



RESEARCH ARTICLE

# Analysis of the antioxidant activity and caffeine content of *Barbula indica* (Hook.) Spreng. (Bryophyta; Pottiaceae)

Supriya Joshi<sup>1</sup>, Emmanuel Iwuala<sup>2</sup> & Afroz Alam<sup>1\*</sup>

<sup>1</sup>Department of Bioscience and Biotechnology, Banasthali Vidyapith, Tonk-304022 (Rajasthan), India

<sup>2</sup>Department of Plant Science and Biotechnology, Federal University Oye Ekiti, Ekiti State, Nigeria

\*Email: [afrozalamsafvi@gmail.com](mailto:afrozalamsafvi@gmail.com)



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## Abstract

The current research aims to investigate the phytochemicals of *Barbula indica* in different solvents, its antioxidant properties and quantification of the caffeine content in a methanolic extract by high-performance thin-layer chromatography (HPTLC). The total alkaloid content was higher in the methanolic extract, 32.06±0.28 mg/g. The antioxidant properties were evaluated by using DPPH and NOSA; the IC<sub>50</sub> values of DPPH and NOSA were 61.09±1.26 µg/mL and 58.04±0.46 µg/mL, respectively. TLC and HPTLC are advanced standardization procedures that provide quantitative and semi-quantitative data about the active compound present in a sample. The development of such a biochemical fingerprint can be used to differentiate the species and adulterants through the analysis of phytochemical content and thus can serve as biochemical markers in pharmacological research and studies related to plant systematics.

## Keywords

Alkaloid, antioxidant, *Barbula indica*, caffeine, HPTLC, phytochemical

## Introduction

India has one of world's most diverse, ancient and distinct culture reating to the medicinal use of plants. A wide variety of medicinally important and valuable phytochemicals are used to treat a variety of illnesses. Mosses, a type of bryophytes, are terrestrial plants found all over the world that play an active role in the cycling of nutrients and water. They also play an important role in regulating weathering and yield many products (1–3). Bryophytes are employed to monitor habitats, in agricultural and urban regions and are in use as bioindicators of atmospheric conditions (4, 5).

Bryophytes do not have advanced protective features like lignified cell walls, thorns, spines, and bark which are possessed by other groups of plants. They however produce a wide range of secondary metabolites that protect plant from insects and infectious microorganisms (6–8). Benzenoids, bibenzyls and other natural products have been identified and described as bryophytes to date (9, 10). Several chemicals that are biologically constituted as antifungal, antiviral, and antidiuretic agents are beneficial in the treatment of burns and bruising (11). *Rhodobryum roseum* and *R. giganteum* are commonly used in China to treat congestive heart failure; *Sphagnum* species have been used in surgical dressing pads due to their excellent permeability; and *Polytrichum commune* has been used as a homeostatic, diuretic and antipyretic substance (12).

The initial step in identifying the phytochemical constituents in a sample is the analysis using TLC and HPTLC which can provide a densitogram and a computerized chromatographic fingerprint which can be used to identify the marker component in a particular plant. Both processes can yield adequate, rapid and consistent reproducible results (13).

Methods based on HPTLC can be a potential substitute as they are being investigated as a useful technique in regular drug analysis. The capacity to evaluate multiple samples simultaneously with a modest amount of mobile phase is a major benefit of HPTLC. This can cut down the amount of time and money spent for chemical analysis. Furthermore, it can reduce the risk of exposure and significantly reduce the challenges associated with the disposal of toxic organic effluents and thus lowering the risk of environmental pollution (14).

Most bryologists in India have so far concentrated studies on morpho-taxonomy and diversification issues (15, 16, 17). As a result, currently there is a lacuna in research relating to phytochemical constituents of bryophytes in India.

With this background, qualitative, quantitative techniques including TLC, and HPTLC were used to determine the phytochemical elements such as alkaloids (caffeine) present in the extract of *B. indica*. The study is found to be crucial and important for scientifically validating folkloric claims about the value of *B. indica*.

## Materials and Methods

### Identification and Collection of Plant sample

*B. indica* (Hook.) Spreng. samples were collected from Nainital, Uttarakhand (India), during December–January 2020 (Fig. ). The specimens were accurately identified based on morphological characters (18). The reference specimen's taxonomic details were submitted to the Banasthali University Herbarium, Rajasthan, India (BURI-1394/2022) at Banasthali Vidyapith.



Fig 1. *Barbula indica* population in the wild

### Extraction

The plant specimens were completely cleaned with water to remove soil and other plant debris. The collected thalli were then placed in liquid nitrogen and transferred to the research lab, where it was stored at  $-80^{\circ}\text{C}$  until further investigation. The thalli of *B. indica* were air-dried at room temperature and pulverized into a powder before extract preparation. Methanol, diethyl ether and hexane solvents were used separately to dissolve 5 g of powder, which was then maintained at  $30^{\circ}\text{C}$  in an orbital-shaker for 48 hours. After filtering, the extract was kept at  $4^{\circ}\text{C}$  until use (19).

### Determination of total alkaloid content

A spectrophotometric technique was used to determine the total anti-oxidant capacity (TAC) (20, 21). This procedure is related to the response linking bromocresol green (BCG) and alkaloids. After mixing the plant sample with different solvents separately, (1 mg/mL and 2 N HCl), it was filtered. 0.1 N NaOH was used to bring the pH of the phosphate buffer combination to neutral. In a separating funnel, 5 mL of BCG solution and 5 mL of phosphate buffer were added to 1 mL of this combination. The mixture was forcefully agitated, and then chloroform was used to extract the resultant complex. The sample was taken and diluted to volume using chloroform in a 10 mL volumetric flask. The complex's absorbance in chloroform was measured at 470 nm.

### Antioxidant Assay

#### Radical scavenging action of DPPH

For determining the effect of the crude methanolic extract on free radicals, the method described by Mukhia *et al.* (22) was used. DPPH states that in 100 mL of methanol, 4 g of DPPH was dissolved. 2 mL of DPPH solution was added to 2 mL of extract. After 30 minutes of incubation period, the decrease in the DPPH free radical was measured using a spectrophotometer (ELICO double beam, SL 210 UV Vis Spectrophotometer) at 517 nm. The scavenging activity of the methanolic extract was determined using the  $\text{IC}_{50}$  value. It's defined as a sample potency that induces a 50% reduction in oxidative radicals. The lower the  $\text{IC}_{50}$  value, the stronger the antioxidant scavenging activity. The % decolorization was used to measure the extract's scavenging activity. The sample's % decolorization was used to determine the sample's scavenging activity.

#### Nitric oxide scavenging assay (NOSA)

The approach of Mukhia *et al.* (22) was used to calculate the nitric oxide scavenging activity of the sample. It was incubated for 150 minutes at  $25^{\circ}\text{C}$  in 0.5 mL of phosphate-buffered saline (1 M; pH 7.4) with 0.5 mL of the extract and 2 mL of 20 mM sodium nitroprusside. After incubation, 3 mL of Griess Reagent was added to the solution compound and allowed to stand for 30 minutes at room temperature. The combination's absorbance was measured at 540 nm. Nitric oxide radical scavenging activity was calculated and expressed as  $\text{IC}_{50}$ .

#### TLC (Thin Layer Chromatography) profile

TLC analysis of the sample was performed with known standards based on the results of the qualitative phyto-

chemical analysis (23). An accurately measured extract was dissolved in methanol solvent to produce a known concentration. The extract was separated into a suitable mobile phase along with the standard silica gel 60F<sub>254</sub> aluminum sheet (3×10 cm). The sample was spotted on the aluminum sheet with a micro-capillary tube. The separation of compounds was done using different combinations of solvent systems and was tested according to their varying polarities. A chromatogram was developed with a solvent system containing a volume ratio of ethyl acetate: methanol (85:15). The TLC sheet was dipped in the solvent chamber of the selected solvent system and allowed to run up to three-fourths of the sheet. It was separated and dried with air. The sheet was kept in the UV-chamber to visualize the spots. Following that, a specific type of compound was completed, with a single compound identified as erectly segregated spots. The retention factor ( $R_f$ ) for each point is comparable to the amount of space moved over the total amount of space covered by the solvent.

### Profile of HPTLC (High-Performance Thin Layer Chromatography)

#### Preparation of Sample

In 1 mL of HPTLC-grade methanol, 5 mg of plant thalli methanolic extract was dissolved and centrifuged for 5 minutes at 3000 rpm. As an HPTLC analysis, this solution was used (24).

#### Developing Solvent System

The HPTLC fingerprint profile for alkaloids was developed using an ethyl acetate: methanol (85:15) solvent solution (25-27).

#### Sample Application

3  $\mu$ L of the reference mix and 2  $\mu$ L of the sample were loaded individually as 5 mm bands on loaded silica gel 60F<sub>254</sub> aluminum sheets (3×10 cm) using a Linomat 5 applicator connected to a CAMAG HPTLC apparatus and Hamilton syringe, which was set up with winCATs software.

#### Chromatogram Development

The chromatogram was collected in a twin-trough glass chamber (20×10 cm) saturated with the appropriate moving phase after the spots were applied.

#### Detection of a Spot

The images were captured using a UV 280 nm visible range and an air-dry plate (CAMAG Reprostar 3 in an image documentation chamber). The winCATs (1.3.4 version) software was used to capture the maximum number, including its height, area, and  $R_f$  values, for fingerprint data.

#### Statistical Analysis

The data are shown as the average of three triplicates ( $n = 3$ ). All the data were analyzed using the IBM SPSS Statistics 20 software. Three-way interactions were applied to the chosen variable. To compare the results, a one-way ANOVA was used. Tukey's  $P$ -value 0.05 Post-Hoc-Tests were used to compare the variance of data for each output variable using multiple comparison. The results are presented as a mean with a standard deviation.

## Results and Discussion

The gametophytes of *B. indica* were examined in this study concerning their phytochemical content, taking the winter season into account and demonstrating their association with water availability in the environment. Alkaloid compounds were also investigated and separated from mixtures of diverse components using TLC and HPTLC.

### Quantitative Analysis of alkaloid

The total alkaloid content of *B. indica* was expressed as caffeine. Alkaloid was highly present in the methanolic extract,  $32.06 \pm 0.28$  mg/g, rather than diethyl ether,  $18.04 \pm 0.10$  mg/g, and hexane,  $9.02 \pm 0.06$  mg/g (Fig. 2). The methanolic extract of the plant produced the best result.

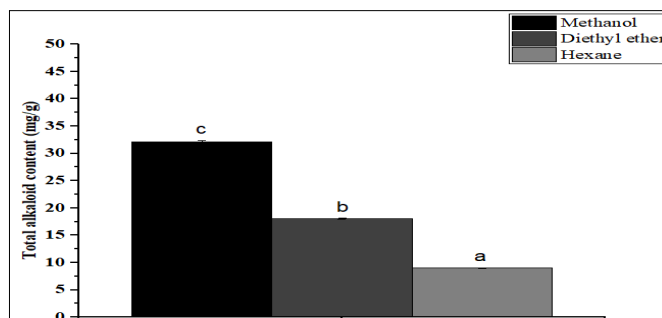


Fig 2. Quantitative analysis of the total alkaloid content of *B. indica*.

### Antioxidant properties

For the DPPH assay, the  $IC_{50}$  value of *B. indica* was  $61.09 \pm 1.26$   $\mu$ g/mL whereas, for the NOSA assay, the  $IC_{50}$  value was  $58.04 \pm 0.46$   $\mu$ g/mL (Fig. 3).

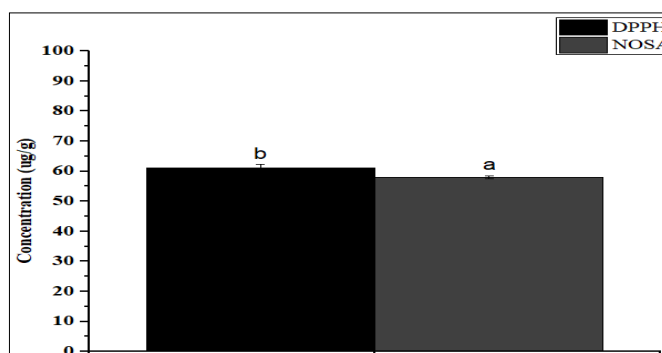


Fig 3. Antioxidant activities of *B. indica* in methanolic extract.

### Thin Layer Chromatography (TLC)

The result of the separation of an alkaloid compound in a methanolic extract of *B. indica* by thin-layer chromatography shows a spot with a light violet color, and  $R_f$  values determined by the distance travelled by the solute and the distance travelled by the solvent are both measured. The standard and sample  $R_f$  values were 0.55 and 0.53, respectively, confirming the presence of an alkaloid such as caffeine (Fig. 4).

### HPTLC (High-performance thin-layer chromatography)

The result from HPTLC fingerprint scanning for alkaloids such as caffeine at wavelength 280 nm in a methanolic extract of *B. indica* (Fig. 5). The  $R_f$  values in ascending order begin at 0.42 and end at 0.48, with the highest caffeine concentration being 23.95 ng, or 24.18 percent, and its corresponding  $R_f$  value being 0.47 (Table 1; Fig. 6).

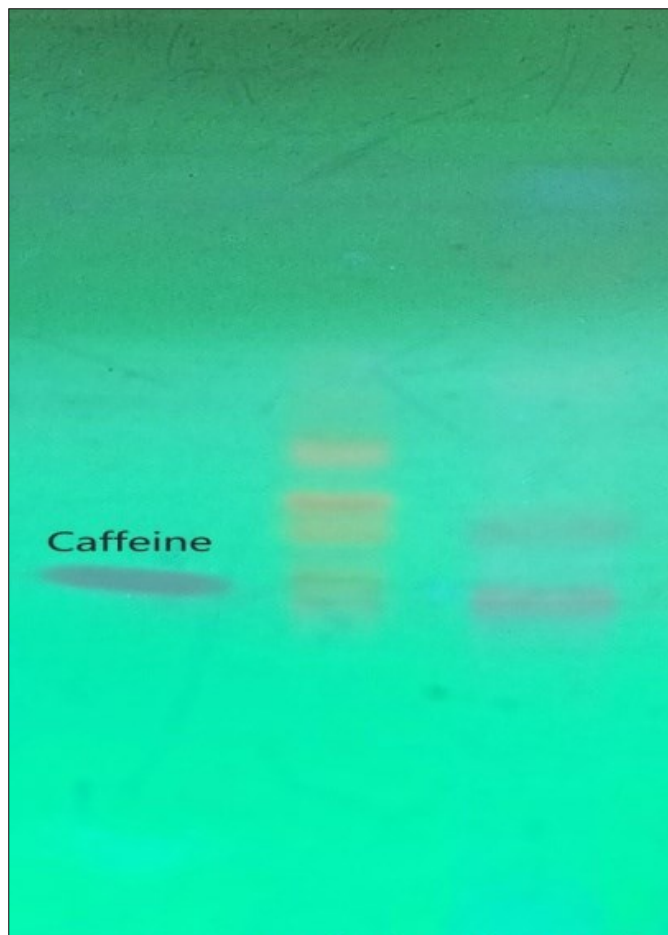


Fig 4. Spot detection of *B. indica* by using TLC

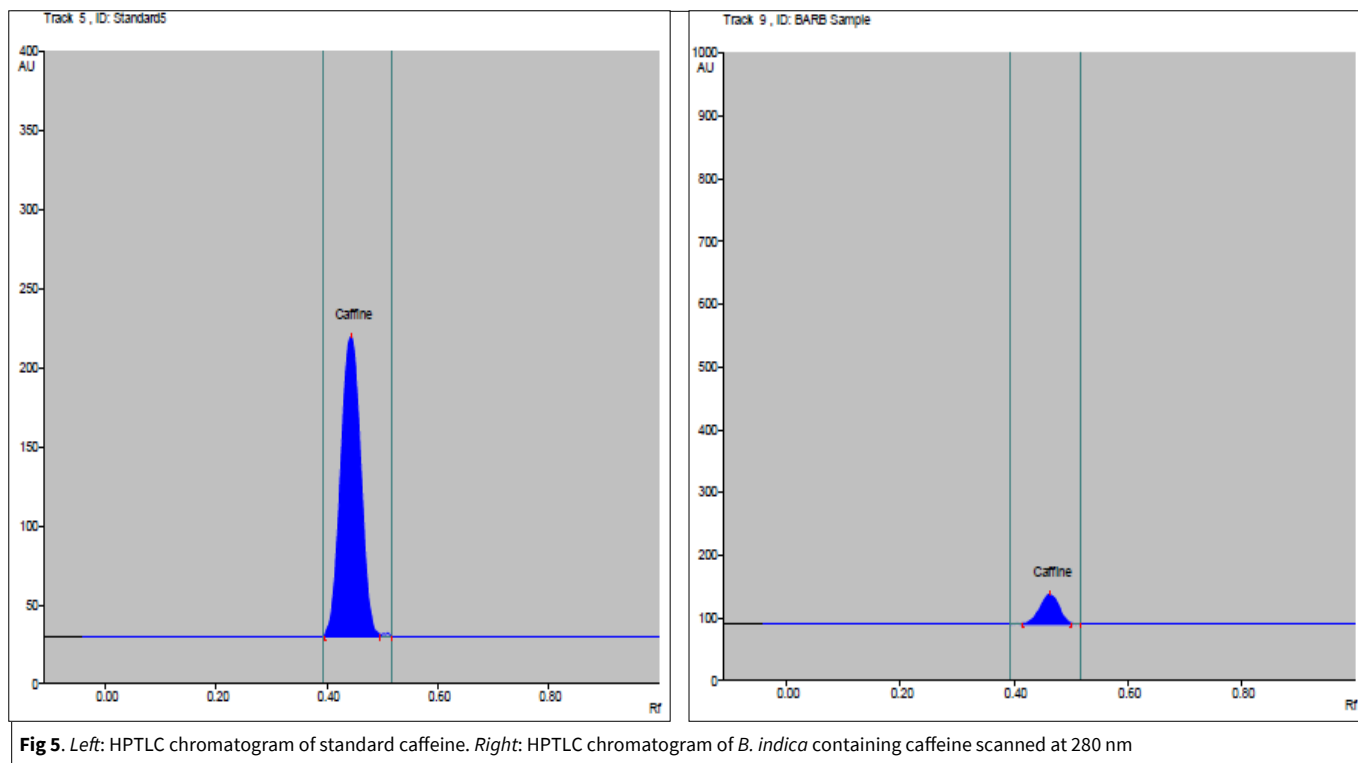


Fig 5. Left: HPTLC chromatogram of standard caffeine. Right: HPTLC chromatogram of *B. indica* containing caffeine scanned at 280 nm

Standardization and authentication measures should be used to ensure the identification, quality, purity, and safety of herbal medications. The chromatographic approach is the most generally utilized technique for general use among the several methods available for separating the plant elements (28).

This study included phytochemical profiling, anti-

oxidant profiling, and HPTLC profiling. The antioxidant capacity of this species could be attributed to the phytochemical content, which can act as a reducing agent, as well as the synergistic effects of the alkaloid. The presence of an active metabolite in the methanolic extract of the study species, *B. indica*, was confirmed by TLC and HPTLC analysis. To separate the bioactive molecule, TLC and HPTLC used relatively high polar solvents such as ethyl acetate and methanol as mobile phases (Figs. 4–5). Many early results (29, 30) reveal that this mobile phase of high-polarity solvents can effectively separate bioactive chemicals in a variety of plant species. Alkaloids are a large and diversified collection of secondary metabolites found in almost all plants at concentrations of 10–15%. At the cellular level, it also has anti-mitotic and allergenic properties. Many alkaloids are bitter, yet they have a physiological effect that makes them useful in therapies for disorders like malaria, diabetes, cancer, and heart failure (31).

### Conclusion

The present study was carried out to determine the phytochemical and antioxidant parameters of *B. indica*, in detail. The presence of diverse phytoconstituents could explain the diverse pharmacological effects of *B. indica*. Apart from other alkaloids, this study also estimated caffeine, in *B. indica*. Although this species is frequently found in the Kumaun region of Uttarakhand and though it is easy to

identify and collect, few people are aware of its therapeutic benefits including that of other bryophytes. This finding can make the plant as an easily accessible choice of caffeine for the tribal people who can use them as a choice whenever caffeine is required for therapeutic purposes. In this way, the present study places this commonly growing moss species as an addition to the local herbology.

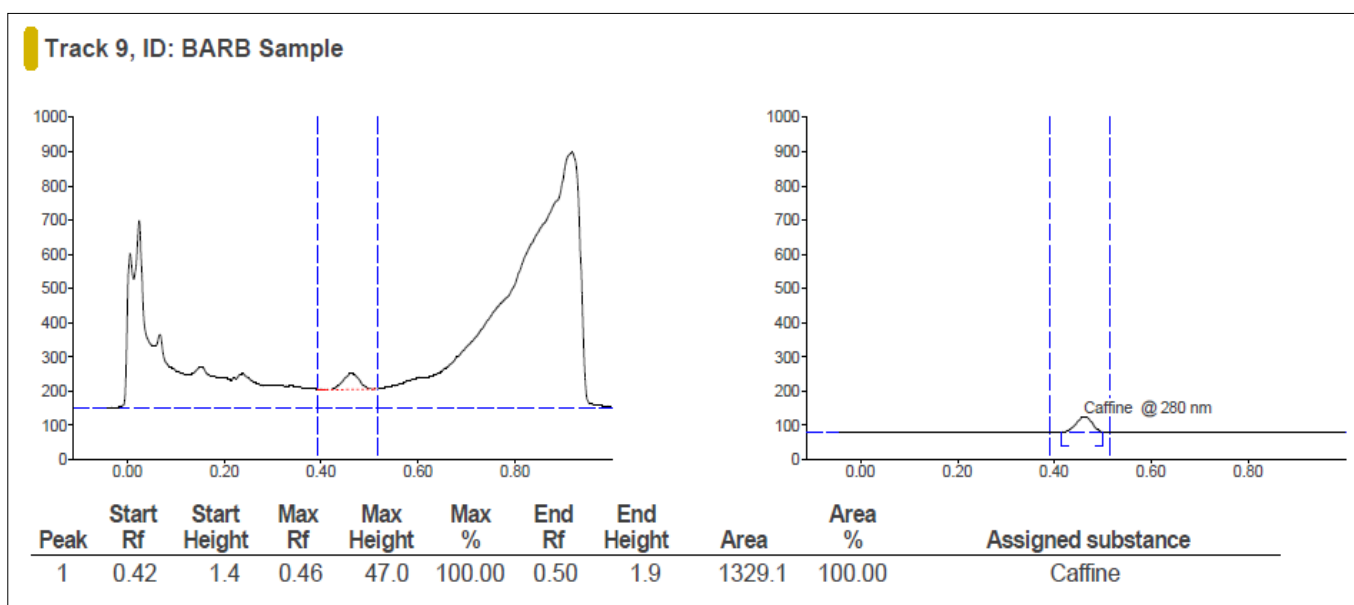


**Table 1.** Height and amount of caffeine present in the standard and sample.

Substance: Caffeine @ 280 nm

Regression via height: Polynomial  $Y = 5.499 + 2.366 * X + -0.004758 * X^2$   $r = 0.99899$   $sdv = 2.44$

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
1	1	0.46	20.00 ng	48.64				
2	1	0.43	40.00 ng	94.68				
3	1	0.44	60.00 ng	131.63				
4	1	0.44	80.00 ng	167.55				
5	1	0.44	100.00 ng	189.30				
6	1	0.45	120.00 ng	219.08				
7	1	0.45	140.00 ng	245.98				
8	2	0.46		27.62	18.00 ng			BARB Sample
9	2	0.46		46.99	18.20 ng			BARB Sample
10	2	0.48		59.43	23.95 ng			BARB Sample

**Fig 6.** HPTLC chromatogram of methanolic extract of *B. indica*

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## Authors contributions

All authors worked together to complete this work. The subject was created, and the plant was selected by AA. The experiments were done, the first draft was written by SJ, and the obtained results were analysed by EI. All authors reviewed and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interests to declare.

**Ethical issues:** None.

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