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RESEARCH ARTICLE

FREE RADICAL SCAVENGING CAPACITY, ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT OF GARDENIA LATIFOLIA

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ABSTRACT

Gardenia latifolia was extracted with ethanol and aqueous using cold-extraction. To evaluate the antiradical and antioxidant abilities of the extracts, four *in vitro* test systems were employed, *i.e*., dpph (1,1,diphenyl-2-picryl hydrazyl), nitric oxide, hydrogen peroxide and reducing power assay. The extracts exhibited outstanding antioxidant activities that were superior to that of standard. The ethanol extract exhibited the most significant antioxidant activities, and cold-extraction under stirring seemed to be the more efficacious method for acquiring the predominant antioxidants. Furthermore, the antioxidant activities and total phenol & flavonoids content of the extracts followed the same order, *i.e*., there is a good correlation between antioxidant activities and phenolic compounds. The results showed that these extracts, especially the ethanol extract, could be considered as natural antioxidants and may be useful for curing diseases arising from oxidative deterioration.

INTRODUCTION

Free radicals are known to be the major cause for various chronic diseases including atherosclerosis, neurodegenerative diseases, cancer, diabetes and inflammatory diseases. Antioxidants can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Plants synthesize an extensive array of chemical compounds and many of them have pharmacological properties. Plants are rich source f free radical scavenging molecules such as vitamins, terpernoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinines, coumarins, alkaloids, amines, betalins and other metabolites which are rich in antioxidant activity. India is the leading producer of medicinal plants and is aptly called the botanical garden of the world (Pal and Shukla, 2003). Since time immemorial ago, plants have been used in traditional medicine for primary health care. In India, more than 2500 plant species are reported by traditional healers and 100 species of plants serve as regular sources of medicine (Pei, 2001). For the last few decades there has been an increasing attention in the study of medicinal plants and their traditional use in different parts of the world (Doss and Anand, 2014, Sasiumar *et al*., 2006; Doss and Anand, 2013). There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases (Sivaraj *et al.,* 2010).

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Gardenia latifolia (Rubiaceae) is commonly known as Indian boxwood or Ceylon boxwood,is a densely foliaceous small tree that occurs throughout the greater parts of Indian common in deciduous forests along the streams. The stem bark and fruits are reported to be used in the treatment of varius ailments such as snake bite,skin diseases, stomach pais,caries in humans and ephemeral fever in live stocks (Reddy et al., 2006; Madava Chetty et al, 2008;Dr.Duke's) Fruits are used for making perfumes (Chandra Prakash, K.2009). Hence, in the present study, the antioxidant activities of aqueous and ethanolic extracts of *G.latifolia* were evaluated using four *in vitro* methods. Total phenolic and flavonoids contents of the crude extracts was also determined

Materials and methods

Plant collection

The fresh aerial plant parts were collected from Kolli hills, Namakkal District, Tamil Nadu, India. The collected plant is identified by Botanical Survey of India (BSI/SRC/5/23/2013/Tech-795 & Serial No. 1), Coimbatore and the voucher specimens were deposited at the herbarium of Department of Botany, National College (Autonomous), Tiruchirappalli-1.

Preparation of extracts

Plant material

Fresh and health leaves were collected from Kolli hills, Tamilnadu, India.

The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were powdered with the help of mechanical blender and used for extraction.

Aqueous extract

Ten grams of powdered leaves were macerated with 100 ml of sterile distilled water in a blender for 24 hrs. The macerate was filtered through Whatmann no.1 filter paper to get pure extract. The extract was preserved aseptically in brown bottles at 4^{o}C until further use.

Ethanol extract

Air dried powder of 10 g was placed in a conical flask containing 100 ml of ethanol plugged with cotton and then kept on a rotary shaker at 200 rpm for 24 hrs. Later, it was filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to make volume one fourth of its original volume.

Chemicals

Ethanol, DPPH, Ascorbic acid and DMSO were of AR grade from Himedia (Mumbai, India), TCA, Ferric chlorite, Gallic acid, Quecetin, Potassium ferric cyanide, Sodium nitroprusside, Sulfanilamide, Naphthylethylenediamine dihydrochloride, Sodium carbonate, Hydrogen peroxide, Folin phenol reagent, Aluminum chloride and Potassium acetate were from SD Fine Chemicals (Mumbai, India).

DPPH assay

DPPH scavenging activity was carried out by the method of Blois (1958). Different concentrations (1000, 500, 250, 125 and 62.5 µg/ml) of crude extracts were dissolved in DMSO (dimethyl sulfoxide) and taken in test tubes in triplicates. Then 5 ml of 0.1mM ethanol solution of DPPH (1, 1, Diphenyl-2-Picrylhydrazyl) was added to each of the test tubes and were shaken vigorously. They were then allowed to stand at 37^{o}C for 20 minutes. The control was prepared without any extracts. Methanol was used for base line corrections in absorbance (OD) of sample measured at 517nm. A radical scavenging activity was expressed as 1% scavenging activity and was calculated by the formula:

Percentage of radical scavenging activity = Control OD – Sample OD/ Control OD

Reducing power assay

Reducing activity was carried out by using the method of Oyaizu (1986). Different concentration (1000, 500, 250,125 and 62.5 µg/ml) of crude extracts were prepared with DMSO and taken in test tube as triplicates. To test tubes 2.5 ml of sodium phosphate buffer and 2.5 ml of 1% Potassium ferric cyanide solution was added. These contents were mixed well and were incubated at 50^{o}C for 20 minutes. After incubation 2.5ml of 10% Trichloroacetic acid (TCA) was added and were kept for centrifugation at 3000rpm for 10 minutes. After centrifugation 5ml of supernatant were taken and to this 5ml of distilled water was added. To this about 1ml of 1% ferric chlorite was added and was incubated at 35^{o}C for 20 minutes. The OD (absorbance) was taken at 700nm and the blank was prepared by adding every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding every other solution but without extract. The reducing power of the extract is linearly proportional to the concentration of the sample.

Hydrogen peroxide

The ability of plant extracts and standards to scavenging hydrogen peroxide was determined according to the method Ruch *et al*. (1998). A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). The extract at different concentrations in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution (0.6 ml, 43 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The percentage of scavenging of hydrogen peroxide of extracts and standard compounds was calculated using the following equation:
Hydrogen peroxide scavenging activity = A0 – A1/A0 X 100.
Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

Nitric oxide

Nitric oxide scavenging activity can be estimated by the use of Griess IlIosvoy reaction (Garrat, 1964).The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO_2 Under aerobic conditions, NO_2 reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations (5 - 200µg/ml) of crude extracts (ethanol &aqueous) were dissolved in methanol and incubated at 30^{0}C for 2 hours. The same reaction mixture without the extracts but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formedduring diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550nm. Inhibition of nitrite formation by the plant extracts and the standard antioxidant ascorbic acid were calculated relative to the control. Inhibition data (percentage inhibition) were linearized against the concentrations of each extract and standard antioxidant.

Total phenolic content

Total phenolic contents were determined by Folin Ciocalteu reagent (McDonald *et al*., 2001). A dilute extract of each crude extracts (0.5 ml of 1:10g ml^{-1}) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/ml solutions of gallic acid in methanol: water (50:50, v/v).

Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference compound.

Determination of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination (Chang *et al*., 2002). Each crude extracts (0.5ml of 1:10 g/ml) in ethanol were separately mixed with 1.5 ml of methanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solution at concentrations 12.5 to 100g ml^{-1}in methanol.

Statistical Analysis

All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Antioxidants are capable of exerting protective effects against oxidative stress in biological systems. They terminate Reactive Oxygen Species chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease and cancer have appeared during last three decades. In current herbal drug scenario, plant derived antioxidants are gaining importance because of their potential health benefits, no toxicity and side effects over synthetic antioxidants like butylhydroxyanisole and butylhydroxytoluene (BHA and BHT, respectively).

The DPPH radical scavenging activity of *G.latifolia* leaf extracts is shown in Table 1. Ethanol extract showed the highest scavenging activity of 87.99 % at 1000 µg/ml concentration.

Table 1. DPPH scavenging of crude extracts of *G.latifolia*

Concentrations	Antioxidant activity (%)		Standard (Ascorbic acid)
	Ethanol	Aqueous	
1000 µg/ml	87.99 ± 0.39	82.43 ± 0.68	
500 µg/ml	59.87 ± 0.46	54.28 ± 0.46	
250 µg/ml	29.33 ± 0.06	25.57 ± 0.46	88.42 ± 1.02
125 µg/ml	13.04 ± 0.44	13.00 ± 0.45	
62.5 µg/ml	8.60 ± 0.33	7.04 ± 0.33	

Values are mean ± Standard deviation

Table 2. Reducing power of crude extracts of *G.latifolia*

Concentrations	Antioxidant activity (%)		Standard (Ascorbic acid)
	Ethanol	Aqueous	
1000 µg/ml	0.889 ± 0.04	0.784 ± 0.03	
500 µg/ml	0.482 ± 0.02	0.424 ± 0.03	
250 µg/ml	0.294 ± 0.02	0.255 ± 0.04	
125 µg/ml	0.157 ± 0.05	0.148 ± 0.01	0.892 ± 0.05
62.5 µg/ml	0.080 ± 0.02	0.070 ± 0.02	

Values are mean ± Standard deviation

Aqueous extract showed a scavenging ability of 82.43 % at a higher concentration of 1000 µg/ml. It is well accepted that the DPPH radical scavenging by antioxidants is due to their hydrogen donating ability (Singh and Rajini, 2004; Goncalves *et al*., 2005). The antioxidant activity may be directly correlated to the phenolic content in different solvent extracts (Singh *et al*., 2002; Cai *et al*., 2004; Kulkarni *et al*., 2004). The crude extracts (ethanol and aqueous) of *G.latifolia* recorded the highest phenol and flavonoid contents and also had the highest DPPH scavenging activity. This suggests that the phenolic compounds contributed significantly to the antioxidant capacity of the investigated plant species. The extracts (ethanol and aqueous) showed potent antioxidant power by reducing power capacity. Results of reducing power assay are shown in table 2. The reducing power ability of the extract and standard were found to be 0.899 % and 0.892% respectively. The aqueous extract showed moderate activity when compared to the standard (0.784 %). Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes so that they can act as primary and secondary antioxidants (Chanda and Dave, 2009)

Table 3. Hydrogen peroxide radical scavenging of crude extracts of *G.latifolia*

Concentrations	Antioxidant activity (%)	
	Ethanol	Aqueous
0.5 µg/ml	14.16 ± 0.22	13.33 ± 0.29
1.0 µg/ml	28.97 ± 0.30	27.56 ± 0.38
1.5 µg/ml	52.53 ± 0.37	47.65 ± 0.44
2.0 µg/ml	62.50 ± 0.46	54.14 ± 0.55
2.5 µg/ml	79.03 ± 0.44	61.24 ± 0.44

Values are mean ± Standard deviation

Table 4: Nitric oxide Scavenging of crude extracts of *G.latifolia*

Concentrations	Antioxidant activity (%)	
	Ethanol	Aqueous
0.5 µg/ml	9.39 ± 0.19	5.79 ± 0.64
1.0 µg/ml	17.44 ± 0.49	12.18 ± 0.76
1.5 µg/ml	31.82 ± 0.33	22.05 ± 0.42
2.0 µg/ml	41.7 ± 0.32	38.21 ± 0.40
2.5 µg/ml	55.33 ± 1.30	48.95 ± 0.32

Values are mean ± Standard deviation

Table 5. Hydroxyl Radical scavenging activity of crude extracts of *G.latifolia*

Concentrations	Percentage of Inhibition	
	Ethanol	Aqueous
10 µg/ml	7	4.05
20 µg/ml	12	8.78
40 µg/ml	16	13.51
60 µg/ml	34	24.32
80 µg/ml	43	38.51
100 µg/ml	58	44.59

Values are mean ± Standard deviation

Table 6: Bioactive compounds of crude extracts of *G.latifolia*

Extracts	Total Phenol*	Flavonoids**
Ethanol	1345	458
Aqueous	982	122

*mg GAE/g dry mateial
** mg QE/g dry material

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it can give rise to hydroxyl radical in the cells.

Thus the removal of H2O2 is very important for antioxidant defence in cell or food systems. The results of the H2O2 radical scavenging activity are given in Table 3. The scavenging activity of the ethanolic extract was found to be the highest, followed by aqueous. This has special significance due to the fact that H2O2 is a potent oxidant. It has a strong tendency to oxidize DNA in the cells, causing mutations. Reactive agents of H2O2 may sometimes even cause cell death due to the generation of free hydroxyl radicals inside the cell. The H2O2 radical scavenging activity is attributed to primary antioxidants, which in case of plants would be phenolics (Dorman *et al*., 2003). NO is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecules in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumour activities.

Nitric oxide is a free radical that exhibits neumerous physiological properties and it is also implicated in several pathological states (Moncada *et al*., 1991). The nitric oxide scavenging activity of crude extracts is shown in table 4. The NO scavenging activity is seen in the order of ethanol and aqueous. Excess production of nitric oxide is associated with several diseases. NO is produced in various cells including neurons, endothelial cells and neutrophils by three isoforms of NO synthase, from L-arginine (Sessa *et al*., 1993). Hydroxyl radicals are very reactive and can be generated in biological cells through the Fenton reaction. Hydroxyl radicals scavenging activity was quantified by measuring inhibition of the degradation of the deoxyribose by free radicals. The hydroxyl radical scavenging ability of *G.latifolia* leaf extracts in ethanol and aqueous is depicted in table 5.There was a scavenging of 58 % hydroxyl activity with ethanol extract at a concentration of 100 µg/ml. Even though aqueous extract also showed significant hydroxyl activity (44.59%). The hydroxyl radical is extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology capable of damaging biomolecules of the living cells (Halliwell, 1997; Walling, 1975).

Some compounds are capable of redox cycling the metal ions required for hydroxyl ions generation, thus increasing the radical production, exhibiting a pro-oxidant activity (Li and Xie, 2000). All the extracts have shown inhibitory activity against hydroxyl radical and there was a significant difference in their activities at similar concentrations. The content of total phenolics in crude extracts were determined using folin ciocalteau assay, calculated from regression equation of calibration curve of Gallic acid equivalent (Table 6). Phenolic content of crude extracts was found to be 982 – 1345 mg gallic acid equivalent / g dry material. The flavonoid content of crude extracts was found to be 122 – 458 mg quercetin equivalent / g dry material. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, flavonoids are wide spread in all natural compounds and posses a broad spectrum of biological activities. The high phenolic and flavonoid content in the present study may be responsible for its free radical scavenging activity. The findings of the present study suggest that *Gardenia latifolia* could be a potential source of natural antioxidant that could have great importance as therapeutic agent in preventing or slowing the oxidative stress related degenerative diseases.

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