Asian Journal of Chemistry; Vol. 28, No. 6 (2016), 1288-1292

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2016.19656

In vitro Antioxidant Potential of Standardized Ethanol Extract of *Clerodendron serratum* Linn. Leaves

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Received: 7 October 2015; *Accepted*: 10 January 2016; *Published online*: 29 February 2016; AJC-17793

Leaves of *Clerodendron serratum* were investigated for their phytochemical constituents like phenolic compounds, flavonoids, tannins, terpenoids, steroids and screened for *in vitro* antioxidant activity against 2,2-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion, hydroxyl radical, nitric oxide radical, total antioxidant capacity and reducing power. Estimation of total phenolic compounds by Folin-Ciocalteu reagent indicated that the extract (1 mg) contained 130 µg of gallic acid equivalents and that of total flavonoids demonstrated 7 µg of quercetin equivalents in 1mg of the extract. DPPH radical, hydroxyl radical, nitric oxide radical and superoxide radical scavenging activities were found to be 211, 138.35, 171.54 and 177.93 µg/mL, respectively. The extract showed significant activities in all antioxidant assays and the reducing power of the extract was found to be significantly higher than ascorbic acid. The results obtained indicate that *in vitro* antioxidant property of *Clerodendron serratum* leaf extract may be due to high content of phenolic compounds and justify the therapeutic applications of the plant in the indigenous and traditional system of medicine for the treatment of inflammatory and neurological disorders.

Keywords: *Clerodendron serratum*, *in vitro* Antioxidant, Phenolic compounds, Flavonoids.

INTRODUCTION

Reactive oxygen species (ROS) which are different forms of activated oxygen consists of free radicals such as hydroxyl (OH^-), superoxide (O_2^-), nitric oxide (NO), peroxyl (ROO^-), lipid peroxyl (LOO^-) radicals and non-free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2^-), ozone (O_3), lipid peroxide (LOOH) [1-3]. Reactive oxygen species produced by all aerobic organisms can easily react with most biological molecules including protein, lipids, lipoproteins, DNA and can generate oxidative stress leading to many pathophysiological disorders such as inflammation, diabetes, cancer, neurodegenerative disorders and genotoxicity [4,5].

It is widely accepted that natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent cellular damage caused by oxidative stress and found to be more safer than their synthetic counterparts [6]. Antioxidant agents of natural origin such as herbal plants, vegetables and fruits containing phenolics, flavonoids, tannins and proanthocyanins have gained special attention because of free radical scavenging abilities [7,8].

Clerodendron serratum (*C. serratum* Linn.) Moon, commonly known as Bharangi in Hindi, Sirutekku in Tamil, Cheruthekku in Malayalam, belongs to family verbanaceae [9]. According to Samhita Kala, Bharangi has been widely used to cure many diseases like Shwasa (breathlessness), Kasa (cough), Vrana (wound), Shotha (swelling) and Vataja disorders (neurological disorders) [10]. It is found to be distributed in the forests of Western Ghats of India and globally in Ceylon, Malay and Penninsula [11].

Main phytochemical constituents reported in the leaves of *C. serratum* are carbohydrates, phenolics, flavonoids, terpenoids and steroids. Flavonoids include catechin, α-spinasterol, luteolin, apigenin, baicalein, scutellarein, 6-OH-luteolin, luteolin-7-0-β-D-glucuronide and phenolic acids found to be reported are caffeic acid and ferulic acid and carbohydrates, which includes a mixture of glucose, arabinose and glucuronic acid [9,12].

EXPERIMENTAL

Collection of plant materials: The leaves of plant *C. serratum* were collected from Attapadi forest, Palakkad district, Kerala. It was taxonomically identified by Department of Botany, University of Kerala and herbarium of the plant is preserved for future reference [Voucher no: 114 10/3 (UCBD)].

Preparation of extract: The leaves were washed and shade dried at room temperature. Dried leaves were coarsely powdered (437 g) and subjected to extraction by cold maceration with 70 % ethanol (7.45 % w/w yield) at room temperature with continuous stirring for 6 days, after defatting with petroleum ether (60-80 °C).The solvents were evaporated with rotary vacuum and stored in a desiccator.

Estimation of total phenolic compounds and total flavonoids: Total soluble phenolic compounds in ethanolic extracts of *C. serratum* were determined with the Folin-Ciocalteu reagent according to the method of Slinkerd and Singleton [13]. 40 µL of ethanolic extract of *C. serratum* were mixed separately with 1 mL of Folin-Ciocalteu reagent and 2 mL of 20 % $NaCO_3$. It was mixed, boiled for 1 h, cooled and centrifuged at 10,000 rpm for 5 min. Absorption was recorded at 575 nm in a spectrophotometer.

Total flavonoids content was estimated by method of Kim [14]. Formation of acid stable complexes with C-4 keto group and either C-3 or C-5 hydroxyl group of flavones and flavonols in addition with *ortho*-dihydroxyl groups in the A or B ring of flavonoids forms the basis of the estimation. For plotting the calibration curve, quercetin was used as standard. To 1 mL of sample:water (50:50 v/v) or standard solution of quercetin (0-500 mg/L) was added to 4 mL of water in a volumetric flask. At zero time, 0.3 mL of 50 g/L sodium nitrite was added to the flask. After 5 min, 0. 3 mL of aluminium trichloride was added. At 6 min, 2 mL NaOH were added to the mixture and immediately diluted with 204 mL of water. Absorbance of the mixture was read at 510 nm *versus* blank.

Total antioxidant activity: Total antioxidant capacity of the extracts was evaluated by phosphomolybdenum method according to the procedure described by Mistuda *et al.* [15]. A 0.3 mL of extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then, the absorbance of the solution was measured at 695 nm using a UV-visible spectrophotometer against blank after cooling to room temperature. Methanol (0.3 mL) in the place of extract was used as the blank. The total antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

DPPH radical scavenging activity: DPPH radical scavenging activity was assessed, according to the method of Blois [16]. DPPH is a stable free radical with red colour which turns yellow colour when scavenged. Antioxidants react with DPPH and reduce it to DPPH-H and as a result the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability. The extract at various concentrations ranging from 10 to 250 µg/mL was mixed in 1 mL of freshly prepared 0.5 mM DPPH ethanol solution and 2 mL of 0.1 M acetate buffer. The resulting solutions were then incubated at 37 °C for 0.5 h and measured colorimetrically at 517 nm. Ascorbic acid was used as positive control under the same assay condition. Negative control was without any inhibitor or extract. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The percentage DPPH radical scavenging activity of extract was calculated from the decrease in absorbance at 517 nm in comparison with negative control.

Hydroxyl radical scavenging activity: Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium based on the method of Halliwell and Gutteridge [17]. This assay is based on the quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by Fe^{3+} ascorbate, EDTA-H_2O_2 system. The reaction mixture containing $FeCl_3$ (200 µM), EDTA [1.04 mM], H_2O_2 (1 mM) and 2-deoxy-D-ribose (2.8 mM) were mixed with or without extract at various concentration (125-2000 µg/mL) in 1 mL final reaction volume made with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 h at 37 °C. The mixture was heated at 95 °C in water bath for 15 min followed by addition of 1 mL each of TCA (2.8 %) and TBA (0.5 % TBA in 0.025 M NaOH containing 0.02 % BHA). Finally, the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of the supernatant liquid was measured at 532 nm. The negative control without any antioxidant or extract was considered 100 % deoxyribose oxidation. The percentage hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive control.

Nitric oxide radical scavenging activity: Nitric oxide was generated from sodium nitroprusside and measured using the Griess reaction. The assay was done according to the method of Marcocci *et al.* [18]. The assay is based on principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which reacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. 320 µL ethanol extract, 360 mL sodium nitroprusside, 216 mL Greiss reagent (1 % sulfanilamide, 2 % H_3PO_4 and 0.1 % naphthylethylene diamine-dihydrochloride) was mixed and incubated at 25 °C for 1 h. Lastly, 2 mL water was added and absorbance was taken at 546 nm and the percentage inhibition was calculated.

Superoxide anion radical scavenging activity: Superoxide anion radical scavenging activity was assessed based on the method of Nishimiki *et al.* [19] with a mild modification. About 1 mL of 156 µM NBT solution in phosphate buffer (100 mM, pH 7.4), 1 mL 468 µM NADH in phosphate buffer (100 mM, pH 7.4) and 0.1 mL of various concentrations of ethanolic extract of *C. serratum* leaves and reference compound (12.5, 25, 50, 100 and 200 µg/mL) were mixed and the reaction was started by adding 100 mL 60 mM PMS in phosphate buffer (100 mM, pH 7.4). The reaction was incubated at 25 °C for 5 min and absorbance at 560 nm was measured against ascorbic acid. All the tests were performed in triplicate and the results average. The percentage inhibition was calculated by comparing the results of reference compound and test sample.

Reducing power activity: The reducing power activity extract was estimated following the method of Oyaizu [20]. Extract solution (2 mL), phosphate buffer (2 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2 mL, 10 mg/mL) were added and then kept at 45 °C for 0.5 h. TCA (2 mL, 100 mg/L) was added to the reaction mixture. A 2 mL aliquot of the above mixtures was added to 2 mL of distilled water and 0.4 mL of 0.1 % w/v ferric chloride in a test tube. The absorbance was measured after 10 min at 700 nm. Increased absorbance of the reaction mixture suggests a high reducing power.

Statistical analysis: Experimental results were mean ± SD of three parallel measurements. Statistical analysis was performed based on student's 't' test and $P < 0.001$ and < 0.05 were regarded as significant.

RESULTS AND DISCUSSION

Phenols exhibit significant antioxidant and free radical scavenging activity due to the presence of hydroxyl groups in its structure. Total phenolic content of ethanolic extract of *C. serratum* were reported as gallic acid equivalents. 1 mg of ethanolic extract of *C. serratum* leaves was found to contain 130 µg of gallic acid equivalents of phenolic compounds. Results showed that high content of phenolic compounds in this plant extract may be an attributing factor for its usage for the management of oxidative stress induced ailments.

Flavonoids which are polyphenolic compounds also exhibit antioxidant activity and free radical scavenging activity as it contain two or more phenol subunits. The total flavonoid content of ethanolic extract of *C. serratum* was reported as quercetin equivalents. 1 mg of ethanolic extract of *C. serratum* leaves was found to contain 7 µg of quercetin equivalents of flavonoids. Diverse studies have shown that quercetin and its glycosides, luteolin and its derivatives and rutin, all belong to flavonoids exert inhibitory activity against lipid peroxidation [21,22]. As flavonoids are also polyphenolic compounds their presence in this plant extract may produce substantiating effect on antioxidant activity.

Total antioxidant capacity: Phosphomolybdate method has been routinely used to evaluate total antioxidant capacity of extract. This assay method is based on the reduction of Mo(VI) to Mo(V) by the sample and subsequent formation of green phosphate/Mo compounds with a maximum absorption at 765 nm. Fig. 1 showed that the ethanolic extract of *C. serratum* leaves with IC_{50} value 168.223 µg/mL had significant ($p < 0.001$) antioxidant activity which increased in a concentration dependent manner. The results suggested that significant antioxidant activity might be due to presence of phenolic compounds present in the extract. Recent studies have shown that many flavonoids and polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants [23,24].

Inhibition of DPPH radical: The model of scavenging stable DPPH free radical is widely used for relatively rapid evaluation of antioxidant activities compared to other methods [25]. Ethanolic extracts of *C. serratum* leaves showed significant scavenging effects on the DPPH radical. The scavenging effect of the ethanolic extract was comparable with that of ascorbic acid, with the IC_{50} value for the ethanolic extract as 211 µg/mL and that of ascorbic acid as 135 µg/mL. The method is based on the reduction of methanol DPPH solution in the presence of hydrogen donating antioxidant resulting in the formation of non-radical form DPPH-H by the reaction. The degree of colour change is proportional to the concentration and potency of antioxidant. Large decrease in absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [26]. The extract was able to reduce the stable radical DPPH to the yellow coloured diphenylpicryl-hydrazine. Fig. 2 of this study suggests that the ethanolic extract of *C. serratum* leaves contain phytochemical constituents that are capable of donating 'H' to a free radical to scavenge the potential damage and it possess significant ($p < 0.001$) DPPH radical scavenging activity.

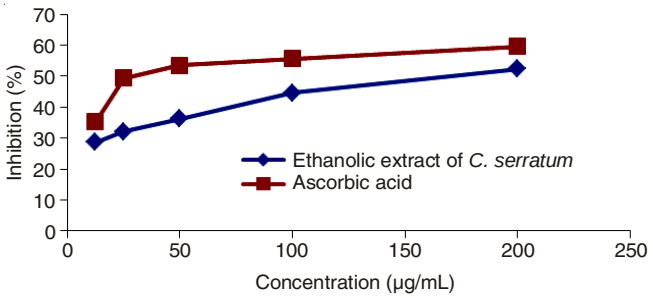

Fig. 1. Total antioxidant activity of ethanolic extract of *Clerodendron serratum*

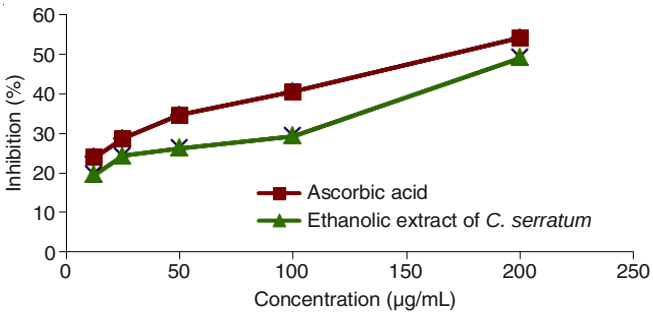

Fig. 2. DPPH radical scavenging activity of ethanolic extract of *Clerodendron serratum*

Inhibition of hydroxyl radical: Hydroxyl radical scavenging activity was quantified by measuring the inhibition of degradation of 2-deoxyribose by the free radicals generated by Fenton reaction. Fig. 3 shows the hydroxyl radical scavenging of the extract and ascorbic acid. The extract showed extremely significant ($p < 0.001$) hydroxyl radical scavenging effect with IC_{50} value of 138.35 µg/mL. Ferric EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA and low pH form a pink chromogen [27,28]. Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules such as protein, DNA and lipids, causes lipid peroxidation. It reacts with poly-unsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [29,30]. This radical is regarded as detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system resulting in carcinogenesis, mutagenesis and cytotoxicity [31]. Thus hydroxyl radical scavenging capacity is directly

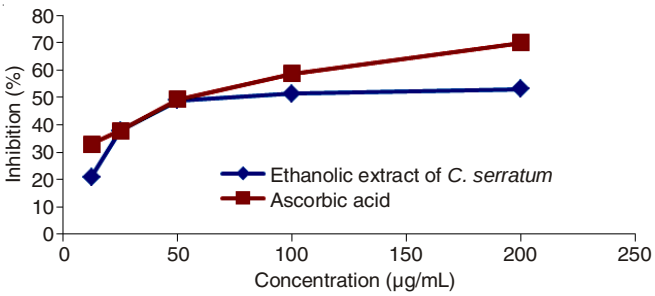

Fig. 3. Hydroxyl radical scavenging activity of ethanolic extract of *Clerodendron serratum*

proportional to its antioxidant activity which is measured by the low intensity of red colour [32]. From the results it can be concluded that ethanolic extract of *C. serratum* leaves scavenged hydroxyl radicals and prevented degradation of 2-deoxy-2-ribose.

Inhibition of nitric oxide radical: Sodium nitroprusside in aqueous solution at physiological pH spontaneously produces nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. Fig. 4 illustrates the percentage inhibition of nitric oxide generation by ethanolic extract of *C. serratum* leaves and gallic acid. The concentration of ethanolic extracts of *C. serratum* needed to produce 50 % inhibition of nitric oxide release was found to be 171.54 µg/mL, whereas 45.73 µg/mL was needed for gallic acid, used as reference compound. The results indicate that ethanolic extract of *C. serratum* leaves had significant ($p < 0.05$) scavenging effect on nitric oxide radicals.

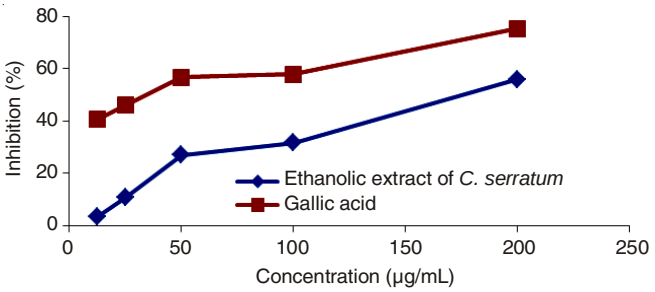

Fig. 4. Nitric oxide radical scavenging activity of ethanolic extract of *Clerodendron serratum*

Inhibition of superoxide anion radical: Superoxide radical is considered as a major biological source of reactive oxygen species [33] and gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [34]. In PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 m with antioxidants, thus indicate the consumption of superoxide anion in the reaction mixture. Fig. 5 shows the superoxide anion radical scavenging activity of ethanolic extract of *C. serratum* leaves was compared with the same doses of ascorbic acid ranging from 12.5 to 200 µg/mL. When compared to ascorbic acid with IC_{50} value of 67.06 µg/mL, the superoxide anion scavenging activity of the ethanolic extract

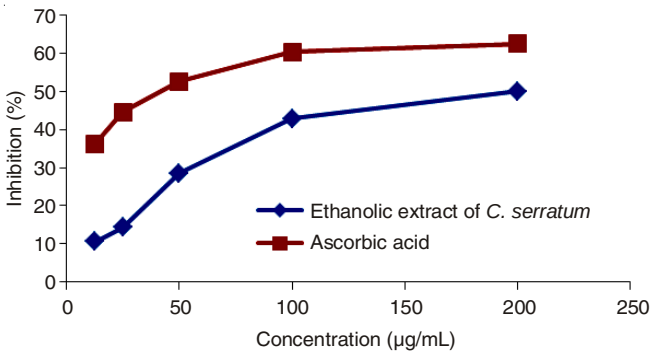

Fig. 5. Superoxide anion radical scavenging activity of ethanolic extract of *Clerodendron serratum*

of *C. serratum* leaves was found to be less (IC_{50} = 177.93 µg/mL). Their scavenging ability depends on the concentration of phenolic compounds and the number of hydroxyl groups [35]. Thus, the results shows that ethanolic extract of *C. serratum* can act as superoxide scavengers.

Reducing power activity: Reducing power of the ethanolic extract has been investigated from the Fe^{3+}-Fe^{2+} transformation using the method performed by Oyaizu [20]. Fig. 6 shows the dose response curves for reducing powers of ethanolic extract of *C. serratum* leaves. It was found that reducing power of the extract and ascorbic acid increased with an increase in the concentration and it was found to be 1.398 and 0.89 at 200 µg/mL, respectively. All doses of ethanolic extract of *C. serratum* showed higher activity than ascorbic acid and the difference was statistically significant ($P < 0.001$). The reducing properties are generally associated with the presence of reductones, which exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [36]. Antioxidant activity of phenolic compounds may be attributed to concomitant reducing power [37,38] and act as significant indicator of its potential antioxidant activity [39]. High content of phenolic compounds in the extract may contribute towards the antioxidant activity due to presence of hydroxyl group which act as hydrogen donor.

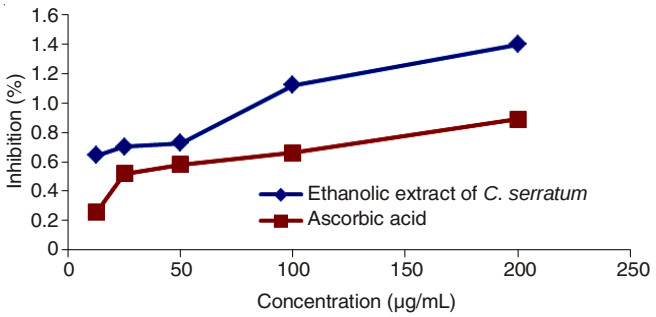

Fig. 6. Reducing power activity of ethanolic extract of *Clerodendron serratum*

Diverse studies mentioned the significance of the polyphenols and flavonoids in the antioxidant activity of different plant extracts [39,40]. Our results are in accordance with the results published by Bhujbal *et al.* [41], while the screening of *in vitro* antioxidant activity of *C. serratum* roots.

Conclusion

Preliminary phytochemical investigation revealed that the extract of *Clerodendron serratum* Linn. contained high content

of phenolic compounds and flavonoids. Antioxidant activity results showed that the ethanolic extract of *C. serratum* leaves markedly scavenge DPPH, hydroxyl, nitric oxide and superoxide radicals and also possess strong metallic reducing power. The significant antioxidant activity could be due to the presence of phenolic compounds mainly bioactive flavonoids. In present study, phenolic compounds mainly flavonoids showed a concentration dependent antioxidant activity in ethanolic extract of *C. serratum* leaves. The scavenging ability of flavonoids is due to the presence of hydroxyl groups. From the results, it can be concluded that significant antioxidant potential of ethanolic extract of *C. serratum* leaves may be attributed to the presence of flavonoids mainly apigenin and luteolin which provides justification for the traditional usage of this plant for the treatment of inflammatory and neurological disorders.

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