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Full Length Research Paper

Antioxidant activity of *Tinospora cordifolia* leaf extracts through non-enzymatic method

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This study was carried out to evaluate the antioxidant activity of leaves through non-enzymatic method. *Tinospora cordifolia* (Guduchi) has several beneficial properties, including antioxidant activity. The leaves of guduchi was extracted with methanol and partitioned in water with ethyl acetate and butanol. The phenolic content of the extracts was determined by Folin-Coicalteu method, and antioxidant activity was assayed through non-enzymatic *in vitro* models such as antioxidant capacity, by radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, reducing power, phosphomolybdenum and metal chelating activity. The phenolic contents of the extracts as gallic acid equivalents were found to be highest in methanol (44.36 mg/g) followed by ethyl acetate (38.73 mg/g), butanol (36.42 mg/g) and water extract (23.60 mg/g). At 250 µg/ml concentration, the antioxidant activity of the free radical scavenging activities of the extracts assayed through DPPH, reducing power, phosphomolybdenum and metal chelating activity were found to be highest with methanol, followed by ethyl acetate, butanol and water extract. The antioxidant activity of BHT was higher than the extracts at each concentration points. The data obtained in the *in vitro* models clearly establish the antioxidant potency of the extracts from the leaves of *T. cordifolia*.

Key words: Antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) activity, metal chelating activity, phosphomolybdenum activity, reducing power, *Tinospora cordifolia.*

INTRODUCTION

Tinospora cordifolia (Wild.) Miers ex Hook. F. and Thomas (Tc), commonly known as Guduchi, is an herbaceous vine of the family Menispermaceae indigenous to the tropical areas of India, Myanmar and Sri Lanka. Guduchi is widely used in veterinary folk medicine/ayurvedic system of medicine for its general tonic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-allergic and anti-diabetic properties (Nadkarni and Nadkarni, 1976; Chopra et al., 1982; Zhao et al., 1991). The plant is used in ayurvedic, “Rasayanas” to improve the immune system and the body resistance against infections. The root of this plant is known for its anti-stress, anti-leprotic and anti-malarial activities (Zhao et al., 1991; Nayampalli et al., 1982).

The active adaptogenic constituents are diterpene compounds including tinosporone, tinosporic acid, cordifolisides A to E, syringe, the yellow alkaloid berberine, giloin, crude giloninand, a glucosidal bitter principle, as well as polysaccharides, including arabinogalactan polysaccharide (Winston and Maimes, 2007; Singh et al., 2003). Picrotene and bergenin were also found in the plant. The active principles of *T. cordifolia*, a traditional Indian medicinal plant, were found to possess anticomplementary and immunomodulatory activities. Recent research has demonstrated that a combination

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of guduchi 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 (Adharyu et al., 2008). Many herbs and medicinal plants have been shown to have antioxidant activity, and this has been exploited by their inclusion in food to prevent deterioration or as neutraceuticals, as medical products and as ingredients in cosmetics (Aburjai and Natsheh, 2003; Frankel, 1999; Rotblatt and Ziment, 2002). In this study the antioxidant activity of different extracts from leaves of *T. cordifolia* was determined through non-enzymatic methods.

MATERIALS AND METHODS

Preparation of the leaf extract

Tinospora cordifolia was collected from the Seoul province, South Korea in 2010. Taxonomic identification was performed by Dr. Ill-Min Chung, Department of Applied Life Sciences, Konkuk University, and a voucher specimen (CT) was deposited in the Herbarium of the Department of Applied Life Sciences, Konkuk University, Seoul province, South Korea. *T. cordifolia* leaves were washed thoroughly in tap water to remove adhering mud particles, rinsed in distilled water, drained, and dried in a hot air oven at 50 ± 2°C. The dried leaves were finely powdered. The dried powder was extracted with 5 × 2 L methanol for 24 h. After removal of the solvent *in vacuo*, the crude extract was suspended in 0.5 L distilled water and extracted with 0.2 L portions of ethyl acetate and butanol until the extracts were nearly colorless. Solvents were removed *in vacuo*, and the extracts were obtained, respectively.

Determination of total phenolic content

The total phenolic content was determined by the Folin-Coicalteu (FC) method (Singleton and Rossi, 1965). Distilled water (3.16 mL) was mixed with a DMSO solution of the test compound (40 μL). Then, 200 μL of FC reagent was added. After 5 min, 600 μL of 20% sodium carbonate solution was added and the solutions were mixed again. The solutions were left at room temperature for 2 h. Then, the absorption of the developed blue colour was determined at 765 nm, using a Macasys Optizen 2120UV plus UV-spectrophotometer (Mecasys, Korea). The concentration of the total phenolic content was determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The estimation of phenolic compounds from the extracts was carried out in triplicate, and the results were averaged.

Determination of total flavonoid content

Total flavonoid content of the extracts was determined by using the aluminium chloride colorimetric method as described by Willet (2002), with some modifications. Extracts (0.5 mL), 10% aluminium chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (4.3 mL) were mixed. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using a Macasys Optizen 2120UV plus UV-spectrophotometer (Mecasys, Korea). Quercetin was used to make the calibration curve. The estimation of total flavonoids in the extracts was carried out in triplicate, and the results were averaged.

Assay of DPPH- radical-scavenging activity

The antioxidant activity of the extracts from guduchi leaves, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH-) free radical, was determined by the method described by Katerere and Eloff (2005). Different concentrations (50 to 250 μg/mL) of the extracts (0.2 mL of extract and BHT) were taken in different test tubes with 4 mL of a 0.006% MeoH solution of DPPH-. Water/methanol (0.2 mL) in place of the extracts was used as control. Absorbance at 517 nm was determined after 40 min. Radical scavenging activity was expressed as the inhibition percentage, and was calculated using the following formula:

Radical scavenging activity (%) = [(A0 - A1) / A0] × 100

Where A0 is the absorbance of the control, and A1 is the absorbance of the extracts/standard.

Assay of reductive potential

The reductive potential of the extracts was determined according to the method of Dorman and Hiltunen (2004). The reaction mixture contained varying concentrations of the extracts and standard (50 to 250 μg/mL) in 1 mL of distilled water, phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reductive potential. All analysis were run in triplicate and averaged.

Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of guduchi leaves extracts was evaluated by the method of Prieto et al. (1999). An aliquot of 0.1 mL of sample solution (1 mg/mL) was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under same conditions as the rest of the sample. For samples of unknown composition, water soluble antioxidant capacity was expressed as equivalents of ascorbic acid (mg/g of extract).

Chelating effects on ferrous ions

The chelating effect was determined according to the method of Dinis et al. (1994). Briefly, 2 mL of various concentrations (50 to 250 μg/mL) of the extracts in methanol was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated by using the formula given below:

Metal chelating effect (%) = [($A_{control}$ - A_{sample}) / $A_{control}$] × 100

Table 1. Total phenolic (expressed as gallic acid equivalents) and flavonoid content (expressed as mg quercetin/g) from leaves extracts of *Tinospora cordifolia.*

Extract	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
Ethyl acetate	38.73±2.26	8.99±0.40
Methanol	44.36±0.65	10.31±1.20
Butanol	36.42±1.15	6.02±0.33
Water	23.60±0.50	1.13±0.02

Values are means of triplicates ± SD.

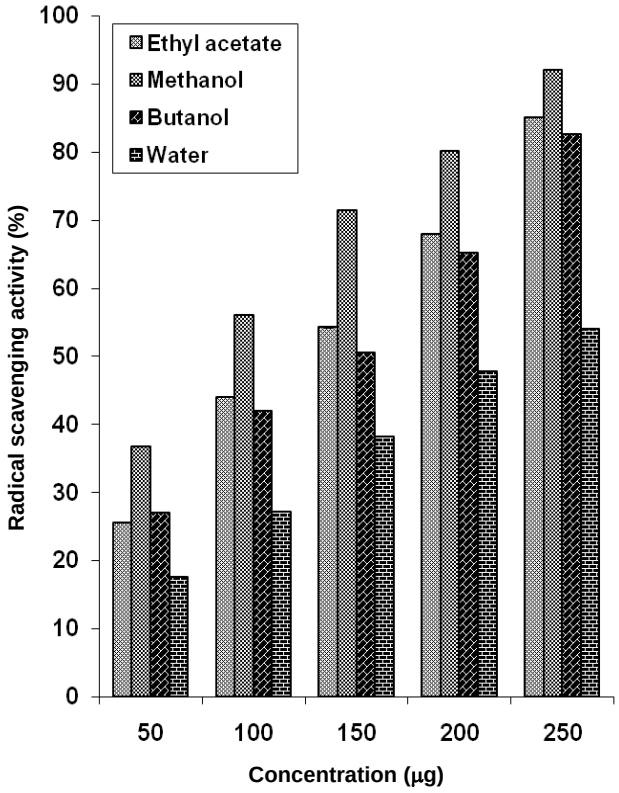

Figure 1. Free radical-scavenging activity of the extracts from *T. cordifolia* leaves at different concentrations by DPPH method. Each sample was assayed in triplicate for each concentration. Values are means of triplicates ± SD.

Where $A_{control}$ is the absorbance of control (the control contains $FeCl_2$ and ferrozine complex formation molecules) and A_{sample} is the absorbance of the test compound. EDTA was used as a standard.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

The total phenolic content of the extracts from leaves 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, methanol extract had the highest (44.36 mg/g) amount of phenolic compounds followed by ethyl acetate (38.73 mg/g), butanol extract (36.42 mg/g) and water extract (23.60 mg/g). The result of the total flavonoid contents of the extracts from *T. cordifolia* leaves is presented in Table 1. The total flavonoid content varied from 1.13 to 10.31 mg quercetin/g weight. The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but 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 (Elliot, 1999). 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 (Prior and Cao, 2000; Zia-Ul-Haq et al., 2008, 2011a, b, 2012).

DPPH- radical-scavenging activity

The free radical-scavenging activity of the extracts was tested through DPPH- method (Katerere and Eloff, 2005) and the results were compared with BHT (Figure 1). DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH- solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extracts were able to reduce the stable radical DPPH- to the yellow-coloured diphenylpicrylhydrazine. The IC_{50} values of the extracts were methanol (89.26 µg/mL), ethyl acetate (138.22 µg/mL), butanol (148.48 µg/mL) and water (231.69 µg/mL), respectively. It has been found that cysteine, glutathione, ascorbic acid for tocopherol, polyhydroxy aromatic compounds (foe example, hydroquinone, pyrogallol, gallic acid), and aromatic amines (for example, ρ-phenylene diamine, ρ-aminophenol), reduce and decolorize 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (Blois, 1958). The positive correlation between polyphenolic content of the extracts and its antioxidant activity is well documented (Huang and Mau, 2006). Therefore, the content of total phenolic compounds in the extracts might explain their high antioxidant activities. In this study, the extracts exhibited a concentration - dependent antiradical activity by

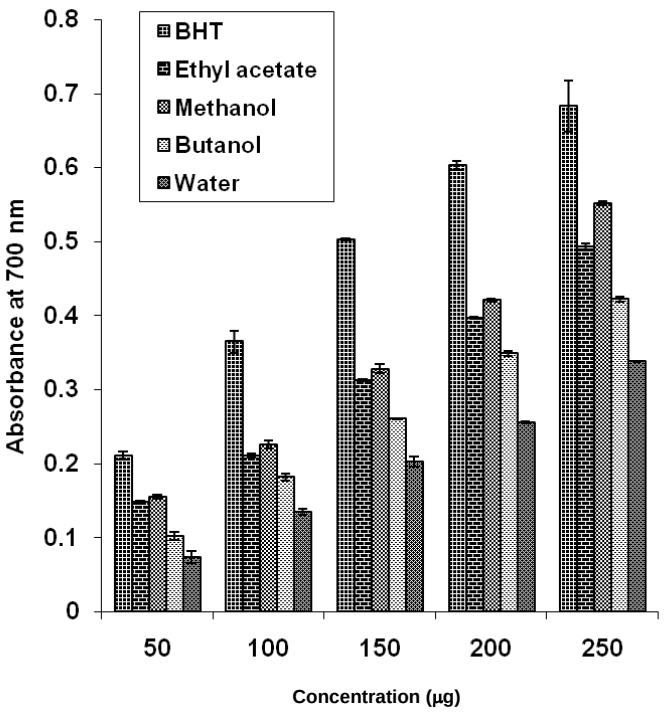

Figure 2. Reducing power of the extracts from *T. cordifolia* leaves at different concentrations. Each sample was assayed in triplicate for each concentration. Values are means of triplicates ± SD.

inhibiting DPPH- radical (Figure 1). Of the different extracts, methanol extract exhibited the highest antioxidant activity of 92.09% at 250 µg/mL concentration, followed by ethyl acetate (84.98%), butanol (82.59%) and water extract (53.95%), respectively at the same concentration (Figure 1). One of the possible mechanisms is polyphenolic-associated compounds (formation of non-extractable complex between high molecular weight phenolics and compounds). Those kinds of phenolic compounds show antioxidant activity due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triple oxygen or decomposing peroxide. BHT (butylated hydroxytoluene) showed higher degree of free radical scavenging activity than that of the extracts at low concentration points. The DPPH activity of BHT exhibited 92.04% at 50 µg/mL concentration (data not shown).

Assay of reductive potential

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Re et al., 1999; Diplock, 1997; Zia-Ul-Haq et al., 2008, 2011a, b, 2012). In the present study, the extracts and BHT exhibited effective reducing capacity at all concentration points. The reducing capacity of the extracts and BHT increased with increase in the concentration (Figure 2). The reducing power of the extracts followed the order: methanol < ethyl acetate < butanol < water. The reducing capacity of BHT was found to be higher than the extracts at each concentration points. Earlier authors (Gao et al., 2000; Amarowicza et al., 2004) have observed a direct correlation between antioxidant activities and reducing capacity of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain, by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing capacity of extracts suggested that reductone-associated and hydroxide groups of compounds can act as electron donors and can react with free radicals to convert them to more stable products, and thereby terminate radical chain reactions.

Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of the extracts was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte, and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the extracts was found to decrease in the order: methanol extract > ethyl acetate > butanol extract > water extract (Table 2).

Metal chelating activity

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 color of the complex. Measurement of color reduction therefore allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi et al., 2000). Metal chelating activity was claimed as one of the antioxidant activity mechanism, since it reduced the concentration of the catalyzing transition metal in lipid peroxidation (Senevirathne et al., 2006). It was reported that chelating agents which form s-bonds with metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Keowmaneechai and McClements, 2006; Yang et al., 2006). In this assay, both the extracts and standard compounds interfered with the formation of ferrous complex with the reagent ferrozine, suggesting that it has

Table 2. Antioxidant capacity of *Tinospora cordifolia* leaves extract by phosphomolybdenum method.

Extract	Antioxidant capacity (%) as equivalent to α-tocopherol (mg/g)
Ethyl acetate	120.13±4.8
Methanol	159.42±5.5
Butanol	98.46±2.01
Water	41.99±1.50

Values are means of triplicates ± SD.

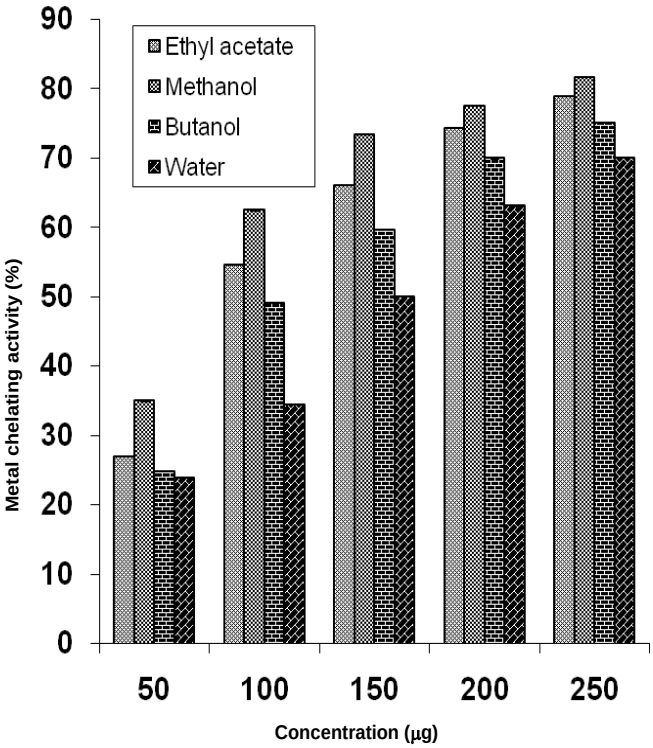

Figure 3. Metal ion chelating effect of the extracts from *T. cordifolia* leaves. Each sample was assayed in triplicate for each concentration. Values are means of triplicates ± SD.

chelating activity and captures the ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex is linearly decreased with the dose taken (from 50 to 250 µg/mL). The percentages of metal scavenging capacity at 250 µg/mL doses of the extracts were found to be methanol (81.62%), ethyl acetate (78.89%), butanol (75.10%) and water (70.05%), respectively (Figure 3). The standard EDTA exhibited 98.59% activity at 100 µg/mL concentration. Metal chelating activity was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al., 1999). The data obtained from Figure 3 reveals that the extracts demonstrate an effective capacity for iron binding, suggesting that its action as antioxidant may be related to its iron-binding capacity.

Conclusion

The extracts from the leaves of *Tinospora cordifolia* exhibited different levels of antioxidant activity in all the models studied. On the basis of the results of this present study, the decreasing order of the antioxidant activity of the extracts assayed through all the four models was found to be methanol > ethyl acetate > butanol > water extract. This order is similar to the phenolic contents of the extracts that showed the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract. In the present study, it is found that methanol extract contains substantial amount of phenolics, and it is the extent of phenolics present in this extract being responsible for its marked antioxidant activity as assayed through various *in vitro* models.

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