

Studies on Antioxidant Activity and Total Phenolic Content of *Tinospora cordifolia* (Miers.) Stem Using *in Vitro* Models

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ABSTRACT

In the present study, we examined the anti-oxidant effects of *Tinospora cordifolia* stem. Dried and powdered stem of *Tinospora cordifolia* was extracted with ethanol and methanol. Total phenolic content of different solvent extracts were determined, to find the correlation between phenols and antioxidant activity. Ascorbic acid was used as standard. Antioxidant assay was carried out by using DPPH (1, 1-Diphenyl-2-picrylhydrazyl) radical scavenging activity. Ethanolic stem extract showed the highest free radical scavenging activity (56.35%). The antioxidant activity of methanolic extract was poor when compared to the ethanolic extract. Ethanolic stem extract had the highest phenol content of 66.28 ± 0.82 mg/g. These results suggest that, Phytocompounds were better extracted in ethanol and there is direct correlation between the total polyphenols extracted and its antioxidant activity.

Keywords: DPPH (1, 1-Diphenyl-2-picrylhydrazyl), Antioxidant assay, *T. cordifolia*, total phenol content.

INTRODUCTION

Free radicals or reactive oxygen species (ROS) are formed in our body as a result of biological oxidation. Oxidation is a natural process in organism for the production of energy to fuel biological cycles. Conversely, the uninhibited production of oxygen derived free radicals cause damage to the body and contributes to oxidative stress^{1,2}.

Oxidation by-products of normal metabolism cause extensive damage to DNA, protein and lipids, constitute a major

contribution to ageing and also to degenerative disease. Oxidative damage of proteins, DNA and lipids is associated with chronic degenerative diseases including cancer, coronary artery disease, hypertension, diabetes etc³.

The relation between free radicals and disease can be explained by the concept of 'oxidative stress' elaborated by⁴. In a normal healthy human body the generation of pro-oxidants in the form of ROS is effectively kept in check by the various

levels of antioxidant defense. However, when it gets exposed to adverse physiochemical, environment or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiations, toxic chemicals, over nutrition, this delicately maintained balance is shifted in favor of pro-oxidants resulting in 'oxidative stress'.

The most effective path to eliminate and diminish the action of free radicals is antioxidant defense mechanism. Antioxidants are those compounds that inhibit or delay the oxidation of molecules by inhibiting the initiation or propagation of oxidizing chain reaction. Natural antioxidants constitute a broad range of compounds including phenolic compound, nitrogen compound and carotenoids⁵.

Natural antioxidants like phenolic compounds, flavonoids which are secondary plant metabolites present in food products of plant origin^{6,7} can trap free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzyme⁸ and also exhibit a wide range of biological effects, including antiageing, antimutagenicity and protective effects on oxidative stress^{9,10,11}.

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer¹². Recently, phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids^{13,14}.

Guduchi (*Tinospora cordifolia* (Wild) Miers ex Hood. F and Thoms) is a large, glabrous, deciduous climbing shrub

belonging to the family Menispermaceae¹⁵. It is distributed throughout tropical Indian subcontinent and China, ascending to an altitude of 300m. In Hindi, the plant is commonly known as Giloe, which is a Hindu mythological term that refers to the heavenly elixir that have saved celestial beings from old age and kept them eternally young.

Guduchi is widely used in veterinary folk medicine/ayurvedic system of medicine for its general tonic, antiperiodic, antispasmodic, anti-inflammatory, antiarthritic, antiallergic and anti-diabetic properties^{15,16}. Earlier studies reported the antiosteoporetic¹⁷, hepatoprotective¹⁸, immunomodulatory¹⁹, antihyperglycaemic²⁰, anti-tumor²¹, anti-HIV^{22,23} properties of *Tinospora cordifolia*.

The stem of *Tinospora cordifolia* is one of the constituents of several ayurvedic preparations used in general debility, dyspepsia, fever and urinary diseases. The stem is bitter, stomachic, diuretic²⁴ stimulates bile secretion, prevents from constipation, burning sensation, vomiting, enriches blood and cures jaundice.

Since scanty work has been done on antioxidant activity of *T. cordifolia* stem, therefore, present study was undertaken to determine its antioxidant activity and total phenolic content in different solvent extracts using standard methods. The finding from this work may add to the overall value of the medicinal potential of the shrub.

MATERIALS AND METHODS

Plant material

The mature stem of *Tinospora cordifolia* was collected from Botanical garden, Department of Botany, Khandari Campus, Dr B.R. Ambedkar University, Agra during 2011-2012. The plant material was identified by the Department of Botany, School of Life Sciences, Khandari Campus, Dr. Bhim Rao Ambedkar University, Agra.

The collected material was kept in polythene bags which were subsequently sealed to protect from dust and microbes. Collected plant material brought to the laboratory and stored in a refrigerator. The stored material was thoroughly washed with tap water followed by sterilized distilled water. After cleaning the stem was dried in shade and grinded into powdered form for further analysis.

Extraction

Organic extract

Organic extract was prepared by soxhlet extraction method²⁵. A thimble was prepared by using 0.5 mm Whatmann No. 1 filter paper. About 100 gm of powdered material was uniformly packed into a thimble and run in soxhlet extractor. It was exhaustible extracted with solvents (ethanol and methanol) for the period of about 48 hour or 22 cycles or till the solvent in the siphon tube of extractor become colourless. After that extracts were filtered with the help of filter paper (Whatmann No. 1) and solvent was evaporated in rotary evaporator to get the syrupy consistency, then after the extract was kept in refrigerator at 4°C to determine antioxidant activity.

Antioxidant assay

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) free radical scavenging assay

The scavenging activity of *Tinospora cordifolia* stem extracts was determined using DPPH assay²⁶.

This method depends on the reduction of purple DPPH to a yellow colored diphenyl picrylhydrazine. The determination of the disappearance of free radicals was done using spectrophotometer. The remaining DPPH which showed maximum absorption at 518nm was measured. Each plant extract sample's stock solution (10 mg/ml) was

diluted to final concentrations of (9, 8, 7, 6, 5, 4, 3, 2 and 1 mg/ml) in DMSO (Dimethyl sulphoxide). 1 ml of a 0.3mM DPPH DMSO solution was added to 2.5 ml of sample solution of different concentrations. These were test solutions. Ascorbic acid was used as positive control and prepared in same manner as above. As DPPH is sensitive to light, it is exposed to the minimum possible light. These solutions were allowed to react at room temperature for 30 minutes. The absorbance values were measured at 518 nm and converted into the percentage antioxidant activity using the following equation:

$$\begin{aligned} & \text{Free radical scavenging activity (\%)} \\ &= \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100 \end{aligned}$$

Where, (Abs = Absorbance)

The test was done in triplicate.

Quantitative phytochemical analysis

Determination of total phenolic content

Total phenolic content (TPC) in the extracts was determined using the Folin-Ciocalteu reagent method²⁷. This method depends on the reduction of FCR by phenols to a mixture of blue oxides which have a maximal absorption in the region of 765 nm using spectrophotometer. Stock solution of stem extracts were prepared to the concentration of 10 mg/ml (50 mg of powdered extract dissolved in 5 ml of ethanol). To 1.0 ml of each extract, 5 ml of Folin – Ciocalteu reagent was added. The solution was vortexed and incubated in dark for 3 minutes. After that 5 ml of sodium carbonate (75 g/l) solution was added to the mixture and mixed thoroughly. The mixture was incubated in the dark for 1 hour. The absorbance was read at 765 nm. Blank consisted of 5 ml Folin – Ciocalteu reagent, 1 ml ethanol and 4 ml sodium carbonate solution. Based on the measured absorbance,

the concentration of phenolics was read (mg/ml) from the calibration line; then total content of phenolic compounds were expressed as tannic acid equivalents (TAE), calculated by the following formula:

$$C = c \times V/m$$

Where, C-total content of phenolic compounds (mg of TA/g of extract)

c - the concentration of tannic acid established from the calibration curve (mg/ml)

V - the volume of extract (ml)

m - the weight of pure plant extract (g)

Statistical analysis

All the assay was performed in triplicates and the absorbance was presented as mean \pm SE. Data were analyzed using the Statistical Analysis System software package. Analysis of variance was performed using ANOVA procedures. Significant differences between means of absorbance were determined using Fisher's least significant difference (LSD) test. The level of significance was used for comparison at 0.01 and 0.05 levels. Student's *t* - test was used for testing significance level between absorbance with ethanol and methanol solvent; P values $>$ 0.05 were considered to be significant.

RESULTS

Antioxidant activity

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) free radical scavenging activity of stem extract

All the concentration of the test solution more or less scavenged the free radicals. Both the stem extracts showed effective scavenging activity against free radicals (Table 1 & 3 and Fig. 1 & 2). In both the extracts tested, ethanolic extract of the stem of *T. cordifolia* displayed an excellent activity against the free radicals. The ethanolic stem extract showed the highest

scavenging activity (56.35%) at 10 mg/ml and lowest (20.08%) at 1 mg/ml (Table 1). The ethanolic stem extract showed the highest absorbance value (0.532 ± 0.0053) at 10mg/ml and lowest (0.187 ± 0.0087) at 1mg/ml (Table 2). It was found that generally when concentration of extract was decreased the absorbance values also get decreased regularly.

The methanolic stem extract of *T. cordifolia* showed comparatively lesser activity with highest scavenging activity (44.92%) at 10mg/ml and lowest (18.09%) at 1mg/ml compared to the ethanolic extract (Table 3). The methanolic stem extract demonstrated the highest absorbance value (0.324 ± 0.0054) at 10mg/ml and lowest (0.172 ± 0.0043) at 1mg/ml and in similar manners, the absorbance value decreased with the decrease in the concentration of the extract (Table 4). The results were found to be statistically significant, at both 0.01 and 0.05 level of significance. In both the test extracts of *T. cordifolia*, ethanol stem extract showed the highest scavenging activity (Fig. 3).

Total phenol content

The total phenol in the extracts was determined with the Folin-Ciocalteu reagent method. Tannic acid was used as a standard compound and the total phenols were expressed as mg/g tannic acid equivalent using the standard curve equation: $y = 0.0909x + 0.0139$, $R^2 = 0.9882$ (Fig. 4).

Total phenolic content of different extracts of stem of *T. cordifolia* is presented in Table 5. It is clear that concentration of polyphenols in the ethanolic stem extract of *T. cordifolia* was higher when compared to methanol extract of stem. This assay supported that there is a positive relation between phenols and antioxidant activity. Phenols increased the antioxidant properties of the test plant. The ethanol stem extract having highest phenol content (66.28 ± 0.82 mg/g) also had the better scavenging or

antioxidant activity when compared to methanolic stem extract.

DISCUSSION

The stable radical DPPH has been used widely for the determination of primary antioxidant activity. The DPPH anti-oxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of anti-oxidant²⁸.

In the present study it was observed that ethanol stem extract possessed stronger free radical scavenging activity (56%). The similar results were also observed by²⁹ who reported that ethanolic extract of stem of *T. cordifolia* growing with the support of *Azadirachta indica* (Neem) showed the highest DPPH free radical scavenging activity (86.36%).

Present study revealed that ethanol was the better extractive solvent for antioxidant activities. The present result coincides with view of³⁰ that ethanol extract of HC9 (A herbal composition comprised of nine different plant materials viz. *Picrorhiza kurroa*, *Cyperus rotundus*, *Zingiber officinale*, *Cedrus deodara*, *Tinospora cordifolia*, *Holarrhena antidysenterica*, *Swertia chirata*, *Cyclea peltata* and *Hemidesmus indicus*) exhibited significant DPPH radical scavenging activity with IC₅₀ value of 172.89 µg/ml as compared to the aqueous extract (HC9 aq.).

These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components. The same relationship was also observed between phenolics and antioxidant activity in roship extracts³¹.

Polyphenols are the major plant compounds with anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids³². It is reported that the phenolics are responsible for the variation in the anti-oxidant activity of the plant³³. They exhibit anti-oxidant activity by

inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals^{34,35}.

In the present study estimation of total phenolic content of *T. cordifolia* revealed that ethanol stem extract had highest phenol content. It suggests that phenol content is responsible for the highest DPPH radical scavenging activity of ethanol stem extract in the present study. Similar results were also reported by³⁶ who observed high correlation between phenolic composition and antioxidant activities of extracts of some medicinal plants viz. *Azadirachta indica*, *Hemidesmus indicus*, *Manilkara zapota*, *Psorelea corylifolia*, *Rubia cordifolia* and *Tinospora cordifolia*.

A dose dependent radical scavenging was observed with all the extracts investigated i.e., when the concentration of the extract was decreased, the free radical scavenging activity was also decreased. These results are in agreement with the reports of²⁹.

CONCLUSION

We have demonstrated that ethanolic stem has better antioxidant activity and contain a noticeable amount of total phenols when compare to methanolic stem. Accordingly in this study, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity.

The result of the present study suggests that *T. cordifolia* can be used as a source of antioxidant for Pharmacological preparation which is very well evidenced by the present work.

ACKNOWLEDGEMENT

Financial assistance received from the Government of Uttar Pradesh (Higher Education Department) in the form of Centre for Excellence to the department of Botany, is

gratefully acknowledged.

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Table 1. Percentage scavenging activity of ethanol stem extract of *Tinospora cordifolia*

Plant part used	Solvent	Concentration (mg/ml)	DPPH Scavenging activity (%)
Stem	Ethanol	10	56.35
		9	53.16
		8	47.67
		7	43.89
		6	39.69
		5	35.07
		4	31.55
		3	29.65
		2	23.41
		1	20.08

Table 2. Antioxidant activity profile of ethanol stem extract of *Tinospora cordifolia*

Plant part used	Solvent	Concentration (mg/ml)	Absorbance at 518 nm
Stem	Ethanol	10	0.532 ± 0.0053
		9	0.489 ± 0.0101
		8	0.394 ± 0.0018
		7	0.386 ± 0.0006
		6	0.368 ± 0.0017
		5	0.348 ± 0.0017
		4	0.295 ± 0.0020
		3	0.268 ± 0.0058
		2	0.242 ± 0.0023
		1	0.187 ± 0.0087

± Standard error

Table 3. Percentage scavenging activity of methanol stem extract of *Tinospora cordifolia*

Plant part used	Solvent	Concentration (mg/ml)	DPPH Scavenging activity (%)
Stem	Methanol	10	44.92
		9	43.12
		8	39.31
		7	35.67
		6	32.42
		5	29.30
		4	27.02
		3	23.54
		2	20.14
		1	18.09

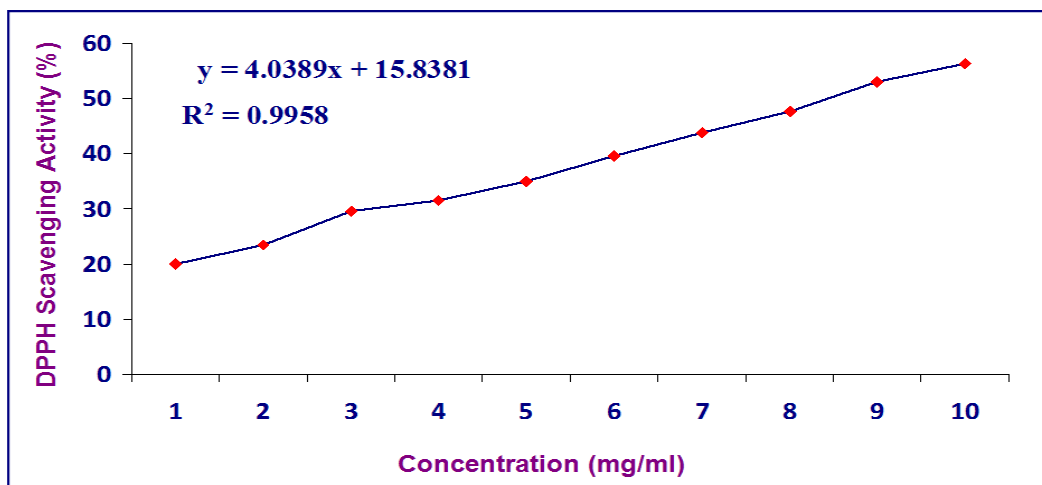
Table 4. Antioxidant activity profile of methanol stem extract of *Tinospora cordifolia*

Plant part used	Solvent	Concentration (mg/ml)	Absorbance at 518 nm
Stem	Methanol	10	0.324 ± 0.0054
		9	0.311 ± 0.0031
		8	0.307 ± 0.0033
		7	0.232 ± 0.0083
		6	0.226 ± 0.0079
		5	0.225 ± 0.0038
		4	0.218 ± 0.0055
		3	0.204 ± 0.0081
		2	0.176 ± 0.0029
		1	0.172 ± 0.0043

Table 5. Phenolic content of different extracts of *Tinospora cordifolia*

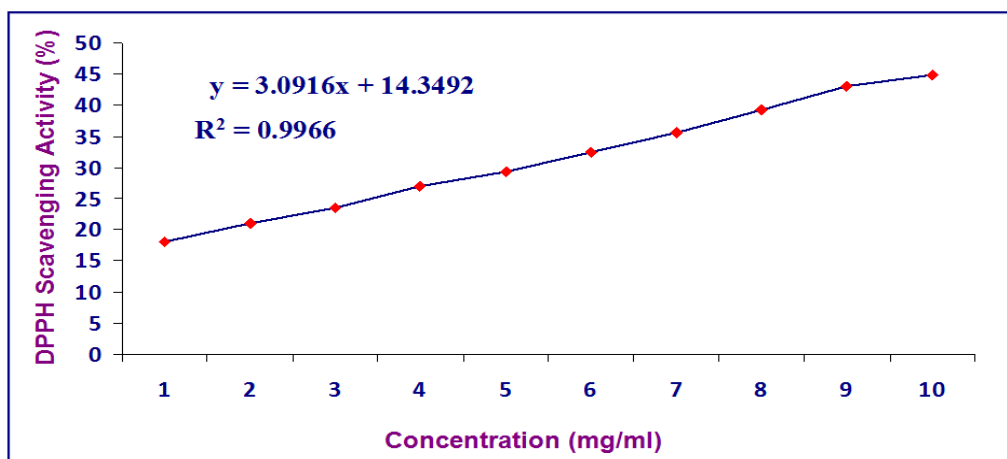
Plant part used	Solvent	Total phenolic content (mg TA/g of extract)
Stem	Ethanol	66.28 ± 0.82
	Methanol	51.86 ± 0.77

TA: Tannic Acid



Linear correlation between the concentration of solution and antioxidant activity of ethanol stem.
Correlation coefficient $r = 0.9979$, coefficient of determination (R^2) = 0.9958.
Correlation is significant at the 0.01 level.

Figure 1. DPPH radical scavenging activity of ethanol stem extract of *Tinospora cordifolia*



Linear correlation between the concentration of solution and antioxidant activity of methanol stem.
Correlation coefficient $r = 0.9983$, coefficient of determination (R^2) = 0.9966.
Correlation is significant at the 0.01 level.

Figure 2. DPPH radical scavenging activity of methanol stem extract of *Tinospora cordifolia*

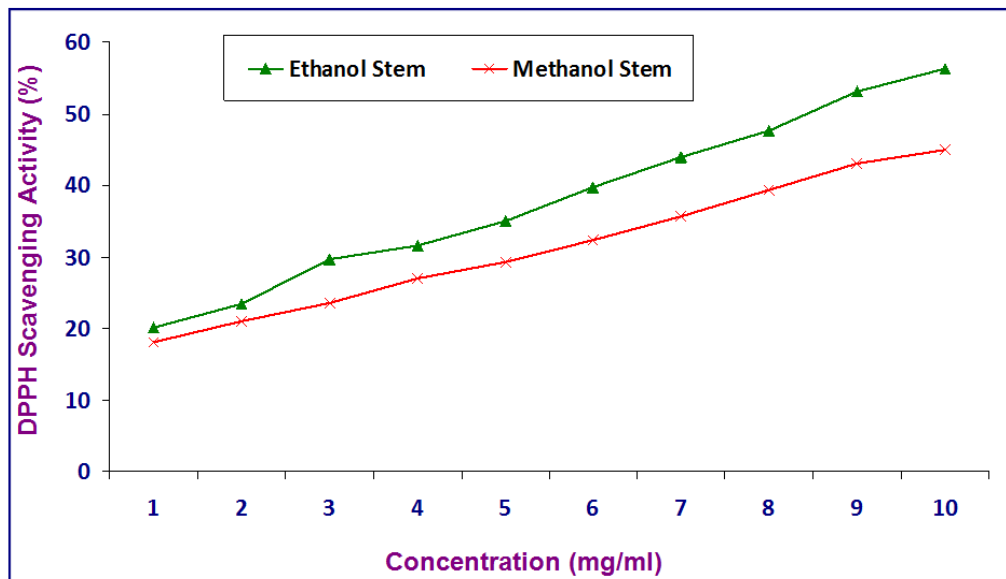


Figure 3. Comparative DPPH radical scavenging activity of different extracts of *Tinospora cordifolia*

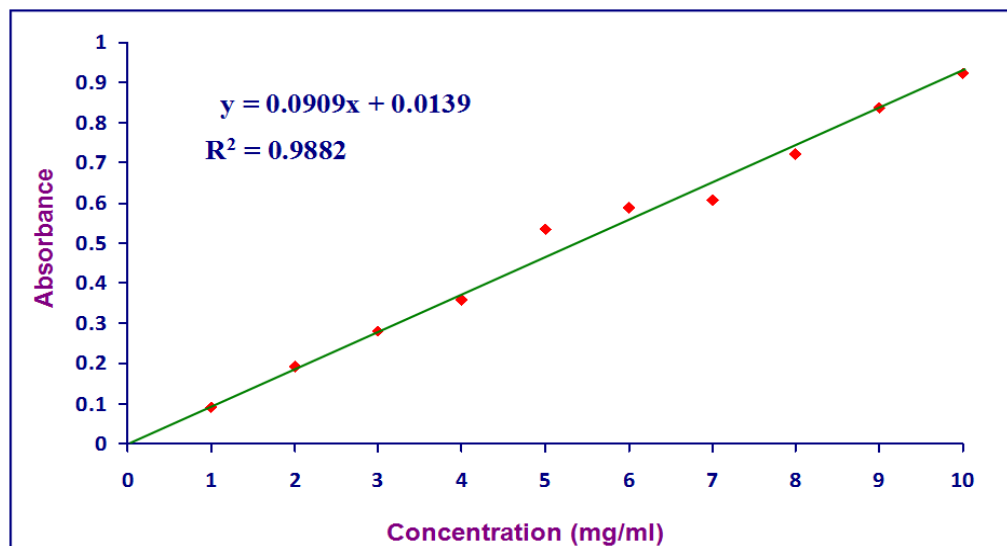


Figure 4. Standard curve of Tannic acid