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PRELIMINARY PHYTOCHEMICAL ANALYSIS OF *RICCIA BILLARDIERI* EXTRACTS IN 5 DIFFERENT SOLVENTS

Mahesh S.[a], Laija S. Nair & Mini I.

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Abstract

Riccia is the largest genus of thallose liverworts in India. The members of the genus are simple, primitive both morphologically and anatomically. The present study analysed the phytochemical profile of *R. billardieri*. Shade dried thalli of Riccia billardieri were extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, methanol and water respectively for 10 hours using Soxhlet hot continuous extraction. The extracts were filtered & concetrated using a rotary evaporator and weighed. Solvent extracts were screened for the presence of different phytochemicals based on the method of Khandelwal (2007). Presence/absence of Alkaloids, Phenols, Flavonoids, Saponins, Tannins, Glycosides, Terpenoids, Steroids and Coumarins were analysed. The study gave extracts with series of compounds having relatively good yields for phenols, terpenoids and flavonoids. Further analyses is needed to clarify the results obtained. Quantification and characterisation of the extracts needs to be done to ascertain the presence of unique compounds and also to derive taxonomic markers if any are present.

Keywords: *R. billardieri,* phytochemical profile.

Introduction

Bryophytes numbering 15,000 to 25,000 and more are the second largest group of ter¬restrial plants. Ecologically important as pioneer vegetation in many succession series, these physically insignificant, ephemeral colonial plants have to survive multifarious threats to complete their lifecycles. Habitat destruction, climatic shifts, temperature fluctuations are but some of the abiotic stresses which they face. Grazing animals form another part of biotic stress faced by them. Yet these plants which lack any sort of secondary protective tissues overcome and survive these adversities. They are veritable chemical factories, producing a plethora of chemical compounds which provide protection from pathogens, grazing animals and stresses. These compounds show a wide range of physiological and biological actions ranging from desiccation tolerance, allelopathic, anti microbial, anti cancerous, anti insectivorous, anti viral etc. Given their insignificant physical presence, the volume of chemicals they produce and their effect should have made this group of plants, favourites of plant researchers. Yet in the past century very few have been analysed from the chemical perspective. Traditional systems of healing have utilised these plants for their curative properties from time immemorial, but scientific validation has been lacking in a majority of cases. It was only from the last decades of the 20th century and the present, that interest in this area has resur¬faced with taxonomic and biochemical prospecting leading the way. Terpenoids, Poly phe¬nols, organic acids, flavonoids, bis-benzyls are but some of the array of chemicals now being unearthed from these plants. Classification of the liverworts is extremely difficult morpho¬logically, and thus a study of their unique secondary metabolites is invaluable in assigning species. Studying the natural products present in liverworts, particularly the oil bodies which produce not only a number of lipophilic terpenoids with a variety of carbon skeletons but also aromatic compounds, especially phenolics (Asakawa, 1982a, 1995, 1997, 2001; Asakawa et al., 2001a; Zinsmeister and Mues, 1990) can be of great importance, phytochemically and taxonomically.

Materials and Methods

Plant Material:

Riccia is the largest genus of thallose liverworts in India. The members of the genus are simple, primitive both morphologically and anatomically, and characterized by usually linear or oblong thalli differentiated into photosynthetic and storage zone and with or without simple air pores, with embedded sessile sporophytes. They are unique in their habits in having the tendency to grow in rosettes (a unique feature of the genus). 36 species of Riccia have been reported from India, 18 each from Western Himalayas and the Western Ghats. Low elevated/plain areas of Punjab & West Rajasthan, Gangetic plains and Central India also show presence of various species. They are mostly terrestrial in habit. About 11 species are endemic to India (Sushil Kumar Singh, 2014).

[a]Dept. of Botany, Christian College, Kattakada, Trivandrum
Corresponding author, e mail: maheshlaija@gmail.com

Soxhlet Extraction:
80g of shade dried thalli of *Riccia billardieri* were extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, methanol and water respectively for 10 hours using Soxhlet hot continuous extraction. The extracts were filtered & concetrated using a rotary evaporator and weighed. The extracts yielded residues as follows, Chloroform (0.27 g), Petroleum ether (0.28 g), Ethyl Acetate (0.58 g), Methanol (0.56 g) and Water (0.56 g).

Qualitative analysis:
Solvent extracts were screened for the presence of different phytochemicals based on the method of Khandelwal (2007). Presence/absence of Alkaloids, Phenols, Flavonoids, Saponins, Tannins, Glycosides, Terpenoids, Steroids and Coumarins were analysed.

Detection of Alkaloids
a) Mayer's test : Mayer's reagent was added to a fraction of the extract. Formation of a cream coloured precipitate confirmed the presence of alkaloids.
b) Dragendorff's test : To a fraction of the extract was added Dragendorff's reagent and an orange - brown precipitate indicated the presence of alkaloids.
c) Wagner's test : Wagner's was added to a fraction of the extract and a reddish-brown precipitate confirmed the presence of alkaloids.

Detection of Phenolic Compounds
a) Ferric chloride test : To a fraction of the extract add Neutral ferric chloride (5%) solution and the presence of a deep blue colour indicated phenolic compounds.
b) Lead acetate test : Addition of lead acetate solution (10%) to a fraction of the extract giving, a white precipitate confirmed the presence of phenolics.

Detection of Flavonoids
a) Aqueous NaOH test : To an aliquot of the extract, 1N aqueous NaOH was added, yellow - orange colour was detected, which indicated presence of flavonoids.
b) Concentrated H2SO4 test : To a small quantity of the extract was added concen trated H2SO4, formation of orange colour confirmed presence of flavonoids.
c) Shinoda test : To an aliquot of extract was added pieces of Magnesium turnings, followed by concentrated HCL and then heated slightly. Development of dark pink colour was detected conforming the presence of flavonoids.

Detection of Saponins
Foam test: A small quantity of the extract was vigorously shaken with water and formation of foam indicated presence of saponins.

Detection of Tannins
a) Gelatin test: The extract was tested with 1 % gelatin solution containing 10% Sodi um chloride, appearance of white precipitate detects tannin.
b) Ferric chloride test: A small amount of the extract was diluted with distilled water in the ratio 1:4, a few drops of 10 % Ferric chloride solution was added to it. Blue or Green colour indicated the presence of tannins.

Detection of Glycosides
Keller killani test: To the extract evaporated to dryness, was added 0.4 ml of glacial acetic acid containing trace amounts of Ferric chloride. 0.5 ml of concentrated H2SO4 was carefully along the side of the test tube, appearance of blue colour in the acetic acid layer indicated glycosides.

Detection of Terpenoids
Liebermann-Burchard test: Extract (1 ml) was dissolved in chloroform and a few drops of acetic anhydride was followed by the addition of a few drops of H_2SO_4. Development of dark green colour detected terpenoids.

Detection of Steroids
To the extract evaporated to dryness, a few drops of acetic anhydride and concentrated H_2SO_4; was added, an array of colour change from yellow, green, and brown finally to black indicated the presence of steroids.

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

Quantitative analysis:
Quantification of phenols
Total phenol content was estimated by the method of Mayer et al., (1995). An aliquot of 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 incubated 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 formed was removed by centrifuging it for few minutes and the absorbance of the clear blue solution was recorded at 650 nm against reagent blank. The reaction between phenols and phosphomolybdate in Folin-ciocalteau reagent results in the formation of a blue complex which is estimated spectrophotometrically.

Quantification of flavonoids
The total flavonoid content of the extracts were determined by AlCl3 method (Mervat et al., 2009), with slight modification. 100µl of extract was mixed with 100µl of 20 % $AlCl_3$ and 2 drops of glacial acetic acid. The mixture was diluted with methanol to 3 ml. After 45 minutes, the OD was read at 415 nm using the extract without $AlCl_3$ as blank. Standard curve was made using quercetin (50-250 µg/ml) in methanol under the same condition. Total flavonoids was expressed as mg quercetin equivalent/g weight.

Table 1. Phytochemical profile of *R. billardieri* using Chloroform, Petroleum Ether, Ethyl acetate, Methanol and Water extracts.

	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Water
Reducing sugar	_	++	_	_	++
Glycosides	_	_	+	_	++
Flavonoids	_	_	_	+++	+++
Alkaloids	_	+++	+++	_	++
Tannins	_	_	_	++	++
Phenol	_	_	_	++	++
Terpenoids	_	++	_	_	++
Steroids	+++	+	++	+++	++
Coumarins	++	+++	+	+++	+
Saponins	++	_	_	+++	++

Quantification of Terpenoids

Ferguson's Method : 10 g of plant powder was taken separately and shaken in alcohol for 24 hours. It was filtered and, the filtrate was extracted with chloroform, this extract was treated as total terpenoids.

Results and Discussion

The bryophytes for their survival depend on plethora of chemicals to overcome adverse environmental conditions, discourage grazing animals and to prevent infection from pathogens. In addition most of the hepatics produce unique secondary metabolites which can be used to demarcate them taxonomically. They are peculiar to liverworts and show interesting biological activities, such as antimicrobial, antifungal, cytotoxic, insect antifeedant, insecticidal, muscle relaxing, some enzyme inhibitory and apoptosis inducing activities (Asakawa, 1981, 1982a, 1988, 1990a,b, 1993, 1995, 1998, 1999; Mues, 2000; Zinsmeister et al., 1991). This assumes great importance especially in a country like India which is rich in Bryophyte Flora but has very scarce taxonomic data regarding them.

Riccia a common thalloid liverwort was made the target of this study, for phytochemical profiling. Qualitative screening of extracts detected a series of compounds in non-polar to polar solvents (Table 1). Quantification was restricted to Phenols, Flavonoids and Terpenoids, as they have been reported earlier to have maximum biological action.

Terpenoids: Kempinski et al., 2015, has reported on the important physiological roles (i.e. hormones, aliphatic membrane anchors, maintaining membrane structure) and ecological roles (i.e. defense compounds, insect/animal attractants) of terpenoids. Pharmaceutical and industrial applications ranging from flavours and fragrances (Schwab et al., 2008) to medicines (Dewick, 2009; Niehaus et al., 2011; Shelar 2011) have been analysed. The pattern of terpenoids and aromatic compounds often depends not only on developmental stage, season and altitudinal distribution, but also on sexual (male, female and sterile) forms of the same species. Complex diterpenoids have been reported from morphologically primitive liverworts, further analysis of the terpenoids from the present study needs to be done to concur or differ from these findings. The study yielded 6.60±0.10 mg/g in chloroform and 4.60±0.27 mg/g in water (Table 2). Classical reports on presence of oil bodies as reserve food source in Riccia is well documented, which probably accounts for the large yields of terpenoids with respect to the other analysed chemicals. Storage terpenoids are usally non volatile and hence do not provide any characteristic smell. Storage terpenpoids have solubility in water, as they are physically present in the cytoplasm as oil bodies. Further studies are required to differentiate and characterise these biologically important compounds.

Sterols: The Ricciaceae comprise two genera, Ricciocarpos and Riccia. (Asakawa, 1982a) reported the presence of phytosterol mixtures from Riccia. Presence of sterols in this study is in concurrence with the above results. Sterols were present in significant volumes in all extracts. Petroleum ether and methanol extracts gave the maximal amount of

sterols ascertained by the qualitative tests. Asakawa, Y., et al.(2012) has reported that the singular presence of sterols in comparison to the other compounds in Riccia, which might make it significant as a bio marker for this genus, among the Marchantiaceae members.

Flavonoids: Flavonoids are ubiquitous water soluble components in bryophytes and have been isolated from or detected in the Marchantiophyta. In comparison to Terpenoids and Phenols the current study gave lesser yields of flavonoids, 0.83±0.02 mg/g in methanol and 2.83±0.22 mg/g in water. Flavonoids were absent in chloroform extracts as their solubility is less in non-polar solvents.

Phenols: Oiso et al., 1999 analysed about 1000 sps of liverworts from around the world and identified specific chemical markers for the majority of genera of liverworts. One of the most characteristic chemical constituents of liverworts are bis-bibenzyls because these types of phenolic compounds have not been found in any other organism, except in a Japanese fern, Hymenophyllum barbata. Phenolics (Asakawa, 1982a, 1995, 1997, 2001; Asakawa et al., 2001a; Zinsmeister and Mues, 1990) can be of great importance, in liverworts both phytochemically and taxonomically. In the current study phenolics were present in methanolic and aqueous extracts to the tune of 5.58±0.07 mg/g and 7.50±0.09 mg/g respectively (Table 2). Characterisation of the phenolic content needs to be done to identify the specific components present. Phenolic compounds have roles in lignin synthesis, (Manoj and Murugan, 201) have reported that many liverworts although lacking in lignified tissue produce relatively great amounts of this compound. The present study gave higher values of phenolic content (Table 2), than reported by them (1.44 to 2.4 mg/g) tissue. Phenolics are present in plants not only as structural components but also in the form of phenolic acids, which are present in the cytosol. The non specific accumulation of phenolic acids, their oxidation and condensation products might be a defence mechanism against invading pathogenic bacteria. Phenolic acids inactivate and degrade pathogenic enzymes.

The present study analysed the phytochemical profile of *R. billardieri*. The study gave extracts with series of compounds having relatively good yields for phenols, terpenoids and flavonoids. Further analyses is needed toclarify the results obtained. Quantification and characterisation of the extracts needs to be done to ascertain the presence of unique compounds and also to derive taxonomic markers if any are present.

Table 2. Total Phenols, Flavonoids & Terpenoids in mg/g tissue. Values are mean ± SD of three replicates.

	Total Phenols (mg/g tissue)	Flavonoids (mg/g tissue)	Terpenoids (mg/g tissue)
Chloroform	_	_	6.60±0.10
Methanol	5.58±0.07	0.83±0.02	-
Water	7.50±0.09	2.83±0.22	4.60±0.27

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