Egyptian Journal of Basic and Applied Sciences 5 (2018) 197–203

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Contents lists available at ScienceDirect

Egyptian Journal of Basic and Applied Sciences

journal homepage: www.elsevier.com/locate/ejbas

Full Length Article

Column chromatography and HPLC analysis of phenolic compounds in the fractions of *Salvinia molesta* mitchell

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ARTICLE INFO

Article history:
Received 7 January 2018
Received in revised form 16 May 2018
Accepted 30 May 2018
Available online 9 June 2018

Keywords:
Salvinia molesta
Antioxidants
Phenolic compounds
Column chromatography
Fractionation
Free radicals

ABSTRACT

Salvinia molesta, commonly known as giant *Salvinia*, is a floating fern belonging to the family of Salviniaceae. In this study the active fractions of the fern extract were separated using column chromatography and phenolic compounds present in the active fractions were determined by RP-HPLC. Ethyl acetate extract was found to possess significant pharmacological activity when compared to other extracts under study and therefore an attempt was made to fractionate ethyl acetate extract. The analysis was performed through two different mobile phases involving solvent A (acetonitrile) and solvent B (0.1% phosphoric acid in water) and solvent A (methanol) and Solvent B (4% acetic acid). HPLC analysis indicated the presence of phenolic compounds namely ascorbic acid, quercetin, gallic acid, resorcinol, catechol, vanillin and benzoic acid with specific retention times. The detected compounds possess antioxidant and antitumour activities. The results of the present study suggests the possibility to use *S. molesta* as a source for a plausible antioxidant agent which could be isolated and used as a lead candidate for the development of antioxidant drugs that help stop or limit damage caused by free radicals and to counteract oxidative stress leading to the prevention of a variety of chronic and degenerative diseases.

1. Introduction

The phenolic compounds are ubiquitous in plant kingdom. They synthesize several thousand different chemical structures and are characterized by hydroxylated aromatic rings. These compounds are secondary metabolites which are derived from the pentose phosphate, shikimate and phenylpropanoid pathways in plants [1]. These are one of the most widely occurring groups of pytochemicals which are of appreciable physiological and morphological importance in plants [2]. A number of studies have been aimed to characterize the health promoting activities of phenolic compounds due to their antioxidant properties. They are useful in treatment and management of cancer, cardiovascular and neurodegenerative diseases or as components in anti-aging or cosmetic products [3].

The antioxidant activity of phenolic compounds are mainly due to their redox potential which empower them to function as reducing agents, donors of hydrogen atoms or electrons, singlet oxygen quenchers or metal chelators [4–6]. Phenolic compounds exhibit a wide range of physiological properties such as anti-allergic, anti-microbial, anti-thrombotic, anti-inflammatory, anti-arthritic, antipyretic, analgesic, antioxidant, cardio-protective, immunomodulatory and vasodilatory effects [7–11]. These activities of phenolic-flavonoidic compounds may be due to the presence of gallic acid, ellagic acid, ascorbic acid, quercetin, tannic acid, vanillin, resorcinol, catechin etc. [12–14].

Modern studies have shown that ferns possess biological properties such as anti-microbial, antioxidant, anti-proliferative, anti-inflammatory, antitussive, antitumor, anti-HIV, enzyme modulation and stimulation, hormonal action, interference of DNA replication and physiological action [15,16]. Iqbal Choudhary et al. [17] have isolated phenolic compounds together with few other phytoconstituents for the first time from the aquatic fern *S. molesta*. The isolated compounds were two glycosides, 6′-O-(3,4-dihydroxy benzoyl)-β-D-glucopyranosyl ester and 4-O-β-D-glucopyranoside-3-hydroxy methyl benzoate, along with five already known compounds viz., methyl benzoate, hypogallic acid, caffeic acid, paeoniflorin and pikuroside. They exhibited potent free radical scavenging activity in a non-physiological assay. These compounds possess interesting characteristics, noteworthy of further study.

Basing on these data the aim of the present study was to fractionate ethyl acetate extract of *S. molesta* using column chromatography and to quantify the phenolic compounds present in the fractions by RP-HPLC with photo diode array detection (PDA). This

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https://doi.org/10.1016/j.ejbas.2018.05.010
2314-808X/

study was the first to quantify seven antioxidant phenolic compounds in the fern extract applying two different mobile phases.

2. Materials and methods

2.1. Chemicals and phenolic standards

Hexane, ethyl acetate, ethanol, methanol, acetone, vanillin-H_2SO_4 spray, acetonitrile, phosphoric acid, acetic acid, chromanorm water, gallic acid, catechol, benzoic acid, resorcinol, ascorbic acid, vanillin, quercetin, silica gel and sea sand. All the above chemicals were of analytical grade and were purchased from Hi media, Pvt. Ltd., Mumbai, India.

2.2. Plant materials

Plants of *S. molesta* were collected from the paddy fields, rivers and ponds of Kalliyad and Kaiyamkulam, Kaithachira, Thrissur, Kerala, India. The specimen was identified and authenticated by Dr. G. Jeya Jothi, Taxonomist, Loyola College, Chennai, Tamil Nadu, India. The voucher specimen (No: LCH-130) of the plant has been preserved in Loyola College Herbarium for further reference. The plant materials were cleansed under running tap water three to four times, after which it was shade dried at room temperature for three weeks. The dried plant materials were pulverized into fine powder, passed through a sieve (mesh No. 40) and were stored in airtight containers [18].

2.3. Preparation of plant extracts

The extraction from the plant materials was performed by maceration. Four different solvents namely hexane, ethyl acetate, ethanol and methanol were used for the sequential extraction starting from low polarity to high polarity. 50 g of the powdered plant materials were soaked in 200 ml of hexane in a stoppered container and was placed on the orbital shaker at 120 rpm for 72 h at room temp. The mixture was then pressed and filtered through Whatman No.1 filter paper and was concentrated under reduced pressure using a rotary evaporator. The same procedure was followed for the other three solvents. The extraction process was carried out in triplicates with each solvent. The dried crude extracts were stored in amber vials and were placed in a refrigerator at 4 °C [18,19].

2.4. Column chromatographic fractionation of ethyl acetate extract

The ethyl acetate extract (EAE) was subjected to Silica gel column chromatography for the isolation of phytoconstituents. A vertical glass column (40 mm width × 60 mm length) made of borosilicate material was used for the fractionation. The column was rinsed well with acetone and was completely dried before packing. A piece of glass wool was placed at the bottom of the column with the help of a glass rod. Sea sand (50–70 particle size) was added to the top of the glass wool to 1 cm height. The sand particles were rinsed down using the solvent. Hexane was poured into the column up to $3/4^{th}$ level by closing the stopcock. 200 g of silica gel (60–120 mesh size) was used as the packing material. Silica slurry was prepared with hexane and was poured from the top of the column approximately $2/3^{rd}$ of the column with simultaneous draining of the solvent to aid proper packing of the column. Sea sand was added to the top of silica slurry to 1 cm height and the sand particles were rinsed down with the solvent. 20 g of EAE was mixed with minimum quantity of hexane and was poured down from the top of the column along the sides and was rinsed down with the solvent. Sea sand was added to the top of the extract to 1 cm height. Solvent level 6 cm from above the extract was maintained to prevent drying of the column. Gradient elution method was followed to separate fractions from EAE by using solvents from low polarity to high polarity (i.e. hexane to methanol) in varying ratios. The flow rate was adjusted to 5 ml/min and 40 ml solvent was collected for each fraction.

2.4.1. TLC of fractions

The fractions were collected separately and subjected to TLC (20 × 20 cm aluminium sheets coated with silica gel 60 F_{254}) to detect the presence of phytocompounds. The TLC plates were sprayed with vanillin-con. H_2SO_4 spray (15 g of vanillin in 250 ml of ethanol + 2.5 ml of con. H_2SO_4) and dried at 100 °C in hot air oven for 20–30 min. The R_f value of each spot was calculated. Fractions with the same R_f values were pooled and concentrated to dryness using rotary evaporator. The dry weight of the fractions was measured. The condensed fractions and EAE were further analyzed by HPLC for the presence of antioxidant phenolic compounds.

2.5. HPLC analyses of fractions and EAE

HPLC profiles of EAE and isolated fractions of *S. molesta* were determined by two methods using two different mobile phases selected on the basis of varying gradations of solvent systems in specific retention times and elute detections [20]. Analysis of all samples was performed using Shimadzu LC-10 AT VP, Luna 5u C18 reverse-phase analytical column (250 × 4.6 mm) with binary gradient mode, SPD-M10A VP photo diode array detector (PDA), injection volume 20 μl, total flow 1 ml/min, column oven temperature 25 °C and detection wavelength 280 nm. 55 mg of EAE and each fraction were dissolved in 3 ml of methanol for the analysis. The solvents used for the mobile phases were previously filtered through millipore and degassed prior to use. Quercetin, ascorbic acid, benzoic acid, gallic acid, vanillin, resorcinol and catechol were used as standard solutions for the quantification of phenolic compounds.

2.5.1. Method A

HPLC analyses of ascorbic acid, benzoic acid, gallic acid, vanillin, resorcinol and catechol were performed by Method A. Gradient elution of two solvents was used for the quantification of ascorbic acid, benzoic acid, gallic acid, vanillin, resorcinol and catechol: Solvent A (acetonitrile) and solvent B (0.1% phosphoric acid in water) [21]. Gradient elution program was begun with 92% of solvent B and was held at this concentration for 0–35 min. This was followed by 78% of solvent B for the next 35–45 min. Total run time was 45 min.

2.5.2. Method B

HPLC analysis of quercetin was performed by Method B. Gradient elution of two solvents was used for the quantification

Table 1
Experimental yield of *S. molesta* fractions.

Number of elutes (aliquots of 40 ml each)	Solvent system	Name of Fractions	Yield of Fractions (g)
1–164	H: EA (100:0 and 90:10)	Fraction A	6.06
165–375	H: EA (80:20, 70:30 and 60:40)	Fraction B	1.24
376–531	H: EA (50:50, 40:60 and 30:70)	Fraction C	2.22
532–583	H: EA (20:80, 10:90 and 0:100)	Fraction D	2.03
584–650	EA: MEOH (100:0, 90:10 and 80:20)	Fraction E	3.62

Table 2
Retention times of phenolic compounds present in EAE and Fraction A of *S. molesta*.

Salvinia molesta ethyl acetate extract					*Salvinia molesta* Fraction A				
Phenolic compounds	Retention time	Area	Height	Concentration	Phenolic compounds	Retention time	Area	Height	Concentration
Ascorbic acid	2.875	52,900	16,881	84.446	Ascorbic acid	2.909	29,381	10,031	46.901
Gallic acid	6.097	3353	227	0.534	Gallic acid	–	–	–	–
Resorcinol	12.850	1638	154	0.625	Resorcinol	–	–	–	–
Catechol	16.200	559,222	26,580	24.276	Catechol	15.966	18,129	869	0.787
Vanillin	28.254	294,220	14,324	22.544	Vanillin	28.116	198,708	9586	15.225
Benzoic acid	39.809	517,865	19,835	348.303	Benzoic acid	40.074	302,338	7069	203.345
Quercetin	13.694	60,048	9719	5.526	Quercetin	14.004	60,473	13,269	5566

Table 3
Retention times of phenolic compounds present in Fractions B and C of *S. molesta*.

Salvinia molesta Fraction B					*Salvinia molesta* Fraction C				
Phenolic compounds	Retention time	Area	Height	Concentration	Phenolic compounds	Retention time	Area	Height	Concentration
Ascorbic acid	2.879	42,561	12,368	67.941	Ascorbic acid	2.888	30,576	10,512	48.810
Gallic acid	5.977	7289	516	1.162	Gallic acid	5.795	1518	159	0.242
Resorcinol	12.629	2841	175	1.083	Resorcinol	12.746	3074	197	1.172
Catechol	15.849	78,369	3542	3.402	Catechol	15.428	488,423	23,863	21.202
Vanillin	27.853	1,838,376	87,042	140.860	Vanillin	28.104	192,995	10,775	14.788
Benzoic acid	40.710	58,049	2684	39.042	Benzoic acid	–	–	–	–
Quercetin	13.859	72,028	10,079	6.629	Quercetin	13.714	621,928	82,695	57.238

Table 4
Retention times of phenolic compounds present in Fractions D and E of *S. molesta*.

Salvinia molesta Fraction D					*Salvinia molesta* Fraction E				
Phenolic compounds	Retention time	Area	Height	Concentration	Phenolic compounds	Retention time	Area	Height	Concentration
Ascorbic acid	2.862	30,467	10,426	48.634	Ascorbic acid	2.868	67,443	22,903	107.661
Gallic acid	–	–	–	–	Gallic acid	5.994	15,293	1254	2.437
Resorcinol	12.469	55,965	2147	21.343	Resorcinol	12.585	6887	579	2.627
Catechol	15.732	2,048,513	104,860	88.926	Catechol	15.849	888,986	46,236	38.591
Vanillin	28.579	302,747	16,275	23.197	Vanillin	28.782	84,590	4880	6.481
Benzoic acid	39.918	8956	650	6.024	Benzoic acid	40.557	12,752	1113	8.577
Quercetin	13.958	90,999	12,975	8.375	Quercetin	13.656	128,313	28,609	11.809

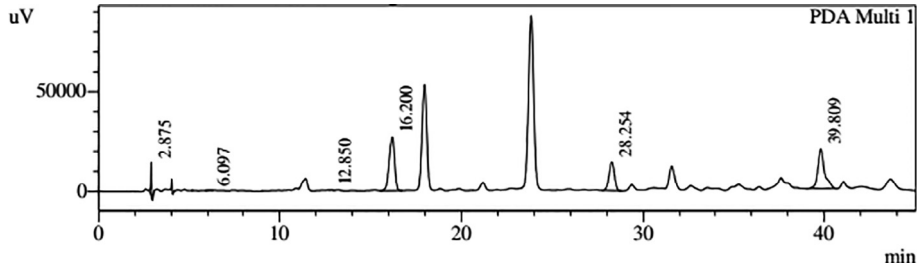

Fig. 1. HPLC profiles of phenolic compounds present in EAE of *S. molesta*.

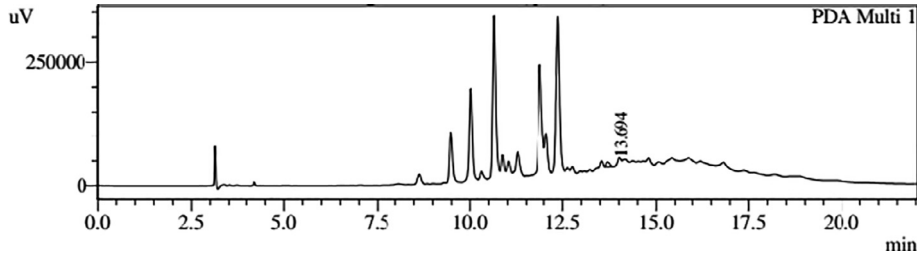

Fig. 2. HPLC profile of quercetin present in EAE of *S. molesta*.

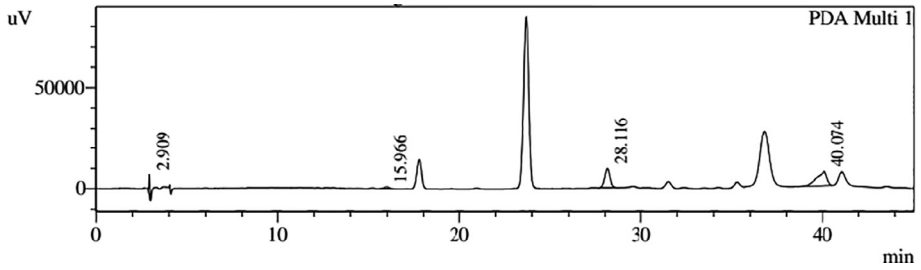

Fig. 3. HPLC profiles of phenolic compounds present in Fraction A of *S. molesta*.

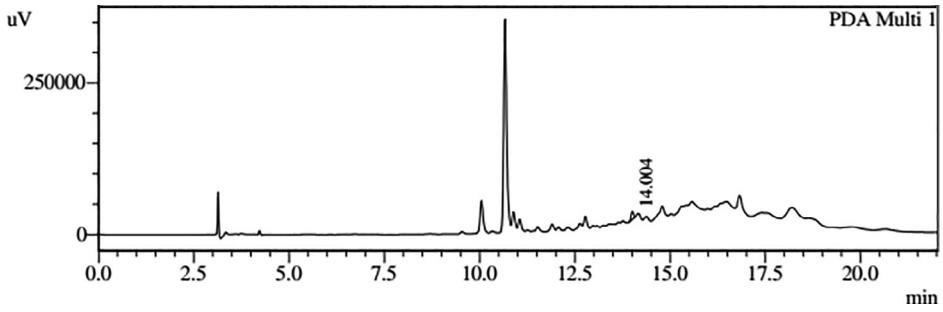

Fig. 4. HPLC profile of quercetin present in Fraction A of *S. molesta*.

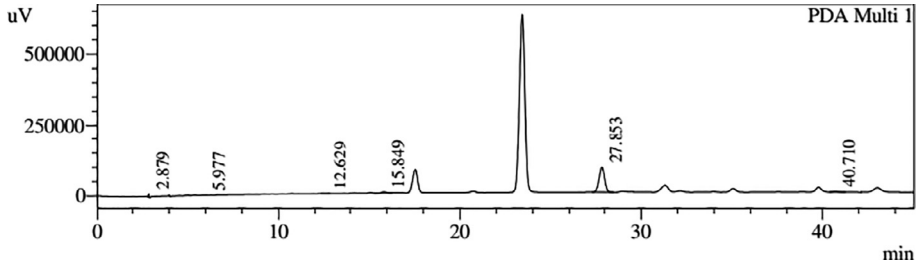

Fig. 5. HPLC profiles of phenolic compounds present in Fraction B of *S. molesta*.

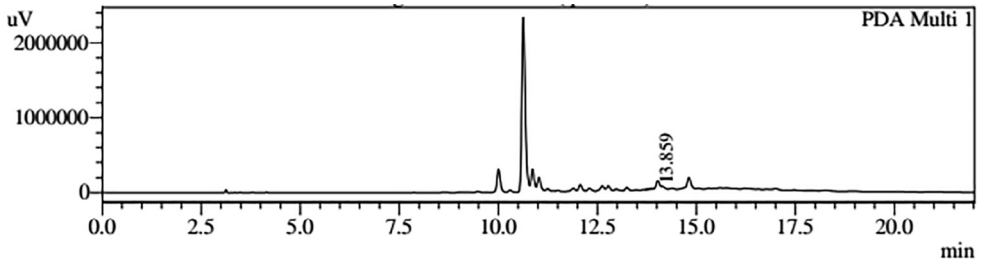

Fig. 6. HPLC profile of quercetin present in Fraction B of *S. molesta*.

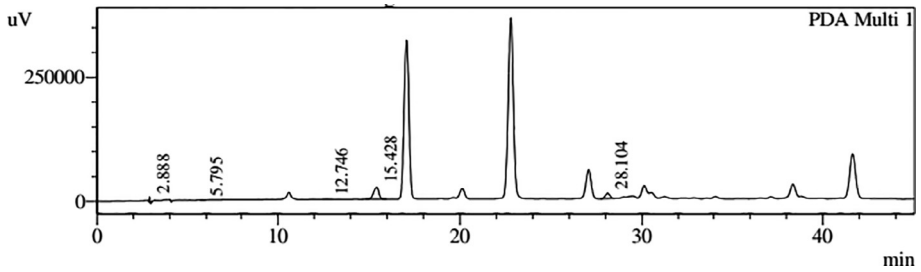

Fig. 7. HPLC profiles of phenolic compounds present in Fraction C of *S. molesta*.

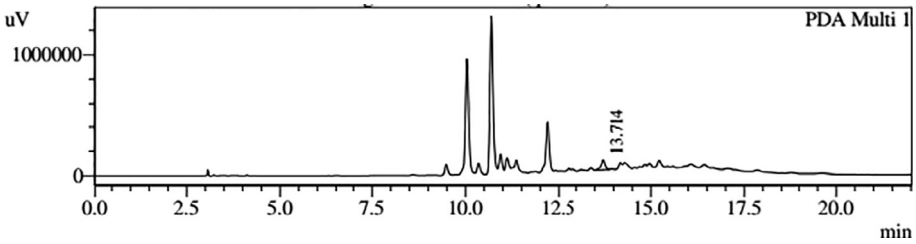

Fig. 8. HPLC profile of quercetin present in Fraction C of *S. molesta*.

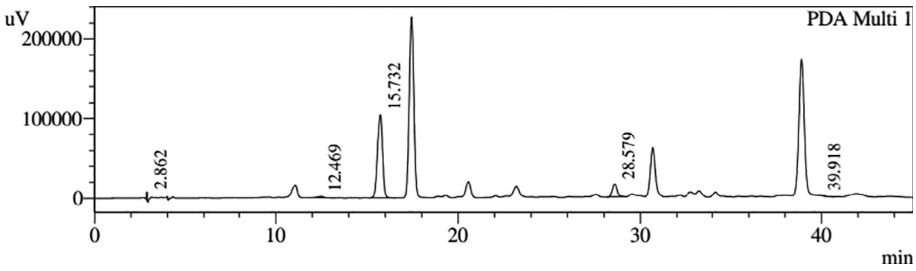

Fig. 9. HPLC profiles of phenolic compounds present in Fraction D of *S. molesta*.

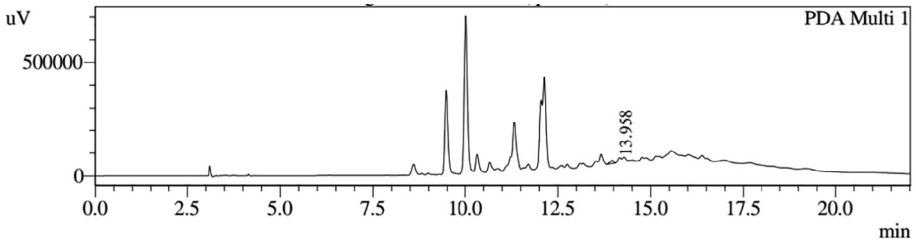

Fig. 10. HPLC profile of quercetin present in Fraction D of *S. molesta*.

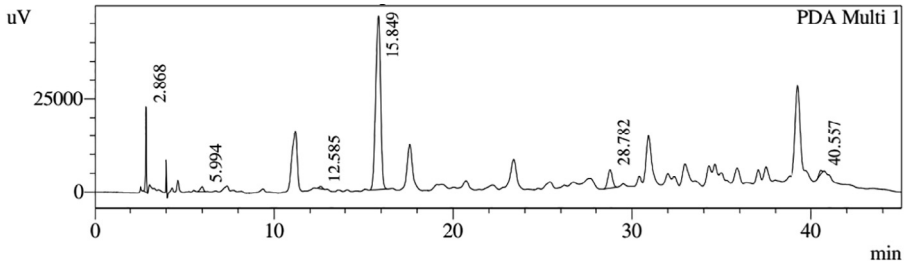

Fig. 11. HPLC profiles of phenolic compounds present in Fraction E of *S. molesta*.

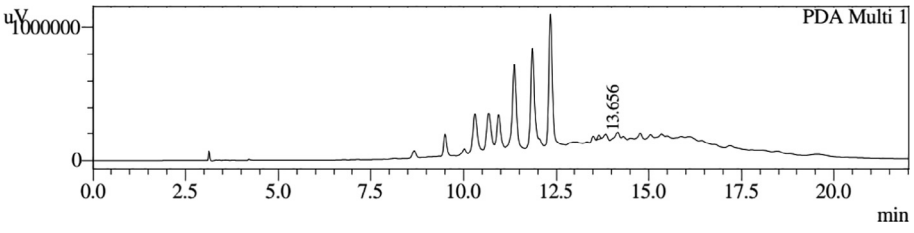

Fig. 12. HPLC profile of quercetin present in Fraction E of *S. molesta*.

of quercetin: Solvent A (methanol) and Solvent B (4% acetic acid) [22]. Gradient elution program was begun with 100% of solvent B and was held at this concentration for 0–4 min. This was followed by 50% of solvent B for 4–10 min and then reduced to 20% of solvent B for the next 10–20 min and then increased to 50% of solvent B for the next 20–22 min. Total run time was 22 min.

3. Results

The fractions obtained from silica gel column chromatography of *S. molesta* EAE were tested for the detection of various phytocompounds using TLC and sprayed with vanillin-con. H_2SO_4 spray and dried at 100 °C in hot air oven for 20–30 min. The phytocompounds showing the same R_f values were pooled into a single fraction. The total number of active fractions obtained after pooling were as follows: The elutes 1–164 aliquots of 40 ml each in solvent systems H:EA (100:0 and 90:10) formed Fraction A; the elutes 165–375 aliquots of 40 ml each in solvent systems H:EA (80:20, 70:30 and 60:40) formed Fraction B; the elutes 376–531 aliquots of 40 ml each in solvent systems H:EA (50:50, 40:60 and 30:70) formed Fraction C; the elutes 532–583 aliquots of 40 ml each in solvent systems H:EA (20:80, 10:90 and 0:100) formed Fraction D and the elutes 584–650 aliquots of 40 ml each in solvent systems EA:MEOH (100:0, 90:10 and 80:20) formed Fraction E. The yields of the fractions obtained are shown in Table 1.

HPLC profiles of *S. molesta* fractions and EAE were analyzed for seven phenolic compounds viz., ascorbic acid, quercetin, gallic acid, resorcinol, catechol, vanillin and benzoic acid. Phenolic compounds present in each fraction and EAE are shown in Tables 2, 3 and 4 with peaks showing different retention times (RT). Phenolic compounds present in EAE (Figs. 1 and 2) were vanillin (28.254 min), benzoic acid (39.809 min), quercetin (13.694 min), ascorbic acid (2.875 min), gallic acid (6.097 min), resorcinol (12.850 min) and catechol (16.200 min). Quercetin (14.0 min), ascorbic acid (2.909 min), catechol (15.966 min), vanillin (28.116 min) and benzoic acid (40.074 min) were present in Fraction A (Figs. 3 and 4). Ascorbic acid (2.879 min), quercetin (13.859 min), gallic acid (5.977 min), resorcinol (12.629 min), catechol (15.849 min), vanillin (27.853 min) and benzoic acid (40.710 min) were present in Fraction B (Figs. 5 and 6). Gallic acid (5.795 min), ascorbic acid (2.888 min), quercetin (13.714 min), resorcinol (12.746 min), catechol (15.428 min) and vanillin (28.104 min) were present in Fraction C (Figs. 7 and 8). Catechol (15.732 min), ascorbic acid (2.862 min), resorcinol (12.469 min), quercetin (13.958 min), vanillin (28.579 min) and benzoic acid (39.918 min) were present in Fraction D (Figs. 9 and 10). Resorcinol (12.585 min), catechol (15.849 min), vanillin (28.782 min), benzoic acid (40.557 min), quercetin (13.656 min), ascorbic acid (2.868 min) and gallic acid (5.994 min) were present in Fraction E (Figs. 11 and 12).

4. Discussion

A major study conducted in *S. molesta* by Li et al. [23] using bioactivity guided fractionation of ethanol extract yielded 50 compounds, including 17 abietane diterpenes (1, 17–22), nine phenolics (2–4, 29–32, 49 and 50), five fatty acids (24–28), five triterpenes (35–39), four apocarotenoids (42–45), two acyclic sesquiterpenoids (6 and 23), two monoterpenes (5 and 46), two jasmonates (33 and 34), two steroids (40 and 41) and two coumarins (47 and 48). All the abietane diterpenes were isolated from *S. molesta* for the first time, and out of the 6 compounds, (1–6), salviniol (1) was a rare abietane diterpene with new ferruginol-menthol coupled skeleton and both salviniside I (2) and salviniside II (3) were novel benzofuran glucose conjugates with unique 10-membered macrodiolide structures. Another study has shown that naringinin was the major phenolic compound present in acetone: methanol (1:1) extract of *S. molesta* which was identified and quantified by HPLC followed by myricetin along with rutin, epicatechin, catechin, quercetin, kaempferol and vanillin. These compounds were also found to have free radical scavenging potential [24].

A study by Cary and Weerts [25] showed that *S. molesta* grew most rapidly in high concentration of phosphorous and nitrogen (2–20 mg N 1^{-1} and 2 mg PO_4-P 1^{-1}). Since this plant can uptake nitrogen and other minerals from the aquatic environment, it is presumed that this plant contains nutritious biomass which could serve as an alternative unconventional plant protein source. It also possesses high crude fiber, tannin, lignin, and ash content which could limit its usage in the non-ruminant animal feeding operations [26]. According to Moozhiyil and Pallauf, [27] the crude protein content of *S. molesta* is relatively high in all stages of growth (young: 32.2%; medium: 37.5% and mature: 36.8%) compared to terrestrial forages. It was also found out that lignin content was as high as 13.7% while the average crude ash was 17.3% and the crude fiber was 35.2%. According to the result of the above study the level of tannin increased as the plant matured. The present study has identified seven penolic compounds such as ascorbic acid, quercetin, gallic acid, resorcinol, catechol, vanillin and benzoic acid in *S. molesta* and, therefore it can be concluded that this plant is one of the plausible natural antioxidants that could be used as a lead candidate for synthesizing antioxidant drugs which can be used for the treatment of many oxidative stress related diseases.

5. Conclusion

The present study has reported the presence of phenolic compounds such as ascorbic acid, quercetin, gallic acid, resorcinol, catechol, vanillin and benzoic acid in the fractions of ethyl acetate extract of *S. molesta*. Ethyl acetate extract was found to possess significant pharmacological activities; hence it was fractionated using silica gel column chromatography using different solvents in varying polarity. The study has found that *S. molesta*, an aquatic fern has promising medicinal properties and is a potent natural antioxidant owing to the presence of a number of phenolic compounds. Therefore, further investigation is needed to purify these phenolic components to be used as lead compounds for the development of novel antioxidant drugs.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgements

This study was financially supported by University Grants Commission Maulana Azad National Fellowship Scheme (F1-17.1/2012-13/MANF-2012-13-CHR-KER-7693), Ministry of Minority Affairs, New Delhi, India. We thank Dr. Jayaraj, CIU, KFRI, Peechi, Thrissur, India, for helping us with HPLC analyses. We are immensely grateful to Prof. Cinzia Forni (Italy), Dr. Sr. Ignatius Mary (France) and Dr. T.V. Poonguzhali (Chennai) for their valuable suggestions and comments.

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