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Evaluation of Antioxidant Activities of *Bergenia ciliata* Rhizome

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Abstract: The study was aimed to evaluate antioxidant activity of methanolic and aqueous extracts of *Bergenia ciliata* (Haw.) Sternb. rhizome. Total phenolic content was determined in both extracts. Free radical ($DPPH^{\bullet}$ and $^{\bullet}OH$) scavenging potential of the extracts revealed that both extracts to be active radical scavengers. Reducing (Fe^{+3}-Fe^{+2}) power and lipid peroxidation inhibition efficiency (TBARS assay) of both extracts were also evaluated and both extracts showed promising activity in preventing lipid peroxidation and might prevent oxidative damages to biomolecules. Furthermore, the ability of the extracts to protect DNA (pBR322) against UV-induced photolysed H_2O_2 – oxidative damage was analysed. Both the extracts were able to protect DNA from oxidative damage. The results obtained suggest that extracts of *B. ciliata* have promising therapeutic potential and could be considered as potential source for drug development by pharmaceutical industries.

Keywords: Phenolics; free radicals; lipid peroxidation; DPPH; TBARS.

1. Introduction

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide, which are generated by normal physiological processes and various exogenous factors initiate peroxidation of membrane lipids as well as a wide range of other biological molecules through a process that is believed to be implicated in the etiology of several disease conditions, including coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer [1, 2]. Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infections and degenerative diseases. However, during recent years people have been more concerned about the safety of their food and the potential effect of synthetic additives on their health. The two most commonly used synthetic antioxidants; butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have begun to be restricted because of their toxicity and DNA damage induction [3, 4]. Therefore, natural antioxidants from plant extracts have attracted increasing interests due to their safety. Antioxidants can either directly scavenge or prevent generation of ROS. Recent researches have been interested in finding novel antioxidants to combat and/or prevent ROS-mediated diseases.

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Bergenia ciliata (Haw.) Sternb., is commonly called winter bergonia. It is an evergreen perennial herb growing to 0.3 m by 0.5 m. *B. ciliata* rhizome extracts is proved to have anti-bacterial and anti-tussive properties [5, 6]. It is reported to be helpful in dissolving kidney stones [7]. *B. ciliata* is used in traditional ayurvedic medicine for the treatment of several diseases in Nepal, India, Pakistan, Bhutan and some other countries. Thus the aim of the present study was to evaluate antioxidant properties of methanolic and aqueous extracts of *B. ciliata* rhizomes by measuring scavenging activity against free radicals, reducing capacity and protection of biological molecules from reactive oxygen species-induced damage.

2. Materials and Methods

2.1 Chemicals

1, 1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), gallic acid, ascorbic acid, trichloroacetic acid (TCA) was purchased from Himedia Laboratories Pvt. Ltd. (India). Folin-Ciocalteau reagent was procured from Sisco Research Lab (India), and pBR322 was purchased from Medox Biotech India Pvt. Ltd. (India). The remaining chemicals and solvents used were of standard analytical grade and HPLC grade, respectively.

2.2 Plant material

B. ciliata rhizomes were collected from their natural habitat in the Himalayas at Rudraprayag (30º28' N, 78º98' E), Uttaranchal, India. The specimens were identified by C. Sathyanarayanan, Arya Vaidya Pharmacy (Coimbatore) Limited, Kanjikode, Palakkad, India. Collected specimens were shade dried, powdered, sieved and stored until further use. Voucher specimens were maintained in laboratory for future reference (Herbarium # VIT/SBCBE/CCL/07/6/07; Dated: June 11, 2007).

2.3 Extraction

50 g of *B. ciliata* rhizome powder was serially extracted with using methanol and water as solvents in a Soxhlet apparatus. The powder:solvent ratio was maintained as 1:6. The extracts obtained were evaporated to dryness at 40 °C in reduced pressure (methanol: 337 mbar and Aqueous: 72 mbar) in a rotary evaporator (BUCHI, Switzerland). The dried extracts were weighed to determine the yield of soluble constituents and stored in a vacuum desiccator.

2.4 Determination of Total Phenolic Compounds

Total phenolic content was determined by the method described by Lister and Wilson (2001) [8]. 50 µg, 100 µg, 150 µg, 200 µg and 250 µg of the extracts were made up to 0.5 mL with distilled water. 2.5 mL of Folin-Ciocalteau reagent (1:10 dilution) and 2 mL of sodium carbonate (7.5% w/v) were added and the tubes incubated at 45 ºC for 15 min. The absorbance was read at 765 nm using a Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA). Gallic acid was used as a standard, and results were expressed in terms of gallic acid equivalence (GAE) in µg.

2.5 Reducing power

The ability of the extracts to reduce Fe^{+3}– Fe^{+2} was accessed by the method of Yildirim et al. (2001) [9]. 50 µg, 100 µg, 150 µg, 200 µg and 250 µg of the extracts were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6; 1.79% NaH_2PO_4 and 1.89% Na_2HPO_4) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 30 min. 2.5 mL of 10% trichloroacetic acid was

later added and the tubes were centrifuged at 3000 rpm for 10 min. 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride added. Absorbance was measured at 700 nm. Increasing absorbance values of the reaction mixture indicated increasing reducing power of the extracts.

2.6 DPPH Radical Scavenging Assay

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay as described by Blois (1958) [10]. 20 µg, 40 µg, 60 µg, 80 µg and 100 µg of the extracts were taken in test tubes and made up to 0.5 mL with the respective solvents. 3 mL of 0.1 mM DPPH$^{\bullet}$ in ethanol was added to each tube and incubated in dark at room temperature for 30 min. The absorbance was read at 517 nm using a Cary 50 UV-Vis Spectrophotometer (Varian Inc., Australia). The percentage inhibition (I %) was calculated using the formula,

$$I\ \% = [Abs\ (Control) - Abs\ (Sample)] / Abs\ (Control) \times 100.$$

2.7 Thiobarbituric Acid Reactive Assay (TBARS)

The assay was performed as described by Halliwell and Gutteridge (1999) [11], in which the extent of lipid peroxidation was estimated from the concentration of malondialdehyde (MDA), a thiobarbituric acid reactive substance (TBARS), which is produced due to lipid peroxidation. The liver for the preparation of homogenate to be used in this assay was obtained from Wistar strain Albino rats after approval of the Institutional Animal Ethical Committee (PSGIMSR/27.02.2008) and was performed in accordance to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985) (NIH, 1985) [12].

50 µg, 100 µg, 150 µg, 200 µg and 250 µg of the extracts were taken in test tubes and were evaporated to dryness at 80 ºC. 1 mL of 0.15 M potassium chloride was added to the tubes followed by 0.5 mL of rat liver homogenate (10% w/v in PBS; calcium, magnesium free). Peroxidation was initiated by the addition of 100 µl of 2 mM ferric chloride. After incubating the tubes for 30 min at 37 ºC, the peroxidation reaction was stopped by adding 2 mL of ice-cold HCl (0.25 N) containing 15% TCA and 0.38% TBA. The tubes were kept at 80 ºC for 1 hour, cooled and centrifuged at 7500 rpm. The absorbance of the supernatant, containing TBA-MDA complex was read at 532 nm. The anti-lipid peroxidation activity (ALP %) was calculated using the formula,

$$ALP\ \% = [Abs\ (Control) - Abs\ (Sample)] / Abs\ (Control) \times 100$$

2.8 Hydroxyl radical scavenging activity

$^{\bullet}$OH radical scavenging activities of the extracts were estimated by the method of Klein et al. (1981) [13]. 50 µg, 100 µg, 150 µg and 200 µg of the extracts were taken in test tubes. 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of 0.018% EDTA and 1 mL of 0.85% (v/v) DMSO (in 0.1 M phosphate buffer, pH 7.4) were added followed by 0.5 mL of 0.22% (w/v) ascorbic acid. The tubes were capped tightly and incubated on a water bath at 85 ºC for 15 min. Post incubation, the test tubes were uncapped and ice-cold trichloroacetic acid (17.5% w/v) was added in each immediately. 3 mL of Nash reagent (7.5 g of ammonium acetate, 300 µl glacial acetic acid and 200 µl acetyl acetone were mixed and made up to 100 mL with distilled water) was added to all the tubes and incubated at room temperature for 15 min. Absorbance was measured at 412 nm. Percentage hydroxyl radical scavenging activity (HRSA %) was calculated by the following formula:

$$HRSA\ \% = [(Abs\ (control) - Abs\ (Sample)/Abs\ (Control)] \times 100$$

2.9 DNA damage inhibition efficiency

Potential DNA damage inhibition by *B. ciliata* extracts was tested by photolysing H_2O_2 by UV radiation in presence of pBR322 plasmid DNA and performing agarose gel electrophoresis with the irradiated DNA [14]. 1 µl aliquots of pBR322 (200 µg/mL) were taken in three polyethylene microcentrifuge tubes. 50 µg of each extract was separately added to two tubes. The remaining tube was left untreated as the irradiated control (C_R). 4 µl of 3% H_2O_2 was added to all the tubes which were then placed directly on the surface of a UV transilluminator (300 nm). The samples were irradiated for 10 min at room temperature. After irradiation, 4 µl of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) was added. The samples in all tubes were then analyzed by gel electrophoresis on a 1% agarose gel in TBE buffer (pH 8). Untreated non-irradiated pBR322 plasmid (C) was run along with the extract-treated UV-irradiated samples (methanolic extract treated = S_M and aqueous extract treated = S_A) and untreated UV-irradiated (C_R) plasmid DNA. The gel was stained in ethidium bromide (1 µg/mL; 30 min) and photographed on Lourmat Gel Imaging System (Vilbar, France).

2.10 Statistical analysis

All data were recorded as mean ± standard deviation of triplicate measurements. The significance of differences among treatment means was determined by one-way ANOVA. MATLAB ver. 7.0 (Natick, MA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

3. Results and Discussion

3.1 Extract yield and total phenolics

50 g of rhizome powder yielded 13.44 g of crude methanolic extract and 3.24 g of crude aqueous extract. Phenolic compounds are considered to contribute to the antioxidant activities of the plant extracts [15]. Total phenolic contents in the methanolic and aqueous extracts were expressed as GAE and are presented in Table 1. The methanolic extract had higher phenolic content than aqueous extract. This may be due to the difference in the polarity of the two solvents, and thereby the different phenolic components differentially eluted [16].

Table 1. Total phenolic contents of methanolic and aqueous extracts of *Bergenia ciliata* rhizome

	GAE ± SD (in µg)[a]	
Amount (µg)	**Methanolic Extract**	**Aqueous Extract**
50	19.89 ± 0.32	7.46 ± 0.04
100	33.51 ± 0.34	9.70 ± 0.09
150	48.00 ± 1.01	11.89 ± 0.02
200	59.59 ± 1.38	14.19 ± 0.13
250	61.44 ± 0.09	14.78 ± 0.08

[a] GAE ± SD at 95% confidence interval

3.2 Reducing power

Reducing capacity of the extract components may serve as a significant indicator of its potential antioxidant activity [17]. Different studies have indicated that the electron donation capacity of bioactive compounds is associated with antioxidant activity [18, 19]. Phenolic compounds are reported to be the major phytochemicals in plants responsible for antioxidant activity of plant materials [20]. Figure 1 shows the reducing activity of both extracts compared with BHT. Reducing power of both extracts increased with an increase in concentration. Methanolic extract displayed a higher reducing activity compared to the aqueous extract, and its activity at 150 µg was close to that of BHT at the same concentration.

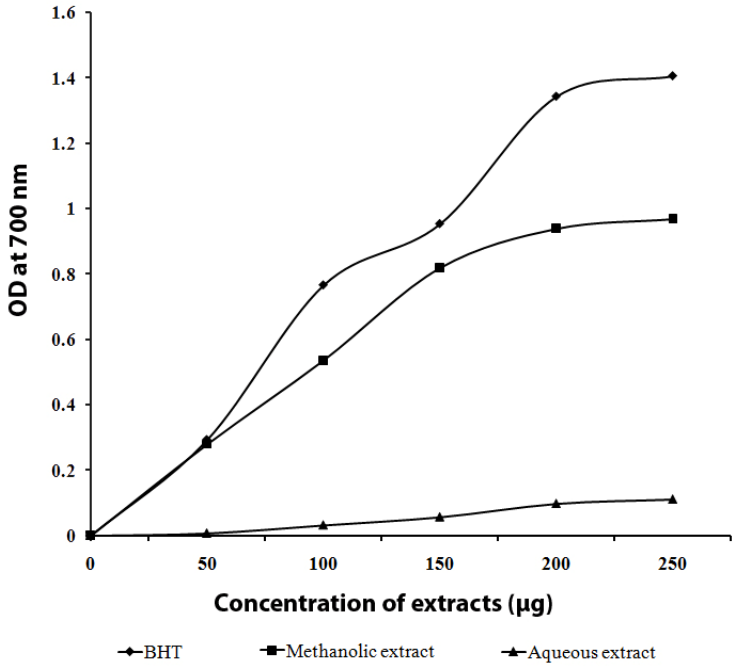

Figure 1. Reducing power of methanolic and aqueous extracts of *B. ciliata* rhizome. BHT was taken as the standard**.**

3.3 DPPH radical scavenging assay

To obtain information about the mechanisms of the antioxidative effects of the extracts, we examined their radical scavenging effects by measuring changes in absorbance of $DPPH^{\bullet}$ radical at 517 nm. Both extracts showed a concentration dependent scavenging of $DPPH^{\bullet}$ radicals. Methanolic extract was found to be more active radical scavenger than aqueous extract, thus confirming a previous report [21]. The rests of $DPPH^{\bullet}$ radical scavenging assay revealed that the extracts might prevent reactive radical species from damaging biomolecules such as lipoproteins, polyunsaturated fatty acids (PUFA), DNA, amino acids, proteins and sugars in susceptible biological and food systems [22]. The activity was compared to ascorbic acid which is employed as the standard, and results were plotted against ascorbic acid equivalence (AAE) in µg (Figure 2).

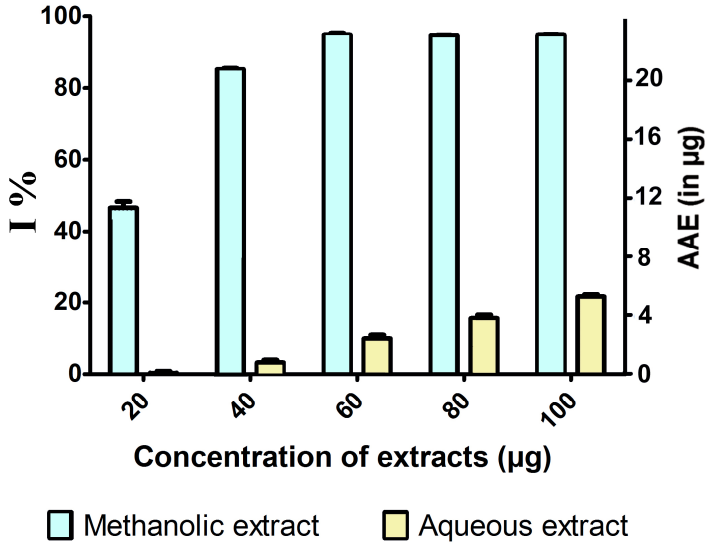

Figure 2. DPPH• scavenging activities of methanolic and aqueous extracts of *B. ciliata* rhizome with AAE (ascorbic acid equivalence) in µg. Data expressed as mean ± SD of $n = 3$ samples ($P<0.05$).

3.4 TBARS assay

Fluidity and permeability of the cell membrane is maintained by its phospholipids, glycerides, and fatty acids. Excess of free radicals attack the lipids of the cell membrane, particularly the polyunsaturated fatty acids, leading to a chain reaction of lipid peroxidation that could lead to cell death [23]. Both extracts were capable of preventing the formation of MDA in a dose-dependent manner. The methanolic extract was observed to be a significantly ($P<0.05$) better inhibitor of lipid peroxidation compared to the aqueous extract. Figure 3A shows the percentage lipid peroxidation (ALP %) potential of the extracts with their corresponding BHT equivalence (in µg).

3.5 Hydroxyl radical scavenging activity

Hydroxyl radical is an extremely reactive species formed in biological systems. It is capable of damaging almost every molecule found in living cells [24]. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, hydroxyl radical is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids [25]. Hydroxyl radical scavenging ability was estimated by generating hydroxyl radicals using ascorbic acid–iron EDTA. Hydroxyl radicals formed by the oxidation react with DMSO to yield formaldehyde, which provides a convenient method for their detection by treatment with Nash reagent [13]. Antioxidant efficiency of the two extracts was determined as their ability to scavenge the free radicals generated. The ability of the extracts to quench hydroxyl radicals can be related to the prevention of lipid peroxidation, and it seems to be a good scavenger of active oxygen species, thus reducing the rate of chain reaction. Figure 3B shows the percentage of hydroxyl scavenging activity (HRSA %) of the two extracts.

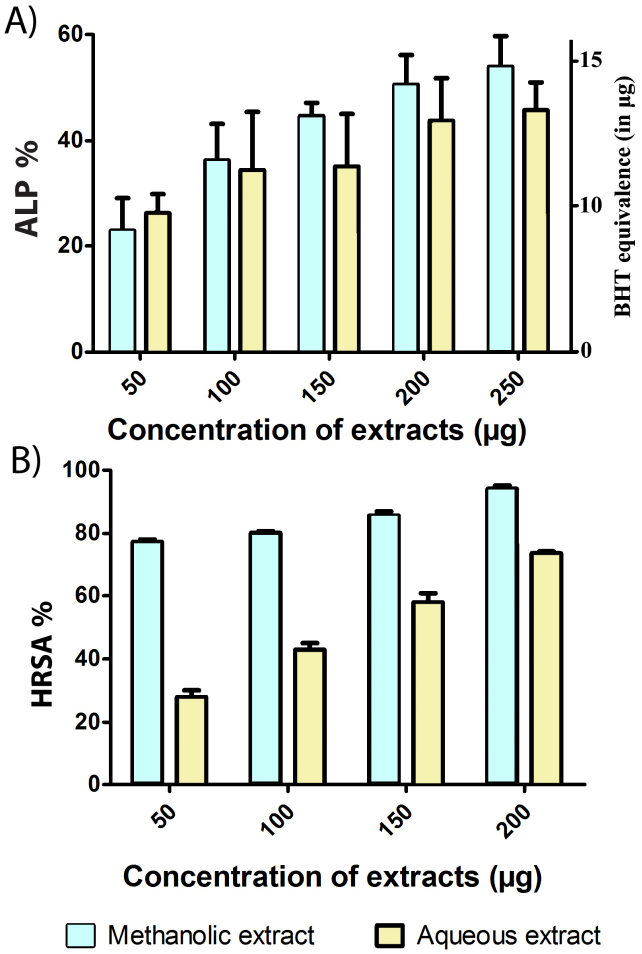

Figure 3. Antioxidant potential of *B. ciliata* rhizome. Data expressed as mean ± SD (n = 3, P<0.05). (A) Anti-lipid peroxidation activities of methanolic and aqueous extracts of *B. ciliata* rhizome with BHT equivalence in µg. (B) Hydroxyl radical scavenging activities of methanolic and aqueous extracts of *B. ciliata* rhizome.

The results obtained in all antioxidant assays showed statistically significant difference between the methanolic and aqueous extracts at P<0.05. Furthermore, to check whether the polyphenols in the extracts are responsible for these activities, correlation and regression analyses were performed. Total phenolic content of both extracts showed significant and strong positive correlation (P<0.05) with free radical (DPPH$^{\bullet}$ and $^{\bullet}$OH) scavenging efficiency and ALP (Figure 4). These results suggest a possible important role that the polyphenolic constituents of the extracts might play in free radical neutralization and lipid peroxidation inhibition.

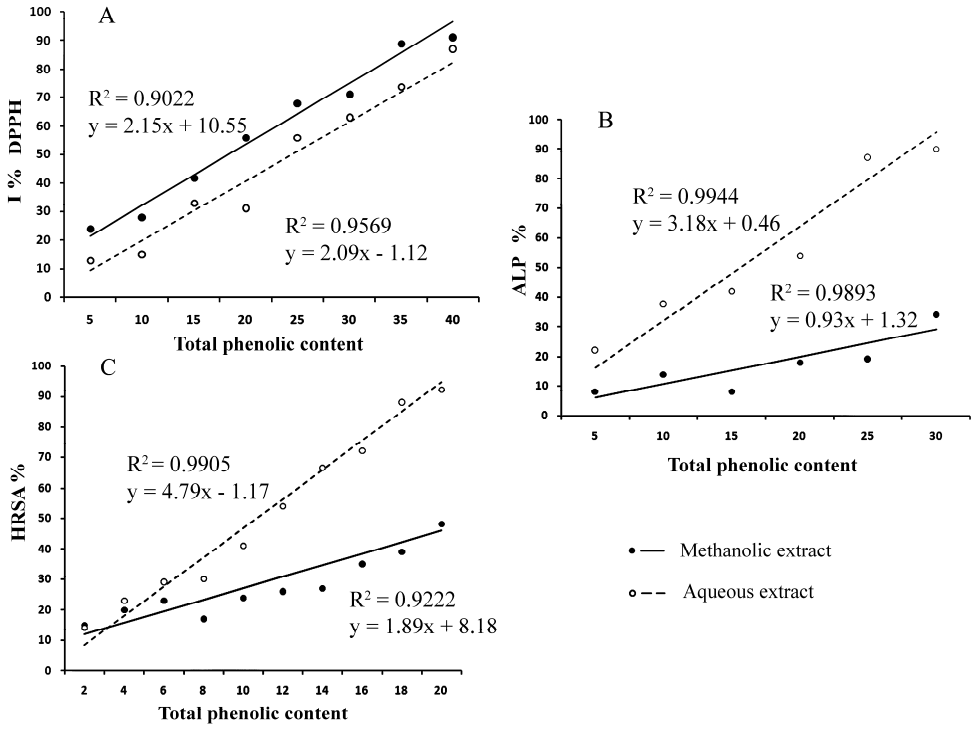

Figure 4. Relationship between total phenolic content of methanolic and aqueous extracts of *B. ciliata* rhizomeand (A) DPPH• radical scavenging potential (I %). (B) Lipid peroxidation inhibition and (C) •OH scavenging potential. All parameters show strong and significant positive correlation with total phenolic content (at P<0.05) for both extracts.

The study also showed ALP % significantly correlating with both I % (of DPPH) and HRSA % at P<0.05 (Figure 5) for both methanolic and aqueous extracts. This infers that both extracts differentially inhibit lipid peroxidation by virtue of their varying degrees of free radical quenching potential.

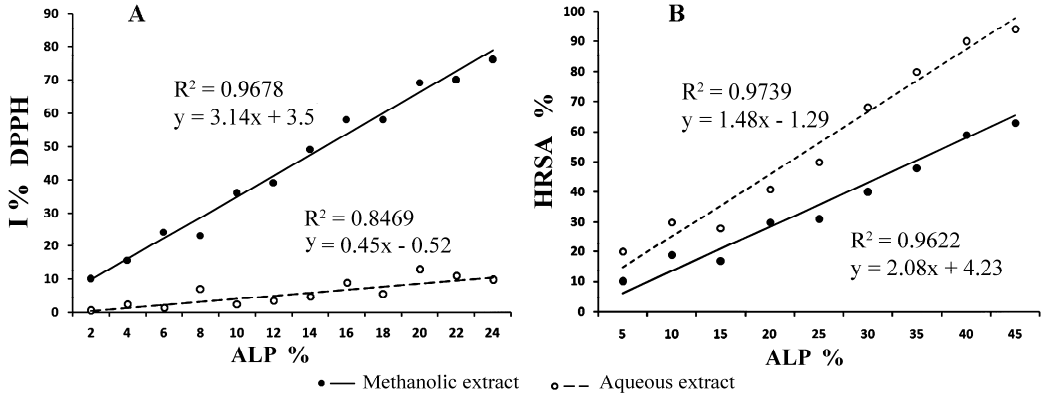

Figure 5. Relationship between total phenolic content of methanolic and aqueous extracts of *B. ciliata* rhizome *and* (A) DPPH• radical scavenging potential (I %) and lipid peroxidation inhibition and (B) OH• scavenging potential and lipid peroxidation inhibition. A strong and significant positive correlation at P<0.05 was observed for both extracts.

3.6 DNA damage inhibition efficiency

Antioxidants are found to play important role in protecting DNA from various ROS mediated damages and may be useful in the treatment of human diseases where oxygen free-radical production is particularly implicated. Extracts from medicinal plants are reported for DNA protection and their protective nature is attributed to the presence of antioxidant components [26].

Figure 6 shows the electrophoretic pattern of pBR322 DNA following UV-photolysis of H_2O_2 in absence (in controls C and C_R) and presence (in samples S_M and S_A) of the extracts. Normal pBR322 (C) showed two bands on agarose gel electrophoresis. The faster moving band represented the native form of supercoiled circular DNA (SC DNA) and the slower moving band corresponded to the open circular form (OC DNA) [14]. Both methanolic and aqueous extracts were able to protect DNA to a certain extent. The aqueous extract displayed considerably better protective activity in comparison to the methanolic extract. UV-photolysis of H_2O_2 in C_R damaged the entire DNA (no bands visible). While S_A showed a SC DNA band, though faint in comparison to C, S_M didn't show any band for SC DNA. Instead, it developed a new intermediate band for linear DNA (LIN DNA). The results infer that UV-photolysed H_2O_2 (3%) treatment of pBR322 obliterated the entire DNA (in C_R), while 50 µg of both the methanolic and aqueous extracts gave partial protection against DNA damage, with latter having higher protective potential.

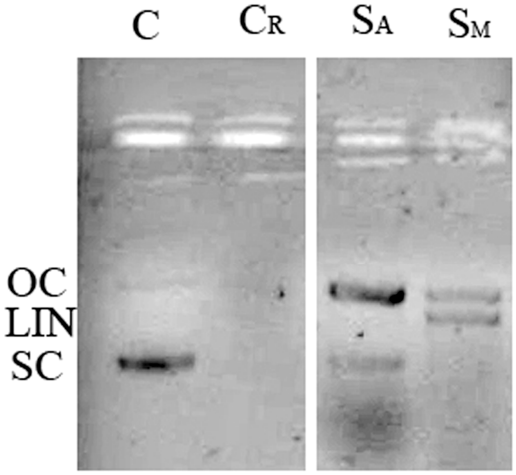

Figure 6. Effect of methanolic and aqueous extracts of *B. ciliata* rhizome at 50 µg concentration on the protection of DNA against $^{\bullet}OH$ radicals generated by photolysis of H_2O_2. Lane 1: untreated DNA (control); lane 2: 3% H_2O_2; lane 3: Aqueous extract + H_2O_2; lane 4: Methanolic extract + H_2O_2. OC = open circular DNA; LIN = linear DNA; SC = supercoiled DNA. Lanes 2, 3 and 4 contained UV-irradiated samples.

4. Conclusion

Methanolic and aqueous *B. ciliata* rhizome extracts were found to possess antioxidant activity, including reducing power, free radical scavenging activity and lipid peroxidation inhibition potential. The methanolic extract displayed greater potential in all antioxidant assays. It is, however, interesting to note that the aqueous extract demonstrated considerably higher DNA protection, albeit lagging behind its methanolic counterpart as an antioxidant. *B. ciliata* rhizome extracts might find use in pharmaceutical industry as precursors of therapeutic drugs that can be implemented as antithesis against oxidative stress and consequent toxicity to cellular biomolecules. Further studies for isolation and identification of active components are in prospect.

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