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IN VITRO ASSESSMENT OF TINOSPORA CORDIFOLIA STEM FOR ITS ANTIOXIDANT, FREE RADICAL SCAVENGING AND DNA PROTECTIVE POTENTIALS

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ABSTRACT

Antioxidants have reactive oxygen species (ROS) scavenging ability with great relevance in the prevention of oxidative stress. The present study aimed to examine the antioxidant and ROS scavenging activities of 70% methanolic extract of *Tinospora cordifolia*. The plant was found a significant source of carbohydrate, alkaloid, ascorbic acid, tannin, phenolic and flavonoid compound. The extract was found to be an antioxidant with a TEAC value of 0.257 ± 0.03. The extract has shown different radical scavenging activity and 128.86± 4.07 µg/ml, 103.17 ± 5.75 µg/ml, 51.98 ± 4.80 µg/ml, 327.38 ± 21.44 µg/ml, were determined as IC_{50} value for hydroxyl radical, superoxide, nitric oxide, and hypochlorous acid respectively. The plant inhibits lipid peroxidation with an IC_{50} of 75.86 ± 2.16 µg/ml. The extract also has good DNA protective potential. In other words, the present results provide evidence that *T. cordifolia* acts as an antioxidant and DNA protector.

KEYWORDS : ***Tinospora cordifolia*, radical scavenging, antioxidant, DNA protection**

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INTRODUCTION

Reactive molecules such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\cdot}$) evolved as a consequence of normal aerobic metabolism and collectively these molecules are called as reactive oxygen species (ROS)[1,2]. ROS damages macromolecules, especially DNA, which undergo strand breakage, change and release of bases as well as modification of sugar moieties[3,4], thus promoting a series of pathological event, viz, cancer and aging[5]. ROS also give rise to emphysema, cirrhosis, arteriosclerosis, inflammation, genotoxicity and other diseased conditions along with pro-oxidants[6]. Various medicinal plants containing antioxidants act through different mechanisms – such as chelating metals that catalyse the formation of free radicals and also by scavenging the same, thus becoming vital for human body due to their ability to fight oxidative damage. Several antioxidants have been synthetically prepared such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) but their use is limited due to possible carcinogenic properties. Thus major attention is being given to isolate natural antioxidants which are present in different kinds of plant materials[7]. Many indigenous herbal plants have been used as natural antioxidant in India[8]. The medicinal value of these plants lies in some chemical substances known as phytochemicals that produce a definite physiological action on human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds[9]. *T. cordifolia*, family 'Menispermaceae', belongs to the group of medicinal plants that grows in the tropical and subtropical regions of India. It is a large glabrous climber with succulent corky stem, subdeltoid cordate leaves, branches sending down, and pendulous fleshy roots. The herb is extensively used in the Indian system of medicine; the extract of different parts of the herb has found wide use in variety of diseases. It is widely used in ayurvedic system of medicine for its general tonic, antiperiodic, antispasmodic, anti-inflammatory, antistress, antileprotic, antimalarial, hepatoprotective, immuno modulatory, antineoplastic activities[10-15, 17, 18], hypoglycaemic, hypolipidaemic[16]. A hydroalcoholic extract of *T. cordifolia* was reported to have aldose reductase inhibitory activity whereas isoquinoline alkaloids from this plant was reported having aldose reductase activity[19, 20]. The present study is aimed to investigate *in vitro* antioxidant, DNA protection role and all the known free radicals' scavenging activity of the extract of *T. cordifolia* stem in 70% methanol in water.

MATERIALS AND METHODS

(i) Chemicals

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), 2-deoxy-2-ribose, mannitol, lipoic acid, quercetin, sodium nitroprusside, 1, 10-phenanthroline and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Folin-ciocalteu reagent, Mercuric chloride, Potassium Iodide, Anthrone, Vanillin, 2, 4-Dinitrophenylhydrazine, Thiourea and *N*,*N*-dimethyl-4-nitrosoaniline were obtained from Merck, Mumbai, India. DPPH, gallic acid, (+) catechin and curcumin were obtained from MP Biomedicals, France. Catalase, Reserpine and Sodium bicarbonate were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue, Lead acetate were purchased from BDH, England. D-glucose was procured from Qualigens Fine Chemicals, Mumbai. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India. Desferal was bought from Novartis Pharma Stein AG, Switzerland.

(ii) Plant material

The stem of *T. cordifolia* was collected from Bankura district of West Bengal, India. The

plant was identified by the Central Research Institute (Ayurveda), Kolkata, India, where specimen of plant was deposited (Specimen No. CRHS 123/08)

(iii) Crude extract preparation of T. cordifolia

The collected plant material was shade dried at room temperature for 2 weeks and grinded to powder. The powder (100 gm) was mixed with 70% methanol (1000 ml) and kept in a shaking incubator overnight (12 hours, 37 °C, 160 rpm). Then the mixture was centrifuged at 2850 x *g* for 15 minutes. The pellet was mixed with 70% Methanol (1000 ml) and kept overnight at the shaking incubator and centrifuged. The supernatant liquid was collected from both the phases and filtered. The resultant filtrate was concentrated in a rotary evaporator under reduced pressure. The concentrated extract was lyophilized and stored at -20 °C until further use.

(iv) Animals

The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/CPCSEA) approved the use of adult male Swiss Albino mice (*Mus musculus)*, weighing 20-25 gm for experimentation. Each polypropylene cage contained 4 mice at a time; supplied with *ad libitum* laboratory diet and water. The mice were kept at 25 ± 2 °C and 60 ± 5% humidity and normal photo cycle (12 hours dark/12 hours light).

(v) Phytochemical analysis

Qualitative tests Phytochemical analysis of *T. cordifolia* stem extracts was carried out using standard qualitative methods as described previously[21, 22]. *Quantification of total phenolic content* Quantification of total phenolic content was done using previously reported method with slight modifications[23]. Briefly, 0.1 ml of extract was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water). The reaction mixture was incubated for 5 min at 22 °C; then 0.06% Na_2CO_3 solution was added to the mixture. After incubation at 22 °C for 90 min, the absorbance was measured at 725 nm. The phenolic content was evaluated from a gallic acid standard curve. *Quantification of total flavonoid content* Total flavonoid content was quantified according to a standard method using quercetin as a standard[24]. The plant extract of 0.1 ml was added to 0.3 ml distilled water followed by 0.03 ml 5% $NaNO_2$. After 5 min of incubation at 25 ºC, 0.03-ml 10% $AlCl_3$ was added. After another 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally the reaction mixture was diluted to 1 ml with water. Then the absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve. *Quantification of carbohydrate content* Quantification of carbohydrate content was carried out using previously described method[25]. 100 mg of the 70% methanolic extract of *T. cordifolia* was weighed into a test tube, hydrolysed by keeping it in a boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cooled to room temperature. The volume was made to 100 ml and centrifuged. 0.25 ml sample extract made up to 0.5 ml distilled water and mixed with anthrone reagent (4 ml) and was incubated at 95 ºC for 8 min. After incubation, cooled rapidly and absorbance was measured of green to dark green colour at 630 nm. All tests were performed six times. The carbohydrate content was evaluated from a glucose standard curve. *Quantification of alkaloid content* Quantification of alkaloid content was carried out using previously reported method[26]. To the 1 ml of extract (1 mg/ml) in water 0.1 ml of $FeCl_3$ (2.5 mM $FeCl_3$ in 0.5 M HCl) was added followed by addition of 0.1 ml 1,10-phenanthroline. After incubation for 30 min at 70 ºC the absorbance was taken at 500 nm. All tests were performed six times. The alkaloids content was evaluated from the reserpine standard graph. *Quantification of ascorbic acid content* This quantification was carried out according to the previously described method[27]. 1 ml aliquots of 70% methanolic extract (1 mg/ml) in water were mixed with 1 ml of '2,4-dinitro-phenylhydrazine reagent' (2% 2,4-dinitrophenylhydrazine and 4% thiourea in 9 (N) H_2SO_4) and was incubated at 95 ºC water bath for 15 min. After incubation 5 ml of 85% H_2S0_4 was added dropwise in an ice bath. Then the mixture was stood for 30 min, the absorbance was

measured at 520 nm. All tests were performed six times. The ascorbic acid content was evaluated from L-ascorbic acid standard curve. *Quantification of tannin content* This was assayed as described by[28] with a slight modification. 0.1 ml aliquots of 70% methanolic extract (1 mg/ml) in water were mixed with the 0.5 ml vanillin hydrochloride reagent (Mix equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol. The solutions were mixed just before use, and avoid using even if it is slightly colored). After incubation for 20 min at room temperature the absorbance was measured of magenta-pink colour at 500 nm. All tests were performed six times. The tannin content was evaluated from the catechin standard graph.

(vi) Antioxidant and free radical scavenging assays

Total antioxidant activity assay

Antioxidant capacity was determined by an improved $ABTS^{\bullet+}$ radical cation decolorisation assay in comparison to trolox standard[29]. The $ABTS^{\bullet+}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution and 2.45 mM potassium persulfate (final concentration) followed by overnight incubation in dark at room temperature. Then 10 µl sample solution was mixed with 1 ml $ABTS^{\bullet+}$ solution and the absorbance was measured at 734 nm. All experiments were repeated 6 times. The Trolox equivalent antioxidant capacity (TEAC) was determined by plotting the percentage inhibition of absorbance as a function of concentration of standard and sample. *DPPH radical scavenging assay* The free radical scavenging activity of the extract was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) using a standard method previously described with slight modifications[30]. Different concentrations (0-100 µg/ml) of the extracts and the standard trolox were mixed with equal volume of ethanol. Then 50 µl of DPPH solution (1 mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solution was kept standing for 2 min before the optical density (OD) was measured at λ = 517 nm. The measurement was repeated with six sets. The percentage radical scavenging activity was calculated from the following formula: % of scavenging [DPPH] = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Hydroxyl radical scavenging assay* Hydroxyl radical scavenging assay was performed by a standard method previously described[29]. Hydroxyl radical was generated by the Fenton reaction using a Fe^{3+}-ascorbate-EDTA-H_2O_2 system. The assay quantifies the 2-deoxyribose degradation product, by its condensation with TBA. All tests were carried out six times. Mannitol was used as a standard compound. Percentage of hydroxyl radical scavenging was evaluated by the following equation: % of scavenged hydroxyl radical = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Superoxide radical scavenging assay* Measurements of superoxide anion scavenging activities of the samples and standard were performed based on the reduction of NBT according to a previously described method[29]. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals. These radicals reduce nitro blue tetrazolium (NBT) into a purple colored formazan which was measured spectrophotometrically at 562 nm. All tests were performed six times. Quercetin was used as a standard superoxide radical scavenger. The percentage of superoxide anion generation scavenging was calculated using the following formula: % of scavenged superoxide = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Nitric oxide radical scavenging assay* Nitric oxide generated from aqueous SNP solution interacts with oxygen to produce nitrite ions at physiological pH, which can be quantified according to the Griess Illosvoy reaction[29]. The pink chromophore generated was measured spectrophotometrically at 540 nm. Curcumin was used as a standard. All tests were performed six times. The percentage of nitric oxide radical generation scavenging was

calculated using the following formula: % of scavenged Nitric oxide = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Hydrogen peroxide scavenging assay* The assay was performed according to the method described previously with slight modifications[31]. 50 mM H_2O_2 and various concentrations (0–2.0 mg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 µl of the H_2O_2-sample solution was mixed with 10 µl HPLC-grade methanol and 0.9-ml FOX reagent was added (prepared by mixing 9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of ferric-xylenol orange complex was measured at 560 nm. All tests were carried out six times and sodium pyruvate was used as a standard H_2O_2 scavenger. The percentage of scavenging of hydrogen peroxide was calculated by the following formula: % of scavenged H_2O_2= $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Peroxynitrite scavenging activity* A previously described standard method was followed to synthesize Peroxynitrite (ONOO-)[32]. Briefly, 5 ml 0.6 M KNO_2 was mixed with an acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) on ice bath for 1 min and 5 ml of ice-cold 1.2 M NaOH was added to the solution. The solution was subjected to treatment with granular MnO_2 prewashed with 1.2 M NaOH to remove the excess H_2O_2. The reaction mixture was left overnight at -20 °C. Peroxynitrite solution was collected from top of the frozen mixture and concentration was measured spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}). To measure peroxynitrite scavenging activity an Evans Blue bleaching assay was used[29]. The percentage scavenging of ONOO- was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as a standard. The percentage of scavenging of peroxynitrite anion was calculated using the following equation: % of scavenged Peroxinitrile = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Singlet oxygen scavenging assay* The assay was performed according to the method described previously with slight modifications[33]. The production of singlet oxygen ($^1O2$) was determined by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2, the bleaching of RNO was read at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2, 50 mM L-histidine, 10 µM RNO and various concentrations (0–100 µg/ml) of sample in a final volume 2 ml. The solution mixture was incubated at 30 ºC for 40 min and the absorbance of RNO was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a standard. All tests were performed six times. Singlet oxygen scavenging activity was calculated by the following formula: % of scavenged (Singlet oxygen) = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Hypochlorous acid scavenging assay* Hypochlorous acid (HOCl) was prepared freshly by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4 and the concentration of HOCl was determined by measuring absorbance at 235 nm using molar extinction coefficient of 100/M cm. The assay was carried out as described previously[29]. All tests were performed six times. Ascorbic acid was used as the standard. All tests were performed six times. Singlet oxygen scavenging activity was calculated by the following formula: % of scavenged (Hypochlorous acid) = $[(A_0 – A_1) / A_0] * 100$ Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard. *Iron (Fe^{2+}) chelation* The ferrous ion chelating activity was evaluated by a standard spectrophotometric method with minor changes[34]. The reaction was carried out in

HEPES buffer (20 mM, pH 7.2). Various concentrations of plant extracts (0–300 µg/ml) were mixed with 12.5 µM ferrous sulfate solution. The reaction was initiated by the addition of ferrozine (75 µM). The mixture was vortexed and incubated for 20 min at room temperature and the absorbance was measured at 562 nm. All tests were performed for six times. EDTA was used as a standard iron chelator. *Measurement of reducing power* The Fe^{3+} reducing power of the plant extract was determined using previously described method with slight modification[35]. Different concentrations (0–1.0 mg/ml) of extract (0.5 ml) were mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate. The solution was incubated at 50 °C in a water bath for 20 min. 0.5 ml of TCA (10%) was then added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was incubated for 10 min at room temperature and absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a standard. *Lipid peroxidation inhibition assay* This assay was carried out according to a previously described method with slight modifications[38]. Swiss albino mice brain homogenate was prepared by centrifuging brain (20 ± 2 g) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 µl aliquot of homogenate supernatant was mixed with the plant extract of various concentrations (0-25 µg/ml), followed by addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid and incubated for 1 h at 37 °C. 500 µl 28% TCA was used to stop the reaction and then 380 µl 2% TBA was added followed by heating at 95 °C for 30 min to generate the colour. Then the samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All tests were performed six times. Trolox was used as the standard.

(vii) DNA protection assay

The protection of the pUC-18 plasmid DNA, damaged by Fenton reaction generated OH radicals, was studied by quantifying the decrease of supercoiled DNA after oxidative attack, using a previously described method[37]. After completion of reaction, 25 µl of each reaction mixture was loaded in 1% agarose gel. After migration, the gel was stained with ethidium bromide and visualized in a UV transilluminator. The DNA bands were quantified through densitometry and the following formulae were used to calculate the percentage of protection. % SC = [1.4 X SC/(OC+(1.4 X SC))] X 100 where, SC = supercoiled; OC = open circular; 1.4 = correction factor % protection = 100 X [(control SC – chelator SC)/(control SC – no chelator SC) – 1] The ability of the plant extract to protect the DNA supercoil can be expressed by the concentration of sample required for 50% protection, designated as the $[P]_{50}$ value.

(viii) Statistical analysis

All data are reported as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula Y = 100*A1/(X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t tests. $P < 0.05$ was considered significant.

RESULTS

Determination of phytochemicals

The total phenolic content and total flavonoid content 70% methanolic extract of *T. cordifolia* was found to be 51.33 ± 0.002 mg/100 mg gallic acid equivalent and 178.67 ± 0.003 mg/100 mg quercetin equivalent per 100 mg plant extract respectively. The carbohydrate content was found to be 9.21 ± 0.02 mg/100 mg glucose equivalent, alkaloid was 45.11 ± 0.36 mg/100 mg reserpine equivalent, 0.61 ± 0.04 mg/100 mg ascorbic acid and tannin was 2.35 ± 0.023 mg/100 mg catechin equivalent (Table 2).

Table 1
Phytochemical analysis (Qualitative) of 70% methanolic extract of T. cordifolia stem

Phytochemicals	Stem extract
Phenolics	+
Flavonoids	+
Carbohydtrate	+
Alkaloids	+
Tannins	+
Terpenoids	+
Triterpenoids	-
Anthraquinones	-
Saponins	-
Glycosides	-

Table 2
Quantitative analysis of phytochemicals of 70% methanolic extract of T. cordifolia stem

Phytochemical	Amount present
Total phenolics (mg/100 mg extract gallic acid equivalent)	51.33 ± 0.002
Total flavonoids (mg/100 mg extract quercetin equivalent)	178.67 ± 0.003
Carbohydtrate (mg/100 mg extract glucose equivalent)	9.21 ± 0.02
Alkaloid (mg/100 mg extract reserpine equivalent)	45.11 ± 0.36
Ascorbic acid (mg/100 mg extract L-ascorbic acid equivalent)	0.61 ± 0.04
Tannin (mg/100 mg extract catechin equivalent)	2.35 ± 0.023

Total antioxidant activity

The antioxidant activity of *T. cordifolia* was measured by using TEAC assay, based on interaction between antioxidant and $ABTS^{\bullet+}$ radical cation which has a characteristic colour showing maxima at 734 nm. Interaction with the extract or standard trolox suppressed the absorbance of the $ABTS^{\bullet+}$ radical cation and the results expressed as percentage inhibition of absorbance are shown in figure 1(a) and figure 1(b), respectively. The TEAC value of the *T. cordifolia* extract was found to be 0.256 ± 0.030.

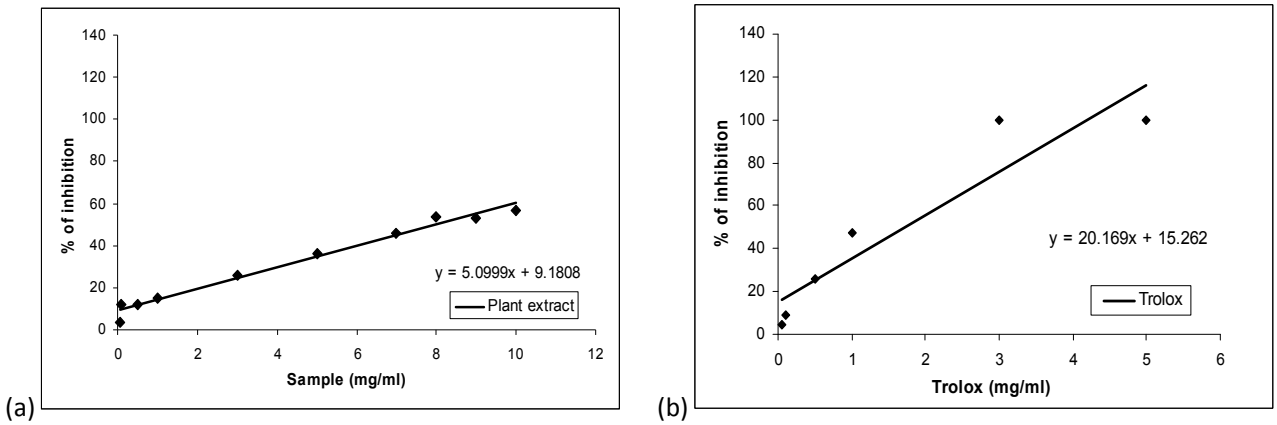

Figure 1
Total antioxidant activity. Effects of (a) T. cordifolia stem extract and (b) reference compound trolox on ABTS radical cation decolorization assay. The percentage of inhibition is plotted against concentration of sample. The value represented as mean ± SD (n = 6)

DPPH radical scavenging assay

The scavenging activity for free radicals of *T. cordifolia* was not at all worth acknowledgeable in the assay. The IC_{50} value for the plant extract was found to be indeterminable, whereas that of the standard ascorbic acid was 5.29 ± 0.28 mg/ml. The data are not shown.

Hydroxyl radical scavenging assay

This assay exhibits the ability of extract and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are displayed in figure 2. The IC_{50} values (Table 3) of the *T. cordifolia* extract and standard mannitol were 128.86 ± 4.07 µg/ml and 571.45 ± 20.12 µg/ml respectively.

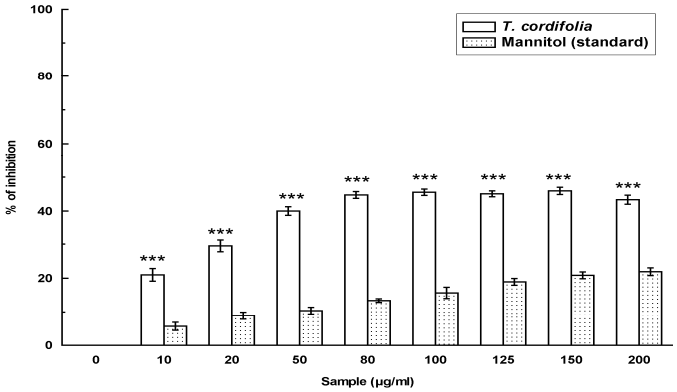

Figure 2

Hydroxyl radical scavenging activity of the T. cordifolia extract and the reference compound mannitol. The data represent the percentage of inhibition of deoxyribose degradation. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs. 0 µg/ml

Superoxide radical scavenging

Superoxide radicals generated from the PMS-NADH coupling, can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. The results are shown in figure 4, the IC_{50} values (Table 3) of the *T. cordifolia* extract and quercetin on superoxide scavenging activity were 103.17 ± 5.75 µg/ml, and 42.06 ± 1.35 µg/ml respectively.

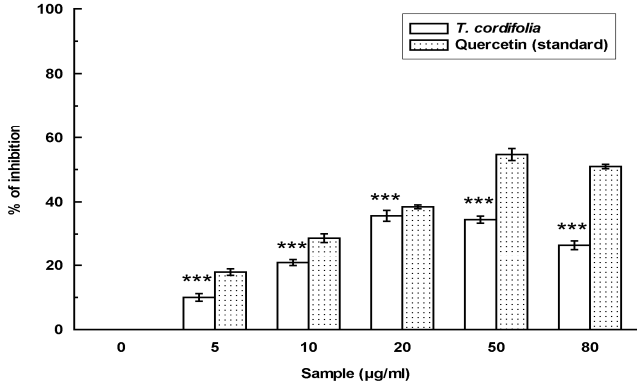

Figure 3

Scavenging effect of T. cordifolia plant extract and standard quercetin on superoxide radical. The data represents the percentage of superoxide radical inhibition. All data are expressed as mean ± S.D. (n=6). ***p < 0.001 vs. 0 µg/ml

Nitric oxide radical scavenging

The extract of *T. cordifolia* was found efficient in scavenging nitric oxide radical (Figure 4). The IC_{50} values for extract and standard curcumin were found 51.98 ± 4.80 µg/ml and 90.82 ± 4.75 µg/ml respectively.

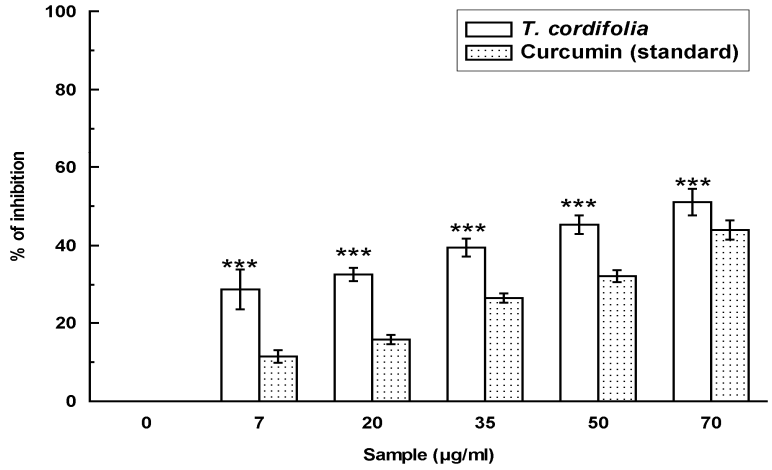

Figure 4

The nitric oxide radical scavenging activity of T. cordifolia extract and standard curcumin. The data represents the % of nitric oxide inhibition. Each value represents mean ± S.D. (n=6). ***p < 0.001 vs. 0 µg/ml

Hydrogen peroxide scavenging

T. cordifolia extract did not show any remarkable result in scavenging hydrogen peroxide in comparison to the standard sodium pyruvate (IC_{50} = 3.24 ± 0.30 mg/ml). So the results and the figure are not provided.

Peroxynitrite scavenging

Figure 6 shows the peroxynitrite scavenging activity of all the extracts in a concentration dependent manner. The calculated IC_{50} values of extract and standard were 6.32 ± 2.03 mg/ml and 0.87 ± 0.056 mg/ml respectively (Table 3). Thus, the IC_{50} value of *T. cordifolia* extract was found very much as compared to the IC_{50} value of standard which indicates extract is a poor scavenger of peroxynitrite.

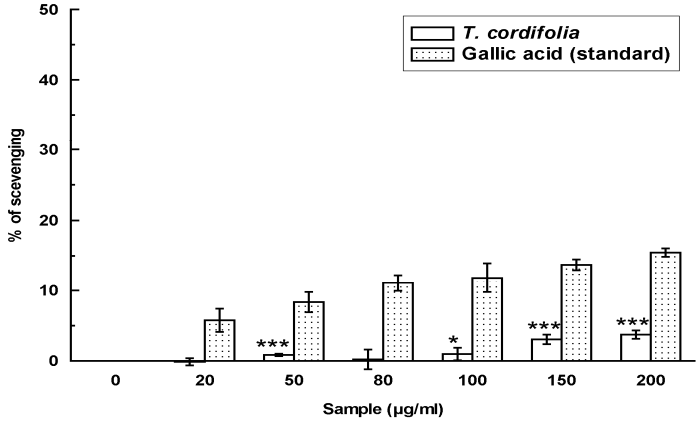

Figure 5

The peroxynitrite anion scavenging activity of T. cordifolia plant extract and standard gallic acid. The data represents the percentage of scavenging of peroxynitrite. All data are represented as mean ± S.D. (n=6). ***p < 0.0001 vs 0 µg/ml

Singlet oxygen scavenging

Extract of *T. cordifolia* showed a moderate dose-dependent scavenging effect of singlet oxygen species (Figure 6) with IC_{50} value of 315.63 ± 45.62 µg/ml (Table 3). Lipoic acid was used as a standard compound and 46.15 ± 1.16 µg/ml lipoic acid was needed for 50% inhibition.

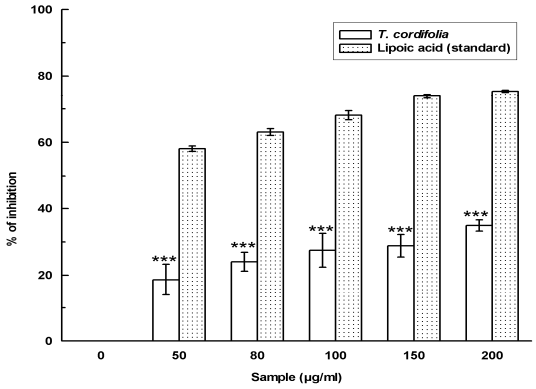

Figure 6

Effect of T. cordifolia plant extract and standard lipoic acid on the scavenging of singlet oxygen. The data represents the percentage of scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs. 0 µg/ml

Hypochlorous acid scavenging

Figure 7 shows hypochlorous scavenging activity of extract of *T. cordifolia* dose-dependently compared to that of standard ascorbic acid. The IC_{50} values *T. cordifolia* extract and ascorbic acid were found to be 327.38 ± 21.44 µg/ml and 235.95 ± 5.75 µg/ml (Table 3).

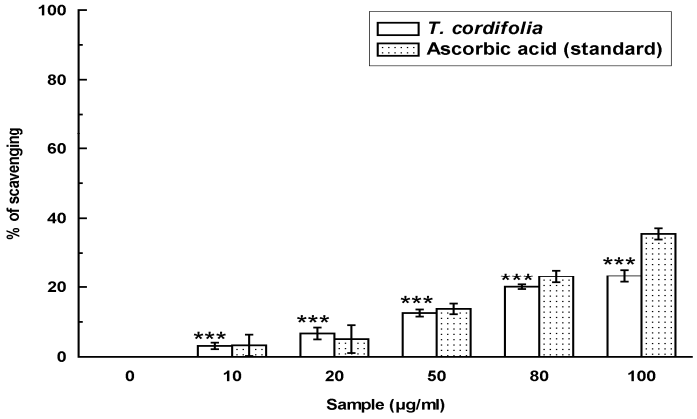

Figure 7

Hypochlorous acid (HOCl) scavenging activity of T. cordifolia stem extract and standard ascorbic acid. All data are expressed as mean ± S.D. (n=6). ***p < 0.001 vs. 0 µg/ml

Iron chelation

Ferrozine along with Fe^{2+} ion generates a violet colored complex. In the presence of a chelating agent, the ferrozine formation is interrupted and the intensity of the violet colour decrease with increase in the concentration of the chelating agent. The result demonstrated that *T. cordifolia* extract is unable to inhibit formation of ferrozine-Fe^{2+} complex while it is inhibited in the presence of reference compound (Data not shown).

Reducing power

The assay for the reducing power showed that the plant extract possess very poor reducing power compared to the standard ascorbic acid; and the results were not shown.

Lipid peroxidation

The IC_{50} value (Table 3) of the *T. cordifolia* extract was found to be 75.86 ± 2.16 µg/ml (Figure 8). Trolox was used as a standard which showed an IC_{50} value of 6.76 ± 0.17 µg/ml showing that the inhibitory efficiency of the plant extract is moderate compared to standard trolox.

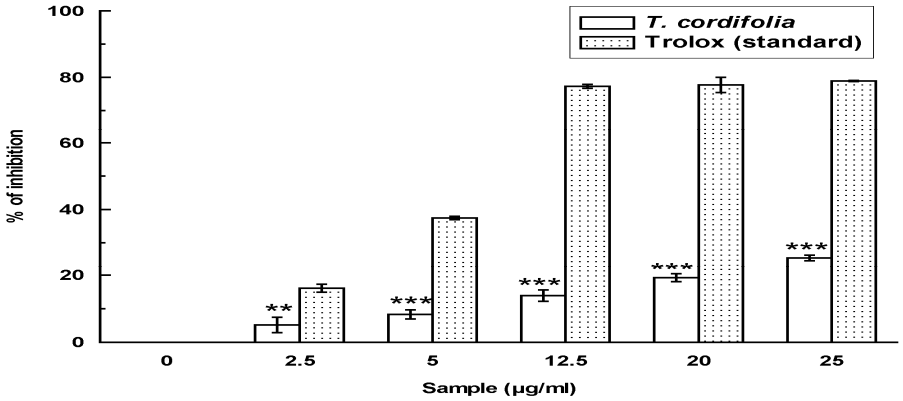

Figure 8

Lipid peroxidation inhibiting capacity of the T. cordifolia extract and the standard trolox. The data is expressed as the % of lipid peroxidation inhibition of brain homogenate, induced by Fe2+/ascorbic acid. Each value represents mean ± S.D. (n=6). *** p < 0.001 vs. 0 mg/ml

DNA protection assay

The protective effect of *T. cordifolia* extract against Fe^{2+}-H_2O_2 mediated DNA breakdown was verified in Figure 10. pUC18 supercoiled DNA was used as control (lane 1). The treatment of supercoiled DNA with Fenton's reagent led to the conversion of DNA to open circular form (lane 2). The addition of increasing concentrations of plant extracts resulting in the restoration of DNA in the supercoiled form (lane 5-9). The results had showed the dose dependant protective effect of extract. The DNA protection activity of extract was found very effective. The $[P]_{50}$ value of the extract was found to be 17.45 ± 0.96 µg/ml (Table 3).

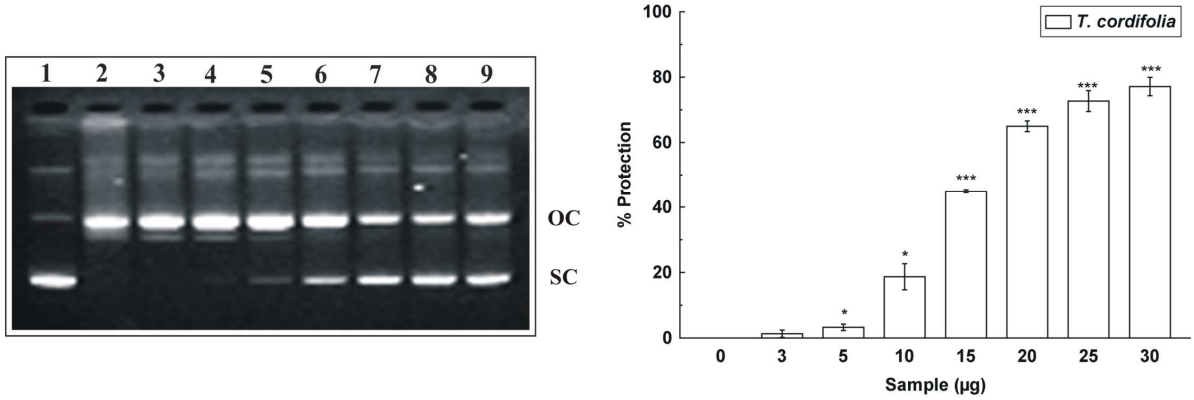

Figure 10

Protection against oxidative damage to pUC18 by T. cordifolia extract. Picture of agarose gel of pUC18 DNA showing bands of supercoiled (SC) and open ciecular (OC) forms. Lanes on the gel represent: (Lane 1) control DNA (no H_2O_2 or Fe^{2+}); (Lane 2) reaction mixture without extract; (Lane 3-9) reaction mixture with extract of increasing concentration (30-300 µg).

Table 3
Relative acitivties of T. cordifolia and reference compounds

Activity	*T. cordifolia*
Trolox equivalent antioxidant capacity (TEAC)	0.257 ± 0.03
Concentration required for 50% DNA protection; $[P]_{50}$	17.45 ± 0.96

Reactive oxygen species scavenging, iron chelating activity and lipid peroxidation inhibition activity (IC_{50} values) of T. cordifolia and reference compounds

Activity	Extract/Reference	IC_{50} (#)
Hydroxyl radical ($OH^{\cdot}$) scavenging	*T. cordifolia*	128.86± 4.07 (6)
	Mannitol	571.45 ± 20.12 (6) ***
Superoxide anion ($O_2^{\cdot-}$) scavenging	*T. cordifolia*	103.17 ± 5.75 (6)
	Quercetin	42.06 ± 1.35 (6) ***
Nitric oxide radical (NO) scavenging	*T. cordifolia*	51.98 ± 4.80 (6)
	Curcumin	90.82 ± 4.75 (6) ***
Peroxynitrite ($ONOO^-$) scavenging	*T. cordifolia*	6328.68 ± 2032.65
	Gallic acid	876.24 ± 56.96 (6) ***
Singlet oxygen ($^1O_2$) scavenging	*T. cordifolia*	315.63 ± 45.62 (6)
	Lipoic acid	46.15 ± 1.16 (6) ***
Hypochlorous acid (HOCl) scavenging	*T. cordifolia*	327.38 ± 21.44 (6)
	Ascorbic acid	235.95 ± 5.75 (6) **
Iron Chelating Activity	*T. cordifolia*	2935.60 ± 370.65 (6)
	EDTA	1.27 ± 0.05 (6) ***
Lipid Peroxidation Inhibition	*T. cordifolia*	75.86 ± 2.16 (6)
	Trolox	6.76 ± 0.17 (6) ***

 # Unit of IC_{50} values of all activities is µg/ml, except H_2O_2 scavenging, where the units are mg/ml.
Data expressed as mean ± S.D. Data in parenthesis indicate number of independent assays.
EDTA represents Ethylenediamine tetraacetic acid.
** p< 0.05.*
*** p< 0.01.*
**** p< 0.001 vs. T. cordifolia.*

DISCUSSION

Phenolic compounds are very important as plant constituent and effective metal chelators and good reducing agents[38]. They can also have radical scavenging ability due to their hydroxyl groups. In addition, flavonoids show their antioxidant feat through scavenging or chelating processes[39]. Ascorbic acid and alkaloids are well known for their pharmacological properties. On the other hand tannins exhibit astringent properties. They exert internal anti-diarrheal, antiseptic, antimicrobial and antifungal effects. Tannins act as radical scavengers, intercepting active free radicals. It also plays a role in treating various degenerative diseases such cancer, multiple sclerosis, atherosclerosis and aging process[40]. Our study showed *T. cordifolia* stem can be considered as source of tannins. The carbohydrate content of studied plant is also found significant. It was described that carbohydrates in food are of major interest in relation to chronic diseases. Different types of carbohydrates give rise to different glycemic responses, and also able to stimulate lipogenesis[41]. Reactive oxygen species damage many of important cellular components like proteins, enzymes, nucleic acids, lipids and carbohydrates and may adversely affect immune functions. Oxidative stress is initiated by free radicals which are generated constantly in the living system and find stability through electron pairing with biological macromolecules such as proteins, lipids and DNA of healthy human cells and

can cause various diseases. In recent years, due to the adverse effects of synthetic drugs, researchers have channelized their attention in isolating natural antioxidants from plants. The present study demonstrates the antioxidant and free radical scavenging activity of 70% methanolic extract of *T. cordifolia*. The reaction between ABTS and potassium persulfate results in the production of a blue colored complex, $ABTS^{\cdot+}$. Addition of the plant extract to this radical cation reduces it to ABTS on a concentration dependent manner. The results are compared with trolox and the TEAC value was found to be 0.257 ± 0.03, which demonstrates the methanolic extract is a potent antioxidant.

Among all free radicals that are generated in biological system hydroxyl radical is the most detrimental[2]. In Fenton reaction, hydroxyl radicals are formed that cause 2-deoxy-2-ribose damage and generate malondialdehyde (MDA) like product. This compound forms a pink chromogen upon heating with TBA at low pH. As the *T. cordifolia* extract or standard mannitol is added to the reaction mixture the hydroxyl radicals are scavenged and thereby sugar damage can be blocked. The results indicate that the plant extract is excellent hydroxyl radical scavenger than standard mannitol. Superoxide radical is also considered as a harmful reactive oxygen species with damaging action on cellular components. PMS-NADH coupling reaction generates superoxide radicals from dissolved oxygen. These superoxide radicals can be measured by its ability to reduce NBT. The results suggest that the plant extract is a moderate superoxide radical scavenger but efficiency is low compared to standard quercetin. The chronic emergence of nitric oxide radical is linked with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis[42]. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract competes with oxygen to react with nitric oxide and thereby inhibits nitrite formation. The present study showed that nitric oxide scavenging activity of *T. cordifolia* extract is better than standard curcumin.

Free radical superoxide and nitric oxide react to form peroxynitrite anion which due to its oxidizing properties can harm cellular components[43]. The study indicates that the scavenging activity of *T. cordifolia* extract is much poor as compared to the standard gallic acid. Furthermore, singlet oxygen is a high energy form of oxygen which is generated in the skin upon UV-radiation. It induces the hyperoxidation, oxygen cytotxicity and decreases the antioxidant activity[44]. The IC_{50} value of the extract was higher than the reference compound. The present study indicates that the *T. cordifolia* extract has singlet oxygen scavenging activity but very poor compared to standard lipoic acid. Hypochlorous (HOCl), another harmful ROS, is produced at the sites of inflammation through the oxidation of Cl- ions by the neutrophil myeloperoxidase[1]. HOCl inactivates the antioxidant enzyme catalase by breaking down its heme prosthetic group. The results show that the *T. cordifolia* extract possesses HOCl scavenging property but the efficiency is less as compared to the standard ascorbic acid. In the process of lipid peroxidation, the iron catalyzed generation of ferry-perferryl complex or hydroxyl radicals accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. The reactive hydroxyl radical reacts with polyunsaturated fatty acid moieties of cell membrane and forms carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. With addition of the *T. cordifolia* extract or standard, the generation of MDA is reduced, hence indicating the ability of the extract, although less than the standard, to inhibit lipid peroxidation. At the cellular level, subjecting cells to oxidative stress can result in severe metabolic dysfunction, including DNA damage with a characteristic pattern of modification of all bases, production of base- free sites, deletions, strand breaks, DNA-protein cross-links, and chromosomal rearrangement. An important reaction involved in DNA damage involves generation of hydroxyl radical through Fenton. Hydroxyl radical is known to react with

all components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone. When DNA was exposed to Fenton reaction, H_2O_2 will be generated to hydroxyl radicals, and then the supercoiled (SC) form of DNA would cleave to give rise open-circular (OC) form. With the occurrence of restored SC form of DNA with increase in *T. cordifolia* extract concentration proposes a evidence that the plant possess excellent DNA protection activity. In conclusion, on the basis of the results obtained from the current study, a 70 % methanolic extract of *T. cordifolia* stem, which contains high amount of phenolics, flavonoids and alkaloids with moderate amount of tannins and carbohydrate, exhibit good total antioxidant activity and moderate free radical scavenging activities. These *in vitro* assays indicate that this plant extract is an important source of natural antioxidant, which might be beneficial to prevent the various oxidative stresses. Therefore, further studies should be carried out to isolate active compounds having antioxidant property.

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