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Original Article

EXTRACTION, ISOLATION AND IDENTIFICATION OF PHYTOCONSTITUENTS PRESENT IN THE HEMIONITIS ARIFOLIA LEAVES

KRISHNA KUMAR K R[1]*, JAYAPRAKASH A P[2], SRINIVASAN K K[3], JYOTI HARINDRAN[1]

[1]University College of Pharmacy, M G University, Cheruvandoor, Ettumanoor 686631, Kottayam, Kerala, India, [2]University College of Pharmacy, M G University, RIMSR, Kottayam, [3]Shri Madhwa Vadiraja Institute of Technology, Vishwothama Nagar, Bantakal, Udupi, Karnataka, India.

Email: krishnakumar_kr@yahoo.co.in

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ABSTRACT

Objective: The investigation was carried out to prepare 95 and 50% ethanol extract of *Hemionitis arifolia* leaves, and for the identification and quantification of the phytoconstituents present in it.

Methods: Soxhlet extraction method was followed to prepare the extracts, followed by standard phytochemical methods to identify the phytoconstituents present in it. Paper chromatography, HPLC, LC MS/MS and LCMS studies were then done for the confirmation, quantification of phytoconstituents and detection of mass values of other unidentified phytoconstituents present in the extract.

Results: Extractive values were found to be 14.6 and 15.6% w/w respectively. In the phytochemical studies, presence of carbohydrates, glycosides, saponins, flavonoids, phenolics, tannins, phytosterols and fixed oils was identified. On doing paper chromatography with Whatman No.1 filter paper using 15% acetic acid solvent system, six different spots with yellow-orange colour were detected under UV visualization. Out of the six spots, four were identified as quercetin, kaempferol, apigenin and rutin by co-chromatography. In the RPHPLC analysis, rutin, quercetin and kaempferol were identified and quantified. In the LC MS/MS analysis, rutin, apigenin and kaempferol were identified and quantified. In the LCMS analysis, ten different m/z values were found, among which m/z 609 was identified as rutin.

Conclusion: The presence of various flavonoids and related glycosides is indicative of potential for medicinal use of this plant.

Keywords: *Hemionitis arifolia*, Phytoconstituents, Paper chromatography, HPLC, LC-MS/MS, HAL - *H. arifolia* leaf, HALH - *H. arifolia* ethanol (50%) extract.

INTRODUCTION

In and around the world, people now depend on a lot of natural/plant medicines through Ayurveda, traditional Chinese medicine (TCM), Homoeopathy and many new products of modern medicine derived from natural sources. Traditional Indian medicine (TIM) and Ayurveda use many of the plants and their parts for the treatment of many diseases and also for good health [1]. Secondary metabolites are special chemicals produced by plants and stored in various plant parts. Globally, around 55,000 species of plants are being used for traditional medicines; but only a small portion has been investigated scientifically to establish their activity [2]. Under the Indian government project AYUSH, many of these uses are being evaluated. Herablome is the project run by the government of China for the evaluation of TCM [3]. WHO also approved the use of traditional medicines. These projects scrutinize the veracity of prevailing claims, examine the potential of more plant products for possible medical solutions and then draw up of a comprehensive scientific catalogue.

The objective of the present study was to extract the shade dried leaves of *H. arifolia* with 95% and 50% ethanol, phytochemical evaluation of extracts including identification and quantification of the phytoconstituents present in it.

MATERIALS AND METHODS

Materials

The leaves of *Hemionitis arifolia* were collected from Thrickalathoor and Aruvappara villages of Ernakulam district during September to December 2009. (Voucher specimen number KRK/UCP/ CMS/506). The plant was identified by Prof. Dr. K V George, Professor of Botany, CMS College, Kottayam. Ethanol from Travancore distilleries, Tiruvalla, Kerala was used for the extraction. In the HPLC, LC-MS and LC-MS/MS studies, reagents of the respective grades were used. Merck laboratory grade reagents were used in all other experiments performed.

Preparation of the extract

The authenticated dried leaves of *H. arifolia* were used for extraction. The dried leaves were made into a fine powder. The powder was packed in a filter paper thimble (400 g in a batch). Ethanol (95%) 2.5 liter was used for extraction. Soxhlet extraction was performed till forty cycles were completed. The procedure was repeated for another two batches (400 gx2) with ethanol (50%) as solvent. The pooled extract was distilled under reduced pressure to get a syrupy consistency material. The extract of *H. arifolia* leaves were defatted with petroleum ether (60-80^{0}C), dried and preserved in a desiccator for further studies. Yield of dry extract was 124.8 g.

Preliminary phytochemical screening

Both 95 and 50% ethanol extract of *H. arifolia* leaves answered the tests for carbohydrates, glycosides, saponins, flavonoids, phenolics, tannins, phytosterols, triterpenoids, fixed oils and fats [4].

Identification of the components by paper chromatography

Sample: defatted HALH extract. Solvent system used: Acetic acid (15%) in distilled water.

Paper used: Whatman no.1 for qualitative work and Whatman no.3 for quantitative work.

Defatted 50% ethanol extract (5 mg) was dissolved in methanol (2 ml) and centrifuged. The centrifugate was spotted on Whatman no.1 filter paper strip 3 cm x 15 cm. It was kept in a chromatographic chamber consisting of a solvent system of 15% acetic acid [5]. The paper was positioned so that 1 mm of the bottom of the strip dipped uniformly in the solvent system. Care was taken to saturate the chromatographic chamber. The chromatogram was run in the ascending mode. When the development was complete, the chromatogram was taken out, dried at room temperature and the spots were visualized under UV before and after exposure to ammonia. The results are depicted in table 1.

The spots were viewed under UV short and long wave lengths. Four different spots were visualized and they were designated as HA-1, HA-2, HA-3 and HA-4 in the ascending order of R_f values. In addition to the four spots mentioned above some, orange material was remaining at the point of application and a faint yellow spot was also present in the solvent front.

The closely moving spots of flavonoids were further separated by preparative paper chromatography with Whatman no. 3 filter paper using 15 % acetic acid as the solvent system.

The separated bands were cut off from the paper chromatogram, air dried and made into small pieces. Each band was eluted with spectroscopic grade methanol. The eluates were filtered and concentrated separately. UV spectrum was recorded in methanol for each of the different bands. The results are depicted in table 2.

HPLC of HALH Estimation of Rutin, Apigenin, Quercetin and Kaempferol in the extract was performed by HPLC method.

Chromatographic conditions

Mobile phase: Solvent A - Phosphate buffer. Prepared the solvent A by dissolving 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 ml of HPLC grade water, 0.5 ml of orthophosphoric acid was added to it and the solution was made up to 1000 ml with water. The above solution was filtered through 0.45 µ membrane and degassed in a soniccator for 3 minutes. Solvent B - Acetonitrile (100%) [6]. The separation was carried out using Shimadzu LC 2010 CHT instruments with C18 column, ODS 0.5 µm, 250 x 4.6 mm (Merck) at ambient temperature. The sample, 20 µl was injected at a time and eluted at a flow rate of 1.5 ml/min. The experiment was performed in gradient mode and run for a period of 40 minutes. The detector was tuned at 370 nm.

Standard and sample preparation

About 1.0 mg each standard was weighed accurately (rutin, apigenin, quercetin and kaempferol) to separate clean and dry 10 ml volumetric flasks. Five ml of methanol was added to each of the volumetric flasks, warmed gently on a water bath, cooled and made up to 10 ml with methanol.

One ml of each standard solution was transferred to different 10 ml volumetric flasks, made up to the mark with methanol (Std. mix).

The test extract (defatted HALH) was weighed accurately (500 mg) and transferred to a 25 ml volumetric flask, 15 ml of methanol was added and soniccated for 6 minutes. Then it was warmed on a water bath for another 5 minutes. Cooled the above solution to room temperature and made up the volume to the mark with methanol. Mixed well and filtered through 0.2 µ membrane filter paper.

LC-MS/MS of HALH

Thermo Scientific TSQ Quantum Access Max Triple quadruple Mass spectrometer is coupled with Thermo Scientific Accela Pump and Autosampler.

Standard and sample preparation

Rutin, quercetin, apigenin and kaempferol Standards 5 mg each was weighed accurately and transferred to four different 50 ml volumetric flasks. After the addition 10 ml of methanol, the mixture was soniccated for about 5 minutes made up the volume with methanol. The solution was diluted to avail the required concentration.

The sample was weighed accurately (100 mg) and transferred it to a centrifuge tube (15 ml). It was vortexed after adding 10 ml of methanol, warmed in a water bath and sonicated for 15 minutes.

HPLC conditions

Purospher STAR Luna RP-18, 2 µm, 2.1x100 mm column were used. Injected 1µl sample at a time. The flow rate was adjusted at 0.15 ml/minute. The column oven temperature was maintained at 40 ^{0}C. The total run time was 20 minutes. Mobile phase used were Acetonitrile 100% (solvent-A) and formic acid in methanol 0.02% (solvent-B). The elution was performed in a gradient manner. The experiment was performed with modifications of the HPLC procedure followed by Thiyagarajan Sathishkumar et al., [7].

In mass spectrometry, vaporization temperature was 250^{0}C and capillary temperature was 350^{0}C. The operation mode was HESI. The spray voltage was 4500 V and three different tube lens 86, 96 and 120 were used.

LCMS of defatted *H. arifolia* leaf extract (HALH)

Mass spectrometer

Thermo Fisher LCQ-Fleet –Ion Trap with Quadrupole Ion Trap Mass Analyser

HPLC

Accela High Speed Pump, Surveyor Plus Auto sampler.

HPLC conditions for HALH The column used was C18, 3 µm, 100 x 4.6 mm. The volume of injection was 5 µL and the flow rate was 350 µl/min. The solvent system used was 0.1% formic acid (Solvent – A) and acetonitrile 100% (Solvent – B). The experiment was performed in gradient mode and the elution was done for twenty minutes. The column was maintained at ambient temperature [7].

Mass spectrometer was working in the ESI MS mode. Sheath gas and auxiliary gas used were nitrogen. The discharge current of the system was 5.0 K µA. The capillary volts used were 7.00V (positive) and -16 V (negative). The tube lens used in the experiment was 55 V (positive & negative). The experiment was performed at ambient temperature.

RESULTS

The four different spots visualized were designated as HA-1, HA-2, HA-3 and HA-4 on the ascending order of R_f values. The spot named HA-1 was having the same R_f value of that of quercetin obtained from Sigma.

The other three spots named HA-2, HA-3 and HA-4 were having matching R_f values respective to kaempferol, apigenin and rutin obtained from Sigma. In addition to that, some orange material was remaining at the point of application and a faint yellow spot in the solvent front. The R_f values of the flavonoids quercetin, kaempferol and apigenin are in accordance to the reports of [8].

Table 1: R_f values of spots obtained in paper chromatography of HALH. Solvent system-15% acetic acid

Spot number	R_f	UV visualization	UV-ammonia visualization
A (HA-1)	0.03	Yellow	Yellow
B (HA-2)	0.04	Dull yellow	Dull yellow
C (HA-3)	0.11	Deep purple	Yellow-green
D (HA-4)	0.56	Deep purple	Yellow

Table 2: UV data determined (in methanol) of the bands separated by preparative chromatography from HALH

Sample code	UV peaks/shoulder at WL in nm.
Band A (HA-1)	253sh, 266, 294sh, 322sh, 367
Band B (HA-2)	255, 269sh, 301sh, 390
Band C (HA-3)	267, 296sh, 336
Band D (HA-4)	259, 266sh, 299sh, 359

HPLC results of HALH

The HPLC chromatogram of the extract is cited as fig.1. It shows around ten different peaks. Earlier, HPLC was run with rutin, quercetin, apigenin and kaempferol reference standard materials. Out of the ten different retention times, retention time of 4.87 minutes was matching with rutin, 12.83 minutes was matching with quercetin and 16.57 minutes was matching with kaempferol. The peak observed at the retention time of the standard apigenin was not sufficient enough to distinguish from noise signals. Quantity of rutin, quercetin and kaempferol were 0.063%w/w, 0.002%w/w and 0.005%w/w of the extract.

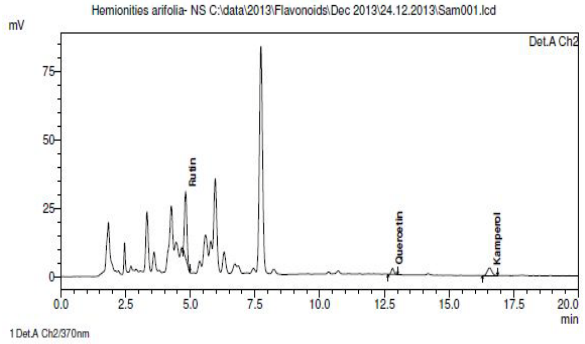

Fig. 1: Fingerprint of defatted HALH

Detector A Ch2 370nm

Peak #	Ret. Time	Name	Area	Area %	Height %
1	4.815	Rutin	155534	75.930	83.062
2	12.814	Quercetin	15313	7.476	7.428
3	16.559	Kamperol	33992	16.594	9.510
Total			204839	100.000	100.000

Result of LC-MS/MS estimation of HALH

In this study, the retention time of the component marked HA-1(6.11 min.), HA-2 (8.58 min.) and HA-3(8.77 min.) matched well with the retention time of rutin, apigenin and kaempferol standards obtained from Sigma. In the MS/MS studies, the quantity of each component present was estimated by comparing with AUC of the respective component. The presence of quercetin was confirmed earlier by HPLC method.

The quantity of rutin, apigenin and kaempferol in the defatted HALH were 0.043, 0.010 and 0.013%w/w of the extract respectively.

LCMS analysis of HALH

In the LCMS analysis [9] of defatted HALH, presence of ten different mass values was observed and is tabulated in table 3. Out of the above ten different mass values, the molecule depicting the mass value 609 was identified as rutin with the UV lambda max. determination, PC, HPLC and LC MS/MS studies. The IR and NMR spectrum also reconfirmed the above findings.

Table 3: Mass values of the components present in defatted HALH

S. No.	Retention time in minutes	m/z	$^{+}$Ve or $^{-}$Ve mode
1	4.30	328.16	$^{+}$Ve
2	4.34	357	$^{-}$Ve
3	5.03	113.97	$^{+}$Ve
4	5.54	609.09	$^{-}$Ve
5	5.74	358	$^{-}$Ve
6	6.21	463	$^{-}$Ve
7	6.41	477	$^{-}$Ve
8	6.87	342	$^{-}$Ve
9	7.08	461	$^{-}$Ve
10	7.22	163	$^{-}$Ve

Note: As the concentrations of the compounds are less, the individual m/z has been extracted and separate chromatograms were generated. The remaining nine molecules present in trace amount need to be identified and quantified.

DISCUSSION

The shade dried leaves of *H. arifolia* were extracted with ethanol 95 and 50%. In paper chromatography studies, six spots were observed. Out of them, four were identified as quercetin, kaempferol, apigenin and rutin. In the HPLC analysis, quercetin, kaempferol and rutin were identified and quantified; but apigenin was not detected in the method used. In the LC-MS/MS analysis, kaempferol, apigenin and rutin were identified and quantified; but quercetin was not detected. In the present study, four different flavonoids were identified. The presence of other flavonoids is clear from the unidentified spots present in the paper chromatogram. The unidentified spots may possibly due to other flavonoids or due to the presence of glycosides of the already identified flavonoid aglycones. In the LC MS studies, ten different mass values were observed. Out of them, m/z 609 is possibly due to rutin. The remaining nine mass values are yet to be identified.

LC-MS analysis of Sample HAA CRUDE

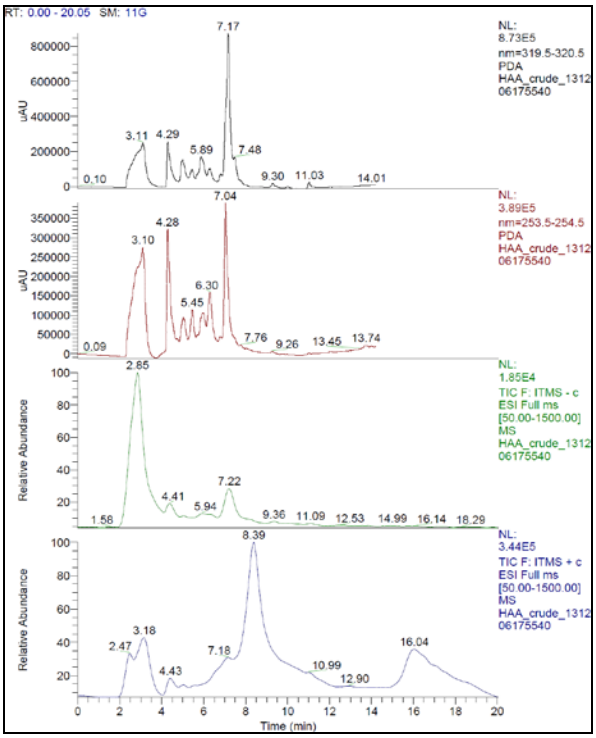

Fig. 2: TIC of the sample HAA CRUDE

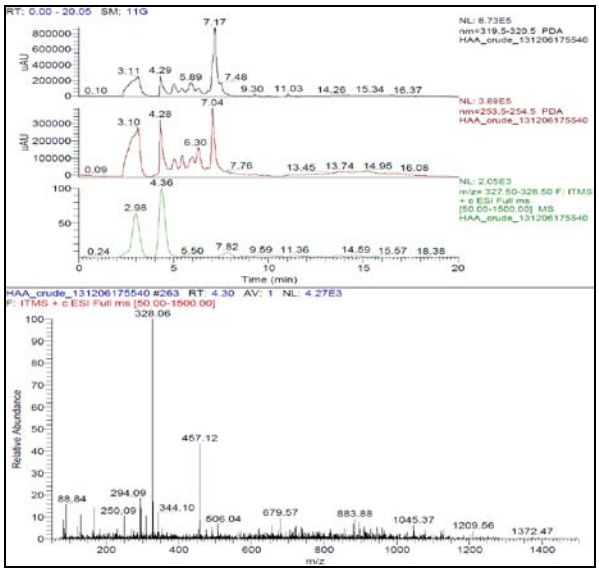

Fig. 3: TIC of the extracted m/z 328 at RT 4.30

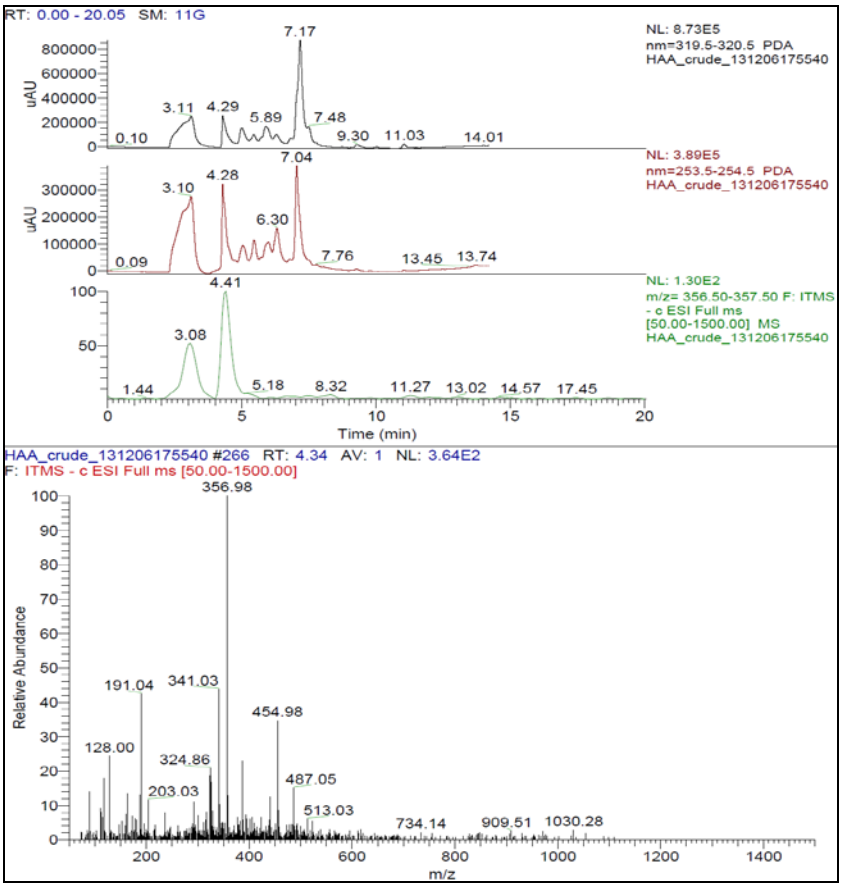

Fig. 4: TIC of the extracted m/z 356.98 (357) at RT 4.34

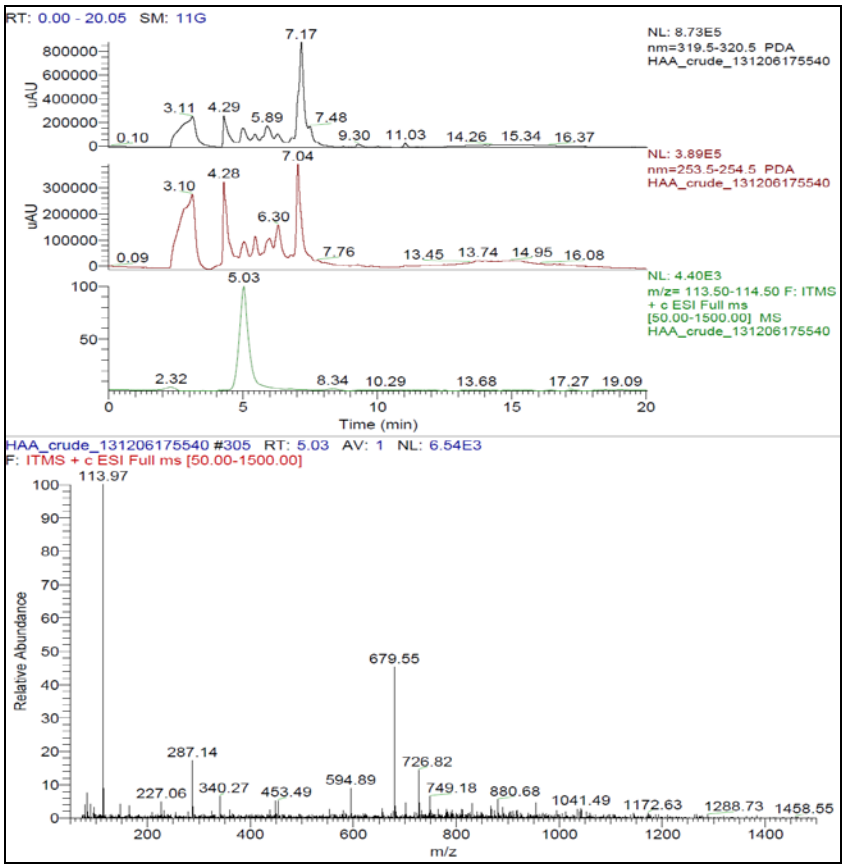

Fig. 5: TIC of the extracted m/z 113.97 at RT 5.03

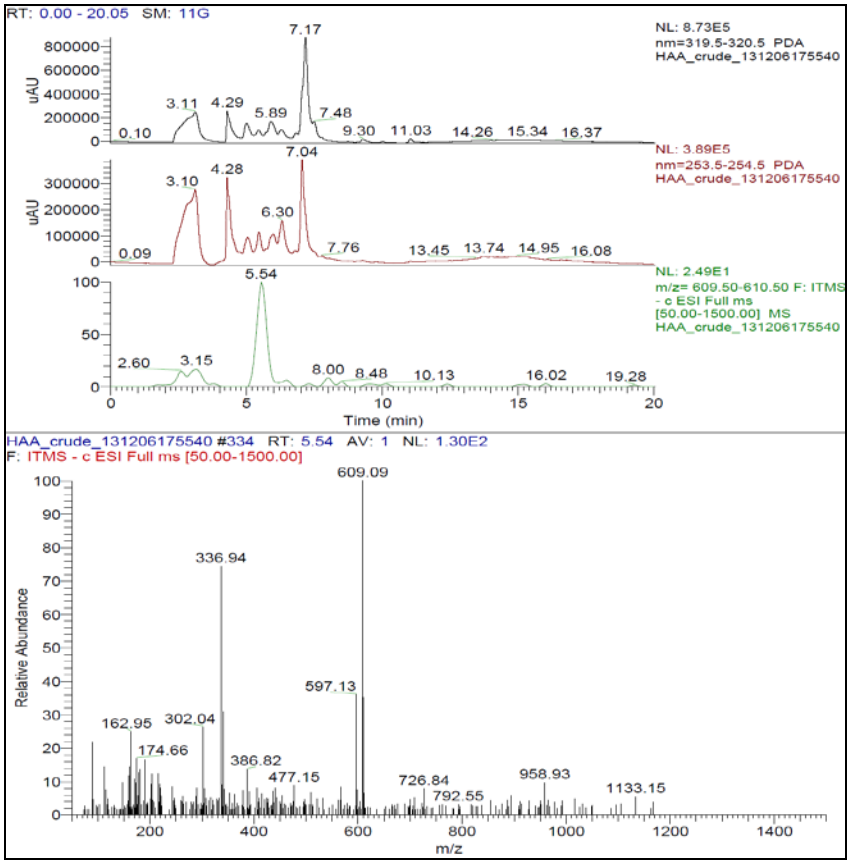

Fig. 6: TIC of the extracted m/z 609.09 at RT 5.54

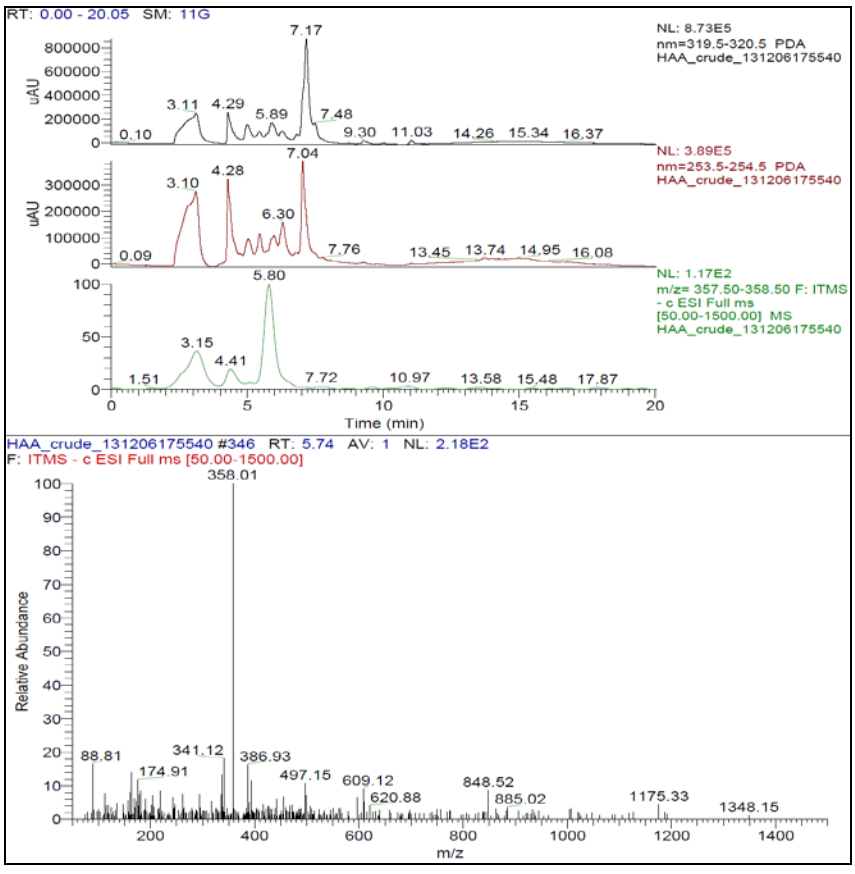

Fig. 7: TIC of the extracted m/z 358 at RT 5.74

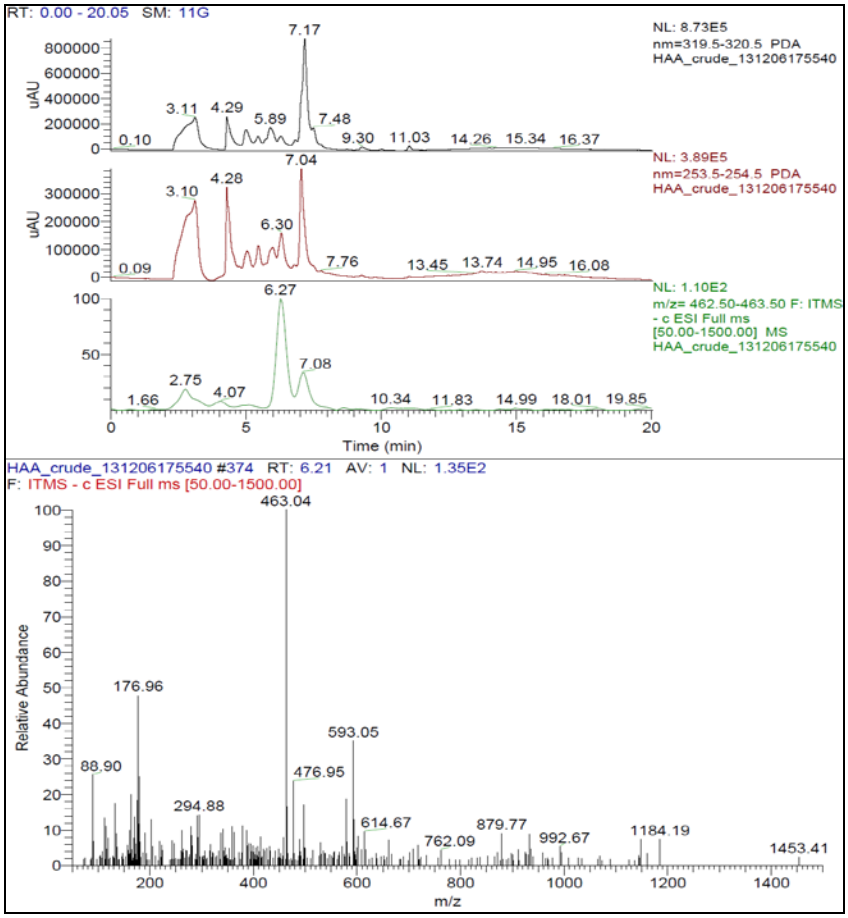

Fig. 8: TIC of the extracted m/z 463.04 at RT 6.21

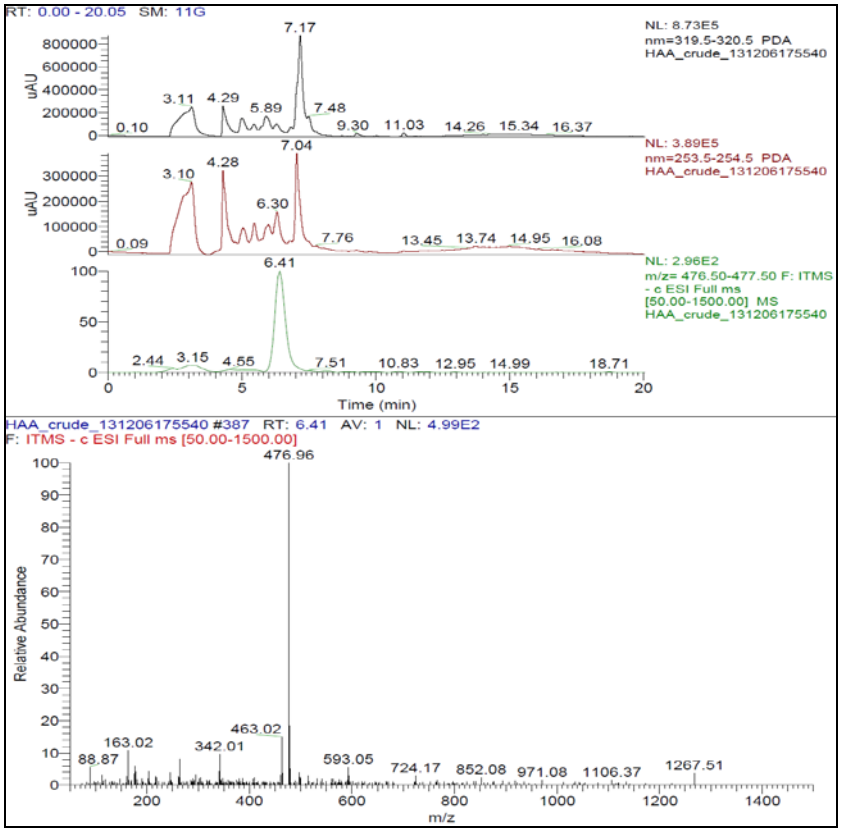

Fig. 9: TIC of the extracted m/z 477 at RT 6.41

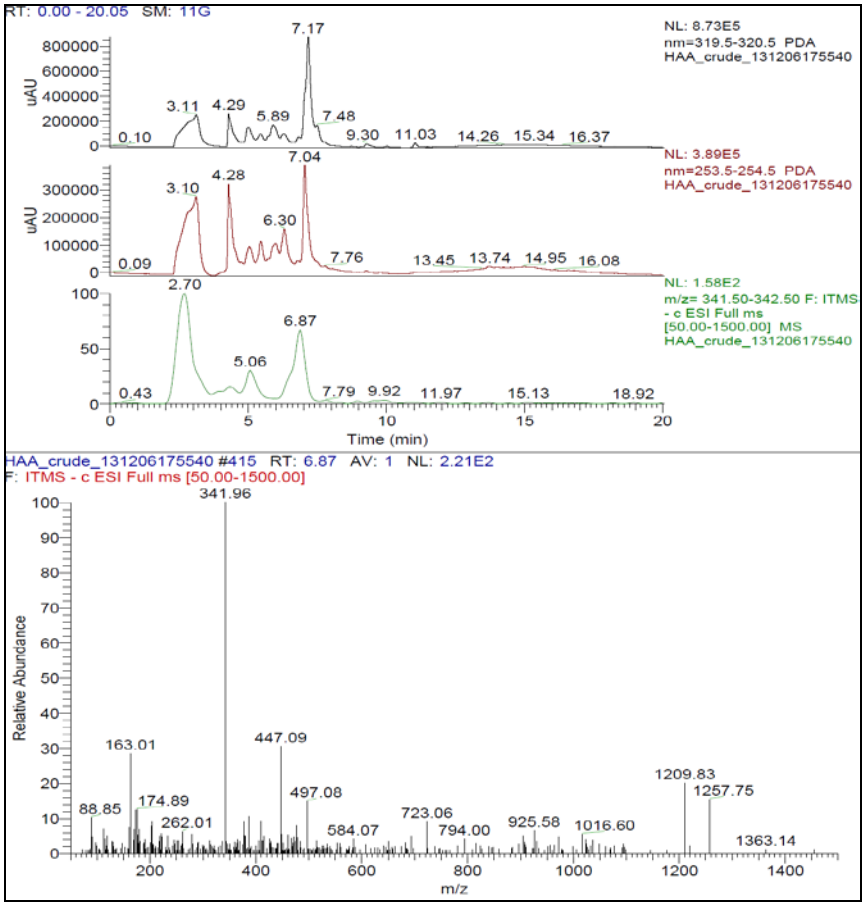

Fig. 10: TIC of the extracted m/z 342 at RT 6.87

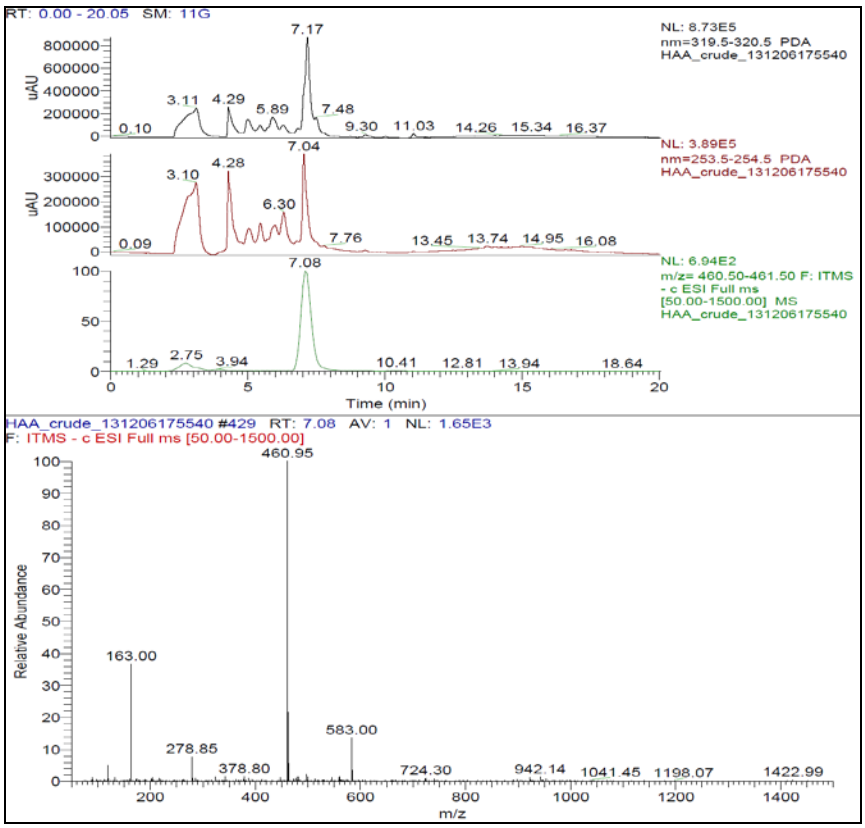

Fig. 11: TIC of the extracted m/z 461 at RT 7.08

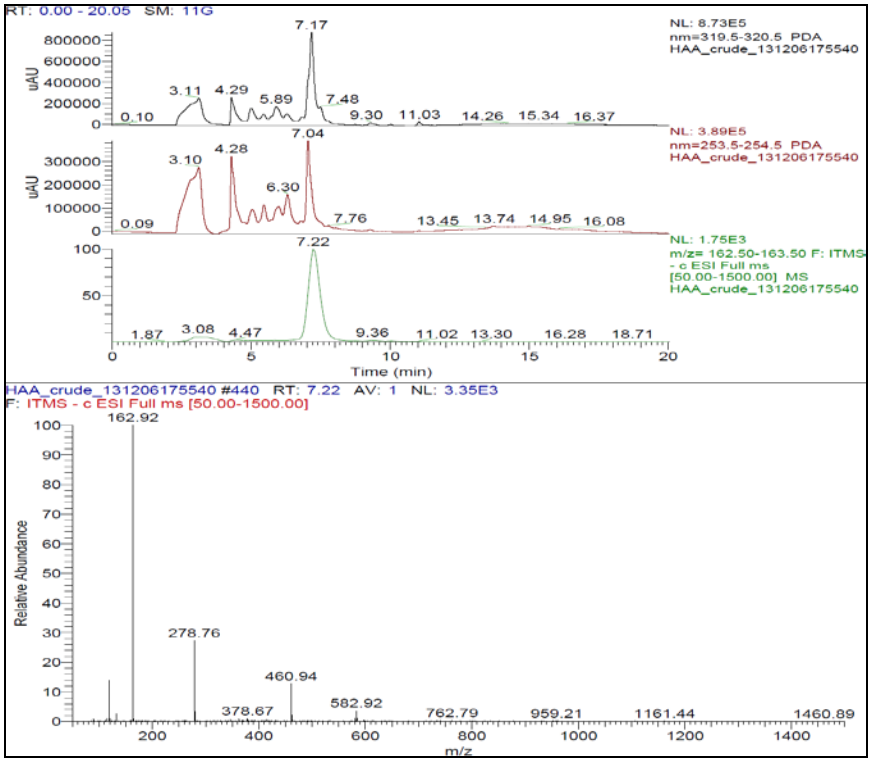

Fig. 12: TIC of the extracted m/z 163 at RT 7.22

CONFLICT OF INTERESTS

Declared None

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