ISSN 0976 – 3333

Available Online at www.ijpba.info.

International Journal of Pharmaceutical & Biological Archives 2010; 1(5):448-456

ORIGINAL RESEARCH ARTICLE

In Vitro Antioxidant Potency of *Tinospora cordifolia* (gulancha) in Sequential Extracts.

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Received 28 Oct 2010; Revised 23 Nov 2010; Accepted 02 Dec 2010

ABSTRACT

Background: Tinospora a medicinal herb used in the Indian system of medicine due to their health benefits. The stem of the plant was studied for its antioxidant and phytochemical constituents. Sequential extraction of the powder was done with different solvents with increasing polarity. The antioxidant activity of polar to non-polar solvents extracts of the herb was evaluated by various antioxidant assays. The phytochemical constituent such as total phenols, flavonoids, tannins, and proximate compositional analysis was carried out.

Results: Methanolic, ethanolic and water extracts showed significant antioxidant potential compared to other solvents and also possess metal chelation and reducing power activity. In the DPPH radical scavenging activity, methanolic extract (98.13%) showed high antioxidant potency, ethanolic extract (90.34%) was a potent scavenger of superoxide radical. At the same time, both methanolic (97.08%) and ethanolic extracts (95.21%) inhibited hydroxyl radical along with other extracts. The metal chelation in methanolic (60.62%), ethanolic (57.62%), aqueous extracts (40.89%) and reducing of ferrous ions was significant found increasing in methanolic, ethanolic and aqueous extracts.

Conclusion: The stem of *Tinospora cordifolia* has potential application in food systems as an antioxidant and probably in biological systems as a nutraceutical.

Keywords: *Tinospora cordifolia*; sequential extracts; *in vitro* antioxidant activity; free radical; scavenging activity

INTRODUCTION

Reactive oxygen species (ROS) and free radicals have attracted increasing attention over the past decade. ROS is produced during normal physiological events and they can easily initiate the per oxidation of membrane lipids, leading to accumulation of lipid peroxides. Though there exists a balance between the generation of ROS and inactivation of ROS by the antioxidant systems in organisms under pathological conditions, ROS is overproduced and results in oxidative stress. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in intercellular molecules. [1, 2]

Natural antioxidants like phenolic compounds, flavonoids which are secondary plant metabolites present in food products of plant origin [3, 4] can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes [5] and also exhibit a wide range of biological effects, including antiageing,

antimutagenicity, and protective effects on oxidative stress. [6, 7, 8] several studies have shown that plant derived antioxidant nutraceuticals scavenge free radicals and modulate oxidative stress related degenerative diseases. [9]

Tinospora cordifolia an indigenous plant used in Ayurvedic medicine is commonly known as (Gulancha) belonging to the family Menispermaceae, is a large spreading glabrous perennial deciduous twiner grown in India. Earlier studies reveal the chemopreventive,[10] anti osteoporetic,[11] hepatoprotective,[12] immuno modulatory[13], Antihperglycaemic, [14] antitumor [15] and antiallergic[16] properties of *Tinospora cordifolia*. The information regarding the *in vitro* antioxidative properties *Tinospora cordifolia* is sparse. Hence here we report the *in vitro* antioxidant activity so that the results of this study

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might be used for the characterization of active compounds.

MATERIALS AND METHODS

Chemicals:

Butylated hydroxyanisole (BHA), nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Phenazine methosulphate (PMS), Nicotinamide adenine dinucleotide reduced (NADH), deoxyribose 4-dimethyl amino cinnamaldehyde, Catechin were purchased from M/s Sigma Chemicals Co. (St. Louis, MO). Thiobarbituric acid (TBA), gallic acid, trichloroacetic acid (TCA), Ethylenediamine tetra-acetic acid (TCA), Ascorbic acid and other chemicals were purchased from M/s Sisco Research Laboratories, Mumbai, India. All reagents procured were analytical grade.

Preparation of the powder and Extraction:

Tinospora cordifolia stem were purchased from reliable local suppliers. They were washed, dried in hot air oven at 40^0C and then finely powdered. The powder was used for extraction. Sequential extraction of the powder was done with different solvents with increasing polarity i.e. hexane, chloroform, ethylacetate, acetone, methanol, ethanol and water. 30g of the powder was extracted in 300ml of the solvent in glass conical flask on a shaker for 24h at room temperature. The extract was filtered with Whatmann filter paper no. 1 and dried by flash evaporation/lyophilisation.

Proximate composition:

The proximate composition of *Tinospora cordifolia* was determined using the Association of Official Chemists method. [17] [18] the mineral content of the stem was analyzed by an atomic absorption spectrophotometer (model AAS Vario 6, Shimadzu, Japan). The vitamin C content was estimated by titrimetric method using 1, 2-dicholorophenol indophenol dye. Vitamin E and total carotenoids in the powdered extract of *Tinospora cordifolia* were estimated as prescribed by Sadasivam and Manickam. [18]

Antioxidant activity by free radical scavenging method:

1,1-diphenyl 2-picrylhydrazyl (DPPH) radical was used for the determination of the antioxidant activity of *Tinospora cordifolia* extract. The method described by Hatano [19] was used with slight modifications. Briefly, 3ml of the reaction mixture contained 0.1ml of different concentration of the extract (1-10mg ml^{-1}) and aliquot of DPPH solution (0.5ml, 0.5g L-1) in methanol. Reaction mixture without the extract was used as control. The mixture was shaken vigorously and allowed to stand in dark for 45min. The antioxidant scavenging activity was measured as the decrease in absorbance of DPPH at 515nm against a blank and calculated using the following formula.

Scavenging Effect (%) = [(A_0 - A_1) / A_0] X 100

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample. All the values expressed are the mean values carried out in duplicates. BHA was used as a positive control.

Superoxide anion scavenging activity:

Measurement of superoxide radical scavenging activity of the T.C extracts was done based on the method described by Liu.[20] The superoxide is generated in 3ml of tris Hcl (16mM pH8.0) containing 1ml of NBT (50µm) solution, 1ml NADH (78µM) solution and sample solution of different extracts in a concentration of 1-10µg in 1ml .The reaction was started by adding 1ml of PMS solution (10um) to the mixture. The reaction mixture was incubated at 25^oC for 5 min. and the absorbance at 560nm was measured against the blank samples. L-Ascorbic acid was used as a control. BHA was used as positive control.

% Inhibition = [(Acontrol - $A_{sample)}$ / $A_{control)}$ x100]

Where $A_{control\ is}$ the absorbance of the L-Ascorbic acid and A_{sample} the absorbance of *Tinospora cordifolia* or standards. The data at each point were the average of two measurements.

Hydroxyl radical scavenging activity:

Non-site specific hydroxyl radical mediated 2-deoxy-D-ribose degradation:

The assay was performed as described by Halliwell, [21] with slight modification. All solutions were freshly prepared. The reaction mixture contained 100µl of 28mM 2-deoxy D ribose (dissolved in KH_2PO_4- K_2HPO_4) phosphate buffer pH 7.4, 500µl solution of various concentration of the extacts (100µg-1000µg/ml), 200µl of 200µM $FeCl_3$ and 1.04mM EDTA (1:1 v/v), 100µl of H_2O_2 (1.0 mM) and 100µl ascorbic acid (1.0mM). After an incubation period of 1hr. at 37^oC the extent of deoxy ribose degradation was measured by the TBA reaction. 1ml of TBA (1% in 50mM NaOH) and 1ml of TCA were added to the reaction mixture, tubes

were heated at 100° C for 20 min. After cooling, the absorbance was read at 532 nm against a blank (containing only buffer and deoxy ribose). The % inhibition was calculated by the formula

Inhibition (%) = $[(A_0 - A_1) / A_0] \times 100$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample. All the values expressed are the mean values carried out in duplicates. BHA was used as a positive control.

Site-specific hydroxyl radical mediated 2-deoxy-D-ribose degradation:

The ability of the extracts to inhibit site specific hydroxyl radical mediated degradation was also carried out to understand its role as a metal chelator. The method was the same as described above except that buffer replaced EDTA.

Reducing Power:

The reducing power of the prepared T.C extract was determined according to method of Oyaizu.[22] Briefly various concentration of the extract (0-5mg/ml) and the standard compound were mixed with PO4 buffer (2.5ml, 0.2mol/L, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$], (2.5ml, 1%). The mixture was incubated at 50°Cfor 20min. To the mixture 10% TCA was added to the mixture, which was then centrifuged at 3000rpm for 10min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml), $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700nm.Increased absorbance of the reaction mixture indicated increased reducing power. α-tocopherol was used as a positive control.

Metal chelating activity:

The assay was determined as described by Dinis.[23] Briefly the extracts were added to a solution of 2mmol/L $FeCl_2$ (.05ml). The reaction mixture was initiated by the addition of 5mmol/L ferrozine (0.2ml), the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562nm. The % of Inhibition of ferrozine-Fe^{2+} complex was given below formula

% Inhibition = $[(A_0 - A_{1)} / A_{0]} \times 100$

Where, A0 was the absorbance of the control & A_1 was the absorbance in the presence of the samples of T.C extracts and standards. The ability of the extract to chelate ferrous ion was compared with the standard BHA.

Total phenolic contents:

Total phenolic content was estimated spectrophotometrically by Folin-Ciocalteau method [24] with some modifications. To 0.1ml extract of different concentration was made up to 3ml with double distilled water, to this 0.5ml of folin-Ciocalteau reagent was added and allowed to stand at room temperature for 10min, to the mixture 2ml of 7% sodium carbonate was added and kept in boiling water bath for 1min, cooled, after which the absorbance was read at 650nm against blank. The concentration was calculated using gallic acid as standard, and the results were expressed as milligram gallic acid equivalents per gram extract.

Flavonoids:

The determination of Flavanoids was carried out according to Delcour.[25] To 1ml of different concentration of the extract, 5ml chromogen reagent (1 g 4-dimethyl amino cinnamaldehyde dissolved in a cooled mixture of 250ml of concentrated HCl and 750ml of methanol, made upto 1L methanol) was added. After 10min, the absorbance was measured at 640nm against a blank consisting of water instead of extract and the flavanoids content was calculated with (±) Catechin and the concentration was expressed as (±) catechin equivalents.

Tannins:

A 0.5g sample was weighed and boiled with 75ml of water for 30min. The contents were centrifuged at 800 X g for 20min and the supernatant was made up to 100ml in a volumetric flask using water. Aliquots were treated with Folin-Denis reagent and absorbance was measured at 700nm.[19]

STATISTICAL ANALYSIS

The statistical processing of the data obtained from all studies is expressed as means ± standard deviation (SD) of three separate experiments using the computer programme Excel.

RESULTS AND DISCUSSION

Proximate Composition and yield of the extracts:

The Proximate Composition of *Tinospora cordifolia* is given (**Table 1**).The herb has good amount of vitamin C, E and carotenoids which contribute to the antioxidant property. The mineral analysis shows significant amount of potassium as compared to other mineral content.

The yield of sequential extracts is presented (**Table 2**). Among the extracts, maximum yield as

well as antioxidant activity was shown by the methanolic, ethanolic and aqueous extracts and therefore, they were chosen for further study.

Table 1: Proximate Composition Of *Tinospora cordifolia*

%, → %,	mg/100g, → mg/100g,	g/L → g/L
Moisture	4.99 ± .001	
Protein	12.075 ± 0.24	
Fat	2 ± .01	
Carbohydrates	36.28 ± 0.23	
Ash	10 ± 0.01	
Crude Fiber	34.65 ± 3.181981	
Vitamin C	18.4 ± 0.001	
Vitamin E	24 ± 1mg/100g	
Total Carotenoids	242.5 ± 6.363961	

Table 2: Mineral content Of *Tinospora cordifolia (mg/100g)*

Iron	*4.92 ± 0.21*
Zinc	*0.832 ± 0.12*
Sodium	*50 ± 0.06*
Potassium	*232.4 ± 0.032*

Table 3: Yield of sequential extracts of *Tinospora cordifolia*

Solvents	Extract yield (g)
Hexane	1.41
Chloroform	3.22
Ethyl acetate	1.26
Acetone	1.51
Methanol	8.96
Ethanol	7.93
Water	6.54

DPPH radicals scavenging activity:

Proton-radical scavenging action is an important attribute of antioxidants, which is measured by DPPH radical scavenging assay. DPPH, a protonated radical, has characteristic absorbance maxima at 517nm which decreases with the scavenging of the proton radical.[26] High radical scavenging activity was observed in methanol, ethanol as well as water extracts of the herb in a concentration dependent manner. Hydrogen-donating ability of the antioxidant molecule contributes to its free radical scavenging nature.[27] The methanol extract activity was more than that of than ethanol and water extracts (**Fig. 1**).

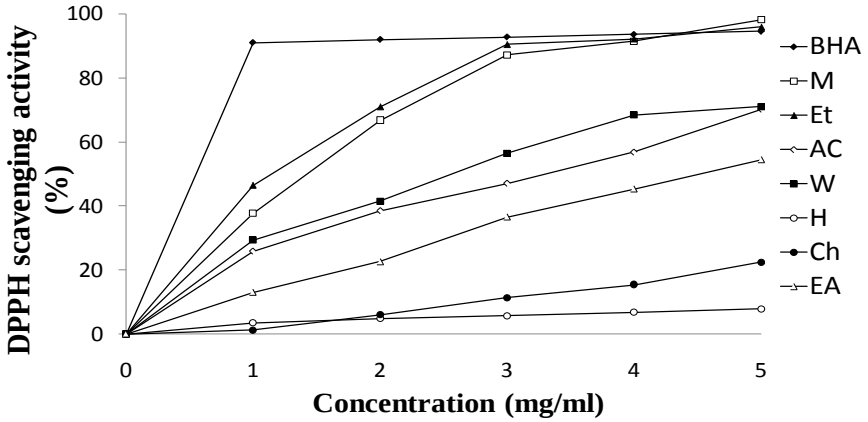

Fig. 1. Scavenging effects of Tinospora cordifolia on DPPH radicals. Each value is presented as standard error (n=3).The vertical bars indicate standard errors where they exceeded the symbol size.

Superoxide anions radical scavenging activity:

Superoxide radical scavenging activity was shown by the extracts in a concentration dependent manner. The ethanolic and methanolic extracts of the herb were markedly a more potent scavenger of superoxide anion compared to other extracts. Aqueous extract also showed superoxide radical scavenging activity, but less than that of ethanolic and methanolic extracts (**Fig. 2**). Superoxide anions play an important role in the formation of the ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA.[28] Superoxide anion derived from dissolved oxygen

by PMS-NADH (phenazine metho sulphate) coupling reaction reduces NBT (nitro blue tetrazolium) in this system. In this method, superoxide anion reduces the yellow dye to produce the blue formazan which is measured spectrophotometrically at 560nm.

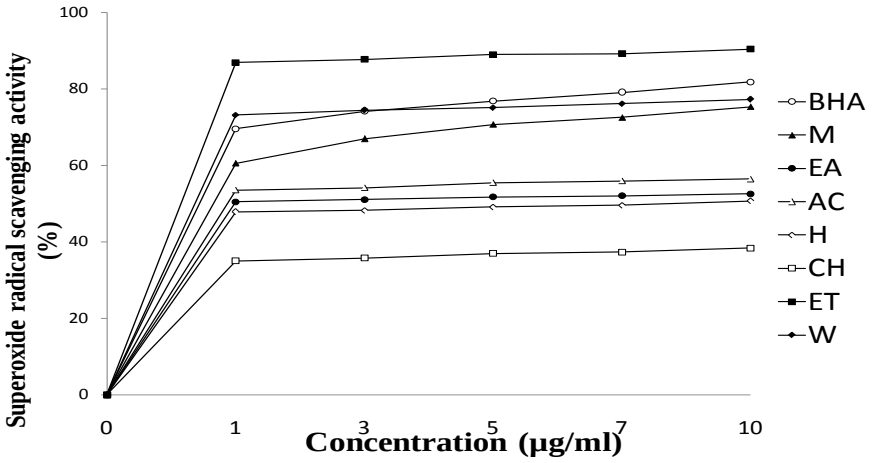

Fig. 2. Superoxide radical-scavenging activity of Tinospora cordifolia. Each value is presented as mean standard error (n=3). The vertical bars indicate standard errors where they exceeded the symbol size.

Hydroxyl radical scavenging assay:

All the extracts except that of chloroform and hexane displayed hydroxyl radical scavenging activity (Figs. 3 and 4). Among them methanolic and ethanolic extracts of the herb showed the highest activity. These extracts inhibited deoxy-ribose degradation induced by hydroxyl radical, scavenging hydroxyl radical directly (non-site specific, **Fig. 3**.) and also via chelating iron ion (site specific, **Fig. 4**). Hydroxyl radicals are produced *in vivo* by Fenton-type reactions; in which transition metals (e.g. iron) reduce hydrogen peroxide. Reducing agents such as ascorbic acid can accelerate $OH^{\cdot}$ formation by reducing Fe^{3+} ions and Fe^{2+}.[29] Here, hydroxyl radicals were generated by a mixture of Fe^{3+}-EDTA,H_2O_2 and ascorbic acid [30] and were assessed by monitoring the degraded fragments of deoxyribose, through malonaldehyde (MDA) formation.[31] Two inferences can be drawn from this experiment carried out under two conditions, that is the role on hydroxyl trapping and role of metal chelation and the conditions referred as "non-site-specific assay" where EDTA added forms a complex with Iron (III), hydroxyl radicals are generated in the solution and the other "site-specific assay" where no EDTA is added, therefore, Iron (III) can bind directly to the deoxyribose molecule and produce hydroxyl radicals at this site itself.

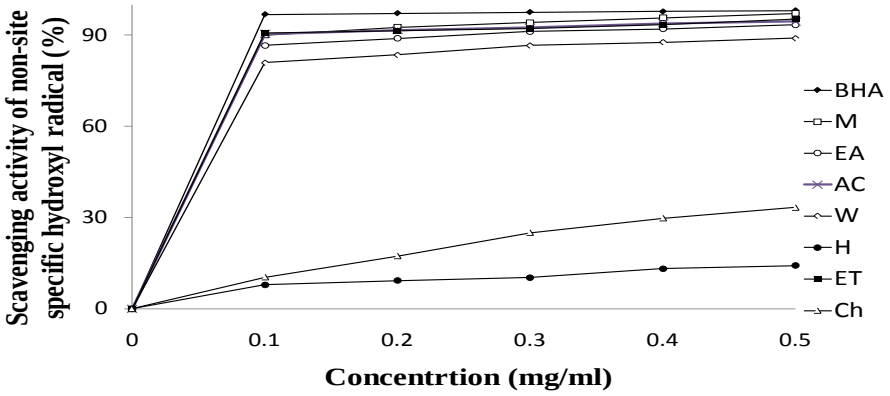

Fig. 3. Inhibitory effects of Tinospora cordifolia on deoxyribose degradation in non-site specific assay. The vertical bars indicate standard errors where they exceeded the symbol size.

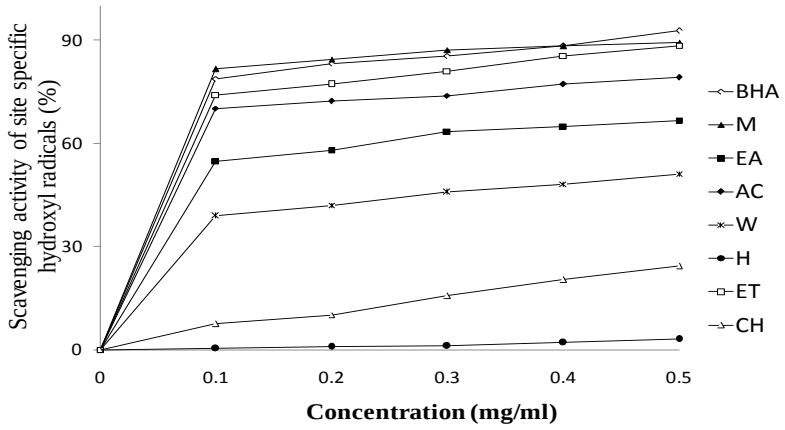

Fig. 4. Inhibitory effects of Tinospora cordifolia on deoxyribose degradation in Site specific assay. Each value is presented as mean ± standard error (n=3). *The vertical bars indicate standard errors where they exceeded the symbol size.*

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Reducing power:

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing properties are generally associated with the presence of Reductones,[32] which exert antioxidant action by breaking the free radical chains, via hydrogen atom donation.[33] Reductones are also reported to prevent peroxide formation, by reacting with certain precursors of peroxide. The reducing power of T. Cordifolia extracts was concentration dependent (**Fig. 5**). Methanolic extract was found to be more active than the ethanolic and aqueous extracts.

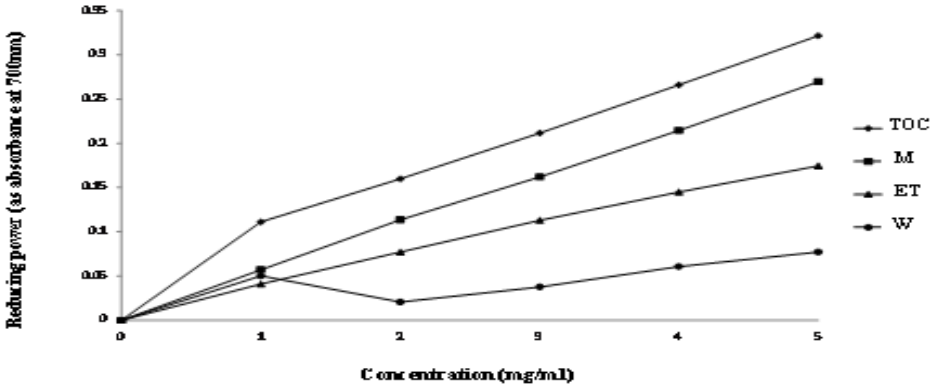

Fig. 5. Reducing power of Tinospora cordifolia. Each value is presented as mean ± standard error (n=3). ***The vertical bars indicate standard errors where they exceeded the symbol size.***

Metal ions chelating activity:

The ferrous ion-chelating effect was shown by both methanolic and ethanolic extracts significantly as compared to water extract (**Fig. 6**). The production of highly ROS such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals is also catalysed by free iron through Haber-Weiss reaction.[34] Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the fenton reaction, Fe^{3+} ion also produces radicals from peroxides although the rate is 10-fold less than that of Fe^{2+} ion.[35] Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions.[36] Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour complex. Therefore, measurement of colour reduction allows estimating the metal chelating activity of the coexisting chelator.

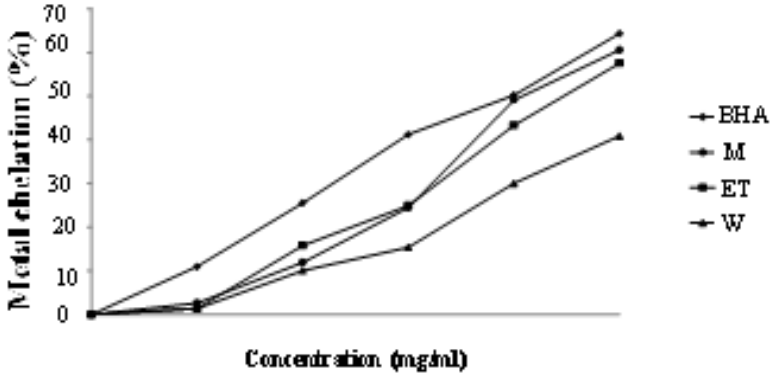

Fig. 6. Metal chelation of Tinospora cordifolia. Each value is presented as mean ± standard error (n=3).). The vertical bars indicate standard errors where they exceeded the symbol size

Polyphenols, tannins and flavonoids:

It is well-known that plant phytochemicals /phenolics, in general, are highly effective free radical scavengers and antioxidants. Hydrogen donating property of the polyphenolic compounds is responsible for the inhibition of free radical induced lipid peroxidation.[37] consequently the antioxidant activities of plant/herb extracts are often explained with respect to their total phenolics and flavonoid contents, with good correlation. The total phenolic content was found to be 8.62 mg/g, flavonoids 3.2 mg/g and tannins 24.84 mg/g (**Fig. 7**).

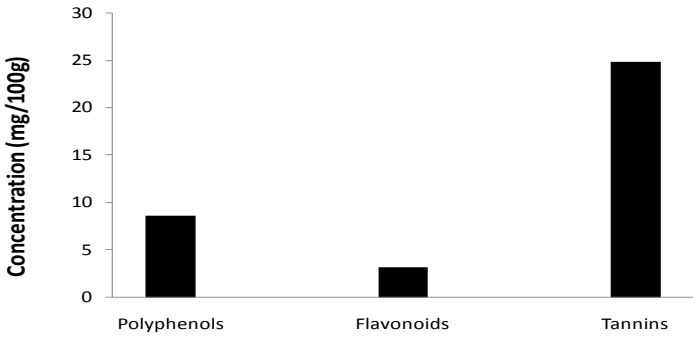

Fig. 7. **Phytochemicals of Tinospora cordifolia. Each value is presented as mean standard error (n=3). The vertical bars indicate standard errors where they exceeded the symbol size.**

Conclusion

Tinospora cordifolia exhibited excellent antioxidant activity in methanol, ethanol and water extracts. They were effective in scavenging superoxide anion radical and inhibited deoxyribose degradation induced by hydroxyl radical, scavenging them directly rather then via chelating iron ion. They significantly acted as strong electron-donating agents in the Fe^{3+} to Fe^{2+} assays and hydrogen donating agents in the DPPH assay. In addition, they possess phytochemicals such as polyphenols, flavonoids and tannins which attribute to a strong free radical scavenging activity.

For the purpose of characterization of antioxidant activity of plant extracts, it is desirable to subject it to an array of tests that evaluates the range of activities. Antioxidant rich plant extracts serve as source of nutraceuticals that alleviate the oxidative stress and therefore prevent or reduce the onset of degenerative diseases.[38, 39] The observed high antioxidant activity of the extracts indicate the potential of the stem as a source of

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natural antioxidants or nutraceuticals to reduce oxidative stress with consequent health benefits.

Acknowledgement

The authors would like to acknowledge Dr. AS Bawa, Director, Defence Food Research laboratory and Dr (Mrs.) Farhath khanum, Project Co-ordinator, Biochemsitry & Nutrition Discilpline, for support and guidance.

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