ANTIOXIDANT STUDIES OF AQUEOUS EXTRACT OF *PHYLLANTHUS RETICULATUS* POIR

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Summary

Free radicals are implicated for many diseases including Diabetes mellitus, arthritis, cancer, ageing. etc. In treatment of these diseases, antioxidant therapy has gained utmost importance. *Phyllanthus reticulatus* Poir (Euphrobiaceae) popularly known as 'potato-bush' is an important medicinal plant and useful in burning sensation, strangury, gastropathy, ophthalmodynia, burns, diarrhoea, skin eruption, diabetes and obesity. Keeping in view of the cited activity, it is contemplated to screen the plant for *in vitro* antioxidant activity using different models *viz.* DPPH radical scavenging, ABTS radical scavenging, iron chelating activity and lipid peroxidation assay, nitric oxide scavenging assay, alkaline DMSO assay, total antioxidant capacity 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 are 20.36 µgm/ml (DPPH radical scavenging), 42.59 µgm/ml (ABTS radical scavenging), 32 µgm/ml (Iron chelating activity) and 41.91 µgm/ml (lipid peroxidation), 122.8 µgm/ml (nitric oxide scavenging) and 2.57 µgm/ml (alkaline DMSO). In total antioxidant capacity assay, 1 mg of extract is equivalent to 51 µg of ascorbic acid. It showed 66.64% inhibition of haemoglobin glycosylation. In all the testing, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals, metal chelation or inhibition of lipid peroxidation. The antioxidant property may be related to the polyphenols and flavonoids present in the extract. These results clearly indicate that *Phyllanthus reticulatus* is effective against free radical mediated diseases.

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

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Introduction

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals[1]. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism[2]. The most common reactive oxygen species (ROS) include superoxide ($0_2^{\cdot}$) anion, hydrogen peroxide (H_20_2), peroxyl ($ROO^{\cdot}$) radicals, and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^{\cdot}$). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome[3]. In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin.

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions[4]. The antioxidative effect is mainly due to phenolic components, such as flavonoids[5], phenolic acids, and phenolic diterpenes[6]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[7]. Many of these phytochemicals possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of cancer in several human populations[4].

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[8, 9, 10]. It contains polyphenols, flavonoid glycosides[11], tannic acid, friedelin, epifriedelinol, betulin, taraxerone, beta-sitosterol, glochidonol, octacosanol, taraxeryl acetate and 21-alpha-hydroxyfriedelan-3-

one[12].*Phyllanthus reticulatus* has also been reported to possess antidiabetic activity[13]. The purpose of this study was to evaluate *Pyllanthus reticulatus* as new potential sources of natural antioxidants and phenolic compounds.

Material and Methods

All chemicals and solvents were of analytical grade and were obtained from Ranbaxy Fine Chemicals, Mumbai, India. 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. UV spectrophotometer (Shimadzu 1650), homogenizer (Remi, India), centrifuge (Remi, India), pH meter (Elico Ltd., India) were the instruments used for the study.

Plant material

The whole plant of *Phyllanthus reticulatus* was collected during August 2007 from Manipal. The plant was identified by Dr. Gopalakrishna Bhat, Botanist, Poorna Prajna College, Udupi, Karnataka. A voucher specimen has been deposited in the museum of department of Pharmacognosy, Manipal.

Plant extract

About 500 g of the powder was taken and extracted with chloroform:water (1:1000) by maceration. The extract evaporated under vacuum gave a dry extract and was stored in a desiccator until further use.

Determination of Total polyphenolic compounds

Total polyphenolic compounds were determined according to a protocol similar to that of *Singleton* and *Rossi*[14]. From the stock solution (1 mg/ml) of the extract, suitable quantity was taken into a 25 ml volumetric flask and mixed with 10 ml of water and 1.5 ml of Folin Ciocalteu's reagent. After 5 minutes 4 ml of 20 % w/v sodium carbonate solution

was added and volume was made up to 25 ml with double distilled water. The Absorbance was recorded at 765 nm after 30 minutes. % of total phenolic was calculated from calibration curve of Gallic acid (50-250 µg) plotted by using the same procedure and total phenolics were expressed as % Gallic acid.

DPPH radical scavenging assay[15,16]

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 1.0 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[15,16]

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.

Iron chelating activity assay[(15-17)]

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[18]

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[15]

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[19]

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[20]

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[21]

The antioxidant activity 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 *Phyllathus 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}) in all models viz. DPPH, ABTS, iron chelating, lipid peroxidation, nitric oxide scavenging and alkaline DMSO were found to be 20.36, 42.59, 32, 41.91, 122.8 and 2.57 µg/ml respectively at 1 mg/ml concentration. In total antioxidant capacity assay, it was found that 1 mg of extract is equivalent to 51 µg of ascorbic acid. It showed 66.64% 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 nitric oxide with an IC_{50} value of 2.57 µg/ml. The activity was moderate in remaining antioxidant models. Total phenolic content was found to be 0.371 mg/ml. The antioxidant activity of the extract is close and identical in magnitude, and comparable to that of standard antioxidant compounds used.

Discussion

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing[3]. Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent disease[4]. 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[22]. 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).

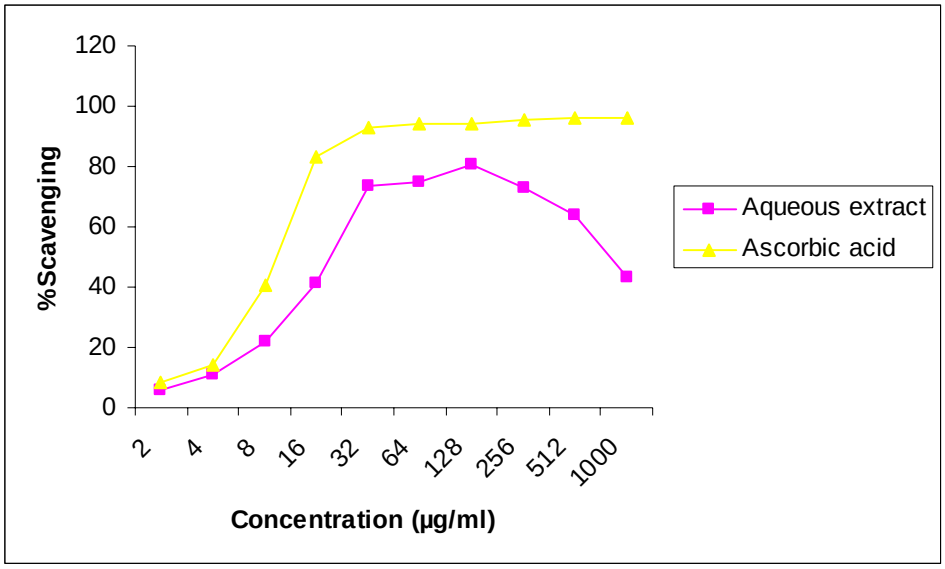

Fig. 1 Antioxidant activity of different concentrations of aqueous extract and ascorbic acid in DPPH radical scavenging method. Each value represents mean ± SEM

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[23] (Fig. 2).

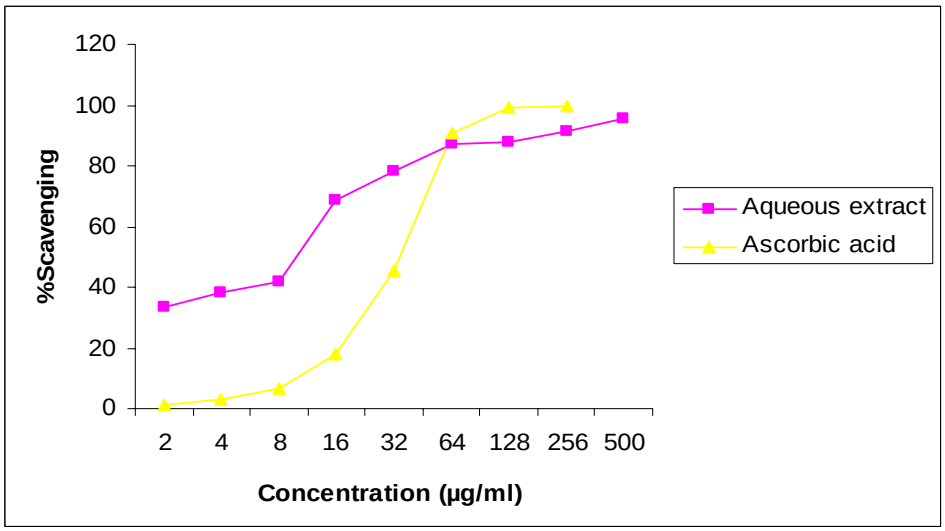

Fig. 2 Antioxidant activity of different concentrations of aqueous extract and ascorbic acid in ABTS radical scavenging method. Each value represents mean ± SEM

Ortho- substituted phenolic compounds may exert pro-oxidant effects by interacting with iron. O-phenanthroline quantitatively forms complexes with Fe^{+2} [24], 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).

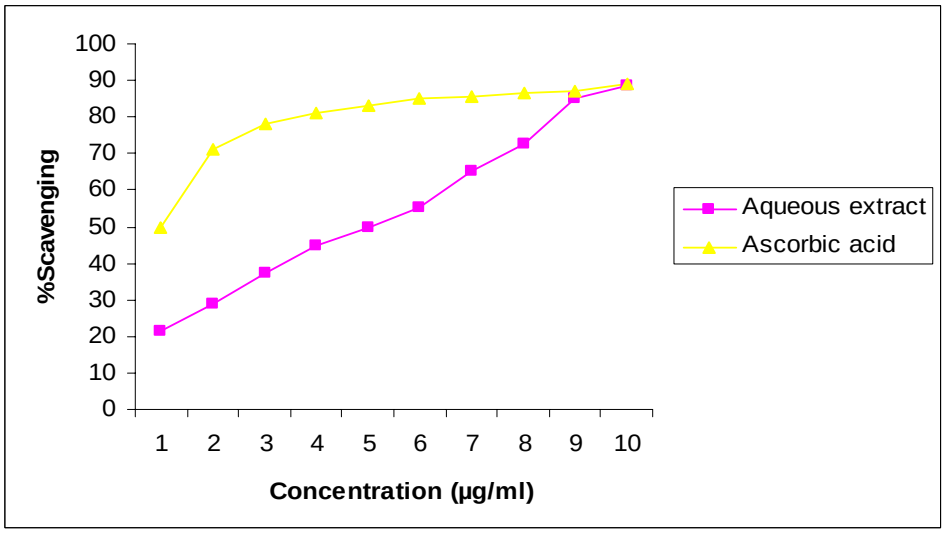

Fig. 3 Antioxidant activity of different concentrations of aqueous extract and ascorbic acid in iron chelating method. Each value represents mean ± SEM

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[25]. 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[26]. 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).

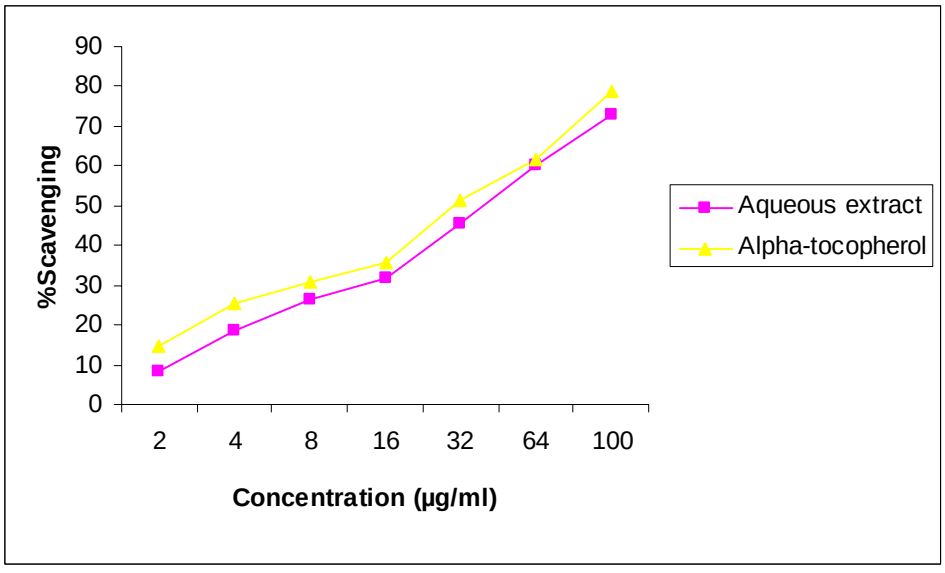

Fig. 4 Antioxidant activity of different concentrations of aqueous extract and ascorbic acid in lipid peroxidation method. Each value represents mean ± SEM

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[27]. *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[28].

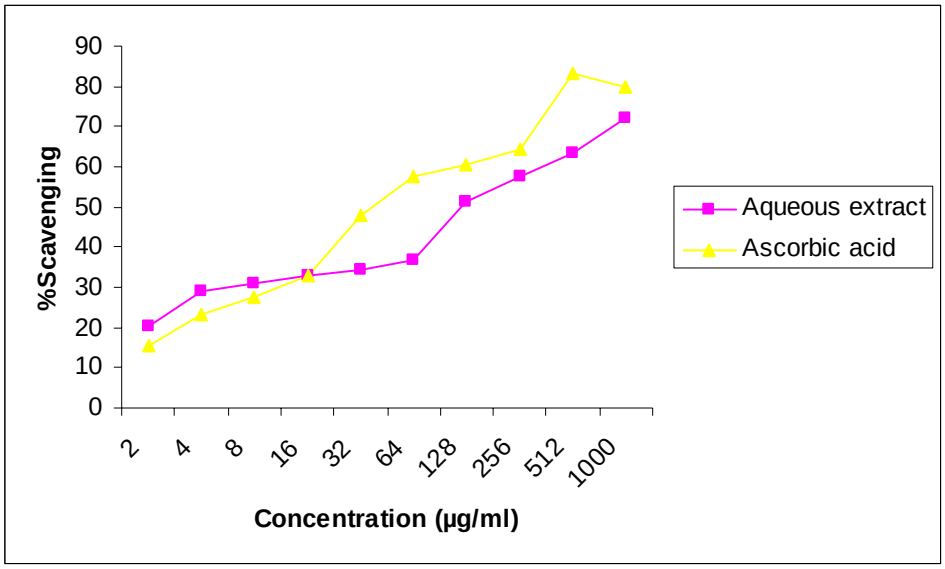

Fig. 5 Antioxidant activity of different concentrations of aqueous extract and ascorbic acid in nitric oxide scavenging method. Each value represents mean ± SEM

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[29]. In our study, alkaline DMSO used for superoxide generation indicates that *P. reticulatus* is a potent superoxide scavenger (Fig. 6).

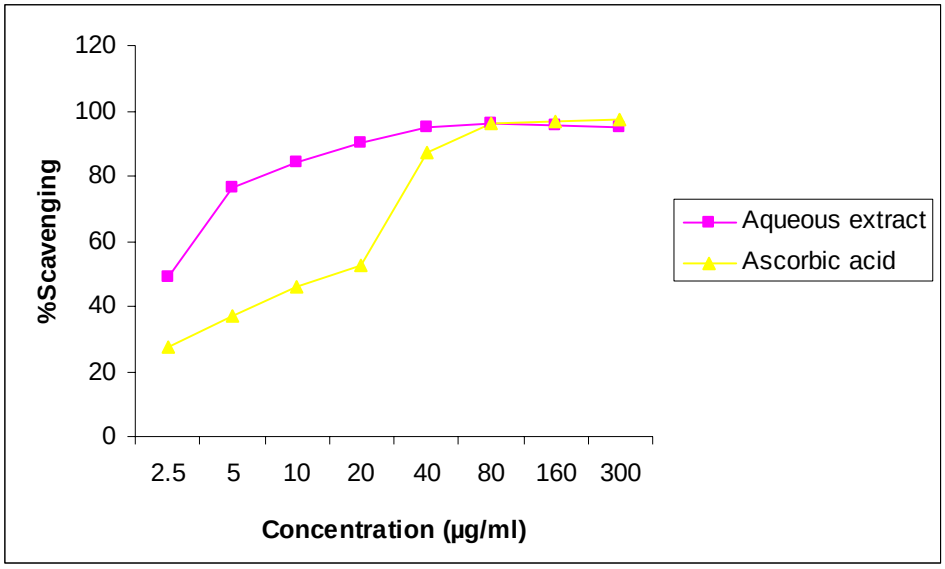

Fig. 6 Antioxidant activity of different concentrations of aqueous extract and ascorbic acid in super oxide scavenging method. Each value represents mean ± SEM

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[21]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[7].

In conclusion, the results of the present study show that the extract of *Phyllanthus reticulatus* which contains highest amount of polyohenols compounds and exhibits the greatest antioxidant activity through the scavenging of free radicals which participate in various pathophysiology of diseases including ageing. It also exerts iron chelating and reducing power activity. Overall, the plant extract is a source of natural antioxidants that can be important in disease prevention, health preservation and promotion of longevity promoter.

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