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Phytochemical evaluation of different solvent mediated extracts of *Riccia billardieri Mont. & Nees*

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Abstract
The past few decades have seen a great wealth of secondary compounds being extracted from the bryophytes. Riccia is a cosmopolitan genus being represented by about 36 species in India. The present study is an attempt to analyse the phytochemical constituents of *Riccia billardieri*. Soxhlet extraction followed by qualitative and quantitative analyses has revealed about ten groups of secondary compounds in varying quantities. Glycosides, alkaloids, saponins, reducing sugars, flavonoids, phenols, terpenoids and coumarins were detected.

Keywords: *Riccia billardieri*, soxhlet extraction, secondary compounds

Introduction
Bryophytes are one of the oldest terrestrial green, spore forming plants, based on the resemblance of the present-day liverworts and the first land plant fossils, the spores of which date back almost 400 million years. The list of secondary compounds being isolated & identified from bryophytes has attained a rapid pace in the past few decades. Terpenoids isolated from the oil-bodies, a unique organelle of the liverworts are responsible for most of the biological properties associated with this group of terrestrial plants. They have been implicated in phytotoxicity, antimicrobial and antifungal activity, activities on insects and molluscs, piscicidal activity, antileishmanial and antitrypanosomal activity, as well as cytotoxicity & anti-inflammatory action. Despite these & many traditional reports, the phytochemistry of bryophytes has been neglected for a long time because they are morphologically insignificant and difficult to collect in large amounts. Being seasonal and dependent on moisture to complete their life cycle they are ephemeral. Bryophytes, as a diverse group, are chemically still incompletely known although; many new compounds for science are being described from them [1]. These tiny plants are in fact superior & cheaper production systems for many recombinant pharmaceuticals [2]. So in a world where pathogens are evolving at much faster rate than the discovery of new antidotes, it is absolutely the need of the hour that such miraculous chemical factories like the bryophytes are researched and exploited for their chemical constituents.

Materials and Methods
Plant material
Riccia billardieri used in this study was authenticated by Dr S K Singh, Scientist, Botanical Survey of India, Northern Regional Centre, Dehradun. The plants were firstly washed gently under running tap water to remove dust and soil and then rinsed with distilled water. 50g shade dried thalli, of *Riccia billardieri* were ground to a powder in a mortar & pestle & extracted with 200 ml of Petroleum ether, Chloroform, Ethyl acetate, Acetone, Ethanol, Methanol & Water respectively for 8 hours using soxhlet hot continuous extraction. The extracts were filtered and concentrated using rotary evaporator at 50° C.

Qualitative Phytochemical Analysis
All the extracts were subjected to preliminary phytochemical screening following standard methods for the detection of the following constituents.

Detection of Alkaloids
The extract was mixed with 10 ml of 2% sulphuric acid for two minutes and filtered. A known quantity of aliquot was treated with a few drops of Dragendorff's reagent. Orange brown precipitate indicated the presence of alkaloids.

Detection of Phenolic Compounds
To a fraction of the extract was added neutral ferric chloride (5%) solution. Deep blue coloured precipitate indicated presence of phenolic compounds.

Detection of Flavonoids
To an aliquot of the extract, was added 1N aqueous NaOH, resulting in an yellow-orange colour. The colour test was proof of flavonoids.

Detection of Saponins
A small quantity of the extract was vigorously shaken with water. Formation of foam indicated presence of saponins.

Detection of Tannins
A small amount of the extract was diluted with distilled water in the ratio 1:4, and a few drops of 10% Ferric chloride solution was added. Appearance of a blue or green colour indicated the presence of tannins.

Detection of Glycosides
To the extract evaporated to dryness, 0.4 ml of glacial acetic acid containing trace amount of Ferric chloride was added.

The solution was then transferred to a test tube and 0.5 ml of concentrated H_2SO_4 was carefully added along the side of the test tube. The acidic layer developed a blue colour indicating presence of glycosides.

Detection of Terpenoids
Extract was (1 ml) dissolved in chloroform and a few drops of acetic anhydride followed by the addition of a few drops of H_2SO_4 gave a dark green colour indicating terpenoids.

Detection of Steroids
To the extracts evaporated to dryness, a few drops of acetic anhydride and concentrated H_2SO were added; an array of colour change from yellow, green and brown to black indicated the presence of steroids.

Detection of Coumarins
The extract dissolved in ethanol, a few drops of alcoholic NaOH was followed by the addition of concentrated HCl through the side of the test tube. The appearance and disappearance of yellow colour indicated the presence of coumarins.

Quantification of Secondary Metabolites
Quantification of Phenol
An aliquot of the chloroform, ethyl acetate, methanol, petroleum ether and aqueous extracts were pipetted out separately and made up to 3 ml with 80% methanol. 0.5 ml *Folin-ciocalteau* reagent was added and kept for 3 minutes. 2 ml of 20 % Na_2CO_3 was added to the mixture and kept in boiling water bath for 1 minute. The white precipitate developed which was removed by centrifuging it for few minutes and the absorbance of the clear blue solution was recorded at 750 nm against the blank containing 3 ml of 80 % methanol, 0.5 ml Folin's reagent and 2 ml of 20 % Na_2CO_3. The reaction between phenols and phosphomolybdate in Folin-ciocalteau reagent resulted in the formation of a blue complex. Gallic acid from 1.56 to 100 µg/ml was used as the standard. Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per g dry plant material. All samples were analysed in triplicate.

Quantification of Flavonoids
The total flavonoid content of the extracts was determined by $AlCl_3$ method. 100µl of the extract was mixed with 100µl of 2% $AlCl_3$ and 2 drops of glacial acetic acid. The mixture was diluted with methanol to 3 ml. After 45 minutes, the absorbance was read at 415 nm using the extract without $AlCl_3$ as blank. Standard curve was made using Quercetin (50-250µg/ml) in methanol under the same conditions. Total flavonoid content of the extracts was expressed as mg quercetin equivalents (QUE) per gram of dry extract (mg/g) [3]. Samples were analysed in triplicate.

Quantification of Terpenoids
. About 50 gm of shade dried powder was soaked in ethanol for 24 hours. It was filtered and filtrate extracted with petroleum ether; in a separating funnel. The ether extract was air dried & treated as total terpenoids [4].

Quantification of Saponins
Saponin content was estimated using anisaldehyde reagent following the method of Shiau *et al*., 2009 [5]. 500µl of sample and 500µl of 0.5% anisaldehyde were mixed and incubated for 10 minutes. 2ml of 50% H_2S0_4 was added along the sides of the tubes and then incubated at 60°C, for 10 minutes in a water bath. After cooling, the absorbency was taken at 435nm. From the standard curve, the saponin content was estimated.

Quantification of Alkaloids
Alkaloids were estimated using the procedure of Sreevidya and Mehrotra, 2003 [6]. Alkaloids were precipitated using Dragendorff reagent. The solution was centrifuged and the supernatant discarded. The residue was then treated with 2 ml disodium sulfide solution. The brownish black precipitate formed was then centrifuged. Completion of precipitation was checked by adding 2 drops of disodium sulfide. To the precipitate 2ml of concentrated HNO_3 was added, shaken and warmed until the precipitate dissolved completely. This solution was diluted to 10 ml in a standard flask with distilled water; 1 ml was then pipetted out, and 5 ml thiourea solution was added to it. The absorbency of the yellow solution developed was measured at 435 nm, and result obtained in mg/gm tissue.

Quantification of Steroids
Steroid content of the plant sample was determined using the method described by Trease and Evans (1989) [7]. A portion of 2 mL was taken from a solution of 2.5 g of powdered plant material prepared in 50 mL of solvent after vigorous shaking for 1 h. The extract solution was washed with 3 mL of 0.1 M NaOH (pH 9) and later mixed with 2 mL of chloroform and 3 mL of ice cold acetic anhydride followed by adding two drops of concentrated H_2SO4 cautiously. The absorbance of both sample and blank were measured spectrophotometrically at 420 nm.

Results and Discussion
The presence & relative abundance of phytochemicals in the solvent extracts of *R.billardieri* are presented in (Table -1). Phenols, flavonoids, terpenoids, coumarins & saponins comprises, the major share of compounds from *R.billardieri*. Water was the best solvent system, as the aqueous extract gave traces of almost all phytochemicals excepting the terpenoids. Phenols, terpenoids, flavonoids, steroids & alkaloids were estimated as these components have been implicated in many previous studies as showing biological activity.
Phenolics are plant substances which possess in common an aromatic ring bearing one or more hydroxyl groups. They possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic as well as the ability to modify gene expression. Phenolics are the largest group of phytochemicals that account for most of the antioxidant activity in plants or plant products [8]. In the current study the maximum yield of phenolics was from the ethanolic fraction (30.4 mg GAE/g), & the minimal yield was (2.5 mg GAE/g) from the petroleum ether fraction. (Table-2). Abu Bakar *et al*., 2015 [9]. had reported earlier that the methanolic & ethanolic fractions of plant extracts derive better phenolic content than the non-polar solvents like petroleum ether.
There are about 8000 naturally occurring plant phenolics and about half of this number is flavonoids. They constitute the largest group of naturally occurring phenolic compounds, occurring both in the free state as well as

glycosides. Flavonoid concentration was estimated as mg Quercetin Equivalents/g tissue. Aqueous, Ethanolic and methanolic fractions gave the maximum flavonoid contents. The aqueous fraction had 28.37 mg QUE/g while the ethanolic & methanolic fraction content was slightly lower at 26.02 & 21.93 mg QUE/g respectively. The flavonoid yield showed a decreasing trend towards the non-polar region of the solvents as they are water soluble compounds which show maximum extractive yield in 70% ethanol. The minimum yield was obtained in ethyl acetate at 3.25 mg QUE/g.

Terpenoids are a large family of isoprenoid compounds, that are lipid soluble components & pigments which are extracted in non-polar solvents like petroleum ether or chloroform. In the current study terpenoids including steroids were detected in the nonpolar solvents. Terpenoid content was restricted to petroleum ether 15.50 mg/g, chloroform 14.6mg/g, ethyl acetate 9.44 mg/g & acetone 7.22 mg/g respectively. Steroid content was also restricted to the above fractions at 6.5 mg/g, 7.98 mg/g, 2.23 mg/g & 1.43 mg/g respectively.

Alkaloids are nitrogen containing compounds which are the most widely distributed among all plant secondary metabolites. Alkaloid presence was detected in almost all extracts, but their maximal values were from the non-polar solvents. Both petroleum ether and chloroform gave almost equal yields, 9.70 mg/g & 9.30 mg/g respectively. The polar solvents yielded less than 1 mg/g.

Table 1: Presence & Relative concentration of Secondary Metabolites, in various solvents

Phytochemicals	Pet. ether	Chloroform	Ethyl acetate	Acetone	Ethanol	Methanol	Water
Reducing Sugar	-	++	-	+	+	-	++
Glycosides	-	-	+	+	++	++	+++
Flavonoids	-	-	+	+	+++	+++	++
Alkaloids	+++	+++	-	+	+	+	+
Tannins	-	-	-	-	+	++	++
Phenols	+	-	-	+	+++	++	++
Terpenoids	+++	+++	++	++	-	-	-
Steroids	+++	+++	++	+	-	-	-
Coumarins	++	+++	+	-	+	+++	+
Saponins	++	-	-	-	+	+++	++
(+, ++, +++): Relative concentration of phytochemicals in the extract.							

Table 2: Secondary metabolites of *R.billardieri*, in various solvents expressed as mg/g tissue.

Solvents	Phenols[a]	Terpenoids	Flavonoids[b]	Steroids	Alkaloids
Pet. Ether	2.5±2.6	15.50±3.1	-	6.5±0.1	9.70±0.9
Chloroform	-	14.6±1.1	-	7.98±0.1	9.30±0.2
Ethyl Acetate	-	9.44±0.7	3.25±0.5	2.23±1.1	-
Acetone	9.30± 0.2	7.22±0.7	3.5±2.5	1.43±1.1	0.67±3.1
Ethanol	30.40±2.1	-	26.02±0.1	-	0.87±3.1
Methanol	21.58±0.5	-	21.93±0.02	-	0.70±0.1
Water	18.92±0.3	-	28.37±0.1	-	0.50±0.1

Values are means of three replicates ±S.D.

[a]Total Phenolic content expressed as mg Gallic acid equivalents (mg GAE/g).

[b]Total Flavonoid content expressed as mg Quercetin equivalents (mg QUE/g).

Conclusion

Qualitative phytochemical screening showed that it is abundant in phytochemicals belonging to glycosides, alkaloids, saponins, reducing sugars, flavonoids, phenols, terpenoids and coumarins. Maximal extracts of all the chemicals were in petroleum ether, ethanol and aqueous fractions. Further tests for biological efficiency, of these extracts needs to be conducted to ascertain whether the extracts have any biological potential.

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