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Original Article

PHYTOCHEMICALS, ANTIOXIDANT AND ANTIPROLIFERATIVE PROPERTIES OF FIVE MOSS SPECIES FROM SABAH, MALAYSIA

FIFILYANA ABDUL KARIM[1,*], MONICA SULEIMAN[1], ASMAH RAHMAT[2], MOHD FADZELLY ABU BAKAR[1,3]

[1]Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia, [2]Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia, [3]Faculty of Science, Technology and Human Development, UniversitiTun Hussein Onn Malaysia (UTHM), 86400 Parit Raja, BatuPahat,Johor, Malaysia
Email: fifilyana1111@gmail.com

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ABSTRACT

Objective: This study was conducted to investigate the phytochemical contents, the antioxidant and antiproliferative properties of 80% methanol, ethanol and aqueous extracts of *Sphagnum cuspidatum* subsp. *subrecurvum*, *Sphagnum cuspidatulum*, *Sphagnum junghuhniannum*, *Pogonatum cirratum* subsp. *fuscatum* and *Pogonatum cirratum* subsp. *macrophyllum*.

Methods: The total phenolic and total flavonoid contents were analysed using Folin-Ciocalteu and aluminium chloride colorimetric methods. The antioxidant properties were evaluated by three different assays, namely, ferric reducing/antioxidant power (FRAP), ABTS and DPPH free radical scavenging assays. MTT assay was used to study the antiproliferative properties against selected cancer cell lines.

Results: The results showed that the aqueous and ethanol extracts of *S. cuspidatum* subsp. *Subrecurvum* has the highest total phenolic and total flavonoid content with the values of 5.42 ± 1.95 mg gallic acid equivalent per g of dry sample and 2.12 ± 0.02 mg catechin equivalent per g of dry sample, respectively. The 80% methanol extracts of *S. junghuhnianum* has the lowest total phenolic and total flavonoid content with the values of 0.80 ± 0.12 mg gallic acid equivalent per g of dry sample and 0.03 ± 0.02 mg catechin equivalent per g of dry sample, respectively. None of the extracts displayed IC_{50} value (concentration that inhibits 50% of free radical) at concentration tested. The 80% methanol extracts of *P. cirratum* subsp. *Fuscatum* induced antiproliferative activity against $CaOV_3$ (ovarian carcinoma) cell line whereas *S. cuspidatulum* induced antiproliferative activity against HepG2 (liver cancer) cell line. None of the extracts induce antiproliferative activity against MDA-MB-231 (non-hormone dependent breast cancer) cell line.

Conclusion: Results obtained indicated that the selected mosses contained considerable amount of phenolics and flavonoids which contribute to antioxidant properties. *P. cirratum* subsp. *Fuscastum* and *S. cuspidatulum* have the potential to be used in pharmaceutical industry due to the ability of these two species to induce antiproliferative activity against $CaOV_3$ and HepG2 cancer cell lines.

Keywords: Phytochemical contents, Antioxidant activity, Antiproliferative activity, Mosses.

INTRODUCTION

Phytochemicals are biologically active compounds that exist naturally in medicinal plants, fruits, vegetables as well as grains. Phytochemicals are believed to be effective in disease prevention due to their antioxidant effect [1]. Antioxidants are any substances that can delay or retard lipids, proteins and nucleic acids oxidation [2]. The examples of antioxidants present in plants are carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols[3]. Antioxidants can be classified into two types known as primary and secondary antioxidants. Primary and secondary antioxidants are categorized due to their protective properties at different stages of the oxidation process and their action by different mechanisms. Primary antioxidants scavenge free radicals whereas secondary antioxidants inhibit the oxidative mechanisms that lead to degenerative diseases [4]. Currently, there has been an upsurge of interest on natural antioxidants in plants to be used in pharmaceutical industry due to carcinogenic properties of some synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene[5].

Mosses and liverworts, in particular, possess certain biological activity and effects [6]. They have been widely used by the Chinese, Europeans, North Americans and Indians as medicine to treat cuts, burns, bruises and diseases such as pulmonary tuberculosis, neurasthenia, tonsillitis, bronchitis, tympanitis and skin disease [7-8]. Bryophytes possess biological compounds that protect them from bacteria or fungi as well as insects [9]. Today, most studies on bryophytes are focused on liverworts as they contain cellular oil bodies, unlike mosses and hornworts. The effects of bryophytes on microbes were studied intensively, but only a few studies were conducted on the phytochemical screening and antioxidant properties of bryophytes. The present paper investigates the phytochemical contents, antioxidant and antiproliferative properties of five mosses species, *Sphagnum cuspidatum* subsp. *subrecurvum*, *Sphagnum cuspidatulum*, *Sphagnum junghuhnianum*, *Pogonatum cirratum* subsp. *fuscatum*, and *Pogonatum cirratum* subsp. *Macrophyllum* collected in Sabah, Malaysia.

MATERIALS AND METHODS

Plant materials and sample preparation

S. cuspidatum subsp. *subrecurvum*, *S. cuspidatulum*, *S. junghuhnianum*, *P. cirratum* subsp. *Fuscatum* and *P. cirratum* subsp. *Macrophyllum* were collected from Crocker Range, Sabah, Malaysia. The herbarium voucher specimens were identified and deposited into BORNEENSIS Herbarium (BORH), Institute for Tropical Biology and Conservation, University Malaysia Sabah, Sabah, Malaysia. The samples were carefully cleaned and rinsed by using distilled water to remove contaminants. Samples were dried in the oven for two days at 40°C [10]. The dried samples were ground into fine powder using a dry grinder. The ground samples were kept in zip lock bag and stored in a freezer (-20°C) for further analysis.

Extraction

A quantity of 0.1 g of sample was mixed with 10 ml of three different solvents which are 80% methanol, ethanol and aqueous solution and extracted using magnetic stirrer for 2 h at room temperature. The mixture was filtered through a Whatman No. 1 filter paper. The

extracts were directly used for phytochemical determinations and antioxidant assays.

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent with modifications [11]. One hundred microliter of extract was mixed with 750 µl of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water), vortex and allowed to stand at room temperature for 5 min. The mixture was then added with 750 µl of sodium bicarbonate (60 g/L) solution. After 90 min at room temperature, absorbance was measured at 725 nm using spectrophotometer. Standards of gallic acid in the concentration ranging from 0 to 100µg/ml were run with the test samples. Results were expressed as mg gallic acid equivalent in 1 g of dried sample (mg GAE/g).

Determination of total flavonoid content

Total flavonoid was measured according to aluminium chloride colorimetric method [12]. Briefly, 1 ml of extract was mixed with 4 ml of distilled water and 0.3 ml of 5% sodium nitrite ($NaNO_2$) solution. After 5 min, 0.3 ml of 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$) was added and allowed to stand for 6 min before 2 ml of 1M sodium hydroxide (NaOH) was added to the mixture. Absorbance was measured at 510 nm using spectrophotometer. Standards of catechin in the concentration ranging from 0 to 100µg/ml were run with the test samples. Results were expressed as mg catechin equivalent in 1 g of dried sample (mg CE/g).

DPPH free-radical scavenging assay

The antioxidant activity of the extracts were measured by using 2,2-diphenyl-1-pycrylhydrazyl (DPPH) as a free radical model [13]. One ml of 0.3 mM* methanol solution of 2,2-diphenyl-1-pycrylhydrazyl (DPPH) was added into 2.5 ml sample or standards. The solution was mixed vigorously and left to stand at room temperature for 30 min in the dark. The mixture was measured spectrophotometrically at 518 nm. The antioxidant activity (AA) was calculated as below:

$$AA\% = 100-[\frac{(\text{Absorbance sample}-\text{Absorbance empty sample})}{\text{Absorbance control}}] \times 100$$

Empty sample = 1 ml methanol + 2.5 ml extract

Control sample = 1 ml 0.3 mM DPPH + 2.5 ml methanol

The percentage of antioxidant activity of all samples was plotted. The final results were expressed as an IC_{50} value (the concentration of sample producing 50% scavenging of the DPPH radical; µg/ml).

FRAP (Ferric reducing/antioxidant power) assay

This method was conducted with slight modifications[14]. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio prior to use in water bath at 37°C. A total of 3 ml FRAP reagent was added to a test tube and a blank reading was taken at 593 nm using spectrophotometer. A total of 100 µl of selected plant extracts and 300 µl of distilled water were added to the test tube. After addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 4 min. The changes in absorbance after 4 min from initial blank reading were compared with standard curve ($FeSO_4$). A standard of known Fe (II) concentrations were run using several concentrations ranging from 200 to 1000 µg/ml. A standard curve was plotted. The FRAP value for the samples was determined by using the standard curve equation. The final result was expressed as the concentration of antioxidant having a ferric reducing ability in 1 g of dry sample (mM/g).

ABTS decolorization assay

The 2,2'-azinobis(3-ethylbenzthiazoline)-6-sulphonic acid or ABTS free radical decolorization assay was carried out with slight modifications[15]. Working ABTS solution (7 mM*) and 2.45 mM potassium persulfate ($K_2S_2O_8$) were added into a beaker. The mixture was allowed to stand for 15 h in the dark at room temperature. The mixture was diluted with solvents used to obtain the absorbance of 0.7 ± 0.02 units at 734 nm. An aliquot of 200 µl of methanolic test solution of each sample was added to 2 ml of ABTS free radical cation solution. It was then vortex vigorously. The absorbance was measured spectrophotometrically at 734 nm. Standards of ascorbic acid in the concentration ranging from 0 to 80 µg/ml were run with the test samples, from which a standard curve was plotted. The radical scavenging activity was expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of dry sample (mg AEAC/g).

Antiproliferative assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was based on the ability of mitochondrial dehydrogenase enzymes from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which is largely impermeable to cell membranes resulting in its accumulation in healthy cells[16]. The MCF-7 (hormone dependent breast cancer), MDA-MB-231 (non-hormone dependent breast cancer) and $CaOV_3$ (ovary cancer) cell lines were cultured in RPMI 1640 medium with L-glutamine, supplemented with 10% of fetal bovine serum and 1% penicillin-streptomycin. The cells were incubated at 37°C under 5% CO_2 in a humidified atmosphere. Trypan blue was used to stain the cells for cell counting. Exponentially growing cells were harvested and counted using haemocytometer and diluted in culture medium to a density of 1 x 10^6cells/ml. From this cell suspension, 100 µl was pipetted into each well of a 96-well plate and incubated for 24 h. The old medium was pipetted out and sample extracts were added into the plate with highest concentration of 100 µg/ml. The plate was then incubated for 72 h. MTT reagent (20 µl) was added into each well and the plate was incubated for 4 h. After that, 100 µl of solubilization solution was added into each well. Reading was taken at 570 nm wavelength using microplate reader, from which cytotoxicity was determined by use of the following formula:

$$\%\ \text{cytotoxicity} = \frac{\text{optical density of sample}}{\text{optical density of control}} \times 100$$

The final results were expressed as an IC_{50} value (the concentration of sample able to inhibit cell proliferation by 50%;µg/ml) that was calculated graphically for each cell proliferation curve.

Statistical analysis

All determinations were carried out in triplicates in three independent experiments. Results were expressed as mean ± standard deviation (SD) using SPSS version 17.0. The data were statistically analysed by two-way ANOVA. Pearson's correlation analysis was carried out to correlate the phytochemicals, antioxidant and anticancer potential between samples. P-value < 0.05 was regarded as significant.

RESULTS AND DISCUSSION

Total phenolic and total flavonoid content

Result obtained in the present study showed that the aqueous extract of *S. cuspidatum* subsp. *subrecurvum* has the highest amount of total phenolic as compared to 80% methanol and ethanol extracts (Table 1, 2 & 3). Polarity of solvents plays an important role in extraction in which the higher the polarity, the better the solubility of phenolic compounds [17]. A study conducted on the phytochemicals of *Ocimum gratssimum* supported that different solvents used in extraction yield different types of phytochemicals in plants since the result showed anthraquinones were detected only in the aqueous extract while alkaloids were detected only in the methanol extracts[1]. Another study conducted on polyherbal formulation also showed that only aqueous extracts revealed the presence of proteins while ethanol extract did not display the presence of proteins [18]. The result of this study is similar to a study on Shiitake mushroom (*Lentinus edodes*) and straw mushroom (*Volvariella volvacea*) in which the water extract of *V. volvacea* had higher phenolic content than the methanol extract [19]. On the other hand, the ethanol extract of *S. cuspidatum* subsp. *subrecurvum* has the highest total flavonoid contents as compared to aqueous and 80% methanol extracts (Table 1,2&3). *S. junghuhnianum* has the lowest total phenolic contents for all extracts.

Phenolic compounds are suggested to contribute to antioxidant properties due to their ability to scavenge free radicals and reactive oxygen species that might be explained by hydroxyl groups of phenolics [20-21]. It has been reported that mosses usually contain flavonoids, apigenin, luteolin, kaempferol and orobol glycosides as well as their dimmers [22]. In the present study, *S. cuspidatum* subsp. *subrecurvum* has high amount of total phenolic contents and total flavonoid contents. The result of this study is in agreement with a study on gingers in which the higher the total phenolic contents, the higher the total flavonoid contents [3].

Table 1: The total phenolic and total flavonoid contents of 80% methanol extracts of selected mosses

Species	Total phenolic content[1]	Total flavonoid content[2]
S. cuspidatum subsp. *subrecurvum*	1.45 ± 0.34[a]	0.55 ± 0.31[a]
P. cirratum subsp. *macrophyllum*	1.38 ± 0.25[a,b]	0.10 ± 0.05[b,e]
S. cuspidatulum	1.28 ± 0.23[a,b]	0.17 ± 0.11[a,e]
P. cirratum subsp. *fuscatum*	1.22 ± 0.12[a,b]	0.08 ± 0.04[c,e]
S. junghuhnianum	0.80 ± 0.12[b]	0.03 ± 0.02[d,e]

Values are expressed as mean ± SD (n=9) which with different alphabets (within column) indicate significant difference ($p < 0.05$).

[1]Total phenolic content was expressed as mg gallic acid equivalent in 1 g of dry sample (mg GAE/g).

[2]Total flavonoid content was expressed as mg catechin equivalent in 1 g of dry sample (mg CE/g).

Table 2: The total phenolic and total flavonoid contents of ethanol extracts of selected mosses

Species	Total phenolic content[1]	Total flavonoid content[2]
S. cuspidatum subsp. *subrecurvum*	2.87 ± 0.35[a]	2.12 ± 0.02[a]
P. cirratum subsp. *fuscatum*	2.13 ± 0.43[a,b]	1.27 ± 0.13[b,c,d]
P. cirratum subsp. *macrophyllum*	1.53 ± 0.21[b,c,d]	1.18 ± 0.35[c,d,e]
S. cuspidatulum	1.12 ± 0.41[c,d]	0.79 ± 0.04[e]
S. junghuhnianum	0.99 ± 0.07[d]	0.88 ± 0.03[d,e]

Values are expressed as mean ± SD (n=9) which with different alphabets (within column) indicate significant difference ($p < 0.05$).

[1]Total phenolic content was expressed as mg gallic acid equivalent in 1 g of dry sample (mg GAE/g).

[2]Total flavonoid content was expressed as mg catechin equivalent in 1 g of dry sample (mg CE/g).

Table 3: The total phenolic and total flavonoid contents of aqueous extracts of selected mosses

Species	Total phenolic content[1]	Total flavonoid content[2]
S. cuspidatum subsp. *subrecurvum*	5.42 ± 1.95[a]	0.28 ± 0.12[b,c,d,e]
P. cirratum subsp. *fuscatum*	4.87 ± 0.62[a]	0.62 ± 0.06[a]
P. cirratum subsp. *macrophyllum*	1.95 ± 0.15[b,c,d]	0.21 ± 0.13[c,d,e]
S. cuspidatulum	1.35 ± 0.14[c,d]	0.13 ± 0.05[d,e]
S. junghuhnianum	0.99 ± 0.12[d]	0.04 ± 0.04[e]

Values are expressed as mean ± SD (n=9) which with different alphabets (within column) indicate significant difference ($p < 0.05$).

[1]Total phenolic content was expressed as mg gallic acid equivalent in 1 g of dry sample (mg GAE/g).

[2]Total flavonoid content was expressed as mg catechin equivalent in 1 g of dry sample (mg CE/g).

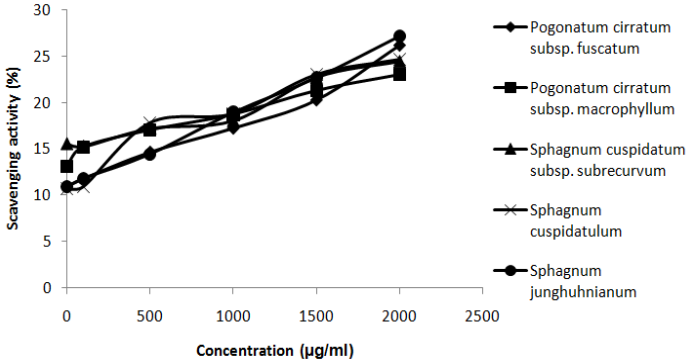

Fig. 1: DPPH radical scavenging activity of 80% methanol extracts of selected mosses

Scavenging activity on 2,2-diphenyl-1-picrylhydrazyl radical

There are few mechanisms to study the antioxidant activities. One of the mechanisms to study the radical scavenging effect is based on the observation of changes in DPPH colour when reacts with radical scavengers. The higher the level of colour transparency indicates higher level of antioxidants. For DPPH assay, none of the extracts of

mosses species displayed scavenging of DPPH free radicals at concentration tested. There is no previous report regarding the antioxidant activity of these species. However, based on the result, it is suggested that the antioxidants of the selected mosses exist in considerable amount. In the present study, none of the extracts displayed IC_{50} value at concentration tested (Figure 1, 2 & 3).

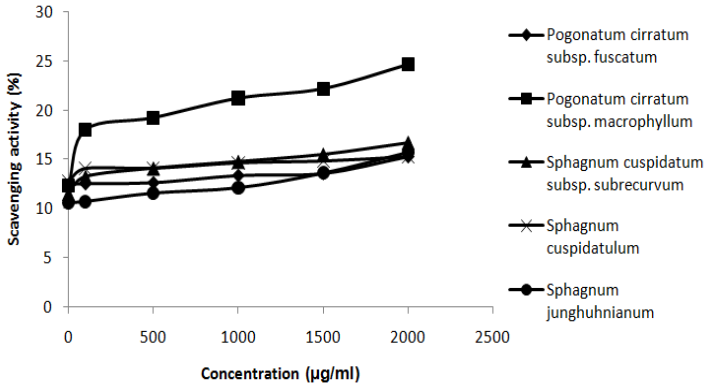

Fig. 2: DPPH radical scavenging activity of ethanol extracts of selected mosses

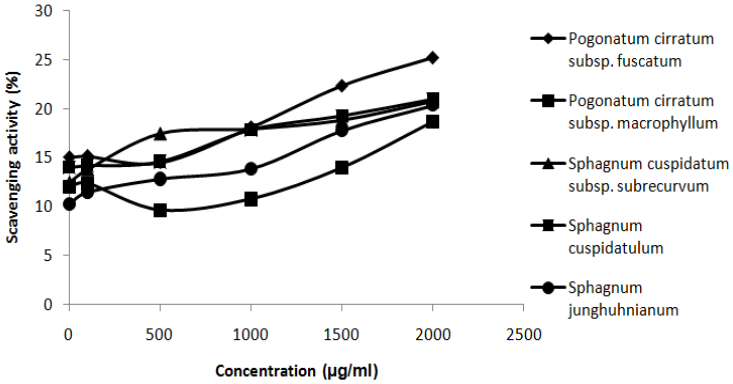

Fig. 3: DPPH radical scavenging activity of aqueous extracts of selected mosses

Table 4: Ferric reducing ability of extracts of selected mosses

Solvent	FRAP assay[1]				
	P. cirratum* subs p. *fuscatum	***S. cuspidatulum***	***S. junghuhnianum***	***S. cuspidatum* subsp. *subrecurvum***	***P. cirratum* subs p. *macrophyllum***
80% methanol	13.74 ± 1.74^{a}	10.21 ± 2.54^{a,b,c}	7.46 ± 0.67^{b,c,d}	7.10 ± 0.71^{c,d}	5.63 ± 0.63^{d}
Ethanol	9.02 ± 0.79^{a}	5.16 ± 1.01^{b,c}	4.85 ± 1.08^{c}	6.94 ± 0.55^{a,b,c}	7.41 ± 0.50^{a}
Aqueous	20.74 ± 2.10^{a}	2.19 ± 0.55^{e}	3.83 ± 0.22^{c,d,e}	2.35 ± 0.43^{d,e}	6.13 ± 1.67^{b,c}

Values are presented as mean ± SD (n=9) which with different alphabets (within row) indicate significant difference ($p < 0.05$).

[1]FRAP was expressed as mM ferric reduction to ferrous in 1 g of dry sample.

Ferric reduction based on FRAP assay

The antioxidant potential was also estimated from the ability of both species to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The reducing ability is due to the compounds in each species breaking the free radical chain by donating hydrogen. Higher reducing power for methanol extracts was in line with the study on commercial mushroom [23]. The reducing ability of aqueous extract of *P. cirratum* subsp. *Fuscatum* was the highest followed by 80% methanol and ethanol extracts. *P. cirratum* subsp. *Fuscatum* displayed higher reducing ability as compared to other moss species. The reducing ability of *S. cuspidatulum* was poor compared to other extracts (Table 4).

ABTS scavenging assay

Another mechanism used in this study to study the antioxidant activity was ABTS radical scavenging activity. Results of this study were almost similar with a study on sage (*Salvia officinalis*) where compounds which have ABTS scavenging activity did not display DPPH scavenging activity[24]. Numerous studies found that antioxidant activity is linearly proportional to the phenolic contents which appear to be the trend in many plant species [4, 25]. However, in the present study, there was no correlation between phytochemical contents and antioxidant activities. The result showed that aqueous extracts displayed higher scavenging activity as compared to 80% methanol and ethanol extracts. The same pattern was observed for the scavenging activity of *P. cirratum* subsp. *Fuscatum* for ABTS assay. The aqueous extract of *P. cirratum* subsp. *fuscatum* displayed the highest scavenging activity (Table 5).

Antiproliferative assay

Antiproliferative assay has been done for 80% methanol extracts of selected bryophytes species. This method is based on the cleavage of yellow tetrazolium salt MTT to purple formazan crystal that occurs in the mitochondria. The changes of yellow colour to purple indicate

of living cells. The result showed that *P. cirratum* subsp. *Fuscatum* induced strong antiproliferative activity against $CaOV_3$ (ovarian carcinoma) cell line. *S. cuspidatulum* also induced antiproliferative activity against HepG2 (liver cancer) cell line. However, none of the extract induces antiproliferative activity against MDA-MB-231 (non-hormone dependent breast cancer) (Table 6).

Table 5: ABTS scavenging activity of selected mosses

Solvent	ABTS assay[1]				
	P. cirratum* subsp. *fuscatum	***S. junghuhnianum***	***S. cuspidatulum***	***P. cirratum*subsp. *macrophyllum***	***S. cuspidatum* subsp. *subrecurvum***
80% methanol	0.12 ± 0.00[a]	0.11 ± 0.00[a]	0.10 ± 0.03[a]	0.04 ± 0.02[b,c]	0.01 ± 0.01[c]
Ethanol	0.08 ± 0.01[a]	0.09 ± 0.02[a]	0.07 ± 0.02[a,c]	0.06 ± 0.01[b,c]	0.04 ± 0.01[c]
Aqueous	0.16 ± 0.00[a]	0.16 ± 0.01[a,b]	0.14 ± 0.00[b]	0.09 ± 0.00[d]	0.10 ± 0.01[c,d]

Values are presented as mean ± SD (n=9) which with different alphabets (within row) indicate significant difference ($p < 0.05$).

[1]ABTS free radical scavenging activity was expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) in 1 g of dry sample.

Table 6: Antiproliferative activities of ≤100 µg/ml of 80% methanol extracts of selected mosses tested against $CaOV_3$ (ovarian carcinoma), HepG2 (liver cancer) andMDA-MB-231 (non-hormone dependent breast cancer)cell lines by using MTT assay

Species	$CaOV_3$	HepG2	MDA-MB-231
*P. cirratum*subsp. *fuscatum*	31.83 ± 5.01	N. D	N. D
*P. cirratum*subsp. *macrophyllum*	N. D	N. D	N. D
*S. cuspidatum*subsp. *subrecurvum*	N. D	N. D	N. D
S. cuspidatulum	N. D	93.00 ± 4.24	N. D
S. junghuhnianum	N. D	N. D	N. D

Data are presented in mean ± SD., N. D = not detected in concentration less than or equal to 100 µg/ml.

CONCLUSION

In conclusion, the results of this study suggest that phenolics and flavonoids are important components in plants that contribute to antioxidant activity. Two selected bryophytes species namely, *P. cirratum* subsp. *Fuscatum* and *S. cuspidatulum,* should be further studied as they have the potential to be used as anticancer agents.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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