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Research Article

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LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-MASS SPECTROMETRY (LC-ESI-MS) BASED IDENTIFICATION OF FLAVONOIDS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) BASED QUANTITATION OF PHENOLIC ACIDS IN INDIAN SUGARCANE (*SACCHARUM OFFICINARUM* L.) STOOLS

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ABSTRACT

Background: *Saccharum officinarum* (L.) (Sugarcane) is cultivated worldwide for sugar and other industrial benefits. Literature review on Phytochemical evaluation revealed that the phenolic acids and flavonoids are reported in abundance in leaves and juice of sugarcane and so far there is no published reports on the phytochemical profile of sugarcane stools. **Objective:**Our objective was to identify and characterize various chemical constituents present in *Saccharum officinarum* (L) stools using chromatographic techniques. **Methods:** Flavonoids present in Indian sugarcane (*Saccharum officinarum* L.) stools were identified using liquid chromatography-electro spray ionization-mass spectrometry (LC-ESI-MS) and phenolic acids were quantified using high performance liquid chromatography (HPLC).**Results and Conclusion:** LC-MS based fingerprinting was developed for identification of phytochemicals in sugarcane stools (*Saccharum officinarum* L.) Ten flavonoids *viz.* vitexin rhamnosyl glucoside, isoorientin 3-*O*-methyl ether, orientin/isoorientin, vitexin, swertisin, isoorientin 7,3'-*O*-dimethyl ether, tricin-7-*O*-neohesperidoside, apigenin, luteolin were identified and isolated schaftoside/isoschaftoside (mixture) as a major phenolic acids which were further quantified with the aid of HPLC. LC-MS based fingerprinting was developed for the identification and characterization of similar chemical entities in Sugarcane stools would be economically viable and an alternative source of Sugar cane leaves and juice for exploring its therpeutic benefits.

Keywords: Saccharam officinalis, Phenolic acids; Flavonoids, Schaftosid and Stools.

1. INTRODUCTION

Saccharumofficinarum (L.) (Sugarcane) is cultivated worldwide for sugar and other industrial items. Phytochemical investigation of sugarcane juice and leaves revealed the presence of flavonoids (swertisin, tricin-7-*O*-neohesperoside-4'-*O*-rhamnoside, tricin-7-*O*-methylglucuronate-4'-*O*-rhamnoside, tricin-7-*O*-methylglucuronide, tricin-7-*O*-β-(6'-methoxycinnamic)-glucoside, luteolin-8-C-rhamnosyl glucoside, tricin-4'-*O*-(erthroguaicylglyceryl)-ether, apigenin, luteolin, orientin, tricin etc.) and phenolic acids (hydroxycinnamic acid, sinapic acid, caffeic acid, coumaric acid etc.) attributing to the free radical scavenging and other pharmacological activities[1-6]. Sugarcane (*Saccharumofficinarum* L.) stools are stalks having viable buds which normally regenerate during advantageous environmental conditions. During sugar production, sugarcane leaves are removed and discarded or burned[7]. Literature supports that several analytical methods have been established for the identification of the metabolites (phenolic acids and flavonoids majorly) in leaves, bagasse and juice of sugarcane[4-10]. However, to the best of our knowledge, there is no single report on the contents of sugarcane stools. Hence, this study was aimed to identify flavonoids present in the sugarcane stools using liquid chromatography electro spray ionization mass spectrometry (LC-ESI-MS) tools in negative mode by applying fragmentation rules and further to quantify phenolic acids (chlorogenic acid and total coumaric acids) with the aid of HPLC.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

All organic solvents were of LC-MS and HPLC grade and were purchased from Thermo Fisher (Hanover Park, IL, USA) and LC-MS grade Water from J.T. Baker (Center Valley, PA, USA). Reagent grade buffers, dimethylsulphoxide-d_6 (99.9% D) for NMR, chlorogenic acid, *m*- and *p*-coumaric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). All aqueous solvents were filtered through 0.45 µM syringe filter and degassed in an ultrasonic bath prior use. NMR (Bruker Avance-300) spectra were recorded in dimethylsulphoxide (DMSO) at 300 MHz spectrometer.

2.2. Sample preparation

S. officinarum(L.)stools were collected from agricultural fields located in Mandya district, Karnataka, India (voucher no. NPD/047/2014) by Dr. Kannan R., a botanist, Pharmacognosy Division, R and D Center, The Himalaya Drug Company, Bengaluru, India. One hundred g of *S. officinarum*(L.)stools powder (60 mesh) was extracted with 400 mL of ethanol (2 x) followed by 400 mL Milli-Q water (1 x) at room temperature for 24 h. The pooled extracted solution was filtered through Whatman filter No.1 and dried *in vacuo* to get the hydroalcholic extract (SOE) (13 g). 2 mg of SOE was dissolved in 1 mL of 70 % methanol, filtered through 0.45 µM syringe filter (Millipore, Bedford, MA).

2.3. LC-ESI-MS analysis

Chromatographic conditions

For the chromatographic separation a Shimadzu LC-20AD series pump and DUG-20A3 series Shimadzu degasser was used. Samples were run on Luna C18 (250 x 4.6 mm, 5µm) Phenomenex column. Eluent A was 0.2% Acetic acid in water and Acetonitrile was eluent B. A linear gradient program was executed at a flow rate of 1.0 mL/min with splitter as follows: 0-18 min of 10% to 40% of Acetonitrile (binary), 18-25 min of 40% Acetonitrile (linear), 25-30 min of 40% to 10% Acetonitrile (binary). The injection volume was 20 µL and injected through SIL-HTC Shimadzu Auto Sampler with total run time of 30 min. The desired temperature of 40 °C was achieved through CTO-10 AS VP column oven. The effluent was monitored at 354 nm.

Mass spectrometric conditions

Mass scans were acquired on API 2000 (Applied biosystem/MDS SCIEX, Canada) mass spectrometer coupled with ESI (Electron spray ionization) source with chromatographic system. Batch acquisition and data processing was controlled by Analyst 1.5 version software.

The MS parameters were optimized with 2 mg/mL of SOE prepared in 70% of methanol solution. Initially, intensity response was checked in both positive and negative ionization mode. Subsequently, good intense response was observed in the negative mode and other parameters like declustering potential (DP) -60v, nebulizing gas (GS1 and GS2) 55 and 65psi, curtain gas (CUR) 30 psi, focusing potential (FP) -400 v, Entrance potential (EP) -10 v and source temperature (TEM) 420 °C and Collision energy (CE) for fragmentation of precursor to product ions were optimized through multiple runs using LC in order to reach most intense precursor to product ion.

Schaftoside/isoschaftoside (**3**) were identified in *S. officinarum* (L.) and isolated from the stools by repetitive preparative HPLC for the first time. The total ion chromatogram and structures of isolated compounds are shown in (Fig. **1** a & b).

2.4. HPLC based quantitation of phenolic acids

For the quantitation of phenolic acids, a Waters e2695 series pump, Waters e2695 degasser with 2998 PDA detector was used. 5 mg of SOE was dissolved in 1 mL of 70 % methanol, filtered through 0.45 µM syringe filter (Millipore, Bedford, MA). Stock samples of phenolic acids (chlorogenic acid, *p*-coumaric acid and *m*-coumaric acid) were prepared in HPLC grade methanol and diluted to appropriate concentrations in order to get calibration curves. Samples were run on Luna C18 (250 x 4.6 mm, 5µm) Phenomenex column. Eluent A was 0.2% Acetic acid in water and Acetonitrile was eluent B. A linear gradient program was executed at a flow rate of 1.0 mL/min with splitter as follows: 0-18 min of 10% to 40% of Acetonitrile (binary), 18-25 min of 40% Acetonitrile (linear), 25-30 min of 40% to 10% Acetonitrile (binary),30-35 min of 10% Acetonitrile (linear). The injection volume was 20 µL and injected through Waters e2695 Auto Sampler. The desired temperature of 40 °C was achieved through Waters e2695 column oven. The effluent was monitored at 310 nm.

2.5. Isolation of Schaftoside/Isoschaftoside

Isolation of Schaftoside/Isoschaftoside (**3**) by semi-preparative high performance liquid chromatography For isolation of schaftosides (**3**), 500 g of *S. officinarum*(L.)stools powder (60 mesh) was extracted with 2000 mL of ethanol (2 x) followed by 2000 mL Milli-Q water (1 x) at room temperature for 24 h. The pooled extracted solution was filtered through Whatman filter No.1 and dried *in vacuo* to get the hydroalcholic extract (SOE) (65 g). 20 g of SOE was loaded on silica gel glass column (30 x 1.25 cm, 120 g) and elution was carried out in gradient mode (500 mL each) with ethyl acetate (EtAc) and methanol (MeOH) to provide five fractions (F1-F5).On the basis of similar thin layer chromatography (TLC) patterns, F4 and F5 were pooled together (6.7 g) and loaded on Diaion HP-20 resin column identification of flavonoids **(**Fig. **2**, Table **1).** Based on mass fragmentation pattern, tentatively ten compounds were identified from the LC-MS-based fingerprint of the crude hydroalcholic extract (SOE) and eluted gradient wise (300 mL each) with 100 % distilled water (H_2O) (Fr. 4A), 1:1 (H_2O:MeOH) (Fr. 4B), 1:3 (H_2O:MeOH) (Fr. 4C), 100 % MeOH (Fr. 4D) to remove color impurities.Fraction 4C (2.3 g) was dissolved in HPLC grade methanol (20 mL) and filtered through 0.45 µm syringe filter. Semi-preparative high-performance liquid chromatography (HPLC) purification of fraction 4C (2.3 g) was carried out in a gradient mode using Chemsil RP_{18} column (250 x 20 mm *i.d*., 5µm, ChemIndia, India) at a flow rate of 8 mL/min.The mobile phase composed of acetonitrile (B)-0.1% aqueous ortho phosphoric acid (A) as follows: 0-18 min of 10% to 40% of Acetonitrile (binary), 18-25 min of 40% Acetonitrile (linear), 25-30 min of 40% to 10% Acetonitrile (binary), 30-35 min of 10% Acetonitrile (linear). The effluent was monitored at 310 nm and the peak fraction was collected at R_t 17.79-18.17.

3. RESULTS AND DISCUSSION

3.1.LC-ESI-MS characterization of flavonoids

There are plethora of literature reports dealing with the chemical characterization of sugarcane juice and leaves. However, as far our knowledge, there is no single report on chemical profile of *S. officinarum* (L.) stools. Several trials with various mobile phases (methanol-Milli-Q water, acetonitrile-water) with different concentrations of buffers (acetic acid, formic acid, and orthophosphoric acid), flow rate, column temperature, particle size and detection wavelengths were carried out to optimize the separation of flavonoids in *S. officinarum* (L.) stools.

Finally, C_{18} column with acetonitrile: 0.2% acetic acid acid in water as mobile phases with column temperature 40 °C at a flow rate of 1 mL/min with detection wavelength 354 nm was finalized.

LC-MS chromatographic profile of crude hydroalcoholic extract (SOE) from *S. officinarum* (L.) stools allowed the possible from the LC-MS-based fingerprint of the crude hydroalcholic extract (SOE).

The compound at R_t 9.62 (Table 1) showed the *m/z* 739.11 with the major fragment ion at *m/z* 593 ($[M-H]^-$–rhamnose) and *m/z* 431 ($[M-H]^-$ rhamnose-hexose) in the mass spectrum. Further, fragment ion at *m/z* 431 $[M-H]^-$ corresponds to the vitexin. Hence, it was confirmed as **vitexin-rhamnosylglucoside** (8-glucopyranosyl-7-[6-*O*-(6-deoxy-mannopyranosyl)-glucopyranosyl]–5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-Benzopyran-4-one)[6].

The compound at R_t 9.93 with molecular ion peak $[M-H]^-$ at *m/z* 562.94, suggested a schaftoside-isoschaftoside pair which are reported to have same fragments $[M-H-120]^-$ in ESIMS, owing to their isomeric nature. Fragment ion at *m/z* 443 $[M-H-120]^-$ relates to the loss of glucose. Hence, they were identified as **schaftoside** (8-arabinopyranosyl-6-glucopyranosyl-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-Benzopyran-4-one) and **isoschaftoside**(6-arabinopyranosyl-8-glucopyranosyl-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-Benzopyran-4 one), respectively[6,10].

The compound at R_t 10.47 showned deprotonated molecular ion the *m/z* 461.02 $[M-H]^-$ with fragment ions at 446 $[M-H]^-$ for loss of methyl group. Further, fragment ion at *m/z* 447$[M-H]^-$ confirmed the mass of iosorientin. Also, losses of 120 amu at *m/z* 327 $[M-H-120]^-$ and 90 amu at *m/z* 357 $[M-H-90]^-$ respectively from *m/z* 447$[M-H]^-$ corroborated to cross-ring cleavages in the sugar unit. This led to the identification of

Isoorientin-3-*O*-methyl ether[4].

Similarly, **orientin/isoorientin** was confirmed at R_t 10.78 with *m/z* of 447.13$[M-H]^-$ and generated fragment ions at 357 $[M-H]^-$ and 327 $[M-H]^-$ for losses of 120 amu and 90 amu respectively[1].

The compound at R_t 11.93 showed $[M-H]^-$ base peak of *m/z* 430.95 and generated fragments at *m/z* 341 and 311 for losses of 120 amu and 90 amurespectively corresponded to the glucosyl ring fracture. This compound was identified as **vitexin** (apigenin-8-*C*-glucoside)[1].

Swertisin (6-glucopyranosyl-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4*H*-1-Benzopyran-4-one) were identified at R_t 12.30. The pseudomolecular ion $[M-H]^-$ was observed at *m/z* 445.01 for loss of one methyl group from the parent molecule while fragment at *m/z* 327 corresponded to loss of glucose (120 amu) a characteristic feature of *C*-glucoside flavonoid[5].

The compound at R_t 12.87 with *m/z* 475.02 $[M-H]^-$ generated the fragment at *m/z* 461 $[M-H-15]^-$ for loss of one methoxy group and at *m/z* 431 $[M-H-30]^-$ for loss of three methoxy groups from *m/z* 475 respectively which led to the identification of **isoorientin-7,3'-*O*-dimethyl ether**(6-glucopyranosyl-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4*H*-1-Benzopyran-4-one)[6].

The compound at R_t 13.98 with molecular ion peak $[M-H]^-$ at *m/z* 637.19 showed the fragments at *m/z* 607 (loss of two methyl groups) and *m/z* 329 (loss of a rhamnose and a hexose) from the *m/z* 637. Hence, the compound was confirmed as **tricin-7-*O*-neohesperidoside** (7-[[2-*O*-(6-deoxy-mannopyranosyl)-glucopyranosyl] oxy]-5-hydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-4*H*-1- Benzopyran-4-one)[6].

Luteolin (*m/z* 284.85) and **Apigenin** (*m/z* 269.01) were identied at R_t 18.99 and 21.85 respectively with generation of $[M-H]^-$ ions after comparison with the mass fragments reported in the literature[4].

Characterization of isolated Schaftoside/Isoschaftoside(3)

Schaftoside/isoschaftoside (**3**). Amorphous yellow powder (EtOH), 563 $[M-H]^-$, 443 $[M-H-120]^-$^{1}H-NMR, δ ppm, 13.64 (s, 1H, 5-OH), 8.00 (d, *J* = 8.4 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.79 (s, 1H), 4.73 (d, *J* = 10.8 Hz, 1H, arabinose anomeric-H), 4.70 (d, *J* = 11.1 Hz, 1H, glucose anomeric-H), 4.00-3.00 (m, sugar-H). The MS and ^{1}H-NMR data were in agreement with the reported data[11].

3.2. HPLC based quantitation of phenolic acids

Previously, phenolic acids (hydroxycinnamic acid, sinapic acid, caffeic acid, coumaric acid etc.) are reported to be present in the various parts of sugarcane attributing to the free radical scavenging and other pharmacological activities. Hence, in the present study, HPLC method was developed to determine the percentage of major phenolic acids in the extract of *S. officinarum* stools (Fig. 3a and 3b) depicts the HPLC chromatogram of *S. officinarum*(L.) extract detected at 310 nm where R_t at 9.106, 10.259 and 11.038 represents chlorogenic acid (0.015 % *w/w*)[12], *m*-coumaric acid[13] and *p*-coumaric acid (0.35% *w/w*)[14] respectively. Total coumaric acids (**13** +**14**) and chlorogenic acid[12] were found to be present in 0.35% *w/w* and 0.015 % *w/w* respectively.

4. CONCLUSIONS

As a regular practice after sugarcane harvesting, stools are normally ploughed out and burnt in the fields in order to prevent sugarcane diseases to the next crop (3). Instead we have developed these stools as value added product to be useful medicinally. Sugarcane leaves and juice shared the similar profile of phenolics acids and flavonoids as seen in sugarcane stools. We

established LC-MS based fingerprinting and identified Vitexinrhamnosylglucoside, Isoorientin 3-*O*-methyl ether, Orientin/Isoorientin, Vitexin, Swertisin, isoorientin 7,3'-*O*-dimethyl ether, tricin-7-*O*-neohesperidoside, Luteolin, Apigenin, chlorogenic acid, *m*-coumaric acid and *p*-coumaric acid as major Phenolic acids. Furthermore, we isolated, identified through H-NMR and quantified schaftoside/isoschaftoside (mixture) from *S. officinarum* (L.) stools for the first time.

Hence, the present study supports the novel usage of sugarcane stools as an economically viable and an alternative source of sugarcane leaves and juice for therapeutic benefits.

CONFLICT OF INTEREST

No competing or financial interest exists for the present work

ACKNOWLEDGEMENTS

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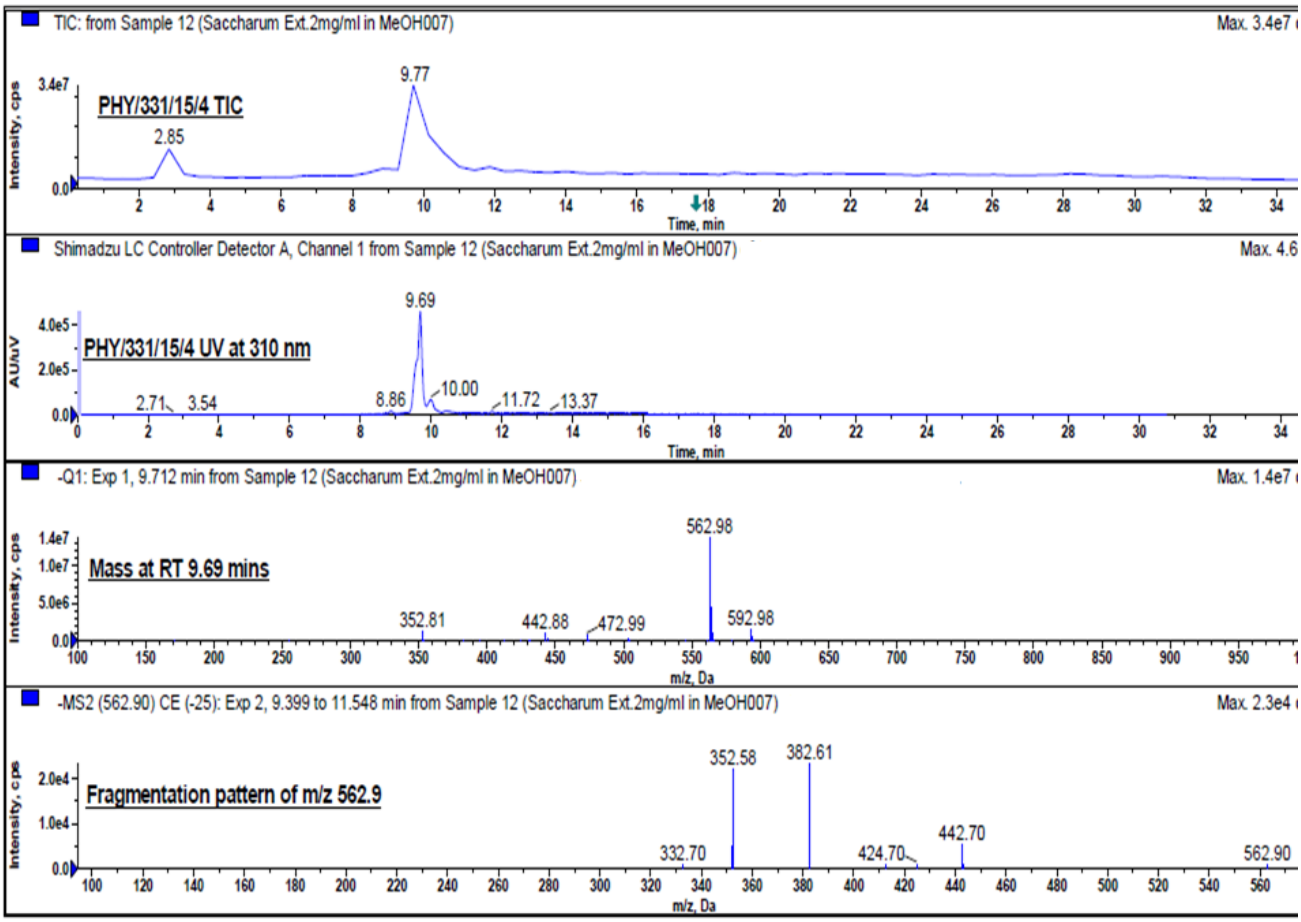
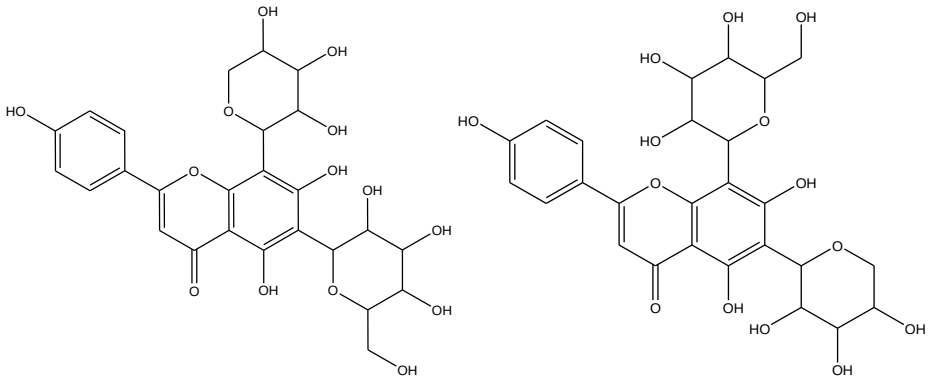


Fig. 1 (a): Total ion chromatogram (TIC) of Schaftoside/Isoschaftoside (3) acquired in (-)-LC-ESI-MS mode (310 nm)



(i) **(ii)**

Fig. 1 (b): Structures of schaftoside (i) and isoschaftoside (ii)

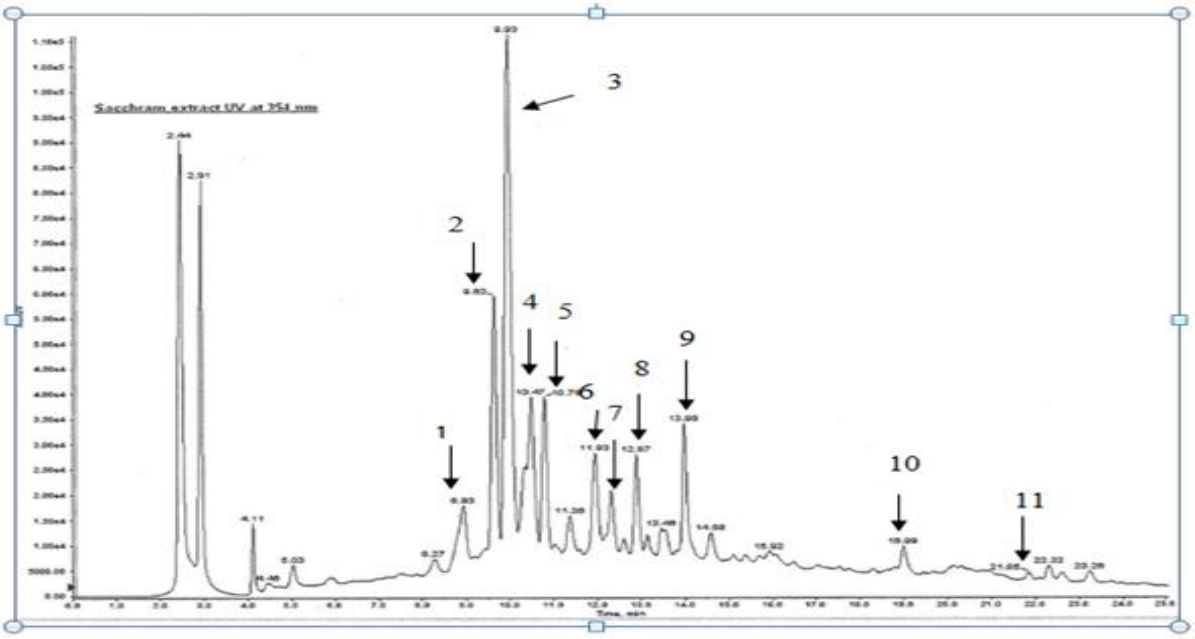


Fig. 2: Extractive ion chromatogram (EIC) of *S. officinarum* (L.) extract acquired in (-)-LC-ESI-MS mode (354 nm)

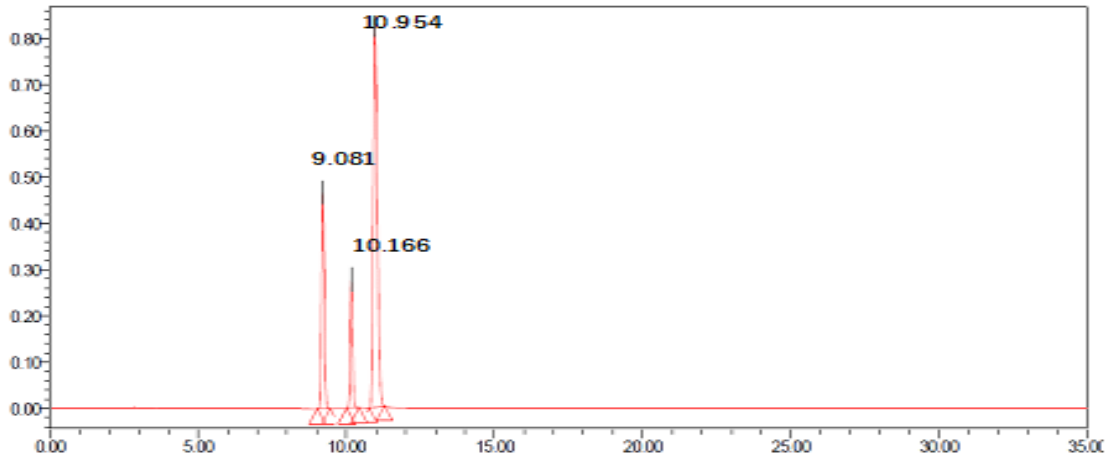


Fig. 3a: HPLC standard chromatogram of at 310 nm. RT at 9.081, 10.166 and 10.954 represents chlorogenic acid (12), *m*-coumaric acid (13) and *p*-coumaric acid (14) respectively.

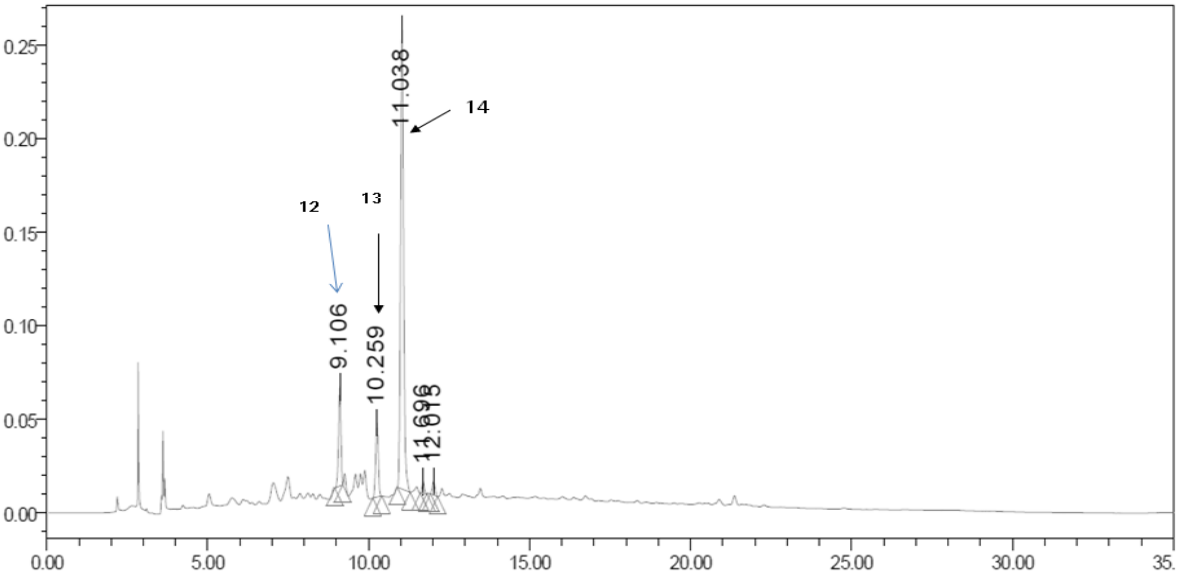


Fig. 3b: HPLC chromatogram of *S. officinarum*(L.) extract at 310 nm. Rt at 9.106, 10.259 and 11.038 represents chlorogenic acid (12), *m*-coumaric acid (13) and *p*-coumaric acid (14) respectively.

Table 1: LC-ESI-MS data of the chromatographic peaks

Peaks no.	$t_{R\ (min)}$	MS $[M-H]^-$ (*m/z*)	MS/MS (*m/z*), (Abundance)	Compound
1	8.93	255.21	156	Unknown
2	9.62	739.11	593,431	Vitexinrhamnosylglucoside
3	9.93	562.94	443	Schaftoside/Isoschaftoside
4	10.47	461.02	446,327	Isoorientin 3'-*O*-methyl ether
5	10.78	447.13	357,327	Orientin/Isoorientin
6	11.93	430.95	311, 341	Vitexin
7	12.30	445.01	327	Swertisin
8	12.87	475.02	461,431	Isoorientin-7,3'-*O*-dimethyl ether
9	13.98	637.19	607,329	Tricin-7-*O*-neohesperidoside
10	18.99	284.85	256, 219,199	Luteolin
11	21.85	269.01	241, 225	Apigenin

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