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Free Radical Scavenging Activity of *Tinospora cordifolia* (Willd.) Miers

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Abstract
Objective: To explore the free radical scavenging activity and total phenolic content of *Tinospora cordifolia* bark extracts and their relation. *Methods:* Extraction of *T. cordifolia* bark was carried out in ethanol and methanol solvents. Free radical scavenging activity was performed by DPPH assay. Total phenolic content was determined by Folin-Ciocalteu reagent. The correlation was observed between antioxidant activity and total phenolic content of the extracts. *Results:* In the free radical scavenging assay, the ethanolic bark extract showed the highest free radical scavenging activity (71.49%). The antioxidant activity of methanolic extract was poor when compared to the ethanolic extract. Ethanolic bark extract had the highest phenolic content of 84.62 ± 0.12 mg/g. *Conclusion:* These results suggest that bark has better antioxidant activity and also better extracted with ethanol and the antioxidant activity (r = 0.99) was positively correlated with the total phenolic content (r = 0.98). These results suggest that ethanolic extract of *T. cordifolia* had potent antioxidant activity and could be explored as a novel natural antioxidant.

Keywords: Antioxidant, Free radicals, Polyphenols, *T. cordifolia*, Total phenolic content.

1. Introduction
Oxidative stress is among the major causative factor for producing free radicals and reactive oxygen species (ROS). ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O_2^-) and hydroxyl radicals (OH^-), as well as non-free radicals (H_2O_2) and singlet oxygen [1]. ROS are formed under normal physiological conditions, but becomes deleterious when not being eliminated by the endogenous system [2] and are known to be the major cause of various chronic and degenerative diseases. Free radicals are derived from two sources: endogenous sources, e.g., nutrient metabolism, ageing process etc. and exogenous sources, e.g., tobacco smoke, ionizing radiations, air pollution, organic solvents, pesticides etc. [3].

ROS are the major sources of primary catalysts that initiate oxidation in vivo and in vitro and create oxidative stress which results in numerous diseases and disorder [4] such as cancer, cardiovascular disease, neural disorder, Alzheimer's disease, mild congnitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative colitis, ageing and atherosclerosis. Oxidative damage of proteins, DNA and lipids are associated with chronic degenerative diseases, including cancer, coronary artery disease, hypertension, diabetes etc. [5].
Recently, phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids [6].

Guduchi (*Tinospora cordifolia* (Wild) Miers ex Hood. F and Thoms) is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae [7]. It is distributed throughout the 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 [7]. The roots of this plant are known for its anti-stress, anti-leprotic and anti-malarial activities [8]. Earlier studies reported the antiosteoporetic [9], hepatoprotective [10], immunomodulatory [11], antihyperglycaemic, anti-tumor [12], anti-HIV [13] properties of *Tinospora cordifolia*.

Keeping in view the above importance of the plant, present study was undertaken to determine the antioxidant activity and total phenolic content of *Tinospora cordifolia* bark 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, since most of the studies have focused on antioxidant activity of *Tinospora cordifolia*.

2. Materials and methods

2.1 Plant material

The mature bark of *Tinospora cordifolia* was collected from Khandari Campus, Agra region during 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. The collected plant material was 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 bark was dried in shade and grinded into powdered form for further analysis.

2.2 Extraction

Organic extract was prepared by soxhlet extraction method [14]. A thimble was prepared by using 0.5 mm Whatman 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 ethanol and methanol (Merck) for the period of about 48 hours 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 (Whatman No. 1) and solvent was evaporated in a rotary evaporator to get the syrupy consistency, then after the extract was kept in refrigerator at 4 ºC to determine antioxidant activity.

2.3. Antioxidant assay

2.3.1. DPPH free radical scavenging assay

The scavenging activity of *Tinospora cordifolia* bark extracts was determined using DPPH assay with some minor modifications [15].

This method depends on the reduction of purple DPPH (Sigma-Aldrich) to a yellow colored diphenyl picrylhydrazine. The determination of the disappearance of free radicals was done using a 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 (Merck) (Dimethyl sulfoxide). One ml of a 0.3 mM DPPH DMSO solution was added to 2.5 ml of sample solution of different concentrations. These are test solutions. Ascorbic acid (HiMedia) was used as positive control and prepared in the 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:

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

Where, (Abs = Absorbance)
The test was done in triplicate.

2.4. Quantitative phytochemical analysis

2.4.1 Determination of total phenolic content

Total phenolic content (TPC) in the extracts was determined using the Folin-Ciocalteu reagent method [16]. 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 a spectrophotometer (Systronics). A stock solution of bark extracts was 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 (Sigma-Aldrich) was added. The solution was vortexed and incubated in the 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 the 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)

2.5. Statistical analysis

All the assay were performed in triplicates and repeated three times at different time points and the absorbance was presented as mean±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 ethanolic and methanolic extracts.

3. Results

3.1. Antioxidant activity

3.1.1. DPPH free radical scavenging activity of bark extract

All the concentration of the test solution more or less scavenged the free radicals. Both the bark extracts showed effective scavenging activity against free radicals (Table 1 & 3 and Fig. 1 & 2). In both the extracts tested, the ethanolic extract of the bark of *T. cordifolia* displayed an excellent activity against the free radicals. The ethanolic extract showed the highest scavenging activity (71.49%) at 10 mg/ml and lowest (44.04%) at 1 mg/ml (Table 1). The ethanolic bark extract showed the highest absorbance value (0.987 ± 0.0053) at 10 mg/ml and lowest (0.516 ± 0.0053) at 1 mg/ml (Table 2). It was found that generally when concentration of extract was decreased the absorbance values also get decreased regularly.

The methanolic bark extract of *T. cordifolia* showed comparatively lesser activity with highest scavenging activity (62.14%) at 10 mg/ml and lowest (21.81%) at 1 mg/ml compared to the ethanolic extract (Table 3). The methanolic bark extract demonstrated the

highest absorbance value (0.612 ± 0.0025) at 10 mg/ml and lowest (0.385 ± 0.0006) at 1 mg/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. Among all the test extracts of *T. cordifolia*, ethanol bark extract showed the highest scavenging activity (Fig. 3).

3.2. Total phenolic content

The total phenols in the extracts was determined by 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 bark of *T. cordifolia* is presented in Table 5. It is clear that concentration of polyphenols in the ethanolic bark extract of *T. cordifolia* was higher when compared to methanol extract of bark. This assay supported that there is a relation between phenols and antioxidant activity. Phenols increased the antioxidant properties of the test plant. The ethanol bark extract having highest phenol content (84.62 ± 0.12 mg/g) also had the better scavenging or antioxidant activity when compared to methanolic extract.

4. Discussion

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [17], with an absorption maximum band around 515–528 nm and thus, it is a useful reagent for evaluation of the antioxidant activity of compounds [18]. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants. The DPPH anti-oxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of anti-oxidant [19].

In the present study it was observed that the ethanolic bark extract possessed stronger free radical scavenging activity (71%). The similar results were also observed by Bhalerao *et al.* [20] 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%). Gowri *et al.* [21] also reported that the leaves and bark of *Acacia nilotica* found to be registered highest reducing activity. Khanam *et al.* [22] have recently studied that alcoholic stem extract of *Tinospora cordifolia* has curative action against cisplatin induced nephrotoxicity. The nephrotoxicity is responsible for oxidative stress and the generation of free radicals.

The present study revealed that ethanol was the better extractive solvent for antioxidant activity. The present result coincides with the view of Premanath and Lakshmidevi [23] that ethanolic leaf extract of *T. cordifolia* showed the highest antioxidant activity. The EC_{50} value of ethanol extract for lipid peroxidation inhibitory activity and DPPH radical scavenging activities were found to be 0.1 and 0.5 mg/ml, respectively. The anti-oxidant activities of other solvent extracts were poor when compared to the ethanol extract. These results suggest that, the active antioxidant compounds are best extracted in ethanol.

The 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 roseship extracts [24]. Phenols are very important plant constituents because of their scavenging ability owing to their hydroxyl groups [25]. The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron donating agents, and metal ion chelating properties [6]. It is well-known that 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 [26]. Polyphenols are the major plant compounds with anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids [27]. They exhibit anti-oxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals [28].

Table 1: Percentage scavenging activity of ethanolic bark extract of *Tinospora cordifolia*.

Plant part used	Solvent	Concentration (mg/ml)	DPPH Scavenging activity (%)
Bark	Ethanol	10	71.49
		9	70.04
		8	66.77
		7	63.32
		6	59.66
		5	55.24
		4	51.08
		3	46.95
		2	45.35
		1	44.04

In the present study estimation of total phenolic content of *T. cordifolia* revealed that the ethanol bark extract had better phenol content. It suggests that phenol content is responsible for the highest DPPH radical scavenging activity of ethanol bark extract in the present study. Similar results were also reported by Kaneria *et al.* [2] who observed high correlation between phenolic composition and antioxidant activities of extracts of some medicinal plants (*Azadirachta indica, Hemidesmus indicus, Manilkara zapota, Psorelea corylifolia, Rubia cordifolia* and *Tinospora cordifolia*).

The extraction of the phytocompounds largely depends on the

solvent time and temperature of extraction as well as the chemical nature of the sample under the same time and temperature conditions, the solvent used and the chemical property of sample is the most important for extraction of compound as factors reported [29].

Since the plant under study had shown potential antioxidant activity, therefore, the findings of the present study support the fact that *T. cordifolia* is a promising source of antioxidant agent and suggest its further advance investigation. The obtained results provide a support for the use of this plant in traditional medicine, as an alternative source for the treatment of degenerative diseases caused by free radicals.

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 of Bhalerao *et al*. [20].

Table 2: Antioxidant profile of ethanolic bark extract of *Tinospora cordifolia*.

Plant part used	Solvent	Concentration (mg/ml)	Absorbance at 518 nm
Bark	Ethanol	10	0.987 ± 0.0053
		9	0.922 ± 0.0040
		8	0.917 ± 0.0035
		7	0.914 ± 0.0092
		6	0.865 ± 0.0098
		5	0.608 ± 0.0062
		4	0.567 ± 0.0095
		3	0.565 ± 0.0014
		2	0.555 ± 0.0070
		1	0.516 ± 0.0053

± Standard error

Table 3: Percentage scavenging activity of methanolic bark extract of *Tinospora cordifolia*.

Plant part used	Solvent	Concentration (mg/ml)	DPPH Scavenging activity (%)
Bark	Methanol	10	62.14
		9	62.02
		8	57.82
		7	51.96
		6	46.94
		5	41.86
		4	36.99
		3	32.00
		2	26.97
		1	21.81

Table 4: Antioxidant profile of methanolic bark extract of *Tinospora cordifolia*.

Plant part used	Solvent	Concentration (mg/ml)	Absorbance at 518 nm
Bark	Methanol	10	0.612 ± 0.0025
		9	0.583 ± 0.0034
		8	0.502 ± 0.0023
		7	0.476 ± 0.0002
		6	0.455 ± 0.0002
		5	0.431 ± 0.0002
		4	0.430 ± 0.0023
		3	0.427 ± 0.0011
		2	0.418 ± 0.0002
		1	0.385 ± 0.0006

± Standard error

Table 5: Total phenolic content of different extracts of *Tinospora cordifolia* bark

Plant part used	Solvent	Total phenolic content (mg T.A/g of extract)
Bark	Ethanol	84.62 ± 0.12
	Methanol	73.46 ± 0.31

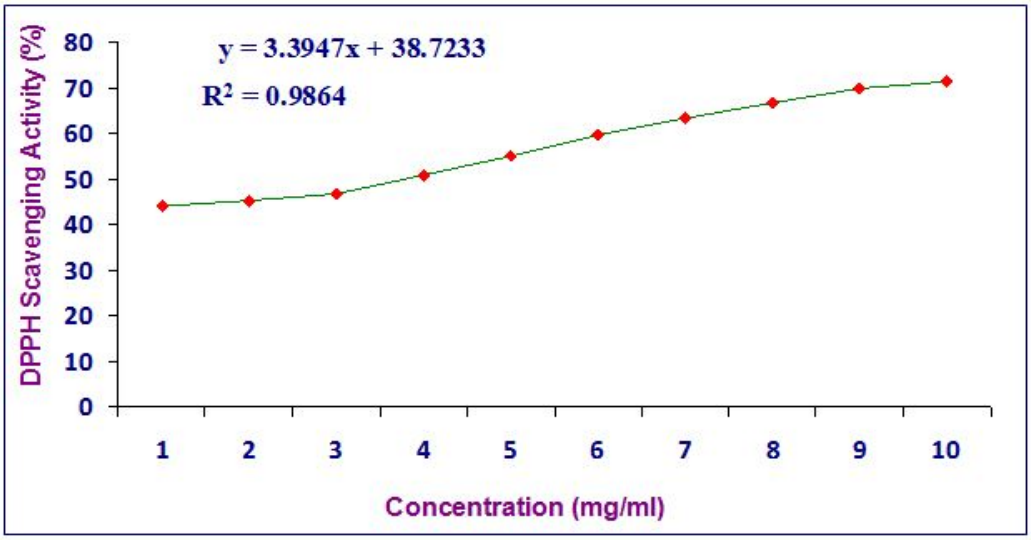

Fig 1: DPPH radical scavenging activity of ethanolic bark extract of *T. cordifolia*
Linear correlation between the concentration of solution and antioxidant activity of ethanolic bark.
Correlation coefficient r = 0.9932, coefficient of determination (R^2) = 0.9864. Correlation is significant at the 0.01 level.

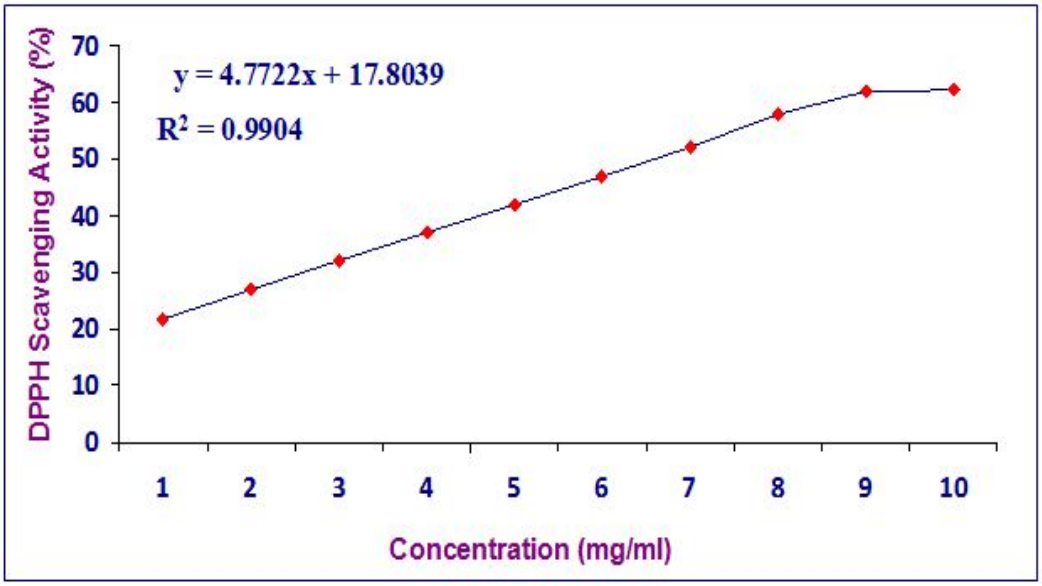

Fig 2: DPPH radical scavenging activity of methanolic bark extract of *T. cordifolia*
Linear correlation between the concentration of solution and antioxidant activity of methanolic bark.
Correlation coefficient r = 0.9952, coefficient of determination (R^2) = 0.9904. Correlation is significant at the 0.01 level.

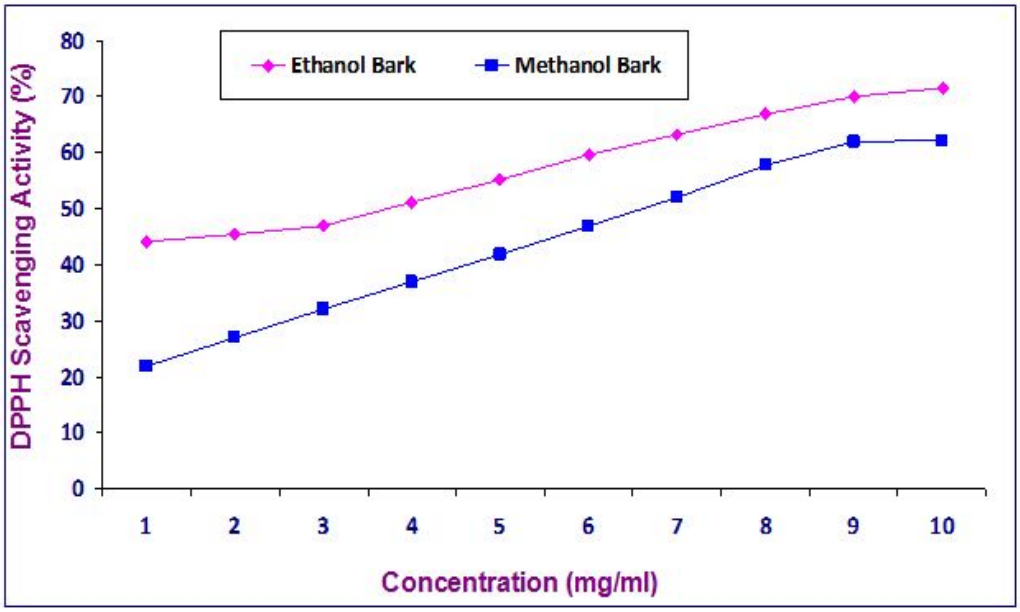

Fig 3: Comparative DPPH radical scavenging activity of different extracts of *T. cordifolia*

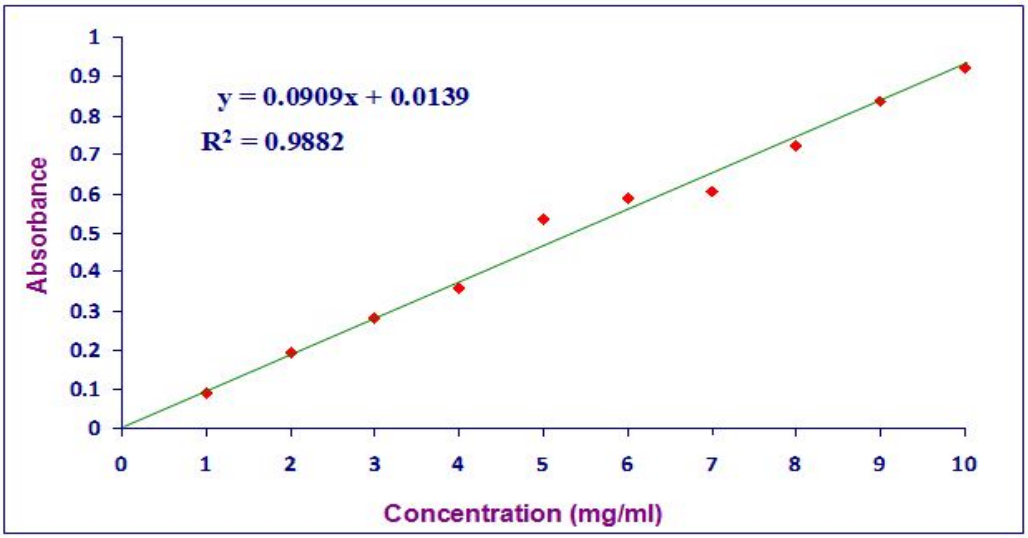

Fig 4: Standard curve of Tannic acid

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