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HPTLC DENSITOMETRIC QUANTIFICATION OF STEROLS IN ETHYL ACETATE EXTRACT OF *EICHHORNIA CRASSIPES* (MART.) SOLMS

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ABSTRACT

Waterhyacinth is one of the fastest growing plants which disrupt the ecological balance. The plant inspite of its obnoxious nature, is a source of various pharmacologically active compounds. Sterols were isolated from the ethyl acetate extract of waterhyacinth and characterized to be a mixture of stigmasterol and β-sitosterol based on UV, IR, 1D and 2D NMR studies. Quantification of the isolated compound in the ethyl acetate extract of waterhyacinth was carried out using High Performance Thin Layer Chromatography. The correlation coefficient obtained for the linearity was 0.9955 for the compound. The amount of sterols were found to be 4.87 mg/100 mg of ethyl acetate extract of waterhyacinth.

KEYWORDS: ***Eichhornia crassipes*, hyacinth, Stigmasterol, β-sitosterol, ethyl acetate extract, HPTLC**

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INTRODUCTION

Eichhornia crassipes (Mart.) Solms commonly known as Waterhyacinth is an aquatic plant noted for its rapid growth and proliferation. Ethyl acetate extract of the plant exhibits reducing power and DPPH radical scavenging ability[1,2], antimicrobial activity[3], wound healing activity[4], anti-inflammatory activity[5]. Number of compounds has been isolated from various extracts of the plant[6]. Sterol mixture consisting of stigmasterol, β-sitosterol and campesterol has been isolated from the acetone extract of the plant[7]. 4α-methyl-5α-ergosta-8,14,24(28)-triene-3β,4β-diol 4α-methyl-5α-ergosta-8,24(28)-diene-3β,4β-diol and 4α-methyl-5α-ergosta-7,24(28)-diene-3β,4β-diol have been isolated from the ethyl acetate of waterhyacinth[8]. 6α-hydroxystigmata-4, 22-dien-3-one has also been isolated from waterhyacinth[9]. Stigmasterol has been isolated from the ethyl acetate extract of waterhyacinth[10]. Sterols found in plants are known as phytosterols and over 250 phytosterols and their related compounds have been identified in foods like plant oils, nuts, seeds, cereals, fruits and vegetables [11].

HPTLC has become a routine analytical technique due to its advantages of reliability in quantification of analytes at micro and even in nanogram levels and cost effectiveness. Low operating cost, high sample throughput and need for minimum sample clean-up are noteworthy. TLC has been known as the fast tool for the detection of compounds. TLC unlike HPLC can detect more compounds disregarding resolution. Moreover, the compounds having no UV absorption, e.g., sugar, still can be detected by spray reagents. Fingerprinting the active compounds in plant extracts can easily be done by TLC chromatogram pattern comparison thus making it an effective tool in the quality control in order to authenticate the extraction of the active compounds. Determination and identification of complex herbal extracts by chromatographic fingerprint analysis is feasible in HPTLC through data analysis system and optimized experimental conditions. Furthermore, the colourful picture like HPTLC image provides extra intuitive parameters of visible colour and or fluorescence and, unlike HPLC and GC, HPTLC can simultaneously determine different samples on the same plate. Thus HPTLC method maintains its innate advantage as well as get over the limitations of developing distance and plate efficiency[12]. HPTLC has been used for the identification and quantification of different sterols rather than other techniques owing to the simple derivatization methods. A large number of HPTLC quantification protocols for sterols have been developed[13-15]. This paper focuses on the isolation of stigmasterol and β-sitosterol mixture from the ethyl acetate extract of waterhyacinth and its quantification by HPTLC densitometry.

MATERIALS AND METHODS

Plant collection and authentication

Waterhyacinth (1050 kg) was collected from Singanallur boat house, Coimbatore, Tamil Nadu in March, 2010. The plant was identified by Dr.G.V.S.Murthy, Scientist F & Head of Office, Botanical Survey of India, Sourthern Regional Centre, Coimbatore- 641 002 with the number BSI/SRC/5/23/2011-12/Tech. The voucher specimen of the plant is maintained in the Department of Chemistry, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu, India.

Extraction of plant material

The roots were cut off and the plant was washed thoroughly. The plant was shade dried for 20 days and 28 kg dry plant was extracted with ethyl acetate (800 L) twice for 6 h and desolvetised yielding ethyl acetate extract (300 g).

Isolation of compound by column chromatography

The desolvetised ethyl extract (300 g) made into a slurry with silica gel was column chromatographed on silica gel (1.5 kg) with petroleum ether and eluted using gradient mixtures of petroleum ether and ethyl acetate (0-100% ethyl acetate). The fractions were monitored by TLC and similar fractions were combined. The fractions from 10% ethyl

acetate were recrystallized using chloroform to give compound 1 (1.5 g).

Preliminary tests
Salkowski test

A few crystals of compound 1 were dissolved in chloroform and a few drops of concentrated sulphuric acid were added to it. The formation of reddish colour in the upper chloroform layer indicated that the compound might be a sterol.

Libermann-Burchard test

A few crystals of compound 1 were dissolved in chloroform and a few drops of concentrated sulphuric acid were added to it followed by the addition of few drops of acetic anhydride. The formation of green colour indicated that the compound might be a sterol.

Characterization of the isolated compound

Melting point was recorded manually using sulphuric acid bath and was uncorrected. UV spectrum was recorded using Systronics 2201. FT-IR spectrum was recorded by KBr method using Schimadzu. NMR spectra were recorded using 500MHz Bruker Avance III spectrometer.

Stigmasterol and β-sitosterol (Compound 1)

Yield: 1.5 g; mp: 148°C; UV (λ_{max}): 210 nm; IR(KBr): 3446 cm^{-1} (hydroxyl group), 3030, 1606, 1458, 1244 cm^{-1} (olefinic group) ; ^{1}H NMR and ^{13}C NMR values are given in Table 1.

Quantification of the isolated compound by HPTLC
Equipment, Instrument and Materials

The analysis was done by using computerized Camag HPTLC system (Camag, Muttenz, Switzerland) consisting of a semiautomatic spotting device connected to a nitrogen tank and WinCATS 4 software (version 4.05, Camag), a TLC scanner III densitometer equipped with mercury, tungsten and deuterium lamp, a 100 µL HPTLC sample syringe (Hamilton, Bonaduz, Switzerland) and a glass twin-trough (10 cm x 10 cm) development chamber, volumetric flasks, measuring cylinders, micro-syringes and ruler. Silica gel $60F_{254}$, (Mean pore size of 60 A^{0} fluorescent excitation wavelength 254 nm) was used for the analysis.

Sample preparation

The working solution was prepared by dissolving the compound (1 mg) and ethyl acetate extract (10 mg) in chloroform (10 mL) in 10 mL volumetric flask.

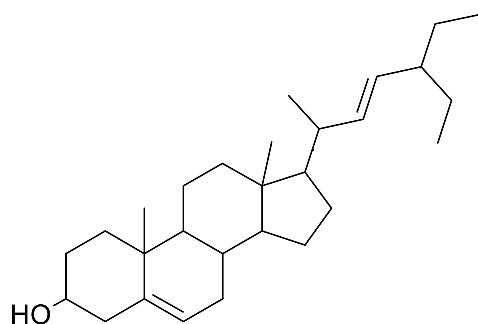
Chromatographic conditions

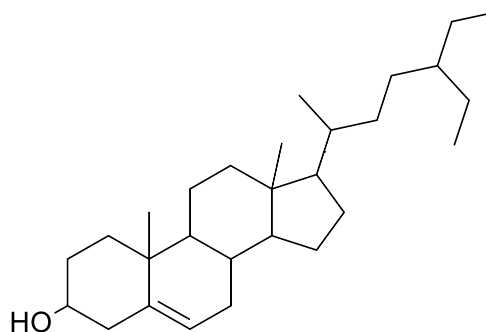
The samples were spotted as bands of width 8 mm with a micro litre syringe on precoated silica gel 60F 254 plate (10 cm x/10 cm) with 200 µm thickness, using Linomat V. A constant application rate of 0.1 µL/s was employed and space between two bands was 15 mm. The slit dimension was kept at 6 mm x 0.30 mm, and 20 mm/s scanning speed was employed. The mobile phase used for the analysis was petroleum ether : ethyl acetate (7:3). Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 40 minutes at room temperature. The length of chromatogram run was 72mm. TLC plates were dried in a current of air with the help of an air-drier prior to the development. Detection was done in UV-visible range. The spotting volume of compound 1 for calibration curve was 2,4,6,8 and 15 µL and that of the ethyl acetate extract was 20 µL. Densitometric scanning was performed on TLC scanner III in the absorbance mode at 540nm. The source of radiation utilized was deuterium and tungsten lamp. Concentrations of chromatographed compound were determined from the intensity of diffusely reflected light. Calibration curve of sample was obtained by plotting peak areas vs concentrations of stigmasterol applied.

RESULTS AND DISCUSSION

Compound 1 was isolated from the ethyl acetate extract of waterhyacinth by column chromatographyon elution with 90:10 petroleum ether :ethyl acetate. Spectral analysis of compound 1 and literature comparison led to the identification of the compound as a mixture of stigmasterol and β-sitosterol. Careful examination of the ^{13}C NMR spectra together with DEPT and HSQC suggested the compound to be a mixture of two sterols. The presence of signals at δ_C 138.31 and δ_C 129.29 indicated the presence of stigmasterol in the mixture. Presence of an

additional signal at δ_C 45.85 correlating with δ_H 0.92 indicated the presence of sitosterol in the mixture. The only difference between the structure of two compounds is the presence of an olefinic bond in the side chain (C-22 and C-23) in stigmasterol which is absent in β-sitosterol. The R_f of the compounds is also the same in most of the solvent systems and hence the difficulty in the separation of the two mentioned sterols[16,17]. The presence of sterol mixture was further confirmed by the lowering of melting point to 148 °C. In the ^{1}H NMR spectrum of compound 1, integration of proton signals at δ_H 5.36 (H-6), δ_H 5.15 (H-22), δ_H 5.02 (H-23) and δ_H 3.53 (H-3) were approximately in the ratio 2:1:1:2. Thus, the ratio of β-sitosterol and stigmasterol might be 1:1. The same ratio of mixture of sterols has been previously isolated from the chloroform extract of *Mammea siamensis* flowers[18] and hexane extract of aerial parts of *Acacia cochliacantha*[19]. Sterol mixture of β-sitosterol and stigmasterol in various ratios has been previously isolated from various plants[17,20-29]. The sterol mixtures show many pharmacological activities including anti-inflammatory effect[28], antihyperglycemic activity[27], antibacterial activity[23].





Stigmasterol ***β-Sitosterol***

Table 1
1H NMR and 13C NMR values of mixture of β-sitosterol and stigmasterol

	β-sitosterol		Stigmasterol	
C no	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
1	1.84	37.2	1.84	37.2
	1.09		1.09	
2	1.86	31.6	1.86	31.6
	1.55		1.55	
3	3.52	71.8	3.52	71.8
4	2.29	42.2	2.29	42.3
5	-	140.7	-	140.7
6	5.35	121.7	5.35	121.7
7	1.97	31.8	1.97	31.8
	1.55		1.55	
8	1.46	31.9	1.46	31.9
9	0.92	50.1	0.92	50.1
10	-	36.5	-	36.5
11	1.49	21.0	1.49	21.0
12	2.01	39.6	2.01	39.7
	1.16		1.16	
13	-	42.3	-	42.3
14	1.01	56.7	1.01	56.8
15	1.55	24.30	1.55	24.36
	1.06		1.06	
16	1.70	28.2	1.70	28.2
	1.27		1.27	
17	1.16	56.0	1.16	56.0
18	1.03	19.3	1.03	21.2
19	0.71	11.8	0.71	11.8
20	1.35	36.1	1.35	40.4
21	0.94	18.9	1.02	21.2
22	1.35	33.96	1.35	138.3
23	1.27	29.70	1.27	129.2
24	0.92	45.85	0.92	51.2
25	1.66	29.17	1.66	31.9
26	0.85	21.08	0.85	21.2
27	0.84	18.9	0.84	18.7
28	1.25	23.0	1.25	23.08
29	0.85	19.8	0.80	19.3

Thin layer chromatography (TLC) is a universally established practical solution used for characterization of raw herbs, active constituent-enriched extracts and their formulations. The procedure can be engaged for the routine analysis of steroids in pharmaceutical formulations and in bulk drug preparations as well as for the quality assurance of related extracts and commercial samples. Focus towards TLC has increased with developments in TLC instrumentation and methods especially in the last few years[30]. HPTLC method has been shown to be an inexpensive method for separation, qualitative detection, or semi-quantitative visual analysis of samples. The use of HPTLC has expanded significantly due to the development of forced flow (FF) and gradient TLC methods, improved selection of stationary and mobile phase, and new methods of quantitation[31]. An HPTLC method was identified to quantify stigmasterol and β-sitosterol mixture in the ethyl acetate extract of *Eichhornia crassipes* (Mart.) Solms. Bands 1-5 corresponded to that of the isolated sterol mixture at different concentrations and band 6 and 7 were that of the ethyl acetate extract. The selected mobile phase showed good resolution. The R_f of the isolated sterol mixture was found to be 0.30. The sterol mixture content of the ethyl acetate extract was found to be 4.87 mg/100 mg of the extract. The densitogram of the isolated stigmasterol and β-sitosterol mixture at white lights is shown in fig 1.

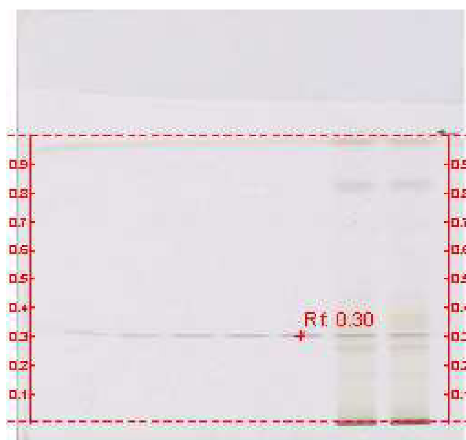


Figure 1
Densitogram of isolated stigmasterol and β-sitosterol mixture at whiter

Linear correlation between the peak area and applied concentration was found to occur in the concentration range of 0.4-1.5 µg for the sterol mixture. The correlation coefficient of the isolated stigmasterol and β-sitosterol mixture was found to be 0.9955. The peak area (*y*) is proportional to the concentration of the sterol mixture (*x*) following the regression equation y = 984.6 + 0.9773x (Fig 2).

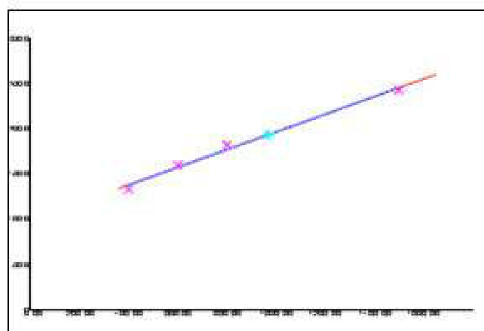


Figure 2
Calibration curve for the isolated stigmasterol and β-sitosterol mixture at 540 nm

CONCLUSION

Mixture of stigmasterol and β-sitosterol was isolated from the ethyl acetate extract of *Eichhornia crassipes*, a hazardous aquatic weed and hence the plant accorded as a rich source of these sterols. The sterol mixture was quantified by HPTLC in the ethyl acetate extract which is an easy and affordable method. This study indicated the importance of this plant in the pharmaceutical industry as the sterols has a notable pharmacological significance.

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