In Vitro Free Radical Scavenging Potential of Methanol Extract of Entire Plant of *Phyllanthus Reticulatus* Poir

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Summary

The herb *Phyllanthus reticulatus* is commonly known as potato bush, which is widely used in the indigenous system of medicine for the treatment of diarrhoea, diabetes, obesity and burns. The methanolic extract of the plant was studied for its *in vitro* free radical scavenging activity in different methods *viz* DPPH radical scavenging assay, ABTS radical scavenging assay, O-phenanthroline assay, lipid peroxidation assay, nitric oxide scavenging assay, superoxide scavenging assay, total antioxidant and non-enzymatic haemoglobin glycosylation assay. The results were analyzed statistically by regression method. Its antioxidant activity was estimated by IC_{50} value and the values were 14.31 µgm/ml (DPPH radical scavenging), 10.51 µgm/ml (ABTS radical scavenging), 22.91µgm/ml (O-phenanthroline assay), 32.16 µgm/ml (lipid peroxidation), 90.51µgm/ml (nitric oxide scavenging) and 4.18µgm/ml (superoxide scavenging). In total antioxidant capacity assay, 1 mg of extract is equivalent to 41.31µg of ascorbic acid. It showed 83.69% inhibition of haemoglobin glycosylation.

Keywords: DPPH, lipid peroxidation, nitric oxide, *Phyllanthus reticulatus*, superoxide

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Introduction

Reactive oxygen species (ROS) can contribute to the etiology of many disorders such as cancer, liver diseases, atherosclerosis, respiratory diseases and inflammatory response syndrome. In recent years there is great deal of interest in developing agents to control damage induced by ROS in biological systems. As plants produce a lot of antioxidants to control the oxidative stress cause by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity[1].

In Indian system of medicine *Phyllanthus reticulatus* Poir (Euphrobiaceae) is an important medicinal plant and popularly known as 'potato-bush'. It is distributed through out India, in hedges or waste places near villages and along streams and canals. Literature survey reveals that the whole plant is astringent, sweet, cooling, diuretic, alternant, stomachic, constipating and attenuant. It is reported to be useful in vitiated condition of *pitta*, burning sensation, strangury, gastropathy, ulemorrhagia, ophthalmodynia, sores, burns, suppuration, diarrhea, skin eruption and obesity[2-4]. It contains polyphenols, flavonoid glycosides[5], tannic acid, friedelin, epifriedelinol, betulin, taraxerone, beta- sitosterol, glochidonol, octacosanol, taraxeryl acetate and 21-alpha-hydroxyfriedelan-3-one[6]. *Phyllanthus reticulatus* has also been reported to possess antidiabetic activity[7]. Though widely used in the indigenous system of medicine, there is however no available data with relation to its antioxidant activity *in vitro* models. The antioxidant property of aqueous extract of the same plant has been already reported. Hence the current paper deals with the antioxidant activity of the methanolic extract of *P. reticulatus* using *in vitro* methods.

Materials and Methods

1, 1-diphenyl, 2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. TBA (Thiobarbituric acid), TCA (Trichloro acetic acid) and BHT (Butylated hydroxytoluene) obtained from Himedia, Mumbai. The other chemicals used were 2,2- azinobis- (3-ethylbenzothiazoline- 6- sulphonate) (ABTS), O-Phenanthroline, ferric chloride, ascorbic acid, sodium nitropruside, dimethyl sulphoxide, NBT(Nitro blue tetrazolium chloride), Folin Ciocalteu's reagent, Gallic acid, sodium carbonate, sodium hydroxide and potassium chloride. All other chemicals and solvents used in the experiment were of analytical grade. The instruments used were UV spectrophotometer (Shimadzu 1650), homogenizer (Remi, India), centrifuge (Remi, India) and pH meter (Elico Ltd., India).

Plant material

The whole plant of *Phyllanthus reticulatus* was collected in the month of August from the surroundings of Manipal. The plant was authenticated by Dr. Gopalakrishna Bhat, Botanist, Poorna Prajna College, Udupi. A voucher specimen (No.561) has been deposited for future reference in the museum of Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal.

Preparation of methanolic extract

About 50 g of the entire plant powder was taken in a soxhlet extractor and extracted with methanol for 72 hours. The extract was filtered and the filterate was distilled off. The residue obtained was stored in the dessicator until its use.

Preliminary phytochemical screening

Preliminary phytochemical screening[8] revealed the presence of phytosterols, polyphenols, flavonoids and carbohydrates.

Preparation of stock solution of methanolic extract

The stock solution was prepared in the concentration of 1000 µg/ml in methanol. From this stock solution, different aliquot concentrations *viz* 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml were prepared in methanol and used for antioxidant studies.

DPPH radical scavenging assay[9,10]

To the methanolic solution of DPPH (1 mM) an equal volume of the test compound dissolved in water was added at various concentrations from 2 to 1000 µg/ml in a final volume of 1 ml. An equal amount of water was added to the control. After 20 min, absorbance was recorded at 517 nm. Experiment was performed in triplicate.

ABTS radical scavenging assay[9,10]

To the reaction mixture containing 0.3 ml of ABTS radical , 1.7 ml phosphate buffer and 0.5 ml drug was added at various concentrations from 2 to 500 µg/ml. Blank was carried out without drug. Absorbance was recorded at 734 nm. Experiment was performed in triplicate.

O-phenanthroline assay [(9-11)]

The reaction mixture containing 1ml O-Phenanthroline, 2ml Ferric chloride, and 2ml drug at various concentrations ranging from 2 to 1000 µg/ml in a final volume of 5 ml was incubated

for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of test compound and Absorbance obtained taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without drug. Experiment was performed in triplicate.

Lipid peroxidation assay[12]

Egg phosphotidylcholine (20mg) in chloroform (2ml) was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5ml) with a vortex mixture. The mixture was sonicated to get a homogeneous suspension of liposome. Lipid peroxidation was initiated byadding 0.05 mM ascorbic acid to a mixture containing liposome (0.1ml). 150 mM potassium chloride, 0.2 mM ferric chloride, drug solution (2-64 µg/ml) were added separately in a total volume of 1ml. The reaction mixture was incubated for 40 min at 37ºC. After incubation, the reaction was terminated by adding 1ml of ice cold 0.25M sodium hydroxide containing 20% w/v TCA, 0.4% w/v TBA and 0.05% w/v BHT. After keeping in boiling water bath for 20 min, the samples were cooled. The pink chromogen was extracted with constant volume of n-butanol and absorbance of the upper organic layer was measured at 532nm. The experiment was performed in triplicate.

Nitric oxide radical scavenging[13]

Sodium nitroprusside 5mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test compound, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 ºC for 5 hr after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The experiment was performed in triplicate.

Superoxide scavenging[14]

Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 ml of alkaline DMSO and 0.2 ml of NBT 20 mM in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate.

Total Antioxidant Capacity[9]

Total antioxidant capacity was measured by spectrophometric method. 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in eppendorf tube with 1 ml of reagent solution

(0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95^0C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid. The experiment was performed in triplicate.

Non-enzymatic haemoglobin glycosylation assay[15,16]

The antioxidant activities of different extracts were investigated by estimating degree of non-enzymatic haemoglobin glycosylation, measured colorimetrically. Haemoglobin, 60 mg/100 ml in 0.01 M phosphate buffer (pH 7.4) was incubated in presence of 2 g/100 ml concentration of glucose for 72 h, in order to find out the best condition for haemoglobin glycosylation. The assay was performed by adding 1 ml of glucose solution, 1 ml of haemoglobin solution, and 1 ml of gentamycin (20 mg/ 100 ml), in 0.01 M phosphate buffer (pH 7.4). The mixture was incubated in dark at room temperature for 72 h. The degree of glycosylation of hemoglobin in the presence of different concentration of extracts and their absence, were measured colorimetrically at 520 nm. The experiment was performed in triplicate.

Statistical analysis

All results are expressed as mean ± S.E.M. Linear regression analysis (Origin 6.0 version) was used to calculate the IC_{50} values.

Results

Several concentrations ranging from 2-1000 µg/ml of the aqueous extract of *Phyllanthus reticulatus* Poir were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner upto the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all models. The maximum inhibitory concentration (IC_{50}) of extract and standard in all models viz. DPPH, ABTS, iron chelating, lipid peroxidation, nitric oxide scavenging and superoxide scavenging has been given in Table 1.

Table: 1 Antioxidant IC_{50} values in different *in vitro* models.

Sl. No.	Assay	Methanolic extract	Ascorbic acid/Tocopherol
1	DDPH scavenging assay	14.31	9.9982
2	ABTS scavenging assay	10.51	35.21
3	Iron chelating assay	22.908	2.017
4	Lipid peroxidation assay	32.16	30.67
5	Nitric oxide scavenging assay	90.51	38.68
6	Superoxide scavenging assay	4.18	15.79

In total antioxidant capacity assay, it was found that 1 mg of extract is equivalent to 41.31 µg of ascorbic acid. It showed 83.69 % inhibition of haemoglobin glycosylation with a concentration of 1.0 mg/ml in non-enzymatic haemoglobin glycosylation assay. On a comparative basis the extract showed better activity in quenching superoxide oxide with an IC_{50} value of 4.31µg/ml. The activity was moderate in remaining antioxidant models. The antioxidant activity of the extract is close and identical in magnitude, and comparable to that of standard antioxidant compounds used.

Discussion

There is extensive evidence to implicate free radicals in the development of degenerative diseases[17]. Free radicals have been implicated in the causation of ailments such as diabetes, liver cirrhosis, nephrotoxicity, cancer etc[18]. Together with other derivatives of oxygen they are inevitable byproducts of biological redox reactions[19]. Reactive oxygen species such as super oxide anions, hydroxyl radical and nitric oxide inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation[20].

DPPH is a stable free radical at room temperature and accept an electron or hydrogen radical to become stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm[21]. DPPH radicals react with suitable

reducing agents, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up (Fig. 1).

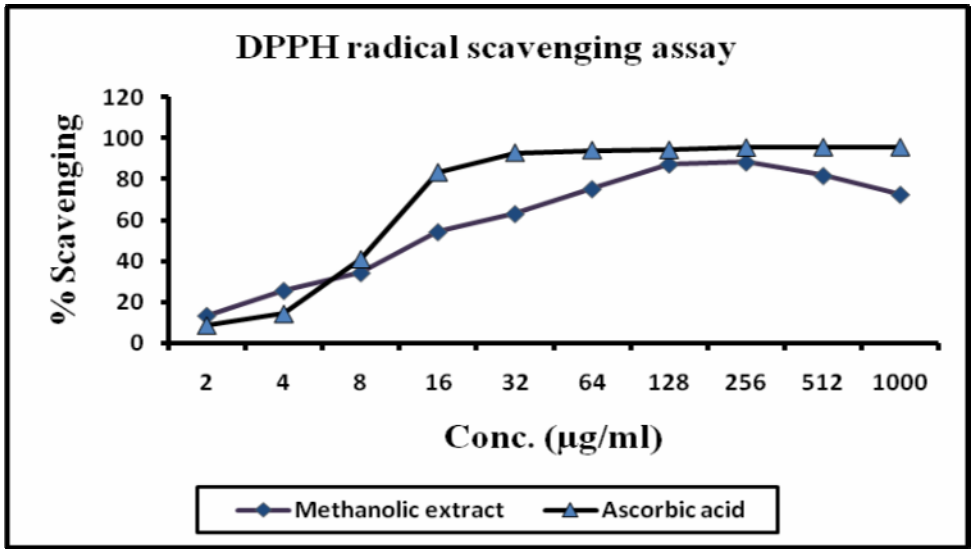

Fig. 1 Antioxidant activity of different concentrations of methanolic extract and ascorbic acid in DPPH radical scavenging assay. Each value represents mean ± SEM.

ABTS is a decolorisation assay, which involves the direct generation of ABTS radical mono cation, which has a long wavelength absorption spectrum without the involvement of any intermediary radical. The antioxidant activity of the extract by this assay implies that action may be by either inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards this radical have been reported in earlier studies[22] (Fig. 2).

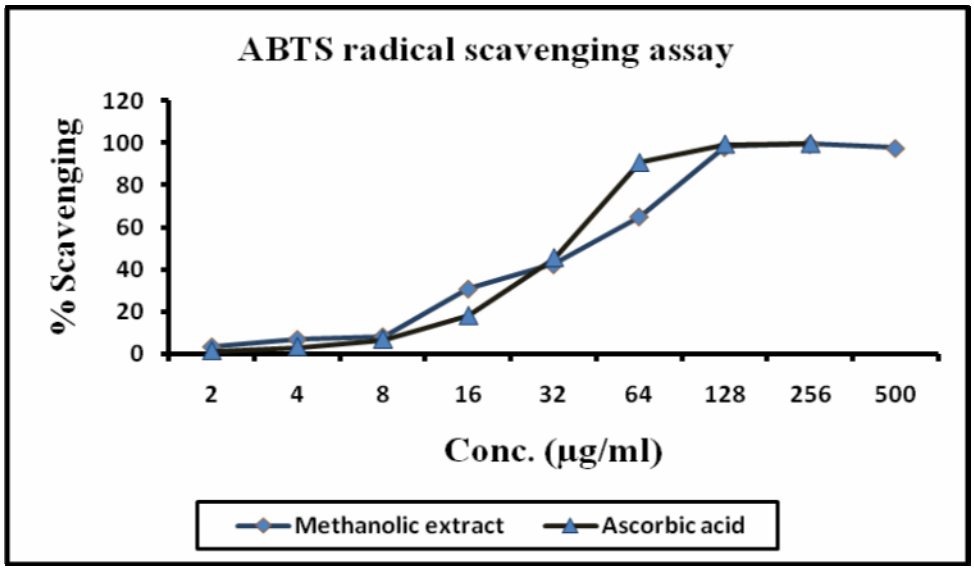

Fig. 2 Antioxidant activity of different concentrations of methanolic extract and ascorbic acid in ABTS radical scavenging assay. Each value represents mean ± SEM.

Ortho- substituted phenolic compounds may exert pro-oxidant effects by interacting with iron. O-phenanthroline quantitatively forms complexes with Fe^{+2} [23], which get disrupted in the presence of chelating agents. The aqueous extract interfered with the formation of ferrous-o-phenanthroline complex, thereby suggesting that the extract has metal chelating activity (Fig. 3).

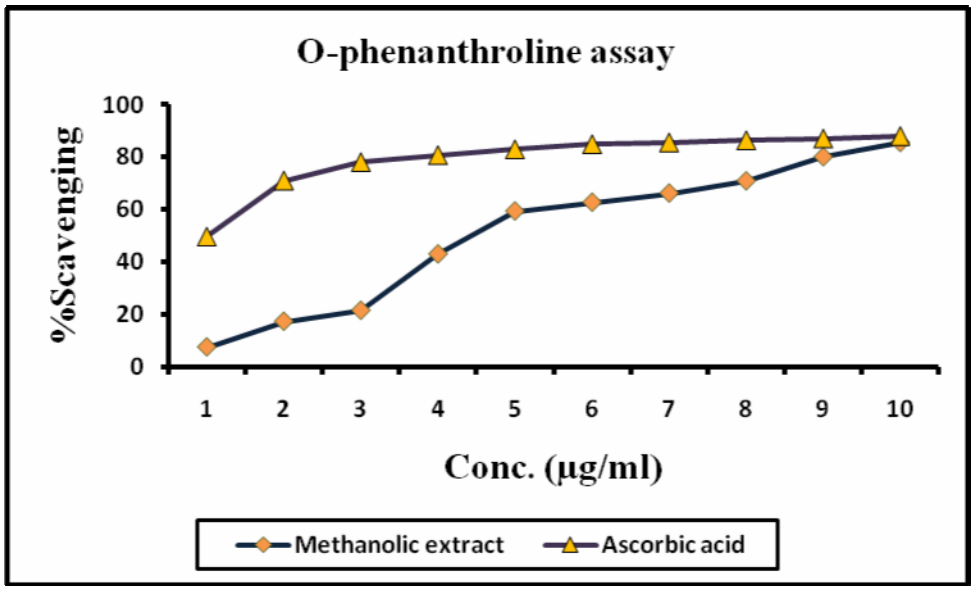

Fig. 3 Antioxidant activity of different concentrations of methanolic extract and ascorbic acid in O-phenanthroline assay. Each value represents mean ± SEM.

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through **.**OH radical by Fenton's reaction. Fig. 4 shows that the extract inhibited lipid peroxidation in egg phophatidylcholine as a dose dependent manner. The inhibition could be caused by absence of ferrylperferryl complex or by scavenging the ·OH radical or the superoxide radicals or by changing the Fe3+/Fe2+ or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. Iron catalyses the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydro-peroxides is produced. Lipid hydro-peroxide can be decomposed to produce alkoxy and peroxy radical which eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases[24]. Thus the decrease in the MDA level in egg phosphatidylcholine with the increase in the concentration of the extract indicates the role of the extract as an antioxidant (Fig. 4).

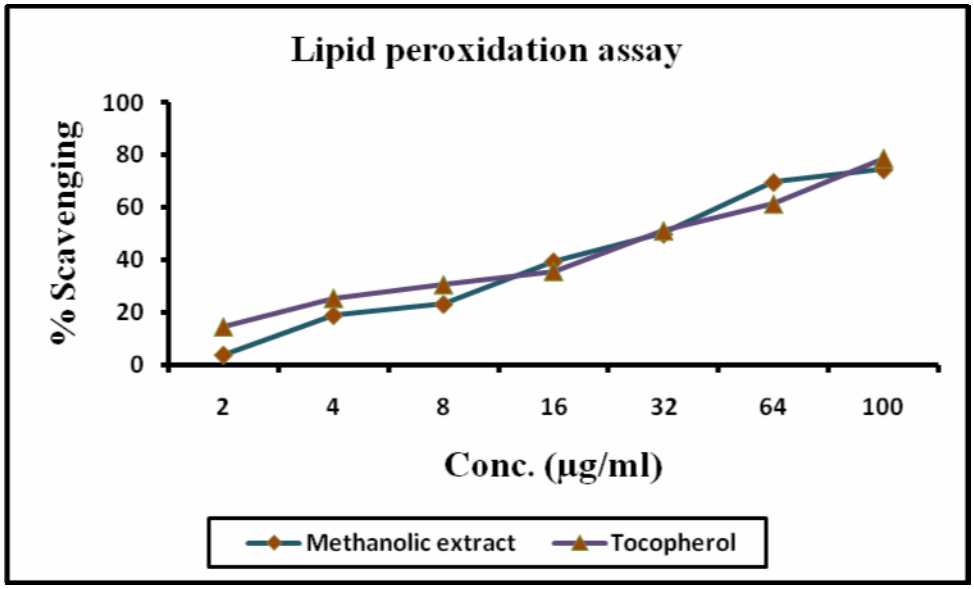

Fig. 4 Antioxidant activity of different concentrations of methanolic extract and ascorbic acid in lipid peroxidation assay. Each value represents mean ± SEM.

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities[25]. *P reticulatus* inhibit nitric oxide in dose dependant manner (Fig. 5). This may be due to antioxidant principles in the extract, which compete with oxygen to react with nitric oxide[26].

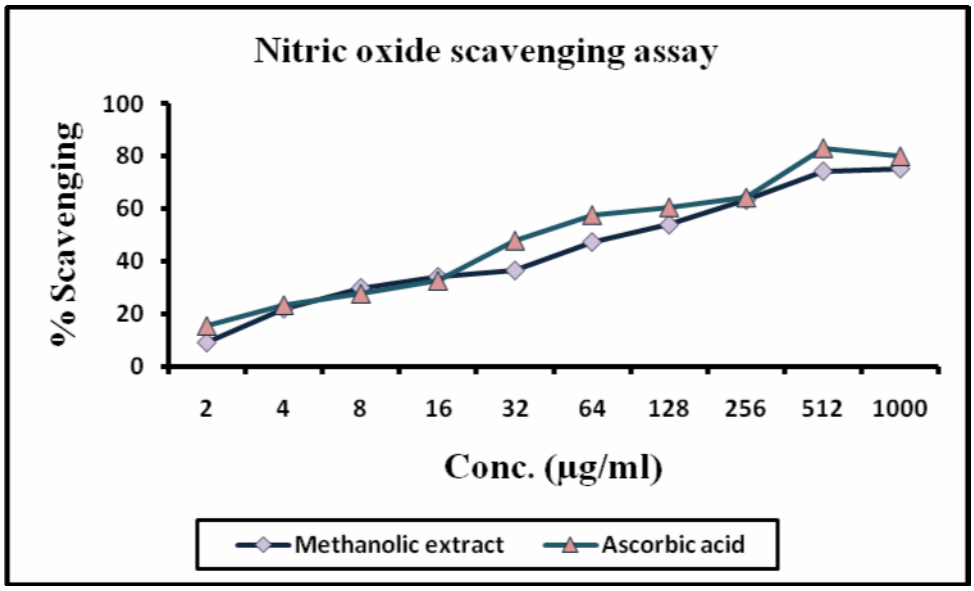

Fig. 5 Antioxidant activity of different concentrations of methanolic extract and ascorbic acid in nitric oxide scavenging method. Each value represents mean ± SEM.

Superoxide is a highly reactive molecule that can react with many substances, produced in various metabolic processes, including phagocytosis. It can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess super oxide dismutase enzymes, which catalyses the breakdown of superoxide radical[20]. In our study, alkaline DMSO used for superoxide generation indicates that *P. reticulatus* is a potent superoxide scavenger (Fig. 6).

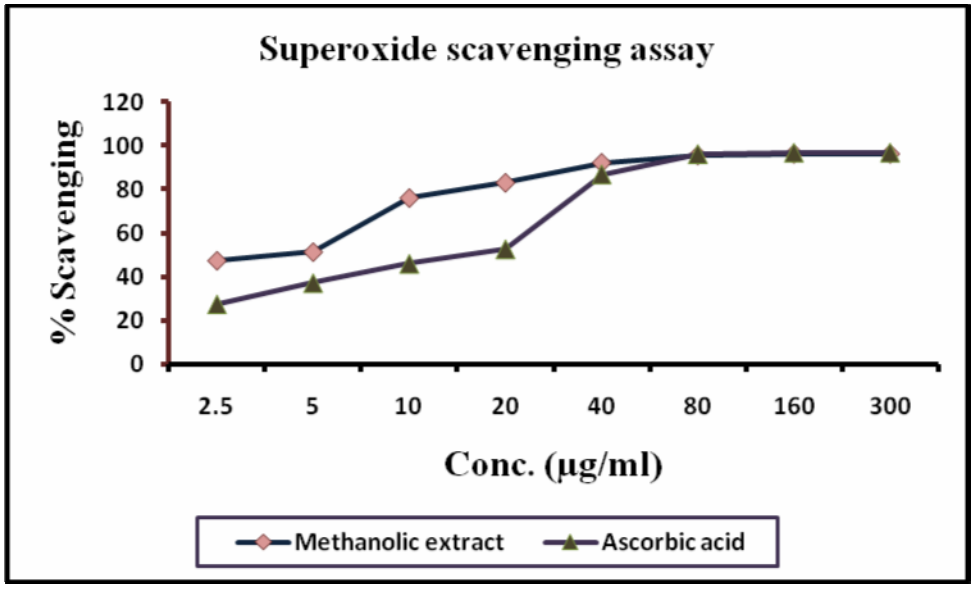

Fig. 6 Antioxidant activity of different concentrations of methanolic extract and ascorbic acid in super oxide scavenging method. Each value represents mean ± SEM.

The total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. Non-enzymatic glycosylation of haemoglobin is an oxidation reaction in which, an antioxidant is expected to inhibit the reaction. The degree of haemoglycosylation *in vitro*, in the presence of different concentration of extract, can be measured colorimetrically[15].

P. reticulatus extract exhibits its antioxidant action in several ways; removal of oxygen, scavenging of reactive oxygen species and nitrogen species or their precursors, inhibiting reactive oxygen species and reactive nitrogen species, binding metal ions needed for catalysis of reactive oxygen generation and up regulation of endogenous antioxidant defences. Antioxidant potential of *P. reticulatus* observed in the study may be due to the presence of methanol extractable phenolic compounds and other constituents responsible for the potent antioxidant activity.

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