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Chemical profiling, *in vitro* antioxidant and antidiabetic assessment of flavonoids from the ethanol extract of *Hermannia geniculata* root

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Abstract

Determination of the *in vitro* antioxidant and the inhibitory potential of flavonoids from *Hermannia geniculata* (FHG) roots on diabetes-linked enzymes was carried out. The chemical profiling of FHG roots extract was investigated using High Pressure Thin Layer Chromatography (HPTLC) fingerprint analysis. The reactive oxygen scavenging potential of the extract was analyzed. Starch solution (1%) was reacted with different concentrations of FHG extract to determine the α-amylase inhibitory potential of the extract while α- glucosidase inhibition assay was carried out through incubation of different concentrations of the extract followed by addition of p-ntrophenyl-α-D-glucopyranoside solution. HPTLC results indicated the presence of flavonoids/ phenol-carboxylic acid, and Kaempferol (Rf 0.80) were detected in the extract with retention factor Rf. ranging from 0.08 to 0.95. FHG extract showed commendable antioxidant properties with IC50 values (3.07± 0.12, 2.13± 0.67) µg/mL for 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic) acid (ABTS) radicals which were lower and significantly different (p<0.05) compared to standard silymarin with IC50: (3.55± 0.10, 2.77± 0.75) µg/mL for DPPH and ABTS respectively. The results indicated mild inhibition of α-amylase with IC50: (5.55± 0.37) µg/mL which was higher and significantly different (p<0.05) from acarbose with IC50: (3.81± 0.29) µg/mL. Moreover, the extract showed 73% inhibition of α-glucosidase. Kinetic studies of FHG extract revealed competitive and mixed non-competitive inhibition of α- amylase and α-glucosidase respectively. This study indicated FHG capabilities of scavenging reactive oxygen species and reducing hydrolysis of starch responsible for post-prandial hyperglyceamia seen in type 2 diabetes mellitus.

Keywords: Antidiabetic, Antioxidant, Flavonoids, *Hermannia geniculate,* HPTLC

Introduction

Diabetes mellitus (DM), a metabolic disease associated with increased hyperglycaemia and subsequent organopathy is one of the most common diseases affecting 463 million people worldwide

(Saeedi *et al*., 2019), while the global prevalence of DM in dogs and cat ranges from 0-3-1.3%. The commonly affected breed of dogs includes Samoyeds, Poodles, Schnauzers, and Bichon Fries (Fall *et al*., 2007). Moreover, feline breeds appearing to be at increased risk of DM are Burmese cat, Norwegian Forest cats, Russian Blues and Abyssinians (Lederer *et al*., 2009; Ohlund *et al*., 2015). Increased oxidative stress resulting from an imbalance in the reactive oxygen species (ROS) and the endogenous antioxidant system, had been implicated in the pathogenesis and progression of DM (Bacanli *et al*., 2019). Studies on ROS has shown that it is involved in inflammation leading to β- cells destruction and/or insulin resistance which is key to diabetic etiology and its associated complications (Tangvarasittichai, 2015). Therefore, use of agents that have antioxidant capabilities may be able to preserve β-cells function through suppression of β-cell apoptosis helping in managing DM (Egea *et al*., 2017; Ogundajo *et al*., 2018; Marañon *et al*., 2020).

Several drugs are in use for treatment of DM type 2 which include acarbose, voglibose, and insulin injection in human, while acarbose, Novolin N, insulin and Glipizide are commonly used in dogs and cats but these drugs are expensive with debilitating side effects associated with their continual use (Nelson, 2000; Mazzaferro *et al*., 2003). Therefore, an alternative or complementary approach using herbal remedies in managing DM is advocated (Sabiu *et al*., 2016). Results of several studies have shown that many plants used in the folkloric treatment of have antioxidant capabilities and can improve β-cells function (Bibi *et al*., 2020; Phukhatmuen *et al*., 2020).

Plants contain different secondary metabolites like flavonoids, phenols, alkaloids, glycoside and terpenes which may be responsible for their pharmacological activities. Flavonoids are biologically active, non-nutritious, non-toxic secondary metabolites of plants that are produced in the cells of the plant through the phenylpropanoid pathway (Shashank and Abhay, 2013). The process of flavonoid biosynthesis starts from the metabolism of phenylalanine into 4-coumaroyl- CoA, (a key intermediate metabolite) which then moves into the main flavonoid biosynthetic pathway (Thomas, 2010; Falcone-Ferreyra *et al*., 2012). Flavonoid biosynthesis occurs in the cell cytosol and the product is stored in the cell vacuole (Agati *et al*., 2012). Flavonoids found in flowering plants are chalcones, flavonols, isoflavones, flavonones, aurones, proanthocyanidins and anthocyanins (Mohotti *et al*., 2020).

In vivo and *in vitro* studies have demonstrated the pharmacological importance of flavonoids, they regulate carbohydrate digestion and possess antioxidant properties (Wang et al., 2018; Claude *et al*., 2020; Rengarajan *et al*., 2020). Several factors contribute to the antioxidant capabilities of flavonoids, they include flavonoids` 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 *et al*., 2013; Shashank & Abhay, 2013). Structural/activity relationship evaluation of flavonoids suggested that the OH at the carbon (C3, C5) plus the –oxo- group at carbon 4 found in flavonols like kaempferol and other flavonoids skeleton may be responsible for its ability to chelate metal ions. Metal ions bind at these 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 31, 41 carbon of the B- ring may be attributed to the antioxidant activities of flavonoids (Rice-Evans *et al*., 1996).

Hermannia geniculata is among the medicinal plant species frequently used in South Africa for the management of different diseases (Balogun *et al*., 2016). It is of the genus of flowering plant from the family *Malnaceae* (Essop *et al*., 2008). The plant is seen in South Africa and its vast majority being found in eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga, it is also found in the Saudi Arabia, West Africa, and north America (Essop *et al*., 2008).

It has been used in the treatment of several diseases like colic, diabetes mellitus, and other oxidative stress-induced illnesses (Moffett, 1993). 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 diarrhea, heartburn, stomach disorder and flatulence called "leletha" in pregnant Sotho women (Moffett, 1993; Balogun *et al*., 2016).

Based on the above knowledge, the present investigation was undertaken to determine the chemical constituents of the extract using HPTLC fingerprinting analysis, evaluate the in vitro antioxidant potentials of the plant, and also evaluate FHG extract capability to inhibit α-glucosidase, α-amylase enzymes and determine the mechanism of action of this plant extract on these carbohydrates metabolizing enzymes. This is because there is still the ongoing search for suitable antidiabetic agents from the plant, due to the adverse effect of the existing synthetic antidiabetic drugs.

Materials and Methods

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 a 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 of flavonoids: The roots were separated, washed under running tap to remove all debris and chopped into small pieces and air-dried at room temperature to a constant weight. The dried root materials were pulverized into fine powder using waring laboratory blender (Labcon, Durban, South Africa) and kept at 4℃ before extraction.

The flavonoids extract from *Hermannia geniculata* roots were obtained through a method described by Liu *et al*. (2015). Briefly, the powdered roots (30 g) was extracted twice with 70% ethanol (1:10, w/v) for 1 h at 25℃. 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℃ before carrying out High-Performance Thin layer Chromatography and other in vitro assays.

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

Chemicals and reagents: The chemicals were purchased from different suppliers: Porcine pancreatic α-amylase, rat intestinal α- glucosidase, 1,1-diphenyl-2-picrylhydrazyl, silymarin, acarbose and para nitrophenyl-glucopyranoside from Sigma-Aldrich 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.

HPTLC finger print analysis of FHG extract: The assay method was as described by Reich & Schibli (2007).

Briefly, 50 mg of FHG extract was weighed accurately with an electronic weighing balance (Afcoset), dissolved in 250 µL of ethanol, and centrifuged at 3000 rpm for 5min. This solution was used as a test solution for HPTLC analysis. Then 2 µL of the test solution and 2 µL of standard solution were loaded as 5 mm band length in the 3 x 10 Silica gel 60 F254 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 it has been 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) to capture 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 a 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 the 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 a 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.

In vitro antioxidant assays: All experiments were conducted in triplicates and all the negative controls (blank) were prepared using the same procedure replacing the extract with distilled water. The percentage inhibition activity of all antioxidant assays was obtained using $[(A0–A1)/A0] \times 100$, where A0 is the absorbance of the control and A1 is the absorbance of the extract/standard. The 50% Inhibition Concentration (IC50) value was calculated and obtained from the linear regression equation using $y = mx + c$, where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC50 value.

2, 2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic) acid (ABTS) radical scavenger 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 a described method (Re *et al*., 1999).

Metal chelating assay: The chelating of ferrous ions by FHG extract was estimated as described by Dinis *et al*. (1994).

Superoxide anion scavenging assay: Determination of superoxide anion radical scavenging potential of FHG extract was achieved according to Liu *et al*. (1997).

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 Mathew & Abraham (2006). Briefly, 100 µL of extract (0.02. 0.04, 0.06, 0.08 and 1.00 µg/mL), 120 µL of deoxyribose (20 mmol/L), 400 µL of phosphate buffer (0.1 mol/L), 40 µL hydrogen peroxide (20 mmol/L) and 40 µL iron two sulphate (500 µmol/L) were added into an Eppendorf tubes (2 mL). Thereafter, there was an addition of 100 µL distilled H_2O and the mixture was incubated at 37℃ for 30 min. Termination of the reaction was carried out by the addition of 400 µL thiobarbituric acid (0.6%) and 0.5 mL trichloroacetic acid (2.8%). About 300 µL of this mixture were dispensed into a 96-well microtiter plate and incubated with boiling water (100°C) in a water bath for 20 min after which the absorbance was measured at 532 using a microplate reader (BIO-RAD, Model 680 Japan). The same procedure was carried using standards (silymarin) in place of the extract. The modification to this experiment was the concentration of the extract used ranging from 0.02-1.0 µg/mL.

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 as described by Braca *et al*. (2001).

In vitro antidiabetic assays: These were carried out by testing the inhibitory effect of FHG extract on α-amylase and α-glucosidase enzymes.

α-amylase inhibitory assay: The α-amylase inhibitory assay was carried out using the procedure of Apostolidis *et al*. (2007). Briefly, 250 mL of different concentrations of FHG extract/standard (0.125, 0.25. 0.50. 0.75, and 1.0) µg/mL prepared in distilled water were placed into a test tube and freshly prepared 250 mL of 0.02 M Na4Po4 buffer (pH 6.9) containing α- amylase solution were added. The resultant mixture was incubated for 10 min at 25℃, this was followed by adding 250 mL of (1%) starch

solution prepared in 0.02 M Na4Po4 buffer (pH 6.9) at a timed interval. The whole mixture was incubated for 10 min at 25℃.
Dinitrosalycylic acid (DNS) reagent (500 mL) was added and the whole mixture boiled in a water bath for 5 min to terminate the reaction. Absorbance was measured after diluting the mixture with 5 mL distilled water. Microplate reader (BIO-RAD, Model 680, Japan) was used to measure absorbance at 540 nm. A similar method was used to prepare the control but distilled water replaced the extract. The procedure was carried out in triplicate.

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). In brief, different concentrations (0.125 – 1.0 µg/mL) of extract/ standard were prepared in distilled water. Then, 50 mL of the extract solution were added into a test tube containing 100 mL of 0.1 M alpha glucosidase solution preincubated in 0.1 M phosphate buffer pH (6.9). The resultant mixture was dispensed into a well-labeled 96-well plate and incubated for 10 min at 25℃. Further addition of 50 mL of 5 nmoL of p-nitrophenyl- α-D-glucopyranoside (pNpG) solution prepared in 0.1 M Na4Po4 buffer (pH 6.9) was made into each well at timed intervals and the reaction mixture incubated for 5 min at 25℃. The absorbance of the mixture which determines the extract inhibitory effect was measured at 405 nm using a microplate reader (BIO-RAD, Model 680 Japan). The same methodology applies to the control, the extract was replaced by distilled water. The experiments were conducted in triplicate.

Kinetic studies
The mechanism of action of FHG inhibition of α-amylase and α-glucosidase was determined.

Mode of α-amylase inhibition: Mode of α-amylase inhibition assay was conducted using a described method by Nagata *et al*. (2015), in brief, 250 mL of (5 mg/mL) standard/extract of FHG were initially incubated with 250 mL of 0.5 mg/mL solution of α-amylase at 25℃ for 10 min in one set of test tubes, while in another test tube α-amylase was pre-incubated with 250 mL of 0.1 M Na4Po4 buffer (pH 6.9). Initiation of the reaction in the two sets of test tube occurred with the addition of 250 mL of (5%) starch solution at an increasing concentration (0.31-50.00) µg/mL. After incubating the mixture for 10 min at 25℃, 0.01 mM of dinitrosalycylic acid (DNS) (500 mL) was added and the reaction was

terminated after boiling for 5 min. Calculation of the amount of reducing sugar liberated was done spectrophotometrically using the maltose standard curve converted to reaction velocities. A double inhibition (Lineweaver & Burk, 1934).

Mode of α-glucosidase inhibition: The kinetics of inhibition of α-glucosidase activities of FHG extract was carried out using a method described by Nagata *et al*. (2015). Briefly, pre-incubation of 50 µL of 5 mg/mL FHG extract with 100 µL of 0.1 M of α-glucosidase solution at 25℃ for 10 min was carried out in one set of the test tube, in another set of test tube pre-incubation of α-glucosidase in 50 µL of 0.1 M Na4Po4 buffer (pH 6.9) was carried out. 50 mL of different concentrations 0.31- 50.00 µg/mL of 0.05 M pNpG were added to both sets of the reaction mixture to start the reactions. Then 500 mL of 0.1 M sodium carbonate at 25℃ for 10 mins were added to terminate the reaction. Calculating the released reducing sugar was done colourimentrically using a p-nitrophenol standard curve.

Statistical data 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 followed by Bonferroni: comparisons of all pair of columns, Statistical significance was considered at P < 0.05.

Results
Flavonoids yield from the extraction of 30 g of plant materials was 1.68 g. The HPTLC fingerprint analysis of the flavonoids was carried out to determine the flavonoids profile present in FHG extract. The result of the different Rf values and their various chromatograms are presented in Table 1 and Figures 1a-1c. The result shows the presence of flavonoid/ phenol carboxylic acid in the FHG extract. The peak seen at Rf 0.80 from the chromatograms confirmed

reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is the substrate concentration was plotted to determine the mode of

the presence of kaempferol in the FHG extract. This is achieved through its similarities with the Rf value of the standard (kaempferol Rf: 0.80). The chemical structure of the identified compound (kaempferol) in the extract is shown in Figure 2.

The results of the in vitro antioxidant potentials of the flavonoid of *Hermannia geniculata* (FHG) are presented in Table 2 and Figures 3a-e. The FHG did scavenge/ chelate and showed remarkable potentials when compared to the standard (silymarin). This is seen by comparing their IC50 values. The IC50 value of FHG extract on DPPH and ABTS radicals were (3.07± 0.12 and 2.13± 0.67) µg/mL respectively which was lower and significantly different (p<0.05) from silymarin with respective IC50: 3.55±0.10 and 2.77±0.75 µg/mL for DPPH and ABTS. The percentage inhibition of FHG extract on DPPH and ABTS radicals was concentration-dependent (Figures 3a and c). The metal chelating activity of the FHG extract was also demonstrated against ferrous ion (Figure 3b). FHG showed great potential with IC50:4.14±0.50 µg/mL which is lower and significantly different (p<0.05) from silymarin with IC50: 5.30± 0.98 µg/mL.

Furthermore, the potential of FHG to scavenge free hydroxyl radical in vitro was also quantified. The result (Table 2, Figure 3e) shows that FHG extract has a similar capability with silymarin in scavenging the hydroxyl radicals with the best activity seen at a concentration of 0.1 mg/mL. The respective IC50 values for FHG extracts and silymarin are 2.51± 0.01 µg/mL and 2.47± 0.01 µg/mL respectively. Moreover, FHG extracts showed the greater capability of scavenging superoxide anion radicals in a concentration dependent manner (Figure 3d) with an IC50 value of 3.21±0.07 µg/mL value which is lower and significantly different (p<0.05) from silymarin with IC50 value of 3.87± 0.14 µg/mL (Table 2).

Table 1: The retention factor (Rf) values of different peaks of flavonoid of *Hermannia geniculata* roots and the standard kaempferol

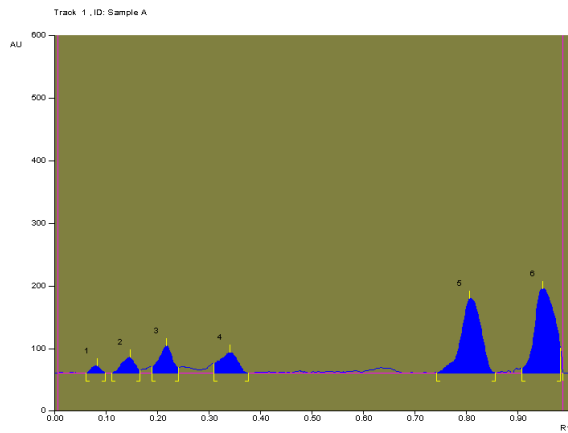| Track	Peak	Rf	Height	Area	Assigned substance
Sample A	1	0.08	11.4	188.9	Unknown
Sample A	2	0.15	25.1	588.3	Unknown
Sample A	3	0.22	43.9	1038.1	Unknown
Sample A	4	0.34	32.9	1043.3	Flavonoid/Phenol carboxylic acid
Sample A	5	0.80	119.8	4231.9	Kaempferol
Sample A	6	0.95	135.4	4767.4	Unknown
STD	1	0.80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

Figure 1a. FHG extract Peak densitogram display (scanned at 254nm). The x- axis is the Rf (retention factor) and the y-axis is AU (area under curve)
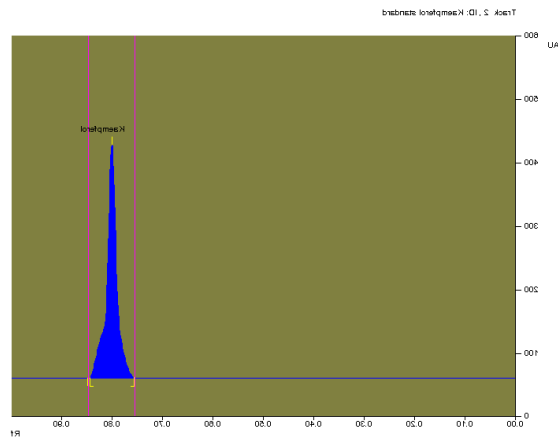

Figure 1b. Flavonoid standard peak densitogram display (scanned at 254nm). The x- axis is the Rf (retention factor) and the y-axis is AU (area under curve)
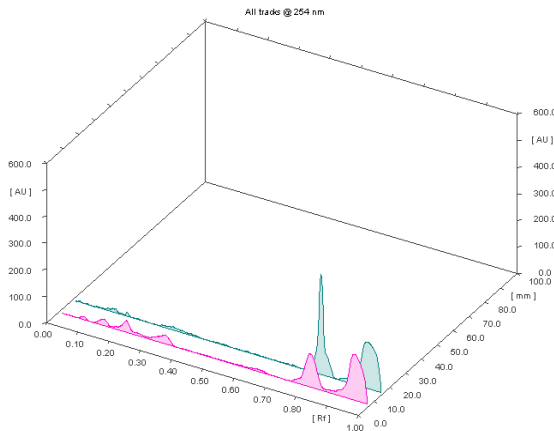

Figure 1c. The 3D display of all Tracts scanned at 254nm. The x- axis is the Rf (retention factor) and the y-axis is AU (area under curve)
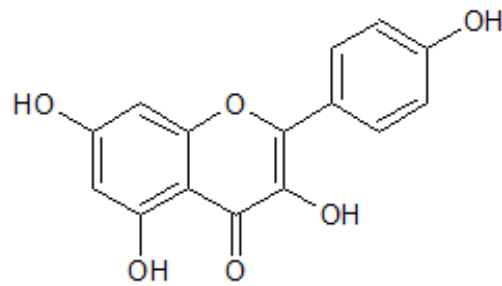

Figure 2. The chemical structure of kaempferol identified in FHG extract

Table 2. The IC_{50} of various antioxidant, α- amylase and α- glucosidase activities of total flavonoid of *Hermannia geniculata* roots extract

Antioxidants	IC_{50} (μg/mL)	Flavonols	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]	2.13± 0.67[a]
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)	Flavonols	Silymarin
α- amylase		5.55± 0.37[a]	3.81± 0.29[b]
α- glucosidase		1.37± 0.19[a]	2.91± 0.42[b]

Values are means of triplicate determination (n=3) ± standard deviation. [ab]Values with different alphabets across the rows are significantly different ($p<0.05$). Silymarin was used as the standard for antioxidant assays while acarbose used the standard for antidiabetic assay

Also, the in vitro enzymatic inhibitory potential of FHG extract on general and specific carbohydrate metabolic enzymes of α-amylase and α-glucosidase was assayed and results displayed in Table 2, Figures 4a and 4b. However, the potential of the extract to inhibit α-amylase activities was lower at all
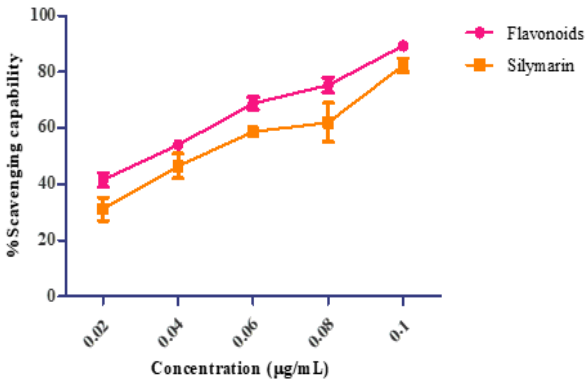
Figure 3a: DPPH radical scavenging capability of *Hermannia geniculata* flavonoids extract. Values are mean± standard deviation (SD) of triplicate determinations
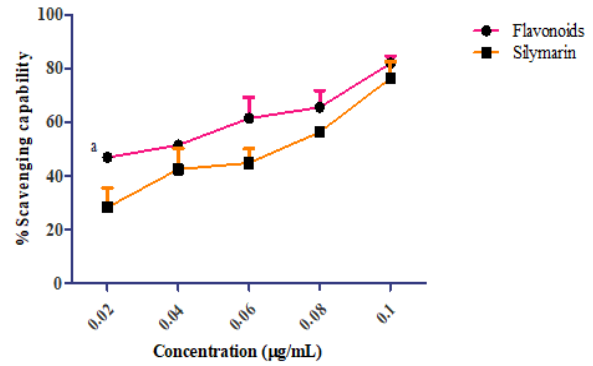

Figure 3b. Metal chelating activities of the flavonoids of *Hermannia geniculata* root extract. Values are mean± standard deviation (SD) of triplicate determinations
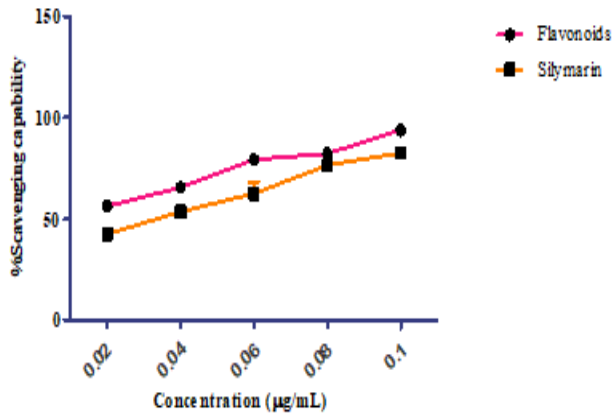

Figure 3c. ABTS radical scavenging activities of the flavonoids of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations
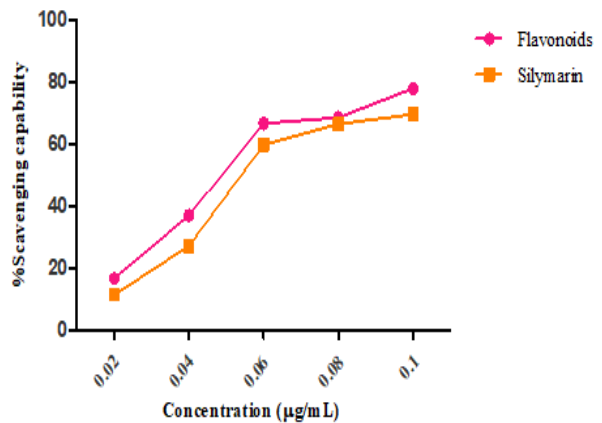

Figure 3d. Superoxide anion radical scavenging capabilities of the flavonoids of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations

Figure 3e. The hydroxyl radical scavenging capabilities of the flavonoids of *Hermannia geniculata* root extracts. Values are mean± standard deviation (SD) of triplicate determinations

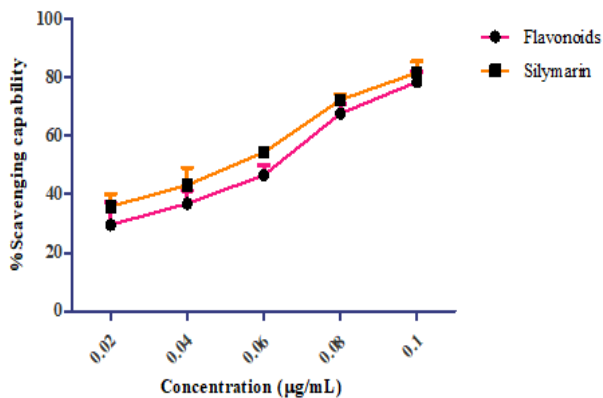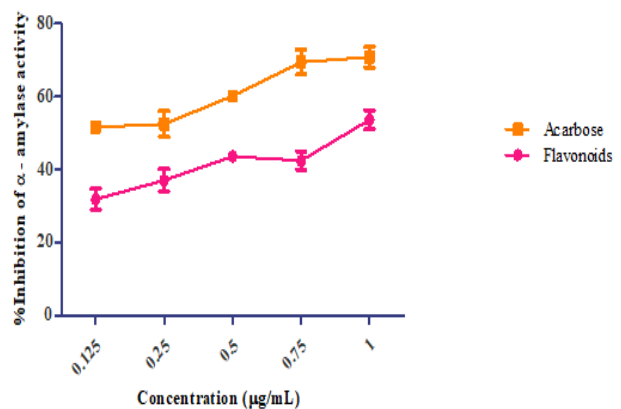**Figure 4a.** Inhibitory potential of *Hermannia geniculata* root flavonoids extract on specific α-amylase activity. Values are Mean ± standard deviation (SD) of triplicate determinations

concentrations compared to the reference compound (acarbose), the IC50 value of FHG extract

was 5.55 ±0.37 µg/mL which is higher and significantly different ($p < 0.05$) from acarbose with a

lower IC50: 3.81 ±0.29 µg/mL but FHG extract exhibited higher inhibition of α-glucosidase enzyme than acarbose with their respective IC50 values of 1.37 ± 0.19 µg/mL and 2.91 ±0.42 µg/mL.

The kinetic study for the probable mode of inhibition of the enzymes displayed in Figure 5a showed that FHG extract exhibited independent inhibition of the 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 a 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 FHG extract (Figure 5b) showed that the Vmax of the extract increased from 0.14 µm/min for the control to 2.70 µm/min for FHG extract while the Km value remained the same at a constant concentration signifying a non-competitive mode of inhibition.

Discussion

High performance thin layer chromatography (HPTLC) fingerprinting is an offline throughput planar chromatography technique used in identifying marker compounds present in medicinal plants (Reich & Schibli, 2007). The presence of flavonoids in the extract were confirmed with Rf values (0.08-0.95). The specificity of the method 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 Rf 0.80 which is similar to the standard kaempferol with Rf 0.80.

Also, similar densitogram and 3D display of the tracts confirmed the detection of kaempferol in the FHG extract. Kaempferol is a flavonol, a product of aromadendrin derived from the flavonoid biosynthetic pathway in a reaction catalyzed by flavonol synthase (Malla *et al*., 2012). Several glycosides of kaempferol derivatives have been identified in medicinal plants (Teffo *et al*., 2010). This will be the first time kaempferol was detected in *Hermannia geniculata* roots extract.

Flavonoids are polyphenolic compounds distributed naturally in plants. They are plant secondary metabolites with reactive oxygen species (ROS) quenching capacity in both plant and animal cells (Agati *et al*., 2012; Rengarajan *et al*., 2020). The unregulated production of ROS by different body cellular mechanisms lead to oxidative stress which is an underlying cause of β-cell destruction and insulin resistance seen in diabetes mellitus type 2 and its various complications (Deo *et al*., 2016). 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 *et al*., 2013; Egea *et al*., 2017). In this study, the results of antioxidant assays showed that FHG extract has a good antioxidant activity which is comparable to the standard silymarin. FHG exhibited a lower IC50 value for DPPH, ABTS, and superoxide anion radicals compared to the standard. The extract also showed iron-chelating capability which will tend to reduce the concentration of transition ion that catalyzes lipid peroxidation in biological systems (Jomova & Valko, 2011). Appreciable hydroxyl radical

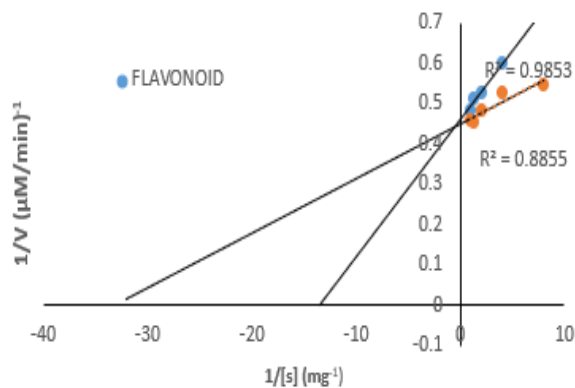**Figure 5a.** Lineweaver-Burk plot of total flavanoid extract eliciting competitive inhibition on α-amylase activity. Results represent mean ± standard deviation (SD); (n=3); (p<0.05)
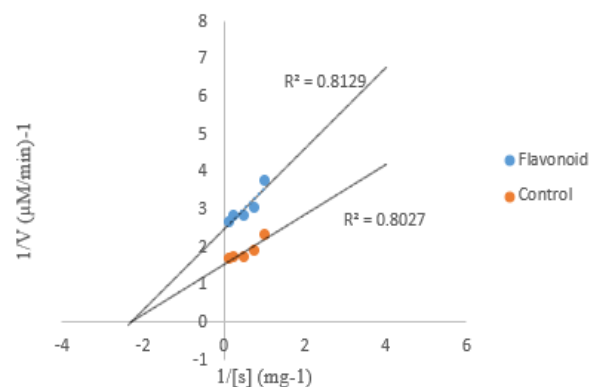
Figure 5b. Lineweaver-Burk plot of total flavonoids extract eliciting non-competitive inhibition on α-glucosidase activity. Results represent mean ± standard deviation (SD); (n=3); (p<0.05)

scavenging capability of FHG was also observed. Hydroxyl radical has been implicated in the production of harmful radicals that cause enormous damage to cellular components (Ogundajo *et al.*, 2018). The standard curve of the percentage inhibition/scavenging/chelating effect and their respective IC50 values in this study showed a decrease in the concentration of the ROS which may be a result of the scavenging effect of the FHG extract. Since the effect of vascular complication induced by hyperglycemia may be due to the reactive oxygen species (ROS) and free radicals in the biological system (Li *et al.*, 2017), the scavenging of ROS and free radicals may be useful in controlling the pathogenesis of diabetes mellitus type 2 in both humans and animals.

α- amylase is an enzyme found in saliva and pancreas, which is responsible for conversion of polysaccharide to disaccharide while the α-glucosidase found in the small intestine catalyzes the conversion of polysaccharides to disaccharides and monosaccharides (Oku *et al.*, 2011). The inhibition of these enzymes delays the breakdown of carbohydrates in the small intestine thus controlling the blood glucose level associated with postprandial hyperglycemia commonly seen in type 2 diabetes mellitus.

The result obtained from α- amylase inhibitory activities in this study showed that FHG extract IC50 value is 5.55 μg/ mL which is higher and significantly different (p<0.05) from acarbose IC50 value of 3.81 μg/ mL. The possession of higher IC50 value of FHG than the standard drug in the inhibition of α-amylase signifies mild inhibition of the enzyme which is a good quality of an antidiabetic agent (Sabiu *et al.*, 2016). This result implies that FHG is a mild inhibitor of α- amylase compared to acarbose, excessive inhibition of α- amylase enzyme by acarbose has been associated with side effect like diarrhea, and stomach upset (Kazeem *et al.*, 2013). This result is consistent with Kim *et al.* (2000) which observed a less potent than acarbose inhibitory effect of flavonoids.

The inhibitory effect of FHG extract on α-glucosidase was also evaluated and, it showed an IC50 value of 1.37 μg/mL which is lower and significantly different (p<0.05) compared to acarbose IC50 value of 2.91 μg/ mL. The lower IC50 value of FHG than the standard drug used in this study indicated stronger inhibition of the α-glucosidase enzyme. FHG extract potent inhibition of α-glucosidase compared with the standard antidiabetic drug in this study showed a great potential of the FHG extract which may be further explored in the quest for potent antidiabetic drug.

Strong inhibition of α-glucosidase will affect the level of carbohydrate catabolism and subsequent postprandial hyperglycaemia seen in type 2 diabetes mellitus. 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 *et al.* (2006) observed that yeast α-glucosidase was potently inhibited by flavonoids like anthocyanidin, isoflavone, flavanol, and flavonol groups than acarbose. The presence of a double bond in the C- ring, the catechol group in the B-ring seen in the flavonoids' the skeleton may be responsible for the potent inhibitory effect on α-glucosidase enzyme (Tadera *et al.*, 2006).

Due to the observed mild and strong inhibitory properties of FHG on α-amylase and α-glucosidase enzymes respectively, the extract was tested for the enzyme kinetics of inhibition with the aid of Lineweaver-Bulk plots. The enzyme kinetic study of α- amylase showed that FHG extract competitively inhibits α- amylase enzyme. The kinetics further revealed an increase in the Km value of the inhibitor while the Vmax remain constant. The lower Km value of the inhibitor means a higher affinity of the inhibitor compared to the substrate for the enzyme thus, competitively inhibiting α- amylase hydrolysis of disaccharide. This is only applicable if both concentrations of the inhibitor and the substrate are kept constant (Johnson & Goody, 2011).

The mode of inhibition of α-glucosidase is mixed 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 an α-glucosidase enzyme by FHG.

From the kinetic studies, competitive and mixed non-competitive inhibition of α- amylase and α-glucosidase enzymes will cause a delay in the absorption of glucose and fructose in the gastrointestinal tract and reduce the gastric emptying rate which alters insulin secretion (Celleno *et al.*, 2007). The effect of reduced starch catabolism will promote weight loss through a reduction in the digestive bioavailability of carbohydrate derived calories (Olaokun *et al.*, 2016).

In this study, the HPTLC fingerprinting analysis of FHG showed the presence of kaempferol and other flavonoids. The FHG extract showed its ROS and free radicals scavenging capabilities, it also demonstrates appreciable inhibition of α- amylase and α-glucosidase enzymes. This suggested that FHG may

be a potential agent that may be explored in order to mitigate oxidative stress and postprandial hyperglycemia seen in diabetes mellitus type 2 in humans and animals. There is a need for further in vivo antioxidant and antidiabetic studies of the plant in diabetic animals.

In conclusion, this study revealed the chemical profile of FHG extract, and also showed that the extract has antioxidants and antidiabetic potential in vitro. It may be deduced from the result that FHG extract has antioxidant and antidiabetic potential. Its probable mechanism of antidiabetic action is through the competitive inhibition of α-amylase and non-competitive inhibition of α-glucosidase enzymes.

Further work on isolation and characterization of the bioactive flavonoid in FHG extract and the in vivo antidiabetic studies in cat and dog should be conducted.

Conflicts of Interest

The authors declare no conflict of interest.

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