BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES OF ROOT EXTRACTS AND ISOLATED COMPOUNDS OF *HERMANNIA GENICULATA*

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BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES OF ROOT EXTRACTS AND ISOLATED COMPOUNDS OF *HERMANNIA GENICULATA*

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A thesis submitted in partial fulfillment of requirement for the award of doctor of philosophy degree (PhD) in Plant Sciences (Phytomedicine and Phytopharmacology)

To

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General abstract

Hermannia geniculata is of the genus of flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae. H. geniculata* has been used in treatment of several diseases like colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Basotho women.

Phytochemical analysis revealed the presence of saponin, phenols, flavonoids, alkaloids, tannin, phytosterol, triterpenes and anthraquinone. Ethanolic extract exhibited the highest free radical scavenging capability with the lowest IC_{50} value (0.52, 0.38, 0.59, 0.63, 0.39 mg/mL) for 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-Azino-bis(3-ethylbenzothiazoline-6-Sulphonic acid (ABTS), hydroxyl radical, superoxide anion radical, metal chelating ability which is significantly lower ($p<0.05$) than the standard silymarin while hydro-ethanol has the highest reducing power showing a significant ($p<0.05$) IC_{50} value of 0.24 mg/mL compared to citrate (IC_{50} value; 0.5 mg/mL). In antidiabetic studies, ethanolic extract was a potent inhibitor of α-glucosidase (IC_{50}; 0.01 mg/mL) which is significantly lower ($p<0.05$) than standard acarbose IC_{50} value (0.52 mg/mL) and hydro-ethanol decoction and aqueous extracts. It also has a milder percentage inhibition of α-amylase enzyme with IC_{50} (0.57mg /mL) which is significantly higher ($p<0.05$) than the standard acarbose IC_{50} (0.047 mg/mL). The mode of inhibition of α-amylase is by competitive inhibition and uncompetitive inhibition of the α-glucosidase enzyme was observed in ethanolic extract. These findings provide an empirical rationalization for the use of the root extract of *Hermannia geniculata* in the management of diabetes mellitus and other oxidative stress induced ailments.

The Vero, HepG2 and RAW 264.7 macrophage cell lines were used to determine the toxicity of the extracts on cells. Similarly, the capabilities of the extract to inhibit 5-lipoxygenase enzyme activities and overproduction of nitric oxide from LPS-activated RAW 264.7 macrophages were evaluated. Results showed selective toxicity of the extracts with LC_{50} values of Vero cells ranges from (0.40-0.57 mg/mL) while the LC_{50} value of HepG2 cells varies from (0.016-0.136 mg/mL). The selectivity index (SI) were (31.87, 18.87, 33.33 and 3.52) for ethanol, hydro-ethanol, decoction and aqueous extracts respectively. The ethanolic extract inhibited NO production in a concentration dependent manner. There was a decrease of 82% at concentration of 0.1 mg/mL and the LC_{50}:3.64 mg/mL is lower and significantly different ($p<0.05$) compared to the reference compound quercetin with LC_{50} value of 8.28 mg/mL. Similarly, the ethanolic extract exhibited potent inhibition of 5-lipoxygenase enzyme with the lowest IC_{50} value of 0.14 mg/mL which is significantly different ($p<0.05$) compared to all other extracts and indomethacin. The GCMS chromatograms revealed five compound (2-keto-butyric-acid, 2, 2-Bis (4-nitrobenzyl)-1-phenylbutane-1,3-dione, n-Undecane, 1,4,5,8-tetrathiadelin and imidazo-1,5-pyrimidine) which has been reported to have antioxidant, anti-inflammatory and antifungal properties. This result suggested that *Hermannia geniculata* roots extract is not toxic and possesses antioxidant, antinflammatory and anticancer activities which could be exploited in development of new safe and effective drugs.

The chemical profiling and *in vitro* biological activities of flavonoids of *Hermannia geniculata* (FHG) roots was also investigated using High Pressure Thin Layer Chromatography (HPTLC) finger print analysis. Antioxidant, antidiabetic anti-inflammatory activities and the ability of FHG extract to inhibit the production of nitric oxide (NO) in lipopolysaccharide (LPS) activated RAW264.7 Macrophage were investigated using standard methods. The selective cytotoxicity of

the extract on Vero and HepG2 cells was also determined. Kaempferol (R_f 0.81) was detected in the extract, its R_f value is similar and comparable with the kaempferol standard used (R_f 0.80). Other flavonoids were also present in the extracts with their R_f values of 0.08-0.95. The FHG extract showed commendable antioxidant properties with IC_{50} values (3.07± 0.12, 2.13± 0.67) for DPPH and ABTS radicals which was lower and significantly different ($p<0.05$) compared to standard silymarin with IC_{50}: (3.55± 0.10, 2.77± 0.75) for DPPH and ABTS respectively. The results indicated milder inhibition of α-amylase with IC_{50}: (5.55± 0.37) which was higher and significantly different from the standard acarbose with IC_{50}: (3.81± 0.29) Nevertheless, the extract exhibited 73% inhibition of α-glucosidase which exerted better inhibitory effect on 5-lipoxygenase enzyme than indomethacin with their respective IC_{50}: (10.15± 0.02 and 12.03± 0.02). Inhibition of NO production was observed in LPS activated RAW 264.7 Macrophages with the highest concentration of 0.1 mg/mL decreasing NO production by 87%. Selective toxicity of Vero and HepG2 cells with their respective LC_{50} value of (>1 and 0.02 mg/mL) was also observed. The antiproliferative potentials of the extract was confirmed with Selectivity Index of 50. This study indicated for the first time that FHG extract was non-toxic to normal cells and possess antioxidant, antidiabetic, anti-inflammatory and antiproliferative activities.

The bioactive constituent and pharmacological activities of phenols extracted from *Hermannia geniculata* (PoHG) roots was investigated using *in vitro* methods. The chemical profile was determined by HPTLC analysis. Antioxidant, antidiabetic, anti-inflammatory and cytotoxic effect of PoHG on Vero and HepG2 cells was carried out using standard procedures. Phenolic compounds were detected in the sample at R_f (0.14, 0.81 and 0.95). PoHG radical scavenging capabilities on DPPH, $ABTS^+$ and superoxide anion radicals were similar to the standard (silymarin). The IC_{50} values were DPPH (0.12± 0.00), ABTS (0.13± 0.01) and superoxide anion

(0.20± 0.00). The values of the metal chelating activity of PoHG extract is lower and significantly different from the standard (silymarin) their respective IC_{50} values were (0.06± 0.00 and 0.18± 0.01). The antidiabetic effect was determined by its ability to mildly inhibit α-amylase and strongly inhibit α-glucosidase enzymes, the respective IC_{50} values obtained were (7.52± 0.23 and 1.76 ± 0.14). PoHG extract exhibited a commendable inhibition of 5-lipoxygenase enzyme with IC_{50} value of (0.15 ± 0.03) which is similar to the IC_{50}: (011 ± 0.01) value for the standard (indomethacin). However, the extract was non-toxic to Vero cells with LC_{50} value of >1.00 mg/mL but highly toxic to HepG2 cells with LC_{50}: 0.08 mg/mL. The selectivity index of 12.50 was recorded. The presence of phenolics/ carboxylic acids were also confirmed in the extract, the result of the antioxidant, antidiabetic and antinflammatory activities of PoHG suggested that the phenols extract may be useful in the management of oxidative stress induce diseases, type 2 diabetes mellitus and asthma. It is also safe for use and its antiproliferative activities can be exploited in search for anticancer agents.

A new xanthene derivative Hermannol (9-(7-methyloctyl)-9H- xanthene-2,3-diol) was isolated from the roots of *Hermannia geniculata* . The structure was elucidated by analysis of their 1D, 2D NMR, MS and IR spectroscopic data. The compound displayed good antioxidant and antidiabetic activities.

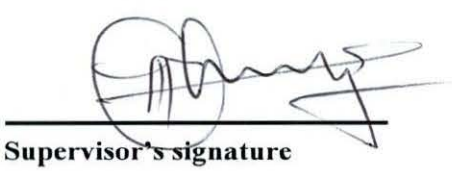
In conclusion, it is evident from the study that different crude extracts of *H. geniculata* roots and its bioactive constituents (flavonoids and phenols), the isolated compound (Hermannol) is non-toxic and possess varied degree of antioxidant, antidiabetic, antiiflammatory and antiproliferative activities which can be exploited for new drug development.

INTELLECTUAL PROPERTY RIGHT AGREEMENT

The plant was sourced from the accredited herb sellers and was rewarded financially with the agreement that the research will be a source of providing information on the biological and pharmacological activities of the plant.

COMPLIANCE STATEMENT

The commercialization of any part of this study in any form has not been done and discouraged. The thesis is written to be a tool for the disseminating information to the traditional healer, researchers and pharmaceutical industries about the use and efficacy of *Hermannia geniculata* roots.



Supervisor's signature



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Chapter One

General Introduction

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General Introduction

As early as the beginning of human existence, early man has familiarized himself with the use of plants in several ways. Primitive man wandering and gathering food also looked for ways of coping with suffering associated with their lifestyle starting from using plant as covering from environmental vagaries, as a shade to rest for the purpose of regaining strength, after which the distinction of medicinal and pharmacological action of plants started to evolve. This relationship between man and plants has grown tremendously and many plants has been used as medicine (Street and Prinsloo, 2013).

Moreover, the leap in growth and knowledge of disease continues at an accelerating pace and several drugs were derived from plants (Baydoun, Wahab, Bano, Imad, & Choudhary, 2016; Khan et al., 2016; Maiese, 2015).

Nature has bestowed Southern Africa with rich plant biodiversity. The South African flora accounted for 9% of world higher plants population, which comprises of more than 30,000 species (Braam Van Wyk, 2000). The use of plants in Southern Africa dated back to about 5000 years ago and it was used for cure and prevention of diseases and to avert evil influences by the ancient tribes (Possa & Khotso, 2015; Shakya, 2016). Many ancient belief that diseases was caused by evil and witchcraft which could be solved with the use of charm and medicinal plants (Possa & Khotso, 2015; Shakya, 2016). This medical system is practiced in other parts of the world as complementary medicine (Shakya, 2016).

Plant based traditional form of medicine play a crucial role in the health care system. World Health Organization have therefore described it as a surest means of attaining total health care coverage for the global population (Mukherjee & Wahile, 2006). Most people in rural communities depends principally on the use of plant to treat mild to moderate ailments. (Modak, Dixit, Londhe, Ghaskadbi, & Paul Devasagayam, 2007; Possa & Khotso, 2015; Shashank & Abhay, 2013).

In the 21st century the demand for herbal based medicine for pharmaceuticals, food supplements, nutraceuticals, health products and cosmetics is increasing worldwide. Phytomedicine or herbal medicine is regarded as the use of plants for medicinal and therapeutic purposes which include curing of ailment and general improvement of human health (Cragg & Newman, 2013; Rates, 2001). Plants have secondary metabolites called phytochemicals. These are compounds present in the plants which it functions to protect the plants against microbial and pest invasion (Mehran & Sangeeta, 2014; Wink, 1988). Phytochemicals are biologically active ingredients in plants which possess therapeutic values and are considered as drugs (Online, Bucar, Wube, & Schmid, 2013; Shen, 2015; Stalikas, 2007).

Classification of phytochemicals is based on their chemical compositions and structures. Most of the phytochemicals are formed by acetate or shikimate pathways (Amorati & Valgimigli, 2015; Dewick, 2002; Pandey & Rizvi, 2009; Sa, Sa, & Em, 2014; Yin, Zhang, Feng, Zhang, & Kang, 2015). These different classes of phytochemicals found in plants include, phenolic compounds, flavonoid, tannins, terpenoids, alkaloids, steroids, saponins, and glycosides (Malla, Koffas, Kazlauskas, & Kim, 2012; Russo, Valentão, Andrade, Fernandez, & Milella, 2015; Sabiu, O'Neill, & Ashafa, 2016; Shashank & Abhay, 2013). Phytochemical show different pharmacological activities and this determine the therapeutic efficacies of a particular plant. The observed variations may be as a result of soil type, genetic composition and environmental stress (Mbhele, Balogun, Kazeem, & Ashafa, 2015; Mfotie, Munvera, Mkounga, Nkengfack, & McGaw, 2017; Olaokun, Mcgaw, Rensburg, Eloff, & Naidoo, 2016; Shashank & Abhay, 2013).

Some phytochemicals have been implicated in the inhibition of enzyme activities. Enzymes like α- amylase, α- glucosidase, lipoxygenase, cyclooxygenase, nitric oxide synthase has been inhibited by different phytochemicals (Alam et al., 2016; Elisha,

Dzoyem, McGaw, Botha, & Eloff, 2016; Mfotie et al., 2017; Ogundajo, Okeleye, & Ashafa, 2017; Sabiu et al., 2016).

Also, plant phytochemicals has been implicated in causing cell's apoptosis through the enhancement of P53 protein expression, induction and upregulation of many pro-apoptotic family like Baz, Bad, Cytochrome C, caspase 8, 3, 9 and inhibition of PI3k/Akt pathway (Bhatia, Mandal, Nevo, & Bishayee, 2015; Machana, Weerapreeyakul, Barusrux, & Nonpunya, 2011; Podolak, Galanty, & Sobolewska, 2010; Tuñón, García-Mediavilla, Sánchez-Campos, & González-Gallego, 2009).

Moreover, phytochemicals have free radical scavenging capabilities (Belmouhoub, Bribi, & Iguer-ouada, 2017; Ogundajo et al., 2017). Free radicals are biomolecules produced *in vivo* and has been involved in causing harmful effect of the body. Free radicals are radical or non-radical derivatives of oxygen or nitrogen and they include singlet oxygen, hydroxyl, superoxide anion, nitric oxide and peroxyl radicals. They are constantly produced in the body by different enzymes like cyclooxygenase, lipoxygenase, xanthine oxidase, aldehyde oxidase cytochrome P-450 monoxygenase and different metabolic processes (Egea et al., 2017; Kurutas, 2016). The presence of these biomolecules in small quantity is beneficial to the body for cellular response and immune function but overproduction lead to oxidative stress (Egea et al., 2017). Although the body system is endowed with endogenous free radicals scavengers like superoxide dismutase, catalyze, glutathione dependent peroxidase, tocopherol, ascorbic acid but when the oxidoreductase equilibrium is unfavourable in pathological conditions it can cause overproduction of reactive species which causes oxidative stress (Angelini et al., 2017; Jomova & Valko, 2011; Maiese, 2015).

Oxidative stress can simply be regarded as an imbalance between pro-oxidant/free radical production and opposing antioxidant process (Shashank & Abhay, 2013). Acute and

chronic oxidative stress have been implicated in many degenerative diseases like diabetes mellitus, insulin resistance, artheriosclerosis, pulmonary diseases, cancer, isheamic perfusion injury, inflammatory diseases, hypertension and ocular degenerative diseases (Amorati & Valgimigli, 2015; Odeyemi, Afolayan, & Bradley, 2015; Rani, Deep, Singh, Palle, & Yadav, 2016; Zhou et al., 2017). In addition, oxidative stress may lead to induction of programmed cell death through apoptosis and autophagy of β-cells (Selassie, Kapur, Verma, & Rosario, 2005).

Challenges involved in the use of herbal drugs include the use of plants without scientific knowledge and guidance for thousands of years, thus the need for scientific validation of the traditional use of these medicinal plants. In addition to this, people consider plants as a natural healing (Ji, Li, & Zhang, 2009; Raskin et al., 2002) but it has been scientifically established that every part of plants have varying medicinal properties and also not all parts of plants are safe for health purposes. Medicinal plant may harbor toxic compounds which can be harmful to the body (Shakya, 2016) therefore it is imperative to conduct exhaustive toxicity studies to determine the safety of medicinal plants (Shakya, 2016). Furthermore, identification of the biologically active phytochemical constituents responsible for the pharmacological activities displayed by the plant could be investigated through the processes of extraction, chemical profiling, isolation and characterization. This processes will assist in the discovery and development of new drugs, quality control, standardization and clinical use of medicinal plants.

The choice of *Hermannia geniculata* Eckl. & Zeyh for the study.

Hermannia geniculata also known by the Basotho tribe as 'kgwakgwa' is among the popular species frequently used for medicinal purpose in South Africa (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008; Moffett, 1993). It is of the genus of flowering plant from the subfamily *Byttnerioideae* in the narrow family *Malnaceae* (Leistner, 2000). It is

a creeping shrubs, the leaves are sub-orbicular, the length of the leaf is about 15 mm, and the leaf texture may be viscid or sticky. *Hermannia geniculata* is readily identified by its hanging flowers, a typically green calyx encloses the base of free petals with five petals which are contorted with transversely expanded filament. The filament is abruptly expanded and contracted beneath the base of the anther into a cruciform filament (Gwynne-evans, 2015).The plant is seen across South Africa its vast majority being endemic in Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga. It is also found in Madagascar, East Africa, North-East Africa and Saudi Arabia. Decoctions of *Hermannia geniculata* is often used in the traditional Basotho medicine (Braam Van Wyk, 2000; Gwynne-evans, 2015). It is used in the management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant BaSotho women. Also, the dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders (Moffett, 1993).

The four main criteria necessary to be considered during the selection or investigation of a plant for drug discovery include random collection, collection based on chemotaxonomy, bio-rational collection and lastly collection based on traditional knowledge (Fabricant & Farnsworth, 2001; Rates, 2001; Shen, 2015). The choice of *Hermannia geniculata* roots to determine its biological and pharmacological activities was based on traditional knowledge collection criterion, this criterion has been described as the basis of Phytomedicine (Rodrigues, 2007).

Objective of the study

The general objectives of the study is to validate the traditional use of *Hermannia geniculata* by scientifically establishing its various pharmacological and biological properties using *in vitro* techniques, conduct the chemical profiling of its biologically active constituents and to discover a drug candidate through isolation and characterization of chemical compound present in the plant roots.

Antihyperglycaemic and Antioxidant Activity of the Plant

Diabetes mellitus is a chronic metabolic disease, it occurs when the pancreas is not producing insulin or the produced insulin cannot be used by the body, this may lead to raise blood glucose levels (Nsiah-kumi et al., 2012). The number of people living with diabetes is expected to rise from 366 million in 2011 to 552 million by 2030 (American Diabetes Association, 2010). There are basically two types of diabetes, type 1 and type 2 diabetes mellitus (Wilkin, 2009). The observed defective insulin production and or insulin resistance observed in type 2 diabetes had been tightly linked to oxidative stress which causes destruction of β-cells. (Bhuiyan, Mitsuhashi, Sigetomi, & Ubukata, 2017). Oxidative stress can be regarded as an imbalance between pro-oxidant/free radical production and opposing antioxidant defenses. Acute and chronic oxidative stresses have been implicated in a number of degenerative diseases, such as atherosclerosis, diabetes mellitus, ischemia/reperfusion (I/R) injury, Alzheimer's disease, inflammatory diseases neurodegenerative diseases, hypertension, ocular diseases, pulmonary diseases, and hematological disorder (Liu et al., 2013; Odeyemi et al., 2015).

Due to the alarming annual growth rate of this disease burden, it has raised concern for the scientific community to do pharmacological evaluation of either raw or isolated natural products in experimental studies (WHO, 2016). *Hermannia geniculata* is among the medicinal plant species frequently used in South Africa for the management of different diseases. (Balogun, Tshabalala, & Ashafa, 2016; Kazeem & Ashafa, 2015; Moffett, 1993). *H. geniculata* has been used in the treatment of several diseases like colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Sotho women (Moffett, 1993). Nevertheless, the is no scientific validation of the use of this plant

therefore this study was undertaken to provide the scientific basis for the traditional use of this plant root through the in vitro studies. Qualitative and quantitative phytochemical screening of *H. geniculata* root extracts was studied through conducted *in vitro* antioxidant and the effect of the extracts on key carbohydrates enzymes of α- amylase and α-glucosidase enzymes. Also, their respective enzyme kinetics was investigated. Porcine pancreatic α-amylase, rat intestinal α- glucosidase, gallic acid, silymarin, acarbose were enzymes and standards used. The products were obtained from (Sigma-Adrich, South Africa). Other chemicals and reagents used were of analytical grade and the water was glass distilled.

In vitro Toxicological and Pharmacological Activities of the Plant

According to world health organization about 80% of people around the world depend on the use of medicinal plant to manage different kind of diseases (Dijk, 2011). Toxicities associated with use of medicinal plants has been documented which ranges from minor to major organopathy (Ernst, 1998; Wojcikowski, Johnson, & Glenda, 2004). Plants that can be considered safe for medicinal plants use should have low toxicities and show no adverse effect on the consumers. Thus, long and short-term effect of biologically active medicinal plants on genome, cells, tissue, organs, and the body system is required in order to increase confidence in the safety to human and in the development of pharmaceuticals.

Cytotoxicity tests using specialized cells have proved most useful when the *in vivo* toxicity of a plant extract is already well established thus the *in vitro* investigations using cell cultures can be used to clarify the mechanisms of toxic action on the target tissue (Ekwall, Silano, & Zucco, 1990). Selective toxicity *in vitro* can also be determined using normal and cancer cells (Badisa et al., 2010). In addition to cytotoxicity testing, cell culture systems are useful to carry out metabolism studies including biotransformation, interaction with endogenous metabolites, binding to cells, and induction of metabolism. This provide

useful insight into the pathogenesis of some human diseases and its probable mechanism of action (Al-qubaisi et al., 2011).

Animal studies have been conducted to evaluate the toxicity profile of the root extract of the plant on Wistar albino rats (Kazeem & Ashafa, 2015). The result showed that there was systemic toxicities after two weeks administration of 5000 mg/kg body weight of the plant extract. However, at a lower dosage of between (75-300 mg/kg) of the extract for a period of 28 days a slight reduction in haematological parameters was identified but histopathological analyses of the organ revealed no significant effect on the heart, liver, lung and kidney.

This current study was undertaken to further investigate the cytotoxic effect of *Hermannia geniculata* roots extracts on African green monkey kidney epithelial cells (Vero). To determines its safety. Its inhibitory activities on 5-lipoxygenase enzyme and hepatocellular carcinoma cells (HepG2) were also determined. To determine its anticancer mechanism of action, its effect on excessive production of NO in a lipopolysaccharides (LPS) activated RAW 264.7 macrophages cells was evaluated. In order to rationally postulate on the anti-inflammatory, antioxidant and anticancer properties of the plant. The chemical constituent of the most active extract (ethanolic extract) was also carried out using GC-MS techniques.

Biological Activities of Flavonoids

Phytomedicine or herbal medicine simply represent the use of plants for treatment and curing of diseases in order to improve human health (Ji et al., 2009; WHO, 2016). Plants contains chemicals and this phytochemicals are product of secondary metabolism which were produced by the plants primarily to protect the plants from herbivores, microbial and pest invasion (Micheal, 1988).

Some of different classes of plant's secondary metabolites which include phenolic compounds (tannins, phenylpropanoids, flavonoids), terpenoids, nitrogen compounds

(alkaloids, indoles, amines, amino acids) steroids, glycoside and saponins (Grotewold, 2006).

Flavonoids are plant secondary metabolites which are non-nutrient, less toxic, effective at low concentration, environmentally friendly and biologically active (Grassi, Desideri, & Ferri, 2010). Flavonoids are biosynthesized in plants through the phenyl propanoid pathway, it occurs through transformation of phenylalanine into 4-coumaroyl-CoA, which finally entered the flavonoid biosynthesis pathway (Martens, Preuß, & Matern, 2010). Major sub-groups of flavonoids that are found in higher plants include chalcones, flavones, flavonols, isoflavones, flavanones, anthocyanins, proanthocyanidins and aurones (Falcone Ferreyra, Rius, & Casati, 2012) In animals, *in vitro* and *in vivo* studies supported the beneficial effect of dietary flavonoids on glucose homeostasis (Cai & Lin, 2009; Jung, Kim, & Choi, 2009) Furthermore, it has been shown to regulate carbohydrate digestion, insulin secretion, insulin signaling and glucose uptake in insulin-sensitive tissues through various intracellular signaling pathways (Hanhineva et al., 2010). Recent findings suggested that flavonoids are safe and possess antioxidant, antidiabetic, anticancer, anti-inflammatory activities (Belmouhoub et al., 2017; Nagulsamy, Ponnusamy, & Thangaraj, 2015; Park et al., 2017; Tafesse, Hymete, Mekonnen, & Tadesse, 2017).

Based on the above knowledge, the present investigation was undertaken for the first time to evaluate the selective cytotoxicity of flavonoids from *Hermannia geniculata* on Vero and HepG2 cells, as well as their antioxidant, anti-inflammatory (through the inhibition of NO production in RAW 264.7 cells activated with lipopolysaccharide and 5-lipoxygenase enzyme), antidiabetic through inhibition of α-glucosidase, α-amylase enzymes and their respective mechanism of action of flavonoids on these carbohydrates metabolizing enzymes to achieve our objectives. The HPTLC finger printing of the extract was carried out to profile its chemical constituents.

Phenols Pharmacological Activities

Plant based traditional medicine system has been playing crucial role in the health care system worldwide (Raskin et al., 2002). The South African flora accounted for 9% of world higher plants population, which comprises of more than 30,000 species (Street & Prinsloo, 2013). Many people depends on the use of medicinal plant due to affordability and unavailability of health care facilities (WHO, 2016).

Hermannia geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases. (Essop et al., 2008; Kazeem & Ashafa, 2015; Moffett, 1993). *H. geniculata* has been used in treatment of several diseases like colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Sotho women (Kazeem & Ashafa, 2015; Moffett, 1993).

Plant contains secondary metabolites called phytochemicals. These phytochemicals yielded pharmacologically active compounds which possess therapeutic efficacies on several endogenous enzymes, biomolecules and reactive oxygen species which are body metabolites capable of causing severe harm to the organism (Egea et al., 2017).

Phenols are plant secondary metabolites which were biosynthesized in plants through two pathways. The acetate/malonate pathway and the shikimate pathways produce intermediate phenylpropanoid precursors like phenyalanine, cinnmic acid, ρ-coumaric acid and ρ-coumaroyl CoA which are product for the biosynthesis of phenolic carboxylic acid and its derivatives (Dewick & Haslam, 1969; Falcone Ferreyra et al., 2012; Thomas Vogt, 2010).

Polyphenols contain numerous variety of compounds like flavonoids, phenolic acids and anthocyanidins. Several studies have reported the medicinal properties of phenolic

compound found in plants such as anti-inflammatory, anticancer and antidiabetic (Ford-Hutchinson, Gresser, & Young, 1994; Ji et al., 2009; Olaokun et al., 2016; Yao, Zhu, Chen, Tian, & Wang, 2013). Research findings described its capabilities to inhibit production or scavenge reactive oxygen and nitrogen species like superoxides and peroxynitrites (Park et al., 2017; Shashank & Abhay, 2013) which may lead to cell death. Phenolic compound has been shown to modulate lipid and carbohydrate metabolism, dyslipidemia, insulin resistance, alleviate inflammatory process and thus a useful agent for treatment of asthma and colitis (Fang, Su, & En, 2008; Busse, McGill, & Horwitz, 1999; Elisha et al., 2016; Moharram & Youssef, 2014; Ullah, 2016).

This study was carried out to determine the activities of the phenols present in *Hermannia geniculata* root. We studied the antioxidant, antidiabetic, antinflammatory and determine the cytotoxicity of the phenolic extract on Vero and HepG2 cells. The chemical profiling of the phenols extract was carried out using HPTLC finger print to achieve our objectives.

Pharmacological activities of New 9-(7-methyloctyl)-9H-xanthene-2,3-diol isolated from the roots of *Hermannia geniculata* Eckl. & Zeyh.

Hermannia geniculata is a genus of flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae* (Essop et al., 2008). It is well adapted to different ecological conditions and can be seen occupying diverse habitats including the Drakensberg Mountains and the sea spray zone of the coastal regions of South Africa. The plant is endemic in Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa. Lesotho, Madagascar, (Essop et al., 2008; Rebelo & Siegfried, 1990) *Hermannia geniculata* is among the medicinal plant species frequently used in South Africa for the management of different diseases. (Balogun et al., 2016; Kazeem & Ashafa, 2015; Moffett, 1993). This study was designed to test the *in vitro*

pharmacological activities *H. geniculata* roots and to isolate, characterized compounds in the crude extract and also test its pharmacological activity

Structure of the thesis

This thesis was compiled as a draft of manuscripts and accepted manuscripts for publication. The literature review of medicinal plant use and various methodologies used to study the biologically active components was presented in chapter two. Chapter three described the scientific validation of the anti-hyperglycaemic and antioxidant activities of *H. geniculata* root extracts. *In vitro* toxicological and pharmacological activities of the plant was presented as chapter four. The Biological activities of flavonoids was described in chapter five and summation of Phenols Pharmacological activities was captured as chapter six. The pharmacological properties of a new xanthene derivatives (Hermannol) isolated from the root of *H. geniculata* was presented in chapter seven while chapter eight is the general discussion, conclusion and recommendations.

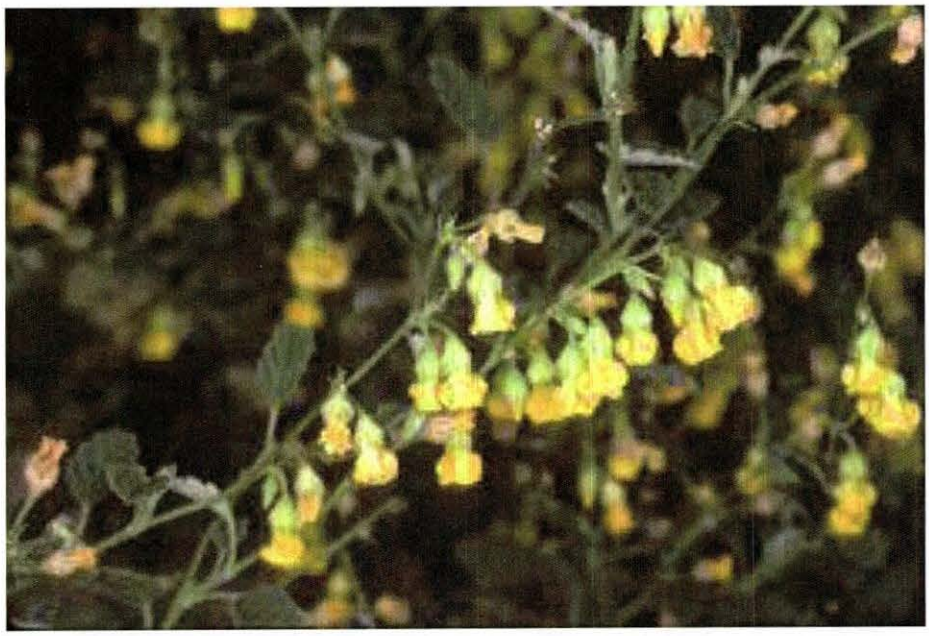


Fig. 1 (A). Pictorial representation of *Hermannia geniculata* Eckl. & Zeyh showing the leaves and flowers (Goldblatt & Manning, 2000).



Fig. 1 (B). Pictorial representation of *Hermannia geniculata* Eckl. & Zeyh showing the roots

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Introduction

Traditional medicine contributes a great role in the life of many people around the world and especially in the African society who do not have access to western medicine (WHO, 2016). Mostly, people in the rural communities and sub urban areas still depend principally on the traditional herb sellers and herbalists for mild to moderate illness and medical emergencies (Balogun, Tshabalala, & Ashafa, 2016; Possa & Khotso, 2015). Also, in developing countries herbs usage is driven by economic issues and accessibility (Possa & Khotso, 2015; Shakya, 2016).

Traditional medicine is defined as the aggregate of skill, knowledge and practices that is based on indigenous culture, belief, theory and experiences whether its scientific or not but can manage and maintain health and also cause an observed improvement in the social, physical, spiritual and mental wellbeing of an individual (WHO, 2016).

Use of herbal remedies is as old as mankind and it continues to evolve over the centuries in different communities and are still preserved as an inherited traditional knowledge which is passed down different generations (Mukherjee & Wahile, 2006).

'Medicinal' means something that has ability to heal and may include drugs, plants, spices, herbs, fruit and seeds (Hornby, Wehmeier, & Ashby, 1995).

Herbs have been used in the treatment of diseases like cold, cough, headaches, infertility, diabetes, inflammatory diseases and various infectious diseases (Braam Van Wyk, 2000; Dijk, 2011; Gwynne-evans, 2015; Moffett, 1993). Herbs preparation may be in concoction, infusion, febrifuge, laxatives and many people practiced self-medication (WHO, 2016).

Hermannia

The genus *Hermannia* is of the family *Malnaceae* in the major group of flowering plant It consist of 65 species (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008; Leistner, 2000).

They are diverse and an attractive group of plants with highly ornamental flowers, fitting into a variety of habitats and growth forms.

Hermannia genus is made up of small shrubs, ranging from upright to sprawling prostrate shrublets. They are characterized by the presence of minute glandular or star-like hairs on the leaves and stems with dark grey bark. The leaves are alternate and entire, lobed or incised. Flowers consist of five petals which are slightly or spirally twisted into an upended rose. Most *Hermannia* species possess a thick woody stem and root, forming an underground stem, which enables the plants to survive dry periods and fires (Gwynne-evans, 2015).

Distribution

The distribution is mainly across the flora of Southern Africa area and it is endemic in all provinces of South Africa (Braam Van Wyk, 2000). The genus is also found in Madagascar, and extends through Kenya, Tanzania, Sudan, Egypt and Saudi Arabia. A single species (*Hermannia tigrensis*) is found in West Africa. There are three species in northern Mexico, United States and Australia. The greatest diversity is within the Western and Northern Cape and Namibia (Balogun et al., 2016; Braam Van Wyk, 2000; Essop et al., 2008; Gwynne-evans, 2015; Moffett, 1993).

Uses of *Hermannia*

The genus was used by the Europeans, Kwena, Tswana, Sotho, Xhosa, Zulu and the San (Essop et al., 2008). The plants were used for diarrhoea, fever and cough. It was also used for treatment of burns and eczema. It serve as diaphoretic, aphrodisiac and prevention of flatulence in pregnant women and it is employed in the treatment of heartburn, colic, haemorrhoids, convulsion and syphilis (Kareru, Gachanja, Keriko, & Kenji, 2007; Watt & Breyer-Brandwijk, 1962). The Xhosa use it for treatment of dysuria. The antispasmodic,

antiplasmodium, antihelminthes, antioxidant and anti-inflammatory activities of the plants has also been recorded (Essop et al., 2008). Many members of the genus are used medicinally for many things ranging from respiratory diseases, coughs, internal aches, stimulants or purgatives, to soothing wounds and cuts (Moffett, 1993). The common name *pleisterbos* (*Hermannia cuneifolia*) refers to the use of the leaves as plasters. In some areas the leaves were infused in a tea, and used to cleanse the blood. A root infusion was used by the early European colonial settlers against epilepsy. A lotion of the leaf was used for eczema and shingles. Certain species have magical significance and are used to drive out spirits and to wash the divining bones. *H. depressa* is used as a protective charm by the Zulus and their antihelmithes, antiplasmodial activities has been documented (Essop et al., 2008; Possa & Khotso, 2015).

Hermannia Species that are commonly found in South Africa include: *Hemannia althaeifolia* L, *H. cristata* Bolus, *H. concinnifolia* l. Verd, *H. cuneifolia* Jacq Var Cuneifolia, *H depressa* N.E Br, *H. desermifolia* Jacg, *H. Hifilifolia* Lf VAr grandicalyx l. verd, *H grandifolia* Aiton, *H. linearifolia* Harv. *H. Saccrifera* (Turez) K. schum *and H. geniculta* Ekyl. Zeyh (Gwynne-Evans, 2015; Leistner, 2000).

Hermannia geniculata

It is of the genus of flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae* (Essop et al., 2008). It is a creeping shrub, the leaves are sub-orbicular crenate, the length of the leaf is about 15 mm, and the leaf texture may be viscid or sticky. *Hermannia* geniculata is readily identified by the hanging flowers, a typically green calyx which encloses the base of five free petals which are contorted with transversely expanded filament. The filament is abruptly expanded and contracted beneath the base of the anther into a cruciform filament (Gwynne-evans, 2015). *Hermannia geniculata* is well adapted to different ecological conditions and it can be seen occupying diverse habitat including

the Drakensberg Mountains, the sea spray zone of the coastal regions of South Africa. The plant is endemic in Eastern Cape, Free State, Guateng, KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa. Lesotho, Madagascar, (Essop et al., 2008; Rebelo & Siegfried, 1990).

Hermannia geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases. (Balogun et al., 2016; Kazeem & Ashafa, 2015; Moffett, 1993). *H. geniculata* roots has been used in treatment of several diseases like colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Sotho women (Moffett, 1993).

Phytomedicine

Phytomedicine simply represent the use of plants for treatment and curing of diseases in order to improve human health (Ji, Li, & Zhang, 2009; WHO, 2016). Plants contains chemicals and this phytochemicals are product of secondary metabolism which were produced by the plants primarily to protect the plants from herbivores, microbial and pest invasions (Micheal, 1988). They are non-nutrient materials which are not useful for plant growth and development. Nevertheless, phytochemicals can be useful as colorant and attractant for pollinators (Mehran & Sangeeta, 2014). Many phytochemicals contains some biologically active ingredients which possess therapeutic values and are considered as drugs or medicine (Mukherjee & Wahile, 2006; Ogundajo, Okeleye, & Ashafa, 2017; Sabiu, O'Neill, & Ashafa, 2016). Classification of phytochemicals is based on their chemical composition and structures. Most are formed either through the acetate pathway or the shikimate pathway (Falcone Ferreyra, Rius, & Casati, 2012; Ji et al., 2009). Some of different classes of plant's secondary metabolites include Phenolic compounds (tannins,

phenylpropanoids, flavonoids), Terpenoids, nitrogen compounds (alkaloids, indoles, amines, amino acids) steroids, glycoside and saponins (Grotewold, 2006).

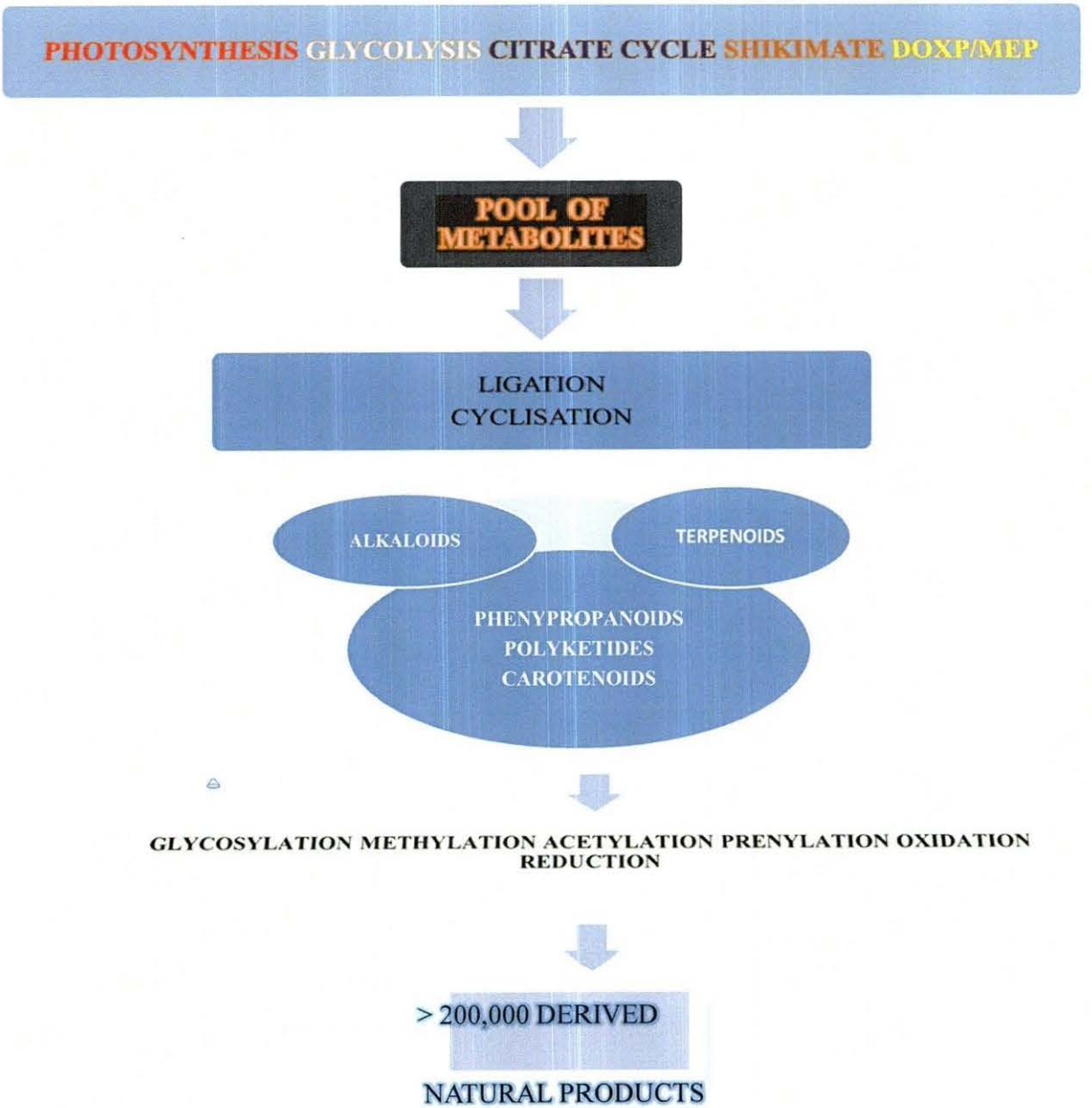


Fig. 2a. Schematic view of the diversity of the secondary metabolites formation (Grotewold, 2006).

Phenolic Compounds

They are plants secondary metabolites which are biosynthesized in the plastid cell cytosol through shikimate, pentosephosphate and phenylpropanoid pathways. Erythrose-4-phosphate and phosphoenol pyruvate from the pentose phosphate and glycolytic pathway respectively enter through the shikimate pathway and were metabolized to 3-deoxy-D-arabinose heptulosanate in a reaction catalyzed by 3-deoxy-D- arabinose heptulosanate (DHAP) synthase which is the rate limiting enzyme in the shikimate pathway (Dewick & Haslam, 1969). The phenolic compounds or polyphenols are made up of several compounds which include anthocyanidins, simple and complex phenolic acids and flavonoids. Simple phenols are large diverse simple monocyclic phenols like gallic acid, ferrulic acid, caffeic, coumaric acid and phenols. Other polymers of phenols that exist in nature include tannins, lignins and melanin (Sherma, 1989; Thomas Vogt, 2010).

Flavonoids

Flavonoids are plants secondary metabolites that possess different metabolic functions in plants and animals. They are the colourants found in fruits, seeds and flowers (Heim, Tagliaferro, & Bobilya, 2002). They contain phenybenzopyran properties. Generally, their chemical structure is C_{15} (C_6- C_3- C_6) forming the skeleton and joined by a chromane ring (Gao, Nishioka, Kawabata, & Kasai, 2004).

The A-ring which is an aromatic ring is fused with the benzopyran ring which is heterocyclic and called the C-ring. It also has a phenyl constituent B-ring. The A-ring may be metatrihdroxylate which typifies phloroglucinol or metadihyroxylated in which case it is resorcinol. Also, the B-ring phenyl constituent may be vicinal-trihydroxylated, orthohydroxylated or monohydroxylated. The C-ring can exist as a ϒ- pyrone, pyran or pyrilium (Augustin, Kuzina, Andersen, & Bak, 2011; Van Acker et al., 1996). Total flavonoid can be extracted by a described procedure (Liu, Wang, & Cai, 2015). The

powdered plant materials will be extracted twice with 85% methanol (1:10, w/v) for 24 h at 50 °C. The extract will then be filtered and the residue obtained after filtration will exhaustively extracted with 50% methanol. Classes of flavonoids that are recognized include anthocyanins, flavones, flavonols, chalcones, aurones, isoflavonoids, flavanones and neoflavonoids (Stalikas, 2007; Tuñón, García-Mediavilla, Sánchez-Campos, & González-Gallego, 2009). Flavonoids may be found in glycosylated form which makes it water soluble and thus increases its biovailabilty.

Flavonoid Biosynthesis in Plants

The biosynthesis of flavonoid is through the phenylpropanoid biosynthetic pathways. The initial reaction involve the transformation of phenylalanine into 4-coumarol CoA which then enters the flavonoid biosynthetic pathways. (Figure 2.1). The by-product of shikimate pathway (phenyalanine) and malonylCoA from the tricarboxylic acid cycle are the first committed step in the biosynthesis of flavonoids. Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamic acid which then undergoes hydroxylation reaction to form p-coumaric acid in a reaction catalyzed by cinnamic acid-4- hydroxylase (CA4H) in the presence of mercaptolethanol, molecular oxygen and a reducing equivalent (NADPH). There is further conversion of p-coumaric acid to 4-coumaroyl-CoA by p-coumarate CoA ligase (4CL). P-coumarol CoA is a substrate for chalcone synthase in the synthesis of the flavonoid skeleton. Through the process of intramolecular aromatization and cyclization, a tetraketide 4, 2^1, 4^1. 6^1 tetrahydroxyl chalcone is formed when three malonyCoA condenses with p-coumaroylCoA. Isomerization of chalcones by chalcone isomerase results in the formation of flavone and isoflavones. Downstream biosynthetic enzymes converts flavones into different flavonoids molecules through the process of glycosylation, acetylation, methylation, hydroxylation

and oxido-reduction reactions (Abdel-Lateif, Bogusz, & Hocher, 2012; Falcone Ferreyra, Rius, & Casati, 2012; Grotewold, 2006; Li, Ou-Lee, Raba, Amundson, & Last, 1993)

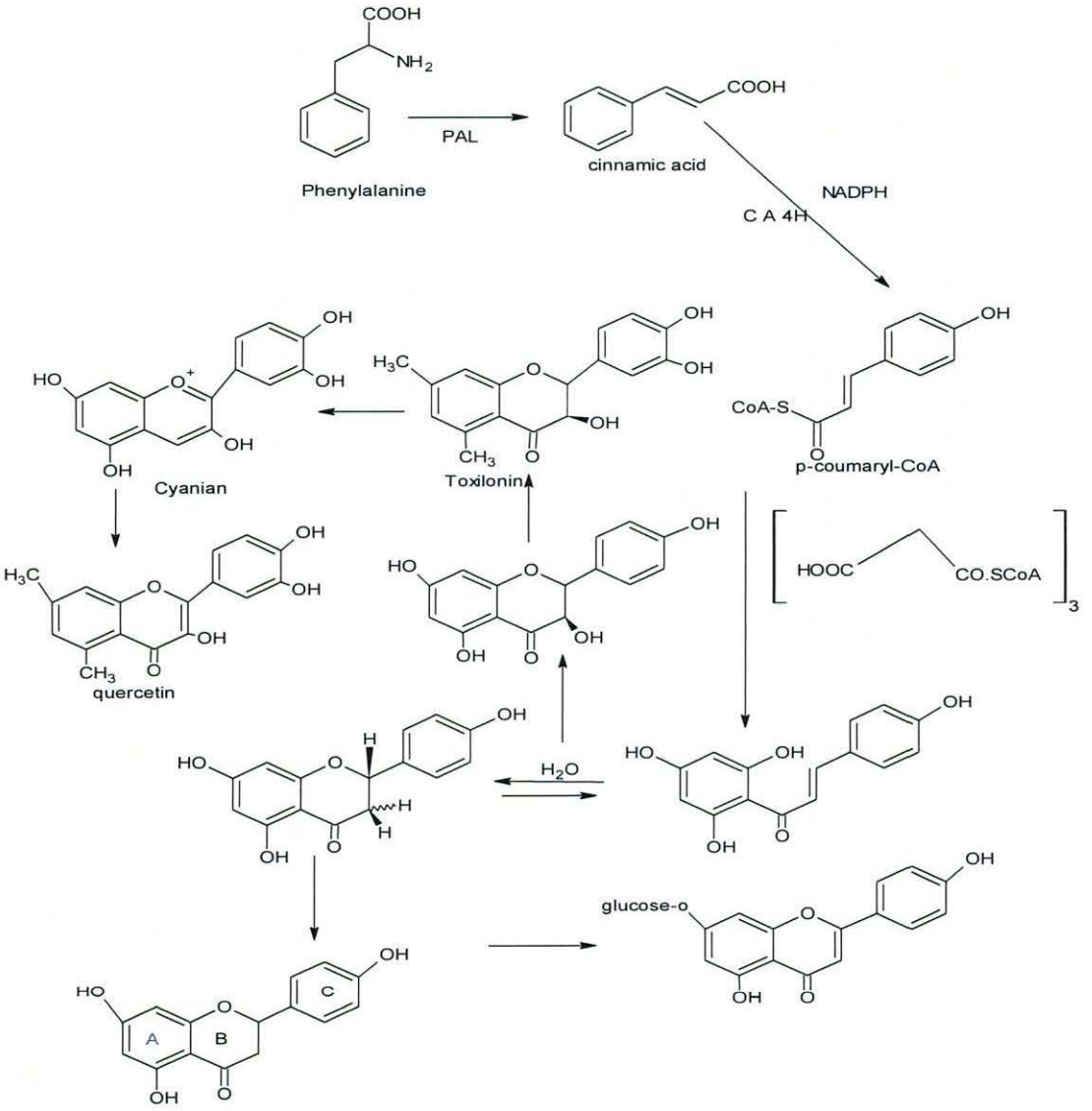


Fig. 2b Biosynthetic pathway of flavonoid (Falcone Ferreyra et al., 2012)

Flavonoids Pharmacological Importance

Pharmacologically, flavonoids has being reported to exhibit a lot of activities, based on different mechanistic pathways. This is responsible for flavonoids activity against degenerative diseases like cancer, diabetics, tumor and cardiomyopathy. Also the ability of flavonoids to inhibit phosphodiesterase and non-selective inhibition of platelet

aggregation factors explains their anti-inflammatory activity (Catherine, 1997; Koteswara, Fang, & Tzeng, 2005; Sofowora, Ogunbodede, Onayade, & Dentistry, 2013).

Flavonoids can modulate molecular events and inhibit transcriptional factors like kappa B, activator protein 1, signal transducers and activator of transcription CCAAT/ enhancer binding protein (Tuñón et al., 2009). Quercetin and kaempferol affect sucrase and maltase enzymes (Fontana Pereira et al., 2011) while the aglycone derivatives like wogonin, apigen inhibit nitic oxide (NO) production in lipopolysaccharide activated RAW 264.7 macrophages (Kim, Cheon, Kim, Kim, & Kim, 1999). It is also known to have a proven activity against *Mycobacterium bovis* BCG (Bernardes et al., 2014). Flavonoids show strong antioxidant activity against DPPH radicals (Lee et al., 2014).

Metabolites of flavonoids act as phosphoinositide -3- kinase (PI3K), Akt protein kinase B, tyrosine kinase, protein kinase C and mitogen activated protein kinase (MAPkinase) signaling cascade modulation either inhibiting or stimulating it (Spencer, 2010; R. J. Williams, Spencer, & Rice-Evans, 2004). Flavonoid helps to stabilize and sustain Islet of Langerhans and insulin secretion (Mohan & Nandhakumar, 2014). Flavonoids inhibit production of interleukin-12 (IL-12), and increases IL-4 production (Messaoudene et al., 2011). Also, flavonoids binds directly to many protein kinases like Akt/protein kinase, B fyn, Janus kinase-1 (JAK-1) Mitogen- activated protein kinase-4 (MKK-4), Raf 1 and protein (ZAP-70) kinase, it also phosphorylate them to regulate multiple cell signaling pathways in carcinogenesis (Hou & Kumamoto, 2010). Flavonoid can improve glucose metabolism, lipid profile, regulates hormones and enzymes thus, helping to protect the body against diseases like diabetes, vascular diseases and cancer. (Babu, Liu, & Gilbert, 2013; Grassi, Desideri, & Ferri, 2010; Mfotie, Munvera, Mkounga, Nkengfack, & McGaw, 2017). The structural activity relationship of flavonoids showed that the presence of the OH in the 5, and 7 carbon of the A-ring, the unsaturation at the 2-3 carbon in the C-

ring and the OH in the 3^1 and 4^1 of the B-ring is responsible for the metal chelating, antioxidant, as well as inhibition of different enzymes like α- amylase, α-glucosidase, lipoxygenase, cyclooxygenase and nitric oxide synthase (Brunetti, Di Ferdinando, Fini, Pollastri, & Tattini, 2013; Devendra et al., 2004; Grassi et al., 2010; Tadera, Minami, Takamatsu, & Matsuoka, 2006; Yao, Zhu, Chen, Tian, & Wang, 2013; P. Zhao et al., 2014)

Tannins

They are found in woody tissue of gymnosperms and angiosperms. Tannins are widely distributed in the plant kingdom. They are common in both angiosperms and gymnosperms. They are located mainly in the bark, wood, fruits, fruit pods, seeds, leaves, roots, and plant galls, in which they are concentrated in the vacuoles or surface wax so as to preclude interference with plant metabolism (Devendra et al., 2004; Krueger, 1964). Chemically, there are two main types of tannins, distributed unevenly throughout the plant kingdom. These are condensed tannins and hydrolysable tannins. The condensed tannins occur almost universally in ferns and gymnosperm and are widespread among the angiosperms, especially in woody species (Grotewold, 2006). By contrast, hydrolysable tannins are limited to dicotyledonous plants and are only found in a relatively few families. Both types of tannins, however, can occur together in the same plant, as they do in oak bark and leaves (Hanhineva et al., 2010).

Biosynthesis of Tannins in plants

Biosynthesis of tannins is through the shikimic acid pathway of flavonoids (Falcone Ferreyra et al., 2012; Silanikove, Gilboa, & Nitsan, 2001). The biosynthesis of tannins is shown in (Fig. 2.2). Condensed tannins or flavolans can be regarded as being formed by the addition of single gallocatechins or catechins to form dimmers and their higher oligomers, with C=C bonds between one 4-8 or 6-8 linkage of one flavan unit to the other one (Schofield, Mbugua, & Pell, 2001; Silanikove et al., 2001; Thomas Vogt, 2010).

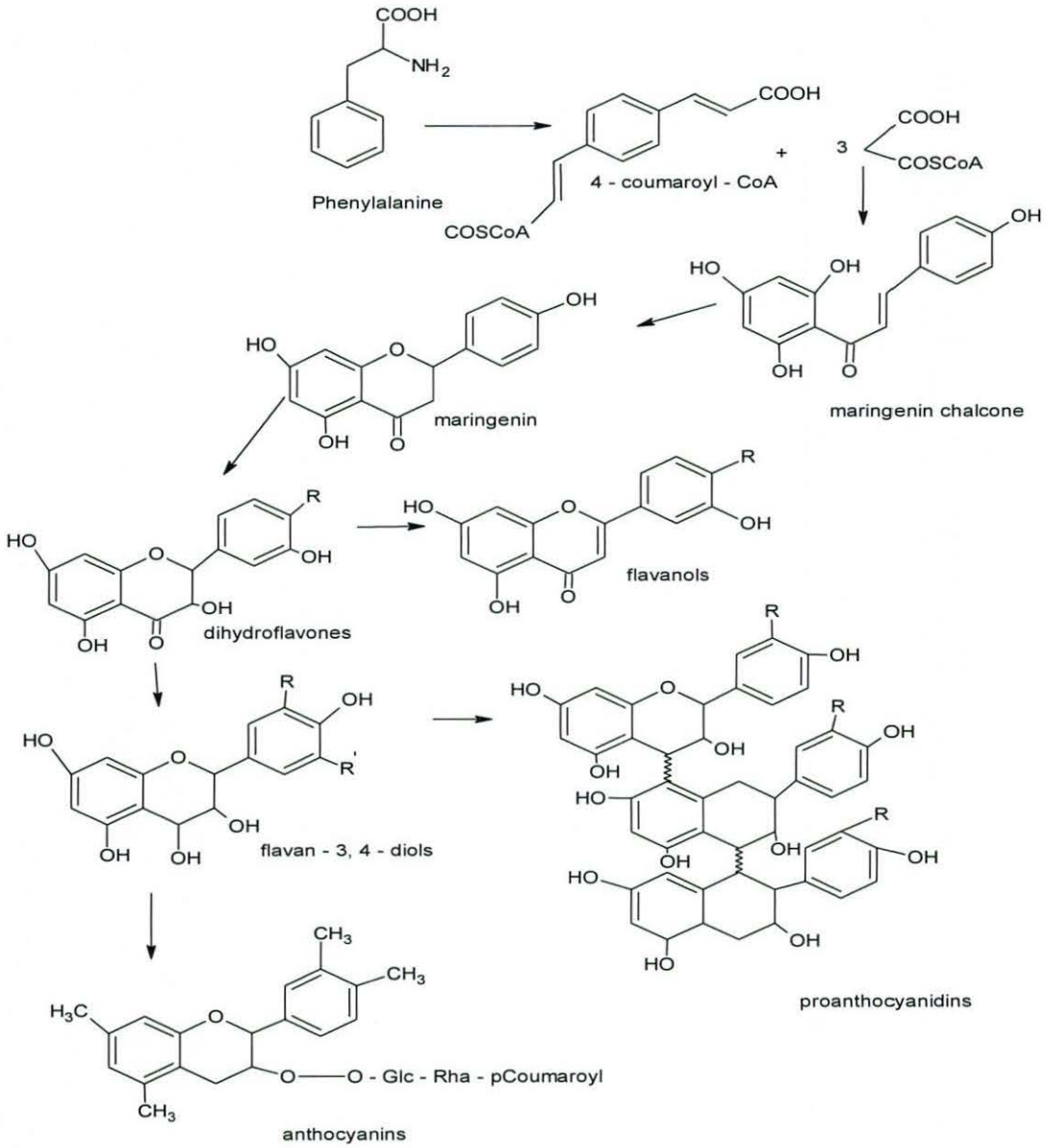
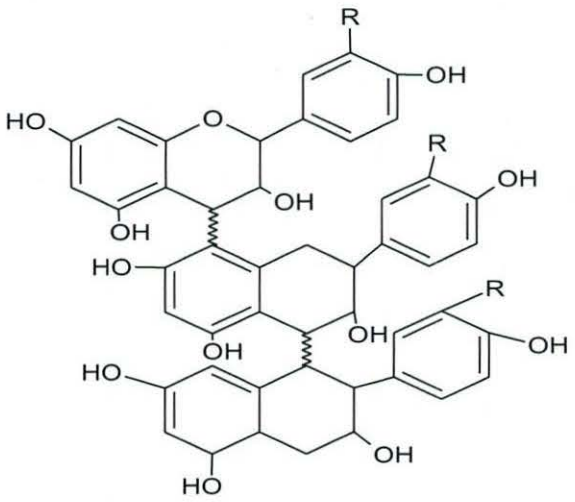


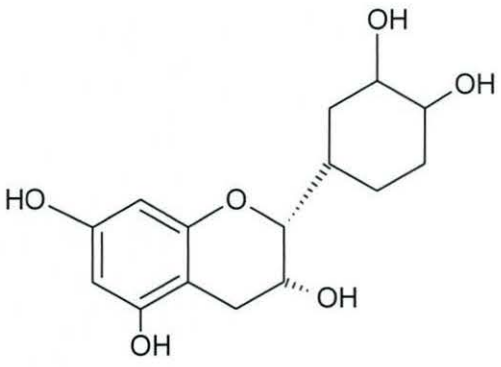
Fig. 2c Biosynthesis of condensed tannin (Silanikove et al., 2001).

Most flavolans have between two and twenty flavan units. The name proanthocyanidin [2.0] is used alternatively for condensed tannins because on treatment with hot acids, some of the carbon-carbon linking bonds are broken and anthocyanidin monomers are released. Anthocyanidins are phyto-flavonoid pigments responsible for the wide range of; ink, red, scarlet, mauve, violet, and blue colours of flowers, leaves, fruits, fruit juices, and wines (Silanikove et al., 2001). Most proanthocyanidins are procyanidins. It means that they yield cyanidins on acid treatment. Prodelphinidins [2.1], profisetnidin [2.2] and

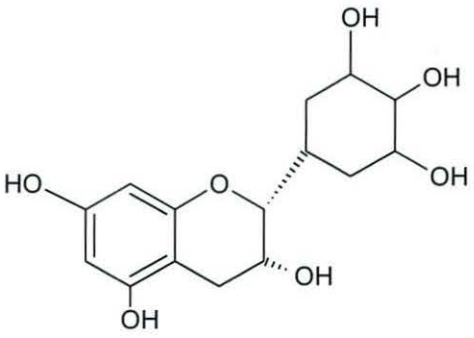
prorobinetinidin [2.3] are also known as mixed polymers. They yield cyanidin and delphinidin on acid degredation (Falcone Ferreyra et al., 2012; Holton & Cornish, 1995; Schofield et al., 2001)



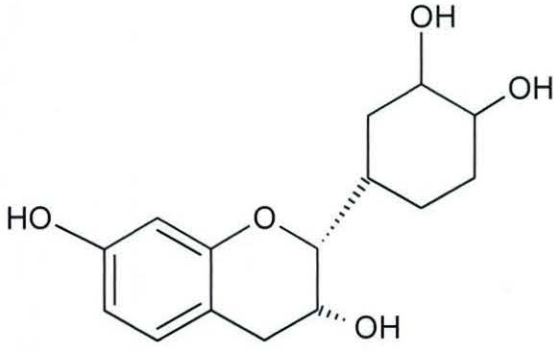
2.0



2.1

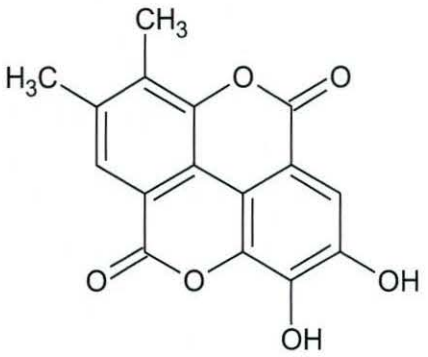


2.2

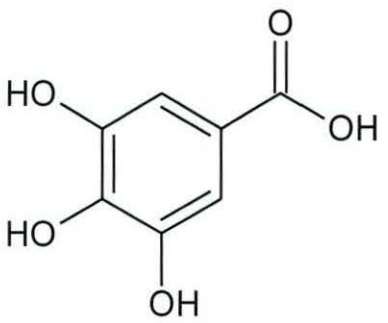


2.3

Hydrolysable tannins are mainly of two classes, the simplest being the galloylglucose depside. It has glucose core surrounded by five or more galloyl ester groups. A second type occurs where the core molecule is a dimer of gallic acid [2.4], namely hexahydroxydiphenic acid, again with glucose attachment on hydrolysis, these ellagitannins yield ellagic acid [2.5]. Within these two classes, it is possible to further subdivide the known compounds according to their biogenetic origin (Smeriglio, Barreca, Bellocco, & Trombetta, 2017; Takuo, Takashi, & Tsutomu, 1992)



2.4



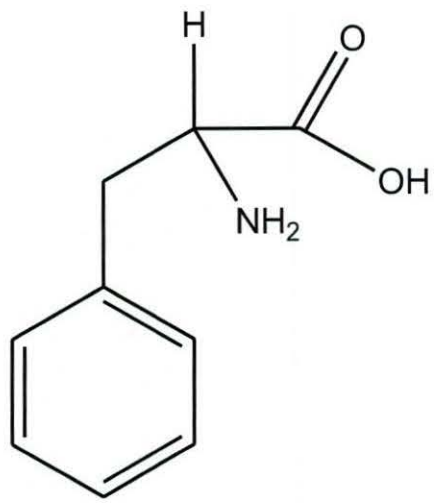
2.5

Pharmacological Activities of Tannins

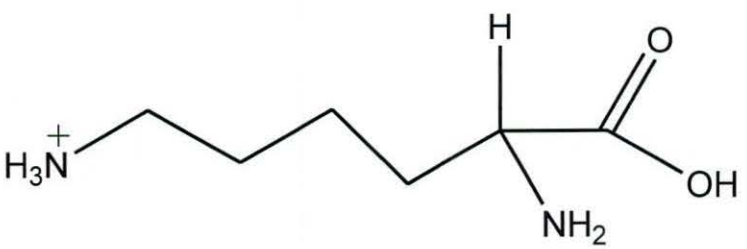
Reports showed that the mechanism of action of tannins involves enzyme inhibition and substrate deprivation, Astringency action on membrane proteins (in the treatment of burns) and metal ions deprivation by chelation (Smeriglio et al., 2017). Tannins are used as antiseptics, anticancer, antidiabetic, anti-inflammatory, haemostatics, and blood coagulating agents, it is also used in the treatment of burns, and skin abrasions. They are also used as astringents in the gastro-intestinal tracts. Their astringency renders proteins unsusceptible to the action of proteolytic enzymes, and microbes (Elisha, Dzoyem, McGaw, Botha, & Eloff, 2016; Hanhineva et al., 2010; Huang, Cai, Zhang, Huang, & Cai, 2017; Orhan, Ergun, & Yeşİlada, 2006; Smeriglio et al., 2017)

Alkaloids

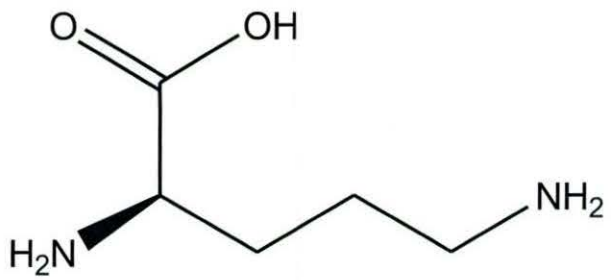
Alkaloids comprise the largest single class of secondary plant substances. Alkaloids are basic substances which contain one or more nitrogen atoms usually in combination or as a part of a cyclic system. Alkaloids are often toxic to man with dramatic physiological activities, hence they are widely used as medicine (Kutchan, 1995). The most common precursors of alkaloids are amino acids, such as phenyalanine [2.6], ornithine [2.7] and lysine [2.8] which is obtained from shikimate biosynthesis pathway (O'Connor & Maresh, 2006).



2.6

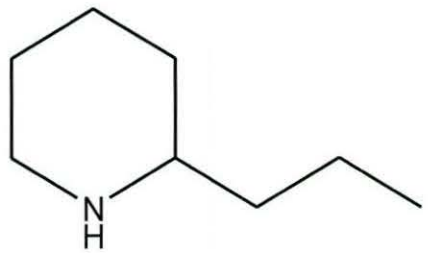


2.7

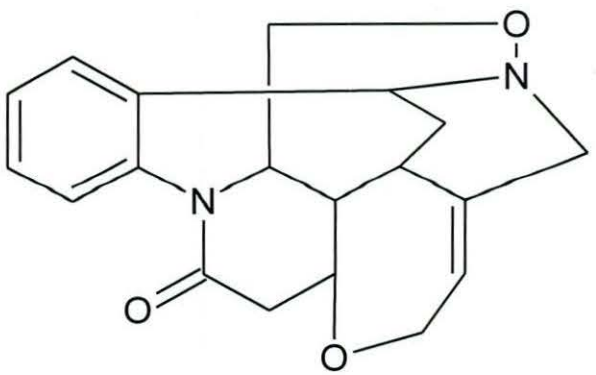


2.8

Chemically, alkaloids range from simple compounds like coniine [2.9], the major alkaloid of hemlock, *conium maculatum* to the pentacyclic structure of strychnine [2.10], the toxin of the *strychnos* bark. Plant purine and pyrimidine bases like caffeine [2.12] are sometimes loosely included in the general term alkaloid. (Ziegler & Facchini, 2008).



2.9



2.10

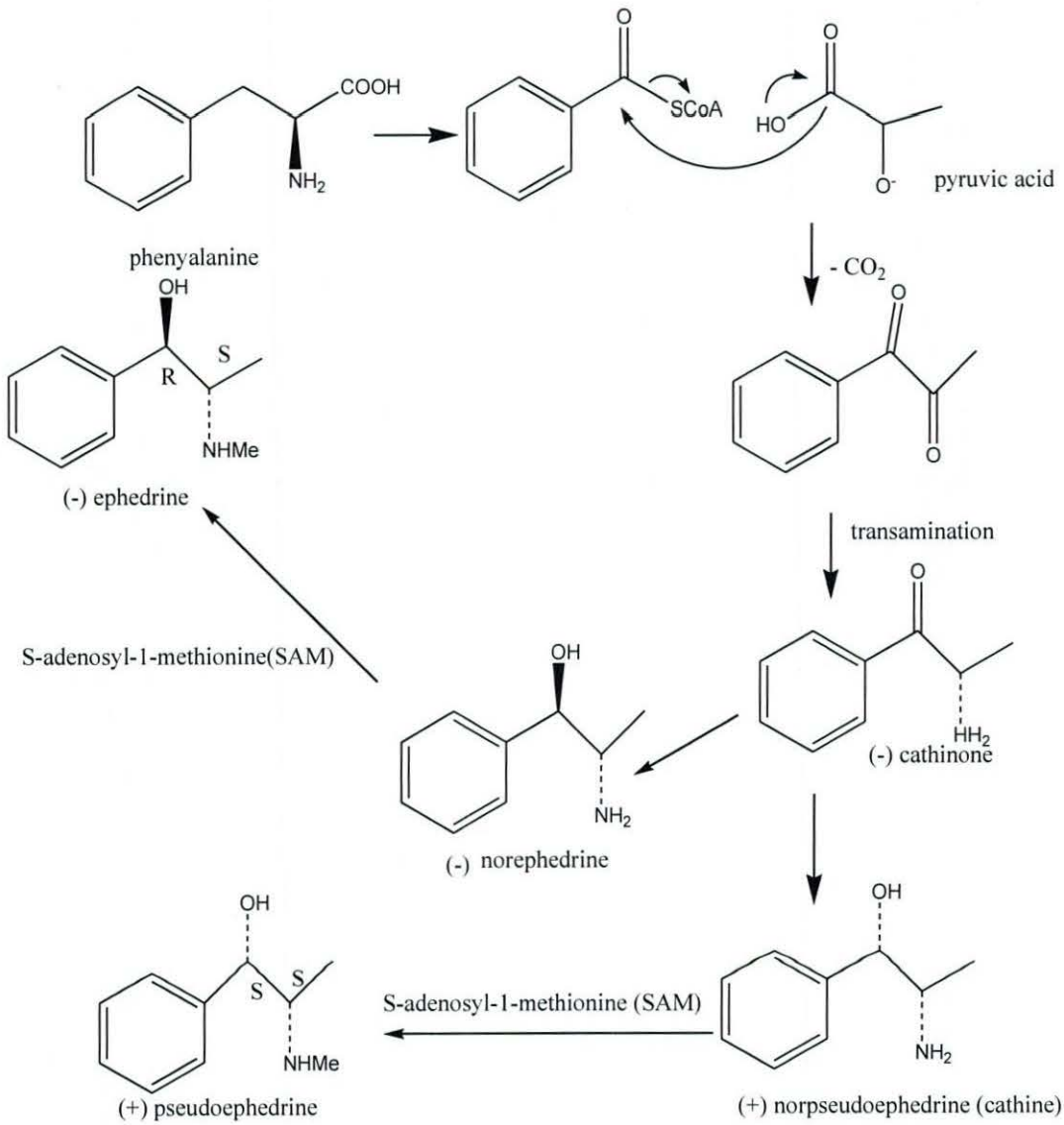


Fig. 2d Biosynthesis of phenylalanine derived alkaloid (Ziegler & Facchini, 2008)

Pharmacological Activities of Alkaloids

Pharmacologically, alkaloids are of immense relevance due to their high toxicity. Many reported indole and pyrrole-imidazole alkaloids play a defensive role and exhibit interesting types of bioactivity, including calmodulin antagonism, cytotoxicity, antimicrobial, antiviral, antiparasitic, anti-inflammatory, and Ca^{2+}-releasing activity (Abdel-Halim, Morikawa, Ando, Matsuda, & Yoshikawa, 2004; Barbosa-Filho et al., 2006; Cortes et al., 2015; López, Bastida, Viladomat, & Codina, 2002; K. Patel, Gadewar, Tripathi, Prasad, & Patel, 2012; Zhang, 2013) Quinine and its related quinoline alkaloids like chloroquine is potent antimalarial agent. Codeine a phenanthrene alkaloid is used as a cough suppressant and as an antidiarrhoeal agent. The opium alkaloids like morphine are an effective pain killer (Elisabetsky, Amador, Albuquerque, Nunes, & Carvalho, 1995).

Terpenoids

Terpenoids are hydrocarbon of plant origin, with general formula $(C_5H_8)_n$. As well as their oxygenated, hydrogenated and dehydrogenated derivatives. The term 'terpene' was given to the compound isolated from terpentine, a volatile liquid isolated from pine tree. Terpenoids are volatile substances which give flower and plant their fragrances. They are universally present in small amounts in living organisms where they play numerous vital roles in plant physiology as well as important functions in all cellular membranes (Theis & Lerdau, 2003). Biogenetically, terpenoids originated through the condensation of the universal phosphorylated derivative of hemiterpene, (IPP) $CH_2C(CH_3)CH_2CH_2OPP$ and dimethylallyl pyrophosphate$(CH_3)_2CCHCH_2OPP$. Isopentenyl pyrophosphate and dimethylallyl pyrophosphate exist at equilibrium in living cells.

Biosynthesis of Terpenes in plants

In biosynthesis, a molecule of isopentenyl pyrophosphate and dimethylallyl pyrophosphate are linked together to give geranyl pyrophosphate, which is the key intermediate in monoterpene formation (Dewick, 2002; Dubey, Bhalla, & Luthra, 2003; Shakya, 2016). Thus, terpenoids biosynthesis is based on the isoprene molecules $CH_2C(CH_3)CHCH_2$, their carbon skeletons are built up from the union of two or more of these C_5-units. The classification of terpenoids ranges from essential oil components, the volatile, mono and sesquiterpenes (C_{10} and C_{15}), through the less volatile diterpenes (C_{20}) to the non-volatile triterpenoids and sterols (C_{30}) and carotenoids pigments (C_{40}). Each of these various classes of terpenoids is significance in either plant growth metabolism or ecology (Arigoni et al., 1997; Crozier, Clifford, & Ashihara, 2006; Dubey et al., 2003).

Pharmacological Activities of Terpenes

The pharmacological potentials of terpenoids include antitumor, antioxidant and antiiflammatory. It also exhibited a moderate *in-vitro* activity towards murine leukemia cell line and selective activities towards several solid tumors and also a penetration enhancer (Shakya, 2016; A. C. Williams & Barry, 2012; M. Zhao et al., 2015). Artemisin, a cadinane sesquiterpene isolated from the plant *Artemisin annua* exhibited strong anitimalarial activity (Liu et al., 2017).

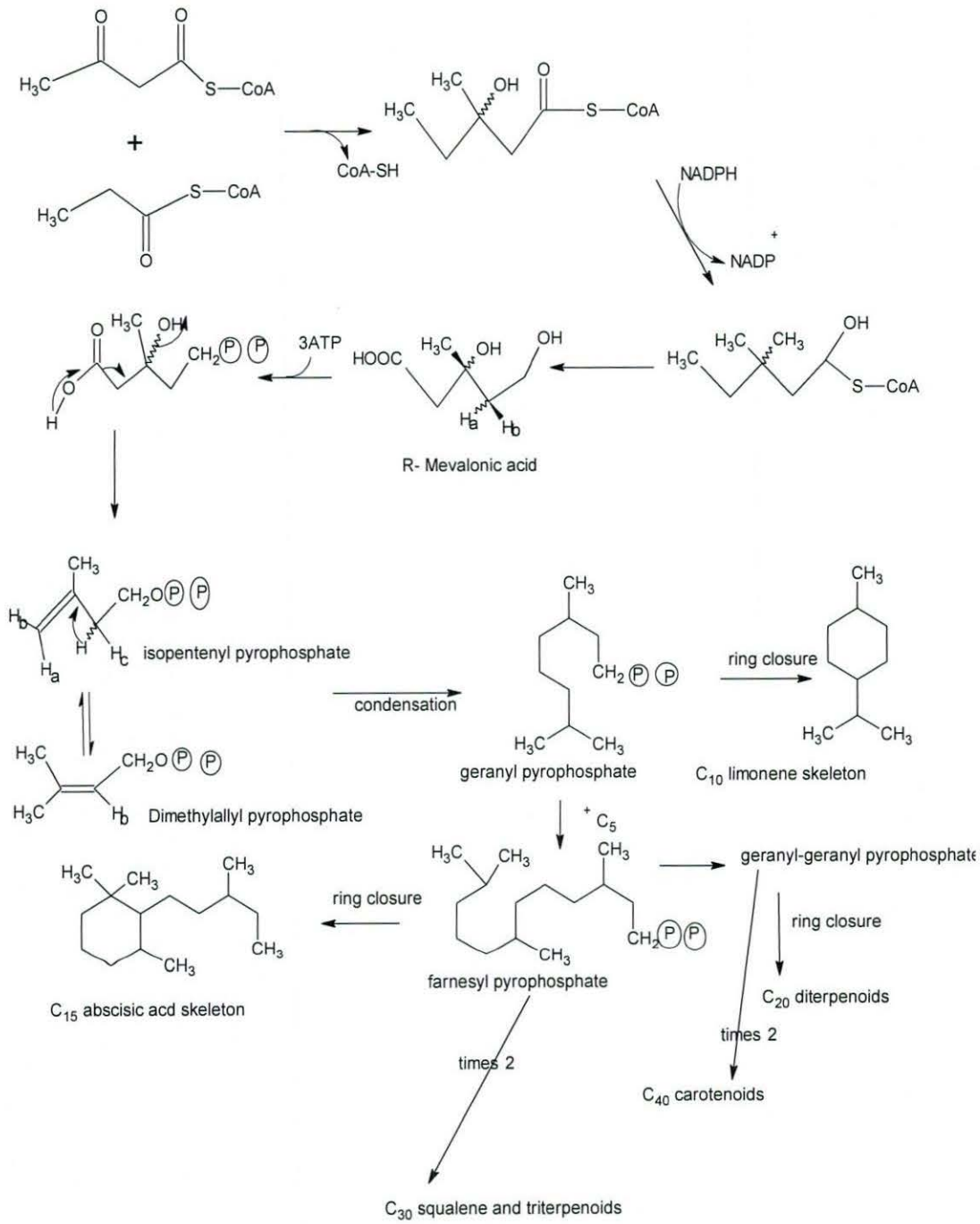
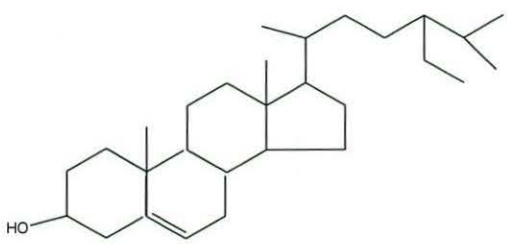


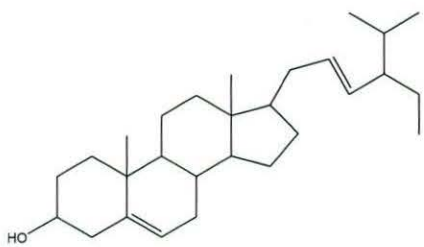
Fig. 2e. Biosynthesis of terpenoids (Dewick, 2002)

Steroids

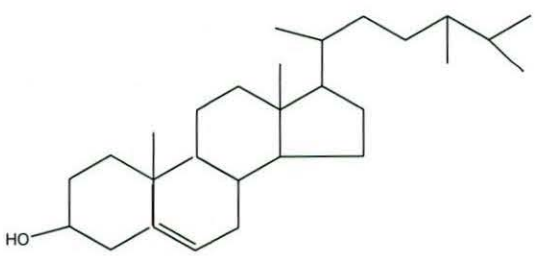
Sterols are triterpenes which are based on the cyclopentane perhydrophenanthrene ring system. They share the same biosynthetic origin with the terpenoids by acetate pathway through the cyclization of squalene (Arigoni et al., 1997). At one time, sterols were mainly considered to be animal substances, but in recent years an increasing number of such compounds have been detected in plant tissues. Plant steroids are called 'phytosterols'which include; β-sitosterols [2.12], stigmasterol [2.13] and campesterol [2.14]. Phytosterols occurred in higher plants as free and simple glycosides.



2.12



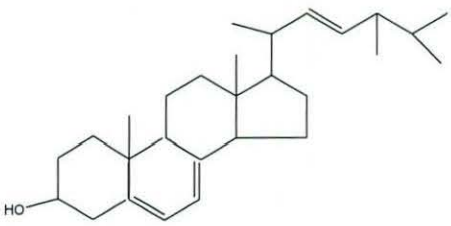
2.13



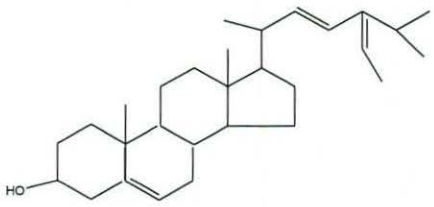
2.14

The occurrence of ergosterol [2.15] is confined to lower plants like yeast and many fungi. Fucosterol [2.16] which is the main steroid of many brown algae occurs mainly in lower plants

but also appears occasionally in higher plants. It has also been detected in the coconut. Phytosterols are structurally distinct from animal sterols.



2.15



2.16

Pharmacological Activities of Phytosterols

Pharmacologically, steroids have been shown to exhibit hormonal and anti-inflammatory activities. They are used as contraceptives, androgenic and anabolic agents. They also exhibit antifungal, antibacterial, antiviral and hypolipidemic activities as found among the saponins (Baydan, 2017; Deo et al., 2016).

Saponins

Saponins are glycosides of both triterpenes and sterols. Saponins have been detected in over seventy families of plant. They are surface-active agents with soap-like properties and can be detected by their ability to cause foaming and to haemolyse blood cells (Sofowora et al., 2013).

The search in plants for saponins has been stimulated by the need for readily accessible sources of sapogenins which can be converted in the laboratory to animal sterols of therapeutic importance, like cortisone and contraceptive estrogen. Saponins are also of economic interest because of their occasional toxicity to cattle, as found in saponins of alfalfa or their sweet taste as found in glycyrrhizin of liquorice root. The glycosidic patterns of the saponins are often complex. Many has five sugar units attached and glucuronic acid as common component (Baydan, 2017).

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Pharmacological Activities of Saponins

Several reports have shown that saponin exhibited a wide range of pharmacological properties. It has be reported used as vaccine adjuvants in the treatment of the Herpes Simplex Virus (HSV), Human Immuno-deficiency Virus (HIV), and influenza. The anticancer and tumour growth inhibitory effects of saponins were also documented (Artun et al., 2016; J. Y. Lee et al., 2006; Nordin et al., 2017; Park et al., 2017; Vahid, Rakhshandeh, & Ghorbani, 2017)

Reactive Oxygen and Antioxidant Assays

In biological systems during respiration, a significant fraction of oxygen is incompletely reduced. Such partially reduced oxygen, and their derivatives, are known as reactive species (RS). They are highly reactive metabolites (Kurutas, 2016). Their reactivity can cause functional damage to biological systems, triggering a number of degenerative diseases, like mutagenesis, carcinogenesis, circulatory disturbances, diabetes and aging (Bartosz, 2010; Bhattacharyya et al., 2014; Tangvarasittichai, 2015).

Types of Free Radicals

Radicals of oxygen (superoxide anion, hydroxyl, alkoxyl, and peroxyl), reactive non-radical species (H_2O_2 and singlet oxygen) and radicals of carbon, nitrogen, and sulphur constitute a variety of reactive molecules that cause oxidative stress to cells. Five percent of the total oxygen metabolism of liver cells results in the production of partially reduced oxygen species. These ROS can be generated both exogenously and endogenously (Egea et al., 2017; Liu, Ooi, & Chang, 1997). Singlet oxygen in an excited form are produced during photochemical reactions, inflammation, phagocytosis, action of cytostatic agents, aromatic nitro compounds and reaction involving secondary lipid peroxyl radical. It can also be formed during the

reaction between superoxide anion and hydroxyl radical (Mittal, Siddiqui, Tran, Reddy, & Malik, 2014).

Peroxyl radicals are produced primarily during lipid peroxidation (Sharma, Al-Omran, & Parvathy, 2007), this is initiated by abstraction of a hydrogen atom from unsaturated lipids. There are several other forms of ROS that contain nitrogen and chlorine in addition to oxygen. Nitrogen dioxide and nitric oxide (NO) are free radicals with odd number of electrons. Nitrogen dioxide is a strong oxidant, NO can react with superoxide to give rise to non-radical peroxynitrite, which is a powerful oxidant. Hypochlorous acid is produced by activated neutrophils from H_2O_2 by heme-containing enzyme myeloperoxidase, a very strong oxidant (Brown & Borutaite, 2001; Singh & Singh, 2008). Other free radicals include sulfhydryl radicals, which undergo electron and hydrogen transfer reactions with a variety of biological molecules (Egea et al., 2017).

Oxidative Stress and Antioxidants

Oxidative stress can be regarded as an imbalance between pro-oxidant/free radical production and opposing antioxidant defenses. Acute and chronic oxidative stresses have been implicated in a number of degenerative diseases, such as atherosclerosis, diabetes mellitus, ischemia/reperfusion (I/R) injury, Alzheimer's disease, inflammatory diseases neurodegenerative diseases, hypertension, ocular diseases, pulmonary diseases, and hematological diseases (Liu et al., 2013; Odeyemi, Afolayan, & Bradley, 2015).

Damage caused by ROS may be due to their attack on membrane lipids, intracellular proteins/enzymes, carbohydrates, and nuclear DNA in cells and tissues. These include undesirable oxidation causing damage to membrane lipids, protein modification, DNA

damage, and cell death induced by DNA fragmentation and lipid peroxidation (Benavente-Garcia & Castillo, 2008).

In biological systems, an antioxidant has been defined as any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or prevents oxidation of the substrate (Sofowora et al., 2013).

Based on chemical nature, antioxidants can be classified as enzyme antioxidants, preventive antioxidants, or scavenging, or chain breaking, antioxidants. The enzyme antioxidants act on specific ROS and degrade them to less harmful product, example include superoxide dismutases (SOD), catalase (CAT), and glutathione Peroxidase (GPx) (Mbhele, Balogun, Kazeem, & Ashafa, 2015).

Based on their mode of action, the antioxidants can be classified as primary, secondary or co-antioxidants. The primary antioxidants are the compounds that are able to donate a hydrogen atom rapidly to a lipid radical, forming a new radical, which is more stable (Nile & Park, 2013). For example, ascorbic acid, flavonoids, and tocopherol can stop chain reactions by donating an electron to the peroxyl radical of the fatty acid to stop propagation. The secondary antioxidants are the compounds that can react with the initiating radicals (or inhibit the initiating enzyme), or reduce the oxygen level (without generating reactive radical species). These can retard the rate of radical initiation reaction by elimination of initiators. This can be accomplished by deactivation of high energy species like singlet oxygen, absorption of UV light, scavenging of oxygen thus reducing its concentration, chelations of metal catalyzing free radical reaction, or by inhibition of enzymes, such as peroxidases, NADPH oxidase, xanthine oxidase (Lyon, 2013; Valko, Morris, & Cronin, 2005).

Antioxidant Activity Assays

The methodologies used for evaluating natural antioxidants must be carefully interpreted based on the system and analytical method for determining the extent of end point of oxidation (Re et al., 1999). Due to difficulty in measuring each antioxidant component separately, and interactions among these different antioxidant components in the network, several methods have been developed to assess the total antioxidant capacity of all the non-enzymatic antioxidant components in a biological sample (Nile & Park, 2013; Re et al., 1999).

The effectiveness of an antioxidant is measured by monitoring the inhibition of oxidation of a suitable substrate under standard conditions and measuring the extent of oxidation (as end point) by chemical, instrumental, or sensory methods. Hence, essential features of any method for such measurement would be a suitable substrate, an oxidation initiator and an appropriate measure of end point (Moharram & Youssef, 2014).

The antioxidant assay methods in biological systems are broadly classified into two groups. In the assays that evaluate lipid peroxidation using lipid or lipoprotein substrate under standard conditions, the degree of inhibition of oxidation is measured and assays that measure free radical scavenging ability necessarily measure the degree of scavenging of the preformed free radicals (Singh & Singh, 2008).

In general, two approaches have been considered. The first are inhibition assays, for which the extent of the scavenging of a free radical by hydrogen atom, or electron donation is the marker of antioxidant activity. These are an indirect measure of total antioxidant power. The second approach include assays involving the presence of antioxidant systems during the generation of the radical, for which the activity is measured and compared with the rate of oxidation of a target molecule in presence and absence of antioxidant system and when

applied to individual compounds, only provide an indication of the number of radicals trapped for each antioxidant molecule introduced into the system (Amorati & Valgimigli, 2015; Bartosz, 2010; Moharram & Youssef, 2014).

In Vitro Antioxidant Methods

2,3-diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH is a stable free radical. The assay is based on the measurement of the scavenging capacity of antioxidants (Blois, 1958). The odd electron of nitrogen atom in DPPH is reduced by a hydrogen atom to form antioxidants to the corresponding hydrazine. The ability is evaluated using electron spin resonance spectroscopy on the basis that the DPPH· signal intensity is inversely proportional to the antioxidant concentration and reaction time (Kedare & Singh, 2011). However, a more frequently used method is the discoloration assay, which evaluates decrease in absorbance at 515–528 nm produced by the addition of antioxidant to DPPH. It has been used to evaluate the antioxidant activity of phenolic compounds by measuring the change in absorbance at 515 to 517 nm (Halliwell & Gutteridge, 1990; Re et al., 1999).

The DPPH method is a valid, easy, accurate, sensitive, and economic method to evaluate scavenging activity of antioxidants of fruits, vegetables juices and plant extracts, since the radical is stable and need not be generated as in other scavenging assays. The results are highly reproducible and comparable to other scavenging methods such as 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Shalaby & Shanab, 2013a).

2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Cation Assay

The ABTS assay is based on the inhibition of the absorbance of cation $ABTS^{+}$ by antioxidants, which shows a main absorption peak at 415 nm and secondary absorption maxima at 660,

734, and 820 nm (Shalaby & Shanab, 2013a). When Trolox (water soluble analogue of Vitamin E) is used as a standard, the method is called TEAC (Trolox Equivalent Antioxidant Capacity). The original TEAC-I method was based on the activation of metmyoglobin with peroxidase and H_2O_2 via the formation of ferrylmyoglobin radical, which oxidizes ABTS to $ABTS^+$. Several different analytical strategies are used for assay: decolorisation and inhibition strategies, in which the absorbance of the reaction is read when the color of the incubation mixture is stable or at a fixed time point; and lag phase strategy, in which the length of lag phase before the antioxidant reaction starts, is measured (Nekvapil et al., 2012).

The ability of an antioxidant to scavenge $ABTS^{\cdot+}$ radical may not reflect its antioxidant activity as other mechanisms effective in food lipids or physiological substrates, including metal chelation and effects of antioxidant partitioning among phases of different polarities, may also contribute to it (Amorati & Valgimigli, 2015; Pietta, 2000).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay directly measures the ability of antioxidant to reduce ferric tripyridyltriazine complex (Fe^{+3}- TPTZ) to ferrous complex (Fe^{+2}-TPTZ) at low pH in FRAP assay (Benzie & Strain, 1996), excess Fe^{+3} is used and the rate-limiting factor of Fe^{+2}-TPTZ and color formation is the reducing ability of the sample (Bolanos De La Torre, Henderson, Nigam, & Owusu-Apenten, 2015). This method virtually permits simultaneous and specific measurement of ascorbic acid concentration and total reducing/antioxidant power in a simple, automated and rapid test.

Metal Chelating Assay

This technique relies on the measurement of the metal-chelating activity of antioxidants, under the conditions of Fenton-like reactions. The method uses a Co (II) complex and hence

evaluates the protecting ability against the formation of hydroxyl radical. Fluorescein is incubated with the sample to be analyzed, and then the Fenton mixture (generating hydroxyl radicals) is added. The initial fluorescence is measured, after which the readings are taken every minute after shaking (Flora & Pachauri, 2010; Jomova & Valko, 2011; Sears & Sears, 2013; Shalaby & Shanab, 2013b).

Antidiabetic Assays

Diabetes mellitus is a chronic metabolic disease, it occurs when the pancreas is not producing insulin or the produced insulin cannot be used by the body, these lead to raise blood glucose levels (Nsiah-kumi et al., 2012). Hyperglycemia are associated with damage to the various organs and tissues. The number of people living with diabetes is expected to rise from 366 million in 2011 to 552 million by 2030 (American Diabetes Association, 2010). There are two types diabetes: type 1 diabetes mellitus and type 2 diabetes mellitus (Wilkin, 2009).

There is great interest in the development of new drugs to reduce the burden of this disease thus, the scientist community has raised interest to evaluate either raw or isolated natural products in experimental studies.

Experimental studies of diabetes in animal models and advanced *in vitro* techniques are essential for the improvement of knowledge and clear understanding of the pathology and pathogenesis, and to find new therapy. Experimental diabetes mellitus studied by several methods that include: chemical, surgical and genetic manipulations.

***In vitro* Antidiabetic Techniques**

Assay of α- amylase inhibition

The *in vitro* α-amylase inhibition can be studied by allowing the test sample to react with α-amylase enzyme when incubated with starch solution. After incubation dinitrosalicylic acid

(DNS) reagent will be added to both the control and test sample. The mixture will then be incubated in boiling water bath for 5 mins and the absorbance taken at 540 nm using spectrophotometer and the percentage of inhibition of α-amylase enzyme will be calculated (Nagmoti & Juvekar, 2013).

A starch solution with potato in sodium phosphate buffer, sodium chloride will be prepared and kept in a boiling water bath for few min. The α-amylase solution is prepared by mixing α-amylase in the same buffer. The colorimetric reagent is prepared by mixing equal volume of sodium potassium tartrate tetra hydrate solution and DNS solution. Starch solution will be mixed with test sample with various concentration or acarbose and α-amylase solution is added and incubated at 25°C to react with the starch solution. DNS reagent will be added to the above solution, and the contents heated for 15 min on a boiling water bath. The final volume is made up with distilled water, and the absorbance measured at 540 nm using spectrophotometer. The percentage inhibition and IC_{50} value will be calculated (Ali, Houghton, & Soumyanath, 2006; Apostolidis, Kwon, & Shetty, 2007; Mccue, Ms, & Shetty, 2016).

Assay for Inhibition of α-glucosidase activity

The α-glucosidase enzyme inhibition activity can be performed by incubating α-glucosidase enzyme solution with phosphate buffer which will contains the test samples at different concentration at 37 °C for 1 h in maltose solution. The reaction mixture will be kept in boiling water for few min and cooled and its absorbance measured at 540 nm to estimate the amount of liberated glucose from maltose by the action of α-glucosidase enzyme. The percentage of inhibition and IC_{50} can be calculated (Mohamed et al., 2012).

Antiinflammatory Assay Methods

Inflammation is protective and defense mechanism of the body. During inflammatory conditions, various pathological changes can take place. The production of active inflammatory mediators may be triggered by microbial products or by host proteins; such as proteins of the complement, kinins, and coagulation systems, which was activated by microbes and damaged tissues. It is a body defense phenomenon in order to eliminate or limit the spread of injurious agent. Various components of inflammatory reaction can lead to various symptoms such as tissue injury, edema formation, and leukocyte infiltration (Zeyda & Stulnig, 2009).

Mediators of inflammation originates either from plasma (complement proteins and kinins) or from cells (histamine, prostaglandins and cytokines). Generally the mediators of inflammation are histamine, prostaglandins (PGs), leukotrienes (LTB4), nitric oxide (NO), platelet-activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines, and growth factors (Ward, 1974). These inflammatory mediator were biosynthesized by the enzymatic activities of lipoxygenase and cyclooxygenase (Goldstein, Kemp, Soczynska, & McIntyre, 2009; Meng et al., 2013).

Lipoxygenase is a non-heme iron dioxygenase that is ubiquitous in plants and animals and catalyzes the dioxygenation of polyunsaturated fatty acids (PUFAs) containing a (1Z,4Z)-pentadiene system such as, for instance, linoleic acid, R-linolenic acid, or arachidonic acid (Ford-Hutchinson, Gresser, & Young, 1994). Typically, the methodologies used for the measurement of lipoxygenase activity are the spectrophotometric assay based on the measurement of the absorbance at 234 nm produced by hydroperoxy lipid product or the oxygraphic assay based on the evaluation of oxygen consumption using an oxygen electrode.

A colorimetric method for determination of lipoxygenase activity applicable to high throughput assays, based on the fact that a lipid hydroperoxide can oxidize Fe^{2+} to Fe^{3+}, which in acidic medium oxidized xylenol orange to a colored forms that absorbs in the region of 500-600 nm. The ferrous oxidation-xylenol orange (FOX) assay method for determination of lipid hydroperoxides is based on the acidic conditions in which Fe^{2+} is oxidized to Fe^{3+} which then oxidizes xylenol orange to a product that absorb at 550 nm. The procedure has been adapted for determination of lipoxygenase activity in plant extracts.

The enzymatic assay is based on a discontinuous determination of lipoxygenase activity using the FOX reagent for colorimetric determination of hydroperoxides accumulated in the medium by a period of incubation that is established by the addition of the extract (start of the reaction) and the addition of FOX reagent (finish of the reaction) (Ford-Hutchinson et al., 1994; Pinto, Tejeda, Duque, & Macías, 2007). The procedure is capable of detecting lipoxygenase and has being especially useful for a rapid visual evaluation of this enzymatic activity.

Cell Line Use in Medicinal Plant Studies

Cell culture can be used to screen for toxicity both by estimation of the basal functions of the cell or by tests on specialized cell functions (Tantengco & Jacinto, 2015). General toxicity tests, aimed mainly at detection of the biological activity of test substances, can be carried out on many cell types (fibroblasts, HeLa and hepatoma cells) (Allen et al., 2005; Babu et al., 2013; Stepanenko & Dmitrenko, 2015). A number of parameters including vital staining, cytosolic enzyme release, cell growth and cloning efficiency are used as end-points to measure toxicity. Organ-specific toxic effects are tested using specialized cells by measuring

alterations in membrane and metabolism integrity and/or in specific cell functions (Vijayarathna & Sasidharan, 2012; Xu, Deng, Yuan, Yang, & Guo, 2011).

Cytotoxicity tests using specialized cells have proved most useful when the *in vivo* toxicity of a chemical is already well established and where *in vitro* investigations using specialized cell cultures have been used to clarify the mechanisms of toxic action on the target tissue (Baydan, 2017). These tests have also provided useful insight into the pathogeneses of some human diseases. For some toxic chemicals, it is the functional status of the cell rather than the cell type that determines the extent to which the inhibition of a given biochemical mechanism is critical to the function and survival of the cell (Ekwall, Silano, & Zucco, 1990).

Selective toxicity *in vitro* can also be determined using normal and cancer cells (Badisa et al., 2011). Cytotoxicity testing of cancer cells are normally used. In addition to cytotoxicity testing, cell culture systems are also useful to carry out metabolism studies including biotransformation, interaction with endogenous metabolites, binding to cells, and induction of metabolism.

Cell Culture Systems and Methods

A primary cell culture is a culture started from cells, tissues or organs taken directly from organisms. A primary culture may be regarded as such until it is successfully sub-cultured for the first time. It then becomes a cell line. A cell line arises from a primary culture at the time of the first successful subculture. The term, cell line, implies that cultures from it consist of numerous lineages of cells originally present in the primary culture. The terms, finite, or continuous, are used as prefixes if the status of the culture is known (Russell, Graham, Smiley, & Nairn, 1977).

A continuous cell line derived from primary cultures or diploid cell lines by transformation processes which are either spontaneous, or induced by viruses, chemical or physical agents (Weyermann, Lochmann, & Zimmer, 2005). Available cell lines are collected by the American Type Culture Collection which provides a catalogue listing of every cell type with its history and information concerning viability, growth medium, growth characteristics, plating efficiency, age of culture since origin, morphology, karyology, sterility tests and virus susceptibility (Skehan et al., 1990).

Primary cell cultures have morphological and biochemical characteristics that are more similar to those of the original tissue; however, problems with obtaining reproducible results may negate these advantages. Nevertheless, primary cultures offer the only possibility for comparative studies of some specialized tissues taken from different animal species where cell lines and strains from the same tissues are not available. Primary cultures are generally more sensitive to the effects of toxic chemicals than are cell lines because, while exposed, they have also to adapt to culture conditions. The main limitations of primary cultures are low homogeneity and a tendency to rapid loss of specialization under culture conditions (Friedrich et al., 2007). Cell lines offer the advantage of being more homogeneous and standardized than primary cultures. They are well characterized, easy to cultivate and reproducible results are easier to obtain.

A growing number of cell types have been shown more recently to retain some specialized functions in culture. Some examples include: Endothelial cells *in vitro* display several specialized functions including a non thrombogenic surface to platelets, Factor VIII antigen, the surface angiotensin converting enzyme, and synthesis of fibronectin and collagen (Connolly, Knight, Harakas, Wittwer, & Feder, 1986).

The use of serum-free media consisting of a nutrient basal medium supplemented with hormones and growth factors is necessary to the various cell types. These selective media facilitate adaptation of cells to the culture and allow a better standardization of experimental conditions. Serum is a very complex and poorly characterized mixture, the composition of which may vary according to the commercial batch; some components essential for cell growth may be absent. Serum may contain naturally-occurring substances or microbiological contaminants (mycoplasma, viruses, endotoxins) that are toxic for certain types of cultures (Denizot & Lang, 1986). Serum-free media facilitates the isolation of the desired cell type and, in setting up primary cell cultures, they almost completely eliminate the overgrowth of fibroblasts that, usually, grow rapidly in serum-supplemented media. Moreover, these selective media are useful to study interactions of cells with hormones or drugs and to perform cell nutrition studies.

Cell lines are more widely used for general toxicity studies than primary cell cultures because they are well characterized and more easily cultured. When the mechanism of toxicity of a chemical is under investigation and it becomes necessary to take into account specific characteristics of specialized cell types, primary cell cultures of the target organ or tissue are often used in conjunction with cell lines from the same origin (Martin, 1981) .

***In Vitro* Testing of Cell Toxicity of Plant Extracts: Methodological Aspects**

The first and most readily observed effect following exposure of cells to toxicants is morphological alteration in the cell layer and/or cell shape in monolayer culture. Therefore, it is not surprising that morphological alterations are used as an index of toxicity. A systematic appraisal of cell injury has been attempted to allow a greater standardization of the observations. A checklist suitable for computer-based programmes has been proposed

(Weyermann et al., 2005). Different types of toxic effects may require investigative tools of different levels of sensitivity. Gross modifications such as blebbing or vacuolization can be observed using light microscopy whereas fine ultrastructural modifications require analysis by transmission or scanning electron microscopy. Another indicator of toxicity is altered cell growth. The effect of chemicals on the capability of cells to replicate is used as an index of toxicity; the concentration of the substances at which 50 per cent of the cells do not multiply is called the median inhibitory dose LC_{50} (Mosmann, 1983; Weyermann et al., 2005).

Other indices of toxicity to basal cell functions involve measurement of biochemical or metabolic cell alterations. Acid phosphatase activity has been used as an index of cell damage (Friedrich et al., 2007). Lactate dehydrogenase activity in the culture medium, usually measured as the NADH-NAD conversion needed to convert pyruvate into lactate has been used as an index of membrane damage (Connolly et al., 1986; Friedrich et al., 2007; Yang, Sinai, & Kain, 1996).

Use of Chromatography Techniques in Phytochemistry

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and composition. In general, chromatography involves moving a sample through the system over a stationary phase (Sherma, 1998). The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Different compounds can be separated from each other as they move through the column. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas

chromatography), paper (paper chromatography), High-performance liquid chromatography (HPLC) and High-performance thin layer chromatography (HPTLC) (Poole, 1999).

Thin layer chromatography

Thin layer chromatography (TLC), involved separating mixtures by eluting them through a planar chromatographic bed and visualizing the separated components by staining or charring. In thin layer chromatography, a solution of the sample in a volatile solvent is applied via glass capillary tubes from the bottom of a uniform layer of inert adsorbent, such as silica gel or alumina, which as uniformly spread over a suitable supporting plate such as glass or plastic and dried under standard conditions. When the spot has dried, the plate is placed vertically in a suitable tank with lower edge immersed in the selected mobile phase. The solvent rises by capillary action and an ascending chromatographic separation is thus obtained, resolving the sample mixture into discrete spots. At the end of the run the solvent is allowed to evaporate from the plate and separated spots are located and identified either by physical methods such as visual inspections, fluorescence or radiation monitoring or by chemically reacting with a developing reagent. Silica gel is the most commonly used adsorbent in TLC studies. It is prepared by the hydrolysis of sodium silicate to polysilicic acid which on further condensation and polymerization, yield silica gel material (Koll, Reich, Blatter, & Veit, 2003; Patel, Patel, & Batel, 2011).

Gas chromatography

Gas chromatography (GC) is one of the most important chromatography techniques. It may be regarded as the forerunner of modern instrumental analysis. There are broadly three modes in which gas chromatography is carried out namely Gas-liquid chromatography (GLC) Capillary column gas chromatography (CGC) and Gas solid chromatography (GSC). Gas

liquid chromatography uses a packed column with liquid stationary phase coated on inert support particle, Capillary column gas chromatography uses open tubular columns with liquid or solid stationary phase coated on the inner walls of the column tubing whiles Gas solid chromatography (GSC) uses packed column with the solid surface of the particles like alumina and cross-linked polymer forming the stationary phase. Gas chromatography uses a gaseous mobile phase transporting components as a vapour over a stationary phase. Separation is affected by interaction of different individual components with stationary phase. Gas chromatography has a wide range of application. These include drugs and consumer products analysis, environmental monitoring of air, water and legislation. Gas column chromatography-mass spectrometry (GC-MS) is advanced gas chromatography technique which uses mass spectroscopic detectors. This allows sample mixtures containing common organic analyte to be separated and identified using a single bench-top instrument (Karasek & Clement, 1988).

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a type of liquid chromatographic technique used to separate and quantifies analytes that have been dissolved in solution. HPLC instrumentation includes a pump, injector, detector and integrator or acquisition and display system. The heart of the system is the column where separation occur, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute into the injector at the end of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a peak on the data displayed on a chart recorder or computer screen and is known as chromatogram.

The HPLC can be achieved by exploiting a variety of sorption processes namely adsorption, partition, ion-exchange and size exclusion. Adsorption chromatography arises from interactions between solutes and surface of the solid stationary phase. The mechanism of separation is based on surface adsorption on the basis of polarity. Adsorbent like alumina and silica gel are employed in adsorption chromatography. The solvent systems used in adsorption chromatography are polar solvents, commonly hexane, containing a small amount of polar solvents modifier, such as 2-propanol, dichloromethane or methyl-t-butyl ether. Partition chromatography involves a liquid stationary phase that is immiscible with the eluent and is coated on an inert support. The mechanism of separation is based on partitioning phases or adsorption interactions between solute and polar bonded phase. Partitioning chromatography takes place under normal phase or reverse phase mode. Reverse phase uses non-polar stationary phase like octadecylsilane (ODS), which is bonded to a silica support via silyl ether (Siloxane) linkage or C-18 hydrocarbon bonded to a silica support. Polar eluents like methanol or acetonitrile with aqueous buffers are employed as the mobile phase. Normal phase uses a stationary phase containing hydrocarbon chain, modified with attachment of polar functional groups like ether, nitrile, nitro, diol or amino substituents to the chain end. The modified hydrocarbon is then chemically bonded to the silica support. Normal phase elution is usually carried out with relatively non-polar solvents such as tetrahydrofuran, diethyl ether, chloroform and hexane. Ion exchange chromatography has a stationary phase with an ionically charged surface that is different from the charge of the sample. The mechanism of analyte separation is based on charge interaction between solute ions and counter ionophores on packing. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where

both pH and ionic strength are used to control elution time. In size exclusion chromatography, molecules are separated based on their sizes, the stationary phases consists of material with precisely controlled pore size. Smaller particles get caught up in the column material, which elute later than the larger particles. Principally, two classes of material are used as stationary phase packing material in size exclusion chromatography, either semi-rigid cross-linked macromolecular polymers or rigid inert porous silica or glasses. Elution can be carried out both with aqueous and polar organic solvents (Ferenczi-Fodor, Végh, Nagy-Turák, Renger, & Zeller, 2001)

High performance thin layer chromatography (HPTLC)

HPTLC is an enhanced form of thin layer chromatography (TLC). It uses HPTLC plates featuring small particles with a narrow size distribution. As a result, homogenous layers with a smooth surface can be obtained. HPTLC uses smaller plates (10 x10 or 10 x 20 cm) with significantly decreased development distance (typically 6 cm) and analysis time (7–20 min). HPTLC plates provide improved resolution, higher detection sensitivity, and improved in situ quantification and are used for industrial pharmaceutical densitometric quantitative analysis. Normal phase adsorption TLC on silica gel with a less polar mobile phase, such as chloroform and methanol, has been used for more than 90% of reported analysis of pharmaceuticals and drugs. Lipophilic C-18, C-8, C-2; phenyl chemically-modified silica gel phases; and hydrocarbon- impregnated silica gel plates developed with a more polar aqueous mobile phase, such as methanol–water or dioxane–water, are used for reversed-phase TLC. Other precoated layers that are used include aluminum oxide, magnesium silicate, magnesium oxide, polyamide, cellulose, kieselguhr, ion exchangers, and polar modified silica gel layers that contain bonded amino, cyano, diol, and thiol groups. Optical isomer separations that are

carried out on a chiral layer produced from C-18 modified silica gel impregnated with a Cu (II) salt and an optically active enantiomerically pure hydroxyproline derivative, on a silica layer impregnated with a chiral selector such as brucine, on molecularly imprinted polymers or on cellulose with mobile phases having added chiral selectors such as cyclodextrins have been reported mostly for amino acids and their derivatives. Mixtures of sorbents have been used to prepare layers with special selectivity properties. HPTLC plates need to be stored under appropriate conditions. Before use, plates should be inspected under white and UV light to detect damage and impurities in the adsorbent. It is advisable to prewash the plates to improve the reproducibility and robustness of the results (Koll et al., 2003; Sherma, 2008)

Spectroscopic techniques in Phytochemistry.

Spectroscopic techniques in organic chemistry involved the application of electromagnetic radiations in elucidating the chemical structure of organic compounds. Although almost all parts of the electromagnetic spectrum are useful, but in organic chemistry only energy absorption from three or four regions of electromagnetic spectrum are employed. These energy region are ultraviolet, infrared and radiofrequency (Nuclear magnetic resonance) absorptions (Harris, 1986).

Ultraviolet spectroscopy

Ultraviolet spectroscopy is a spectroscopy technique which involved the absorption of ultraviolet radiation (200-400 nm) by an organic molecule. This absorption leads to excitations of the electron and energy transition from highest occupied molecular orbital (low energy level) to the lowest unfilled orbital (high energy level). The ultraviolet radiation that is absorbed has energy exactly equal to the energy difference (ΔE) between the excited and ground state. The characteristic energy of a transition and the wavelength of radiation

absorbed reflect the properties of a group of an atom in the analyte. The group of atoms producing such absorption is called a chromophore. The principle of ultraviolet spectroscopy is govern by beer lambert's law which explain that the greater the number of molecule capable of absorbing light of a given wavelength, the greater the extent of light absorption. Furthermore, the more effectively a molecule absorbs light of a given wavelength, the greater the extent of light absorption. These guiding idea lead to the empirical expression, known as Beer-Lambert Law. Beer-Lambert law relates absorption to the concentration of absorbing solute ($\log (I_O/I)=\varepsilon cl$). It also relates the total absorption to optical path length ($\varepsilon=A/cl$). The instrument used in measuring ultraviolet absorption of organic molecule is ultraviolet-visible spectrophotometer. The major parts of ultraviolet –visible spectrophotometer are a light source, usually a deuterium lamp which emit electromagnetic radiation, a monochromator which spread the beam of light to its component wavelenghts and detector which records the transmitted light (I). The sample cell is constructed with quartz, a material which is transparent to ultraviolet radiation. The ultraviolet spectrum is generally recorded as a plot of absorbance versus wavelength (Kolarovic & Traitler, 1985).

Infrared Spectroscopy

Infrared (IR) spectroscopy is one of the most common spectroscopy techniques used by organic and inorganic chemist. Infrared radiations span a section of electromagnetic spectrum having wavenumbers from roughly 13,000 to 10 cm^{-1} or wavelengths from 0.78 to 100 μm. The technique involved the absorption measurement of different infrared frequencies by a sample positioned in the path of an infrared beam. Molecules are excited to higher energy state when absorbed infrared radiations. The frequencies of infrared radiation absorbed, corresponds to the stretching and vibration frequencies of the bonds in the molecules. Infrared

spectroscopic analysis is used to determine the chemical functional groups in organic compound. Different functional groups absorb characteristics frequencies of infrared radiation. The instrument used in determining infrared absorption spectrum for a compound is called an infrared spectrometer. The Infrared absorption information of a compound obtained from an infrared spectrometer is presented in the form of a spectrum with wavelength or wavenumber on the x-axis and absorption intensity or percent transmittance (T) on the y-axis. Transmittance is the ratio of radiant power transmitted by sample (I) to the radiant power incidence on the sample (I_o). Absorbance (A) is the logarithm to the base ten of the reciprocal of the transmittance (Ho et al., 2003).

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a spectroscopic method that involved the study of magnetic nuclei. Most common magnetic nuclei that are being studied in organic compounds are hydrogen and carbon nuclei. Nuclear magnetic resonance gives information about the number of magnetically distinct types of hydrogen and carbon present in the analyte. It also gives information regarding the nature of the immediate environment of the hydrogen and carbon nuclei present. The nuclear magnetic resonance phenomenon is based on the principle that the nucleus of hydrogen (^1H) and carbon (^{13}C) behaves as tiny magnet, because they possesses both electric charge and mechanical spin, therefore they respond to the influence of an external field, and tend to align itself to the direction of the field with a processional motion at low energy state. If the precessing nucleus is irradiated with radiofrequency energy of the same frequency with the precessing nuclei, the low energy precessing nuclei absorbed energy from the radiofrequency, move to high energy state and occupied an opposite direction to the applied field. This absorption energy transmission is

then recorded and processed to yield a nuclear magnetic resonance spectrum. Nuclear magnetic spectrum is obtained on a horizontal scale called chemical shift, which ranges from 0 to 14 ppm for Proton (^{1}H) and 10 to 220 ppm for carbon (^{13}C) nuclei. Chemical shift is the shift in frequency experienced by magnetic nuclei, based on their nature and chemical environment. Chemical shift are expressed in δ (delta) units. The instrument designed for the measurement of nuclear magnetic resonance is called nuclear magnetic resonance spectrometer. The basic features of the instrument include a magnet, radiofrequency source and a detection unit (Klampfl & Himmelsbach, 2015).

Two-Dimensional Nuclear Magnetic Resonance Spectroscopy

Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) is a set of nuclear magnetic resonance spectroscopy (NMR) methods which give data plotted in a space defined by two frequency axes rather than one. Two-dimensional NMR spectra provide more information about a molecule than one-dimensional NMR spectra and are especially useful in determining the structure of a molecule, particularly for molecules that are too complicated to work with using one-dimensional NMR. Types of 2D NMR can be classified as Homonuclear through-bond correlation methods which include correlation spectroscopy (COSY), Heteronuclear through-bond correlation methods, which include heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC), and through-space correlation methods, such as nuclear overhauser effect spectroscopy (NOESY) experiment (Harris, 1986).

Homonuclear correlation spectroscopy (COSY): It is the first and most popular two dimension which is used to identify spins which are coupled to each other. It consists of a single radiofrequency pulse (p1) followed by the specific evolution time (t1), followed by a

second pulse (p2) and followed by a measurement period (t2) (Heeschen, 1968). The two-dimensional spectrum that results from the COSY experiment shows the frequencies for a single isotope, most commonly hydrogen (^{1}H) along both axes. COSY spectra show two types of peaks namely diagonal peak and cross peak. Diagonal peaks have the same frequency coordinate on each axis and appear along the diagonal of the plot, while cross peaks have different values for each frequency coordinate and appear off the diagonal. Diagonal peaks correspond to the peaks in a 1D-NMR experiment, while the cross peaks indicate couplings between pairs of nuclei (Wasson & Corvan, 1978).

Another related COSY techniques is double quantum filtered COSY (DQF COSY). DQF COSY uses a coherence selection method such as phase cycling or pulsed field gradients, which cause only signals from double-quantum coherences to give an observable signal. This has the effect of decreasing the intensity of the diagonal peaks and changing their lineshape from a broad "dispersion" lineshape to a sharper "absorption" lineshape. It also eliminates diagonal peaks from uncoupled nuclei. These all have the advantage that they give a cleaner spectrum in which the diagonal peaks are prevented from obscuring the cross peaks, which are weaker in a regular COSY spectrum (Harris, 1986).

Heteronuclear single quantum coherence (HSQC): This is a CH correlation experiment which uses proton detection of the ^{13}C signals using an INEPT sequence. It shows higher resolution in the C-dimension than does the related HMQC experiment. HSQC detects correlations between nuclei of two different types which are separated by one bond. This method gives one peak per pair of coupled nuclei, whose two coordinates are the chemical shifts of the two coupled atoms. The HSQC works by transferring magnetization from the *I* nucleus (usually the proton) to the *S* nucleus (usually the hetero atom) using the INEPT pulse

sequence; this first step is done because the proton has a greater equilibrium magnetization and thus this step creates a stronger signal. The magnetization then evolves and then is transferred back to the *I* nucleus for observation. An extra spin echo step can then optionally be used to decouple the signal, simplifying the spectrum by collapsing multiplets to a single peak. The undesired uncoupled signals are removed by running the experiment twice with the phase of one specific pulse reversed, this reverses the signs of the desired but not the undesired peaks, so subtracting the two spectra will give only the desired peaks. Heteronuclear multiple-quantum correlation spectroscopy (HMQC) gives an identical spectrum as HSQC, but using a different method. The two methods give similar quality results for small to medium sized molecules, but HSQC is considered to be superior for larger molecules (Harris, 1986).

Mass Spectrometry

Mass Spectrometry is an analytic technique that utilizes the degree of deflection of charged particles by a magnetic field to find the relative masses of molecular ions and fragments. It is a powerful method because it provides a great deal of information and can be conducted on tiny samples. Therefore, mass spectroscopy allows quantitation of atoms or molecules and provides structural information by the identification of distinctive fragmentation patterns. The instrument used in mass spectrometry analysis is mass spectrometer. The mass spectrometer operation can be divided to three part namely, creation of gas-phase ions, separation of the ions on their mass-to-charge ratio and measurement of the quantity of ions of each mass-to-charge ratio. These three phases of operation are carried out by suitable ionisation source, mass analysers and detector respectively (Ma & Ouyang, 2016).

The Ionisation Source: Ionisation source converts gas phase sample molecules into ions. Examples include Chemical Ionisation (CI), Electron Impact (EI), Electrospray Ionization (ESI), Fast Atom Bombardment (FAB), Field Desorption/Field Ionisation (FD/FI), Matrix Assisted Laser Desorption Ionisation (MALDI) and Thermospray Ionisation (TI) (Bruins, 1998).

Electron Impact Ionisation: This is obtained by passing a beam of electrons through a gas-phase sample and collides with neutral analyte molcules (M) to produce a positively charged ion or a fragment ion. This method is applicable to all volatile compounds and gives reproducible mass spectra with fragmentation to provide structural information (Bruins, 1998).

Electrospray Ionization (ESI): Electrospray Ionisation is obtained by nebulizing solution under atmospheric pressure and exposed to a high electrical field which creates a charge on the surface of the droplet. The production of multiple charged ions makes electrospray extremely useful for precise mass measurement (Ho et al., 2003).

Fast Atom Bombardment (FAB): Fast Atom bombardment method of ionisation generates ion by using a high current of bombarding particle to bombard the analyte which is in low volatile liquid matrix. This is a soft ionisation technique and is suitable for analysis of low volatility species (Ho et al., 2003).

Chemical Ionisation: Chemical ionisation method employed the ionisation of a reagent gas by electron impact and then subsequently reacts with analyte molecules to produce analyte ions. This method gives molecular weight information and reduced fragmentation in comparison to EI (Klampfl & Himmelsbach, 2015).

The mass analyzer: Mass analyser sorts ions by their masses by applying electromagnetic fields. Examples include quadrupoles, Time-of-Flight (TOF), magnetic sectors, fourier transform and quadrupole ion traps (Bruins, 1998).

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Chapter Three

Kinetics of α-amylase and α-glucosidase inhibitory potential of *Hermannia geniculata* Eckl. & Zehl root extracts used in the Basotho traditional medicine

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This chapter has been formatted to be submitted in a peer review journal

Kinetics of α-amylase and α-glucosidase inhibitory potential of *Hermannia geniculata* Eckl. & Zehl root extracts used in Basotho traditional medicine.

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ABSTRACT

The scientific investigation of the folkloric use of *Hermannia geniculata* roots in the management of diabetes mellitus was conducted. Phytochemical analyses, *in vitro* antioxidant and hyperglycaemic studies were carried out on the crude extracts of *H. geniculata.* Qualitative phytochemical analysis revealed the presence of saponins, phenols, flavonoids, alkaloids, tannin, phytosterol, triterpenes and anthraquinones. The ethanolic extract exhibited the highest free radical scavenging capability with the lowest IC_{50} value (0.52, 0.38, 0.59, 0.63, 0.39 mg/mL) for 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-Azino-bis(3-ethylbenzothiazoline-6-Sulphonic acid (ABTS), hydroxyl radical, superoxide anion radical and metal chelating ability which is significantly different ($p<0.05$) from the standard (silymarin). In antidiabetic studies, ethanolic extract is a potent inhibitor of α-glucosidase (IC_{50}: 0.15 mg/mL) which is lower and significantly different ($p<0.05$) from the standard (acarbose) IC_{50} value of (0.52 mg/mL). Ethanolic extract exhibited a milder inhibition of α-amylase enzyme with IC_{50} (0.57mg/mL) which is higher and significantly different ($p<0.05$) from acarbose with IC_{50} (0.47 mg/mL). Kinetic studies revealed *H. geniculata* ethanolic roots extract exhibited competitive inhibition of α-amylase and uncompetitive inhibition of α-glucosidase enzymes. All these findings provided the scientific basis which support the use of

the root extract of *H. geniculata* in the management of diabetes mellitus and other oxidative stress induced ailments by the Basotho traditional medicine.

Keywords: *Hermannia geniculata*; antioxidant; diabetes mellitus; enzymes.

1.0 INTRODUCTION

Medicinal plant is an important part of traditional health care system and a veritable health care source for the vast majority of the world population. It was estimated that 70-80% of people worldwide use herb for management of mild to moderate illnesses (Chaudhary, Gadhvi, & Chaudhary, 2010; Geun Kim & Sook Oh, 2012; Jokar, Noorhosseini, Allahyari, & Damalas, 2017; Jütte *et al.*, 2017; Yea, Kim, Kim, & Yi, 2017).

The phenomenal rise of the alternative medicine industry responds to some of these shortcomings in what modern medicine has to offer. In several North American and European countries, the production and sale of herbal medicines, dietary supplements, and other so-called "natural" products have become a huge and profitable industry, amounting to $32 billion a year in the USA alone (Margaret, 2016). Therefore, South Africa with about 9% of the world vegetation (Street & Prinsloo, 2013) must tap into this emerging market through conscious and systematic investigation of medicinal plants in her domain. Thus, the need for scientific investigation of *Hermannia geniculata* which has been frequently used in the folkloric medicine for the cure of several diseases like diabetes, colitis, severe wound, gastrointestinal disorder and skin diseases (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008; Mutiu Idowu Kazeem & Ashafa, 2015; Moffett, 1993).

It is a flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae* (Gwynne-Evans, 2015; Leistner, 2000). The plant is seen across South Africa and its vast majority being endemic in Eastern Cape, Free State, Guateng, KwaZulu-Natal, Limpopo and Mpumalanga.

It is also found in Madagascar, East Africa, North-East Africa and Saudi Arabia and North America (Gwynne-Evans, 2015).

Studies on safety and toxicity of the aqueous roots extract of *H. geniculata* on rats showed that it is safe (Mutiu Idowu Kazeem & Ashafa, 2015). However, there is dearth of scientific information to authenticate its wide use as a medicine by the Basotho tribe.

This present study seek to validate the folkloric use of *H. geniculata* in the management of diabetes mellitus and several oxidative stress induced diseases. It also revealed the kinetics of α-amylase and α-glucosidase inhibitory potentials of *H. geniculata* root extracts.

2.0 **MATERIALS AND METHODS**

2.1 Plant collection, Preparation and Extraction.

Hermannia geniculata roots were purchased from a local market in Puthaditjhaba, Qwaqwa, Northern Free State Province, South Africa. Confirmation of the species identity was carried out with the herbarium specimen with voucher specimen file number (5056.000-10700) (Moffett, 1993) at the University of Free State, Qwaqwa Campus, South Africa. It was also compared with our earlier Voucher specimen (Ash/med/05/2013/QwHB) (Mutiu Idowu Kazeem & Ashafa, 2015) at the herbarium.

The roots were separated, washed under running tap to remove all debris and chop into smaller pieces before being dried in the oven for nine days at 45°C to a constant weight. The dried roots material was pulverised into fine powdered using waring laboratory blender (Labon, Durban, South Africa.

30g each of the dried powdered material was extracted in 300 mL of distilled water, warm water of 40°C (decoction), ethanol and hydro-ethanol (50-50), with constant shaking on a Labcon platform shaker (Laboratory Consumables, PTY, Durban, South Africa).The mixture

was filtered using No. 1 Whatman filter paper. The ethanol extract was concentrated to dryness *in vacuo* at 40°C using a rotary evaporator (Cole-Palmer, South Africa). All other extracts were air dried and stored at -4°c until use.

2.2 **Chemicals and reagents**

Porcine pancreatic α-amylase, rat intestinal α- glucosidase, 1,1-diphenyl-2-picrylhydrazyl, gallic acid, acarbose and paranitrophenyl-glucopyranoside were products of Sigma-Adrich, South Africa while starch soluble (extra pure) was obtained from J.T BakerInc., Phillipsburg, USA. Other chemicals and reagents were of analytical grade and the water used was glass distilled.

2.3 **Measurement of percentage yield.**

The percentage yield of the extract was calculated as ((c-b)/a) x100. Where a = weight of sample; b = weight of beaker and c= weight of beaker + sample.

2.4 **Qualitative Phytochemical screening**

Using described procedure (Sofowora, 1982) the root extracts of *Hermannia geniculata* was subjected to qualitative phytochemical screening. Alkaloids, anthraquinones, flavonoids, phenols, saponins, tannins, triterpenes, phytosterols were screened for.

2.5 **Quantitative phytochemical analysis**

2.5.1 **Assessment of total phenolic content**

The quantification of phenolic content of *H. geniculata* extracts was carried out using the procedure reported by (Wolfe, Wu, & Liu, 2003) An aliquot of the extract fractions (1 mL) was mixed with 5 mL Folin Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15s and allowed to stand for 30 min at 40°C for colour development. Absorbance was read at 765 nm using a

spectrophotometer (Beckman, DU 7400, USA). Extracts were evaluated at a final concentration of 1 mg/mL. Gallic acid was used as standard. Total phenolic content was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.

2.5.2 **Determination of total flavonoids**

The total flavonoids of the extracts were determined using the method already adopted (Samatha, Shyamsundarachary, Srinivas, & Swamy, 2012). Briefly, to 0.5 mL of the sample and standard (1 mg/mL), 2 mL distilled water was followed by 0.15 mL of 5% of $NaNO_2$ and allowed to stand at 25°C for 5 - 6 min. 0.15 mL of 10% $AlCl_3$ was added and allowed to stand for another 6 minutes. After which 1 mL of 4% NaOH was added to the mixture and make up to 5 mL with distilled water, vortexed for 15 minutes and colour change observed. The absorbance was measured at 420 nm using spectrophotometer. Total flavonoid contents were calculated as quercetin (mg/g) equivalent using the equation obtained from a calibration curve of quercetin

2.6 ***In vitro*** **antioxidant assays**

All experiments were conducted in triplicates and all the negative control (blank) was prepared using the same procedure replacing the extract with distilled water

2.6.1 **1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity**

The free radical scavenging activity of the H. geniculata root extracts were evaluated based on its scavenging activities on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The method was described by (Braca *et al.*, 2001). Briefly, 150 µL of the varying concentration of plant extract/ standard (0.02 – 0.1 mg/mL) was added separately to 150 µL of 0.004% methanolic solution of DPPH in a 96-well microtiter plate. The absorbance at 517

nm was determined after 30 min using a 96-well microplate reader (BIORAD, model 680, Japan), and the percentage inhibitory activity of the extract/standard was calculated using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. The half maximal inhibitory concentration (IC_{50}) value were calculated from the linear regression equation using $y = m\ x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.6.2 **Hydroxyl radical scavenging ability**

The ability of the plant extracts to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the modified method of (Mathew & Abraham, 2006). Briefly, 100 µL of different concentrations (0.02-01 mg/mL) of *H. geniculata* root extracts and standard, 120 µL of 20 mM deoxyribose, 400 µL of 0.1 M phosphate buffer, 40 µL of 20 mM hydrogen peroxide and 40 µL of 500 µM ferrous sulphate each were taken and mixed in 2 mL Eppendorf tubes. Then 100 µL of distilled water was added and incubated for 30 min at 37 °C. Subsequently, 0.5 mL 2.8% of trichloroacetic acid (TCA) and 400 µL of 0.6% thiobarbituric acid (TBA) solutions were added to stop the reaction. From the mixture, 300 µL of the resultant mixture was dispensed into a 96-well micro titer plate and incubating in boiling water for 20 min. The absorbance was taken at 532 nm using a microplate reader (BIO-RAD, model 680, Japan). The percentage inhibition of FHG extract/standard was obtained using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and obtained from the linear regression equation using $y = m\ x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.6.3 **Superoxide Anion Scavenging Assay**

Determination of superoxide anion radical scavenging potential of *H. geniculata* root extracts were achieved according to (Liu, Ooi, & Chang, 1997). Superoxide radicals were generated in 50 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 50 mL of NBT (50 mM) solution, 50 mL of NADH (78 mM) solution and different concentrations (0.02–0.1 mg/mL) of FHG extract and standard. The reaction was initiated by adding 1 mL of phenazine methosulphate (PMS) solution (10 mM) to the mixture. The reacting mixture was incubated at 25 ^{0}C for 5 min, then the absorbance was measured at 560 nm using a microplate reader (BIO-RAD, model 680, Japan). IC_{50} was then evaluated from calibration curve following estimation of percentage superoxide anion scavenging capacity of the tested extract of FHG using the expression: Percentage Scavenging (S %)=$[(A_{control}-A_{extract})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control, $A_{extract}$. The IC_{50} value was obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value

2.6.4 **Metal Chelating Assay**

The chelating of ferrous ions by root extracts of *H. geniculata* was estimated as described by (Dinis, Madeira, & Almeida, 1994). Briefly, 40 µL of the different concentrations of the extract and standards (0.02 –0.1 mg/mL) was dispensed into a 96-well microtiter plate, 200 µL of 2 mM $FeCl_2$ solution was afterwards added to the mixture. The reaction was initiated by the addition of 80 µL 5 mmol/L ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 mins. The absorbance of the solution was then read at 562 nm using a BIO-RAD (model 680, Japan) microplate reader. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the

absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and obtained from the linear regression equation using y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.6.5 **Ferric ions reducing power**

Ferric ions reducing power (FRAP) of the extracts and standards were determined according to the method adopted by (Müller, Fröhlich, & Böhm, 2011). Varying concentrations of the extracts (0.125 - 1.0 mg/mL) were mixed with 2.5 mL of 20 mM phosphate buffer and 2.5 mL1%, w/v potassium ferricyanide ($KFe(CN)_6$), and then the mixture was incubated at 50 °C for 30min. Afterwards, 2.5 mL of 10%, w/v trichloroacetic acid (TCA) and 0.5mL 0.1%, w/v ferric chloride were added to the mixture in 96 well plate and which was kept aside for 10 min. The absorbance was measured at 700 nm, gallic acid and silymmarine were used as standard antioxidants.

2.6.6 **2,2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Radical Scavenging Determination**

The ability of *H. geniculata* root extracts to scavenge ABTS cation chromophore obtained from the oxidation of ABTS solution and potassium persulphate was determined according to already adopted method (Re *et al.*, 1999). Briefly, 50 mL each of 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulphate leaving the mixture for 4-16 h until the reaction was completed and absorbance was stable. The resultant mixture was diluted with ethanol to get an absorbance of 0.700±0.05. The absorbance reading was taking at 734 nm using microplate reader (BIO-RAD, model 680, Japan). 20 µL of different concentration (0.02-0.1 mg/mL) of FHG and standard was then mixed with 200 µL ABTS solution in a 96-well microtiter plate and absorbance was read at 734 nm using a microplate reader (BIO-RAD,

model 680, Japan) after 15 mins of incubation at 25°C. The percentage inhibition activity was obtained using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

The IC_{50} value was calculated and obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.7 *In vitro* Antidiabetic Assays

2.7.1 α-Amylase Inhibitory Assay

This assay was carried out using the procedure of (Apostolidis, Kwon, & Shetty, 2007). 250 mL of varying concentration of extract/ standard (0.125 – 1.0 mg/mL) was placed in a test tube and 250 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was incubated at 25°C for 10 min, followed by addition of 250 mL of starch (1%) solution in 0.02 M sodium phosphate buffer (pH 6.9) at timed intervals, the resulting reaction mixture was then incubated at 25°C for 10 min. The reaction was terminated by adding 500 mL of dinitrosalicylic acid (DNS) reagent before incubating the tubes in boiling water for 5 min and cooled to 25°C. 5 mL distilled water was added to the reacting mixture after cooling and the absorbance was measured at 540 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was done in triplicate and was calculated as percentage inhibition, thus; % Inhibition = (Absorbance (control) – Absorbance (extract))/ Absorbance (control) ×100. Concentrations of extract resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y

= m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.7.2 α-Glucosidase Inhibitory Assay

The effect of the FHG extract on α-glucosidase activity was determined according to the method described by (Apostolidis *et al.*, 2007) with slight modification. In brief, different concentrations (0.125 – 1.0 mg/mL) of extract/ standard were prepared in distilled water. Then, 50 mL from the stock solution was mixed with 100 mL of 0.1 M phosphate buffer (pH6.9) containing 1.0 M of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25°C for 10 min. Following this, 50 mL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. The inhibitory effect of extract/standard on the enzyme activities were determined by measuring the absorbance of the mixtures at 405 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The experiments were conducted in triplicate and the α-glucosidase inhibitory activity was expressed as % inhibition using the expression:

%Inhibition= [(Acontrol-Aextract)/Acontrol]×100, where Acontrol and Aextract are the absorbance's of the control and extract respectively. Concentrations of extract/standard resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.8 Kinetic Studies

2.8.1 Mode of α-Amylase Inhibition

This assay was conducted using modified methods (Ali, Houghton, & Soumyanath, 2006). Briefly, 250 mL of the (5 mg/mL) extract of FHG/ standard were pre-incubated with 250 mL of 0.5 mg/mL α-amylase solution for 10 min at 25°C in one set of tubes while α-amylase was pre-incubated with 250 mL of 0.1M phosphate buffer (pH 6.9) in another set of tubes. The reaction of the two sets of the mixtures was initiated by adding 250 mL of 5% starch solution at increasing concentrations (0.31 – 50.00 mg/mL). The mixture was then incubated for 10 min at 25°C, followed addition of 0.01mM DNS (500 mL). The reaction was terminated after boiling for 5 min. The amount of reducing sugars released was determined spectrophotometrically using maltose standard curve and converted to reaction velocities. A double reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted to determine the mode of inhibition.

2.8.2 Mode of α-Glucosidase Inhibition

The kinetics on inhibition of α-glucosidase activity by FHG extract using modified methods of (Nagmoti & Juvekar, 2013) was adopted. Briefly, 50 µL of 5 mg/mL extract was pre-incubated with 100 µL of 0.1M α-glucosidase solution for 10 min at 25°C in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated with 50µL of 0.1M phosphate buffer (pH6.9). 50 mL of 0.05M pNPG at different concentrations (0.31 – 50.00 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10min at 25 °C, and 500 mL of 0.1M Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined colourimetrically using a p-nitrophenol standard curve. Reaction rates (v) were thereafter calculated and double reciprocal plots of enzyme

kinetics. Km and Vmax values were also calculated from Lineweaver-Burkplot (1/v versus 1/[S]) (Lineweaver & Burk, 1934).

2.9. **Statistical Analysis**

Statistical analysis was performed using a Graph Pad Prism 5 statistical package (Graph Pad Software, San Diego, MA, USA). Data were expressed as means of replicate determinations ± SD, for all assays and was subjected to one-way analysis of variance (and nonparametric) followed by Bonferroni: compare all pair of column. Statistical significance was considered at $P < 0.05$.

3.0 **RESULTS**

The percentage yield by the different solvent used in the extraction is presented in Table i.

3.1 **Phytochemicals**

The qualitative and quantitative analyses of the root extracts of *H. geniculata* are presented in Tables ii and iii respectively. Saponins, phenols, flavonoids, anthraquinones, alkaloid, tannins, triterpenes and phytosterols were detected at varying degree in all the tested extracts while anthraquinone and phytosterol were found in trace amount in the ethanol and hydro-ethanol extracts.

3.2 **Antioxidant activity**

The *in vitro* antioxidant potentials of the root extracts of *H. geniculata* are shown in Figures 1-6. The extracts scavenged/inhibited/chelated the generated radicals/ions/metals in all assays evaluated. Ethanolic extracts showed better capability to scavenge DPPH and hydroxyl radicals in a dose dependent manner (0.125-1.00) mg/mL Figures i and ii. Its corresponding IC_{50} value is (0.52 and 0.59) mg/mL which is lower and significantly different ($p<0.05$) from the standard (silymarin) IC_{50}: (1.09 and 1.12) mg/mL as seen in Table iv.

However, hydro-ethanol showed remarkable capability in scavenging superoxide anion radical, its IC_{50} value is 0.49 mg/mL which is comparable to silymarin with IC_{50}:1.12 mg/mL. *H. geniculata* root extracts also showed significant metal chelating potential against ferrous ion (Figure 3) and the respective IC_{50} value when compared with the standard (citrate) is presented in Table iv. The reducing power and ABTS cation scavenging capability of the extracts competed well with silymarin in a dose dependent manner (0.125- 1) mg/mL with the highest dose of 1mg/mL showing the best activity Table iv, Figures 5 and 6 respectively.

3.3 ***In vitro*** **antidiabetic assays**

The inhibitory potentials of *H. geniculata* root extracts on both α- amylase and α- glucosidase enzymes is dose dependent (0.125-1) mg/mL, the percentage inhibition is presented in Figures 7 and 8. Ethanolic extract has the lowest IC_{50} (0.15) mg/mL which is significantly different ($p<0.05$) from all other extracts and acarbose (Table v). Ethanol and decoction extracts show milder inhibition of α-amylase with their respective IC_{50} value of (0.57 and 0.62) which is higher and significantly different ($p< 0.05$) from acarbose and hydro-ethanol (IC_{50}:0.47 and 0.42) mg/mL respectively.

4.0 **DISCUSSION**

The use of plants in treating diseases is as old as civilization (Fabricant & Farnsworth, 2001) and herbal medicine is still a major part of habitual treatment of different diseases (Cragg & Newman, 2013). The process in the preparation of herbs like pulverization, extraction and solvents deployed in the extraction of raw material for drugs affects the percentage yield of the biologically active compound present in the extracts. In this experiment, we use the local solvents deployed in herbs preparation such as ethanol, hydro-ethanol, decoction and distil water as solvent for extraction. The percentage yield indicated that hydro-ethanol has the

highest yield of 29.71% from the 30g dry weight of the plant sample extracted while decoction extract yield 8.05% of the 30g dry weight of the plant sample. It is worthy of note that the traditional healer use decoction (boil the dry root of *Hermannia geniculata*) as their method of extracting the biologically active component of the plant (Moffett, 1993). It may be suggested that this method of extraction accounted for low yield of extract which may contain less biologically active compound.

Result of the quantitative phytochemical assays indicated the concentration of the different quantity of the phytochemicals (PC) found in the root extracts of *H. geniculata* though, its bioavailability is unpredictable in *in vivo* study, because a lot of factors like absorption barrier of the PC in the gastro intestinal tract (GIT), the effects of different enzymes such as the glucosidase, esterase, oxidase and hydrolases originating from the host and the mycobiota which may inhibit PC activity in the GIT (Sousa, *et al.,* 2008). Phytochemicals are known to possess varying antioxidant activities (Egea *et al.*, 2017; Huang, Cai, Zhang, Huang, & Cai, 2017; Iranshahy *et al.*, 2017; Y. Liu, Wang, & Cai, 2015; Tafesse, Hymete, Mekonnen, & Tadesse, 2017). Antioxidant activity of a medicinal plant cannot be concluded based on a single antioxidant test model (Egea *et al.,* 2017) as such several *in vitro* antioxidant tests were conducted on the extracts using silymarin as positive control for all assays except metal chelating assay where citrate was used as the standard. We determine the free radical scavenging capability of *H. geniculata* on the molecules of DPPH radicals, ABTS cations radical, the reducing power, superoxide anion radicals. We also assay for hydroxyl radical which is one of the most potent reactive oxygen species in the biological system that react with polyunsaturated fatty acid moieties of cell membrane phospholipid causing cellular damage.

The result of the assay showed that ethanol extract of *Hermannia geniculata* has better performance in free radical scavenging activity compared to the standard and other extract tested for DPPH, hydroxyl radical and metal chelating activities while hydro ethanol showed superior activity compared to the standard and other extracts tested in ABTS, superoxide anion and reducing power. All these predictions is based on the standard curve of percentage inhibition/scavenging effect and IC_{50} value of the tested extract which revealed a decrease in concentration of the reactive oxygen species (ROS) which may be due to the scavenging ability of *H. geniculata* extracts. Similar findings has been documented for the antioxidant and anti-inflammatory properties of some *Hermannia* species (Essop *et al.*, 2008). It is noteworthy that the tested extract demonstrated ability to neutralize the ROS at different degree which may be because of the presence of phytochemicals like polyphenols which has capability to directly scavenge superoxide and other ROS like hydroxyl and peroxyl radicals (Azofeifa *et al.*, 2013; Medini, Fellah, Ksouri, & Abdelly, 2014; Pandey & Rizvi, 2009). Saponins, triterpenes and phytosterol has been demonstrated to scavenge superoxide anion (Dufour, Lavoie, Laprise, & Legault, 2007; Repetto, Llesuy, & Aires, 2002; Zhao *et al.*, 2013). Flavonoid are currently receiving attention as a potential protector against variety of human disease, major flavonoid has been shown to have neutralizing effect on free radical and ROS like hydroxyl radical, superoxide radical, hydrogen peroxides (Egea *et al.*, 2017; Kim, Moon, Choi, Kim, & Lee, 2013; Repetto *et al.*, 2002; Shakya, 2016).

Marked postprandial hyperglycaemia is important in the pathogenesis of type 2 diabetes, it induces mitochondrial superoxide overproduction which potently inhibit the glycolytic enzyme glyceraldehyde-3-phosphate (GAPDH) thus, diverting upstream metabolites from glycolytic pathway into pathway of glucose overutilization resulting in formation of

diacylglycerol (DAG) from dihydroxylacetone phosphate (DHAP) a potent activator of protein kinase C (PKC) which ultimately causes β-cells destruction and insulin resistance (King & Loeken, 2017; Tiwari, Pandey, Abidi, & Rizvi, 2013; Ullah, 2016). The unregulated hydrolysis of starch by α- amylase and α-glucosidase which catalyze the rate limiting step in the conversion of oligosaccharides and disaccharides into monosaccharide's is responsible for the elevated blood glucose seen in type 2 diabetes mellitus. Therefore, controlling hyperglycaemia via inhibition of carbohydrate hydrolysing enzymes is an important strategy in the management of type 2 diabetes mellitus (Mbhele, Balogun, Kazeem, & Ashafa, 2015; Mohamed *et al.*, 2012; Sabiu, O'Neill, & Ashafa, 2016). *In vitro* evaluation of the inhibitory effect of our extracts α- glucosidase and pancreatic α- amylase enzymes was carried out on all the extract using acarbose as the standard to determine its percentage inhibition and their respective IC_{50} value. Mild inhibition of α- amylase and strong inhibition of α- glucosidase enzymes is targeted as a way of reducing postprandial hyperglycaemia, and elimination of the unwanted effect like gastrointestinal discomfort flatulence, diarrhoea associated with the use of acarbose (Kazeem, Adamson, & Ogunwande, 2013; Sabiu *et al.*, 2016). In this study, ethanol and decoction extracts mildly inhibit α- amylase with their respective IC_{50} values of (0.57 and 0.62) which is higher and significantly different ($p<0.05$) from acarbose with lower IC_{50} (0.47 mg/mL. The result of the inhibitory potentials of the extracts on α- glucosidase showed ethanol and decoction extracts has potent inhibition of the enzyme activity. Thus, may be employed in the management of postprandial hyperglycemia. This finding is consistent with findings of many authors (Mbhele *et al.*, 2015; Mohamed *et al.*, 2012; Olaokun, Mcgaw, Rensburg, Eloff, & Naidoo, 2016) who described moderate inhibition of α- amylase and strong inhibition of α-glucosidase as a better therapeutic approach to be deployed in the delay

and regulation of carbohydrate hydrolysis in the intestine which is responsible for glucose toxicity observed in type 2 diabetes mellitus.

The ethanolic extract which possess the highest IC_{50} for α- amylase enzyme and lowest IC_{50} for α- glucosidase compared to acarbose and other tested extracts of *H. geniculata* was used to determine the mode of inhibition of α- amylase and α- glucosidase enzymes in other to investigate its enzyme inhibition kinetics.

Result for the mode of inhibition of α- amylase enzyme showed that the ethanolic extract is competitively inhibiting the breakdown of disaccharides and oligosaccharides which are substrate for α- amylase. The Vmax values obtained with inhibitor and without inhibitor in the reaction pathway is the same, the Km values decreased from $4.85x10^{-2}$ $(\mu M)^{-1}$for reaction pathway without inhibition to $1.44x10^{-2}$ $(\mu M)^{-1}$ with inhibitor. Decreased Km value signify increase affinity. This result suggested competitive mode of inhibition.

However, the mode of inhibition of α- glucosidase by ethanolic extract of *H. geniculata* is by uncompetitive inhibition. The propose model is the binding of the *H. geniculata* extract (inhibitor) to a site other than the active site and only when the substrate is binding to ES complex thereby inhibiting the formation of product. The kinetic further shows that there is a decrease in Km from ($7.10x10^{-2}$ $(\mu M)^{-1}$ to $4.69x10^{-2}$ $(\mu M)^{-1}$without inhibitor and with inhibitor respectively) and also a decrease in Vmax from 19.76 μM/min without inhibition to 14.66 μM/min with inhibition which suggests a 39.74% decrease in overall activity of α- glucosidase enzyme in the presence of ethanolic extract of *Hermannia geniculata*.

Conclusion

Our findings scientifically justify the use of *H. geniculata* in Basotho traditional medicine. Moreover, the overall result revealed better performance of the extracts *in vitro* than all the

standards (acarbose/silymarin/citrate) in both antidiabetic and antioxidant assays. Therefore the root extract of *Hermannia geniculata* may play an important role in the development of nutraceuticals and also in the management of oxidative stress induced illnesses and diabetes.

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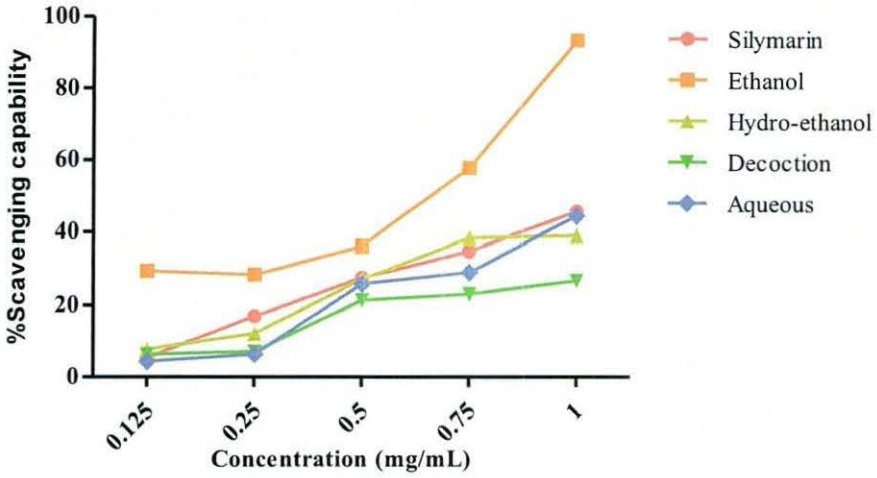


Figure 1. DPPH scavenging effect of the root extracts of *Hermannia geniculata.* Values are mean ± standard deviation (SD) of triplicate determinations. N=3; ($p<0.05$).

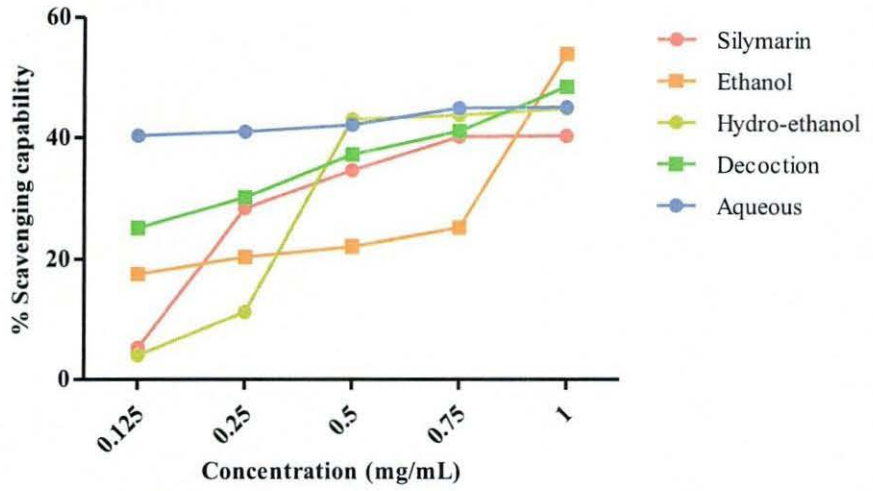


Figure 2. Scavenging effect of the root extracts of *Hermannia geniculata* on hydroxyl radical Values are mean ± standard deviation (SD) of triplicate determinations. N=3; ($p<0.05$).

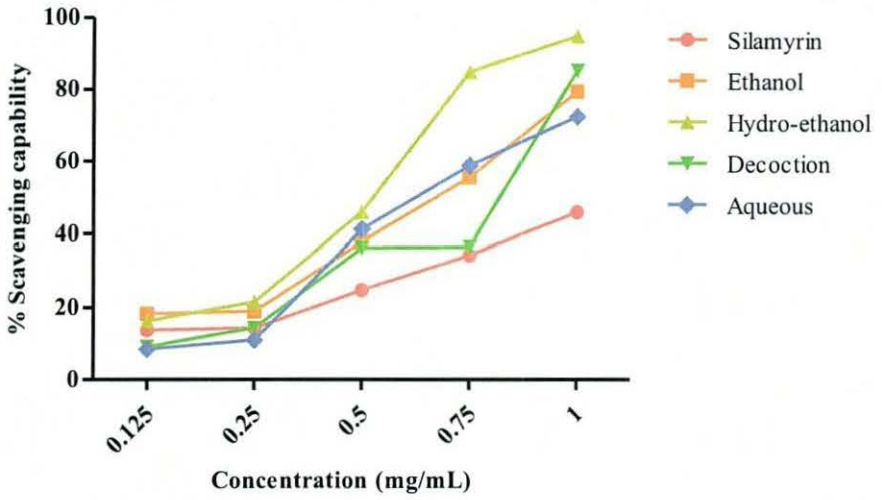


Figure 3. Scavenging effect of the root extracts of *Hermannia geniculata* on superoxide anion radical Values are mean ± standard deviation (SD) of triplicate determinations. N=3; ($p<0.05$).

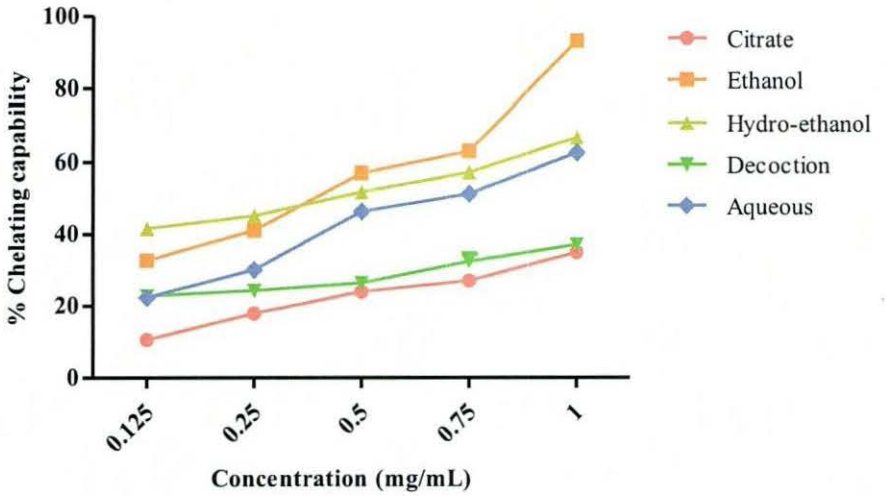


Figure 4. Metal chelating capability of the root extracts of *Hermannia geniculata.* Values are mean ± standard deviation (SD) of triplicate determinations. N=3; ($p<0.05$).

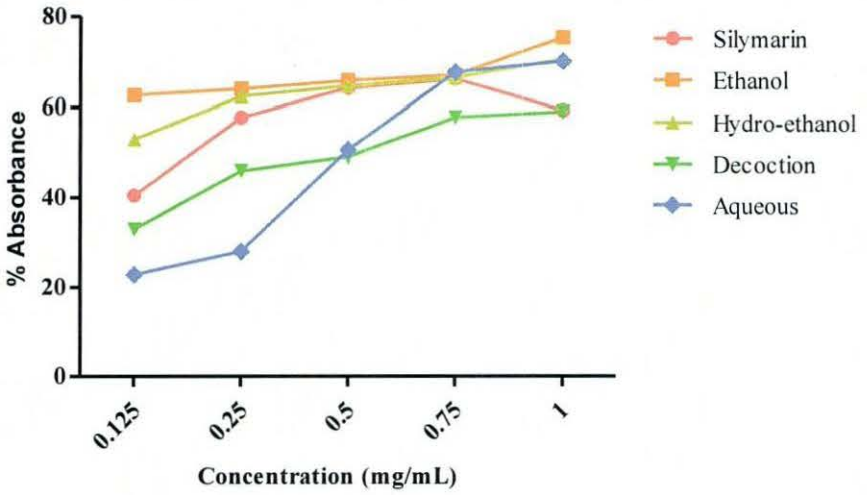


Figure 5. Reducing potentials of the root extracts of *Hermannia geniculata.* Values are mean ± standard deviation (SD) of triplicate determinations. N=3; ($p<0.05$).

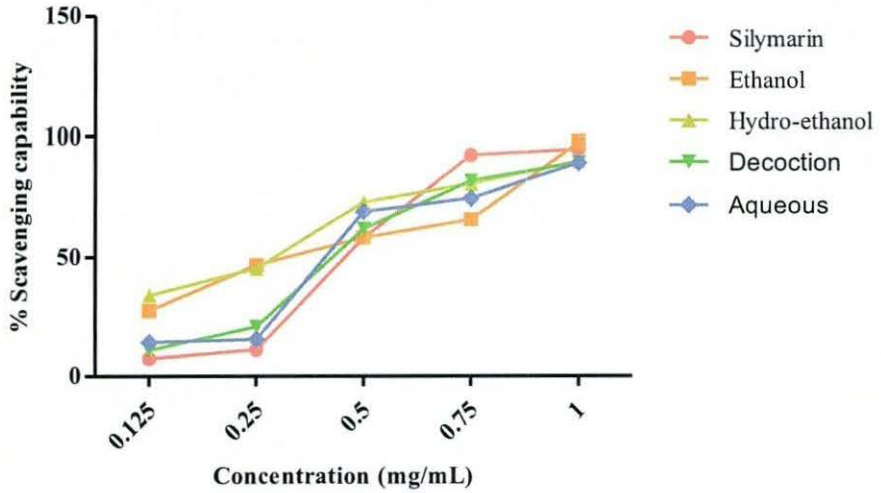


Figure 6. ABTS scavenging effect of the root extracts of *Hermannia geniculata.* Values are mean and standard deviation (SD) of triplicate determinations. N=3; ($p<0.05$).

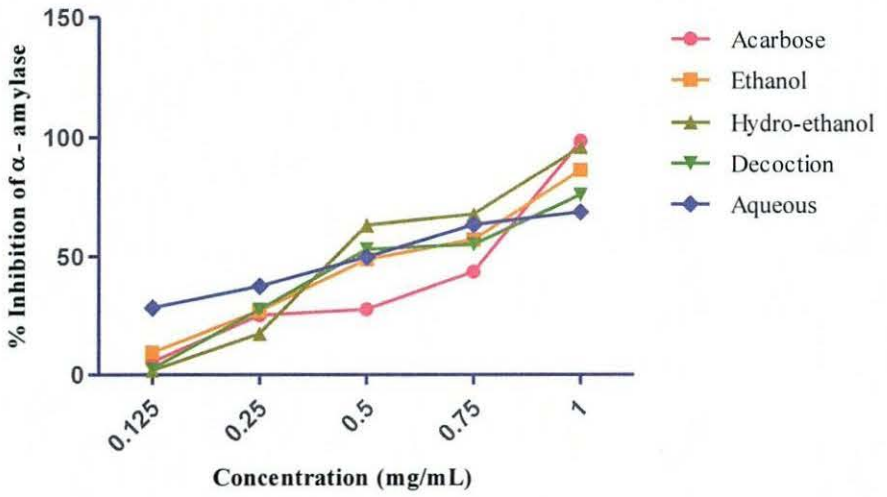


Figure 7. The inhibitory potentials of *H. geniculata* root extracts on α-amylase activity. Value are mean and standard deviation (SD) of triplicate determination. N=3; ($p<0.05$).

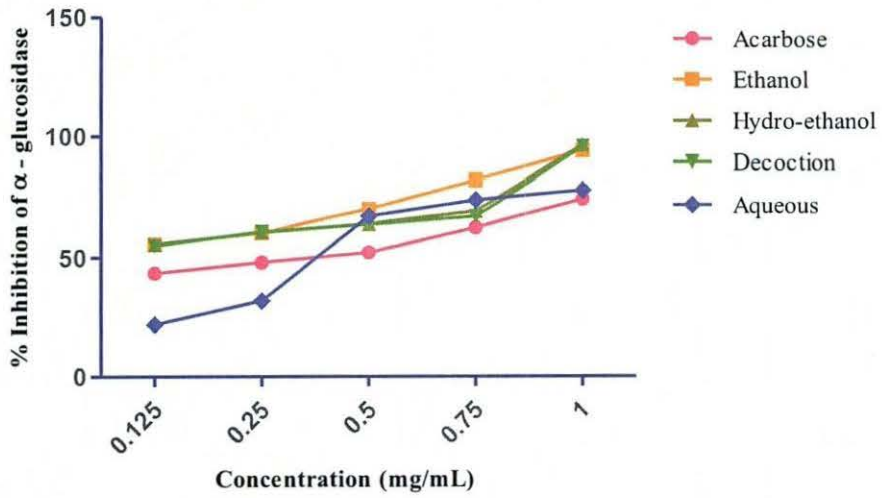


Figure 8. The inhibitory potentials of *H. geniculata* root extracts on α-glucosidase activity. Value are mean and standard deviation (SD) of triplicate determination. N=3; ($p<0.05$).

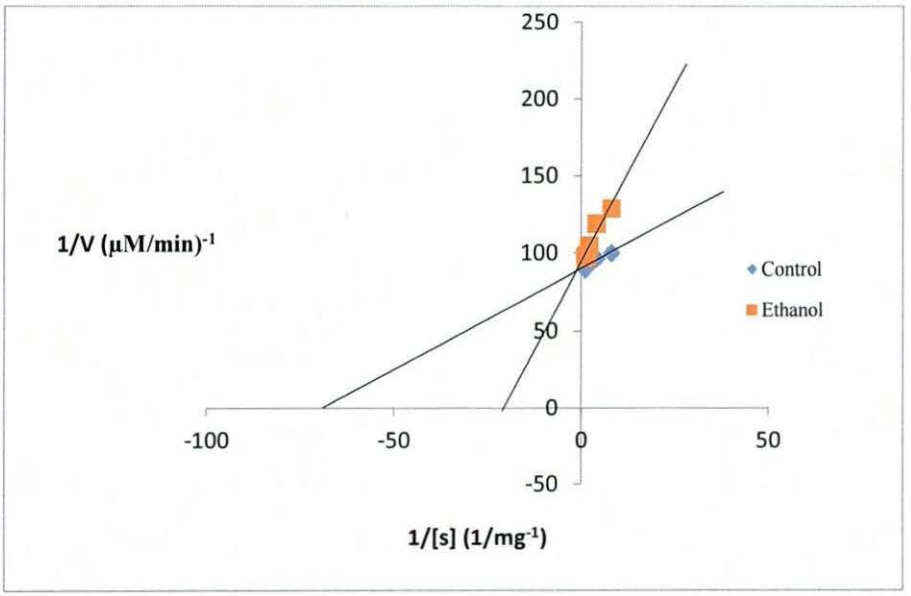


Figure 9. Lineweaver-Burk plot of ethanolic extract of *Hermannia geniculata* eliciting competitive inhibition on α- amylase activity. Result represent mean± standard deviation: (n=3; ($p<0.05$)

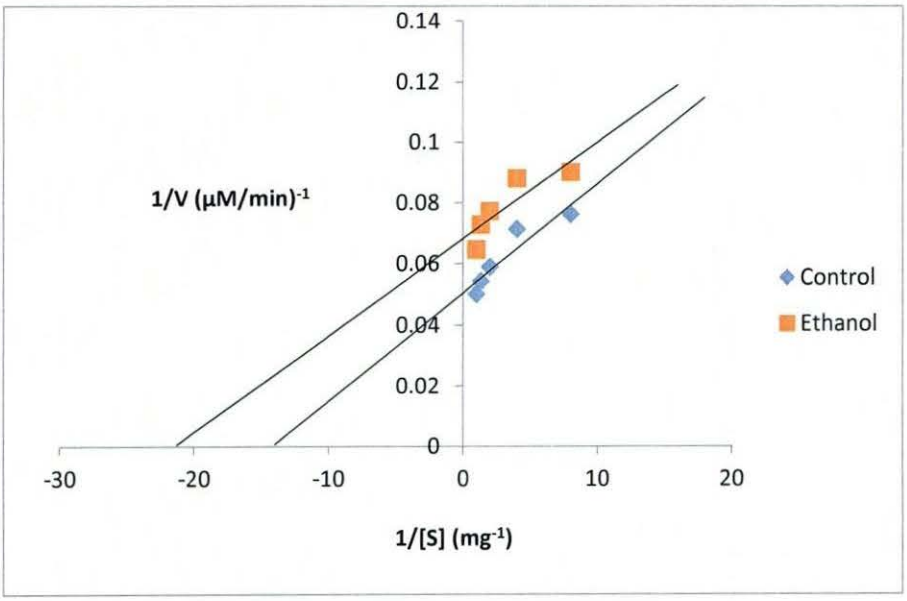


Figure 10. Lineweaver-Burk plot of ethanolic root extract of *Hermannia geniculata* eliciting uncompetitive inhibition on α-glucosidase activity. Result represent mean± standard deviation: (n=3; ($p<0.05$)

TABLES

Table i: The percentage yield from different extracting solvents used in the root extract of *Hermannia geniculata*

	Ethanol	Hydro-ethanol	Decoction	Aqueous
Percentage yield (%)	11.12	29.71	8.05	18.55

Table ii. Phytochemical constituents of the root extracts of *Hermannia geniculata.*

Phytochemicals	Ethanol	Hydro-ethanol	Decoction	Aqueous
Alkaloids	+++	+++	++	++
Phenols	+++	++++	+++	++
Flavonoids	+	+	-	-
Anthraquinones	++	++	+++	+++
Tannins	++++	++++	++	++
Triterpenes	-	++	+++	++++
Phytosterol	_	++	+++	+++

Key: +: detected; +++: degree of intensity; -: not detected or in trace amount.

Table iii. The result of the quantitative phytochemical screening of *Hermannia geniculata* root extracts.

Phytochemicals	Ethanol	Hydro-ethanol	Decoction
Aqueous			
Total Flavonoid (mg quercetin in g-1)	0.36	1.10	0.61
0.30			
Total phenol (mg gallic acid g-1)	8.35	10.29	10.79
10.41			

Table iv. The IC_{50} values of the free radical scavenging/chelating capabilities of different extracts of *Hermannia geniculata*.

Samples	EC_{50} (µg/mL)				
	DPPH	ABTS	Hydroxyl	Superoxide	Metal Chelating
Silymarin	1.09 ± 0.02^{a}	0.39 ± 0.05^{a}	1.12 ± 0.02^{a}	1.12 ± 0.01^{a}	
Citrate					1.5 ± 0.01^{a}
Ethanol	0.52 ± 0.05^{b}	0.38 ± 0.02^{a}	0.59 ± 0.01^{b}	0.63 ± 0.10^{b}	$0.39 \pm .01^{b}$
Hydro-ethanol	1.15 ± 0.03^{a}	0.30 ± 0.02^{b}	0.94 ± 0.01^{a}	0.49 ± 0.00^{c}	$0.41 \pm .05^{b}$
Decoction	1.78 ± 0.01^{c}	0.49 ± 0.02^{c}	1.03 ± 0.01^{a}	0.57 ± 0.01^{b}	$1.73 \pm .02^{c}$
Aqueous	1.05 ± 0.01^{a}	0.49 ± 0.05^{c}	1.76 ± 0.01^{c}	0.60 ± 0.01^{b}	$0.67 \pm .01^{d}$

The values are expressed as mean ± standard deviation (SD) of triplicate determination. Means down vertical column not sharing a common superscript are significantly different ($p<0.05$) from each other. Silymarin is the standard antioxidant agent for all the antioxidant assays except metal chelating that has citrate as the standard.

Table v. The IC_{50} values for the root extracts of *Hermannia geniculata* on specific activities of α-amylase and α-glucosidase enzymes

Samples	IC_{50} (µg/mL)	α- glucosidase	α-amylase
Acarbose		0.52 ± 0.04^{a}	0.47 ± 0.01^{a}
Ethanol		0.15 ± 0.00^{b}	0.57 ± 0.01^{b}
Hydro-ethanol		0.39 ± 0.00^{a}	0.42 ± 0.05^{a}
Decoction		0.46 ± 0.01^{a}	0.62 ± 0.03^{d}
Aqueous		0.45 ± 0.04^{a}	0.53 ± 0.08^{a}

The values are expressed as mean ± standard deviation (SD) of triplicate determination. Means down vertical column not sharing a common superscript are significantly different ($p<0.05$) from each other.

Chapter Four

In vitro Pharmacology and Toxicological Studies and Anticancer Mechanism of Root Extracts of *Hermannia geniculata* Eckl. & Zeyh

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This chapter is under review in Asian Pacific Journal of Tropical Biomedicine

***In vitro* Toxicological Studies and Anticancer Mechanism of Root Extracts of *Hermannia geniculata* Eckl. & Zeyh.**

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ABSTRACT

Objective: To investigate the pharmacological and toxicological effect of the extracts of *Hermannia geniculata* roots. **Methods**: *In vitro* cytotoxicity of the extract was carried out on Vero, HepG2 and RAW 264.7 macrophage cell lines using MTT assay. Also, the capabilities of the extract to inhibit 5-lipoxygenase enzyme activities and overproduction of nitric oxide from LPS-activated RAW 264.7 macrophages were evaluated. **Results**: The MTT assay showed selective toxicity of the extracts to normal and cancer cell. The LC_{50} values of Vero cells ranges from (0.40-0.57 mg/mL) while the LC_{50} value of HepG2 cells varies from (0.016-0.136 mg/mL). The selectivity index (SI) were (31.87, 18.87, 33.33 and 3.52) for ethanol, hydro-ethanol, decoction and aqueous extracts respectively. The ethanolic extract inhibited NO production in a dose dependent manner. There was a decrease of 82% at concentration of 0.1 mg/mL and the LC_{50}:3.64 mg/mL is lower and significantly different ($p<0.05$) compared to the reference compound quercetin with LC_{50} value of 8.28 mg/mL. Similarly, the ethanolic extract exhibited potent inhibition of 5-lipoxygenase enzyme with the lowest IC_{50} value of 0.14 mg/mL which is significantly different ($p<0.05$) compared to all other extracts and indomethacin. The GCMS chromatograms revealed five compound (2-keto-butyric-acid, 2, 2-Bis (4-nitrobenzyl)-1-phenylbutane-1,3-dione, n-Undecane, 1,4,5,8-tetrathiadelin and imidazo-1,5-pyrimidine) which has been reported to have antioxidant, anti-inflammatory and

antifungal properties. **Conclusion**: This result suggested that *Hermannia geniculata* roots extract is not toxic and possesses antioxidant, antinflammatory and anticancer activities which could be exploited in development of new, safe and effective drugs.

Keywords: Cytotoxicity; nitric oxide; 5-lipoxygenase; *Hermannia geniculata;* antioxidant.

INTRODUCTION

Traditional medicine is being defined as the aggregate of skill, knowledge and practices that is based on indigenous culture, belief, theory and experiences whether its scientific or not but can manage and maintain health and also cause an observed improvement in the social, physical, spiritual and mental wellbeing of an individual [1]. Use of herbal remedies is as old as mankind and it continues to evolve over the centuries in different communities and are still preserved as an inherited traditional knowledge which is passed down different generations [2].

'Medicinal' means something that has ability to heal and may include drugs, plants, spices, herbs, fruit and seeds [3]. In addition to the curative properties, plant extracts has been severally use as a poison. They also possess selective toxicity in different organisms. For instance, pyrethenoid has been reported to affect invertebrates cells but not mammalian cells [4]. Reports of selectivity were documented against oncogenic and normal cells [5].

Furthermore, plant contains secondary metabolites which are large array of organic compounds that are produced in plant but have no direct function in their growth and development [6,7]. They were divided into three different chemical group which include terpenes, phenolics and nitrogen containing compounds. This secondary metabolites are biosynthetic products of shikmic acid, malonic acid, mevalonic acid and methyerythritol 4-phosphate (MEP) pathways [8]. In plant, secondary metabolites serve protective function

against microbes and herbivores [9]. They can also inhibit enzymes, biomolecules like nitric oxide, leukotrienes and these activities has been exploited for therapeutic purposes.

Nitric oxide (NO) is a signaling molecule which is useful to the body at a normal concentration but unregulated production of NO becomes a pathological condition. Medicinal plants has proven to be effective in inhibiting the excessive production of the molecule. Also, leukotrienes (LTs) produced by leucocytes are potent pro-inflammatory mediators biosynthesized from 5-hydroxyeicosatetraenic acid (5-HETE) in a reaction catalyzed by 5-lipoxygenase enzyme [10]. They have been implicated in the pathogenesis and progression of inflammatory diseases, diabetes and cancer [11–13]. The detection of plant with biologically active components that can inhibit synthesis of biomolecules like 5-HETE implicated in the pathogenesis and progression of cancer and without adverse effect to the normal body cells is of great importance.

According to world health organization about 80% of people around the world depends on the use of medicinal plant to manage different kind of diseases [14]. Toxicities associated with use of medicinal has been documented which ranges from minor to major organopathy [15,16]. Plants that can be considered safe for medicinal use should have low toxicities and show no adverse effect on the consumers. Thus, long and short term effect of bioactive medicinal plants on genome, cells, tissue, organs, and the body system is required in order to increase confidence in the safety to human and also in the development of pharmaceuticals.

Hermannia geniculata roots is a genus of flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae.* It is a creeping shrub, the leaves are sub-orbicular broad crenate, the length of the leaf is about 15mm, and leaf texture may be viscid or sticky. *Hermannia* geniculata is readily identified by the hanging flowers, a typically green calyx encloses the

base of free petals with five petals which are contorted with transversely expanded filament. The filament is abruptly expanded and contracted beneath the base of the anther into a cruciform filament [17,18]. The plant is seen across South Africa and its endemic in Free State, KwaZulu-Natal, Eastern Cape, Mpumalanga Limpopo and Guateng. It is also found in Madagascar, Kenya, North-East Africa, Saudi Arabia and North America. [19,20]. Animal studies have been conducted to evaluate the toxicity profile of the root extract of the plant on Wistar albino rats [21]. The result showed that there was systemic toxicities after two weeks administration of 5000 mg/kg body weight of the plant extract. However, at a lower dose of between (75-300 mg/kg) of the extract for a period of 28 days a slight reduction in haematological parameters was identified but histopathological analyses of the organ revealed no significant effect on the heart, liver, lung ,and the kidney.

This study was carried out to further interrogate the cytotoxic effect of *Hermannia geniculata* roots extract on African green monkey kidney cells (Vero) to determine its safety. We tested its inhibitory activities on 5-lipoxygenase enzyme and hepatocellular carcinoma cells (HepG2) in order to determine its anticancer mechanism of action, we also evaluated its effect on excessive production of NO in a lipopolysaccharides activated RAW 264.7 macrophages cells so that we can rationally postulate on the anti-inflammatory, antioxidant and anticancer properties of the plant.

2.0 Materials and Methods

2.1 Plant Collection, Preparation and Extraction.

Hermannia geniculata roots were purchased from registered herbal vendor in Puthaditjhaba Sesting market, Qwaqwa, Northern Free State Province, South Africa. Confirmation of the species identity was carried out with the herbarium specimen with voucher specimen file

number (5056.000-10700) [22] at the University of Free State, Qwaqwa Campus, South Africa. It was also compared with our earlier Voucher specimen (Ash/med/05/2013/QwHB) [21] at the herbarium.

2.2 Preparation of Extract

The roots were washed to remove all debris and chopped into small pieces. It was further air dried to a constant weight. The dried root material was pulverised into fine powdered using waring laboratory blender (Labon, Durban, South Africa.

30 g each of the dried powdered material was extracted in 300 mL of distilled water, hot water at 45° C (decoction), ethanol and hydro-ethanol (50-50), with constant shaking on Labcon platform shaker (Laboratory Consumables, PTY, Durban, South Africa) for 72 h and the mixture was filtered using Whatman No. 1 filter paper. The extracts were concentrated to dryness *in vacuo* at 40° C using a rotary evaporator (Cole-Palmer, South Africa).

2.3 Chemicals

Sodium dodecyl sulphate, bovine serum albumin (BSA), sodium chloride (NaCl), MgCl2·6H2O, Foetal calf serum (FCS), penicillin/ streptomycin/fungizone (PSF) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Highveld Biological Products (South Africa). Phosphate buffered saline (PBS) and trypsin were purchased from Whitehead Scientific (South Africa). Quercetin, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich St. Louis, MO, USA.

2.4 Cell culture of the Vero and HepG2

Human hepatocellular carcinoma (HepG2) and normal African green monkey kidney epithelial (Vero) cell lines were maintained at the department of paraclinical science cell line

laboratories, University of Pretoria, (South Africa) The cell culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were cultured at 37°C under a humidified atmosphere containing 5% CO2.

2.5 Cytotoxic of Vero and HepG2 cells

Viable cells growth after incubation of Vero and HepG2 cells incubated with *Hermannia geniculata* extracts was determined using the tetrazolium-based colorimetric (MTT) assay [23]. Briefly, cells of a sub-confluent culture were harvested and centrifuged at 200 rpm for 5 mins and re-suspended in growth medium for 5 x 10^4 cells/ mL. The growth medium used was Minimal Essential medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). A total of 200 µL of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96– well microtitre plate. MEM (200 µL) was added to wells of columns 1 and 12 to maximize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37° C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells which were then washed with 150 µL phosphate buffer saline (PBS, Whitehead Scientific) and replaced with 200 µL of at differing concentration in quadruplicate. The serial dilutions of the *H. geniculata* extracts at differing concentration in quadruplicate was carried out. The serial dilutions of the test extracts were prepared in MEM. The cells were disturbed as little as possible during the aspiration of medium and addition of *H. geniculata* extracts. The microtitre plates were incubated at 37° C in a 5% CO_2 incubated for 48 h with *Hermannia geniculata* extracts. Untreated cells and positive (doxorubicin chloride, Pfizer Laboratories) were included as controls.

After incubation, 30 µL MTT (Sigma, stock solution of 5mg/ mL in PBS) was added to each well and the plates incubated for a further 4 h at 37° C. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µL DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a micro plate reader (Biotek Synergy) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cell were used to blank the reader. The LC_{50} values were calculated as the concentration of *H. geniculata* extracts resulting in a percentage reduction of absorbance compared to untreated cells.

2.5 Selectivity index (SI)

The degree of selectivity of the compounds was expressed by its SI value as suggested by [24]. High SI value (>2) of an extract suggests selective toxicity against cancer cells, while a compound with SI value <2 is considered to give general toxicity which can also cause cytotoxicity in normal cells [5,25]. Each SI value was calculated using the formula: SI = IC_{50} normal cell/IC_{50} cancer cell.

2.6 Assay of nitric oxide production and viability of LPS- activated RAW 264.7 macrophages

Cell culture of RAW 264.7 Macrophages

The RAW 264.7 macrophage cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in a plastic culture flask in DMEM containing L-glutamine supplemented with 10 % FCS and 1 % PSF solution under 5 % CO2 at 37 °C. Nitric oxide (NO) inhibitory activity and viability of LPS-activated RAW 264.7 macrophages were

determined. The RAW 264.7 macrophages cells were seeded in 96 well-microtitre plates and was activated by incubation in medium containing 1 μg/mL LPS alone (control) or lipopolysaccharide with different concentrations of the samples of *H. geniculata* was dissolved in DMSO. Quercetin served as a positive control NO inhibitor for the reduction of NO production [26].

2.7 **Measurement of nitrite**

Nitric oxide released from macrophages was determined as described by [27] is by measuring the nitrite concentration in culture supernatant using the Griess reagent. After 24 h incubation, 100 μL of supernatant from each well of cell culture plates was transferred into 96-well microtitre plates and an equal volume of Griess reagent was added. The absorbance of the resultant solutions was determined on a BioTek Synergy microplate reader after 10 min at 550 nm. The concentrations of nitrite were derived from regression analysis using serial dilutions of sodium nitrite as a standard. Percentage inhibition was calculated based on the ability of compounds to inhibit nitric oxide formation by cells compared with the control (cells in media without compounds), which was considered as 0 % inhibition.

2.8 **Cell viability**

To determine whether the observed nitric oxide inhibition was not due to cytotoxic effects, MTT assay was also performed on the macrophage cells as described by [23]. 30 μl MTT (Sigma, stock solution of 5mg/ mL in PBS) was added to each well and the plates incubated for a further 4 h at 37° C. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 μL DMSO at each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by

detecting absorbance in a micro plate reader (Biotek Synergy) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cell were used to blank the reader. The LC50 values were calculated as the concentration of *H. geniculata* extracts resulting in a percentage reduction of absorbance compared to untreated cells.

2.6 Soybean 5-Lipoxygenase Inhibition Assay

This procedure was carried out according to the method described [28] the basis of the assay is measurement of Fe^{3+} xylenol orange formed at 560 nm spectrometrically. Different concetration (0.00078-0.1 mg/mL) of the standard/extracts was incubated for 5 min at 25°C. I40 µM of linoleic acid in (50 mM, pH 7.4) tris-buffer was added and the resultant mixture incubated in the dard for 20 min at 25°C. 100 µL of FOX reagent (100 µM of Fe_2so_4, 30 mM H_2so_4, and 100 µM of xylenol orange), water/ methanol (1:9). Evaluation of the inhibitory effect of the extract/standard on 5-LOX was calculated using percentage inhibition of H_2O_2 production from absorbance change measured at 560 nm after 30 min at 25°C.

The % inhibition = (Absorbance $_{control}$- Absorbance $_{sample}$)/ Absorbance $_{control}$ X 100.

Graphically we can determoine the % inhibition of LOX enzyme by a lnear regression equation of y=mx+c Where y is the % activity +50, m= slope, c= intercept and the x=IC_{50} value.

2.7 Gas Chromatography– Mass Spectrometry (GC–MS) analysis of *Hermannia geniculata* roots ethanolic extract

The GC-MS analysis of the chemical constituents from *Hermannia geniculata* roots extract was carried out on Agilent Technologies 6890 Series gas chromatograph together with an Agilent 5973 Mass Selective detector which is operated by Agilent Chemstation software. A

(thickness 0.25 μm, internal diameter 30x0.25mm) is an agilent eHP-5MS. The glass flow rate/velocity is 0.57 mL/min / 27.5 cm/sec and is made up of ultra-pure He. Temperature of the injector was fixed at 250°C.The beginning temperature (50°C) was set to rise to 250°C at 15°C/min with a hold time 4 min at each increment. Spitless mode with the spit ratio 20:1 were made and 1μL was injected. Ionization mode of the MS was 70 eV and electron multiplier voltage at 1859 V. Other set of mass spectrometric operating factors were done. National institute of standards and Technology/ Wiley libraries were used to compare the mass spectra data, retention time and fragmentation pattern to identify the components of the sample analyzed.

3.0 RESULTS

The result of cell viability of Vero cells is displayed in Tables 1 and 4, the viability of Vero cells decreases with increasing the concentration of the extracts. The lowest extracts concentration of 0.05 mg/mL has the highest cells viability which ranges from 88-96 % and the LC_{50} value of the extracts decreases as follows: ethanol (0.57±0.05) > hydroethanol (0.50± 0.00) > aqueous (0.48± 0.02) > decoction (0.40± 0.02). The effect of the plant extract on HepG2 seen in Tables 2 and 4 showed that at concentration of 0.75 mg/mL after 48 h exposure, the viability of the cancer cell was reduced to about 2.2-7.6% for all the tested extracts. Their respective LC_{50} values were (0.012± 0.000, 0.016± 0.001, 0.027± 0.003, 0.137± 0.001 and 0.032± 0.002) mg/mL for decoction, ethanol, hydro-ethanol, aqueous extracts and doxorubicin. There was similarities in the LC_{50} values of ethanol, hydro-ethanol, decoction and doxorubicin with their respective values of (0.012± 0.000, 0.016± 0.001, 0.027± 0.003, and 0.032± 0.002). The selectivity of the extracts to normal and tested cancer cells was

measured using selectivity index. From Table 4, the SI values were (3.52, 18.51, 31.87, 33.33 and 31.25) for aqueous, hydro-ethanol, ethanol, decoction and doxorubicin respectively.

The result of NO inhibitory effect of the extract show that ethanolic extract has lower and significantly different ($p<0.05$) IC_{50} value (3.64 ± 0.123 mg/mL) compared to the reference compound quercetin with (IC_{50}: 8.28 ± 0.045) However the extract did not affect the viability of extract with between 83-95% RAW 264.7 cells exposed to Ethanolic extracts were viable (Table 3). *H. geniculata* extracts exhibited a pronounced inhibition of 5-LOX enzyme compared to the standard indomethacin, their respective IC_{50} value decreased in the following order, ethanol< hydro-ethanol< decoction< aqueous with their respective IC_{50} values are (0.14± 0.06, 2.06± 0.00, 3.85± 0.02, 7.15± 0.13 and indomethacin IC_{50}: 3.24±0.12). The IC_{50} value for ethanol is lower and significantly different ($p<0.05$) from all the other extracts and indomethacin.

GCMS result of the ethanolic extract of *H. geniculata* shown in Table 4, Figure 6, indicated that the presence of five compounds.

4.0 **DISCUSSION**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT reduction assay was conducted on both Vero and HepG2 cells to measure the effect of the extracts on the cells during a 48 h exposure in order to detect changes in the mitochondrial functionality. The result of cell viability of Vero cells was displayed in Tables 1 and 4. It showed that the viability of the cells decreases with increase in the concentration of the extracts. The lowest extracts concentration of 0.05 mg/mL has the highest cells viability which ranges from 88-96 %. The average LC_{50} value of 0.50 mg/mL showed that the extract is not toxic. Extract with LC_{50}

below 0.02 mg/mL are considered to be cytotoxic [29]. *Hermannia* species has been reported to be non-toxic and safe for use [19,21].

Plant mechanism of causing apoptosis in cells include the enhancement of P53 protein expression, induction and upregulation of many pro-apototic family like Bax, Bad, Cytochrome c, caspase 8, 3, 9 and inhibition of PI3k/Akt pathway [30,31]. These death stimuli activates cascade of intrinsic and/or extrinsic death cellular mechanism [31]. The effect of the plant extract on HepG2 seen in Tables 2 and 4 showed that at concentration of 0.75 mg/mL after 48 h exposure, the viability of the cancer cell was reduced to about 7-9% for all the tested extracts. Plant extracts has been reported to be specific and highly selective on tumor cells rather than normal and non-transformed cells [32]. The selectivity of the extracts was measured using selectivity index. From Table 4, the SI values were (3.52, 18.51, 31.87 and 33.33) for aqueous, hydro-ethanol, ethanol and decoction. Selective toxicity >2 signifies that the agent has potential anticancer activity [24,29]. Therefore, the recorded SI suggest the anticancer capability of the plant extract.

Nitric oxide (NO) is synthesized from L-arginine by three isoform of NO synthase (NOS), two are constitutive and the third is induced nitric oxide synthase (iNOS) which is activated during inflammation and produces high level of NO for a long period [33–35]. NO diffuses very rapidly through water and cell membranes and easily combine with superoxide anion ($.O_2^-$) to form peroxynitrite ($ONOO^-$) a potent mutagenic oxidant. NO production may contribute to DNA damage, renal hyperfilteration and hyperperfusion observed in the pathogenesis of diabetes complication [33]. Therefore, NO production inhibitors are useful agent in preventing cell death and inflammatory diseases. Plant extracts has been used in suppressing NO production in LPS- stimulated RAW 264.7 murine macrophages cell line

[27,36]. The extent of NO production can be determined by measuring the concentration of nitrite, a stable oxidized product [36]. The IC_{50} values of NO production refers to Table 3 indicated that ethanolic extract has lower and significantly different ($p<0.05$) IC_{50} value (3.64 ± 0.123 mg/mL) compared to the reference compound quercetin with (IC_{50}: 8.28 ± 0.045). The highest concentration of the extract (0.1 mg/mL) inhibited NO production by 90%. Hydrolysable tannins, flavonoids, compounds isolated from alkaloids and saponins have been reported to have inhibitory effect on NO production in LPS activated RAW 264.7 Macrophage [37–39]. The result of RAW 264.7 macrophage cells viability (Table 3) showed that there was no significant difference ($p>0.05$) in the percentage viability of the cells at all concentration of the extracts tested. Therefore, the observed reduction of NO production seen at higher concentration of the extracts may be due to the inhibitory effect of the extract on iNOS enzymes. This result for the first time confirm the NO inhibitory activities of *H. geniculata* root extracts.

Inhibition of 5-lipoxygenase (LOX) enzyme block the development of several disease including cancer, diabetes and inflammatory diseases. *H. geniculata* root extracts exhibited a pronounced inhibition of 5-LOX enzyme compared to the standard indomethacin, their respective IC_{50} value decreased in the following order as shown in Table 4. The comparisms of the means on a two way ANOVA showed that IC_{50} value for ethanol is lower and significantly different ($p<0.05$) from all the other extracts and indomethacin which suggest that ethanol was able to extract more bioactive components responsible for the observed activity in the plant roots materials which has potent inhibitory effect on 5-LOX enzyme activity. Report from other studies showed that *Hermannia* species has good anti-inflammatory activities [19]. Inhibition of 5-LOX enzyme blocked the biosynthesis of 5-

Hydroxyeicosatetraenoic acid (5-HETE) which is an essential factor in the survival of several cancer cells [40] thus one of the effective mechanism of action of *H. geniculata* root extracts on Human hepatocellular carcinoma cell is through the inhibition of 5-LOX which is the enzyme that catalyzes the biosynthesis of 5-HETE an essential factor in the survival and proliferation of the liver cancer cells. Also, observed inhibition of 5-lipoxygenase enzyme suggested that extracts of *H. geniculata* roots may be used as an inflammatory agents. The inhibition of 5-Lox indirectly inhibit production of leucotrienes which a proinflammatory mediators.

The GCMS result of the ethanolic extract of *H. geniculata* shown in Table 4, Figure 6, indicated that the presence of five compounds. 2-keto-butyric-acid has been reported to be able to stimulate the biosynthesis of fibronectin by fibroblast, endothelial cells, astrological cells and hepatocyte as a sequela to inflammation and tissue repair [41]. Also, 2, 2-Bis (4-nitrobenzyl)-1-phenylbutane-1,3-dione and n-Undecane are excellent reducing equivalents in has antioxidant properties [42,43]. The antifungal activities of 1,4,5,8-tetrathiadelin has documented [44]. Imidazo-1,5-pyrimidine pharmacological importance has been documented. It was reported to have antiulcer, antihypertensive and antitrypanosomal activities [45–47]. Furthermore, it has been reported to have activity against NF-Kb and AP-1 protein expression [46].

CONCLUSION

The selective toxicity, antilipoxygenase and inhibitory potentials of *H. geniculata* confirmed that the plant is safe and may be exploited for its anti-inflammatory, anticancer, and antioxidant properties.

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Table 1. The percentage viability of Vero cells at different concentrations.

Concentration (mg/mL)	1.00	0.50	0.10	0.05
Extracts	% viability of Vero cells			
Ethanol	16± 1.24	30± 2.01	61± 2.87	96± 1.08
Hydro-ethanol	17± 2.30	20± 3.01	75± 3.25	94± 4.22
Decoction	12± 0.10	17± 0.19	42± 1.21	84± 1.41
Aqueous	12± 1.37	16± 2.01	48± 3.21	88± 2.75

Table 2. The percentage viability of HepG2 cell at different concentrations.

Concentration (mg/mL)	0.75	0.375	0.1875	0.09375	0.46875
Extracts	% viability of HepG2 cells				
Ethanol	7.6± 0.42	10.2±1.02	21.1± 0.14	26.4± 2.45	38.2± 4.32
Hydro-ethanol	2.2± 0.01	8.0±1.00	10.2± 1.80	12.2± 1.10	15.7± 2.11
Decoction	2.4± 0.06	9.4± 2.01	11.2± 2.01	14.5± 1.42	15.0± 1.03
Aqueous	7.1± 0.25	10.2± 1.22	21.5±1.34	27.4± 3.21	33.1± 1.82

Table 3. The percentage viability of RAW 264.7 macrophages cells and the % inhibition of NO production at different concentrations

Concentration (mg/mL)	1	0.5	0.25	0.125
Extract/standard	% viability of RAW264.7 macrophages cells			
Ethanol	83± 2.30	88± 2.15	92± 0.24	95± 2.34
Quercetin	67± 1.22	70± 1.40	77± 2.10	88± 2.20

Concentration (mg/mL)	0.1	0.075	0.050	0.025	0.0125
Extract/standard	% nitric oxide production				
Ethanol	10 ± 2.50	25 ± 3.75	33± 2.50	37± 3.00	40± 2.00
Quercetin	20± 1.00	25± 1.12	29± 2.17	47± 1.00	49± 2.10
Control	80± 2.45	80± 2.00	80± 1.00	81± 3.12	81± 2.75

Table 4: Showing cytotoxic activity expressed as LC_{50} (mg/mL) of *Hermannia geniculata* root extracts

LC_{50}: Values: (mg/mL)	Vero cell	HepG2 cell	SI
Ethanol	0.57± 0.052[a]	0.016 ± 0.001 [a]	31.87
Hydro- ethanol	0.50± 0.001[a]	0.027 ± 0.003[a]	18.51
Decoction	0.40± 0.022[a]	0.012± 0.000[a]	33.33
Aqueous	0.48± 0.021[a]	0.136± 0.001[b]	3.52
Doxorubicin	>1.00± 0.125[b]	0.032± 0.002[a]	31.25

Table 5. Compounds identified from the ethanol extract of *Hermannia geniculata* roots from Gas Chromatography- Mass Spectrometry (GC-MS) chromatogram.

S/N	Constituents	Retention time (Min)	Area %	Molecular formular	Molecular weight (g/mol)
1	2-keto-butyric-acid	11.058	1.32	$C_4H_6O_3$	102
2	2,2-Bis(4-nitrobenzyl) -1-phenylbutane-1,3-dione	14.143	1.36	$C_{24}H_{20}N_2O_6$	432
3	n-Undecane	15.529	1.71	$C_{11}H_{24}$	156
4	1,4,5,8-tetrathiadelin	26.961	3.41	$C_6H_{10}S_4$	210
5	Imidazo(1,5-a)pyrimidine	29.865	92.20	$C_6H_5N_3$	119

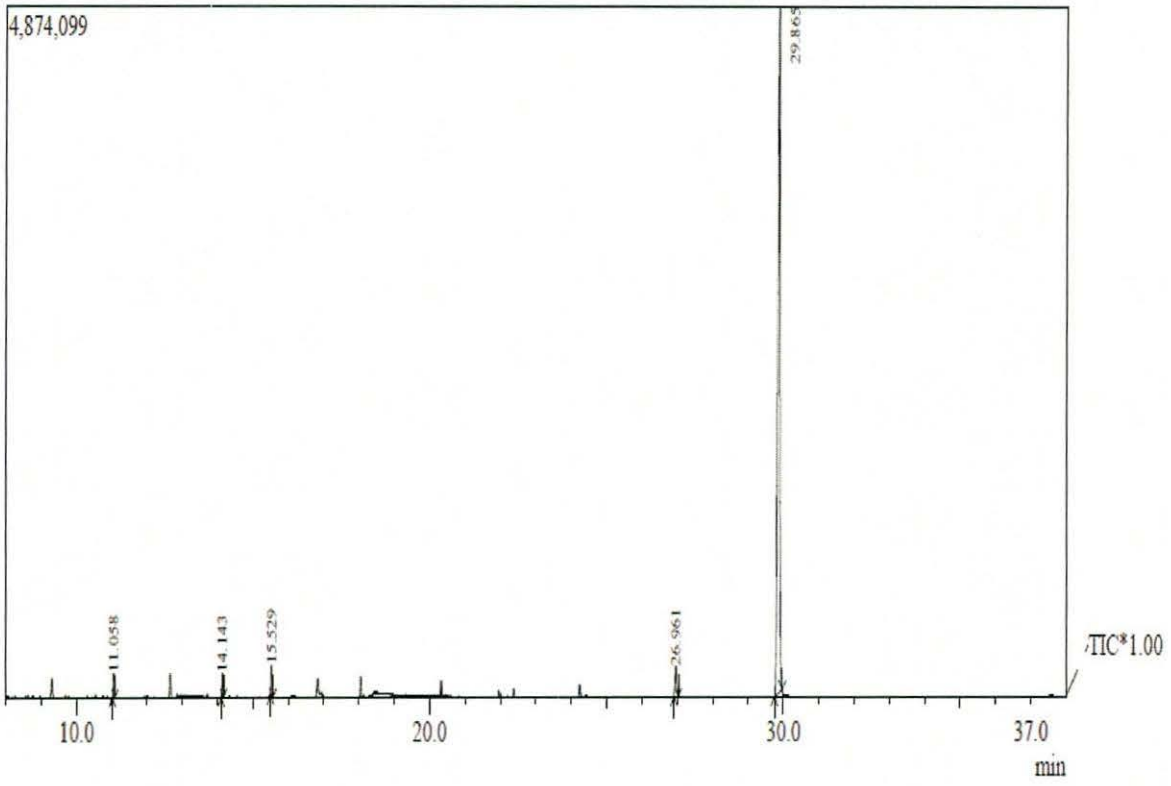


Figure 1. Showing the Gas Chromatography- Mass Spectrometry (GC-MS) chromatogram of ethanolic extract of *Hermannia geniculata* roots extract.

Chapter Five

Understanding the impact of flavonoids from *Hermannia geniculata* Eckl. & Zeyh roots extract on normal cells, antioxidant, antidiabetic, anti-inflammatory and its antiproliferative activities

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This chapter is a draft manuscript to be submitted for peer review journal

Understanding the impact of flavonoids from *Hermannia geniculata* Eckl. & Zeyh roots extract on normal cells, antioxidant, antidiabetic, anti-inflammatory and its antiproliferative activities

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ABSTRACT

The chemical profiling and *in vitro* biological activities of flavonoids of *Hermannia geniculata* (FHG) roots was investigated using High Pressure Thin Layer Chromatography (HPTLC) finger print analysis. Antioxidant, antidiabetic anti-inflammatory activities and the ability of FHG extract to inhibit the production of nitric oxide (NO) in lipopolysaccharide (LPS) activated RAW264.7 Macrophage were investigated using standard methods. The selective cytotoxicity of the extract on Vero and HepG2 cells was also determined. HPTLC results indicated the presence of flavonoids/ phenol -carboxylic acid and Kaempferol (R_f0.81) were detected in the extract with Retention factor R_f ranging from (0.08-0.95). The FHG extract showed commendable antioxidant properties with IC_{50} values (3.07± 0.12, 2.13± 0.67)

for DPPH and ABTS radicals which was lower and significantly different ($p<0.05$) compared to standard silymarin with IC_{50}: (3.55± 0.10, 2.77± 0.75) for DPPH and ABTS respectively. The results indicated milder inhibition of α-amylase with IC_{50}: (5.55± 0.37) which was higher and significantly different from the standard acarbose with IC_{50}: (3.81± 0.29) Nevertheless, the extract exhibited 73% inhibition of α-glucosidase which exerted better inhibitory effect on 5-lipoxygenase enzyme than indomethacin with their respective IC_{50}: (10.15± 0.02 and 12.03± 0.02). Inhibition of NO production was observed in LPS activated RAW 264.7 Macrophages with the highest concentration of 0.1 mg/mL decreasing NO production by 87%. Selective toxicity of Vero and HepG2 cells with their respective LC_{50} value of (>1 and 0.02 mg/mL) was also observed. The antiproliferative potentials of the extract was confirmed with Selectivity Index of 50. This study indicated for the first time that FHG extract was non-toxic to normal cells and possess antioxidant, antidiabetic, anti-inflammatory and antiproliferative activities.

Keywords: Anti-inflammatory, Antidiabetic, Nitric Oxide, Antioxidant, Flavonoids.

1.0 INTRODUCTION

Flavonoids are plant secondary metabolites which are non-nutrient, less toxic, effective at low concentration, environmentally friendly and biologically active. Flavonoids are biosynthesized in plants through the phenyl propanoid pathway, it occurs through transformation of phenylalanine into 4-coumaroyl-CoA, which finally entered the flavonoid biosynthesis pathway (Martens, Preuß, & Matern, 2010). Flavonoids are synthesized in the cytosol, stored in vacuoles and they are also known to be present in external rhizosphere through exudation(Landry, Chapple, & Last, 1995; Li, Ou-Lee, Raba, Amundson, & Last, 1993). Major sub-groups of flavonoids that are found in higher plants include chalcones,

flavones, flavonols, isoflavones, flavanones, anthocyanins, proanthocyanidins and aurones ((Falcone Ferreyra, Rius, & Casati, 2012)

Diverse functions of flavonoids in plants include UV protection, sexual reproduction process, defense and flower coloring. Some flavonoids provide stress protection, by acting as scavengers of free radicals such as reactive oxygen species (ROS), as well as chelating metals that generate ROS via the Fenton reaction (Williams, Spencer, & Rice-Evans, 2004)

In animals, *in vitro* and *in vivo* studies also supported a beneficial effect of dietary flavonoids on glucose homeostasis (Cai & Lin, 2009; Jung, Kim, & Choi, 2009) Furthermore, it has been shown to regulate carbohydrate digestion, insulin secretion, insulin signaling and glucose uptake in insulin-sensitive tissues through various intracellular signaling pathways (Hanhineva et al., 2010) Diabetes mellitus (DM) is the most common health problem of the world in the current century. Nowadays more than 366 million people suffer from DM and 552 million are expected to be affected by diabetes by 2030 (American Diabetes Association, 2010). Presently, there is no doubt that DM and its related complications are associated with increased oxidative stress resulting from the imbalance in the production of free radicals such as reactive oxygen species (ROS) and the body's antioxidant defense system, (Abdollahi, Ranjbar, Shadnia, Nikfar, & Rezaie, 2004). The ROS have been implicated in inflammation and β- cells destruction and/or insulin resistance which has an important role in the etiology of diabetes and its complications (Rahimi, Nikfar, Larijani, & Abdollahi, 2005). Therefore, the use of antioxidant and anti-inflammatory therapy can preserve β-cell function by suppression of β-cell apoptosis (Rahimi et al., 2005) thus helps in the management of diabetes. However, many drugs are in use for the management of DM but many problems including cost, unavailability and the adverse side effects were associated with their continuous use for

patients who must use them for a long time (Hasani-ranjbar, Larijani, & Abdollahi, 2008). Therefore, it seems very logical to think of alternative approaches for the management of diabetes.

In the recent years, a number of studies have been conducted on the use of herbal/natural products in diabetes. Studies in the last decade have shown that most antidiabetic herbs have high antioxidant power and can improve β-cell function and increase secretion of insulin from Langerhans islets (Hasani-Ranjbar, Larijani, & Abdollahi, 2009; Hosseini & Abdollahi, 2012).

Hermannia geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases (Balogun, Tshabalala, & Ashafa, 2016). It is of the genus of flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae* (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008). The plant is seen across South Africa and its vast majority being endemic in Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga. It is also found in Madagascar, East Africa to North-East Africa and Arabia (Essop et al., 2008).

It has been used in treatment of several diseases like colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders. It is also used in the management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Sotho women (Balogun et al., 2016; Moffett, 1993).

Based on the above knowledge, the present investigation was undertaken for the first time to evaluate the selective cytotoxicity of flavonoids from *Hermannia geniculata* on Vero and HepG2 cells, as well as their antioxidant, anti-inflammatory (through the inhibition of NO

production in RAW 264.7 cells activated with lipopolysaccharide and 5-lipoxygenase enzyme), antidiabetic through inhibition of α-glucosidase, α-amylase enzymes and their respective mechanism of action of flavonoids on these carbohydrates metabolizing enzymes. The HPTLC finger printing of the extract was also carried out to profile its chemical constituents.

2.0 MATERIALS AND METHODS

2.1 Plant Collection, Preparation and Extraction.

Hermannia geniculata roots were purchased from local market in Puthaditjhaba, Qwaqwa, Northern Free State Province, South Africa. Confirmation of the species identity was carried out with the herbarium specimen with voucher specimen file number (5056.000-10700) (Moffett, 1993) at the University of Free State, Qwaqwa Campus, South Africa. It was also compared with our earlier Voucher specimen (Ash/med/05/2013/QwHB) (Kazeem and Ashafa 2013) at the herbarium.

2.2 Extraction of Flavonoids.

The roots were separated, washed under running tap to remove all debris and chopped into small pieces and air dried at a room temperature to a constant weight. The dried root materials was pulverised into fine powdered using waring laboratory blender (Labcon, Durban, South Africa) and kept at 4°C before extraction.

The flavonoids extract from *Hermannia geniculata* roots was obtained through a method described (Y. Liu, Wang, & Cai, 2015). Briefly, the powdered roots (30 g) was extracted twice with 70% ethanol (1:10, w/v) for 1 h at 25 °C. The extraction solution was centrifuged at 3,000 rpm for 5 min and passed through 0.45 μm Millex-HV syringe filter unit and the residue obtained after filtration was further extracted with 50% ethanol. The extract was

filtered with Whatman No. 1 filter paper and freeze dried. The freeze dried extract was dissolved in distilled water (300 mL) and stored at -4°C before carrying out High Performance Thin layer Chromatography and other *in vitro* assays.

2.3 Determination of Extraction Yield

The extraction yield of flavonoids of *Hermannia geniculata* roots (FHG) was calculated using the following formula: Extraction yield % = P0/ P 1 x100.

P0: weight of the powder before extraction: P1: weight of the dry extract after extraction

2.4 Chemicals and Reagents

The chemicals were purchased from different suppliers were: Linoleic acid (Merck, Darmstadt, Germany), xylenol orange, ferric chloride (Searle Company, England), indomethacin, sodium dodecyl sulphate, sodium nitrite, ferrous sulphate, and 5-lipoxygenase (Glycine max) (Sigma, Germany). Porcine pancreatic α-amylase, rat intestinal α- glucosidase, 1,1-diphenyl-2-picrylhydrazyl, silymarin, acarbose and paranitrophenyl-glucopyranoside were products of Sigma-Adrich Co., St Loius, USA while starch soluble (extra pure) was obtained from J.T BakerInc., Phillipsburg, USA. Other chemicals and reagents were of analytical grade and the water used was glass distilled.

2.5 *In vitro* Antioxidant Assays

All experiments were conducted in triplicates and all the negative control (blank) was prepared using the same procedure replacing the extract with distilled water

2.5.1 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Radical Scavenging Determination

The ability of the FHG extract to scavenge ABTS cation chromophore obtained from the oxidation of ABTS solution and potassium persulphate was determined according to already

adopted method (Re et al., 1999). Briefly, 50 mL each of 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulphate leaving the mixture for 4-16 h until the reaction was completed and absorbance was stable. The resultant mixture was diluted with ethanol to get an absorbance of 0.700±0.05. The absorbance reading was taking at 734 nm using microplate reader (BIO-RAD, model 680, Japan). 20 µL of different concentration (0.02-0.1 mg/mL) of FHG and standard was then mixed with 200 µL ABTS solution in a 96- well microtiter plate and absorbance was read at 734 nm using a microplate reader (BIO-RAD, model 680, Japan) after 15 mins of incubation at 25°C. The percentage inhibition activity was obtained using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

The IC_{50} value was calculated and obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.5.2 **Metal Chelating Assay**

The chelating of ferrous ions by FHG extract was estimated as described by (Dinis, Madeira, & Almeida, 1994). Briefly, 40 µL of the different concentrations of the extract and standards (0.02 –0.1 mg/mL) was dispensed into a 96-well microtiter plate, 200 µL of 2 mM $FeCl_2$ solution was afterwards added to the mixture. The reaction was initiated by the addition of 80 µL 5 mmol/L ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 mins. The absorbance of the solution was then read at 562 nm using a BIO-RAD (model 680, Japan) microplate reader. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and

obtained from the linear regression equation using y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.5.3 **Superoxide Anion Scavenging Assay**

Determination of superoxide anion radical scavenging potential of FHG extract was achieved according to (F. Liu, Ooi, & Chang, 1997). Superoxide radicals were generated in 50 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 50 mL of NBT (50 mM) solution, 50 mL of NADH (78 mM) solution and different concentrations (0.02–0.1 mg/mL) of FHG extract and standard. The reaction was initiated by adding 1 mL of phenazine methosulphate (PMS) solution (10 mM) to the mixture. The reacting mixture was incubated at 25 ^{0}C for 5 min, then the absorbance was measured at 560 nm using a microplate reader (BIO-RAD, model 680, Japan). IC_{50} was then evaluated from calibration curve following estimation of percentage superoxide anion scavenging capacity of the tested extract of FHG using the expression: Percentage Scavenging (S %)=$[(A_{control}-A_{extract})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control, $A_{extract}$. The IC_{50} value was obtained from the linear regression equation using y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value

2.5.4 **Hydroxyl radical scavenging ability**

The ability of the plant extracts to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the modified method of (Mathew & Abraham, 2006). Briefly, 100 µL of different concentrations (0.02-01 mg/mL) of FHG extract and standard, 120 µL of 20 mM deoxyribose, 400 µL of 0.1 M phosphate buffer, 40 µL of 20 mM hydrogen peroxide and 40 µL of 500 µM ferrous sulphate each were taken and mixed in 2 mL Eppendorf tubes. Then 100 µL of distilled water was added and incubated for 30 min at 37 °C. Subsequently, 0.5 mL

2.8% of trichloroacetic acid (TCA) and 400 µL of 0.6% thiobarbituric acid (TBA) solutions were added to stop the reaction. From the mixture, 300 µL of the resultant mixture was dispensed into a 96-well micro titer plate and incubating in boiling water for 20 min. The absorbance was taken at 532 nm using a microplate reader (BIO-RAD, model 680, Japan). The percentage inhibition of FHG extract/standard was obtained using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.5.5 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging activity of the FHG extract was evaluated based on its scavenging activities on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The method was described by (Braca et al., 2001). Briefly, 150 µL of the varying concentration of plant extract/ standard (0.02 – 0.1 mg/mL) was added separately to 150 µL of 0.004% methanolic solution of DPPH in a 96-well microtiter plate. The absorbance at 517 nm was determined after 30 min using a 96-well microplate reader (BIORAD, model 680, Japan), and the percentage inhibitory activity of the extract/standard was calculated using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. The half maximal inhibitory concentration (IC_{50}) value were calculated from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.6 *In vitro* Antidiabetic Assays

2.6.1 α-Amylase Inhibitory Assay

This assay was carried out using the procedure of (Apostolidis, Kwon, & Shetty, 2007). 250 mL of varying concentration of extract/ standard (0.125 – 1.0 mg/mL) was placed in a test tube and 250 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was incubated at 25°C for 10 min, followed by addition of 250 mL of starch (1%) solution in 0.02 M sodium phosphate buffer (pH 6.9) at timed intervals, the resulting reaction mixture was then incubated at 25°C for 10 min. The reaction was terminated by adding 500 mL of dinitrosalicylic acid (DNS) reagent before incubating the tubes in boiling water for 5 min and cooled to 25°C. 5 mL distilled water was added to the reacting mixture after cooling and the absorbance was measured at 540 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was done in triplicate and was calculated as percentage inhibition, thus; % Inhibition = (Absorbance (control) – Absorbance (extract))/ Absorbance (control) ×100. Concentrations of extract resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.6.2 α-Glucosidase Inhibitory Assay

The effect of the FHG extract on α-glucosidase activity was determined according to the method described by (Apostolidis et al., 2007) with slight modification. In brief, different concentrations (0.125 – 1.0 mg/mL) of extract/ standard were prepared in distilled water. Then, 50 mL from the stock solution was mixed with 100 mL of 0.1 M phosphate buffer

(pH6.9) containing 1.0 M of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25°C for 10 min. Following this, 50 mL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. The inhibitory effect of extract/standard on the enzyme activities were determined by measuring the absorbance of the mixtures at 405 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The experiments were conducted in triplicate and the α-glucosidase inhibitory activity was expressed as % inhibition using the expression:

%Inhibition= [(Acontrol-Aextract)/Acontrol]×100, where Acontrol and Aextract are the absorbance's of the control and extract respectively. Concentrations of extract/standard resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.6.3 **Kinetic Studies**

2.6.3.1 **Mode of α-Amylase Inhibition**

This assay was conducted using modified methods (Ali, Houghton, & Soumyanath, 2006). Briefly, 250 mL of the (5 mg/mL) extract of FHG/ standard were pre-incubated with 250 mL of 0.5 mg/mL α-amylase solution for 10 min at 25°C in one set of tubes while α-amylase was pre-incubated with 250 mL of 0.1M phosphate buffer (pH 6.9) in another set of tubes. The reaction of the two sets of the mixtures was initiated by adding 250 mL of 5% starch solution at increasing concentrations (0.31 – 50.00 mg/mL). The mixture was then incubated for 10 min at 25°C, followed addition of 0.01mM DNS (500 mL). The reaction was terminated after

boiling for 5 min. The amount of reducing sugars released was determined spectrophotometrically using maltose standard curve and converted to reaction velocities. A double reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted to determine the mode of inhibition.

2.6.3.2 **Mode of α-Glucosidase Inhibition**

The kinetics on inhibition of α-glucosidase activity by FHG extract using modified methods of (Nagmoti & Juvekar, 2013) was adopted. Briefly, 50 µL of 5 mg/mL extract was pre-incubated with 100 µL of 0.1M α-glucosidase solution for 10 min at 25°C in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated with 50µL of 0.1M phosphate buffer (pH6.9). 50 mL of 0.05M pNPG at different concentrations (0.31 – 50.00 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10min at 25 °C, and 500 mL of 0.1M Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined colourimetrically using a p-nitrophenol standard curve. Reaction rates (v) were thereafter calculated and double reciprocal plots of enzyme kinetics. Km and Vmax values were also calculated from Lineweaver-Burkplot (1/v versus 1/[S]) (Lineweaver & Burk, 1934).

2.7 **Soybean 5-Lipoxygenase Inhibition Assay**

The assay was performed according to previously described procedure (Pinto, Tejeda, Duque, & Macías, 2007) with slight modifications. The assay is based on the formation of the complex Fe^{3+}/xylenol orange with absorption at 560 nm. 5-lipoxygenase from *Glycine max* was incubated with different concentration of the extract or standard (0.00078-0.1 mg/mL) at 25°C for 5 min. Then linoleic acid (final concentration, 140 µM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25°C for 20 min in the dark. The assay was

terminated by the addition of 100 µL of FOX reagent [sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM), methanol/water (9:1)]. The lipoxygenase inhibitory activity was evaluated by calculating the percentage of the inhibition of hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25°C. % inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance control] x100. The 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.8 Cell Culture of Vero and HepG2

The Vero cell and HepG2 cells were maintained at Department of Paraclinical Sciences, cell line laboratories, Onderstepoort campus, University of Pretoria, South Africa. The culture medium used for the cell culture was DMEM supplemented with 10% FBS. 100 µg/mL streptomycin and 100 units/mL penicillin was added to the culture medium. The cell culture environmental condition was 5% $C0_2$ humidified atmospheric condition at 37°C.

2.9 Cytotoxicity of Cell Lines

Viable cells growth after incubation of African green monkey (Vero) cells and Human hepatocarcinoma (HepG2) with FHG extract was determined using the tetrazolium-based colorimetric (MTT) assay (Mosmann, 1983). Briefly, cells that have reached sub-confluent in their culture medium were harvested, centrifuged and re-suspended in the growth medium at 5 x 10^4 cells/ mL. MEM was used as the growth medium and it was supplemented with FCS (5%) and 0.1% gentamicin (Vibrae). MEM (200 µL) was added to wells of columns 1 and 12 to maximize the "edge effect" and maintain the relative humidity. The 96 well plates were incubated at 37°C until the exponential growth phase of the cells were reached. MEM in the

plates was removed carefully without disturbing the cells and washed in 150 µL PBS. It is important to minimize the disturbance of the cells during the aspiration of MEM. The serial dilutions of the FHG extract at differing concentrations of 0.05 – 1.0 mg/mL were made in quadruplicate. The serial dilutions of the test extract were all prepared in MEM and the mixture were added to the wells. The microtititre plates were incubated at 37°C in 5% CO_2 for 48h. The cells that were not treated and doxorubicin chloride were used as negative and positive control respectively.

After 48 h of incubation of the plates, 30 µL MTT (stock of 5 mg/mL in PBS) was put into all the wells and the plates were subjected to 4 h of further incubation at optimum temperature of 37°C. After incubation, the MTT in the culture medium in each of the cell was removed gently without distorting the MTT crystals. The formazan crystals formed by MTT were dissolved by the addition of DMSO in each of the well. The plates were gently shaken to facilitate better dissolution of the MTT crystals. The MTT reduction by the cells was measured by taking their absorbance using a microplate reader (Synergy Multi-Mode Reader, BioTek) at 570nm and a reference wavelength of 630nm. The reader was blanked using the column 1 well which contains only MTT and medium. The LC_{50} values was determined as the concentration of *Hermannia geniculata* extracts resulting in a 50% reduction of absorbance compared to untreated cells.

2.10 **Selectivity Index (SI)**

The degree of selectivity of FHG extract was expressed by its SI value as suggested by **(Badisa et al., 2011)**. High SI value (>2) of an extract suggests selective toxicity against cancer cells, while a compound with SI value <2 is considered to give general toxicity which can also cause cytotoxicity in normal cells (Machana, Weerapreeyakul, Barusrux, &

Nonpunya, 2011)(Awang, Aziz, Kamaludin, & Chan, 2014). Each SI value was calculated using the formula: SI = IC_{50} normal cell/IC_{50}

2.11 Nitric oxide (NO) Production LPS-activated RAW 264.7 Macrophage Viability Assays.

2.11.1 Cell culture of RAW 264.7 Macrophage

The RAW 264.7 macrophages cells was bought from the American Type Culture Collection. (Rockville, MD, USA). It was cultured in a DMEM containing L-glutamine and supplemented with 1% PSF, 10% FCS and 5% CO_2 at 37°C. The ability of the extracts to inhibit the nitric oxide produced from the stimulation of cell by bacteria lipopolysaccharides and the toxicity of the extracts on the cells were carried out.

The cells were seeded in 96 well plates and were activated by incubation in medium containing 1 µg/mL Lipopolysaccharides. Extract at different concentration ranging from (0.75 mg/mL-0.02344 mg/mL) were added to the wells. Quercetin was used as positive control (Mu *et al.*, 2001).

2.11.2 Measurement of nitrite

The released nitrite oxide from the 264.7 RAW macrophages cells were determined as described by (Mu et al., 2001). Briefly, the concentration of nitrite in the cultured supernatant was aspirated from each well and was put in a new 96 well plate, after which 100 µL of the Griess reagent was added to the new well. The absorbance of the resultant solution was read on microplate reader (Biotek Synegy) at 550nm after ten minutes. The nitrite concentration was evaluated using regression analysis of the serial dilution of the sodium nitrate standard. The ability of the extract to inhibit the nitrite production was determined in relation to % inhibition of the negative control. (0% inhibition of nitrite

2.11.3 **RAW 264.7 Macrophage Cell Viability**

In other to know whether the observed NO inhibition was not due to the toxic effect of the extracts on cells, MTT assay was carried out on the RAW 264.7 macrophages cells as described by (Mosmann, 1983). 30 µL MTT (stock of 5 mg/mL in PBS) was put into all the wells and the plates were subjected to 4 h of further incubation at optimum temperature of 37oC. After incubation, the MTT in the culture medium in each of the cell was removed gently without distorting the MTT crystals. The formazan crystals formed by MTT were dissolved by the addition of DMSO in each of the well. The plates were gently shaken to facilitate better dissolution of the MTT crystals. The MTT reduction by the cells was measured by taking their absorbance using a microplate reader (Synergy Multi-Mode Reader, BioTek) at 570nm and a reference wavelength of 630nm. The reader was blanked using the column 1 well which contains only MTT and medium. The LC50 values was determined as the concentration of Hermannia geniculata extracts resulting in a 50% reduction of absorbance compared to untreated cells.

2.12 **HPTLC Finger Print Analysis of FHG Extract**

The assay method was described by (Reich & Schibli, 2007). Briefly, 50 mg each of FHG extract was weighed accurately in an electronic balance (Afcoset), dissolved in 250 µL of ethanol and centrifuged at 3000 rpm for 5min. This solution was used as test solution for HPTLC analysis. Then 2 µL of test solution and 2 µL of standard solution were loaded as 5 mm band length in the 3 x 10 Silica gel 60 F_{254} TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

The sample loaded plate was kept in Thin Layer Plate (TLC) twin trough developing chamber (after saturated with solvent vapor) with respective mobile phase (Flavonoid) and the plate was developed in the respective mobile phase up to 90 mm.

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at visible light, ultraviolet (UV) 254 nm and UV 366 nm. The developed plate was sprayed with respective spray reagent (Flavonoid) and dried at 100°C in hot air oven. The plate was photo-documented in visible light and UV 366 nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber. Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254 nm. The Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version. Toluene-Acetone-Formic acid (4.5: 4.5: 1) was used as the mobile phase while 1% ethanolic aluminum chloride was used as the spraying reagent.

2.12 **Statistical analysis**

Statistical analysis was performed using a Graph Pad Prism 5 statistical package (Graph Pad Software, San Diego, MA, USA). Data were expressed as means of replicate determinations ± SD, for *in vitro* assays and was subjected to two-way analysis of variance (and non-parametric) followed by Bonferroni: compare all pair of column. Statistical significance was considered at $P < 0.05.3.0$

RESULTS

The yield of total flavonoids was 1.68 g from the extraction of 30g of plant materials.

The result of the *in vitro* antioxidant potentials of the flavonoid of *Hermannia geniculata* (FHG) is presented in (Table 1) and (fig. 1-5). The FHG dose dependently scavenge/ chelate

and showed remarkable potentials when compared to the standard (silymarin). The IC_{50} value of FHG extract on DPPH and ABTS radicals were (3.07± 0.12 and 2.13± 0.67) respectively which was lower and significantly different ($p<0.05$) from silymarin with respective IC_{50}: 3.55±0.10 and 2.77±0.75 for DPPH and ABTS. The percentage inhibition of FHG extracts was dose dependent and they were capable of inhibiting radical formation between 40-80% as shown in Figure 1 and 3.

The metal chelating activity of the FHG extracts was also demonstrated against ferrous ion (Figure 2). FHG showed great potential in a dose dependent manner with IC_{50}:4.14±0.50 which is lower and significantly different from silymarin with IC_{50}: 5.30± 0.98.

Furthermore, the potential of FHG to scavenge other free radical *in vitro* was also quantified. The result is displayed in Table 1 which showed that FHG extract has similar capability with silymarin in scavenging for the hydroxyl radicals with the best activity seen at concentration of 0.1mg/mL. The respective IC_{50} values for FHG extracts and silymarin is 2.51± 0.01 and 2.47± 0.01. Moreover, FHG extracts showed greater capability of scavenging superoxide anion radicals with a lower IC_{50}:3.21±0.07 which is significantly different ($p<0.05$ from silymarin with IC_{50}: 3.87± 0.14.

Also, the *in vitro* enzymatic inhibitory potentials of FHG extract on general and specific carbohydrate metabolic enzymes of α-amylase and α-glucosidase was assayed and results displayed in Table 1 and Figure 7 and 8. However, the potential of the extract to inhibit α-amylase activities were lower at all concentration compared to the reference compound (acarbose), the IC_{50} value of FHG extract was 5.55 ±0.37 which is higher and significantly different ($p<0.05$) from acarbose with a lower IC_{50}: 3.81 ±0.29 but FHG extract exhibited

higher inhibition of α- glucosidase enzyme which is significantly different ($p<0.05$) from acarbose with their respective IC_{50}: values of 1.37 ± 0.19 and 2.91 ±0.42.

The kinetic study for the probable mode of inhibition of the enzymes displayed in Figure 8 and 9 showed that FHG extract exhibited independent inhibition of α- amylase enzyme. The double reciprocal plot revealed a decrease in the km value of the extract compared to the control while their velocities remain constant thus suggesting competitive mode of inhibition. The Vmax is 2.5 μm/min and the km values reduce from 32 mg to 14 mg for the control and the extract respectively.

The mode of inhibition of α-glucosidase enzyme by the FHG extract showed that the Vmax of the extract increased from 0.14μm/min for the control to 2.70μm/min for FHG extract while the Km value remains the same at a constant concentration signifying a non-competitive mode of inhibition.

In Table 1 and Figure 10, the effect of FHG extract on 5-lipoxygenase enzyme involved in arachidonic acid metabolism showed a dose dependent inhibition of the enzyme with the highest concentration of 0.1mg/mL showing about 89% inhibition. The IC_{50} value of the extract is higher and significantly different ($p<0.05$) from the reference compound (indomethacin). The respective IC_{50} value for FHG extract and indomethacin were 10.15± 0.12 and 12.03± 0.42.

The cytotoxicity of the extract was tested on two cell lines, the normal African green monkey kidney epithelium cells (Vero cells) and human hepatocellular carcinoma cells (HepG2 cells) to determine the viability of the cells exposed to the extracts. The result was shown in Table 2 and Figures 12-13. Doxorubicin was used as the positive control. MTT assay was conducted to determine cells viability after 48 h of exposure to the extract. It was observed that 65% of

the Vero cells were viable at the highest concentration of 1mg/mL of FHG extract and the LC_{50} value is 1 mg/mL. Furthermore, the percentage viability of the HepG2 cells is lowest at the highest concentration of 0.75mg/mL with less than 5% of the cell viable. The LC_{50} value is 0.02 mg/mL.

The result of the selectivity index was shown in Table 2, the SI for FHG extract is 50 which is higher than the SI of doxorubicin value of 31.25.

The inhibitory potentials of FHG extract on excessive and uncontrolled production of pro inflammatory molecule nitric oxide (NO) was assayed. The result was shown in Figure 13 and 14. FHG extract significantly reduced the production of NO from LPS activated RAW264.7 Macrophages at the highest concentration of 100 µg/mL and the inhibition is dose dependent. The IC_{50}: 3.61 mg/mL was lower and significantly different ($p<0.05$) from reference compound (quercetin) with IC_{50} value of 6.72 mg/mL. We also determined the percentage viability of the extract to ascertain the viability of the cell using MTT assay. The LC_{50} value of the FHG extract on the cell is >1.00mg/mL.

The HPTLC finger print analysis of the flavonoids was carried out to determine the flavonoids profile present in FHG extract. The result of the different R_f values and their various chromatograms were presented in Table 3 and Figures 15-19. It showed the presence of flavonoid/ phenol carboxylic acid in the FHG extract. The peak at the R_f of 0.81 from the chromatograms confirmed the presence of kaempferol in the FHG extract due to the similarities in the R_f value compared to that of the standard used also confirmed that (kaempferol with R_f :0.80)

The chemical structure of the identified compound (kaempferol) in the extract is shown in Figure 20.

4.0 DISCUSSION

High performance thin layer chromatography (HPTLC) finger printing is an offline throughput planar chromatography technique used in identifying marker compounds present in medicinal plants (Reich & Schibli, 2007). The result of the HPTLC profiling of FHG extract is shown in Table 3 and Figures 15-20. The presence of flavonoids in the extract was confirmed with R_f values (0.08-0.95). The specificity of the methods was ascertained by analyzing the standard and the sample with similar chromatographic conditions. Chromatograms revealed similarities for both the standard and the test samples. Moreover, the detection of kaempferol in the FHG extract was confirmed with R_f 0.81 which is similar to the standard kaempferol with R_f 0.80. Also, similar densitogram, baseline and 3D display of the Tracts confirmed the detection of kaempferol in the FHG extract. The chemical structure of kaempferol can be seen in Figure 21. It is a flavonol, a product of aromadendrin derived from flavonoid biosynthetic pathway in a reaction catalyzed by flavonol synthase (Malla, Koffas, Kazlauskas, & Kim, 2012). Several glycosides of kaempferol derivatives have been identified in medicinal plants (Teffo, Aderogba, & Eloff, 2010). This will be the first time it was detected in *Hermannia geniculata* roots extract.

3.1 *In vitro* Antioxidant Activities

Flavonoids are poly phenolic compounds distributed naturally in plants. They are plant secondary metabolites with reactive oxygen species (ROS) quenching capacity in both plant and animal cells (Spencer, 2010). The unregulated production of ROS by different body mechanism lead to oxidative stress which is an underlying cause of β-cell destruction and insulin resistance seen in diabetes mellitus and its various complications (Modak, Dixit, Londhe, Ghaskadbi, & Paul A Devasagayam, 2007). The DPPH scavenging methods, ABTS

decolorization assay, hydroxyl radical averting capacity, superoxide anion radical scavenging assay and metal chelating capabilities are models for evaluating the antioxidant potency of medicinal plants.(Alam, Bristi, & Rafiquzzaman, 2013; Egea et al., 2017). Results of antioxidant assays showed that FHG extract has good antioxidant activity which is comparable to the standard silymarin. The standard curve of the percentage inhibition/scavenging/chelating effect and their respective IC_{50} values showed that there was a decrease in the concentration of the ROS may be as a result of the scavenging effect of the FHG extract. Several factors contribute to the antioxidant capabilities of flavonoids which include a low redox potentials in the range of (2.13-1.0V) capable of reducing highly oxidized free radicals like superoxide, hydroxyl, peroxyl and alkoxyl radicals (Mishra, Kumar, & Pandey, 2013; Shashank & Abhay, 2013). Also the structural/activity relationship evaluation of flavonoids suggested that the OH at the carbon 3, 5 plus the –oxo- group at carbon 4 found in flavonols like kaempferol and other flavonoids may be responsible for its ability to chelate metal ions. Metal ions binds at this specific positions giving flavonoids the iron stabilizing properties (Van Acker et al., 1996). In addition to this, the unsaturation in the 2-3 bond of the C-ring and also the OH in the 3^1, 4^1 carbon of the B- ring may be attributed to the antioxidants activities of flavonoids (Rice-Evans, Miller, & Paganga, 1996).

3.2 *In vitro* Antidiabetic Studies.

The result obtained from α- amylase inhibitory activities showed that total flavonoid from *Hermannia geniculata* extract IC_{50} value is 5.55 mg/ mL is higher and significantly different ($p<0.05$) from acarbose IC_{50}: 3.81 µg/ mL. This showed that flavonoid is a mild inhibitor of α- amylase compared to acarbose. This result is consistent with the work of (Kim, Kwon, & Son, 2000) who observed a less potent than acarbose inhibitory effect of flavonoid. The

enzyme kinetic study of α- amylase showed that FHG extract competitively inhibit the enzyme. The kinetics further revealed an increase in the Km value of the inhibitor while the Vmax remain constant. The lower Km value for the inhibitor means a higher affinity of the inhibitor compared to the substrate for the enzyme thus, competitively inhibiting enzyme catalysis of disaccharide. This is only applicable if both concentration of the inhibitor and the substrate is kept constant (Johnson & Goody, 2011). The inhibitory effect of total flavonoid extract on α-glucosidase was also evaluated, the IC_{50}: 1.37 mg/mL is lower and significantly different ($p>0.05$) compare to that of acarbose (2.91 mg/ mL).

This showed that FHG extract was a more potent inhibitor of α-glucosidase than acarbose. This finding is consistent with the work of (Kim et al., 2000) who observed Leutonin-7- o-glycoside isolated from flavonoids extract was a more potent inhibitor of α-glucosidase enzyme than acarbose. Also, (Tadera, Minami, Takamatsu, & Matsuoka, 2006) observed that yeast α-glucosidase was potently inhibited by flavonoids like anthocyanidin, isoflavone, flavanol and flavonol groups than acarbose. The presence of double bond in the C- ring, the catechol group in the B-ring seen in kaempferol and other flavonoids may be responsible for the potent inhibitory effect on α-glucosidase enzyme (Tadera et al., 2006).

The mode of inhibition of α-glucosidase is non-competitive. The Km values remain constant while there is a change in the Vmax. This is consistent with (Tadera et al., 2006) who described non-competitive inhibition of α-glucosidase enzyme by flavonoids.

In this work we observed a commendable inhibition of α- amylase and α-glucosidase enzymes by FHG extract. This suggested that it may be a potential agent that can be used to mitigate against postprandial hyperglycemia seen in diabetes mellitus. The use of this agent may have less side effect like abdominal discomfort, diarrhea, meteorism and megacolon associated

with the use of other inhibitors of carbohydrate hydrolyzing enzyme like acarbose, voglibose and miglitol. Our result showed that the total flavonoid is a moderate inhibitor of α- amylase enzyme with IC_{50} value of 5.5 mg/ mL and a potent inhibitor of α-glucosidase IC_{50}: 1.37 mg/ mL compare to acarbose with α- amylase and α-glucosidase (IC_{50}: 3.81 mg/ mL and 2.91 mg/ mL) respectively. This finding is consistent with (Mbhele, Balogun, Kazeem, & Ashafa, 2015; Sabiu, O'Neill, & Ashafa, 2016). From the kinetic studies, competitive and mixed non-competitive inhibition of α- amylase and α-glucosidase enzymes will cause delay in absorption of glucose and fructose in the gastro intestinal tract and reduce the gastric empting rate which alter insulin secretion the effect of reduced starch metabolism will promote weight loss through reduction in the digestive availability of carbohydrate derived calories (Celleno, Tolaini, D'Amore, Perricone, & Preuss, 2007; Olaokun, Mcgaw, Rensburg, Eloff, & Naidoo, 2016)

3.3 ***In vitro*** 3.3 ***In vitro*** **Anti-inflammatory Assay.**

The result of the *in vitro* anti-inflammatory assay is presented in Table 1 and Figure 13. FHG extract exhibited a varying degree of inhibitory effect on 5-lipoxygenase enzyme. The inhibition of lipoxygenase was dose dependent with highest concentration of the extract showing the highest degree of inhibition. This anti-inflammatory result supported the traditional use of *Hermannia geniculata* roots in the management of different inflammatory diseases (Balogun et al., 2016; Moffett, 1993). The IC_{50} value of FHG extract is 0.15 mg/ mL which demonstrate also the good activity of FHG extract against the enzyme. The IC_{50} value is comparable to the positive control indomethacin which has an IC_{50}: 0.11 mg/ mL. Flavonoids have been demonstrated to have antilipoxygenase activity (Elisha, Dzoyem, McGaw, Botha, & Eloff, 2016). Flavonoids are also known to affect a number of regulatory

enzymes which are essential to inflammation and immune response. Inhibition of 5-lipoxygenase enzyme by FHG extract will inhibit the oxidation of arachidonic acid from membrane phospholipid to leukotrienes which is a molecule require in the biosynthesis of several proinflammatory cytokines (Shim & Paige, 2012).

3.4 *In vitro* Cytotoxicity

We investigated the cytotoxicity effect of FHG extract on HepG2 and Vero cell lines. Doxorubicin, a known anticancer drug was used as the positive control. The respective LC_{50} values and their selectivity index (SI) were presented in table 1. For HepG2 assay, the LC_{50} value of FHG extract was (0.02 mg/mL) which is comparable to the value of Doxorubicin. The percentage viability of the cells to different concentration of the extract is presented in Figure 12. It showed that antiproliferative activity of the extracts is dose dependent and the highest concentration of 0.75 mg/mL produces the highest cytotoxicity in the tested extract. The extracts are less toxic to Vero cells with LC_{50} value >1 mg/mL. The percentage viability presented in Figure 13, it showed that FHG extract was not toxic to the cells at all concentration. This result showed that all the extract may be safe at concentration of 1 mg/mL. The selectivity index represents the overall activity of each extracts (Badisa et al., 2011). As seen in Table 3, the extract SI value is 50. This selective index greater >2, suggested that the extract may have anticancer properties. This is similar to work of Badisa *et al.*, (2011) where compounds having SI greater than 2 is being accepted to have promising anticancer activity.

3.5 NO inhibitory Activity.

NO scavenging is important because it is a proinflammatory molecule, which can diffuse across the cell membranes. Low grade NO production is beneficiary for maintaining the normal body functions but unregulated production of NO reacts with superoxide anion

generating peroxynitrite which causes oxidation of low density (lipoprotein LDL) and this is a key process that lead to cell apoptosis, subsequent inflammation and artheriosclerosis (Ohshima & Bartsch, 1994; Smith & Lassmann, 2002). Therefore, NO inhibitors are useful agent in preventing cell death and inflammatory diseases which is a sequela to cell apoptosis. (Smith & Lassmann, 2002) has described the activity of quercetin in suppressing NO production in LPS- stimulated RAW 264.7 murine macrophages cell line. The extent of NO production can be determined by measuring the concentration of nitrite, a stable oxidized product (Elisha et al., 2016; Putri, Elya, & Puspitasari, 2017). In our present work RAW 264.7 macrophages were treated with LPS and various concentration of FHG extract. Quercetin serve as the positive control. The NO production and the cell viability were measured.

The decrease in the NO produced from the percentage inhibition and the lower IC_{50} value 6.71 mg/mL compared to the value of quercetin IC_{50}: 8.28 mg/mL in Table 1 and Figure 14 suggested that FHG extract may be able to inhibit NO production from LPS stimulated macrophages cells. This results was similar to the work of (Elisha et al., 2016) where flavonoids were described to be able to inhibit NO production. Also, (Mfotie Njoya, Munvera, Mkounga, Nkengfack, & McGaw, 2017) attributed the capacity of extracts to inhibit NO production to the phenolic content of the extracts which regulate the synthesis of inducible nitric oxide synthase (iNOS) by inhibiting the nuclear transcription factor NFk-ß. Furthermore, the hydroxyl group on position 3 of C-ring, two hydroxyl groups in B-ring are important site for NO scavenging (Glebska, Pulaski, Gwozdzinski, & Skolimowski, 2001; MacMicking, Xie, & Nathan, 1997). The cytotoxicity of the extracts against RAW 264.7 cells was also tested by MTT assay. The result was presented in Figure 15. It showed that there was

no significant cytotoxic effect of the extracts on the RAW 264.7macrophages cells at all concentrations of the extract which may account for the observed inhibition of NO production.

4.0 CONCLUSION

The antioxidants, antidiabetic, anti-inflammatory and selective cytotoxicity of FHG extracts indicated that the pharmacological potentials of *Hermannia geniculata* in the treatment of oxidative stress related disorders. Further works on the in vivo biological activities is ongoing in the laboratory.

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Figures

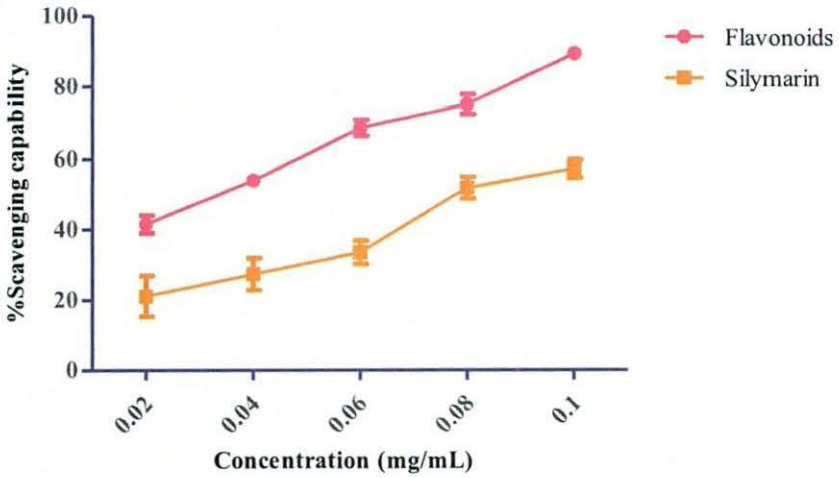


Fig. 1. DPPH radical scavenging capability of *Hermannia geniculata* flavonoids extract.

Values are mean± standard deviation (SD) of triplicate determinations.

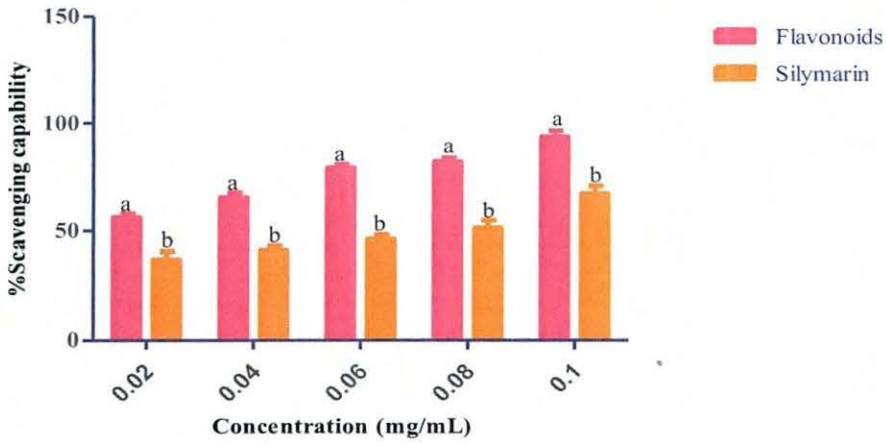


Fig. 2. Showing the metal chelating activities of the flavonoids of *Hermannia geniculata* root extract.

Values are mean± standard deviation (SD) of triplicate determinations. [ab]Values with different alphabets are significantly different ($p<0.05$)

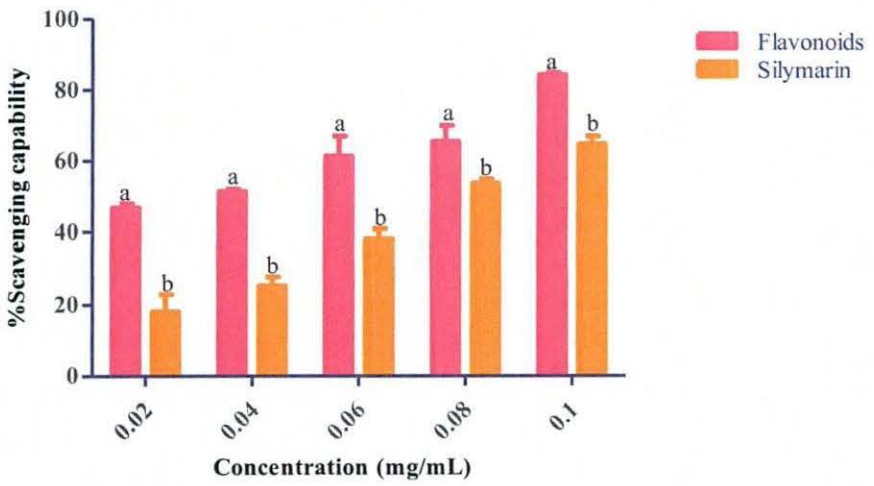


Fig. 3. Showing the ABTS radical scavenging activities of the flavonoids of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations. [ab]Values with different alphabets are significantly different ($p<0.05$)

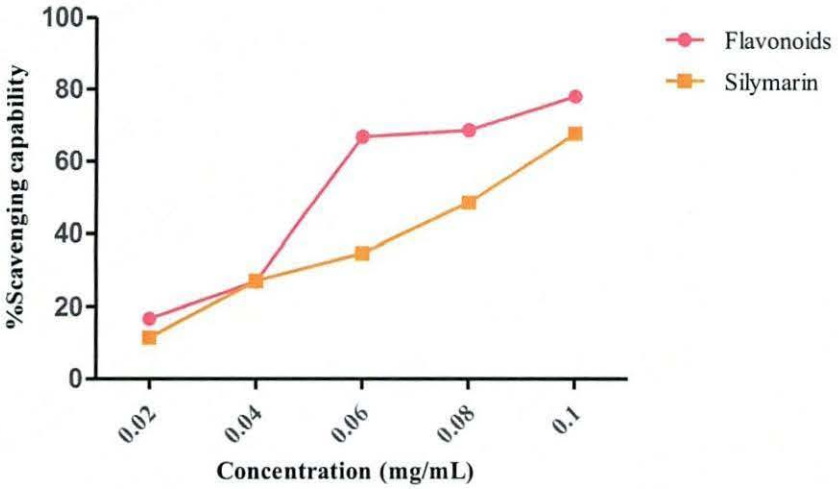


Fig. 4. Showing superoxide anion radical scavenging capabilities of the flavonoids of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations

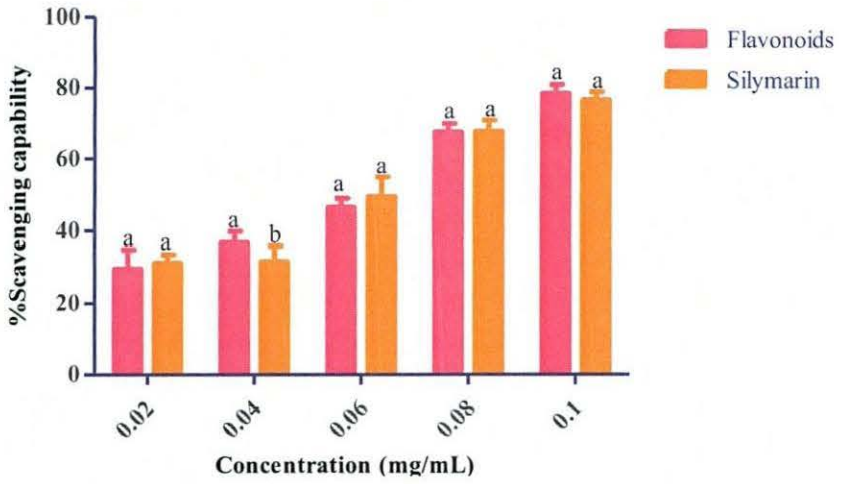


Fig. 5. Showing hydroxyl radical scavenging capabilities of the flavonoids of *Hermannia geniculata* root extracts.

Values are mean± standard deviation (SD) of triplicate determinations. [ab]Values with different alphabets are significantly different ($p<0.05$)

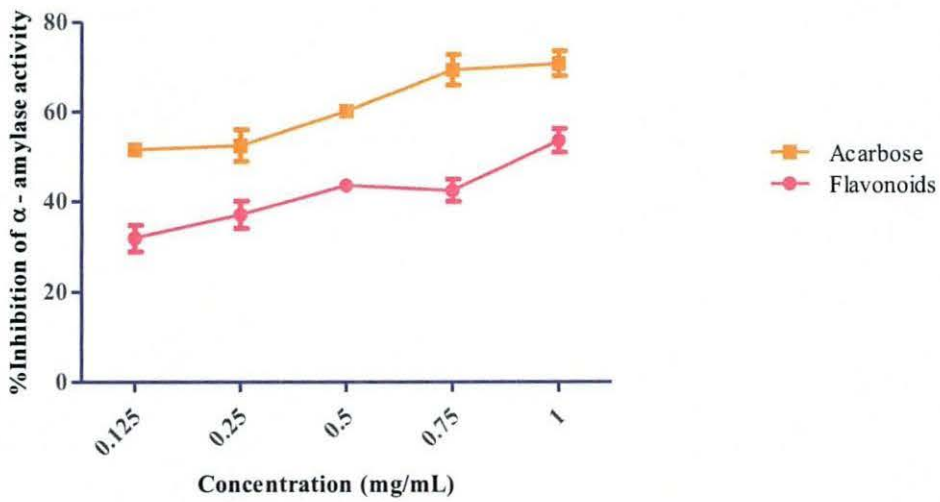


Fig. 6. Inhibitory potential of *Hermannia geniculata* root flavonoids extract on specific α-amylase activity. Values are Mean ± standard deviation (SD) of triplicate determinations.

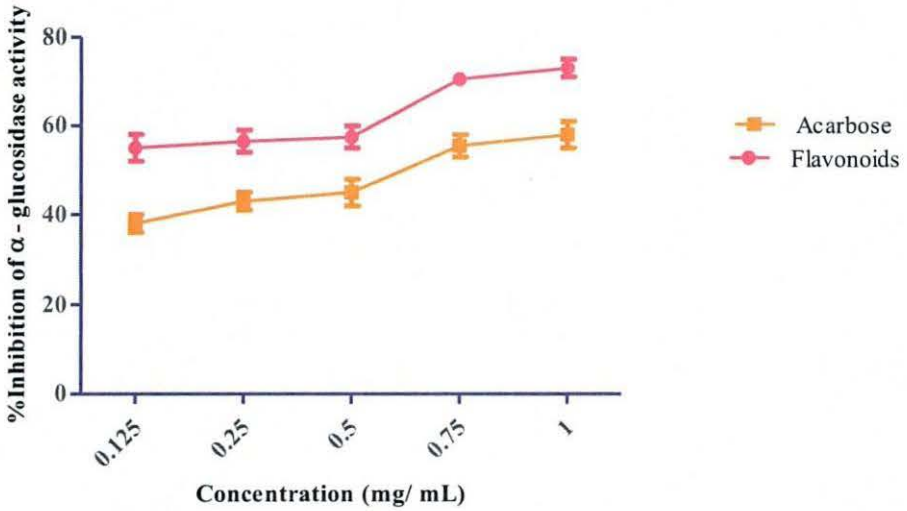


Fig. 7. Inhibitory potential of *Hermannia geniculata* root flavonoids extract on specific α-glucosidase activity.

Values are Mean ± standard deviation (SD) of triplicate determinations

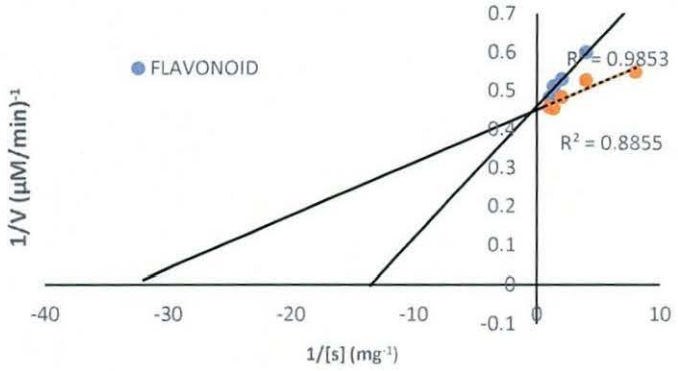


Fig. 8. Lineweaver-Burk plot of total flavanoid extract eliciting competitive inhibition on α-amylase activity. Results represent mean± standard deviation (SD); (n=3); ($p>0.05$).

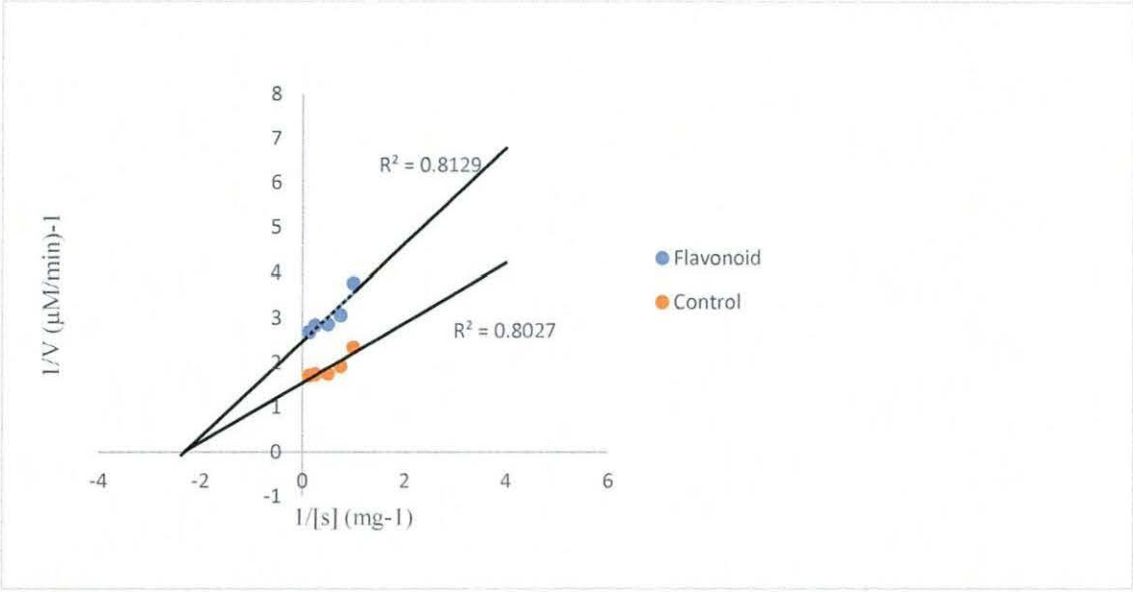


Fig. 9. Lineweaver-Burk plot of total flavonoids extract eliciting non-competitive inhibition on α-glucosidase activity. Results represent mean± standard deviation (SD); (n=3); ($p>0.05$).

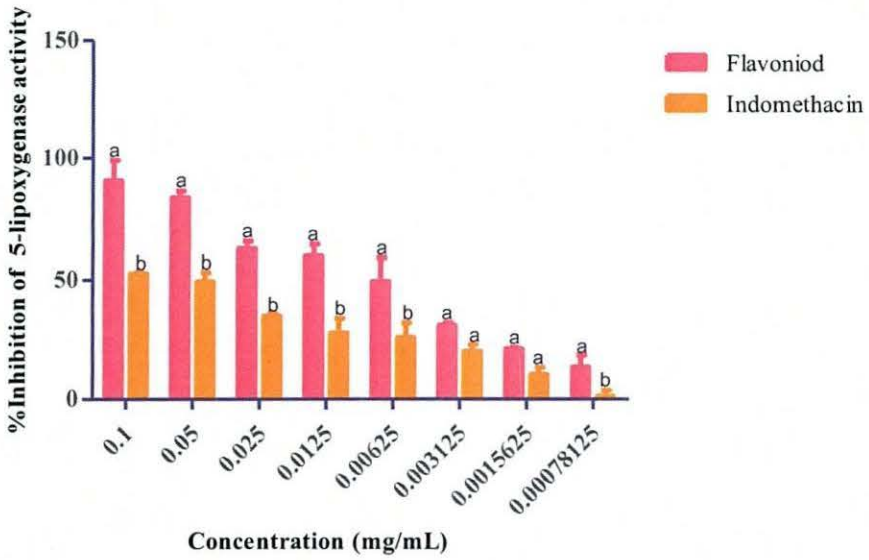


Fig. 10. Showing the antilipoxygenase activity of flavonoids of *Hermannia geniculata* root extract.

[ab]Values reprensent values that are significantly different ($p<0.05$).

Results represent three triplicate value, mean± standard deviation (SD).

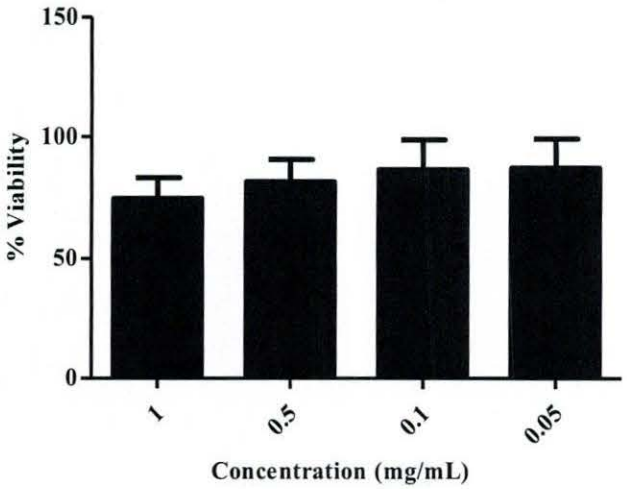


Fig. 11. Showing the percentage viability of Vero cell viability at different concentrations of the FHG extract. Data represent the mean ± SE (standard deviation) of three independent experiments.

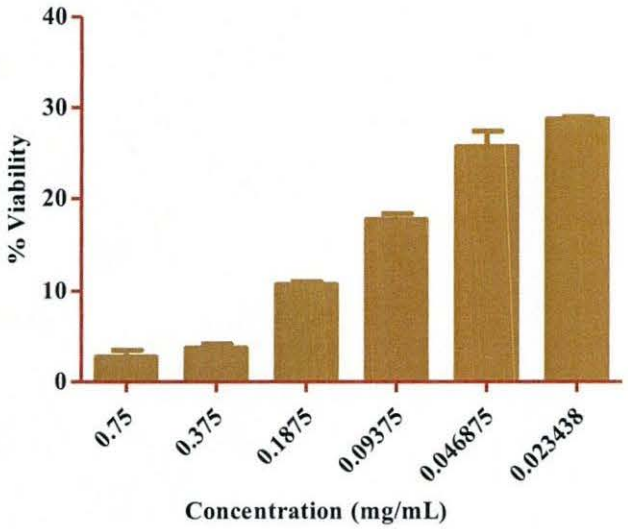


Fig. 12. Showing the percentage viability of HepG2 cell viability at different concentrations of the FHG extract. Data represent the mean ± SD (standard deviation) of three independent experiments.

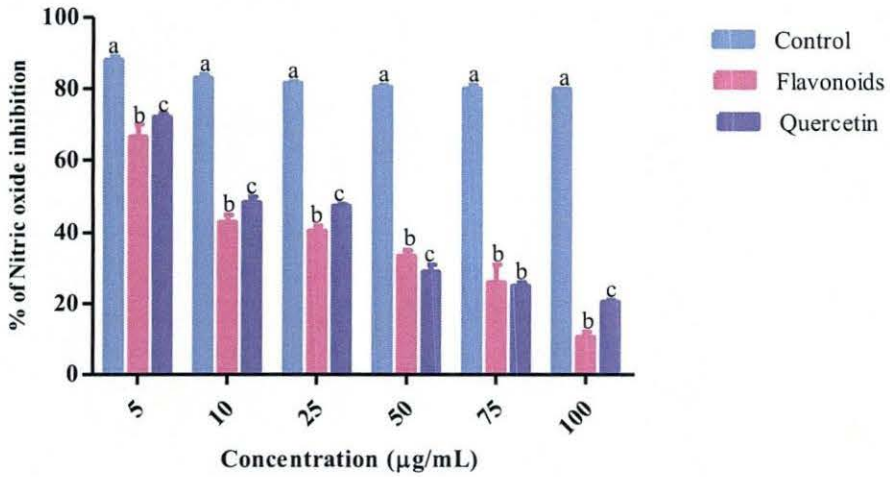


Fig. 13. Nitric oxide inhibitory activity of the extract from FHG on RAW 264.7 macrophages

Data represent the mean ± SE (standard error) of three independent experiments, [abc]Means significantly different ($p < 0.05$).

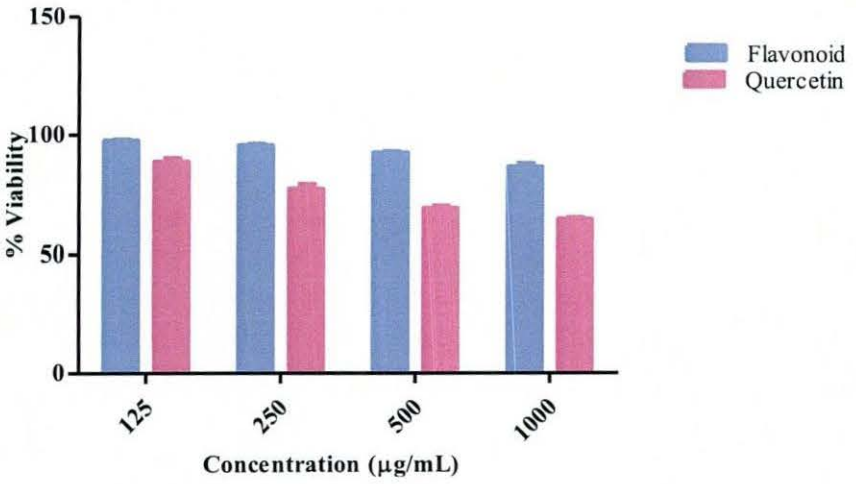


Fig. 14. Nitric oxide inhibitory activity of the extract from FHG on RAW 264.7 macrophages with respect to their cell viability. Data represent the mean ± SD (standard deviation) of three independent experiments.

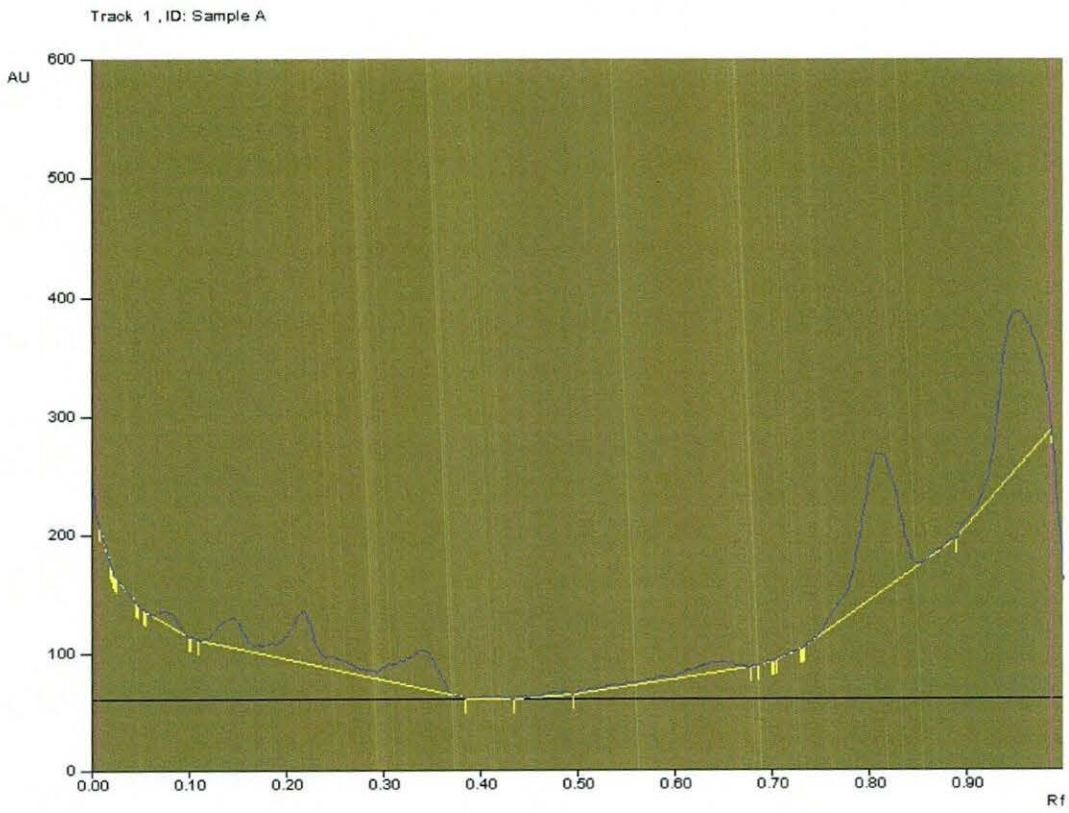


Fig. 15. Showing Track A – sample A the FHG extract Baseline display (scanned at 254nm)

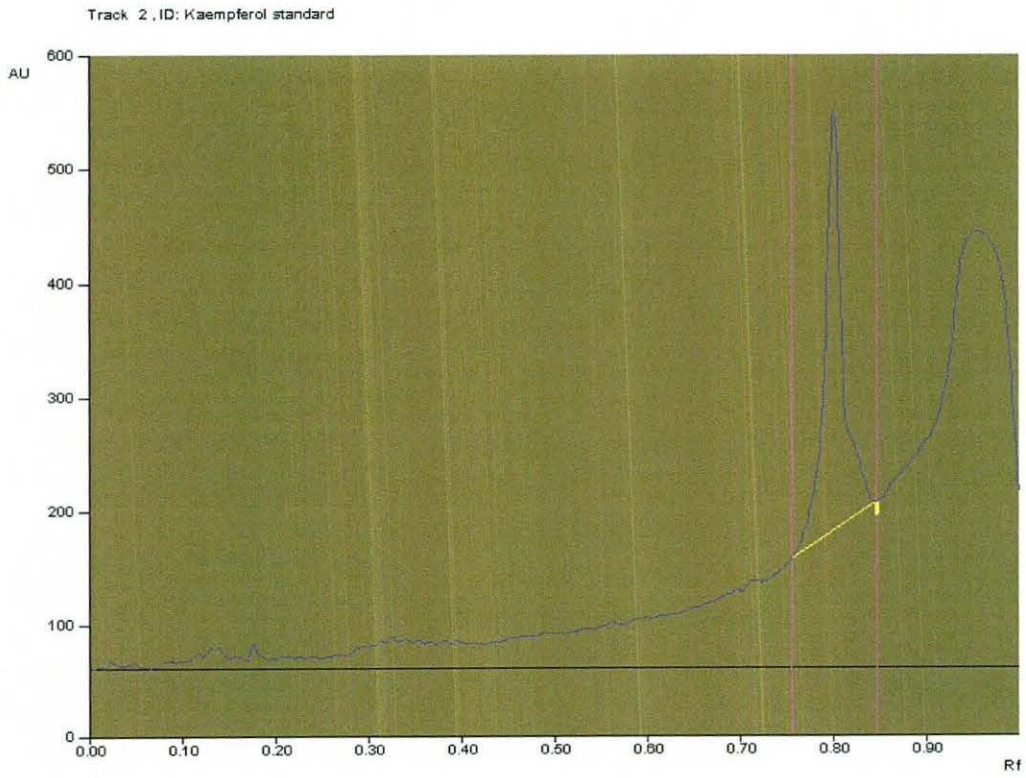


Fig. 16. Showing Track STD – Flavonoid standard Baseline display (scanned at 254nm)

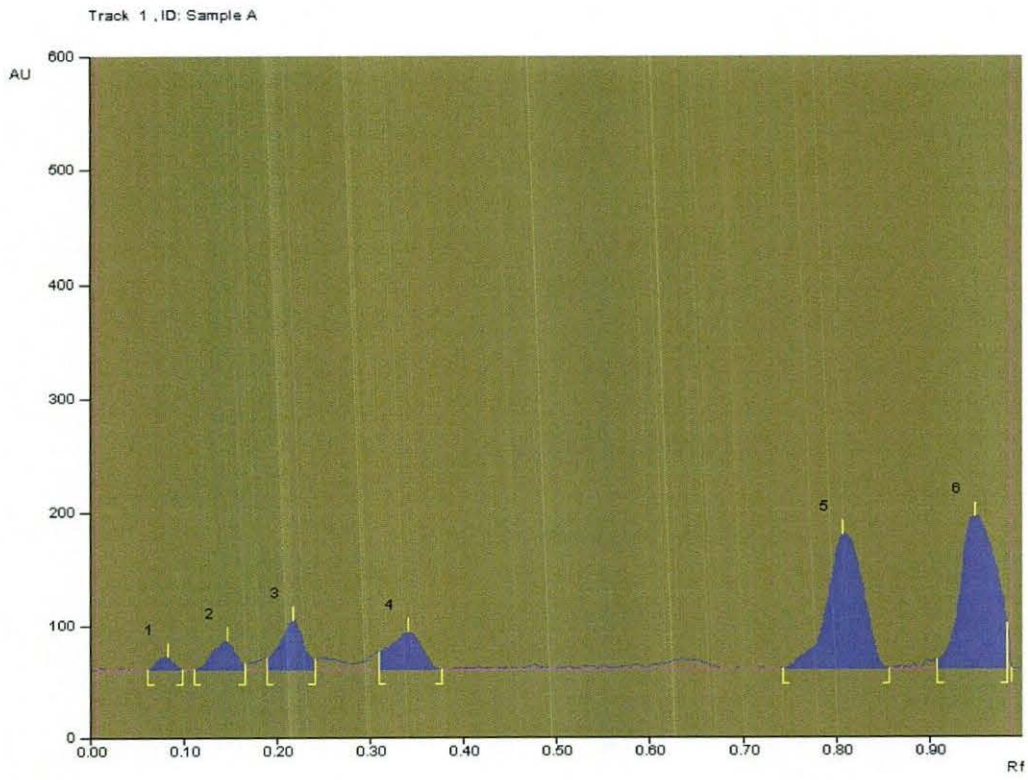


Fig. 17. Showing Track A- sample A the FHG extract Peak densitogram display (scanned at 254nm)

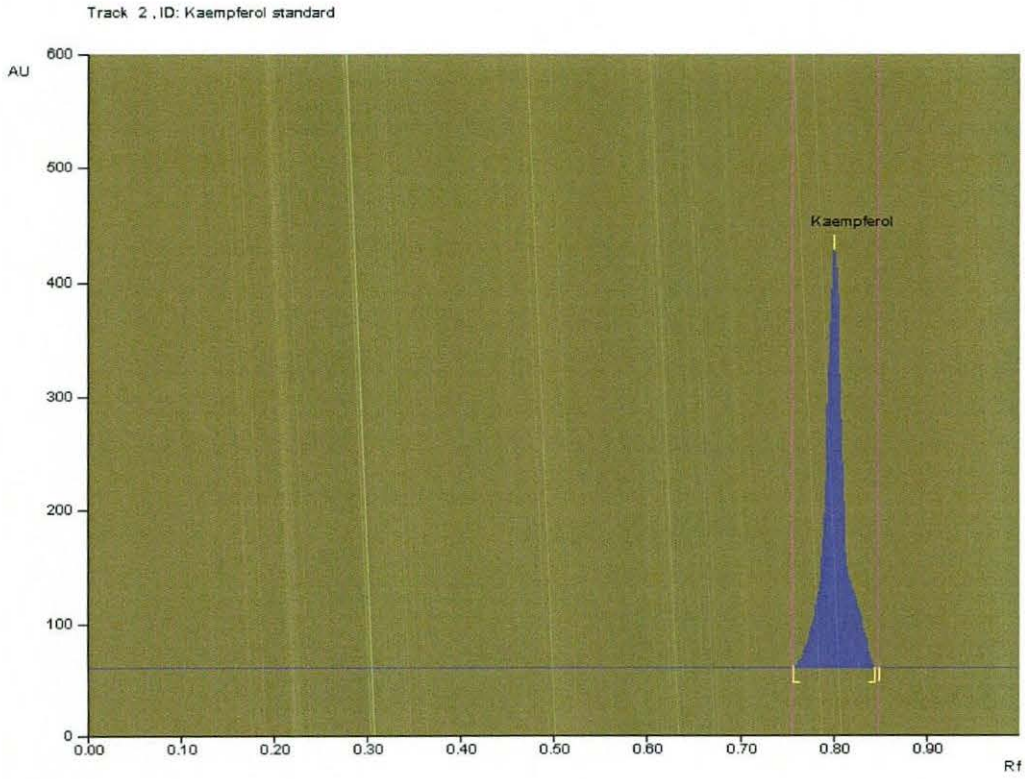


Fig.18. Showing Track STD – Flavonoid standard peak densitogram display (scanned at 254nm)

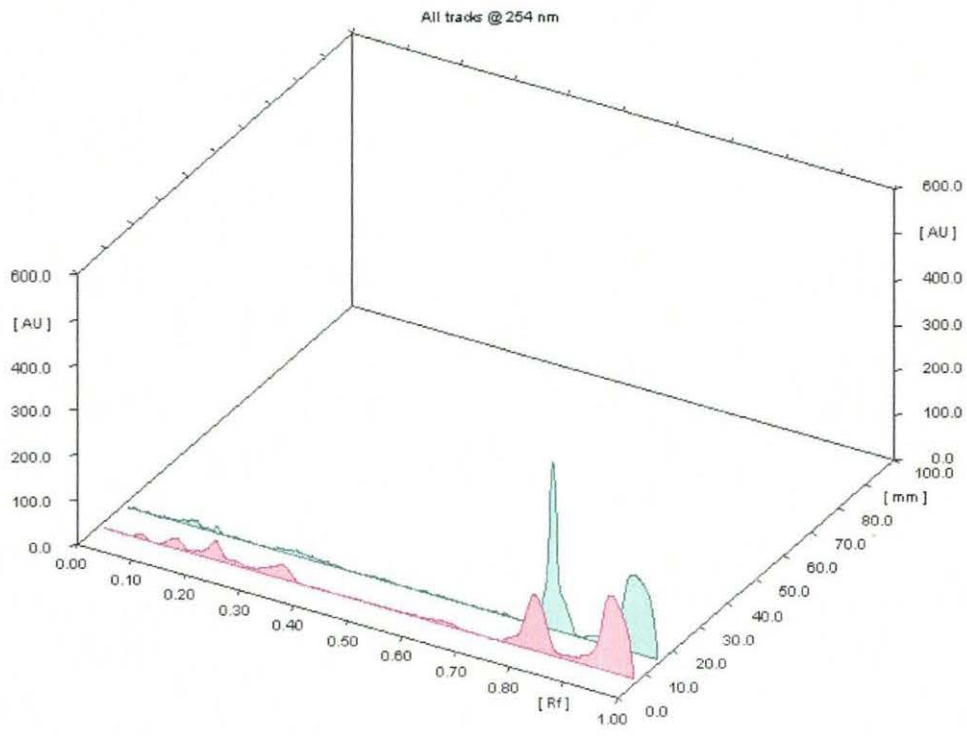


Fig. 19. Showing the 3D display of all Tracts scanned at 254nm.

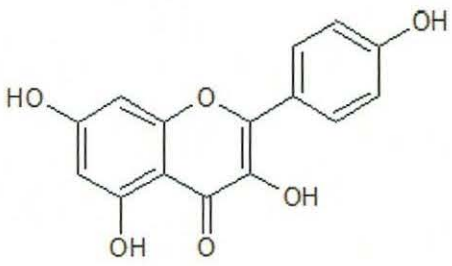


Fig. 20. Showing the chemical structure of kaempferol identified in FHG extract.

Tables

Table 1. The IC_{50} of various antioxidant, α- amylase, α- glucosidase and 5-lipoxygenase activities of total flavonoid of *Hermannia geniculata* root extract.

Antioxidant	IC_{50} (ug/mL)	Flavonoids	Silymarin
DPPH		3.07 ±0.12[a]	3.55± 0.10[b]
Metal Chelating		4.14± 0.50[a]	5.30 ± 0.98[b]
ABTS		2.13± 0.67[a]	2.77± 0.75[b]
Reducing Power		2.72± 0.13[a]	2.62± 0.99[a]
Hydroxyl Radical		2.51± 0.01[a]	2.47± 0.01[a]
Superoxide anion		3.21± 0.07[a]	3.87± 0.14[b]
Antidiabetic	IC_{50} (µg/mL)	Flavonoids	Acarbose
α- amylase		5.55± 0.37[a]	3.81± 0.29[b]
α- glucosidase		1.37± 0.19[a]	2.91± 0.42[b]
Anti-inflammatory	IC_{50} (µg/mL)	Flavonoids	Indomethacin
05- lipoxygenase		10.15± 0.12[a]	12.03±0.02[b]
NO production		6.71± 0.03	8.28± 0.05

Values are means of triplicate determination (n=3) ± standard deviation. [ab]No significant difference between extract with the same value, but significant difference $p>0.05$ between different value

Table 2: showing cytotoxic activity expressed as LC_{50} (mg/mL) of FHG extract

LC_{50}: Values: (mg/mL)	Vero cell	HepG2 cell	RAW264.7	SI
Flavonoid	>1.00± 0.268	0.020± 0.003	>1.00	50
Doxorubicin	>1.00± 0.125	0.032± 0.002	>1.00	31.25

Data are the means ± SD (standard deviation) n=8. Selectivity Index (SI) = IC_{50} Vero cell/ IC_{50} HEPG2 cell. SI value > 2 indicating high selectivity.

Table 3. Showing the R_f values of different peaks of flavonoid of *Hermannia geniculata* roots and the standard kaempferol

Track	Peak	Rf	Height	Area	Assigned substance
Sample A	1	0.08	11.4	188.9	Unknown
Sample A	2	0.15	25.1	588.3	Unknown
Sample A	3	0.22	43.9	1038.1	Unknown
Sample A	4	0.34	32.9	1043.3	Flavonoid/Phenol carboxylic acid 1
Sample A	5	0.81	119.8	4231.9	Kaempferol
Sample A	6	0.95	135.4	4767.4	Unknown
STD	1	0.80	371.8	6972.0	Kaempferol standard

Chapter six

Pharmacological activities of *Hermannia geniculata* roots phenols: HPTLC finger printing, antioxidant, antidiabetic, antinflammatory, cytoxicity of Vero and HepG2 cells.

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This chapter is a draft manuscript to be submitted for peer review journal

Pharmacological activities of *Hermannia geniculata* roots phenols: HPTLC finger printing, antioxidant, antidiabetic, antinflammatory, cytoxicity of Vero and HepG2 cells.

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ABSTRACT

The bioactive constituent and pharmacological activities of phenols extracted from *Hermannia geniculata* (PoHG) roots was investigated using *in vitro* methods. The chemical profile was of the extract was determined by HPTLC analysis. Antioxidant, antidiabetic, anti-inflammatory and cytotoxic effect of PoHG on Vero and HepG2 cells was carried out using standard procedures. Phenolic compounds were detected in the sample at R_f (0.14, 0.81 and 0.95). PoHG radical scavenging capabilities on DPPH, $ABTS^+$ and superoxide anion radicals were similar to the standard (silymarin). The IC_{50} values were DPPH (0.12± 0.00), ABTS (0.13± 0.01) and superoxide anion (0.20± 0.00). The values of the metal chelating activity of PoHG extract is lower and significantly different from the standard (silymarin) their respective IC_{50} values were (0.06± 0.00 and 0.18± 0.01). The antidiabetic effect was determined by its ability to mildly inhibit α-amylase and strongly inhibit α-glucosidase enzymes, the respective IC_{50} values obtained were (7.52± 0.23 and 1.76 ± 0.14). PoHG extract exhibited a commendable inhibition of 5-lipoxygenase enzyme with IC_{50} value of (0.15 ± 0.03) which is similar to the IC_{50}: (011 ± 0.01) value for the standard (indomethacin). However, the extract

was non-toxic to Vero cells with LC_{50} value of >1.00 mg/mL but highly toxic to HepG2 cells with LC_{50}: 0.08 mg/mL. The selectivity index of 12.50 was recorded. The presence of phenolics/ carboxylic acids were confirmed in the extract, the result of the antioxidant, antidiabetic and antinflammatory activities of PoHG suggested that the phenols extract may be useful in the management of oxidative stress induce diseases, type 2 diabetes mellitus and asthma. It is also safe for use and its antiproliferative activities can be exploited in search for anticancer agents.

Keywords: Phenols; antioxidants; antidiabetes; antinflammatory; HPTLC; *Hermannia geniculata.*

INTRODUCTION

As early as the beginning of human existence, man has been familiar with the use of plants in variety of ways. The relationship that existed between plant and man has grown and several plants has been used as medicine. There is enormous growth in the knowledge and pace of using plants for management of several diseases (Bairi, Ouzir, Agnieszka, & Khalki, 2017; Jokar, Noorhosseini, Allahyari, & Damalas, 2017; Jütte et al., 2017; Shakya, 2016; Yea, Kim, Kim, & Yi, 2017). Plant based traditional medicine system has been playing crucial role in the health care system worldwide (Alam et al., 2016; Raskin et al., 2002; Tupec et al., 2017; Twilley, Langhansová, Palaniswamy, & Lall, 2017). The South African flora accounted for 9% of world higher plants population, which comprises of more than 30,000 species (Street & Prinsloo, 2013). Many people depends on the use of medicinal plant due to affordability and unavailability of health care facilities (WHO, 2016).

Hermannia geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases. (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008;

Kazeem & Ashafa, 2015; Moffett, 1993). It belongs to the genus of flowering plant from the subfamily *Byttnerioideae* in the family *Malnaceae*. It is a creeping shrubs with sub-orbicular broad crenate leaves, the length of the leaf is about 15mm and the texture may be viscid or sticky. *Hermannia* geniculata is readily identified by the hanging flowers, a typically green calyx encloses the base of free petals with five petals which are contorted with transversely expanded filament. The filament is abruptly expanded and contracted beneath the base of the anther into a cruciform filament. (Gwynne-evans, 2015). The plant is endemic in Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa. The plant can also be found in Madagascar, Kenya, Lesotho and Saudi Arabia (Essop et al., 2008; Gwynne-Evans, 2015).

H. geniculata has been used in treatment of several diseases like colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Sotho women (Kazeem & Ashafa, 2015; Moffett, 1993).

Plant contains secondary metabolites called phytochemicals which are primarily used by the plant for protection against microbial and pest invasion (Micheal, 1988). Phytochemicals includes flavonoids, phenols, saponins, anthraquinones, alkaloids and glycosides (Mena et al., 2016). These phytochemicals yielded pharmacologically active compounds which possess therapeutic efficacies on several endogenous enzymes, biomolecules and reactive oxygen species which are body metabolites capable of causing severe harm to the organism (Egea et al., 2017; Hasani-Ranjbar, Larijani, & Abdollahi, 2009; Z. Liu, Liu, Xiong, Feng, & Tang, 2016).

Phenols are plant secondary metabolites which were biosynthesized in plants through two pathways. The acetate/malonate pathway and the shikimate pathways produce intermediate phenylpropanoid precursors like phenyalanine, cinnmic acid, ρ-coumaric acid and ρ-coumaroyl CoA which are product for the biosynthesis of phenolic carboxylic acid and its derivatives (Dewick & Haslam, 1969; Falcone Ferreyra, Rius, & Casati, 2012; Thomas Vogt, 2010).

Polyphenols contain numerous variety of compounds like flavonoids, phenolic acids and anthocyanidins. Several studies have reported the medicinal properties of phenolic compound found in plants such as anti-inflammatory, anticancer and antidiabetic (Ford-Hutchinson, Gresser, & Young, 1994; Ji, Li, & Zhang, 2009; Olaokun, Mcgaw, Rensburg, Eloff, & Naidoo, 2016; Yao, Zhu, Chen, Tian, & Wang, 2013). Research findings also described phenols capabilities to inhibit production or scavenge reactive oxygen and nitrogen species like superoxides and peroxynitrites (Park et al., 2017; Shashank & Abhay, 2013). Phenolic compounds can modulate lipid and carbohydrate metabolism, dyslipidemia, insulin resistance, alleviate inflammatory process and oxidative stress (Elisha, Dzoyem, McGaw, Botha, & Eloff, 2016; Moharram & Youssef, 2014). Studies also revealed that phenolic compounds induces caspase mediated apoptosis and is cytotoxic to several cancer cells (Nandi & Vracko, 2007). Experimental studies attributed the activities of polyphenols to the presence of individual phenolic compounds present in the plant (Mfotie Njoya, Munvera, Mkounga, Nkengfack, & McGaw, 2017). Therefore the activities of phenolic compound depends on its different phenolic constituents.

This study was carried out to determine the biological activities of the phenols present in *Hermannia geniculata* root. We studied the antioxidant, antidiabetic, antinflammatory and also determine the cytotoxicity of the phenolic extract on Vero and HepG2 cells.

2.0 MATERIALS AND METHODS

2.1 Plant Collection, Preparation and Extraction.

Hermannia geniculata roots were purchased from local market in Puthaditjhaba, Qwaqwa, Northern Free State Province, South Africa. Confirmation of the species identity was carried out with the herbarium specimen with voucher specimen file number (5056.000-10700) m(Moffett, 1993) at the University of Free State, Qwaqwa Campus, South Africa. It was also compared with our earlier Voucher specimen (Ash/med/05/2013/QwHB) (Kazeem & Ashafa, 2015) at the herbarium.

2.2 Extraction of Phenols.

The roots were separated, washed under running tap to remove all debris and chopped into small pieces before air dried at room temperature to a constant weight. The dried root materials was pulverised into fine powdered using waring laboratory blender (Labcon, Durban, South Africa) and kept at 4°C prior to extraction.

Phenols extraction was carried out using shake extraction method (Li et al 2011). Briefly, 85:15 (v/v) mixture of 95% ethanol and 1 M hydrochloric acid was added to 5 g of the plant materials, and the suspension was shaken on an orbital (rotary) shaker for 1 h at 30°C. The mixture was centrifuged at 3000 rpm for 10mins, the mixture was then sieved with 0.45 µm Millex-HV syringe filter unit and the filtrate was evaporated under reduced pressure. For further analysis, the dry residue was used after dissolving it in a 50% methanolic solution and

stored at -4°C before carrying out high performance thin layer chromatography and other *in vitro* assays.

2.3 Chemicals and Reagents

The chemicals were purchased from different suppliers were: Linoleic acid (Merck, Darmstadt, Germany), xylenol orange, ferric chloride (Searle Company, England), indomethacin, sodium dodecyl sulphate, sodium nitrite, ferrous sulphate, and 5-lipoxygenase (Glycine max) (Sigma, Germany). Porcine pancreatic α-amylase, rat intestinal α- glucosidase, 1,1-diphenyl-2-picrylhydrazyl, silymarin, acarbose and paranitrophenyl-glucopyranoside were products of Sigma-Adrich Co., St Loius, USA while starch soluble (extra pure) was obtained from J.T BakerInc., Phillipsburg, USA. Other chemicals and reagents were of analytical grade and the water used was glass distilled.

2.4 *In vitro* Antioxidant Assays

All experiments were conducted in triplicates and all the negative control (blank) was prepared using the same procedure replacing the extract with distilled water

2.4.1 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Radical Scavenging Determination

The ability of the PoHG extract to scavenge ABTS cation chromophore obtained from the oxidation of ABTS solution and potassium persulphate was determined according to already adopted method (Re et al., 1999). Briefly, 50 mL each of 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulphate leaving the mixture for 4-16 h until the reaction was completed and absorbance was stable. The resultant mixture was diluted with ethanol to get an absorbance of 0.700±0.05. The absorbance reading was taking at 734 nm using microplate reader (BIO-RAD, model 680, Japan). 20 µL of different concentration (0.02-0.1

mg/mL) of PoHG and standard was then mixed with 200 µL ABTS solution in a 96- well microtiter plate and absorbance was read at 734 nm using a microplate reader (BIO-RAD, model 680, Japan) after 15 mins of incubation at 25°C. The percentage inhibition activity was obtained using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

The IC_{50} value was calculated and obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.4.2 **Metal Chelating Assay**

The chelating of ferrous ions by PoHG extract was estimated as described by (Dinis, Madeira, & Almeida, 1994). Briefly, 40 µL of the different concentrations of the extract and standards (0.02 –0.1 mg/mL) was dispensed into a 96-well microtiter plate, 200 µL of 2 mM $FeCl_2$ solution was afterwards added to the mixture. The reaction was initiated by the addition of 80 µL 5 mmol/L ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 mins. The absorbance of the solution was then read at 562 nm using a BIO-RAD (model 680, Japan) microplate reader. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.4.3 **Superoxide Anion Scavenging Assay**

Determination of superoxide anion radical scavenging potential of PoHG extract was achieved according to (Liu, Ooi, & Chang, 1997). Superoxide radicals were generated in 50 mL of Tris-

HCl buffer (16 mM, pH 8.0) containing 50 mL of NBT (50 mM) solution, 50 mL of NADH (78 mM) solution and different concentrations (0.02–0.1 mg/mL) of PoHG extract and standard. The reaction was initiated by adding 1 mL of phenazine methosulphate (PMS) solution (10 mM) to the mixture. The reacting mixture was incubated at 25 ^{0}C for 5 min, then the absorbance was measured at 560 nm using a microplate reader (BIO-RAD, model 680, Japan). IC_{50} was then evaluated from calibration curve following estimation of percentage superoxide anion scavenging capacity of the tested extract of PoHG using the expression: Percentage Scavenging (S %)=$[(A_{control}-A_{extract})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control, $A_{extract}$. The IC_{50} value was obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value

2.4.4 **Hydroxyl radical scavenging ability**

The ability of the plant extracts to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the method of (Mathew & Abraham, 2006). Briefly, 100 µL of different concentrations (0.02-01 mg/mL) of PoHG extract and standard, 120 µL of 20 mM deoxyribose, 400 µL of 0.1 M phosphate buffer, 40 µL of 20 mM hydrogen peroxide and 40 µL of 500 µM ferrous sulphate each were taken and mixed in 2 mL Eppendorf tubes. Then 100 µL of distilled water was added and incubated for 30 min at 37 °C. Subsequently, 0.5 mL 2.8% of trichloroacetic acid (TCA) and 400 µL of 0.6% thiobarbituric acid (TBA) solutions were added to stop the reaction. From the mixture, 300 µL of the resultant mixture was dispensed into a 96-well micro titer plate and incubating in boiling water for 20 min. The absorbance was taken at 532 nm using a microplate reader (BIO-RAD, model 680, Japan). The percentage inhibition of PoHG extract/standard was obtained using $[(A_0–A_1)/A_0] \times 100$,

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and obtained from the linear regression equation using y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.4.5 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging activity of the PoHG extract was evaluated based on its scavenging activities on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The method was described by (Braca et al., 2001). Briefly, 150 µL of the varying concentration of plant extract/ standard (0.02 – 0.1 mg/mL) was added separately to 150 µL of 0.004% methanolic solution of DPPH in a 96-well microtiter plate. The absorbance at 517 nm was determined after 30 min using a 96-well microplate reader (BIORAD, model 680, Japan), and the percentage inhibitory activity of the extract/standard was calculated using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. The half maximal inhibitory concentration (IC_{50}) value were calculated from the linear regression equation using y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.5 *In vitro* Antidiabetic Assays

2.5.1 α-Amylase Inhibitory Assay

This assay was carried out using the procedure of (Apostolidis, Kwon, & Shetty, 2007). 250 mL of varying concentration of PoHG extract/ standard (0.125 – 1.0 mg/mL) was placed in a test tube and 250 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was incubated at 25°C for 10 min, followed by addition of 250 mL of starch (1%) solution in 0.02 M sodium phosphate buffer (pH 6.9) at timed intervals,

the resulting reaction mixture was then incubated at 25°C for 10 min. The reaction was terminated by adding 500 mL of dinitrosalicylic acid (DNS) reagent before incubating the tubes in boiling water for 5 min and cooled to 25°C. 5 mL distilled water was added to the reacting mixture after cooling and the absorbance was measured at 540 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was done in triplicate and was calculated as percentage inhibition, thus; % Inhibition = (Absorbance (control) – Absorbance (extract))/ Absorbance (control) ×100. Concentrations of extract resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.5.2 α-Glucosidase Inhibitory Assay

The effect of the PoHG extract on α-glucosidase activity was determined according to the method described by (Apostolidis et al., 2007). In brief, different concentrations (0.125 – 1.0 mg/mL) of extract/ standard were prepared in distilled water. Then, 50 mL from the stock solution was mixed with 100 mL of 0.1 M phosphate buffer (pH6.9) containing 1.0 M of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25°C for 10 min. Following this, 50 mL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. The inhibitory effect of extract/standard on the enzyme activities were determined by measuring the absorbance of the mixtures at 405 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The experiments were conducted in

triplicate and the α-glucosidase inhibitory activity was expressed as % inhibition using the expression:

%Inhibition= [(Acontrol-Aextract)/Acontrol]×100, where Acontrol and Aextract are the absorbance's of the control and extract respectively. Concentrations of extract/standard resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.5.3 **Kinetic Studies**

2.5.3.1 **Mode of α-Amylase Inhibition**

This assay was conducted using modified methods (Ali, Houghton, & Soumyanath, 2006). Briefly, 250 mL of the (5 mg/mL) extract of PoHG/ standard were pre-incubated with 250 mL of 0.5 mg/mL α-amylase solution for 10 min at 25°C in one set of tubes while α-amylase was pre-incubated with 250 mL of 0.1M phosphate buffer (pH 6.9) in another set of tubes. The reaction of the two sets of the mixtures was initiated by adding 250 mL of 5% starch solution at increasing concentrations (0.31 – 50.00 mg/mL). The mixture was then incubated for 10 min at 25°C, followed addition of 0.01mM DNS (500 mL). The reaction was terminated after boiling for 5 min. The amount of reducing sugars released was determined spectrophotometrically using maltose standard curve and converted to reaction velocities. A double reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted to determine the mode of inhibition.

2.5.3.2 **Mode of α-Glucosidase Inhibition**

The kinetics on inhibition of α-glucosidase activity by PoHG extract using modified methods of (Nagmoti & Juvekar, 2013) was adopted. Briefly, 50 µL of 5 mg/mL extract was pre-

incubated with 100 μL of 0.1M α-glucosidase solution for 10 min at 25°C in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated with 50μL of 0.1M phosphate buffer (pH6.9). 50 mL of 0.05M pNPG at different concentrations (0.31 – 50.00 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10min at 25 °C, and 500 mL of 0.1M Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined colourimetrically using a p-nitrophenol standard curve. Reaction rates (v) were thereafter calculated and double reciprocal plots of enzyme kinetics. Km and Vmax values were also calculated from Lineweaver-Burkplot (1/v versus 1/[S]) (LineweaverandBurk, 1934).

2.6 **Soybean 5-Lipoxygenase Inhibition Assay**

The assay was performed according to previously described procedure (Pinto, Tejeda, Duque, & Macías, 2007). The assay is based on the formation of the complex Fe^{3+}/xylenol orange with absorption at 560 nm. 5-lipoxygenase from *Glycine max* was incubated with different concentration of the extract or standard (0.00078-0.1 mg/mL) at 25°C for 5 min. Then linoleic acid (final concentration, 140 μM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25°C for 20 min in the dark. The assay was terminated by the addition of 100 μL of FOX reagent [sulfuric acid (30 mM), xylenol orange (100 μM), iron (II) sulfate (100 μM), methanol/water (9:1)]. The lipoxygenase inhibitory activity was evaluated by calculating the percentage of the inhibition of hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25°C. % inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance control] x100. The 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation $y = m x + c$,

where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.7 Cell Culture of Vero and HepG2

The Vero cell and HepG2 cells were maintained at Department of Paraclinical Sciences, cell line laboratories, Onderstepoort campus, University of Pretoria, South Africa. The culture medium used for the cell culture was DMEM supplemented with 10% FBS. 100 µg/mL streptomycin and 100 units/mL penicillin was added to the culture medium. The cell culture environmental condition was 5% $C0_2$ humidified atmospheric condition at 37°C.

2.8 Cytotoxicity of Cell Lines

Viable cells growth after incubation of African green monkey (Vero) cells and Human hepatocarcinoma (HepG2) with PoHG extract was determined using the tetrazolium-based colorimetric (MTT) assay (Mosmann, 1983). Briefly, cells that have reached sub-confluent in their culture medium were harvested, centrifuged and re-suspended in the growth medium at 5 x 10^4 cells/ mL. MEM was used as the growth medium and it was supplemented with FCS (5%) and 0.1% gentamicin (Vibrae). MEM (200 µL) was added to wells of columns 1 and 12 to maximize the "edge effect" and maintain the relative humidity. The 96 well plates were incubated at 37°C until the exponential growth phase of the cells were reached. MEM in the plates was removed carefully without disturbing the cells and washed in 150 µL PBS. It is important to minimize the disturbance of the cells during the aspiration of MEM. The serial dilutions of the PoHG extract at differing concentrations of 0.05 – 1.0 mg/mL were made in quadruplicate. The serial dilutions of the test extract were all prepared in MEM and the mixture were added to the wells. The microtitre plates were incubated at 37°C in 5% CO_2 for

48h. The cells that were not treated and doxorubicin chloride were used as negative and positive control respectively.

After 48 h of incubation of the plates, 30 µL MTT (stock of 5 mg/mL in PBS) was put into all the wells and the plates were subjected to 4 h of further incubation at optimum temperature of 37°C. After incubation, the MTT in the culture medium in each of the cell was removed gently without distorting the MTT crystals. The formazan crystals formed by MTT were dissolved by the addition of DMSO in each of the well. The plates were gently shaken to facilitate better dissolution of the MTT crystals. The MTT reduction by the cells was measured by taking their absorbance using a microplate reader (Synergy Multi-Mode Reader, BioTek) at 570nm and a reference wavelength of 630nm. The reader was blanked using the column 1 well which contains only MTT and medium. The LC_{50} values was determined as the concentration of *Hermannia geniculata* extracts resulting in a 50% reduction of absorbance compared to untreated cells.

2.9 Selectivity Index (SI)

The degree of selectivity of PoHG extract was expressed by its SI value as suggested by **(Badisa et al., 2011)**. High SI value (>2) of an extract suggests selective toxicity against cancer cells, while a compound with SI value <2 is considered to give general toxicity which can also cause cytotoxicity in normal cells (Awang, Aziz, Kamaludin, & Chan, 2014; Machana, Weerapreeyakul, Barusrux, & Nonpunya, 2011). Each SI value was calculated using the formula: SI = IC_{50} normal cell/IC_{50}

2.10 HPTLC Finger Print Analysis of PoHG Extract

The assay method was described by (Reich, Schibli, & DeBatt, 2008). Briefly, 50 mg each of PoHG extract was weighed accurately in an electronic balance (Afcoset), dissolved in 250 µL

of ethanol and centrifuged at 3000 rpm for 5min. This solution was used as test solution for HPTLC analysis. Then 2 µL of test solution and 2 µL of standard solution were loaded as 5 mm band length on a 3 x 10 Silica gel 60 F_{254} TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

The sample loaded plate was kept in Thin Layer Plate (TLC) twin trough developing chamber (after saturated with solvent vapor) with the mobile phase (Flavonoid) and the plate was developed in the respective mobile phase up to 90 mm.

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at visible light, and ultraviolet (UV) at 254 and 366 nm. The developed plate was sprayed with r spraying reagent (Flavonoid) and dried at 100°C in hot air oven. The plate was photo-documented in visible light and UV (366 nm) mode using Photo-documentation (CAMAG REPROSTAR 3) chamber. Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254 nm. The Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version. Toluene-Acetone-Formic acid (4.5: 4.5:1) was used as the mobile phase while 1% ethanolic aluminum chloride was used as the spraying reagent.

2.11 **Statistical analysis**

Statistical analysis was performed using a Graph Pad Prism 5 statistical package (Graph Pad Software, San Diego, MA, USA). Data were expressed as means of replicate determinations ± SD, for *in vitro* assays and was subjected to two-way analysis of variance (and non-parametric) followed by Bonferroni: compare all pair of column. Statistical significance was considered at $P < 0.05.3.0$

3.0 **Result and Discussion**

High performance thin layer chromatography (HPTLC) finger printing provide improved resolution, higher detection sensitivity, and improved in situ quantification of phytochemicals. They are necessary tool used for medicinal plant densitometric quantitative analysis. The result of HPTLC profiling of PoHG confirmed the presence of phenols in the PoHG extract with R_f values (0.14-0.95). The specificity of the method was ascertained by analyzing the standard and the test samples within similar chromatographic conditions. The chromatograms obtained showed similarities for both the standard and the test samples.

3.1 **Antioxidant activities of PoHG**

DPPH decolorization assay was use to evaluates the antioxidant activities of the phenolic compound using change in absorbance in a standard procedure which determine the radical scavenging capability of the PoHG extract. The IC_{50} value calculated from the regression line showed that PoHG has a similar IC_{50} value with the standard (silymarin) with their respective IC_{50} values of (0.12± 00 and 0.38± 0.02) mg/mL. Similarity between the IC_{50} values was also observed in $ABTS^+$ and superoxide anion radical scavenging assays as displayed in Table 1, Figure 1, 2 and 3.

(Martins, Barros, & Ferreira, 2016; Roleira et al., 2015) documented phenolic compounds with antioxidant activities. The variation in the pharmacological activities of phenolics from different plant families has been attributed to the genotypic and environmental differences like climate, location, temperature, fertility, microbial and pest exposure within species, choice of the parts tested, time of taking samples (Devendra et al., 2004; K. H. Kim, Moon, Choi, Kim, & Lee, 2013).

Furthermore, the type of phenolic constituents present in plant is also responsible for the antioxidant activities. Rosemaric acid, ferrulic acid, and caffeic acid have been reported to have high antioxidant capability if present in a plant sample (Wojdyło, Oszmiamki, & Czemerys, 2007). The role of pH of the solvent in the activities of the phenolic compound is also important in determining it's *in vivo* antioxidant activities. Phenolics mechanism of reducing oxidation is by transferring of H^+ from the polyhydroxyl group to the carbon carrying ROO^- radicals. Therefore, the antioxidant capabilities of phenols are strongly reduced by hydrogen bonded accepting molecules. Thus, the solvent effects is determined by the solute acidity and solvent basicity (Foti, 2007; Medini, Fellah, Ksouri, & Abdelly, 2014).

Phenolic compound with high antioxidant activities will have O-H bond in positions with lower bond dissociation constants. The dissociation of the hydroxyl functional groups in flavonoid which is an example of phenols present in PoHG extract occur in the following sequence 7-OH>4^1-OH>5-OH (Al-sehemi & Irfan, 2017; Rice-Evans, Miller, & Paganga, 1996). This may also contribute to the observed antioxidant properties of PoHG.

The result of the metal chelating capabilities of PoHG as shown in Table 1and Figure 2 showed that phenol has a better activity compared to the standard with the IC_{50} value of (0.06± 0.00) mg/mL which is lower and significantly different ($p<0.05$) from the standard with IC_{50} value of 0.18± 0.01 mg/mL. Scavenging of the reactive species is very crucial for cell survival because the reactive oxygen species can attack membrane lipids causing lipid peroxidation, cause protein modification, DNA damage leading to apoptosis, all this were involved in the etiopathogenesis of oxidative stress that is implicated in several neurodegenerative disease like artheriosclerosis, diabetes mellitus and cancer (Egea et al., 2017).

From this results, PoHG has a good radical scavenging activity, the inhibition observed from the graph suggested that the extract was able to inhibit the production of reactive oxygen. Therefore, PoHG can be a useful agent in ameliorating oxidative stress induce illnesses.

3.2 **Antidiabetic activity of PoHG**

Diabetes is a global social disease and threaten to reach a pandemic level by 2030 (Ezzati, 2016). Chronic hyperglycemia or glucotoxicity which results from unregulated activity of carbohydrate metabolizing enzymes (α-glucosidase and α-amylase) stimulate hyperinsulinemia, insulin resistance and formation of glycation end products. All this further aggravate β- cell secretory failure and apoptosis (King & Loeken, 2017; Liu et al., 2013). Strong and mild inhibition of α-glucosidase and α-amylase enzymes has been suggested to be an effective strategy to control rise in postprandial plasma glucose level which may be seen in type 2 diabetes (Olaokun et al., 2016; Sabiu, O'Neill, & Ashafa, 2016).

From the result of this study as reflected in Table 2 Figure 7 and 8, PoHG extract exhibited strong inhibition of α-glucosidase with lower IC_{50} value of (1.76 ± 0.14) mg/mL which is significantly different ($p<0.05$) from acarbose with IC_{50}: (4.38 ± 0.25) mg/mL. However, PoHG showed mild inhibition of α-amylase enzyme with an IC_{50} value of (7.52 ±0.23) mg/mL. The effect of the extract on α-glucosidase is similar to observation of (Ademiluyi & Oboh, 2013) where bound and free phenols exert strong inhibition on α-glucosidase and α-amylase enzymes.

The higher inhibition of α-glucosidase and mild inhibition of α-amylase is of great pharmaceutical importance, it helps to address the side effect like diarrhoea, flatulence and abdominal discomfort associated with some available synthetic antidiabetic drugs including acarbose and voglibose which are two important drugs that is currently in use for the

management of type 2 diabetes (J. Kim, Kwon, & Son, 2000; Tundis, Loizzo, & Menichini, 2010).

For this research, rat intestinal α-glucosidase was used, it has been reported that inhibitors of rat intestinal α-glucosidase can be substituted for evaluation of human enzymes (Oku, Tanabe, Ogawa, Sadamori, & Nakamura, 2011). Therefore, we seek to obtain results applicable to human enzymes. The results obtained from this study suggested that PoHG extract may be a useful agent in the management of type 2 diabetes through its inhibitory effects on the two key carbohydrate metabolizing enzymes that has been implicated in post-prandial hyperglycaemia seen in type 2 diabetes.

Kinetics of α-amylase inhibition by PoHG extract showed uncompetitive inhibition of the enzyme, binding of the inhibitor to the ES complex occur when the ES is exposed. Binding of the inhibitor to the ES complex increases the affinity of the enzyme for the substrate thereby reducing the Km value. The decrease in the value of Vmax showed that there is reduced enzyme turnover. The Vmax and Km decreases at the same rate. The velocity of the enzyme decreased from 0.155 to 0.150 mM/mins while the Km value decreased from 15.2 to 14.0 mM.

Intestinal α-glucosidase has four catalytic domains (Roskar et al., 2015) its mode of inhibition by PoHG extract was by non-competitive inhibition. The inhibitor does not affect the availability of the binding site to the substrate (Km is constant). However, it has significant inhibitory effect on the speed of the reaction by reducing the Vmax. The maximum velocity was reduced from 1.82 to 0.95 mM/mins while the Km remain constant.

3.3 **Antiinflammatory activities of PoHG**

Use of conventional drug like corticosteroid, salphasalazine which inhibit leukotriene biosynthesis through 5-lipoxygenase (5-LOX) inhibition has been in use for the management of inflammatory bowel diseases, acute colitis and asthma (Rask-Madsen, Bukhave, Laursen, & Lauritsen, 1992; Raskin et al., 2002). These antinflammatory agents currently in use effectively manage acute inflammatory reactions but in chronic stage, the long term use causes a lot of side effects which necessitate the need for search of medicinal plant with inhibitory effect on 5-LOX enzyme. This study was designed to examine the inhibitory effect of PoHG on 5-lipoxygenase enzymes. The result shown in Table 2 and Figure 5 revealed 83% inhibition of 5-LOX by PoHG. The PoHG IC_{50} value of (0.15±0.01)mg/mL which is similar to IC_{50}: (0.11± 0.03) mg/mL value of indomethacin. Several report of the inhibition of 5-lipoxygenase by phenolic extracts has been (Pergola & Werz, 2010; Schaible et al., 2016; Schneider & Bucar, 2005; Werz & Steinhilber, 2006). 5-LOX catalyze metabolism of arachidonic acid to form (5-hydroxyecosateteraenic acid) HETE which is a substrate for leukotriene production. Leukotriene B4, C4, D4 and E4 contribute to cellular adhension, degranulation, chemotaxis of neutrophils and inflammation by increasing vascular permeability and this are important step in the pathophysiology of asthma and acute colitis (Banoglu et al., 2016; Busse, McGill, & Horwitz, 1999; Gür, Çalişkan, & Banoglu, 2017; Hofmann & Steinhilber, 2013; Kuhn, Banthiya, & Van Leyen, 2015; Werz, Gerstmeier, & Garscha, 2017). Therefore, based on the observed effect of PoHG on 5-LOX, it may be an effective agent in the management of asthma and can accelerate healing in cases of acute colitis.

Cytotoxicity of PoHG was tested on Vero and HepG2 cells. The result seen in Table 3, Figure 11 and 12 showed a selective toxicity of the cells. However there was no toxicity noticed at

all concentration of the extracts (0.05-1) mg/mL on Vero cells, the LC_{50} value is >1.00 mg/mL. Nevertheless, toxicity of HepG2 cells was recorded with less than 10% cell viability seen at concentration of 0.75 mg/mL the LC_{50} value of 0.08 mg/mL. The selectivity Index was 12.50. This findings showed that PoHG is non-toxic to normal kidney cells and also possesses an antiproliferative properties.

Conclusion

The *in vitro* activities of the PoHG showed that the extract can be used in the management of asthma, diabetes and colitis. *In vivo* studies of the extract is ongoing in the laboratory. Also, the isolation and elucidation of the bioactive constituents of the extract is at an advance stage. This results validate the traditional use of the plant in the treatment of inflammatory diseases.

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Results

Table 1. Showing IC_{50} values for the free radical scavenging abilities of phenolic extract of *Hermannia geniculata* roots.

	Phenols	Silymarin
Antioxidants	IC_{50} values (mg/mL)	
DPPH	0.12± 0.00[a]	0.38± 0.02[a]
Metal Chelating	0.06± 0.00[a]	0.18 ± 0.01[b]
ABTS	0.13± 0.01[a]	0.11± 0.01[a]
Hydroxyl Radicals	0.02± 0.00[a]	0.12± 0.01[b]
Superoxide anion	0.20± 0.00[a]	0.20± 0.01[a]

The values are expressed as mean ± SEM of triplicate determination. [ab]Means along the row not sharing a common superscript are significantly different ($p<0.05$) from each other. Silymarin is the standard antioxidant agent for all the antioxidant assay.

Table 2. Showing IC_{50} values of the inhibitory capabilities of different extracts of *Hermannia geniculata* phenols on α-amylase, α-glucosidase and 05-lipoxygenase enzymes.

	Phenols	Acarbose
Antidiabetes	IC_{50} values (mg/mL)	
α-amylase	7.52 ± 0.23	7.62 ± 0.12
α-glucosidase	1.76 ± 0.14[a]	4.38 ± 0.25[b]
	Phenols	Indomethacin
Antiinflammatory	IC50 values (mg/mL)	
05- lipoxygenase	0.15 ± 0.03	0.11 ± 0.01

The values were expressed as Mean ± SEM of triplicate determination. [ab]Means across the horizontal row not sharing a common superscript are significantly different ($p<0.05$) from each other. Acarbose is the standard antidiabetic agent for antidiabetic assay and indomethacin is the standard anti-inflammatory agent used in this assay.

Table 3. Showing cytotoxic activity of the phenols of *Hermannia geniculata* on Vero and HepG2 cells expressed as LC_{50} (mg/mL) of plant extracts

LC_{50}	Vero	HepG2	SI
Phenols	>1.00 ± 0.02	0.08	
12.50			
Doxorubicin	>1.00 ± 0.01	0.03	
33.33			

Data are the means ± SD (standard deviation) n=8. Selectivity Index (SI) = IC_{50} Vero cell/ IC_{50} HEPG2 cell. SI value > 2 indicating high selectivity.

Table 3. Showing the R_f values of different peaks of phenols of *Hermannia geniculata* roots and the standard quercetin

Track	**Peak**	**R_f**	**Height**	**Area**	**Assigned substance**
Sample A	1	0.14	12.4	193.8	Phenolic 1
Sample A	2	0.81	93.5	2398.8	Phenolic/carboxylix acid
Sample A	3	0.95	207.0	9065.5	Unknown
STD	1	0.54	198.9	14512.0	Quercetin standard

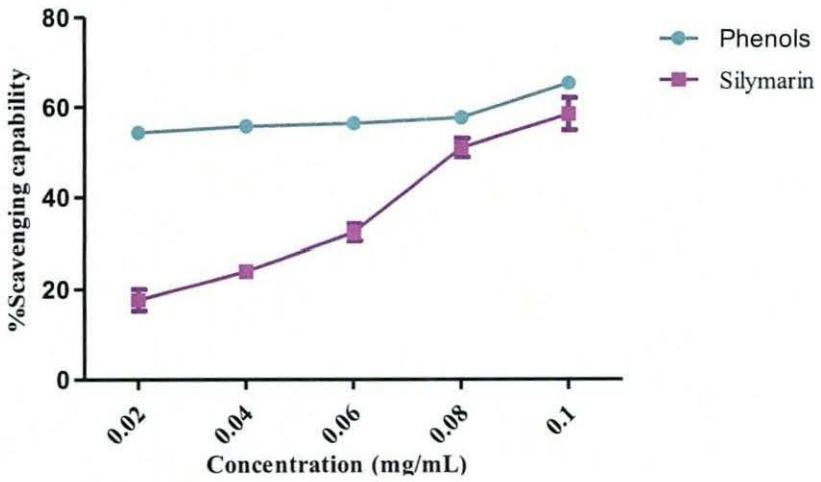


Figure 1. DPPH radical scavenging capability of *Hermannia geniculata* phenols extract. Values are mean± standard deviation (SD) of triplicate determinations.

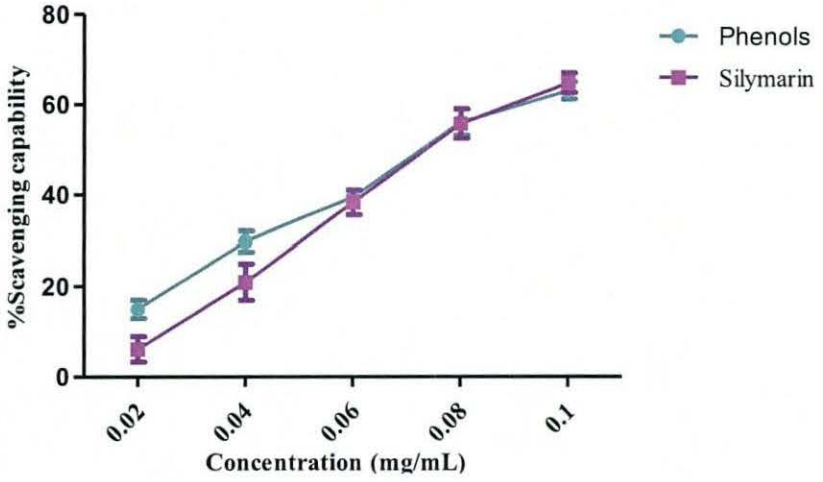


Figure 2. Showing the ABTS radical scavenging activities of the phenols of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations.

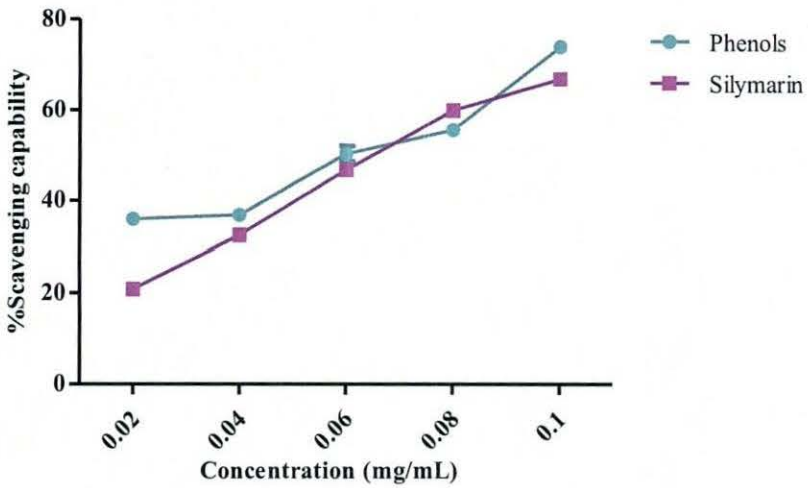


Figure 3. Showing the Metal chelating activities of the phenols of *Hermannia geniculata* root extract. Values are mean± standard deviation (SD) of triplicate determinations.

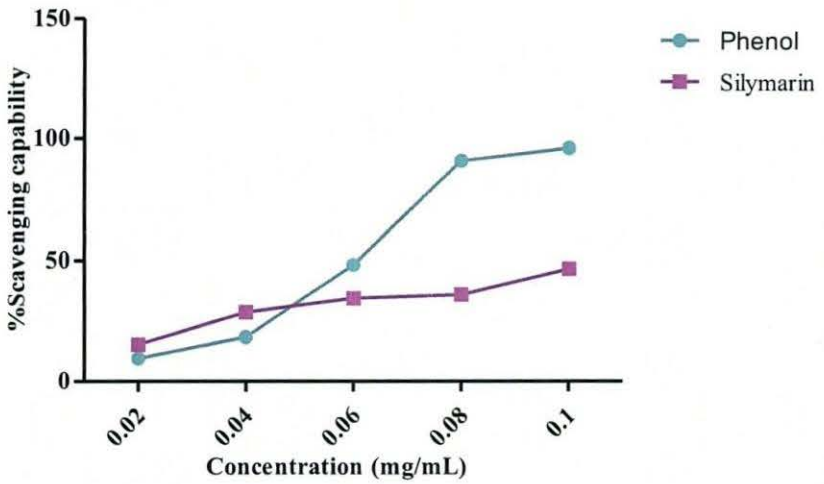


Figure 4. Hydroxyl radical scavenging capabilities of the phenols of *Hermannia geniculata* root extracts.

Values are mean± standard deviation (SD) of triplicate determinations

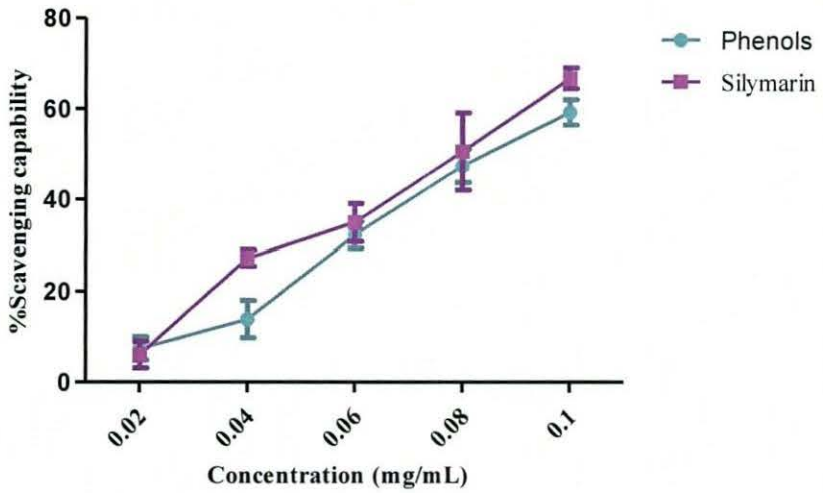


Figure 5. Superoxide anion radical scavenging capabilities of the phenols of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations

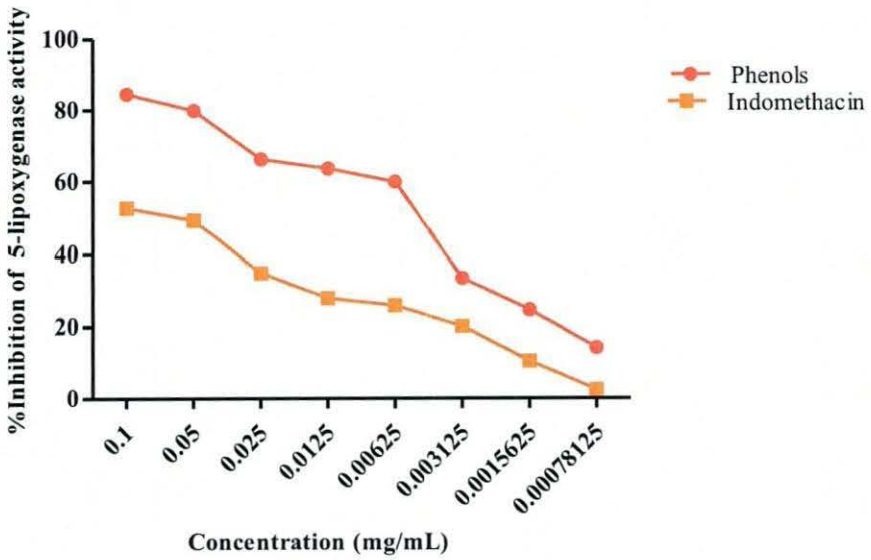


Figure 6. Anti-lipoxygenase activity of phenols of *Hermannia geniculata* root extract.

Results represent three triplicate value, mean± standard deviation (SD).

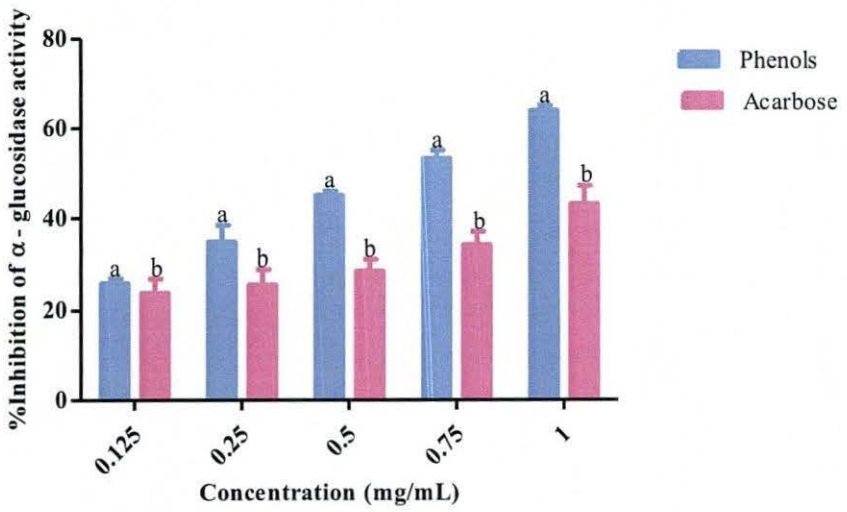


Figure 7. Inhibitory potential of *Hermannia geniculata* root phenols extract on specific α-glucosidase activity. Values are Mean ± standard deviation (SD) of triplicate determinations. [ab] represent values that are significantly different ($p<0.05$).

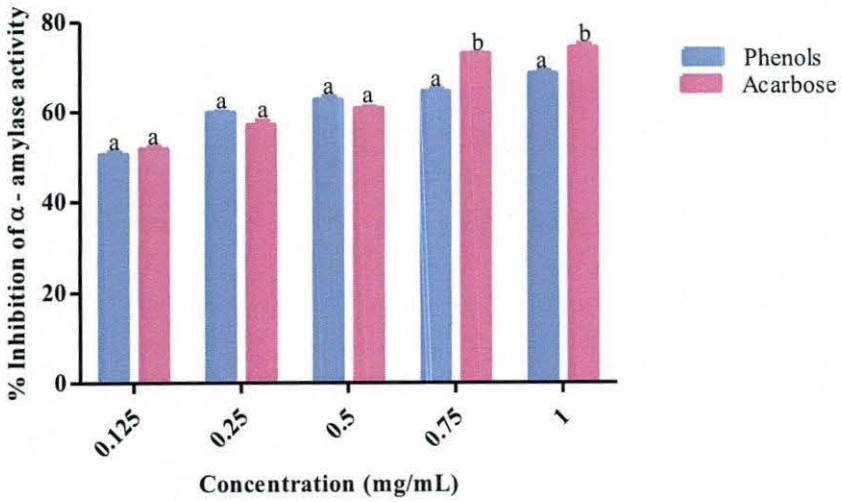


Figure 8. Inhibitory potential of *Hermannia geniculata* root phenols extract on specific α-amylase activity. Values are Mean ± standard deviation (SD) of triplicate determinations. [ab] represent values that are significantly different ($p<0.05$).

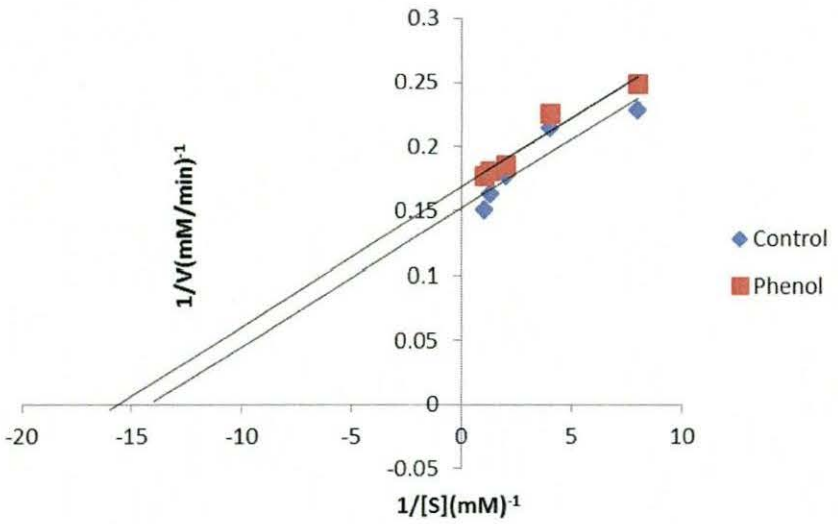


Figure 9. Lineweaver-Burk plot of total phenols extract eliciting uncompetitive inhibition on α-amylase activity. Results represent mean± standard deviation (SD); (n=3); ($p>0.05$).

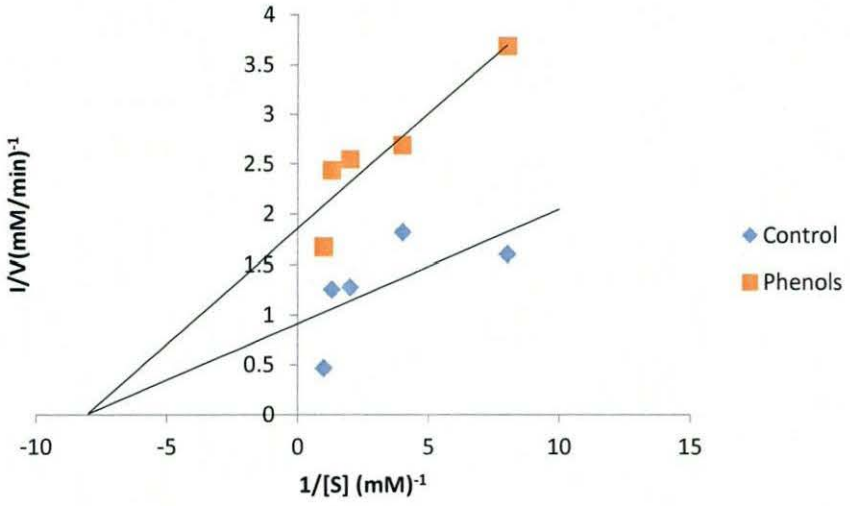


Figure 10. Lineweaver-Burk plot of total phenols extract eliciting non-competitive inhibition on α-glucosidase activity. Results represent mean± standard deviation (SD); (n=3); ($p>0.05$).

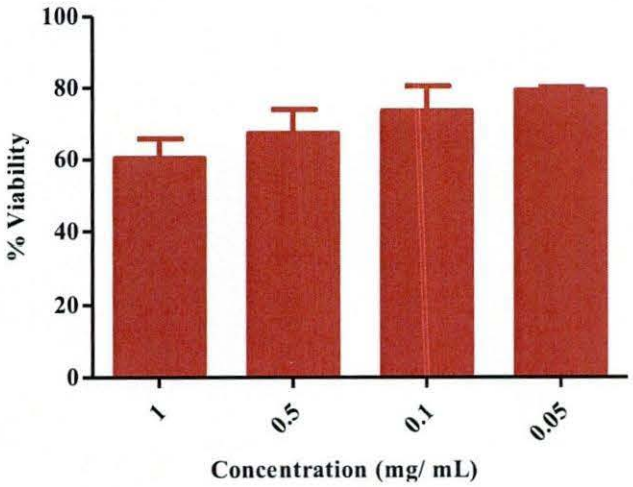


Figure 11. Percentage viability of Vero cells at different concentrations of the Phenols of *Hermannia geniculata* roots extract. Data represent the mean ± SE (standard deviation) of three independent experiments.

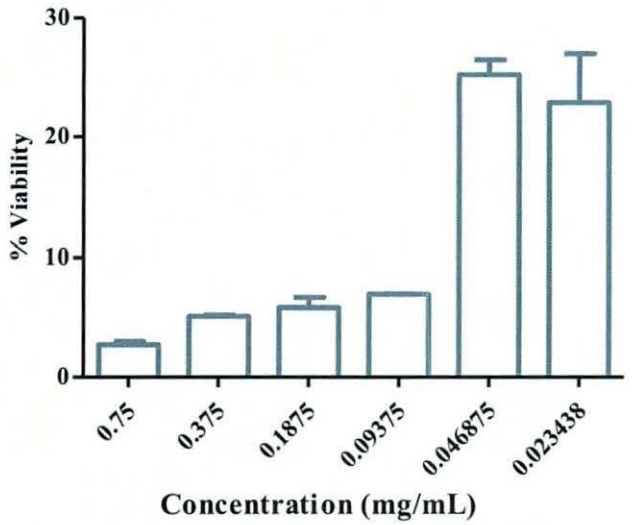


Figure 12. Percentage viability of HepG2 cell viability at different concentrations of the Phenols of *Hermannia geniculata* roots extract. Data represent the mean ± SD (standard deviation) of three independent experiments.

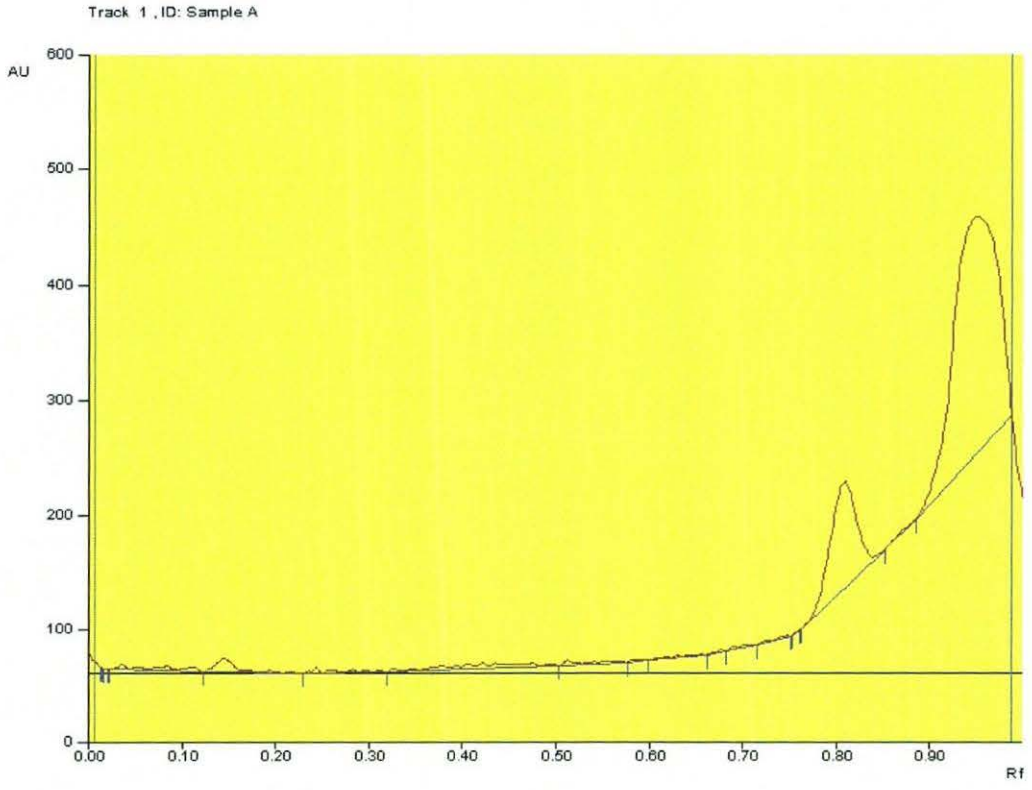


Figure 13. Track A – Baseline display (Scanned at 254nm) of the sample of Phenols of *Hermannia geniculata* roots extract

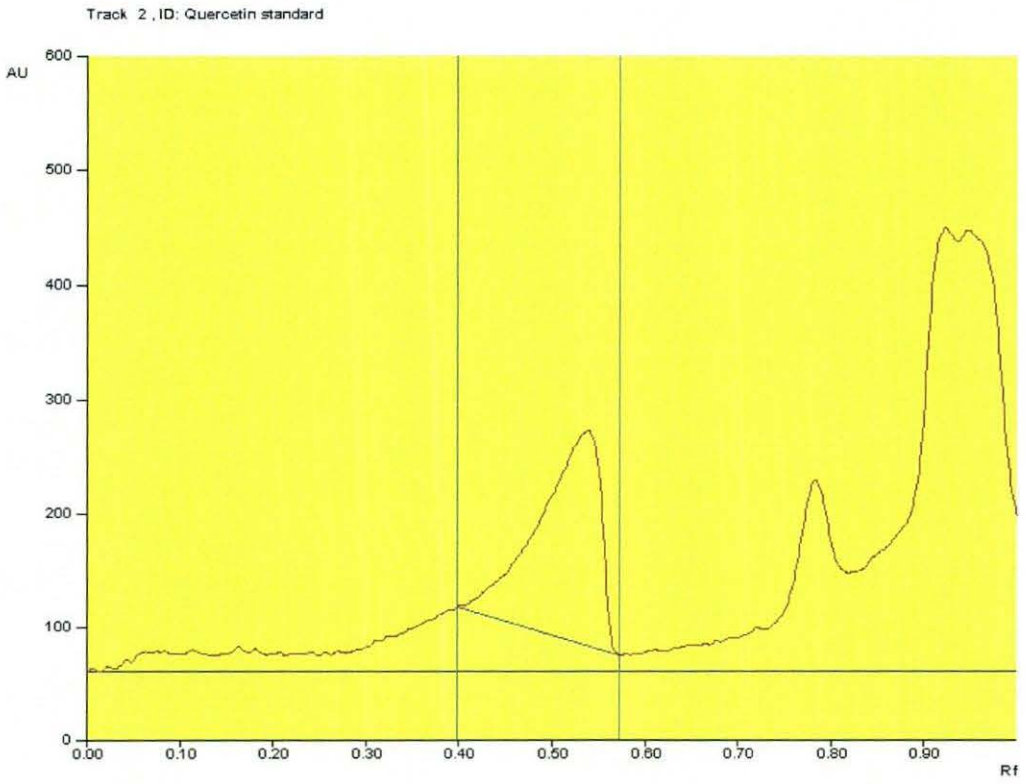


Figure 14. Track STD – Phenolic standard Baseline display (Scanned at 254nm) of quercetin.

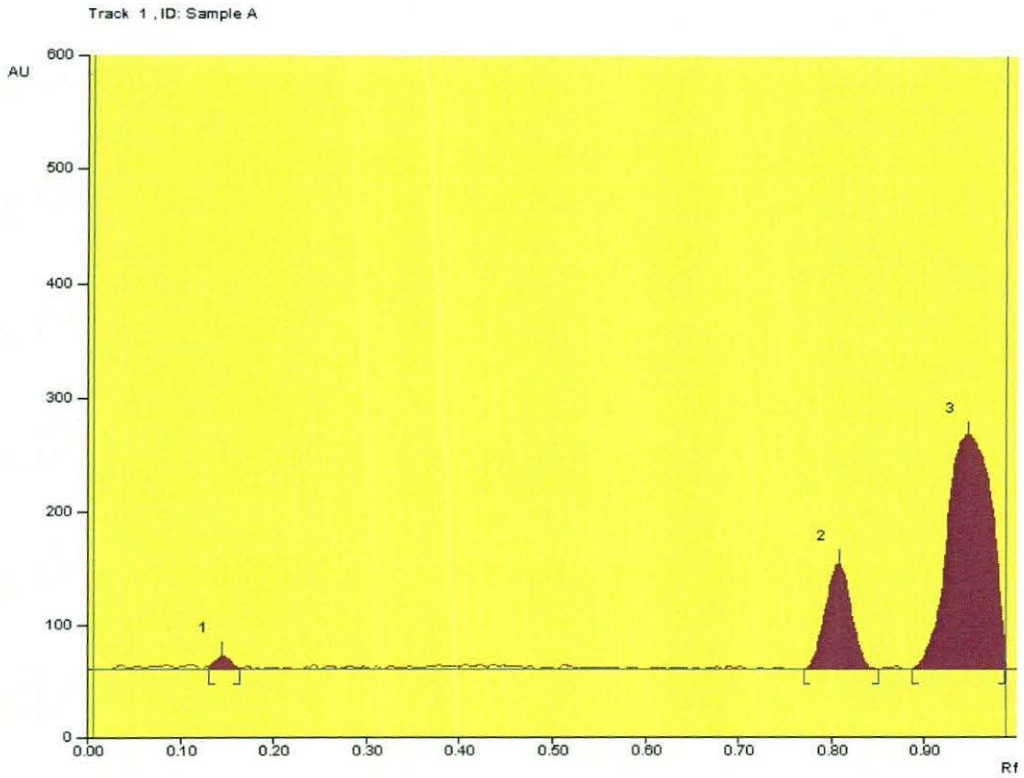


Figure 15. g Track A – Sample A peak densitogram display (Scanned at 254nm) of Phenols of *Hermannia geniculata* roots extract

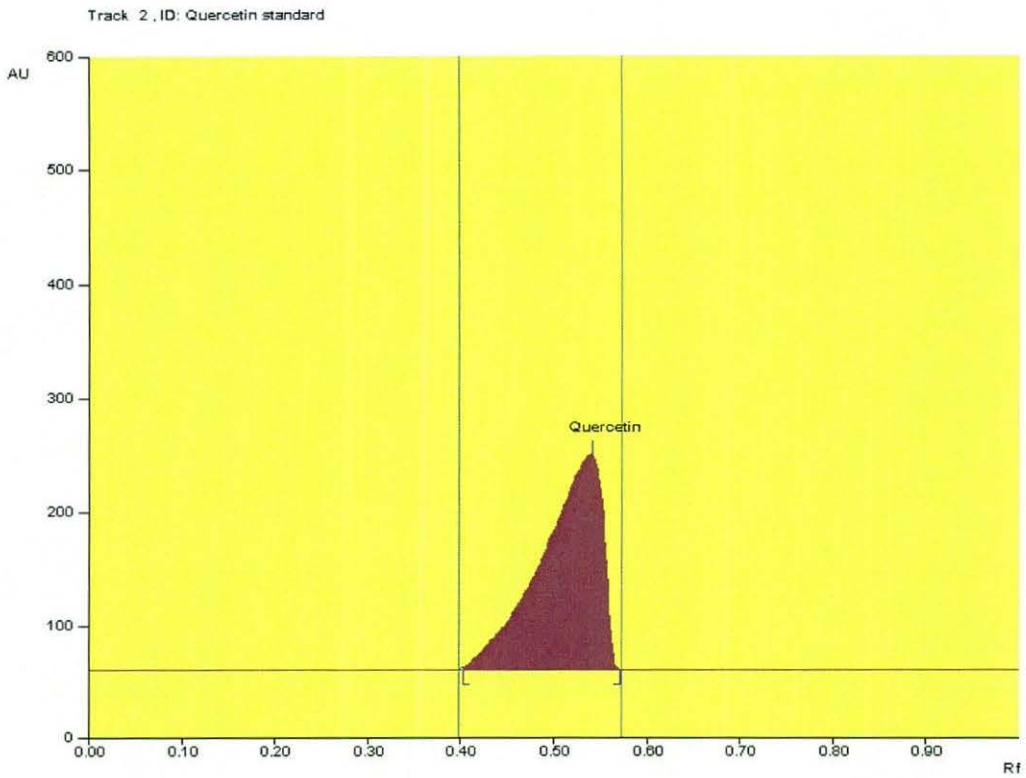


Figure 16. Track STD – Phenolic standard Peak densitogram display (Scanned at 254nm) of the standard quercetin.

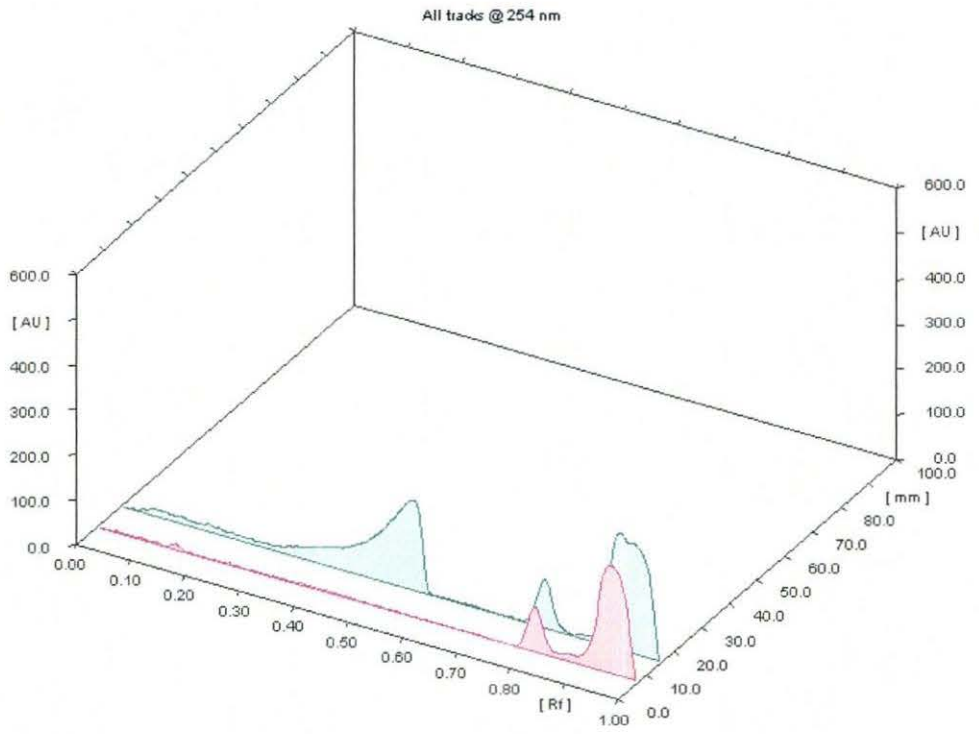


Figure 17. 3D display of all Tracks of Phenols of *Hermannia geniculata* roots extract and the standard quercetin

Chapter Seven

Pharmacological activities of Hermannol (9-(7-methyloctyl)-9H-xanthene-2,3-diol), a new Xanthene derivative isolated from the roots of *Hermannia geniculata* Eckl. & Zeyh.

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This chapter is under review in Natural Product Communication

Chapter Seven

NPC Natural Product Communications

Pharmacological activities of Hermannol (9-(7-methyloctyl)-9H-xanthene-2, 3-diol), a new Xanthene derivative isolated from the roots of Hermannia *geniculata* Eckl. & Zeyh.

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A new xanthene derivative Hermannol (9-(7-methyloctyl)-9H- xanthene-2, 3-diol) was isolated from the roots of *Hermannia geniculata*. The structure was elucidated by analysis of their 1D and 2D NMR, MS and IR spectroscopic data. The compound displayed good antioxidant and antidiabetic activities.

Keywords: Hermannia geniculata, Hermannol, antidiabetic antioxidant, structural elucidation.

Hermannia geniculata is a genus of flowering plant from family *Malnaceae* and subfamily *Byttnerioideae* (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008). It is a creeping shrub, the leaves are sub-orbicular crenate, the length of the leaf is about 15 mm, and the leaf texture may be viscid or sticky. *Hermannia geniculata* is well adapted to different ecological conditions and it can be seen occupying diverse habitat including the Drakensberg Mountains, the sea spray zone of the coastal regions of South Africa. The plant is endemic in Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa. It can also be found in Lesotho and Madagascar (Essop et al., 2008; Rebelo & Siegfried, 1990).

Hermannia geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases. (Balogun, Tshabalala, & Ashafa, 2016; Kazeem & Ashafa, 2015; Moffett, 1993). *H. geniculata* roots has been used in treatment of several diseases which include colic, diabetes mellitus and other oxidative stress induced illnesses. The dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders, also used in the management of diarrhoea, heartburn, stomach disorder and flatulency called "leletha" in pregnant Sotho women (Moffett, 1993).

Result and discussion

Roots of Hermannia geniculata (600 g) was extracted exhaustively using ethanol to obtain ethanol crude extract. Fractionation of the ethanol extract (15 g) employing application of silica gel column, and Preparative Thin Layer chromatography techniques yielded 52 g of a new compound of Xanthene derivative, Hermannol (9-(7-methyloctyl)-9H-xanthene-2,3-diol). Hermannol was obtained as a yellow

powder. HRESIMS showed molecular ion at m/z 339.1960 [calculated for $C_{22}H_{27}O_3$, 340.2038] suggesting a molecular formula of $C_{22}H_{28}O_3$. An inspection of 1H, 13C, COSY and HSQC NMR spectra suggested a xanthene skeleton

The IR broad absorption at 3400 cm-1, couple with the absorptions at 3000 cm-1 indicated the presence of O-H stretch signal for hydroxyl and C-H stretch signal for aliphatic hydrocarbon respectively. The C-O bonds of the ether bridge between the two benzene ring of the xanthene skeleton is reflected at the absorption band of 1100cm-1 while the absorption at 2923, 1465, and 757 cm-1 suggested the presence of benzene ring of the xanthene skeleton. An inspection of 1H, 13C, and HMQC NMR spectra suggested a xanthene skeleton, with 1H MNR spectrum displayed 6 aromatic methine protons at δH 6.92 (d, J= 12 Hz, H-3), 8.09 (d, J= 12 Hz, H-2), 7.98 (d, J= 6 Hz, H-5), 7.52 (d, J= 6 Hz, H-5), 7.43 (s) and 7.08 (s). The protons H-2 and H-3 exhibited ortho coupling to give a doublet signals each, this is confirmed by similarities in their coupling constants (J) (12 Hz). The aliphatic methine proton of the xanthene skeleton was revealed at position H-13 with chemical shift value (δ) of 4.05 ppm. The methine protons of 2-methylOctyl moiety attached to the xanthine skeleton [-CH2CH2CH2CH2CH2CH2CH(CH3)2] was observed at position H-20 (δ 0.87 m). The signal of six overlapped methylene protons H-14, H-15, H-16, H-17, H-18 and H-19 of -CH2CH2CH2CH2CH2CH2CH(CH3)2 moiety corresponds to the signal at δ 1.2ppm. The heteronuclear multiple bond connectivities (HMBC) between the six overlapped methylene protons further confirmed the assignment. 1H-NMR spectrum also revealed two methyl protons (H-21 and H-22) signal of -CH2CH2CH2CH2CH2CH2CH(CH3)2 moiety at 0.92 ppm. Two hydroxyl proton signal were observed at 1.6ppm

The 13C NMR spectrum exhibited 22 carbons signal corresponding to two methyl, six methylene, eight methines and six quaternary carbons. The quaternary carbon signals are seen at position C-1 (δ 151.1), C-6 (δ 134.4), C-7 (δ 145.1), C-8 (128.4) C-9 (δ 144.5) and C-10 (δ 140.9). Carbons at C-9 and C-10 are oxygenated carbons carrying the hydroxyl substituents on the xanthene skeleton while C-1 and C-7 are the oxygenated carbon of the C-O-C moiety of the xanthene skeleton. The presence of these oxygenated carbons is reflected in the downfield shift of their respective chemical shift values. Eight methine carbons were observed at positions C-2 (δ 118.0), C-3 (δ 126.0), C-4 (δ 122.4), C-5 (δ 128.0), C-8 (δ 107.1), C-11 (δ 115.0), C-13 (δ 39.02) and C-20 (δ 22.86). The signal at positions C-2, C-3, C-4, C-5, C-8 and C-11 corresponds to aromatic methane carbon of xanthene skeleton while C-13 and while the signal at C-13 and C-20 are aliphatic methine carbon of xanthene skeleton and –CH2CH2CH2CH2CH2CH2CH(CH3)2 moiety respectively. Six methylene carbon signal were shown at position C-14 (δ 32.09), C-15 (δ 26.91), C-16 (δ-30.33), C-17 (δ 30.33), C-18 (δ 29.52) and C-19 (δ 33.38). The six methylene carbon signals at due to the presence of –CH2-CH2CH2CH2CH2CH2CH(CH3)2 moiety. The HSQC spectrum permitted the one-bond proton-carbon correlations that facilitated the assignment of the methyl, methylene and methine signals. Assembly of the various moieties of the compound was principally achieved from the HMBC CH correlations (Tables 1). The compound was established as a xanthene derivatives based on the information deduced from the spectral data. Extensive literature search and comparison with known xanthene derivatives established elucidated compounds as new, therefore the compound 9-(7-methyloctyl)-9H- xanthene-2,3-diol, was trivially named as Hermannol. To the best of our knowledge, this compound is being reported for the first time. The NMR data and key HMBC correlations of the compound measured in CDCl3 are presented in Tables 1. The proposed structures of Hermannol and its HMBC correlations are displayed in Figures 1 and 2 respectively.

The result of the antioxidant activity Figure 3, 4 and Table 3 showed that the compound has good radical scavenging capabilities against DPPH radicals with IC50 value of 0.29 which is similar with the IC50 value of the standard. The metal chelating properties was also tested the compound demonstrated commendable metal chelating properties with IC50 value of 0.28 which is similar to the IC50 values of the reference compound (silymarin).

The inhibitory properties of Hermannol against α-amylase and α-glucosidase enzymes, Figure 5, and 6 and Table 2 showed a milder inhibition of α-amylase with IC50 value of 0.59 mg/mL which is higher and significantly different ($p<0.05$) from acarbose with IC50: 0.43 mg/mL. Further results of the assay revealed that it has better inhibition of α-amylase enzyme with IC50 value of 0.04 mg/mL which is lower and significantly different from acarbose with IC50 value of 0.16 mg/mL. This result showed that Hermannol, a new compound isolated from the root of H. geniculata may be used in the management of oxidative stress induced diseases and can substitute acarbose in the treatment of post pandrial hyperglycaemia.

In summary, Hermannol is a new 9-(7-methyloctyl)-9H-xanthene-2,3-diol isolated from the roots of Hermannia geniculata and showed good in vitro antioxidant and antidiabetic activities.

Table 1. $_1$H (600 MHz) and ^{13}C (150 MHz) NMR Data and key HMBC correlations of Hermannol in $CDCl_3$ (d=doublet; m=multiplet; om=overlap multiplet; s=singlet

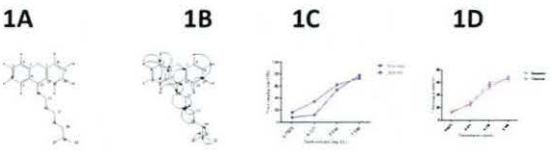
Positions	13C	Dept	δ^1H	J(Hz)	COSY	HMBC
1	155	C				H2
2	118	CH	6.92 d	12	H-3	H3
3	126	CH	8.09 d	12	H-2	
4	122.4	CH	7.98 d	6	H-5	
5	128	CH	7.52 d	6	H-6	H3
6	131	C				
7	145	C				
8	145	CH	7.43 s			H10
9	144	C				
10	140	C				
11	115	CH	7.08 s			H10
12	125					
13	39.02	CH	4.02 t			H7
14	32.09	CH_2	1.20 om			H15
15	26.99	CH_2	1.20 om			
16	30.33	CH_2	1.20 om			H15
17	30.33	CH_2	1.20 om			H15
18	29.02	CH_2	1.20 om			H15
19	33.38	CH_2	1.20 om			
20	22.85	CH	0.87 m			H19
21	14.28	CH_3	0.92 m			H20
22	14.28	CH_3	0.92 m			H20
		2OH	1.6 s			

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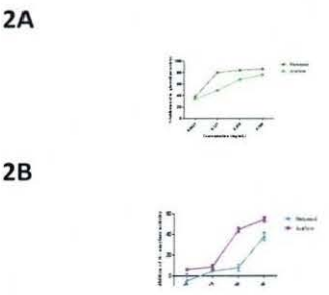
Table 2: The inhibitory activities of Hermannol on oxidants and key carbohydrate metabolizing enzymes.

IC_{50}(mg/mL)	Hermannol	Silymarin	Acarbose
DPPH	0.29± 0.00	**0.24± 0.00**	
Metal chelating	0.28± 0.00	**0.29± 0.00**	
α-amylase	0.59± 0.02		0.43± 0.01
α-glucosidase	0.04± 0.00		0.16± 0.00

Values represent mean and standard deviation of triplicate determination.



Figures 1 A: The structure of Hemannol, B: The HMBC correlation of Hemmanol, C: The DPPH radical scavenging capability of Hemannol, Values are means and standard deviation of triplicate determination. N=3; $p<0.05$. D: The metal chelating capability of Hemannol, Values are means and standard deviation of triplicate determination. N=3; $p<0.05$.



Figures 2: A: The inhinbitory effect of Hermannol on α-glucosidase enzyme, Values are means and standard deviation of triplicate determination. N=3; $p<0.05$. B: The inhibitory effect of Hermannol on

α-amylase, Values are means and standard deviation of triplicate determination. N=3; $p<0.05$.

Experimental

General experimental procedure. IR spectral was measured on Jasco Fourier Transform IR spectrometer FT-IR model 410) loaded with OMNIC software. NMR spectra were recorded on a Varian Inovan spectrometer. All chemical shifts were quoted on the δ scale in ppm using residual solvent as then internal standard ($CDCl_3$: 7.24 ppm for ^1H NMR, 77.0 ppm for ^{13}C NMR) coupling constant (J) are reported in Hz. HRESIMS were measured on a Shimadzu LCMS-IT-TOF mass spectrometer. The chemicals used for antioxidant and antidiabetic assays were purchased from (Sigma-Aldrich, South Africa). All chemicals were of analytical grade and distilled water was used for *in vitro* pharmacological activities.

Plant materials. Fresh *Hermannia geniculata* roots were purchased from local market in Puthaditjhaba, Qwaqwa, Northern Free State Province, South Africa. Confirmation of the species identity was carried out with the herbarium specimen with voucher specimen file number (5056.000-10700) (Moffett, 1993) at the University of Free State, Qwaqwa Campus, South Africa. It was also compared with our earlier Voucher specimen (Ash/med/05/2013/QwHB) (Kazeem & Ashafa, 2015) at the herbarium.

Extraction and Isolation.

The powdered roots of *H. geniculata* 600 g was extracted with 100% ethanol which yield 72 g of crude extract. 15 g of the crude ethanol extract was then fractionated on a silica gel column (5x60 cm) eluted with mixture of EtOAc and hexane (70: 30 to 100:0 at 50 mL each) to yield 62 subfractions. Fraction 27 was further purified using preparative TLC eluted with EtOAc/Hexane (90/10) to afford a pure compound Hermannol (52 mg).

Hermannol: A yellow powder (52 mg); Melting point: 671.55 (K); . IR (film):ν_{max} 3400, 3000, 2923, 1465, 1100 and 757 cm^{-1}. ^1H NMR ($CDCL_3$, 600Hz) and ^{13}C NMR ($CDCL_3$, 150Hz) see Table 1; (+)-HREIMS m/z 339.1960 $[M+H]^+$ [calculated for $C_{22}H_{28}O_3$, 340.2038].

Antioxidant Assays

1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging activity of the Hermannol was evaluated based on its scavenging activities on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical using a described method (Braca et al., 2001). Briefly, 150 µL of the varying concentration of Hermannol/ standard (0.02 – 0.1 mg/mL) was added separately to 150 µL of 0.004% methanolic solution of DPPH in a 96-well microtiter plate. The absorbance at 517 nm was determined after 30 min using a 96-well microplate reader (BIORAD, model 680, Japan), and the percentage inhibitory activity of the extract/standard was calculated using $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the Hermannol/standard. The half maximal inhibitory concentration (IC_{50}) value were calculated from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

Metal Chelating Assay

The chelating of ferrous ions by Hermannol was estimated as described by (Dinis, Madeira, & Almeida, 1994). Briefly, 40 µL of the different concentrations of Hermannol and standards (0.02 –0.1 mg/mL) was dispensed into a 96-well microtiter plate, 200 µL of 2 mM $FeCl_2$ solution was afterwards added to the mixture. The reaction was initiated by the addition of 80 µL 5 mmol/L ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 mins. The absorbance of the solution was then read at 562 nm using a BIO-RAD (model 680, Japan) microplate reader. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The IC_{50} value was calculated and obtained from the linear regression equation using $y = m x + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

***In vitro* Antidiabetic Assays**

α-Amylase Inhibitory Assay

This assay was carried out using the procedure of (Apostolidis, Kwon, & Shetty, 2007). 250 mL of varying concentration of Hermannol/ standard (0.125 – 1.0 mg/mL) was placed in a test tube and 250 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was incubated at 25°C for 10 min, followed by addition of 250 mL of starch (1%) solution in 0.02 M sodium phosphate buffer (pH 6.9) at timed intervals, the resulting reaction mixture was then incubated at 25°C for 10 min. The reaction was terminated by adding 500 mL of dinitrosalicylic acid (DNS) reagent before incubating the tubes in boiling water for 5 min and cooled to 25°C. 5 mL distilled water was added to the reacting mixture after cooling and the absorbance was measured at 540 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing Hermannol with distilled water. The α-amylase inhibitory activity was done in triplicate and was calculated as percentage inhibition, thus; % Inhibition = (Absorbance (control) – Absorbance (extract))/ Absorbance (control) ×100. Concentrations of extract resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

α-Glucosidase Inhibitory Assay

The effect of the FHG extract on α-glucosidase activity was determined according to the method described by (Apostolidis et al., 2007) with slight modification. In brief, different concentrations (0.125 – 1.0 mg/mL) of Hermannol/ standard were prepared in distilled water. Then, 50 mL from the stock solution was mixed with 100 mL of 0.1 M phosphate buffer (pH6.9) containing 1.0 M of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25°C for 10 min. Following this, 50 mL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. The inhibitory effect of extract/standard on the enzyme activities were determined by measuring the absorbance of the mixtures at 405 nm using a microplate reader (BIO-RAD, model 680, Japan). The control was prepared using the same procedure replacing the extract with distilled water. The experiments were conducted in triplicate and the α-glucosidase inhibitory activity was expressed as % inhibition using the expression: %Inhibition= [(Acontrol-Aextract)/Acontrol]×100, where Acontrol and Aextract are the absorbance's of the control and extract respectively. Concentrations of extract/standard resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the linear regression equation y = m x + c, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

Compound name

MP: 671.55 (K).

Rf: (EtOAc/Hexane 9:1).

IR (film):ν_{max} 3400, 3000, 2923, 1465, 1100 and 757 cm^{-1}.

^1H NMR (600 MHz, $CDCl_3$): δ_H 6.92 (d, J= 12 Hz, H-3), 8.09 (d, J= 12 Hz, H-2), 7.98 (d, J= 6 Hz, H-5), 7.52 (d, J= 6 Hz, H-5), 7.43 (s) and 7.08 (s) H-13: (δ) 4.05, H-14, H-15, H-16, H-17, H-18 and H-19: (δ) 1.2, H-20: δ (0.87), H-21, H-22: (δ) 0.92 and OH: (δ) 1.6.

^{13}C NMR (150 MHz $CDCL_3$): C-1 (δ 151.1), C-6 (δ 134.4), C-7 (δ 145.1), C-8 (128.4) C-9 (δ 144.5) C-10 (δ 140.9), C-2 (δ 118.0), C-3 (δ 126.0), C-4 (δ 122.4), C-5 (δ 128.0), C-8 (δ 107.1), C-11 (δ 115.0), C-13 (δ 39.02) and C-20 (δ 22.86).

HRESIMS showed molecular ion at m/z 339.1960 [calculated for $C_{22}H_{27}O_3$, 340.2038].

Anal. Calcd for $C_{32}H_{50}BrP$: C, 70.44; H, 9.24. Found C, 70.32; H = 9.43.

52 mg of Hermannol yield was gotten from fractionation of 15 g of ethanol root extract **of *H. geniculata.***

^1H NMR: (600 MHz, $CDCl_3$) δ_H 6.92 (d, J= 12 Hz, H-3), 8.09 (d, J= 12 Hz, H-2), 7.98 (d, J= 6 Hz, H-5), 7.52 (d, J= 6 Hz, H-5), 7.43 (s) and 7.08 (s) H-13: (δ) 4.05, H-14, H-15, H-16, H-17, H-18 and H-19: (δ) 1.2, H-20: δ (0.87), H-21, H-22: (δ) 0.92 and OH: (δ) 1.6.

^{13}C NMR: (150 MHz $CDCL_3$): C-1 (δ 151.1), C-6 (δ 134.4), C-7 (δ 145.1), C-8 (128.4) C-9 (δ 144.5) C-10 (δ 140.9), C-2 (δ 118.0), C-3 (δ 126.0), C-4 (δ 122.4), C-5 (δ 128.0), C-8 (δ 107.1), C-11 (δ 115.0), C-13 (δ 39.02) and C-20 (δ 22.86).

Supplementary data: The spectra are attached as a supplementary files.

Acknowledgments – The 1D and 2D NMR analyses was carried out in the department of chemistry, University of Stellenbosch.

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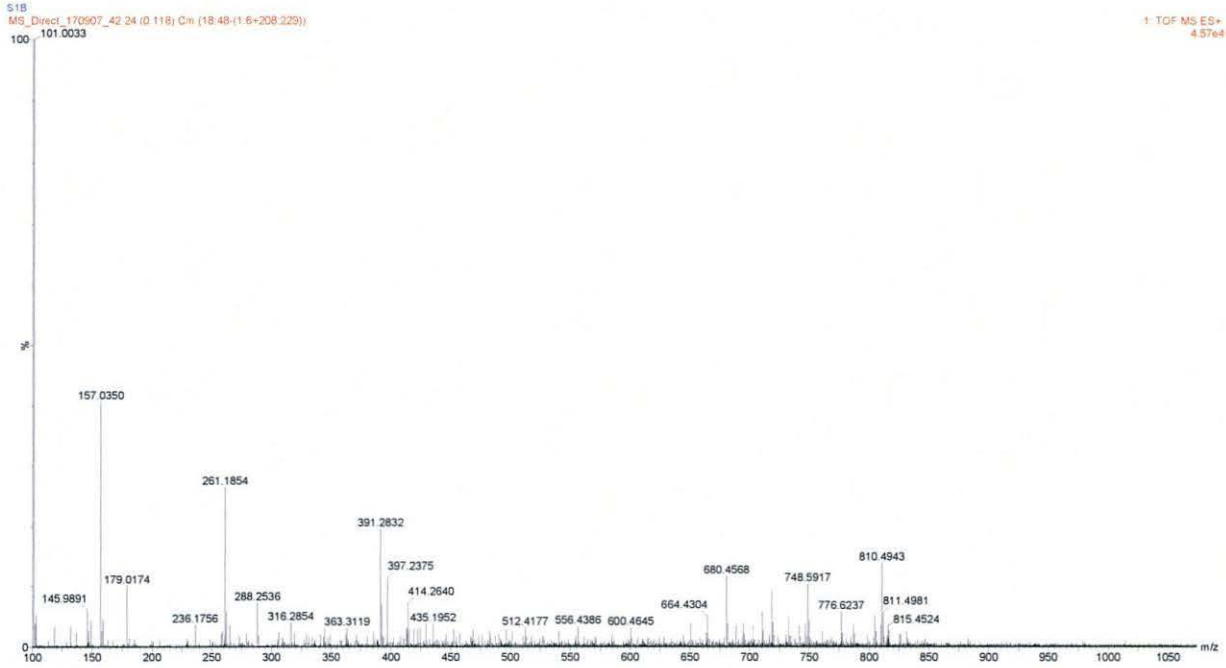
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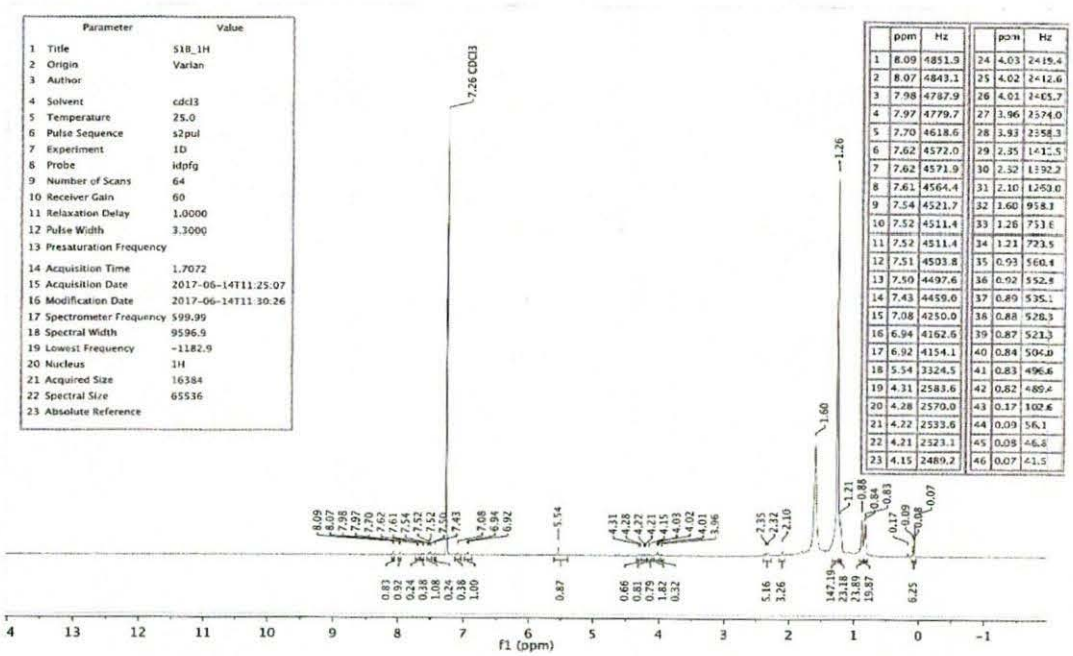
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Supplementary data.

Instrument: Waters Synapt G2, ESI probe injected into a stream of acetonitrile, ESI positive, Cone Voltage 15V

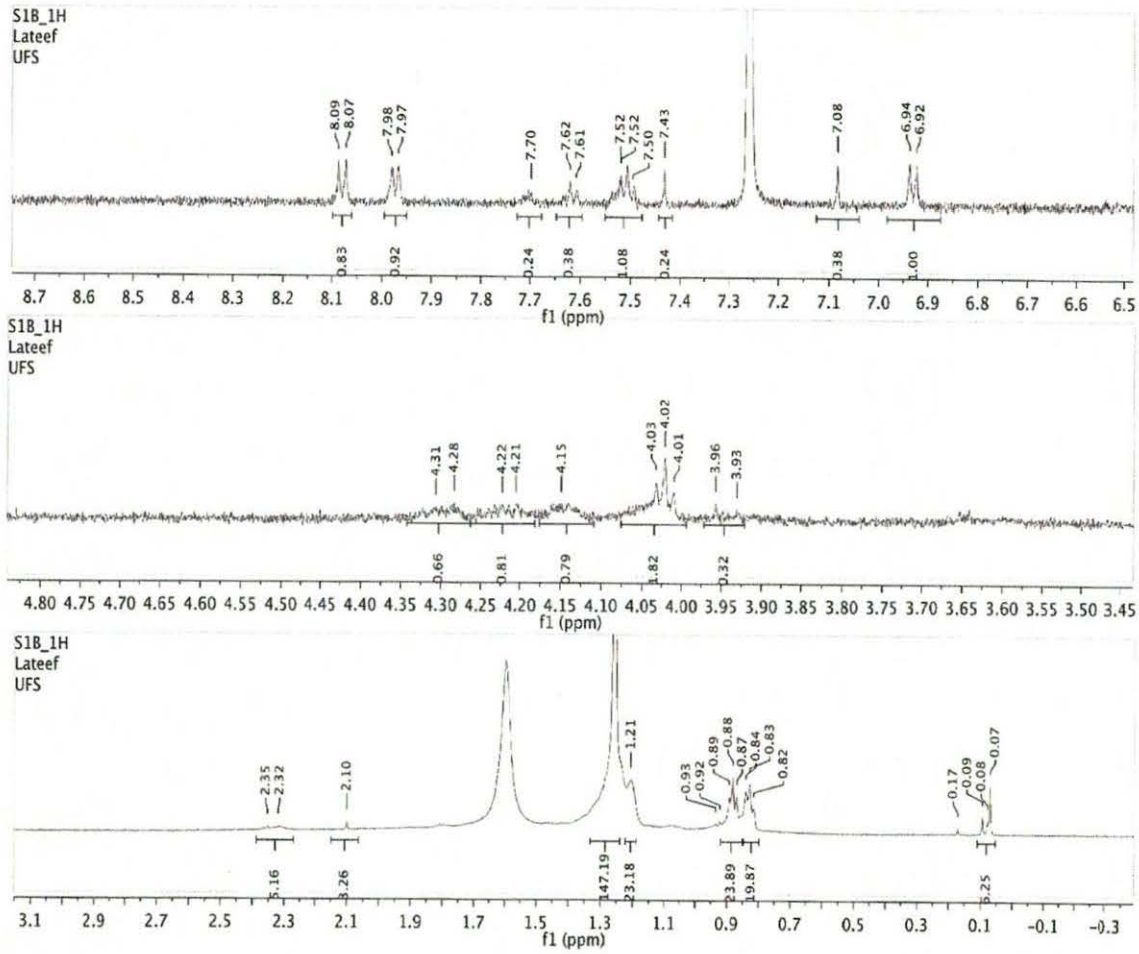


Appendix 1: Mass Spectra of Hermannol

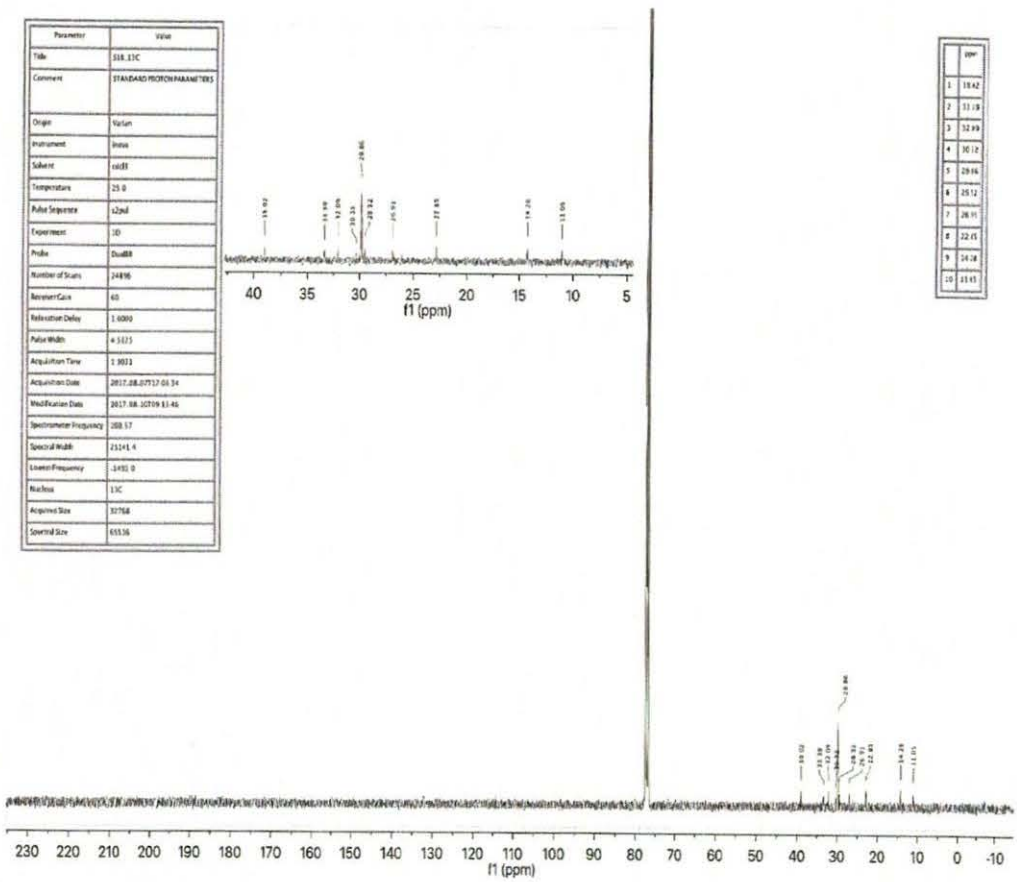


	ppm	Hz		ppm	Hz
1	8.09	4851.9	24	4.03	2419.4
2	8.07	4843.1	25	4.02	2412.6
3	7.98	4787.9	26	4.01	2405.7
4	7.97	4779.7	27	3.96	2374.0
5	7.70	4618.6	28	3.93	2358.3
6	7.62	4572.0	29	2.35	1411.5
7	7.62	4571.9	30	2.32	1392.3
8	7.61	4564.4	31	2.10	1260.0
9	7.54	4521.7	32	1.60	958.3
10	7.52	4511.4	33	1.26	753.6
11	7.52	4511.4	34	1.21	723.5
12	7.51	4503.8	35	0.93	560.4
13	7.50	4497.6	36	0.92	552.8
14	7.43	4459.0	37	0.89	535.1
15	7.08	4250.0	38	0.88	528.3
16	6.94	4162.6	39	0.87	521.3
17	6.92	4154.1	40	0.84	504.0
18	5.54	3324.5	41	0.83	496.6
19	4.31	2583.6	42	0.82	489.4
20	4.28	2570.0	43	0.17	102.6
21	4.22	2533.6	44	0.09	56.1
22	4.21	2523.1	45	0.08	46.8
23	4.15	2489.2	46	0.07	41.5

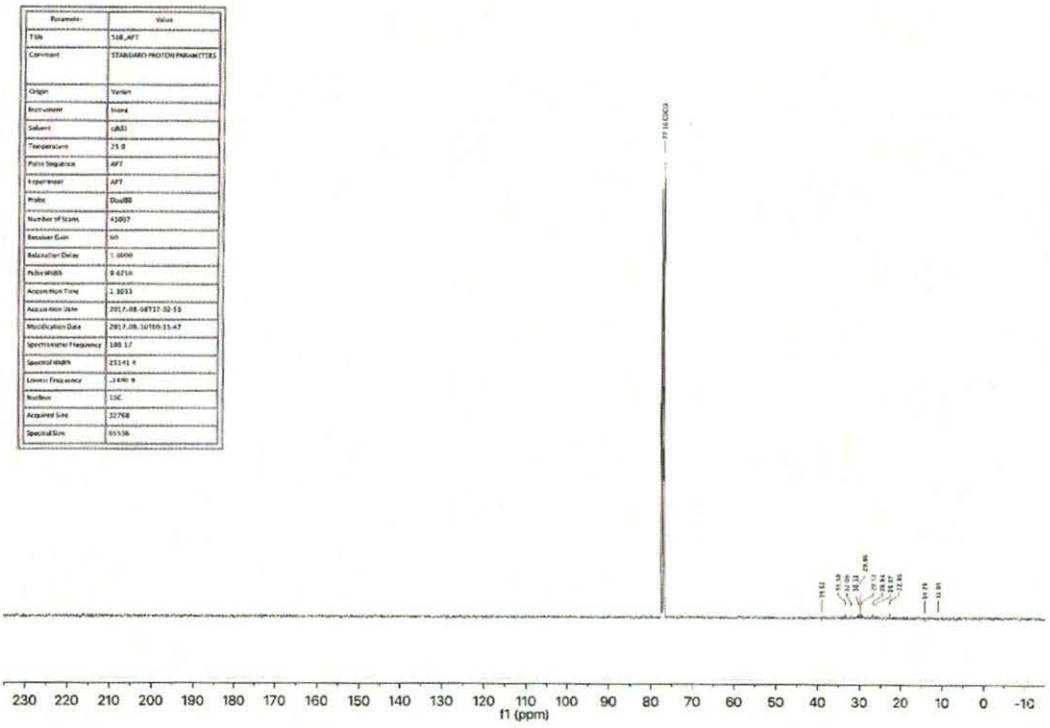
Appendix 2: ^{1}H NMR spectra of Hermannol



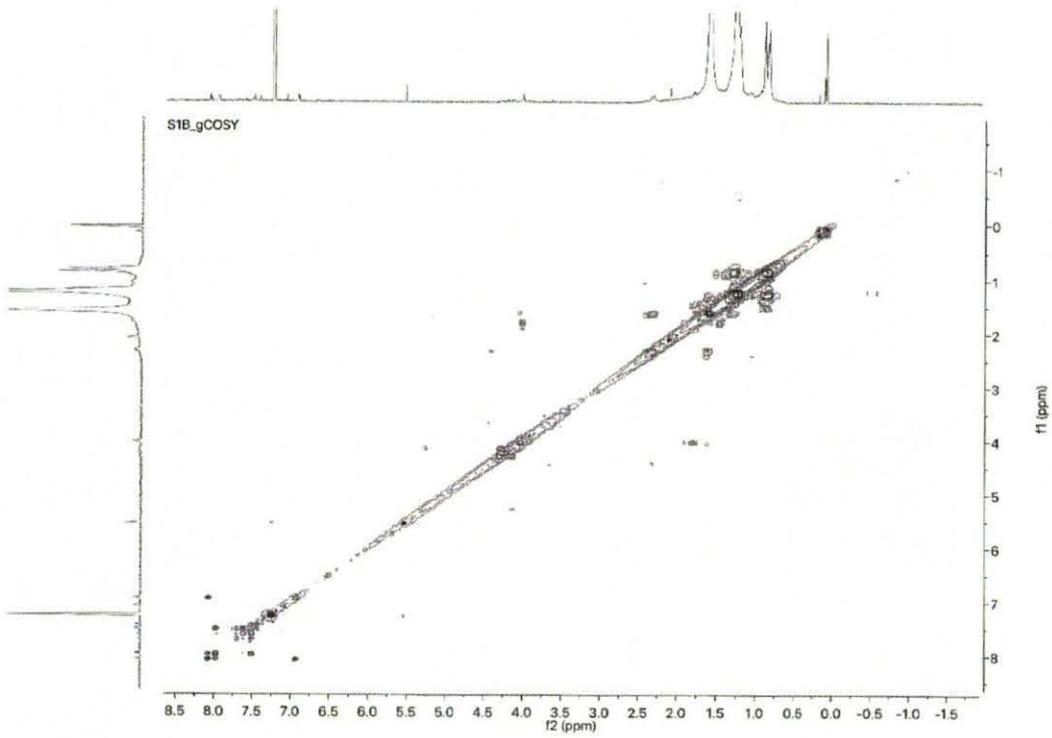
Appendix 3: Expansion and Integration of ^{1}H NMR spectra of Hermannol



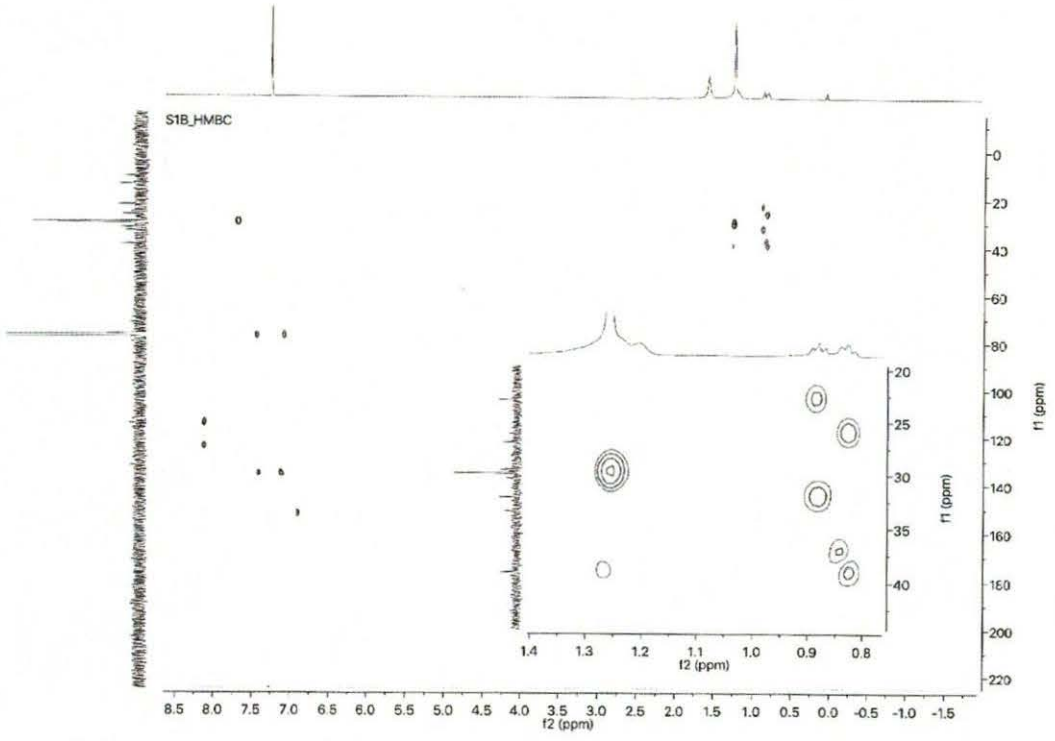
Appendix 4: ^{13}C NMR spectra of Hermannol



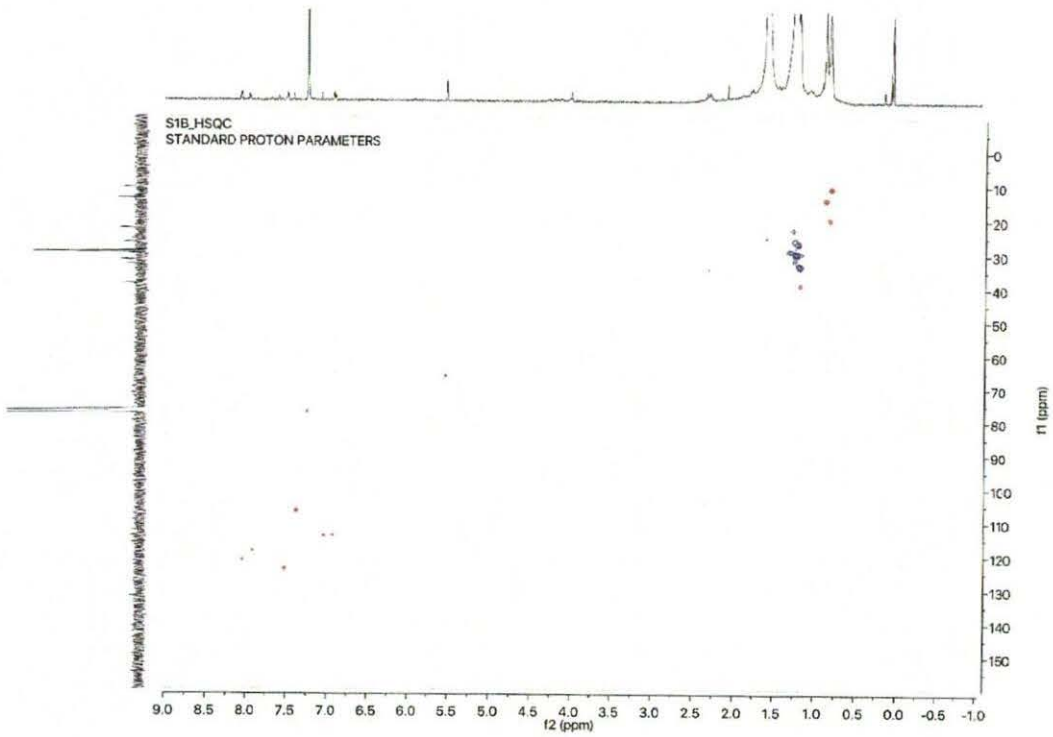
Appendix 5: Attack Proton Test (APT) NMR spectra of Hermannol



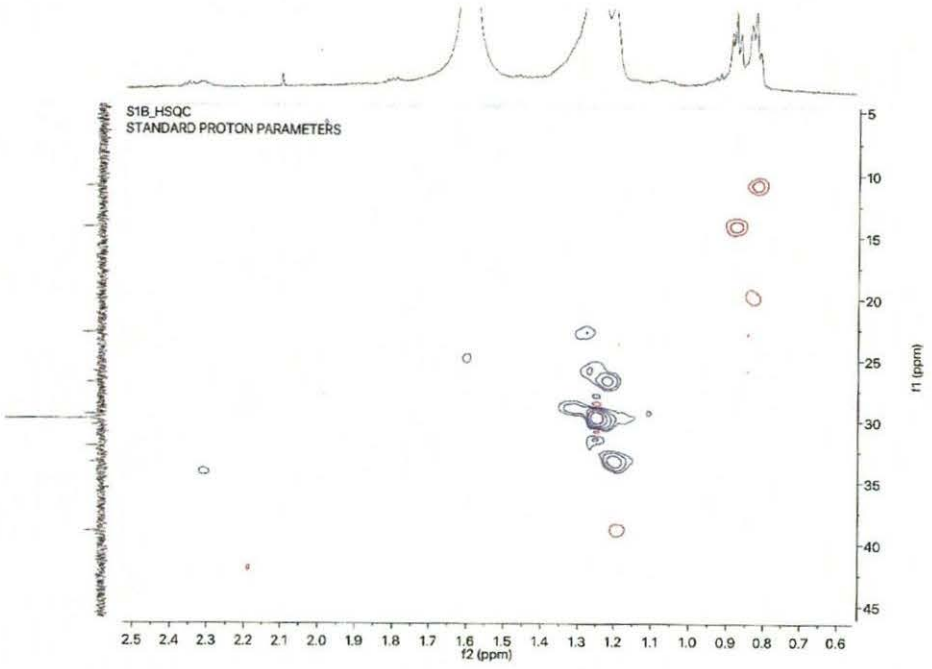
Appendix 6: 2Dimensional Correlation Spectroscopy (COSY) NMR spectra of Hermannol



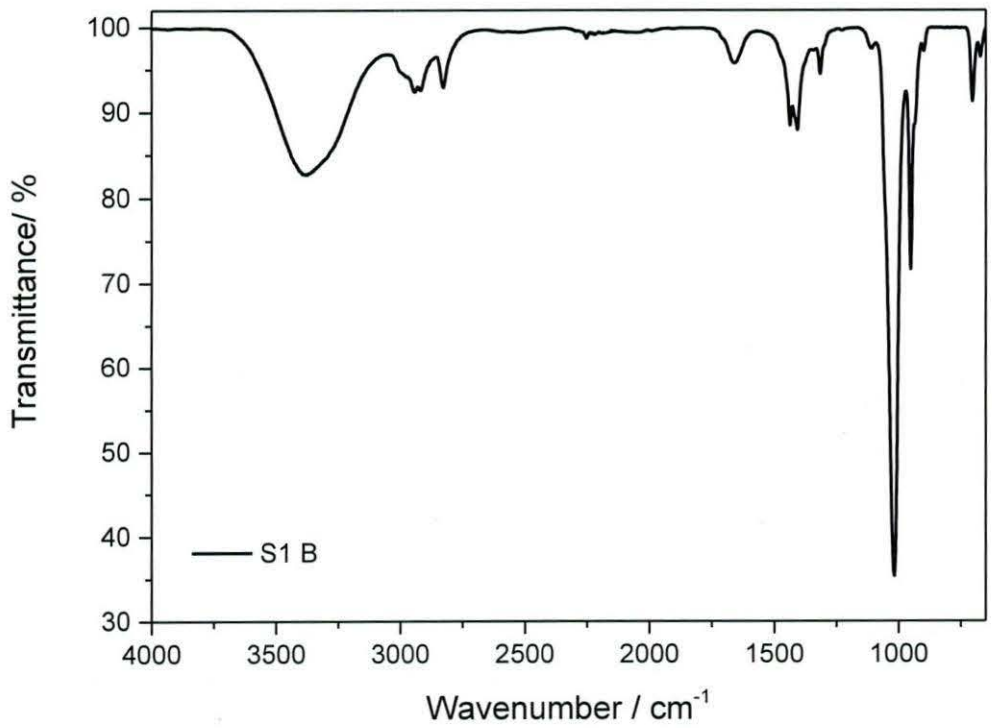
Appendix 7: Heteronuclear Multiple Bond Correlation (HMBC) NMR spectra of Hermannol



Appendix 8: Homonuclear Single Quantum Coherence (HSQC) 2D NMR spectra of Hermannol



Appendix 9: Expansion of Homonuclear Single Quantum Coherence (HSQC) 2D NMR spectra of Hermannol



Appendix 10: FTIR absorption spectrum of Hermannol

Chapter Eight

General discussion and recommendations

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General discussion

Medicinal plant is an important part of traditional health care system and a veritable health care source for the vast majority of the world population. It was estimated that 70-80% of people worldwide use herb for management of mild to moderate illnesses (Chaudhary, Gadhvi, & Chaudhary, 2010; Geun Kim & Sook Oh, 2012; Jokar, Noorhosseini, Allahyari, & Damalas, 2017; Jütte et al., 2017; Yea, Kim, Kim, & Yi, 2017).

The phenomenal rise of the alternative medicine industry responds to some of these shortcomings in what modern medicine has to offer. In several North American and European countries, the production and sale of herbal medicines, dietary supplements, and other so-called "natural" products have become a huge and profitable industry, amounting to $32 billion a year in the USA alone (Margaret, 2016). Therefore, South Africa with about 9% of the world vegetation (Street & Prinsloo, 2013) must tap into this emerging market through conscious and systematic investigation of medicinal plants in her domain. Thus, the need for scientific investigation of *Hermannia geniculata* which has been frequently used in the folkloric medicine for the cure of several diseases like diabetes, colitis, severe wound, gastrointestinal disorder and skin diseases (Essop, Zyl, Vuuren, Mulholland, & Viljoen, 2008; Kazeem & Ashafa, 2015; Moffett, 1993).

Oxidative stress caused by reactive oxygen species (ROS) which may be produced *in vitro* or *in vivo* has been implicated as an underlying factor in the pathogenesis of several diseases like cancer, diabetes, and Alzheimer's (Abdelrahman, Mahmoud, El-Sayed, Tanaka, & Tran, 2017; Bairi, Ouzir, Agnieszka, & Khalki, 2017; Deo et al., 2016; Mfotie Njoya, Munvera, Mkounga, Nkengfack, & McGaw, 2017; Vahid, Rakhshandeh, & Ghorbani, 2017). Medicinal plants has been reported to have good antioxidant potentials and thus used to ameliorate

oxidative stress induced diseases (Asgari Lajayer, Ghorbanpour, & Nikabadi, 2017; Jdey et al., 2017; Maleki, Ghorbanpour, & Kariman, 2017; Twilley, Langhansová, Palaniswamy, & Lall, 2017).

The results of the antioxidant and antidiabetic studies of the crude extracts of *H. geniculata* (chapter three) showed that ethanolic extract exhibited the highest antioxidant activities. The IC_{50} value 0.52 mg/mL for DPPH radical is lower and significantly different from the standard (silymarin) IC_{50}:1.04 mg/mL. The results indicated that ethanol (an organic polar solvent) can extract the biologically active component in the plant materials effectively because of its radical scavenging capabilities. Many authors have observed good antioxidant activities of plants extracts (Jdey et al., 2017; Simirgiotis, Quispe, Bórquez, Mocan, & Sepúlveda, 2016; Twilley et al., 2017). Phytochemistry of *H. geniculata* roots extracts revealed the presence of phenols, flavonoids, terpenes, anthraquinone, saponins, tannins and alkaloids. These phytochemicals have been demonstrated to have good antioxidant activities (Bairi et al., 2017; Huang, Cai, Zhang, Huang, & Cai, 2017).

Structural activities relationship studies revealed that the presence of polyhydroxyl in the 7, 5, 3, 3^1, 4^1 with the unsaturation in the C-ring and the presence of the –oxo- group in the 4 carbon of the C-ring of the flavonoids were responsible for the metal chelating and free radical scavenging activities of the flavonoids present in the plant extracts (Eric *et al.,* 1993).

All the extracts used in this study showed high activity in inhibiting key carbohydrate hydrolyzing enzymes of α- amylase and α-glucosidase. This result was in agreement with findings reported by several researchers who had recorded good activities against this enzymes (Duraiswamy, Shanmugasundaram, Sheela, Cherian, & Mammen, 2016; El, Hussein, Alm-eldeen, Hafez, & Mohamed, 2016; Sabiu, O'Neill, & Ashafa, 2016). Moreover,

we determined the kinetics of inhibition using ethanolic extract which has the best activity against the enzymes. The result showed that ethanolic extract inhibition of α- amylase is through competitive inhibition while it exhibited uncompetitive inhibition of α- glucosidase enzymes.

These results validates the folkloric use of *H. geniculata* for the treatment of hyperglycemia and oxidative stress induced illnesses.

The toxicity of the crude extract was carried out using normal kidney epithelial cells, RAW 264.7 macrophages and HepG2 cells, this was captured in chapter four.

The result revealed that crude extracts were not toxic to Vero cells with LC_{50} values ranges from (0.40-0.50) mg/mL and the viability of RAW 264.7 macrophages was >1.0 mg/mL for the ethanolic extract. This result confirmed that the extracts are non-toxic.

However, toxicity to cancer cells was observed with LC_{50} values of all the extracts ranging from (0.016-0.136) mg/mL. The selectivity index (SI) varied from (3.52- 33.33). High extract selectivity >2 showed that the plant has antiproliferative activities (R. Badisa et al., 2011) while LC_{50} value of 0.03 mg/mL is considered to have anticancer activity (R. B. Badisa, Badisa, Walker, & Latinwo, 2007).Therefore, decoction and the ethanolic extracts possess anticancer activities. In other to define the mode of action of the anticancer activity of the extract, we conducted an assay on 5-lipoxygenase (5-LOX) enzyme which catalyzes the production of 5-hydroxyecosatetraenenic acid (5-HETE) an essential factor in cancer growth and survival (Ford-Hutchinson, Gresser, & Young, 1994; Ghosh & Myer, 1998; Meng et al., 2013; Pergola & Werz, 2010; Schneider & Bucar, 2005). The result showed that all the extracts inhibited 5-LOX enzymes. Their percentage inhibition ranges between 65-83%. The result of inhibition of nitric oxide (NO) production by lipopolysaccharide activated RAW

264.7 macrophages by ethanolic extract indicated that the IC_{50} value of 3.64 mg/mL which is lower and significantly different ($p<0.05$) compared to the standard (quercetin) IC_{50}: 8.28 mg/mL. These observations further confirmed the antioxidant, anti-inflammatory and anticancer potentials of crude extracts of *H. geniculata* roots.

The biological activities of medicinal plants have been linked to the phytoconstituents of the plant. This study used the extracted flavonoids from the plant materials and evaluated the *in vitro* biological activities as reported in chapter five. The result of the chemical profile of the extracted flavonoids revealed the presence of flavonoids, phenolic/carboxylic acid and kaempferol. Commendable antioxidant activity of the extract was observed against DPPH and ABTS with their respective IC_{50} value of (3.07 and 2.13) mg/mL which is lower and significantly different from the standard (silymarin). The antioxidant activities of the flavonoid was observed with the inhibition of NO production by LPS stimulated RAW 264.7 macrophages cells. The IC_{50} value was 6.71 mg/mL which was lower and significantly different ($p<0.05$) to the standard IC_{50} value of 8.28 mg/mL. NO scavenging is important because it is a proinflammatory molecule, which can diffuse across the cell membranes. Low grade NO production is beneficiary for maintaining the normal body functions but unregulated production of NO reacts with superoxide anion generating peroxynitrite which causes oxidation of low density (lipoprotein LDL) and this is a key process that lead to cell apoptosis, subsequent inflammation and artheriosclerosis (Hendrix et al., 2017; Lai, Chu, Lakshminrusimha, & Lin, 2016; Mfotie, Munvera, Mkounga, Nkengfack, & McGaw, 2017). Therefore, NO inhibitors are useful agent in preventing cell death and inflammatory diseases which is a sequela to cell apoptosis (Hendrix et al., 2017; Irer et al., 2007).

The low redox potentials in the range of (2.13-1.0V) capable of reducing highly oxidized free radicals like superoxide, hydroxyl, peroxyl and alkoxyl radicals (Mishra, Kumar, & Pandey, 2013; Shashank & Abhay, 2013). The catechol groups in the A and B carbon rings couple with the unsaturation and presence of –oxo-group in the C rings have been further explained as the reason for their antioxidant activities (Eric *et al.*, 1993).

The activities of flavonoids on α-amylase and α-glucosidase enzymes showed that it had mild inhibitory effect on α-amylase enzyme. The IC_{50} value of 5.55 mg/mL which is higher and significantly different from acarbose with IC_{50}:3.81 mg/mL. Moreover, Flavonoids extract showed a stronger inhibition of α- glucosidase with the IC_{50}: 1.37 mg/mL which is lower and significantly different from IC_{50}: 2.91 mg/mL of acarbose. Stronger inhibition of α-glucosidase and milder inhibition of α-amylase had been reported to neutralize the observed side effect associated with the use of acarbose in the management of type 2 diabetes (Sabiu, O'Neill, Ashafa, & Ashafa, 2016; Vahid et al., 2017). The documented side effect include, diarrheoa, abdominal disturbances and flatulence which has been associated with strong inhibition of α-amylase enzymes by acarbose and voglibose (Deo et al., 2016; Kim et al., 2017; Olaokun, Mcgaw, Rensburg, Eloff, & Naidoo, 2016; Tafesse, Hymete, Mekonnen, & Tadesse, 2017). Therefore, the flavonoid extract of *H. geniculata* may likely be a better agent that can be exploited for the management of diabetes occasioned by post-prandial hyperglycaemia.

Furthermore, the study of the activities of the extract on 5-LOX enzyme indicated commendable inhibition of 5-LOX enzyme. The IC_{50} value for flavonoid and indomethacin was similar (10.15 and 12.03) mg/mL respectively. This result showed the importance of the anti-inflammatory potential of flavonoids from this study. Several authors have documented

the anti-inflammatory activities of flavonoids (Huang et al., 2017; Shakya, 2016; Verma & Hussain, 2017). Chronic low grade inflammation has been attributed to destruction and loss of β-cells (Gerber & Rutter, 2017), malfunctioning β-cells produces less insulin which causes hyperglycaemia, the raised glucose level causes advanced glycation end products and several other cascades that produces ROS which affects insulin receptor leading to insulin resistances and more destruction of β-cells, therefore setting a stage for type 2 diabetes mellitus (Ullah, 2016). Inhibition of 5-LOX enzyme can therefore help in protecting the integrity of β-cells which may be highly beneficial in the management of diabetes (Mohan & Nandhakumar, 2014).

The effect of flavonoids extract on Vero and RAW 264.7 macrophages cells indicated that the extract is non-toxic to both cells. The LC_{50} value for both cells is >1.0 mg/mL. Nevertheless, toxicity to HepG2 cells was observed with the LC_{50}: 0.02 mg/mL. The selectivity index is 50. The high SI value suggested that the extract may have anticancer activity (R. Badisa et al., 2011). Flavonoid has also been documented to possess anticancer activity (Bairi et al., 2017; Twilley et al., 2017). The outcome of these studies showed that flavonoid extracted from *H. geniculata* contained kaempferol, safe and possesses antioxidant, antidiabetic, anti-inflammatory and anticancer properties.

In our search for more probable bioactive constituent in *H. geniculata* root we extracted the Phenols and the activities of the extracted phenols was presented in chapter six. The phenols radical scavenging effect on DPPH, ABTS and superoxide anion radicals were similar with the standard (silymarin) but better metal chelating activities compared to the standard was observed. The inhibition of α-amylase and α-glucosidase enzymes was also similar with acarbose. This study also observed the inhibition of 5-LOX enzyme with IC_{50} value of (0.15)

mg/mL. Inhibition of 5-LOX is essential for the inhibition of leukotrienes which are downstream metabolic product o of 5-LOX enzymes (Ahmed et al., 2012; Antoniu, 2014; Martel-Pelletier, Lajeunesse, Reboul, & Pelletier, 2003; Pergola et al., 2014; Werz, Gerstmeier, & Garscha, 2017). Leukotrienes are pro-inflammatory biomolecules which has been implicated in asthma and bronchitis because of the high concentration of leukotrienes in the thoracic region (Antoniu, 2014; Gür, Çalişkan, & Banoglu, 2017; Putri, Elya, & Puspitasari, 2017; Rådmark, Werz, Steinhilber, & Samuelsson, 2015; Van Buul & Taube, 2016). Thus the phenols of *H. geniculata* may therefore be a good agent in treatment of asthma.

Selective cytotoxicity of the Vero and HepG2 cells was observed with their respective LC_{50} values were (>1.0 and 0.08) mg/mL. The selectivity index was 12.50 which signified the antiproliferative properties of the extract (R. Badisa et al., 2011).

From this result, phenols is safe and non-toxic and possess antioxidant, antiasthmatic and anticancer properties.

In line with all the results of the studies, we have discovered the effectiveness of the plant extracts and some of its phytochemicals against several diseases in *in vitro* experiments models using standard.

Finally, in search of cheap and effective drug candidates from the root of *H. geniculata*. We carried out isolation and structural elucidation of a pharmacologically active compound. Roots of *H. geniculata* (600 g) was extracted exhaustively using ethanol to obtain ethanol crude extract. Fractionation of the ethanol extract (15 g) employing application of silica gel column, and Preparative Thin Layer chromatography techniques yielded a new compound of Xanthene derivative, Hermannol (9-(7-methyloctyl)-9H-xanthene-2,3-diol). Hermannol was obtained as

a yellow powder. HRESIMS showed molecular ion at m/z 339.1960 [calculated for $C_{22}H_{27}O_3$, 340.2038] suggesting a molecular formula of $C_{22}H_{28}O_3$. An inspection of ^1H, ^{13}C, COSY and HSQC NMR spectra suggested a xanthene skeleton. The IR broad absorption at 3400 cm^{-1}, couple with the absorptions at 3000 cm^{-1} indicated the presence of O-H stretch signal for hydroxyl and C-H stretch signal for aliphatic hydrocarbon respectively. The C-O bonds of the ether bridge between the two benzene ring of the xanthene skeleton is reflected at the absorption band of 1100cm^{-1} while the absorption at 2923, 1465, and 757 cm^{-1} suggested the presence of benzene ring of the xanthene skeleton. An inspection of ^1H, ^{13}C, and HMQC NMR spectra suggested a xanthene skeleton, with ^1H MNR spectrum displayed 6 aromatic methine protons at δ_H 6.92 (d, J= 12 Hz, H-3), 8.09 (d, J= 12 Hz, H-2), 7.98 (d, J= 6 Hz, H-5), 7.52 (d, J= 6 Hz, H-5), 7.43 (s) and 7.08 (s). The protons H-2 and H-3 exhibited ortho coupling to give a doublet signals each, this is confirmed by similarities in their coupling constants (J) (12 Hz). The aliphatic methine proton of the xanthene skeleton was revealed at position H-13 with chemical shift value (δ) of 4.05 ppm. The methine protons of 2-methylOctyl moiety attached to the xanthine skeleton [$-CH_2CH_2CH_2CH_2CH_2CH_2CH(CH_3)_2$] was observed at position H-20 (δ 0.87 m). The signal of six overlapped methylene protons H-14, H-15, H-16, H-17, H-18 and H-19 of $-CH_2CH_2CH_2CH_2CH_2CH_2CH(CH_3)_2$ moiety corresponds to the signal at δ 1.2ppm. The heteronuclear multiple bond connectivities (HMBC) between the six overlapped methylene protons further confirmed the assignment. ^1H-NMR spectrum also revealed two methyl protons (H-21 and H-22) signal of $-CH_2CH_2CH_2CH_2CH_2CH_2CH(CH_3)_2$ moiety at 0.92 ppm. Two hydroxyl proton signal were observed at 1.6ppm

The ^{13}C NMR spectrum exhibited 22 carbons signal corresponding to two methyl, six methylene, eight methines and six quaternary carbons. The quaternary carbon signals are seen

at position C-1 (δ 151.1), C-6 (δ 134.4), C-7 (δ 145.1), C-8 (128.4) C-9 (δ 144.5) and C-10 (δ 140.9). Carbons at C-9 and C-10 are oxygenated carbons carrying the hydroxyl substituents on the xanthene skeleton while C-1 and C-7 are the oxygenated carbon of the C-O-C moiety of the xanthene skeleton. The presence of these oxygenated carbons is reflected in the downfield shift of their respective chemical shift values. Eight methine carbons were observed at positions C-2 (δ 118.0), C-3 (δ 126.0), C-4 (δ 122.4), C-5 (δ 128.0), C-8 (δ 107.1), C-11 (δ 115.0), C-13 (δ 39.02) and C-20 (δ 22.86). The signal at positions C-2, C-3, C-4, C-5, C-8 and C-11 corresponds to aromatic methane carbon of xanthene skeleton while C-13 and while the signal at C-13 and C-20 are aliphatic methine carbon of xanthene skeleton and $-CH_2CH_2CH_2CH_2CH_2CH_2CH(CH_3)_2$ moiety respectively. Six methylene carbon signal were shown at position C-14 (δ 32.09), C-15 (δ 26.91), C-16 (δ-30.33), C-17 (δ 30.33), C-18 (δ 29.52) and C-19 (δ 33.38). The six methylene carbon signals at due to the presence of $-CH_2-CH_2CH_2CH_2CH_2CH_2CH(CH_3)_2$ moiety. The HSQC spectrum permitted the one-bond proton-carbon correlations that facilitated the assignment of the methyl, methylene and methine signals. Assembly of the various moieties of the compound was principally achieved from the HMBC CH correlations (Tables 1). The compound was established as a xanthene derivatives based on the information deduced from the spectral data. Extensive literature search and comparison with known xanthene derivatives established elucidated compounds as new, therefore the compound 9-(7-methyloctyl)-9H- xanthene-2,3-diol, was trivially named as Hermannol. To the best of our knowledge, this compound is being reported for the first time. The NMR data and key HMBC correlations of the compound measured in $CDCl_3$ are presented in Tables 1. The proposed structures of Hermannol and its HMBC correlations are displayed in Figures 1 and 2 respectively.

The result of the antioxidant activity Figure 3, 4 and Table 3 showed that the compound has good radical scavenging capabilities against DPPH radicals with IC_{50} value of 0.29 which is similar with the IC_{50} value of the standard. The metal chelating properties was also tested the compound demonstrated commendable metal chelating properties with IC_{50} value of 0.28 which is similar to the IC_{50} values of the reference compound (silymarin).

The inhibitory properties of Hermannol against α-amylase and α-glucosidase enzymes, Figure 5, and 6 and Table 2 showed a milder inhibition of α-amylase with IC_{50} value of 0.59 mg/mL which is higher and significantly different ($p<0.05$) from acarbose with IC_{50}: 0.43 mg/mL. Further results of the assay revealed that it has better inhibition of α-glucosidase enzyme with IC_{50} value of 0.04 mg/mL which is lower and significantly different from acarbose with IC_{50} value of 0.16 mg/mL. This result showed that Hermannol, a new compound isolated from the root of *H. geniculata* may be used in the management of oxidative stress induced diseases and may be a good drug candidate in the management of post prandial hyperglycaemia.

The GCMS result of the ethanolic extract of *H. geniculata* shown in chapter four, indicated the presence of five compounds. 2-keto-butyric-acid which has been reported to be able to stimulate the biosynthesis of fibronectin by fibroblast, endothelial cells, astrological cells and hepatocyte as a sequela to inflammation and tissue repair (Tanaka & Nishida, 1985). Also, 2, 2-Bis (4-nitrobenzyl)-1-phenylbutane-1,3-dione and n-Undecane are excellent reducing equivalents and they possess antioxidant properties (Azzena, Demartis, & Melloni, 1996; Mzé-Ahmed, Hadj-Ali, Dagaut, & Dayma, 2012). The antifungal activities of 1,4,5,8-tetrathiadelin has been documented (Adeogun, Adekunle, & Ashafa, 2016). Imidazo-1,5-pyrimidine pharmacological importance has also has been reported to have antiulcer, antihypertensive and antitrypanosomal activities (Novinson et al., 1976; Patil, Ganguly, &

Surana, 2008; Rovnyak et al., 1992). Furthermore, it has been reported to have activity against NF-Kb and AP-1 protein expression ((Rovnyak et al., 1992)

In addition, the use of high performance thin layer chromatography (HPTLC) finger printing provided improved resolution, higher detection sensitivity, and improved *in situ* quantification of phytochemicals (Reich & Schibli, 2007) to determine the chemical profile of the flavonoid and phenol extracts. HPTLC was used to identify marker compounds present in the extracts. The result of the HPTLC profiling of the extract is shown in chapter five. The presence of flavonoids in the extract was confirmed with R_f values (0.08-0.95). The specificity of the methods was ascertained by analyzing the standard and the sample with similar chromatographic conditions. Chromatograms revealed similarities for both the standard and the test samples. Moreover, the detection of kaempferol in the extract was confirmed with R_f 0.81 which is similar to the standard kaempferol with R_f 0.80. Also, similar densitogram, baseline and 3D display of the Tracts confirmed the detection of kaempferol in the FHG extract. Several glycosides of kaempferol derivatives have been identified in medicinal plants (Teffo, Aderogba, & Eloff, 2010). It was noteworthy that kaempferol was detected in *H. geniculata* roots extracts in this study for the first time in South Africa.

The result of HPTLC profiling of Phenol presented in chapter six confirmed the presence of phenols in the PoHG extract with R_f values (0.14-0.95). The specificity of the method was ascertained by analyzing the standard and the test samples within similar chromatographic conditions. The chromatograms obtained showed similarities for both the standard and the test samples.

Conclusion

The plant extracts proved to be effective but these tests are only sufficient for drug discovery which is the main focus of this study. For drug development, there is need to completely conclude on the efficacy of the plant against the diseases we experimented in this study and also conduct clinical trials using *in vivo* studies.

Therefore, the following submission could be made:

- The four crude extracts (ethanol, hydro-ethanol, decoction and aqueous) had good antioxidant activities
- Ethanol extract was the most active in free radicals scavenging
- All the extracts had good activities against α- amylase and α-glucosidase enzymes
- All the extracts were non-toxic and safe
- All extracts possessed anticancer properties
- Ethanolic extract, flavonoids and phenols have good inhibitory activity on NO production by LPS stimulated RAW 264.7 macrophages.
- Flavonoids possess good antioxidant, antidiabetic, anti-inflammatory and showed selective toxicity to normal and cancer cells
- Phenols possessed antioxidant and good activity against pancreatic α-amylase, intestinal α-glucosidase and 5-LOX enzymes
- Phenol showed selective toxicity to Vero, RAW 264.7 macrophages and HepG2 cells
- The activities shown by the crude extracts validates its use for diabetes and oxidative stress induced diseases.
- Hermannol a new xanthene derivative that has good antioxidant and antidiabetic activity was isolated and characterized.

- Hermannol may be a good antidiabetic drug candidate

Recommendation

The plant is not among the threatened species despite its medicinal usefulness. However, caution should be taken about overutilization and exploitation of the plant species following the information provided by this study.

Further studies should be conducted on the isolated compound both *in vitro* and *in vivo* like genotoxicities and reproductive toxicities.

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Thank you.

Adeniran Lateef Ariyo

September, 2017

Dedication

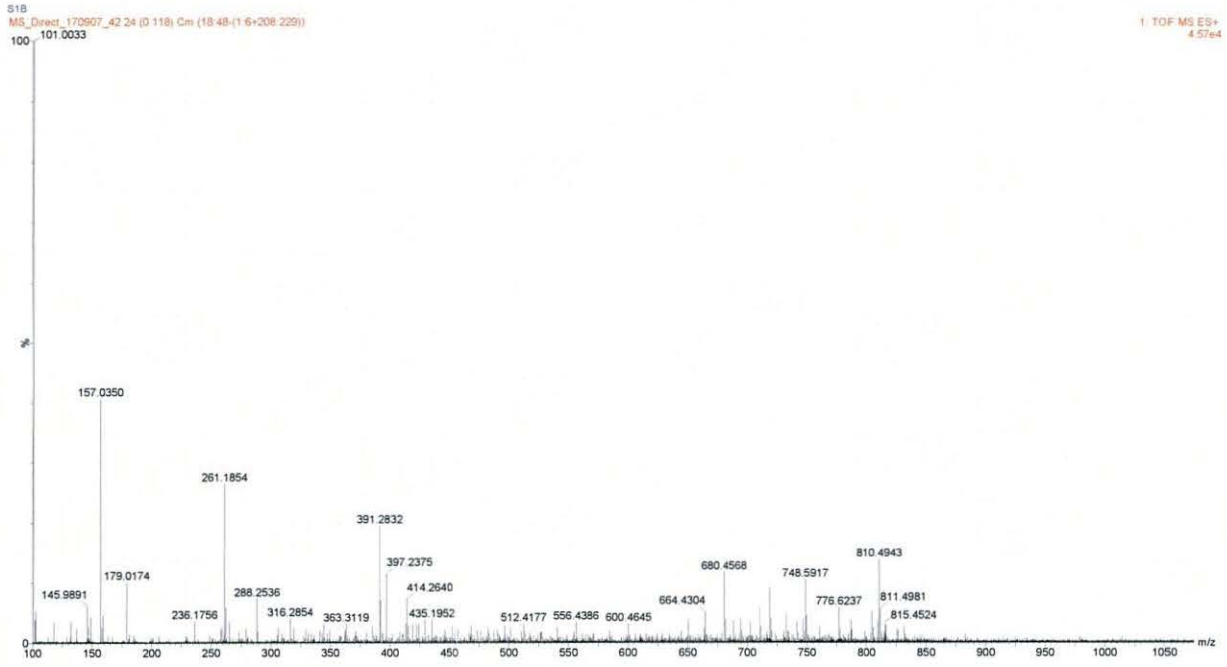
This research is dedicated

To

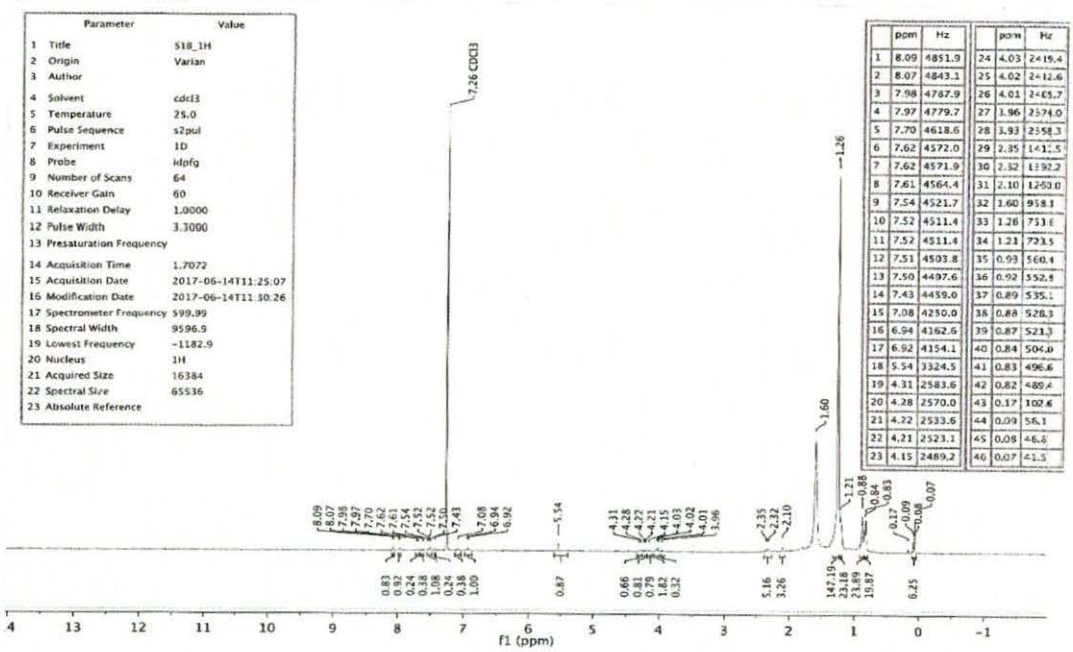
All those who believe in me that I can make something out of going to school

Appendices

Instrument: Waters Synapt G2, ESI probe injected into a stream of acetonitrile, ESI positive, Cone Voltage 15V

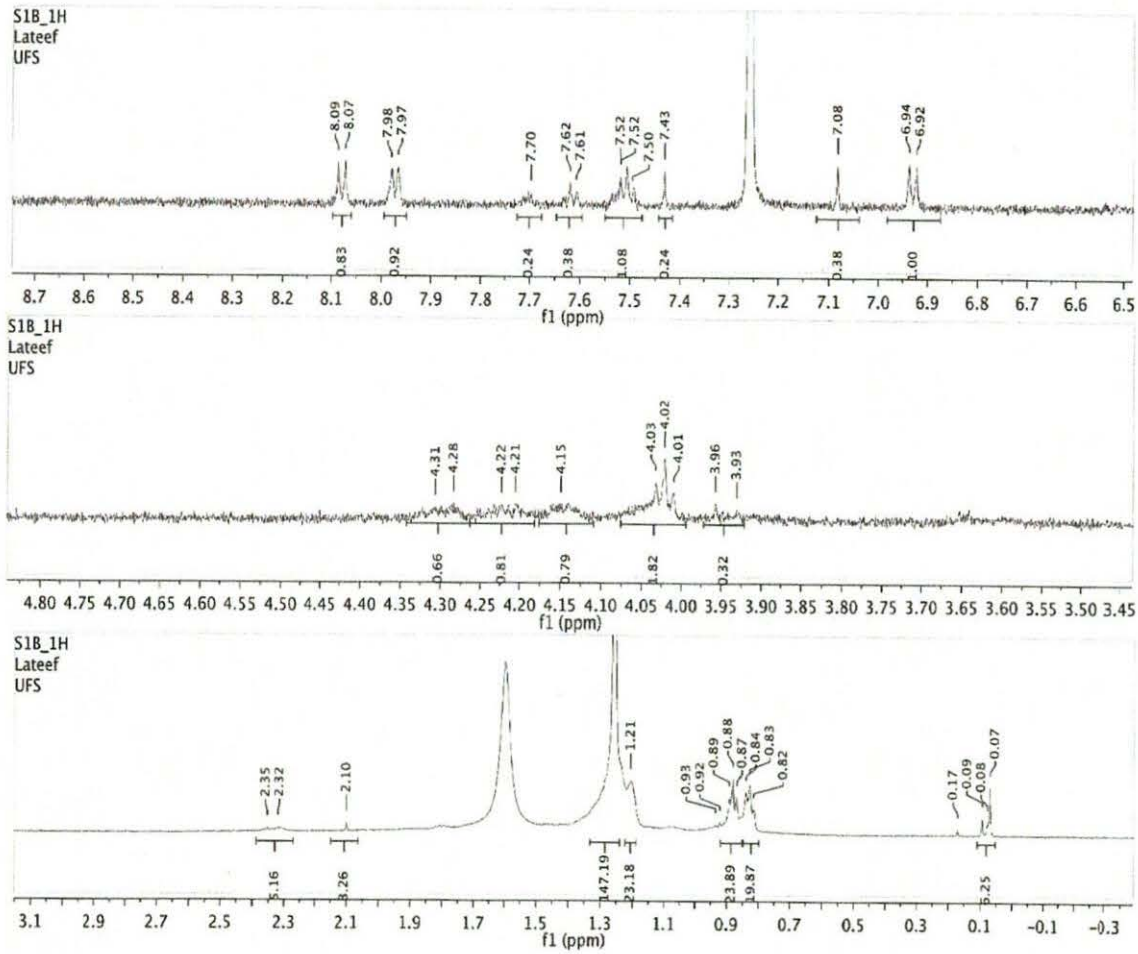


Appendix 1: Mass Spectrum of Hermannol

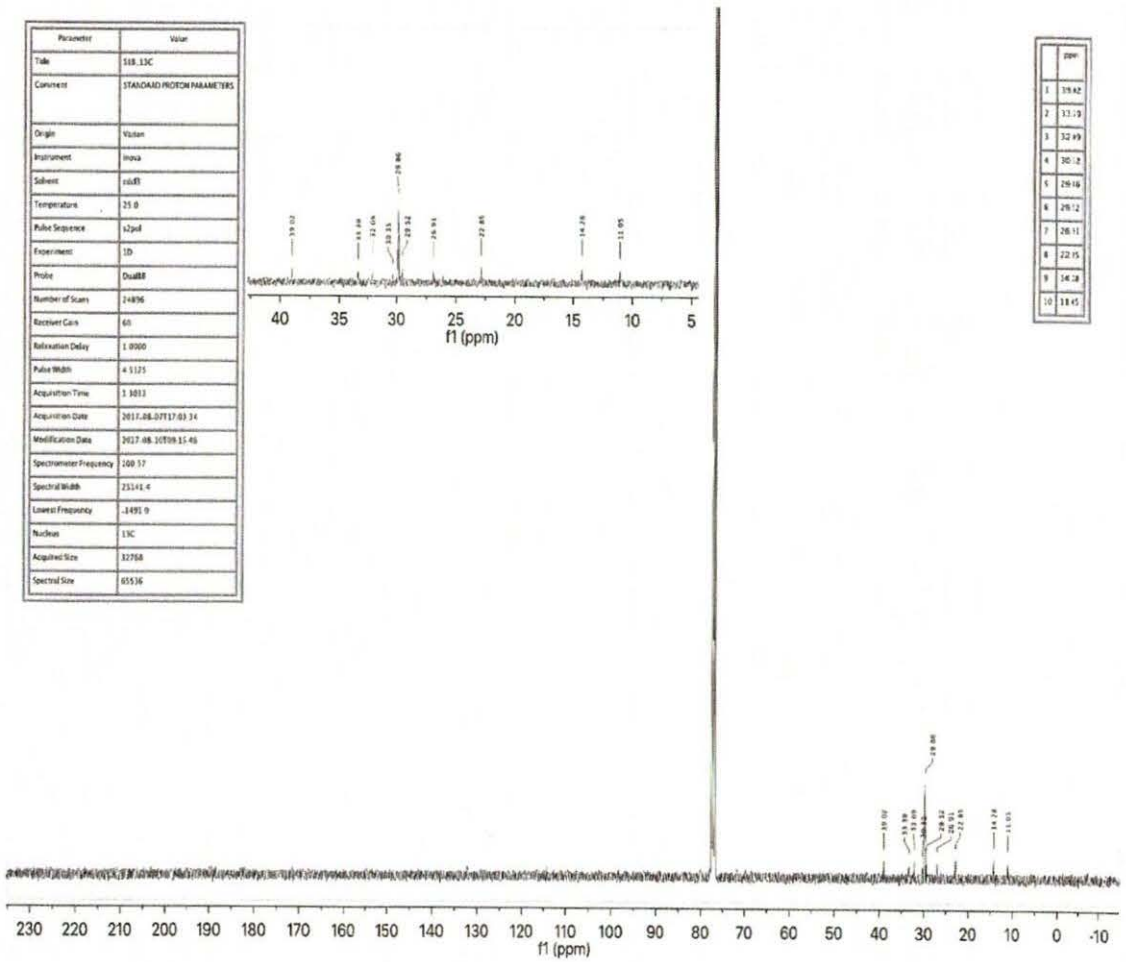


	ppm	Hz		ppm	Hz
1	8.09	4851.9	24	4.03	2419.4
2	8.07	4843.1	25	4.02	2412.6
3	7.98	4787.9	26	4.01	2405.7
4	7.97	4779.7	27	3.96	2374.0
5	7.70	4618.6	28	3.93	2358.3
6	7.62	4572.0	29	2.35	1411.5
7	7.62	4571.9	30	2.32	1392.2
8	7.61	4564.4	31	2.10	1260.0
9	7.54	4521.7	32	1.60	958.3
10	7.52	4511.4	33	1.26	753.8
11	7.52	4511.4	34	1.21	723.5
12	7.51	4503.8	35	0.93	560.4
13	7.50	4497.6	36	0.92	552.5
14	7.43	4459.0	37	0.89	535.1
15	7.08	4250.0	38	0.88	528.3
16	6.94	4162.6	39	0.87	521.3
17	6.92	4154.1	40	0.84	504.0
18	5.54	3324.5	41	0.83	496.6
19	4.31	2583.6	42	0.82	489.4
20	4.28	2570.0	43	0.17	102.6
21	4.22	2533.6	44	0.09	56.1
22	4.21	2523.1	45	0.08	46.8
23	4.15	2489.2	46	0.07	41.5

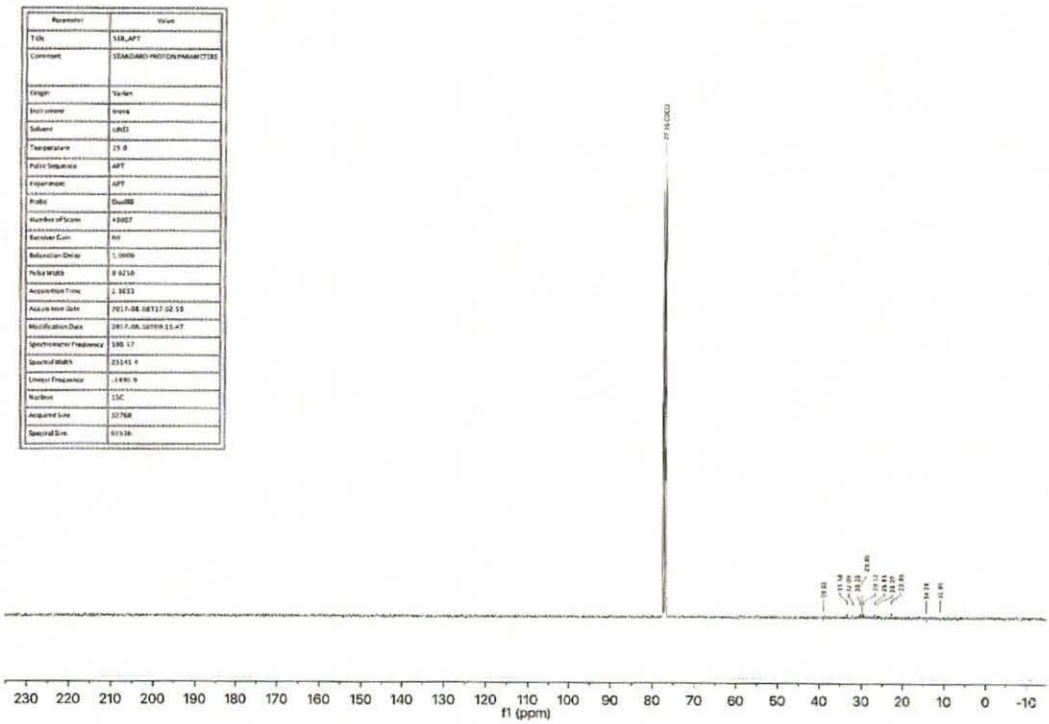
Appendix 2: ^{1}H NMR spectrum of Hermannol



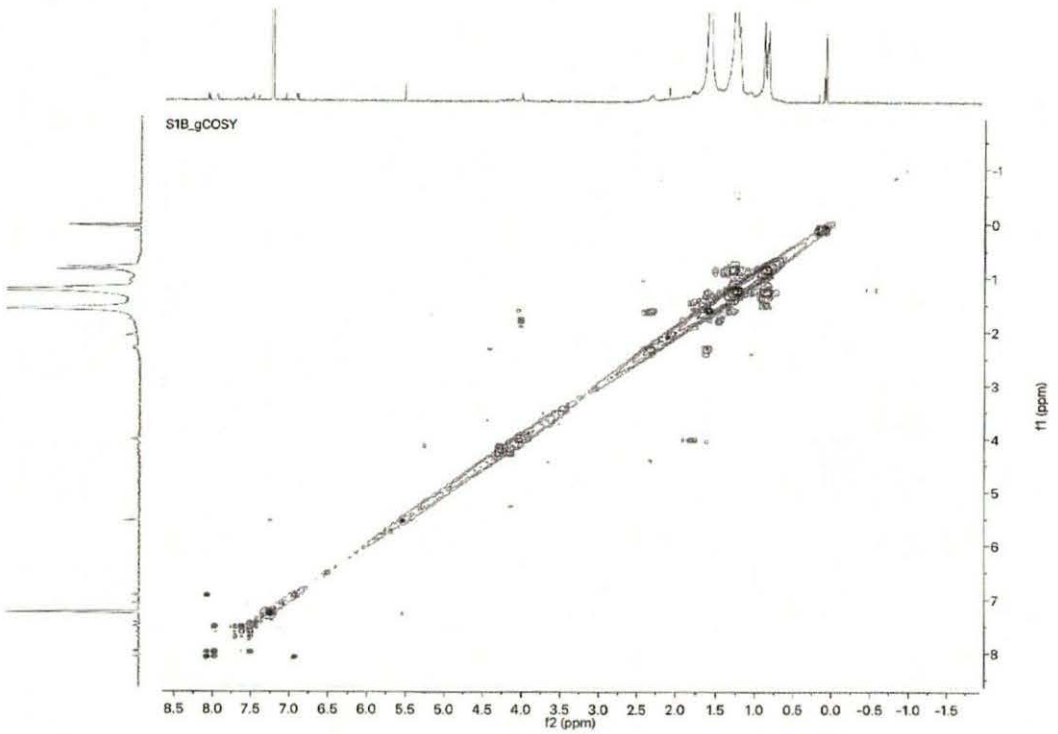
Appendix 3: Expansion and Integration of ^{1}H NMR spectrum of Hermannol



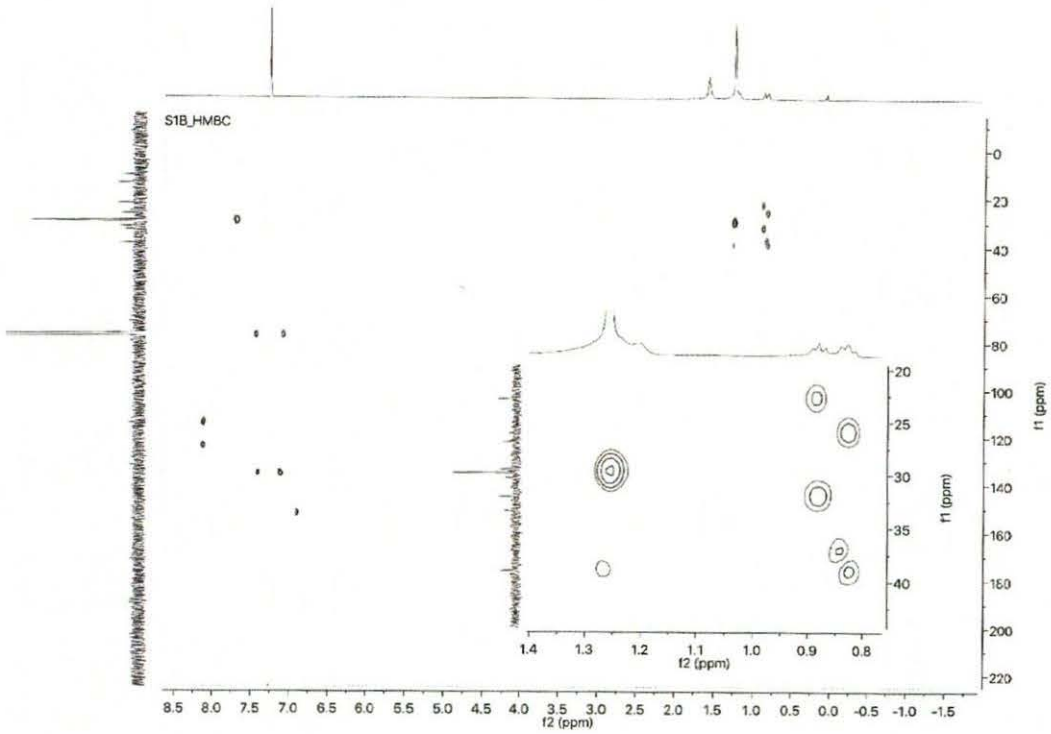
Appendix 4: ^{13}C NMR spectrum of Hermannol



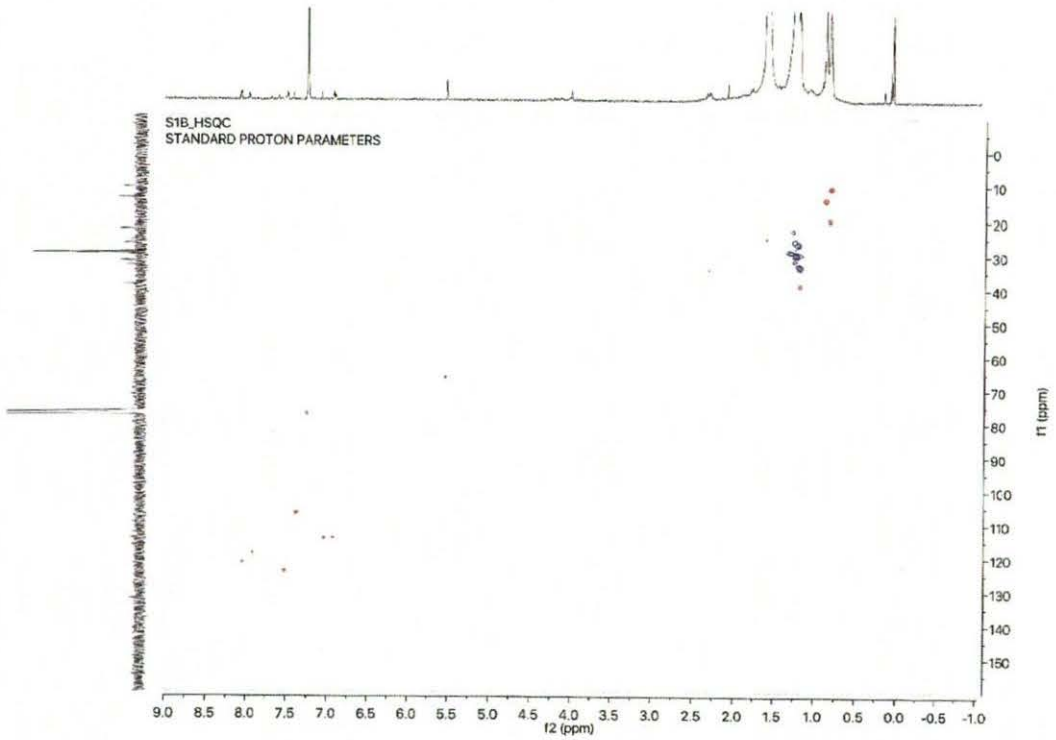
Appendix 5: Attack Proton Test Spectrum (APT) of Hermannol



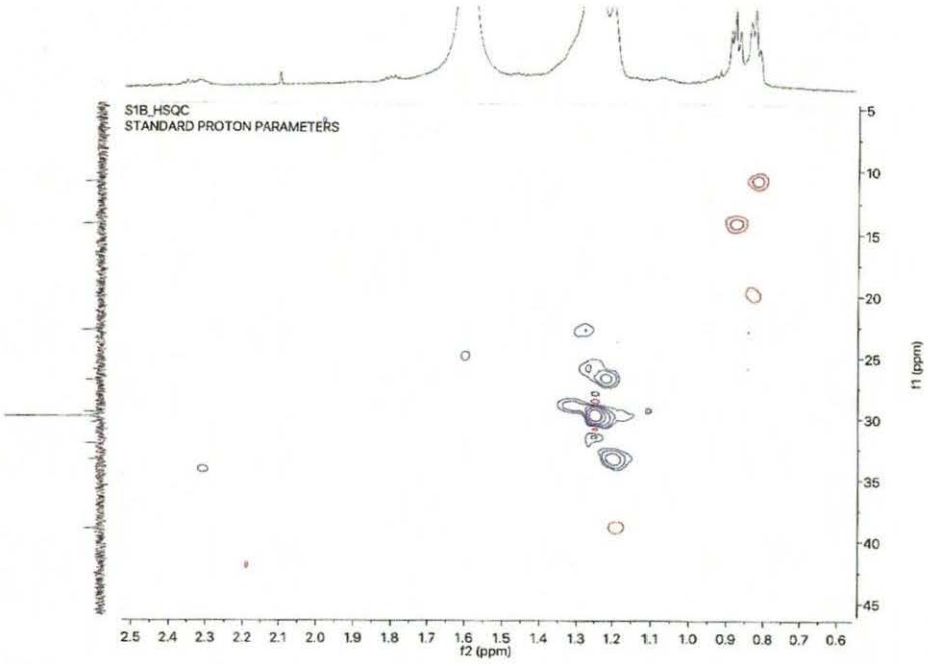
Appendix 6: Homonuclear Correlation Spectrum (COSY) of Hermannol



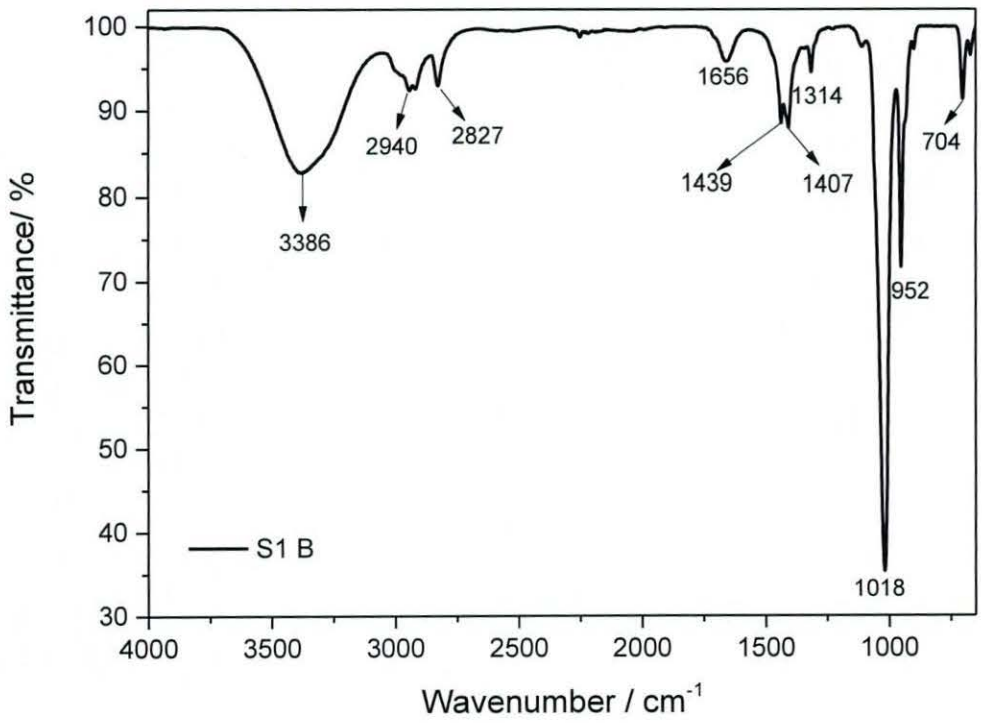
Appendix 7: Heteronuclear Multiple Bond Correlation Spectrum (HMBC) of Hermannol



Appendix 8: Heteronuclear Single Quantum Coherence Spectrum (HSQC) of Hermannol



Appendix 9: Expansion of Heteronuclear Single Quantum Coherence spectrum (HSQC) of Hermannol



Appendix 10: Fourier Transform Infrared Spectrum of Hermannol

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