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In- vitro Evaluation of Free-radical Scavenging Potential of Pakhenbed Leaves [*Bergenia ciliata* (Haw.) Sternb.]

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Abstract

Pakhenbed [*Bergenia ciliata* (Haw.) Sternb., Saxifragaceae] leaves were successively extracted with hexane (H), benzene (B), chloroform(C), diethyl ether (D), ethyl acetate (EA), acetone (A), ethanol (E), methanol (M) and double distilled water (W) respectively using silica gel column chromatography. The antioxidant activity of purified extracts was determined by measuring the scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, lipid peroxidation and superoxide anion generation. The fractions with significant free radical scavenging activity were further evaluated through TLC for determining the specific R_f value of bioactive components. Ethyl acetate fraction was found (99.30% inhibition) to be the best scavenger of DPPH radical at a dose of 100 µg/ml among 33 extracts which was comparable with two standards, Quercetin and Ascorbic Acid (44.67 and 47.78% inhibition respectively). NBT-NADH-PMS based highest super oxide scavenging activity (48.91% inhibition) was observed in ethyl acetate:acetone :: 1:3 fraction, which was also comparable with the Quercetin standard (72.67 %). Optimum anti-lipid peroxidation was 44.30% in ethyl acetate:acetone :: 3:1 and minimum 0.36% was observed in methanol fraction in water (1:3). The observations from this study suggest the ethno medicinal use of Pakhenbed which could be commercially exploited by the pharmaceutical industry for natural antioxidant.

Keywords: Antioxidant activity, Lipid peroxidation, TLC fingerprint, DPPH scavenging assay, Superoxide scavenging.

INTRODUCTION

The therapeutic properties of pakhenbed, *Bergenia ciliata* (Haw.) Sternb., family Saxifragaceae [BC] has been known for decades in the Indian Ayurvedic medicines and used as medicine by the tribal Hill people of Darjeeling district. It is widespread in the forest of Darjeeling Hills. It is a perennial herb with stout rootstock. The leaves are variable, sparsely hairy to glabrous, broadly obovate or elliptic, finely or sparsely denticulate or shallowly sinuate-dentate. The flowers are white, pink or purple, long cymose panicles, capsules are rounded. The out sides of rhizome are buff brown colour and pinkish brown inside. All parts of the plant are slightly aromatic in odour.

The increasing interest in alimentary applications of herbal components rich in antioxidant is due to the possible correlation between the oxidant action of free radicals and the onset of some important pathologies like fever, hoemoptysis etc [1]. This kind of correlation will lead the pharmaceutical industry to seek antioxidants that may be used in the formulation of preventive phyto pharmaceuticals. Recently consumer preference has also shifted from synthetic to natural antioxidants. It is also reported that natural components rich in antioxidant have proved to be less toxic than synthetic molecules [2].

BC leaves are well known for their medicinal properties and thus have been used to treat many diseases like pulmonary infection, dysentery, ulcer, dysuria, spleen enlargement, cough and fever. A high amount of bergenin, gallic acid, glucose, mucilage, and β-sitosterol are present in the BC leaves. For the past few decades, several medicinal preparations of BC leaves have been used in Ayurveda and also ethno botanical application as tonic and astringent. Most of the biological activities and active constituents of this plant may be related to its antioxidant nature. However, no information is available on its antioxidant properties. Based on this idea the *in vitro* antioxidant activity of extract of BC leaves was evaluated and reported hereunder. More particularly, in the present study, the distribution profile of antioxidants from non-polar to polar fractions of BC leaves were examined in different ROS scavenging and lipid peroxidation assays and further characterized through TLC based fingerprinting in order to determine their specific R_f values. The design of experiments were chosen in such a way so that the information related to the concentration and separation of antioxidants in a specific solvent system has been elucidated which will assist the pharmaceutical industry to purify the same efficiently.

MATERIAL AND METHODS

Chemicals

Solvents used for column chromatography; hexane (H), benzene (B), chloroform(C), diethyl ether (D), Ethyl acetate (EA), ethanol-80 %(E) and methanol (M). TLC plate obtained from Merck (Germany). Silica gel G60, 2,2-diphenyl-1-picryl hydrazyl(DPPH), nitro blue tetrazolium(NBT), reduced nicotinamide adenine dinucleotide phosphate sodium salt monohydrate(NADPH), phenazine methosulphate (PMS), trichloro acetic acid (TCA), thiobarbituric acid (TBA), FeSO4, 7 H2O, KOH, KH_2PO_4, formic acid, glacial acetic acid, quer-

cetine and ascorbic acid were either purchased from Sigma Chemicals (USA) or of Merck analytical grade.

Plant material

BC (Pakhenbed) leaves were collected from Kurseong local area, Darjeeling, West Bengal, India in May 2007. Taxonomic position was authenticated by Prof A.P. Das, Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The material has been deposited in the 'NBU Herbarium' recorded against the accession number 9483 dated 12-05-2008.

Animal material

Goat liver, used for determination of lipid peroxidation were purchased from slaughter house immediately after slay and was used for experimental purposes within one hour of purchase.

Leaf extracts, purification and standard preparation

A sample of 20 g of BC leaves was crushed with 100 ml of methanol within 24 h of collection. The extracts were refluxed for 2 h in a hot plate with glass beads .The flask containing methanol extracts were kept over night for cold percolation .Then the extracts were filtered through double layer of muslin cloth and volume were reduced by heating over water bath. The residual part was treated with chloroform and filtered through the same, residue was washed 3 times with chloroform again and extracts were concentrated by heating at 40^{o} C using water bath. Finally the condensed filtrate was again filtered and filtrate was used for purification of components from hydrophobic to hydrophilic in the silica gel column chromatography (volume of the column was 47.10 cc). Different solvents were used for elution one after another in four different percentage compositions with two adjacent solvents like 100:0, 75:25, 50:50 and 25:75 respectively. Solvent elution profile was monitored in accordance with the following flowchart: H (50 ml) → H: B (25 ml, 3:1)→H: B (25ml, 1:1)→H: B (25 ml, 1:3)→B (50 ml) H: B (25 ml, 3:1)→H: B (25ml, 1:1)→H: B (25 ml, 1:3)→C (50 ml)→B: C (25 ml, 3:1)→B: C (25ml, 1:1) →B: C (25 ml, 1:3) →D (50 ml)→D: EA (25 ml, 3:1)→D: EA (25ml, 1:1)→D: EA (25 ml, 1:3)→EA (50 ml)→EA: A (25 ml, 3:1)→EA: A (25ml, 1:1)→EA: A (25 ml, 1:3)→A (50 ml)→A: E (25 ml, 3:1) →A: E (25ml, 1:1)→A: E (25 ml, 1:3) →E (50 ml)→E: M (25 ml, 3:1) → E: M (25ml, 1:1)→E: M (25 ml, 1:3)→M (50 ml)→M: W (25 ml, 3:1)→M: W (25ml, 1:1)→M: W (25 ml, 1:3)→W (50 ml). After removal of solvents in vacuo, extractive values were measured and the fractions were reconstituted in methanol at a concentration of 100 µg/ml and used for antioxidant assays.

DPPH based free-radical scavenging activity

The free radical scavenging activities of each fraction were assayed using a stable DPPH, following standard method [3]. The reaction mixture contained 2 ml of 0.1mM DPPH and 0.1 ml of each methanol fraction. Simultaneously a control was prepared without sample fraction. The reaction mixture was allowed to incubate for 5 min at room temperature in the dark and scavenging activity of each fraction was quantified by decolarization at 515 nm. Percentage of free radical scavenging activity was expressed as % inhibition from the given formula:

DPPH scavenging effect (%) = A0-A1 / A0 x 100

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample.

Superoxide radical scavenging assay

The superoxide radical scavenging assay was performed using standard method [4] followed by slight modification. The reaction mixture contained 1 ml of NBT solution (156 µM prepared in phosphate buffer, pH-7.4), 1ml of NADH solution (468 µM prepared in phosphate buffer, pH-7.4) and standardized 50 times methanol diluted different fraction of the sample were added. Finally, reaction were accelerated by adding 100 µL PMS solution (60 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixture were shaken and incubated at 25^{o} C for 5 min and absorbance at 560 nm was measured against blank sample. Decreasing value of absorbance of reaction mixture indicates increasing superoxide anion scavenging activity. Percentage inhibition was calculated using the formula given above.

Anti-lipid peroxidation (ALP) assay

The degree of lipid peroxidation was evaluated by estimating the thiobarbituric acid-reactive substances (TBARS) using the standard method [5] after minor modifications. Briefly, different extracts at a concentration of 100 µg/ml were added to the goat liver homogenate. Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration of $FeSO_4$ was 0.5 mM/ml). After 30 min, 100 µl of this reaction mixture was placed in a tube containing 1.5 ml of 10% trichloroacetic acid and centrifuged after 10 min. The supernatant was separated and mixed with 1.5 ml of 0.67% thiobarbituric acid in 50% acetic acid. The mixture was heated in a 100 °C water bath at 85 °C for 30 min to complete the reaction. The intensity of the pink coloured complex was measured at 535 nm in a UV-VIS spectrophotometer (Systronics, India). ALP % was calculated using the following formula:

ALP % = {(A2-A1) / (A2-A0)} x 100 where A0 was the absorbance of control, A1 was the absorbance of sample and A2 was the absorbance of iron (Fe^{2+}) induced lipid peroxidation.

TLC purification of components, assay of antioxidants and finger printing profile

Fractions (1) EA:A :: 1:1 (2) A (3) A:E :: 1:1 (4) A:E :: 1:3 (5) D:EA :: 3:1(6) D:EA :: 1:1(7) EA:A :: 1:3 and (8) A:E::3:1 were run for TLC purification of components. Extracts were concentrated to 1 mg/ml in methanol and 1 µl of sample was spotted on two precoated TLC plates with micropipette. The plate was developed in the solvent system ethyl acetate:formic acid:glacial acetic acid:double distilled water :: 50:7:5.5:13. Plates were dried at room temperature and sprayed with 0.2% DPPH methanol solution and left for 30 min. R_f values and diameter of each spot was recorded.

Statistical analysis

All the experiments were repeated three times and the data were represented as mean ± SD and were analyzed by SPSS (version11.0). One way ANOVA was used to determine the difference among the fractions. The Duncan's multiple range test (DMRT) was used for making comparison [6]. P value < 0.05 was regarded as significant.

RESULTS AND DISCUSSION

The tribal apothecary prescribed BC leaves and formulated active medicaments for the relief of patients' sufferings from many diseases at Hills of Darjeeling. As suggested by many authors, herbal antioxidants prevent various stress induced dis-

eases in human beings. It is well known fact that free-radicals play an important role in auto-oxidation of fatty acid; as for example oxidation of muscle cholesterol may be initiated by reactive oxygen species generated during oxidation of polyunsaturated fatty acid [7]. DPPH is used as a free-radical to evaluate antioxidative activity of natural materials and degree of its discolourisation is attributed to hydrogen donating ability of test compounds, which is indicative of their scavenging potential [8]. Significant DPPH antioxidant activity of BC leaf extracts with 33 solvents at different ratio were observed (Table1).

Table 1: DPPH free radical scavenging activity in the different solvent fractions of BC leaves*.

Solvent Fraction (v/v)	*100 %*	*75 %*	*50 %*	*25 %*
Hexane in benzene	24.73 ± 0.51^{M}	20.40 ± 0.26^{O}	18.53 ± 0.50^{P}	21.87 ± 0.51^{NO}
Benzene in chloroform	22.73 ± 1.86^{N}	25.50 ± 0.26^{M}	18.77 ± 1.15^{P}	24.77 ± 0.68^{M}
Chloroform in diethyl ether	20.93 ± 1.82^{O}	20.50 ± 1.55^{O}	25.87 ± 0.40^{M}	87.40 ± 0.46^{D}
Diethyl ether in ethyl acetate	89.50 ± 0.75^{C}	92.70 ± 0.36^{B}	89.63 ± 1.26^{C}	89.50 ± 0.46^{C}
Ethyl acetate in acetone	99.30 ± 0.26^{A}	99.00 ± 0.44^{A}	82.37 ± 0.40^{E}	79.57 ± 0.59^{F}
Acetone in ethanol	74.57 ± 0.25^{G}	86.67 ± 0.35^{D}	75.40 ± 0.53^{G}	47.64 ± 0.35^{H}
Ethanol in methanol	28.17 ± 1.10^{L}	35.70 ± 0.26^{K}	25.40 ± 0.78^{M}	35.90 ± 0.10^{K}
Methanol in water	20.60 ± 0.30^{O}	42.00 ± 0.92^{J}	36.67 ± 1.26^{K}	12.63 ± 0.35^{Q}
Aqueous fraction	5.53 ± 0.40^{R}			
Quercetin standard	44.67 ± 1.53^{I}			
Ascorbic acid standard	47.78 ± 0.93^{H}			

* Values indicate percentage inhibition of DPPH free radical @ dose of 100 µg / ml over control ± standard deviations (n = 3), symbolized by the same alphabet/s (in superscript) are not significantly different at $P < 0.05$. Critical Difference (at 5% level) = 1.1337.

Comparison of scavenging effects of successive extraction on DPPH radical showed the consistently higher radical scavenging activity at all the fraction of ethyl acetate and acetone. The data showed that the extracts are free-radical inhibitors and act as primary antioxidants. Similar trends of free-radical scavenging potential were observed in seabuckthorn (*Hippophae rhamnoides L.*) fruit and leaf extracts [9]. Recently DPPH assay has also been used to determine antioxidant activity in *Tanacetum subspecies, Dracocephalum moldavica* and *Phyllanthus amarus* [10]. DPPH accept an electron or hydrogen radical to become a diamagnetic molecule. As the electron becomes paired of in the presence of free-radical scavenger the colour absorption vanishes and resulting discoloration stoichiometrically coincides with respect to the number of electrons taken up. The bleaching of DPPH absorption is a representative of the capacity of the test materials. DPPH reacts with specific antioxidant molecule which involves reduction of DPPH, primary reaction involved in this respect is $Z^{\cdot}$ (DPPH) + AH (donor molecule) $\rightarrow ZH + A^{\cdot}$.

Noticeable effect of scavenging of free-radicals was observed in the eight fractions which were further established by TLC (Table 4 and Figure 1).The R_f values of the yellow spots with purple background on the TLC plate developed after the application of DPPH (0.2% in methanol) is the recognition of antioxidant molecules present in that fraction [11].

Superoxide anion scavenging activities in the fractions were determined by NBT-NADH-PMS system. In this assay only 6 fractions showed significant superoxide scavenging activity which were comparable with reference standards. Some fractions beyond 100 µg/ml did not show considerable superoxide scavenging property which may be due to the colour masking [12]. Decrease of optical density values against control is the indication of the presence of bioactive compounds possessing super oxide radical scavenging activity. The ascending order of super oxide scavenging activity in the fractions was shown in (Table 2).

Table 2: Superoxide radical scavenging activity in the different solvent fractions of BC leaves†.

Solvent Fraction (v/v)	*100 %*	*75 %*	*50 %*	*25 %*
Hexane in benzene	3.27 ± 0.06^{M}	2.23 ± 0.31^{NO}	3.27 ± 0.06^{M}	0.26 ± 0.06^{S}
Benzene in chloroform	1.20 ± 0.10^{PQR}	0.90 ± 0.10^{R}	1.10 ± 0.10^{QR}	1.77 ± 0.15^{OP}
Chloroform in diethyl ether	4.17 ± 0.15^{L}	5.43 ± 0.15^{K}	4.17 ± 0.15^{L}	6.10 ± 0.10^{J}
Diethyl ether in ethyl acetate	42.57 ± 0.31^{D}	1.53 ± 0.35^{PQ}	28.13 ± 0.76^{G}	40.77 ± 0.51^{E}
Ethyl acetate in acetone	33.47 ± 0.81^{F}	42.77 ± 0.25^{D}	46.67 ± 0.32^{C}	48.90 ± 0.17^{B}
Acetone in ethanol	14.97 ± 0.85^{I}	22.20 ± 0.17^{H}	0.92 ± 0.12^{R}	1.47 ± 0.32^{PQR}
Ethanol in methanol	2.43 ± 0.21^{N}	2.33 ± 0.06^{N}	2.40 ± 0.17^{N}	3.50 ± 0.30^{M}
Methanol in water	2.37 ± 0.32^{N}	1.43 ± 0.21^{PQR}	1.67 ± 0.25^{PQ}	1.27 ± 0.21^{PQR}
Aqueous fraction	3.17 ± 0.15^{M}			
Quercetin standard	72.67 ± 0.32^{A}			

† Values indicate percentage inhibition of Superoxide radical @ dose of 100 µg/ml over control ± standard deviations (n = 3), symbolized by the same alphabet/s (in superscript) are not significantly different at $P < 0.05$. Critical Difference (at 5% level) = 0.4428

Superoxide radical requires a slight input of energy that is often provided by NADPH in biological systems. It can be formed enzymatically or non-enzymatically. Superoxide may decrease the activity of other antioxidant defense, an enzyme like catalase. Super oxide dismutase and catalase enzyme activity strengthen body defence mechanisms against harmful effects of oxygen free-radicals, because SOD and catalase is the key enzyme in scavenging the superoxide radicals which helps in cleaning the H_2O_2 formed during incomplete oxidation [13].

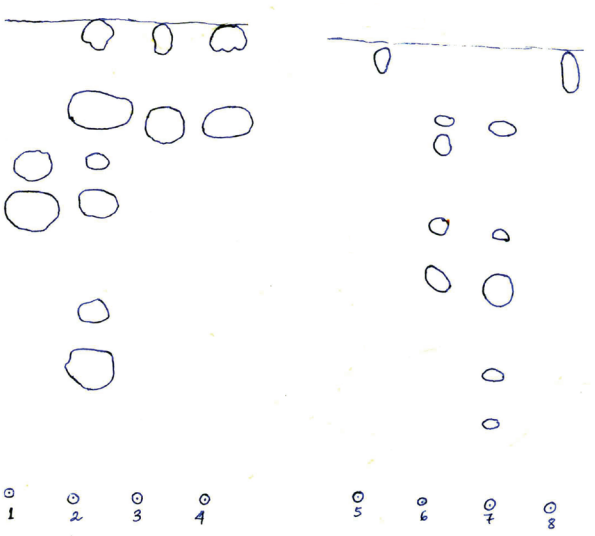

Figure: 1 TLC Fingerprint

Besides enzymatic defenses, BC leaf extract may be alleviating non-enzymatic antioxidants in the biological system Other plants which have proved their activity against superoxide anion are *Cissus quadrangularis* [14], *Orthosiphon stamineous* [15],

Rosa domacena [16] and *Helichrysum italicum* [17].

Lipid peroxidation is another important physiological event in a variety of ailment including aging, cancer, cardiovascular and respiratory diseases and rheumatoid arthritis. Hence, current interest has focused on the potential role of antioxidant in the treatment and prevention of these diseases. The lipid peroxidative degradation of biomembrane is one of the principle cause of toxicity of synthetic chemicals [18], lipid peroxidation also yield a wide range of cytotoxic products most of which are aldehyde. The BC leaves have tremendous anti-lipid peroxidation activity (Table 3) which may protect the tissues from lipid peroxidation. It is known that cleavage products of lipid peroxidation accumulate in the central nervous system and in cardiac muscle fibers [19]. BC leaf extracts have been found to reduce lipid peroxidation of microsomal tissues in goat liver. Generally malonaldehyde formed during the lipid peroxidation, may be inhibited by the antioxidant molecules present in BC extract. The inhibition could be caused by the inhibition of formation of ferryl perferryl complex [20].

Table 3: Percentage of anti-lipid peroxidation in the different solvent fractions of BC leaves†.

Solvent fractions v/v	***100%***	***75%***	***50%***	***25%***
Hexane in benzene	14.07 ± 0.21^{I}	7.73 ± 0.06^{L}	3.20 ± 0.17^{NO}	3.50 ± 0.10^{MN}
Benzene in chloroform	2.17 ± 0.15^{OPQR}	2.73 ± 0.21^{NOP}	1.53 ± 0.06^{QR}	2.03 ± 0.20^{PQR}
Chloroform in diethyl ether	2.47 ± 0.47^{NOPQ}	6.80 ± 0.10^{L}	2.47 ± 0.10^{OPQ}	12.37 ± 0.12^{J}
Diethyl ether in ethyl acetate	35.97 ± 0.95^{D}	39.60 ± 0.17^{C}	25.10 ± 0.17^{F}	21.17 ± 0.91^{H}
Ethyl acetate in acetone	23.97 ± 0.89^{G}	44.30 ± 0.15^{B}	35.90 ± 0.06^{D}	32.53 ± 0.40^{E}
Acetone in ethanol	11.33 ± 0.12^{K}	23.60 ± 0.56^{G}	2.47 ± 0.15^{NOPQ}	11.17 ± 0.15^{K}
Ethanol in methanol	14.10 ± 0.17^{I}	24.50 ± 0.25^{FG}	4.28 ± 0.07^{M}	2.17 ± 0.06^{OPQR}
Methanol in water	0.41 ± 0.09^{S}	1.37 ± 0.21^{QRS}	2.37 ± 0.32^{OPQ}	0.36 ± 0.04^{S}
Aqueous fraction	1.20 ± 0.10^{RS}			
Curcumin Standard	46.83 ± 0.76^{A}			

† Values indicate percentage inhibition of Lipid Peroxidation @ dose of 100 µg/ml over control ± standard deviations (n = 3), symbolized by the same alphabet/s (in superscript) are not significantly different at $P < 0.05$. Critical Difference (at 5% level) = 0.7881

The other plants show antioxidant activities mainly due to the presence of phenyl propanoid derivatives, like polyphenols; besides other secondary metabolites widely distributed in plant kingdom. The antioxidant activities of BC leaves may be due to its polyphenol content. The antioxidant properties are generally associated with the presence of reductones. The activity of reductones is believed to break radical chain by donation of a hydrogen atom, indicating that anti oxidative properties are concomitant with the development of reducing power [21]. Therefore, the marked antioxidant properties in different fraction may be related with its higher reducing power.

Considering all aspects of free-radicals and anti-lipid peroxidation effects, this study does confirm the effectiveness reached by the popular application of BC leaf extract as natural antioxidant in the region of Darjeeling.

In conclusion, the extracting solvents significantly affected the free-radical scavenging property and anti-lipid peroxidation of BC leaves extract. Optimal antioxidants can only be obtained by extracting BC leaves with medium polar solvents like ethyl acetate or acetone. Highly non-polar or polar solvents may not be suitable for this extraction process. Thus, our results indicate that selective extraction from natural material, by an appropriate solvent, is important for obtaining fractions with high antioxidant activity and anti-lipid peroxidation. This is a first screening report of free-radical scavenging properties of BC leaves in successive solvent fraction. The information from this study may direct the pharmaceutical industry to concentrate the antioxidants from BC leaves and also consider the same as corroboration of Ayurveda claims as phytophermaceuticals. TLC fingerprint data can also help the apothecary to recognize the true BC leaf drugs from its adulterants. Further studies are already been under taken for the characterization of individual components of bioactive fractions of BC leaf to elucidate the mechanisms underlying the bioactive properties (both in terms of phytochemistry and pharmacology).

Table 4. DPPH antioxidant activity in solvents fraction observed on TLC plate.

Spot serial No.	*Solvent fraction*	*Ratio*	*R_f values*	*Diameter of the spot (cm).*
1	EA:A	1:1	0.646 0.73	1.50 1.10
2	A		0.306 0.420 0.640 0.720 0.906 0.993	1.40 0.90 1.10 0.60 2.55 1.05
3	A:E	1:1	0.824 0.750	1.20 1.00
4		1:3	0.825 0.950	1.30 1.00
5	D:EA	3:1	0.993	0.85
6	D:EA	1:1	0.986	0.65
7	EA:A	1:3	0.513 0.420 0.805 0.720	0.80 0.60 0.60 0.45
8	A:E	3:1	0.540 0.290 0.527 0.590 0.820	0.45 0.60 0.95 0.40 0.65

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