INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

EVALUATION OF PHYTOCHEMICAL CONSTITUENTS OF CRYPTOGAMS OF ALMORA DISTRICT, UTTARAKHAND FOR THEIR TOTAL PHENOLIC CONTENT, TOTAL FLAVONOID CONTENT AND ANTIOXIDANT POTENTIAL

Shweta Singh[1*], Yogesh Joshi[2] and Ajay Kumar Singh Rawat[1]

[1]Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow-226001, Uttar Pradesh, India.

[2]Department of Botany, S.S.J. Campus, Almora-263601, Uttarakhand, India.

ABSTRACT

Cryptogams are non-flowering plants having no advance vascular tissues. The Western Himalayan region is the natural niche of some rare species of cryptogams having diverse group of secondary metabolites which are widely known for their therapeutical properties and medicinal uses. In the present study, ethanolic extract of three cryptogamic plants viz *Everniastrum cirrhatum* (lichen), *Brachythecium procumbens* (bryophyte) and *Selaginella bryopteris* (pteridophyte) were used to evaluate total phenolic content (TPC), total flavonoid content (TFC) and *in vitro* antioxidant potential by DPPH and ABTS method. The TPC of aforesaid three cryptogams ranged from 25 to 105 mg GAE/gm extract and the TFC ranged from 20 to 37.5 mg QE/gm extract. The highest TPC was found in *E. cirrhatum* while maximum TFC was observed in *S. bryopteris*. A comparison of antioxidant results indicated that *E. cirrhatum* was the most effective in the DPPH (IC_{50} 0.437 mg/ml) and ABTS (IC_{50} 0.009 mg/ml) assays. A positive correlation was noted between the TPC and both the antioxidant assays. The present study suggesting that the level of antioxidant activity varies from lichen to bryophytes. Experimental results indicated that the *E. cirrhatum* may be an important source of polyphenolic compounds with high antioxidant capacity. However, proper biological identification and isolation of compounds responsible for the activity using chemical and pharmacological assays needs to be done to utilize these cryptogams for the benefit of the society.

Keywords: Antioxidant, Cryptogams, Total flavonoid content, Total phenolic content.

INTRODUCTION

Free radicals are created in form of super oxide anion, hydroxyl radical and hydrogen peroxide when cells use oxygen to generate energy. However, all living organisms have intrinsic cell management which kept under strict control by several enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase systems that scavenge free radicals. At low or moderate amount free radicals and reactive oxygen species play important physiological roles; they are essential for production of energy, synthesis of biologically essential compounds, and phagocytosis, a critical process of the immune system[1-3]. Moreover, the production of excessive free radicals stimulate oxidative damage to cell molecules (DNA, RNA, protein etc.) and such

situation contribute to more than one hundred disorder in humans including atherosclerosis, coronary heart disease, neurodegenerative disorder, cancer and they playing major role in the aging process[4,5]. The search for plant-derived antioxidants has received much attention and effort in order to identify the compounds that has high capacity in scavenging free radicals related to various diseases[6]. The most effective components seems to be flavonoids and phenolic compounds which scavenges free radicals such as peroxide, hydroperoxide of lipid hydroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases[7,8].

The Western Himalayan segment is the ecological niche of some native endemic and rare species of cryptogams[9]. The entire region is endowed with a wide range of biological wealth. It is suggested that growth conditions of medicinal plants could affect the antioxidant properties of herbal remedy through associated environmental factors such as UV stress, waterlogged conditions, and the amount of plant competition etc.[10]. Plants found in this region produce characteristic and unique secondary metabolites. The most common compounds found in cryptogams species are comprised depsidones, diphenyl ethers, dibenzofurans; flavonoids, phenylpropanoids, and bibenzyl derivatives flavonoids; quercetin, luteolin and apigenin derivatives which are beneficial for many human diseases[11-13].

In our present investigation, we explored some cryptogams to determine the total phenolic, total flavonoid contents and antioxidant activity of three cryptogamic plants (Fig. 1) viz. *Everniastrum cirrhatum* (Fr.) Hale ex Sipman. (lichen), *Brachythecium procumbens* (Mitt.) A. Jaeger. (bryophyte) and *Selaginella bryopteris* (L.) Bak. (pteridophyte) of Almora district, Kumaun Himalaya, Uttarakhand.

MATERIALS AND METHODS

Chemicals and Reagents

2,2′-Diphenyl-2-picryllhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazine-6-sulfonic acid) (ABTS), potassium persulphate, gallic acid, quercetin, ascorbic acid, aluminium chloride, Folin-Ciocalteu's phenol reagent and sodium carbonate were purchased from HiMedia and MP Biomedicals, Mumbai, India. Ethanol and other organic analytical grade solvents were used (Merck Chemicals, India).

Plant material

All the three cryptogams were collected from the region of Almora district, Kumaun Himalaya, Uttarakhand. The voucher specimen of aforesaid plant samples were confirmed by Dr. D.K Upreti (Lichenology lab), Dr. A.K. Asthana (Bryology lab) and Dr. P.B. Khare (Pteridology lab) and deposited in Herbarium of CSIR-National Botanical Research Institute, Lucknow for future identification and authentication prospect. The collected plant materials were air-dried in darkness at room temperature (20^{o}C). Dried plants were cut up and stored in tight-seal dark containers until needed.

Extraction of Plant Samples

Powdered plant materials (50 gm) was extracted with 200 ml of ethanol and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residues were re-extracted with equal volume of solvents. This process was repeated three times. Combined supernatant was evaporated to dryness under vacuum at 40^{o}C using rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4^{o}C.

Determination of Total Phenolic Content (TPC)

The concentration of phenolics in plant extracts was determined using spectrophotometric method[14]. Ethanolic solution of the extract in the concentration of 10 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of ethanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% sodium carbonate. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of sodium carbonate. The samples were thereafter incubated in a thermostat at 45^{o}C for 45 min. The absorbance was determined using spectrophotometer at λ_{max} = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration curve. Phenolic content in extracts was expressed in terms of gallic acid equivalent (mg of GAE/g of extract).

Determination of Total Flavonoid Content (TFC)

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method[15]. The sample contained 1 ml of ethanol solution of the extract in the concentration of 10 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in ethanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λ_{max} = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration curve; then, the content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg of QE/gm of extract).

Determination of Antioxidant Activity

2, 2-Diphenyl-2-picryllhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging capacity of the samples was determined by Blois[16] (1958) with some modifications. To evaluate antioxidant activity, solution of 0.135mM DPPH in ethanol was prepared and 2.9 ml of this solution was mixed with 0.1 ml of extract in ethanol containing 0.25-1 mg of the extract. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30min. The absorbance of the mixture was measured at 517nm using Double beam UV-V is Spectrophotometer. Ascorbic acid was used as reference standard. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Abs control - Abs sample) / (Abs control)] X 100

Where Abs control is the absorbance of DPPH radical + ethanol; Abs sample is the absorbance of DPPH radical + sample extract/standard. IC_{50} value (mg/ml) was calculated through linear graph equation.

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Radical Scavenging Assay

The ABTS radical scavenging capacity of the samples was determined by Arnao et al. [17] (2001) with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL ethanol to obtain an absorbance of 0.706±0.01 units at 734 nm using a spectrophotometer. A fresh ABTS solution was prepared for each assay. Different concentrations (1 mL) of dry extracts were allowed to react with 1 mL of the ABTS solution, and the absorbance was taken at 734 nm after 5 min. Ascorbic acid was used as positive controls. The percentage of ABTS inhibition was calculated using the formula:

ABTS radical scavenging activity (%) = [(Abs control - Abs sample) / (Abs control)] X 100

Where Abs control is the absorbance of ABTS radical + ethanol; Abs sample is the absorbance of ABTS radical + sample extract/standard. IC_{50} value (mg/ml) was calculated through linear graph equation.

Statistical analysis

All experiments were conducted in triplicate and values expressed as mean ± standard deviation using Microsoft excel®.

RESULTS

Determination of Total Phenolic Content (TPC)

Plants sources are effective antioxidant agents due to the presence of polyphenolics compounds. These compounds are ubiquitously present in plants and some of their parts have high content of phenolics. Phenolic compounds are good antioxidant because of presence of hydroxyl and hydrogen groups which effectively scavenge free radicals including reactive oxygen species[18]. Folin-Ciocalteau (F-C) assay was used to determine TPC of ethanolic extract of three cryptogams using Gallic acid as a standard phenolic compound. F-C method is based on oxidation of phenolics by a molybdotungstate in F-C reagent to yield a colored product with λ max 745-750 nm[19]. The results of TPC were demonstrated in table 1 and fig. 2. The results indicate that *E. cirrhatum* had highest total phenolic content at 105±1.527 mg GAE/gm extract. Phenolic content of *E. cirrhatum* (lichen) is four times higher than *B. procumbens* (bryophytes) 25±2.08 mg GAE/gm extract and two fold higher than *S. bryopteris* (pteridophyte) 47±4.041 mg GAE/gm extract. The present values of TPC were represented as mean ± SD up to three determinations.

Determination of Total Flavonoid Content (TFC)

Flavonoids are secondary metabolites, widely distributed in plants. In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation[20]. In our study, total flavonoid content of samples was determined by method of Quettier et al.[15] (2000). TFC of three different plants are shown in table 1 and figure 2. Flavonoid content of *S. bryopteris* (37.5±1.808 mg GAE/gm extract) was found to be highest followed by *B. procumbens* (36.5±1.212 mg GAE/gm extract) and *E. cirrhatum* (20±1.000 mg GAE/gm extract).

DPPH radical scavenging assay

The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability on the DPPH radical is determined by the decrease in its absorbance at 517 nm induced by antioxidants. It loses this absorption when it accepts an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow[21]. IC_{50} values of ethanolic extract of all three cryptogams were determined as shown in table 2 and figure 3. All extract show noticeable antioxidant activity in DPPH assay as compared to ascorbic acid. *E. cirrhatum* extract showed highest activity at IC_{50} 0.437 mg/ml followed by *S. bryopteris* and *B. procumbens*. The results suggested that ethanolic extract of cryptogams have good hydrogen donating capacity for free radical scavenging.

ABTS radical scavenging assay

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals[22]. The proton radical scavenging property of ethanolic extract of aforesaid plant samples were determined by ABTS assay. The IC_{50} values of radical scavenging were determined as shown in table 2 and figure 3. According to the result, *E. cirrhatum* (IC_{50} value of 0.009 mg/ml) was found to possess highest radical scavenging activity followed by *S. bryopteris* (IC_{50} value 0.030 mg/ml) and *B. procumbens* (IC_{50} 0.059 mg/ml).

Correlation of $1/IC_{50}$ values of antioxidant activities with TPC and TFC

Furthermore, quantitative analysis was also used for investigating the correlation between TPC, TFC and $1/IC_{50}$ value of antioxidant activities in all the three extracts. As the 1/IC50 (not IC50) value showed parallelism with antioxidant activity, it was therefore calculated (Table 2) and used for evaluating antioxidant activity. These four correlations were represented by plotting graphs between $1/IC_{50}$ values (DPPH and ABTS), total phenolic contents and total flavonoid content shown in figure 4. While, Table 3 shows the Pearson's correlation (R) and correlation coefficient (R^2) values of the both assays versus total phenolic and flavonoid contents. Careful analysis of correlation data revealed that there is positive relation between TPC and $1/IC_{50}$ values of both antioxidant assays with R^2 (0.959) for DPPH and R^2 (0.988) for ABTS assay. Similarly, close positive relation were seen between TFC and $1/IC_{50}$ of antioxidant assay.

DISCUSSION

Widely uses of herbal sources as a potent natural agent of antioxidant draws our attention for the search of more biologically active plants. Several researches prove that lower groups of plants contain metabolites with significant free radical scavenging properties including superoxide and reactive oxygen species scavenging[18]. Therefore, in our study, we collected samples of cryptogamic plants from the high altitude regions of Western Himalayan segment of Uttarakhand for the screening of their phytochemical and antioxidant property. Reported literature shows that cryptogams of this region contain significant amount of polyphenolic compounds as secondary metabolites which was further validated by previous study which demonstrated that stressful geographical conditions like high UV radiation etc.[10]. Promote production and accumulation of such bioactive compounds in these lower groups of plants as a protectant. The results of our study shows that cryptogamic plant samples also contain varying amount of TPC and TFC. There antioxidant activities were also different for each plant extract in which lichen plant proves to be contained significant amount of antioxidant compounds. Further, study of correlation between TPC and TFC on antioxidant properties shows positive correlation between them, suggested that polyphenolics and flavonoids content was directly linked with

radical scavenging properties. This is because polyphenolics and flavonoids posses' structural composition of aromatic rings with one or more hydroxyl and hydrogen functional groups provided ability to scavenge large number of free radicals[10].

Table 1: Total phenolic content and Total flavonoid content of three cryptogams of Almora district, Kumaun Himalaya, Uttarakhand.

Sample Name	TPC (mg GAE/ gm ext)	TFC (mg QE/ gm ext)
E. cirrhatum (Lichen)	105 ± 1.527	20 ± 1.000
B. procumbens (Bryophyte)	25 ± 2.080	36.5 ± 1.212
S. bryopteris (Pteridophyte)	47 ± 4.041	37.5 ± 1.808

Table 2: Antioxidant activities (IC_{50} and $1/IC_{50}$ value) of three cryptogams of Almora district, Kumaun Himalaya, Uttarakhand.

Sample Name	DPPH Assay (mg/ml)		ABTS Assay (mg/ml)	
	IC_{50} value	$1/IC_{50}$ value	IC_{50} value	$1/IC_{50}$ value
E. cirrhatum (Lichen)	0.437	2.288	0.009	111.111
B. procumbens (Bryophyte)	1.114	0.898	0.059	16.949
S. bryopteris (Pteridophyte)	0.653	1.531	0.030	33.333
Ascorbic acid	0.004	250	0.0009	1111.111

Table 3: Correlation between Total phenolic content (mg GAE/gm ext), Total flavonoid content (mg QE/gm ext) and $1/IC_{50}$ value of antioxidant activities (DPPH and ABTS assays)

	DPPH assay		ABTS assay	
	R	R^2	R	R^2
TPC	0.980	0.959	0.994	0.988
TFC	-0.866	0.750	-0.977	0.954

E. cirrhatum

B. procumbens

S. bryopteris

Fig. 1: Three cryptogams in Almora district, Kumaun Himalaya, Uttarakhand.

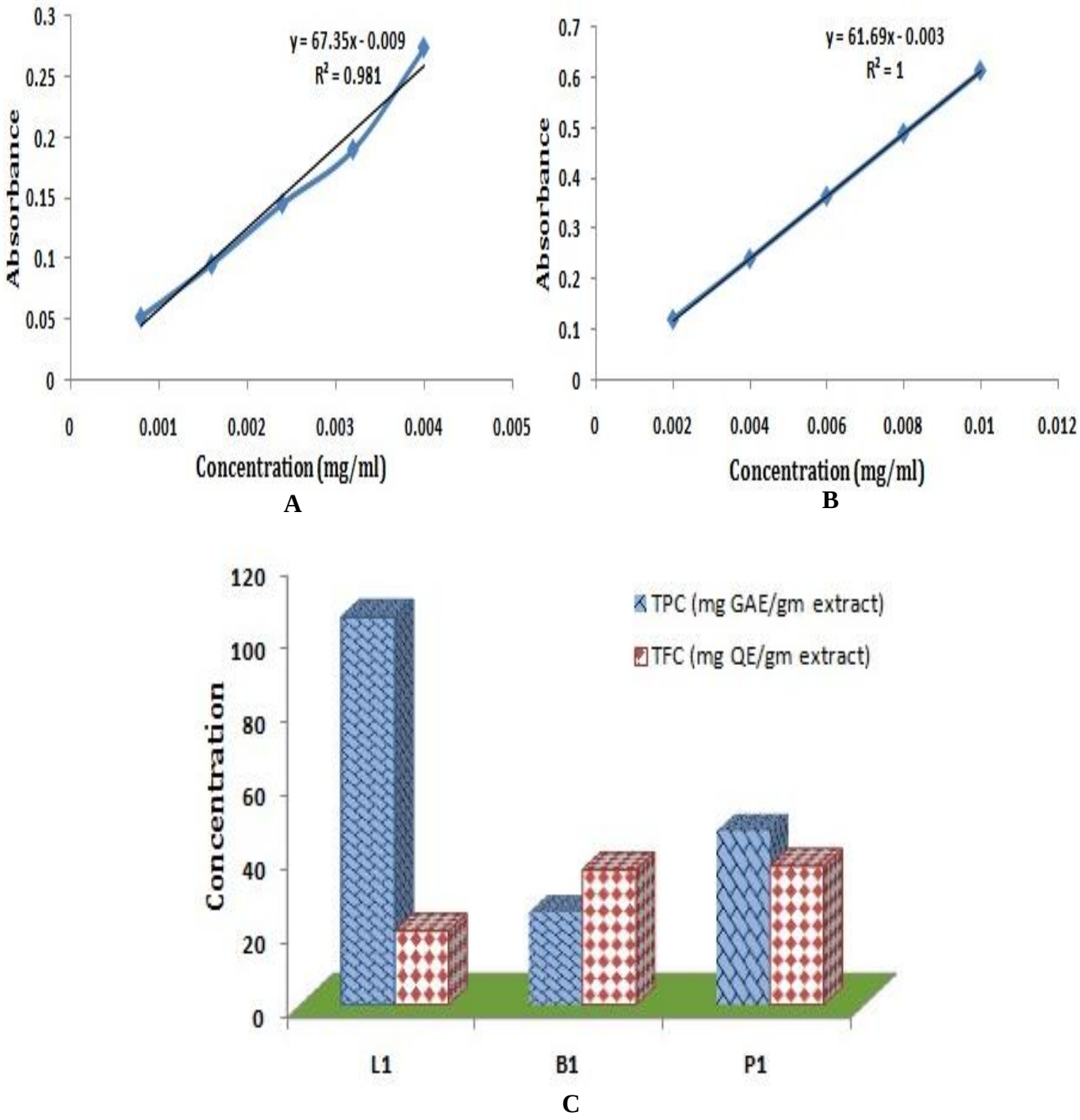
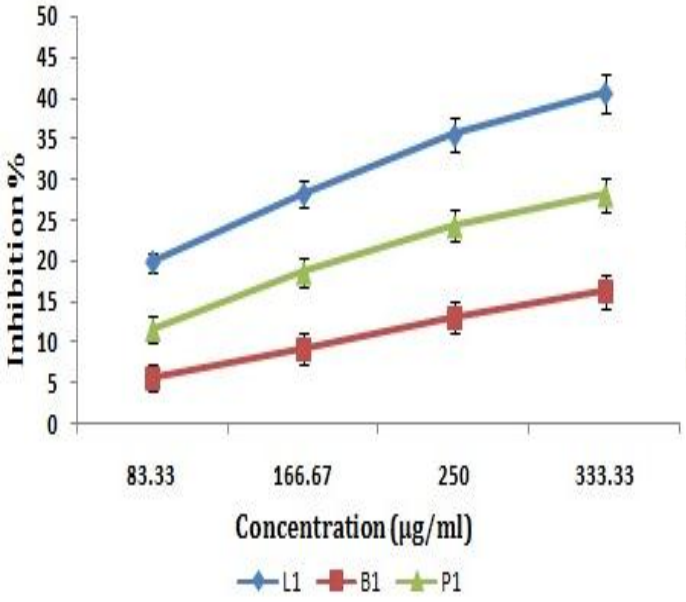
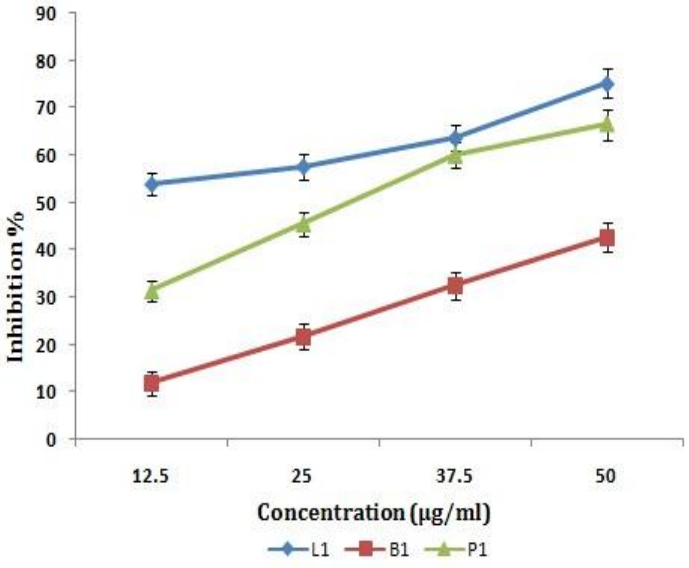


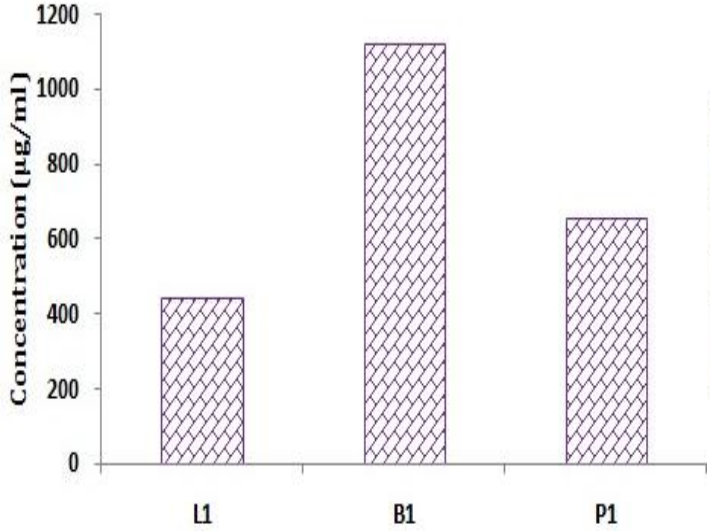
Fig. 2: A-Calibration curve of Gallic acid, B-Calibration curve of Quercetin, C-Graph of Quantification of Total phenolic content (TPC) and Total flavonoid content (TFC) of three cryptogams, L1- *E. cirrhatum*, B1- *B. procumbens*, P1- *S. bryopteris*


Inhibition %
50
45
40
35
30
25
20
15
10
5
0
83.33
166.67
250
333.33
Concentration (µg/ml)
L1
B1
P1

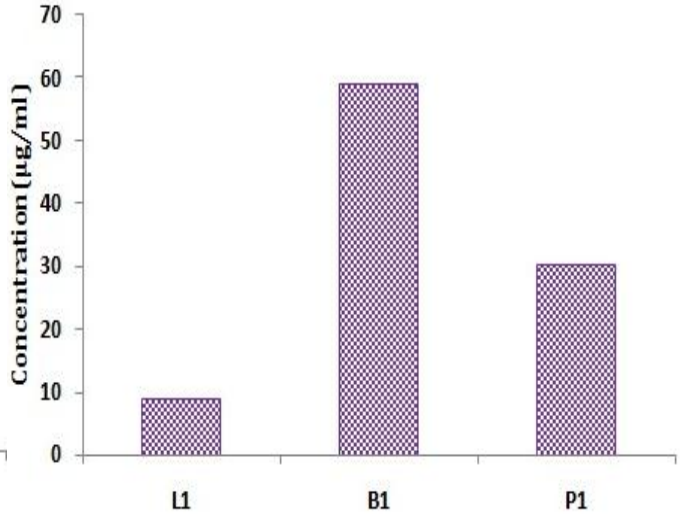
A1


Inhibition %
90
80
70
60
50
40
30
20
10
0
12.5
25
37.5
50
Concentration (µg/ml)
L1
B1
P1

B1


Concentration (µg/ml)
1200
1000
800
600
400
200
0
L1
B1
P1

A2


Concentration (µg/ml)
70
60
50
40
30
20
10
0
L1
B1
P1

B2

Fig. 3: Antioxidant activity of three cryptogams viz. L1- *E. cirrhatum*, B1- *B. procumbens*, P1- *S. bryopteris*, A- DPPH Free radical scavenging Activity, B- ABTS Free radical scavenging

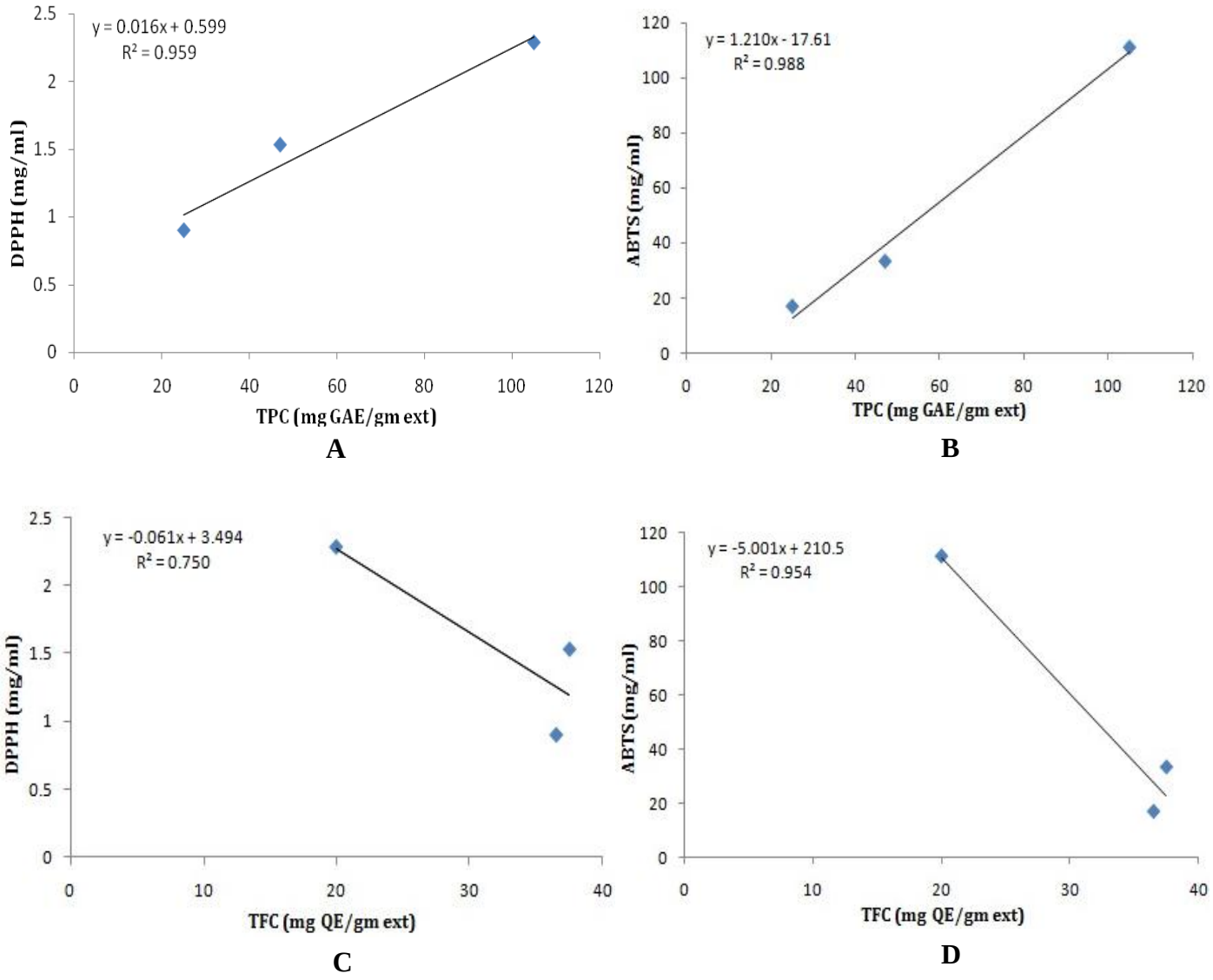


Fig. 4: Linear correlation graph between (A) TPC and DPPH $1/IC_{50}$ (B) TFC and 1/DPPH IC_{50} (C) TPC and 1/ABTS IC_{50} (D) TFC and 1/ABTS IC_{50}

CONCLUSIONS

Plant samples of cryptogams groups used in our study shows significant amount of phenolics and flavonoids contents. DPPH and ABTS test proved that these lower groups of plants having medicinal properties and could be used as potent herbal source of antioxidant. However, proper biological identification and isolation of responsible compound using latest methods

needs to be done to utilize these medicinal plants for human benefit.

ACKNOWLEDGMENTS

The authors are thankful to the Director, CSIR-National Botanical Research Institute, Lucknow for providing facilities and encouragement throughout the work.

REFERENCES

1. Pham-Huy LA, He H and Pham-Huyc C. Free Radicals, Antioxidants in Disease and Health. Inte J Biomed Sci. 2008; 4(2): 89-96.
2. Hui Wang, Xia Zhang, and Fusheng Cheng. Antioxidant Activity of Bioactive Polysaccharides Isolated from Natural Sources. J Anim Sci Biotechnol. 2011; 2(4): 230-238.
3. Halliwell B and Gutteridge JMC. Free Radicals in Biology and Medicine. Oxford University Press, Oxford. 2007.
4. Pong K. Oxidative stress in neurodegenerative disease: Therapeutic implication for superoxide dismutase mimetics. Expert Opin Biol Ther. 2003; 3: 127-139.
5. Sandhya B, Manoharan S, Sirisha Lavanya G, Manmohan CR. Lipid peroxidation and antioxidant status in prostate cancer patients. Indian J Sci Technol. 2010; 3(1): 83-86
6. Silva EM, Souza JNS, Rogez H, Rees JF and Larondelle Y. Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. Food Chem. 2007; 101: 1012-1018.
7. Frankel EN, Huang SW, Aeschbach R and Prior E. Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acis, in bulk oil and oil-in-water emulsion. J Agric Food Chem. 1996; 44: 131-135.
8. Hertog MG, Feskens EJM, Hollman PCH, Katan MB and Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet. 1993; 342: 1007-1011.
9. Mishra R and Verma DL. Antifungal Activity of Some Rare Himalayan Bryophytes. Res J Pharm Technol. 2011; 4(3): 474-475.
10. McCune LM and Johns T. Antioxidant activity relates to plant part, life form and growing condition in some diabetes remedies. J Ethnopharmacol. 2007; 112: 461-9.
11. Xie CF and Lou HX. Secondary Metabolites in Bryophytes: An Ecological Aspect. Chem Biodiv. 2009; 6(3): 303-12.
12. Andrade JM, Passos Cdos S, Dresch RR, Kieling-Rubio MA, Moreno PR, Henriques AT. Chemical analysis, antioxidant, antichemotactic and monoamine oxidase inhibition effects of some pteridophytes from Brazil. Pharmacogn Mag. 2014; 10(1): S100-S109.
13. Huneck S and Yoshimura I. Identification of lichen substances. Verlag Berlin Heidelberg: Springer. XII, 1996; 493.
14. Singleton VL, Orthofer R and Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 1999; 299: 152-178.
15. Quettier DC, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx MC, Cayin JC, Bailleul, F and Trotin F. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. J Ethnopharmacol. 2000; 72: 35-42.
16. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181: 1199-200.
17. Arnao M, Cano A and Acosta M. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem. 2001; 73: 239-44.
18. Razali N, Razab R, Mat Junit S and Abdul AA. Radical scavenging and reducing properties of extracts of cashew shoots (*Anacardium occidentale*). Food Chem. 2008; 111: 38-44.
19. Prior RL, Wu X and Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agricl Food Chem. 2005; 53: 4290-4303.
20. Galeotti F, Barile E, Curir P, Dolci M and Lanzotti V. Flavonoids from carnation (*Dianthus caryophyllus*) and their

antifungal activity. Phytochem Lett. 2008; 1: 44.
21. Hseu YC, Chang WH, Chen CS, Liao JW, Huang CJ, Lu FJ, Chia YC, Hsu HK, Wu JJ, Yang HL. Antioxidant activities of *Toona Sinensis* leave extract using different antioxidant models. Food Chem Toxicol. 2008; 46(1): 105-114.
22. Mathew S and Abraham ET. In vitro antioxidant activity and scavenging effects of *Cinnamomum verum* leaf extract assayed by different methodologies. Food Chem Toxicol. 2004; 44: 198-206.