ANTIHYPERGLYCEMIC AND ANTI-OXIDANT POTENTIAL OF ETHANOL EXTRACT OF*VITEXTHYRSIFLORA*LEAVES ON DIABETIC RATS

ABSTRACT:

The antihyperglycemic effect of an ethanol extract of *Vitexthyrsiflora* leaves was investigated in normal male rats and streptozotocin-induced diabetic male rats and its antioxidant potentialwas evaluated. After preparation of the extract, it was subjected to a phytochemistryscreening, and tested on male rats made hyperglycemic in the Oral Glucose Tolerance Test and in streptozotocindiabetic rats. Glibenclamide (10 mg/kg) was served as a positive control in both experiments. during both experiments, the extract was tested at 200 and 300 mg/kg. The evaluation of the antioxidant potential was done through the determination of the Total Polyphenolsand Total Flavonoid Contents, and by usingthe Ferric Reducing Antioxidant Power assay and the Free radical scavenging activity on DPPH method. The results show that the extract contains alkaloids, flavonoids, steroids, saponins and phenolic compounds. The test on hyperglycemic rats inOGTT showed that, the extract was effective (at a dose of 200 mg/kg)to significantly decreased glucose-induced hyperglycemia (**$p < 0.01$). The extract was ineffective on streptozotocin-induced diabetic rats. The study of the antioxidant potential showed that, polyphenols and flavonoids increase with the concentration of the extracts. IC_{50} value was found to be 65.97, based on the log (inhibitor) vs. normalized response-Variable slope. FRAP appears to be significantlyhighly correlated with Total Polyphenols Content and Total Flavonoids Content.this shows that the ethanol extract of*V.thyrsiflora* leaves could be servedto prevent acute hyperglycema, but not a chronic hyperglycemic state.

Keys words: antihyperglycemic, antioxidant, diabetic rats,*Vitexthyrsiflora*.

INTRODUCTION

The genus *Vitex* (Verbenaceae) is constituted by 250 species of small trees and shrubs which occur in tropical and subtropical regions[1]. *Vitexthyrsiflora* Baker (Verbenaceae) is a glabrous under shrub that is widely distributed in Cameroon[2]. This plant is reported to be useful in the treatment of orchitis[2]. The rootbarks are used in the treatment of stomach pains, sexual sterility and wounds[3]. Previous chemical investigations on the leaves and fruits resulted in the isolation of phytoedysteroids[3]. However, the investigation of some other *Vitex* species have resulted in the isolation of iridoids[4,5,6], diterpenes[7,8]; steroids[9], flavonoids[6] and triterpenoids[9]. Several of previous compounds isolated from *Vitex*genushave shownantioxidants activities[10, 11].Among all secondary metabolites, phenolic antioxidants appear to be the most important since they have shown promising antioxidant activity in both *in vivo* and *in vitro*investigations. Plant phenolics are mainly classified into five major groups, phenolic acids, flavonoids, lignans, stilbenes and tannins[12, 13, 14].The antioxidant activities of these compounds have been attributed to various mechanisms which have been established by various assay procedures[15].Antioxidant activity has a fundamental role in cellular protection during an inflammation process. The identification of phytochemical compounds in plant species has been exploited in recent years, due to the growing popularity of herbal medicines and consumers growing enthusiasm for foods with bioactive characteristics that aid in preventing and fighting disease[16, 17].

Diabetes is characterized by chronic hyperglycemia, a source of increased oxidative stress and tissue oxidative damage. In particular, hyperglycemia promotes the glycation of proteins resulting in the formation of advanced glycation products (AGE). Thus antioxidant molecules

appearas an opportunity for a strategy to fight not only against hyperglycemia, but also against complications related to diabetes mellitus[18, 19].

Then, the objectives of this study based on these findings, was first to evaluate the antihyperglycemic effect of an ethanol extract of *V.thyrsiflora* leaves in glucose loaded rats and in STZ-diabetic rats and secondto determine the Total Polyphenols Content (TPC), Total Flavonoids Content (TFC), the Ferric Reducing Antioxidant Power Assay (FRAP) and the DPPH free radical scavenging assay of this extract.

MATERIAL AND METHODS

Experimental

Drugs and Chemicals

Streptozotocin, quercetin and catechin were obtained from Sigma Chemicals (St. Louis, MO). Glibenclamide (Glycomin®) was obtained from StridesArcolat Ltd. Bangalore, India. All other chemicals used were of analytical grade. Spectrophotometric measurements were doneusing the equipment available at Institute of Medical Research and Medicinal Plant Studies.

Plant material

The leaves of *V. thyrsiflora* Baker (Verbenaceae) were collected at Melong, in the Littoral Region of Cameroon in November 2009. Authenthification was performed by Mr Nana who compared it with a Voucher Specimen (No 34861 HNC), in the Cameroon National Herbarium, Yaoundé.

Extraction

The extract was obtained by maceration of air-dried and powdered leaves of *V. thyrsiflora* (3.0 kg) with EtOH (3 x 15 L, 72 h) at room temperature, to obtain a crude extract (150 g).

Phytochemical screening of secondary metabolites

The *V. thyrsiflora* leaves ethanol extract was also subjected to phytochemical analysis according to the methods of Harborne[20] and Evans [21].

Experimental Animal

Healthy adult male *Wistar* rats weighing 200 - 250 g were used in the present study. The animals were housed in clean grill cages and maintained in a well ventilated temperature controlled room at the animal house of Institute of Medical Research and Medicinal Plants studies, Yaoundé, Cameroon, with a constant 12h light/dark schedule. The animals were fed with standard rat pellet diet and clean drinking water was made available *ad libitum*.

Induction of diabetes

Rats were fasted overnight (16h) before inducing diabetes with streptozotocin. Streptozotocin was prepared in freshly prepared sodium chloride solution 0.9% and was injected intraperitonially at a concentration of 55 mg/kg body weight in a volume of saline of about 500µl/200g b.w by applying the protocol of Szkudelski[22]. Control rats were injected with saline solution only. The diabetic state was confirmed 72 h after streptozotocin injection. Threshold value of fasting blood glucose was taken as ≥ 200mg/dl. Diabetic rats were weigh matched for body weight and divided into f 5groups consisting of 5 animals each.

Antihyperglycaemic effect of ethanol extract of *V.thyrsiflora* leaves on diabetic rats

The approval of the Institutional Animal Ethics Committee was obtained before starting the study. An international protocol for conducting experiments on animals were followed.

Experimental design

The Diabetic rats were divided into 5 groups with five rats each: (A, B, Cand D). Group A rats received DMSO 3%, those of group B and Cwere treated with ethanol extract of *V.thyrsiflora*at the doses of 200 and 300 mg/kg b.w respectively, and Group Dreceived glibenclamide (10 mg/kg b.w).

Blood sampleswere collected before the commencement of treatment with the extract and then after, at 1h, 3h and 5h intervals.

Oral Glucose Tolerance Test on normoglycemic rats

This study was carried out on normal male rats with normal blood glucose level, according to the method of Schoenfelder*et al*.[23]. The animals were fasted for 16 h prior to the study. Five groups with 5 animals each were constituted and the animals received a dose of 3 g/kg of glucose by oral route 60 min after haven been treated with the extract as follows: Group I: vehicle (DMSO 3%; 10 ml/kg b.w, negative control), Group II: water-ethanol extract (200 mg/kg b.w), Group III: Ethanol extract (300 mg/kg b.w), Group IV: Ethanol extract (400 mg/kg b.w) and Group V: glibenclamide (10 mg/kg b.w), was served as positive control.

Blood was collected from the animals before administration of the extract (-60 min), and then after at 0, 30, 60, 90 and 150 min post administration.

Blood Glucose Estimation

Blood samples were obtained by tail prick and fasting blood glucose levels were estimated with a One Touch Ultra glucometer (Life Scan, Inc., Milpitas, CA, USA) in all animals. Blood glucose levels were expressed in mg/dL.

Dosage of phenolic compounds and antioxidants evaluation

Determination of Total phenolic content (TPC)

The ability of the extracts to reduce the phosphomolybdic-tungstate chromogene in FolinCiocalteu with maximum absorbance at 760 nm (Total phenolic content, TPC) was assessed as earlier described by Vinson*et al*.[24]. Data were reported as mean ± SD for triplicate measurements. Catechin was used as control and the results were expressed as mg Catechin equivalent/g (mg CE/g).

Determination of Total flavonoid content (TFC)

The total flavonoid content (TFC) was measured as earlier described[25]. Total Flavonoid Content of the extract were expressed as mg Quercetin Equivalent/g (mg QE/g) through the calibration curve with quercetin. Data were reported as mean ± SD for triplicate measurements.

Determination of ferric reducing antioxidant power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) of an extract measures the ability of the extract to reduce ferric tripyridyltriazine to ferrous tripyridyltriazine, yielding a blue coloration with maximum absorbance at 593 nm. FRAP assay was performed like previously described by Benzie & Strain[26]. The FRAP value was calculated and expressed as mg Catechin Equivalent/g (mg CE/g) based on a plotted calibration curve , using Catechin as standard, at a concentration ranging from 50 to 600 µmol.

DPPH free radical scavenging assay

The ability of the extract to scavenge free radicals by converting DPPH (purple in color) into di-phenyl hydrazine (yellow in color), was measured at 517 nm as earlier described[27].

The percentage (%) radical scavenging effect of extract was calculated as follows:

% radical scavenging effect = [(Abs1-Abs2)/Abs1] x 100

Where Abs1 is the absorbance of the control (containing all reagents except for the extract), and Abs2 is the absorbance of plant extract (is the optical density in the presence of the extract). All tests were performed in triplicates.

The parameter IC_{50} (concentration at which the DPPH radicals were scavenged by 50%) (mg/ml) was also calculated. IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data was presented as mean values ± standard deviation (n = 3).

Statistical Analysis

Results were expressed as mean ± SD. Statistical analysis were carried out using one way Analysis of variance (ANOVA) followed by Dunnet test for comparison to vehicle control or followed by Newman-Keuls Multiple Comparison Test for antioxidant parameters, using GraphPad Prism 5.03 software. A value of $p \leq 0.005$. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ was considered to be significant.

RESULTS

Phytochemical screening revealed that the ethanol extract of *V. thyrsiflora*leaves contained alkaloids, flavonoids, steroids, phenolics compound, saponosids, catechictanins and anthraquinones (**Table 1**).

The Area Under Curve (AUC) associated with the effect of ethanol extract of *V.thyrsiflora*leaves on Oral Glucose Tolerance Test (OGTT) in normoglycemic male rats was significantly decreased (**$p \leq 0.01$) at 200mg/kg, when compared to vehicle control group (474 ± 26.93 to 388.60 ±16.05), as well as in the group receiving glibenclamide at 10mg/kg, where the AUC was decreased from 474 ± 26.93 to 392.50± 19.45 (**Figure 1b**).

The administration of ethanol extract of *V.thyrsiflora*leaves at 200mg/kg or 300mg/kg to diabetic rats didn't show any difference in the blood glucose level, according to the AUC associated with each group tested. Even glibenclamide didn't show a significant effect on lowering the high blood glucose level state induce by streptozotocin **(Figure 2).**

The Total Polyphenol Content (TPC) appears to be significantly dose dependent on the concentrations of the extract, until 40 mg/kg (79.36±0.3717 mg Catechin/g), where the TPC became stable and not different to that of 50 mg/kg**(Figure 3)**.

The Total Flavonoid Content (TFC) evaluated in*V.thyrsiflora*ethanol extractshowed a significant dose dependent increase when compared to 2.5mg/kg. The content was the same at doses of 5mg/kg and 10 mg/kg (**Figure 4**).

The correlation betweenFerric Reducing Antioxidant Power (FRAP) and Total Flavonoid Content (TFC) showed a very strong positive correlation (Pearson r = 0.9223 with R^2 = 0.8507, p** = 0.0088) (**Figure 5**).

The correlation between FRAP and TPC was significantly high (p** = 0.0010) (**Figure 6)**.

DISCUSSION

The aim of this work was to evaluate the antioxidant and the antihypeglycemic potential of the ethanolextract of*V.thyrsiflora*leaves. The phytochemical screening reveals that the ethanol extract of *V.thyrsiflora*leaves possesses alkaloids, flavonoids, steroids, phenolic compounds, catechicstanins, anthraquinones and saponosids in traces.

The evaluation of the effect of *V.thyrsiflora*ethanol leaves extract on hyperglycemic normal ratsin OGTTshowed that, the 200mg/kg dose was the most active to lower the AUC induced by glucose loaded in normal rats male (**$p \leq 0.01$). Glibenclamide (10 mg/kg), a widely used antidiabetic drug was effective to decrease the AUC observed in the vehicle group (**$p \leq 0.01$).

The results of OGTT in normal rats could be correlated with the ability of the extract to probably enhance the secretion of insulin in the likely manner of sulfonylureas and inhibit α-glucosidases present in the border brush of the small intestine[28, 29]. Enhanced tissue uptake of blood glucose induced by *V.thyrsiflora* might also be taken into consideration as an alternative possibility.

In our case, the decrease in blood glucose at 200 mg/kg and not at 300 mg/kg could be explained by the presence of hyperglycemic compounds which have become the majority at this dose. Some studies showed that,antagonistic relationship among phytochemicals would affect the efficacy of crude extracts as used in traditional medicine, like observed by Milugo*et al*.[30] on antagonistic effect of alkaloids and saponin.

Furthermore, the results show that administration of the extract is associated with no effect on blood glucose level in STZ-diabetic rats. Since streptozotocin selectively destroys ß-cells of the pancreas, we would expect the extract to exert no effect on plasma glucose concentrations in STZ diabetic rats if the mode of action is mediated through insulin production.

Furthermore, the observations in the present study indicate that the hypoglycaemic effect of the extract as well as that of glibenclamide were far more pronounced in OGTT rats than in STZ-diabetic rats. This result is probably in line with the early suggestion that glibenclamide was effective in moderatelystreptozotocin-induced diabetic animal and ineffective in severe diabetic rats[31, 32]; the extract could act in the same way as glibenclamide. These observations taken together suggest that ethanol extract of *V.thyrsiflora*leaves has a potential in the management of acute hyperglycaemia.

Antioxidant parameters showed a dose-dependent rise in the concentration of the extract. The TPC and the TFC showed a positive correlation with the FRAP test, which could suggest that this method would act through polyphenols and flavonoids.

The Pearson correlation coefficient (R^2) is often used for measuring and describing the degree of linear regression between two continuous quantitative variables that are normally distributed. In our study, the R^2 value of the correlation between the iron reduction technique "FRAP" (Ferric Reducing Antioxidant Power)and the Total Phenolic Content was equal to 0.9477 with p** = 0.0010 and R^2 = 0.8507 with p** = 0.0088 for Total Flavonoid Content respectively. These results suggested that a great part of the antioxidant capacity of the ethanol extract of *V.thyrsiflora* leaves is attributed to the Total Polyphenol Content,mainly to Flavonoid Content in the extract, which have the hydrogen-donor ability to reduce iron. Similar studies suggested a linear relationship between antioxidant capacity and flavonoid contents of the plant extract[33, 34].

The DPPH assay is often used to evaluate the ability of antioxidant to donate hydrogen or to scavenge free radicals. However, DPPH scavenging activity is best represented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution[35]. In our study IC_{50} was 65.97 mg/mL, R^2 = 0.9945 for the *V.thyrsiflora* leaves ethanol extract based on the log (inhibitor) vs normalized response - variable slope, which can be considered to be low antioxidant activity (IC_{50} from 50.62 to 110.46 mg/ml) according tothe scale of Surinut*et al*[36];this might suggest that the antioxidant activity of this extract would not be mediated by donating hydrogen to scavenge free radicals but by the reduction of iron.

CONCLUSION

Based on the results of the present study, we conclude that the plant extract possesses antioxidant potential. The findings of the present study also suggest that *V.thyrsiflora*ethanolleaf extract could be a potential natural source of antioxidants and could have greater importance as therapeutic agent in preventing or slowing oxidative stress related degenerative diseases. However, this work is the first report which evaluate antihyperglycemic effect of ethanol extract of *V.thyrsiflora*leaves and its antioxidant potential. Further studies should be carried out to evaluate α-amylase inhibitory and beta-glucosidase inhibitory activities, isolate and identify active compounds, to understand the mechanism of action against hyperglycemia.

CONFLICT OF INTEREST

“No conflict of interest associated with this work”.

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Table 1: Phytochemical screening of ethanol extract of *V. thyrsiflora leaves*

Phytochemicalconstituentstested	Results
Alkaloids	+
Flavonoids	+++
Tri Terpenoids	-
Steroids	+++
Phenolics compound	+++
Saponosids	+ +
Catechic tanins	+++
Anthraquinones	++
Glucosides	-

(+) = indicates presence of phytochemicals and (-) = indicates absence of phytochemicals. +++ = shows high concentration; ++ = shows moderate concentration; + = shows small concentration.

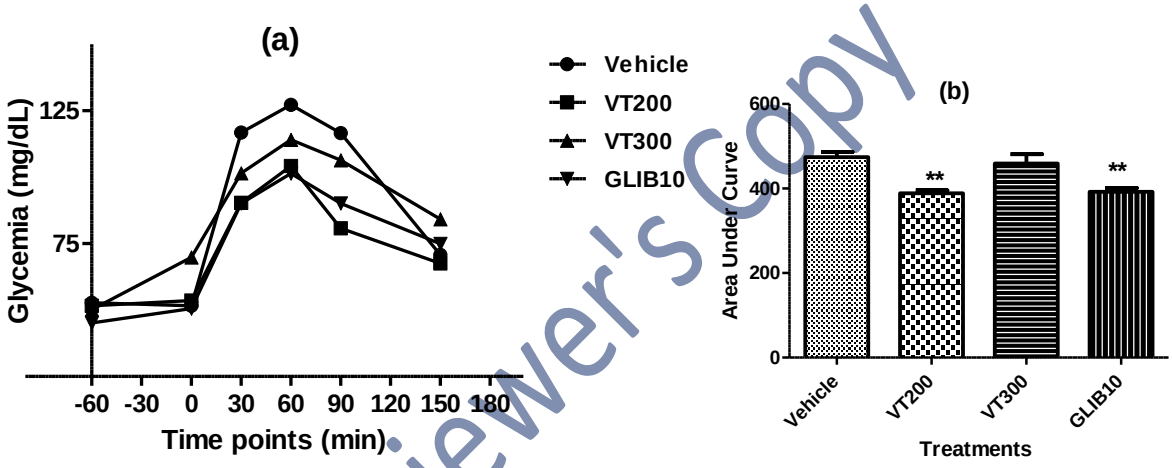

Figure 1: a. Effect of ethanol extract of *V. thyrsiflora*leaves on glycaemia during Oral Glucose Tolerance Test (OGTT) and **b.** Area Under Curve associated with this effect of ethanol extract of *V. thyrsiflora*leaves.
Data are expressed as means ± S.D (n = 5). $^{**}p \leq 0.01$ compared with the corresponding value for vehicle control rats. VT200: *V. thyrsiflora*(200 mg/kg); VT300: *V. thyrsiflora* (300 mg/kg); Glib10: Glibenclamide (10 mg/kg).

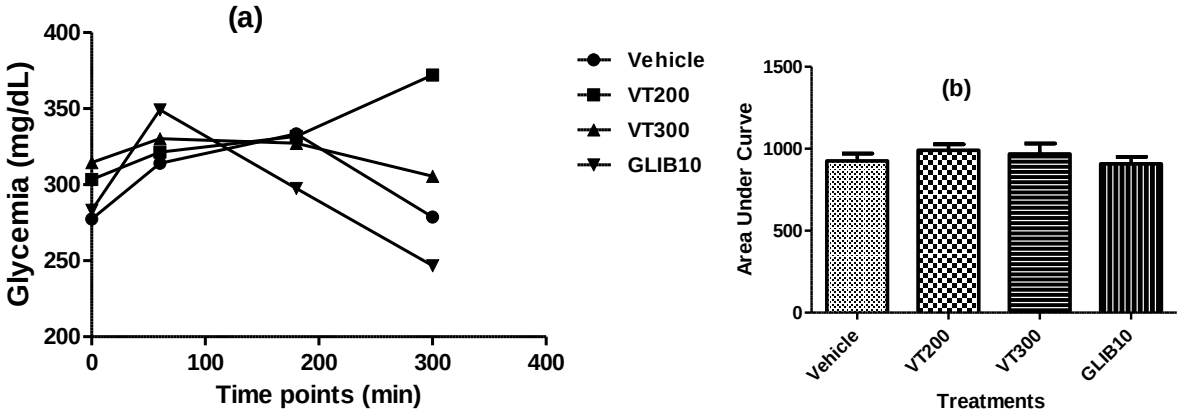

Figure 2: a. Effect of *V. thyrsiflora*ethanol extract on glycemia of STZ-induced diabetic rats; **b.** Area Under Curve associated with the effect of ethanol extract of *V. thyrsiflora*leaves on glycemia of STZ-induced diabetic.

Data are expressed as means ± S.D (n = 5). $p \leq 0.001$ compared with the corresponding value for vehicle control rats. VT200: *V. thyrsiflora*(200 mg/kg); VT300: *V. thyrsiflora* 300 mg/kg); Glib10: Glibenclamide (10 mg/kg).

Total Polyphenol Content (TPC)

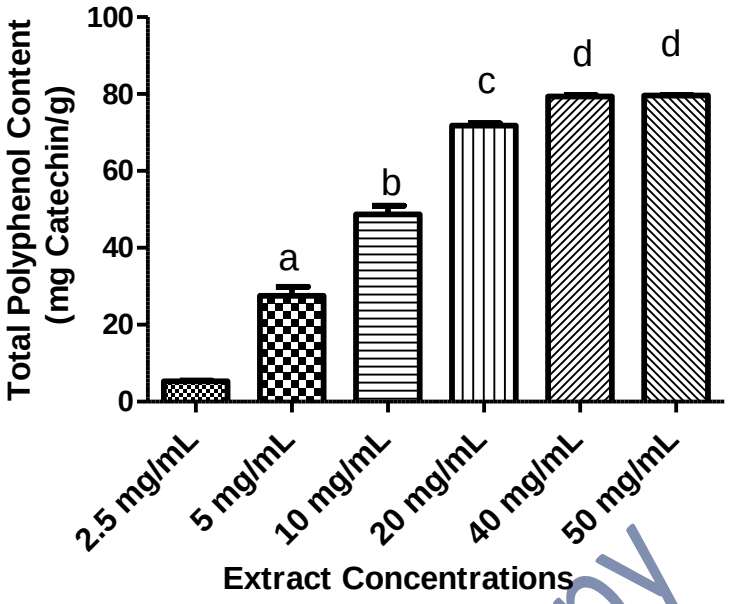

Figure 3: Total Polyphenol Content of ethanol extract of *V. thyrsiflora*.

Data are expressed as means ± S.D (n = 3); a significantly different from 2.5 mg/ml, b significantly different from 2.5 mg/ml and 5 mg/mL, c significantly different from 2.5 mg/ml, 5 mg/ml and 10 mg/ml, d significantly different from 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml.

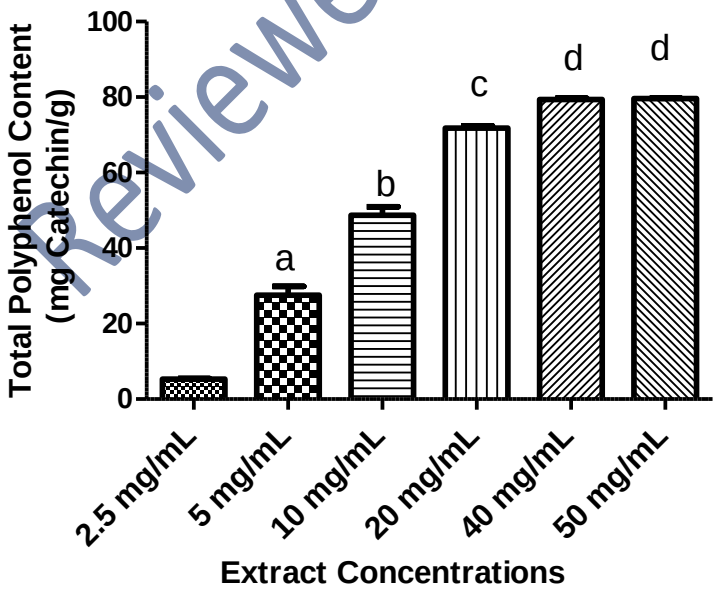

Figure 3: Total Polyphenol Content of ethanol extract of *V. thyrsiflora*.

Data are expressed as means ± S.D (n = 3); a significantly different from 2.5 mg/ml, b significantly different from 2.5 mg/ml and 5 mg/mL, c significantly different from 2.5 mg/ml, 5 mg/ml and 10 mg/ml, d significantly different from 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml.

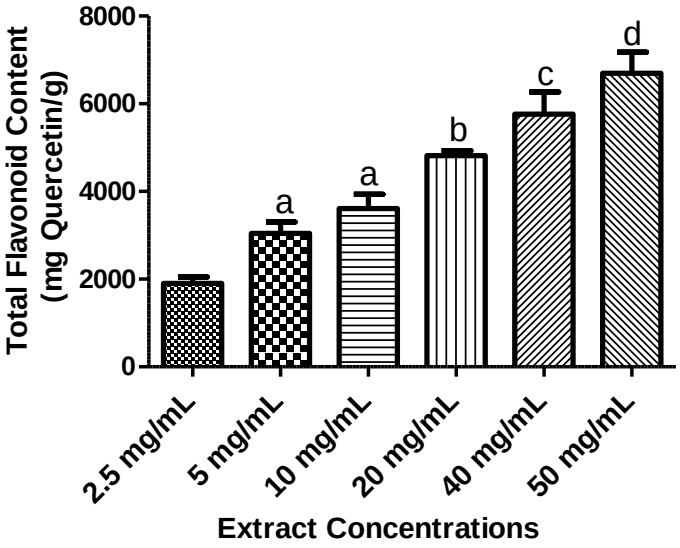

Figure 4: Total Flavonoid Content (TFC) of ethanol extract of *V. thyrsiflora*.
Data are expressed as means ± S.D (n = 3); a significantly different from 2.5 mg/mL, b significantly different from 2.5 mg/ml, 5 mg/ml and 10 mg/ml, c significantly different from 2.5 mg/ml, 5 mg/ml, 10 mg/mL and 20 mg/ml, d significantly different from 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/mL and 40 mg/ml.

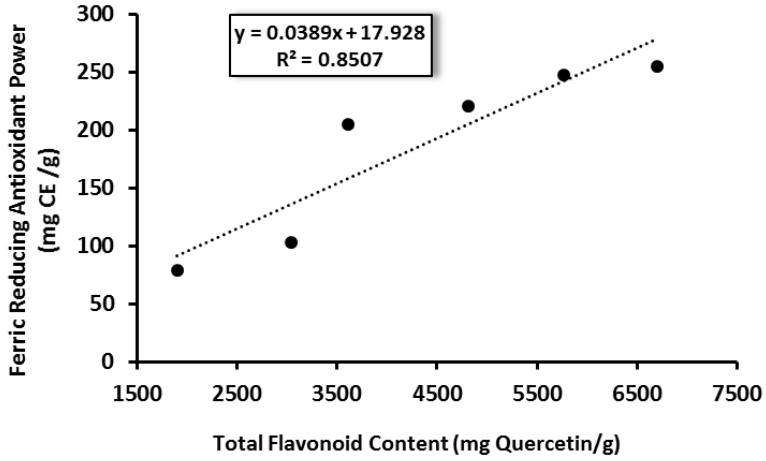

Figure 5: Correlation between FRAP and the Total Flavonoid Content (mg QE/g).
(Pearson r =0.9223; R^2 = 0.8507, p** = 0.0088 ($p < 0.01$).

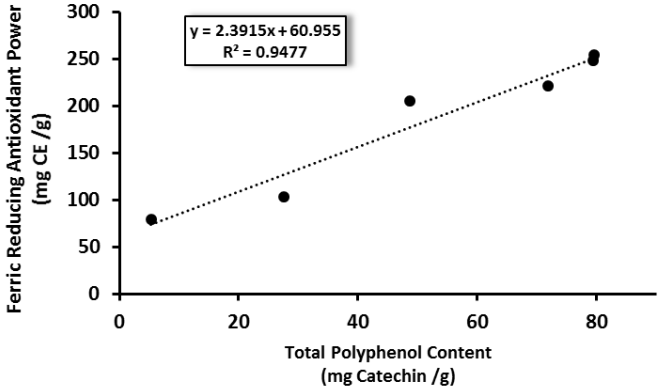

Figure 6: Correlation between FRAP and the Total Polyphenol Content (mg QE/g)
(Pearson r = 0.9735; R^2 = 0.9477, p** = 0,0010($p < 0.01$).