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IN-VITRO ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF STEPHANIA GLABRA (ROXB.) MIERS TUBERS

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ABSTRACT

Aim: The present study was investigated to evaluate, the In vitro antioxidant activity of ethanolic extract of Stephania glabra (SGE) tubers using various antioxidant assay.

Materials and Methods: Antioxidant activity was evaluated by different assays, including 2, 2-diphenlyl-1-picrylhydrazyl (DPPH) radical scavenging, nitric oxide scavenging, hydrogen peroxide scavenging, metal chelating activities, reducing power, beta-carotene bleaching assay and linoleic acid system using FTC.

Results: Various antioxidant assays were compared to standard antioxidants such as, butylated hydroxytoluene (BHT), and ascorbic acid (Vitamin C). The SGE exhibited strong antioxidant activity in the DPPH assay (IC_{50}, 543.16 µg/mL), 10 µg/mL of extract exhibited 52.88% inhibition of Hydrogen peroxide, 150 µg/mL of extract exhibited 61.0% inhibition of nitric oxide, respectively and effective inhibition against linoleic acid system using FTC and TBARS. The extract of SG has effective reducing power, and metal chelating activities.

Conclusion: The results obtained in this study suggests that the ethanolic extract of Stephania glabra tubers possesses in vitro antioxidant activity. Further study is required for the isolation of active compounds, which can be used for the treatment of various disorders related to oxidative stress.

INTRODUCTION

It has been suggested that natural antioxidants are more safe and healthy than synthetic antioxidants which used in food (Mahfuz et al., 2007). The oxidative damage caused by ROS may generate various diseases in the human body, such as aging, arthritis, cancer, inflammation, and heart diseases (Abe et al., 1998). Stephania glabra (Roxb.) Miers is

a large, climbing shrub, indigenous to lower Himalayas of India. The tubers of S. glabra (Roxb.) Miers has been used as antidysenteric, antipyretic, antiasthamatic and antituberculosis agent (Chopra et al., 1958) and also used as psychomedicine by natives in India (Khanna et al., 1972, Gaur 1999, Kirtikar and Basu., 2004). More than thirty alkaloids of different classes have been isolated from the plant such as gindarudine (Semwal and Rawat, 2009a, b), protoberberine, palmatine (Bhakuni and Gupta 1982), and 11-hydroxypalmatine (Semwal et al., 2010b). But there is no information about in vitro antioxidant activity of Stephania glabra tubers. The purpose of present study was to evaluate the in vitro antioxidant activity of Stephania glabra extract.

MATERIALS AND METHODS:

CHEMICALS

Ammonium thiocyanate, 1,1-diphenyl- 2-picryl-hydrazyl (DPPHd), 3-(2-Pyridyl)-5,6-bis (4- phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), Griess reagent were purchased from Sigma chemical (St. Louis, MO). Butylated hydroxytoluene (BHT), Ferrous chloride, Ascorbic acid (Vitamin C), EDTA, β-carotene, Tween-80 trichloracetic acid (TCA) were purchased from Himedia Co. All others unlabelled chemicals and reagents were analytical grade.

PLANT MATERIAL

Fresh tubers of Stephania glabra were collected from the Champawat region of Uttarakhand in the month of April 2012. The tubers were authenticated by Dr. A.K. Gupta, Reader, Department of Botany, Meerut College, Meerut. The voucher specimen (MCM/Bot-1/2012) of the plant material has been deposited in the Department.

PREPARATION OF PLANT EXTRACT

Stephania glabra tubers were dried under shade, coarsely powdered and passed through 40 mesh sieves. The powdered material (80 g) was extracted with ethanol using soxhlet apparatus at room temperature for 24 h to produce the original extract. The solvent was evaporated by rotary evaporation at 35°C, and the remaining water was removed by Lyophilization, yielding a dark brown color mass (12.35g) and stored at 4°C. The yield of the ethanolic extract was found to be 15.43%.

IN VITRO ANTIOXIDANT ACTIVITY

DPPH RADICAL SCAVENGING ASSAY

The free radical scavenging activity was evaluated using an improved DPPH assay (Lillian et al., 2008). 2.7 mL (0.2 mM) DPPH, was added to 0.3 mL of the extract at various concentrations. The mixture was shaken vigorously and incubated at room temperature for 1 h before the absorbance was measured at 517 nm.

$$\textbf{Percentage inhibition} = [(As - Ai)/As] \times 100$$

where As is the absorbance of pure DPPH and Ai is the absorbance of DPPH in the presence of various extracts. BHT and Vitamin C concentrations identical to the experimental samples were used as references.

REDUCING POWER ASSAY

This method described by Oyaizu (1986), 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of (1% w/v) $K_3Fe(CN)_6$ were added to 1.0 mL of exract in various concentrations (25-100 µg). The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 mL of Trichloro acetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of $FeCl_3$ (0.1% w/v). The absorbance was measured after 10 min. at 700 nm against blank sample. The increased absorbance of the reaction mixture indicated increased reducing power. BHT and Vitamin C were used as standards for comparison.

ASSAY OF METAL ION CHELATING ACTIVITY

The chelating activity of a metal ion (Fe^{2+}) was measured according to the method reported by Dinis et al. (1994). The reaction mixture, containing 1.0 mL of the SGE extract, 0.05 mL of ferrous chloride ($FeCl_2$) solution (2 mM), 0.2 mL of ferrozine solution (5 mM), and 2 mL of distilled water, was shaken well and incubated at room temperature for 10 min. The absorbance of the Fe^{2+}–ferrozine complex was measured at 562 nm against blank. The chelating activity was calculated using the following equation:

Metal chelating ability (%)= [(As−Ai)/As]×100

where As is the absorbance of the control and Ai is the absorbance in the presence of the extract. Disodium ethylenediamine tetraacetic acid (EDTA-2Na) was used as the control.

Beta-CAROTENE BLEACHING ASSAY

Antioxidant activity was measured using the Beta-carotene linoleate model system (Shon et al., 2003) with a slight modification. Briefly, 6 mg of β-carotene was dissolved in 20 mL of chloroform, and 4 mL of this solution was pipetted into a 500 mL round-bottomed flask containing 80 mg linoleic acid and 800 mg Tween-80. After removing chloroform using a rotary evaporator, 200 mL of distilled water was added slowly to the mixture with vigorous agitation to form a stable emulsion. Then, 3 mL aliquots of the emulsion were transferred into different test tubes containing 0.2 mL of the samples (500 µg/mL) and were incubated in a water bath at 50°C for 2 h. BHT and Vitamin C were used as standards for comparison. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. Measurement of absorbance was continued until the colour of Beta-carotene disappeared; every 30 minutes upto 2 hr.

ANTIOXIDANT ACTIVITY IN A LINOLEIC ACID SYSTEM USING FTC

The ferrothiocyanate (FTC) test was conducted as described by Osawa and Namiki (1981). Extracts (400 µg) in 4 mL ethanol, 4 mL of 2.5% linolenic acid dissolved in ethanol, 8 mL of phosphate buffer (50 mM, pH 7.0), and 4 mL distilled water were mixed in a vial with a screw cap. The mixed solution was placed in a dark oven at 40°C. Exactly 0.1 mL of the solution was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. After 3 min, 0.1 mL of ferrous chloride (0.02

M) in 3.5% hydrochloric acid was added to the reaction mixture. The absorbance of the resulting red colour was measured at 500 nm every 24 h until the day after the absorbance of the control reached maximum value. A mixture without the plant sample was used as the control. BHT and Vitamin C served as the reference antioxidants. All data are the average of triplicate analyses.

HYDROGEN PEROXIDE SCAVENGING (H_2O_2) ASSAY

The hydrogen peroxide scavenging activity was estimated according to the method of Ruch et al. (1989). Hydrogen peroxide (40 mM) solution was prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (5-15 µg/mL) in distilled water was added to hydrogen peroxide (0.6 mL) solution and absorbance at 230 nm is determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. The percentage of hydrogen peroxide scavenging is calculated as follows:

% scavenged (H_2O_2) = (Ai – At) / Ai x 100

where Ai is the absorbance of control and At is the absorbance of test.

ASSAY OF NITRIC OXIDE SCAVENGING ACTIVITY

Nitric oxide scavenging activity was determined by the method of (Marcocci et al., 1994) using Griess reagent. 2 mL of 10 mM sodium nitropruside dissolved in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations (50- 200 µg/mL). The mixture was then incubated at 25°C. After 150 min of incubation, 0.5 mL of the incubated solution was taken and mixed with 0.5 mL of Griess reagent [(1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was then incubated at room temperature for 30 min and its absorbance was measured at 546 nm. The amount of nitric oxide radical inhibition was calculated following this equation:

% inhibition of NO radical = (A_0 - A_1)/ A_0 x 100

where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place with Griess reagent.

THIOBARBITURIC ACID (TBA) ASSAY

The TBA value of the extract was determined using the method by Kikuzaki and Nakatani (1993). Up to 2 mL of 20% trichloroacetic acid and 2 mL of 0.67% 2-thiobarbituric acid were mixed thoroughly with 1 mL of the extract solution and were placed in a boiling water bath (100°C) for 10 min. As it cooled, the mixture was centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm. The lower absorbance indicates the strong antioxidant activity.

RESULTS AND DISCUSSION

DPPH RADICAL SCAVENGING ASSAY

The molecule 1, 1-diphenyl-2-picrylhydrazyl (a,a-diphenyl-bpicrylhydrazyl; DPPH) is

characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals (Soares et al., 1997). The delocalization of electron also gives rise to the deep violet color. The normal purple colour of DPPH will turn into yellow when its single electron is paired with a hydrogen atom coming from a potential antioxidant (Manzocco et al., 19998). DPPH is a free radical compound and has been widely accepted to estimate the scavenging capacity of antioxidants. The DPPH scavenging activities of different concentrations of SGE are presented in **Fig. 1**. Apparently, Vitamin C showed the highest scavenging activity (IC_{50} = 124.93 µg/mL). SGE exhibited a dose-dependent DPPH radical scavenging activity. SGE showed a higher activity than BHT, with IC_{50} values of 543.16 µg/mL and 570.74 µg/mL, respectively, which suggests that SGE has a strong scavenging activity.

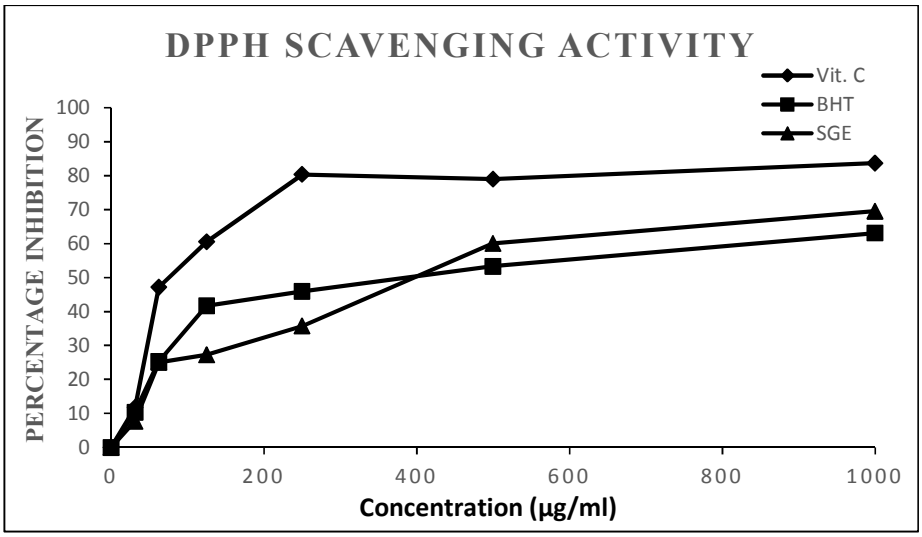

Fig. 1. Antioxidant activity of the Stephania glabra ethanolic extract as determined by the DPPH radical scavenging assay. SGE, Stephania glabra extract Vit. C, ascorbic acid; BHT, butylated hydroxytoluene.

It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins, and aromatic amines (p-phenylene diamine, p-aminophenol, etc.), reduce and decolourise DPPH by their hydrogen donating ability (Yokozawa et al., 1998).

REDUCING POWER ASSAY

This method is based on the principle of increase in the absorbance of the reaction mixtures indicates an increase in the antioxidant activity. In this method, antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700 nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples (Jayaprakash et al., 2001). To measure the reducing capacity of SGE, its ability to

transform Fe^{3+} into Fe^{2+} was investigated. Fe^{2+} formation can be monitored by measuring Prussian blue formation. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990).

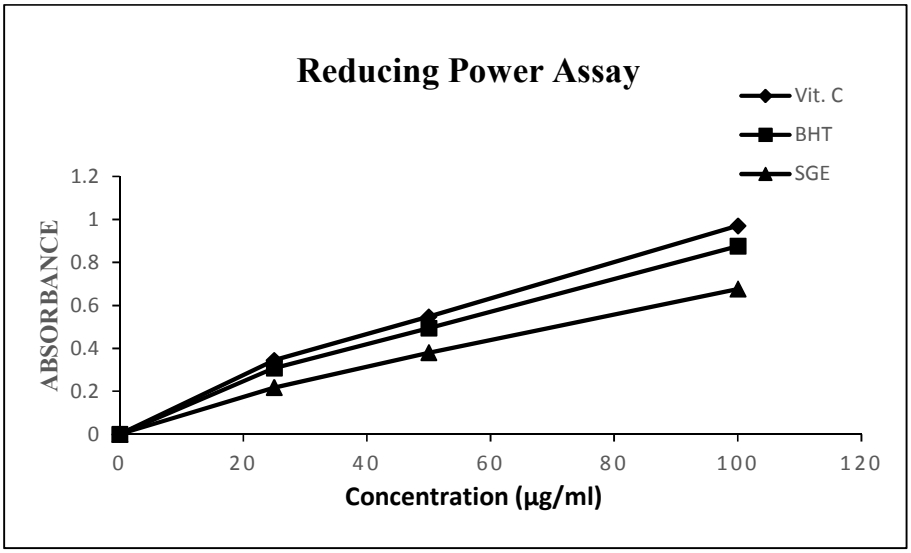

Fig. 2. Reducing power assay of Stephania glabra. High absorbance indicates strong antioxidant activity. SGE, Stephania glabra extract; Vit. C (ascorbic acid); BHT, butylated hydroxytoluene.

As shown in **Fig. 2,** the reducing capacities of the extract increased with increasing sample concentration and indicate that the order of reductive potential is Vitamin C > BHT > SGE.

ASSAY OF METAL ION CHELATING ACTIVITY

Ferrozine can form a complex with a red color by forming chelates with Fe2+. Measurement of the color reduction determines the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000).

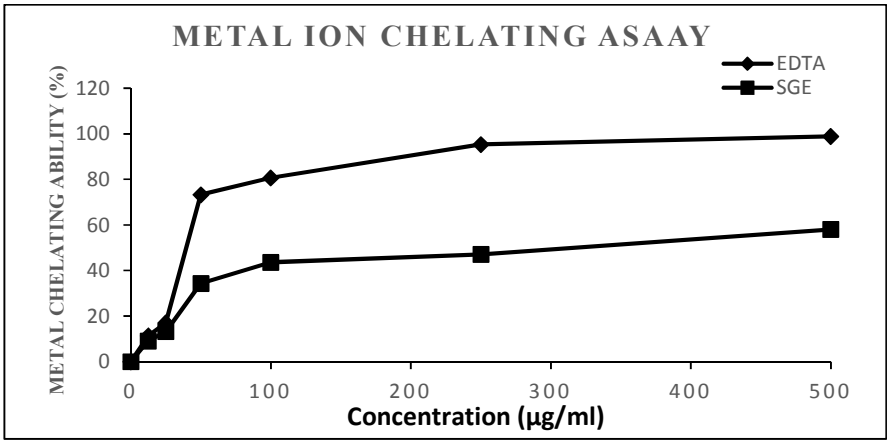

Fig. 3. Antioxidant property of Stephania glabra ethanolic extract determined as the Fe^{2+} chelating ability. SGE,Stephania glabra extract; EDTA, ethylenediaminetetraacetic acid.

Metal chelation is an important antioxidant property because it reduces the concentration of the catalyzing transition metal in Lipid peroxidation. **Fig. 3** showed ferrous chelating

abilities of SGE, when compared with that of EDTA. The values demonstrated that EDTA has the strongest chelating capacity 80.68 ± 1.59 % at 100 µg/mL. The chelating ability of the SGE was 9.17– 58.04 % at concentrations ranging from 12.5- 500 µg/mL. The results suggested that the SGE complexes with the ferrous ion in a dose-dependent manner.

Beta-CAROTENE BLEACHING ASSAY

The antioxidants can hinder beta-carotene bleaching by neutralizing free radicals (Juntachote and Berghofer, 2005). Linoleic acid, which releases a free radical with a hydrogen atom, causes the Beta-carotene to lose its chromophore and orange color**.** As shown in **Fig. 4,** that the absorbance.of the control and Vitamin C decreased rapidly when SGE and BHT were much slower. By this we can conclude that SGE as effective as BHT as an antioxidant, and much more effective than Vitamin C. The SGE represents excellent antioxidant ability.

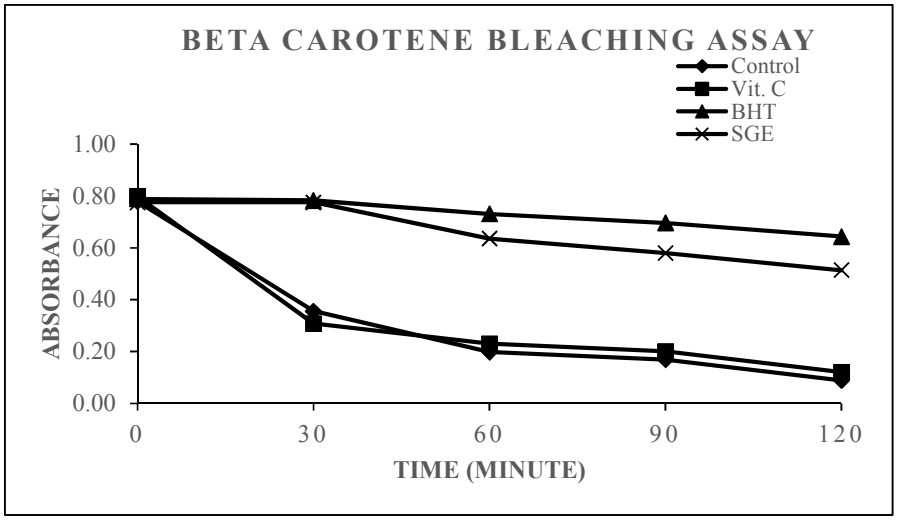

Fig. 4. Antioxidant activity of Stephania glabra extract in the beta-carotene bleaching assay system. High absorbance indicates strong antioxidant activity. SGE, Stephania glabra extract; Vit. C (ascorbic acid); BHT, butylated hydroxytoluene.

ANTIOXIDANT ACTIVITY IN A LINOLEIC ACID SYSTEM USING FTC

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. Peroxides formed during linoleic acid oxidation may be detected and quantified by their ability to oxidize Fe^{2+} _ Fe^{3+}. Fe^{3+} ions form a thiocyanate complex with SCN−, which has a maximum absorbance at 500nm (Liu and Yao, 2007). **Fig. 5** shows the time-course plots for the antioxidant activity of the SGE using the FTC assay. Therefore, samples with high antioxidant activity are revealed by low absorbance values. The absorbance of the control showed a steady increase and reached peak value in day 9. BHT showed a high capacity to inhibit linoleic acid peroxidation with almost constant absorbance of 0.23. The absorbance of SGE increased slowly, which implies a strong potential for antioxidant ability. The absorbance of the SGE was higher than that of BHT but lower than that of Vitamin C. The SGE showed a strong antioxidant activity in comparison to Vitamin C, but it was less efficient than BHT.

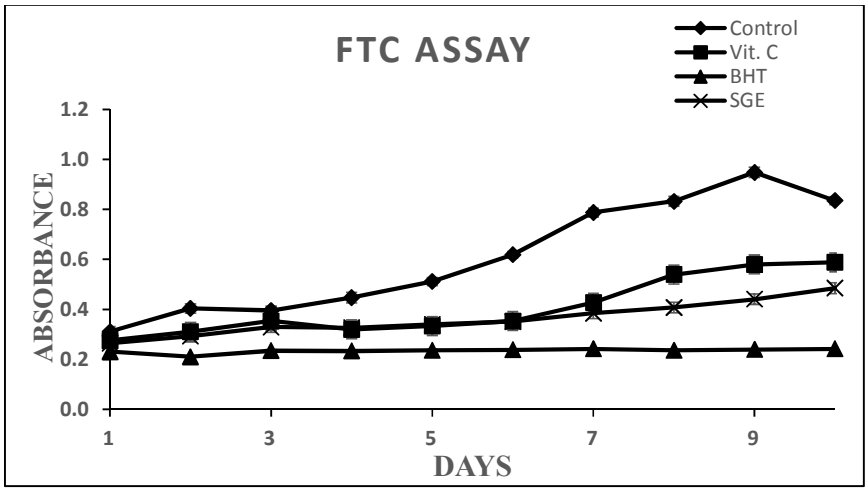

Fig. 5. Antioxidant activity of Stephania glabra extract assayed using the FTC method. Low absorbance indicates strong antioxidant activity. Results are mean ± SEM of three parallel measurement. SGE, Stephania glabra extract; Vit. C (ascorbic acid) ; BHT, butylated hydroxytoluene.

HYDROGEN PEROXIDE SCAVENGING (H_2O_2) ASSAY

Human beings are exposed to H_2O_2 indirectly via the environment nearly about 0.28 mg/kg/day with intake mostly from leaf crops. Hydrogen peroxide may enter into the human body through inhalation of vapor or mist and through eye or skin contact (Halliwell 1985). H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH^-) that can initiate lipid peroxidation and cause DNA damage in the body (Ruch et al., 1989). The scavenging ability of SGE extract with hydrogen peroxide is shown in **Fig. 6** and compared with the standards, BHT and ascorbic acid. It has been noticed that all the extracts are capable of scavenging hydrogen peroxide in a dose-dependent manner. The percentage of scavenging hydrogen peroxide determined with 10 µg/ml of the ethanolic extract of the SGE, BHT and Vitamin C, was found to be 52.88%, 41.45% and 19.18% respectively. The hydrogen peroxide scavenging effect of the SGE extract and the standards follows the trend, SGE > BHT > Vit.C.

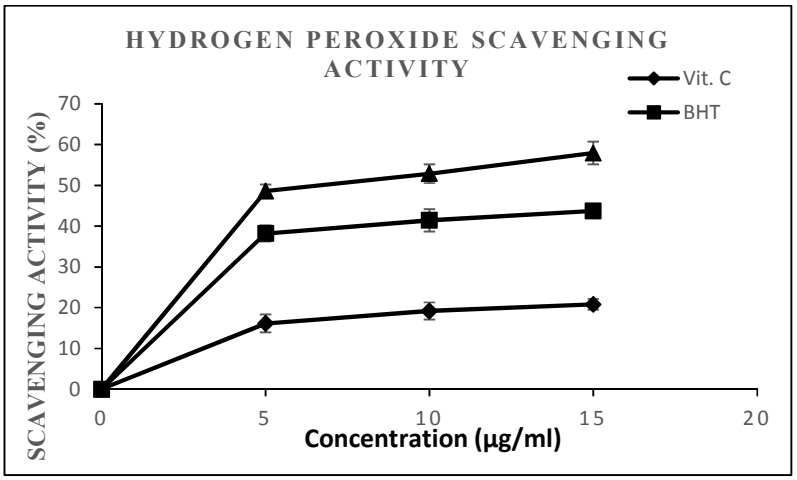

Fig. 6. Scavenging activity of ethanolic extract of Stephania glabra on hydrogen peroxide. Results are mean ± SEM of three parallel measurement. SGE, Stephania glabra extract; Vit. C (ascorbic acid); BHT, butylated hydroxytoluene.

ASSAY OF NITRIC OXIDE SCAVENGING ACTIVITY

Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions (Moncada et al., 1991). From **Fig. 7**, it is observed that scavenging of nitric oxide by the extract is concentration dependent. The percentage of scavenging Nitric oxide determined with 150 µg/ml of the ethanolic extract of the SGE, BHT and Vitamin C, was found to be 61.0%, 32.42% and 28.52% respectively. The hydrogen peroxide scavenging effect of the SGE extract and the standards follows the trend, SGE > BHT > Vit.C.

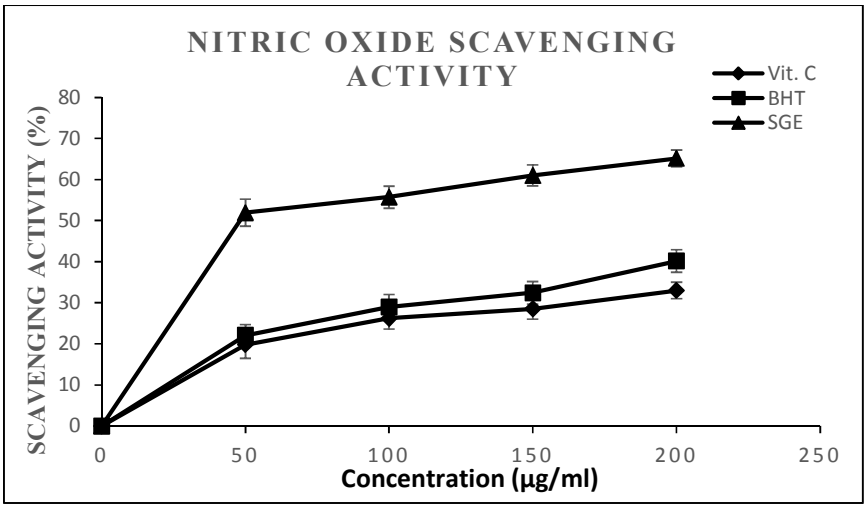

Fig. 7. Scavenging activity of ethanolic extract of Stephania glabra on Nitric Oxide. Results are mean ± SEM of three parallel measurement. SGE, Stephania glabra extract; Vit. C (ascorbic acid); BHT, butylated hydroxytoluene.

THIOBARBITURIC ACID (TBA) ASSAY

During the acid-heat treatment, the lipid peroxides decomposed to MDA, which can also be formed during the oxidation process. The extent of Lipid peroxidation was also assayed by detecting the amount of MDA. MDA can react with TBA to produce a red complex that can be measured by its absorbance at 532nm (Guillén-Sans and Guzmán- Chozas, 1998). The results of the TBA assays are shown in **Fig. 8**. The absorbance values obtained for BHT, SGE, and Vitamin C were 0.997 ± 0.086, 1.133 ± 0.078, and 1.237± 0.120, respectively, compared with a control value of 1.573 ± 0.099. Thus, the antioxidant activity of SGE is stronger than that of Vitamin C but weaker than that of BHT.

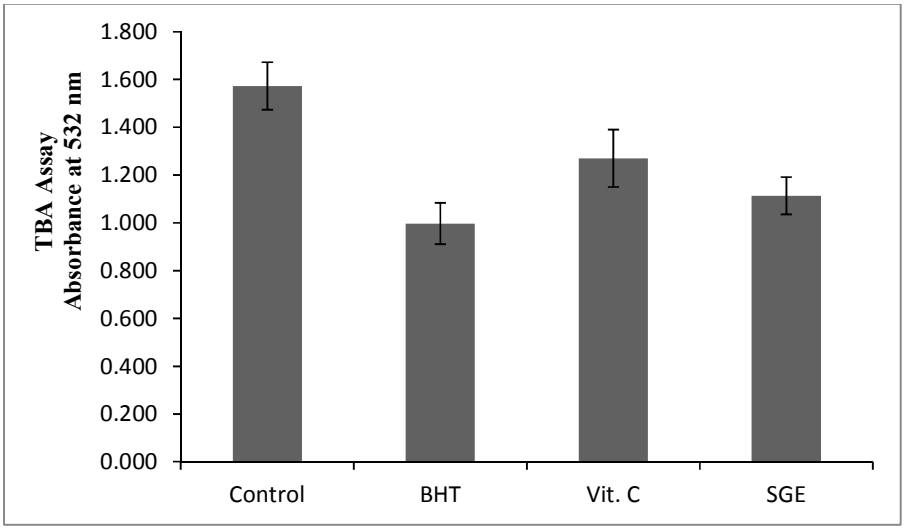

Fig. 8. Antioxidant activity of Stephania glabra extract assayed using the TBA method. Low absorbance indicates strong antioxidant activity. Results are mean ± SEM of three parallel measurement. SGE, Staphania glabra extract; Vit. C (ascorbic acid) ; BHT, butylated hydroxytoluene.

CONCLUSION

The current study suggests that Stephania glabra has higher amounts of berberine and palmitine alkaloid, which scavenge free radicals. According to the results of this study, it is concluded that the ethanolic extract of Stephania glabra possesses strong in vitro antioxidant activity, which support the traditional use of this plant against diseases related to oxidative stress. Further studies are required for the isolation and identification of individual alkaloid compounds and also in vivo studies are needed for understanding their mechanism of action as an antioxidant.

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