Egyptian Journal of Basic and Applied Sciences xxx (2018) xxx–xxx

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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 xxxx

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.
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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
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Please cite this article in press as: Gini TG, Jeya Jothi G. Column chromatography and HPLC analysis of phenolic compounds in the fractions of *Salvinia molesta* mitchell. Egyp. Jour. Bas. App. Sci. (2018), https://doi.org/10.1016/j.ejbas.2018.05.010

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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 3/4th 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 2/3rd 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 of quer-

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

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