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## Phytochemical screening and *in-vitro* antioxidant potential of two ethnomedicinally important mosses of Dicranaceae from Darjeeling hills

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### Abstract

*Leucobryum bowringii* Mitt. and *Dicranum scoparium* Hedw., belonging to family- Dicranaceae, are widely distributed epiphytic mosses in Darjeeling hills and are reported to have significant ethnomedicinal uses. These two mosses were analysed to determine their phytochemical composition and antioxidant potential. Phytochemical investigation demonstrated abundance of terpenoids in chloroform extract and presence of flavonoids and other phenolics in methanolic and ethanolic extracts of both the plants. Higher abundance of these compounds were observed in *Leucobryum bowringii*. Free radical scavenging activity and ferric ion reducing property indicated significant antioxidant potential of ethanol, methanol and chloroform extracts of both mosses. Bioactivity of the extracts may be imparted by the phytochemical constituents of those fractions. So, these mosses can be presumed to have significant pharmaceutical prospect which can be a promising source of bioactive chemicals and drugs.

**Keywords:** Bryophytes, Darjeeling, Dicranaceae, ethnomedicine, phytochemicals, antioxidant

### 1. Introduction

Bryophytes are one of the earliest and largest group of land plants having approximately 8000-9000 moss members, 6000 species of liverworts and 100 species of hornworts, existing worldwide (Castaldo *et al.*, 1998) [8]. Different bryophyte species have been recorded to have traditional uses as medicinal plants and their folk classification has been executed in ethnobotanical literatures (Asakawa, 2007; Harris, 2008) [4, 23]. Ethnic uses of bryophytes was first reported by Flowers (1957) [19] among the Gosiute people of Utah. Most of those species have been recorded as Traditional Chinese Medicine and were also used by Native North Americans for medicinal purposes. Recently, different bryophytes have been thoroughly evaluated to identify the active principles and their biological activities (Glime, 2007) [21]. A number of new compounds have been isolated from liverworts and few mosses and their structures have been elucidated (Asakawa, 2001, 2013) [5, 31]. From the reports of ethnomedicinal uses of bryophytes and the recent works on chemical composition of some of the plant members, the mosses are presumed to have significant biological activities (Singh *et al.*, 2006) [35]. Previous investigations demonstrated that bryophytes possess extremely high amounts of secondary metabolites such as terpenoids, phenolics (flavonoids and bibenzyl derivatives), glycosides, fatty acids as well as some rare aromatic compounds (Jockovic *et al.*, 2008; Sabovljevic *et al.*, 2010) [24, 32]. Evidences of mosses having bioactive phytochemicals has attracted the attention of botanist and pharmaceutical industry. Alkaloids, flavonoids, biflavonoids, and isoflavonoids from bryophyte extract possess effective antibacterial, antifungal activity against pathogenic micro-organisms (Neto *et al.*, 2011; Dan *et al.*, 2008) [29, 16]. Volatile phenolics derivatives of bryophytes act as antioxidants and may therefore have health-promoting effect in the prevention of degenerative diseases as well as aging (Shahidi, 1997) [34].

Eastern Himalayan Biodiversity zone is a rich reservoir of diverse types of mosses. In spite of their high pharmaceutical potential these mosses have rarely been investigated. Very few Indian reports are available on the phytochemical analyses of mosses to understand their pharmaceutical potential (Talai Mukhopadhyay *et al.*, 2013) [38]. Different mosses, especially those belonging to the order- Dicranales, were reported to have ethno-medicinal importance (Chandra *et al.*, 2016; Sabovljevic *et al.*, 2016) [10, 33]. In spite of high availability of Dicranaceae mosses, there is only one previous report on phytochemical analysis of Dicranaceae member from Darjeeling hills (Mitra, 2017) [28]. So, the present work was mainly framed to evaluate the chemical composition and bioactivity of two extensively grown moss members which also have ethnomedicinal reports.

## 2. Materials and methods

### 2.1. Plant material

The moss materials- *Leucobryum bowringii* Mitt. and *Dicranum scoparium* Hedw., both belonging to the family Dicranaceae are abundantly grown epiphytic mosses in Darjeeling hills. Ethnomedicinal uses of *Dicranum scoparium* by The Balkans as a treatment of body pain was reported by Pavletic and Stilinovic (1963) [30]. *Leucobryum bowringii* is also used by Kani tribes of Southern-Western Ghats of India as a traditional medicine for body pain (Lubaina *et al.*, 2014) [26].

### 2.2. Collection of plant materials

The mosses grow as cushion forms on the bark of *Cryptomeria sp.* The samples were collected from Lamahatta of Darjeeling district hill region of Eastern Himalayas during July 2016. The clumps of each moss material were carefully sampled keeping all the plant parts intact. Clumps collected from different colonies of a single population were considered for replica analyses. After sampling, mosses were transferred in air-tight zip-lock bag and were transported to laboratory and were kept in ambient temperature before further chemical analyses. Chemical analyses were carried out within 24 h of sampling to avoid any change in chemical composition due to long term storage. The mosses were identified on the basis of taxonomic guidelines and morpho-anatomical features following the key for identification described in the monograph 'Mosses of Eastern India and Adjacent Regions' (Gangulee, 1969) [20]. Voucher specimens are kept in herbarium of Department of Botany, Darjeeling Government College.

### 2.3. Extraction of phytochemicals

Mosses were carefully cleaned with distilled water after removal of rhizoidal portions and were dried. Dry biomass of 200 mg was homogenized with 10 ml distilled water, methanol, ethanol, diethyl ether, chloroform and hexane separately. The obtained extracts were centrifuged and the supernatants were evaporated to dryness. A volume of 10 ml of respective solvent was added before further analysis.

### 2.4. Qualitative test of phytochemicals

Chemical tests for screening and identification of phytochemicals in different plant extracts were executed following the standard procedures as described by Harborne (1973) [22], Sofwara (1993) [37]. Qualitative evaluation of steroids, terpenoids, glycosides, coumarins, phenolics, flavonoids, tannins and saponins were performed.

### 2.5. Quantitative estimation of phytochemicals

#### 2.5.1. Estimation of phenols

Total phenolics content was estimated following the method of Singleton and Rossi (1965) [36] from all the solvent fractions. A volume of 1 ml of each extract was added to 0.5 ml of Folin reagent diluted with distilled water (1:1). After incubation for 5 min, 2 ml of 20% sodium bicarbonate was added. The mixture was kept in boiling water bath for 10 minutes followed by cooling at room temperature. The absorbance of the developed colour was measured at 650 nm by UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu). Calibration curve was prepared using different concentrations of gallic acid and the content was expressed as mg of gallic acid equivalent (GAE) g<sup>-1</sup> dry weight.

#### 2.5.2. Estimation of flavonoids

Total flavonoid contents of all the extracts were determined following the method of Chang *et al.* (2002) [11]. A volume of 0.5 ml of extract was mixed with 1.5 ml of methanol and 0.1 ml of 10% aluminium chloride followed by the addition of 0.1 ml of 1M potassium acetate. The mixture was then diluted with 2.8 ml of distilled water and was incubated at room temperature for 30 min. The absorbance of the developed colour was recorded at 415 nm. The calibration curve was prepared using different concentrations of rutin and the content was expressed as mg rutin equivalent (RE) g<sup>-1</sup> dry weight.

#### 2.5.3. Estimation of triterpenoids

Total triterpenoid content was estimated following the method of Fan and He (2006). A volume of 0.2 ml plant extract was added to 0.3 ml of 5% vanillin/glacial acetic acid solution and 1 ml perchloric acid. The mixtures were incubated at 60° C for 45 min followed by cooling in ice water bath. A volume of 5 ml glacial acetic acid was again added to it. Absorbance of the developed colour was measured at 548 nm. Calibration curve was prepared using different concentrations of linalool and the content was expressed as mg of linalool equivalent (LE) g<sup>-1</sup> of extract.

### 2.6. Evaluation of antioxidant activity

#### 2.6.1. Free radical scavenging activity assay

Antioxidant activity of the plant extracts were evaluated in terms of their 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability (Blois, 1958). A volume of 3 ml DPPH solution was added to 0.2 ml of each solvent extract and the mixture was incubated for 30 min at room temperature. The absorbance was recorded at 517 nm. DPPH without any plant extract was considered as control. Antioxidant activities of propyl gallate and ascorbic acid served as reference. Percentage of DPPH scavenging activity was calculated using the following formula:

$$\% \text{ DPPH scavenging activity} = \frac{[(\text{control absorbance} - \text{sample absorbance}) \times 100]}{\text{Control absorbance}}$$

The inhibition values were calculated for different concentrations of crude extract. The radical scavenging activity was expressed as IC<sub>50</sub> value which denotes the concentration of sample extract which is required to scavenge 50% of DPPH radical.

#### 2.6.2. Ferric reducing antioxidant potential (FRAP) assay

Antioxidant activity was also assessed as ferric ion reducing ability of the extracts which was determined using FRAP assay according to the method of Dudonne *et al.* (2009) [17]. FRAP reagent was prepared by mixing 10 ml of 300 mM acetate buffer (pH- 3.6) with 1 ml of 10 mM TPTZ [2,4,6-tri(2-pyridyl-s-triazine)] in 40 mM HCl and 1 ml of 20 mM ferric chloride. A volume of 0.1 ml plant extract was added with 3 ml of FRAP reagent. The mixture was incubated for 30 min at 37° C. Absorbance of the developed colour was measured at 593 nm. Difference between absorbance of sample and blank were used to calculate the FRAP value and was expressed as mmol of Fe<sup>2+</sup> ion produced ml<sup>-1</sup> of extract using ferric chloride as standard curve. Ascorbic acid and propyl gallate were used as reference antioxidant for comparison.

The results are expressed as mean value ± SD of three determinants. Each moss sample which was taken from three different colonies of a population, were considered for replica analyses.

### 3. Results and discussion

#### 3.1. Phytochemical analysis

Qualitative evaluation revealed the presence of all the studied phytochemical classes in different solvent extracts (Table 1). Methanol extract exhibited presence of most of the chemical

groups. Flavonoids were presumed to be the major extracted chemical in methanol extract of both the mosses as observed from the colour intensity of the reaction mixture. Chloroform extract contained mostly terpenoids along with steroids and glycosides.

**Table 1:** Qualitative evaluation of phytochemicals in *Leucobryum bowringii* and *Dicranum scoparium*.

Phytochemicals	<i>Leucobryum bowringii</i>						<i>Dicranum scoparium</i>					
	Aqueous	Methanol	Ethanol	Chloroform	Diethyl ether	Hexane	Aqueous	Methanol	Ethanol	Chloroform	Diethyl ether	Hexane
Steroids	+	++	++	+	+	+	++	++	+	+	+	+
Terpenoids	-	+	t	++	-	t	-	+	t	++	-	t
Glycosides	+	-	-	+	-	-	+	-	-	+	-	-
Coumarins	-	+	+	-	-	-	-	+	t	-	-	-
Phenolics	+	+	+	t	+	-	+	+	+	t	+	-
Flavonoids	t	++	++	-	-	-	+	++	++	-	-	-
Tannins	+	+	+	-	-	-	+	+	+	-	-	-
Saponins	-	-	+	-	-	-	-	-	t	-	-	-

\*Note: '++'- Present with high abundance; '+'- Present; 't'- Trace; '-'- Absent.

Among the phytochemical groups, contents of total phenolics, flavonoids and triterpenoids were evaluated which are considered to be biologically active constituents. Phenolics and their derivatives are important metabolites that have different biological activities including stress responses. Methanol and ethanol extracts of both the mosses contained high amount of phenolics. *Leucobryum* contained  $28.09 \pm 4.72$  and  $37.71 \pm 4.56$  mg of GAE phenolics  $g^{-1}$  of methanol and ethanol extracts respectively. Methanol and ethanol extracts of *Dicranum* demonstrated  $26.17 \pm 6.02$  and  $26.41 \pm 5.01$  mg of GAE phenolics  $g^{-1}$  dry weight (Table 2). Moderate content of phenolics was accounted in aqueous extracts of both the mosses.

Flavonoids are one of the major class of polyphenolic compounds which are found in diverse groups of plants including bryophytes (Chebil *et al.*, 2007) [12]. These compounds perform a key role in modulating reactive oxygen species levels in plants and therefore have antioxidant

potential (Cooks *et al.*, 1996; Lee *et al.*, 2002) [15, 25]. Crude polar solvent extracts of both mosses exhibited high contents of flavonoids. Highest flavonoid content was observed in ethanol fraction of *Leucobryum* ( $30.45 \pm 5.65$  mg of RE  $g^{-1}$  of extract) followed by methanol and aqueous extracts. In *Dicranum* also, ethanol extract contained highest flavonoids ( $22.42 \pm 0.21$  mg of RE  $g^{-1}$  of extract) followed by methanol and aqueous extracts.

Terpenoid is an important group of metabolite having pharmaceutical potential. Chloroform extract of both the mosses contained highest triterpenoids which is equivalent to  $31.67 \pm 4.04$  mg of linalool  $g^{-1}$  extract in *Leucobryum bowringii* and  $27.6 \pm 5.26$  mg of linalool  $g^{-1}$  extract in *Dicranum scoparium*. Medium to low contents of triterpenoids were accounted in methanol, ethanol and hexane extracts of both the mosses. Therefore, chloroform seems to be the most suitable solvent to extract terpenoids from mosses.

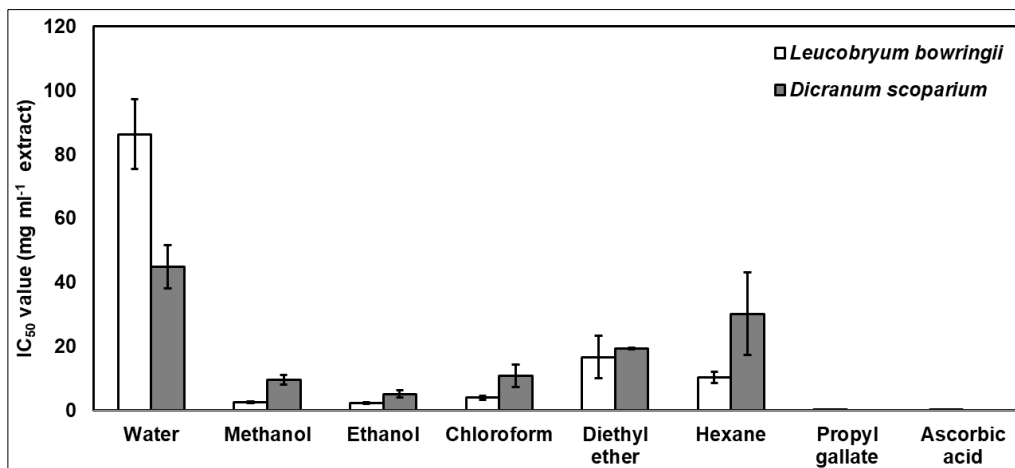
**Table 2:** Phenolics, flavonoids and triterpenoids contents in two plant samples represented as mg of standard equivalent  $g^{-1}$  dry weight  $\pm$  SD (n=3).

	<i>Leucobryum bowringii</i>			<i>Dicranum scoparium</i>		
	Phenolics	Flavonoids	Triterpenoids	Phenolics	Flavonoids	Triterpenoids
Water	$10.55 \pm 1.47$	$6.42 \pm 2.89$	0	$8.96 \pm 0.35$	$3.64 \pm 0.77$	0
Methanol	$28.09 \pm 4.72$	$21.71 \pm 3.49$	$11.87 \pm 5.41$	$26.17 \pm 6.02$	$16.08 \pm 0.08$	$8.23 \pm 1.24$
Ethanol	$37.71 \pm 4.56$	$30.45 \pm 5.65$	$1.89 \pm 0.58$	$26.41 \pm 5.01$	$22.42 \pm 0.21$	$4.33 \pm 0.25$
Chloroform	$0.02 \pm 0.01$	$0.01 \pm 0.002$	$31.67 \pm 4.04$	$0.07 \pm 0.01$	$0.001 \pm 0.0001$	$27.6 \pm 5.26$
Diethyl ether	$9.07 \pm 3.86$	$0.01 \pm 0.004$	0	$5.25 \pm 0.99$	$0.005 \pm 0.001$	0
Hexane	--	0	$5.07 \pm 2.66$	--	0	$3.47 \pm 0.51$

#### 3.2. Antioxidant potential

Antioxidant activity is considered as one of the major aspect of bioactivities and are in focus of interest to pharmacists. Free radicals play the major role in pathogenesis of diseases and are important components of stress signal cascades (Castro and Freeman, 2001) [9]. Different solvent extracts of both the mosses showed dose dependent antioxidant activities as revealed from DPPH radical scavenging and ferric ion reducing properties. DPPH radical scavenging activity is represented as  $IC_{50}$  values which demonstrates the concentration of extract that can inhibit 50% of radicals. Assay revealed higher antioxidant activity of *Leucobryum*

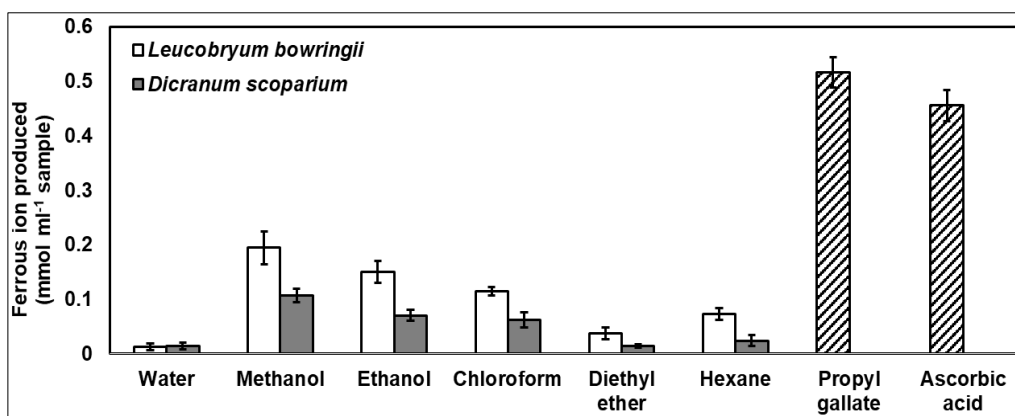
*bowringii* than *Dicranum scoparium*. Bioactivities of ascorbic acid and propyl gallate were also measured which acted as positive control. Highest free radical scavenging potential was accounted by ethanol and methanol extracts of *Leucobryum bowringii* having  $IC_{50}$  values of  $2.21 \pm 0.2$  mg  $ml^{-1}$  and  $2.46 \pm 0.18$  mg  $ml^{-1}$  respectively (Figure 1). Chloroform extract also showed decent scavenging potential with  $IC_{50}$  value of  $3.93 \pm 0.67$  mg  $ml^{-1}$ . In *Dicranum scoparium*, methanol extract exhibited higher antioxidant potential having  $IC_{50}$  value of  $5.10 \pm 1.05$  mg  $ml^{-1}$  followed by methanol and chloroform extracts with  $IC_{50}$  values of  $9.53 \pm 1.49$  mg  $ml^{-1}$  and  $10.67 \pm 3.53$  mg  $ml^{-1}$  respectively.



**Fig 1:** DPPH radical scavenging potential of solvent extracts of the mosses expressed as IC<sub>50</sub> values; error bars represent SD values (n=3).

FRAP assay has also revealed antioxidant potential which can be estimated in terms of formation of Fe<sup>2+</sup> ions from Fe<sup>3+</sup> ions by reducing ability of crude plant extracts. In the positive controls, 0.516 ± 0.03 and 0.455 ± 0.03 mmol Fe<sup>2+</sup> ions generated ml<sup>-1</sup> propyl gallate and ascorbic acid, respectively. In comparison with control, *Leucobryum bowringii* exhibited high bioactivity with generation of 0.194 ± 0.03 mmol Fe<sup>2+</sup>

ml<sup>-1</sup> of crude methanol extract and 0.149 ± 0.02 mmol Fe<sup>2+</sup> ml<sup>-1</sup> of crude ethanol extract (Figure 2). Chloroform extract also showed good bioactivity in FRAP assay. In *Dicranum scoparium*, methanol extract was revealed to have high antioxidant potential followed by ethanol and chloroform extracts.



**Fig 2:** Ferric reducing antioxidant potential of solvent extracts of the mosses expressed as the amount of ferrous ion produced; error bars represent SD values (n=3).

Previous researches exhibited antioxidant activities of solvent extracts of different other mosses. Ethanol extracts of *Atrichum undulatum*, *Polytrichum formosum*, *Pleurozium schreberi* were shown to have antioxidant properties (Chobot *et al.*, 2006, 2008) [14, 13]. Aqueous extract of moss *Bryum moravicum* was also observed to have good antioxidant potential (Pejin *et al.*, 2013) [31]. In the present study, *Leucobryum bowringii* demonstrated more antioxidant potential than *Dicranum scoparium* in both the assays which is justifiable from higher contents of flavonoids, terpenoids and phenolics in the former moss. Antioxidant activity of ethanol and methanol extracts of both the samples may be due to higher phenolics and flavonoid contents in these fractions. Antioxidant property of chloroform extracts may be imparted by the terpenoids of this fraction. Phytochemical data of present study is comparable with some previous reports on mosses (Adebisi *et al.*, 2012) [2]. Polyphenols with antibiotic properties were previously detected in *Atrichum*, *Mnium*, *Polytrichum*, *Sphagnum* (McCleary and Walkington, 1966) [27]. Among the flavonoids, luteolin, apigenin and orobol glycosides and their dimers are usually found in mosses which have bioactive potential (Basile *et al.*, 1999) [6]. Although *Leucobryum bowringii* has not yet been studied for

its phytochemical composition, *Dicranum scoparium* has previously been reported to have antimicrobial activity (Abay *et al.*, 2015) [1]. So, the outcome of the present study may be an important addition to the researches with ethnomedicinally significant mosses.

#### 4. Conclusion

Antioxidant potential of crude solvent extracts of the mosses signifies their other bioactive potential which may be imparted by the phytochemical constituents. Ethnomedicinal uses of the two investigated mosses can be justified by correlating phytochemical composition and bioactivity study. Therefore these mosses can have significant pharmaceutical prospect and can be subjects for future research.

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