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IDENTIFICATION OF POTENTIAL ANTI-CANCER LEADS FROM SIVAKARANTHAI-*SPHAERANTHUS AMARANTHOIDES*-USING TANDEM MASS AND THEIR *IN-SILICO* STUDIES

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ABSTRACT: *Sphaeranthus Amaranthoide,* commonly known as "Sivakaranthai" in Tamil. The pharmacological evaluation of this plant extract shows antibiotic, anti-inflammatory and analgesic activities, also exhibit anti-bacterial activity against Gram-positive as well as Gram-negative bacteria. This plant extract is also used by herbal practitioners towards the treatment of specific cancer. The present research has been carried out to identify the potential leads towards the treatment of cancer. As the first attempt, we identified two major compounds, Decahydro-6-(iminomethyl)-4a-methylnaphthalen-2-ol and its isomer with MW:195Da using LCMS technique from the Sivakaranthai-whole plant collected during Panguni-uthiram, a full moon day. This identification activity has been presented and published as a poster at 62nd American Society for Mass Spectrometry (ASMS) conference on Mass Spectrometry and Allied Topics held during June 15 - 19, 2014 at Baltimore, Maryland, USA. Subsequently, the identification of phytochemicals has been carried out for the Sivakaranthai whole plant collected during the other normal day and three major compounds with MW: 516Da, MW: 360Da and 374Da are identified. Further, this study has been extended to Sivakaranthai-kulithailam and the molecular masses of the four major phytoconstituents identified are MW: 390Da, MW: 254Da two isomers and MW: 315Da, reported in this research article. Induced fit docking study has been carried out for all the four identified compounds and it was found that the compound with molecular mass 516Da binds better than that of co-crystal and all other three compounds with the breast cancer target - phosphoglycerate dehydrogenase (PHGDH) (PDB ID: 5NZP).

KEYWORDS: Sivakaranthai; Sphaeranthus Amaranthoide; Phytochemical identification; Herbal medicine; Cancer treatment; Indian herb.

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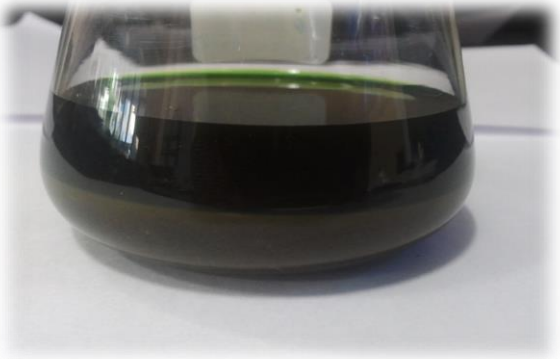
1.INTRODUCTION

The Indian Holistic rejuvenator herb [1-4], sivakaranthai and its kulithailam have been used for the treatment of various cancers by Konganar Vedic Academy and Herbal Research Centre, Palani, India. Sivakaranthai is basically a weed of paddy field of south India [5-6]. The successful outcome of the usage of the above plant prompted us to identify the phytochemical compositions behind the cure. It is believed that the sivakaranthai-whole plant collected during the 'full-moon' day has more effect in the treatment rather than when collected during the normal day. Hence, the study has been planned and HPLC, LCMS & LCMSMS analyses for the methanolic extract of sivakaranthai-whole plant collected during the 'full-moon' day were carried out and we identified two major phytoconstituents as an isomer of one another. The picture of the sivakaranthai-whole plant, sivakaranthai-whole plant (dried), sivakaranthai-whole plant (powdered) and its methanolic extract are shown in Fig.1.

Sivakaranthai whole Plant

Sivakaranthai whole Plant (dried)

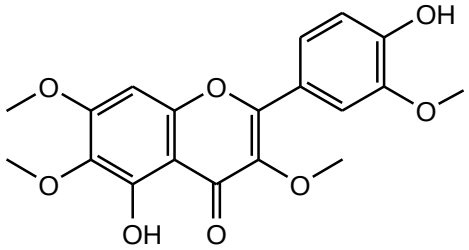
Sivakaranthai Powder



Methanolic Extract

Fig 1: The picture of sivakaranthai whole plant, dried, powdered and its methanolic extract

The methanolic extract of sivakaranthai has been subjected to HPLC analysis and we found two major peaks eluting at retention time of 36.5min and 37.7min with percentage area of 58.44% and 30.84%, respectively, under area normalization method. The identified peak has been isolated through prep-HPLC and subjected to LCMS and LCMSMS analyses and the major compounds were identified as an isomer of one another, namely, Decahydro-6-(iminomethyl)-4a-methylnaphthalen-2-ol having molecular mass as 195Da. Both the isomers are found to have identical LCMSMS fragments in nMS^2 analysis under different collision energies during MSMS fragmentation. Based on the LCMS and LCMSMS data, the structure has been identified and reported for the first time as a poster at the 62nd American Society for Mass Spectrometry (ASMS) Conference held during June 15 - 19, 2014 at Baltimore, Maryland, USA. The methanolic extract of sivakaranthai whole plant, collected in the 'normal day' has also been subjected to HPLC analysis and three major peaks with masses of MW: 516Da, MW: 360Da and MW: 374Da were identified. The peak with mass 360Da has been identified and reported as a chemopreventive agent, namely, chrysosplenol D [7] by Gayatri et al (2016). Further, the peak with MW: 374Da identified by us has the mass difference of 14 units with the reported one and this difference is due to the one additional methyl group leading to the prediction of the structure for the MW 374 to be the one shown in Fig.2.

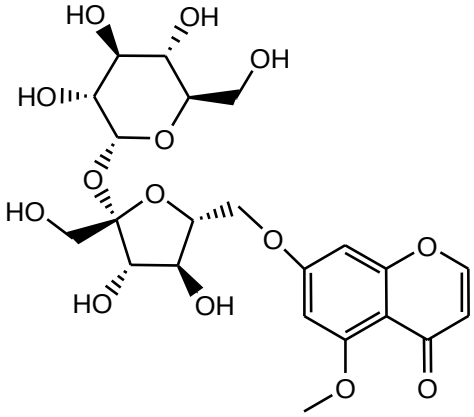


$C_{19}H_{18}O_8$
Mol. Wt.: 374 Da

5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,6,7-trimethoxy-4*H*-chromen-4-one

Fig 2: Structure of chrysosplenol D

There is also a possibility of the presence of methyl group in the 3rd and 4th position of hydroxyl group. Based on the LCMS and LCMSMS data, the structure of the compound with molecular mass 516Da is shown below.



7-(((2R,3S,4S,5S)-3,4-dihydroxy-5-(hydroxymethyl)-5-((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)tetrahydrofuran-2-yl)methoxy)-5-methoxy-4H-chromen-4-one

Fig 3: The structure of the compound with MW: 516Da

The Sivakaranthai-kulithailam has also been subjected to HPLC analysis and four major peaks were found with concentration of 39.97% at RT 4.7min, 18.59% at RT 10.6min, 7.72% at RT 14.81min and 12.20% at RT 16.7min having the molecular masses of 390Da, 315Da, 255Da and 255Da, respectively. All the four peaks were subjected to MSMS analysis and data were depicted in this article.

2. MATERIALS AND METHODS

2.1 Source of Sivakaranthai whole plant and Sivakaranthai-kuzhithailam

The sivakaranthai whole plant was collected during the special day of 'Panguni Uthiram – a full moon day' in the area of Palani Hills, Tamil Nadu by Rangasamy Siddhar, the herbal medicine practitioner. Also, the sivakaranthai plant was collected on a normal day for the study. The sivkaranthai-kuzhithailam has been prepared and provided by Rangasamy Siddhar.

2.2 HPLC, LCMS and LCMSMS methodology

The Liquid Chromatographic analysis was carried out using RPC18 column of particle size 5µm (150mm x 4.6mm) with mobile phase consisting of 0.01M Ammonium formate in water: acetonitrile with the gradient flow rate of 800µL/min. The outlet of the Shimadzu NEXERA UHPLC-PDA detector was connected to the Shimadzu LCMS-8040 Triple Quadrupole Mass Spectrometer System of APCI interface having the corona needle voltage of ±4.5kV, interface temperature 420°C, desolvation line temperature 270°C, heat block temperature 280°C. Nitrogen gas was used as nebulizer and drying gas with the flow rate of 2.80L/min and 14.00L/min, respectively. The ESI interface is also used in this research. The detection mass range was set from 50Da to 2000Da. Argon gas was used as a collision gas while carrying out the MSMS operations to obtain characteristic fragmentations of phytoconstituents of sivakaranthai.

3. RESULTS AND DISCUSSION

3.1 Identification of major phytoconstituents in Sivakaranthai collected during the Panguni Uthiram- a full moon day

The sivakaranthai plant was shade dried and made as a powder using home appliance mixer. This powder was soaked for 24Hrs in methanol as solvent. The dark green methanolic extract was subjected to the reversed phase HPLC analysis using C18 column. A close observation of LC chromatogram showed the presence of more polar constituents eluting between 24.0min and 30.0min. The total content of these constituents was found to be about 11.0% area monitored at wavelength 330nm. Two major peaks were observed as relatively non-polar constituents appearing at the retention time of 36.5min and 37.7min, respectively with a total concentration of about 89.0%area. The HPLC chromatogram of the methanolic extract is shown in Fig 4.

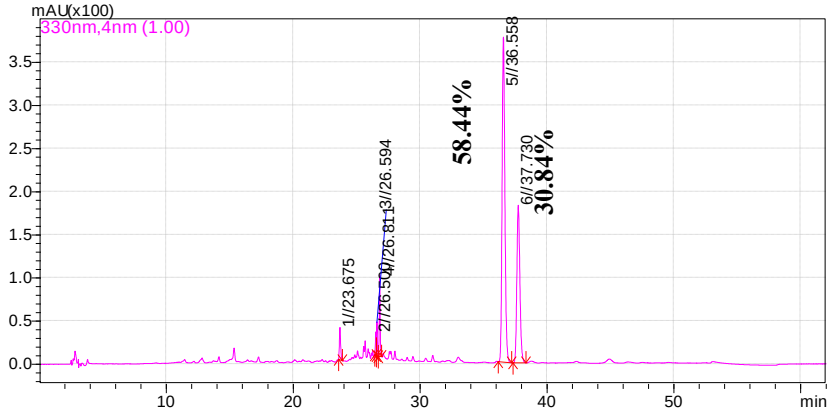


Fig 4: HPLC chromatogram of methanolic extract of sivakaranthai

The above identified peaks were isolated through preparative HPLC using Enable semi-prep C-18 (250 x 20mm, 10µm) column. The mobile phase consisting of water:methanol was used with the flow rate of 10mL/min. The typical preparative HPLC chromatogram (stacked) is shown in Fig 5. The isolated compound-1 and compound-2 were analysed in HPLC, the chromatographic purity was found to be 98.59% & 98.02% area, respectively. The HPLC chromatograms of isolated pure compound-1 and compound-2 are shown in Fig.6.

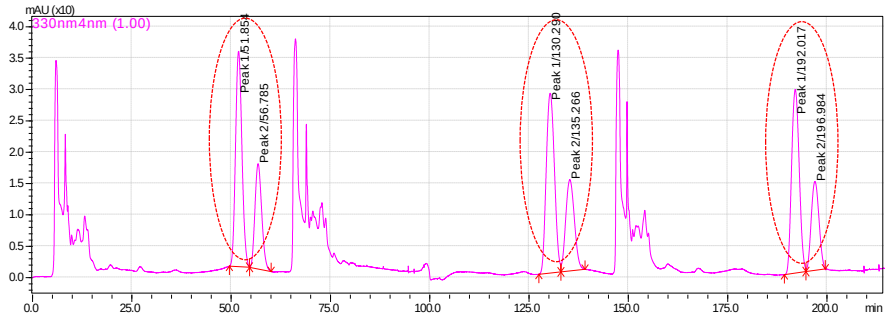
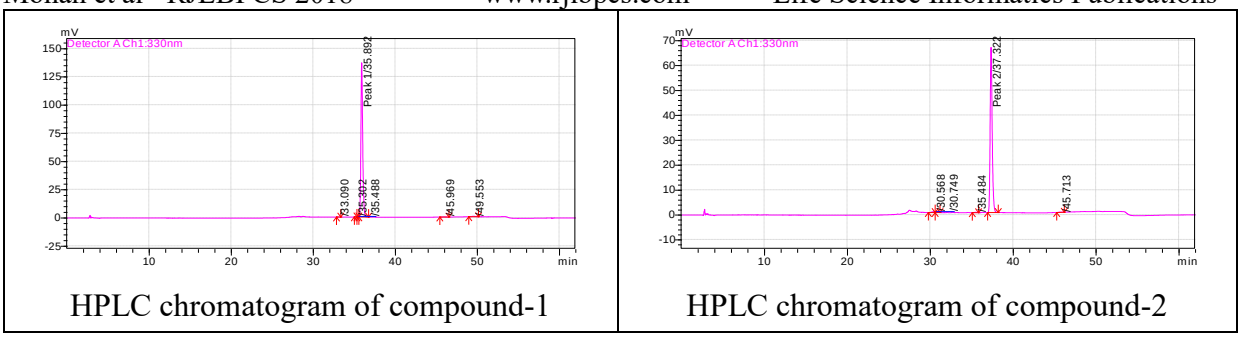


Fig.5: The preparative HPLC stacked injection chromatogram



HPLC chromatogram of compound-1 | HPLC chromatogram of compound-2

Fig.6: The HPLC chromatograms of isolated pure compound-1 and compound-2

The sivakaranthai methanolic extract was subjected to LCMSMS analysis [24-27] for the possible determination of molecular masses of the above two major constituents using both ESI & APCI techniques. Since ESI analysis did not show any interpretable mass spectrum, APCI technique was applied both in the SCAN mode as well as MSMS mode. In the SCAN mode APCI analysis, the molecular mass of both the constituents had been observed for the first time as 195Da, confirming that one peak is the isomer of the other. The mass spectrum of compound-1 and compound-2 along with its extracted ion chromatograms are shown in Fig 7 and Fig 8 respectively.

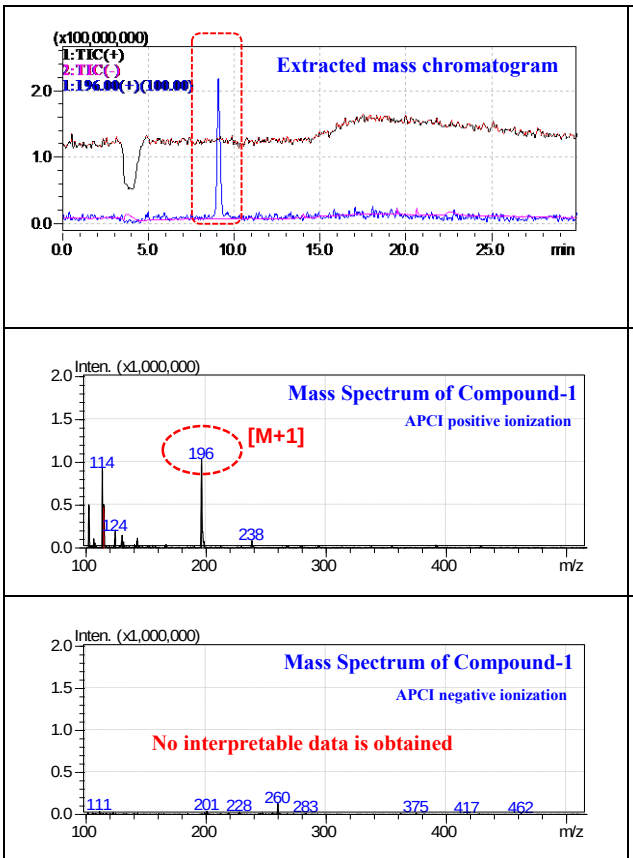


Fig.7:The Extracted TIC and Mass Spectrum of compound-1

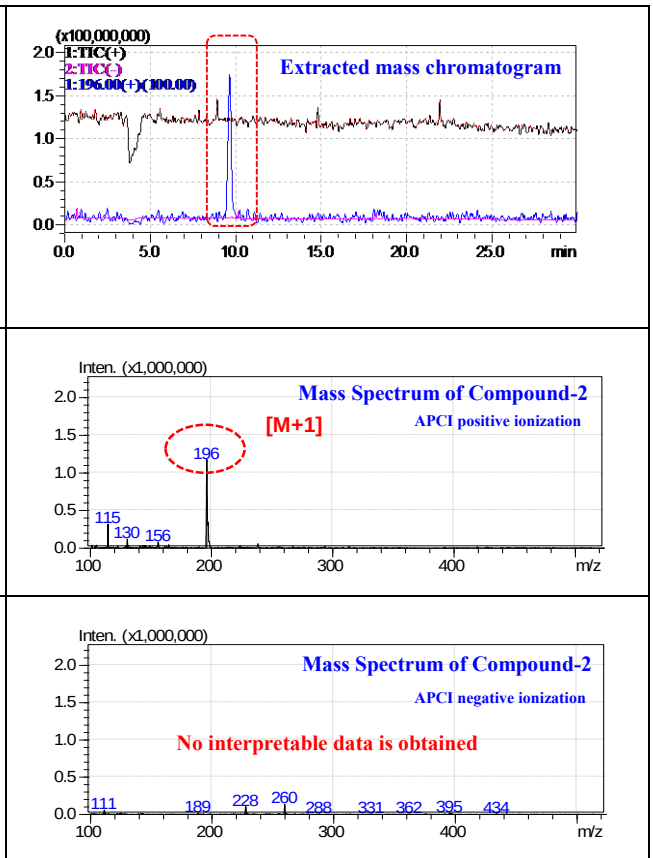


Fig.8:The Extracted TIC and Mass Spectrum of compound-2

The identified molecular ion m/z 196 of the compound-1 and compound-2 were subjected to the MSMS analysis [32-33] in APCI positive ionization mode under different collision energies to obtain the nMS^2 data. The major fragments at m/z169, m/z151, m/z137 and m/z126, are identical for both the compounds (shown in Fig 9 and Fig 10)

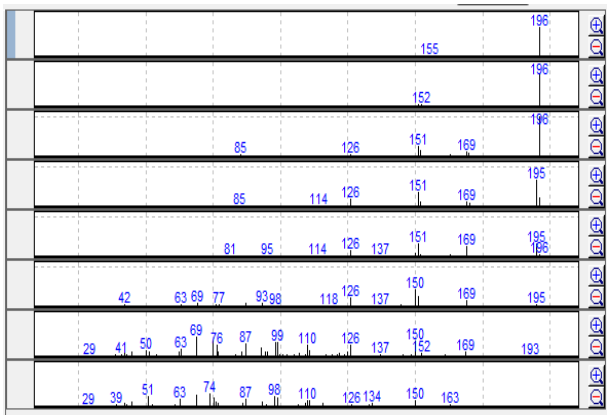


Fig 9. The MSMS spectrum of compound-1 in APCI positive ionization mode

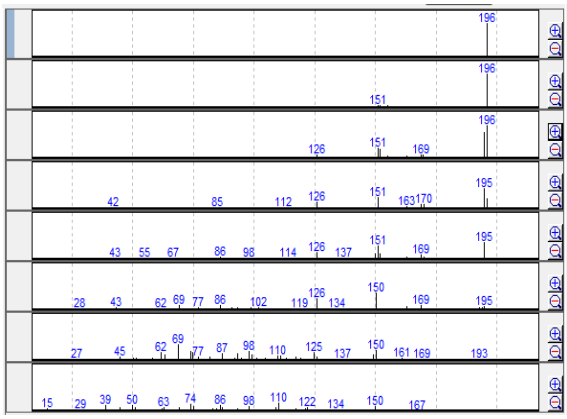
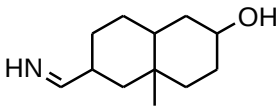


Fig 10. The MSMS spectrum of compound-2 in APCI positive ionization mode

The identified major possible phytoconstituents in sivakaranthai is Decahydro-6-(iminomethyl)-4a-methylnaphthalen-2-ol, whose structure is shown in Fig.11.



Mol. Mass: 195Da

Fig.11: The structure of Decahydro-6-(iminomethyl)-4a-methylnaphthalen-2-ol

The proposed MSMS fragmentation pathway is shown in Fig.12.

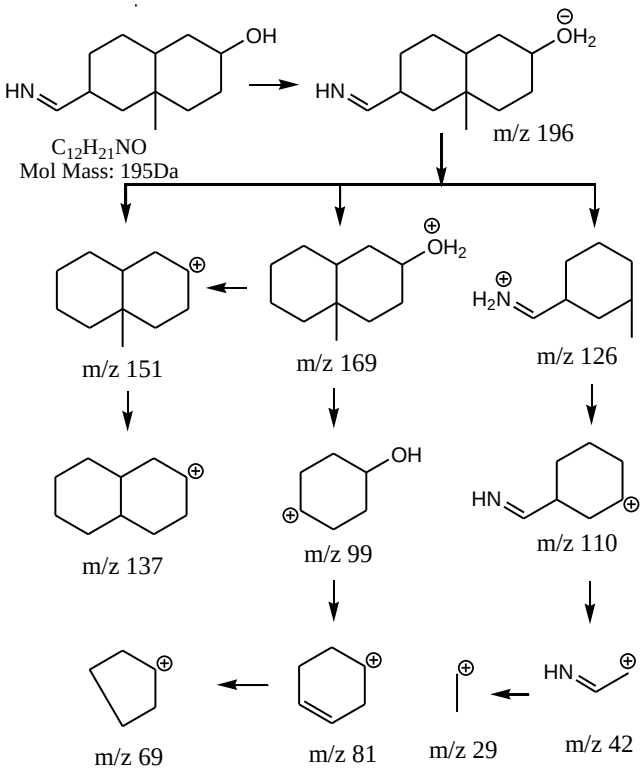


Fig.12: The proposed MSMS fragmentation pathway of compound-1 & compound-2

The isomeric nature of the isolated compound-1 and compound-2 has the same MSMS fragmentation pathway. The protonated hydroxyl group of the compound leads to the formation of the quasi-molecular ion m/z 196. The loss of CN from the ion m/z 196 produces the ion m/z 169; further loss of H_2O gives the ion m/z 151. The loss of CH3 from the ion m/z 151 produces the ion m/z 137. Further fragmentation of the major ions produce the ion m/z 110, m/z 99, m/s 81, m/z 69, m/z 42 and m/z 29. The fragmentation pattern of the predicted structure has been well explained for its confirmation.

3.2 Identification of major phytoconstituents in sivakaranthai collected during the normal day

The sivakaranthai whole plant collected during the normal day has been shade dried and made as a powder using home appliance mixer. This powder was soaked for 24Hrs in methanol as solvent. The dark green methanolic extract was subjected to the reversed phase HPLC analysis using C18 column. The HPLC chromatogram is shown below in Fig.13

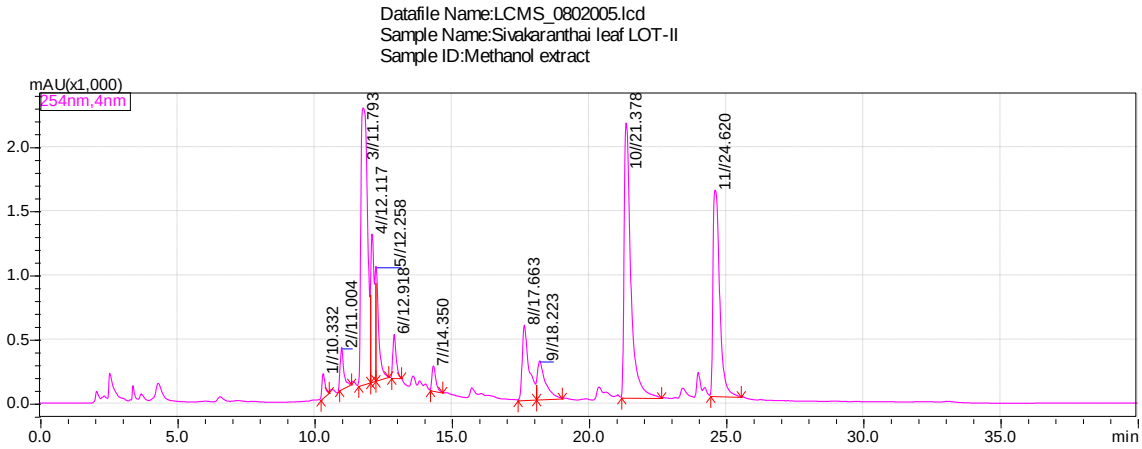
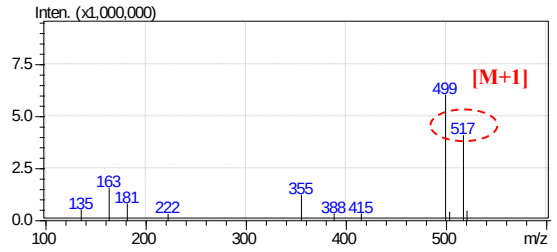


Fig.13: The HPLC chromatogram of methanolic extract of sivakaranthai collected during normal day

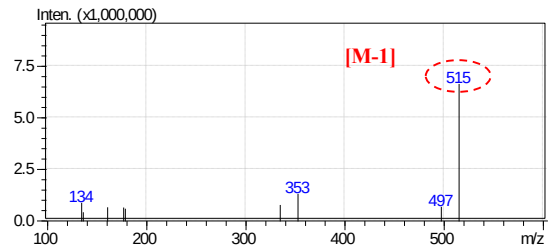
The HPLC analysis showed three major peaks at RT 11.79min, 21.38min and 24.62min. The same extract has been subjected to LCMS analysis and we identified the molecular masses as 516Da for peak RT 11.79min, 360Da for peak RT 21.38 and 374Da for peak RT 24.62. The identified molecular masses have been subjected to LCMSMS analysis under different collision energies in ESI positive ionization mode to obtain nMS^2 stable fragments. The mass spectral data and the proposed MSMS fragments are shown in fig.14 for peak RT 11.79min, Fig.15 for peak at RT 21.38min and Fig.16 for peak at RT 24.62min

The Mass spectra of peak RT 11.79min

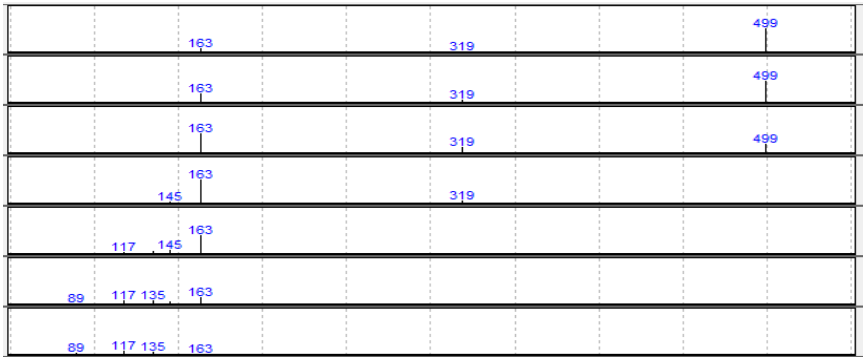
ESI Positive ion mass spectrum



ESI Negative ion mass spectrum



The MSMS spectra of m/z 517 under different collision energies



The molecular ion m/z517 is highly fragile in nature and produced the stable product ion of m/z499 and further fragments.

The proposed MSMS fragmentation pathway is

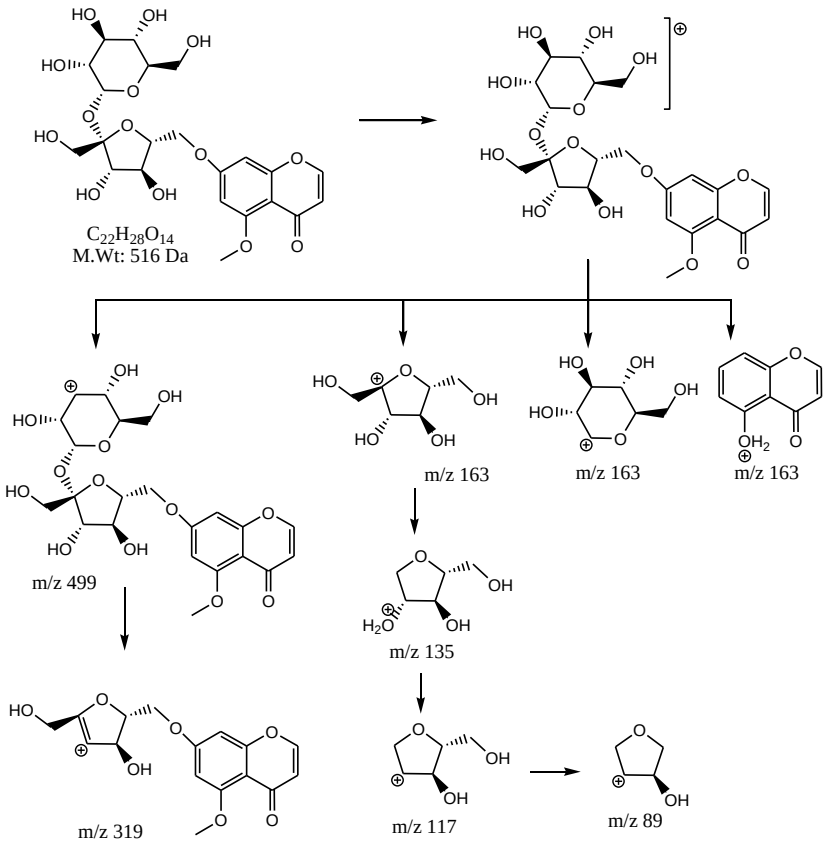
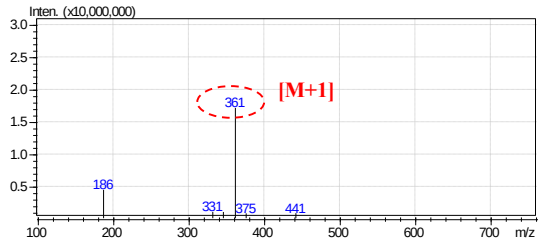


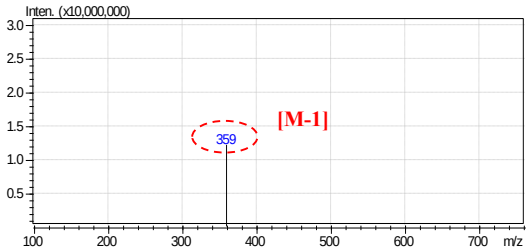
Fig.14: The MS and MSMS Spectra and its proposed fragmentation pathway of peak RT 11.79min

The Mass spectra of peak RT 21.38min

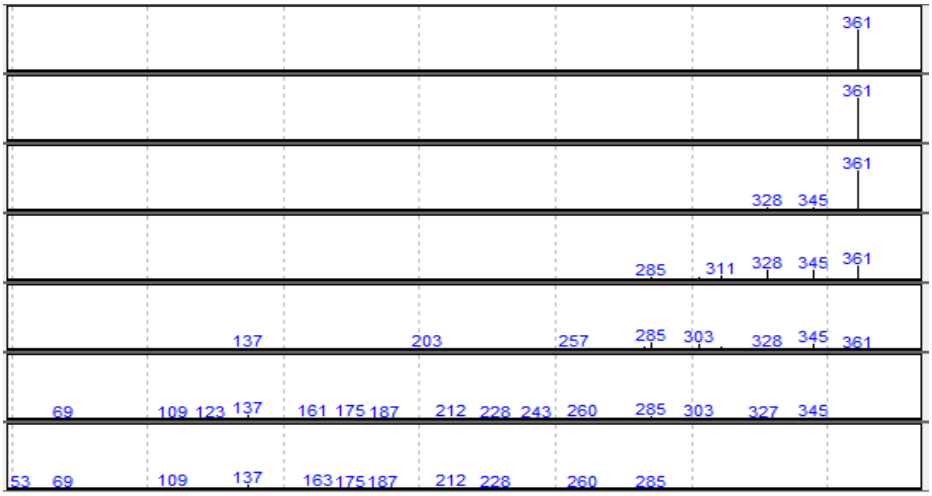
ESI Positive ion mass spectrum



ESI Negative ion mass spectrum



The MSMS spectra of m/z 361 under different collision energies



The proposed MSMS fragmentation pathway is

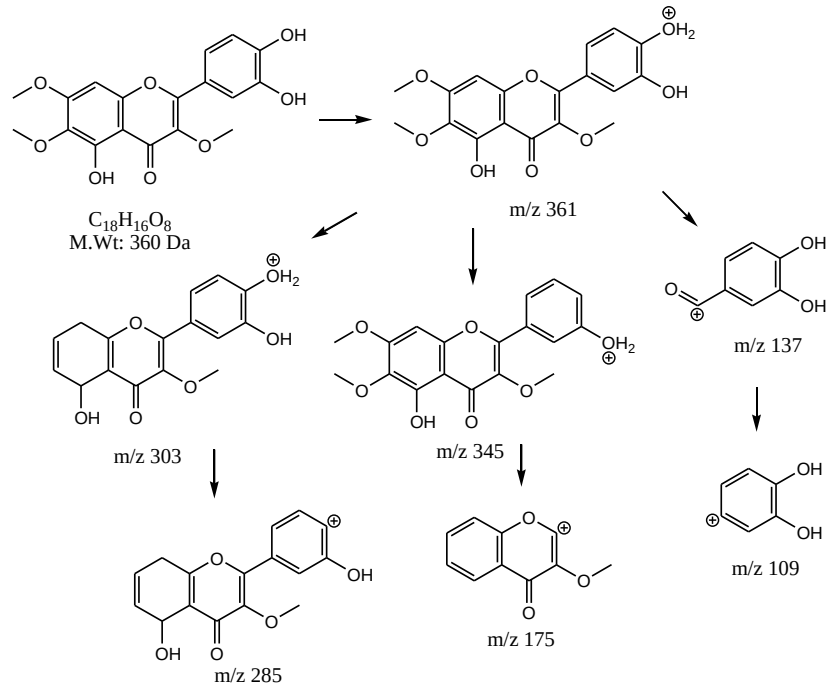
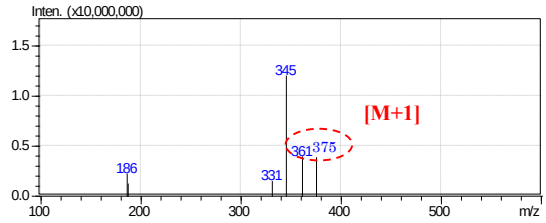


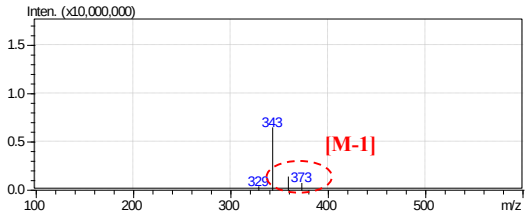
Fig.15: The MS and MSMS Spectra and its proposed fragmentation pathway of peak RT 21.38min

The Mass spectra of peak RT 24.62min

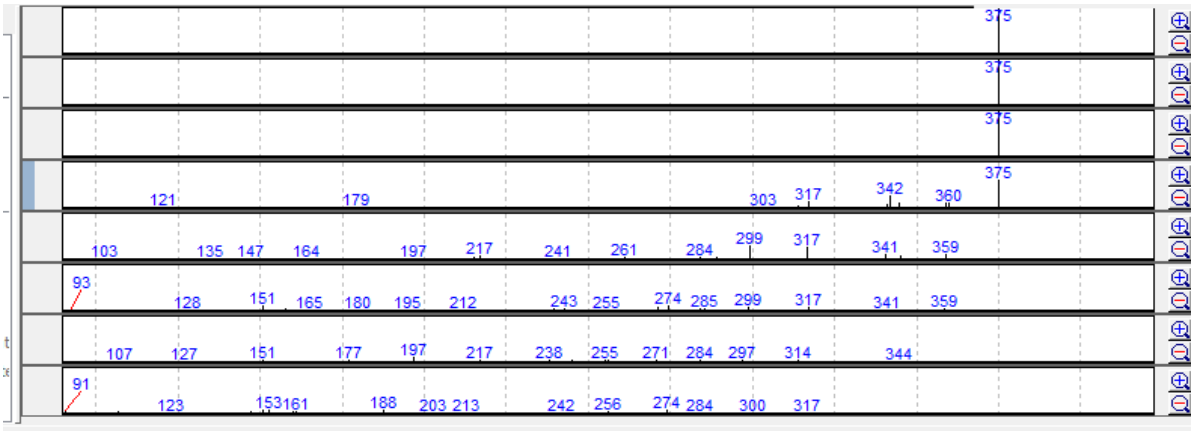
ESI Positive ion mass spectrum



ESI Negative ion mass spectrum



The MSMS spectra of m/z 375 under different collision energies



The proposed MSMS fragmentation pathway is

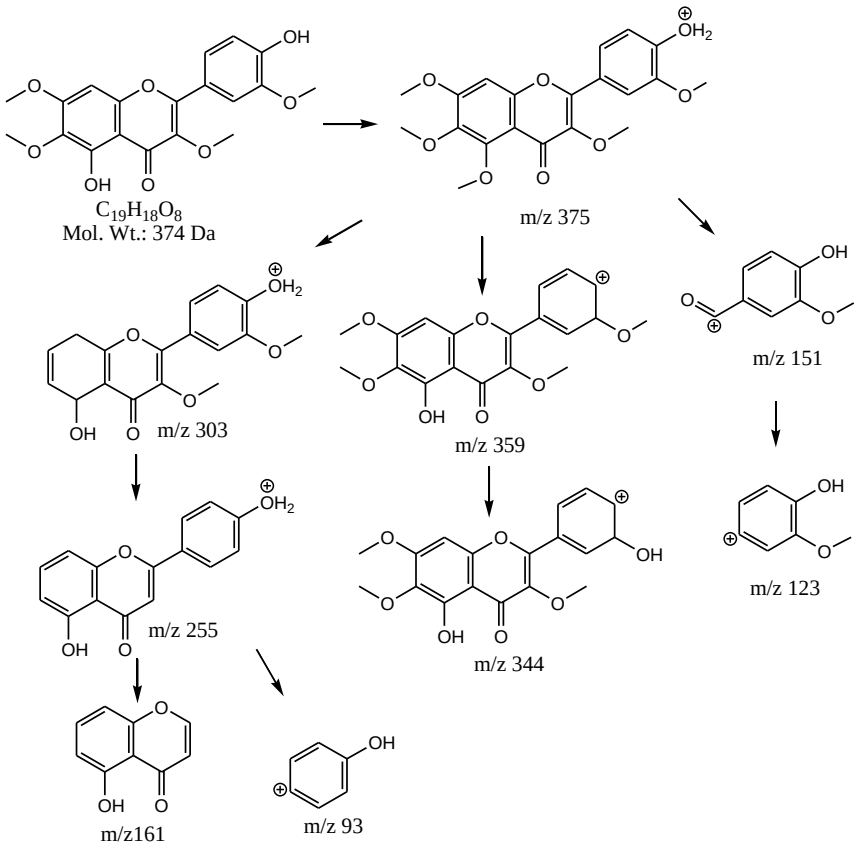


Fig.16: The MS and MSMS Spectra and its proposed fragmentation pathway of peak RT 24.62min

Fig.17 shows the structural confirmation of the newly identified compound with the molecular mass 374Da through MSMS fragments

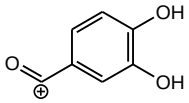
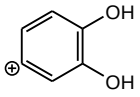
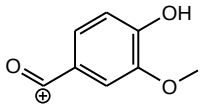
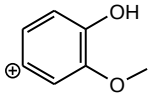
Marker MSMS fragments from the ion m/z 360	Marker MSMS fragments from the ion m/z 375
 m/z 137  m/z 109	 m/z 151   m/z 123

Fig.17: The MSMS marker fragments which distinguishes the structure of the mass 360Da and 374Da

The above marker fragments propose the methyl group present in the 3rd Hydroxyl or 4th Hydroxyl group attached with the phenyl ring, which is confirmed by the addition of 14 units in the marker fragment of m/z 137 + 14 = m/z 151 and m/z 109 + 14 = m/z 123.

3.3 Identification of major phytoconstituents in sivakaranthai kulithailam

The appearance of the sivakaranthai-kulithailam is a dark brown colour oily liquid. Since this is an oily material, combinations of solvents were used for its complete dissolution (MDC:Hexane:Ethanol:Formic acid (77:20:1:2)). A drop of oil was dissolved in 1 mL of composite solvents and subjected to HPLC analysis under Normal Phase chromatography [28-31]. The HPLC chromatogram is shown in Fig.18 and the percentage area of the phytoconstituents are shown in the Table-1.

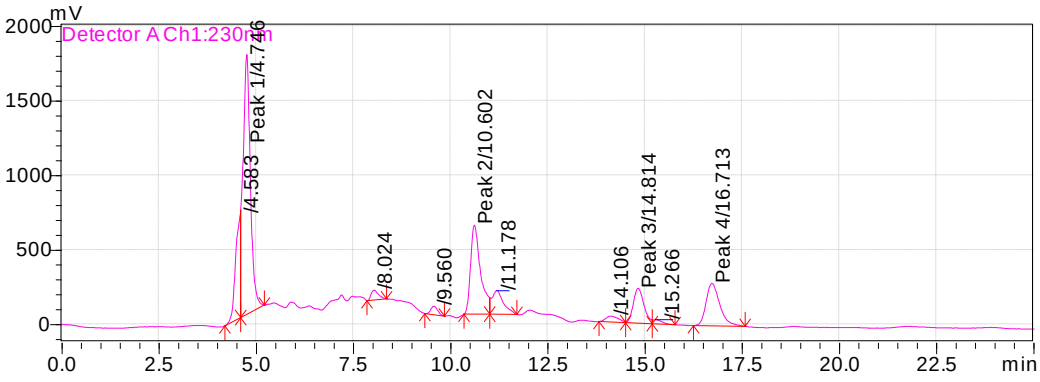


Fig.18: HPLC chromatogram (Normal Phase) of sivakaranthai-kulithailam

PEAK TABLE
Detector A Ch1 230nm

Peak #	Ret. Time	Area	Area %	Name
1	4.58	6161997	10.71	
2	4.75	22994594	39.97	Peak 1
3	8.02	874084	1.52	
4	9.56	688477	1.20	
5	10.60	10691868	18.59	Peak 2
6	11.18	3305539	5.75	
7	14.11	895520	1.56	
8	14.81	4440431	7.72	Peak 3
9	15.27	456431	0.79	
10	16.71	7019015	12.20	Peak 4
Total		57527956	100.00	

Table 1: The percentage of the phytoconstituents of Sivakaranthai-kulithailam

Since the Normal Phase chromatography technique will not be compatible for the LCMS analysis, we have made an attempt and isolated the four compounds through preparative HPLC using the combinations of solvents in the mobile phase. The Inertsil-Silica preparative column with 5.0mL/min flow rate has been used in the Shimadzu Preparative HPLC and we isolated few mg of all the four peaks. The preparative HPLC chromatogram is shown in Fig.19

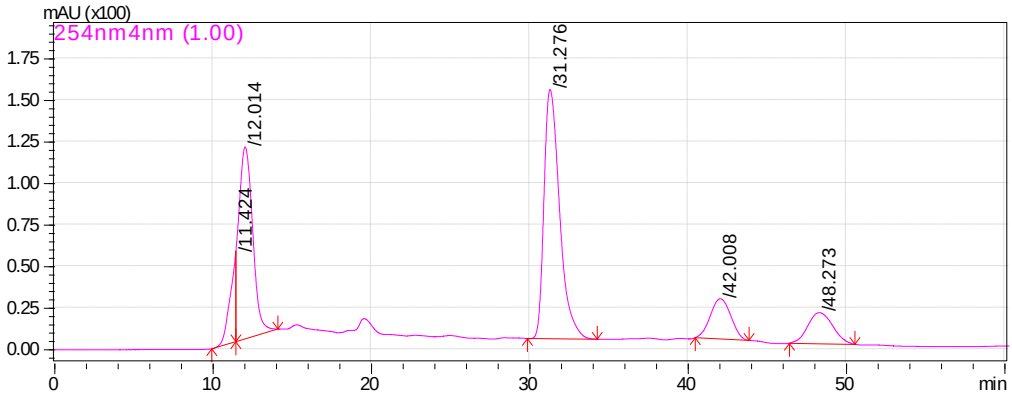


Fig.19: The prep-HPLC chromatogram of Sivakaranthai-kulithailam

The isolated peaks were analysed in HPLC to know the purity of the peaks. The isolated peaks were subjected to LCMS analysis and we identified the molecular masses as 390Da for peak at RT 4.7min, 315Da for peak at RT 10.6min, 256Da for peak at RT 14.8min and 256Da for peak at RT 16.7min. The corresponding HPLC purity chromatogram, Mass spectrum and MSMS data are given in the Fig.20, Fig.21, Fig.22 and Fig.23, respectively.

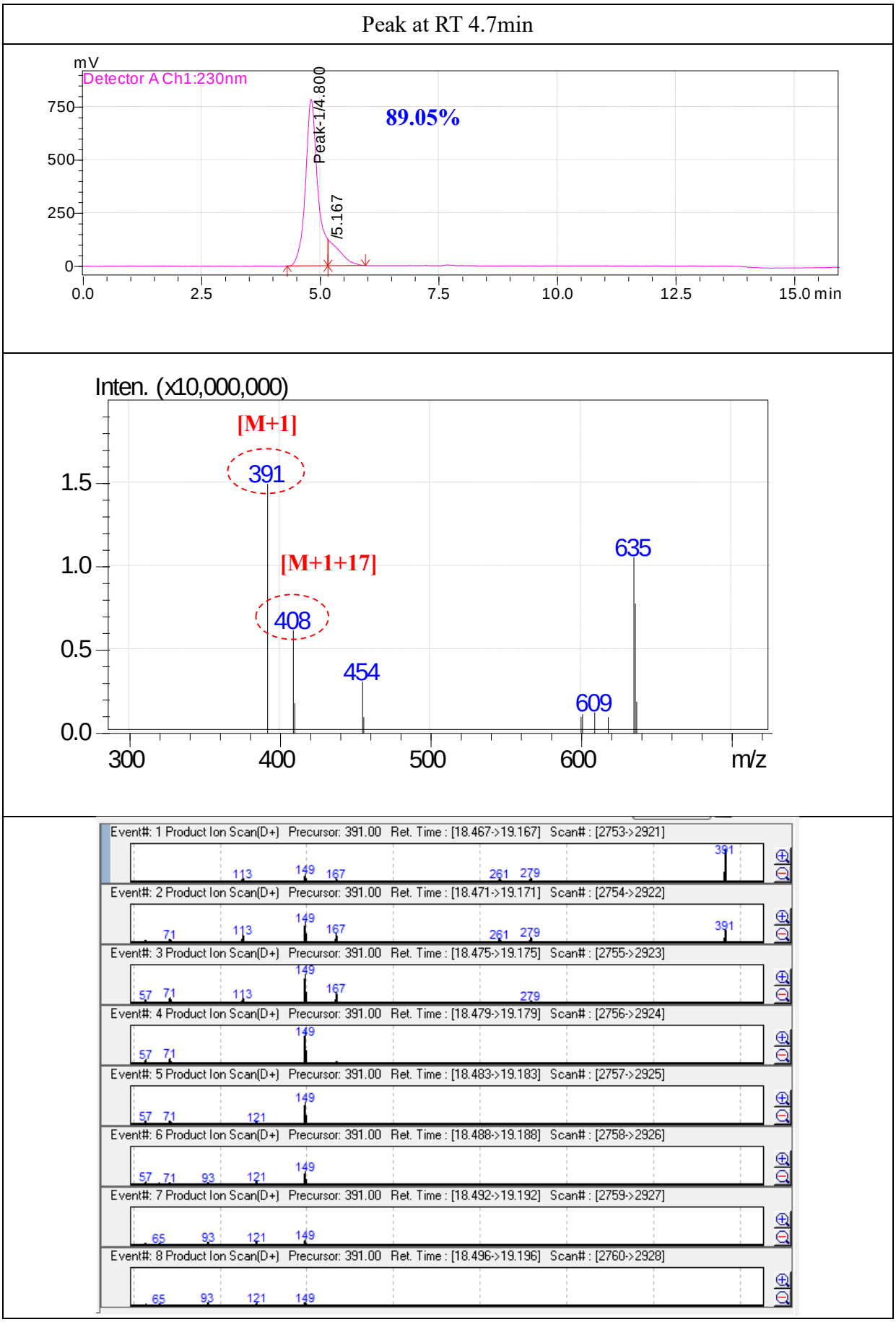
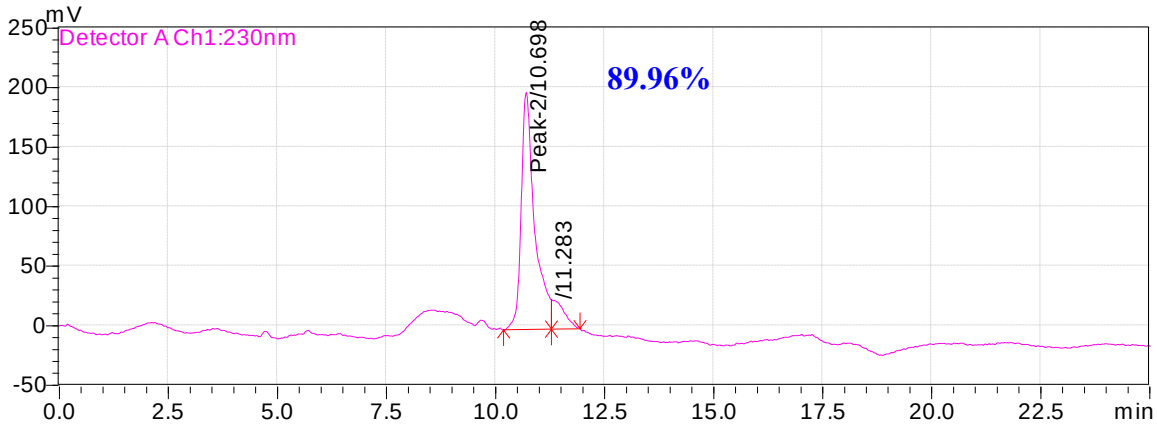
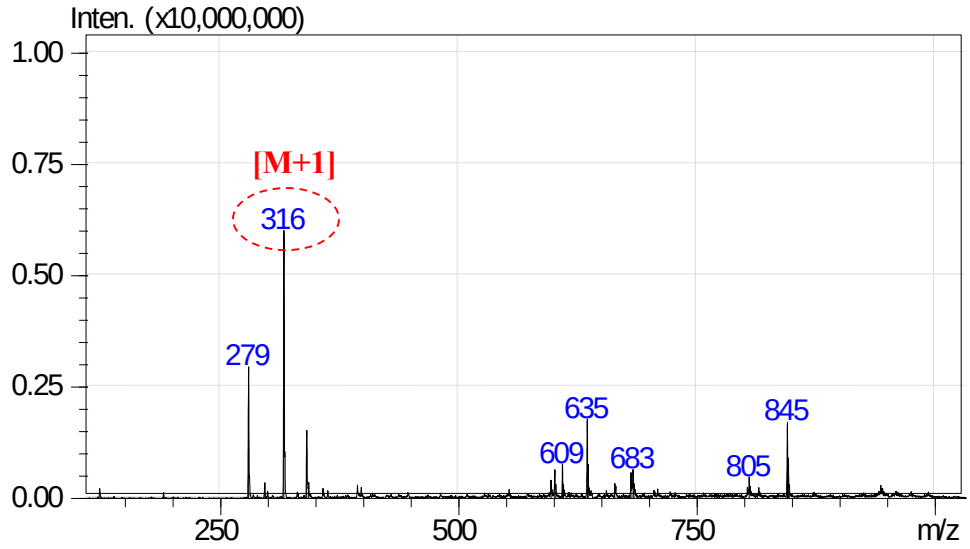


Fig.20: The HPLC purity chromatogram, Mass spectrum and MSMS data of the peak RT 4.7min

Peak at RT 10.6min





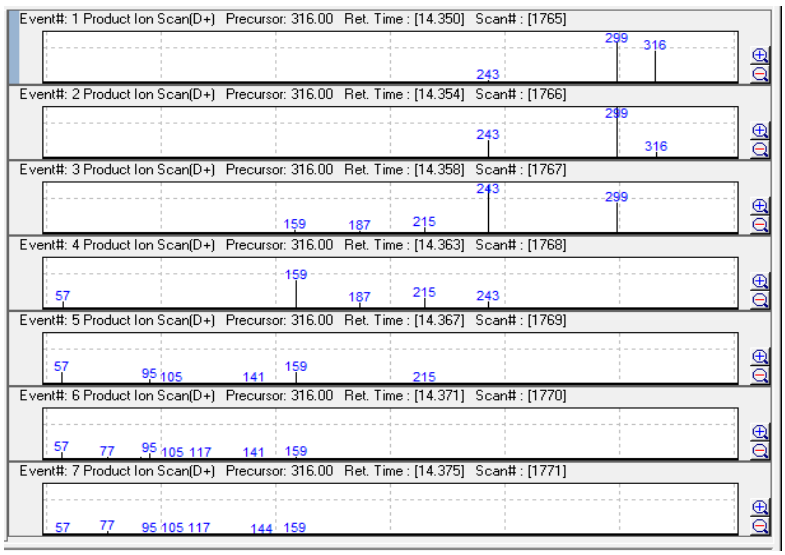


Fig.21: The HPLC purity chromatogram, Mass spectrum and MSMS data of the peak RT 10.6min

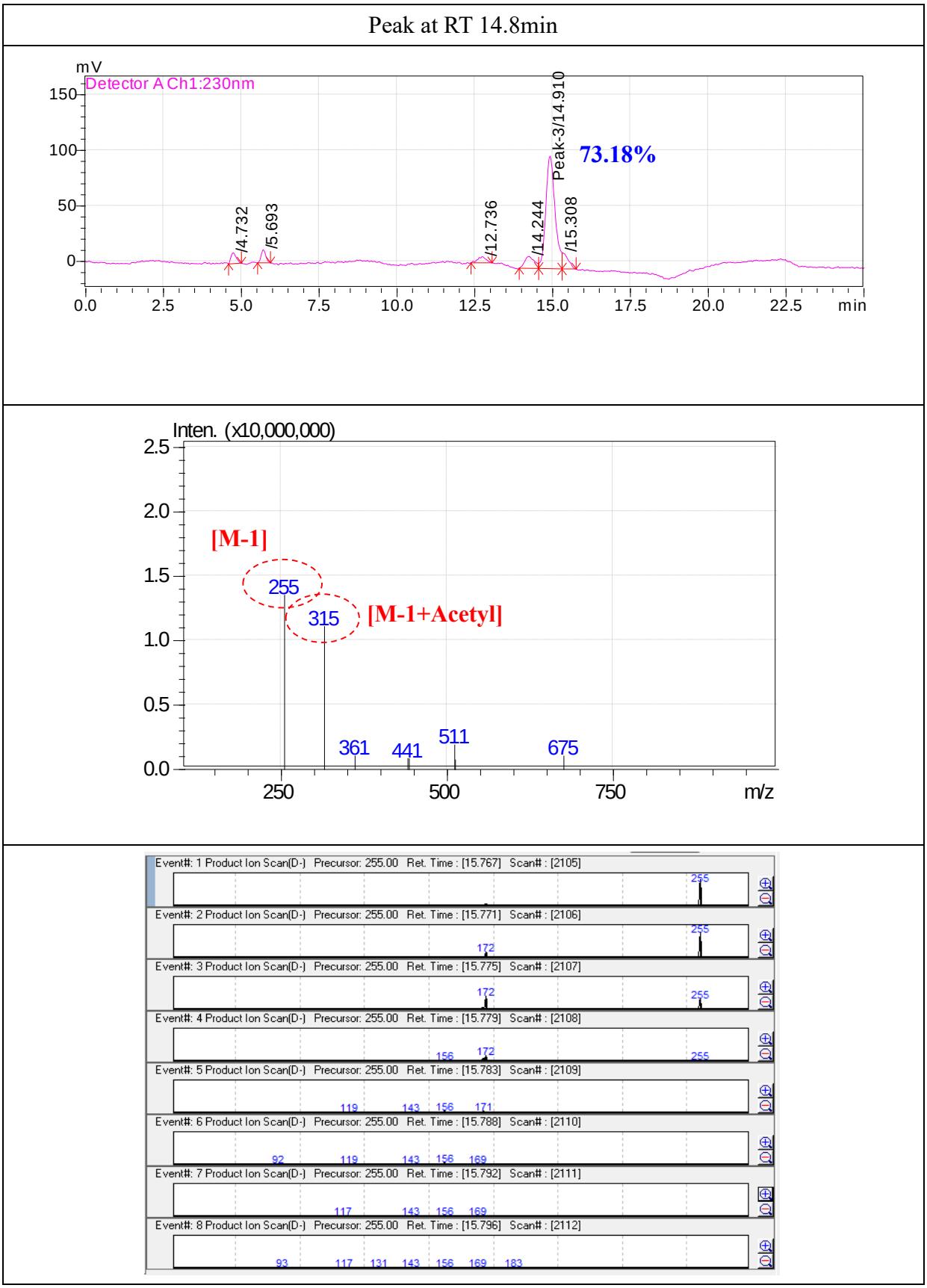


Fig.22: The HPLC purity chromatogram, Mass spectrum and MSMS data of the peak RT 14.8min

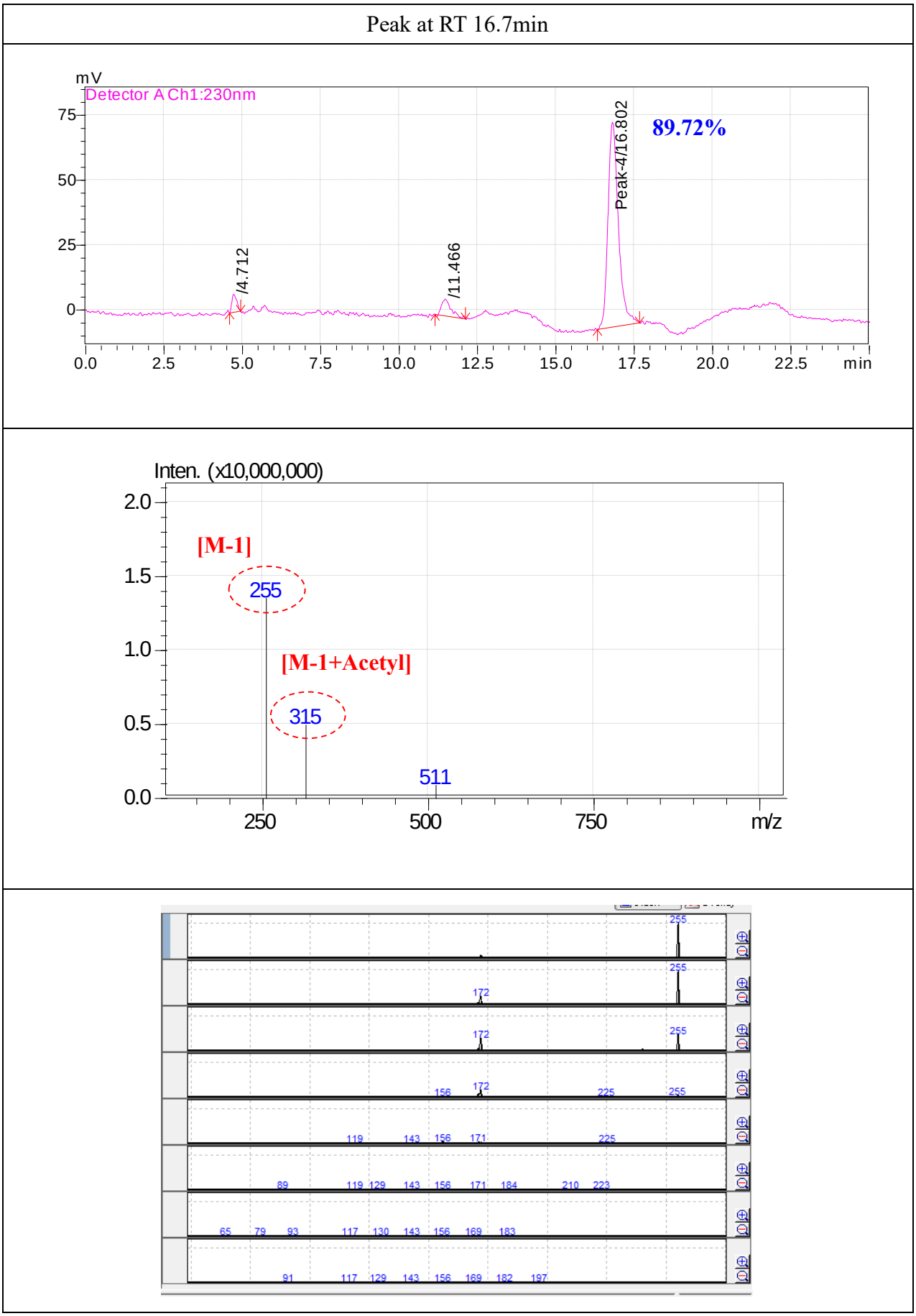
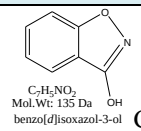
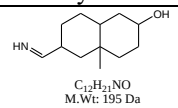
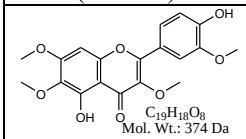
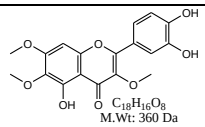
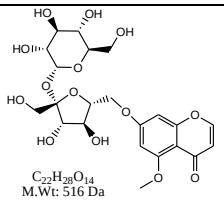


Fig.23: The HPLC purity chromatogram, Mass spectrum and MSMS data of the peak RT 16.7min

3.3 Induced Fit Docking of the identified compounds against Breast Cancer target - Phosphoglycerate dehydrogenase (PHGDH) (PDB ID – 5NZP)

The identified compounds from sivakaranthai [compound-1 and 2 (isomers): Decahydro-6-(iminomethyl)-4a-methylnaphthalen-2-ol,compound-3:5-hydroxy-2-(3,4-dihydroxyphenyl)-3,6,7-trimethoxy-4H-chromen-4-one, compound-4: 5-hydroxy-2-(4-hydroxy-3-methoxy phenyl)-3,6,7-trimethoxy-4H-chromen-4-one and compound-5:7-(((2R,3S,4S,5S)-3,4-dihydroxy- 5-(hydroxymethyl)-5-((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)tetrahydrofuran-2-yl)methoxy)-5-methoxy-4H-chromen-4-one have been subjected to molecular docking in induced fit mode with the breast cancer target Phosphoglycerate dehydrogenase (PHGDH) PDB ID: 5NZP to understand the mechanistic pathway of interaction between the target protein and ligand. The Glide program of commercial Schrodinger suite (Schrodinger LLC, USA 2014) [8] was used. The values of docking scores and Glide energies are listed in Table-2. The *in-silico* model study shows that the identified compound with molecular mas 516 Da (compound-5) is found to be better than that of the co-crystal.

Table 2: The results of induced fit docking

Ligand / ID	Docking score	Glide Energy (kcal/mol)	Interaction	Distance Å D…A	Hydrophobic Interaction
 $C_7H_5NO_2$ Mol.Wt: 135 Da benzo[*d*]isoxazol-3-ol Co-crystal	-6.256	-26.262	Asp175(O-H...O)	3.15	Leu151, Leu153, Tyr174, Pro176, Ile177, Pro208, Leu210 and Leu216
 $C_{12}H_{21}NO$ M.Wt: 195 Da 1 & 2 (isomers)	-7.858	-35.680	Thr207(O-H…N) (O-H…O)Ser212	3.11 2.86	Leu151, Leu153, Tyr174, Pro176, Ile177, Leu193, Pro208 and Leu216
 $C_{19}H_{18}O_8$ Mol. Wt.: 374 Da 3	-6.061	-45.305	Ser212(O-H...O)	2.86	Leu151, Leu153, Tyr174, Pro176, Ile177, Leu193, Pro208, Leu210 and Leu216
 $C_{18}H_{16}O_8$ M.Wt: 360 Da 4	-6.209	-43.285	Ser212(O-H...O) (O-H...O)Ser212	3.06 2.94	Leu151, Leu153, Tyr174, Pro176, Ile177, Leu193, Pro208, Leu210 and Leu216
 $C_{22}H_{28}O_{14}$ M.Wt: 516 Da 5	-7.361	-58.523	(O-H...N)Glu194 (O-H...O)Glu194 Trp197(O-H...O) (O-H...O)Ser212 Ser212(O-H...O)	3.12 3.06 2.87 3.01 3.11	Leu151, Tyr174, Pro176, Ile177, Pro192, Leu193, Trp197, Pro208, Leu210 and Leu216

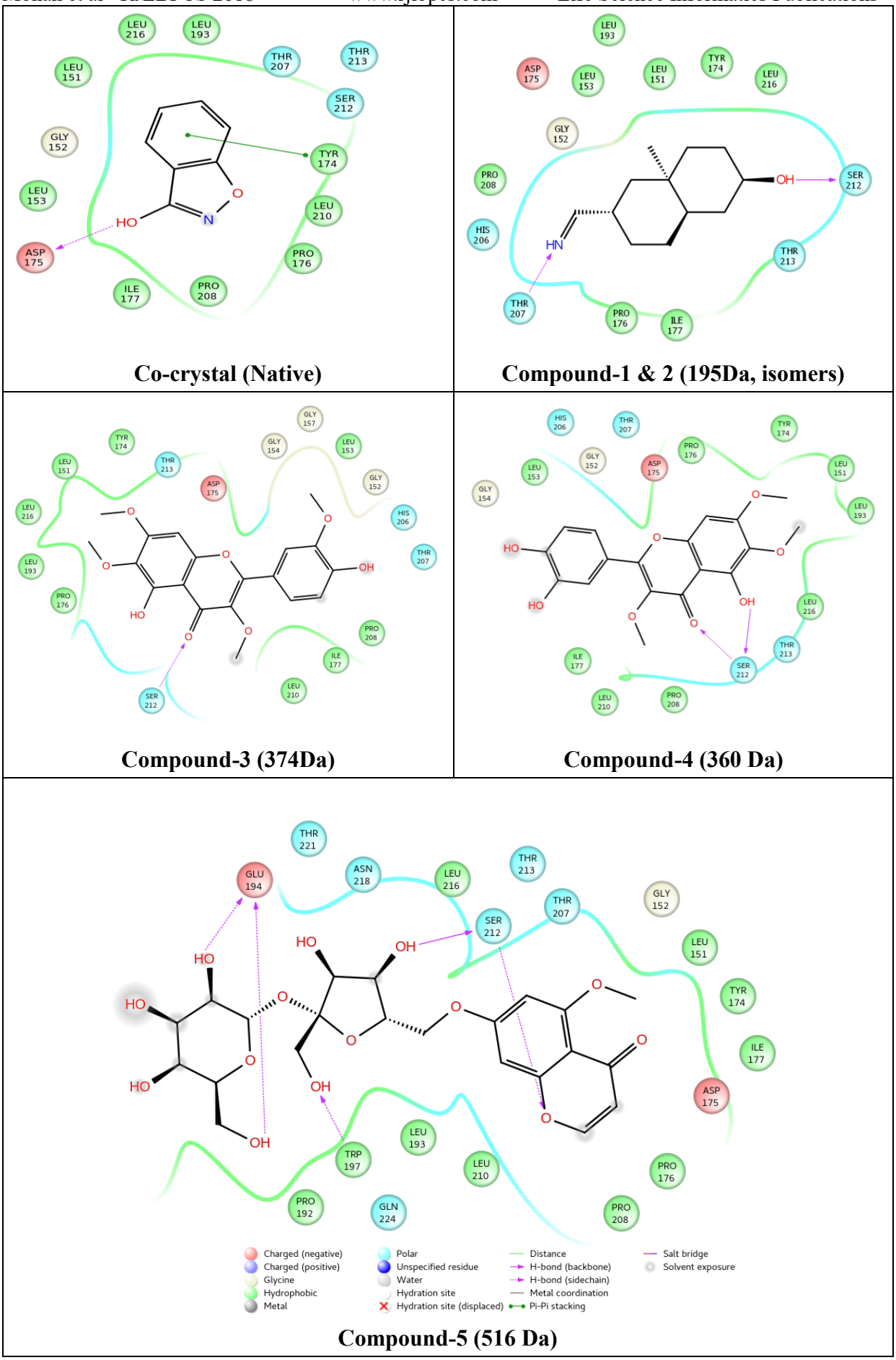


Fig.24: The ligand interaction diagram of the identified compounds

Fig.24, the ligplot diagrams show the active site residues involved in hydrogen bonds and hydrophobic interactions (green spheres). Most of the hydrophobic interactions of the co-crystal (Benzo[d]isoxazol-3-ol, anti-breast cancer drug) are maintained in all the four ligands as hydrophobic interactions suggesting the anti-breast cancer nature of the herb sivakaranthai [9-23].

4. CONCLUSION

This research article confirms that the Sivakaranthai-an Indian Rejuvenator herb has the potential anti-cancer leads, which we have identified and characterized through LCMS, LCMSMS (Tandem Mass Spectrometry) study data. The *in-silico* study confirms that the identified compounds show similar hydrophobic interactions as that of the co-crystal complex used from the PDB ID: 5NZP of breast cancer as the target. Based on the Glide energy, the compound-5 of molecular mass 516Da is found to be the better inhibitor than that of the other ones.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this research study.

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