Asian Journal of Chemistry; Vol. 23, No. 9 (2011), 4017-4020

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

In Vitro Antioxidant Potential of Aqueous and Organic Extracts of *Clematis connata*

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(*Received*: 13 November 2010; *Accepted*: 14 May 2011) AJC-9958

The aerial parts of *Clematis connata* were extracted in methanol-water (90:10) and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol successively using partition chromatography. Total phenolic contents of all extracts were determined, using Folin-Ciocalteu reagent and ranged between 15.9 ± 0.8 to 265.7 ± 1.4. The antioxidant potential of extracts was evaluated *viz.*; DPPH, FRAP, ABTS and total antioxidant models. Ethyl acetate extract showed highest activity in DPPH (93 ± 1.6 %, IC_{50} 104 ± 1.5 µg) FRAP (6.3 ± 0.3), ABTS (0.405) and total antioxidant (1.098 ± 0.05). A strong correlations observed between total phenols, total antioxidant activity, DPPH and FRAP with R^2 values ranged from 0.7881-0.8827.

Key Words: *Clematis connata*, FRAP, DPPH, Phosphomolybdate.

INTRODUCTION

Reactive oxygen species (ROS) cause injury by reacting with biomolecules such as lipids, proteins and nucleic acids, as well as by depleting the body of endogenous enzymatic and non-enzymatic antioxidants. Health problems such as heart disease, diabetics, cancer etc are all contributed by oxidative damage. Antioxidants are substances which can prevent or slow down the oxidative damage to human body. They act as "free radical scavengers", hence prevent and repair damage done by free radicals. Antioxidants may arbitrate at different levels in the oxidative process (*e.g.*, by scavenging for free radicals and lipid peroxyl radicals, removing oxidatively damaged biomolecules and having other types of action)[1,2]. Antioxidants may also enhance immune defense and therefore low the risk of cancer and infection. Antioxidants protects against the development of chronic diseases, including coronary heart disease, atherosclerosis and certain forms of cancer.

A number of assays have been introduced for the measurement of total antioxidant activity of plant extracts and their purified constituents[3]. Two types of approaches have been taken: (i) the inhibition assays; in which the extent of scavenging by hydrogen or electron-donation of pre-formed free radicals is the marker of the antioxidant activity and (ii) assays involving the presence of antioxidant system during the generation of the radical.

Clematis connata also known as Himalayan Clematis, belonging to the family Ranunculaceae, is a deciduous climber found in Himalayas from Pakistan and Kashmir to Bhutan, Tibet and Western China. The genus Clematis, having more than 300 species, has been reported to show antioxidant, antifungal, antibacterial and antignorrheal activities[4,5], but no work has been reported on this species. Therefore, the present study was designed to evaluate the various extracts of *Clematis connata* as antioxidants using various *in vitro* models.

EXPERIMENTAL

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and gallic acid were purchased from Sigma-Aldrich (USA). All other chemicals and reagents of analytical grade were purchased from Merck (Germany).

Clematis connata was collected from P.O.K. identified by Dr. Zaheer-ud-Din Khan (Taxonomist) and a voucher specimen was deposited at Sultan Herbarium at Department of Botany, GC University, Lahore, Pakistan.

Extraction: The plant material was shade dried, powdered and extracted by percolation method for 7 days at room temperature (3.0 kg) with 10 L methanol-water (90:10). The crude extract (CM); 212.8 g, 7.09 %, was filtered through Whatman filter paper No. 40, concentrated using rotary evaporator and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol which resulted in CH (36.4 g, 17.1 %), CC (18.4 g, 8.6 %), CE (15.2 g, 7.1 %) and CB (70.1 g, 32.9), respectively.

Determination of total phenolics: The total phenolics in extracts of *Clematis connata* were determined using Folin-Ciocalteu reagent[6]. The extract (20 mg) was dissolved in 20 mL of methanol, 300 µL of each sample was mixed with 200 µL

of Folin-Ciocalteu reagent and 0.8 mL of 10 % sodium carbonate solution. The mixture was allowed to stand for 0.5 h and the absorption was measured at 765 nm against a blank which contained 300 µL of methanol in place of sample. The total phenolics content was expressed as gallic acid equivalents in mg/g of extract (Table-1). Correlation studies between total phenolic content and antioxidant activities in the DPPH, FRAP and phosphomolybdate assays were performed.

DPPH radical scavenging assay: The radical scavenging ability of different extracts was measured using the method of Shahwar *et al.*[6]. Methanol solution (0.2 mL) of all the extract at various concentrations (25-300 µg/mL) was added to 1 mL (0.2 mg/mL) methanol solution of DPPH and kept in dark for 0.5 h at room teperature. The absorbance was measured at 517 nm was using UV/VIS spectrophotometer.

The percentage scavenging of radical was determined by the following formula

$$\text{DPPH Inhibition (\%)} = \frac{\text{Abs. of blank} - \text{Abs. of sample}}{\text{Abs. of blank}} \times 100$$

Ferric reducing antioxidant power: Ferric reducing antioxidant power (FRAP) of the extracts was carried out using method of Shahwar *et al.*[7]. 150 µL of FRAP (FeIII-TPTZ) reagent and different concentration (50-200 µg/mL) of sample were mixed. Read the absorbance at 592 nm after 8 min addition of reagent.

Total antioxidant capacity by phosphomolybdate method: The total antioxidant capacity of the plant extracts was evaluated by the method of Pe *et al.*[8]. An aliquot of 0.2 mL (500 µg/mL) of the sample solution was mixed with 2 mL of the reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was heated at 95 ºC for 1 h and absorbance was measured at 695 nm against a blank contained 2 mL of reagent solution.

$ABTS^{\cdot+}$ assay: Stock solutions of ABTS (50 mM) and sodium persulfate (7 mM) in phosphate buffered saline (pH = 8.0) were prepared. The stock solution of sodium persulfate and ABTS was mixed in ratio of 1:99. The mixture was stored in the dark for 16 h. 100 µL (2 mg/mL) of extract was added to test tube containing 2.5 mL of $ABTS^{\cdot+}$ solution. The reaction mixture was incubated at 37 ºC for 0.5 h and read the absorbance at 734 nm using UV/VIS spectrophotometer[9].

Statistical analysis: All assays were carried out in triplet form and standard deviation (± SD) was calculated using computer program MS Excel.

RESULTS AND DISCUSSION

Pakistan has a strong tradition of herbal remedies and like most developing countries its rural population still depends mainly on the indigenous system of medicine for their health related matters[10]. It was, therefore, seemed interesting to evaluate scientifically and determine the efficacy of the aerial parts of *Clematis connata*. Antioxidant potential of this plant was evaluated using four different reported assays.

Total phenolic contents: Phenolics are the most wide-spread secondary metabolites in the plant kingdom, which have received much attention as potential natural antioxidant in terms of their ability to act as efficient radical scavengers and to exhibit strong antioxidant activity. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers[11]. Total phenolic contents in the plant extract were calculated using FC method and expressed as gallic acid equivalents mg/g of extract. Present results showed that all the extracts have moderate amount of total phenols which ranged from 63.2 ± 0.4 to 265.7 ± 1.4, among them, ethyl acetate extract contained highest contents of phenols and *n*-hexane extract has the least one.

Antioxidant activities: DPPH forms a stable molecule on accepting an electron or a hydrogen atom and thus has applications in the determination of radical scavenging activity of natural products[12,13]. The ethyl acetate extract showed maximum antiradical activity (93 ± 1.6 %), while *n*-hexane extract showed lowest activity (46 ± 1.1) as shown in Fig. 1. All other extracts exhibited remarkable scavenging activity. The chloroform extract showed least IC_{50} value 35 ± 0.8 µg with 92 ± 1.3 % DPPH inhibition (Table-1, Fig. 2). Phenolic compounds are the principal antioxidant constituents of natural products and are composed of phenolic acids and flavonoids, which are potent radical terminators[14] by donating hydrogen to radicals. The high potential of polyphenols to scavenge free radicals may be because of their many phenolic hydroxyl groups[15].

The reducing power of the extracts was determined using standard protocols such as ABTS, FRAP and phosphomolybdate assays. All extracts showed significant antioxidant behaviour and it was deduced from the results that ethyl acetate extract exhibited highest activities in all three antioxidant assays; ABTS (0.405), FRAP (6.3 ± 0.3) and total antioxidant activity (1.098 ± 0.05). FRAP and total antioxidant activity was measured at different concentration of extract to check the dose effect on the activities. It was observed from results that both assays

TABLE-1
TOTAL PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITIES OF *Clematis connata*

Extract	Total phenols[a]	DPPH (%)	IC_{50} (µg)	FRAP[b] (µM)
CM	63 ± 1.8	63 ± 1.2	125 ± 1.9	4.1 ± 0.2
CH	15.9 ± 0.8	46 ± 1.1	–	2.1 ± 0.1
CC	210.9 ± 2.1	92 ± 1.3	35 ± 0.8	5.4 ± 0.2
CE	265.7 ± 1.4	93 ± 1.6	104 ± 1.5	6.3 ± 0.3
CB	163.9 ± 1.1	90 ± 1.1	107 ± 1.0	3.8 ± 0.4
CA	99 ± 0.6	74 ± 0.8	150 ± 1.2	2.6 ± 0.2
Gallic acid[c]	–	94 ± 0.9	14 ± 0.2	7.5 ± 0.1

[a]mg GAE/g of extract, [b]Equivalent to $FeSO_4 \cdot 7H_2O$, [c]Positive control, – = Not calculated.

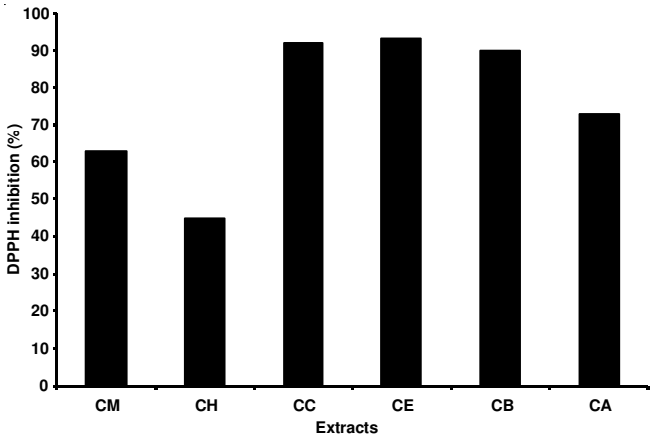

Fig. 1. DPPH scavenging activity of extracts of *Clematis connata*

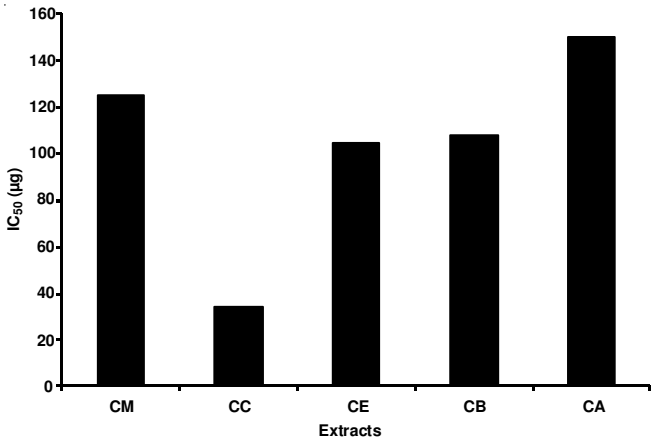

Fig. 2. IC_{50} values of extracts of *Clematis connata*

are concentration dependent (increasing the conc. of the extract enhanced the ability to reduce the ferric and molybdenium ion) as shown in Figs. 3-5.

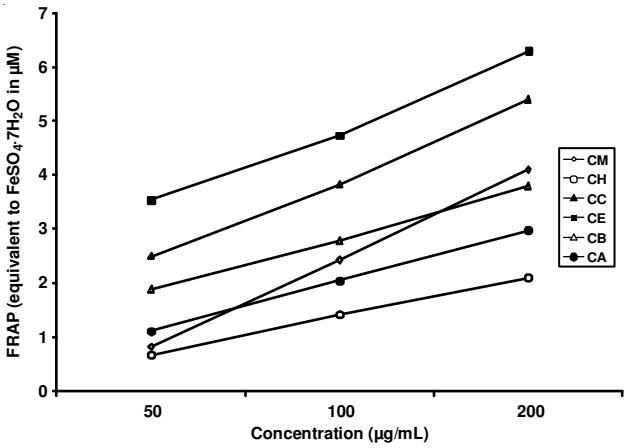

Fig. 3. FRAP assay of extracts of *Clematis connata*

The correlation studies between total phenols and DPPH, FRAP and total antioxidant were also carried out. The results revealed that there was a strong correlation between phenolics and antioxidant activities of the extracts (R^2 = 0.8827 for DPPH, 0.7915 for total antioxidant and 0.7881 for FRAP) as shown in Figs. 6-8, the extract which contained more phenolic contents showed more antioxidant activities. The total phenolics contents didn't incorporate all the antioxidants, there is

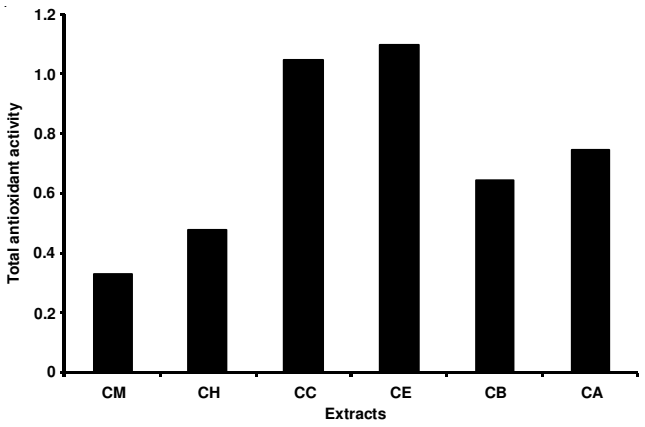

Fig. 4. Total antioxidant activity of extracts of *Clematis connata*

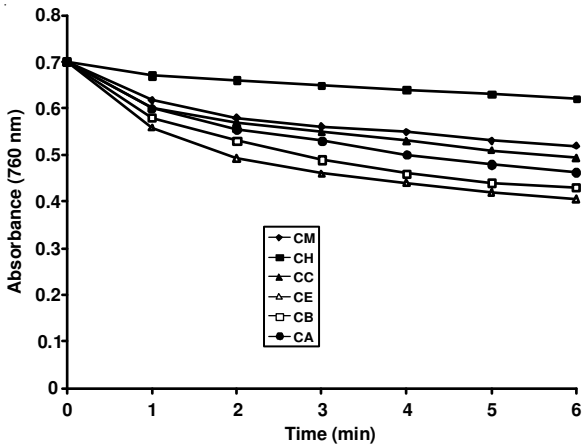

Fig. 5. ABTS assay of extracts of *Clematis connata*

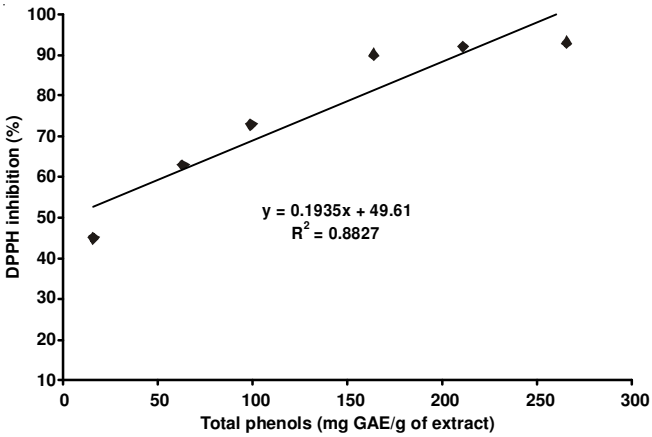

Fig. 6. Correlation between total phenols and DPPH scavenging activity of extracts of *Clematis connata*

synergism between antioxidants in the mixture. In addition, the antioxidant activities also depend upon the structure and interaction between the antioxidants. It was reported in literature that presence of - CH=CH-COOH groups, hydroxylated cinnamates ensured greater H-donating ability and subsequent stabilized the free radical than the carboxylate group in the hydroxyl benzoate[16].

Conclusion

It is well known that free radicals are the principal cause of several diseases, including Parkinson's disease, coronary

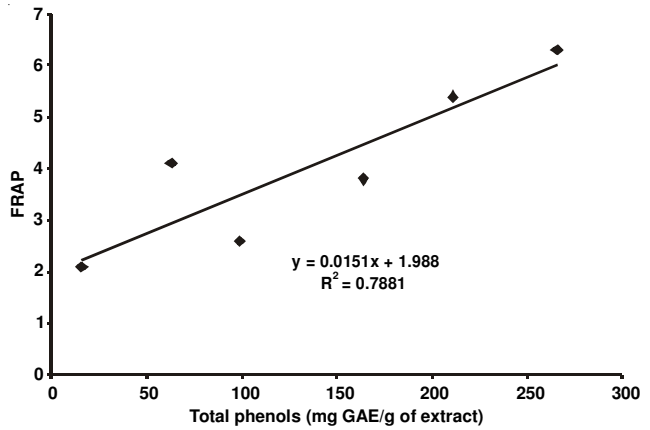

Fig. 7. Correlation between total phenols and FRAP of extracts of *Clematis connata*

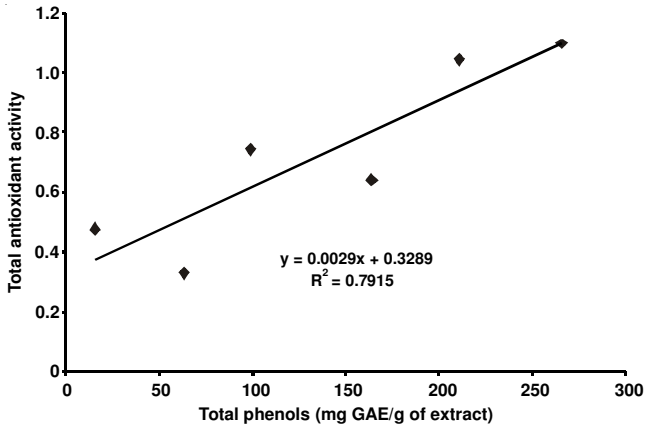

Fig. 8. Correlation between total phenols and total antioxidant activity of extracts of *Clematis connata*

heart disease, cancer and Alzheimer's disease. The results obtained in the present study indicated that aqueous and organic soluble extracts of *Clematis connata* exhibited excellent antioxidant activities. The phytochemical studies of this genera showed the presence of caffeic acid and it was concluded that antioxidants activities may be due to such type of compounds in the under consideration plant. The findings of the present study suggested that *C. connata* could be a potential source of natural antioxidant that could have great importance as therapeutic agent in preventing or slowing the process of aging and age associated oxidative stress and related degenerative diseases.

ACKNOWLEDGEMENTS

The authors are thankful to Mr. Ajaib, Department of Botany, GC University, Lahore for his cooperation regarding collection of plant materials.

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