The genus consists of more than 150 species, and species such as *E. microphylla, E. accuminata, E. laevis* have been reported on their medicinal prowess. Distribution of *Ehretia* in Southern Africa is found among a variety of habitats such as the lush forests of the Eastern Cape and Arid parts of Namibia. In South Africa, two species have been identified, namely *E. rigida* and *E. obtusifolia* and are used in traditional medicine. African and Asian countries traditional medicine is highly recommended because of its affordability. The aim of the study was to establish a baseline and compare different biological activities and phytochemical properties exhibited by the two South African species.

In the study, phenolics, saponins, flavonoids, and tannins were detected in bark, roots, and leaves, of both species, but no detection of alkaloidsf. *E. obtusifolia* had a higher quantity of flavonoids than *E. rigida.* Both species exhibited high phenolic quantities in leaves with *E. rigida* having the highest quantity. Condensed tannins were found with a higher content in leaves than roots and bark for both species, with *E. rigida* containing higher quantities.

E. rigida had the lower MIC's compared to *E. obtusifolia* (0.195 mg/ml against *M. luteus* from ethyl-acetate root extracts). *E. rigida* had more samples with a MIC lower than 1 mg/ml than *E. obtusifolia.* The lowest MIC for leaves was 0.39 from ethyl-acetate extracts against *S. aureus* while methanol bark extracts also achieved 0.39 mg/ml against *M. luteus*. *E. obtusifolia's* lowest MIC was 0.195 mg/ml from methanol leaf extracts against *K. pneumoniae.* Activity against *C. albican* was not as good as against the bacterial strains, as the lowest MIC was 0.78 mg/ml for both species.

E. rigida and *E. obtusifolia* had dose-dependent antioxidant activity, with methanol and ethyl-acetate bark, leaf, and root extracts having the highest activities for both species. This study revealed that in comparison to literature, the activity achieved was similar or better when compared to the likes of *E. laevis* extracts.

The α-glucosidase inhibitory activity reported in this study was dose-dependent. The relationship between antioxidant activity and antidiabetic activity is well documented and this study found that extracts with high antioxidant activity also had similarly high α-

glucosidase inhibitory activity. It was *E. rigida* methanol extracts from bark and roots which exhibited the highest activities compared to *E. obtusifolia*. However, based on the dose-dependent activity, *E. obtusifolia* is more potent because of the higher activity observed at the lowest concentrations.

The study demonstrated that both species have good ethnopharmacological properties and were rich in phytochemicals, particularly phenolics and flavonoids. With *E. rigida* being the least studied of the two species with only one reported study, it was important to carry out this investigation as it has yielded further evidence that the genus *Ehretia* has multiple species with medicinal potential.

Chapter 1: Literature review: Aims, objectives and hypothesis

1.1 Introduction

The *Ehretia* genus according to **Miller (1989**) belongs to the Boraginaceae family and contains approximately 150 species (**Gottschling & Hilger., 2004; Shukla & Kaur., 2018**). All species are trees or shrubs, and are distributed mainly across Asia, Africa, Australia, Europe, and North America (**Retief & Van Wyk., 2001**). In countries such as China, Japan and India, the different plant parts such as the fruits, leaves, bark, roots, and heartwood are used in traditional medicine (**Li et al., 2010; Chien et al., 2012; Sivasankari et al., 2013**). Species of *Ehretia* produce small fruits and therefore are visited by avian frugivores. Some of the species are said to be valuable supplementary nutritional feedstuff for ruminants, due to their *in vitro* fermentation traits and low fibre (**Shukla & Kaur, 2018**). The most common or highly reported species of the genus are used in China and India - *E. microphylla, E. accuminata* and *E. laevis* because of their excellent response in biological activities (antibacterial, antioxidant, anti-inflammatory, antidiabetic) according to **Li et al (2010) and Sivasankari et al (2013)**. The following secondary metabolites have been detected in the genus- phenolic acids, lignans, flavonoids, nitrile glycosides, steroids, triterpenoids, and pyrrolozidin alkaloids (**Sivasankari et al., 2013; Shukla & Kaur., 2018**).

1.2 *Ehretia* genus description and ecology

Ehretia has a wide distribution in Southern Africa, growing in a variety of habitats ranging from lush forests of the Eastern Cape, to the arid part of the Namibia (**Carruthers., 1997; Steyn., 1998**). Boraginaceae has two subfamilies, Boraginaceae s.str and Ehretiaceae (which includes woody taxa such as *Cordia* and *Ehretia*) according to **Miller (1989**). Various characteristics and traits of the genus, such as pollen morphology, woodiness and structure have revealed the two subfamilies to be more closely related, thus should not be separated (**Miller., 1989**). Trees and shrubs are predominantly found in the genus, with shrubs which grow up to 0.4 m such as species as *E. obtusifolia* and *E. rigida* with a habitat regarded as a transitional growth form into herbaceous shrubs (**Retief & Van Wyk, 2001**).

1.2.1 Leaf description

Ehretia leaves vary based on their distribution and habitat, however South African investigated species have leaves which are broadly ovate and vary in size (**Miller., 1989**). The variation of the blade is from 6-95 X 3-56 mm, with the largest leaves/leaf blade found in the *E. amoena* along with species which grow in moist conditions of the Eastern regions, Northern Province, Mpumalanga, and Swaziland (**Steyn., 1998**). Species growing in drier and central regions, for example *E. alba* have smaller leaves, and the reduction in leaf size is an observed adaptation to less transpiration under stressful condition, and is predicted to be drought tolerant (**Retief & Van Wyk., 2001**).

1.2.2 Flower and inflorescence

Ehretia species of Southern Africa have an inflorescence with scorpoid cymules stood apically on young shoots or at the apex of short branchlets (**Carruthers., 1997**). However, the inflorescence of *E. rigida subsp. rigida* is supported apically on the shoots of young shoots and found together in clusters, forming a corymb-like structure. In species such as *E. obtusifolia*, the peduncle of the inflorescence may be very short with flowers clustered at the end of shortened branchlet. The difference in trichome complements, is used to separate the difference between the species, where *E. coerulea*, *E. obtusifolia* and *E. namibensis* are characterised by a simple, multicellular, capitate glandular trichome, while *E. amoena*, *E. rigida and E. alba* do not have such trichomes (**Steyn., 1998**). Furthermore, the presence of unexpected glandular trichomes in species suggested a hybridisation phenomenon (**Retief & Van Wyk., 2001**).

1.2.3 Fruit

The family Boraginaceae is not characterised only by its cymes, but also by its fruit display which plays an important role in its identification (**Gottschling & Hilger., 2004**). The fruits of most species in the Boraginaceae family are drupaceous with four nutlets with a thin to leathery exocarp, a fleshy mesocarp and bony endocarp (**Retief & Van Wyk., 2001**). According to **Miller (1989),** based on fruit structure the genus may be divided into two groups: Old world (Southern Africa) and New world species (America and Asia). The fruits are bright red to orange in colour and attract birds which are also responsible for seed dispersal.

1.2.4 Species description: Ehretia rigida

E. rigida is a multi-stemmed shrub growing up to 12 m with a single inflorescence predominantly on every new shoot with densely clothed hairs. The corolla tube is white and cylindrical with pale lilac or purple lobes. *E. rigida* is distributed east of Southern Africa, namely, North Zimbabwe, South Africa and east of Mozambique. *E. rigida* grows in the valley, bush clumps, thornveld, and or open flat habitats (**Retief & Van Wyk., 2001**).

1.2.5 Species description: Ehretia obtusifolia

E. obtusifolia is a small tree or shrub growing up to 3 m, with a leaf blade of 17-25 X 10-12 mm in dimension and has an obtuse apex with entire leaf margins. The inflorescence is a branched cyme with short branchlets densely covered with setae and glandular trichomes (**Retief & Van Wyk., 2001**). The corolla tube is cylindrical, white in colour and 5 mm long. The lobes are mauve or blue (**Carruthers., 1997**). The fruits are globous with 3-5 mm long pyrenes and flowering time between September to December. *Ehretia obtusilofia* is closely related to *E. amoena* and *E. rigida* and are readily distinguished, but in areas where they are sympatric, specimens with intermediate features may occur. *E. obtusifolia* is well distributed across the Northern Province, Mpumalanga, Gauteng, Swaziland and KwaZulu Natal, into North Africa and Asia, to the likes of Ethiopia, Iran, Afghanistan, Pakistan, and India. It grows in the bushveld, woodland, grassland, hill slopes, bush clumps, stony soil, and sandy loam habitats (**Retief & Van Wyk, 2001**).

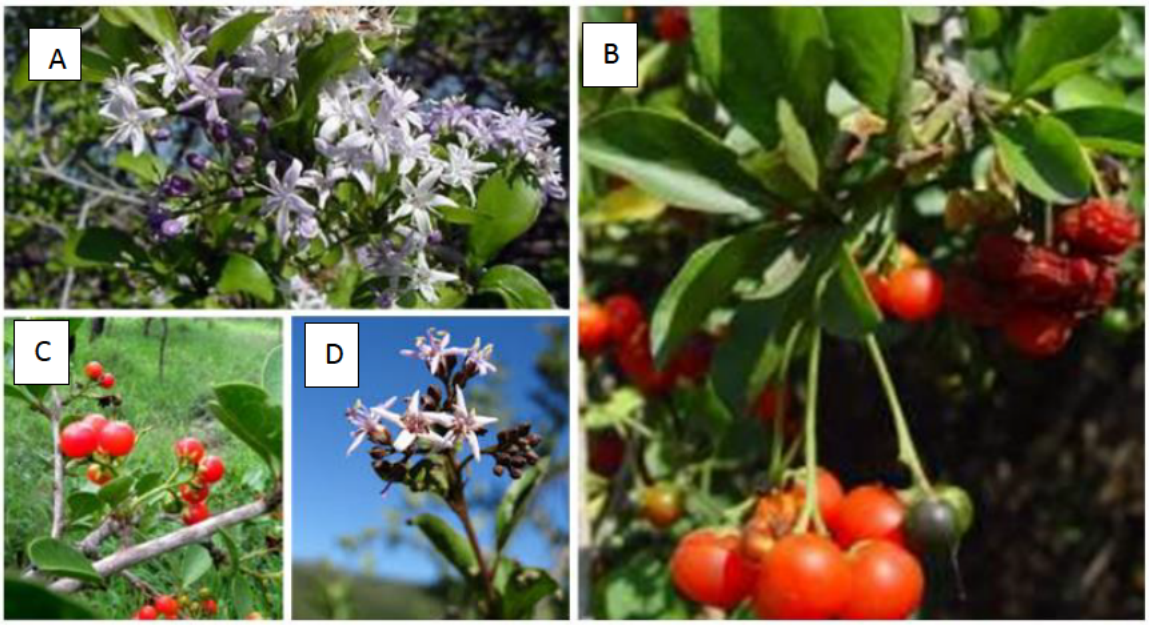

Figure 1.1: *Ehretia rigida flowers* (A) and fruits (B) along with *Ehretia obtusifolia* flowers (D) and fruits (C). Photographer: Geoff Nichols

Figure 1.2: Drawing of leaves, fruits, and flowers of (A) *E. rigida* (Kerr collection) and (B) *E. obtusifolia* (Kirktikar & Basu., 1918)

1.3 Germination

Seed germination in *Ehretia* genus has not been thoroughly investigated, with only two species been investigated on germination, namely *Ehretia anacua* and *Ehretia cymosa. E. anacua* is a south Texas native shrub which extends all the way into Mexico (**Jahrsdoerfer & Leslie., 1988; Vora., 1989**). It is consumed by birds and animals, acts as a nest site for white winged doves (*Zenaida asiatica*). A majority of land is being lost through cultivation and land use practices, which has led to a loss of habitats for most species (**Jahrsdoerfer & Leslie., 1988**). The State and wildlife agency in America has been attempting to restore habitats, and *E. anacua* is one of the five important woody plants in habitat restoration for white winged doves (**Vora., 1989**). Its low seed germination due to seed dormancy has hampered its establishment efforts. Germination studies reported *E. anacua'*s optimal temperature for germination to be 30^{o} C post soaking in concentrated sulfuric acid for 2 hr (**Fulbright et al., 1986**).

Dormancy is of great concern in *Ehretia* and is partly caused by a deficiency of endogenous gibberellins (GA). A study to enhance seed germination for *E. anacua* was conducted by applying the following treatments: (1) substrata moistened in GA solutions, (2) chilling imbibed seeds prior to exposing to warm temperature and (3) different storage times (**Fulbright et al., 1986**). The study showed that germination increased with an increase in seed storage, however for optimal germination, duration of storage and conditions was not determined (**Fulbright et al., 1989**). Fruiting plants are visited by many species of frugivores, some of which are thieves and other dispersers who assist in seed germination by ingesting the seeds, but there is insufficient evidence stating how frequently germination in fleshy-fruited plants is affected by frugivore ingestion. In this group of plants, seed germination by ingestion from frugivores has been studied, where **Lieberman and Lieberman (1986)** found that ingestion from bats, starlings, and parrots had no effect on germination.

1.4 Biological activities

The practice of plant based traditional medicine is predicated on hundreds of years of belief and observation. Today's modern medicine was mostly derived from ancient herbal traditions (**Lie et al., 2010**). In rural Africa and Asia, medicinal plants have been prescribed by traditional healers, and because of their affordability and availability they have become fundamental in the African health care system (**Yamamura et al., 1995; Mahomoodally., 2013**). Plants

contain abundant natural products that exhibit different biological activities (**Valle Jr et al., 2015**). In the genus *Ehretia,* literature has reported a wide variety of biological activities from extracts and isolated compounds. A study by **Joshi et al (2020)** reported that heartwood in *Ehretia sp* is a source of food for humans while the leaves are applied to ulcers and used to treat headaches (**Gumgumjee & Hajar., 2015**). The most common biological activities in the genus include - antibacterial, antifungal, and antiviral therapy, anti-inflammatory, antidiabetic, antioxidant, antitubercular, antiallergic and antitrypanosomal and antiprotozoal activity (**Shukla & Kaur., 2018**).

1.4.1 Antimicrobial

Resistant bacteria have become a detrimental problem in hospitals and remain an unsolved problem that carries with it a heavy burden on health care systems (**Rimando et al., 1987; Jyothirmai et al., 2016).** Even with advances in antibiotic therapy, antibiotic resistance continues to be a challenge among hospitalised patients (**Mutha et al., 2015)**. In the spread of multi-drug resistance, doctors tend to resort to second or third choice drugs to treat patients, unfortunately this may cause patients to be at an even greater risks due to the drugs potentially creating more harmful side-effects. To address these complications, scientists have taken to controlling the use of antibiotics by understanding the genetic mechanisms of resistance and effectively developing new ones (**Valle Jr et al., 2015**).

In the second half of the 20th century (1950-2000), there emerged a greater understanding and acceptance to the use of medicinal plants as alternative treatments in health care (**Rimando et., 1987)**. With an increase in microbial resistance to common antibiotics, researchers were led to investigate antimicrobial activities exhibited in medicinal plants (**Mutha et al., 2015; Valle Jr et al., 2015**). Plants with antimicrobial activity have enormous therapeutic potential, because the compounds found in these plants are effective in treating infectious diseases while simultaneously mitigating many of the side effects associated with synthetic drugs (**Valle Jr et al., 2015**; **Gumgumjee and Hajar., 2015**).

There has been an increase in reports on the use of medicinal plants in different parts of the world, particularly Asia, Africa and many other developing countries which still rely on or use medicinal plants to treat various diseases (**Rimando et al., 1987**). It is hypothesised that medicinal plant extracts that can target sites which antibiotics cannot, will be active against

multidrug resistant bacteria, however very little information is known on such activity (**Rimando et al., 1987; Valle Jr., 2015**). The rare cases of plants infected by microbes is an indication of a well-developed defence mechanism which suggested compounds that can inhibit (through disrupting the life cycle) the growth of bacteria and or even kill them without toxicity or minimal toxicity to the host cells, are candidates for the development of new drugs (**Valle Jr et al., 2015; Mutha et al., 2015**).

1.4.2 Antioxidant

There is growing interest to find/mine naturally occurring antioxidants for them to be used in food and medicine to replace synthetic ones which have side-effects, such as carcinogenicity (**Li et al., 2009; Dzoyem & Eloff., 2015**). Plant stress causes production of harmful ROS such as singlet oxygen, superoxide radicals, hydrogen peroxide and the most harmful one being the hydroxyl radical, which is formed by the combination superoxide and hydrogen peroxide molecules in the presence of Fe^{+2} and Fe^{+3}. The OH^- radical damages organelles, proteins, DNA, lipids thus affecting plant metabolism and reduces plant growth. (**Hasanuzzaman et al., 2013; Hatfield & Prueger., 2015**).

Free radicals are a major cause of many disorders in humans, including atherosclerosis, arthritis, ischemia, central nervous system injury, gastritis, cancer and even AIDS (**Sivasankari et al., 2013**). Free radicals caused by environmental pollutants such as radiation, chemical toxins, deep fried and spicy foods result in a depleted immune system, change in gene expression and induced abnormal proteins (**Sivasankari et al., 2013; Dzoyem & Eloff., 2015**). The oxidation process is one of the main pathways of generating free radicals in foods, drugs and living systems, and by enhancing the body's natural antioxidant defences, one can reduce the risk of chronic diseases. (**Li et al., 2009; Shukla & Kaur., 2018**). In this group of plants, leaf extracts from 1-butanolic and chloroform fractions have exhibited good antioxidant activity, along with ethyl-acetate fractions from fruit extracts (**Shukla & Kaur., 2018**).

The use of antioxidant substances to scavenge and eradicate reactive oxygen species (ROS) can prevent and minimise diseases related to oxidation, suggesting that Alzheimer's which is an oxidative stress related disorder can be treated with antioxidants and free radical scavengers (**Dzoyem & Eloff, 2015**). Since synthetic antioxidants have low solubility and moderate antioxidant activity, a practical way to control chronic diseases could be through a daily dietary intake of fruits and vegetables rich in antioxidants and active compounds

(**Adebayo et al., 2015**). Food is vital in maintaining health and can be used to one's advantage, by consuming foods rich in naturally occurring antioxidant because they are more stable and active when they occur in plant-based foods and are therefore superior to synthetic ones (**Sivasankari et al., 2013; Adebayo et al 2015., Ogundajo & Ashafa., 2017**).

1.4.3 Anti-inflammatory

Inflammation is a biological response of vascular tissues to harmful stimuli such as pathogens and damaged cells (**Levick et al., 2007; Chung et al., 2009**). Cell damage caused by inflammation releases arachidonic acid in the cell membrane via two metabolic pathways: the first pathway involves cyclooxgenase 1 (COX-1) and cyclooxygenase 2 (COX-2) to produce prostoglandins and thromboxanes (**Levick et al., 2007**). The second pathway is the lipoxygenase pathway, which involves lipoxygenase 5 (LOX-5), lipoxygenase 12 (LOX-12) and lipoxygenase 15 (LOX-15), enzymes that produce leukotrienes and hydroperoxyl fatty acids (**Chung et al., 2009**). The earliest record of treating inflammation came in 30 AD by Celsius when he used willow (*Salix alba*) leaf extract on infected area (**Adebayo et al., 2015**). This led to the discovery of acetyl salicylic acid, an active constituent found in aspirin, and a major of anti-inflammatory drugs used in clinical practices with other non-steroidal anti-inflammatory drugs (NSAIDs) suggests **Adebayo et al (2015)**

NSAIDs are the most effective medications at managing pain, fever, swelling and redness (**Ferreira., 2002; Chung et al., 2009**). NSAIDs can achieve these effects by inhibiting COX-1 and COX-2 activity. While COX-1 inhibitors are associated with many side-effects such as gastrointestinal erosions and renal and hepatic deficiency, while newer COX-2 inhibitors are said to be more effective and present with less side-effects, except for COX-2 inhibitor Vioxx which was withdrawn because cardiovascular events (**Iqbal et al., 2005; Levick et al., 2007**).

A study reported four *E. obtusifolia* compounds isolated from ethyl-acetate fractions to inhibit lipoxygenase in a concentration dependent manner (**Jyothirmai et al., 2016)**. Further literature by **Dong et al** (**2000**) provided pharmacological experimental evidence, on an isolated compound in *E. dicksonni* ((10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid methyl ester) used to reduce inflammation on mice ears at a dose of 500 ug/ml (**Dong., et al 2000; Li et al., 2010**).

1.4.4 Antidiabetic

Diabetes is one of the major metabolic disorders, with micro and macro-vascular complications that result in significant morbidity and is among the five causes of death in the world (**Ogundajo and Ashafa., 2017**). Diabetes is caused from a dynamic interaction between defects in insulin secretion and insulin action (**Tundis et al., 2010**). The defects lead to increased concentration of blood glucose which in turn damage bodily systems, particularly blood vessels (**Tundis et al., 2010**). Diabetic disorders include retinopathy, nephropathy, neuropathy and angiopathy (**Sarkodie et al., 2015**).

Diabetes is spreading rapidly in African countries because of the presumed unhealthy lifestyle, uncontrolled urbanisation, westernisation and eating habits (**Sarkodie et al., 2015**). In diabetic individuals, the high blood sugar (hyperglycemia) generates reactive oxygen species (ROS) that damage cell membranes, cause lipid peroxidation, which leads to secondary complications such as heart attack, kidney failure, retinopathy, and nerve damage (**Tundis et al., 2010; Ogundajo and Ashafa, 2017**). Antioxidants have been shown to prevent damage and deterioration of β-cells by inhibiting the peroxidation chain reaction, therefore may serve to protect against development of diabetes. Plants containing natural antioxidants can preserve β cell function and stop diabetes induced ROS formation (**Li et al., 2010; Ogundajo and Ashafa., 2017**)

Inhibiting the enzymes involved in diabetes (α-amylase and α-glucosidase) is one of the ways of controlling the postprandial glycaemic reaction (**Adisakwattana et al., 2010**). Currently, enzyme inhibitors include acarbose, miglitol and voglibose (**Tundis et al., 2010**). However, as effective as they are, these glycaemic agents have side effects which include swelling, abdominal discomfort, diarrhoea, and flatulence (**Ogundajo and Ashafa., 2017**). It is therefore vital that new potent glycaemic agents with have less side effects are discovered, and studies

Table 1.1: List of *Ehretia* species and their use in traditional medicine (Shukla & Kaur., 2018)

Botanical name	Common name	Part used	Traditional use
Ehretia acuminata R.Br.	Pudila, Nara, Koda	1. Leaves	1. Leaf extracts in water taken orally 2-3 times for acute dysentery
		2. Bark	2. Bark juice used to treat sores on tongue and fever
Ehretia laevis Roxb.	Chamror (Punjab)	1. Leaves	1. Leaf juice is applied in ulcer, skin disease, and headache
		2. Root	2. Root used against venereal diseases
		3. Bark	3. Inner bark used as food and as gargle to treat throat infection
		4. Fruit	4. Fruit used against infections of urinary passages, diseases of lungs and spleen
Ehretia microphylla Lam.	Pala.	1. Leaves	1. Dry leaf decoction are used to cure coughs and treatment of bloody discharge and dysentery
		2. Root	2. Roots are used against syphilis and as cure vegetable poisoning
Ehretia obtusifolia.		1. Leaves	1. Decoction of leaves and bark against Malaria
Ehretia cymosa		1. Leaves	1. Different parts of plant are used against diarrhoea
		2. Bark	2. The extract of leaves used against Malaria 3. Bark and leaves used against epilepsy
Ehretia amoena		1. Leaves	1. Plant works against trypanosomiasis

suggest they can be found from natural sources (**Li et al., 2010**). Plant extracts have been used for ethnomedicinal treatments of diabetes and *Ehretia cymosa* have demonstrated promising antidiabetic effects and leaf extracts are reported to treat measles, pain, epilepsy, convulsions, and spasms. (**Sarkodie et al., 2015; Ogundajo & Ashafa., 2017**).

1.5 The production of secondary metabolites in *Ehretia*

For the past two centuries, modern chemistry and biology has described the role of primary metabolites being associated with cell division and growth, respiration, storage, and

reproduction (**Bourgaud et al., 2001**). Later secondary metabolites were described based on their low abundance of less than 1% of total carbon, or a storage chemical usually occurring in dedicated cells or organs (**Torssell., 1997; Bourgaud et al., 2001; Akula & Ravishankar., 2011**). With the improvement and use of analytical techniques such as chromatography, these molecules have been recovered and identified as being part of the phytochemistry of plants (**Bourgaud et al., 2001; Hussain et al., 2012**).

Plant secondary compounds/metabolites are classified according to their biosynthetic pathways and the largest phytochemical families include phenolics, terpenes and steroids and alkaloids (**Harborne., 1999; Hussain et al., 2012**). Angiosperms have a variety of secondary metabolites synthesised from primary metabolites (Lipids, proteins, and carbohydrates) according to **Akula & Ravishankar** (**2011**). The most widespread family are the phenolics because of their involvement in the synthesis of lignin, a common organic macromolecule found in all higher plants (**Torssell., 1997; Harborne., 1999**). Alkaloids however are an exclusive family of compounds, sparsely distributed and are only found on specific plant genera and species. Secondary metabolites are also contributors to tastes and odours in plants and are also a unique source of additives in food. Additionally, they are industrially important pharmaceuticals due to their many biological activities. (**Hussain et al., 2012**).

1.6 Aims and objectives

The aim of this study was to determine ethnopharmacological and phytochemical potential of two Southern African *Ehretia* species - *E. rigida* and *E. obtusifolia,* and to make a detailed comparison with other species, in order to increase understanding on the medicinal potential of the genus *Ehretia*

The objectives of this study were to investigate and compare:

1. The biological activities (Antibacterial, antifungal, antioxidant, and anti-diabetic) exhibited by different plant organs of *E. rigida* and *E. obtusifolia*
2. The phytochemical composition of the different plant organs of *E. rigida* and *E. obtusifolia*

Chapter 2: Phytochemical study of bark, leaves, and roots of *Ehretia rigida* and *Ehretia obtusifolia*

2.1 Introduction

Plants play a crucial role in a person's everyday diet, and for many years they have been studied intensively, with reports of nutritional value and this makes them indispensable (**Thakur et al., 2019**). Higher plants are well known for their synthesis of primary metabolites such as proteins, carbohydrates, lipids and in addition they synthesise secondary metabolites such as alkaloids, anthocyanins, flavonoids, quinones, lignans, steroids and terpenoids (**Bourgaud et al., 2001**; **Karuppusamy., 2009; Shukla and Kaur., 2018**). Plant secondary metabolites did not have a recognized significant role in maintaining fundamental life processes in plants before, however with acknowledgements to improvements in biochemical techniques and increased knowledge in molecular biology, secondary metabolites have been reported to play a role in a plant's interaction with the environment, and act as defence chemicals to protect plants from insects, pests, herbivores and phytopathogens (**Bourgaud et al., 2001; Thakur et al., 2019**).

Secondary metabolites are produced in small amounts of less than 1% per dry mass, the physiological and developmental stage of the plant determines the quantity of secondary metabolites (**Karuppusamy., 2009**). Although present in all plants, not all plants have the capacity to produce the various classes of the secondary metabolites according to **Bourgaud et al** (**2001**) and the plant family and genus determines which phytochemicals will be present. Over-time these phytochemicals have been used as a source of medicine for humans and animals (**Chien et al., 2012**) and subsequently used as agro-chemicals, flavours, fragrances, colours, biopesticides and food additives (**Karuppusamy., 2009; Pavlovic et al., 2012**).

2.1.1 Phytochemicals found in plants and their medicinal purposes

2.1.1.1 Alkaloids

Alkaloids are the rarest and most sought of the secondary metabolites, particularly in the pharmaceutical and cosmetic industries (**Chien et al., 2012; Shukla and Kaur, 2018**). Alkaloids have been the source of many of the modern drugs used to treat various disorders such as heart failure, blood pressure and cancer (**Joshi and Wagh., 2018a)** Several alkaloids such as vinblastine and camptothecin have been isolated from plants exhibiting antimetastisis and

antiproliferation effects on various types of cancer (**Chien et al., 2012; Shukla and Kaur., 2018**). Other popular alkaloids used as drugs nowadays include morphine isolated from *Papaver somniferum* for pain relief, nicotine, isolated from the nightshade family plants, *Nicotiana tabacum* which is used in insecticides and is a powerful neurotoxin, reserpine from *Rauwolfa* for controlling high blood pressure, quinine, and artemisinin for the treatment of malaria (**Omokhua et al., 2015**).

2.1.1.2 Phenolics

Phenolic acids are a group of secondary metabolites that possess a benzene ring with a hydroxyl group attached to it. They are universally present in plants with over 8000 known phenolic structures (**Velappan & Thangaraj., 2014; Joshi & Wagh., 2018**). The phenolic group includes other classes of secondary metabolites such as flavonoids and tannins (**Shukla & Kaur., 2018**). They are popular for their antioxidant activity because they act as reducing agents, quenchers, free radical scavengers, ion chelators, enzyme cofactors and terminators of radical chain reactions (**Thakur et al., 2019**). Phenolic compounds found in dietary products such as vegetables and fruits have a unique taste and contain health promoting properties (**Omokhua et al., 2015**). The nutritional benefits of phenolics are largely related to their antioxidant and anticarcinogenic effects. Other biological activities exhibited by phenolic compounds include anti-inflammatory, anti-apoptosis, anti-atherosclerosis, anti-aging and endothelial function improvement and inhibition of angiogenesis and protection of the cardiovascular system (**Pavlovic et al., 2012**). In *Ehretia obtusifolia* phenolic compounds such as caffeic anhydride, methyl 2-O-feruloyl-1a-O-vanillactate and methyl rosmarinate have been identified and isolated primarily because of their antioxidant and possible anti-carcinogenic activity (**Shukla & Kaur., 2018**)

2.1.1.3 Flavonoids

Flavonoids are a group of polyphenolic compounds found in different plant parts and have a wide range of biological activities. They are found in fruits, nuts, spices, herbs and derived in products such as wine, tea, and chocolate (**Velappan & Thangaraj., 2014**). There are more than 6000 flavonoid compounds found in nature and comprise of the following classes: flavonols (quercetin, kaempferol, myricetin and tangertin), flavones (apigenin, luteolin and tangeretin), flavanones (hesperetin, naringenin and eriodictyol), flavanols (catechin and epicatechins), anthocyanidins (cyanidin, delphinidin) and isoflavones (genistein and glycitein)

(**Chien et al., 2012; Shukla and Kaur., 2018**). Studies have revealed flavonoids have beneficial effects in treating and managing diseases such as cancer, cardiovascular and neurodegenerative disorders (**Zara et al., 2012; Joshi and Wagh., 2018a**), in addition they are active against microbial infections and diarrhoea. Like phenolic acids, flavonoids can prevent injuries by scavenging free radicals. They carry out this process by directly scavenging for ROS by activating antioxidant enzymes, metal chelating activity, inhibiting activity of oxidase enzymes, reducing α-tocopheryl radicals and by increasing uric acid (**Li et al., 2010**).

2.1.1.4 Tannins

Tannins are a subclass of phenolics present in plants and exist in a condensed or hydrolysed form (**Chien et al., 2012**). Tannins have been identified as one of the most beneficial medicinal properties in plants, and exhibit antiseptic activity against gastrointestinal nematodes, antimicrobial activity, antioxidant and antiviral (anti-human immunodeficiency virus) effects (**Li et al., 2010**).

2.1.1.5 Benzoquinone

Quinones plant-derived secondary metabolites that include benzoquinones. They are mostly in higher plant families such as the Polygonaceae, Rubiaceae, Leguminosae, and Boraginaceae, but are more common in fungi. *Ehretia* is well documented for producing potent benzoquinones with significant anti-cancer and antibacterial activity (**Shukla and Kaur., 2018**).

Different classes of secondary metabolites in *Ehretia* have been documented in different species, with the most prevalent being phenolics, flavonoids and benzoquinones. The aim of this investigation is to determine secondary metabolite production of different plant parts (leaves, bark, and roots) of *E. rigida* and *E. obtusifolia.*

2.2 Materials and Methods

3.2.1 Plant collection

Five *Ehretia obtusifolia* and three *Ehretia rigida* plants were purchased at the National Botanical garden, Pietermaritzburg KwaZulu Natal South Africa. The bark of *Ehretia rigida* was collected from the tree grown at the National Botanical garden in University of KwaZulu Natal Pietermaritzburg.

2.2.2 Plant extract preparation

The leaves of *E. rigida* and *E. obtusifolia* were carefully collected and washed with distilled water. Bark of *E. rigida* was peeled off using a blade and branches of *E. obtusifolia* were scraped to remove bark. Roots were washed with distilled water to remove soil until there were only roots and root hairs remaining. The different plant parts were placed in the oven to dry at 45^{o}C for 72 h. Once dried, the different plant parts were ground into fine powder using a mill. Ground material was stored in sealed plastic zip-lock bags and kept at room temperature until ready for use.

2.2.3 Qualitative determination of phytochemicals in different plant organs between *E. obtusifolia* and *E. rigida*

2.2.3.1 Phenolic detection

To determine the presence of phenolics in plant organs of *E. rigida* and *E. obtusifolia,* the ferric chloride test was carried out. In triplicates, 0.1 g of leaf, bark, and root ground material of *E. rigida* and *E. obtusifolia* were extracted with 10 ml 50% MeOH. The various extracts were measured into test-tubes (5 ml) and a few drops of 2 % of ferric chloride were added. The presence of phenolic compounds was confirmed by a dark green colour in the test tube.

2.2.3.2 Flavonoid detection

Detection of flavonoid compounds was determined by the sodium hydroxide (NaOH) method by **Trease and Evans., 2002)**. Leaf, bark, and roots (0.1 g) were suspended in 5 ml distilled water and thereafter the mixtures were filtered. NaOH (10% of 2 ml) was added in extract mixtures and the appearance changed to a yellow colour, which disappeared after an addition of a few drops of HCL thus indicated the presence of flavonoids. The test was done in triplicates

2.2.3.3 Tannin detection

Ground leaf, bark, and root (0.5 g) of *E. obtusifolia* and *E. rigida* material were weighed in test-tubes. The mixtures were filtered and a few drops of 1% ferric chloride were added to 2 ml of each filtrate. Appearance of blue-black colouration confirmed the presence of tannins.

2.2.3.4 Saponin detection

A Froth test as described by **Tadhani and Subhash** (**2006**) taken from **Omokhua et al** (**2015**) was used to detect saponins. Bark, leaf, and root ground samples from *E. obtusifolia* and *E. rigida* were weighed (0.1 g) in test-tubes in triplicates. MeOH (5 ml of 50 %) was measured

and added in the test-tubes, the test-tubes thereafter were corked and shaken vigorously for 2 min. The appearance of a stable froth after allowing the mixture to stand for 45 min confirmed the presence of saponins

2.2.4 Quantitative determination of phytochemicals in different plants organs of *E. rigida* and *E. obtusifolia*

2.2.4.1 Folin-Ciocalteu (Folin-C) assay for total phenolics

The Folin-C assay for total phenolics was conducted as described by **Makkar et al** (**2007**) with modification, using gallic acid as a standard. The reaction mixture was made with 50 µl of plant extract transferred into a test-tube, 950 µl of distilled water was added, followed by 500 µl of 1 N Folin-C reagent and 2.5 ml of 2% sodium carbonate ($NaCO_3$) were added in the dark. A blank made of 50% MeOH without the gallic acid and plant extract was similarly prepared in triplicates. The test-tubes containing the mixture were incubated in the dark for a period of 40 min at room temperature, and subsequently the absorbance reading was measured at 725 nm using a Carey Varian 50 UV- visible spectrophotometer. The total phenolic content was determined against the gallic acid concentration giving an equivalent per gram of dry weight (GAE). A student t- test or Man Whitney test if t-test assumptions are not was carried to determine difference in phenolic content in organs between *E. rigida and E. obtusifolia.* A One-Way ANOVA or Kruskal-Wallis test if assumptions of ANOVA are not met was used to determine difference in phenolic content between bark, leaf, and roots of *E. obtusifolia and E. rigida* using SPSS version 25.

2.2.4.2 Calorimetric assay for determining flavonoid content

The method used to determine total flavonoid content was one described by **Zhishen et al** (**1999**) and modified by **Marinova et al** (**2005**). The aluminium chloride assay uses catechin as the standard. The experiment was conducted in triplicates, with a reaction mixture made of 250 µl of 50 % MeOH plant extracts, 75 µl of 5% sodium nitrite ($NaNO_3$), 75 µl of 10 % aluminium chloride ($AlCl_3$), and 500 µl of sodium hydroxide (NaOH). Six hundred µl of distilled water was used to adjust the volume of the reaction mixture to 2.5 ml was added and the test-tubes were incubated for 40 min at room temperature. 50 % MeOH was used as a blank and the absorbance readings were measured at 510 nm using a Carey 50 UV-visible spectrophotometer. The flavonoid content was expressed as catechin equivalent (CE) per dry weight. A One-Way ANOVA or Kruskal Wallis test if ANOVA assumptions are not met was used

to determine difference in flavonoid content between bark, leaf, and roots of *E. obtusifolia and E. rigida* using SPSS version 25. A t-test or Man Whitney test if t-test assumptions are not met was performed to determine significant difference between *E. obtusifolia* and *E. rigida* in flavonoid content present in bark, leaf, and roots

2.2.4.3 Butanol-HCL assay for determination of condensed tannins (proanthrocyanidin)

The butanol-HCL assay was conducted to determine condensed tannins according to the method described by **Makkar** (**1999**). A butanol-HCL (95:5) reagent was made and 3 ml was added in the test-tube along with 500 µl of plant extract, and 100 µl of ferric reagent (2 % ferric ammonium sulphate). The reaction mixtures were vortexed and left to incubate in boiling water (100^{o}C) for 1 hour. The reaction mixtures were left to cool down for one hour, and the absorbance readings were measured at 550 nm using a Carey 50 UV-visible spectrophotometer. The experiment was done in triplicates. The concentration of condensed tannins was calculated and expressed as leucocyanidin equivalent using the formular described by **Porter, Hrstrich and Chan** (**1986**) and **Makkar** (**1999**):

$$\% = (A550\ X\ 78.26)\ X\ \frac{dilution factor}{\%dry\ matter}\ X\ 10$$

A550 = absorbance at 550 nm

Dilution factor = 1.0 for all plant extract

2.3 Results

Table 2.3.1: Phytochemical detection in leaves, bark, and roots of *Ehretia rigida*

Phytochemicals	Leaves	Bark	Roots
Alkaloids	-	-	-
Flavonoids	+++	+++	+++
Phenolics	+++	++	+++
Saponins	++	++	++
Tannins	+++	+++	+++

Table 2.3.2: Phytochemical detection in leaves, bark, and roots of *Ehretia obtusifolia*

Phytochemicals	Leaves	Bark	Roots
Alkaloids	-	-	-
Flavonoids	+++	+++	+++
Phenolics	+++	++	++
Saponins	++	++	++
Tannins	+++	+++	+++

- = Absent; + = Present; ++ = Moderate; +++ = Abundant

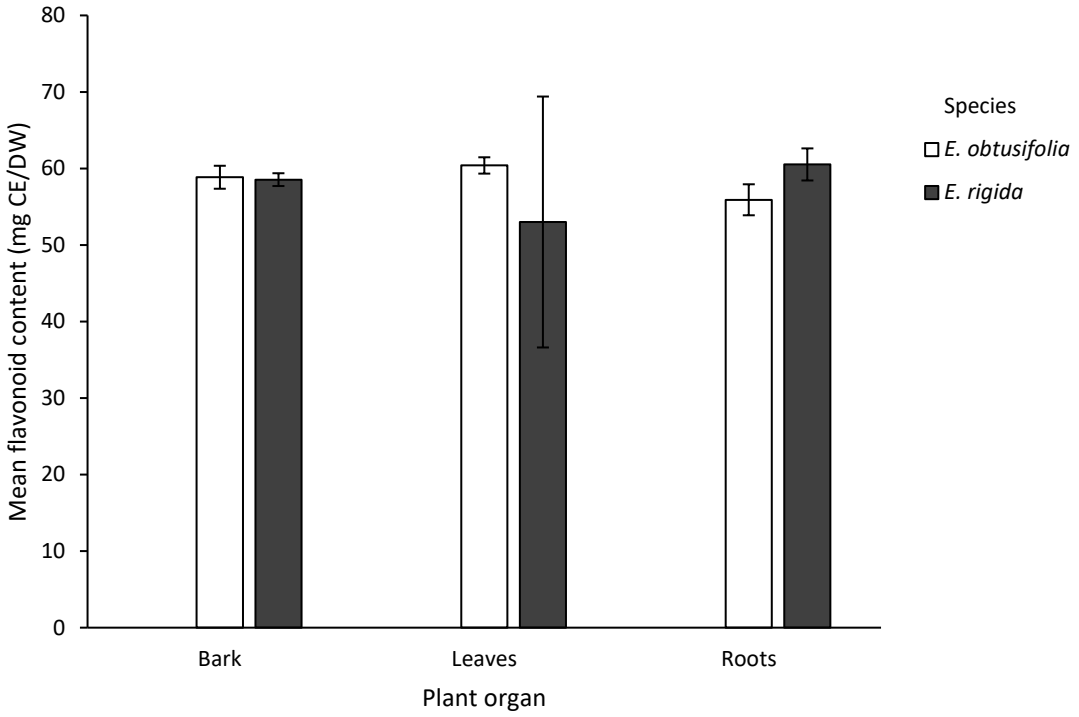

Figure 2.3.1: Comparison of total flavonoid content in leaves, bark, and roots of *E. obtusifolia* and *E. rigida*

E. obtusifolia leaves, bark, and roots were significantly different in total flavonoid content ($F_{2,9}$ = 8.3; p = 0.009) and according to the Kruskal-Wallis test, the total flavonoid content was not significantly different in leaves, bark, and roots of *E. rigida* (H =2.46; df = 2; p = 0.276). There was no significant difference in bark flavonoid content between *E. rigida* and *E. obtusifolia* (t = -3.61; df = 6; p = 0.73). The total flavonoid content in bark was 58.54±0.84 and 58.85±1.50 mg CE/DW for *E rigida* and *E. obtusifolia* respectively. Total flavonoid content in leaves of *E. obtusifolia* (60.40±1.06 mg CE/DW) was higher than that of the total flavonoid content in *E. rigida* leaves (53.01±16.40 mg CE/DW.) and the total flavonoid content in leaves was not significantly different according to the Man Whitney U test (*U* = 7*;* $n_1 = n_2 = 4$; p = 0.773). *E. rigida* had a higher flavonoid content in roots than *E. obtusifolia*, the total flavonoid content present in roots for both species was 60.53±2.09 mg CE/DW and 55.91±2.02 mg CE/DW respectively. There was a significant difference in total flavonoid content in roots between *E. rigida* and *E. obtusifolia* (t = 3.17; df = 6; p = 0.019).

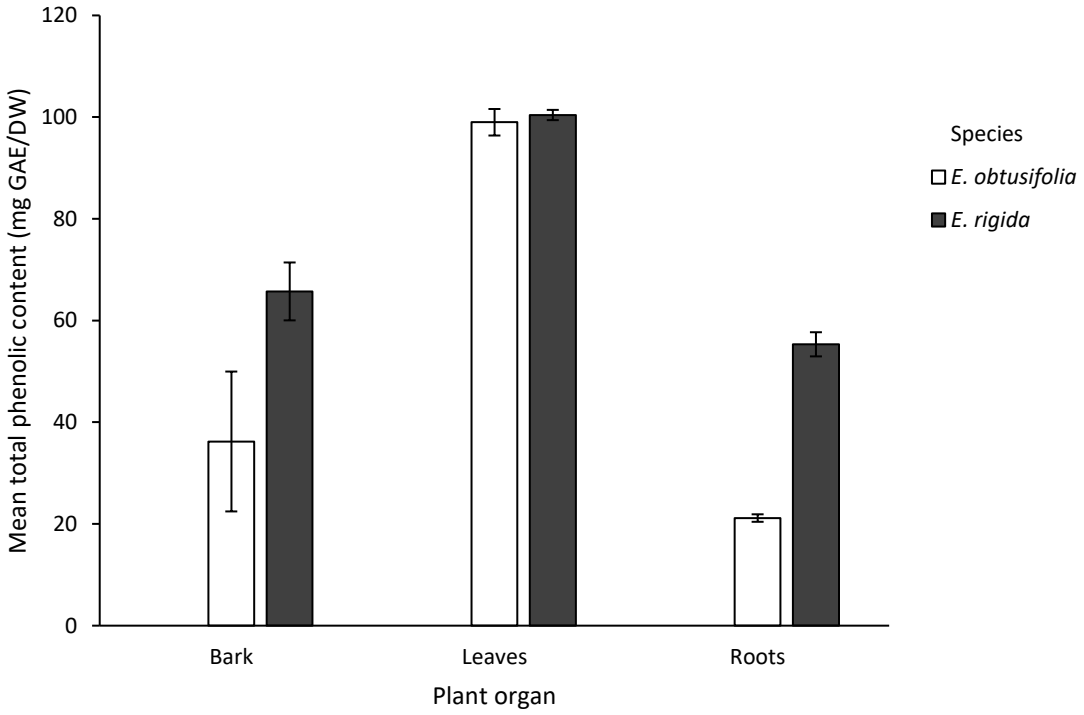

Figure 2.3.2: Total phenolic content present in leaves, bark, and roots of *E. obtusifolia* and *E. rigida*

Kruskal -Wallis test showed there was a significant difference in phenolic content when comparing leaves, bark, and roots of *E. rigida* (H = 9.26; df = 2; p = 0.010) and there was no statistically significant difference in phenolic content in *E. obtusifolia* leaves, bark, and roots (H= 2.46; df = 2; p=0.292), however Figure 2.3.2 indicated otherwise. There was a statistically significant difference in phenolic content between leaves of *E. obtusifolia* and *E. rigida* according to the Man Whitney test (U = 0.00; $n_1 = n_2 = 4$; p = 0.021). The lowest phenolic content was 21±16 mg GAE/DW found in *E. obtusifolia* roots. *E. rigida* had a marginally high phenolic content of 100.42±1.01 mg GAE/DW in leaves while *E. obtusifolia* had 98.99 mg GAE/DW, bark and roots of *E. rigida* had 65.72±5.69 and 55.32±2.37 mg GAE/DW respectively. Total phenolic content in bark between *E. obtusifolia* and *E. rigida* was significantly different (t = 3.96; df = 6; p = 0.007) and the total phenolic content in roots between *E. obtusifolia* and *E. rigida* was also significantly different (t = 27.47; df = 6; p = 0.000).

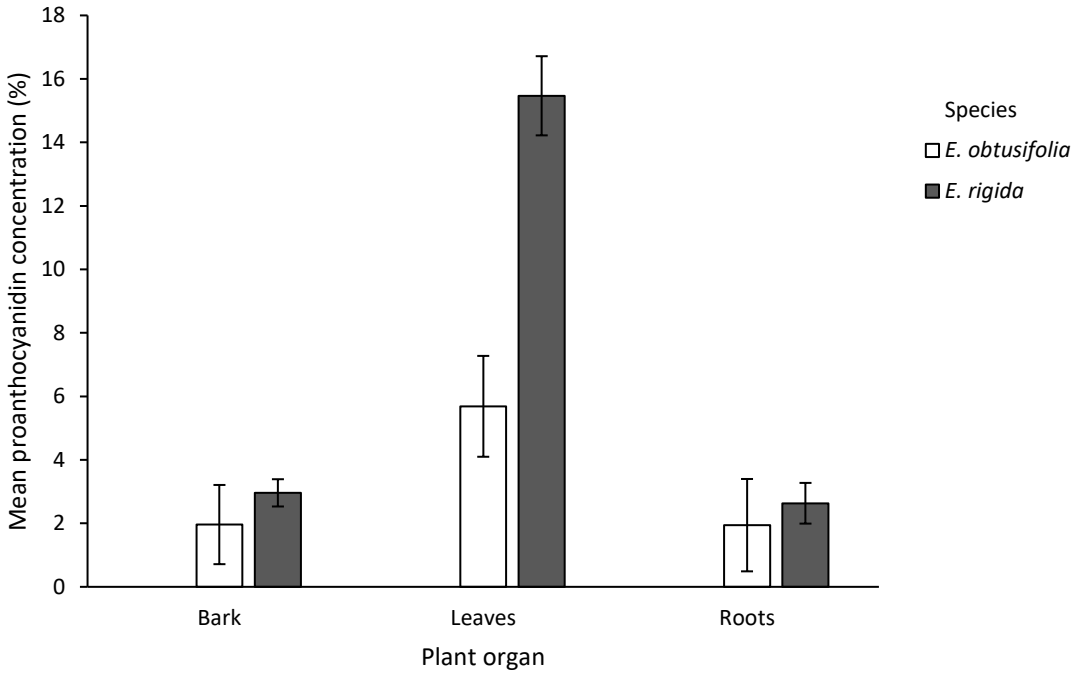

Figure 2.3.3: Concentration of proanthocyanidin (condensed tannins) in leaves, bark, and roots of *E. obtusifolia* and *E. rigida*

There was a significant difference in condensed tannins found in leaves, bark, and roots of *E. obtusifolia* according to results of A One-Way ANOVA ($F_{2,9}$= 9; p = 0.007). A One-Way ANOVA showed there was a significant difference in condensed tannins present between leaves, bark, and roots of *E. rigida* ($F_{2,9}$ = 298.9; p = 0.000). *E. rigida* recorded the highest total of condensed tannins in leaves of 15.46±0.62 % and 5.94±0.79 % was found in *E. obtusifolia.* The concentration of condensed tannins between *E. obtusifolia* and *E. rigida* leaves was significantly different (t = 9.69, df = 6, p<0.05). Condensed tannins in bark were not significantly between *E. obtusifolia* and *E. rigida* (t = 1.515; df = 6; p = 0.18). The mean concentration of condensed tannins in bark of *E. rigida* (2.96±0.21%) was marginally higher than *E. obtusifolia* (1.96±0.6 2%). *E. rigida* concentration of condensed tannins in roots was marginally higher than *E. obtusifolia* (2.63±0.32 and 1.94±0.72 % respectively) and there was no significant difference in the concentration of condensed tannins comparing roots of *E. obtusifolia* and *E. rigida* (t= 0.86; df = 6; p = 0.429).

2.4 Discussion

For many years' plants have been a source of food, used in manufacturing of cosmetics, dying clothes, and used as medicine to treat various diseases (**Chien et al., 2012**). Medicinal plant extracts have an assortment of biological activities, which is credited to the presence of phytochemicals or secondary metabolites that are said to be responsible for a plants defence against pathogenic bacteria, fungi, viruses, and physical attacks like herbivory (**Pavlovic et al., 2012**). Secondary metabolites are produced in minute amounts of less than 1% dry weight which makes their extraction difficult and expensive especially for the rare ones such as alkaloids (**Thakur et al., 2019**). Alkaloids in bark, leaves or roots were not detected in both *E. rigida* and *E. obtusifolia* (Table 2.3.1 and 2.3.2), however in the genus *Ehretia* the presence of alkaloids has been detected and isolated in *E. aspera* (ehretinine) along with glyoxylic acid *E. thrysiflora* (allantoin) as reported by **Shukla & Kaur (2018**). Phenolics were detected abundantly in in both species, while flavonoid detection was only moderate for both species (Table 2.3.1 and 2.3.2). Saponins were also detected along with tannins and were found to be abundant in the plant organs of both *E. obtusifolia* and *E. rigida.* Of the two species, *E. rigida* had the greater abundance of phytochemicals present in bark, leaves and roots, thus biological activities are speculated to be more distinct or noticeable in *E. rigida*.

A qualitative analysis to determine the presence of phytochemicals is not enough and may be subjective to one's perception. Therefore, to obtain a deeper understanding of the presence of phytochemicals, the study aimed to quantify and determine total phenolic and flavonoid content. Quantifying alkaloids, naphthoquinones, polyphenols, phytosterols terpenoids and fatty acids is a priority which leads to the step of extracting and isolating compounds (**Chien et al., 2012)**). Polyphenols made of phenolic acids and flavonoids are well distributed in the plant family and their diverse pharmaceutical activities such as antioxidants, anti-inflammatory, antiviral, antibacterial, and hepato-protecting activity are critical to healthcare. Of the many secondary metabolites, many polyphenols are known to be the contributors to consumer goods such as tea, red wine, beverages, plant foods and in traditional health therapies **(Li et al., 2010; Velappan & Thangaraj., 2014**).

The study established a baseline for the quantity of phytochemicals in *E. rigida* and *E. obtusifolia* and revealed *E. rigida* had higher phenolic content in bark and roots than *E. obtusifolia* (Figure 2.3.2). Phenolics are important in plants because they are responsible for

defence against herbivory and pathogens such as fungi, attracting pollinators, absorbing harmful UV rays and or reducing the competitive growth of nearby plants (**Ynalvez et al., 2018**). The mechanism of phenolics when acting as a defence mechanism includes, phenolic toxicity to microorganisms via enzyme inhibition by the oxidised compounds or by building hydrogen, hydrophobic and ionic bonds, thus modulating their 3D structures and therefore their bioactivities (**Chien et al., 2012**).

Polyphenols such as phenolic acids and flavonoids distributed in the Boraginaceae family have been reported to have different pharmaceutical activities, primarily antioxidant, anti-inflammatory, antiviral, antibacterial, and hepato-protecting effects (**Li et al., 2010; Maskovic et al., 2016).** Phenolics are associated with excellent antioxidant activity because of their role in absorbing radicals, quenching oxygen singlets and triplets, and are responsible for decomposing peroxides (**Joshi and Wagh., 2018b**).

E. obtusifolia had a moderately higher quantity of flavonoids in leaves and bark, than *E. rigida* and *E. rigida* had a higher quantity of flavonoids in roots (Figure 2.3.1). The study showed that in leaves, bark, and roots of each single species, there was a higher recorded quantity of flavonoids per gram of dry weight than phenolics, particularly in *E. obtusifolia*, which makes *E. obtusifolia* the favoured species for isolating flavonoids. Flavonoids are considered to have high antioxidant activity because of their chemical structure (**Pavlovic et al., 2012)** which makes flavonoids excel in radical scavenging activities, therefore they are useful in therapeutic and prophylactic applications (infections, inflammation, burns or radiation activity) (**Maskovic et al., 2016**).

Leaf methanolic and aqueous extracts from *Ehretia laevis* were studied for their total phenolic content (TPC) by **Joshi and Wagh** (**2018a**) and the results of the study indicated relatively high quantity of phenolic content. The comparative study conducted between *E. obtusifolia* and *E. rigida* revealed that the leaves, bark, and roots had higher phenolic content than that of *E. laevis* (89.55±0.67 mg GAE/DW for methanol bark extracts and 53.55±0.29 mg GAE/DW for aqueous bark extracts) (**Ragnathrao and Shanmugasundaram., 2018)**. Other studied species of *Ehretia* include *E. serrata* and *E. thyrsiflora* and have reported high total phenolic and flavonoid content present in their leaves and fruit. The study by **Zara et al., 2012** showed that *E. serrata* had phenolic content of 38.58 ± 2.8 and 264.18 ± 1.7 mg GAE/DW in fruit and leaves respectively, and for flavonoid content in fruit in leaves, it reported 209.366 ± 4.5 and 493.409

± 1.6 respectively. When compared to *E. rigida* and *E. obtusifolia¸ E. serrata's* total phenolic and flavonoid content in leaves was significantly higher.

The concentration of proanthocyanidin (%) was higher in *E. rigida* than *E. obtusifolia* with leaves having higher quantity of condensed tannins than any other plant organ (Figure 2.3.3). A study investigating *Onosma aucheriana* from the Boraginaceae family reported methanolic extracts containing flavonoid monomers and condensed tannins with high antioxidant activity and hydrolysable tannins (**Maskovic et al., 2016**). Hydrolysable tannins have a polyhydric alcohol at their core, the hydroxyl groups are partially or fully esterified with gallic acid. On hydrolysis they break down into their constituent phenolic acids and carbohydrates. Whereas condensed tannins are dimers, the simplest being procyanidin. Studies suggest that tannins are said to be 15 -30 times more effective in quenching peroxyl radicals than phenolics (**Hill., 2003**).

2.5 Conclusion

The study revealed that leaves in both species are ideal organs to extracts secondary metabolites (Figures 2.3.1 - 2.3.3). *E. rigida* generally had higher quantity of phenolic content compared to *E. obtusifolia* and this was detected in roots and bark. For condensed tannins *E. rigida* had higher content in all organs. For flavonoids, *E. obtsusifolia* had a higher content. Leaves showed to be suitable candidates for sustainable harvesting of secondary metabolites compared to roots and bark. Both species had high content of secondary metabolites and based on the establishment of the quantity of secondary metabolites, biological activities such as antimicrobial and antioxidant in chapter 3 and 4 are predicted to be high.

Chapter 3: Antimicrobial activity exhibited by different plant organs of *Ehretia rigida* and *Ehretia obtusifolia*

3.1 Introduction

According to the World Health Organisation (WHO), infectious diseases caused by pathogenic bacteria and deep invasive fungal infections are the leading cause of morbidity and mortality (**Shaik et al., 2014**). These microorganisms are a major health care problem and are said to be the cause of more than 14 million deaths worldwide (**Shaik et al., 2014; Sarkodie et al., 2015)**. In the past, infectious diseases such as pneumonia, malaria, typhoid fever were successfully treated with antibiotics, however microorganisms have the ability to evolve and adapt to the course of treatment provided to fight of infection. As a result, multidrug resistant bacteria have become a reality, and there is no treatment that can be used to combat such pathogenic bacteria (**Gamboe et al., 2008**).

Bacteria are the most abundant unicellular organisms on earth, made of prokaryotic cells with a rigid double cell wall that protects them from osmotic damage (**Ogunseitan., 2016**). Bacteria are categorised as either being Gram-positive or Gram-negative, and what makes them different is in the structure of their cell wall. Gram-negative bacteria have an outer membrane that is high in lipopolysaccharide (a single peptidoglycan and a periplasmic space to separate from cytoplasmic membrane) and gram-positive bacteria have a multilayer of peptidoglycan outside the cell membrane which retains the crystal violet stain when washed with alcohol (**Chien et al., 2012**). Bacteria play an important role in everyday human activity, such as aiding in digestion, preventing formation of colonies of pathogenic bacteria and they are also helpful in enriching soil, and fermentation of alcoholic beverages and cheese (**Shaik et al., 2014**).

3.1.1 Fungal infection

Candida albicans is one of the most common pathogens infecting humans, it accounts for more than 90 % of fungal infections in humans (**Zida et al., 2017; Svetaz et al., 2010; Pretorius et al., 2003**). Fungal infection in humans can be superficial or deeply invasive and the most common diseases are deep mycoses and vulvo-vaginal candidiasis. Common antifungal drugs have become less effective against some fungal infections because of antifungal resistance, and this has called for the study of underlying mechanisms in antifungal resistance causing failure of drug treatments in fungal infections (**Shaik et al., 2014**). *C. albicans* is polymorphic with two distinct morphological forms: yeast and filamentous. The ability of *C. albicans* to

replicate is linked with yeast hyphal transition, and biofilm formation whereby the hyphal forms are important for invading the tissue and escaping macrophages, and the biofilms are important for producing antifungal tolerant persistence, which leads to persistent fungal infections (**Zida et al., 2014)**. The dimorphism and formation of biofilms by *C. albicans* with its ability to cause infections related to biofilms has encouraged a search for new antibiofilm substances as one of the leading efficient avenues of treatment (**Zida et al., 2014; Pretorius et al., 2003**).

3.1.2 Antibacterial and antifungal activity

For a very long-time mankind has used plants to treat diseases with some of the remedies still being used and practiced today (**Jyothirmai et al., 2016**). Folk medicine is largely based on plants and today still holds a respectable position in developing countries because of a lack of available modern health care system (**Mutha et al., 2015; Shaik et al., 2014**). Plant based remedies are said to be effective because of compounds which are produced by plants to defend themselves when they are infected or physically damaged (**Pretorius et al., 2003**). These said compounds are called secondary metabolites, and their identification and quantification were studied in chapter 2. Some of these compounds such as phenolic and flavonoid substances generally have significant antimicrobial activity, however when considering traditional remedies, one needs to carry thorough *in vitro* analyses because these treatments may carry adverse effects (**Mutha et al., 2015; Shukla & Kaur., 2018**)

In the past three decades, evidence supporting the potential of medicinal plants to treat human diseases has been accumulating according to **Shaik et al** (**2014**), and numerous plants have been documented to be rich in secondary metabolites (phenolics, tannins, alkaloids, and flavonoids) with *in vitro* antimicrobial properties (**Gumgumjee & Hajar., 2015**). Scientists in the pharmaceutical industry (drug developers and clinical microbiologists) are interested in antimicrobial plant extracts because – 1) most of the phytochemicals find their way to the list of antimicrobial drugs prescribed, and several of them are already being tested on humans. The effective short life span of antibiotics renders them useless once bacteria develop resistance, and hence new sources especially plant-based sources are being investigated. 2) The public is becoming increasingly educated on the over-prescription and misuse of traditional antibiotics and prefer more independence over their medical care **(Shukla & Kaur., 2018; Waheed et al., 2019**). Many plant compounds are of unreliable purity however, they

are already easily available from herbal suppliers and in food stores for self-medication (**Pretorius et al., 2003**).

3.2 Materials and methods

3.2.1 Extract preparation

The leaves of *E. rigida* and *E. obtusifolia* were carefully collected and washed with distilled water. Bark of *E. rigida* was peeled off using a blade and branches of *E. obtusifolia* were scraped to remove bark. Roots were washed with distilled water to remove soil until there were only roots and root hairs remaining. The different plant parts were placed in the oven to dry at 45^{o}C for 72 h. Once dried, the different plant parts were ground into fine powder using a mill. Twelve grams of leaf, bark, and root ground material was weighed into conical flasks and in each flask, 100 ml of pure methanol was added and thereafter sonicated for 1 hour. Filtrate was filtered through size 1 Whatman filter paper using an industrial vacuum pump into a clean conical flask and left under fume hood and fan to dry. Drying took 5-7 days and once dried, extract was weighed. The procedure was carried with dichloromethane and ethyl-acetate as well.

3.2.2 Microdilution: antibacterial assay

To determine the antibacterial activity exhibited by leaves, bark, and roots of *E. obtusifolia* and *E. rigida*¸ a microdilution assay according to **Eloff** (**1998)** with modification by **Ndhlala et al** (**2013**) was used. The assay was conducted to determine the minimum inhibitory concentration (MIC) of methanol (MeOH), dichloromethane (DCM), and ethyl-acetate (EA) crude extracts against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Micrococcus luteus* (ATCC 4698), and *Klebsiella pneumoniae* (ATCC 13883) bacterial cultures. Cultures were prepared overnight in McCartney bottles, and the absorbance readings were obtained at 600 nm and readings had to be between 1.6 -1.8 absorbance, thereafter, diluted with MH Broth at 1:100 (200µl bacteria in 20ml MH Broth). If absorbance was not between 1.6-1.8 the quantity of bacteria in the broth was adjusted according to ($x = \frac{200 \times Abs}{1.8}$).

Dried and weighed extratcs of methanol, bark, and roots of *E. rigida* and *E. obtusifolia* were dissolved with 50% methanol to a concentration of 25 mg/ml. One hundred µl of sterile water was added into each well of a 96 well microplate, followed by 100 µl of plant extracts to mix in well A. One hundred µl of mixture in well A was removed and added in well B and was

subsequently two-fold serially diluted until well H, and 100 µl of mixture from well H was discarded. Neomycin standard was prepared in an Eppendorf tube (48 0µl water and 20µl of neomycin to make concentration of 10 mg/ml) and was used as a positive control. One hundred µl of diluted bacterial culture was added in every well and the plates were covered with parafilm and incubated at 37^{o}C overnight.

After 24 hours of incubation, an indicator solution called INT (2-(4-idophenyl)-3-(4-nitrophenyl)-5-phenytetrazolium chloride) was prepared at 0.2 mg/ml and 50 µl was added into every well. The microplate was incubated at 37°C for 24 hr. Wells where there was no bacterial inhibition appeared red or pink and those that indicated inhibition did not change colour.

3.2.3 Microdilution antifungal assay

Minimum inhibitory concentration of *Candida albicans* (ATCC 10231) was determined with leaf, bark, and root methanol (MeOH), dichloromethane (DCM) and ethyl-acetate (EA) crude extracts. Extraction procedure the same as described in 3.2.1. The experiment was conducted as described by **Eloff** (**1998)** and modified by **Ndhlala et al (2013)**. Extracts were resuspended to 25 mg/ml with 50% methanol. Five ml of fungal culture were prepared over-night in McCartney bottles. Four hundred µl of fungal culture was diluted with sterile saline water (0.85%) and the absorbance reading was taken at 530 nm. Stock of diluted fungal culture was prepared at 1:1000 with Yeast Malt (YM) broth (20 µl of diluted fungal culture in 20 ml of YM broth).

In a 96 well microplate, 100 µl of sterile water was added in each of the wells using a multichannel micropipette, followed by 100 µl of extracts in well A, and Amphotericin B standard solution was used as a positive control which was prepared at 0.25 mg/ml and added in well A. One hundred µl of the mixture in well A was two-fold serially diluted until well H. In the final well (well H), 100 µl was discarded. One hundred µl of fungal dilution was added in every well and plates were covered with parafilm and incubated at 37^{o}C overnight.

After 24 hours of incubation, an indicator solution 2-(4-idophenyl)-3-(4-nitrophenyl)-5-phenytetrazolium chloride (INT) was prepared at 0.2 mg/ml and 50 µl was added to every well. The microplate was incubated at 37°C for 48 hr. Wells where there no fungal inhibition bacteria appeared red or pink and those which indicated activity, did not change colour

3.3 Results

Table 3.3.1: MIC of *Ehretia rigida* leaf, bark and root extracts suspended at 25 mg/ml

Plant part	Solvent	Bacterial strains			
		K. pneumoniae	*S. aureus*	*E. coli*	*M. luteus*
Leaves	Methanol	1.56	1.56	1.56	3.123
	DCM	**0.78***	3.12	1.56	1.56
	Ethyl acetate	1.56	**0.39***	1.56	**0.78***
Bark	Methanol	**0.78***	3.12	1.56	**0.39***
	DCM	**0.78***	1.56	6.25	**0.78***
	Ethyl acetate	>6.25	>6.25	>6.25	>6.25
Roots	Methanol	**0.39***	>6.25	1.52	**0.39***
	DCM	>6.25	>6.25	>6.25	>6.25
	Ethyl acetate	**0.78***	1.56	**0.39***	**0.195***
Control	Neomycin	0.025	0.0125	0.049	0.049

*MIC values below 1 mg/ml are considered to have good activity

Samples which showed good antibacterial activity in *E. obtusifolia* were from methanol leaf extracts against *K. pneumoniae* with a MIC of 0.195 mg/ml, and *S. aureus* and *M. luteus* with a 0.78 mg/ml respectively. Dichloromethane (DCM) leaf extracts were active against *M. luteus* and *K. pneumoniae* exhibiting a MIC of 0.78 mg/. MIC of 1.56 mg/ml was exhibited by methanol leaf extracts against *E. coli* which is considered good activity against *E. coli* because it's one of the most common infectious bacteria (Table 2.2). In *E. rigida* the lowest MIC was 0.195 mg/ml from ethyl-acetate root extracts against *M luteus.* Ethyl-aetate root extracts were also active against *E. coli* with a MIC of 0.39 mg/ml and a MIC of 0.78 mg/ml against *K. pneumoni*ae. Methanol root extracts had a MIC of 0.39 mg/ml against *K. pneumoniae* and *M. luteus. E. rigida* bark methanol and dichloromethane extracts were active against *M. luteus* and achieved a MIC of 0.39 mg/ml and 0.78 mg/ml respectively. The bark extracts were also equally active against *K. pneumoniae* with MIC of 0.78 mg/ml. *E. rigida* leaf ethyl-acetate leaf

extracts had MIC of 0.39 mg/ml against *S. aureus* and 0.78 mg.ml against *M. luteus.* DCM leaf extracts had a MIC of 0.78 mg/ml against *K. pneumoniae.*

Other *E. rigida* extracts with considerable activity were from methanol leaf extracts against *K. pneumoniae, S. aureus* and *E. coli,* along with ethyl-acetate leaf extracts against *K. pneumoniae* and *E. coli*, methanol bark extracts *E. coli* and dichloromethane bark extracts against *S. aureus* all extracts had MIC of 1.56 mg/ml (Table 2.1). Neomycin exhibited the lowest MIC of 0.0125 mg/ml against *S. aureus and* 0.025 mg/ml against *K. pneumoniae*, while *M. luteus* and *E. coli* were susceptible to 0.049 mg/ml of neomycin.

Good antifungal activity was observed from *E. obtusifolia* methanol and ethyl-acetate leaf extracts that had a MIC of 0.78 mg/ml. *E. obtusifolia* DCM bark extracts had antifungal activity of 1.56 mg/ml. *E. rigida* samples with good activity were exhibited by methanol leaf and root extracts with a MIC of 0.78 mg/ml. *E. rigida* methanol bark along with DCM and ethyl-acetate root extracts had MIC of 1.56 mg/ml. Amphotericin B had a MIC of 0.0097 mg/ml and a MFC 0.0781 mg/ml (Table 3.3).

Table 3.3.2: MIC of *Ehretia obtusifolia* leaf, bark and roots extracts suspended at 25 mg/ml

Plant part	**Solvent**	**Bacterial strains**			
		K. pneumoniae	*S. aureus*	*E. coli*	*M. luteus*
Leaves	Methanol	**0.195***	**0.78***	1.56	**0.78***
	DCM	**0.78***	1.56	3.125	**0.78***
	Ethyl acetate	>6.25	>6.25	>6.5	6.25
Bark	Methanol	1.56	>6.25	3.125	3.125
	DCM	3.125	>6.25	>6.25	>6.25
	Ethyl acetate	1.56	1.56	>6.25	3.125
Roots	Methanol	1.56	>6.5	>6.25	3.125
	DCM	6.25	>6.25	>6.5	6.25
	Ethyl acetate	6.25	3.125	1.56	1.56
Control	Neomycin	0.025	0.0125	0.049	0.049

Table 3.3.3: MIC and MFC of *Ehretia obtusifolia* and *Ehretia rigida* leaf, bark, and root extracts

Species	Plant part	Solvent	*Candida albicans*	
			MIC	MFC
E. obtusifolia`	**Leaves**	Methanol	**0.78**	3.125
		DCM	3.125	3.125
		Ethyl acetate	**0.78**	1.56
	Bark	Methanol	6.25	6.25
		DCM	1.56	3.125
		Ethyl acetate	>6.25	
	Roots	Methanol	6.25	
		DCM	>6.25	
		Ethyl acetate	3.125	6.25
E. rigida	**Leaves**	Methanol	**0.78**	3.125
		DCM	3.125	3.125
		Ethyl acetate	3.125	6.25
	Bark	Methanol	1.56	1.56
		DCM	6.25	6.25
		Ethyl acetate	>6.25	
	Roots	Methanol	**0.78**	1.56
		DCM	1.56	1.56
		Ethyl acetate	1.56	1.56
	Control	Amphoterecin B	0.0097	0.0781

*MIC values below 1 mg/ml are considered to have good activity

3.4 Discussion

Naturally occurring substances found in plants and animals have been a source of medicine since humans began, with the plant kingdom being the most beneficial in treating multiple ailments (**Heyman et al., 2009**). Over time, scientists have performed experiments that have distinguished which plants have medicinal properties, which are toxic, and which of those are inactive. Through trial and error, scientists have been able to apply medicinally beneficial plants to treat diseases and the applied biotechnology process has evolved over the past century, such that over 20 000 medicinal plants have been screened and tested for medicinal properties (**Ynalvez et al., 2018)**. Drug resistant pathogen infections are estimated to cause 10 million annual deaths by 2050. These include the contribution of antibiotic resistance in addition of failure of anti-malarial drugs and antiviral therapy (**Ynalvez et al., 2018**).

Antimicrobial activity was determined in *E. obtusifolia* and *E. rigida* extracts, and the results showed that more of *E. rigida* samples were active achieving a low MIC (<1 mg/ml) than *E. obtusifolia* extracts. Not only did *E. rigida* have more extracts activity lower than 1 mg/ml, but it came from different plant parts and inhibited more than two of the bacterial strains. This speaks well of *E. rigida's* diversity and wide range capacity in antibacterial activity**.** Bark and root organs of *E. rigida* exhibited the best activity (<1 mg/ml) respectively with *K. pneumonia*, *E. coli*, and *M. luteus* being the most susceptible bacteria (Table 2.1). Differences in sensitivity to the extracts from Gram-positive (*M. luteus* and *S. aureus*) and Gram-negative (*E. coli* and *K. pneumoniae*) bacteria was likely due to the microorganisms' morphological difference in the membranes. Since the outer membrane of the cell wall of the Gram-negative bacteria acts as a barrier to many substances including antibiotics, therefore it could have prevented any natural substances in the extracts from passing through (**Alrumman et al., 2012**).

According to **Sarkodie et al** (**2015**), *E. cymose* is used by poultry and guinea fowl farmers to treat birds suffering from diarrhoea or loss of appetite. They further conducted an antimicrobial study on *E. cymose* leaf extracts that showed that *Pseudomonas aeruginosa*, *E. coli, Bacillus subtilis*, and *S. aureus* were inhibited by leaf extracts, and *E. coli* was the most susceptible (MIC of 0.0108 ug/ml) and *P. aeruginosa* (MIC of 0.1744 ug/ml) being the least susceptible. *E. coli* is the most common bacteria responsible for causing diarrhoea in animals and humans, hence any plant extract inhibitory activity against *E. coli* may serve as an alternative to some of the already marketed antibiotics.

In this study carried out there was also a notable difference in activity between extracts from different solvents, where all three solvents used produced extracts with good activity, particularly ethyl-acetate and methanol. This suggested that ethyl-acetate and methanol were the most suitable solvents to extract the ideal active compounds. Ethyl-acetate was the most suitable solvent for extracts of *E. rigida,* and methanol was the best solvent for extracts of *E. obtusifolia.* The yield of a plants extract is dependent on several things, namely the solvent used, extraction method, and the conditions of the extraction (**Razali at al., 2012; Muhamad et al., 2014**). Successful extraction of bioactive compounds (phytoconstituents) is largely dependent on the solvent used declares **Muhamad et al (2014)**. During the process of extraction, the solvent diffuses into the solid material and solubilises compounds with similar polarity. It has been recorded that different extracts from the same plant material may have different results in respect to antioxidant, total phenolic and flavonoid content, and other biological activities **(Alrumman et al., 2012; Razali at al., 2012; Muhamad et al., 2014)**.

A study by **Joshi et al (2020)** showed that most pure solvents had a weak extraction power. Pure solvents in this study such as DCM, with low relative polarity compared to ethyl acetate and aqueous methanol resulted in less activity because of less compounds with the same polarity (Table 3.3.1 – 3.3.3). The results of **Muhamad et al (2014**) suggested that aqueous solvents had better extraction power than pure solvents, since mixing non-poplar solvents with water increased the polarity index and therefore increased the extraction efficiency. This was the case found in this study, where 80% aqueous methanol resulted in increased activity than pure solvents, suggesting higher extraction power.

Individuals with *Candida* fungal infection are diagnosed by the presence of creamy-white card like particles on oral mucosal surfaces or on their tongues and these particles are removed by scrapping. If the particles are left untreated, they may lead to difficulty swallowing and or diarrhoea, which subsequently causes weight loss in the individual (**Motsei et al., 2003**). An antifungal assay was conducted to observe antifungal activity in *E. obtusifolia* and *E. rigida,* and both species achieved the same MIC suggesting they had an equal inhibitory effect (Table 3.3.3). Methanol leaf extracts produced the lowest MIC in both species, while ethyl-acetate leaf extracts of *E. obtusifolia* also achieved the same MIC. This suggested that both methanol and ethyl-acetate are suitable solvents for extracting antifungal properties in leaves of *E. obtusifolia. E. rigida* showed to be the most suitable species in terms of plant organs with

antifungal activity (leaves and roots), while in *E. obtusifolia* it was only leaves**.** It was also observed in the antifungal assay that methanol and ethyl acetate extracts were more active than DCM, with the root and bark extracted with said solvents being active for *E. rigida* samples and the leaf extracts being the most active for *E. obtusifolia.* With respect to antifungal activity, *E. rigida* was the more active.

A study conducted in South Africa revealed that *Candida* infection is alarming in patients with HIV-AIDS, such that even with treatment being provided, patients continue to come back with recurring symptoms after 2-3 weeks (**Motsei et al., 2003**). *Candida albicans* occupies the mucosal membranes of most mammals and bird species. The disease is also associated with hormonal deficiencies. The condition is frequently diagnosed in infants, diabetics, patients on antibiotics, catheters and steroids, and mammary glands of lactating cows (**Ynalvez et al., 2018**). *Candiasis* is prevalent and considering HIV-AIDS being highest in South Africa, finding alternative treatment to curve its infection rate and *E. rigida* and *E. obtusifolia* phytoconstituents can assist.

Plant compounds responsible for antimicrobial activity are found in minute amounts (**Pretorius et al., 2003**). In this study the concentration of the extracts resuspended was 25 mg/ml and for neomycin and Amphotericin B controls it was 10 mg/ml and 0.25 mg/ml respectively. This made the extract concentration significantly higher than the controls and it was predicted that there would be activity. There was significant antibacterial activity but for antifungal assay, the activity was not as significant.

Studies have indicated that a lack of antifungal activity from crude extracts may be due to the absence of biologically active antifungal compounds i.e., specific compounds which inhibit mycelial growth of fungi (**Qasem & Abu-Blan., 1995)**. Extracts were resuspended in 50 % methanol and if there are any stimulating growth factors for the growth of fungi, that negates the effects of possible inhibitory substances that could been present suggests **Qasem & Abu-Blan (1995)**. However, growth promoting substances are concentration specific and would need to be in high concentrations to implicate the absence of active compounds, hence identifying the compound responsible will result in precise inhibitory activity.

C. albicans was the only fungus tested, to get further conclusive results of the antifungal activity exhibited by these two species, a wider range of fungi will need to be tested. *C.*

albicans was chosen for its high incidence which accounts for 70% of fungal infectious diseases. Other fungi that could have been included in the study are *Cryptococcus neoformans* which are just as problematic throughout the world because they cause fatal cryptococcal meningitis and cryptococcal pneumonia in patients with AIDS (**Svetaz et al., 2010**).

3.5 Conclusion

Pretorius et al (2003) tested multiple plant species for antibacterial activity and found similar results to this study, that plant pathogenic fungi are more resistant to natural plant extracts that pathogenic bacteria, and a lower MIC was observed for antibacterial activity than antifungal activity. In another study conducted to screen 60 crude extracts, found 21 % of extracts inhibited growth of bacteria and 8.33 % inhibited fungal growth, which suggested that when it comes to antimicrobial activity, plants primarily exhibit antibacterial activity than antifungal activity (**Heisey & Gorman., 1992**).

Chapter 4: Antioxidant and antidiabetic activity of *Ehretia* species in South Africa (*E.rigida and E. obtusifolia)*

4.1 Introduction

Several physiological and biochemical processes in the human body produce free radicals and reactive oxygen species (ROS) as by-products (**Cai et al., 2004; Sharma et al., 2012**). An overproduction of free radicals causes oxidative damage to biomolecules such as lipids, proteins, and DNA, which ultimately leads to chronic diseases in humans such as diabetes, atherosclerosis, ischemia, arthritis, cancer, AIDS, and reperfusion injury in many tissues (**Cai et al., 2004; Pourmorad et al., 2006**). Environmental pollutants (methane, lead, particulate matter, sulphur-dioxide, radiation, chemicals, toxins, deep fried and spicy foods, and physical stress, result in free radicals that cause a depletion of the immune systems antioxidants. This causes a change in gene expression that brings about abnormal proteins (**Hajhashemi et al., 2010**). Antioxidants such as vitamin C or E, and glutathione to name a few; can remove potentially damaging free radical in the living system and prevent these inevitable oxidation processes (**Sivasankari et al., 2013**).

The process of oxidation (the gain/addition of an oxygen atom) is the most important pathway for producing free radicals in food, drugs and living organisms (**Hajhashemi et al., 2010).** Natural antioxidants in the body such as catalase and hydroperoxides convert hydrogen peroxide and hydroperoxides into non-radical forms, therefore help to prevent an increase of free radicals in the body (**Sharma et al., 2012**). Due to a depletion of naturally occurring antioxidants in the body by different maladies (mitochondrial respiration, peroxisomal metabolism, phagocyte activity and arachidonate pathways), the consumption of antioxidants is one way to increase antioxidants in the immune system become free radical scavengers (**Pourmorad et al., 2006**).

Synthetic antioxidants, for example butylated hydroxy anisole, butylated hydroxy toluene (BHT), tertiary butylated hydroquinon and gallic acid esters have been suspected to have negative health consequences such as affecting the proper functioning of the liver, kidney, and lungs, as well as blood coagulation (**Cai et al., 2004**). Therefore, their use is strictly prohibited and have rather been substituted with naturally occurring antioxidants such as those found in plants. A variety of plants (fruits, vegetables, medicinal herbs etc) contain free radical scavenging molecules such phenolic compounds, nitrogen compounds, vitamins and

terpenoids and other secondary metabolites rich in antioxidant activity (**Ogundajo and Ashafa., 2017**). Antioxidant compounds are of great importance in healthcare/medicine because of their potential anti-inflammatory, antiatherosclerosis, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (**Dzoyem and Eloff., 2015; Ogundajo and Ashafa., 2017**). Hence an intake of antioxidants is associated with reduced risk of cancer, cardiovascular diseases, diabetes, and other diseases associated with aging (**Cai et al., 2004**).

4.1.1 ROS production and oxidative stress

ROS are derived from oxygen, and an estimated 1 % of oxygen in plants is used to produce ROS at different production sites such as chloroplast, mitochondria, and peroxisomes (**Hajhashemi et al., 2010; Sharma et al., 2012**). ROS have been recognised for playing dual roles in plants, depending on their concentration. At high concentrations ROS are harmful - causing peroxidation of lipids, protein oxidation, nucleic cell damage, enzyme inhibition, and activation of programmed cell death. The accumulation of damage caused by free radicals is thought to be one of the causes of aging. At low concentration they act as secondary messengers in intracellular signalling cascades that mediate responses in plant cells under environmental stress (drought, salinity, heat) according to **Sharma et al (2012).** Every living system must protect itself from excess ROS and is damages, and this defence mechanism is known as passive ROS function (**Li et al., 2010; Hasanuzzamah et al., 2013; Sivasankari et al., 2013**).

4.1.2 Defence and elimination of ROS

There are number of ways that antioxidants exert defence mechanisms, among them 1) Enzymatic systems which catalyse the removal of free radicals and ROS. 2) Sacrificial antioxidants which donate oxygen to free radicals. 3) Proteins that minimise the availability of prooxidants. 4) Proteins that protect molecules by other mechanisms e.g heat shock proteins. In the enzymatic system, superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) from ascorbate glutathione cycle, and glutathione reductase (GR) are responsible for removing or breaking down ROS. Ascorbate, carotenoids, tocopherols and phenolics serve as potent non-enzymatic antioxidants in a cell.

4.1.3 Antidiabetic

Inhibiting α-amylase and α-glucosidase enzymes which are involved in the break down glucose and starch in the body, is one of the ways of controlling the postprandial glycaemic reaction (**Adisakwattana et al., 2010**). Currently, enzyme inhibitors include acarbose, miglitol and voglibose (**Tundis et al., 2010**). However, though affective, these antiglycaemic agents have side effects which include swelling, abdominal discomfort, diarrhoea, and flatulence (**Ogundajo and Ashafa., 2017**). It is therefore vital that new potent glycaemic agents with less side effects are discovered, and studies suggest they can be found from natural sources (**Li et al., 2010**). Plant extracts have been used for ethnomedicinal treatments of diabetes and *Ehretia cymosa* has demonstrated promising antidiabetic effects from leaf extracts that reported to treat measles, pain, epilepsy, convulsions, and spasms. (**Sarkodie et al., 2015; Ogunajo & Ashafa., 2017**).

4.2 Materials and methods

4.2.1 Antidiabetic activity: α - glucosidase assay

A microplate serial dilution assay was conducted according to **Ting et al** (**2005**) to investigate the inhibition of α-glucosidase to determine antidiabetic activity from *E. obtusifolia* and *E. rigida* leaf, bark, and root extracts. Stock solutions of the reagents required for the experiment were prepared. 0.1 M of phosphate buffer was prepared by dissolving 13.124 g of sodium monohydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$) followed by 7.043 g of sodium dihydrogen phosphate monohydrate ($Na_2H_2PO_4H_2O$) in 800 ml of distilled water, once completely dissolved pH was adjusted 6.8 and the solution was made to 1000 ml to make the phosphate buffer. Extracts were dissolved with 100 % dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml. Phosphate buffer (20 µl) was added in every well, followed by 20 µl of extracts in the first wells. A two-fold serial dilution was performed by removing 20 µl in well A until well G, and the final 20 µl of extract from well G was discarded. 20 µl of α-glucosidase enzyme was added in every well, subsequently 40 µl of p-Nitrophenol-α-glucopyronoside (pNPG) substrate. The microplates were placed in an incubator at 37 ^{o}C for 40 min and after incubation, 80 µl of sodium carbonate ($NaCO_3$) was added to terminate the reaction. Absorbance of plate was determined using a Chromate spectrophotometer at wavelength of 405 nm.

$$\%\ \alpha\ glucosidase\ activity = \frac{corrected\ Ab\ of\ test}{Ab\ of\ control}\ X\ 100$$

4.2.2 (DPPH) 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity assay

E. rigida and *E. obtusifolia* methanol, ethyl-acetate, and dichloromethane extracts were investigated, or antioxidant activity based on the scavenging activity of the stable DPPH free radical, with ascorbic acid used as the standard. The method was adopted from **Moyo et al (2010)** and was modified. The $DPPH^-$ solution was prepared freshly before the assay. The assay was performed in triplicates as follows; dried extracts of *E. obtusifolia* and *E. rigida* bark, leaves and roots were re-dissolved in 50% aqueous methanol to 1 mg/ml. 10–100 µl of each plant extract was diluted with methanol to make up a volume of 750 µl. For activity at a concentration of 30 ug/ml, 30 µl of sample + 720 µl of 50 % MeOH were diluted in test-tube and subsequently, 750 µl of methanolic DPPH solution (750 µl, 0.1 mM) was added. The final concentration of $DPPH^-$ in the final reaction was 50 µM (**Sharma and Bhat., 2009**). The reaction mixtures were prepared under dim light in test tubes, shaken well, and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was recorded at 517 nm against a blank. The percentage (%) inhibition of free radical species into the presence of plant extracts and synthetic antioxidants was calculated as:

$\%RSA = [1 - (Abs_{extract} - Abs_{background}/Abs_{control})]\ X\ 100\ \%$

4.3 Results

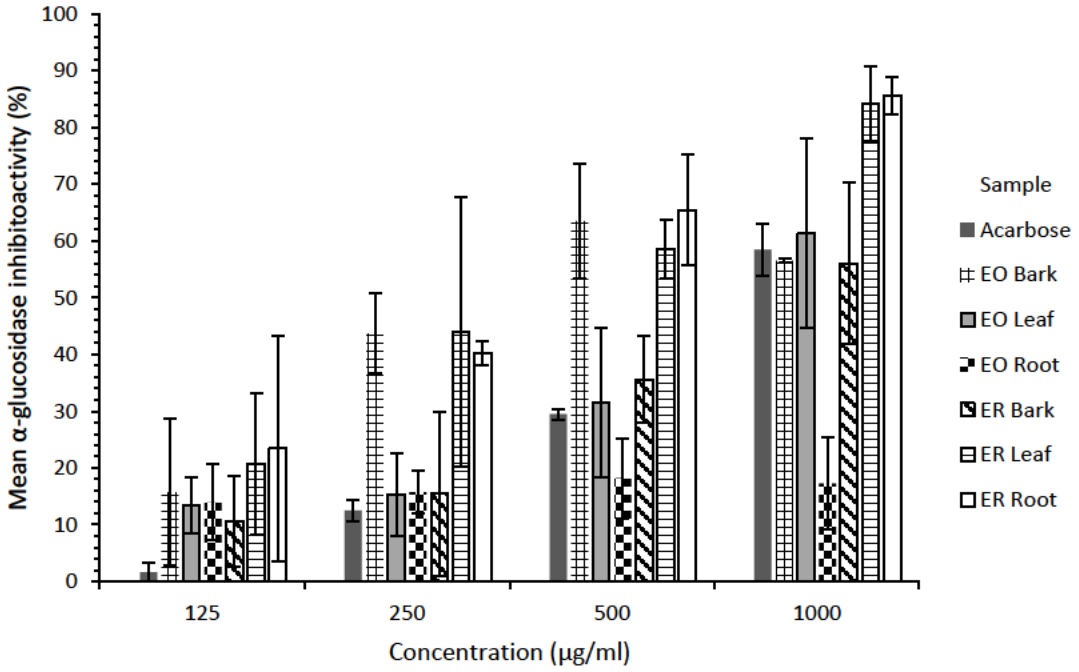

Figure 4.3.1: α-glucosidase inhibition of *E. obtusifolia* and *E. rigida* leaf, bark, and root methanol extracts at different concentrations

Results observed at Figure 4.3.1 are described below. *E. rigida* root, *E. rigida* leaves, and *E. obtusifolia* bark reported the highest α-glucosidase inhibitory activities of 23±20, 21±13, and 16±12 % respectively at 125 µg/ml and there was no significant difference in activity between leaf, bark, and root extracts from both species ($F_{6,14}$ = 1.198; p = 0.366). At 250 µg/ml, 44±27, 44±7 and 40±2 % were the highest recorded α-glucosidase inhibitory activities by *E. rigida* leaf, *E. obtusifolia* bark and *E. rigida* root extracts respectively ($F_{2,6}$ = 5.77; p = 0.005). 65±9, 63±10 and 59±6 % were the high α-glucosidase inhibitory activities reported by *E. rigida* root, *E. obtusifolia* bark, and *E. rigida* leaf extracts respectively at 500 µg/ml (*H* = 10.892; df = 6; p = 0.092). At 1000 µg/ml *E. obtusifolia* leaf extract recorded 61±16 % activity, while *E. rigida leaf* and root MeOH extracts recoded the highest α-glucosidase inhibitory activity of 84±6 and 86 ±4 % respectively (*H* = 15965; df = 6; p = 0.014).

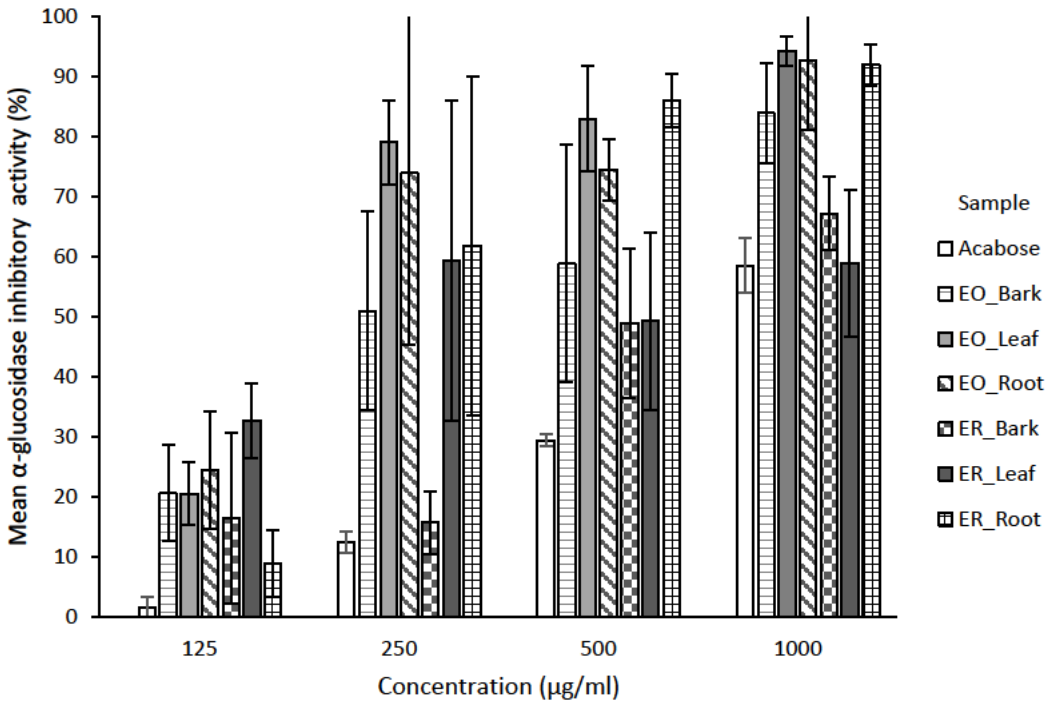

Figure 4.3.2: α-glucosidase inhibitory activity of *E. obtusifolia* and *E. rigida* leaf, bark, and root ethyl-acetate extracts at different concentrations

The results of the α-glucosidase inhibitory activity for ethyl-acetate extracts observed in Figure 4.3.2 are described below. At 125 µg/ml *E. rigida* ethyl-acetate leaf extracts had the highest α-glucosidase inhibitory activity of 32.73±5.73 %, followed by *E. obtusifolia* root and *E. obtusifolia* bark extracts that reported activities of 24.40±10.4 and 20.54±7.54 % ($F_{6,14}$ = 6.32; p = 0.002). *E. obtusifolia* leaf ethyl-acetate extracts reported the highest activity at 250 µg/ml of 79.04±5.15 %, while *E. obtusifolia* roots had an activity of 73.96 ±28.63% and 61.77±27 % was reported by *E. rigida* root extracts (*H* = 14.32; df = 6; p = 0.026). *E. rigida* root extracts had the highest activity of 86.04 ±4.55% at 500 µg/ml and *E. obtusifolia* leaf extracts reported the second highest activity of 82.93±2.93 %. *E. obtusfolia* root extracts had activity 74.49 ± 5.51 % and 58.82±19.77 % from *E. obtusifolia* bark extracts were recorded. According to the Kruskal Wallis H test, α - glucosidase inhibitory activity was significantly different between the two species (*H* = 17.126, df = 6; p = 0.009). At 1000 µg/ml, *E. obtusifolia* ethyl-acetate leaf extracts and root extracts reported 94.32±15 and 92.61±11.51 activity. *E. rigida* root extracts recorded activity of 92±3.46% and *E. obtusifolia* bark extracts had 83.90±25 % activity (*H* = 16.517; df = 6; p = 0.011).

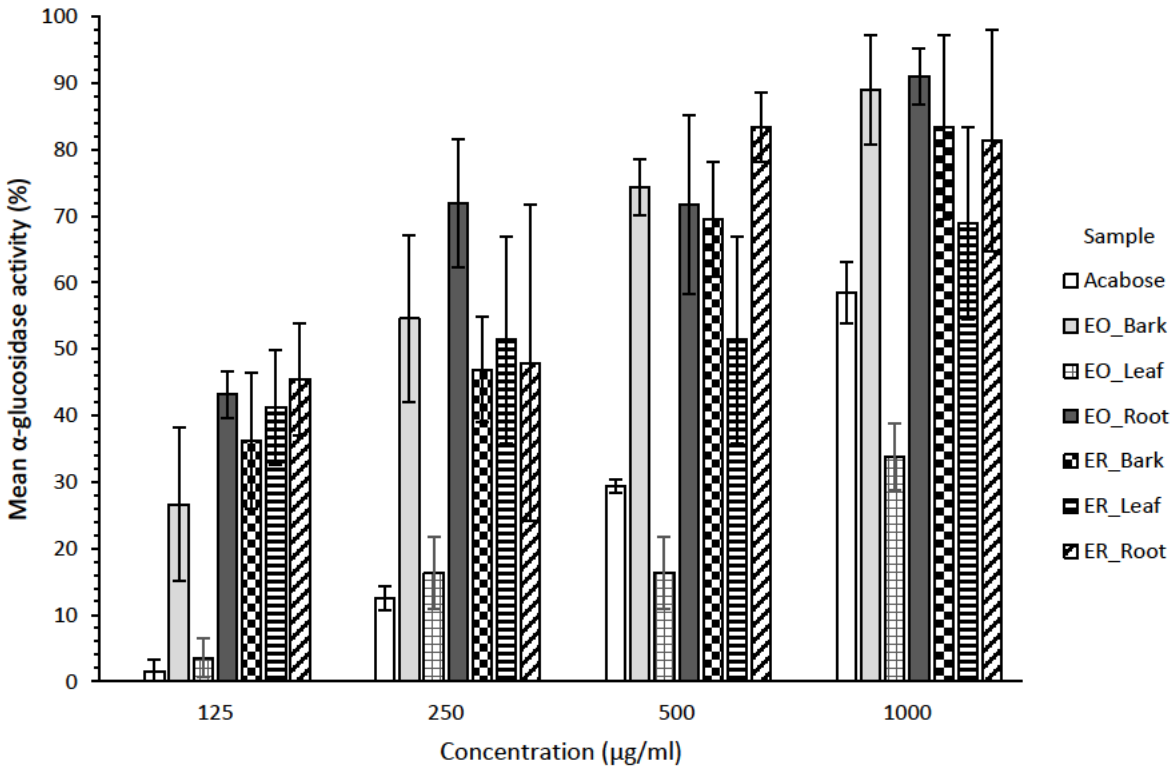

Figure 4.3.3: α-glucosidase activity of *E. obtusifolia* and *E. rigida* leaf, bark, and root dichloromethane extracts at different concentrations

The α - glucosidase inhibitory activity at 125 µg/ml was determined, and *E. rigida* dichloromethane root extracts had the highest activity of 45±8 % followed by *E. obtusifolia* root extracts with 43±4 %. *E. rigida* leaf and bark extracts had activity of 41±7 and 36±9 % and there was a significant difference in activity between *E. rigida* and *E. obtusifolia* extracts ($F_{6,13}$ = 19.93; p = 0.00). At 250 µg/ml, 72±10 % was the highest activity achieved by *E. obtusifolia* dichloromethane root extracts. *E. obtusifolia* had the highest inhibitory activity from bark extracts of 55±13 % and *E. rigida* leaf extracts had 51±13 % activity. According to the Kruskal Wallis H test *E. obtusifolia* and *E. rigida* extracts were significantly different (*H* = 14.32; df = 6; p = 0.026). At a concentration of 500 µg/ml, 83±5 % was the highest activity by *E. rigida* dichloromethane root extracts. *E. obtusifolia* dichloromethane bark extracts achieved 74±4 %, while *E. obtusifolia* root extracts had 72±13 %, and *E. rigida* bark and leaf extracts had 69±10 and 51±15 % respectively (*H* = 17.697; df = 6; p = 0.007). At 1000 µg/ml 91±2.5 % was the highest activity achieved by *E. obtusifolia* root extracts, followed by 89±8 % by *E. obtusifolia* bark extracts. 83±5 % activity was exhibited by *E. rigida* bark extracts and 81±10

% by *E. rigida* root extracts. In addition, *E. rigida* leaf extracts had an activity of 69±13 % ($F_{6,14}$ = 14.4; p = 0.000).

The following result observed in Figure 4.3.4 - 4.3.6 depict antioxidant activity via DPPH radical scavenging activity

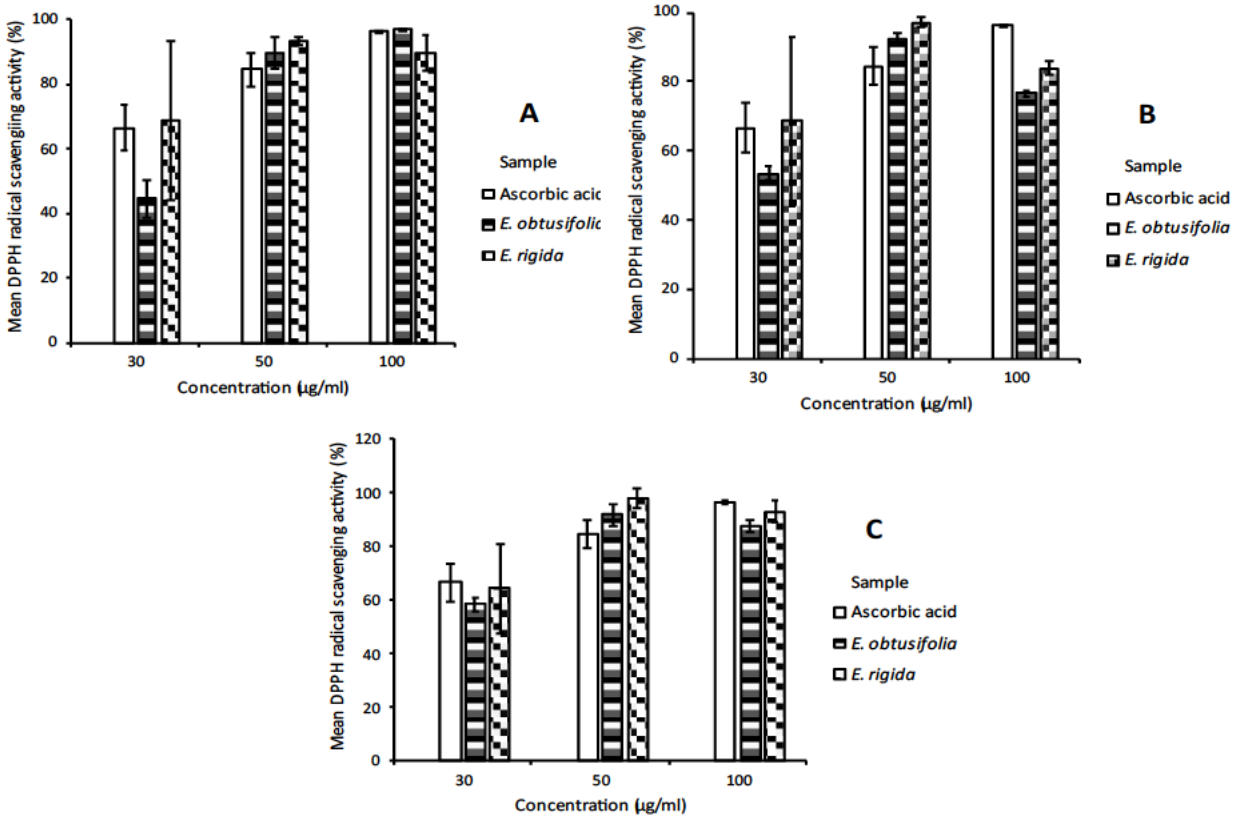

Figure 4.3.4: Antioxidant activity using the DPPH assay in ethyl-acetate bark (A), leaf (B), and root (C) extracts of *E. rigida* and *E. obtusifolia*.

Compared with 100 µg/ml of ascorbic acid, *E. rigida* and *E. obtusifolia* ethyl-acetate bark extracts were statistically significantly different according to the Kruskal-Wallis H test (H = 7.2, df = 2, p = 0.027), but ascorbic acid and *E. obtusifolia* were not significantly different and the highest activity recorded 97±0.5 % by *E. obtusifolia* ethyl-acetate bark extract (A). There was no significant difference in activity of *E. obtusifolia* and *E. rigida* bark extracts compared to ascorbic acid at 50 µg/ml ($F_{(2;6)}$ = 4.67; p = 0.06), and *E rigida* reported high activity of activity of 93±1 %. There was no significant difference in activity of *E. obtusifolia* and *E. rigida* ethyl acetate bark extracts compared with ascorbic acid at 30 µg/ml (*H* = 5.6, df = 2, p = 0.06), and the highest activity was 69±25 % by *E. rigida*. At a low concentration of 30 µg/ml, *E. rigida* had

the highest DPPH activity of 69±27 % in leaf extracts (B), and there was no significant difference in activity (*H* = 4.62, df = 2, p = 0.099). *E. rigida* ethyl-acetate leaf extract (B) had the highest activity at 50 µg/ml of 97±1 % and there was a significant difference in activity (*H* = 7.2, df = 2 p = 0.027). At 100 ug/ml there was a significant difference in DPPH activity of *E. obtusifolia* and *E. rigida* ethyl-acetate leaf extracts compared to ascorbic acid ($F_{2,6}$ = 15.04, p = 0.005), and *E. rigida* had the highest activity of 84±2.5 % while *E. obtusifolia* achieved activity of 77±0.3 %(B). *E. rigida* had the highest activity compared to *E. obtusifolia* at all concentration for ethyl-acetate root extracts (C), reporting 64±16 % at 30 µg/ml; 98±4 at 50 µg/ml; and 93±4 % at 100 µg/ml. At 100 µg/ml there was a significant difference in activity between ethyl-acetate root extracts of *E. rigida* and *E. obtusifolia* when compared to ascorbic acid (*H* = 6.49, df = 2, p = 0.03). At 50 µg/ml there was a significant difference ($F_{2,6}$ = 8.55, p = 0.017) and at 30 µg/ml there was no significant difference in activity between root ethyl-acetate root extracts of *E. rigida, E. obtusifolia* with ascorbic acid (*H* = 1.8, df = 2, p = 0.393).

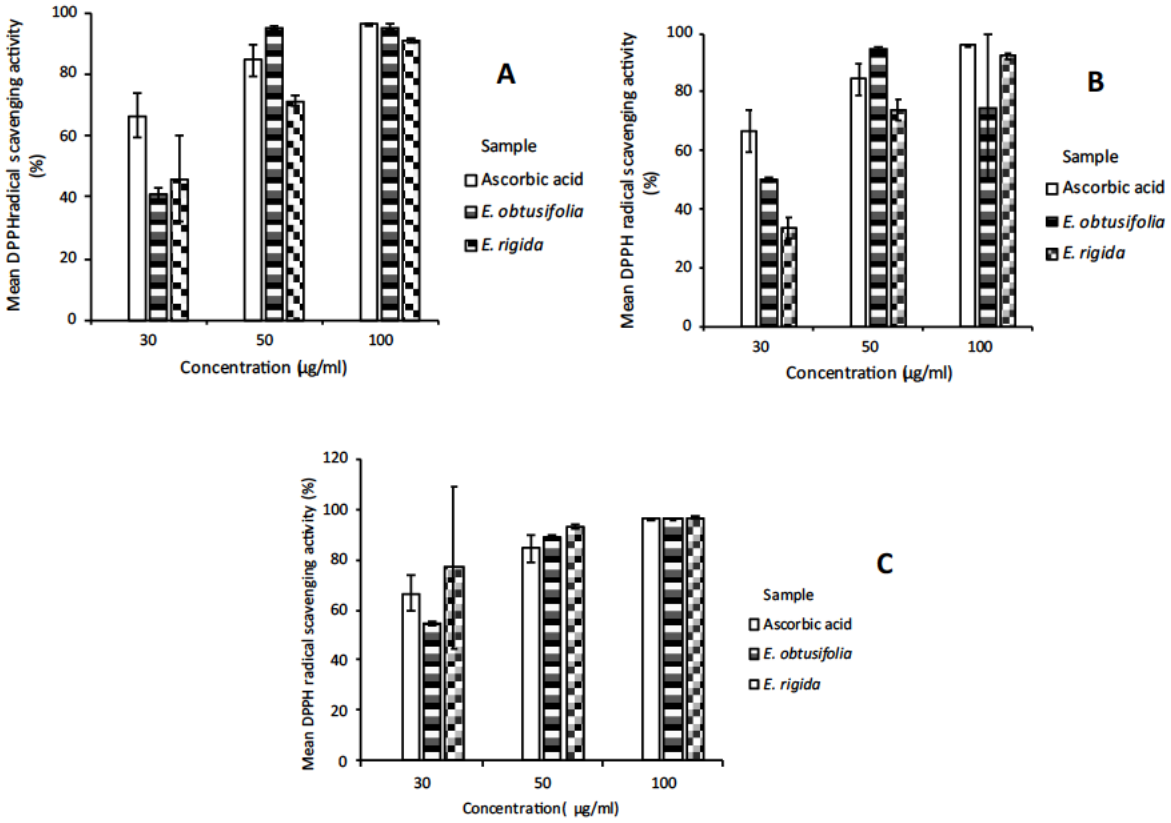

Figure 4.3.5: Antioxidant determination by DPPH assay in methanol bark (A), leaf (B), and root (C) extracts from *E. rigida* and *E. obtusifolia*.

E. obtusifolia methanol bark extracts reported the highest activity of 95±1% at 100 µg/ml and there was a significant difference in DPPH activity between the bark extracts of *E. obtusifolia* and *E. rigida,* and ascorbic acid ($F_{1,2}$ = 71.63, p = 0.000). At 50 µg/ml *E. obtusifolia* methanol bark extracts had activity of 95±1 % which was higher than that of *E. rigida* and there was a significant difference in DPPH activity between the species and ascorbic acid ($F_{2,6}$ = 54.16; p = 0.000). At 30 µg/ml, *E. rigida* had a higher DPPH activity of 46±14 % than *E. obtusifolia,* and there was a significantly different ($F_{2,6}$ = 8.99; p = 0.016). At 100 µg/ml *E. rigida* methanol leaf extracts reported higher DPPH activity than *E. obtusifolia* of 92±1 % (B), and Kruskal Wallis H test showed there was no significant difference in activity between *E. rigida, E. obtusifolia* and ascorbic acid (*H* = 2.4, df = 2, p = 0.301). At 50 µg/ml *E. obtusifolia* methanol leaf extracts had the highest activity of 95±1 % compared to *E. rigida* and there was a significant difference in DPPH activity between the species and ascorbic acid ($F_{2,6}$ = 30.23; p = 0.000). *E. obtusifolia*

had higher activity than *E. rigida* from leaf extracts of 50±0.5 % at 30 µg/ml and there was a significant difference $F_{2,6}$ = 51.26; p = 0.000 (B). *E. rigida* methanol root extracts at 30 µg/ml had high activity of 77±32 % (C) and there was a significant difference ($F_{2,6}$ = 19.95; p = 0.002) and at 50 µg/ml the activity reported was 93±1 % for *E. rigida* and 89±0.8 % for *E. obtusifolia* ($F_{2,6}$ = 54.16; p = 0.000). At 100 µg/ml *E. rigida* had high activity of 97±1 % which was marginally higher than that of *E. obtusifolia* and ascorbic acid which both had 96±1% activity (*H* = 7.2; df = 2; p = 0.027).

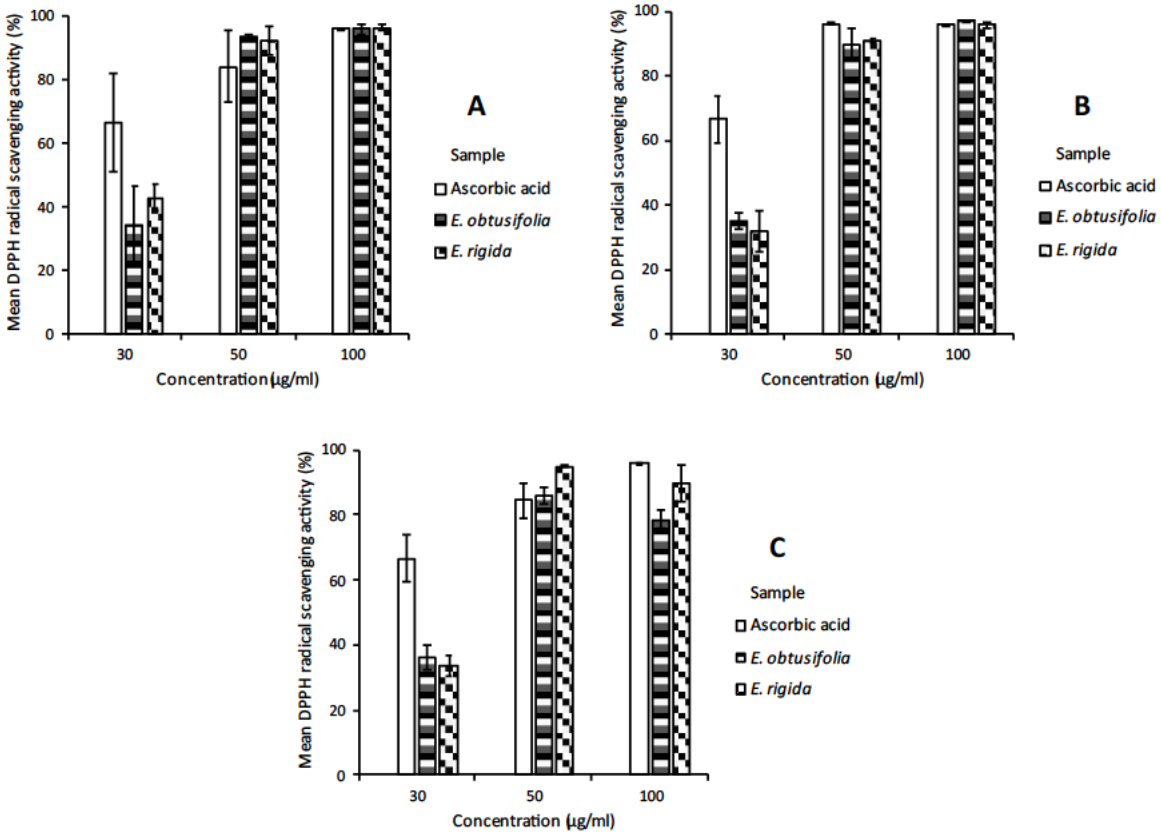

Figure 4.3.6: Antioxidant activity using the DPPH assay in bark (A), leaf (B), and root (C) dichloromethane extracts of *E. rigida* and *E. obtusifolia*.

According to Figure 4.3.6 *E. rigida* dichloromethane bark extracts (A) had activity of 43±4 % at 30 µg/ml, and there was a significant difference in activity between the species and ascorbic acid ($F_{2,6}$ = 37.66; p = 0.000). At 50 µg/ml *E. obtusifolia* was marginally higher with activity of 94±1% ($F_{2,6}$ = 9.08; p = 0.013). 96±1 % activity was recorded in *E. obtusifolia* and 97±1 % activity for *E. rigida* at 100 µg/ml for dichloromethane bark ($F_{2,6}$ = 0.73; p = 0.519). *E.*

obtusifolia had marginally higher activity than *E. rigida* of 98±1 % for DCM leaf extracts (B) at 100 µg/ml (*H* = 5.6, df = 2; 0.061). *E. obtusifolia* dichloromethane leaf extracts had activity of 35±2 % at 30 µg/ml, the activity was marginally higher than of *E. rigida* and there was no significant difference in activity (*H* = 5.9; df = 2; p = 0.081). *E. rigida* leaf DCM extracts were fractionally higher at 50 µg/ml with activity of 91±0.5 % and there was no significant difference (*H* = 5.42; df = 2; p = 0.06) (B). In root DCM extracts, *E. rigida* had higher activity at 100 µg/ml and the DPPH activity between ascorbic acid, *E. obtusifolia* and *E. rigida* was significantly different (H = 7.2; df = 2; p = 0.027). At 50 µg/ml antioxidant activity of 85±6 and 95±1 % was found in *E. obtusifolia* and *E. rigida* respectively and there was a significant difference in activity between the species and ascorbic acid ($F_{2,6}$ = 10.88; p = 0.010). Dichloromethane root extracts had activity of 36±3 and 33±2.8 for *E. obtusifolia* and *E. rigida* respectively, and a Kruskal Wallis H test showed there was no significant difference between the two species and ascorbic acid (*H* = 56, df = 2; p= 0.061).

4.4 Discussion

The overall observation in radical scavenging activity of DPPH between *E. rigida* and *E. obtusifolia* was that both species had good to excellent antioxidant activity. Studies on antioxidant activity in *E. obtusifolia* organs have been reported, however only a single case presented by **Dzoyem and Eloff** (**2015**) has reported antioxidant activity in *E. rigida.* Therefore, a comparison of the two South African species will do well to increase information on the genus and other native South Africa species with medicinal properties such as antioxidant activity. *Ehretia* species are used traditionally in Asia countries to treat different illnesses, and in South Africa, *E. rigida* is traditionally known to alleviate pain and inflammation says **Dzoyem and Eloff** (**2015**). However, in Zimbabwe *E. obtusifolia* is used to treat various illnesses such as sore throat, teething pains for infants, menstrual pain, abdominal pain, and infertility in women (**Zara et al., 2012; Sivasankari et al., 2013**).

A comparison of *E. rigida* and *E. obtusifolia* bark, leaf and root methanol extracts showed that *E. obtusifolia* bark extracts had higher antioxidant activities at 50 and 100 µg/ml, however at 30 ug/ml, *E. rigida* was marginally higher. A study by **Dzoyem and Eloff** (**2015**) reported an IC_{50} of 171.75 ±3.46 µg/ml for *E. rigida* methanol leaf extracts. To our knowledge this was the only study conducted that evaluated *E. rigida* in the world. Other plant or tree organs (bark, roots, stems, and branches) had not been investigated, and it's worth noting that from this

study, multiple methanol extracts of *E. rigida* had activities ≥60 % at 50-100 µg/ml (Figure 4.3.5). Fifty ug/ml was the concentration with highest antioxidant for bark and leaf methanol extracts, suggesting that 50 µg/ml was the optimum concentration for excellent radical scavenging activity. Observing methanol root extracts there was marginal difference in activity between the two species, where *E. rigida* had higher activity at 30 ug/ml.

A conducted which investigated antioxidant activity via radical scavenging activity of DPPH molecule was reported by **Legaspi and Bagaoisan (2020)** and determined that *E. microphylla* methanol fruit extracts had favourable antioxidant activity because of the carotenoid and ascorbate content found in fruit. It also suggested high antioxidant enzyme activity from enzymes such as peroxidase, catalase, and superoxide dismutase. A study has found that amongst the plant extracts of *E. laevis,* methanol extracts had the most potent DPPH scavengers and had activity of up to 87.13% at 200 µg/ml (**Joshi and Wagh., 2018b**). While **Sivansankari et al (2013)** found *E. laevis* ethyl-acetate fruit extracts had an IC_{50} of 122.56 µg/ml for radical scavenging of DPPH and a IC_{50} of 342.16 µg/ml for methanol fruit extracts. All these studies were deemed to have good activity and further investigation of the *Ehretia* genus was required. Therefore, this makes the results from this study conducted on *E. obtusifolia* and *E. rigida* important because the South African species had activity better than species mentioned in the above studies.

Ethyl-acetate extracts rom both species had good activities, and nearly with similar/higher activity than ascorbic acid (Figure 4.3.4). The overall results showed that *E. rigida* had higher activity than *E. obtusifolia,* but the difference in activities attributed by leaf and root extracts was not significant between the two species, to separate and conclude which one is more suitable than the other. Ethyl-acetate stem extracts from *E. microphylla* showed maximum DPPH radical scavenging activity along with ethanol stem extracts, and leaf aqueous extracts which showed a dose-dependent activity in scavenging DPPH (**Legaspi and Bagaoisan., 2020**). These findings by **Legaspi and Bagaoisan (2020) were** like the results of *E. obtusfolia* and *E. rigida* of this study (Figure 4.3.4). *E. serrata* ethyl - acetate fractions had the highest antioxidant activities amongst fruits and leaves and its high activity was credited by phenolic content. Ethyl-acetate leaf fraction of *E. serrata* had a EC_{50} of 120.499 µg/ml and was the highest radical scavenging activity. Fruit extracts reported a EC_{50} of 450.213 ug/ml, while methanol and aqueous fruit fractions had 2500 µg/ml EC_{50} which was their lowest in

antioxidant potential (**Zara et al., 2012**). Compared to *E. obtusifolia* and *E. rigida,* both species presented higher and more potent activity.

When investigating antioxidant activity, it is has become common to determine TFC and TPC because various studies have shown that plants rich in phenolics and flavonoid also have excellent antioxidant activity. A study showed that there was a weaker pattern in scavenging capacity for ethyl-acetate compared to methanol. DCM extracts had lower activity than ethyl-acetate and methanol (**Razali at al., 2012**). Pure solvents such as methanol with a higher polarity index of around 5.1-5.2 yield higher TFC, TPC, and antioxidant content. Studies have revealed that since water has a polarity index of 9.0, it can enhance solubility of both methoxylated and hydroxylated compounds, therefore improves overall extraction yield than most solvents **(Razali at al., 2012)**. In this study methanol and ethyl-acetate with higher polarity index than DCM had higher extraction efficiently because of the higher antioxidant activity depicted in Figure 4.3.1 - 4.3.2.

Radical scavenging activity was high for *E. rigida* and *E. obtusifolia* at 50 and 100 µg/ml as was expected for dichloromethane extracts of both species. The decrease in activity at 50 – 30 µg/ml was sharp, and the activity was only slightly different between leaf and bark extracts. At the highest concentration (100 µg/ml), *E. rigida* root extracts had higher activity which was possibly due to the presence of phenolics. Phenolics and flavonoid compounds are said to be responsible for antioxidant activity because of their redox properties that act as reducing agents, hydrogen donors and singlet oxygen quenchers. The phenolic and flavonoid content was investigated in Chapter 2, however not all of antioxidant activity is a result of phenolics as a correlation will not always be found between the two quantitative analyses. Therefore, antioxidant activity may also be a result of a mixture of other phytochemicals or antioxidants (Vitamin C and E, beta-carotene, carotenoids) (**Dzoyem and Eloff., 2015)**.

Dietary sugars such as starch can be broken and degraded into oligosaccharides by α-amylase and then into monosaccharides by α-glucosidase in the small intestine to be absorbed into the blood, and hence increases the blood glucose content. Therefore, inhibition of α-amylase and α-glucosidase decreases blood glucose levels for type 2 diabetic patients (**Deng et al., 2020)**. Treatment of diabetes mellitus (DM) continues to be one of the most challenging chronic diseases, with scientists still searching for the safest and effective medication to

overcome the effects hyperglceamia, hyperinsulinaemia, hyper-lipidaemia, oxidative stress, inflammation, atherosclerosis, and other complications. Vitamin E and betaine are antioxidants which have been shown to have good clinical implications in the reduction of DM severity and the protection of the organs from diabetes mellitus induced damage (**Abimbola et al., 2021**).

There is a relationship between antioxidant and anti-diabetic activity is well established, such that the reactions which cause or lead to oxidative damage, and an increase in free radicals are related or work similarly to the ones which cause an increase in glucose levels (defect of insulin secretion and insulin action). Phytochemical constituents as already mentioned in chapter 2 have a wide capacity in combating different illness, infections, and or chronic diseases such as diabetes. Antihyperglycemic activity in medicinal plants is a result of their capacity to restore the function of pancreatic tissues by causing an increase in insulin output to reduce or inhibit intestinal absorption of glucose, through the assistance of phytochemicals in insulin dependent processes. Glycosides, alkaloids, terpenoids, flavonoids, carotenoids have been identified in plants which have antidiabetic activity (**Sarkodie et al., 2015).**

A study to determine the effect of administered *E. anacua* leaf extracts on blood glucose in rats, showed that the extracts improved the structure of pancreatic tissue and in extension restored pancreatic function. The change in physiology of the pancreas due to inflammation of the cells by alloxan intoxication, was restored because of the extract. Pancreatic cells of the rat were preserved due to the administration of *E. anacua* leaf extracts, furthermore the this indicated the plants potential as a free radical scavenger and lipid peroxidation inhibitor, which helps to maintain cell membrane integrity and prevent oxidative stress caused by free radicals (**Abimbola et al., 2021)**. This study highlighted the relationship between antioxidant activity and antidiabetic activity, such that if there is good antioxidant activity, the same extracts will exhibit antidiabetic activity. Similar findings were discovered int this study, where extracts with high antioxidant activity also had high antidiabetic activity (**Abimbola et al., 2021**).

Examining the α-glucosidase experiments it was observed that methanol extracts of *E. rigida* had higher activities than bark, leaf, and root methanol extracts of *E. obtusifolia*. Besides *E. obtusifolia* methanol bark extracts which had activity similar or equal to *E. rigida,* bark extracts

of *E. rigida* leaf and root methanol extracts had higher activities than *E. obtusifolia* by direct comparison. However, ethyl-acetate produced different results and it was observed that *E. obtusifolia* leaf and root extracts had higher activity than *E. rigida*. The results of this study showed that methanol, ethyl-acetate, and dichloromethane had dose dependent α-glucosidase activity for *E. rigida* and *E. obtusifloia,* which agreed with *E. cymosa* ethyl-acetate and methanol fractions **(Ogundajo and Ashafa., 2017).**

Based on the dose-dependent activity observed for *E. obtusifolia* and *E. rigida* extracts, *E. obtusifolia* is more potent because of higher activity at the lowest. Their activities declined slowly with a decrease in concentration. The quantity of phytochemicals which were identified in chapter 2 such phenolics, flavonoids, saponins, and tannins could be the reason behind to the reported high α-glucosidase activity. The capability of phytochemicals acting as antioxidants is well documented thus also their capability to be antidiabetic agents say (**Ogundajo and Ashaf., 2017**). The α-glucosidase activity from dichloromethane *E. rigida* and *E. obtusifolia* extracts was dose-dependent. *E. rigida* bark, leaf and root extracts had good activity, with root and leaf extracts having the highest activity of the organ at the lowest concentration of 125 ug/ml and bark and root extracts had marginally higher activity at higher concentration.

4.5 Conclusion

The activity demonstrated by *E. rigida* and *E. obtusifolia* showed that extracts had good antioxidant and antidiabetic activities and makes both species suitable drug candidate and their activity was similar to that of ascorbic acid and acarbose. *E. cymosa* ethyl-acetate and methanol extracts had similar findings with the current study and are deemed to have little to no side effects compared to synthetic drugs and therefore are a suitable alternative. Phytochemicals such as poly phenols play a role as antidiabetic agents by interacting with intestinal enzymes and transporters that control absorption in postprandial glucose. When medicinal plants are consumed through food or drink, their break down and release of phytochemicals in the intestines accumulate to high concentrations of approximately hundreds of micromoles, which will be enough to inhibit glucose absorption (**Sarkodie et al., 2015).**

Chapter 5: Conclusions

5.1 Introduction

The aim of this project was to determine the pharmacological potential of two South African species of *Ehretia,* namely *E. rigida* and *E. obtusifolia*. This was achieved by undertaking an assortment of phytochemical analyses and assays to determine biological activity. The genus has been previously studied especially with Asian species, and the findings have reported the genus being rich in species with a variety of medicinal properties, and these plants have been used traditionally for centuries. In the first chapter the different biological activities and phytochemicals present in genus *Ehretia* were reported and the lack of research concerning Southern African species was highlighted. The findings of this study have uncovered and provided further understanding on the medicinal potential of the entire genus and not just in species concentrated in Asia, but also species found in Southern Africa.

5.2 Phytochemicals (phenolics, flavonoids and tannins)

The comparative phytochemical study between *E. obtusifolia* and *E. rigida* outlined the high content of phytochemicals present, particularly phenolics and flavonoids in the leaves, bark, and roots. These phytochemicals also exhibited strong antimicrobial and antioxidant activity observed in Chapter 3 and 4. The results indicated the two species to be worthy of further investigation and have the potential to be a new source of natural antioxidants. Future investigations can include isolation and characterisation of the antioxidant compounds in these plants.

It is important to determine the molecular mechanics of how bioactive compounds are synthesised in plants. This knowledge about phytochemicals will help breeders, conservation biologists, and biotechnologists in creating protocols to enhance the production of these

compounds in plants, without placing their diversity and availability at risk (**Ynalvez et al., 2018)**. Previous study has indicated the presence of phenolics and flavonoids in *Ehretia* to be linked with antioxidant and anti-inflammatory activities. This makes *E. rigida* and *E. obtusifolia* attractive to scientists who should pay more attention to South African species of *Ehretia* including *E. amoena* (**Li et al., 2010**). The results of the study present an opportunity to South African (provided the pharmacological potential of the species are investigated) researchers and industries to undertaking further research to substantiate earlier studies, and possibly commercialise compounds found in the genus.

High performance liquid chromatography (HPLC) to isolate and purify active compounds responsible for the activity in *E. rigida* and *E. obtusifolia*, along with mass spectrophotometry for identification of specific phytochemicals responsible for any biological activity would improve our understanding of these valuable plants. Furthermore, isolating and identifying the compounds offers an exciting and interesting avenue that will have relevance in the field of discovering bioactive products. (**Ynalvez et al., 2018**).

5.3 Antimicrobial

Antibacterial and antifungal findings in Chapter 3 confirmed the medicinal potential of *E. rigida* and *E. obtusifolia.* This was the first report of antimicrobial activity in *E. rigida* and *E. obtusifolia,* the phenolics and flavonoids observed in Chapter 2 could either act together in synergy or individually for the proven antimicrobial activity.

The relationship between the presence of phytochemicals and their biological activities has caught the attention of scientists who are coming up with more innovative ways to utilize the relationship. This has broadened our understanding and opened additional fields of research as speculated by **Joshi and Wagh (2018b**). Drug resistant microorganism are becoming more prevalent and the need for alternative sources of treatments with fewer side-effects compared to the current antibiotics. This inspires the need for identifying and isolating of active compounds in *E. rigida* and *E. obtusifolia* (**Mutha et al., 2015**).

The extraction power and polarity of the solvent used, determines what bioactive compounds can found in plants. A study by **Muhamad et al (2014**) found that there was higher antioxidant activity, and lower MIC of bacteria and fungi from aqueous extracts. The findings suggested

that a 50:50 ratio of the solvents allows equal extraction of polar and non-polar phytoconstituents and results in better activity than using “pure” solvents. This study used only “pure” solvents, perhaps the results would have been different if aqueous solvents were used for extraction.

5.4 Antioxidant and antidiabetic

Diabetes is a serious illness affecting millions of people globally, with the majority of patients diagnosed with type 2 diabetes which affects insulin resistance because liver, muscle, and fat cells cannot respond normally to insulin. Commercial inhibitors such as acarbose, volibose and migitol help reduce glucose levels but have many side effects (flatulence, abdominal pain, diarrhoea) according to **Promyos et al (2017)**. Both *E. rigida* and *E. obtusifolia* demonstrated good α-glucosidase activity. The activity was higher than that of acarbose in some cases which means both species could to be of use in the management of diabetes.

Oxidative stress is the cause of morbidity and this is also associated with diabetes, therefore the use of antioxidants can help treat diabetes along with tocopherol, ascorbic acid, tannins, flavonoids, phenolics and carotenoids (**Ogundajo and Ashafa., 2017)**. It is important to note that synthetic antioxidants have low solubility and moderate antioxidant activity, hence they are not best suited for treatment of some of the triggers of diabetes, and rather a daily dietary intake of fruits and vegetables rich in antioxidants is recommended (**Sivansankari et al., 2013)**. It was clear from the antioxidant and α-glucosidase assays in Chapter 4 that both *E. rigida* and *E. obtusifolia* had good inhibotory activity from leaf, bark, and root extracts. *E. rigida* had marginally better activity.

E. rigida and *E. obtusifolia* species have higher activity than some of the investigated and favoured species of *Ehretia.* It would be interesting to identify and isolate active compound and determine if they are the same as the ones found in other species of *Ehretia* (*E. laevis, E. microphylla, E. thrysifolia and E. serrata*) and compare their biological activity potency.

5.5 Conclusion

E. rigida has not been previously studied comprehensively, this project has established a baseline regarding its medicinal potential, while *E. obtusifolia* has been researched, it serves as a good comparison with *E. rigida*. The project determined the biological activities of leaves, bark, and roots and used different solvents to extract phytochemicals. Both species have good

biological activities, particularly antioxidant and antibacterial activity. Further research using GC-MS should establish the identity of phytochemicals responsible for the activity.

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