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Research Article

In vitro Enzyme Inhibitory Evaluation and Free Radical Scavenging Potential of Ethanolic Leaf Extract of *Macrotyloma uniflorum* (L.)

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ABSTRACT
Diabetes mellitus is a metabolic disorder and it is one of the free radical mediated diseases characterized by hyperglycemia. Postprandial hyperglycemia caused by the hydrolysis of carbohydrates by pancreatic enzymes, α-amylase and intestinal α-glucosidase is a severe condition and inhibition of these enzymes is an efficient way in the management of diabetes mellitus. Among the various anti-diabetic therapeutic approaches one major strategy is reducing gastrointestinal absorption of glucose by inhibition of carbohydrate metabolizing enzymes alpha-amylase and alpha-glucosidase. In the present study the ethanolic extract of *Macrotyloma uniflorum* leaves have been assessed for the inhibition of alpha-amylase and alpha-glucosidase, additionally *in vitro* antioxidant analysis of ethanolic leaf extract of *M. uniflorum* was performed by 1,1-diphenyl-2-picryl hydroxyl (DPPH), 2,2' azinobis-3 ethylbenzothiozoline-6 sulfonic acid (ABTS+) cation decolourization test, hydroxyl radical (OH.), hydrogen peroxide (H_2O_2), nitric oxide radical (NO), superoxide radical scavenging assays and also metal chelating activity, ferric reducing antioxidant power assay (FRAP) and reducing power assay. Saturation in the enzyme inhibition and free radical scavenging activity has been attained in concentration dependent manner. This study indicates significant alpha-amylase and alpha-glucosidase inhibition, free radical scavenging potential of *M. uniflorum* leaves which can be exploited for the treatment of various free radical mediated diseases like diabetes mellitus.

Keywords: Diabetes, *M. uniflorum*, α-amylase, α-glucosidase, Antioxidant

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies in which chronic hyperglycemia is caused by defect or alterations in either the discharge or action of insulin results in disturbances in carbohydrate, fat and protein metabolism. Oxidative stress is known to play a significant role in the development and progression of Diabetes mellitus[1]. Hydrolysis of starch by pancreatic α-amylase and uptake of glucose by intestinal α-glucosidase causes rapid rise in blood glucose levels or hyperglycemia in type-2 diabetes patients. Inhibition of these enzymes is an effective approach for type-2 diabetes management[2]. Free radicals are chemical groups which contain one or more unpaired electrons and due to that unpaired electrons they are highly unstable hence cause damage to other molecules by extracting electrons from them in order to accomplish stability. Free radicals are persistently produced in the human body, as they are essential for energy supply, chemical signaling detoxification, and immune function[3]. Oxidative damage to the cellular biomolecules such as lipids, proteins and DNA are playing a critical role in the prevalence of numerous chronic diseases[4, 5]. Nowadays, research has focused on medicinal plants to extort new natural antioxidants that can replace artificial additives[6, 7]. Large amount of medicinal plants have been investigated for their antioxidant properties. A great number of plants worldwide showed a strong antioxidant activity and evidence for a powerful scavenging activity against free radicals[8, 9]. Natural antioxidants either in the form of raw extracts or their chemical constituents are very efficient to prevent the critical processes caused by oxidative stress[10]. In recent times awareness has increased considerably in finding naturally occurring antioxidants for the use in foods or medicinal materials to substitute synthetic antioxidants which are deadly restricted due to their side effects such as carcinogenicity[11]. Antioxidants from natural resources possess multifacetedness in their massive amount and magnitude of activity that provide massive scope in correcting imbalance[12]. The role of medicinal plants in disease prevention or control has been attributed to the antioxidant properties of their constituents such as vitamins, terpenoids, steroids, phenolic acids, stilbenes, tannins, flavanoids, quinines, coumarins, alkaloids[13]. Horse gram (*Macrotyloma uniflorum* (Lam.) Verdc. is a minor legume used as a pulse crop in India and has been established to have good nutritional value[14,15]. Horse gram seeds have recently

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been shown to prevent atherosclerosis and hyperlipidaemic atherosclerosis in rats[16]. This plant has been used in traditional system of medicine for treating haemorrhoids, tumours, bronchitis, cardiopathy, nephrolithiasis, urolithiasis, splenomegaly, ophthalmopathy, verminosis, inflammation and liver problem. An effort was made to investigate the hepatoprotective activity of *Macrotyloma uniflorum* in Wistar albino rats[17]. The present study aims to evaluate the ethanolic extract of *M. uniflorum* leaves for antioxidant potential, free radical scavenging activity and enzyme (α amylase and α glucosidase) inhibitory activity in order to understand the usefulness of the plant in the treatment of diabetes.

MATERIALS AND METHODS

Collection of plant material

The plant specimens for the proposed study were collected from Kothavadi village, Coimbatore district, Tamil Nadu, India. The plant was taxonomically authenticated by Dr. G.V.S Moorthy, Botanical Survey of India, TNAU campus Coimbatore, with the voucher number BSI/SRC/5/23/2013-14/Tech/1309.

Sample extraction

50 g of powdered plant material was weighed and extracted with 250 ml of ethanol for 72 hours using occasional shaker. Repeated extraction was done with the same solvent till clear colorless solvent was obtained. Obtained extract was evaporated to dryness in room temperature and stored at 0-4˚C in an air tight container.

In vitro free radical scavenging activity

DPPH radical scavenging assay was estimated by Blois method[18], 2,2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) radical activity was estimated by the method of Re, et al.,[19] the hydroxyl radical scavenging activity was measured according to the method of Klein, et al.,[20] hydrogen peroxide radical scavenging assay was determined by Ruch method[21],Nitric oxide (NO) scavenging activity of the extract was determined by the method of Green, et al.,[22] measurement of superoxide radical scavenging activity was done using the standard method of Liu, et al.,[23] and metal chelating activity was performed by the method Dinis[24]. The reducing power of the whole plant extract was quantified according to the method of Oyaizu[25] and the total antioxidant potential of sample was determined using ferric reducing antioxidant power (FRAP) by the method of Benzie and Strain[26].

In vitro α-amylase inhibition study

The α-amylase inhibitory activity was determined according to the method described by[27]. Briefly, the total assay mixture containing 200 µl of 0.02M sodium phosphate buffer, 20 µl of enzyme, and the plant extracts in the concentration range 20-100µg/ml were incubated for 10 min at room temperature followed by addition of 200 µl of 1% starch in all the test tubes. The reaction was terminated with addition of 400 µl of 3, 5 dintrosalycylic acid (DNSA) colouring reagent, then placed in boiling water bath for 5 minutes, cooled at room temperature and diluted with 15 ml of distilled water and the absorbance measured at 540nm. The control samples were also prepared accordingly without any plant extracts and were compared with the test samples containing various concentrations of the plant extracts prepared with DMSO. The results were expressed as % inhibition calculated using the formula:

$$\text{Inhibition activity (\%)} = \frac{\text{Abs (control)-Abs (extract)}}{\text{Abs (control)}} * 100$$

Acarbose was taken as reference standard with same concentration as that of sample. The percentage inhibition vs. concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as IC_{50} value.

In vitro α-glucosidase inhibition assay

The α-glucosidase was dissolved in 100 mM phosphate buffer at pH 6.8 was used as enzyme source; 10 mM paranitrophenyl-α-D-glucopyranoside (PNPG) was used as substrate. *Macrotyloma uiflorum* extract powder was weighed and mixed with dimethylsulfoxide to get a concentration of 20-100 µg/ml. The different concentration of plant extract was mixed with 320 µl of 100 mM phosphate buffer (pH 6.8) and 50 µl of 10 mM PNPG in the buffer and then it was incubated at 30°C for 5 minutes. After the incubation, 20 µl of the buffer containing 0.5 mg/ml of the enzyme was added and further incubated at 30°C for five minutes. Finally, 3.0 ml of 50 mM sodium hydroxide was added to the mixture and the absorbance (A) was measured at 410 nm on a spectrophotometer. The enzyme without plant extract was used as a control[28].

$$\text{\% Inhibition} = \frac{A_{410}\text{ control} - A_{410}\text{ test}}{A_{410}\text{ control}} * 100$$

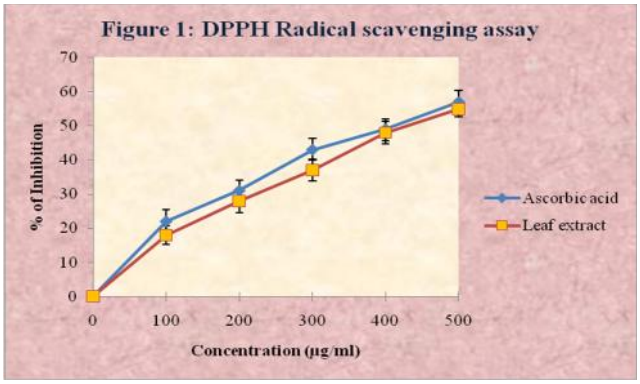
Acarbose was taken as reference standard with same concentration as that of sample. The percentage inhibition vs. concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as IC_{50} value.

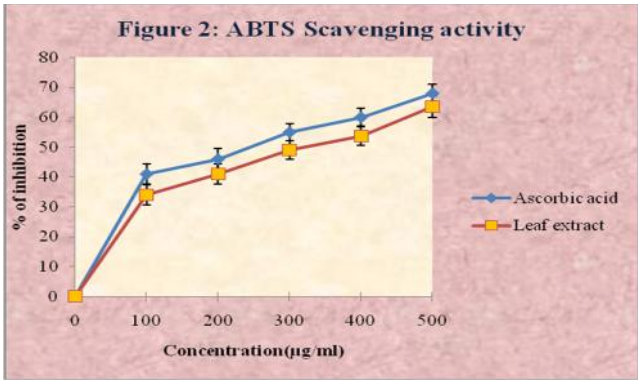
Statistical analysis

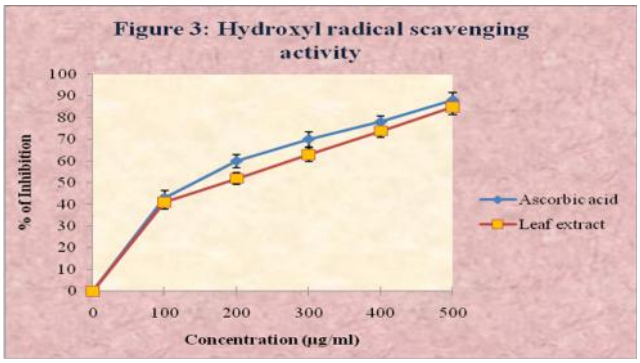
All the experimental results were entered using three parallel measurements of the Mean ± Standard deviation (n=3).

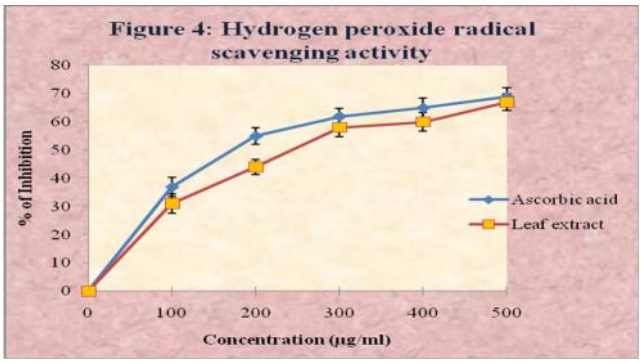
RESULTS AND DISCUSSION

Many bioactive principles from plants have been reported to have hypoglycemic effect which includes alkaloids, flavonoids, triterpenoids and carbohydrates[29-36]. More number of phytochemicals have been identified from the leaves of *Macrotyloma uniflorum* in earlier studies[5, 14]. Medicinal plants are rich sources of bioactive compounds and thus serve as an significant raw material for drug production and have become a target for the search of novel drugs[37]. Many scientists have investigated the plants containing various phytochemicals that reveal additive and synergistic interaction in antidiabetic properties which exert optimistic health promoting









effects[38]. Antioxidant tests could be based on the evaluation of lipid peroxidation or on the capacity of free radical scavenging potency (hydrogen-donating ability). The use of DPPH radical provides an easy, rapid and convenient method to assess the antioxidants capacity. Free radicals are believed to play a vital role in the aging process and in disease progression. Many aromatic, medicinal and spice plants contain compounds that acquire definite strong antioxidative components[39- 42]. α-amylase catalyzes the hydrolysis of starch and α-glucosidase catalyzes the final step in carbohydrate digestion which leads to postprandial hyperglycemia. Inhibitors of α-amylase and α- glucosidase are significant in the control of hyperglycemia as they delay carbohydrate digestion causing reduced glucose absorption rate which as a result reduces the postprandial plasma glucose rise[43].

Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating capacity. DPPH is stable nitrogen centered free radical containing an odd electron in its structure that can accept an electron or hydrogen radical to develop into a stable diamagnetic molecule[44]. The high DPPH activity could be correlated with high phenolic content. Literature survey exposed that high level of phenolic content showed rapid decrease in absorbance of DPPH radical[45]. Thus, the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in the absorbance of DPPH elucidation[46]. Figure 1 illustrates the significant decrease in the DPPH radical which is due to the scavenging ability of the plant extract and the standard ascorbic acid. The ethanolic leaf extract of *M. uniflorum* showed maximum activity of 55% at the concentration of 500µg/ml, whereas at the same time the ascorbic acid at maximum concentration exhibited 57% inhibition. The IC_{50} values were found to be 425 ± 0.55 µg/ml for ethanolic extract and 410 ± 0.76 µg/ml for ascorbic acid respectively. These results indicated that the *M. uniflorum* extract exhibited the ability to quench the DPPH radical, which indicated that the extract is having good antioxidant as well as radical scavenging activity.

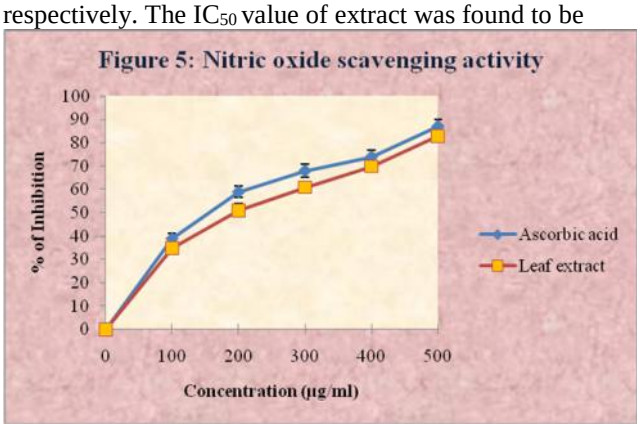
2, 2´- azino-di [3-ethylbenzthiazolin sulfonate] (ABTS) radical scavenging activity

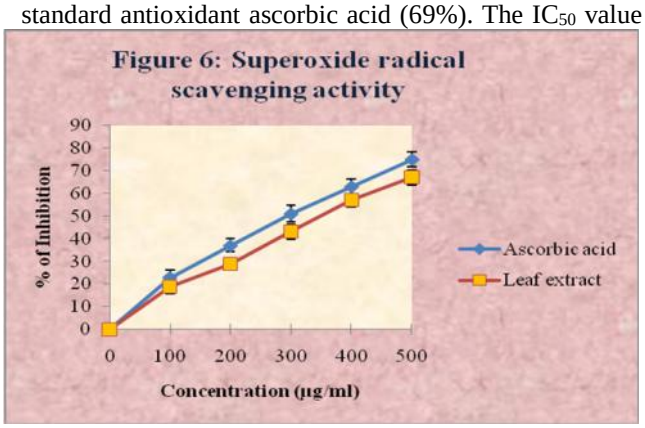
ABTS assay is an outstanding tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants[47]. Figure 2 indicates that the ethanolic extract of *M. uniflorum* on ABTS+ radical assay shows a significant antioxidant activity at a concentration of 500µg/ml with an inhibitory activity of 63% as compared to that of standard ascorbic acid (68%). The extract showed better activity in quenching ABTS radical with an IC_{50} value of 310 ± 0.72 µg/ml comparable to the standard ascorbic acid of 245 ± 0.76 µg/ml.

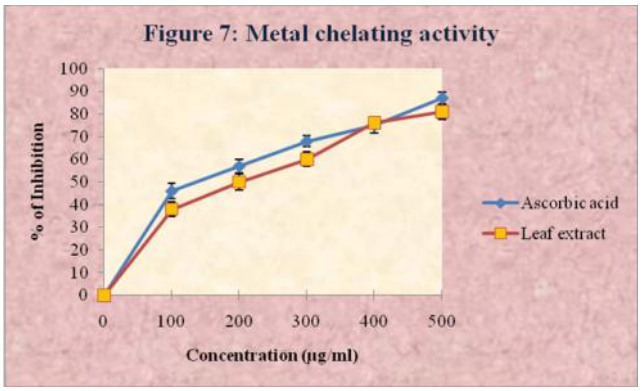
Hydroxyl radical scavenging activity

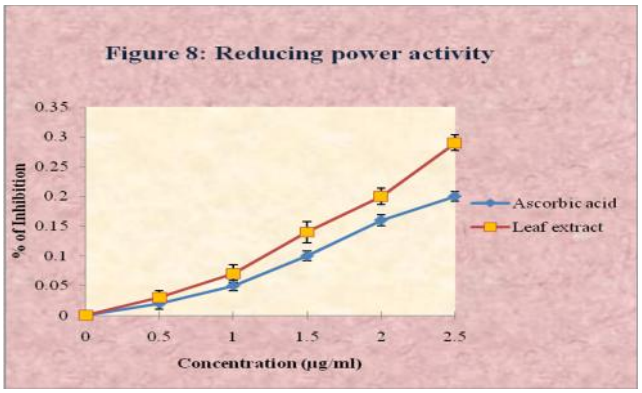
The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins[48]. The hydroxyl radical in the cells can simply cross the cell membrane at specific sites, react with the majority of biomolecules and furthermore can cause tissue damage and finally lead to cell death. Thus, removing hydroxyl radical from the body is very important for the protection of living systems[49]. The results of hydroxyl radical scavenging powers of the plant extract and ascorbic acid were depicted in Figure 3. The ethanolic leaf extract of *M. uniflorum* as well as standard exhibited noticeable scavenging ability in a dose-dependent manner. At the concentration of 500 µg/ml, the ethanolic extract exhibited 85% inhibition whereas with

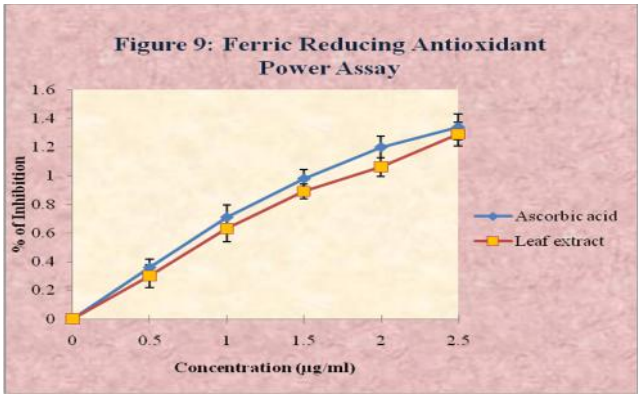
the standard antioxidant showed 88% inhibition respectively. The IC_{50} value of extract was found to be 180 ± 0.36 µg/ml and the reference standard was found to be 140 ± 0.5 µg/ml. The extract and ascorbic acid exhibited strong scavenging effects for hydroxyl radicals which could inhibit lipid damage at different concentrations. The results of ethanolic extract of *M. uniflorum* seemed to be excellent scavengers of reactive oxygen species. The percentage of hydroxyl radical scavenging activity increased as the concentration of the extract increased.











Hydrogen peroxide radical scavenging activity

Hydrogen peroxide is a feeble oxidizing agent that inactivates a small number of enzymes directly by the oxidation of essential thiol (-SH) groups[50]. Hydrogen peroxide scavenging depends upon the phenolic content of the extract which can donate electrons to H_2O_2 consequently neutralizing it into water[51]. The scavenging of hydrogen peroxide by the extract increased in a dose dependent manner is illustrated in Figure 4. The percentage inhibition of ethanolic extract was found to be 67% at 500 µg/ml concentration when compared with standard antioxidant ascorbic acid (69%). The IC_{50} value of plant extract and the standard was found to be 240 ± 0.87 µg/ml and 175 ± 0.53 µg/ml respectively. Thus proves the ethanolic extract of the *M. uniflorum* was accomplished of scavenging H_2O_2 in a dose dependent manner.

Nitric oxide (NO) scavenging activity

Nitric oxide (NO) is a potent pleiotropic moderator of physiological progression such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays numerous role as an effector particle in miscellaneous biological systems including neuronal messenger, vasodilation, antimicrobial and antitumor activities[52]. Excess concentration of NO is associated with several diseases. The *in vitro* inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the creation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce

nitrite ions that can be measured by using Griess reagent[53]. The percentage inhibition of ethanolic extract was found to be 83% at 500 µg/ml concentration when compared with standard antioxidant ascorbic acid (87%). The IC_{50} value of plant extract and the standard was found to be 155 ± 0.75 µg/ml and 190 ± 0.66 µg/ml respectively.

Superoxide radical scavenging activity

Superoxides are formed from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction such as antioxidation by catecholamines[54]. The decrease in absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The superoxide scavenging activity of ethanolic extract of *M. uniflorum* was found to increase in a concentration dependent manner (Fig.6). The extract showed maximum scavenging activity of 67% at a concentration of 500µg/ml and the standard reported 75% scavenging activity at a concentration of 500µg/ml. The IC_{50} value of extract was found to be 350 ± 0.58 µg/ml and the reference standard was found to be 295 ± 0.61 µg/ml.

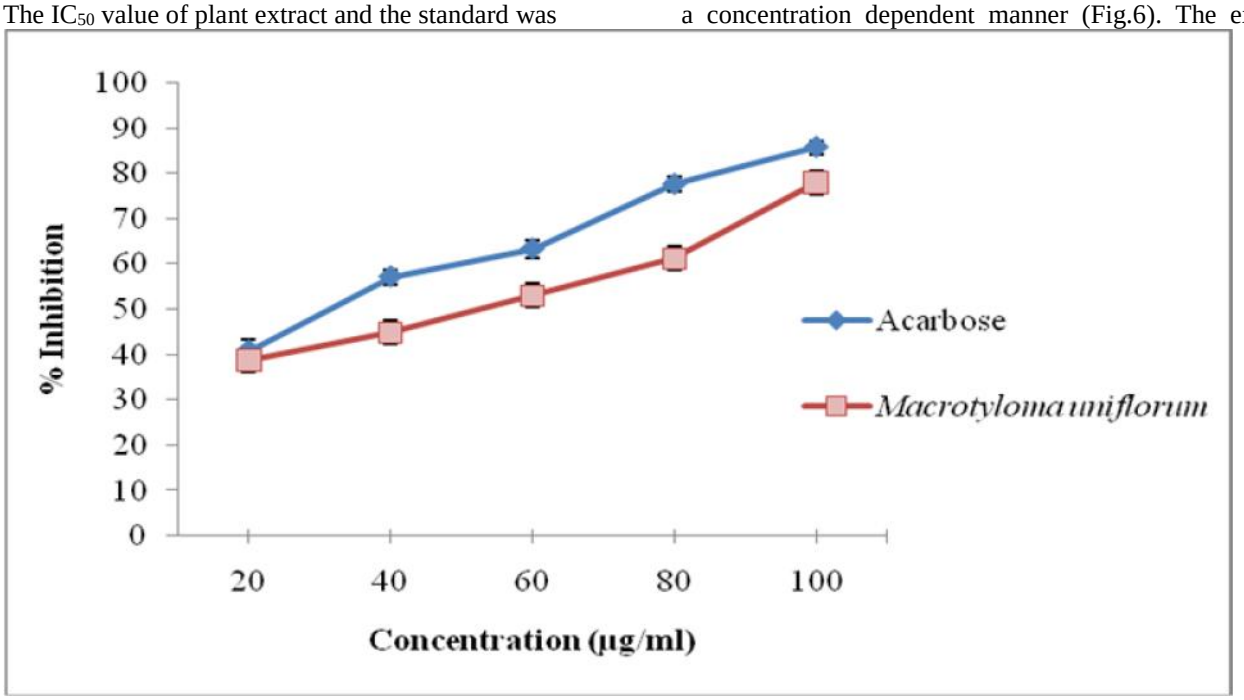


Figure 10: The percentage inhibition of α-amylase by ethanolic extract of *Macrotyloma uniflorum* (Values are expressed as Mean ± SD (n=3)

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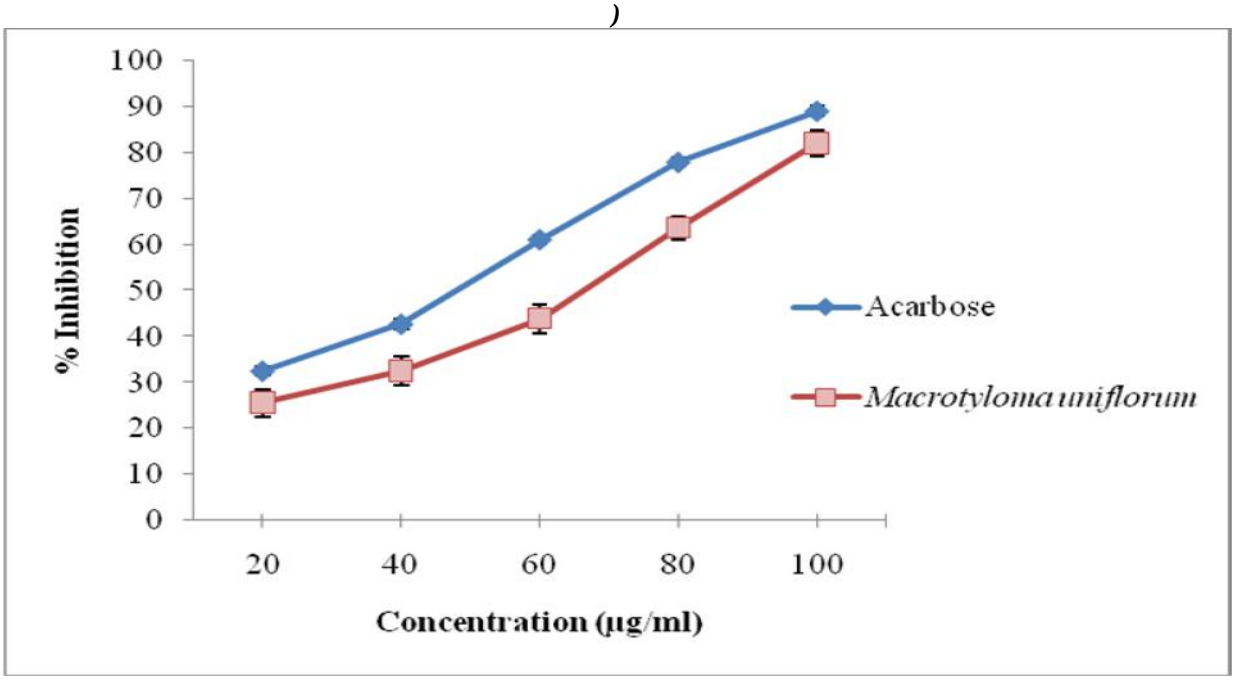

Figure 11: The percentage inhibition of α-glucosidase by ethanolic extract of *Macrotyloma uniflorum* (Values are expressed as Mean ± SD (n=3)

Metal ion chelating activity

Transition metal ions, especially iron can stimulate lipid peroxidation by Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} OH^- + OH^-$) and can also accelerate lipid peroxidation by decaying lipid hydro peroxides into peroxyl and alkoxyl radicals that can be responsible for the chain reaction. Metal ion chelating capacity is momentous since it reduces the concentration of the transition metal that catalyzes lipid peroxidation[55]. The main approach to avoid reactive oxygen species generation is associated with redox active metal catalysis involving chelating of the metal ions. At the concentration of 500 µg/ml, the ethanolic extract exhibited 81% inhibition whereas with standard antioxidant it showed 87% inhibition respectively. The IC_{50} value of the extract was found to be 200 ± 0.76 µg/ml and the reference standard was found to be 135 ± 0.57 µg/ml. The iron chelating activity of the plant extract is of great significance, because it has been projected that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders like Alzheimer's and Parkinson's diseases[56, 57].

Reducing power assay

Antioxidants are capable of donating electrons to reactive radicals that render them into more constant and unreactive species[58]. The reducing capacity of a compound may serve up a significant indicator of its potential antioxidant activity. Standard curve of ascorbic acid as well as the ethanolic extract of *M. uniflorum* was shown in figure 8 in which the ethanolic extract reducing ability increases with increasing concentration (100-500 µg/ml) like the antioxidant activity of standard curve. This result showed that the extract consists of hydrophilic compound that causes reducing power ability[59].

Ferric reducing antioxidant power assay

Generally, the reducing properties are linked with the existence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction[60]. The FRAP scavenging capacity of the ethanolic extracts of *Macrotyloma uniflorum* at five different concentrations (100-500 µg/ml) exhibited optical density like 0.3, 0.63, 0.89, 1.06 and 1.29 respectively at 595nm which is depicted in figure 9. Since FRAP assay is easily reproducible and linearly interrelated to molar concentration of the antioxidant present, it can be reported that ethanolic extract may act as free radical scavenger, proficient of transforming reactive free radical species into established non radical products[61].

In vitro α-amylase and α-glucosidase inhibitory activity

Increasing concentrations (20, 40, 60, 80 and 100 µg/ml) of *M. uniflorum* ethanolic leaf extracts were prepared for their inhibitory enzyme activities. Figure 10 and 11 shows the % inhibition of α- amylase and α-glucosidase activity of *M. uniflorum* ethanolic leaf extract along with the standard, acarbose. There was a dose dependent increase in the percentage inhibitory activity against α-amylase with 38.78% at a concentration of 20 µg/ml and 78% at 100 µg/ml concentration. The IC_{50} values of the plant extract and the standard acarbose were found to be 23 ± 0.54 µg/ml and 52 ± 0.61 µg/ml respectively. Phenols and flavonoids inhibit amylase, sucrase and also Sodium Glucose Transporter- 1 of intestinal brush boundary cells which diminish the absorption of glucose that ultimately reduce the hyperglycemia[62]. This result is in concurrence with previous reports which indicated that excessive inhibition of pancreatic α- amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon and therefore gentle α-amylase inhibition activity is desirable[63]. In this context our plant extract shows moderate activity which is preferable. In the case of α-glucosidase, 25.57% and 82.1% inhibition was observed at 20 µg/ml and 100 µg/ml concentrations. The positive control, acarbose has exerted the highest persuasive inhibitory action against α-amylase (85.71%) and α- glucosidase (89.14%). The IC_{50} value for plant extract was found to be 67 ± 0.47 µg/ml whereas α-glucosidase inhibitory activity of positive control acarbose produced percentage of 51 ± 0.77 µg/ml. One of the strategies and methods adopted to cure diabetes mellitus involves the inhibition of carbohydrate digesting enzymes such as α-amylase and α-glucosidase in the gastrointestinal glucose absorption thereby lower the postprandial glucose level[64]. An effective means of lowering the levels of postprandial hyperglycemia has been obtainable by α-amylase and α- glucosidase inhibitors. Several inhibitors of α-amylase and α-glucosidase have been isolated from medicinal plants to serve as an alternative drug with augmented potency and lesser adverse effects than existing synthetic drugs[65, 66].

CONCLUSION

Ethanolic extract of the *Macrotyloma uniflorum* produced momentous antioxidant activity, demonstrating that the extract exhibited a potential free radical scavenging ability. In the current study, *M. uniflorum* confirmed α-amylase and α-glucosidase inhibitory potential which may serve as a lead for the isolation and identification of compounds responsible for it. However, the active ideology responsible for inhibitory action of α-amylase and α-glucosidase need to be acknowledged and characterized for the improvement of indigenous botanical possessions for the development of novel hypoglycemic drug. The result obtained from the present *in vitro* study will be confirmed by taking up *in vivo* studies in future. Due to the possession of secondary metabolites this study justifies that there is hypoglycemic activity of *M. uniflorum* leaves and this can be used in the management of diabetes.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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