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In vitro antioxidant activity of various extracts of *Aristolochia bracteolata* leaves

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SUMMARY

Aristolochia bracteolata is being used in traditional medicine for a variety of ailments. Three successive and two crude extracts of its leaves were subjected for *in vitro* antioxidant activity using seven different methods. The successive ethyl acetate and crude methanol extracts have shown potent antioxidant activity in ABTS method with IC_{50} values of 17.08 ± 0.44 and 28.12 ± 2.87 µg/ml, respectively. The crude methanol and successive chloroform extracts have shown moderate antioxidant activity in scavenging of hydroxyl radical by p-NDA method. All the extracts have shown moderate to low antioxidant activity by other methods.

Key words: *Aristolochia bracteolata*; Extracts; Scavenging; Antioxidant

INTRODUCTION

Aristolochia bracteolata Lam, (Family Aristolochiaceae) is a perennial herb found as a weed in black soil in Bengal, Western Peninsula, Deccan, Kerala and Karnataka, India. Its roots, leaves and juice of the whole plant are used medicinally (Anonymous, 1985). The leaves are reported to possess anthelmintic and catatonic properties. It is also used in the treatment of syphilis, gonorrhoea, eczema, boils, foul ulcer and in other skin diseases (Satyavati *et al.*, 1976). It is one of the prominent ethnobotanical antidotes for snake bite. Allantoin and aristolochic acid, isolated from this were found to be potent inhibitiors of several basic enzymes of some snake venoms (Garg, 2000).

In vitro antioxidant activity of its ethanol extract was carried out using DPPH and nitric oxide methods (Shirwaikar and Somashekar, 2003). The extract has also shown potent wound healing, antiinflammatory and antioxidant properties (Shirwaikar *et al.*, 2003). A significant increase in the levels of two powerful antioxidant enzymes, SOD and catalase was observed in the granuloma tissue (Shirwaikar *et al.*, 2003). *Aristolochia giberti*, another species also exhibited potent antioxidant activity (Velazquez *et al.*, 2003). The present study was carried out to confirm the antioxidant activity in several models and to know the nature of the potent extract. Three successive and two crude extracts of *Aristolochia bracteolata* leaves were screened for *in vitro* antioxidant studies using seven different methods.

MATERIALS AND METHODS

Collection

Aristolochia bracteolata was collected during September 2003 from village Sayala, Surendranagar District,

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Gujarat, and was authenticated by Dr S Rajan, Medicinal Plants Survey and Collection Unit, Government Arts College, Ootacamund, India.

Extraction

Successive extraction

The fresh leaves were shade dried, powdered and extracted (100 g) successively with 600 ml each of petroleum ether (60 - 80°C), chloroform, ethyl acetate and methanol in a Soxhlet extractor for 18 - 20 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40 - 50°C). The petroleum ether extract yielded a dark brown sticky solid, weighing 2.849 g. The chloroform extract yielded a black semi-solid residue weighing 0.374 g. Similarly, the ethyl acetate and methanol extracts yielded black and dark brown semi-solid residues, weighing 0.350 g and 0.542 g, respectively.

Crude extraction

The powdered leaves (100 g) were also subjected to extraction with methanol (500 ml) in a Soxhlet extractor for 18 - 20 h. The extract was concentrated similarly, to yield a green coloured semisolid residue, weighing 22.950 g. Similarly, a crude distilled water extract was also prepared by heating leaves powder (25 g) in a 500 ml round bottom flask under reflux for 2 h with 250 ml of distilled water. The mixture was cooled, filtered and the filtrate was concentrated similarly to yield a black solid residue, weighing 10.705 g. All the extracts were preserved in a refrigerator till further use. The petroleum ether extract was not studied for antioxidant activity due to its insolubility in dimethyl sulfoxide (DMSO).

Preparation of test and standard solutions

All the five extracts of *Aristolochia bracteolata* and three known antioxidants ascorbic acid, rutin and butylated hydroxy anisole (BHA) were dissolved in distilled DMSO separately and used for the *in vitro* antioxidant testing using six different methods, except the hydrogen peroxide method. For the hydrogen peroxide method, the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain the lower dilutions.

Chemicals

2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) diammonium salt were obtained from Sigma Aldrich Co, St Louis, USA. Rutin and p-nitroso dimethyl aniline (p-NDA) were obtained from Acros Organics, New Jersey, USA. Ascorbic acid, Nitro blue tetrazolium (NBT) and BHA were obtained from SD Fine Chemicals Ltd., Mumbai, India. 2-Deoxy-D-ribose was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Sodium nitroprusside was obtained from Ranbaxy Laboratories Ltd., Mohali, India. Naphthyl Ethylene Diamine Dihydrochloride was obtained from Roch - Light Ltd., Suffolk, England. Sulphanilic acid was obtained from E-Merck (India) Ltd., Mumbai India. All chemicals used were of analytical grade.

***In vitro* antioxidant activity**

DPPH radical scavenging method

The antioxidant activity of various extracts of *Aristolochia bracteolata* and the standard compounds were assessed on the basis of radical scavenging effect on the DPPH stable free radical (Shreejayan and Rao, 1996; Hwang *et al.*, 2001). The extract or standard solution (10 μl) was added to DPPH in methanol solution (200 μl) in a 96-well microtitre plate (Tarsons Product (P) Ltd., Kolkata, India). After incubation at 37°C for 30 min, the absorbance of each solution was determined at 490 nm using Elisa microtitre plate reader (Bio Rad Laboratories Inc, California, USA, Model 550). The corresponding blank readings were also taken and the remaining DPPH was calculated. IC_{50} value is the concentration of sample required to scavenge 50% DPPH free radical.

Nitric oxide radical inhibition assay

Nitric oxide is a free radical and scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marcocci *et al.*, 1994). Nitric oxide was generated from sodium nitroprusside and measured by the modified Griess Ilosvog reaction (Garrat, 1964; Badami *et al.*, 2003).

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml) and extracts or standard solutions (1 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was removed, 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completing diazotisation, and 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min. A pink coloured chromophore was formed in diffused light. The absorbance was measured at 540 nm against the corresponding blank solutions in microtiter plates using ELISA reader. IC_{50} values were calculated.

Scavenging of ABTS radical cation

The principle behind the technique involves the reaction between ABTS and potassium persulphate to produce the ABTS radical cation, a bluegreen chromogen. In the presence of the antioxidant reductant, the colored radical cation is converted back to colourless ABTS, the absorbance of which is measured at 734 nm (Re *et al.*, 1999). To 0.2 ml of various concentrations of the extracts or standards, added 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution. Absorbance of these solutions was measured spectrophotometrically, after 20 min at 734 nm. Percentage scavenging was calculated from the control where no extract or standard was present. IC_{50} values were calculated.

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS), pH 7.4. Various concentrations of the extracts or standards in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm after 10 min against a blank solution that contained extracts or standards in PBS without hydrogen peroxide (Guddadarangavvanahally *et al.*, 2004). IC_{50} values were calculated.

Scavenging of hydroxyl radical by deoxyribose method

The sugar deoxyribose (2-deoxy-D-ribose) is degraded on exposure to hydroxyl radicals, generated by irradiation or by Fenton systems. If the resulting complex mixture of products is heated under acid conditions, malondialdehyde (MDA) is formed and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen (Barry *et al.*, 1987).

To the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer, pH 7.4 (20 mM), were added 0.2 ml of various concentrations of extract or standard in DMSO to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37°C. After incubation, ice-cold trichloroacetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v), in 0.25 N hydrochloric acid were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and absorbance was measured at 532 nm. A control was performed without adding the extract or standard. IC_{50} values were calculated.

Scavenging of hydroxyl radical by p-NDA method

Hydroxyl radical scavenging is measured by the inhibition of p-NDA bleaching. Hydroxyl radicals generated through Fenton reaction can bleach p-NDA specifically. Scavenging activity was measured by the extent of inhibition of bleaching in the presence and absence of the extract solutions (Elizabeth and Rao, 1990).

To a solution mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic

acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and p-NDA (0.01 mM, 0.5 ml) in phosphate buffer pH 7.4 (20 mM), were added various concentrations of extract or standard in distilled DMSO (0.5 ml), to give a final volume of 3 ml. Absorbance was measured at 440 nm. Percentage scavenging was calculated from the control where no extract or standard was present. IC_{50} values were calculated.

Scavenging of super oxide radicals by alkaline DMSO method

Super oxide was generated according to the alkaline DMSO method (Elizabeth and Rao, 1990). The reduction of NBT by super oxide was determined in the presence and absence of extracts. To the reaction mixture containing 0.1 ml of NBT (0.1 mg) and 0.3 ml of extract or standard in DMSO, added 1 ml of alkaline DMSO (1 ml, 1% water, 5 mM NAOH) to give a final volume of 1.4 ml and the absorbance was measured at 560 nm. IC_{50} values were calculated.

RESULTS

All the five extracts of *Aristolochia bracteolata* have shown potent or moderate antioxidant activity in the seven methods tested. The successively ethyl acetate and the crude methanol extracts have shown potent antioxidant activity with IC_{50} values of 17.08 ± 0.44 and 28.12 ± 2.87 μg/ml, respectively (Table 1) in ABTS method. The other extracts have shown moderate antioxidant activity in this method. Most of the extracts have shown moderate antioxidant activity in DPPH, nitric oxide and hydrogen peroxide methods. The crude methanol extract and the successive chloroform extracts have shown moderate and weak antioxidant activity, respectively in scavenging of hydroxyl radical by p-NDA method. In the scavenging of super oxide radical by alkaline DMSO method and hydroxyl radical by deoxy ribose method, the extracts exhibited weak or no activity. However, the IC_{50} values obtained for all the extracts in all the methods were found to be higher than the standards used, indicating their low activity compared to the standards.

DISCUSSION

Lipid peroxidation has gained more importance nowadays because of its involvement in the pathogenesis of many diseases like atherosclerosis, cancer, diabetes mellitus, myocardial infarction, immunological incompetence, neurodegenerative disorders and also in aging. Protective role of antioxidants against free radical mediated toxicity

Table 1. *In vitro* antioxidant activity of successive and crude extracts of *Aristolochia bracteolata*

Sample	IC_{50} Value ± SE* (μg/ml) by method						
	DPPH	Nitric Oxide	ABTS	H_2O_2	Deoxyribose	p-NDA	Alkaline DMSO
SUCCESSIVE EXTRACTS							
Chloroform	400.00 ± 6.19	80.00 ± 2.60	117.00 ± 5.00	176.66 ± 3.33	438.30 ± 7.80	360.00 ± 12.09	455.00 ± 0.50
Ethyl acetate	96.87 ± 1.87	96.87 ± 1.87	17.08 ± 0.44	103.33 ± 1.66	655.00 ± 6.13	>1000	770.00 ± 0.24
Methanol	91.00 ± 4.23	92.75 ± 4.99	122.33 ± 1.33	435.00 ± 2.89	>1000	>1000	>1000
CRUDE EXTRACTS							
Methanol	123.00 ± 5.02	138.50 ± 3.12	28.12 ± 2.87	177.00 ± 9.17	>1000	184.37 ± 4.12	720.00 ± 0.12
Water	130.00 ± 4.75	>1000	146.00 ± 2.30	425.00 ± 12.59	880.00 ± 7.63	>1000	>1000
STANDARDS							
Ascorbic acid	2.79 ± 0.08	-	11.25 ± 0.49	-	-	-	>1000
Rutin	3.81 ± 0.11	98.45 ± 2.30	0.50 ± 0.01	36.16 ± 0.16	-	205.83 ± 0.83	>1000
BHA	-	-	-	24.75 ± 0.14	74.66 ± 1.45	-	>1000

*Average of three determinations

is now well established (Halliwell, 1994). Several plant extracts and phytoconstituents have shown potent antioxidant activity (Tripati *et al.*, 1996).

In the present study, five extracts of *Aristolochia bracteolata* were studied for *in vitro* antioxidant activity using seven standard methods. The successive ethyl acetate and crude methanol extracts have shown potent antioxidant activity in ABTS method. These two extracts were shown moderate antioxidant activity in all other methods. The crude water extract is found to be the least active among all the extracts. The variations in activity may be due to the fact that diversity in the basic chemical structure of phytoconstituents possesses different degree of antioxidant activity against different free radicals. The preliminary phytochemical investigation revealed the presence of phenolic compounds in the polar extracts of the plant. Plant phenolics are known to exhibit potent antioxidant activity (Jaggi and Kapoor, 1999). Hence, the observed antioxidant activity of the extracts of *Aristolochia bracteolata* may be due to the presence of these constituents. The ethanol extract of *Aristolochia bracteolata* has shown wound healing, antiinflammatory and antioxidant properties (Shirwaikar *et al.*, 2003; Shirwaikar and Somashekar, 2003). The present study also confirms the antioxidant property of the plant. The observed anti-inflammatory and wound healing activity in these studies may be due to its antioxidant nature. However, further studies are required to confirm the same. The plant merits further investigation to isolate its active constituents and to establish the activity in animal models.

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