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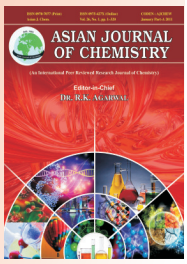

Evaluation of Two Bryophytes (*Funaria hygrometrica* and *Polytrichum commune*) as a Source of Natural Antioxidant

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Extraction of two Bryophyte plants *i.e. Funaria hygrometrica* and *Polytrichum commune* was done through methanol in order of their increasing polarity using Soxhlet apparatus. Total phenolic contents were determined with Folin-Ciocalteu reagent which ranged from 30.5 to 547 mg GAE/g of extract. *Polytrichum* showed significant but slightly less phenolics contents while that of *Funaria* showed the high phenolic contents having a value of 1630 for *Funaria* and 975 for that of *Polytrichum.* Antioxidant activities of these extracts were evaluated through DPPH$^{\bullet}$ radical scavenging, Ferric thiocyanate method, ABTS$^{\bullet+}$ Assay, FRAP Assay, Superoxide Anion Radical Scavenging Activity, Total phenolics and total flavonoids determination assay, Metal chelating activity, metal chelating effect and ferric thiocyanate (FTC) methods. In terms of TEAC values the extracts of *Funaria* and *Polytrichum* had the TEAC value as 97.5 and 71.06. The results of DPPH assay showed that *Funaria* and *Polytrichum* showed least IC_{50} values, hence they have a greater potential. The methanolic extract of *Polytrichum* showed a very high concentration of flavonoids, 3636.36 of *Polytrichum* while the other plant sample (*Funaria*) contained significant but slightly less quantities of flavonoids as 1751.8.

Keywords: DPPH, ABTS, FRAP, FTC, Radical-scavenging, Total phenolics and Total flavonoids.

INTRODUCTION

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury[1]. The major constituents of biological membranes are lipids and proteins. Reactive oxygen species can easily initiate damage of the cell membrane constituent that is, phospholipids and lipoproteins by propagating a reaction cycle[2,3]. It has been mentioned by many authors that antioxidant activity of plants is due to their phenolic compounds[4-7]. Bryophytes are considered amongst the oldest land plants. Lack of economic importance, insignificant number and size and inconspicuous distribution have made bryophytes apparently of no use when compared to their tracheophyte cousins. Bryophytes have been investigated extensively for active constituents and pharmacological activity. Bryophytes possess strong antioxidative enzymatic machinery which helps them to cope up with extreme climates and stresses[8,9]. In longer term, plant species identified as having high levels of antioxidant activity *in vitro* may be of value in the design of further studies to unravel novel treatment strategies for disorders associated with free radicalsinducedtissue damage. A number of synthetic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) have been added to foodstuffs but, are reported to cause liver disorders[10]. Therefore, attention has been directed towards the purification of natural antioxidants from botanical sources, especially edible plants. The study was conducted to evaluate the antioxidant activity of these two bryophyte plant species by applying different antioxidant assays like FTC, FRAP, DPPH radical scavenging, *etc*.

EXPERIMENTAL

Folin-Ciocalteu (FC) reagent, DPPH, BHT, α-tocopherol, gallic acid, linoleic acid and potassium thiocyanate were purchased from Fluka and Sigma-Aldrich (USA). All other chemicals and solvents were analytical grade.

Preparation of plant extracts: Whole plant samples were collected from Donga Galli Murree, Pakistan during their growing season. The plant material was authenticated at Botany Department GCU Lahore. After collection, plants were kept in laboratory (shade) at room temperature for drying. These plants were then ground in pestle and mortar to fine powdered form then extracted in methanol using Soxhlet apparatus. Crude extracts were filtered and concentrated at reduced temperature using rotary evaporator.

Determination of total phenolic content: Total phenolic contents of the extracts were determined by Folin-Ciocalteu reagent[11]. 0.1 mL of extract was combined with 2.8 mL of 10 % Na_2CO_3 and 0.1 mL of 2 N Folin-Ciocalteu reagents. After 40 min, absorbance was measured at 725 nm using UV-visible spectrophotometer (CECIL-7200). The results were determined as mg equivalent of gallic acid per gm of extractby computing with standard calibration curve ($R^2 = 0.9909$ value) constructed for different concentrations of gallic acid.

FTC assay: The antioxidant activity of extracts on inhibition of linoleic acid per oxidation was assayed by ferric FTC[12]. 0.1 mL of the ethanolic solution of the extract (5 mg/mL) was mixed with 10 mL of absolute ethanol, 10 mL of 0.2 M phosphate buffer (pH 6.0) and 2 mL of 2 % (v/v) linoleic acid. All the samples were incubated at 40 °C. At regular intervals, (48 h) 5 mL ethanol, 0.1 mL 0.02 M ferrous chloride in 3.5 % HCl and 0.1 mL of aq. 20 % ammonium thiocyanate was added in the above solution and absorbance was recorded at 500 nm. Gallic acid, BHT and α-tocopherol were used as standard reference in 2 mg/mL concentration.

DPPH radical scavenging assay: The antioxidant activity of extracts was measured in termsof radical scavenging ability by DPPH method[13]. Methanolic solution (1 mL) of different extracts at 100 µg/mL concentration was added to 1 mL methanolic solution of DPPH (2 mg/mL). The absorbance was measured at 517 nm after 0.5 h.The results were evaluated as percentage scavenging of radical (% scavenging of $DPPH^{\bullet}$ = Abs. of blank – Abs. of sample/Abs. of blank × 100). IC_{50} value (concentration of sample where absorbanceof DPPH decreases 50 % with respect to absorbance ofblank) of extracts were determined. The results were compared with standards (gallic acid and BHT).

$ABTS^{\bullet+}$ assay protocol: $ABTS^{\bullet+}$ Assay was initially developed by Miller & Rice in 1993 and later improved by[14]. For the evaluation of antioxidant activity $ABTS^{\bullet+}$ assay was done as described by Re *et al.*[14]. 7 mM solution of ABTS was prepared in double distilled water, which generated $ABTS^{\bullet+}$ while reacting with 2.45 mM potassium persulfate after 24 h on standing under dark. The $ABTS^{\bullet+}$ stock solution was diluted with PBS buffer of pH 7.4 or methanol to an absorbance of 0.70 + 0.02 at 734 nm. For the evaluation of antioxidant activity, added 10 µl of sample to 2.99 mL of diluted solution of $ABTS^{\bullet+}$ (A = 0.70 + 0.02) and noted the change in absorbance after every 1 min interval for 8 min. Appropriate solvent blank was run in parallel. All the samples were run in triplicate and mean values of absorbance were calculated. A dose response curve of Trolox was prepared by plotting its absorbance at 734 nm and % age inhibition for each sample was calculated by using this formula

$$\% \text{ age inhibition (at 734 nm)} = (1\text{-}A^f/A^0) \times 100$$

Ferric reducing antioxidant power: The ferric reducing ability of plant sample was determined by the method proposed by[15]. The FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer of pH 3.6, 2.5 mL of 10 mM TPTZ solution in 40 mM HCl and 20 mM ferric chloride (2.5 mL). The absorbance was measured at 593 nm after the mixing of 3 mL FRAP reagent, 300 µl of distilled water and 100 µl of sample. FRAP value was calculated from the standard curve of iron (II) sulphate.

Determination of total flavonoid: The TFC content of plant fractions was determined by following method developed by[16]. 0.25 mL of plant sample/quercetin standard solution was mixed with 1250 µl of distilled water in a test tube then added 75 µl of $NaNO_2$ solution. After 5 min stay added 0.5 mL of 1 M NaOH and raised the volume with distilled water to a final volume of 2.5 mL. The absorbance of this sample solution was measured at 510 nm. TFC contents were determined from the standard curve of quercetin and expressed as mg of quercetin equivalents per gram of sample.

Metal chelating activity: The ability of plant extracts to chelate Iron (II) was determined by the known method[17]. The reaction mixture was prepared by mixing 100 µL of sample with 0.05 mL of $FeSO_4$ and 0.2 mL of 5 mM ferrozine and raised its volume to 4 mL with double distilled ethanol. This reaction mixture was allowed to stand for 10 min at room temperature. Then the absorbance of the solution at 562 nm was noted. The results were expressed as the %age of bound iron, which can be calculated from theformula shown below or in terms of EDTA standard:

$$\% \text{ age bound iron} = [(\text{A control-A sample})/\text{A control}]$$

Superoxide anion radical scavenging activity: This activity of plant extracts was evaluated by the known method[18]. In this method, NADH-PMS system was employed for the *in vitro* generation of superoxide radical anions. The reaction mixture was prepared by mixing100 µl of sample, 624 µM of NADH, 200 µM NBT and 80 µM PMS in phosphate buffer (0.1M) of pH 7.4. Then absorbance of reaction mixture was measured at 560 nm. The % age scavenging of each sample was calculated from the formula:

$$\% \text{ age scavenging} = [1\text{- AS/AB} \times 100]$$

RESULTS AND DISCUSSION

Total phenolic contents: Phenolic compounds are commonly present in both edible and non-edible plants and exhibit multiple biological effects including antioxidant activity[19]. The phenolic contents of the selected plant extracts were determined by FC reagent and expressed as gallic acid equivalents in mg/g of crude extract. It is thought that phenolic compounds can attribute to the total antioxidant activity. The methanol extract of *Funaria hygrometrica* showed the highest phenolic contents (1630), while other plant sample (*Polytrichum commune*) contained relatively less phenolic contents (975). Total phenolic contents of solvent extracts are shown in Fig.1.

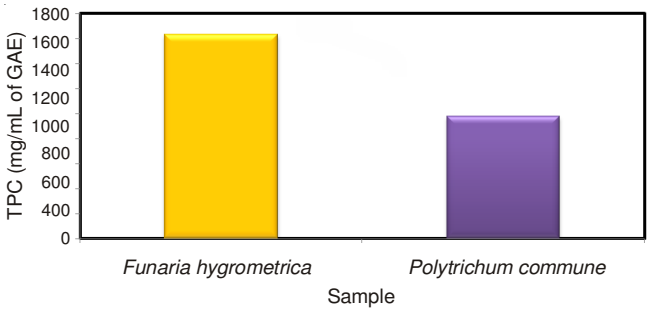

Fig. 1. Total phenol contents

Antioxidant activities

DPPH radical scavenging assay: Antioxidants react with DPPH•, which is a stable freeradical and convert it to 1,1′-diphenyl-2-picryl hydrazine[20]. The degree of decolourization of the purple coloured solution of DPPH• indicated the scavenging potential of the antioxidant compound. It was found that the radical scavenging activity of *Funaria* was more than that of BHT (78.9 %) and α-tocopherol (77.4 %), whereas for *Polytrichum* the (94.7 %) and α-tocopherol (80.6 %) (Figs. 2-3).

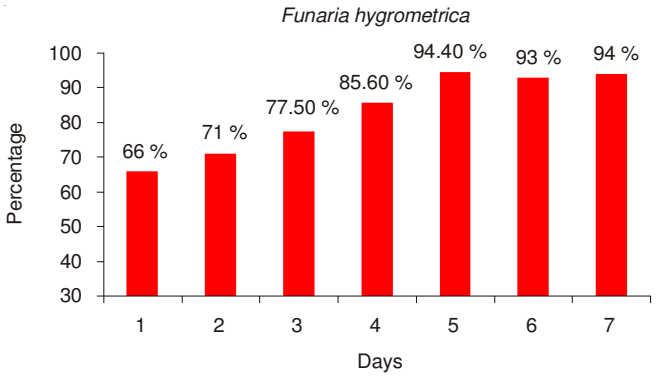

Fig. 2

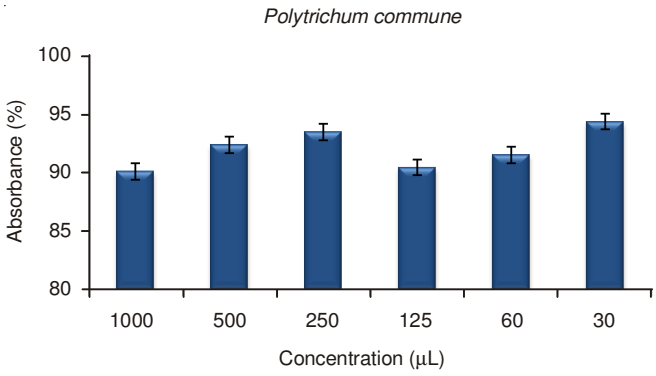

Fig. 3

Ferric thiocyanate assay: The methanolic extract of *Polytrichum* showed a maximum value of 95 % during the seven days experimentation while the lowest value was 82.1 % and *Funaria* showed a maximum value of 94.4 % and a lowest value of 66 % during the seven days. This is in agreement with the previous reported work[21-24]. The results were comparable with BHT and α-tocopherol (Figs. 4 and 5)

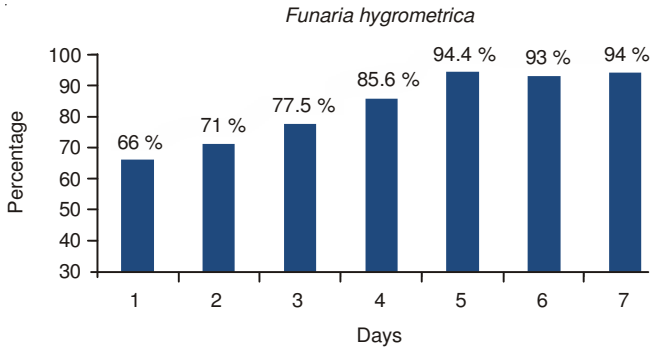

Fig. 4

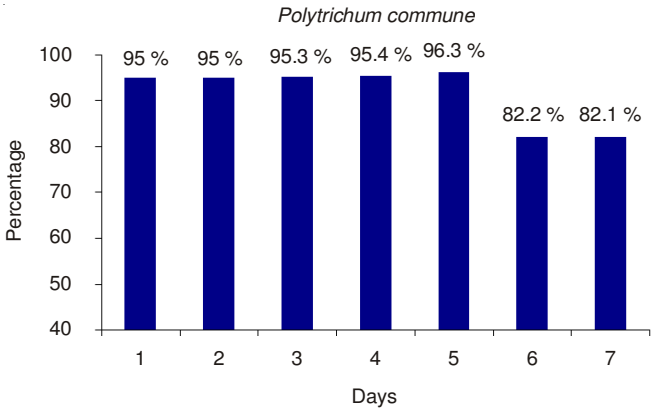

Fig. 5

ABTS•+ assay: 2,2'-Azinobis(3-ethylbenzo thiazoline)-6-sulphonic acid (ABTS) is a phenothiazine drug, when reacting with potassium persulfate, forms a green coloured radical cation. The results of ABTS assay were expressed in terms of TEAC value. As TEAC is the measure of effective antioxidant activity of the substance and stands for "Trolox equivalent antioxidant capacity". A higher TEAC value referred to a greater antioxidant potential of sample. As all the samples had a great antioxidant potential, but the extract of *Polytrichum commune* showed relatively lower potential of antioxidant activity having the TEAC value of 71.06 mM of Trolox equivalence. While the extracts of *Funaria hygrometrica* had greatly higher antioxidant potential in terms of TEAC values that is 97.5 (Fig. 6).

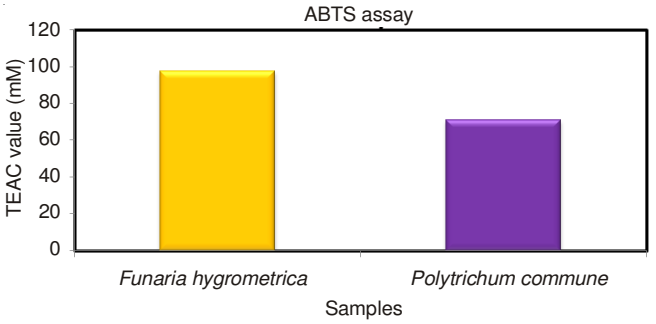

Fig. 6.

Total flavonoid contents: The principle of this colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the *ortho*-dihydroxyl groups in the A- or B-ring of flavonoids which absorbs maximally at 400 nm. The total flavonoid contents of plant are expressed in terms of quercetin equivalents. The methanol extract of *Polytrichum commune* showed a high concentration of flavonoids (3636.36) while the other plant sample (*Funaria hygrometrica*) contained significant but slightly less quantities of flavonoids (1751.8) (Fig. 7).

Superoxide radical scavenging activity: This assay employed an *in vitro* generation model [PMS/NADH/O_2] of superoxide radical anions and nitro blue tetrazolium (NBT) as a probe. The results of this assay are expressed in terms of

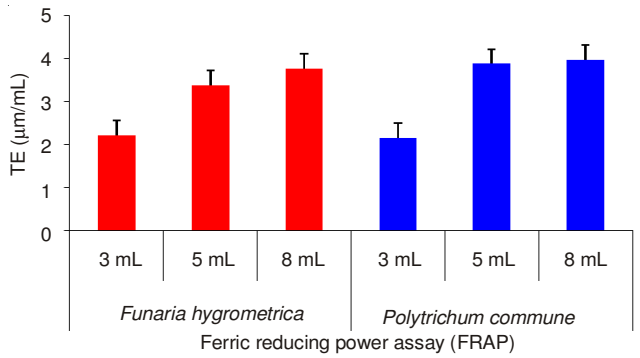

Fig. 7

% age scavenging of superoxide radical anion. The decrease in absorbance at 560 nm with the sample and the standard compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. The results of this assay showed that % age scavenging of superoxide radical anion is higher for *Polytrichum commune* (56.3). The methanol extract of *Funaria hygrometrica* showed a slightly less % age scavenging of superoxide radical anion (17.32) (Fig. 8).

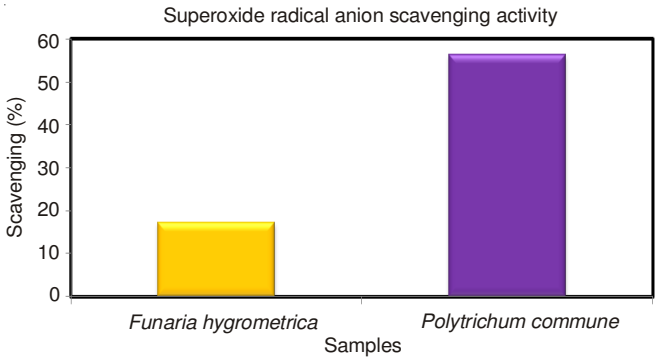

Fig. 8

Metal chelating activity: Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal. The methanol extract showed the maximum value of percentage of bound iron with *Funaria hygrometrica* (49.29), while the other plant sample (*Polytrichum commune*) also show metal chelating activity but the value of percentage of bound iron is lesser (41.9) (Fig. 9).

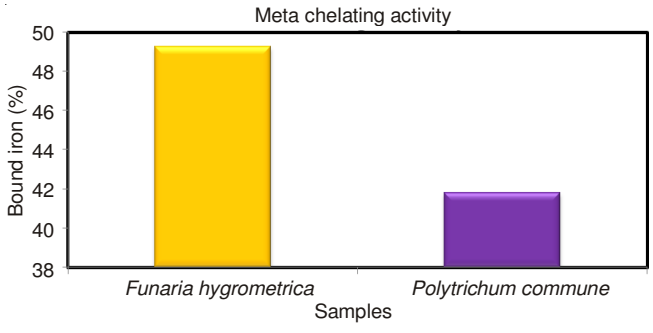

Fig. 9

FRAP Essay: The FRAP (Ferric reducing antioxidant power) assay employed ferrictripyridyltriazine complex Fe (III)-(TPTZ)2 Cl3 (pale yellow in colour) as an oxidizing agent, When it came in contact with an antioxidant, it gets reduced to Fe (II)-$(TPTZ)_2Cl_3$ (Blue in colour) and absorbs at 593 nm. The results of FRAP assay are often expressed in FRAP units. A FRAP unit can be defined as the reduction of ferric(III) to ferrous(II). A higher FRAP value referred to a higher reducing ability of the sample which means a higher antioxidant potential. The methanolic extract of *Polytrichum commune* showed the highest FRAP value (3.96 TEµM/mL), referring to highest antioxidant potential. While the other methanol extract have a comparatively less antioxidant potential (3.761 TE µM/mL) (Fig. 10).

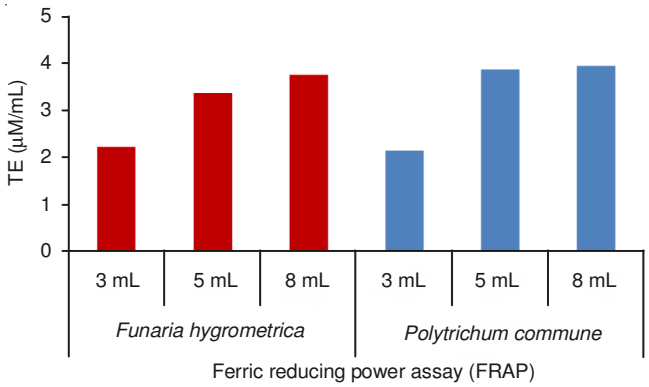

Fig. 10

Conclusion

In the past few years interest in the search of new natural antioxidants has grown because reactive oxygen species (ROS) production and oxidative stress is linked to many diseases. The use of synthetic antioxidants generally leads to problems of toxicity. In this study, antioxidant potential of methanol extracts of two different plants from the bryophytes were assayed by FTC, DPPH, ABTS, TFC, TPC, TCA, FRAP and metal chelating effect methods. The antioxidant activity of the extracts was also studied using linear regression analysis and found strongly correlated with total phenolic contents.

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