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ORIGINAL ARTICLE

High performance Thin Layer Chromatography-Automated Multiple Development Bioautography of Phytoconstituents and Quantification of Stigmasterol in *Monochoria vaginalis* and *Monochoria hastata* with Antioxidant Potential

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ABSTRACT

Context: *Monochoria vaginalis* and *Monochoria hastata* belonging to *Pontederiaceae* are edible aquatic herbs commonly used by ethnic communities of India for treating various afflictions. **Objective:** The main objective of the study is to propose a simple and a rapid bioautographic fingerprinting profile using high-performance thin-layer chromatography (HPTLC) with gradient elution for screening the phytochemicals for the antioxidant property. Quantification of stigmasterol is carried in selected parts of *Monochoria* genus. **Materials and Methods:** Stigmasterol content of the three different parts of the species were quantified and validated by HPLTC. Fingerprint analysis was carried out using HPTLC-automated multiple development 2 (HPTLC-AMD2)-based gradient elution technique. Bioautography was done using 1-diphenyl-2-picrylhydrazyl (DPPH) solution to check the antioxidant property. Phytochemicals such as phenols, flavonoids, sterols, and saponins were estimated using 96-well plate and antioxidant potential were confirmed by DPPH, hydroxyl and nitric oxide scavenging activity. **Results:** The developed HPTLC-AMD2 method showed clear separations resulting in sharp, intense peaks. Phytoconstituents were determined for the first time in the *Monochoria* species. The results indicate that both species are rich in various bioactive contents with potent antioxidant potential. Stigmasterol was found to be present in all the selected parts with varying concentration. **Conclusion:** This study reveals that *Monochoria* species are rich in phytoconstituents with potent antioxidant activity and the developed method is efficient, simple, rapid, and reliable for separating the phytoconstituents of *Monochoria* providing a passport data of extracts. A positive, significant linear relationship between antioxidant activity and total phenol content and total flavonoid content showed that phenolic compounds and flavonoids were the dominant antioxidant components present in the extracts.

Key words: Antioxidant activity, gradient elution, high-performance thin-layer chromatography-automated multiple development, *Monochoria*, stigmasterol

SUMMARY

- *Monochoria vaginalis and Monochoria hastata* are ethnomedicinally claimed to be used for various ailments such as diabetes and neurological disorders
- Fingerprint profiling by high-performance thin-layer chromatography (HPTLC) gradient elution method showed the presence of phytoconstituents such as phenols, steroids, and flavonoids
- Quantification of major phytoconstituents such as phenols, flavonoids, saponins, sterols, and triterpenoid contents using 96-well plate method, showed their presence in all the parts
- Stigmasterol has been reported and quantified for the first time in *Monochoria* species
- HPTLC Bioautography using 1-diphenyl-2-picrylhydrazyl (DPPH) provided that most of the phytoconstituents present in the extracts had antioxidant activity
- Antioxidant activity was further confirmed by DPPH, nitric oxide, and hydroxyl radical scavenging activity.

Abbreviations used: HPTLC-AMD: High-performance Thin-layer Chromatography-Automated Multiple Development; MHL-*Monochoria hastata* leaf, MHS-*Monochoria hastata* stem; MHR-*Monochoria hastata* Rootstock; MVL-*Monochoria vaginalis* leaf; MVS-*Monochoria vaginalis* stem; MVR-*Monochoria vaginalis* Rootstock. TPC-Total phenol content; TFC-Total flavonoid content; TSC-Total sterol content; TSAC-Total saponin content; TTC-Total triterpenoid content.

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INTRODUCTION

Monochoria genus comprises of two species commonly found throughout India, *Monochoria vaginalis* C. Presl and *Monochoria hastata* (L.) Solms belongs to the family *Pontederiaceae* (Water hyacinth family). Both the species are morphologically similar and gregarious in nature. *Monochoria* in the Ayurvedic literatures such as *Charaka samhita*, *sushruta samhita*, and *astanga hridya* is called as Indivara, Neelotpala, and karambha and are claimed to be used for the treatment of diabetes and neurological disorders. The juice of fresh leaves of *M. vaginalis* is used for the treatment of diabetes by the Paniya and kattunaikar tribes

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of Wayanad district of Kerala.[1] The leaves were also claimed to possess analgesic properties and used as dentifrices.[2] The rootstock or rhizome is used for the treatment of asthma. Decoction of fresh root is given for nausea and digestive disorders.[3] Leaves and roots of *M. hastata* have been used as digestive and as uterine tone.[4] Reported secondary metabolites include cerebrosides, sterols, triterpenoids, and phenolic compounds.[5] Literature reports on *M. vaginalis* claims protection against acetaminophen-induced nephrotoxicity, alloxan-induced diabetes, and hepatoprotective properties. Both the species are medicinally potent with strong Ayurvedic and traditional claims, which paves an interest in the identification and quantification of the bioactive contents.

Integration of Ayurvedic wisdom and traditional claim is the linchpin for the evolution of new chemical entity from the natural source. The power of advanced scientific techniques and traditional knowledge can bring a new hope in the field of herbal drug discovery.[6] Due to lack of scientific evidence on quality control and standardization, most of the herbal drugs could not attain the drug status. Aquatic plants contain a rich source of complex mixtures with a wide range of polarity, which makes the separation, identification, and quantification of phytoconstituents a challenging task. Fingerprinting of phytoconstituents is an important parameter in the drug developing process. In recent years, the profiling of phytoconstituents has been identified to be a convenient and effective method for the quality control and standardization of phytopharmaceuticals, especially when there is a lack of authentic standards for the identification of active components present in the natural products. The matrix independence of AMD separations is a useful feature for this application. HPTLC bioautography is a combinatorial assay, which combines the chromatographic separation and *in situ* biological activity determination. This makes it an ideal method to facilitate the localization and target-directed isolation of active constituents in complex matrix samples. AMD is highly recommended in case of samples containing substances of wide polarity or those being structural analogs.[7]

AMD is a technique that uses repeated development of HPTLC plates with decreasing solvent strength on the increasing distance. After each development, the plate is vacuum dried. The development starts with the most polar solvent and concludes with the least polar solvent.[8] Gradient development with linear eluotropic profile leads to a band reconcentration which improves the separation.[9] Due to the complex nature of herbal extracts, separation of each antioxidant compound and analyzing it individually is costly and inefficient, notwithstanding the possible synergistic interactions among the antioxidant compounds in a mixture. Therefore, the concept of coupling chromatographic fingerprints with biological finger printing analysis has gained attention for the quality control of plant extracts. Thin-layer chromatography with postchromatographic derivatization using a methanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) can be a valuable tool in such analysis.[10,11]

In the present study, a simple and rapid six-step gradient elution method for fingerprint profiling of the hydroalcoholic extracts of *Monochoria* species was developed. Phytochemicals such as phenolic compounds, flavonoids, sterols, saponins, and triterpenoids have been determined for the first time, and their antioxidant potential has been explored.

MATERIALS AND METHODS

Plant materials

Fresh plants were collected from the marshy lake (*M. hastata*) and from the streams (*M. vaginalis*) of Ambalavayal, Wayanad District, Kerala, India, in December 2012 and were authenticated by Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. Voucher specimens of *M. vaginalis* (BSI SRC/5/23/2011–12) and *M. hastata* (BSI SRC/5/23/2012–2013) have been preserved in the laboratory for the future reference. Three parts of two species, namely, *Monochoria vaginalis* leaf (MVL), *Monochoria vaginalis* stem (MVS), *Monochoria vaginalis* rootstock (MVR) of *M. vaginalis* and *Monochoria hastata* leaf (MHL), *Monochoria hastata* stem (MHS), *Monochoria hastata* rootstock (MHR) of *M. hastata* were used as plant materials. The plant parts were carefully separated and washed in running tap water to clean the foreign particles and cut into small pieces with a sharp knife to facilitate drying and shade dried in room temperature for about 3 days, pulverized and passed through a 60-mesh sieve, and stored dry until use. All results were expressed by dry weight (DW).

Plant identification using DNA Barcoding

DNA isolation

Genomic DNA was isolated by following the protocol of Saghai-Maroof *et al.*[12] with minor modifications. Polymerase chain reaction (PCR) amplification of DNA barcode marker was done using 50 ng of total genomic DNA as template and the commonly used primers for matK. The purified PCR products were sequenced from both the ends using the same PCR primers in 31306l Genetic analyzer (Applied Biosystems, CA, USA). The sequences were manually edited using Sequence Scanner Software v. 1.0 (Applied Biosystems, CA, USA) and full-length sequences were assembled.

Data analysis

Database search for species identification were done using basic local alignment search tool against nonredundant nucleotide database at NCBI (www.blast.ncbi.nlm.nih.gov/blast.cgi). DNA sequence obtained was matched with the existing database in the library.

Extract preparation

Cleaned plant parts were cut in to small pieces with a sharp knife to facilitate drying and shade dried in room temperature for about 3 days, pulverized and passed through a 60-mesh sieve, and stored dry until use. The air-dried plant parts were extracted with hydroalcohol (30:70) by cold maceration. Extracts were then filtered through a paper filter (Whatmann No. 1) and concentrated under reduced pressure using Rotovac. The dry mass was stored in glass vials for further analysis.

High-performance thin-layer chromatography-automated multiple development

HPTLC was performed on aluminum sheets coated with Silica gel 60F, 20 cm × 10 cm (Merck, 254 Darmstadt, Germany). Plates were activated before use by heating in an oven for 30 min at 110°C. A volume of 2, 4, and 8 μL of the hydroalcoholic extracts of MVL, MVS, MVR, MHL, MHS, and MHR were sprayed with compressed air, as 8-mm narrow bands using a 100 μL syringe with a Linomat 5 semi-automatic sample applicator (Camag, Muttenz, Switzerland), 8 mm from the lower edge, with the 10-mm distance from each side and track distance of 7 mm, that is, 18 applications per plate.

HPTLC plates were developed in Automated Multiple Development Chamber (AMD2, Camag) with six-step gradient elution method [Figure 1]. Images of plates were captured using a TLC-Visualizer (Camag, Muttenz, Switzerland) with a 12 bit camera (Camag) under UV light 254 nm, 366 nm before derivatization and at 540 nm, after derivatization with anisaldehyde sulfuric acid. winCATS 3.0 planar chromatography manager software was used for quantitative evaluation of plates and to transform images into chromatogram.

High-performance thin-layer chromatography-bioautography

The plates were developed by the optimized six-step gradient elution method. After development, the plates

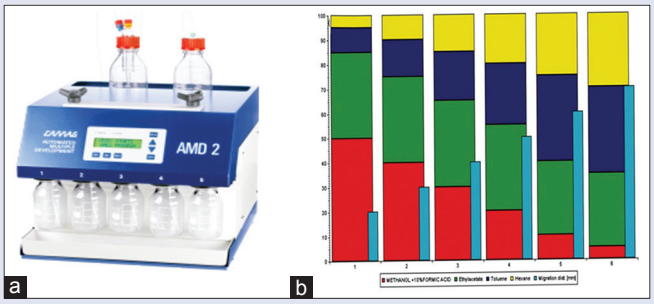

Figure 1: (a and b) Automated Multiple development Chamber, Polarity gradient graph

were air-dried for 15 min and immersed in the DPPH reagent (0.05% [2, 2-diphenyl-1-(2, 4, 6-trinitrophenyl) hydrazyl radical] DPPH in methanol) for 1 s and then dried for 1 min at room temperature in the fume hood. The dried plates were wrapped in an aluminum foil and kept in the dark for 30 min. The antiradical activity of each component was estimated from the intensity of disappearance of the violet/purple background of the plate and densitometric scanning was done at 517 nm. Free-radical scavenging zones were readily identified as yellow band against a light violet/purple background.

Quantification and validation of stigmasterol

Hydroalcoholic extracts of the different parts (leaf, stem, and rhizome) of both the species were filtered and vacuum dried at 45°C. The dried extracts were separately redissolved in 1 ml of methanol and sample of varying concentration (530 µl) of stigmasterol was spotted for quantification. A volume of 1 mg of standard (stigmasterol) was prepared separately in 1 ml of methanol, and different amounts of (1000–6000 ng) Stigmasterol was loaded on HPTLC plate to get the calibration curve.

Calibration curve was established using 5 analyte concentrations of the TLC standard representing µg of stigmasterol. Standard and sample solutions were applied in the form of bands on precoated HPTLC silica gel plates 60 F-254 (10 cm × 10 cm with 250 µm thickness) by means of Linomat V automated spray-on band applicator. The mobile phase consisted of Chloroform: Methanol (10 ml) (8:2 v/v).

Ascending development of the plates was carried out in 10 cm × 10 cm Camag HPTLC twin trough chamber saturated with mobile phase for 15 min at room temperature. Plates were developed to a distance of 7 cm beyond the origin. Development time was 10 min. After development, the plates were air-dried for 5 min and derivatized with anisaldehyde-sulfuric acid reagent for stigmasterol, heated at 105°C for 5 min. Densitometric scanning was performed on Camag TLC scanner III in the reflectance mode at 540 nm for stigmasterol. Slit dimension was kept 6 mm × 0.1 mm in absorbance mode using tungsten lamp. The entire program was operated using winCATS planar chromatography manager. This method was validated as per the International Conference on Harmonisation (ICH) guidelines (1994, 1996, and 2005). The method validation parameters checked were linearity, precision, accuracy and recovery, limit of detection, limit of quantification, specificity, robustness, and ruggedness. All measurements were performed in triplicates.

Quantification of bioactive contents

Determination of total phenolic content

Total phenol content (TPC) was determined according to the Folin–Ciocalteu method using 96-well plates.[13] Total phenolic content was expressed as mg gallic acid/100 g of the DW of the extract.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined according to the method described by Herald *et al.*,[14] Briefly, the standard/sample solution was mixed with sodium nitrite, and aluminum chloride in 96-well plates and the absorbance was measured at 510 nm. Total flavonoid content was expressed as mg quercetin equivalent/100 g of dry weight of the extract.

Determination of total sterol content

Total sterol content (TSC) was determined according to Liebermann–Burchard colorimetric method,[15] using cholesterol as calibration standard. The results were expressed as mg cholesterol equivalent/100 g of DW of the extract.

Determination of total saponin content

Total saponin content (TSAC) was determined based on the method described by Xu and Chang,[16] using diosgenin as the calibration standard in UV spectrophotometer. The results are expressed as mg diosgenin equivalent/100 g of DW of the extract.

Determination of total triterpenoid content

The total triterpenoid content (TTC) of the sample was determined according to the method of Ni,[17] with some modifications, using urosolic acid as calibration standard, and the absorbance was measured at 548 nm in a UV spectrophotometer. The results were expressed as mg urosolic acid equivalent/100 g of DW of the extract.

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) of the extracts was assessed by the phosphomolybdenum method as described by Prieto *et al.*[18] Standard/sample solution was mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After incubation in boiling water bath for 90 min, the absorbance was measured at 695 nm. The results were expressed as mg ascorbic acid equivalent/100 g of DW of the extract.

Determination of 1-diphenyl-2-picrylhydrazyl free-radical scavenging activity

The DPPH radical scavenging activity was determined in 96-well plates according to the method introduced by Blois.[19] An aliquot of 2 µL of each extract was mixed with 198 µL methanolic solution of DPPH (75 µM). Decolourization of purple free-radical DPPH solution was measured at 517 nm after 30 min incubation in the dark at room temperature. Ascorbic acid was used as the calibration standard.

Determination of nitric oxide scavenging activity

Nitric oxide scavenging activity was carried out based on the 96-well plate method described by Panda.[20] The absorbance was measured at 546 nm using microplate reader. Gallic acid was used as calibration standard.

Determination of hydroxyl radical scavenging activity

The ability of the extracts to inhibit nonsite-specific hydroxyl radical-mediated peroxidation was carried out according to the method described by Hinneburg *et al.*[21] The extent of oxidation was estimated from the absorbance of the solution at 532 nm. Gallic acid was used as calibration standard.

Data analysis

Results were expressed as mean ± standard deviation (SD) of triplicate data. Correlations were analyzed using Pearson's correlation in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Probability $P \leq 0.05$ indicates significance.

RESULTS AND DISCUSSION

DNA barcoding

Authentication at the DNA level provides more reliability because DNA is a stable macromolecule which is not affected by external factors and is predominant in all tissues. Therefore, development of DNA-based marker is important for the authentication of medicinal plants. Identification of the *Monochoria* species was confirmed by DNA barcoding of both the plants. Success of PCR amplification and sequence recoverability is an important criterion for assessing the utility of DNA barcodes. rbcL barcode marker was amplified using universal primer pairs and standard protocols. There was no variation in sequence length for rbcL; bidirectional sequencing recovered the 365 base pair target sequence for all the PCR amplifications. The rbcL fragment was successfully amplified for both *M. vaginalis* and *M. hastata*. High bidirectional sequences obtained from rbcL for both the plants confirmed its identity at genus level. NCBI search confirmed their identity by giving 100% match for *M. hastata* and 99% match for *M. vaginalis*.

Fingerprint profiling

Fingerprint profiling of *Monochoria vaginalis* and *Monochoria hastata* has been developed using six-step gradient elution technique in HPTLC-AMD2 [Table 1]. Fingerprints and quantification of individual components are useful to identify phytoconstituents present in plant extracts, which helps in determining the authenticity of phytopharmaceuticals. Quality and safety regulatory bodies of herbal drugs have accepted the fingerprint analysis as the major identifying step in the standardization of botanicals and particularly for the herbs with unknown compound matrix.[22] The matrix independence separation feature of AMD separations is a useful in the fingerprinting. Earlier Di *et al.*[23] has detected different varieties of mushrooms in the commercial product, Lingzhi, similarly, Galand *et al.*[24] used the fingerprints of triterpenoic acids present in the Ganoderma (Lingzhi) mushrooms to authenticate Lingzhi preparations. Gocan and cimpan[25] reviewed the different HPTLC techniques such as AMD, TLC, and OPTLC for the analysis of medicinal plants and had concluded that AMD showed a higher resolution rate due to lower spot diffusion. Simultaneous AMD separation and comparison of six extracts from different parts of *Monochoria* species, containing different classes of compounds; fatty acids, saponins, flavonoids, phenols, alkaloids, etc. were carried out.[24] Owing to the large number of phytoconstituents, which coexist in a plant extract, the separation of an unknown number of unidentified compounds being sensitive to small structural changes and the wide differences between the polarities of the unknown compounds, normal phase HPTLC with suitable gradient was required.

Universal gradient system with various mobile phase compositions was checked for separation of phytoconstituents of crude extracts, but universal gradient system did not give an optimum separation for the selected extracts, due to high polarity fractions. To improve the separation ethylacetate was included in the eluent composition of all the steps. Best separation was obtained in six-step gradient composition. Preconditioning of the plate with modifier (25% ammonia solution) was carried out before each step to prevent peak tailing, which frequently occurs in plant samples with wide polarity phytoconstituents [Table 1]. Exposure of the developed plate under UV 254 and 366 nm showed the presence of polar and nonpolar UV active compound in all the extracts [Figure 2a]. Derivatization of the plate with DPPH revealed the presence of active constituents with antioxidant activity. Anisaldehyde sulfuric acid derivatized showed the presence of terpenoids and phenolic compounds. Figure 2b showed the AMD–HPTLC provided a good separation for polar substances in the lower part of the plate and the less polar compounds in the upper part with varied Rf values and sharp, intense peaks of the separated compounds, hence an appropriate gradient system for the fingerprint analysis of the crude extracts of *Monochoria* species with better simultaneous separations. Many literatures have been reported on the separation of phytoconstituents using 25 steps Universal gradient solvent system for qualitative and quantitative determination of phytoconstituents. The major drawbacks of the AMD2 separations are analysis duration with complex combinational ratios with 25 steps; and

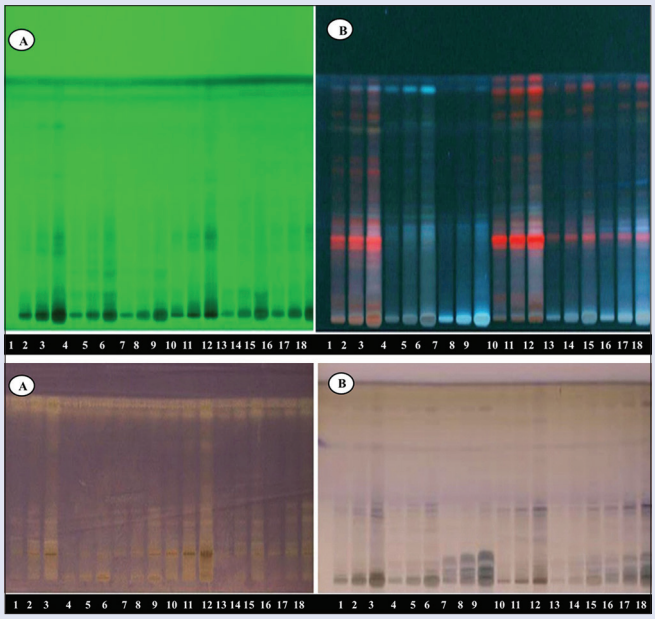

Figure 2: (A) High-performance thin-layer chromatography plate at 254 nm (A), 366 nm (B); (B), High-performance thin-layer chromatography plate derivatized in 1-diphenyl-2-picrylhydrazyl (A), High-performance thin-layer chromatography plate derivatized in anisaldehyde–sulfuric acid (B), Tracks [1–3]-2, 4, and 8 µl of hydroalcoholic extract of *Monochoria hastata* leaf, Tracks [4–6]-2, 4, 8 µl of hydroalcoholic extract of *Monochoria hastata* stem, Tracks [7–9]-2, 4, 8 µl of hydroalcoholic extract of *Monochoria hastata* rootstock, Tracks [10–13]– 2, 4, 8 µl of hydroalcoholic extract of *Monochoria vaginalis* leaf, Tracks [14–16]-2, 4, 8 µl of hydroalcoholic extract of *Monochoria vaginalis* stem, Tracks [16–18]-2,4,8 µl of hydroalcoholic extract of *Monochoria vaginalis* rootstock

Table 1: Gradient mobile phase for automated multiple development 2

Gradient steps	Solvent concentration (volume %)				Migration distance (mm)	Drying time (min)	Preconditioning with ammonia
	Methanol + 10% formic acid	Ethyl acetate	Toluene	Hexane			
1	50.0	20.0	30.0	0.0	20	3	Yes
2	40.0	35.0	25.0	0.0	30	3	Yes
3	30.0	40.0	30.0	0.0	40	2	Yes
4	20.0	40.0	30.0	5.0	50	2	No
5	10.0	40.0	40.0	10.0	60	2	No
6	5.0	35.0	50.0	15.0	70	2	No

hence, the present work focused on developing a fingerprint technique with reduced number of runs to separate the maximum number of phytoconstituents.

Antioxidant potential of the extracts was preliminarily screened using the TLC-DPPH bleaching method. The results showed that all the extracts showed a yellow spot against purple background signifying the antioxidant property of the extracts [Figure 2a]. Since the finger print analysis showed many bands with different polarities, an effort was taken to quantify the phytochemical present in the extract and also to study their antioxidant potential *in vitro*.

Quantification and validation of stigmasterol

HPTLC could provide adequate information and parameters for comprehensive identification and differentiation of the two closely related herbal medicines. Experimental conditions, such as mobile phase composition, scan mode, scan speed, and wavelength detection were optimized to provide accurate and precise results for the quantification of stigmasterol. Development with the mobile phase, Chloroform: Methanol (10 ml) (8:2 v/v) on the precoated HPTLC plates produced compact, flat, bands of stigmasterol (R_f 0.3), when derivatized with anisaldehyde– sulfuric acid reagent [Figure 3]. The content of stigmasterol varied in all the extracts, and the results were summarized in [Table 2]. Validation data for the developed quantitative HPTLC method meet the acceptance criteria for accuracy, precision, linearity, detection, and quantification limits set by ICH [Table 2a].

Table 2: Quanification report of stigmasterol by high-performance thin-layer chromatography

Serial number	Samples	Concentration (%w/w)
1	MHL	0.214
2	MHS	2.017
3	MHR	2.592
4	MVL	0.176
5	MVS	2.131
6	MVR	1.927

MHL: *Monochoria hastata* leaf; MHS: *Monochoria hastata* stem; MHR: *Monochoria hastate* rootstock; MVL: *Monochoria vaginalis* leaf; MVS: *Monochoria vaginalis* stem; MVR: *Monochoria vaginalis* rootstock

Table 2a: Validation summary of stigmasterol by high-performance thin-layer chromatography

Parameters	Values
Linearity range	1000-5000 ng
Correlation coefficient (R)	0.9950
LOD (ng/spot)	80
LOQ (ng/spot)	200
RSD (%) of intraday precision (*n*=3)	2.43
RSD (%) of interday precision (*n*=3)	2.94
Recovery (%)	99.77±0.92

LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation

Determination of bioactive contents

The preliminary phytochemical evaluation showed the presence of various phytochemicals such as flavonoids, polyphenols, saponins, and glycosides in the hydroalcoholic extracts of all the parts. The results of total phenolic content in the selected extracts were given in Table 3. The content of total phenols in the extract expressed as gallic acid equivalents (GAE) varied between 134.8 and 64.75 mg/100 g of dry extract. As shown in the [Table 3], the pattern of variation in TFC was similar with TPC, with the highest content of TFC in rootstock of both the plants and lowest in leaf extract. The content of total flavonoids in the extract was expressed as QE varied between 98.5 and 27.4 mg/100 g of DW of the extract. The triterpenoid content of the extracts was determined were given in the [Table 3], equivalent to urosolic acid. The content of TTC varied between 2137.2 and 1637.4 mg/100 g of DW. The TSC of the extract was expressed as cholesterol equivalents. The TSC varied between 429.5 and 214 mg/100 g of DW of the extract. The TSC of the extracts varied significantly. The highest content was found in rootstock followed by stem and leaf of both the species. *M. vaginalis* contains higher sterol content than *M. hastata.* The result of the TSAC determination was given in Table 3. The contents of total saponins in the extract were expressed as diosgenin equivalent (Ds). The saponin content was found to be highest in MHS (110.3214 mg/100 g of DW) and the lowest in MVL (53.4214 mg/100 g of DW).

In vitro antioxidant activity

In vitro antioxidant activity was done by DPPH radical scavenging activity, TAC, and hydroxyl radical scavenging activity and nitric oxide

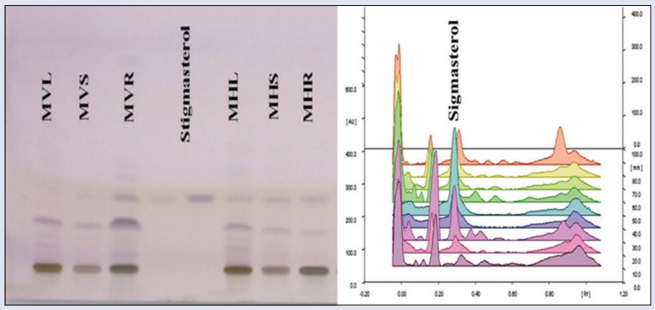

Figure 3: High-performance thin-layer chromatography plate after derivatization with anisaldehyde sulfuric acid reagent revealing the presence of stigmasterol in the plant extracts

Table 3: Phytochemical contents of monochoria species

Samples	TPC[a]	TFC[b]	TSC[c]	TSAC[d]	TTC[e]
MHL	117.33±1.97	76.85±2.72	310.02±3.67	57.32±1.22	1732.50±13.02
MHS	64.75±1.47	27.42±3.26	382.12±2.12	110.35±2.10	2137.21±9.88
MHR	134.8±2.56	98.58±3.15	419.72±2.06	94.12±1.88	1966.08±16.32
MVL	104.50±2.01	68.74±2.88	214.05±2.44	53.47±1.36	1637.44±11.76
MVS	68.83±3.13	33.87±3.92	360.55±3.18	86.66±2.72	1918.02±21.51
MVR	106.44±2.35	57.56±2.44	429.52±1.68	107.89±3.17	1873.28±16.33

[a]TPC: Expressed as mg gallic acid/100 g of dry extract; [b]TFC: Expressed as mg quercetin/100 g of dry extract; [c]TSC: Expressed as mg cholesterol/100 g of dry extract; [d]TSAC: Expressed as mg Diosgenin/100 g of dry extract; [e]TTC: Expressed as mg urosolic acid/100 g of dry extract, [f]TAC: Expressed as mg gallic acid/100 g of dry extract. TPC: Total phenolic content; TFC: Total flavonoid content; TSC: Total sterol content; TSAC: Total saponin content; TTC: Total triterpenoid content; MHL: Monochoria hastata leaf; MHS: *Monochoria hastata* stem; MHR: *Monochoria hastata* rootstock; MVL: *Monochoria vaginalis* leaf; MVS: *Monochoria vaginalis* stem; MVR: *Monochoria vaginalis* rootstock; TAC: Total antioxidant capacity

radical scavenging activity. TACs of the extracts were expressed as GAE. The phosphomolybdenum method was based on the reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/Mo complex. The antioxidant capacity of rootstock of MHR was higher than all the other extracts [Table 4]. Many methods are used to determine the radical scavenging effect of antioxidants. The free radical scavenging activity of extracts depends on the ability of antioxidant compounds to lose hydrogen and the structural conformation of these components.

TAC expressed as mg gallic acid/100 g of dry extract. DPPPH, Hydroxyl radical (HO), Nitric oxide radical (NO)-expressed as mg ascorbic acid/100 g of dry extract. Values are given as mean ± SD of three replicates.

The DPPH radical scavenging effect of hydroalcoholic extracts of all the parts of both the species and the standard (Ascorbic acid) on the DPPH radical were studied. The scavenging effect of the hydroalcoholic extracts and the reference compound on the DPPH radical expressed as IC_{50} value was in the following order: the results indicated that MHR (1.387 ± 3.6 µg/mL) had the lower IC_{50} value and MVS (4.553 ± 1.6 µg/mL) being highest. The DPPH radical scavenging activity of the hydroalcoholic extract of rootstock of *M. hastata* showed the highest scavenging activity which was comparable to the reference compound (ascorbic acid). Free-radical scavenging activity also increased with an increasing concentration. The ability of root extracts to scavenge DPPH radicals suggests that they are electron donor and can react with free radicals to convert them to more stable products and terminate radical chain reactions. The nitric oxide radical scavenging activity of the hydroalcoholic extracts were determined and the IC_{50} values of the extracts showed that, MHR had lower IC_{50} (379.3 ± 1.4 µg/mL) value and MVS (544.9 ± 1.4 µg/mL) being the highest among the extracts. Percentage inhibition of the extracts increased with increasing concentration of the extract. However, the activity of ascorbic acid was more pronounced, than that of the extracts. All the extracts of both the species showed potential inhibitory effect on hydroxyl radical scavenging activity. The most potent being MHR with an IC_{50} value of 466.1 ± 7.3 µg/mL and less potent being MVS with an IC_{50} value of 574.8 ± 4.1 µg/mL. The results showed that all the extracts exhibited a good inhibitory activity against hydroxyl radicals in a dose-dependent manner. The ability of the abovementioned extract to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction. The antioxidant activity increased with an increase in concentration of the extracts reaching a plateau.

Table 4: Antioxidant properties of hydro alcoholic extracts of *Monochoria* species

Samples	TAC	DPPH	HO	NO
MHL	232.03±1.52	2.332±1.0	488.5±2.0	451.2±3.4
MHS	128.14±3.04	3.232±5.0	515.6±0.5	494.2±1.2
MHR	312.08±2.16	1.387±3.6	466.1±7.3	379.3±1.4
MVL	138.20±1.07	3.53±4.9	541.2±4.5	491.5±2.6
MVS	77.29±1.60	4.553±1.6	574.8±4.1	544.9±1.4
MVR	144.72±2.04	3.382±1.5	480.8±3.4	472.6±3.1

DPPH: 1, 1-diphenyl-2-picrylhydrazyl; MHL: *Monochoria hastata* leaf; MHS: *Monochoria hastata* stem; MHR: *Monochoria hastate* rootstock; MVL: *Monochoria vaginalis* leaf; MVS: *Monochoria vaginalis* stem; MVR: *Monochoria vaginalis* Rootstock; TAC: Total antioxidant capacity; HO: Hydroxyl radical; NO: Nitric oxide radical

Pearson's correlation among the phytochemicals and the *in vitro* antioxidant activity

Correlations between antioxidant activity and TPC, TFC, TTC, TSC, and TSAC were evaluated in the present study and the results are shown in [Table 5]. TAC was highly significant with the total phenolic and flavonoid contents of the extracts. Total steroidal content showed a high positive correlation with DPPH, whereas phenol content, flavonoid content, and steroidal content showed a good correlation with DPPH. In nitric oxide scavenging activity, TPC, TFC, TSC, TTC and TSC showed a high-positive correlation. TSC showed a good correlation with nitric oxide scavenging activity, whereas phenol, flavonoid, and triterpenoid showed a moderate correlation. TSAC showed a nonsignificant correlation with all the antioxidant methods, signifying that it does not played much role in the antioxidant activity [Table 5].

CONCLUSION

Our work is the introductory approach of characterization of *Monochoria* species of India. The reports of these investigations revealed the complex phytoconstituents present and their potential as free radical scavengers. *Monochoria* species are gregarious in nature and look similar morphologically, which makes the identification difficult. Establishment of chromatographic profiling, identification and quantification of stigmasterol in the plant parts revealed for the first time, and free-radical scavenging property provides the feasibility to systematically investigate the plant phytoconstituents. Utilization of the developed method allows a search for the species that comprise free-radical scavengers, which can be adapted for the search of phytoconstituents with antioxidant potential. In conclusion, a new, rapid chromatographic method has been developed to study the nature of phytoconstituents present in the different species of *Monochoria*. The developed HPTLC-DPPH empowered the performance of rapid screening of phytochemicals present in the different parts of Monochoria. The method can be used for preparation of monograph of *Monochoria,* since it plays a major role in the Ayurvedic medicine.

Table 5: Correlation matrix (Pearson's correlation coefficients) of phytochemical contents with antioxidant property

Variable	DPPH#	NO	#OH	TPC	TFC	TAC	TSC	TSAC	TTC
DPPH*	1	0.9346**	0.9601***	0.8989**	0.8995**	0.7595*	0.9825***	0.5606 NS	0.8995**
NO		1	0.8033*	0.9958***	0.9959***	0.9368**	0.9842***	0.3074 NS	0.9959***
HO*			1	0.7492*	0.7500*	0.5715 NS	0.8928**	0.7500*	0.7500*
TPC				1	1.000***	0.9647***	0.9640***	0.2492 NS	0.9980***
TFC					1	0.9643***	0.9643***	0.2500 NS	0.9146**
TAC						1	0.8624**	0.1072 NS	0.8433**
TSC							1	0.4285 NS	0.3293**
TSAC								1	0.9216**
TTC									1

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$. NS: Nonsignificant, TPC: Total phenolic content; TFC: Total flavonoid content; TSC: Total sterol content; TSAC: Total saponin content; TTC: Total triterpenoid content; DPPH: 1, 1-diphenyl-2-picrylhydrazyl; Total antioxidant capacity; TAC: Total antioxidant capacity; HO: Hydroxyl radical; NO: Nitric oxide radical

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Conflicts of interest

There are no conflicts of interest.

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