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Original Article

Analysis of Antioxidant, Acetylcholinesterase Inhibitory Properties and Chemical Composition of Some Indian Himalayan Species of *Berberis*: A Comparative Study

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ABSTRACT

Background: Methanolic extracts of the stems of six species of *Berberis* e.g., *Berberis umbellata*, *B. vulgaris* (Syn: *B. nepalensis*), *B. insignis*, *B. asiatica,* and *B. aristata* were studied for their antioxidant properties and acetylcholinesterase inhibitory properties. **Materials and Methods :** Antioxidant property was studied in different systems of assay e.g., DPPH radical scavenging assay, superoxide radical scavenging assay, nitric oxide scavenging assay, assay to chelate metal. Acetylcholinesterase (AChE) inhibitory property was measured using AChE from electric eel. **Results:** It was observed that all the extracts scavenged DPPH, superoxide and nitric oxide radical and chelated metal ion in a dose dependent manner. The antioxidant properties were correlated with total phenol content, total flavonoid content, berberine and palmatine content. The AChE inhibitory activities of the extracts were also correlated well with berberine, palmatine, total phenol and total flavonoid content. A few phenolic compounds were detected by GC-MS. Highest activity in all respect was observed in *B. aristata*. Conclusion: Methanolic extracts of the stems of *B. aristata* exhibited the highest antioxidant activity.

Keywords: Antioxidant, Acetylcholinesterase, *Berberis*, Berberine, Palmatine, Phenols.

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INTRODUCTION

Different species of *Berberis* (Berberidaceae) are commonly used in many traditional systems of medicines. The crude extracts of various parts and pure isolates of *Berberis* species were reported to possess hypoglycemic, anticancer, antifatigue, anticoagulant, antipyretic, local anesthetic, antiprotozoal, anti-bacterial, hypotensive, anti-inflammatory, and CNS-depressant activities. *Berberis* species have been found to possess tonic, stomachic, astringent, diaphoretic properties and are also useful in the treatment of jaundice, enlargement of spleen.[1]

In Ayurvedic system of medicine, *B. aristata* DC is known as 'Daruharidra'. This plant is used in various systems of indigenous medicine for treating a variety of ailments such as eye and ear diseases, rheumatism, jaundice, diabetes, stomach disorders, skin disease, malarial fever and as tonic.[2] *B. asiatica* Roxb. ex. DC. is a very common substitute to "Daruharidra".[3] *Berberis vulgaris* L. is a medicine for cystitis.[4] Extracts of Indian *Berberis* species were found to have antimicrobial properties.[5]

The generations of oxygen radicals are associated with accelerating aging, cancer, cardiovascular and neurodegenerative diseases.[6] The neuropathological occurrence associated with memory loss is a cholinergic deficit which has been correlated with the severity of Alzheimer's disease (AD).[7] Approaches to enhance cholinergic function in AD have included simulation of cholinergic receptors or prolonging the availability of acetylcholine (ACh) released into the neuronal synaptic cleft by inhibiting ACh hydrolysis by acetylcholinesterase (AChE); the latter may be achieved through the use of AChE inhibitors.[8] In the present paper we report a comparative study of alkaloid and phenol contents, antioxidant and acetylcholinesterase inhibitory properties of the stems of five Indian *Berberis* sp. e.g. *B. aristata*, *B. asiatica*, *B. umbellata* Wall. ex G. Don, *B. vulgaris* L. (Syn. B. nepalensis (DC.) Spreng.), *B. insignis* Hook. f. and Thomson.

MATERIALS AND METHODS

Plant Materials

The stems of *Berberis umbellata* (Voucher no. Bot 332B-4), *B. vulgaris* (*B. nepalensis*) (Voucher no. Bot 332B-2), *B. insignis* (Voucher no. Bot 332B-3), *B. asiatica* (Voucher no. Bot 332B-5) and *B. aristata* (Voucher no. Bot 332B-1) were collected from Darjeeling and adjoining areas of India. The extracts were made from the dried ground materials by refluxing with 100% methanol for 5 hr. The extracts were then evaporated to dryness.

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Chemicals

5,5' Dithiobis (2 nitrobenzoic acid), acetylthiocholine iodide were obtained from Sisco Research Laboratories PVT. Ltd., India. Acetylcholinesterase from *Electropus electricus* (electric eel) was purchased from Sigma. All other reagents were of analytical grade. Berberine and palmatine were gifts from Prof. *B. talapatra*, University of Calcutta.

DPPH Radical Scavenging Activity

The antioxidant activity of the extracts on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined following the method described by Braca *et al*. (2001).[9] The extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated as [(Ao- Ae)/Ao]x100 (Ao = Absorbance without extract; Ae = absorbance with extract).

Superoxide radical (O2-.) scavenging activity

Superoxide radical scavenging activity was determined following the method used by Dasgupta and De (2004)[10] in the riboflavin-light-nitrobluetetrazolium (NBT) system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer pH 7.8, 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT (75 µM) and 1 ml sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks.

Metal Chelating Effect (Ferrous ion)

The method is based on the chelation of ferrous ions by the plant extract.[11] Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of plant extract (chelating agents) the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelators. Fe^{2+} chelating ability is due to antioxidant activity of the plant extract.

Nitric Oxide Scavenging Activity

Nitri oxide scavenging activity was measured modifying the methods of Sumanout *et al*., (2004).[12] Sodium nitroprusside (100mM) (0.2 ml) dissolved in phosphate buffer saline (pH 7.4) (PBS) and different concentrations of the crude extract dissolved in PBS (1.8 ml) were incubated at 25°C for 2.5 hr. Nitric oxide generated was detected by Griess reagent (2 ml). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm.

Determination of Total Antioxidant Capacity

The assay is based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH.[13] The antioxidant capacity is expressed as ascorbic acid equivalent (AAE). Extract (0.3ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95°C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank. Total antioxidant capacity of plant extract was measured from the regression equation (y=0.0114x + 0.0664) prepared from the concentration versus optical density of ascorbic acid.

Acetylcholinesterase Inhibitory Activity

Acetylcholinesterase inhibitory property was measured modifying the method of Ellman *et al*., (1961)[14] AChE from electric eel was used for assay. Different concentrations of methanolic solutions of plant extract (0.01ml) were added to 0.02 ml AChE (19.93 unit/ml buffer, pH 8) and 1ml of buffer. The reaction was started by adding 0.01 ml 0.5 mM 5,5' dithiobis (2 nitrobenzoic acid) (DTNB) and 0.02 ml. 0.6mM acetylthiocholine iodide solution. The reaction mixture was incubated at 37°C for 20 min. The optical density was measured at 412 nm immediately. The percentage inhibition of AChE activity by plant extract was calculated.

Determination of Total Phenol Content

Phenol content was determined by Folin-Ciocalteau reagent in alkaline medium and was expressed as gallic acid equivalents (GAE).[15] Total phenol content was calculated from the regression equation (y = 0.0193x – 0.0006) prepared from a range of concentrations of gallic acid and optical densities for the concentrations.

Determination of Total Flavonoid Content

Total flavonoid content was determined following Kim *et al*.[16] and was expressed as catechin equivalent (CE), calculated from the regression equation prepared from a range of concentrations of catechin and optical densities for the concentrations.

Thin Layer Chromatography

The dried methanol extracts were dissolved in methanol and the aliquots were chromatographed, along with the authentic samples berberine and palmatine on TLC plates (10 x 20 cm) precoated with silica gel 60 F254 (0.25 mm thickness). Samples (2-10 µl) and standard compounds (2 µl) were applied on plates by means of linomat 5 applicator (Camag, Switzerland) with the nitrogen flow providing a delivery speed of 150 nLsec-1 from the syringe. and the plates were run in the solvent system n-propanol: water: formic acid (90:8.0:0.4).[17] The compounds were identified by superimposing the UV spectra of the samples and the standards within the same R*f* values. Quantitative analyses of the compounds were done by scanning the plates using Camag TLC scanner model 3 equipped with Wincats software (Camag) (silt width condition 5.00x0.45 mm, at wavelength 266nm). Concentrations of berberine and palmatine were calculated from the peak areas in the sample extract and peak areas of known concentrations of authentic samples (regression equation for berberine: Y=30736x+79.91; regression equation for palmatine: Y=1859x+71.20).

GC-MS Analysis

The methanol extracts were dissolved in methoxyamine hydrochloride in pyridine. Internal standard ribitol was added. The samples were derivatized with N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA). A mixture of fatty acid methyl ester markers (2 µl) was added. HP-5MS capillary column of Agilent was used for GC-MS analysis using Agilent 5975 gas chromatography system. The following oven temperature programme for 37.5 min run was prefixed: oven ramp 60°C (1 min hold) to 325°C / min, 10 min hold before cool down. Other adjustments like injection temperature at 250°C, MS transfer line at 290°C and ion source at 230°C were set.[18] Helium was used as the carrier gas (carrier linear velocity 31.141 cm / s). Metabolites were identified on the basis of mass fragmentation pattern, retention time, retention index compared with Fiehn Metabolomics Library.

Statistical Analysis

The results were statistically analyzed by ANOVA (one way) and by paired *t*-test to show significant differences in activity. *P* values <0.05 were regarded as significant. Correlation coefficients to determine the relationship between two variables (concentrations and % inhibition; activity and total phenol/flavonoid content) were calculated using MS Excel software. Each experiment was repeated three to five times. Regression equations were prepared from the concentrations of the extracts and percentage inhibition due to activity. IC_{50} values (concentration of sample required for 50% inhibitory activity) were calculated from these regression equations. IC_{50} value is inversely related to the activity.

RESULTS

Our present study showed that stems of all the five species of *Berberis* e.g., *B. umbellata*, *B. vulgaris, B. insignis, B. asiatica,* and *B. aristata* contained both berberine and palmatine. Berberine content was highest in *B. aristata* followed by *B. insignis, B. asiatica, B. vulgaris, B. umbellata.* Palmatine content was also recorded highest in B. *aristata* followed by *B. vulgaris, B. insignis, B. asiatica, B. umbellata* (Table 1). Total phenol and flavonoid content in the five species of *Berberis* were also measured (Table 1). Total phenol content was highest in *B. aristata* and lowest in *B. umbellata*. Total flavonoid content was highest in *B. aristata* and lowest in *B. umbellata*. Roots, stem and leaves of eight species of *Berberis* collected from different altitudes in Garhwal, Himilaya, were analyzed for berberine by comparing colour intensity.[19] By applying HPLC, four alkaloids including berbamine, jatrorrhizine, berberine and palmatine were determined in methanol extracts from root bark, root, stem bark and stem of *Berberis* plant from different area, different species and different parts.[20] Berberine content in the root and stem bark of three different *Berberis* species *B. asiatica, B. aristata* and B. *lycium* has been quantitatively determined by HPTLC.[17] In addition, total flavonoid content and total phenol content were measured. GC-MS anslysis detected phenolic compounds in different species of *Berberis* (Table 2).

During the present study we have measured the antioxidant activity in different systems of assay e. g. DPPH radical scavenging assay, superoxide radical scavenging assay, metal chelation capacity, nitric oxide scavenging activity and total antioxidant capacity. DPPH radical absorbs at 517 nm. The colour changes from purple to yellow in the non-radical form and its absorbance at wavelength 517 decreases. Methanol extracts of the stems of different *Berberis* sp. quenched DPPH free radical in a dose dependent manner [R2 in all cases being < 0.95 (p = 0.01)]. IC_{50} values are shown in Table 3. One-way ANOVA and Dunnett's Multiple Comparison Test revealed that in this system the DPPH radical scavenging activities of the five species of *Berberis* stem extracts were not significantly different from each other. However paired t-test revealed that the activity of each species was significantly different from that of *B. aristata*. It has been found that the activities in different species were correlated (Table 4) with berberine content (R^2 = 0.928), total phenol content (R^2 = 0.619), total flavonoid content (R^2 = 0.668), and palmatine content (R^2 = 0.634).

Superoxide radical ($O^{2\cdot-}$) is of interest because it is involved *in vivo* in different disease conditions. Superoxide radical scavenging assay was based on the capacity of the extracts to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in presence of riboflavin-light-NBT system. Our results show that the stem extracts of *Berberis* species contained scavengers of superoxide radicals and that these reacted in a dose dependent manner (R^2 value being < 0.96 in all species). One-way ANOVA and Dunnett's Multiple Comparison Test revealed that the activities in different species were not significantly different. However, paired *t*-test revealed that the activity of each species was significantly different from that of *B. aristata*. The activities in different species were well correlated (Table 4) with palmatine content (R^2 = 0.901) and less correlated with berberine content (R^2 = 0.7), total phenol content (R^2 = 0.412) and total flavonoid content (R^2 = 0.601).

Table 1: Palmatine and Berberine content in *Berberis* species.

Plant Material	Palmatine content	Berberine content	Total Phenol content	Total flavonoid content
	(µg / µg extract ± SD)	(µg / µg extract ± SD)	(µg GAE / mg plant extract ± SD)	(µg CE / mg plant extract ± SD)
B. aristata	0.066 ± 0.003	0.107 ± 0.003	535.29 ± 3.66	64.38 ± 2.64
B. asiatica	0.012 ± 0.00	0.035 ± 0.001	376.56 ± 4.31	33.91 ± 0.54
B. insignis	0.015 ± 0.00	0.051 ± 0.005	411.01 ± 16.30	41.40 ± 0.27
B. umbellata	0.011 ± 0.001	0.015 ± 0.002	286.30 ± 10.86	27.39 ± 0.48
B. vulgaris	0.035 ± 0.004	0.030 ± 0.004	510.79 ± 16.35	54.90 ± 1.14

Table 2: Phenols identified from different species of *Berberis*.

	B. asiatica	*B. aristata*	*B. insignis*	*B. umbellata*	*B. vulgaris*
Benzoic acid	+	+	+	+	-
Catechol	-	-	-	+	-
Hydroquinone	-	+	-	-	+
Pyrogallol	-	-	+	-	+
4-Hydroxybenzoic acid	+	+	+	+	+
Shikimic acid	+	+	+	+	-
Quinic acid	+	+	+	+	+
Coniferyl alcohol	-	-	-	-	+
Gallic acid	-	+	+	+	-
Sinapyl alcohol	+	+	+	+	+
Ferulic acid	-	+	-	-	+
Caffeic acid	+	-	+	+	+
Kaempferol	-	-	+	+	-
Chlorogenic acid	+	-	+	+	-
Isoquercitrin	-	-	-	+	-

Table 3: IC_{50} values of *Berberis* species.

Plant Materials	Experiments				
	DPPH Radical	Super-oxide radical	Metal chelation	Nitric Oxide Inhibition	AChE inhibition
B. aristata	69.68 µg/ml	71.28 µg/ml	680.8 µg/ml	40.32 µg/ml	50.44 µg/ml
B. asiatica	114.98 µg/ml	207.96 µg/ml	2052.79 µg/ml	150.31 µg/ml	129.48 µg/ml
B. insignis	97.48 µg/ml	213.7 µg/ml	1460.85 µg/ml	98.61 µg/ml	115.35 µg/ml
B. umbellata	139.55 µg/ml	187.86 µg/ml	2255.25 µg/ml	206.7 µg/ml	152.09 µg/ml
B. vulgaris	114.38 µg/ml	177.76 µg/ml	1146.67 µg/ml	67.03 µg/ml	102.68 µg/ml

Table 4: Correlation (R^2) between activity and alkaloid and phenol content.

Activity	Berberine	Palmatine	Total phenol	Total flavonoid
DPPH radical	0.923	0.634	0.619	0.668
Superoxide radical	0.700	0.901	0.412	0.601
Metal chelation	0.646	0.818	0.917	0.971
Nitric oxide	0.553	0.663	0.961	0.921
AChE Inhibition	0.853	0.914	0.827	0.915

Ferrous ion chelating property of different *Berberis* species were compared. Percentage metal chelating activity was proportional to the concentration of the extract in all species (R^2 <0.98; p = 0.001). One-way ANOVA and Dunnett's Multiple Comparison Test revealed that the activities in different species were not significantly different. The activities in different species were well correlated (Table 4) with palmatine content (R^2 = 0.818), total phenol content.

Antioxidant activities of five different species of *Berberis* were also tested *in vitro* for nitric oxide scavenging activity. The extracts scavenged nitric oxide and the activity was proportional to the concentration. On the basis of IC_{50} values highest activity was observed in *B. aristata* followed by *B. insignis, B. vulgaris, B. asiatica, B. umbellata*. One-way ANOVA and Dunnett's Multiple Comparison Test revealed that the activities in different species are not significantly different. However paired *t*-test revealed that the activity of each species was significantly different from that of *B. aristata*. The activities in different species were well correlated (Table 4) with total phenol content (R^2 = 0.961) and total flavonoid content (R^2 = 0.921) and less correlated with berberine content (R^2 = 0.553) and palmatine content (R^2 = 0.663) content. Total antioxidant capacity of the methanolic extracts of the stems of different *Berberis* sp. was measured (Table 5). Total antioxidant capacity of 1 mg extract was highest in *B. aristata* being equivalent to the activity of 27.90 µg ± 1.73 ascorbic acid and lowest in *B. umbellata* being equivalent to 15.26 µg ± 0.74 ascorbic acid.

Methanol extracts of all the *Berberis* Species studied inhibited AChE activity in a dose dependent manner. One-way ANOVA and Dunnett's Multiple Comparison Test showed that the activities in different species were significantly different. The activities in different species were well correlated with palmatine and berberine content (Table 3). It was found that the activity was proportional to the berberine and palmatine content (R^2 = 0.853 and R^2 = 0.914 respectively) in the species studied and the activities in different species were also well correlated (Table 3) with total phenol content (R^2 = 0.827) and total flavonoid content (R^2 = 0.915).

DISCUSSION

Previous studies have shown that a few *Berberis* species had antioxidant activity. *B. vulgaris* fruits demonstrated antioxidant activity and reduced cell viability of human liver cancer cell line.[21] *B. aristata* was effective scavenger of DPPH free radical as well as an effective inhibitor of polyphenol oxidase.[22] The blood glucose lowering potential of *B. aristata* stem bark extract (methanolic extract) in alloxan-induced diabetic rats as well as its *in vitro* antioxidant property has been reported.[23] Cytoprotective and hydroxyl radical scavenging activity of root bark[24] of B. *vulgaris* have been reported. Antioxidant activity of *B. koreana* bark,[25] roots, twigs, and leaves of *B. vulgaris* and *B. croatica*,[26] B. *microphylla* fruit[27] have been reported. Different phenols and flavonoids are reported to have antioxidant properties.[6] Little work has been done on the phenols and flavonoids in *Berberis* species. Activity-guided fractionation of the MeOH extract from the root bark of *B. vulgaris* led to the isolation and identification of three phenolic compounds, N-(*p*-trans-coumaroyl) tyramine, cannabisin G and (+/-)-lyoniresinol. Of these, cannabisin G and (+/-)-lyoniresinol exhibited hydroxyl radical scavenging activity and cytoprotective activity in cultured MCF-7 cells modulated by hydrogen peroxide.[24] According to Koncic *et al.*[26] antioxidant activity of *B. vulgaris* and *B. croatica* correlated well with the content of phenols and flavonols. Berberine and palmatine have previously been reported to have antioxidant properties.[28] From the Table 3, it is apparent that there is a correlation between antioxidant activity and total phenol content, total flavonoid content and the alkaloid contents. Synergistic activity between alkaloids and phenols are suggested in different *Berberis* species studied for their antioxidant activity.

Table 5: Total antioxidant capacity.

Plant material	Total antioxidant capacity (µg AAE / mg plant extract ± sd)
B. aristata	27.90 ± 1.73
B. asiatica	15.57 ± 0.62
B. insignis	17.62 ± 0.27
B. umbellate	15.26 ± 0.74
B. vulgaris	20.22 ± 0.49
B. vulgaris	20.22 ± 0.49

Most of the pharmacological activities of *Berberis* were studied in respect of berberine, palmatine and the related alkaloids. Different *Berberis* sp. has been reported to contain berberine[19] and other alkaloids jatrorrhizine and palmatine etc. Berberine and palmatine are isoquinoline alkaloids having different pharmacological properties. Previously different plant extracts containing berberine and palmatine have been reported to have AChE inhibitory properties.[28] Berberine ameliorated the spatial memory impairment in the rat model of Alzheimer's disease.[29] Phenols like gallic acid, kaempferol.[30] Caffeic acid and chlorogenic acid[31] were found to have high acetylcholinesterase inhibitory properties. More detailed work about the other constituents particularly the phenolic compounds should be carried out.

CONCLUSION

Methanol extracts of all the species of *Berberis* e.g., *B. umbellata*, *B. vulgaris, B. insignis, B. asiatica,* and *B. aristata* showed dose dependent antioxidant and acetylcholinesterase inhibitory activities. *B. aristata* exhibited highest activity. The activities were correlated with berberine, palmitine, total phenol and total flavonoid contents. Analysis of chemical composition identified the alkaloids and a number of phenolic metabolites. The role of phenolic metabolites in such bioactivities should be studied further.

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