Enzymatic and Non-enzymatic bioassays for the Comparative analysis of free radical scavenging activity of various extracts of *Tinospora cordifolia* (Willd.) Hook. f. and Thoms.] and their Protective Effect on Oxidation of Biomolecules.

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Abstract

Tinospora cordifolia (Willd) is a medicinal plant, commonly known as Guduchi or Giloy. It has multifaceted implications as an immunomodulatory, anti-inflammatory and antioxidant drug. The present study was carried out for the Comparative analysis of free radical scavenging activity of various extracts of Tinospora cordifolia stem and their Protective Effect on Oxidation of biomolecules. In the present study, the antioxidant potential of different solvent extracts of stem were evaluated by both enzymatic and non-enzymatic methods. The stem of Tinospora cordifolia was extracted with ethanol and distilled water through both hot and cold extraction methods. The total phenolic and flavonoid content of the extracts were determined by Folin-Coicalteu method and aluminium chloride colorimetric method respectively. The In-vitro bioassays were done to evaluate the antioxidant activity through ABTS radical scavenging assay, Reducing ability (FRAP) assay, Lipid peroxidation assay (LPO) and Peroxidase assay. The phenolic contents of the extracts as gallic acid equivalents were found to be highest in ethanolic extract of test drug. In ATBS assay and FRAP assay, the free radical scavenging activity was found to be highest with ethanolic extract followed by aqueous extract of test drug. Protective effect on biomolecule oxidation can be inferred by Peroxidase assay and Lipid peroxidation assay which is also found to be highest with ethanolic extract. The data obtained from this study clearly establish the higher antioxidant potency of the ethanolic extract of T. cordifolia stem compared to its aqueous extract.

Keywords: Antioxidant activity, Free radical scavenging, Tinospora cordifolia, Guduchi, ABTS assay.

Introduction

Tinospora cordifolia (Wild.) Miers ex Hook. F. and Thomas (Tc), commonly known as Guduchi or Giloy, is indigenous to the tropical areas of India, Myanmar and Sri Lanka. It is a large glabrous, succulent, climbing shrub belonging to the family of Menispermaceae. Leaf, stem and roots of this plant have been shown to possess various therapeutic actions. Guduchi is widely used in Ayurvedic system of medicine for its anti-spasmodic, anti-inflammatory, anti-arthritic, anti-allergic and anti-diabetic properties[1]. The plant is used in Ayurvedic, “Rasayanas” to improve the immune system and the body resistance against infections[2].

The active adaptogenic constituents are diterpene compounds including tinosporone, tinosporic acid, cordifolisides A to E[3], the yellow alkaloid berberine[4], crude giloninand, a glucosidal bitter principle, as well as polysaccharides, including arabinogalactan polysaccharide[5]. Picrotene and bergenin were also found in the plant. The active principles of T. cordifolia were also found to possess immunomodulatory activities[6]. Recent research has demonstrated that a combination of T. cordifolia extract and turmeric extract is effective in preventing the hepatotoxicity, which is otherwise produced as a side effect of conventional pharmaceutical treatment for tuberculosis using drugs, such as isoniazid and rifampicin[7].

Increasing evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated in several degenerative diseases like cancer, asthma, diabetes[8], arthritis, and cardiovascular problems[9].Production of reactive oxidants such as superoxide,

hydroxyl radicals and hydrogen peroxide in living cells is an inevitable process of normal oxygen metabolism[10]. Mechanism responsible for the radical mediated injuries to cells and tissues mainly include lipid peroxidation, oxidative DNA damage and protein oxidation[11]. As a result, investigations on these biomolecules oxidation and their detrimental effects have been in focus for many years[12]. Therefore, antioxidants particularly from herbal sources have a great relevance in the prevention and therapeutics of such diseases for being safer and more effective in the context of their efficiency and non-toxicity than the synthetic antioxidants[13]. Many herbs and medicinal plants have been shown to have antioxidant activity[14]. Despite its long usage as testified in traditional folklore, the biological properties of various extracts of plant parts of T. cordifolia including free-radical scavenging ability and its effect on biomolecule oxidation are least explored. The present study was carried out for the Comparative analysis of free radical scavenging activity of different solvent extracts of Tinospora cordifolia stem and their Protective Effect on Oxidation of biomolecules by both enzymatic and non-enzymatic methods.

Materials and Methods

Collection and Authentication of Test drug

Tinospora cordifolia stem was collected from the Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi, U.P., India. Taxonomic identification and Authentication was done in Department of Dravyaguna, Institute of Medical Sciences, Banaras Hindu University, Varanasi, U.P., India.

Preparation of the stem extract

Tinospora cordifolia stem were washed thoroughly in tap water to remove adhering mud particles, the papery bark were removed, then rinsed in distilled water, drained, and dried in a hot air oven at 50 ± 2°C. The dried stem were crushed. The extraction was done in ethanol and distilled water. Hot extraction was done using soxhlet apparatus for 36 hours. Cold extraction was also done for 36 hours using shaking incubator. Solvents were removed in vacuum, and total four different extracts were obtained, respectively.

Determination of Total Phenolic content

Total phenolic compound contents were determined by the Folin-Ciocalteu method (McDonald *et al.*, 2001; Ebrahimzadeh *et al.*, 2008a, b; Nabavi *et al.*, 2008). The extract samples (0.5 mL, 1:10 diluted) were mixed with Folin Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 mL, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200 and 250 mg mL^{-1} solutions of gallic acid in methanol. Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass) which is a common reference compound. Total phenolic content can be calculated from the equation:

$$T = \frac{CV}{M}$$

Where:

T = Total phenolic concentration
C = Concentration of gallic acid from caliberation curve (mg mL-1)
V = Volume of extract (mL)
M = Wt. of plant extract

Determination of total flavonoid content

Total flavonoid content was determined by using aluminium chloride colorimetric method ($AlCl_3$) according to the known method (Dewanto *et al.,* 2002; Sakanaka *et al.,* 2005) with slight modifications using Quercetin as standard. One milliliter of test material was added to 10 mL volumetric flask containing 4 mL of water. To above mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1M NaOH was added and the total volume was made upto 10 mL with distilled water. Then the solutions were mixed well and absorbance was measured against blank at 510 nm. Total flavonoid content of the extracts was expressed in milligram of quercetin equivalents g^{-1} DW. Total flavonoid content can be calculated from the equation:

$$T = \frac{CV}{M}$$

Where:

T = Total flavonoid concentration
C = Concentration of gallic acid from calibration curve (mg mL-1)
V = Volume of extract (mL)
M = Wt. of plant extract

Determination of Reducing ability (FRAP assay):

The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (0.3 M acetic acid and 0.3 M sodium acetate), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM Ferric chloride ($Fecl_3.6H_2O$) solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $Fecl_3.6H_2O$. The temperature of the solution was raised to 37°C before use. Plant extracts (100 µL each of extract were allowed to react with 2900 µL of the FRAP solution for 30 min in the dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 100-1000 µM $FeSO_4$. Results are expressed in mM Fe (II) g^{-1} dry mass and compared with that of BHT, ascorbic acid, quercetin and catechin.

Determination of Peroxidase assay:

The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase when catalyzed by peroxidise at 420 nm and at 20°C. Plant sample (200 mg) was homogenized with 10 mL of phosphate buffer and refrigerated centrifuged at 10000 rpm for 20 min. The clear supernatant was taken as the enzyme extract. The activity was assayed after the method of Chance and Maehley (1955) with following modifications. The 2.4 mL of phosphate buffer, 0.3 mL pyrogallol (50 µM) and 0.2 mL of H_2O_2 (30%) were added. The

amount of purpurogallin formed was determined by taking the absorbance at 420 nm immediately after adding 0.1 mL enzyme extract. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used in calculating the enzyme activity that was expressed in terms of mM min^{-1} g^{-1} DW.

Determination of Lipid peroxidation assay (LPO):

0.5 g of dry material was homogenized with 10 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged for 5 min (15000 g, 4°C). Supernatant was collected and 1 mL of supernatant was mixed with 4 mL of 0.5% (w/v) TBA (Thiobarbituric acid) in 20% (w/v) TCA and then incubated in water bath at 95°C for 30 min. Reaction was quickly ended by incubating on an ice bath. In case the solution is not clear, centrifuge at 10000 g for 10 min and the absorbance was measured at 532 and 600 nm. OD_{600} values were subtracted from MDA-TBA complex values at 532 nm and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient εM = 155 mM^{-1} cm^{-1}. Results were presented as µmols MDA g^{-1}.

Determination of ABTS radical scavenging assay:

To determine ABTS radical scavenging assay, the method of Re *et al*. (1999) was adopted. The stock solutions included 0.002M ABTS solution and 0.07 M potassium persulphate solution. The working solution was then prepared by mixing the 25 mL of ABTS stock and 0.1 mL of potassium persulphate stock and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing ABTS solution with ethanol to obtain an absorbance of 0.706±0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 mL) at varying concentration were allowed to react with 3 mL of the ABTS solution and the absorbance was taken at 734 nm after 6 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as:

$$\text{Inhibition (\%)} = 1 - \frac{ABS_{sample}}{ABS_{control}} \times 100$$

where, $ABS_{control}$ is the absorbance of ABTS radical+ methanol, ABS_{sample} is the absorbance of ABTS radical +sample extract/standard.

Results and Discussion

No.	Sample (Tinospora cordifolia stem extract)	(FRAP assay)	(Peroxidase assay)	(Lipid peroxidation assay)	(ABTS radical scavenging activity)	Total phenol content	Total flavonoid content
1.	Hot Ethanolic extract	124.21 ±12	0.19±0.2	12.29±1.2	712±12	17.12±1.4	40.00±2.5
2.	Hot Aqueous extract	100.12 ±15	0.09±0.3	9.50±1.4	400±15	15.3±1.9	38.0±2.4
3.	Cold Aqueous extract	84.00± 10	0.04±0.2	8.40±1.0	350±10	7.5±1.4	19.3±2.1
4.	Cold Ethanolic extract	112.00 ±14	0.15±0.4	11.20±1.1	625±04	9.2±1.5	12.4±2.0

Table 1. Antioxidant activity of various extracts of Tinospora cordifolia stem.

Total phenolic and flavonoid content

The total phenolic content of the extracts from stem of T. cordifolia was determined by Folin-Coicalteu method and the results are expressed as equivalents of gallic acid (Table 1). Among the four extracts, ethanol extract (hot extraction) had the highest (17.12 mg/g) amount of phenolic compounds followed by hot aqueous extract (15.3 mg/g), cold ethanol extract (9.2 mg/g) and cold aqueous extract (7.5 mg/g). The result of the total flavonoid contents of the extracts from T. cordifolia stem is presented in Table 1. Among the four extracts, hot ethanol extract had the highest (40.0 mg quercetin/g) amount of flavonoids followed by hot aqueous extract (38.0 mg quercetin/g), cold aqueous extract (19.30 mg quercetin/g) and cold ethanol extract (12.42 mg quercetin/g). The levels of total phenolics determined in this way are in fact based on their chemical reducing capacity relative to gallic acid. It has been observed that the phenol antioxidant index is a combined measure of the quality and quantity of antioxidants in vegetables[15]. The phenolic compounds are the dominant antioxidants that exhibit scavenging efficiency on free radicals, and reactive oxygen species are numerous and widely distributed in the plant kingdom[16].

Antioxidant activities

ABTS radical scavenging activity

ABTS radical scavenging activities of stem extracts were evaluated (Table 1). Hot ethanolic extract was found to be the most effective radical scavenger of ABTS radical among all the stem extracts followed by cold ethanolic extract, hot aqueous extract and cold aqueous extract. Since phenolic compounds have been studied extensively earlier as important contributors to the antioxidant properties[17] ,the highest radical scavenging ability of the potent extracts can be directly correlated to the presence of high content of polyphenols as well as tannins. The hydrogen donating ability of these compounds is responsible for their effective antioxidant property and used for protecting against cellular oxidative damage[18].

Ferric reducing antioxidant power (FRAP)

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity[19]. Higher absorbance indicates higher reducing/antioxidant power of the plant samples[20]. Table 1 clearly shows that hot ethanolic extract is the most potent reducing agent among stem extracts (124.21 mM Fe(II) g^{-1}) followed by aqueous extract (hot extraction), ethanolic extract (cold extraction), and aqueous extract (cold extraction) The antioxidant activity of typical antioxidants has been attributed by various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activity[21].The data on the ferric reducing power for the extracts suggest that it contributes significantly toward the observed antioxidant effect and Protective effect on biomolecule oxidation.

Peroxidase assay

The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase when catalyzed by peroxidise at 420 nm and at 20°C. Table 1 clearly shows that ethanolic extract (hot extraction) shows highest oxidation among stem extracts (0.19 mM min^{-1} $g^{-1)}$ followed by ethanolic extract (cold extraction), aqueous extract (hot extraction) and aqueous extract (cold extraction) respectively.

Lipid peroxidation assay (LPO)

Lipid peroxidation assay (LPO) of stem extracts were evaluated and the results are depicted in Table 1. Hot ethanolic extract shows maximum LPO activity among stem extracts (12.29 μmols MDA g^{-1}) followed by hot aqueous extract, cold ethanolic extract and cold aqueous extract.

Conclusion

The results of present study indicate that the ethanolic extract (hot extraction) of stem possess potent free radical scavenging activity compared to other solvent extracts. These extracts also conferred significant protection against oxidation of biomolecules such as proteins and lipids. The radical scavenging ability of the extracts could be due to the presence of phenolic compounds and flavonoids. Therefore ethanolic extract of stem of Tinospora coridifolia can be considered as a potential source of natural antioxidants.

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