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Quantitative evaluation and *in vitro* free radical scavenging ability of ethanolic stem extract of *Macrotyloma uniflorum* L.

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ABSTRACT

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, metal chelating activity, ferric reducing antioxidant power assay (FRAP) and reducing power assay using established procedure. Additionally total phenolics and tannin were estimated. The ethanolic stem extract showed better ability to scavenge superoxide radical (IC_{50} value of 435 ± 0.45 µg/ml), hydroxyl radical (IC_{50} value of 270 ± 0.51 µg/ml), 2,2-diphenyl-1-picryl hydroxyl radical (IC_{50} value of 485 ± 1.05 µg/ml), 2,2' azinobis-3 ethylbenzothiozoline-6 sulfonic acid (IC_{50} value of 310 ± 0.78 µg/ml), hydrogen peroxide (IC_{50} value of 400 ± 0.76 µg/ml), nitric oxide radical (IC_{50} value of 335 ± 0.76 µg/ml) and metal chelating activity (IC_{50} value of 300 ± 0.58 µg/ml). The reducing power and ferric reducing antioxidant power abilities increased with respect to increase in concentration of extract. In all the assays, ascorbic acid was used as standard.

Keywords: *Macrotyloma uniflorum*, Free radicals, Antioxidants

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INTRODUCTION

Medicinal plants are the bio resources gifted by natural world used to heal an assortment of human diseases to determine their probable sources for new drugs [1,2]. In living systems free radicals are produced as part of the body's regular metabolic process, and the free radical chain reactions are regularly produced in the mitochondrial respiratory chain, through xanthine oxidase activity, atmospheric pollutants, from intermediary metal catalysts, xenobiotics and drugs. In addition, chemical draft of fat stores under different conditions such as lactation, fever, infection and fasting can result in increased radical activity and cell damage. Oxygen free radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structural arrangement and function of collagen basement and other membranes, and play a role in the long-term complication of diabetes [3,4]. The advantageous medicinal effects of plant materials normally result from the combinations of secondary products present in the plants. The medicinal actions of plants are unique to particular plant species or groups are dependable with this concept as the combination of secondary products in a particular plant is taxonomically distinct.

Oxidative stress is known to be a constituent of molecular and cellular tissue damage mechanisms in an extensive spectrum of human diseases like cancer, aging, neuro degenerative diseases, atherosclerosis and pathological events [5,6]. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might fabricate highly reactive oxygen species, which are capable of oxidizing biomolecules, resultant in tissue damage and cell death. Therefore, the great interest has been recently paying attention on the natural foods, medicinal plants and phytocostituents due to their well known abilities to scavenge free radicals (i.e. antioxidant power) [7,8].

Macrotyloma uniflorum has been used in traditional system of medicine for treating haemorrhoids, tumours, bronchitis, cardiopathy, nephrolithiasis, urolithiasis, splenomegaly, strangury (irritation at the base of the bladder), ophthalmopathy, verminosis (disease caused by parasitic worms), inflammation and liver problem. An attempt was made to investigate the hepatoprotective activity of *Macrotyloma uniflorum* in Wistar albino rats [9]. Horse gram seeds have recently been shown to prevent atherosclerosis in rats and may be a probable functional food for the prevention of hyperlipidaemic atherosclerosis [10]. Leaves and stems were reported to have more number of amino acids and lectin like glycoprotein. Presence of coumesterol and psoralidin were also reported. Seeds of *M. uniflorum* contain lectins, glycoprotein, agglutinin, anti-A phytoagglutinin, glycosidase enzymes, allantoinase, diuretic dipeptide and pyroglutamyl glutamine [11]. Literature survey showed that Dolichin A & B, pyroglutaminylglutamine along with several flavonoids were secluded from this plant [12].

The use of antioxidants in management of oxidative stress related pathologies is a possible therapeutical strategy for the future. Natural product with antioxidant properties may perhaps trigger this goal. The aim of this *in vitro* study is to assess the antioxidant activity of the ethanolic stem extract of *Macrotyloma uniflorum* using various *in vitro* scavenging assays.

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 is obtained. Obtained extract was evaporated to dryness by using a rotary vacuum evaporator at 40-50˚C 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 [13], 2,2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) radical activity was estimated by the method of Re, et al., [14] the hydroxyl radical scavenging activity was measured according to the method of Klein, et al., [15] Hydrogen peroxide radical scavenging assay was determined by Ruch method [16], Nitric oxide (NO) scavenging activity of the extract was determined by the method of Green, et al., [17] Measurement of superoxide radical scavenging activity was done using the standard method of Liu, et al., [18] and metal chelating activity was performed by the method Dinis [19]. The reducing power of the whole plant extract was quantified according to the method of Oyaizu [20] and the total antioxidant potential of sample was determined using ferric reducing antioxidant power (FRAP) by the method of Benzie and Strain [21].

Estimation of total phenol

Total phenolic content was carried out by the method of Singleton and Rossi [22]. The plant sample (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin-Ciocalteu reagent was added. After 3 minutes 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath exactly for one minute. It was then cooled and the absorbance was measured at 650 nm using spectrophotometer against the reagent blank. Standard curve of gallic acid solution and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

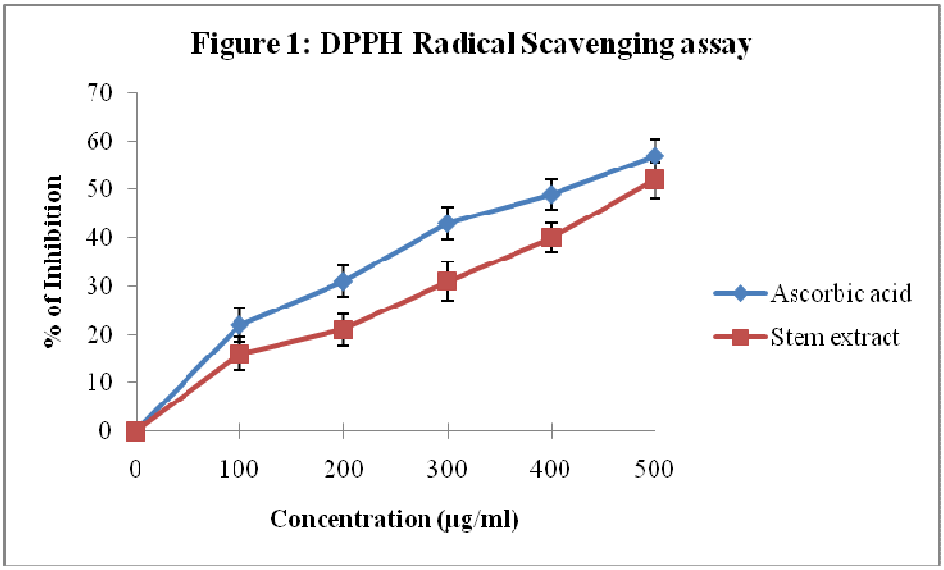
Estimation of total tannin

Total tannin content was determined in the method of Schendrel [23]. 0.2-1.0 ml of standard tannic acid solution was pipetted out in to a series of test tubes. To another test tube 0.5 ml of extract solution was taken. The volumes of all the tubes were made up to 3.0 ml with distilled water. 3.0 ml of distilled water was taken as blank. To all the tubes added 2.0 ml of 20% Na_2CO_3 followed by the addition of 0.5 ml of Folin-Ciocalteu reagent and incubated at room temperature for 30 minutes. The absorbance was read against reagent blank at 700 nm. From the standard graph, the amount of tannin present in the sample was calculated.

RESULTS AND DISCUSSION

The oxidative damage caused by reactive oxygen species (ROS) such as the super oxide radical ($O_2^{\bullet-}$) and by hydroxyl radicals ($HO^{\bullet}$) on lipids, proteins, and nucleic acids may generate various diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, immune deficiency diseases and aging [24-26]. Natural and synthetic antioxidants have been shown to improve product stability, quality and lead long life. Many research works have mentioned the inconvenience of synthetic antioxidants [27-30]. Indeed, the use of synthetic antioxidant in food products has decreased due to their volatility, as well as their possible poisonous and carcinogenic effects on health [28]. Nowadays, research has an alert on using medicinal plants to extract new natural antioxidants that can replace synthetic additives in addition, the public's conviction that phytochemicals are intrinsically safer than synthetic chemicals [31]. Herbal medications gain popularity due to an awareness that there is a lower incidence of adverse reaction to plant preparation compound than synthetic pharmaceuticals [32].

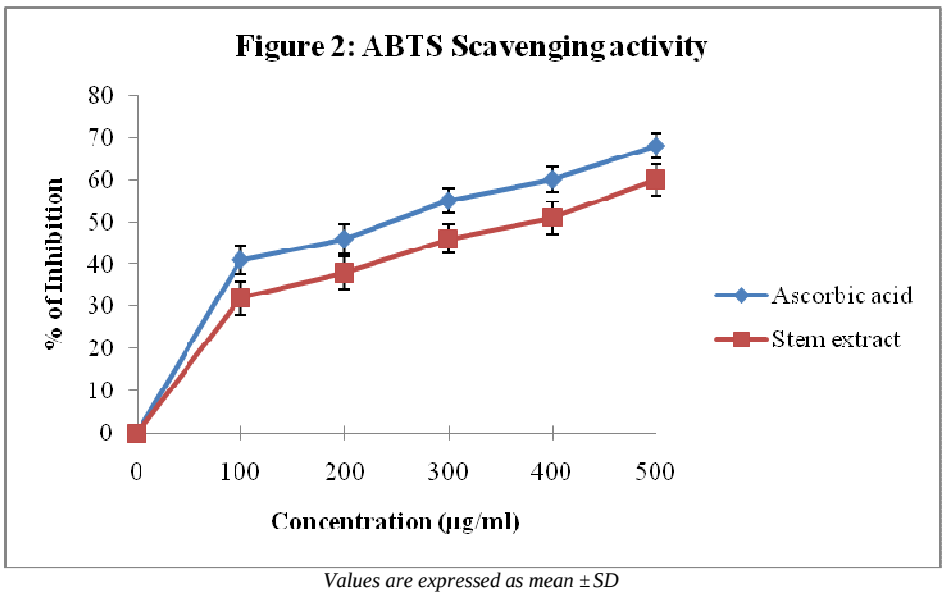
DPPH radical scavenging test is based on the exchange of hydrogen atoms between the antioxidant and the constant DPPH free radical. DPPH is a stable free radical at room temperature which accepts an electron or hydrogen radical to form a steady diamagnetic molecule. DPPH radical is condensed to the corresponding hydrazine, a colour change of the solution from violet to yellow indicates the scavenging behavior of the crude plant sample due to bioactive compounds such as phenolic compounds, flavonoids, terpenoids and their derivatives [33,34]. Further reduction of DPPH radical is related to the high scavenging activity of the particular extract [35]. In figure 1 ethanolic stem extract of *Macrotyloma uniflorum* showed maximum activity of 60% at 500 µg/ml where as ascorbic acid at the same concentration exhibited 68% inhibition respectively. IC_{50} values were found to be 485 ± 1.05 µg/ml and 410 ± 0.76 µg/ml for the extract and the reference standard ascorbic acid.



Values are expressed as mean ±S.D

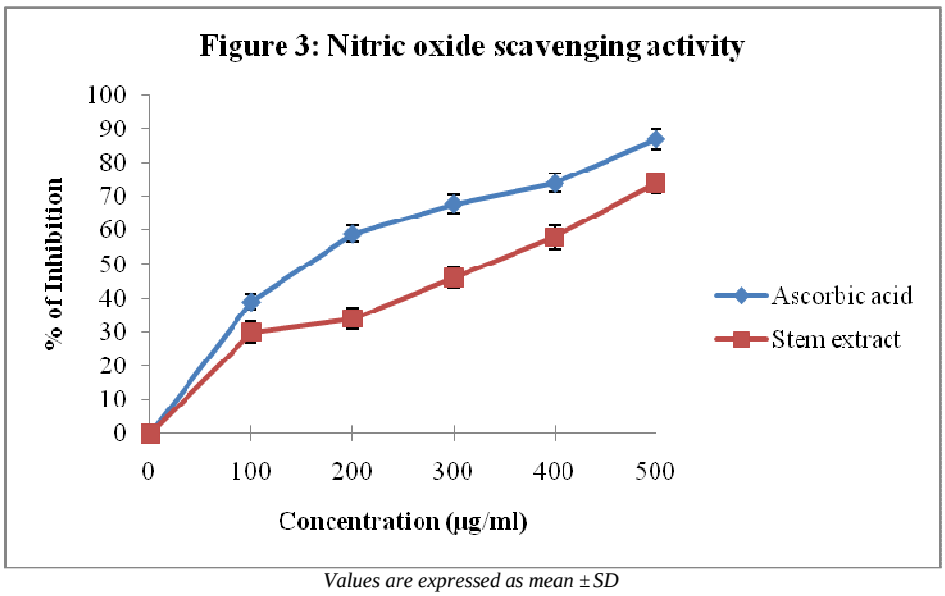
The ABTS assay is based on the inhibition of the absorbance of the radical cation ABTS, which has a distinctive wavelength fascination spectrum [36]. The ABTS chemistry involves nonstop generation of ABTS radical mono cation with no contribution of any intermediary radical. It is a decolorization assay; consequently the radical cation is performed prior to the addition of antioxidant test system, rather than the production of the radical taking place

frequently in the existence of antioxidants. The results obtained this assay imply the action of the extract either by inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards ABTS radicals have been previously reported [37]. 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 ± 0.78 µg/ml comparable to the standard ascorbic acid of 245 ± 0.76 µg/ml.



Values are expressed as mean ±SD

Nitric Oxide (NO) is a diffusible free radical that plays many effectual roles in assorted biological systems together with neuronal messenger, vasodilatation, antimicrobial and antitumor activities [38]. The nitric oxide radical scavenging activities of *M. uniflorum* stem extract were shown in figure 3. The IC_{50} value of the extract was found to be 335 ± 0.76 µg/ml where as the standard ascorbic acid exposed 155 ± 0.66 µg/ml. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. *M. uniflorum* extract also moderately inhibits nitrite formation by directly competes with oxygen to react with nitric oxide.



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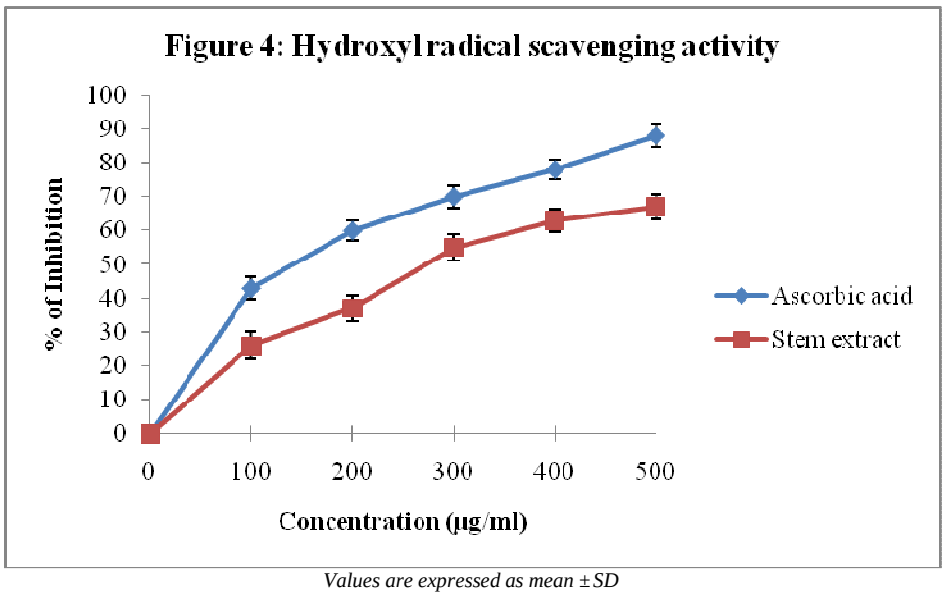
Hydroxyl radical is a tremendously reactive oxidizing radical that will react with most of the biomolecules at diffusion controlled rates. It has extremely short half life but is competent of causing damage inside a small radius of its place of production. A single hydroxyl radical can result in development of many molecules of lipid hydro peroxides in the cell membrane, which may rigorously interrupt its function, and finally lead to cell death [39]. In addition, hydroxyl radical is considered to be one of the quick initiators of the lipid peroxidation progression, abstracting hydrogen atoms from unsaturated fatty acids [40]. The results of hydroxyl radical scavenging powers of the plant extract and ascorbic acid were depicted in figure 4. 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 67% inhibition whereas with the standard antioxidant showed 88% inhibition respectively. The IC_{50} value of extract was found to be 270 ± 0.51 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 slow down 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.



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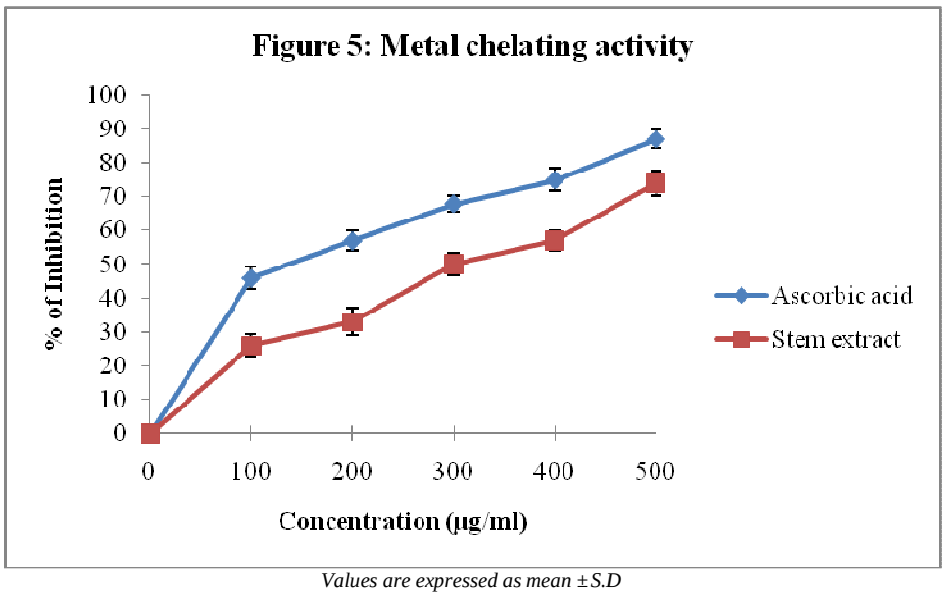
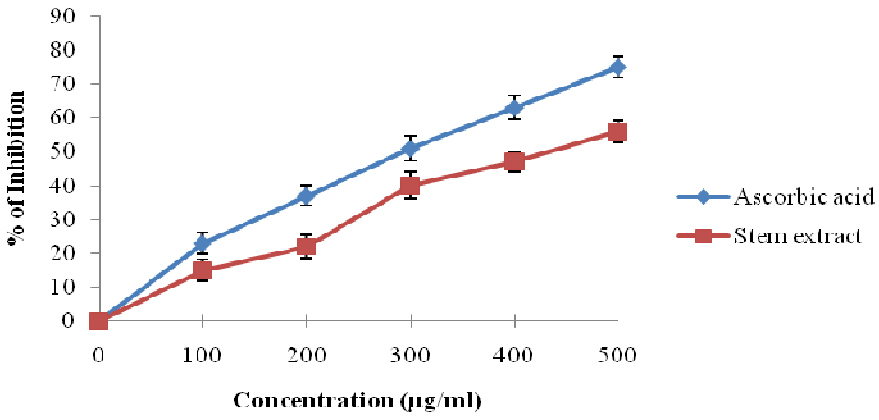


Values are expressed as mean ±S.D

Fe^{2+} has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reaction affords fortification against oxidative damage. Chelating agents can inhibit radical generation by stabilizing transition metals, accordingly reducing free radical damage. In addition, phenolic compounds have the effective potential to bind to metal ions due to their chemical structures, and have been shown to exhibit antioxidant activity through the chelation of metal ions [41]. At the concentration of 500 µg/ml, the ethanolic extract exhibited 74% inhibition whereas with standard antioxidant showed 87% inhibition respectively (Figure 5). The IC_{50} value of the extract was found to be 300 ± 0.58 µg/ml and the reference standard was found to be 135 ± 0.57 µg/ml.

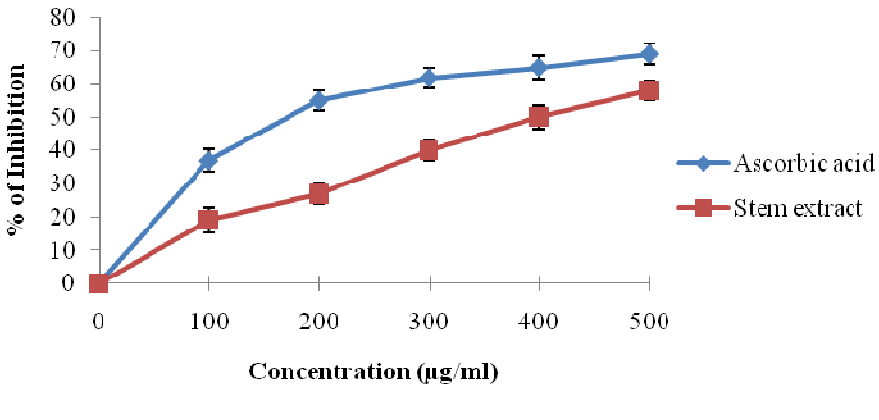
Superoxide anion is also another dangerous reactive oxygen species as it damages cellular components in biological systems. This species is formed by a number of enzyme systems in auto oxidation reactions and by non enzymatic electron transfers that univalently diminish molecular oxygen and also reduce certain iron complexes such as cytochromes [42]. Figure 6, reveals that a considerable dose response relationship is found in the superoxide free radical scavenging activity in the plant extract. Maximum scavenging activity (56%) was observed at 500 µg/ml concentrations and the IC_{50} value of the extract and ascorbic acid found to be 435 ± 0.45 µg/ml and 295 ± 0.61 µg/ml respectively.

Figure 6: Superoxide radical scavenging activity



Values are expressed as mean ± SD

Figure 7: Hydrogen peroxide radical scavenging activity

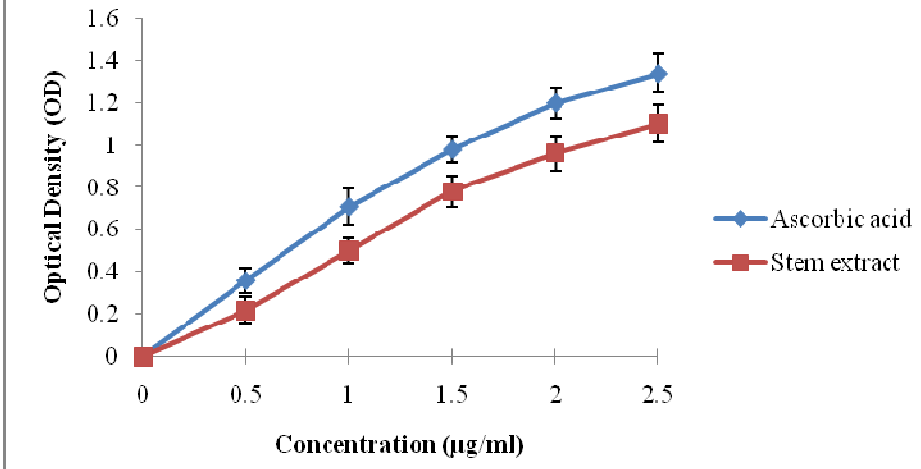


Values are expressed as mean ± SD

Hydrogen peroxide, even though not a radical species play a function to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally occurring iron complexes within the cell believed to react with H_2O_2 *in vivo* to generate extremely reactive hydroxyl radicals and this may be the beginning of many of its toxic effects [43]. The scavenging of hydrogen peroxide by the extract increased in a dose dependent manner is illustrated in figure 7. The percentage inhibition of ethanolic extract was found to be 58% 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 400 ± 0.76 µg/ml and 175 ± 0.53 µg/ml respectively. Thus proves the ethanolic extract of the *M. uniflorum* was able to scavenging H_2O_2 in a dose dependent manner.

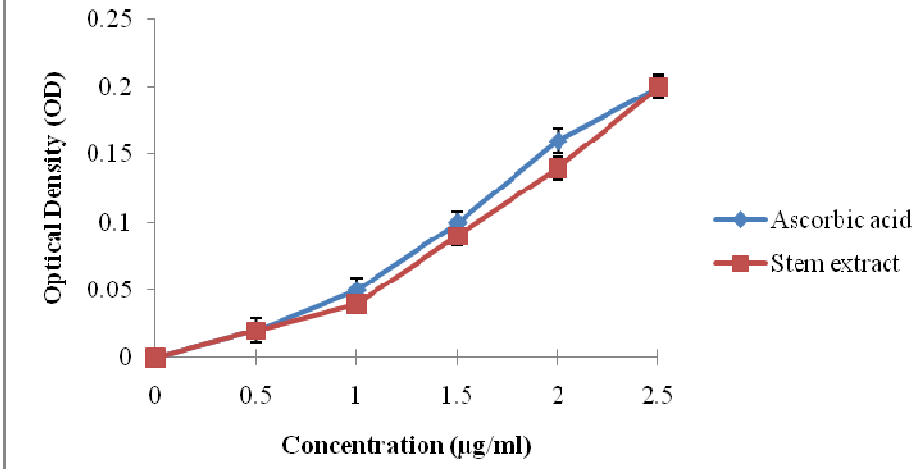
Ferric Reducing Antioxidant Power (FRAP) assay is easily reproducible and linearly interrelated to molar concentration of the antioxidant present and it was reported that its act as free radical scavenger, proficient of transforming reactive free radical species into conventional non radical products [44]. The FRAP scavenging capacity of the ethanolic extracts of *Macrotyloma uniflorum* at five different concentrations (100-500 µg/ml) exhibited optical density like 0.22, 0.5, 0.78, 0.96 and 1.1 respectively at 595nm which is depicted in figure 8.

Figure 8: Ferric Reducing Antioxidant Power Assay



Values are expressed as mean ±SD

Figure 9: Reducing power activity



Values are expressed as mean ±SD

Reducing power of the fractions was assessed using ferric to ferrous reducing action as determined spectrophotometrically from the configuration of Perl's Prussian blue colour complex [45]. In the measurement of the reducing ability, it has been investigated from the Fe^{3+} to Fe^{2+} transformation. Fe^{3+} reduction is frequently used as an indicator of electron donating activity, which is a significant mechanism of phenolic antioxidant action and can be strongly interrelated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating hydrogen atom [46,47]. Standard curve of ascorbic acid as well as the ethanolic extract of *M. uniflorum* was shown in figure 9 in which the ethanolic extract reducing ability increases with increasing concentration (100-500 µg/ml) like the antioxidant activity of standard curve.

Total Phenolic Content (TPC)

Phenolic compounds are ubiquitous secondary metabolites in plants and they are known to have antioxidant activity also it is likely that the activity of these extracts is due to these compounds [48]. Phenolics are vital plant secondary metabolites with antioxidant activity owing to their redox potential, which play an important role in fascinating and neutralizing the free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [49]. In the present study, the total phenol content was estimated and it was found that the ethanolic extract of *M. uniflorum* contained 400 ± 0.64 mg/g of dried extract equivalent to standard Gallic acid.

Table 1: Results of quantitative estimation of total phenolics and total tannin content of *M. uniflorum*

S.No	Parameters	Content (mg/g)
1.	Phenolics	400 ± 0.64
2.	Tannin	130 ± 0.76

The values are expressed as mean ± SD of triplicates

Total Tannin Content (TTC)

The toxic or anti-nutritional effects tend to accelerate the nervous tension when a very large quantity of the diet having high concentration of tannins. Thus consumption of foods naturally having antioxidant activity is the most resourceful way of combating such tissue injuries, undesired transformations and preventing health risks [50]. In the present study, the total phenol content was estimated and it was found that the ethanolic extract of *M. uniflorum* contained 130 ± 0.76 mg/g of dried extract equivalent to standard tannic acid.

CONCLUSION

From this results we find that a good amount of phenol and tannin were present in *M. uniflorum* stem extract. The extracts when tested for their free radical scavenging activity were found to have considerable antioxidant potential. This activity is due to their elevated phenol and tannin content. Hence this plant extract is good source for the development of natural antioxidant medicines for diseases involving free radicals. From the observed results it could be concluded that the ethanolic stem extract of *M. uniflorum* may be a probable source of natural antioxidant reveled from various *in vitro* assays.

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