Antioxidant and antidiabetic potentials of *Medicago laciniata* (L) Mill root extracts: *in vitro* investigations

Tshabalala, B.D. (BSc. Hons)

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A master's degree dissertation submitted to the Department of Plant Sciences, Faculty of Natural and Agricultural Sciences, University of the Free State, Qwaqwa campus

Supervisor:

Dr AOT Ashafa

Co-supervisors:

Dr Balogun FO and Dr Sabiu S

Department of Plant Sciences, University of the Free State

Declaration

I declare that the dissertation hereby submitted for the Master of Science degree at the University of the Free State is an original work by Tshabalala, Bongani Donnycath under the supervision of Dr AOT Ashafa, Dr FO Balogun and Dr S Sabiu. I therefore cede the copyright of this dissertation in favour of the University of the Free State.

Student Signature……

Supervisor Signature……..

Co-supervisor…….

Co-supervisor…….

Dedication

To God, my family and friends.

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Without their individual inspiration and impact in my life as well as in this study most of things would have not worked flawlessly. Firstly, I thank Almighty God for giving me strength and wisdom to tackle everyday obstacles. I thank the Nation Research Fund for the bursary given to me in order to pursue my study. This to me is a rare opportunity and privilege.

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Acronyms used

CPM	crude plant material
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
DPPH	2.2-diphenyl-1-picrylhydrazyl
GAE	gallic acid equivalence
IC_{50}	½ inhibitory concentration
OHAs	oral hypoglycemic agents
PBS	phosphate buffer saline
*p*NPG	*p*-nitrophenyl-α-D-glucopyranoside
QE	quercetin equivalence
RE	rutin equivalence
TCA	trichloroacetic acid

Abstract

Diabetes mellitus (DM) is one of the chronic ailments that contribute to high mortality rate worldwide. Synthetic drugs used to control and manage this disease have several constrictions like prohibitive price to the unemployed class and or low-income earners, disturbing side effects such as use during pregnancy. Due to these constraints and others, an alternative approach to control and manage DM is highly required. The aim of the current study was to investigate the *in vitro* antioxidant and antidiabetic potentials of *Medicago laciniata* (L) Mill root extracts. Solvents used for extraction of the plant material were hydro-ethanol, ethanol, water and acetone. Assays carried out in this investigation were phyto-chemical screening (qualitative and quantitative methods), antioxidant assays (DPPH radicals, reducing power, metal chelating, hydrogen peroxide, hydroxyl radicals and nitric oxide assays), and antidiabetic assays (alpha amylase and alpha glucosidase inhibition). Results showed that *M. laciniata* root possesses several medicinal phyto-chemicals like alkaloids, flavonoids, flavonols, saponins, tannins, cardiac glycosides and phenols. Quantification of phyto-chemicals showed 0.632

(µg/mL), 0.151(mg/g), 0.035 (mg/g) and 0.032 (mg/g) contents of total flavonoids, total flavonols, total phenols and total tannins respectively, while alkaloids and saponins showed 27 and 78% respectively. Antioxidant results revealed varied IC_{50} values of extracts in different assays performed. The lowest IC_{50} values recorded were 0.602±0.034 mg/mL, 0.712±0.072 mg/mL, 0.512±0.002 mg/mL, 0.306±0.021 mg/mL, 0.513±0.041 mg/mL and 0.455±0.164 mg/mL in DPPH radicals, reducing power, metal chelating, hydrogen peroxide, hydroxyl radicals and nitric oxide assay respectively. Hydro-ethanol extract showed the strongest alpha glucosidase inhibition with the lowest IC_{50} value of0.07±0.014 mg/mL while acarbose (standard) showed 0.24±0.17 mg/mL. All extracts showed poor alpha amylase inhibitory potential as compared to acarbose which was recorded to have the lowest IC_{50} value of 0.60±0.191 mg/mL. Among other extracts, ethanol extracts showed better alpha amylase inhibition with an IC_{50} value of 2.11±0.026mg/mL. Results obtained from different assays in this study suggest that *Medigaco laciniata* (L) Mill have antioxidant and antidiabetic potentials; may

also possess other medicinal properties due to the variety of phyto-chemical discovered in this plant. Hence the study justified the folkloric use of this plant.

Chapter 1

1.0 Introduction

Plants are self-dependent organisms, that are capable of self-sustaining themselves in relation to the nourishment and health distress in the natural environment. This distinctive manner does not only benefit them but other organisms such as animals, insects and man. Plants are therefore primarily referred to as food factories because of the presence of molecules like starch (energy rich molecule) which is generated by photosynthetic processes. However non-photosynthesizing organisms acquire energy for daily activities from starch molecule (Buragohain, 2015). Plants are furthermore capable of synthesizing secondary metabolites, which ensures the smooth operation of the system. This is normally achieved by combating foreign substances like micro-organisms from disrupting the normal-functioning of the plant system (Farnsworth and Morris, 1976). The application of metabolites is widely increasing in various fields like veterinary, agriculture, health practices and others. This high awareness and importance of plants illustrate that they play a major role in the well-being of mankind with their medicinal potentials (Khanum *et al.,* 2012). In reality, the cycle of life and natural systems are definitely deficient exclusive of plants existence.

Plants possessing medicinal properties are referred to as medicinal plants. They have valuable properties particularly in preventing, curing and sustaining

health of human-beings. It is known that even most modern drugs applied today are derived from phyto-chemicals obtained from different parts of plants including roots, stems, leaves, seeds, bark and fruits (Dewick, 1996). Flavonoids, phenols, saponins, tannins and alkaloids are some of the numerous bioactive compounds synthesized by medicinal plants.

Medicinal Plants are readily available and accessible to every-being regardless of their financial or nutritional background, probably because of their efficacies and lesser side effects due to the presence of inherent naturally synthesized phyto-chemicals (Yadav and Agarwala, 2011). One of the most important factors coming from chemicals produced by medicinal plants is that they have holistic approach when combating ailments. After all, it is known that most ailments particularly chronic diseases are characterized by a group of complications.

The practice or use medicinal plants, is well acknowledged throughout the world for the treatment and control of chronic, sub-chronic, temporary and mild diseases (Malviya *et al.,* 2010). South Africa is a country composed of people with different races and cultures, where medicinal plants are used to treat various kinds of ailments. Chronic diseases are categorized by their disturbing behavior that causes the patient to suffer from negative effects for a very long time and sometimes to life time. Therefore patients suffering from this kind of diseases need

to apply or administer medication for a very long period of time of their life in order to control the complications caused by chronic condition. Thus, treating these diseases may be very costly, might leave toxic residue, and or trigger addiction especially since today's health practices utilizes laboratory synthesized drugs (Shyam and Ganapaty, 2013).

The use of medicinal plants is generally increasing; although most of the plants have not yet been scientifically validated (da Costa *et al.*, 2000). However some of these plants may also have documented scientific evidence on their medicinal properties, despite the unknown negative impact and mutilations they may cause in the long run (Fennell *et al.*, 2004).Occasionally factors like temperature, period of administration, method of preparation, amount of dosage and ratio composition of compounds can modify a healthy remedy into a possible toxin (Fennel *et al.*, 2004). Therefore, studies involving identification of phyto-constituents and ratio composition of compounds are vital in ensuring the safety and efficacy of these plants, especially when dealing with acute and chronic ailments distressing human beings (Parekh and Chanda, 2008).

The human system like any other organism is maintained by cellular metabolism (catabolism and anabolism), where in most cases leads to the release of free radicals (Young and Woodside, 2001).Production of free radicals is normal, at least when the body tries to stabilize and control chemical reactions taking place

during metabolic processes. However, excessive production of free radicals can be a potential constrain to the functioning of the cell. Uninterrupted production of excess free radicals may result and support the onset of many chronic diseases including cancer, heart disease, ageing, diabetes mellitus and others (Boynes, 1991) arising from the alteration, damage and destruction of cells and tissues. Hence, the presence of antioxidants in medicinal plants when ingested may go a long way in inhibiting the agile and destructive activities of excess free radicals, by scavenging, converting and reducing their power to react and damage useful molecules (Pourmorad *et al*., 2006). These acts eventually assist in battling and controlling persistent diseases.

Many chronic diseases are very dangerous and may result in fatality (Grover *et al*., 2002). Diabetes mellitus (DM) is one of the disorders that are considered to be extremely serious and poses serious threat to the present and next generation of mankind. DM is known to be a group of metabolic diseases characterized by hyperglycemia (high blood glucose), arising from insufficient insulin secretion, inaction or both thus resulting in symptoms and or complication such as polyuria, polydipsia, polyphagia and in severe cases, destroys the cells of nervous system, heart, and blood vessels (Malviya *et al.*, 2010; Deepa *et al.*, 2013). DM can be classified into two major types namely Type 1(insulin dependent diabetes mellitus, IDDM) and Type 2 (non-insulin dependent diabetes mellitus, NIDDM). Type 1

diabetes occurs due to inability of the body to produce insulin: therefore patients need to be injected with synthetic insulin and this affects about 10% of reported diabetic cases worldwide. On the other hand type-2 diabetes contribute to the remaining 90% of all reported diabetic cases, thus rates type 2 diabetes as one of the five leading non-communicable disorders globally (Kavishankar, 2011).

There are several factors that may contribute to the risk of developing type-2 diabetes, this include but not limited to sedentary life style, auxiliary stress and lower testosterone level. Eating too much of carbohydrates without enough insulin is also one of important factors contributing to complications related to DM. Interestingly insulin is a hormone responsible for controlling glucose level in the blood stream by ensuring the absorption of glucose molecule by target cells. Thus many synthetic anti diabetic drugs control DM by influencing insulin production, or by inhibiting activities of α-amylase and α-glucosidase enzymes, which are the two prominent enzymes accountable for constant break-down of carbohydrates (starch) in the small intestine and absorption of glucose into tissues (Rajaram and Sureshkumar, 2011).Synthetic antidiabetic drugs such as acarbose (glucosidase inhibitor) are said to have unpleasant side effects (Kwon *et al.*, 2007) such as hypoglycemia (low blood sugar), weight gain, nausea, and gastro-intestinal disturbances(Balogun *et al*., 2016; Balogun and Ashafa, 2017). Due to these highlighted side effects, researchers are looking for alternative therapy in

medicinal plants similar to synthetic drugs but with lesser or no side effects. Intriguingly plants contain many medicinal properties including antioxidants and antidiabetic properties among other activities (Kwon *et al*., 2006).

Metabolites assist in the growth and survival of the plant, while secondary metabolites are mostly employed in battling foreign invaders from disrupting the functioning of the plant (Yadav and Agarwala, 2011).Glycosides, alkaloids, terpenoid, flavonoids and carotenoids are secondary metabolites that have been reported to have hypoglycemic activity (Malviya *et al*., 2010). This ropes the importance of evaluating the chemical composition of medicinal plants.

Numerous plant species have been documented and others suspected to be hypoglycemic agents, but this information alone is not sufficient, hence there is a need to study and discover more medicinal plants vis-à-vis their relationship to DM, or their antidiabetic potentials(Ahmad, 2012).The dull part of this regarding information about some medicinal plants is that they are only known, circulated and practiced within a pertinent tribe, while people in nearby regions may suffer from same illness due to lack of information. Thus, more scientific investigations and reports are required in ensuring information about medicinal plants are shared to the world at large. *Medigaco laciniata* (L) Mill (Figure 1) is one of the plants used in treating and controlling DM by Basotho tribe in the Eastern Free State. However this claim is not yet proven scientifically.

The genus *Medicago* is composed of flowering plants in the legume family with at least 87 species (Steele *et al.*, 2010). Most members of this family are characterized as low creepers that resemble clover but have burs. They are also known by specializing in production of bioactive compounds such as flavonoids (Gholami, 2014).While many of the plant species in this genus have not been reported to have medicinal and antidiabetic potential, a related species, *Medicago sativa* is reported to have promising antidiabetic activity (Kundan and Anupam, 2011). However local healers and herbal sellers in Qwaqwa township of Free State Province (Figure 2), have confidence in the root extract of *Medicago laciniata* for controlling blood sugar level of diabetic patients.

M. laciniata root usually forms a symbiotic relationship with bacterium *Sinorhizobium meliloti* which is capable of nitrogen fixation. The plant is mostly found in less arid area towards the top of the slopes and beds of the valleys (Heyn, 1963) and is classified as a winter annual plant. Medicinal properties of this plant have not been scientifically investigated and reported. Basotho tribe in the Eastern Free State also normally uses this plant for patients that are under high psychological stress. The root of the plant is chewed like chewing gum, and then the saliva with extracts from the root is swallowed.

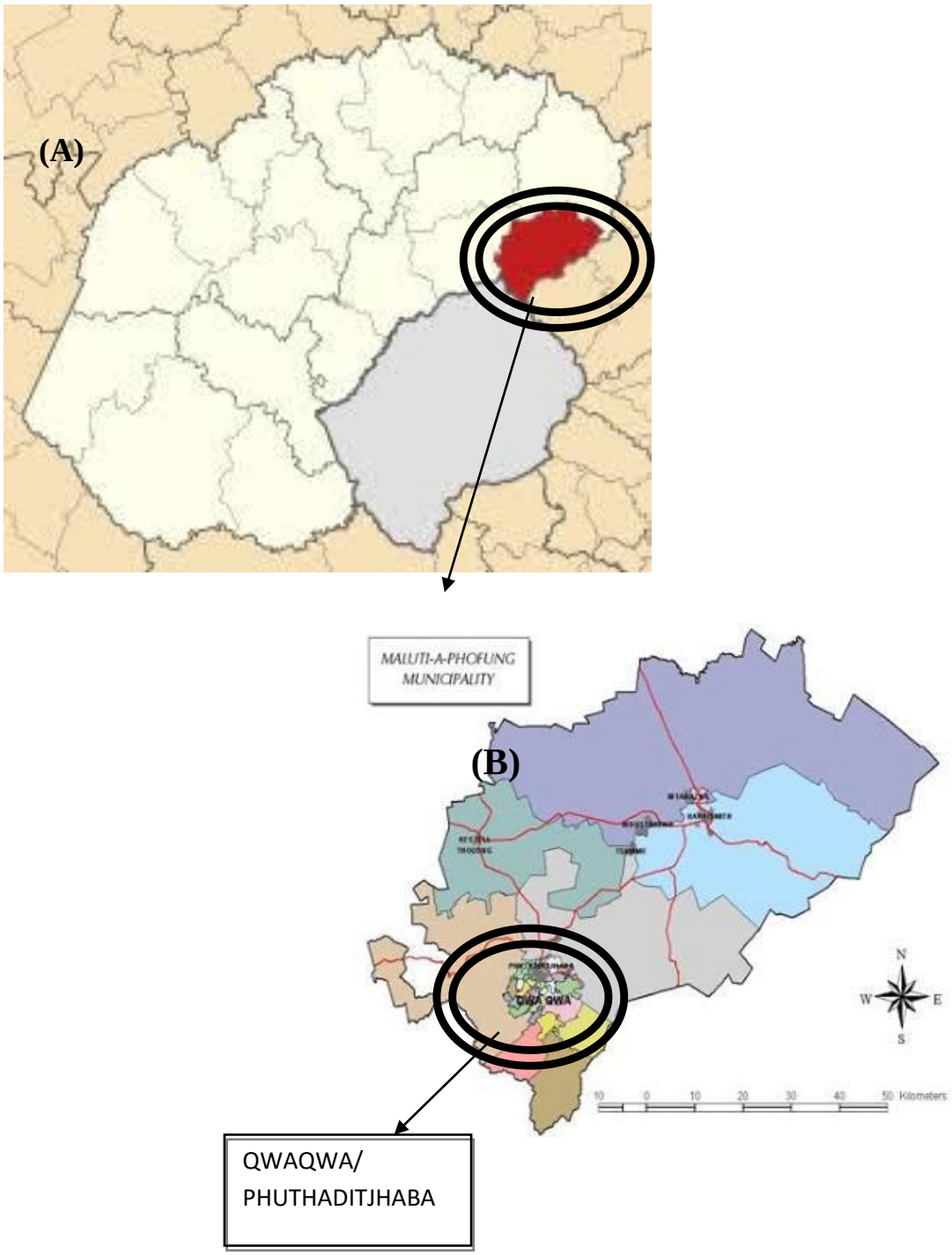

Figure1. Free Statemap highlighting (A) Maluti-A-Phofung municipality and (B) Qwaqwa town where*M. laciniata* root Mill (L) was located and collected

(A)*https://en.m.wikipedia.org/wiki/Maluti-a-Phofung_Local_Municipality*.

(B)*https://map.gov.za/about-Maluti-A-Phofung*.

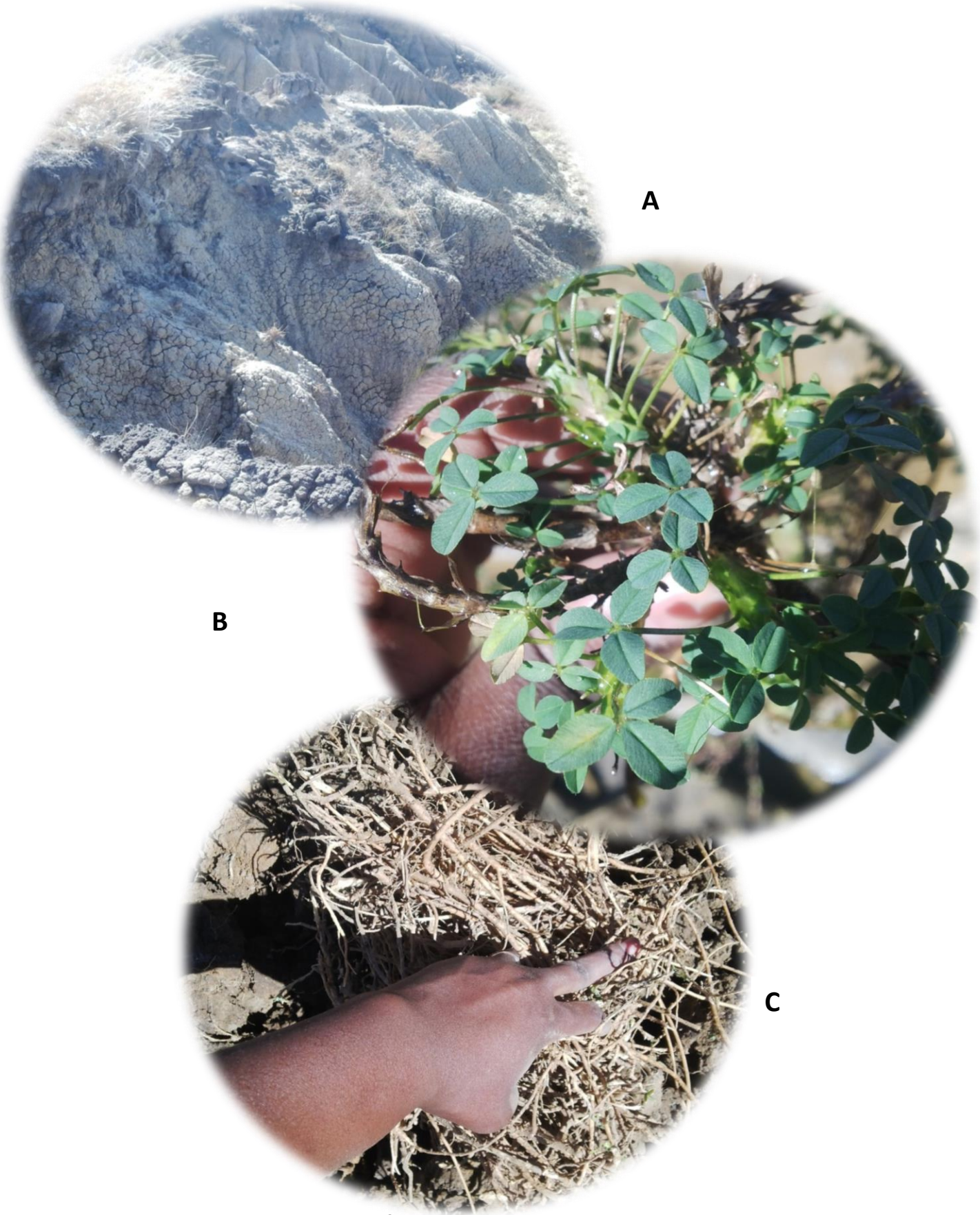

Figure 2. Pictorial representation of (A) the aggregation habitat, (B) the shoot part, and (C) the root part of *Medigaco laciniata* Mill (L).

Tshabalala B, 2016.

1.1 Justifications for the study

Diabetes mellitus is considered a chronic disease responsible for major cause of hospitalization. The prevalence of the menace increases with population size and the cost of treatment has become a major setback to the sufferers. The use of oral hypoglycemic agents (OHAs) such as biguanides and sulphonylureas is one of the treatment modalities developed for diabetes control. However, the drugs are expensive and unaffordable particularly to the rural dwellers like Qwaqwa populace. Hence, it is imperative that studies and search for alternative drugs from medicinal plants are continuous. As searches and the uses of medicinal plants intensely increase, there is also a great need for evaluation of their toxicological effects to ascertain their safety. Moreover, ratio and concentration of phyto-chemicals, safe dosages of extracts, and method of preparation should be well understood.

1.2 Aims and objectives

The aim of the study is to reveal antioxidant and antidiabetic potentials of *Medicago laciniata* (L) mill root extracts via in *in vitro* investigations which would be achieved through the following specific objectives:

1. To conduct phyto-chemicals profiling of extracts by qualitative and quantitative and methods

2. To assess the *in vitro* anti-oxidative potentials of the extracts
3. To investigate the *in vitro* hypoglycemic activity of extracts via α-amylase and α-glucosidase inhibitions.

Chapter 2

2.0 Materials and Methods

2.1 *Collection and preparation of plant material*

Medicago laciniata root materials were collected from the field (Damp valleys) near and around University of The Free State, Qwaqwa Campus (S 28° 48’ 34.4” and E 28° 82’ 42.5”). Preparation and storage of plant material were adopted from the method described by Hussain *et al*. (2013). The whole plant was washed under clean running tap water and shade-dried naturally at room temperature. The root part was separated from aerial part and then pulverised into fine powder by a mechanical grinder and stored in closed container until use.

2.2 *Plant Extraction*

Four solvents (acetone, ethanol, water and hydro-ethanol) were utilized to extract root sample as detailed by Ashafa *et al*. (2008). Exactly four portions (25g each) of root sample were extracted with 250 mL of each solvent. The solutions obtained were allowed to mix properly on a platform shaker (Labcon ®, South Africa) at a speed of 110rpm for 24 h. Extracts were filtered through Whatman No.1 filter paper. Filtrates of acetone, ethanol and hydro-ethanol were concentrated under reduced pressure at 40°C using rotary evaporator (Cole-

Parmer, China, Model AC230BV). Water extract was freeze-dried using (Virtis SP Scientific) lyophilizer. Obtained crude extracts were kept in an air-tight container and stored at 4°C until use for bioassay.

2.3 *Phyto-chemical screening*

The presence of different phyto-constituents was revealed by employing different tests described by Mudi and Ibrahim (2008) and Tiwari *et al*. (2011) with slight modifications.

2.3.1 *Qualitative determination of phytoconstituents*

Dragendroff's test was used to indicate the presence of alkaloids. Root extracts (1 mg) were dissolved in 10 mL dilute hydrochloric acid (10%) and filtered. The filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodine) and formation of red precipitate indicated the presence of alkaloids.

Presence of saponins was detected using froth foam test: 0.5 mg of extract was shaken with 2 mL of distilled water. The foam produced which persisted for ten minutes indicated the presence of saponins.

Presence of tannins was detected using Gelatin test, 1% gelatin solution containing sodium chloride was added to plant extract. The formation of white precipitate indicated the presence of tannins.

Flavonoids were detected using lead acetate test. The extracts were treated with few drops of lead acetate solution and formation of yellow color precipitate indicated the presence of flavonoids.

To test for the presence of phlobatannins, 2 mL of each plant's fraction was added to 5 mL HCl. Formation of turbidity/precipitate indicated the presence of phlobatannins.

Test for cardiac glycosides, 2 mL of each fraction was added in succession to 3mL 3.5% Iron (III) Chloride ($FeCl_3$) and then followed by adding 3mL ethanoic acid. This formed a green precipitate and a dark colored solution respectively. Finally, concentrated H_2SO_4 was carefully poured down the side of the test tube which results in the formation of brownish-red layer, at the interface. This confirmed the presence of cardiac glycosides.

Test for steroids (Harbone, 1998) using Liebermann's test: 2 mL of ethanoicacidanhydride was added to 2 mL of each extract. The content was then cooled on ice for 5min and 1mL of concentrated H_2SO_4 was added along the walls

of the test tube. The change in color from violet to blue and then to green indicated the presence of steroidal nucleus (i.e. aglycone component of cardiac glycosides).

2.3.2Quantitative analysis of phytoconstituents

2.3.2.1 Determination of flavonoids content

Total flavonoid content was estimated following the method described by Lin and Tang (2007). Briefly 10 mg of quercetin was dissolved in 100 mL of methanol (100 µg/mL) and then further diluted to 10, 20, 40, 80 and 160 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of methanol, 0.1 mL of aluminium chloride (10%), 0.1 mL of potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with UV/VIS spectrophotometer. The amount of aluminium chloride (10%) was substituted by the same amount of distilled water in the blank.

Quantification was done on the basis of standard curve of quercetin. A standard curve of absorbance against quercetin concentration was prepared and the results are expressed as w/wflavonoids content (%w/w) = $QE \times V \times D \times 10^{-6} \times 100/W$, where QE-quercetin equivalent (µg/mL), V-total volume of sample (mL), D-dilution factor, W-sample weight (g).

2.3.2.2Determination of flavonols content

The flavonols content was determined using the method described by Kumaran and Karunakaran(2007). Rutin calibration curve was prepared by mixing 2 mL of varying concentrations of rutin (200- 1000 µg/mL) with 2 mL (20 g/L) $AlCl_3$ and 6 mL (50 g/L) sodium acetate. The absorbance at 440 nm was read after 2.5 hr at 20°C. The same procedure was carried out with 2 mL of plant extracts instead of Rutin solution. All determinations were carried out in triplicates. The flavonols content was obtained from Rutin calibration curve and expressed as rutin equivalents (mg/g).

2.3.2.3Quantification of saponins

Saponins content was determined following the method described by Obadoni and Ochuko(2001). Powered sample (5 g) was suspended in 20% ethanol (20 mL ethanol in 80 mL distilled water) and vigorously agitated on a Labcon Platform Shaker (Laboratory Consumables, South Africa) for 30 min. The resulting infusion was then heated in a water bath (55°C) for 4 hr before filtration (Whatman No.1 filter paper). The residue obtained was thereafter re-extracted in 200 mL of the same solvent (20% ethanol). Following this, the resulting mixture was concentrated to about 40 mL in a water bath (90°C). Using a separating funnel (250 mL capacity), the concentration obtained was then extracted twice with

diethyl ether (20 mL) and the ether layer from each partition was discarded while the aqueous layer retained. Exactly 60 mL of *n*-butanol was added and the infusion obtained was concentrated and washed twice with 5% sodium chloride (10 mL). The experiment was performed in triplicate. Following substantial evaporation, the sample was oven-dried (40°C) to constant weight and the % saponins content was estimated using the expression:

$$\%\text{Saponins} = (FW/IW)\times 100$$

FWand IW represented the final and initial weights of the sample respectively. The values obtained were finally converted to g/100g of the sample.

2.3.4.3Quantification of Alkaloids

The experiment was conducted by adapting the method described by Ulubelen(1994). Alkaloids were extracted from 2 g sample of the dried plant root with n-hexane (60 mL) followed by 60 mL methanol (MeOH). The extraction was done at room temperature and each cycle with hexane or MeOH was left for 24 hr. The extract was evaporated in a water bath (40°C). At the last step, each tube was rinsed with 15 mL distilled water, which was then added to the flask. The pH wasadjusted to 2.0 with 5% H_2SO_4 and the contents were extracted with chloroform (CH_3Cl) to separate the non-alkaloids in the mixture. The acidic

aqueous solution was made alkaline (pH 8 to 10) by adding 10% NaOH and the contents were extracted with chloroform to obtain the alkaloids.

Alkaloids were then estimated following the expression:

%Alkaloids = (FW/IW)×100.

FW and IW represented the final and initial weights of the sample respectively. The values obtained were finally converted to g/100g of the sample.

2.3.2.5Quantification of total phenols

Folin-Ciocalteau micro method for total phenols was adapted based on the method described by Slinkard and Singleton (1977).

Brief, from each calibration solution, the sample or blank, 20 µL was pipettedinto separate cuvettes, and to each of the cuvette, 1.58 mL of water was added, and then 100 µL of the Folin-Ciocalteu reagent (1 mL Folin-C with 9 mL distilled water), the solution was mixed well and was left for 8 min, this was then followed by the addition of 300 µL of sodium carbonate solution. Thereafter the mixture was shaken for proper mixing and then left at 20°C for 2 hr. The absorbance of each solution was read at 765nm on a spectrometer (BIO-RAD Model 680) against the blank (the "0 mL" solution) and absorbance vs. concentration was plotted. Calibration curve created with standards and levels of

samples were determined. Results were reported as mg/g gallic acid equivalent (GAE).

2.4Antioxidant assays

2.4.1DPPH radical scavenging assay

The antioxidant activity of the extracts was determined by measuring its capacity of bleaching the purple-colored ethanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as described by Turkoglu *et al*.(2007). Briefly, 100 µLof various concentrations (0.2 – 1.0 mg/mL) of extracts in methanol were added to 1 mL of 0.002 mol/L sample of DPPH in methanol. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. The percentage inhibition (I%) on the DPPH radical was calculated using the expression:

Percentage Inhibition (I%) = $[(A_{control} - A_{extracts})/ A_{control}] \times 100$,

Where $A_{control}$ is the absorbance of the control, $A_{extracts}$ is the absorbance of the extract.

2.4.2Nitric oxide scavenging assay

Nitric oxide radical stalling activity of the extracts was assayed according to the method of Garret (1964). Briefly, 2 mL of 10 mM sodium nitroprusside in 0.5

mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of various concentrations of extracts, and then incubated at 25°C for 2 hr. From the incubated mixtures, 0.5 mL was taken and added to 1 mL of sulfanilic acid reagent (0.33%sulfanillic acid in 20% glacial acetic acid) and further incubated at room temperature for 5 min. Following this, 1mL naphthyl-ethylenediamine dihydrochloride (0.1% w/v) was introduced into the mixtures and the resulting solutions were incubated at room temperature for 30 min. The absorbance was taken at 540 nm and the IC_{50} was evaluated from calibration curve following estimation of percentage nitric oxide radical scavenging capacity of the extracts using the expression:

Percentage scavenging (S%) = $[(A_{control} - A_{extract})/A_{control}] \times 100$,

Where $A_{control}$ is the absorbance of the control, $A_{extract}$ is the absorbance of the extract.

2.4.3Metal chelating assay

The metal chelating potential of the extract with particular reference to ferrous ion was estimated following the method of Dinis *et al*. (1994). Briefly, 0.1 mL of the extracts (different concentrations) was added to 0.5 mL of 0.2 mM of ferrous chloride solution. The reaction was initiated by adding 0.2 mL of ferrozine (5 mM) and incubated at room temperature for 10 min prior to absorbance readings

at 562 nm. Citrate was used as a control and measurement of color reduction determines the chelating activity of the extract to compete with ferrozine for the ferrous ions and IC_{50} value was therefore estimated from the calibration curve.

2.4.4Reducing power method

The reducing power of extracts was evaluated by adopting the method of Isabel *et al*. (2007). Varying concentrations of extracts (0.2-1.0 mg/mL) were suspended in 1 mL of distilled water and mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferrocyanide ($K_3Fe(CN)_6$). The mixtures were incubated at 50°C for 20 min prior to the addition of 2.5 mL of trichloroacetic acid (TCA). Following centrifugation at 3000 rpm for 10 min, 2.5 mL of the supernatant was mixed with an equal amount of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the resulting solutions was read at 700 nm.

2.4.5Hydrogen peroxide scavenging assay

The modified method of Ruch *et al*. (1989) was used to estimate the scavenging activity of hydrogen peroxide. In brief, 1.7 mL of different concentrations (200-1000 µg/mL) of extracts in phosphate buffer saline (PBS: pH 7.4) were carefully mixed with 300 µL of 40 mM H_2O_2andincubated at 25°C for 10 min. For the standard, 1.7 mL of vitamin C was used. The absorbance in each case

was subsequently taken at 230 nm and the percentage H_2O_2 inhibitory potential of extracts was evaluated from the expression:

$\%H_2O_2$ scavenged = $[A_{control} – (A_{sample} – A_{extract})/A_{control}] \times 100$. $A_{control}$ is the absorbance of the mixture without extract.A_{sample} and $A_{extract}$ represent the absorbance of the mixture with the extract and that of the extract alone, respectively. The IC_{50} value was therefore estimated from the calibration curve.

2.4.6 *Hydroxyl radical scavenging assay*

The OH radical scavenging capability of the extracts was determined by adopting the method described by Smirnoff and Cumbes (1996). In brief, 200 µL of vitamin C (200-1000 µg/mL) was mixed with 600 µL of $FeSO_4$ (8 mM), 500 µL of H_2O_2 (20 mM) and 2 mL of salicylic acid (3 mM) in the test tube. Following 30 min incubation period at 37°C, distilled water (0.9 mL) was added and the resulting mixture centrifuged (4472 x *g*, 10 min). The absorbance was subsequently read at 510 nm and the IC_{50} value was evaluated from the calibration curve following estimation of the percentage OH* radical scavenging capacity of the extract as per the expression:

%hydroxyl radical scavenged= $[A_{control} – (A_{sample} – A_{extract})/A_{control}] \times 100$. $A_{control}$, A_{sample} and $A_{extract}$ represent the absorbance of the mixture without extract, mixture with extract and that of extract alone, respectively.

2.5*In vitro* Antidiabetic activities

2.5.1 General α-glucosidase inhibition and kinetic methods

Different concentrations (0.25-1.00 mg/mL) of sample were prepared in dimethyl sulphoxide (DMSO). Then, 50 µL from the aliquot was mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) 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 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) solution in 0.1 M phosphate buffer (pH 6.9) was added to each well. The reaction mixture was incubated at 25°C for 20 min and thereafter the reaction was terminated by adding 2 mL of 0.1 M Na_2CO_3. The absorbance was read at 405 nm using a micro-plate reader (BIO-RAD, Model 680) and the values were then compared with a control which contained 50 µL of the DMSO instead of the extracts. Acarbose (Bayer Medicals, Germany) was prepared in distilled water at the same concentrations as the extracts and was used as the control. The experiments were performed in triplicate. The α-glucosidase inhibitory activity was expressed as %inhibition using the expression:

$$\%\text{Inhibition}=[(\Delta\text{Acontrol}-\Delta\text{Aextract})/\Delta\text{Acontrol}]\times 100$$

WhereΔAcontrol and ΔAextract are the changes in absorbance of the control and the extract sample respectively.

For the enzyme kinetics on the inhibition of α-glucosidase activity by aqueous root extracts of *Medigaco laciniata* (L.) Mill, modified method of Dnyaneshwar and Archana (2013) was adopted. Briefly, 50 µL of 5 mg/mL extracts were pre-incubated with 100 µL of 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 50 µL of phosphate buffer (pH 6.9). This was followed by the addition of 50 µL of *p*NPG at concentrations (0.125 – 2.0 mg/mL) to both sets of reaction mixtures to start the reaction. The mixtures were then incubated for 10 min at 25°C, and 500 µL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a *p*-nitrophenol standard curve converted to reaction velocities at 405 nm wavelength. Reaction rates (V) were thereafter calculated and double reciprocal plots of enzyme kinetics was constructed according to Lineweaver and Burk method to study the nature of inhibition. Km and Vmax values were also calculated from Lineweaver-Burk plot (1/v versus $1/[S]$) (Lineweaver and Burk, 1934).

2.5.2 *α-amylase inhibition and kinetic studies*

The α-amylase inhibitory activity and mode of inhibition were evaluated according to method described by Vijayalakshmi *et al*. (2014). Briefly, varying concentrations (0.25 – 10.0 mg/mL) of extracts were prepared in DMSO and 500

µL of each was mixed with 500 µL of 0.02 M sodium phosphate buffer (pH6.9) containing 0.5 mg/mL of α-amylase solution and incubated in test tubes at 25°C for 10 min. After pre-incubation, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH6.9) was added to each test tube. The reaction mixture was incubated at 25°C for 10 min and stopped with 1.0 mL of dinitrosalicyclic acid color reagent. The test tubes were then incubated in boiling water bath for 5 min and subsequently cooled to room temperature. The reaction mixture was then diluted with distilled water (15 mL), the absorbance reading was measured at 540 nm using a spectrophotometer (Biochrom WPA Biowave II, Cambridge, England) and the values were compared with the control which contained 500 µL of buffer instead of the extracts.

Acarbose was prepared in distilled water at same concentrations as extracts and was used as control. The experiments were conducted in triplicate and the α-amylase inhibitory activity was expressed as percentage (%) inhibition. The concentration of extract causing 50%inhibition (IC_{50}) of α-amylase activity was estimated from its standard calibration curve

The method of Ali *et al.* (2006) was used to determine the mode of inhibition of *M. laciniata* root extract. Briefly, 250 µL of extract (5 mg/mL) was pre-incubated with 250 µL α-amylase solution for 10 min at 25 °C in one set of 5 test tubes. 250 µL of phosphate buffer (pH 6.9) was also pre-incubated with 250

µL of α-amylase solution in another set of 5 test tubes and starch solution (250 µL) of increasing concentrations (0.300–5.00 mg/mL) was added to both sets of reaction mixtures to commence the reaction. The whole mixture was then incubated for 10 min at 25 °C and then suspended in boiling water bath for 5 min after addition of 500 µL of DNS to stop the reaction. A maltose standard curve was used to spectrophotometrically (540 nm) determine the amount of reducing sugars released and converted to reaction velocities. A double reciprocal plot (1/ [V] versus 1/ [S]) where V is the reaction velocity and S is the substrate concentration was plotted. The mode of inhibition of the extract on α-amylase activity was thereafter determined using Lineweaver and Burk (1934).

2.6 Statistical analysis

One-way analysis of variance followed by Dunnet's multiple comparison tests was used for data analysis. While all experiments were carried out in triplicate, the results were expressed as mean± standard error of mean (SEM). GraphPad Prism 5 (GraphPad software, San Diego, California, USA) was used for all data manipulation and analysis.

Chapter 3

3.0 Results

3.1 Qualitative phyto-chemical screening of *M. laciniata* root.

Phyto-chemical screening of hydro-ethanol, ethanol, acetone, and water extracts of *M. laciniata* root revealed the presence of some secondary constituents that could be active exclusively or in unit association with other relative compounds in assays conducted in this study. Table 1revealed moderate to high presence of saponins and tannins in both water and organic extracts. The hydro-ethanol seemed to have higher extraction potential as compared to other solvents. This information is supported by noticeable presence of flavonoids, flavonols, saponins and tannins in this extract. Acetone had the least extraction potential as compared to other extracts, but alkaloids which seemed to be rare in this plant, was detected only in acetone extracts. Phenols were moderately detected in ethanol and hydro-ethanol extracts, while it was slightly present in acetone and water extracts. Interestingly, steroids and terpenoids were totally absent in all the extracts.

It is worthy of mention that information provided by phyto-chemical screening assays was important in determining the level and ratio composition of compounds present in the root part of *M. laciniata*. This is because knowing the

level or percentage of compounds could support and or give guidance on which compounds are active or relatively inactive in the respective assays. These results could also be helpful in future studies where isolation of compounds of *M. laciniata* is intended to be carried-out.

3.2 Quantitative phyto-chemical analysis of *M. laciniata* root extracts

The quantification of the detected phyto-chemicals such as flavonols, flavonoids, alkaloids, phenols, saponins and total tannins in all the extracts of *M. laciniata* root are represented in Table 2.Hydro-ethanol extract revealed the high quantity of total flavonoids as compared to other extracts tested in this study; the content was estimated to be 0.632 µg QE/100mL extract. This was followed by water with 0.402µg QE/100mL extract with the least extract being acetone having flavonoids content of 0.200µg QE/100mL extract, all results are expressed as quercetin equivalence (QE).

Similarly, the estimation of flavonols was found to be the highest in hydro-ethanol extract (0.151 mg/g) with acetone extract (0.105 mg/g) being the least when compared with other extracts. All values as expressed as rutin equivalence (mg RE/100g extracts).

Quantitative analysis revealed total phenolic content was estimated to be the highest in hydro-ethanol extract (0.035 mg/g), as established in the two previous

phyto-chemicals, followed by acetone extract with about 0.031(mg/g) content. Results are expressed as mg/g gallic acid equivalence (mg GAE/100g extracts).

The estimated content of total tannins was almost similar to the content of total phenols, since again hydro-ethanol extract showed the highest estimation of total tannins content as compared to other extracts, the content was estimated to be 0.032(mg/g). On the other hand acetone extracts seemed to have the lowest total tannins content related to its counterpart, the content was estimated to be 0.019(mg/g) recorded as milligram per gram gallic acid equivalence (mg GAE/100g extracts).

The content percentage of alkaloids and saponins was calculated from extraction of crude plant material (CPM). Thus the extracts used in this study were not involved in the calculation. With alkaloids the percentage extracted was 27%, while saponins were much higher with 78%. Even though alkaloids were only detected in acetone extract it was still valuable to estimate the content of them, especially since they may contribute to medicinal activity of this plant.

3.3 Antioxidant activity of *M. laciniata* root extracts.

The antioxidant activity of *M. laciniata* root was linked to the presence of secondary constituents revealed in this study. It was observed that water extract showed the best activity in four (nitric oxide, metal chelating, hydroxyl radical and

reducing power) of six tested free radical assays as revealed from half inhibitory concentration(IC_{50}) values of 0.431, 0.512,0.513 and 0.712 mg/mL respectively when statistically ($p< 0.05$) compared with other extracts. However, the activity of the standard in the reducing power, metal chelating and nitric oxide assays was significantly better than that of water extracts (Table 3). DPPH and hydrogen peroxide assays indicated that ethanol and hydro-ethanol extracts (IC_{50}: 0.602, 0.306 mg/mL) had the best activity respectively and significantly different when compared to other extracts.

DPPH radical scavenging assay is one of the most important assays in evaluating the antioxidant potential of medicinal plants. There was an increase in inhibition of DPPH radical with increasing concentration of the extracts (Hydro-ethanol, ethanol, acetone and water) and they all showed good activity which compared favorably with the standard. The ethanol extracts revealed the best inhibition of DPPH radical at 75 % followed by hydro-ethanol at 63 % at the highest concentration of 1 mg/mL though vitamin C showed the highest inhibition at almost 90% (Figure 3).

Regarding reducing power assay, all the extracts (hydro-ethanol, ethanol, acetone, and water) showed good activity which was comparable to the standard used. Water and ethanol extracts showed good reducing power activity at 54 and 47 % respectively at highest concentration of 1 mg/mL even though the standard

showed the highest inhibition at 76 % (Figure 4). All extracts showed concentration dependent activity except acetone extracts which showed to have poor inhibition (36 %) at the highest concentration (1 mg/mL).

Metal chelating assay was conducted to determine the capability of extracts to convert Fe^{3+} to Fe^{2+}. The standard used showed highest percentage inhibition at highest concentration used (1 mg/mL) with 84 %. However, all extracts showed concentration dependent activity except acetone extracts which showed poor percentage inhibition at 41 % at 1 mg/mL though its highest inhibition was witnessed at 0.6 mg/mL (45%). Fruitfully, water and ethanol extracts revealed better percentage inhibition following the standard at 75 and 72 %respectively as compared to other extracts (Figure 5).

There was an increase in percentage inhibition of hydrogen peroxide radicals with increasing concentration of most of the extracts (0.2 – 0.8 mg/mL), and they all showed good activity which compared favorably with the standard used (Figure 6). However, hydro-ethanol extracts showed better percentage inhibition at 62 % (0.8 mg/mL), which was followed by ethanol extracts 58 %. Acetone established the poorest percentage inhibition (44%) as compared to other extracts used. The standard used was better than all extracts tested; it showed best inhibition activity at 83 % (Figure 6).

Hydroxyl radical scavenging assay was premeditated to determine whether hydroxyl radicals can be scavenged by the four *M. laciniata* root extracts in comparison with the standard used in this assay. The standard used showed good scavenging potential with 79 % inhibition at the highest concentration used (1 mg/mL). However, water extracts surpassed this inhibition to give 89 % and in terms of extract activity, it was followed by hydro-ethanol with 46 % inhibition. Ethanol extract which seemed to be performing well in the previous antioxidant assays showed the weakest percentage inhibition at 36 % (Figure 7).

Nitric oxide scavenging assay also showed good concentration dependent activity of all *M. laciniata* extracts as well as the standard used (citrate). At the highest concentration of 1 mg/mL, citrate depicts the highest percentage inhibition at 89 % followed by hydro-ethanol extract with 75 % inhibition. Water extract showed to be closely comparable with hydro-ethanol extract with 72 % inhibition. However, ethanol extract presented the least and poorest activity at 63 % inhibition (Figure 8).

3.4 Antidiabetic activity of *M. laciniata* root extracts.

The inhibition of alpha glucosidase by *M. laciniata* root was tested on the four extracts (hydro-ethanol, ethanol, water and acetone) to depict the extracts with the best potential to inhibit the activity of this enzyme. It was observed that there

was an increase in the inhibition of the enzyme with increasing concentration of the extract; hydro-ethanol showed the best percentage inhibition with 84 % at the highest concentration used (0.8 mg/mL). Excluding the standard (74 %), hydro-ethanol extract was followed by acetone with 44 %, while water extract showed the poorest inhibition at 31 % (Figure 9).

Going by half inhibitory concentration (IC_{50}) value, hydro-ethanol extract again revealed the best activity as revealed from the lowest IC_{50} valueof0.07 mg/mL which is statistically significant ($p<0.05$) when compared with other extracts and the standard. This was followed by acetone extracts with 0.75 mg/mL IC_{50} value. Ethanol and water extracts showed poor inhibition of alpha glucosidase with IC_{50} value of0.87 and 1.22 mg/mL respectively (Table 4).

Hydro-ethanol extract is said to be accorded the best hypoglycaemic activity as established from strongest α-glucosidase inhibition and mild alpha amylase assays, thus qualified it for mode of inhibition (Table 4). The mode of inhibition of hydro-ethanol extract on α-glucosidase activity was determined using Lineweaver-Burk plot which showed that the extract exhibited uncompetitive inhibition of enzyme activity (Figure 10).

The inhibition of alpha amylase by *M. laciniata* root was similarly examined on four extracts (hydro-ethanol, ethanol, water and acetone) to depict the extracts with the best potential at inhibiting the activity of the enzyme. Ethanol extracts showed better percentage inhibition with 71 % at the highest concentration used (1 mg/mL), it was then followed by hydro-ethanol with 63 %. Meanwhile acetone extract showed the poorest percentage inhibition with 33 %. However, acarbose gave better percentage inhibition of α-amylase enzyme than all *M. laciniata* root extracts with 88% at the highest concentration tested (Figure 11).

Going by IC_{50} value, ethanol extract showed good α-amylase enzyme inhibiting potential when statistically compared ($P< 0.05$) to other extracts with IC_{50} value of 2.11 mg/mL. However, acarbose showed to have best inhibition of this enzyme with lowest IC_{50} value of 0.60 mg/mL. Aside acarbose, ethanol extract was followed by hydro-ethanol extract which showed moderate IC_{50} value of 3.38 mg/mL. Meanwhile acetone extract revealed to be weakest inhibitors of α-amylase with an IC_{50} value of 6.78 mg/mL (Table 4). Statistical analysis showed significant difference ($P< 0.05$) among all extracts and the standard used in this assay.

Acetone extract showed the mildest activity against α-amylase, though followed by hydro-ethanol and since this extract established the strongest inhibition against alpha glucosidase thus qualified it as a good antihyperglycaemic agent, hence, adopted for mode of inhibition assays. The mode of inhibition of

hydro-ethanol extract on α-amylase activity was determined using LineWeaver-Burk plot which showed that the extract exhibited uncompetitive inhibition of enzyme activity (Figure 12).

Table 1. Qualitative phytochemical screening of extracts from*M. laciniata* root.

Inference				
Phyto-chemicals	Hydro-ethanol	Ethanol	Acetone	Water
Alkaloids	-	-	+	-
Flavonoids	+++	++	-	++
Flavonols	++	+	-	++
Saponins	+++	++	++	+++
Tannins	++	++	++	++
Steroids	-	-	-	-
Terpenoids	-	-	-	-
Cardiac glycosides	+	+	+	+
Phenols	++	++	+	+

(-) not present, (+) present, (++) moderately present, (+++) highly present.

Table 2**.** Quantitative phyto-chemical analysis of extracts and crude plant material of *M. laciniata* root.

Extracts	T.flavonoids	T.flavonols	T.phenols	T.tannins	Alkaloids	Saponins
HeE	0.632 ± 0.02^{a}	0.151 ± 0.0^{a}	0.035 ± 0.02^{a}	0.032 ± 0.02^{a}		
EE	0.267 ± 0.01^{b}	0.129 ± 0.0^{b}	0.026 ± 0.02^{b}	0.028 ± 0.02^{a}		
AE	0.200 ± 0.01^{b}	0.105 ± 0.0^{c}	0.031 ± 0.04^{c}	0.019 ± 0.01^{b}		
WE	0.402 ± 0.01^{c}	0.148 ± 0.4^{a}	0.026 ± 0.01^{b}	0.025 ± 0.01^{a}		
PS1					27%	
PS2						78%

Values were performed in triplicate and represented as mean±SEM.

Values with different superscript letters along the same column are statistically different from each other at ($p<0.05$). HeE: Hydro-ethanol extract, EE: ethanol extracts, AE: acetone extracts, WE: water extracts, CPM: crude plant material and is presented in percentage, T: total, mg QE/100mL extracts, mg RE/100g extracts, mg GAE/100g extracts,

Table 3.Antioxidant capacity (IC_{50}) of extracts from*M. laciniata*root

Extracts	IC_{50} values (mg/mL)					
	DPPH radicals	**Reducing power**	**Metal chelating**	**Hydrogen peroxide**	**Hydroxyl radicals**	**Nitric oxide**
Standards	0.648±0.557[a]	0.205±0.03[a]	0.454±0.16[a]	0.152±0.05[a]	0.570±0.15[a]	0.255±0.16[a]
HeE	0.645 ±0.102[a]	1.340±0.10[b]	0.636±0.04[b]	0.306±0.02[b]	1.235±0.11[b]	0.644±0.11[b]
EE	0.602±0.034[b]	0.950±0.17[c]	0.620±0.09[b]	0.384±0.02[b]	1.059±0.18[c]	0.657±0.04[b]
AE	0.795±0.077[c]	1.050±0.03[d]	2.580±0.06[c]	0.685±0.03[c]	1.099±0.03[c]	0.817±0.04[c]
WE	0.838±0.087[d]	0.712±0.07[e]	0.512±0.00[d]	0.405±0.01[d]	0.513±0.04[a]	0.431±0.14[d]

Values are represented as mean±SEM, of triplicates determinations. HeE: hydro-ethanol, EE: ethanol, AE: acetone, WE: water. Values with different superscript letters along the same column are statistically different from each other at ($p<0.05$)

Table 4.Inhibitory effects of *M. laciniata* root extracts on the activities of α-amylase and α-glucosidase enzyme

IC $_{50}$ values (mg/mL)		
Extracts	**Parameters**	
	α-Glucosidase	**α-Amylase**
Standard	0.24 ± 0.17^{a}	0.60 ± 0.19^{a}
HeE	0.07 ± 0.01^{b}	3.38 ± 0.01^{b}
EE	0.87 ± 0.01^{c}	2.11 ± 0.03^{c}
AE	0.75 ± 0.02^{d}	6.78 ± 0.10^{d}
WE	1.22 ± 0.03^{e}	5.97 ± 0.13^{e}

Values are represented as mean±SEM, and were performed in triplicate. HeE: hydro-ethanol, EE: ethanol, AE: acetone, WE: water.Values with different superscript letters along the column are statistically different from each other at ($p<0.05$)

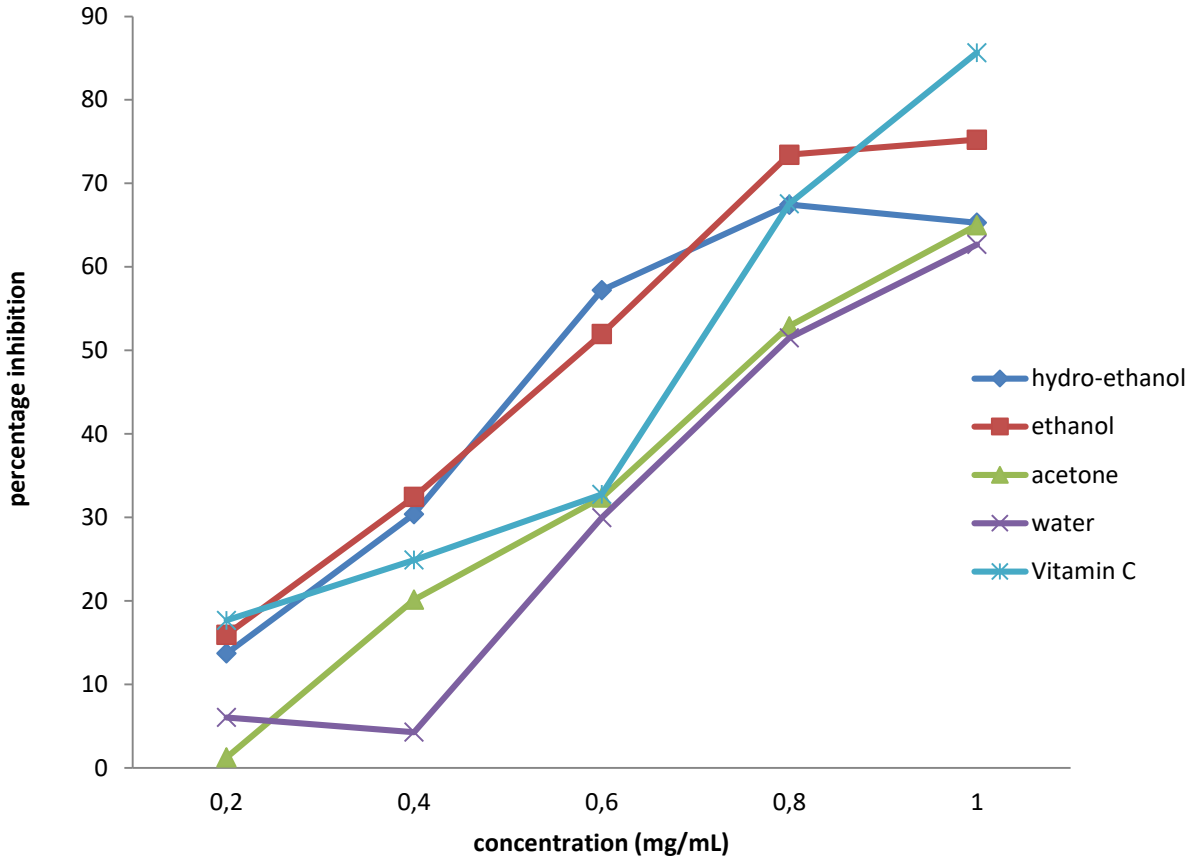

Figure 3. Percentage inhibition on the DPPH radical assay of hydro-ethanol, ethanol, acetone and water extracts of M.*laciniata* root.

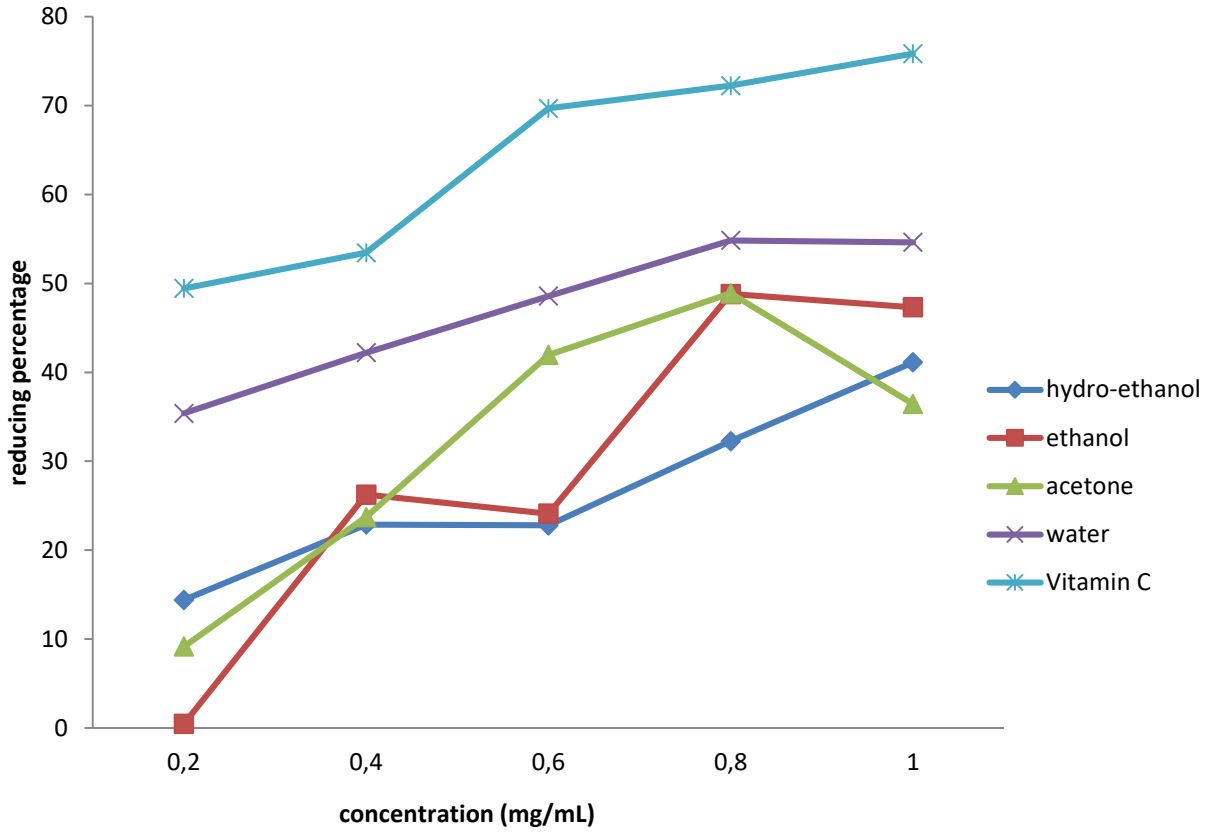

Figure 4. Percentage inhibition of hydro-ethanol, ethanol, acetone and water extracts of M.*laciniata* root on reducing power of free radicals.

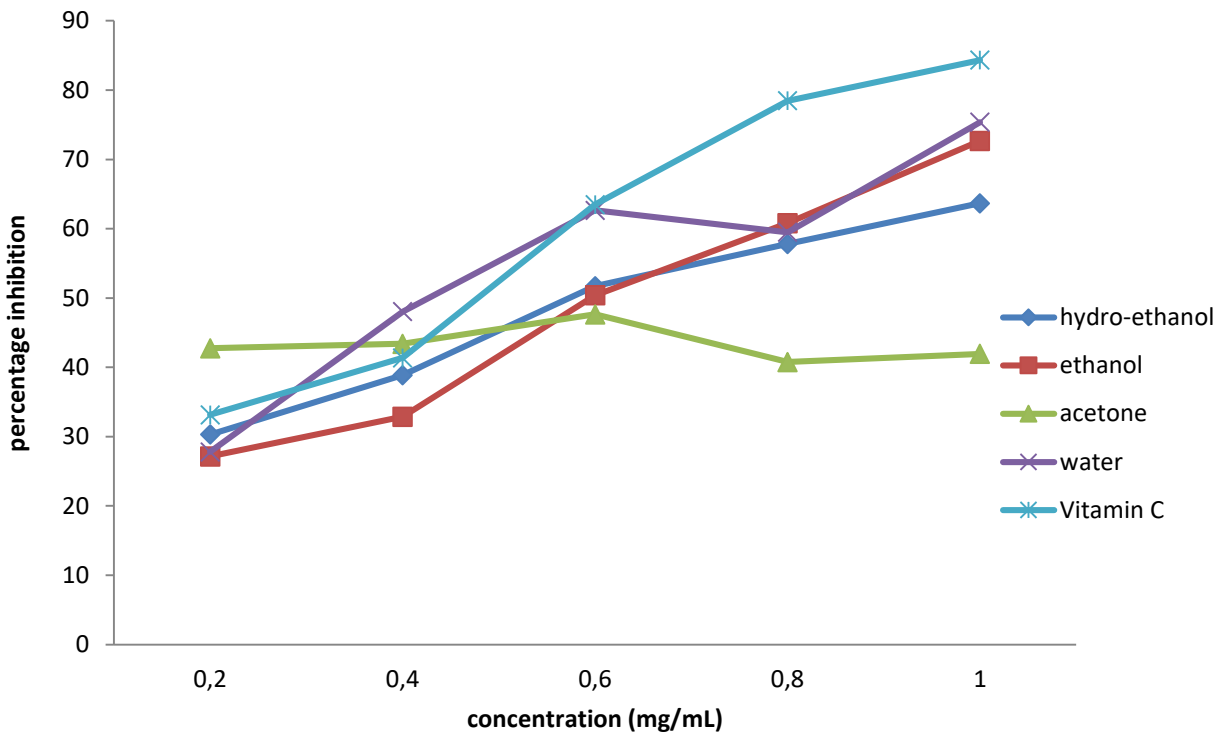

Figure 5. Percentage inhibition of metal free radicals by hydro-ethanol, ethanol, acetone and water extracts of M.*lacianta* root.

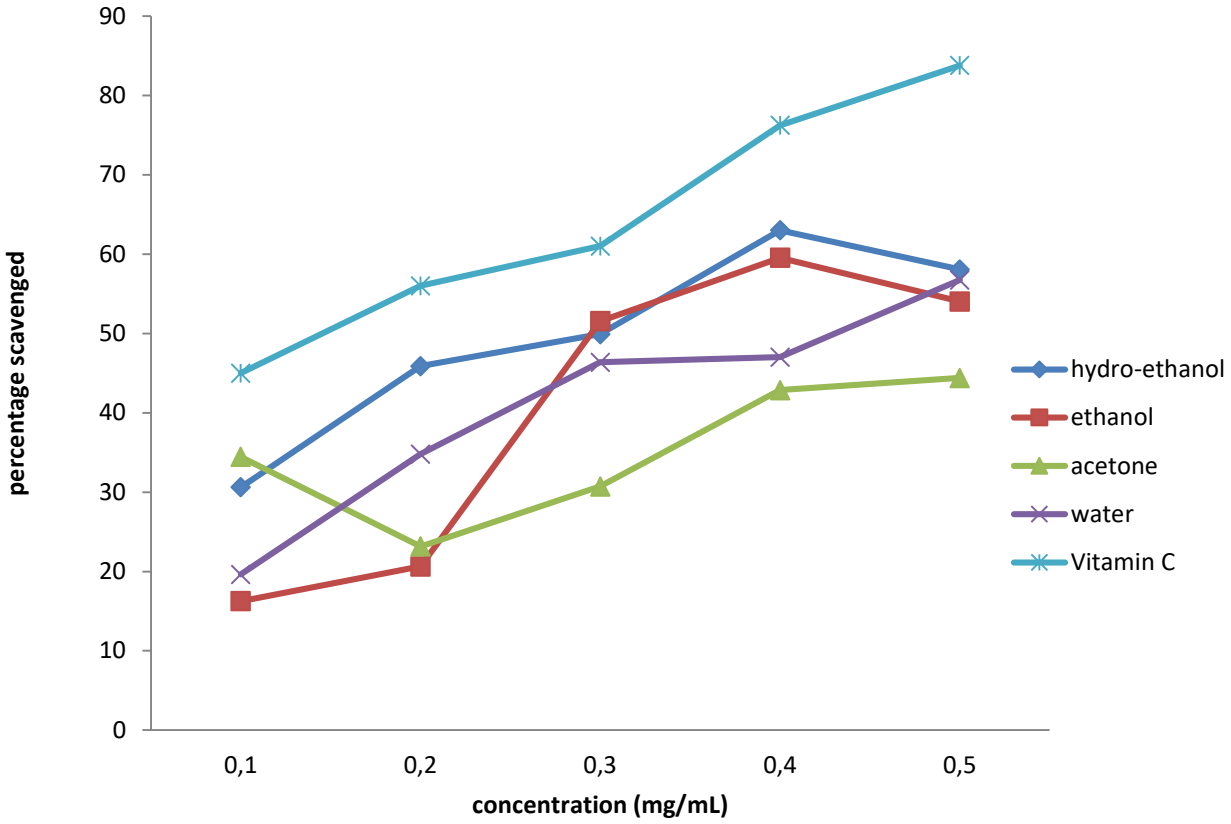

Figure 6. Percentage inhibition of hydrogen peroxide radicals by hydro-ethanol, ethanol, acetone and water extracts of M. *laciniata* root.

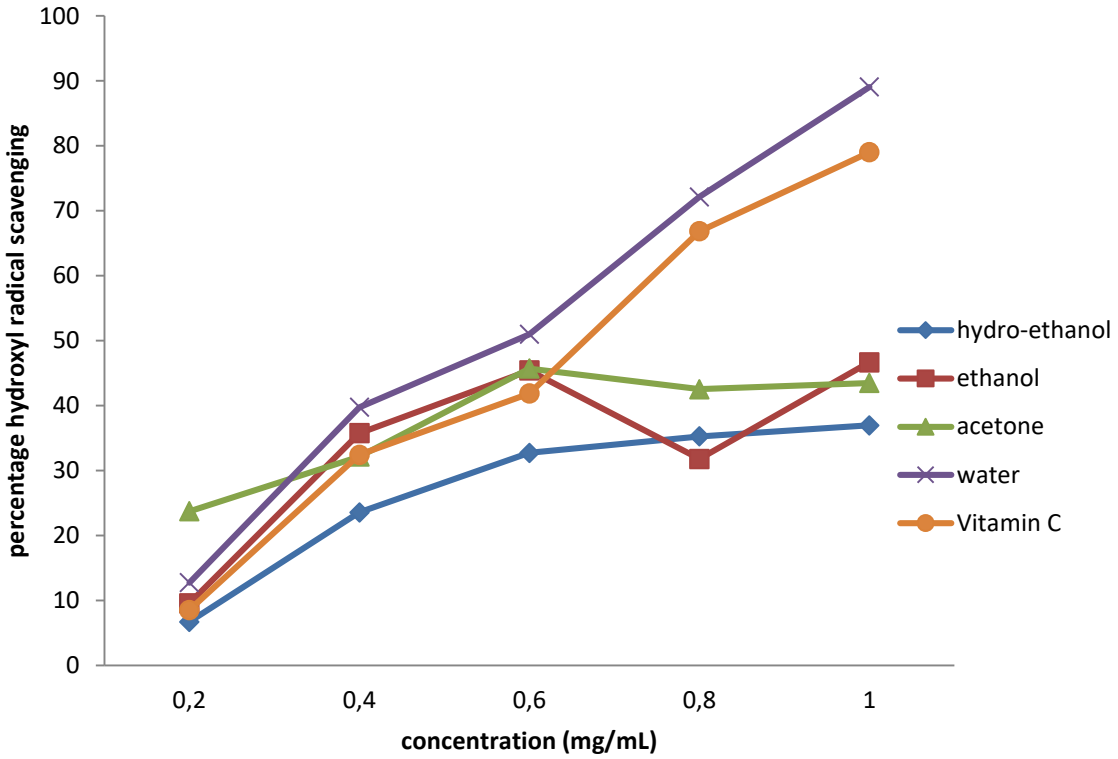

Figure 7**.** Percentage inhibition of hydroxyl radical activity by hydro-ethanol, ethanol, acetone and water extratcs of M.*laciniata* root.

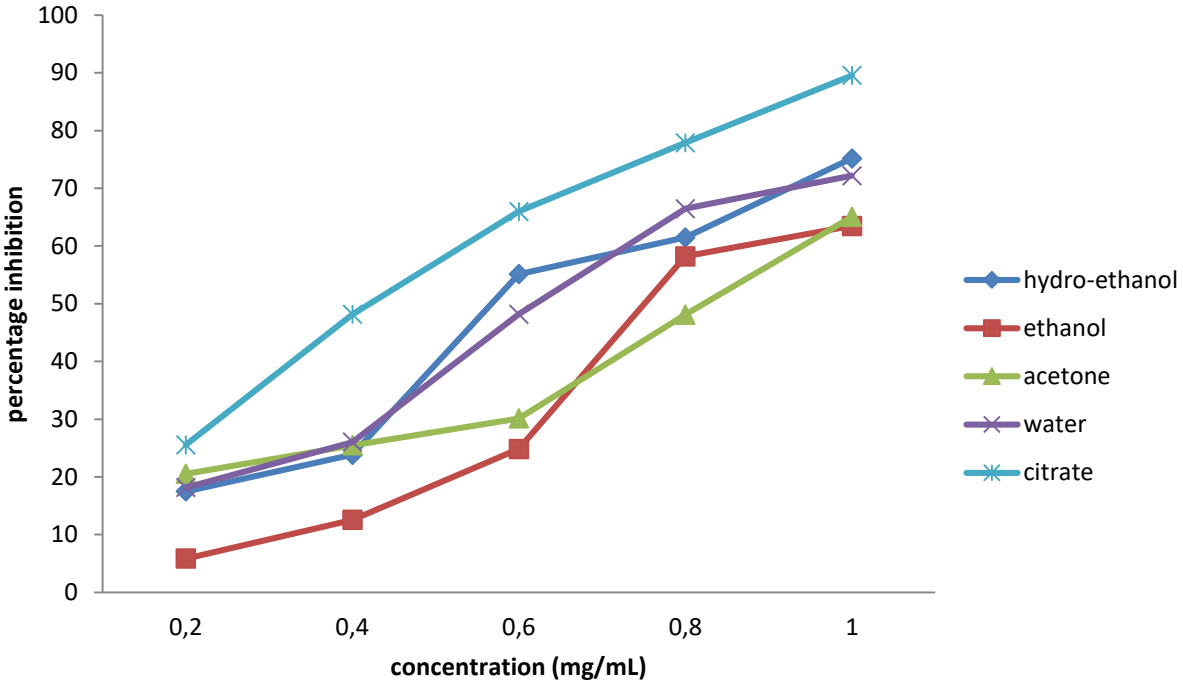

Figure 8**.** Percentage inhibition of nitric oxide radical activity by hydro-ethanol, ethanol, acetone,and water extracts M.*laciniata* root.

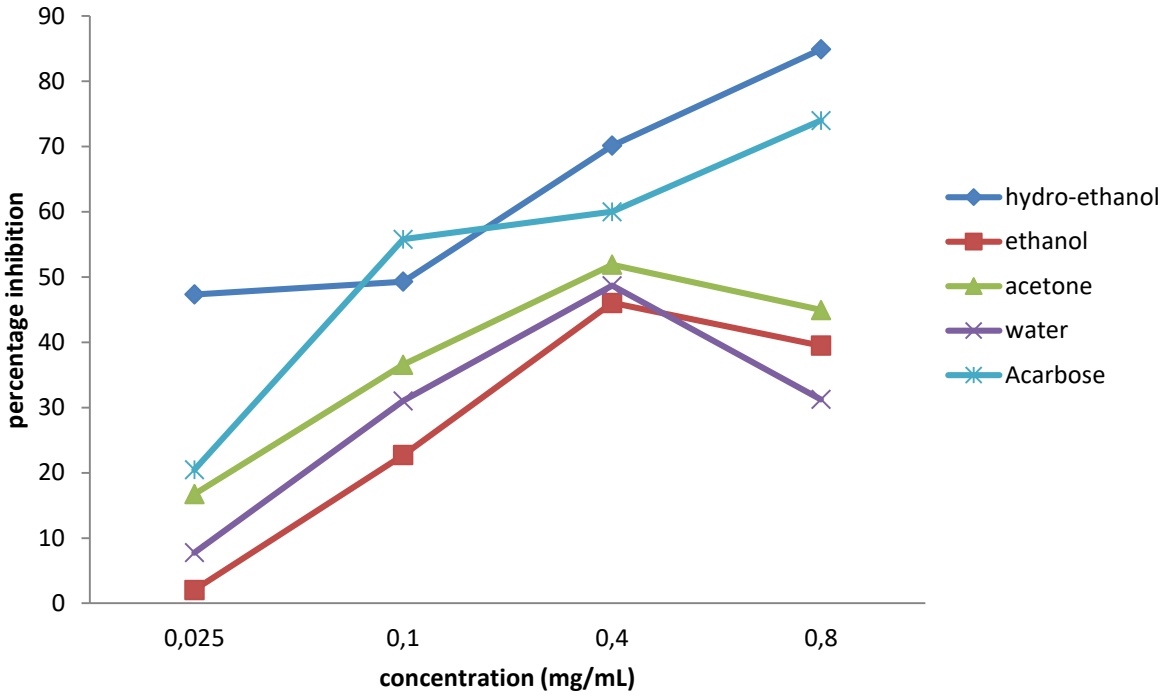

Figure 9**.** percentage inhibition of glucosidase activity by hydro-ethanol, ethanol, acetone, and water extracts of M.*laciniata* root.

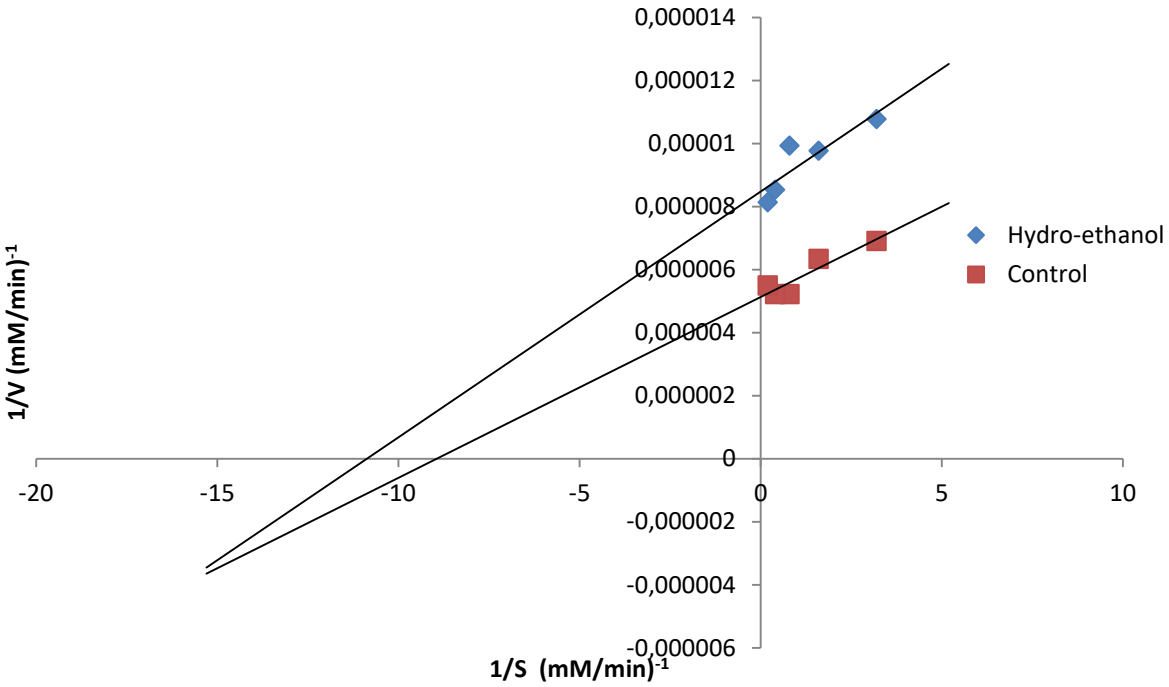

Figure 10. Uncompetitive inhibition of α-glucosidase by hydro-ethanol extract of *M. laciniata* root.

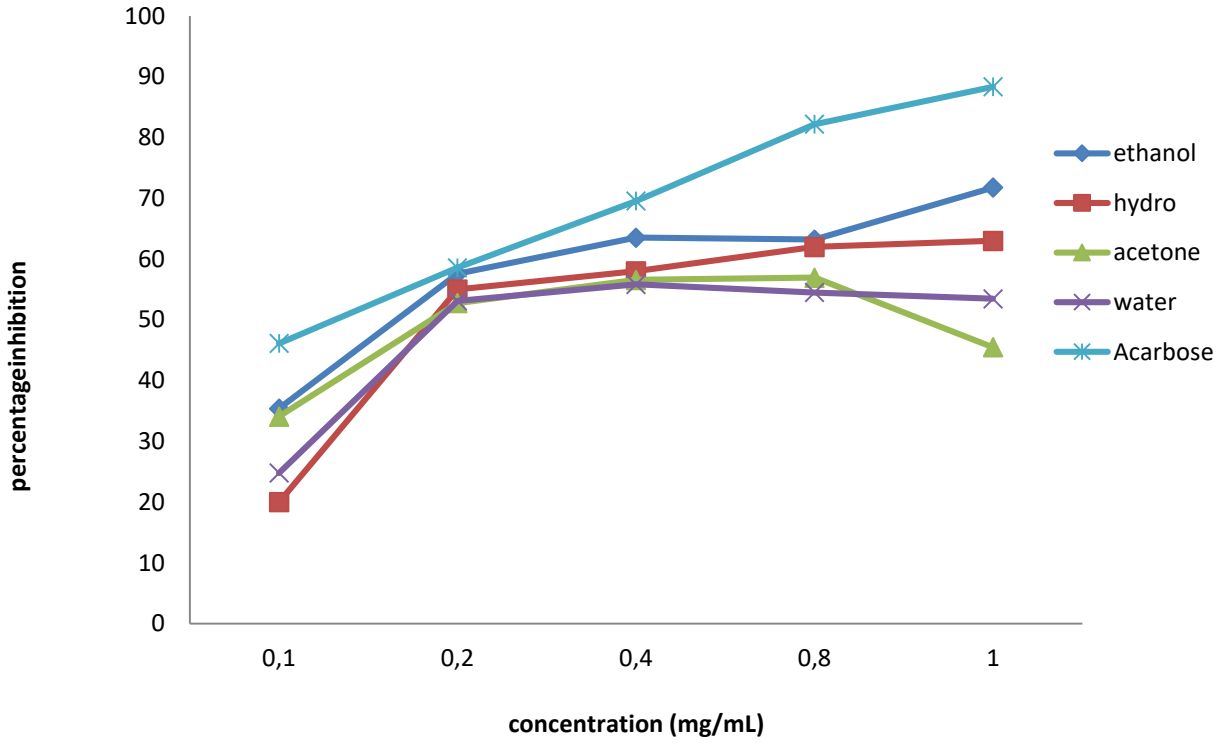

Figure 11. Percentage inhibition of amylase activity by hydro-ethanol, ethanol acetone and water extracts of *M.laciniata* root.

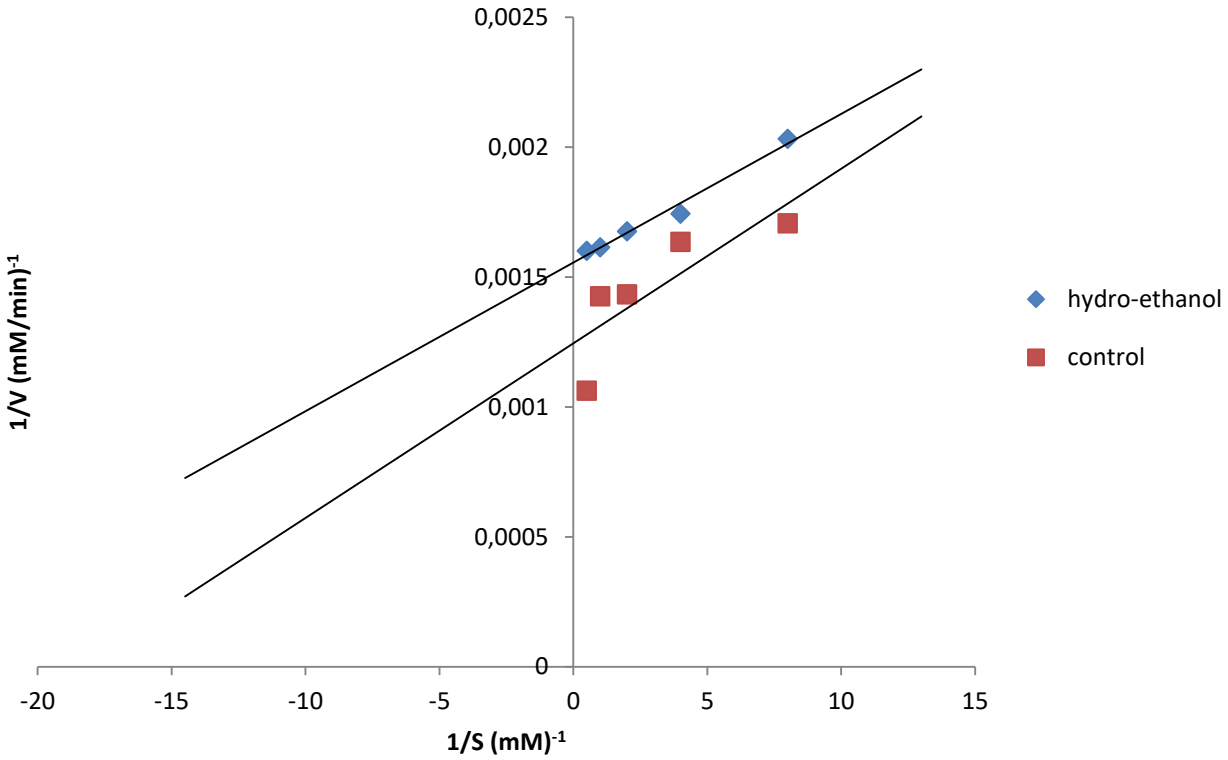

Figure 12. Uncompetitive inhibition of α-amylase by hydro-ethanol extract of *M. lacinicta* root.

Chapter 4

Discussion

Phyto-chemical screening is one of the essential methods in phyto-medicine especially since plants of the same species may differ in terms of constituents available, these differences may normally be triggered and influenced by external factors like climatic condition, as well as landscape (Kokate *et al*., 2004). Moreover, medicinal properties of plants lies in their phyto-chemical composition, which have a defined physiological action on man's body structure (Deepa *et al*., 2013).

In this study phyto-chemical screening was conducted to evaluate different bio-active compounds present in *Medigaco laciniata* (L) Mill root extracts, which may be responsible for the medicinal property of the plants (Ahmad *et al.*, 1999). The evaluation of phyto-constituents of *M. laciniata* root revealed the presence of flavonoids, flavonols, saponins, alkaloids, tannins, and phenols while steroids and terpenoids were not detected.

Flavonoids are known for their ability to act as anti-inflammatory, anti-allergic, and anti-cancer agents. Additionally, they have been reported to have strong antioxidant activity (Buragohain, 2015). Flavonols are one of the subclasses

of flavonoids, thus have and share closely related properties like the ones mentioned above.

Saponins, well known for their variety of benefits such as reduction in blood cholesterol level, lowering cancer risk and bone loss, as well as exhibiting antioxidant potentials may also be capable of improving immune response in animals (Madhu *et al*., 2016). Additionally, Manickam *et al.* (1997) reported that saponins could be used as hypoglycemia, anti-inflammatory, and weight loss agent.

Alkaloids are characterized by having nitrogen and they are said to have the ability to reduce stress and depression symptoms. However in higher doses they maybe poisonous, producing abnormal excitation related nerves disorder (Jisika *et al*., 1992; Obochi, 2006; Madhu *et al*., 2016,). Moreover, previous reports revealed that alkaloids have been used as both anti-bacterial and antidiabetic properties, and showed to be active in these pharmacological activities (Nadu, 2016)

Tannins may be dangerous or beneficial depending on the amount and type consumed or used. A lower to moderate concentration is likely to improve the functioning of digestive system by reducing degradation of protein in the small intestine (Butter *et al*., 2000).

Phenols are the largest metabolites found in medicinal plants. They are able to treat and control many disorders like aging, inflammation, carcinogen,

atherosclerosis, and cardiovascular diseases. Moreover, they are also associated with having antioxidant property by being able to inactivate lipid free radicals or by preventing the decomposition of hydrogen peroxide into free radicals (Nasapol *et al*., 2010; Senguttuvan *et al*., 2014). Additionally, phenolic compounds are known for their nutritional value, and to increase appetite by providing color, taste and aroma (Memnune *et al*., 2009) to food substances.

Many chronic disorders like cirrhosis, atherosclerosis, cancer, diabetes and others are associated with oxidative stress which is caused by uncontrolled lethal oxidative reactions (Wilson, 1988). Oxygen species also referred as free radical, have the potential to stimulate, escalate, and empower complications related to DM. However, oxidants have a vital function endogenously; they normalize the processes of metabolism in target cells, thus are produced by the body in order to perform this function. However, excess production and accumulation of these oxidants in the system can lead to cellular damages which directly and indirectly contribute to complications associated with chronic diseases like DM (Sen *et al*., 2010). On the other hand, anti-oxidative agents are capable of scavenging free radicals or oxygen species, consequently halting and inhibiting the destruction and alterative activity of healthy cells and molecules (Yamagishi and Matsui, 2011).

The present study revealed that *M. laciniata* root have antioxidative properties going by the different radical scavenging assays like DPPH, reducing power, metal chelating, hydrogen peroxide, hydroxyl radical, and nitric oxide employed. However, according to the results collected from the above mentioned assays, extracts did not show convincing correlation from one assay to the other, this behavior of extracts was also reported by Sabu and Kuttan (2002). The reason behind this behavior might be attributed to the relationship between the presence and amount of required metabolite(s) to scavenge a specific free radical(s) or oxygen species. Most of the metabolite identified and quantified in this investigation are said to have anti-oxidative activity, but these oxidants are characterized into several groups depending on the oxygen species each group independently contain.

The contradicting results between phyto-chemical screening assays and antioxidant assays in this investigation are mostly observed between water and hydro-ethanol extracts. Hydro-ethanol extracts showed convincing content of metabolites while water extracts showed moderate content of metabolites. However, in terms of antioxidant assays, water extracts showed better scavenging potential than hydro-ethanol and other extracts. The reason for this contradiction might be due to the type(s) of metabolite(s) extracted since different solvents (hydro-ethanol, ethanol, water, and acetone) with varying polarity were used for

extraction in this study. It has been reported that polarity level plays a major role in extraction of metabolite (Chasemzadeh *et al*., 2001). Hydro-ethanol is a mixture of water and ethanol, therefore it might have occurred that metabolites extracted by this solvent were many but were also not 100 percent untainted, hence hydro-ethanol extracts confirmed most of the vital metabolites but did not show best scavenging potential of free radical. In antioxidant assays, Johanna and Jyh-lun (2007) mentioned that metabolites like phenols, alkaloids and flavonoids are large compounds comprised of sub-classes that fall under their names. It is suspected that metabolites extracted by hydro-ethanol might be sub-classes of highly inactive scavengers of specific free radicals. To support the present findings, Sabu and Kuttan (2002) also reported that potential of extract to scavenge oxygen radicals depend upon the type of radical encountered.

The results discovered from respective assays in this study show a noticeable relationship between phyto-chemical, antioxidant and antidiabetic assays, even though few contradiction are also spotted. Metabolites quantified during phyto-chemical screening can be linked to antidiabetic potentials of medicinal plants (Atangwho *et al.*, 2009). Similarly, antioxidant activities revealed in this study can be attributed to the elicited antidiabetic potentials of *M. laciniata* root. Previous reports suggest that the etiopathogenesis of chronic disorder like DM, cancer,

aging and others are linked to oxidative stress caused by free radicals of oxygen species resulting in cell or tissue damage (Aiyegoro and Okoh, 2010).

Diabetes mellitus (DM) is one of serious leading diseases capable of killing human race (Narkhede *et al*., 2011), thus causing serious or severe health challenges to the current population and future generation. It was estimated that more than 171 million people globally, are suffering from complications of DM (Kamtekar *et al*., 2014). In fact, World Health Organization (WHO) projected in one of their reports that the prevalence of the disease is expected to increase to 366 million by 2030 (Wild *et al*., 2004).Diabetes is characterized by several symptoms like weight gain, sores, fatigue, excessive thirst, and others. Control measures in diabetes and management of these symptoms include regular exercise, the use of OHAs such as sulphonyl ureas (glibenclamide), biguanides (metformin), insulin etc, as well as following a particular diet regimen such as limiting the consumption of carbohydrates and fats or oil. It is interesting to note that the failure to comply with the above directives or dosage or dietary regimen would or might trigger complications attributed to hyperglycemia (high blood glucose) including but not limited to nerve damage (neuropathy), kidney failure (nephropathy), eye disease (retinopathy), foot problems caused partly due to poor blood flow, as well as skin problem such as bacterial and fungal infections.

Hyperglycemia results perhaps due to failure of target cells to absorb adequate amount of carbohydrates and fats or oil end products (glucose) molecules from the blood stream, thus results in high concentration of this saccharide in the blood stream. Lack of absorption of these molecules into cells is associated to low insulin concentration in the blood stream, and or the rejection of insulin by target cells (Sivajothia *et al*., 2008). Alpha amylase and alpha glucosidase enzymes are responsible for breaking down carbohydrates into maltose, sucrose and or eventually glucose. Thus, complications related with or emanating from DM may be controlled or managed by inhibiting the activities of these enzymes. In the past or many decades ago, synthetic OHAs are used to combat and control this condition as well as other related conditions but they come with side effects and do not restore glucose homeostasis (Akanksha *et al*., 2010). Moreover DM is a chronic disease that requires longer time to manage, thus the possibility of these conventional drugs leaving side effects behind is eminent. In line with the afore-mentioned, the use of medicinal plants with little or no side effects is in recent times embraced as panacea to ill-effects of synthetic medicines. Therefore, the current study also ropes the importance of medicinal plants as antidiabetic agents.

Inhibition of α-amylase and α-glucosidase enzymatic activities is believed to reduce or control the blood glucose level whose elevated concentration in the system is related to diabetic conditions. A condition arising either from

dysfunctional insulin, or lack of insulin secretion due to damaged pancreatic beta cells and or high rate of hydrolysation of starch to maltose in a chemical reaction catalyzed by alpha amylase. Subsequently, alpha glucosidase catalyse the conversion of disaccharides into glucose available for absorption into the blood stream. Antidiabetic results from *M. laciniata* root showed dose-dependent inhibition of both alpha glucosidase and amylase in all extracts used. Additionally, hydro-ethanol extracts showed best inhibition activity of glucosidase as compared to other extracts. Contrary, ethanol extracts showed to be better than hydro-ethanol extracts on inhibition of amylase activity. Contrary behavior of these two extracts might be triggered by different acts, composition and location of glucosidase and amylase enzyme respectively. Therefore complicity of amylase might overpower the inhibition activity of hydro-ethanol may due to being semi-volatile, and be suppressed by ethanol extracts may be simply because ethanol is more volatile, thus attained complete constituents. During phyto-chemical screening assays hydro-ethanol extract contained higher quantity of flavonols, flavoinoids, phenols and tannins as compared to other extracts. As a result, there is significant relationship between metabolites extracted and inhibition of alpha amylase enzyme. The same collaboration behavior between metabolites and inhibition of metabolic enzymes like alpha amylase and glucosidase was reported by Kamtekar *et al*., (2014). Results from this investigation suggest that *M. laciniata* root have

antioxidant and antidiabetic properties, and therefore may be one of the alternatives for the management and control of type 2 diabetes mellitus.

Chapter 5

Conclusion

The support and evidences provided by relevant assays in this study, help in concluding that *Medicago laciniata* (L) Mill root extracts contain useful bioactive secondary metabolites, like flavonoids, phenols, tannins, alkaloids and saponins. Additionally, holistic approach of these metabolites in combating free radicals and stabilizing oxidative stress is highly recognized. The study also buttressed that more than one solvent (aqueous and alcoholic) could be employed to extract metabolites from this plant as this might ensure high medicinal activity since all or both hydrophobic and hydrophilic metabolites may possibly have a chance to be extracted and surface their true medicinal potential. Thus suggests that medicinal plants like *M. laciniata* root can be used as alternative or to derive healthier synthetic drugs to manage oxidative stress, and act as antidiabetic agents. However, since Basotho tribe of Eastern Free State use this plant to control DM, findings from this study therefore supports the folkloric use of *M. laciniata* root.

Future studies are suggested to be conducted on *Medicago laciniata* (L) Mill extracts with respect to isolation of bio-active compounds responsible for elicited antidiabetic activity.

Contribution to Knowledge

- Adding knowledge to the science world, more specifically on medicinal plants studies
- Sharing information with locals on laboratory proven facts about *M. laciniata* root.
- Contributed positively to ingredient of newer healthier drugs to fight against the disablers and killers like diabetes mellitus and other diseases.

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Appendix

Preparation of reagents.

1. Rutin Preparation

20 g of aluminium chloride and 50 g of sodium acetate anhydrous powder were separately dissolved in little quantity of distilled water and made up to 1.0 L with distilled water respectively

2. Gallic acid stock solution

Gallic Acid Stock Solution was prepared as follows: in a 10 mL volumetric flask, 0.05 g of dry gallic acid was dissolved in 1 mL of ethanol and diluted to volume with distilled water.

3. Sodium carbonate

Sodium carbonate (0.1 M) was prepared as follows: 20 g of anhydrous sodium carbonate was dissolved in 80 mL of water and brought to boil. After cooling, few crystals of sodium carbonate were added to the solution. The solution was filtered 24 hrs later and 100 mL of distilled water was added to the filtrates.

4. Calibration curve

Preparation of a calibration curve was constructed as follows: 0.1, 0.2, 0.3, 0.5, and 1 mL of above phenol stock solutions was added to 10 mL volumetric flasks respectively, then diluted to the volume with distilled water. These solutions were then having phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/mL gallic acid, the effective range of the assay. Leftover gallic acid solution was poured down the drain.