ANTIBACTERIAL, ANTIOXIDANT AND PHYTOCHEMICAL INVESTIGATION OF *ACACIA ARENARIA, ALOE ESCULENTA,* AND *PECHUEL-LOESCHEA LEUBNITZIAE*.

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ABSTRACT

Natural products present in medicinal plants are an important source of therapeutic agents and many research groups are currently screening different biological activities of plants. This study aimed to investigate the antibacterial, antioxidant properties and phytochemical analysis of *Acacia arenaria* (Fabaceae), *Aloe esculenta* (Alliaceae) and *Pechuel-Loeschea leubnitziae* (Asteraceae) that are used in traditional settings. The antimicrobial activity of the methanol-dichloromethane (DCM) (1:1) extracts of these medicinal plants were tested against *Shigella sonnei* (ATCC 25931), *Serratia marcescens* (ATCC 8100), *Enterococcus faecalis* (ATCC 7080) and *Alcaligenes faecalis* (ATCC 8750). The minimum inhibitory concentration (MIC) of the extracts against afore mentioned microbes were determined. The antioxidant activity of the extracts was determined using α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging and reducing power assays. The phytochemical analysis of *A. arenaria, A. esculenta* and *P. leubnitziae* was done using qualitative and quantitative phytochemical analysis. Extracts demonstrated anti-microbial activity against *S. sonnei, A. faecalis, E. faecalis* and *S. marcescens*. The best activity was recorded with *A. arenaria* leaves extract, with MIC values ranging from 0.1 to 5.0 mg/mL against all tested bacteria. All extracts exhibited antioxidant activity for both assay techniques. The antioxidant activity correlated with the quantitative phytochemical presence of phenolic content ranging from 0.5 ± 0.1 to 5.7 ± 0.1 mg of GAE/g extract obtained in the extracts as well as condensed tannins ranging from 0.1 ± 0.0 to 72.6 ± 0.0 mg of TAC/g extract. Qualitative phytochemical analysis showed the presence of steroids, terpenoids, saponins, flavonoids, anthraquinones, coumarins, alkaloids, phenols and tannins. The results obtained in the present study indicated that the leaves, stem and roots of *A. esculenta*, and *A. arenaria, P. leubnitziae* are potential sources of natural antioxidants

and antimicrobials. Further studies could look into the characterization of the bioactive compounds present in these plants.

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DECLARATION

I, Daniel Ndongo, declare hereby that this is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

Signature.....Date.....2017

Daniel Ndongo

DEDICATION

I dedicate this work to my family and friends for always believing in me and encouraging me even in instances when I felt like giving up. To my brothers and sisters, where there is a will there is a way. Thus, you can go as far as your imagination goes. Do not give up on your dreams.

ABRIVIATION

AIDS: Acquired Immune Deficiency Syndrome

ATP: Adenosine Triphosphate

CAT: Catalase

DCM: Dichloromethane

DNA: Deoxyribonucleic Acid

$DPPH^{\bullet}$: α, α-Diphenyl-β-picrylhydrazyl radical

GAC: Gallic Acid Equivalent

IC_{50}: 50% Inhibition Concentration

MeOH: Methanol

MET: Ministry of Environment and Tourism

MIC: Mimimum Inhibitory Concentration

NBRI: National Botanical Research Institute

TAC: Tannic Acid Equivalent

TCT: Total Condensed Tannins

TPC: Total Phenolic Content

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RPC: Research and Publication Committee

SOD: Superoxide Dismutase

WHO: World Health Organization

INTRODUCTION

1.1. Orientation of the study

Medicinal plants are reservoirs of curative elements used by a large population of Africans in the treatment of various infections (Okigbo, Anuagasi, & Amadi, 2009; Akhter, Hossain, Haque, Shahriar, & Bhuryan, 2012). A medicinal plant is any plant with healing capacity. For centuries, man has used plants to treat common communicable diseases (Alabri, Musalami, Hossian, Weli, & Al-Riyami, 2013). Furthermore, an herbal product from medicinal plants is produced through a number of processes such as extraction, fractionation, purification, and concentration of plant materials (Alabri et al., 2013). According to recent studies conducted by the World Health Organisation (WHO), about 80% of the world's population relies on traditional medicine (Moudgil & Khalil, 2016). There is over 350,659 medicinal plants globally of which only 2% have been explored and tested for their bioactivity (Ghosh, Ramakrishna, & Ramakrishna, 2014).

Medicinal plants reportedly contain biologically active secondary metabolites that include saponins, tannins, essential oils, flavonoids and alkaloids (Okigbo et al., 2009). In many cases, these metabolites serve as plant defence mechanisms against predation by microorganisms, insects and herbivores (T. S. Geetha & Geetha, 2014). Some of these plant-derived compounds have demonstrated good antiviral, antibacterial, antioxidant activities just to mention few (Suneetha, Gopinath, Divya, Amaashankar, & Narasimha, 2013). A number of *in vitro* studies on medicinal plants have revealed antiviral as well as antibacterial potential (Patel, Bessong, & Liu, 2011; Fobofou et al., 2015; Zohra, Meriem, Samira, & Alsayadi, 2012). In addition, many medicinal plants have also shown preservative effects due to the presence of antioxidants (Akbarirad, Gohari, Kazemeini, & Mousavi, 2016).

Namibia reportedly has over 4,334 plant taxa some of which are utilized as herbal medicines (Chinsembu, Hedimbi, & Mukaru, 2011). In the present study antibacterial, antioxidant activities and phytochemical profiles of *A. arenaria, A. esculenta* and *P. leubnitziae* were investigated. *Acacia arenaria* and *A. esculenta* have never been investigated for antibacterial, antioxidant properties neither their phytochemical screening was conducted. On the other hand, phytochemical screening and antibacterial study of *P. leubnitziae* were previously reported (Hedimbi, Kaputjaza, Hans, Mumbengegwi, & Böck, 2012a; Hedimbi, Ndjoze-Sirika, & Han, 2012b). However, there were no studies on the antioxidant activity done on *P. leubnitziae*. *Acacia arenaria, A. esculenta* and *P. leubnitziae* were selected based on known traditional uses reported in literature such as cold, cough and intestinal related conditions (Curtis & Mannheimer, 2005; Mannheimer & Curtis, 2009; Steyn, 2007; Von Koenen, 2001).

1.2. Statement of the problem

Many people in African, Asian communities greatly rely on medicinal plants to alleviate symptoms of various diseases. In Namibia, many residents in rural areas rely on medicinal plants to treat several diseases (Chinsembu, Hijarunguru, & Mbangu, 2015). In most cases, the exact mechanism of action of the extracts is unknown; hence, it is therefore crucially important to determine whether actual pharmacological effects support the traditional claims. *A. arenaria*, *A. esculenta* and *P. leubnitziae* are traditionally used as antibacterial agents in both humans and animals, Von Koenen (2001) but minimal scientific knowledge is available to support the traditional claims. Studying these plants therefore assisted in approving or disapproving the anecdotal use, and contributed to the on-going search for new antibacterial agents because of the continuous emergence of drug resistant bacterial strains.

1.3. Objectives of the study

The specific objectives of this study were to:

a) Investigate phytochemicals present in the crude extracts of *A. arenaria*, *A. esculenta* and *P. leubnitziae* using qualitative and quantitative methods.

b) Investigate the antibacterial property of *A. arenaria* and *A. esculenta* extracts against *Alcaligenes faecalis, Shigella sonnei, Serratia marcescens* and *Enterococcus faecalis*

c) Determine the antioxidant activity of *A. arenaria*, *A. esculenta,* and *P. leubnitziae* using DPPH antiradical scavenging and reducing power assays

1.4. Research hypothesis

a) H_0: The crude extracts of *A. arenaria*, *A. esculenta* and *P. leubnitziae* do not have phytochemicals.

   H_a: The crude extracts of *A. arenaria*, *A. esculenta* and *P. leubnitziae* have phytochemicals.

b) H_0: Crude extracts of *A. arenaria, A. esculenta* and *P. lebnitziae* do not exhibit antibacterial against *A. faecalis, S. sonnei, S marcescens* and *E. faecalis*.

   H_a: Crude extracts of *A. arenaria*, *A. esculenta*, and *P. leubnitziae* exhibit antibacterial property against *A. faecalis, S. sonnei, S. marcescens* and *E. faecalis*.

c) H_0: Crude extracts of *A. arenaria, A. esculenta* and *P. leubnitziae* do not exhibit antioxidant property.

   H_a: Crude extracts of *A. arenaria*, *A. esculenta* and *P. leubnitziae* exhibit antioxidant property.

1.5. Significance of the study

The findings of this study were expected to provide *in vitro* evidence to justify the traditional use of *A. arenaria*, *A. esculenta* and *P. leubnitziae*. The antibacterial and antioxidant results from the present study would support for further investigation to identify active compounds through bioassay guided fractionation from the bioactive extracts of *A. arenaria*, *A. esculenta* and *P. leubnitziae*. Synergistic interactions between compounds in crude extracts have been reported to take place and that is why it was important to first test crude extracts before purification of bioactive constituents. The study findings will also create more awareness on the Namibian *A. arenaria*, *A. esculenta*, and *P. leubnitziae* and add to the scientific value of these species.

LITERATURE REVIEW

2.1. Plants description, geographical location and scientific studies done

2.1.1. *Acacia arenaria*

Acacia arenaria, commonly known as Sand-thorn in English and *Omulyamenye* in Oshiwambo, belongs to the sub-family Mimosoidae under the family Fabaceae (Curtis & Mannheimer, 2005; Mannheimer & Curtis, 2009; Steyn, 2007). According to Steyn (2007), about 1350 Fabaceae species have been described of which 954 are found in Australia, 130 in Africa and the rest elsewhere (Abdel-Farid, Sheded, & Mohamed 2014). However, regardless of the large number of *Acacia* species, minimal research has been done on the phytochemistry of these plants (Abdel-Farid et al., 2014). *Acacia arenaria*, a small tree with a short stem, has zigzag branches with thin thorns (Curtis & Mannheimer, 2005; Mannheimer & Curtis, 2009; Steyn, 2007; Von Koenen, 2001). The plant have bipinnate leaves, whitish flowers that are borne in heads, and sickle-shaped pod fruit somewhat constricted between seeds (Von Koenen, 2001). *Acacia arenaria* is commonly distributed across northern Namibia (Curtis & Mannheimer, 2005; Mannheimer & Curtis, 2009).

Chemical composition of *Acacia* seeds has been reported in Botswana, since Acacia trees are of significant to livestock nutrition in that country (Aganga, Tsopito, Yeboah, Mokgoko, & Manne, 1997). *Acacia arenaria* seeds reportedly have dry matter (92.55%), dry matter digestibly (53.13%), crude protein (19.93%), ash (4.05%) and extractable fat (5.00%) in g/100 g dry matter (Aganga et al., 1997). The mineral composition of the seeds has been reported: calcium (0.37), phosphorus (0.48), potassium (1.39), sodium (0.05) and magnesium (0.20) in g/100g dry matter. The microminerals determined were copper (21), iron (77), manganese (9) and zinc (54) in mg/kg dry

matter (Aganga *et al*., 1997). Figure 1 shows *A. arenaria* picture that was taken during sample collection.

Figure 1: *Acacia arenaria* (whole plant)

2.1.2. *Aloe esculenta*

Aloe esculenta, which belongs to the family Alliaceae, is commonly known as *endombo* in *Oshiwambo.* There are 400 species of this plant of which 31 are found in Namibia (Retief, 2013; Sahu et al., 2013). *Aloe esculenta* is a succulent herb of 80-100 cm in height, which matures in 4-6 years and survives for about 50 years under favourable conditions (Sahu et al., 2013). This plant belongs to the great masses of plants found on the sandy flat soils (Klopper, Matos, Figueiredo, & Smith, 2009). Von Koenen (2001) described *A. esculenta* as a perennial succulent plant with a short stem inside a rosette of leaves. The candelabra-like inflorescence of this plant reportedly bears orange-yellowish flowers, and the capsule contains a number of seeds (Von Koenen, 2001). In Namibia, *A. esculenta* can only be found in the northern part of the country (Klopper et al, 2009).

There exist few reports in literature on experimental studies conducted on *A. esculenta. Aloe esculenta* extract has demonstrated antiplasmodium activity (Amoo, Aremu, & Staden, 2014). The most studied *Aloe* species is *Aloe vera*, and vast literature is available on this plant. The chemical composition of *A. vera* revealed that the active components possibly present in this plant are anthraquinones, chromones, polysaccharides, enzymes, vitamin C, vitamin E, lecithin, glycoprotein and amino acids (Ghayempoura, Montazera, & Rad, 2016; Sahu et al., 2013). In addition, the medicinal properties of *A. vera* include antioxidant, antitumor, anti-ulcer, anti-neoplastic, antiviral and antiiflammatory activities (Ghayempoura et al., 2016; Soltanizadeh & Ghiasi-Esfahani, 2014). There are about 75 active components in *A. vera* that makes it attractive to cosmetic and medicinal industries (Soltanizadeh & Ghiasi-Esfahani, 2014). Figure 2 below shows the picture of *A. esculenta* that was taken during sample collection.

Figure 2: *Aloe esculenta* (whole plant)

2.1.3. *Pechuel-Loeschea leubnitziae*

Pechuel-Loeschea leubnitziae belongs to the family Asteraceae (Curtis & Mannheimer, 2009; Hedimbi et al., 2012b). This shrub is commonly known as Bitter bush in English and *Oshizimba* in *Oshiwambo* (Hedimbi et al., 2012a). It is a small shrub with silver-grey felt-like pubescence on stems and leaves, with leaves tapering petiole-like towards the base, lanceolate, entire and up to about 3 cm long (Von Koenen, 2001). The plant is found throughout Namibia except in the far north-eastern part (Mannheimer & Curtis, 2009).

Phytochemical analysis of *P. leubnitziae's* roots, leaves and stem extracts reportedly contain saponins, anthraquinones, flavonoids, and polyphenols (Hedimbi et al., 2012a). Moreover, stem, leaves, and roots extracts of this plant were reported to possess antimicrobial activities against a number of microbes such as *Neisseria meningitidis*, *Shigella flexneri*, *Proteus vulgaris*, *Pseudomonasa aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (Hedimbi et al., 2012a; Hedimbi et al., 2012b). Figure 3 below shows the *P. leubnitziae* picture that was taken during sample collection.

Figure 3: *Pechuel-Loeschea leubnitziae* (whole plant)

2.2. Traditional uses of *A. arenaria, A. esculenta* and *P. leubnitziae*

The use of traditional medicine has been widely witnessed in most developing countries as a first prescription for the maintenance of good health (Cheikhyoussef, Mapaure, & Shapi, 2011). The plants investigated in this study are used by rural people in the treatment of various diseases in both human and animals (Von Koenen, 2001).

2.2.1. *Acacia arenaria*

The *Aawambo* people, one of the big tribes in Namibia, have used *A. arenaria* roots decoction for coughs, and colds (Von Koenen, 2001). Goats, and cattle consume leaves and shoots of this plant (Curtis & Mannheimer, 2009). Furthermore, *A. arenaria* has also been used by *Aawambo* people for the treatment of intestinal conditions in cattle (Von Koenen, 2001).

2.2.2. *Pechuel-Loeschea leubnitziae*

The Herero people of Namibia reportedly use *P. leubnitziae* to delay menstruation by administering steam baths with leaf decoctions of the plant, which are used for thorough washing of the abdomen (Von Koenen, 2001). *Pechuel-Loeschea leubnitziae* shrub is an effective mosquito repellent. *Ovahimba* are indigenous people living in the Kunene region of Namibia, and also in the neighbouring country Angola use *P. leubnitziae* finely grinded roots for cosmetic purposes (Curtis & Mannheimer, 2005). The Nama tribe of Namibia stuff *P. leubnitziae* into their shoes to alleviate sweaty feet (Von Koenen, 2001). Boiled roots and leaves are used for enemas in cases of intestinal conditions, or as steam bath with colds (Von Koenen, 2001). In addition, *Aawambo* people in Namibia, heat the plant materials, and use it as facial compress for cold to treat infection (Von Koenen, 2001).

2.2.3. *Aloe esculenta*

The *Aawambo* people in Namibia, use *A. esculenta* for various traditional applications. Its leaf sap can be applied to cuts and burns (Von Koenen, 2001). *Aloe esculenta* are also used to manage skin rashes and decoction is taken orally to treat coughs (Chinsembu, Negumbo, Likando, & Mbangu, 2014; Chinsembu et al., 2015). Dried roots are pulverized and mixed with water to make a paste that is massaged onto painful and swollen body parts (Von Koenen, 2001). It is also used in facilitating weaning of babies/toddlers by applying very bitter leaf sap to the mother's nipples (Retief, 2013). In addition, *A. esculenta* is used in the treatment of red water disease and other ailments in cattle and sheep. The plant is also added to drinking water to improve the general health of animals and by reducing parasite load, as well as blood purification by making it sour for invading pathogens to treat acne in animals (Retief, 2013).

2.3. Natural products as sources of drugs

Secondary metabolites are defined as low-molecular weight compound that do not play a role in growth and development but rather play a major in defensive system of the plant (T. S. Geetha & Geetha, 2014). However, so far only roughly 20% of higher plants are investigated. A natural product is defined as a chemical component or substance produced by a living organism that is found in nature (Khalil, Diab, & Moudgil, 2016). Natural products may have pharmacological or biological activities that can be of therapeutic benefit in treating diseases (Khalil et al., 2016). Natural products are seldom utilized as precursors or starting materials for new drugs discovery due to their efficacy and safety (Khalil, et al., 2016).

Plants have been the source of crude drugs/extracts as well as precursors of synthesized compounds that have made large influences to human health and well-being. Their role has

significantly increased in the upgrading of new drugs (T. S. Geetha & Geetha, 2014): (1) they may turn out to be the base for the development of a medicine, a natural blueprint for the development of new drugs, or; (2) a phytomedicine to be utilized for the treatment of disease. There are several examples of plant derivative drugs such as vinblastine, vincristine, vinca alkaloids, comptothecin and so forth (Gurung, Bhattacharjee, & Ali, 2016).

Many drugs derived from plants on the market were discovered based on their traditional uses (Phani, Raj, Rupesh, & Shashi, 2010). For thousands of years, natural products have played a critical role in health care and prevention of diseases (Ilonga, 2012). The earliest known written document is a 4000-year-old Sumerian clay tablet that records remedies for various illnesses (Moudgil & Khalil, 2016). It is estimated that approximately 80 % of the entire world population rely on traditional medicine for their primary health care (Alabri et al., 2013; T. S. Geetha & Geetha, 2014). Nowadays people all over the world are trying to keep away from poor eating habits, pollution and synthetic drugs, addictions (tobacco and alcohol) and so forth because all these lead to chronic oxidative stress (Glód, Wantusiak, Piszcz, Lewczuk, & Zarzycki, 2014; Phani et al., 2010).

Roughly about 5-18% drugs given to patients in the world today are derived from natural sources, whereby 90% of which are either directly or indirectly from plant sources (Moudgil & Khalil, 2016). Moreover, 47% of anticancer drugs on the market are derived from natural products or are mimics of natural product (Moudgil & Khalil, 2016). These anticancer drugs include podophyllotoxin, etoposide, teniposide, vincristine, vinblastine and paclitaxel (Gurung et al., 2016; Popoola et al., 2016).

An interest in natural antioxidant and antibacterial agents has risen recently due to their positive impact on the human body (Glód et al., 2014, Kaur & Mondal, 2014). Plant based products are reportedly rich in bioactive compounds such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavanoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites which are good antioxidants (Annapoorani & Manimegalai, 2013; Jayanthi & Lalitha, 2011; Ugochukwu, Uche, & Ifeanyi, 2013). Antioxidants scavenge a variety of free radicals and it can be extremely important in inhibiting oxidative mechanisms that lead to degenerative diseases (Glod et al., 2014).

The increasing interest in natural products derived therapeutic agent is becoming skeptical for various reasons. Reasons for doubting includes lack of proper documentations about source and formulation used, standardization of the composition, batch-to-batch consistency, documented safety of herbal products, and information about the mechanisms of action of these products (Moudgil & Khalil, 2016).

2.4. Phytochemicals

The three major groups of phytochemicals are nitrogen-containing substance, terpenes and phenolics. Roughly over fourteen thousand nitrogen containing secondary metabolites have been described so far. The main compounds of this group are: alkaloids, amines, non-protein amino acids, cyanogenic glycoside and glucosinolate (Nafuka, 2014).

More than 4000 phytochemicals have been cataloged and are classified by their function, structural and chemical characteristics (Scalbert et al., 2011). However, only about 150 phytochemicals have been studied in detail (M. Saxena, Saxena, Nema, Singh, & Gupta, 2013). Phytochemicals are

divided into various classes and each class's description is based on the structure and function (Irhhaiya et al., 2014).

Medicinal plants contain bioactive compounds that can be used in the treatment of various animals and human diseases (Heneman & Zidenberg-Cherr, 2008). Phytochemicals are divided into two categories which are primary and secondary metabolites (Ilonga, 2012). Primary metabolites are such as chlorophyll, proteins sugar and amino acids on the other hand Secondary metabolites contain terpenoids, phenolics, alkaloids and so forth (Wadood et al., 2013). According to literature, phytochemicals are responsible for medicinal activity of plants (Savithramma, Rao, & Suhrulatha, 2011). Phytochemicals are plant constituents responsible for protecting it from diseases and damage and contribute to the plant's color, aroma and flavour (Saxena et al., 2013). Studies carried have revealed that phytochemicals have an important role in preventing chronic diseases such as cancer, diabetes, coronary heart diseases (Saxena et al., 2013; Saidulu, Venkateshwar, & Gangadhar, 2014). Phytochemicals act by neutralizing free radicals, inhibiting enzymes that activate carcinogens and activate enzymes that detoxify carcinogen (Saidulu et al., 2014).

The classes of phytochemicals investigated in the present study are described in the following subsections.

2.4.1 Phenolics

Phenolics are the largest group of phytochemicals and the most widely distributed in the plant kingdom **(**Irchhaiya et al., 2014; Kabera, Semana, Mussa, & He, 2014). The three most essential groups of dietary phenolics are polyphenols, phenolics acids and flavonoids (Irhhaiya et al., 2014). Among the different classes of phenolic compounds, phenol is considered the smallest class

(Kabera et al., 2014; Saxena et al., 2013). Phenolic reportedly to exhibit antibacterial and antioxidant activities (Pratibha, Sushma, & Gupta, 2012).

Phenolic acids is a diverse group that is divided into two groups namely hydroxybenzoic and hydroxycinnamic acids (Irhhaiya et al., 2014; Kabera et al., 2014). Phenolic polymers, termed tannins, are also phenolic compounds with higher molecular weight and are divided into two groups; hydrolysable and condensed tannins (Saxena et al., 2013). Phenolic compounds are gaining more attention with researchers due to their medicinal properties against oxidative stress. Oxidative stress is the cause of various degenerative diseases such as coronary heart diseases, hypertension, cancer and diabetes

2.4.2. Flavonoids

Flavonoids are the largest group of plant phenolics and most studied as compared to other phenolics due to their pharmacological and biological activity (Saxena et al., 2013). Flavonoids are phenolic compounds that are ubiquitous within the plant kingdom (Nafuka, 2014). About 3000 flavonoid compounds have been described (Percival, 1998). They function as protective agents against environmental stress in plants (Percival, 1998). Flavonoids are also regarded as the most abundant dietary polyphenolic and medicinal phytochemical (Nafuka, 2014). They have gained more interest due to their biological and pharmacological activities (Saxena et al., 2013). This group of compounds reportedly has multiple biological properties including: antibacterial, cytotoxicity, antioxidant, antiinflammatory, antitumor, anti-allergenic, anti-carcinogenic, anti-aging and antiviral activities (Saxena et al., 2013).

Flavonoids are categorized according to chemical structures, into flavones, flavonols, flavanals, inflavanones, isoflavones, isoflavones and anthocyanins (Iikasha, 2016; Nafuka, 2014). Figure 1 show the major classes of flavonoids (Iikasha, 2016).

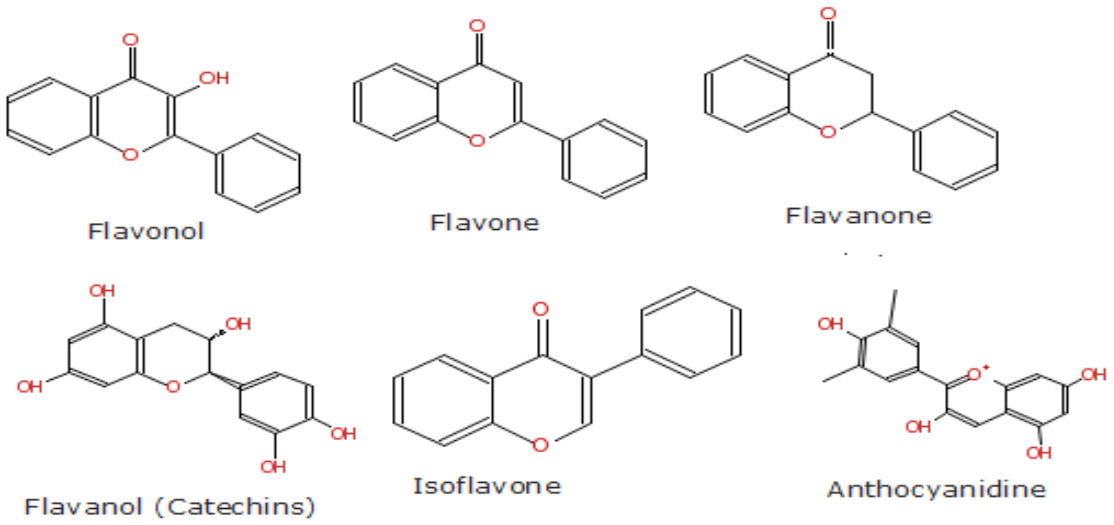


Figure 4: Example of major classes of flavonoids isolated from plants (Adapted from Iikasha, 2016).

2.4.3. Alkaloids

Alkaloids are basic compounds containing one or more heterocyclic nitrogen atoms, derived from amino acids and are pharmacologically active (Saxena et al., 2013). Alkaloids are mostly synthesized by plants but some can also be found in animal tissues e.g. amphibian's skin serves as a source of alkaloids (Nafuka, 2014). Alkaloids are associated with a wide range of pharmacological activities such as anesthetics, central nervous system stimulants, antibacterial, anticancer and so forth (Saxena et al., 2013).

2.4.4. Terpenoids

Terpenoids are defined as a group of natural products derived from five-carbon isoprene units (Irchhaiya et al., 2014; Saxena et al., 2013). Carotenoids, steroids and gibberellic acid are just some of its members (Kabera et al., 2014). In addition, they are classified as mono-, di-, tri-, and sesquiterpenoids depending on the number of carbon atoms they have. Moreover, terpenoids reportedly have medicinal properties such as anti-carcinogenic, antimalarial, antihypertensive, insecticidal, antifungal, antiviral, anti-ulcer and antibacterial (Kabera et al., 2014; Saxena et al., 2013).

2.4.5. Saponins

Saponins are a group of phytochemicals widely distributed in the plant kingdom (Saxena et al., 2013; Sidana, Singh & Sharma, 2016). Saponins are defined as glycosides of steroids or terpenes and include the group of cardiac glycosides as well as steroidal alkaloids Elekofehinti, 2015; Mikołajczyk-Bator, Błaszczyk, Czy˙zniejewski, & Kachlicki, 2016). Saponins reportedly have antibacterial, antifungal, antidiabetic, adjuvant, immunostimulant, hypocholesterolemic, antitumor and antiinflammatory activities (Ajayi, Ajibade, & Oderinde, 2011, Elekofehinti, 2015; Ramachandran, Kamaraj, Subramani, & Jeyakumar 2014; Sidana et al., 2016).

2.4.6. Tannins

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins and other macromolecules (Salminen & Karonen, 2011; Saxena et al., 2013). The pharmacological activities of tannins include antioxidant, anticancer and antibacterial (Ramachandran et al., 2014).

2.4.7. Anthraquinones

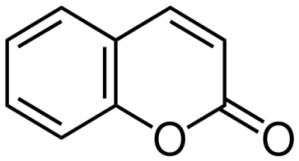
Anthraquinones are secondary metabolites nitrogen atom and about 750 types are reported in higher plants (Irchhaiya, et al., 2014). Anthraquinones are characteristics of the plant families: Fabaceae, Asphodelaceae, Liliaceae, Hypericaceae, Polygonaceae, Rhamnaceae, Rubiaceae, Scrophulariaceae, and Zanthorrhoeaceae (Wink, 2015). Most anthraquinones possess a phenolic OH group that makes them possible to interact with proteins and metals thereby forming hydrogen and ionic bonds respectively in the same way polyphenols do thereby modulate their conformation which explain their broad biological activities (Wink, 2015). The protective benefits of anthraquinones include antifungal, antibacterial, laxative, hepatoprotective, antioxidant, antiinflammatory, anticancer, antidiabetic and antiviral activities (Duvala, Pecher, Poujol, & Lesellier, 2016).

2.4.8. Coumarins

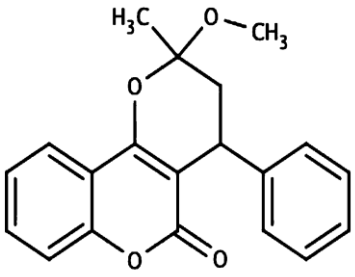
Coumarins are plant derived compounds which have attracted more interest due to their diversity and pharmacological properties (Skalicka-Woźniak, Orhan, Cordell, Nabavi, & Budzyńska, 2015). Coumarins can be identified by the presence of a benzopyrone ring as the shown in figure 2A. Coumarins are further categorized into four sub groups namely: simple coumarins, furanocoumarins, pyranocoumarins and pyrone-substituted coumarins (Nafuka, 2014). Currently only about 2% of coumarins search in plants can be obtained as they are only common in certain genera of the following families Apiaceae, Fabaceae, Poaceae and Rubiaceae (Wink, 2015). Coumarins and its derivatives possess antiinflammatory, anti-edemic, anti-tumor, antiviral, anti-coagulant, antioxidant effect, enzyme inhibition and antibacterial properties (M. R. Patel & Patel,

2011). Caumarins are aromatic and can be incorporated in cosmetics and beverages (Wink, 2015). The diagrams below show the common core structures of coumarins:

A



B



C

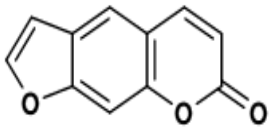


Figure 5: Core chemical structures of coumarins. A= simple coumarins, B = pyrano coumarins, C = furanocoumarins (Adapted from Nafuka, 2014).

2.4.9. Steroids

Steroids is a subgroup belonging to the class terpenoids and have been found to possess a broad spectrum of biological activities (Du preez, 2012). Du preez (2012), further stated that due to their chemical structure they show pharmaceutical activities including some antimalarial activity *in vitro*. Steroids specifically exert multiple biological effects due to their antioxidant and free radical scavenging abilities (Ilonga, 2012). The mechanism of action of these bioactive compounds as antimalarial agents is that they exerted through the prevention stage of the developmental stages

of plasmodium parasites from rings to trophozoites (Du preez, 2012). Figure 6 below shows the general structure of steroids.

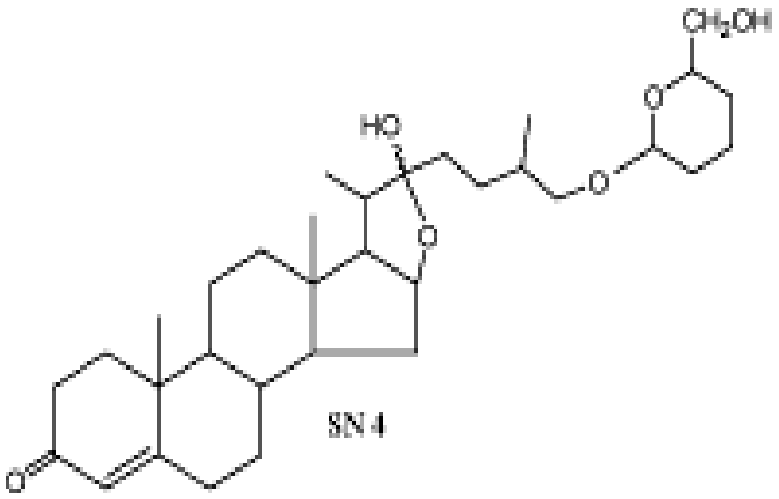


Figure 6: Structure of a typical steroid (Adapted from Du preez, 2012).

2.5. Oxidative Stress

Oxidative stress may be defined as the imbalance between the production of free radicals and the ability to neutralize their harmful effects by antioxidants (Lephart, 2016). Oxidative stress has been found to lead to several diseases such as cancer, heart diseases, brain dysfunction, age related degenerative condition, declination of the immune system, coronary arteriosclerosis, carcinogenesis, gastric ulcer and DNA damage (Kambli, Patil, Chithrashree, & Keshava, 2014). Natural antioxidants such as phenolics, flavonoids and tannins can delay or inhibit the oxidation of biomolecules by regulation of oxidative chain reactions (Mothana, Abdo, Hasson, Althawab, Alaghbari, & Lindequist, 2010).

2.5.1. Free radicals

Free radicals are atoms or molecules with an unpaired electron, they are highly reactive molecular species with an unpaired electron (Buonocore, Perrone, & Tataranno, 2010; Sivanandham, 2011).

Free radicals react with and modify protein, nucleic acids and fatty acids in plasma membrane and lipoprotein (Buonocore et al., 2010; Kammeyer & Luiten, 2015). Free radicals' unpaired electron, causes them to seek out and capture electrons from other substances in order to neutralize themselves (Sen, Chakraborty, Sridhar, Reddy, & De, 2010).

Free radicals are well studied and documented for playing a major role in the body as both deleterious and beneficial species (Sen et al., 2010). In their low or moderate concentrations, free radicals partake in normal physiological functions but in their excess and/or decrease of antioxidants level, it can lead to oxidative stress (Sen et al., 2010). Numerous synthetic drugs taken for the treatment of some diseases are reported to generate free radicals, as a result causing other diseases such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction (Sen et al., 2010; Sivanandham, 2011). Plants are found to be rich in antioxidants that can work in scavenging free radicals thereby terminating oxidative damage of the body (Kapewangolo, Tawha, Nawinda, Knott, & Hans, 2016; Sen et al., 2010). Phytochemicals reportedly possess fewer side effects therefore, more funds are needed to be spend on these types researches (Sen et al., 2010).

There are different sources of free radicals and oxygen is one of them (Sarma, Mallick, & Ghosh, 2010). Oxygen is an essential element needed by aerobic organisms for the process of respiration to occur (Lephart, 2016). When oxygen is supplied from the atmosphere through the lungs at a high concentration it becomes toxic. Oxygen in its ground state is unreactive; but its partial reduction gives rises to active oxygen species (AOS) such as singlet oxygen, super oxide radical anion and hydrogen peroxide (Thomas, S. D. Kamat, & Kamat, 2014). Other sources of free radicals include (Mandal, S. Yadav, Yadav, & Nema, 2009; Sarma et al., 2010; Sen et al., 2010; Sivanandham, 2011)**:**

- Metal-catalyzed reactions
- UV radiations, X-rays, gamma rays and microwave radiations.
- Neutrophils stimulated by exposure to microbes
- In mitochondria-catalyzed electron transport reaction, oxygen free radicals produced as by product.
- Oxygen free radicals in the atmosphere considered as pollutants.
- Inflammation initiates neutrophils and macrophages to produce ROS and RNS
- ROS generated by the metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells.
- ROS formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidases, and neutrophils and by lipid peroxidation
- Industrial effluents excess chemicals, alcoholic intake, certain drugs, asbestos, certain pesticides and herbicides, some metal ions, fungal toxins and xenobiotics.
- Interaction with chemicals, automobile exhausts fumes, smoking of cigarettes, cigars, beedie.
- Burning of organic matter during cooking, forest fires, volcanic activities.

Examples of radicals are such as radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$); and non-radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1\Delta g$), peroxynitrate ($ONOO^-$), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH)[11] (Sen et al., 2010). Non radicals are also termed as oxidants and capable to lead free radical reactions in living organisms easily. Radicals derived from oxygen are characterized as the most important class of radical species generated in

living systems (Sen et al., 2010). Free radicals can be formed from both exogenous and endogenous substances. They are continuously forming in cell and environment.

2.5.2. The role of antioxidants in disease prevention

Natural products represent a rich source of biologically active compounds. Specifically, plants kingdom offers a wide range of natural antioxidants (Addai, 2016; Garcia et al., 2012). Antioxidants prevent cell damage by neutralizing or scavenging the free radicals interactively and synergistically to form harmless by products (Jiménez-Zamora, Delgado-Andrade, & Rufián-Henares, 2015). In the body, an antioxidant may be water-soluble, lipid-soluble, and insoluble or bound to the cell wall (Marinova & Batchvarov, 2011). Plants are found to be rich sources of free radical scavenging molecules (antioxidants) namely terpenoids, phenolic acids, vitamins (A, C & E), stilbenes, lignins, tannins, alkaloids, quinones, coumarins, flavonoids, amines, betalains and other metabolites which possess antioxidant activity (Jayanthi & Lalitha, 2011).

There are two types of antioxidants the synthetic and the natural one. However, most researchers are diverting from synthetic antioxidants due to their toxicity (Elaasser, Abdel-Aziz, & El-Kassas, 2011). Natural antioxidants play a major role in health maintenance as well as prevention of chronic and degenerative diseases namely atherosclerosis, cardiac, cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Akbarirad et al., 2016). A human body can naturally produce antioxidants but they can be are insufficient to suppress the oxidation stress. Plants reportedly contain enough antioxidants that man can use to cover antioxidant deficiency. Therefore, eating of fruits and vegetables and the use of natural supplement is highly recommended.

Within a human body there are three main defence systems namely free radicals detoxifying enzymes, metal binding protein and antioxidants (Lephart, 2016). The free radicals detoxifying enzymes are: catalase which is involved in the conversion of hydrogen peroxide to harmless water and molecular oxygen. Superoxide dismutase (SOD) is an enzyme involved in the conversion of superoxide ($^{\bullet}O_2$) to either molecular oxygen or hydrogen peroxide (Lephart, 2016). Glutathione peroxide is another class of enzymes involved in converting hydrogen peroxide into water and oxygen (Krishnamurthy & Wadhwani, 2012). Metal binding protein such as ferritin, albumin, lactoferrin, and ceruloplasmin that seize free iron and copper that are capable of catalysing oxidative stress reaction (Lephart, 2016).

Dietary antioxidants, namely vitamin C, vitamin E, and beta-carotene are the most widely studied antioxidants (Mandal et al., 2009). Vitamin C is considered the most significant hydrophilic antioxidants in the extracellular fluid. It has the potential of neutralizing free radicals in the aqueous phase prior lipid peroxidation is initiated (Percival, 1998). Vitamin E a major hydrophobic antioxidant embedded in the cell membrane where it functions to protect the membrane fatty acids from lipid peroxidation (Percival, 1998). Vitamin C has also been reported to possess the potential of regenerating vitamin E. Moreover, beta-carotene other carotenoids are also believed to offer antioxidant protection to the lipid-rich tissue. It is reported that beta-carotenes work synergistically with vitamin E (Henríquez et al., 2010).

According to Phani et al., (2010) there are four most commonly used antioxidants namely butylated hydroxyamisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG) and tert-butylhydroxytoluene (TBHQ). Nevertheless, these antioxidants are suspected of being responsible for liver damage and carcinogenesis in laboratory animals (Phani et al., 2010). Therefore, the

development and utilization of more effective and less toxic antioxidants of natural origin particularly from plants are encouraged (Elaasser et al., 2011; Phani et al., 2010).

Antioxidants neutralize free radicals through two different mechanisms. Both mechanisms lead to similar reduction but differ in their kinetics and propensity (Marxen et al., 2007). The two mechanisms are single electron transfer whereby the antioxidant transfer an electron to the radical and hydrogen atom transfer whereby the antioxidant quench the free radicals by donating a hydrogen atom (Marxen et al., 2007; Senguttuvan, Paulsamy, & Karthika, 2014).

2.6. α, α-Diphenyl-1-picrylhydrazyl radical scavenging activity

α, α- diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging offers the first approach for determining the antioxidant capacity of a compound, extract or biological substances (Kedare & Singh, 2011; MacDonald-Wicks, Wood, & Garg, 2006; Moon & Shibamoto, 2009). DPPH molecule is classified as a stable free radical due to that there is delocalization of the spare electron over the entire molecule, so that the molecule does not dimerize like most other free radicals (Kedare & Singh, 2011). The delocalization gives rise to the deep violet colour, with an absorption in ethanol solution at around 520 nm. The following equation $Z^{\bullet} + AH = ZH + A^{\bullet}$ represents the primary equation of free radical neutralization. In the equation $Z^{\bullet}$ represent DPPH radical and AH represent the donor molecule leading to ZH reduced form and $A^{\bullet}$ is free radical produced in the first (Kedare & Singh et al., 2011).

DPPH is a commercial standard free radical often used in measuring the antioxidant activity of various chemical components including plant extracts like in case of this study. To determine the antioxidant potential of a chemical component different experimental approaches are always used.

In this study two methods were used to screen for antioxidant activity; DPPH radical scavenging assay and reducing power method.

DPPH radical scavenging assay is said to be simple, accurate, inexpensive, rapid, sensitive and a convenient method independent of samples' polarity, for screening many samples for radical scavenging activity (Kedare & Singh, 2011; Marinova & Batchvarov, 2011; Marxen et al., 2007). The fore mentioned advantages made the DPPH method suitable for testing medicinal plants as natural sources of scavengers of free radicals thereby discovering lead candidates for commercial purposes. Methanol and ethanol are the most used solvents for determination of radical scavenging activity by DPPH (Marinova & Batchvarov, 2011).

The DPPH assay was developed by Blois in 1958 with the aim to determine antioxidant capacity using α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M= 394.33) (Kedare & Singh, 2011). The principle behind DPPH is that the odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants. (Kedare & Singh, 2011; MacDonald-Wicks et al., 2006; Moon & Shibamoto, 2009). Figure 7 shows the mechanism of how 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) was neutralized to DPPH by an antioxidant.

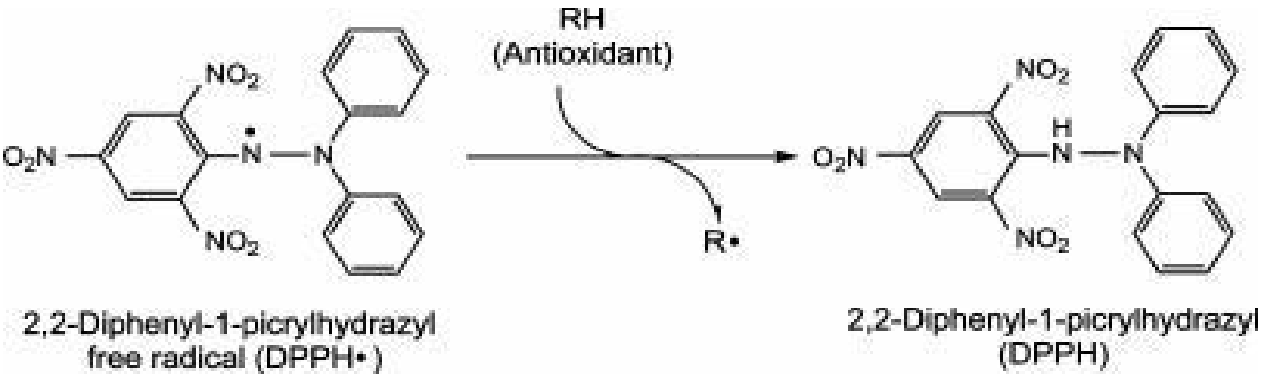


Figure 7. DPPH• free radical conversion to DPPH by anti-oxidant compound (Adapted from Moon & Shibamoto, 2009).

2.7. Reducing Power Assay

The principle behind reducing power assay lies in the substances such as antioxidant with reduction potential to react with potassium ferricyanide (3^{+}) forming potassium ferrocyanide that may serve as important indicators of antioxidant activity (Senguttuvan et al., 2014). In this assay, there is a change of colors from yellow to blue and green depending on the reducing power of the extract or specimen (Jayanthi & Lalitha, 2011). Furthermore, in the presence of reductones (antioxidants), free radicals are being scavenged by accepting hydrogen atom (Jayanthi & Lalitha, 2011). In case of the plant extracts, the reductones in the extract cause the reduction of Fe^{3+}/ Ferric cyanide complex to ferrous form that is Fe^{2+}, which can be measured spectrophotometrically at 700 nm (Senguttuvan et al., 2014). The rationale behind reducing power assay is that there is a direct relationship between reducing power and samples/ extracts concentration such that reducing power increases with an increase in extracts concentration. This is a significant indicator of its potential antioxidant activity (Senguttuvan et al., 2014).

2.8. Quantitative determination of phytochemicals

The discovery of new drugs from natural products is normally based on phytochemical and pharmacological approaches (Santhi & Sengottuvel, 2016). These chemicals are such as phenolics, terpenoids, alkaloids, flavonoids, tannins, anthraquinones etc. (Kaur & Mondal, 2014). In addition bioactive chemicals of plant have various activities such as antibacterial, antioxidant, antiinflammatory, antifungal, antiviral etc, (Obiang et al., 2016). Quantitative phytochemicals screening is the first step toward extraction, purification and identification the bioactive compounds for useful aspects (T. S. Geetha & Geetha, 2014).

2.8.1. Total Phenolic content

Phenolic compounds are found to be good antioxidants (Azlim et al., 2010). Therefore, it is important to determine the content of phenolic compounds present in an extract or sample. The high the content of phenolic compounds the greater antioxidant activity a plant has. The total phenolic content (TPC) assay using the Folin-Ciocalteu (FC) reagent is one of the most regularly utilized methods to quantify the phenolic content of a plant extract (Henríquez et al., 2010). Upon reaction with reducing agents, a blue-coloured complex is formed between the molybdenum and tungsten present in FC, which can then be measured through spectrophotometry (Tan & Lim, 2015). The high sensitivity, reproducibility and convenience of this single electron transfer-based assay has made it prevalent in routine screening of natural products (Tan & Lim, 2015).

2.8.2. Total condensed tannins

The present study only focused on condensed tannins (proanthocyanidins) as they are proven to be one of the groups of compounds with promising health-promoting properties (Zarin, Wan, Isha, & Armania, 2016). Furthermore, condensed tannins reportedly assist in wound healing, reducing pain for pancreatitis, reduce insulin resistance in diabetics, assist in protection from drug toxicity and also can help lower the levels of low- density lipoprotein or in other word bad cholesterol (Zarin et al., 2016). Vinillin (in 4% methanol) assay is a reported method used often to determined total condensed tannins (Rebaya et al., 2014).

Condensed tannins are thought to have strong antioxidant properties making them potential phytoconstituents in preventing diseases, health promoters and anti-ageing agents (Tamilselvi, Krishnomoorthy, Phamotharan, Arumagam, & Sagadevan, 2012). In addition to antioxidant properties, condensed tannins reportedly possess antiinflammatory, anti-asthmatic, anticancer,

antiviral, anti-carcinogenic, anti-allergy, antibacterial, antihypertension and cardiovascular system protective properties (Zarin et al., 2016).

2.9. Phytochemicals as antibacterial agents

Bacterial infections are of particular concern globally due to current bacterial strains that are becoming resistant to antibiotics (Tchinda, Voukeng, Beng, & Kuete, 2016). Epidemic due to bacteria strains resistance is still an issue even in developed nations with understanding in microbiology and their control (Dabur et al., 2007). Plants have been a good source of antibacterial agents and still continue to be highly effective instruments in fighting bacterial infections (Tchinda et al., 2016). Phytochemical derivatives have shown pronounced promise in the relief of intractable infectious diseases including opportunistic Acquired Immune Deficiency Syndrome (AIDS) infections (Rebaya et al., 2014).

Antibacterial drugs are substances that interfere with the growth and spreading of microbes in the host (Saga & Yamaguchi, 2009). Antibacterial action can be bacteriostatic such that it can inhibit the growth of the microbes or bactericidal meaning that it can kill the microbe (Taukoorah, Lall, & Mahomoodally, 2016). Historically the first antibacterial was a synthetic drug discovered by Ehrlich in 1910 for the remedy of syphilis "Salvarsan" (Saga & Yamaguchi, 2009). In 1935 another synthetic drug "Sulfonamide" was discovered by Domagk and other researchers (Saga & Yamaguchi, 2009). Since both of these drugs were synthetic their major limitations included safety and efficacy (Saga & Yamaguchi, 2009).

Antibacterial drugs derived from medicinal plants are such atropine, quinine, morphine and tubocurarine (Abdallah, 2011). Investment in natural product antibacterial drugs studies have been neglected and significantly decreased over the past two decades (Brown, Lister, & May-Dracka,

2013). According to records, only seven new natural chemical entities have been approved for therapy for the treatment of bacterial infections (Brown et al., 2013). The significant decline in investment in natural products antibacterials as well as approval of antibacterial agents could be due to certain factors which includes change of drug regulatory procedures, increased drugs safety standard and failure of modern drugs discovery techniques i.e., high throughput screening against certain bacterial genome and so forth (Brown et al., 2013).

Microorganisms investigated in the study

2.9.1. *Shigella sonnei*

According to Massenet, Vohod, Hamadicko, and Caugant (2011), *Shigella* species are found to be the major cause of diarrhoea worldwide. *Shigella sonnei* is a gram negative pathogenic bacterium that can cause an infection called shigellosis, also known as bacillary dysentery, an infection of the digestive system (Anand, Pande, & Gore, 2013). *Shigella* species are non-spore forming, rod shaped bacteria and are a member of Enterobacteriaceae family (Gaurav, Singh, Gill, Kumar, & Kumar, 2013). These species are more prevalent in human than in any other living organisms and they are often found in small children. However, this species cannot survive long enough outside the host (Baydack & Ens, 2011).

Shigella species are found in the faeces of infected person and often spread through the faecal-oral route by direct contact with an infected person, eating contaminated food by food handlers, eating contaminated food by flies carrying infection, drinking water (Baydack & Ens, 2011). Outbreaks are also reported to occur in areas with severe crowding and/or poor hygiene (Gaurav, Singh, Gill, Kumar, & Kumar, 2013). *S. sonnei* is 1 serotype it causes mildest form of foodborne disease (Massenet et al., 2011).

2.9.2 *Alcaligenes faecalis*

The taxonomy of the genus *Alcaligenes* is closely linked with the taxonomy of the genus *Achromocbater*, and several *Alcaligenes* species have now been reclassified as *Achromobacter* species (Win et al., 2006). *Alcaligenes faecalis*, a species of gram-negative, rod shaped bacterium commonly found in the environment is the most frequently isolated member of the Alicaligenaceae family in the clinical laboratory (Mordi, Yusuf, Onemu, Igeleke, & Odjadjare, 2013). It is reported that members of this family produce strong alkaline reaction in all carbohydrates media. Most of these strains form characteristic colonies with a thin spreading irregular edge (Win et al., 2006). *Alcaligenes faecalis* is reported to cause meningitis in new born, bacteremia in cancer patients, has been associated with pancreatic abscess and corneal ulcer (Mordi et al., 2013).

2.9.3. *Enterococcus faecalis*

Members of the genus *Enterococcus*, collectively called enterococci, are recognized by the as indicator organisms for bacteriological water quality in fresh and saline waters (Harwood et al., 2004). Their presence, specifically at an increased level, is an indication of faecal pollution from animal or humans (Tyne, Martin, & Gilmore, 2013). These organisms are normally found in the gastrointestinal, biliary tracts, in lower number of the vigina and male urethra (Win et al., 2006). Enterococci are increasingly becoming significant agents of many human diseases mainly because of their resistance to antibacterial agents (Tyne et al., 2013). Enterococci are found to be the second most prevalent cause of nosocomial urinary tract and wound infections (Sood, Malhotra, Das, & Kapil, 2008; Win et al., 2006). Even though *Enterococcus faecalis* was once regarded as nonpathogenic, this opportunistic gram-positive coccus now ranks among the most worrisome hospital pathogens (Ogihara, Saito, Sawabe, Hagihara, & Tohda, 2016). It has an instinct resistance

to many antibiotics and a remarkable potential for developing resistance to others (Jain, Mulay, & Mullany, 2016). These species are resistant to pencicillins, cephalosporins, and aminoglycosides and have developed resistance to vancomycin (Win et al., 2006).

2.9.4. *Serratia marcescens*

The *Serratia* species are special among the Enterobacteriaceae family (Yang, Cheng, Hu, Zhu, & Li, 2012). Furthermore, these species are resistant to colistin and cephalothin (Win et al., 2006). Ten species of *Serratia* genus were recognised whereby 7 of which have been recovered from human clinical specimens (Win et al., 2006).

Serratia marcescens, a gram-negative bacterium, is the most significant of the genus *Serratia* and in many cases is associated with a variety of human infections specifically pneumonia and septicaemia in patients with reticuloendothelial malignancies who are taking chemotherapeutic agents (Win et al., 2006). This microorganism was used as a harmless relationship where one organism benefit living another unaffected to trace environmental contamination, basically due to their characteristic red pigmentation of some strains was easy to spot in culture media (Win et al., 2006). Currently, *S. marcescens* is considered an important pathogen with invasive properties and they have a tendency to resist many prevalently utilized antibiotics (Koh et al., 2007). *S. marcescens* can also be considered a significant nosocomial opportunist as evidenced by a case of childhood meningitis following the utilization of contaminated benzalkonium chloride disinfectant solution (Win et al., 2006).

METHODS

3.1. Research design

The study investigated antibacterial, antioxidant and screened for phytochemicals profile of *A. esculenta, A. arenaria* and *P. leubnitziae* using qualitative and quantitative methods. Figure 8 below shows the schematic research design flow diagram.

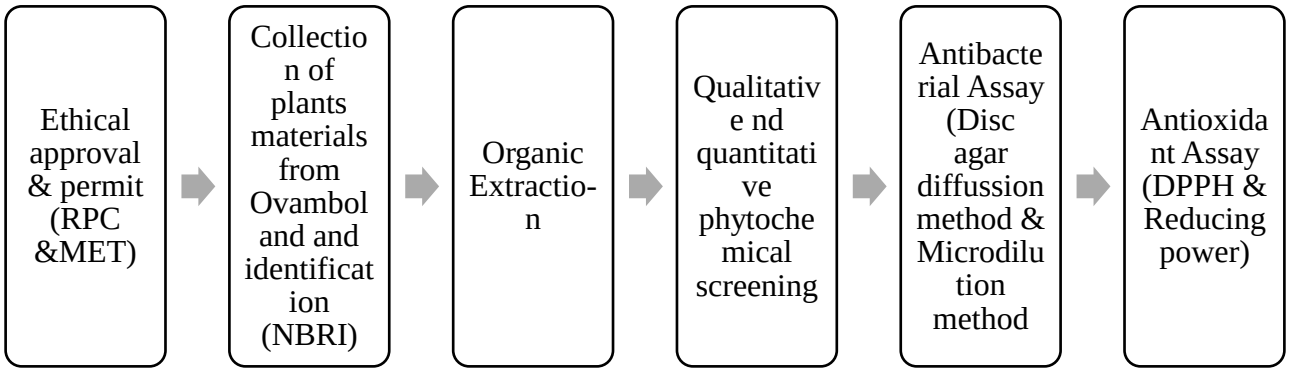


Figure 8: Schematic research design flow diagram

3.2. Plant specimen and collection

The fresh leaves, stems and roots of *A. arenaria*, *A. esculenta* and *P. leubnitziae* were collected from Omutala village in Oshana region, Namibia in November 2014. Research ethical approval was obtained from the University of Namibia (Appendix D). Collection permit was obtained from the Ministry of Environment and Tourism (Appendix E) and Research and Publication Committee of the University of Namibia (RPC) (Appendix F). The plants were identified and authenticated at the National Botanical Research Institute (NBRI), Windhoek. Voucher specimen numbers: *A. arenaria* (300), *A. esculenta* (305) and *P. leubnitziae* (310). The fresh leaves, stems, and roots were air dried at room temperature. The dried plant leaves, stems and roots were powdered using a blender and stored at room temperature until further analysis.

3.3. Phytochemical screening

Phytochemical tests were carried out on the on the powdered specimen using standard procedures to identify constituents (Ajayi et al., 2011; Alabri et al., 2013; Alaga, Edema, Atayese, & Bankole, 2014; Arya, Thakur, & Kashyap, 2012; Maregesi, Mwakalukwa, Mwangomo, & Nondo, 2013; Shakeri, Hazeri, Vlizadeh, Ghasemi, & Tavallae, 2012; Suneetha et al., 2013; Upadhyay, Singh, & Kumar, 2010)

3.3.1. Test for steroids: the powder samples (1 mg) were dissolved in chloroform (10 ml) and added concentrated sulphuric acid (1 ml) into the test tube by wall sides. The colour of the upper layer turned red and the sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

3.3.2. Test for tannins: the grounded 0.5 g of each sample was separately boiled with 10 ml distilled water for five minutes in a water bath and was filtered while hot. 1 ml of cool filtrate was distilled to 5 ml with distilled water and a few drops (2-3) of 10% ferric chloride were observed for any formation of precipitates and any colour change. A bluish-black or brownish-green precipitate indicated the presence of tannins.

3.3.3. Test for saponins: the blended 1 g of each dried stain was separately boiled with 10ml of distilled water in a bottle bath for 10min. The mixture was filtered while hot and allowed to cool. The following test was then carried out. Demonstration of frothing**:** 2.5 ml of filtrate was diluted to 10ml with distilled water and shaken vigorously for 2minutes (frothing indicated the presence of saponin in the filtrate).

3.3.4. Test for terpenoids: exactly 5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated Sulphuric acid (H_2SO_4) was then added to form a layer. A reddish brown precipitate colouration at the interface formed indicated the presence of terpenoids.

3.3.5. Test for flavonoids: the powdered 1 g of dried leaves of each specimen was boiled with 10 ml of distilled water for 5 minutes and filtered while hot. Few drops of 20 % sodium hydroxide solution were added to 1 ml of the cooled filtrate. A change to yellow colour which on addition of acid changed to colourless solution depicted the presence of flavonoids.

3.3.6. Test for anthraquinones: chloroform (5 ml) was added to 0.5 g of the powdered dry samples of each specimen. The resulting mixture was shaken for 5 mins after which it was filtered. The filtrate was then shaken with equal volume of 10 % ammonia solution. The presence of a bright pink colour in the aqueous layer indicated the presence of free anthraquinones.

3.3.7. Test for alkaloids: one gram of powdered sample of each specimen was separately boiled with water and 10 ml hydrochloric acid on a water bath and filtered. The pH of the filtrate was adjusted with ammonia to about 6-7. A very small quantity of Mayer's reagent was added to about 0.5 ml of the filtrate in two test tubes and observed. The test tubes were observed for coloured precipitates or turbidity.

3.3.8. Test for coumarins: With each concentrated alcoholic extract of drugs, few drops of alcohol ferric chloride ($FeCl_3$) solution were added. The formation of deep green colour, which turned yellow on the addition of concentrated Nitric acid (HNO_3), indicated the presence of coumarins.

3.3.9. Test for Phenols: the extracts were dissolved in alcohol and a drop of neutral ferric chloride was added to these. The intense colour indicated the presence of phenolic compounds.

3.4. Extraction of dried powder samples

The extraction of the sample was carried out according to a method previously described by Naz and Bano (2013), with minor modification. The dry powder samples (20 g) were extracted with MeOH-DCM (1:1) solvent (200 mL) for 48 h. After extraction, the samples were filtered using filter paper (Whatman No.1). The extraction solvent was evaporated using a rotary evaporator (Heidolph Instruments GmbH & Co. KG, country) at 40^{o}C. The filtrate was transferred with little volume of ethyl acetate into pre-weighed vails for further drying at room temperature.

3.5. Quantitative phytochemical analysis

3.5.1. Total phenolics content

The total phenolic content was estimated by Folin Ciocalteu method as described by Rebaya et al. (2014) with slight modifications. The extracts (1mL) or the standard solution of Gallic acid (Sigma-Aldrich, Germany) (100, 200, 300, 400, and 500μg/ml) were mixed with 5 mL of distilled water, 1 mL of sodium carbonate (20%) and 1mL of Folin Ciocalteu reagent (Merck, Germany). The mixture was allowed to stand in a water bath for 30 min at 40°C. The absorbance was measured at 765 nm using microplate reader spectrophotometer. The total phenolic content was expressed as mg of gallic acid equivalents per g dry matter (mg GAE. g^{-1}DM). The experiment was run in triplicate and analysis was carried out.

3.5.2. Total Condensed Tannins

The tannin content was determined by a method described by Rebaya et al. (2014) with minor modification, using tannic acid (Sigma-Aldrich, Germany) as a reference compound. A volume of 400 µL of extract was added to 3 mL of a solution of vanillin (4% in methanol) and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation the absorbance was read at 500 nm using micro plate reader. The condensed tannin was expressed as mg TAE/g.

3.6. Antioxidant activity

Due to the complex nature of phytochemicals as well as the antioxidant activity determination dependence on reaction mechanisms, it is advisable for antioxidant activity to be evaluated using multiple assays (Zou, Chang, Gu, & Qian, 2011). Therefore, in this study the antioxidant property of plant extracts was determined using two antioxidant methods; DPPH free radical scavenging assay and reducing power assay.

3.6.1. DPPH radical scavenging assay

The DPPH radical scavenging activity assay described by Azlim et al. (2010) was adopted with slight changes. Different concentrations of extracts (7.81, 15.63, 31.25, 62.50, 125, 250, 500 µg/mL) were prepared. DPPH solution (0.012 mg/mL) was prepared by dissolving 1.18mg of DPPH (Sigma-Aldrich, Germany) in 100 mL ethanol in a foil covered bottle. Distilled water was added in the fi all the wells. Extracts and controls were added to the first row of wells. Ascorbic acid (Sigma-Aldrich, Germany) was used as a standard. Double dilute 100 µL from the first row downwards (discard the final 100 100 µL). DPPH solution (100 µL) was added to all triplicate wells except the wells of the extract control. The mixture was shaken vigorously and was left covered with a foil to stand in the dark for 30 min. The absorbance of the resulting solution was

measured spectrophotometrically at 517 nm using micro plate reader (manufactured by Molecular Devices LLC).

3.6.2. Reducing power assay

The reducing power was assayed as described by Deore et al. (2009) with some modifications. Different concentrations of ethanolic extracts (0.0625, 0.125, 0.25 and 0.5 mg/mL) were mixed with 1.25 ml of phosphate buffer (50 mM, pH 7.0) and 1.25 mL of 1% potassium ferricyanide. The mixture was then incubated at 50^{o}C for 20 min. After incubation, 1.25 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 1.25 mL of the supernatant was mixed with 1.25 mL of distilled water and 0.25 mL $FeCl_3$ solution (0.1%, w/v). The absorbance was measured at 700 nm. The assays were carried out in triplicate and the results were expressed as mean values ± standard deviations.

3.7. Antibacterial Activity

3.7.1. Disk diffusion assay

Antibacterial screening was performed using the disk diffusion method (Zarin et al., 2016). Antibacterial activity was based on clear zone formed around the disk. Complete inhibition was indicated by a clear zone, while partial inhibition was indicated by a semi-clear zone. Imipenem (10 µg) (OXOID LTD, England) was used as the reference antibiotic for antibacterial activity. Sterile distilled water (dH_2O) was used as negative control. The test microorganisms: *A. faecalis* (ATCC 8750), *E. faecalis* (ATCC 7080), *S. sonnei* (ATCC 25931) and *S. marcescens* (ATCC 8100) obtained from Department of Biological Sciences of the University of Namibia were seeded onto nutrient agar medium at 25^{o}C by using sterilized micropipette. The filter paper discs impregnated with the extracts of different concentrations (6.3, 3.2 and 1.6 mg/mL) were placed on

test microorganism-seeded plates. The plates were incubated at 37^{o}C for 24 h. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

3.7.2. Minimum inhibitory concentration (MIC).

MIC was determined by microdilution method with nutrient broth and Muller Hinton agar with few modification (Obiang et al., 2016). Briefly, nutrient broth (100 µL/wells) was distributed into wells of a microplate. One hundred microliters of extracts were added to the first row of wells and a two-fold dilution was added into other wells to make up 0.1-5.0 mg/mL concentration of each extract. Ninety microliters of nutrient broth and 10 µL of inocula were added into wells to prepare a total volume of 200 µL in each well. The plates were slightly shaken and incubated 37 °C overnight. Inhibition was assessed by observing the absence of turbidity in the wells. Wells without extract were used as negative control and Gentamycin (OXOID LTD, England) was used as a positive control.

3.8. Data analysis

The experimental results were expressed as mean ± standard deviation. All experiments were replicated three times. The 50% inhibitory concentration (IC_{50}) values were calculated using a nonlinear regression analysis in the Graphpad prism 6 software (Graphpad Software Inc., California, USA).

RESULTS

4.1. Phytochemical Screening

The present study revealed that extracts of leaves, roots and stem of *A. arenaria, A. esculenta*, and *P. leubnitziae* contained steroids, terpenoids, saponins, flavonoids, anthraquinones, coumarins, alkaloids, phenols, coumarins and tannins (Table 1). However, anthraquinones were only detected in *A. esculenta* root and stem as presented in Table 1. On the other hand, phytochemical such as steroids, phenols and tannins were found to be present in all extracts. In addition, tannins and phenols were detected in appreciable amounts (+++). Most phytochemicals were found to be present with less amount (+) in the *A. arenaria* root and stem extracts and were in appreciable amount (+++) in leaves extract (Table 1). Appreciable was measured according to the change in colours' intensity.

Table 1: Phytochemical screening results *A. esculenta, A. arenaria,* and *P. leubnitziae*'s roots, stems and leaves.

Plant constituent	**Plants Extracts**								
	Aloe esculenta			***Acacia arenaria***			***Pechuel-Loeschea leubnitziae***		
	Leaves	Root	Stem	Leaves	Root	Stem	Leaves	Root	Stem
Steroids	+	+++	+++	++	+	++	++	+	++
Terpenoids	++	–	–	+	–	–	–	+	+++
Saponins	–	+	–	+++	+	–	+	++	+
Flavonoids	+	++	–	+++	–	–	+++	+	–
Anthraquinones	–	+++	++	–	–	–	–	–	–
Coumarins	+	+	+	+	+	+	+	+	+
Alkaloids	+	++	+	+	+	+	–	+	+
Phenols	+++	+++	++	+++	+	+	+++	++	+
Tannins	+++	+++	++	+++	+	+	+++	++	+

(-) = absent, (+) = present, (++) = moderately present, (+++) = appreciable amount

4.2. Quantitative phytochemical analysis

4.3.1. Total phenolic and total condensed tannins content

The total phenolic content (TPC) was expressed as gallic acid equivalents (Table 2). The presence of TPC among the nine tested crude extracts was observed. The TPC was in the range of (0.5 ± 0.1 to 5.7 ± 0.1 mg GAE/g extract). Phenolic compounds are ubiquitous secondary metabolites in plants. They are known to possess antioxidant activity and it is likely that the activity of these extracts is due to this compounds (Rebaya et al., 2015; Motlhanka & Nthoiwa, 2012). The results

obtained in this study showed the greatest total phenolic content in *A. arenaria* leaves extract (5.7±0.1 mg of GAE/g extract) followed by *A. esculenta* root extract (4.8 ± 0.0 mg of GAE/g extract) and *A. esculenta* stem was the least with (0.5 ± 0.1 mg of GAE/g extract) as presented in table 2. The results on the other hand showed that *P. leubnitziae* stem, *A. arenaria* leaves and *A. esulenta* leaves extracts had high levels of TCT and low levels of TPC. The results also showed *A. esculenta* stem extract with the least TCT (0.1 ± 0.0 mg of GAE/g extract) table 2 below show the results.

Table 2: Total phenolic content and total condensed tannins of leaves, roots and stem extract from *A. arenaria, A. esculenta* and *P. leubnitziae*.

Samples	TCT (mg of TAE/g Extract)±SD	TPC (mg of GAE/g Extract)±SD
P. leubnitziae		
Stem	71.6 ± 0.0	1.6 ± 0.1
Root	1.0 ± 0.3	1.6 ± 0.0
Leaves	2.1 ± 0.1	3.1 ± 0.0
A. arenaria		
Stem	0.8 ± 0.2	2.9 ± 0.1
Root	0.1 ± 0.0	2.0 ± 0.1
Leaves	66.5 ± 0.1	5.7 ± 0.1
A. esculenta		
Stem	0.1 ± 0.0	0.5 ± 0.1
Root	0.9 ± 0.1	4.8 ± 0.0
Leaves	4.2 ± 0.0	1.4 ± 0.0

TCT: Total condensed tannins; TAE: Tannic acid equivalent; TPC: Total phenolics content; GAE: Gallic acid equivalent; SD: Standard deviation

4.3. Antibacterial Activity

4.3.1. Disc diffusion results

In vitro antibacterial activity of MeOH-DCM (1:1) crude extracts of nine extracts against the four bacteria was assessed on the presence or absence of inhibition zones. The various crude extracts from dry leaves, stems and roots of *A. arenaria*, *A. esculenta* and *P. leubnitziae* exhibited antibacterial potential against one gram positive bacterium (*E. faecalis*) and three gram negative bacteria (*A. faecalis*, *S. marcescens* and *S. sonnei*) at three concentrations; 6.3, 3.2 and 1.6 mg/ml. Not all the tested concentrations inhibited the tested microbes. However, *A. arenaria* root and *A. esculenta* leaves extracts showed the greatest inhibition (4.5 ± 0.7 mm) and (4.5 ± 0.0 mm) against *S. sonnei* and *A. faecalis* respectively at 6.3 mg/ml. Furthermore, *A. arenaria* stem extract showed inhibition (4.0 ± 0.0 mm) against *S. marcescens* and *E. faecalis* at 3.2 and 1.6 mg/ml respectively. In a similar manner *P. leubnitziae* root and *A. esculenta* leaves extracts showed (4.0 ± 1.4 mm) and (4.0 ± 0.0 mm) respectively against *E. faecalis* microbe. Additionally, *A. esculenta* root extract inhibited most microbes at different concentrations while *A. arenaria* leaves extract inhibited less microbes at different concentrations as compared to other extracts. Table 3 below shows the (inhibition zone±Standard Deviation) measurements were done in doublet and averaged. As expected, Imipenem the positive control demonstrated good inhibition at 10 µg/mL concentration.

Table 3: Antimicrobial screening of plants against *A. faecalis* (ATCC 8750), *E. faecalis* (ATCC 7080), *S. sonnei* (ATCC 25931) and *S. marcescens* (ATCC 8100) using disc diffusion method.

	Extract concentrations (mg/mL)											
	6.3				3.2				1.6			
Extract	Af	Ss	Ef	Sm	Af	Ss	Ef	Sm	Af	Ss	Ef	Sm
AcaL	-	-	3.0 ± 1.4	-	2.5 ± 0.7	-	-	-	-	-	3.0 ± 1.4	-
AcaR	3.0 ± 0.0	4.5 ± 0.7	2.0 ± 0.0	-	2.0 ± 0.0	-	-	2.0 ± 0.0	2.5 ± 2.1	-	1.5 ± 0.7	-
AcaS	1.0 ± 0.0	3.5 ± 2.1	2.5 ± 0.7	-	-	-	-	4.0 ± 0.0	-	-	4.0 ± 0.0	2.5 ± 0.7
PecL	2.5 ± 0.7	-	2.0 ± 0.0	-	2.5 ± 0.7	-	-	-	2.5 ± 0.7	2.5 ± 0.7	2.0 ± 0.0	-
PecR	3.0 ± 1.4	-	4.0 ± 1.4	1.0 ± 0.0	-	-	-	2.0 ± 0.0	-	-	3.0 ± 0.0	-
PecS	-	3.5 ± 3.5	1.3 ± 0.4	3.5 ± 0.7	-	-	-	-	3.5 ± 3.5	-	2.0 ± 0.0	-
AloL	4.5 ± 2.1	-	4.0 ± 0.0	-	3.5 ± 0.7	-	-	-	-	2.5 ± 2.1	2.0 ± 0.0	2.0 ± 0.0
AloR	3.5 ± 0.7	-	2.0 ± 0.0	-	3.5 ± 0.7	-	-	2.0 ± 0.0	2.5 ± 0.7	3.0 ± 1.4	3.5 ± 2.1	3.5 ± 0.7
AloS	3.5 ± 0.7	3.0 ± 0.0	-	2.0 ± 0.0	3.0 ± 1.4	-	3.0 ± 0.0	-	-	-	-	3.5 ± 0.7
IMI	14.0 ± 0	24.0 ± 0	-	14.0±0.0								

AcaL: *Acacia* leaves, AcaR: *Acacia* roots, AcaS: *Acacia* Stem, PecL: *Pechuel* leaves, PecR: *Pechuel* roots, PecS: *Pechuel* stem, AloL: *Aloe leaves*, AloeR: *Aloe roots*, AloS: *Aloe* stem, Af: *A. faecalis*, Ss: *S. sonnei*, Ef: *E. faecalis*, Sm: *S. marcescens*, (-) = no activity detected, units for values are in mm and IMI: Imipenem antibiotic.

4.3.2. MIC data

The result showed in Table 4 revealed the MIC values of MeOH-DCM (1:1) extracts of *A. arenaria, A. esculenta* and *P. leubnitziae.* The best activity was recorded with *A. arenaria* leaves extract, with MIC values ranging from 0.1 to 5.0 mg/mL against all tested bacteria. MIC values below or equal to 5.0 mg/mL were also recorded with *P. leubnitziae* leaves extract and Gentamycin antibiotic respectively against *E. faecalis*, *S. marcescens* and *S. sonnei*. The lowest MIC value (0.1 mg/mL) was recorded with the extract from *A. arenaria* leaves against *S. marcescens* and *S. sonnei* as well as the standard gentamycin against *S. sonnei*.

Table 4: MIC values (mg/mL) as determined by the microdilution method

Extract	*A. faecalis*	*E. faecalis*	*S. marcescens*	*S. sonnei*
	MIC	MIC	MIC	MIC
Acacia leaves	5.0	5.0	0.1	0.1
Acacia stem	>5.0	>5.0	>5.0	>5.0
Aloe leaves	>5.0	>5.0	>5.0	>5.0
Aloe stem	>5.0	>5.0	>5.0	>5.0
Aloe root	>5.0	>5.0	>5.0	>5.0
Pechuel leaves	>5.0	>5.0	>5.0	5.0
Gentamycin	>5.0	5.0	1.3	0.1

4.4. Antioxidant Activity

4.4.1. DPPH Assay

The free radical scavenging activity of *A. arenaria, A. esculenta* and *P. leubnitziae* crude extracts was assessed by the DPPH assay. Figure 9, 10 and 11 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of *A. arenaria, A. esculenta* and *P. leubnitziae.* The results show that *P. leubnitziae* roots extract had the highest DPPH radical scavenging activity with an IC_{50} value of 25.4 µg/mL followed by *A. arenaria* leaves with an IC_{50} value of 26.6 µg/mL. *A. arenaria* roots showed the least IC_{50} value of 196.8 µg/mL as shown in (table 5). The IC_{50} value of the positive control ascorbic acid was 83.7 µg/mL.

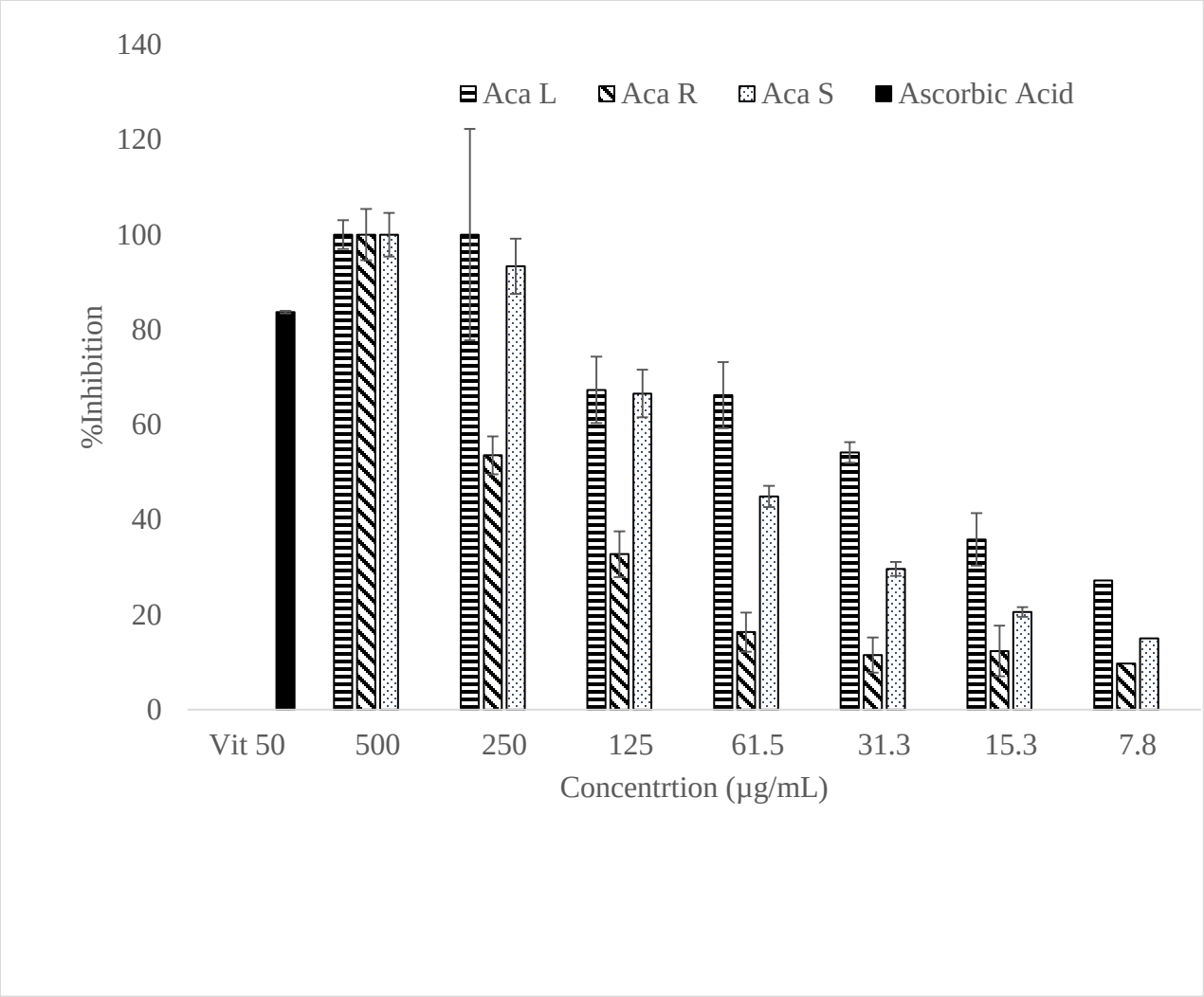


Figure 9: DPPH scavenging activity of *A. arenaria's* roots, leaves and stem extracts as compare to Ascorbic Acid. Aca L: *A. arenaria* leaves, Aca R: *A. arenaria* root and Aca S: *A. arenaria* stem.

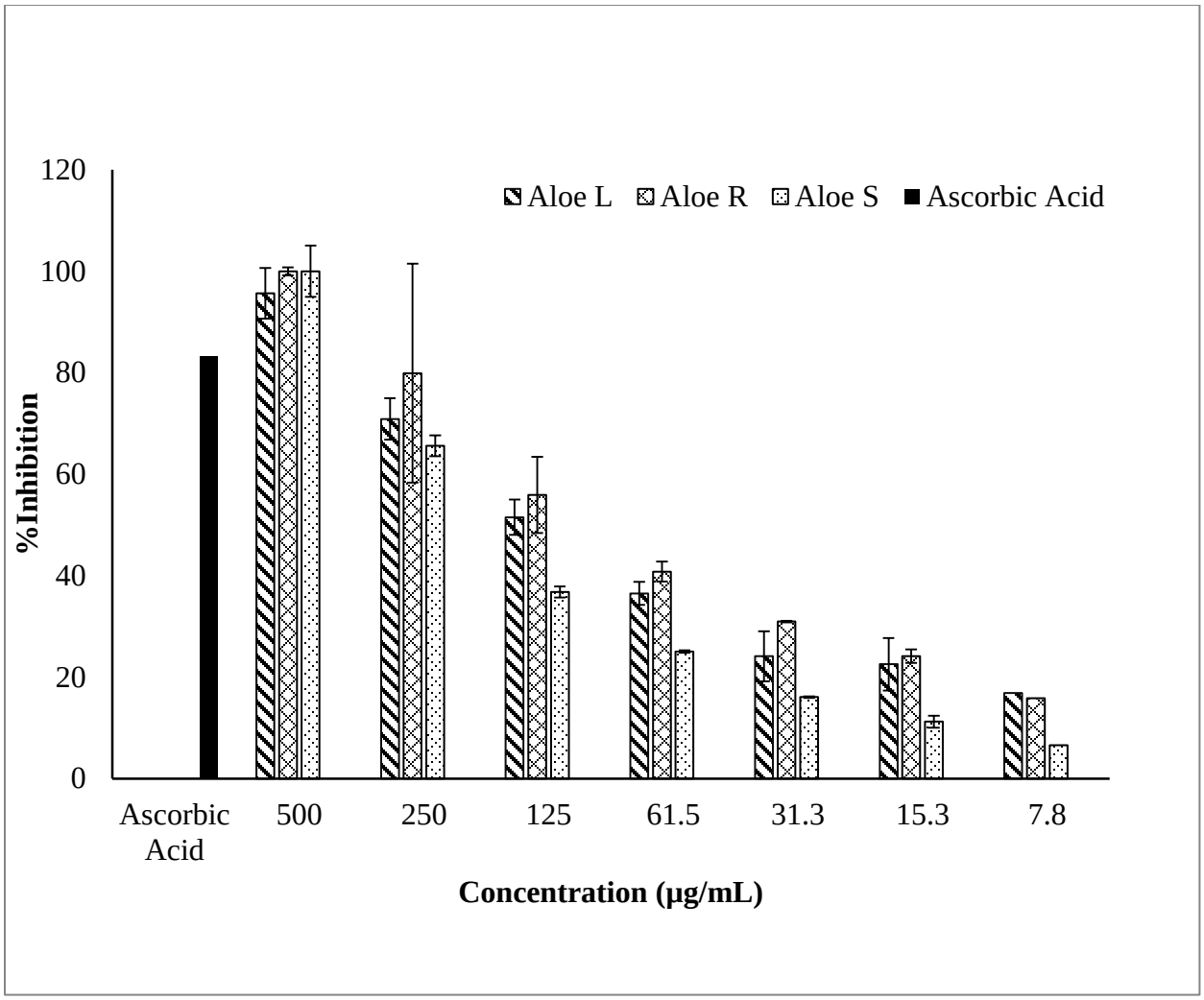


Figure 10: DPPH scavenging activity of *A. esculenta's* roots, leaves and stem extracts as compare to vitamin C. Aloe L: *A. esculenta* leaves; Aloe R: *A. esculenta* roots; Aloe S: *A. esculenta* stem; STD: standard, Ascorbic Acid.

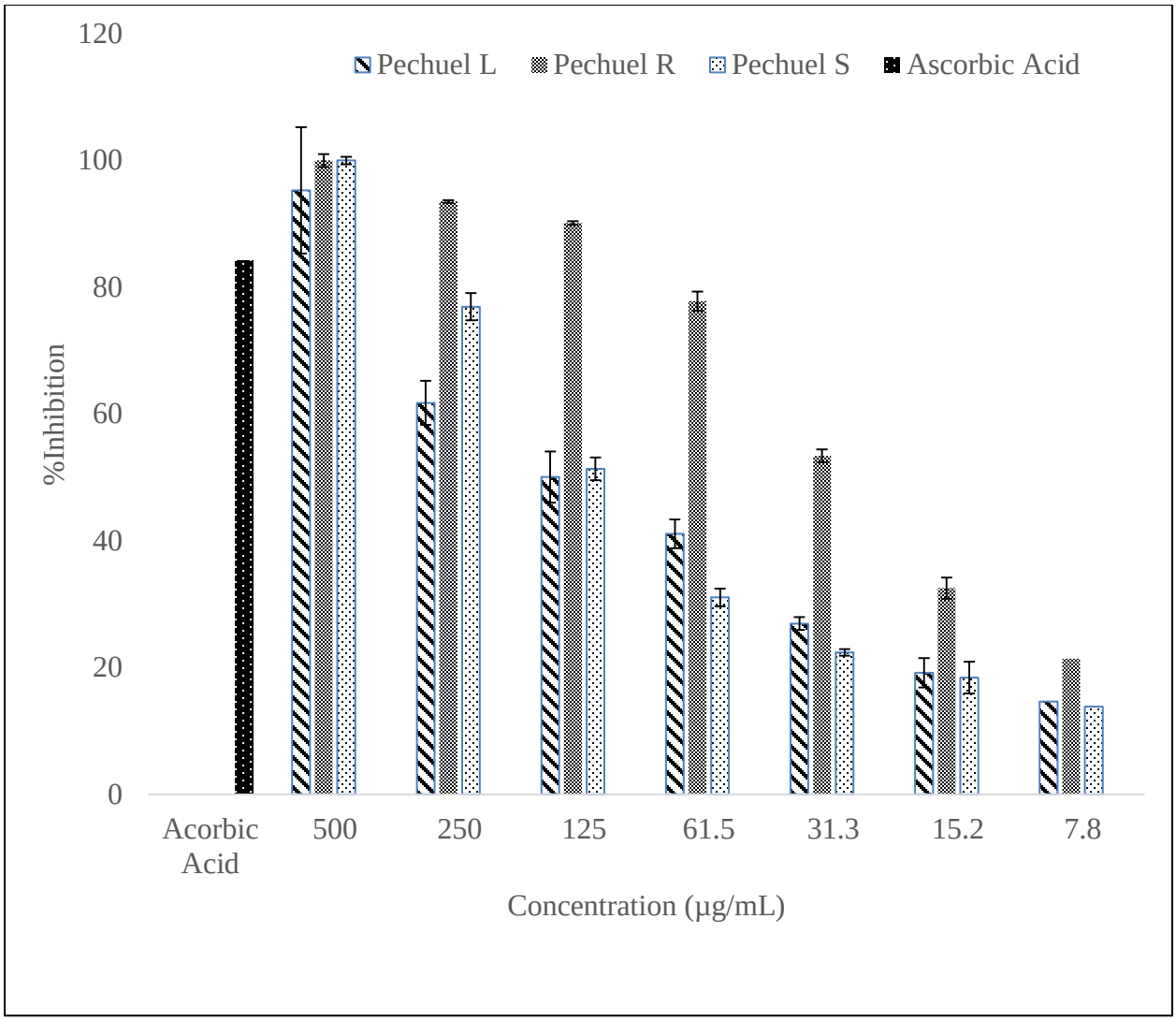


Figure 11: DPPH scavenging activity of *P. leubnitziae* roots, leaves and stem extracts as compared to vitamin C. Pechuel L: *P. leubnitziae* leaves; Pechuel R: *P. leubnitziae* root; Pechuel S: *P. leubnitziae* stem; STD: standard, Ascorbic Acid.

Table 5 is showing the IC_{50} values of leaves, roots and stem extracts of *A. arenaria*, *A. esculenta* and *P. leubnitziae*. A comparable scavenging activity was observed between the extracts and the standard, Ascorbic Acid. The IC_{50} of extracts ranged between (25.4 ± 0.7 µg/mL) of the root extract of *P. leubnitziae* greater than that of the Standard and (196.8 ± 14.6 µg/mL) of the root extract of *A. arenaria* lower than that of the standard.

Table 5: DPPH free radicals *A. arenaria, A. esculenta* and *P. leubnitziae* leaves, roots and stem extracts.

Sample	**IC_{50}±SD (µg/mL)**
A. arenaria	
Leaves	26.6 ± 12.9
Roots	196.8 ± 14.6
Stem	59.0 ± 2.1
A. esculenta	
Leaves	97.9 ± 4.3
Roots	68.0 ± 5.9
Stem	139.2 ± 3.2
P. leubnitziae	
Leaves	96.0 ± 3.6
Roots	25.4 ± 0.7
Stem	95.5 ± 2.0

4.5.2. Reducing power assay

The reducing power was found to be concentration dependent (Fig. 12-14). The reducing power of *A. arenaria, A. esculenta* and *P. leubnitziae* leaves, root and stem extracts except *P. leubnitziae* leaves increased with an increase in concentration. At 0.5 mg/mL, the leaves extract of *A. arenaria* showed an absorbance of 0.269 (Fig. 12) as the highest followed by *P. leubnitziae* leaves extract at 0.062 mg/mL with an absorbance of 0.083 (Fig. 13). *Aloe esculenta* and *P. leubnitziae* root extracts exhibited the lowest reducing ability at 0.125 mg/mL and absorbance of 0.00 (Fig. 13 and 14).

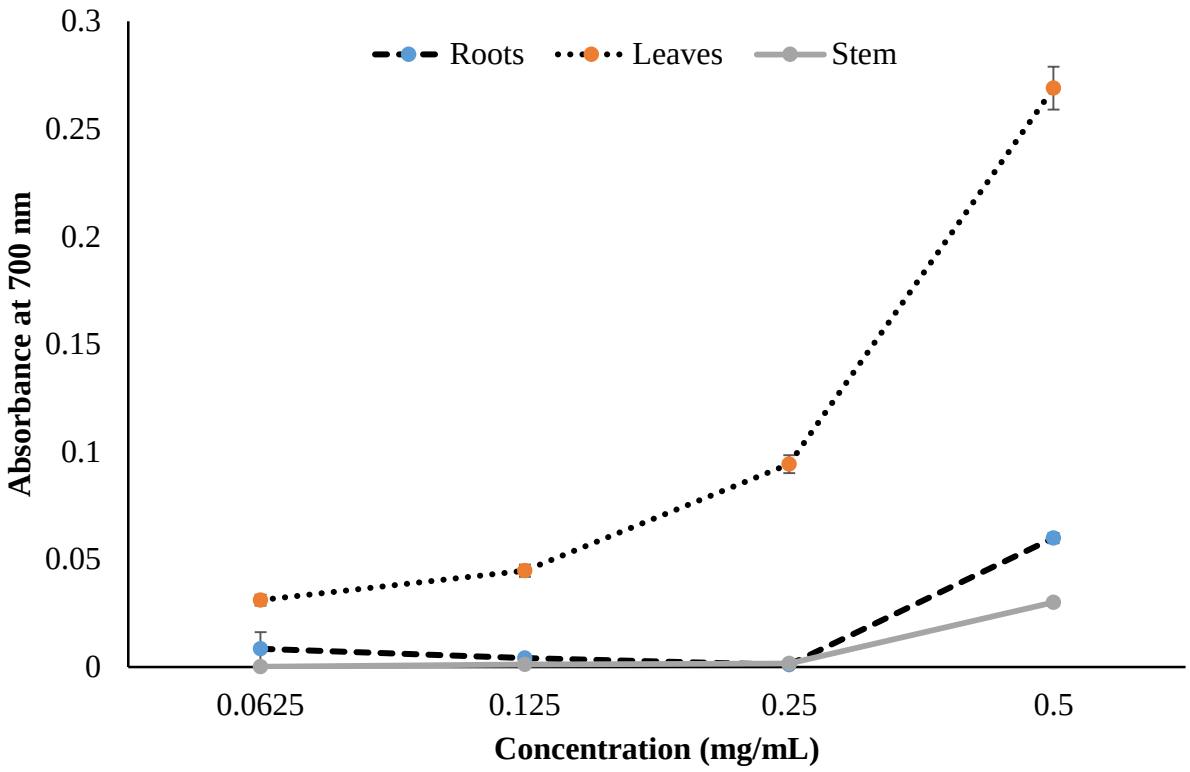


Figure 12: Reducing power activity of *A. arenaria* roots, leaves and stem extracts. Aca R- *A. arenaria* roots; Aca L- *A. arenaria* leaves and Aca S- *A. arenaria* stem.

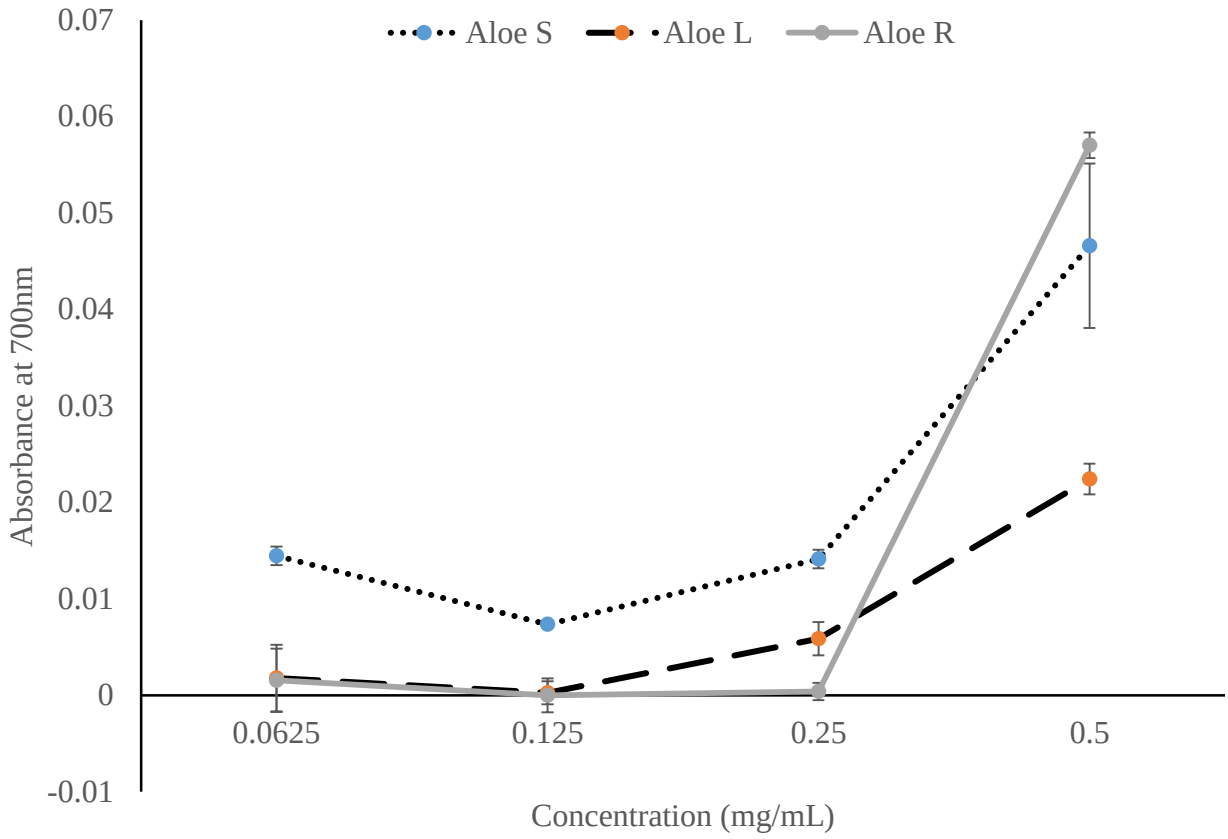


Figure 13: Reducing power activity of *A. esculenta* roots, leaves and stem extracts. Aloe S- *A. esculenta* stem; Aloe L- *A. esculenta* leaves; Aloe R- *A. esculenta* roots.

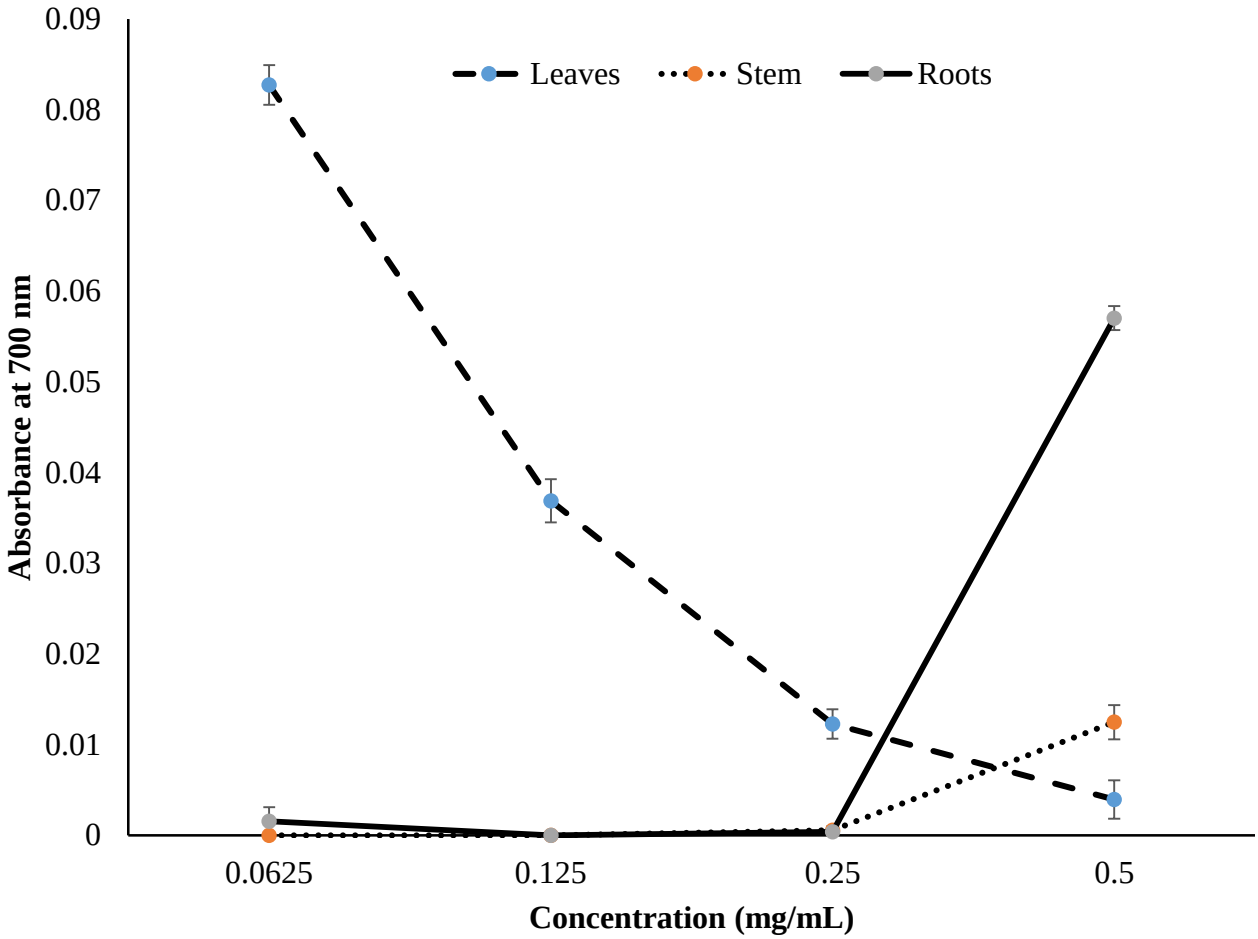


Figure 14: Reducing power activity of *P. leibnitziae* roots, leaves and stem extracts. Bitter L- *P. leubnitziae* leaves; Bitter R- *P. leubnitziae*; Bitter S- *P. leubnitziae* stem.

DISCUSSION

Phytochemicals are bioactive, non-nutrient and naturally occurring compounds in plants (Cheikyoussef, Summers, & Kahaka, 2015). Several phytochemical compounds detected in *A. arenaria, A. esculenta* and *P. leubnitziae* plants extracts (Table 1) are known to have health benefits, physiological activities and medicinal significance (Iikasha, 2016). Steroids, saponins, terpenoids, flavonoids, coumarins, phenols and tannins were tested positive in all three plants. These results are in agreement to the previous studies done on other species by Abdel-Farid et al. (2014) and Atiku, Oladipo, Forcados, Usman, and Mancha (2016) who found that *Acacia nilotica* and *Acacia seyal* have saponins, phenolics and flavonoids and *Acacia seiberian*a possess flavonoids, tannins, steroids, alkaloids and saponins. It is also in agreement with Hedimbi et al. (2012a) who reported on the presence of saponins, flavonoids and polyphenols in *P. leubnitziae*. On the contrary the qualitative phytochemical results of *A. esculenta* slightly differed with those of *Aloe vera*, the extensively research *Aloe* plant. Most phytochemicals detected in this study in *A. esculenta* were not reported in *A. vera* (Lawrence, Tripathi, & Jeyakumar, 2009). However, anthraquinones were only found present in *A. esculenta* roots and stem extracts but absent in *A. arenaria* and *P. leubnitziae*. The results suggested that there are similarities and differences among species of the same genus. This is in agreement with Cheikyoussef et al. (2015) who indicated that there are differences and similarities in the chemical profiles of two species as well as variation between populations from different regions.

In the present study, quantitative phytochemical results clearly indicated (Table 2) that amongst all test extracts, *A. arenaria* leaves extract had the highest amount of phenolic compounds and the lowest phenolic content was obtained in *A. esculenta* stem extract. The high phenolic content in the *A. arenaria* leaves extract correlates with those in other species from the same family such as

Acacia nilotica (9.5 mg GAE/g), *Acacia seyal* (10.2 mg GAE/g) and *Acacia laeta* (9.9 mg GAE/g) (Abdel-Farid et al., 2014). The high content of phenolic compounds in *Acacia* species may be the reason for increasing the potentialities of theses extracts as antioxidants. Since antioxidant activity of many plants extracts is attributed to the presence of phenolic compounds (Abdel-Farid et al., 2014). Condensed tannins are polyphenolic compounds that play an important role in stabilizing lipid oxidation and are also associated with antioxidative property (Tamilselvi et al., 2012). The condensed tannin content of the extracts in tannic acid equivalents was obtained. The highest condensed tannin content was observed in *P. leubnitziae* stem extract and the lowest was observed in *A. esculenta* stem extract. The quantified TPC and TCT correlated to the observed antioxidant activity in all the tested extracts. Tannins reportedly have a good stringent properties (Pratibha et al., 2012). In addition, tannins are known to fasten the healing of wounds and inflamed mucous membrane (Yadav, Chatterji, Gupta, & Watal, 2014). Condensed tannins have been associated with the antioxidant activity of food and plant extracts (Cheikyoussef et al., 2015).

The *in vitro* antioxidant activity of extracts under study were determined using the DPPH assay. Antioxidant activity of all extracts showed that when gradually increasing the samples concentration there was an increase in the % inhibition. A lower IC_{50} value is an indicative of good antioxidant activity (Pratibha et al., 2012). The dose-dependent from previous study showed that IC_{50} for *A. nilotica* and *A. seyal* leaves extracts were higher than that of *A. arenaria* leaves extract (Abdel-Farid et al., 2014). The *A. esculenta* leaves extract (97.9 µg mL^{-1}) (table 5) was also found to possess a lower IC_{50} than that of the *A. vera* leaves extract (5, 200 µg mL^{-1}) the most studied species of *Aloe* species (Bawankar et al., 2013). In the present study, the experimental data indicated that though all the tested extracts demonstrated good radical scavenging activity, the highest DPPH radical scavenging activity was observed in *P. leubnitziae*, followed by *A. arenaria* leaves and *A.*

arenaria stem whereas *A. arenaria* roots' extract demonstrated moderate DPPH radical scavenging activity. *Acacia arenaria, A. esculenta* and *P. leubnitziae* extracts showed a dose-dependent reducing power activity.

Screening for *in vitro* antibacterial activity using imipenem (10 µg) as a positive control clearly indicated that *A. arenaria, A. esculenta* and *P. leubnitziae* leaves, stem and roots extracts had antibacterial activities against the four test microorganisms namely *A. faecalis, E. faecalis, S. sonnei* and *S. marcescens*. According to Alabri et al. (2014), the antibacterial activity of plant crude extracts depends on the dose and the type of bacterial strains employed. This might be the reason why variation in the inhibition zones at different concentration was obtained. The highest zone of inhibition (4.5 mm) was observed for *A. arenaria* root extract and *A. esculenta* (6.3 mg/mL) against *S. sonnei* and *A. faecalis* (table 3). Little ethyl acetate was used to remove concentrated extracts from the round bottom flask the study inhibition zones are correlating to the values of ethyl acetate *A. vera* extract (1.0-9.0 mm) inhibition against gram negative and positive bacteria (Thiruppathi, Ramasubramanian, Sivakumar, & Thirumalai, 2010). According to Thiruppathi et al. (2010), ethyl acetate extract was found to give the best antibacterial results against most pathogenic bacteria. Furthermore, generally the extracts showed greater antibacterial activity against Gram-positive as compared to Gram-negative bacteria (Lawrence et al., 2009). The reason behind this is that, Gram-negative bacteria have additional lipopolysaccharide layer (Lawrence et al., 2009). In this study, results of the extracts at 1.6 mg/mL are almost in agreement to the above reason. A strong relationship between the total phenolic content and antioxidant activity due to its attachment to the cell walls, cell membranes and interference with membranes functions like electron transport, protein synthesis and enzyme activity it made it to be a good antibacterial agents. Thus, phenolic

compounds could lead to the destruction of pathogens (Pratibha et al., 2012), which explains the antibacterial activity observed in the present study.

The findings of this study also showed a difference in MIC of plants extracts against *S. sonnei, S. marcescens, A. faecalis* and *E. faecalis*.It is important to note that MIC value lower or equal to 5 mg/mL was recorded for *A. arenaria* leaves extract against all tested bacteria. The lowest MIC value recorded was 0.1 mg/mL with *A. arenaria* and gentamycin against *S. marcescens* and *S.sonnei*. The MIC values ranged from 0.1-5.0 mg/mL of the acacia leaves were almost closer to that of *A. nilotica* in previous study which ranged from 1.6–3.1 mg/mL (Sadiq et al., 2017).

Although traditional uses for selected plants in this study are documented, scientific validations, antioxidant and antibacterial efficacies of *A. arenaria,* and *A. esculenta* do not exist. In addition antioxidant efficacy of *P. leubnitziae* also does not exit. Nevertheless, the information provided by traditional healers and other knowledge holders during ethanobotanical surveys serves as an important goal toward documentation of the uses, of many medicinal plants. All these validates that traditional plant knowledge is of important value, not just for local uses, but also to researchers for the development of novel antioxidant and antibacterial medicines. The findings of this study served as justification to validate the use of *A. arenaria, A. esculenta* and *P. leubnitziae* in combating infections causing pathogens as well as scavenging free radicals in Namibia. On the other hand, it verified the presence of phytochemical compounds associated with antioxidant and antibacterial activities.

CONCLUSION

This study contributed to the evaluation of Namibian ethnomedicinal plants for search of new chemical entities with known antioxidant and antibacterial properties. Alkaloids, flavonoids, terpenoids, saponins, phenols, tannins and steroids were found to be present in the *A. arenaria, A. esculenta* and *P. leubnitziae*. Furthermore, anthraquinones were only found present in *A. esculenta*. In addition, the study demonstrated total phenolics and condensed tannins contents in three selected plants. All plants showed good antibacterial activity against *A. facaelis, E. faecalis, S. sonnei* and *S. marcescens* and also possessed antioxidant activity. Alkaloids, saponins, phenols, tannins, steroids, terpenoids, flavonoids, anthraquinones and coumarins were identified as possible classes of phytochemicals responsible for the antibacterial and antioxidant activity of *A. arenaria, A. esculenta* and *P. leubnitziae*.

The study findings supported the use of these plants to treat bacterial infections in traditional settings. The further identification of the bioactive components in these plants will serve as a basis for in-depth pharmacological evaluation and isolation of antibacterial and antioxidant phytochemicals for possible drugs development. In addition, the results can assist in herbal drug formulations of the plants studied.

RECOMMENDATION

A number of aspects of this research need to be further investigated to confirm the observed activities. The assays used in this study provided good antioxidant and antibacterial results. In order to confirm the activities of the promising extracts, more in-depth studies are necessary. These includes making use of other antioxidant assays such as total antioxidant, total flavonoids, superoxide etc. The antioxidant potential of the extracts will then be compared through different methods. A bioassay-guided fractionation and purification of the crude extracts is also recommended as this will give more information on the specific compounds active in the crude extracts. Testing several Namibian medicinal plants (as numerous as possible) should be carried out in order to establish a national database of possible lead plants. Several solvents can be used for extraction to ensure both polar and non-polar compounds are extracted. Due to escalating deforestation in our country there is a need that the communities be educated on the importance of these plants in order to preserve them.

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APPENDIX A: L-Ascorbic Acid Calibration Curve

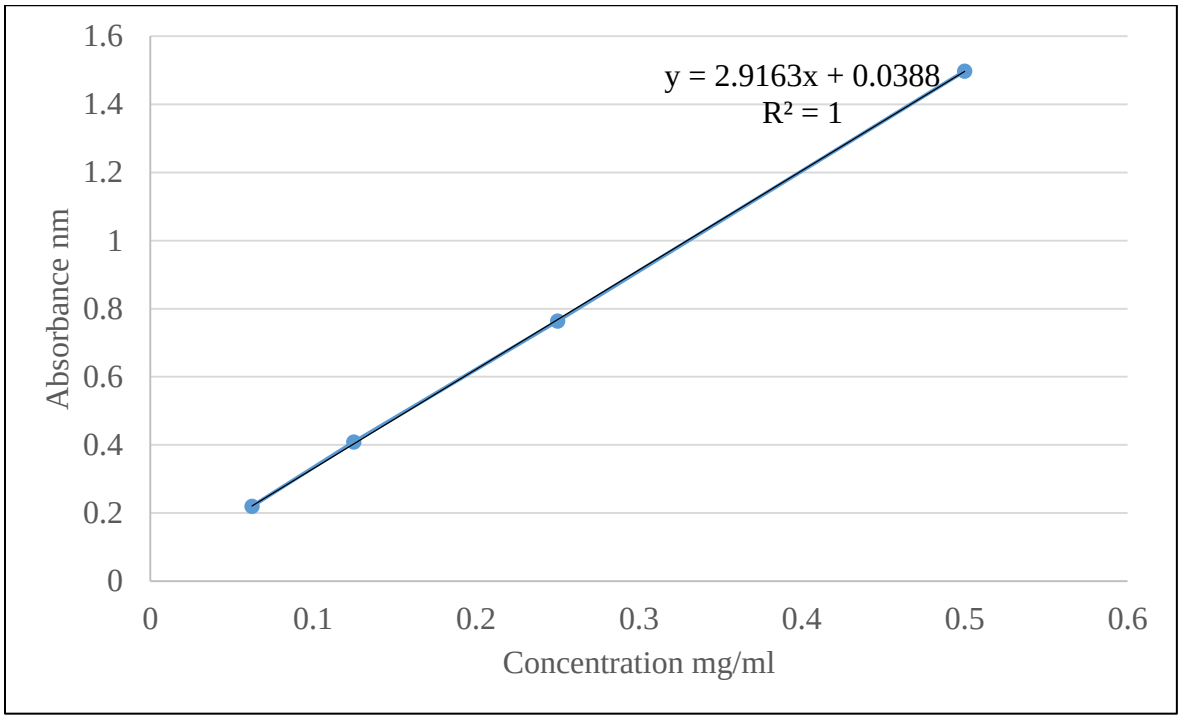

Figure 15: L- Ascorbic Acid Calibration Curve

APPENDIX B: Gallic Acid Calibration Curve

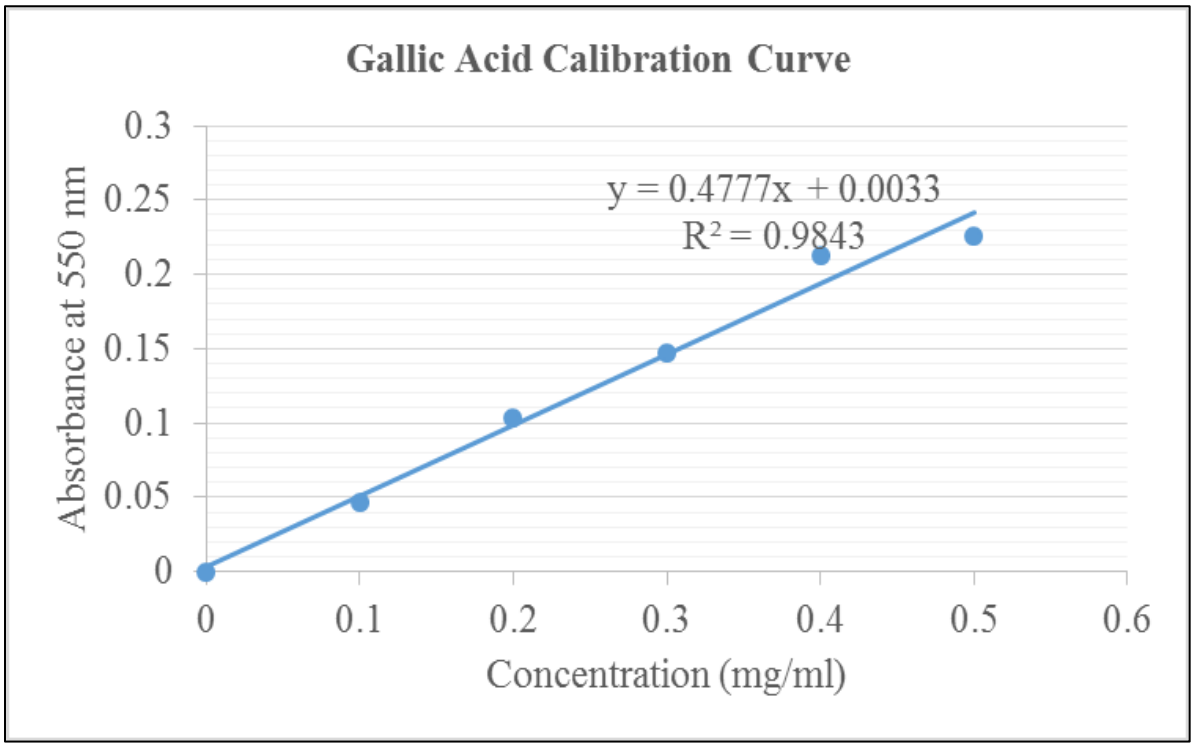

Figure 16: Gallic Acid Calibration Curve

APPENDIX C: Tannic Acid Calibration Curve

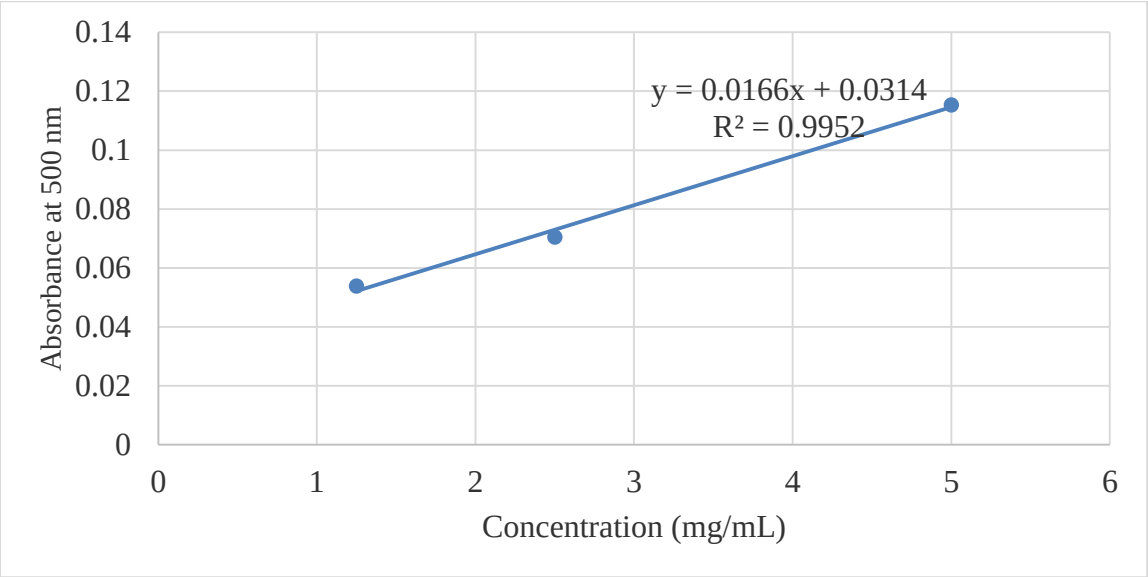

Figure 17: Tannic Acid Calibration Curve

APPENDIX D: Ethical clearance certificate

UNAM
UNIVERSITY OF NAMIBIA

STUDENT ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: FOS/51/2015 **Date: 17 August, 2015**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's **Research Ethics Policy and Guidelines. Ethical** approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

Title of Project: Antiviral, Antibacterial and Antioxidant Investigation of Acacia Arenaria, Aloe Esculenta and Pechuel-Loeschea Leubnitzuae
Nature/Level of Project: Masters

Principal Researcher: Daniel Ndongo

Student Number : 200732838
Host Department & Faculty: Faculty of Science

Supervisors : Dr. P. Kapewangolo (Main) Dr. M. Kandawa-Schulz; Ms. C. Mukakalisa (Co)

Take note of the following:

(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.
(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.
(c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.
(d) The UREC retains the right to:
   (i). withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
   (ii). request for an ethical compliance report at any point during the course of the research.

UREC wishes you the best in your research.

Prof. I. Mapaure
UNAM Research Coordinator
ON BEHALF OF UREC

APPENDIX E: Research/collecting permit.

MINISTRY OF ENVIRONMENT AND TOURISM

RESEARCH/COLLECTING PERMIT

Permit Number 1992/2014
Valid from 1 January 2015 to 31 December 2015

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:

NAMIBIAN POLICE
WANAHEDA
04 MAR 2015
CHARGE OFFICE

Name: **Dr. P. Kapewangolo**
Address: **Department of Chemistry and Biochemestry**
Faculty of Science
Universtity of Namibia
Windhoek
Coworkers: **M. Kandawa- Schulz and M. Hedimbi**

Biological screening of medicinal plants / herbs used traditionally for antiviral treatment at the University of Namibia, subject to attached conditions.

IMPORTANT: This permit is not valid if altered in any way.

MINISTRY OF ENVIRONMENT AND TOURISM, REPUBLIC OF NAMIBIA
2014 -12- 12
WINDHOEK
Private Bag 13306, Windhoek
Tel: 284 2111 • Fax: 258851

Authorizing Officer

IMPORTANT
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.

Enquiries: Warden, email clouw@met.na
Private Bag 13306, Windhoek, Namibia

I certify that this document is a true reproduction/ copy of the original which was examined by me and that from my observations the original has not been altered in any manner.
Signature

EPPENDIX F: Research permission letter

☎ (+264 61) 206 3111
Website: www.unam.na

340 Mandume Ndemufayo Avenue
Private Bag 13301
Windhoek
NAMIBIA

Inspiring minds & shaping the future

Date: **8 October 2015**

TO WHOM IT MAY CONCERN

RE: RESEARCH PERMISSION LETTER

1. This letter serves to inform that student: **Daniel Ndongo**, (Student number: **200732838**) is a registered student in the Department of Chemistry and Biochemistry at the University of Namibia. His/her research proposal was reviewed and successfully met the University of Namibia requirements.
2. The purpose of this letter is to kindly notify you that the student has been granted permission to carry out postgraduate studies research. The School of Post- graduate Studies has approved the research to be carried out by the student for purposes of fulfilling the requirements of the degree being pursued.
3. The proposal adheres to ethical principles.

Thank you so much in advance and many regards.

Yours truly,

Name of Main Supervisor: **Dr. Petrina Kapewangolo**

Signed: _____

Dr. C. N.S. Shaimemanya

Signed: _____

Director: School of Postgraduate Studies
Tel: 2063523
E-mail: cshaimemanya@unam.na