

Identification of flavonoids from the aquatic pteridophyte *Azolla microphylla* using High Performance Thin Layer Chromatography

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Among the several phytochemical constituents flavonoids are important as they exhibit considerable antioxidant activity and are also reported to have antimicrobial activity. Hence in this study HPTLC analysis was carried out to detect flavonoids using Chloroform-Ethyl acetate-Glacial acetic acid as mobile phase and quercetin and kaempferol as standard. The whole plants of *Azolla microphylla* showed at least eight different types of flavonoids with R_f values ranging between 0.06 to 0.97 and the flavonoids identified in the extracts were quercetin and kaempferol.

[**Key words:** *Azolla microphylla*, biofertilizer, flavonoids, HPTLC, kaempferol and quercetin]

Introduction

Azolla is an important constituent of rice paddy fields and contribute significantly to nitrogen economy. Ability of nitrogen fixation is due to the presence of heterocystous cyanobacterium *Anabaena azollae* which is confined to the dorsal leaf cavity of the fern¹. *Azolla* is known as green gold mine due to its multifaceted uses such as H₂ gas producer, weed suppressor, cattle and poultry feed, water purifier, human food etc². *Azolla* is a rich source of protein and essential amino acids. In addition to this it contains several vitamins such as vitamin-A, vitamin B-12 and beta carotene. It is also rich in minerals such as calcium, phosphorous, potassium, magnesium, copper and zinc. Protein content of *Azolla* is 25-35% on dry weight basis and is easily digested by poultry³. A variety of animals have been found to feed on *Azolla* but the chemical nature of the constituents generally determines the desirability of the food source in animals^{4,5}. In general, many plants use flavonoids as signals, growth inhibitors and feeding deterrent to other organisms⁶. Keeping in view of the importance of the organism as feed supplement there is a need to investigate the flavonoid profile of the organism. Phytochemical analysis showed the presence several compounds of antimicrobial

properties⁸. Hence the organism may also be exploited for bio-molecules involved in plant protection. In the present study an attempt was made to elucidate the flavonoid profile of *Azolla microphylla* using high performance thin layer chromatography (HPTLC).

Materials and Methods

Azolla microphylla was grown out door in cemented tanks (5m²) at the Centre for Collection, Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute, New Delhi. Fresh material collected from the tanks were brought to the laboratory and cleaned to remove the debris. It was then washed several times in tap water followed by washing in double distilled water. Plants were then air dried in shade for three weeks and the dried material was made to a powder and the dried and powdered material (50 g) was extracted successively with methanol (300 ml) for 24 h at a temperature that did not exceed the boiling point of the solvent. Extracts were then collected by filtration by Whatman filter paper (No.40). The filtrates were then concentrated in vacuum at 40°C using rotary evaporator (Hahnshin Scientific Co., Korea). The final volume of the filtrate was made to 100 ml. This was partitioned against equal volumes of anhydrous ethyl acetate (fraction I) and diethyl

ether (fraction II) followed by hydrolysis for 1 h each. Fractions were further concentrated in vacuum at 30°C using rotary evaporator and reconstituted in the respective solvent and dried in desiccator. Residues were then stored at -20°C till further use.

HPTLC studies were carried out following the method of Misra *et al.*⁷. For HPTLC analysis extract (100mg) was weighed accurately and then dissolved in 1ml of HPLC Grade Methanol, followed by centrifugation at 3000 rpm for 5 min. Standards used for flavonoids include quercetin and kaempferol. Samples and standards (2 µl) were spotted in the form of bands of width 6 mm with a Hamilton syringe on a pre-coated silica gel aluminum plate 60F₂₅₄ (30 cm x 10 cm) of 200µm thickness and a particle size of 25µm (Sigma-Aldrich, India) using a Camag Linomat 5, Switzerland). Sample loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase and the plate was developed in the respective mobile phase up to 90 mm. Chloroform-Ethyl acetate-Glacial acetic acid (60 : 35 : 5) was employed as mobile phase for the detection of flavonoids. Developed plate was dried by hot air to evaporate solvents from the plate. Plate was kept in Photo-documentation chamber (Camag Reprostar 3) and the images were captured in visible light, 254nm and 366 nm. The developed plate was sprayed with respective spray reagent (Flavonoid) and dried at 100°C in Hot air oven for 5 min. Plate was photo-documented in visible light and 366nm mode using Photo-documentation (Camag Reprostar 3) chamber. Before derivatization, the plate was fixed in scanner stage (Camag TLC Scanner 3) and scanning was done at 254 nm. Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version.

Results and Discussion

Natural products of plant origin such as flavonoids have diverse biological activities including antibacterial potential. Many studies have been conducted in the past to study the phytochemical composition of pteridophytes^{8,9,10}. However, detailed

information on the phytochemical composition of the secondary metabolites in the aquatic *Azolla-Anabaena* system is still lacking.

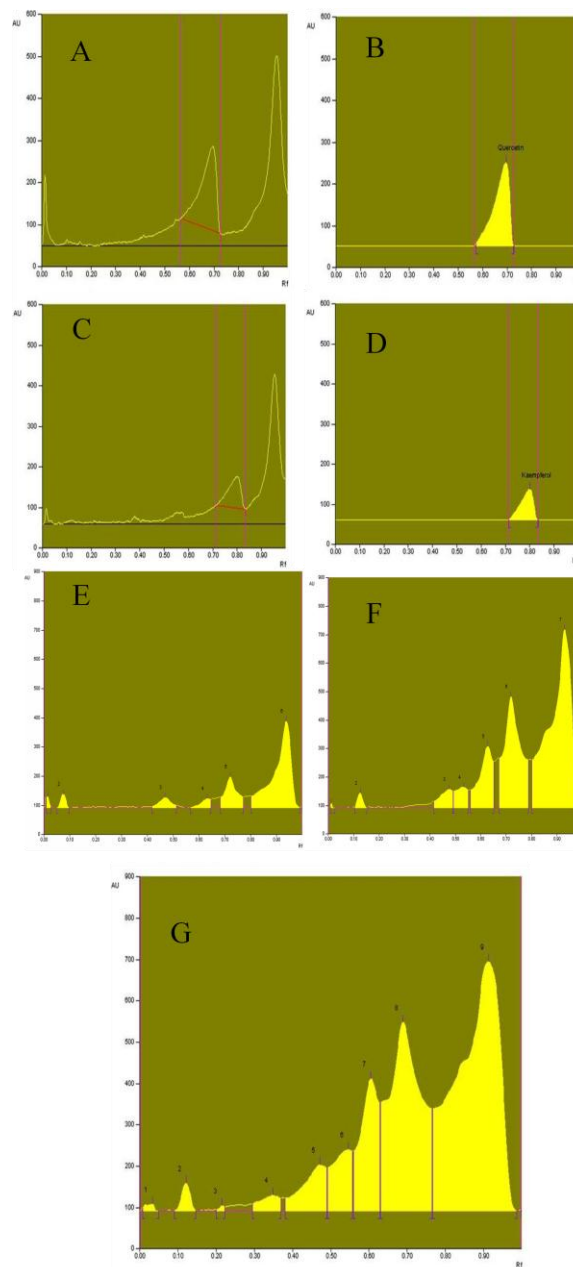


Fig. 1 A-G. HPTLC chromatogram of *Azolla microphylla*. A-D: HPTLC chromatogram and densitogram of standard quercetin and kaempferol at 254 nm; E-G: Densitogram of methanolic, ethyl acetate and diethyl ether extracts at 254 nm

The methanolic extracts of *A. microphylla* were partitioned against ethyl acetate and diethyl ether and the different fractions such as

methanolic, ethyl acetate and diethyl ether fractions were tested for the presence of flavonoids using high performance thin layer chromatography (HPTLC). Similar studies have been performed earlier to study the concentration and ratio of components using standard markers in medicinal plants using HPTLC¹¹. Analysis resulted in peaks of high resolution which were reproducible using Chloroform-Ethyl acetate-Glacial acetic acid (60: 35: 5) as the mobile phase. Fig. 1A-G shows the presence of at least eight 8 different types of flavonoids with corresponding R_f values in the range of 0.06 to 0.97.

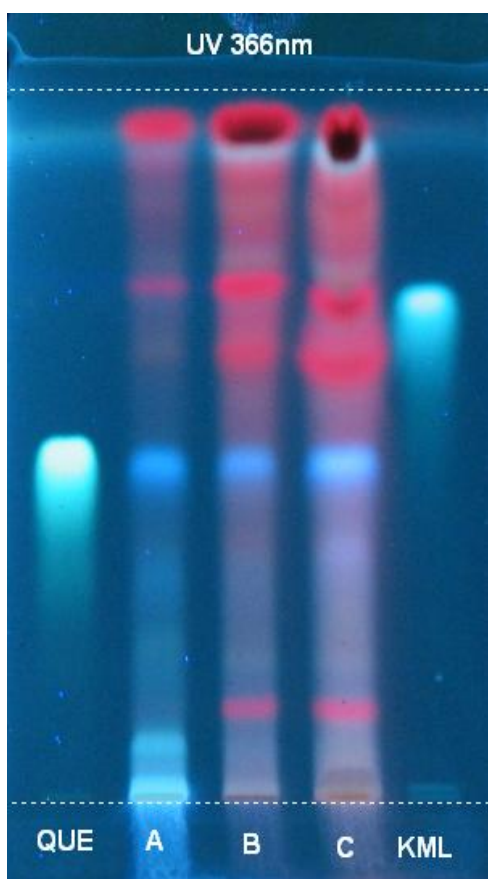


Fig. 2. HPTLC chromatogram of the various fractions of *Azolla microphylla* under UV (366 nm) showing the presence of flavonoids (QUE & KML: quercetin and kaempferol standards and A-C: showing the possible presence of flavonoids such as quercetin and kaempferol in methanolic, ethyl acetate and diethyl ether fractions respectively)

The HPTLC chromatogram of *A. microphylla* extracts is presented in Fig. 2. The analysis involved the comparison between standards and

samples and the presence of flavonoids in the sample was confirmed at 366 nm present in the tracks of the chromatogram and after derivatization, quercetin and kaempferol were the identified flavanoids from *A. microphylla*. The secondary metabolites are important and have been reported to act against herbivores¹². Mithraja *et al.*¹³ screened *Azolla pinnata* for their phytochemical composition and observed the presence of flavonoids. In the present study, use of HPTLC technique led to the identification of flavonoids from various extracts of *A. microphylla*. Singh *et al.*¹⁴ observed antibacterial potential of flavanoids from the pteridophyte *Pteris vittata*. Shan *et al.*¹⁵ observed antibacterial activity by plant extracts having phenolic constituents including flavanoids. Recently, Abraham and Aeri¹⁶ identified the presence of several phytochemicals including flavonoids from *Azolla microphylla*. Abraham¹⁷ reported antibacterial activity by the methanolic extracts of *A. microphylla* against the plant pathogenic bacteria *Xanthomonas oryzae*. The results obtained on the phytochemical composition of *Azolla* are promising and attempts could be made to further evaluate its biocontrol potential. Results of the present study may be extended to study the potential of disease suppression of plant pathogenic bacteria and fungi. This will help to enhance its utility in agriculture as biofertilizer as well as bio-control agent for rice.

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References

1. Moore, A.W., *Azolla*: Biology and agronomic significance, *Bot. Rev.*, 35 (1969) 17-35.
2. Wagner, G.M., *Azolla*: a review of its biology and utilization, *Bot. Rev.*, 63(1) (1999) 1-26.
3. Parashuramulu, S., Swain, P.S. and Nagalakshmi, D., Protein fractionation and *in vitro* digestibility of *Azolla* in ruminants, *J. Animal and Feed Res.*, 3(3) (2013) 129-132.
4. Lejeune, A., Caguan, A. and Van Hove, C., *Azolla* research and development: recent trends and priorities, *Symbiosis.*, 27 (1999) 333-351.
5. Cohen, M.F. and Meziane, T., Tsuchiya M & Yamasaki H, Feeding deterrence of *Azolla* in relation to

- deoxyanthocyanin and fatty acid composition, *Aquat. Bot.*, 74 (2002) 181-187.
6. Cohen, M.F. and Yamasaki, H., Flavonoid induced expression of a symbiosis related gene in the cyanobacterium *Nostoc punctiforme*, *J. Bacteriol.*, 182 (2000), 4644-4646.
 7. Misra, H., Mehta, B. K. and Jain, D. C., Optimization of extraction conditions and HPTLC-UV methods for determination of quinine in different extracts *Cinchona* sp bark, *Rec. Nat. Prod.*, 2(4) (2008) 107-115.
 8. Manikam, V.S., Benniamin, A. and Irudayaraj, V., Antibacterial activity of leaf extracts of *Christella practica* (L.) Lev, *Ind. Fern J.*, (2005) 87-88.
 9. Dalli, A. K., Saha, G. and Chakraborty, U., Characterization of antimicrobial compounds from a common fern *Pteris biauria*, *Ind. J. Exp. Biol.*, 45 (2007) 285-290.
 10. Maridas, M. and Raju, G., Investigation of phytochemical and antibacterial activity of *Huperzia* sp, *Pharmacologyonline.*, 3 (2009) 688-692.
 11. Yamuna, D.M., Wesley, E.G. and Johnson, M., Phytochemical studies of the terpenoids of medicinally important plant *Aerva lanata* L. using HPTLC, *Asia Pac. J. Trop. Biomed.*, (2011) S220-S225.
 12. Keeling, C.I. and Bohlmann, J., Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens, *New Phytol.*, 170 (2006) 657-675.
 13. Mithraja, M.J., Antonisamy, J. M., Mahesh, M., Paul, Z.M. and Jeeva, S., Phytochemical studies on *Azolla pinnata* R. Br., *Marsilea minuta* L. and *Salvinia molesta* Mitch, *Asia Pac. J. Trop. Biomed.*, (2011) S26-S29.
 14. Singh, M., Govindarajan, R., Rawat, A.K.S. and Khare, P.B., Antimicrobial flavanoid rutin from *Pteris vittata* L. against pathogenic intestinal microflora, *Am. Fern J.*, 98(2) (2008), 98-103
 15. Shan, B., Cai, Y.Z., Brooks, D.J. and Corke, H., The *in vitro* antibacterial activity of dietary spice and medicinal herb extracts, *Int J Food Microbiol.*, 117 (2007) 112-119.
 16. Abraham, G. and Aeri, V., A preliminary examination of the phytochemical profile of *Azolla microphylla* with respect to seasons, *Asia Pac. J. Trop. Biomed.*, (2012) S1392-S1395.
 17. Abraham, G., Evaluation of antimicrobial activity of methanolic extracts of the aquatic pteridophyte *Azolla microphylla*, *Vegetos.*, 26(1) (2013) 200-204.