FREE RADICAL SCAVENGING ACTIVITY OF AQUEOUS EXTRACT OF *SPHAERANTHUS INDICUS* (LINN).

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Summary

The free radical scavenging potential of the plant *Sphaeranthus indicus* Linn. was studied by using different antioxidant models of screening. The aqueous extract at 1000 µg/ ml showed maximum scavenging of the radical cation, 2,2- azinobis- (3- ethylbenzothiazoline- 6- sulphonate) (ABTS) observed upto 26.21 % followed by the scavenging of the stable radical 1,1- diphenyl, 2- picryl hydrazyl (DPPH) (24.95 %), superoxide dismutase (17.74 %) and nitric oxide radical (10.63 %) at the same concentration. However, the extract showed only moderate scavenging activity of iron chelation (9.62 %). Total antioxidant capacity of the extract was found to be 127. 85 nmol/g ascorbic acid. The findings justify the therapeutic applications of the plant in the indigenous system of medicine, augmenting its therapeutic value.

Key Words: *Sphaeranthus indicus***,** Antioxidant**,** Free radicals

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Introduction

There is extensive evidence to implicate free radicals in the development of degenerative diseases[1]. Free radicals have been implicated in causation of ailments such as diabetes, liver cirrhosis and nephrotoxicity etc[2]. Together with other derivatives of oxygen they are inevitable byproducts of biological redox reactions[3]. Reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot -}$), hydroxyl radical (**.**OH) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent binding [4], and thus have been shown to augment collagen synthesis and fibrosis. The increased production of toxic oxygen derivatives is considered to be a universal feature of stress conditions. Plants and other organisms with a variety of antioxidant molecules and enzymes have evolved a wide range of mechanisms to contend with this problem.

Sphaeranthus indicus Linn. (Asteraceae) popularly known as 'Gorakmundi' is cultivated all over India[5] for its medicinal values. Preliminary phytochemical screening of underground plant parts (roots and stolon) revealed the presence of flavonoids, carbohydrates, alkaloids, gums and mucilage[6]. The whole herb is used in ayurvedic preparations to treat epilepsy and mental disorders[7]. The oil prepared using the plant roots is reportedly useful in treating scrofula and as an aphrodisiac. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy. It also treats piles and hepatitis[8]. Several tribal populations in northern India use this plant to cure diabetes.

Essential oil, obtained by steam distillation of the whole herb, contains ocimene, α-terpinene, methyl-chavicol, α-citral, geraniol, αionone, ß-ionone, d-cadinene, p-methoxycinnamaldehyde[9] and an alkaloid sphaeranthine[10]. The alcoholic extract of powdered caputula contains stigmasterol, ß-sitosterol,[11] hentriacontane, sesquiterpene lactone,[12] sesquiterpine glycoside, spharntholide[13] and flavone and isoflavone glycoside [14].

There is extensive evidence to implicate free radicals in the development of degenerative disease. These free radicals have been implicated in causation of ailment such as diabetes, liver cirrhosis, nephrotoxicity etc. Plants and other organisms have evolves a wide range of mechanisms to contend with this problem, with a variety of antioxidant molecules and enzymes[15]. Hence there is no doubt that phytochemicals deserve a proper position in the therapeutic armamentarium. As a detailed review of literature afforded no information on the antioxidant potential of the plant, it was therefore thought worthwhile to investigate the antioxidant potential of *Spaheranthus indicus* (*S. indicus*).

Materials 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. The other chemicals used were 2,2- azinobis- (3- ethylbenzothiazoline- 6-sulphonate) (ABTS), sodium nitroprusside, sulphanilamide, potassium superoxide, O-phosphoric acid, napthyl ethylene diamine dihydrochloride, potassium chloride (KCl), ferrous sulphate ($FeSO_4$).

Plant material

The underground portions of *S. indicus* were collected during July 2005 from Manipal and shade dried. The plant was identified by Dr. Gopalakrishna Bhat, Botanist, Poorna Prajna College, Udupi, Karnataka. A voucher specimen (No.PP 552) 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 yield of 18 g and was stored in a dessicator until further use.

Preparation of S. indicus

S .indicus stock solution was prepared in concentration of 1000µg/ml in water. From the stock solution different concentration viz. 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000 µg/ml were prepared in water and used for antioxidant studies.

ABTS radical cation decolorization assay[16]

ABTS radical cation ($ABTS^{\cdot +}$) was produced by reacting ABTS solution (7 mM) with 2. 45 mM ammonium persulfate and the mixture was allowed to stand in dark at room temperature for 12- 16 hr before use. For the study, 0.5 ml of the aqueous extract were added to 0.3 ml of ABTS solution and the final volume was made up with ethanol to make 1 ml. The absorbance was read at 745 nm and the experiment was performed in triplicate.

DPPH radical scavenging activity[17]

To 1 ml of various concentrations of aqueous extract,1 ml solution of DPPH, (0.1 mM) was added. An equal amount of methanol and DPPH served as control. After 20 minutes incubation in the dark, absorbance was recorded at 517 nm. The experiment was performed in triplicate and the percentage scavenging was calculated.

Scavenging of superoxide radical **(Potassium superoxide assay)**[18-19]

The scavenging activity towards the superoxide radical ($O_2^{\cdot -}$) was measured in terms of inhibition of generation of $O_2^{\cdot -}$. The method was performed by using alkaline DMSO method[20]. Potassium superoxide and dry DMSO were allowed to stand in contact for 24 hr and the solution was filtered immediately before use. 200 µl of the filtrate was added to 2.8 ml of an aqueous solution containing NBT (56 µM), EDTA (10 µM) and potassium phosphate buffer (10 mM). Aqueous extract 1 ml were added and the absorbance was recorded at 560 nm against a control in which pure DMSO was added instead of alkaline DMSO.

Scavenging of nitric oxide radical[20-21]

Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction as described previously[22-23]. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations of the aqueous extract dissolved in phosphate buffer saline (0.025 M, pH: 7.4) and the tubes were incubated at 25º C for 5 hr. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess' reagent (1 % sulphanilamide, 2 % O- phosphoric acid and 0.1 % napthyl ethylene diamine dihydrochloride). 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 repeated in triplicate.

Iron chelating activity[24-25]

The reaction mixture containing 1ml 0.05% O-phenathroline in methanol, 2 ml ferric chloride 200 µM and 2 ml of the aqueous extract was incubated at ambient temperature for 10 min and the absorbance of the same was measured at 510 nm. The experiment was performed in triplicate.

Total Antioxidant Capacity[26]

Total antioxidant capacity was measured by spectrophometric method. 0.1 ml of the aqueous 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.

Statistical analysis

Linear regression analysis was used to calculate the IC_{50} values.

Results

Several concentrations ranging from 2 - 1000 µg/ml of the aqueous extract of *S. indicus* 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 Inhibitory Concentration (IC_{50}) in all models *viz.* ABTS, DPPH, superoxide dismutase, nitric oxide and iron chelating, were found to be 26.21, 24.95, 17.74, 10.63 and 9.62 respectively.. Total antioxidant capacity of the extract was found to be 127. 85 nmol/g ascorbic acid.

IC_{50} was calculated by using formulae $b=\sum x.y/x^2$

$$a = \bar{y} - b\,\bar{x}$$

$$IC_{50} = a + b\,(50)$$

Where, b = Regression coefficient of x on y

a = Interception of the line

x = Concentration in µg/ml

y = % scavenging

$\underline{x}$ = mean of concentration

$\bar{y}$ = mean of % scavenging

On a comparative basis the extract showed better activity in quenching ABTS with an IC_{50} value of 26.21 µg/ml and DPPH radicals with an IC_{50} value of 24.95 µg/ml. However, the extract also showed encouraging response in quenching nitric oxide radicals (IC_{50} value – 10.63 µg/ml). The activity was moderate in remaining antioxidant models.

Discussion

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing[2]. Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent disease[28].

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which has a characteristic long wavelength absorption spectrum[17]. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is a decolorization assay, thus the radical cation is performed prior to addition of antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant .The results obtained imply the activity of the extract either by inhibiting or scavenging the $ABTS^{\cdot+}$ radicals since both inhibition and scavenging properties of antioxidants towards $ABTS^{\cdot+}$ radicals have been reported earlier[27]. (Fig. 1).

DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical $DPPH^{\cdot}$. From our study it may be postulated that *S. indicus* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles[17]. 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[28] (Fig. 2).

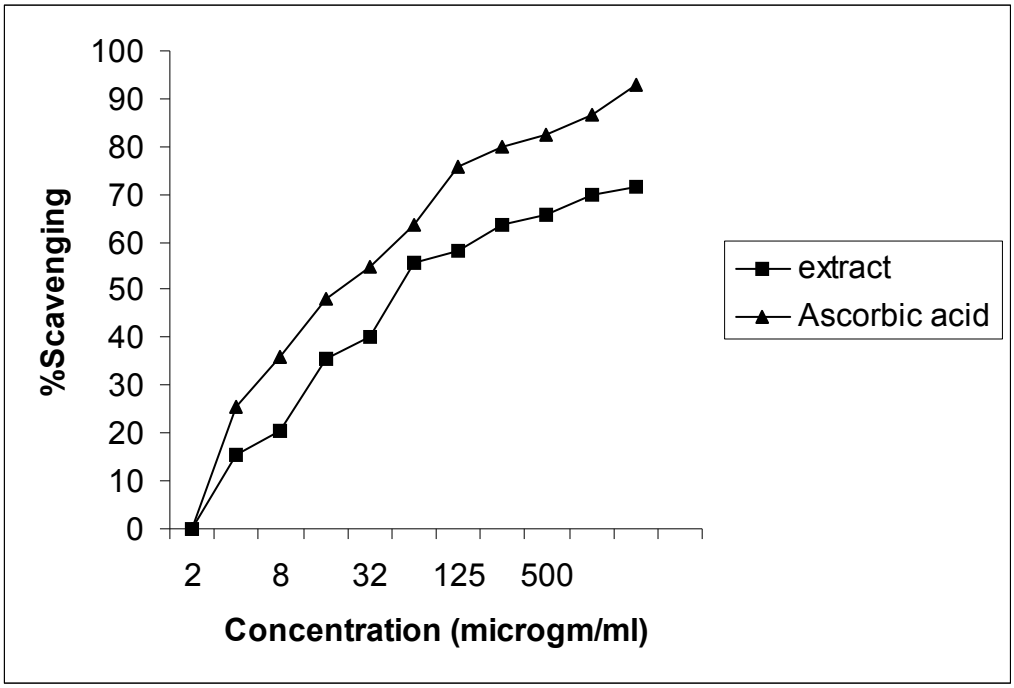

Fig. 1 Free radical scavenging activity of different concentrations of aqueous extract and ascorbic acid in ABTS radical scavenging method.

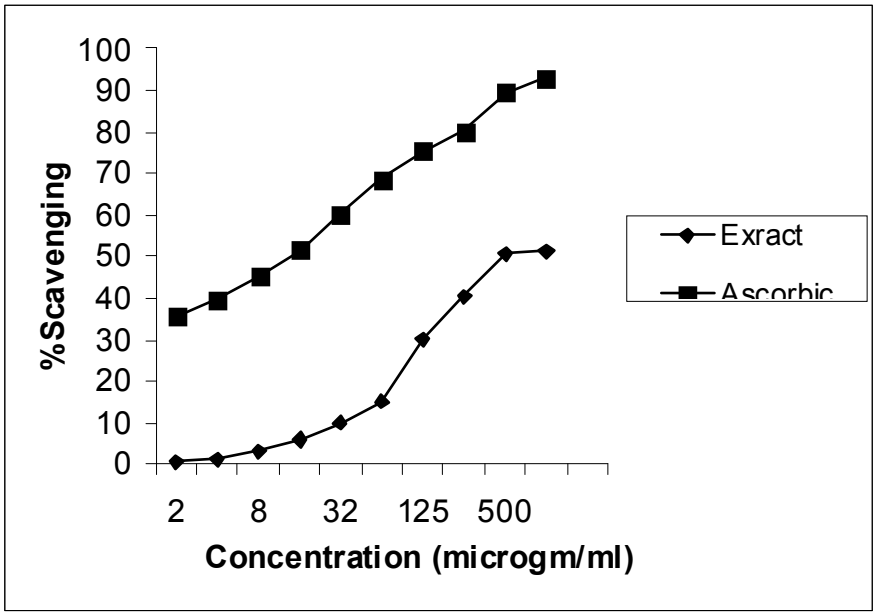

Fig. 2 Free radical scavenging activity of different concentrations of aqueous extract and ascorbic acid in DPPH radical scavenging method.

Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide[29]. Superoxide anion is the first reduction product of oxygen[30] which is measured in terms of inhibition of generation of $O_2^{\cdot -}$. (Fig. 3)

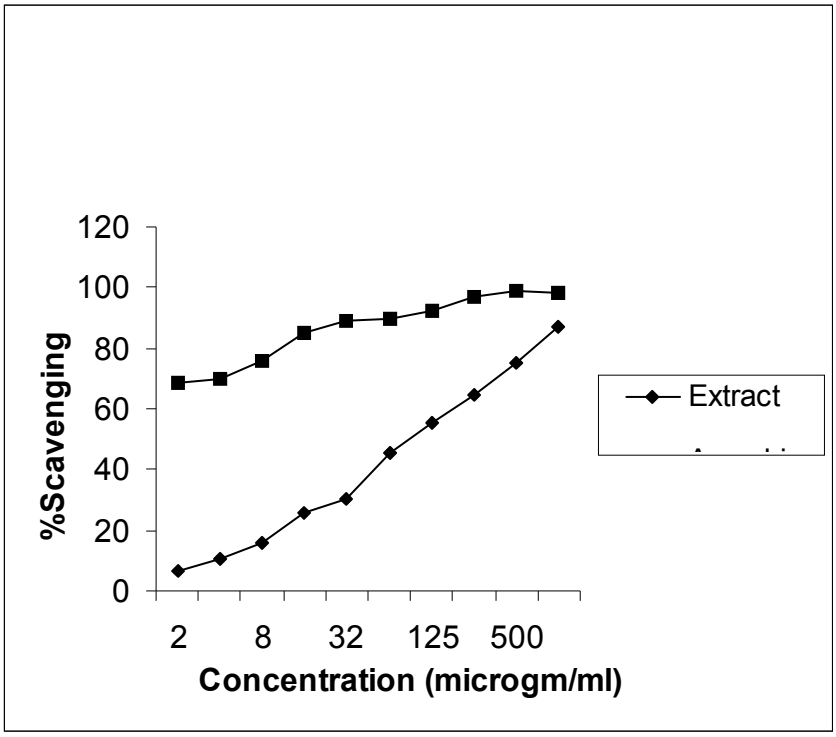

Fig. 3 Free radical scavenging activity of different concentrations of aqueous extract and ascorbic acid by NBT method.

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases[31]. Nitric oxide is a very unstable species under aerobic condition. It reacts with O2 to produce stable product nitrate and nitrite through intermediates NO_2, N_2O_4 and N_3O_4. It is estimated by using Griess reagent. In the presence of test compound which is a scavenger the amount of nitrous acid will decrease. In our study the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate saline buffer at 25º C was reduced by the aqueous extract of *S. indicus*. This may be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide[32] and thus inhibits the generation of nitrite. (Fig. 4)

Ortho- substituted phenolic compounds may exert pro-oxidant effects by interacting with iron. O-phenanthroline quantitatively forms complexes with Fe^{+2} [33], 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. 5). The total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically at 695 nm.

It is reported that flavanoids are natural products which have been shown to possess various biological properties related to antioxidant mechanisms[16] Thus in our study, the antioxidant potential of *S. indicus* may be attributed to the presence of flavanoids and the other constituents present therein[6].

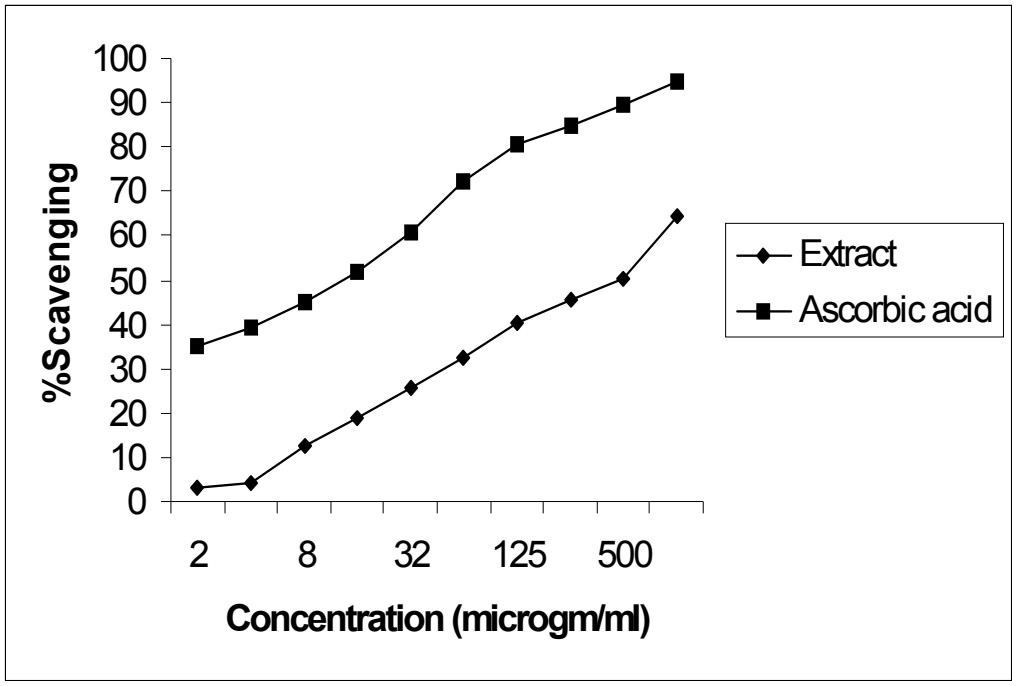

Fig. 4 Free radical scavenging activity of different concentrations of aqueous and ascorbic acid in nitric oxide radical scavenging method.

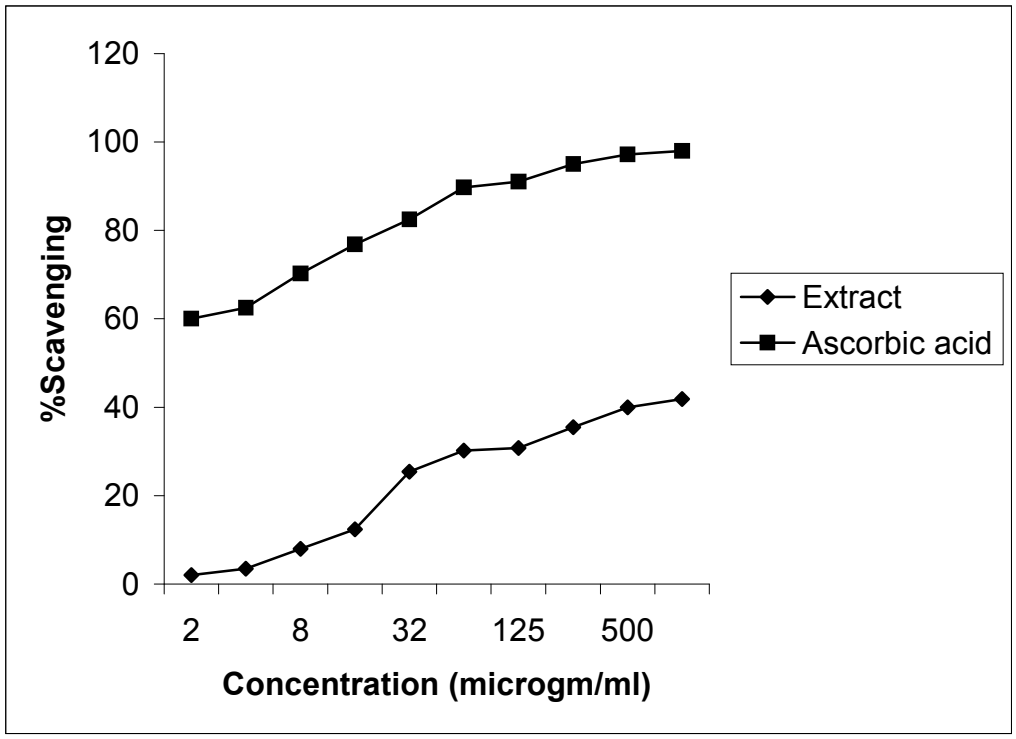

Fig. 5 Free radical scavenging activity of different concentrations of aqueous extract and ascorbic acid in iron chelating (O-phenanthroline) radical scavenging method.

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