Bioassay- and liquid chromatography/mass spectrometry-guided acetylcholinesterase inhibitors from *Picriafel-terrae*

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ABSTRACT

Background: Picria fel-terrae is a traditional Chinese medicine. **Materials and Methods:** A new approach to the search for acetylcholinesterase (AChE) inhibitors from Picria fel-terrae is presented. **Results:** Bioassay- and LC-MS-guided fractionation of the ethyl acetate extract was from traditional Chinese medicine P.fel-terrae. Following primary extraction, the ethyl acetate extracts fraction of P.fel-terrae showed strong AChE inhibitory activities. So the sample was separated using highperformance liquid chromatography (HPLC). The effluent was split towards two identical 96-well fraction collectors, and the presence of the biologically interesting portion and chromatographic fractions could be readily detected by analyzing selected ion chromatograms through an electrophoresis-electrospray ionization mass spectrometry (ESIMS) system for accurate mass measurement. One 96-well plate was used for a bioassay (AChE-inhibitory assay) and detected the bioactivity and position of the relevant peak in the chromatogram. The positive well in the second 96-well plate was used for identification by LC-(+) ESIMS. **Conclusion:** As abovementioned, the AChE inhibitory constituents from P.fel-terrae by LC-bioassay-ESIMS were rapid identified. Liquid chromatography/ mass spectrometry (LC-MS) screening detected the presence of six active compounds, identified as picfeltarraenin IA (1), picfeltarraenin IB (2), picfeltarraenin IV (3), picfeltarraenin X (4), picfeltarraenin XI (5), and one unknown compound. The structures were further determined by 13C NMR. The six compounds expressed stronger AChE inhibition than the known AChE inhibitorTacrine. Above all, the value of this LC-bioassay-ESIMS methodology is highlighted by the finding and structure elucidation of the active constituents from many other structural families of natural products..

Keywords: Acetylcholinesterase inhibitory activity,LC-bioassay-ESIMS, *Picriafel-terrae*

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder with impairment of cognitive function and personality. Current pharmacological strategies, aimed at increasing cholinergic activity include acetylcholinesterase (AChE) inhibitors, cholinergic agonists, acetylcholine (ACh) releasers and stimulants of nerve growth factors. AChE inhibitors, physostigmine and Tacrine can slow the decline of cognitive function and memory in some patients with mild or moderate AD. But they had some disadvantages, such as peripheral cholinergic hyperactivity and liver toxicity with Tacrine.[1]

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Plant extracts are widely used as pesticides and in folk medicine in tropical and subtropical areas. The vast history of experience of traditional Chinese medicine with medicinal plants may facilitate the identification of new bioactive compounds. In order to study possible applications of extracts or compounds derived from extracts, methods to screen for biological activity, and separation techniques to isolate the active principles have to be established. Scientists found the combination of separation methods with bioassays and spectroscopic techniques such as NMR and MS to be a very attractive tool.[2] Highperformance liquid chromatography/ mass spectrometry (LC-MS) was considered as the method of choice because of its sensitivity and selectivity in comparison to wavelength-specific UV detection. The method of LC-MS and LC-MS^n usually is used in finding new compounds, analyzing complex components from

nature, and drug analysis, etc.

In this study, we reported on LC-MS analyses of the extracts, isolation of bioactive compounds *via* bioassay-guided separation techniques including LC-bioassays, and structure determination by MS of the bioactive principles obtained from *Picriafel-terrae* Lour (*P.fel-terrae*). *P. fel-terrae* is an annual plant mainly distributed in southern China, used as a folk medicine for the treatment of herpes infections, cancer and inflammation.[3,4] The plant material was collected in Guangxi and successively extracted with ethanol. And the ethanol extract was extracted with petrol ether and ethyl acetate. Acetylcholinesterase (AChE) inhibitory bioassay coupled HPLC of the ethyl acetate extract gave six compounds, including picfeltarraenin IA (**1**), picfeltarraenin IB (**2**), picfeltarraenin IV (**3**), picfeltarraenin X (**4**), picfeltarraenin XI (**5**), and one unknown compounds. They expressed stronger AChE inhibition than Tacrine, which was a known AChE inhibitor.

MATERIALS AND METHODS

Apparatus and chemical reagents

Over 100,000 analytical balances 1712 were purchased from Sartorius, Germany. HPLC system with LC-10ATvp pump and SPD-10Avp UV/Vis detectorwas purchased from Shimadzu Corporation (Japan). LC-MS chromatograms and spectra for dereplication were measured on a LCQ DECA XPLiquid Chromatography-mass Spectrometry (Thermo-Finnigan, San Jose, CA, USA.). Mass spectrometric detection was performed on a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization (ESI) source. DMSO, HPLC grade methanol and acetic acid, other chemicals and solvents were of highest purity grade and purchased from Dikma, China. Ultra-pure water was obtained from a Milli Q-plus system (Billerica, MA, USA), and Milli-Q water was used for the assays and HPLC analysis. Tacrine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). True Choline esterase assay kit (50T) was purchased from Nanjing Jiancheng Bioengineering Institute, China.

Plant material

The root of *P. fel-terrae* was collected in Guangxi, China. Authentic reference samples were previously isolated from *P. fel-terrae* and the structures identified by 1D NMR and FABMS.

Extraction and fractionation

The dried root pieces of *P. fel-terrae* (200g) were refluxed with ethanol three times for 6h. The organic solvent was removed in vacuo to give 45 g of ethanol extract. The ethanol extract was suspended in 500 ml of water and further partitioned in succession with water, petroleum ether and ethyl acetate, affording 7.2, 4.6, and 7.7 g of the respective fractions.

Bioactivity-guided fractionation and isolation

The ethyl acetate exact fraction of *P. fel-terrae* was separated using HPLC. The eluate was collected using a fraction collector at 300 µl/well in 96-well plates. The solvent in each well was removed in a vacuum oven, and the residue in each well was tested for AChE inhibitory activity [Figure 1].

AChE inhibitory activity

AChE inhibitory activities of compounds 1-6 were measured by the spectrophotometric method developed by Ellmen. Acetylthiocholine iodide was used as substrate in the assay. The reaction mixture contained 1500 µl of (100 mM) tris buffer (pH 7.8), 1000 µl of DTNB, 200 µl (50, 100, 150, 200, 250 µg/ml) of test-compound solution and 200 µl of acetyl cholinesterase solution (erythrocytes), which were mixed and incubated for 15 min (25°C). The reaction was initiated by the addition of 200 µl acetylthiocholine. The hydrolysis of acetylthiocholine was monitored at 412 nm after 30 min. Tacrinewas used as positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows (E is the activity of the enzyme without test-compound, S is the activity of enzyme with test-compound):

% age inhibition = (E-S)/ E × 100

Sample separation by HPLC-DAD

HPLC analyses were performed at 25 ± 1°C using sample solutions filtered through 0.45 µm membrane (Whatman's syringe filter) and analysed (10 µL injected volume) using a Shimadzu (Japan) system equipped with SPD-10A VP Shimadzu system controller, SPD-M10A VP Shimadzu diode array detector, LC-10AT VP Shimadzu liquid

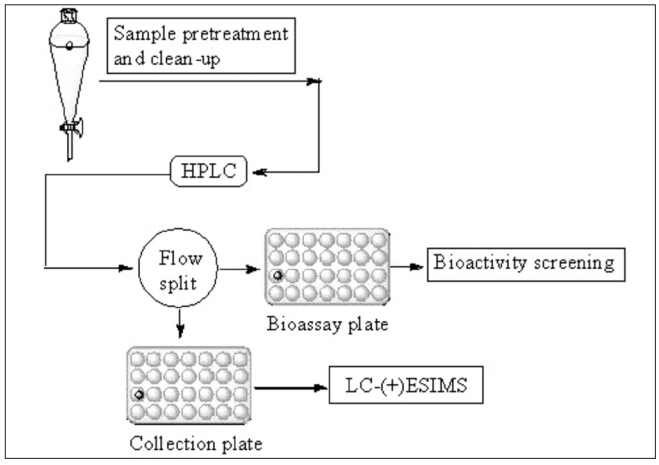

Figure 1: Generic set-up for the fractionation and identification of unknown bioactive substances using LC-bioassay -(+) ESIMS

chromatography pump, Class VP software, and a Rheodyne injector with 20-µL loop. Separation was achieved on a Water's XTerra^{Ò} RP C_{18}-column, 4.6 × 250 mm, 5-µm particle size; isocratic elution was carried out using the mobile phase of 1% acetic acid in methanol: water (30: 70 v/v) at a flow rate of 1mL/min.

Compound analysis by LC-MS

LC-MS conditions were: capillary temperature, 250°C; damping gas helium, sheath gas (N_2) flow, 80; auxiliary gas flow, 30 arbitrary units; source voltage,+3kW; capillary voltage, +24V; tube lens offset, +35V. A Waters Symmetry C_{18} column (250 ´ 4.6 mm i.d., 5.0 µm particles) was used for LC-MS. The ESI source was set at the positive ionization mode. The samples were separated by gradient elution of the mobile phase consisting of 0.01% acetic acid solution in methanol: water (10: 90 to 90: 10, v/v, for 60 min) at the flow rate of 1 ml/min at room temperature(20 µL injected volume). Data acquisition was performed by Xcalibor 1.3 software (Thermo Finnigan, USA.) Peak integration and calibration were performed using LCQuan software (Thermo Finnigan).

RESULTS AND DISCUSSION

Bioactivity-guided fractionation and isolation

HPLC chromatograms were analyzed in three wavelengths 254, 280, and 323 nm through diode-array detector (DAD). The number of peaks was maximum, so we confirmed 254 nm of measurement wavelength. Above all, HPLC was injected 20 µL sample solution, and 66 min chromatogram was recorded [Figure 2]. The ethyl acetate exact fraction was eluted several parts by HPLC, every part was analyzed AChE inhibitory activity, and six compounds showed strong inhibitory activity.

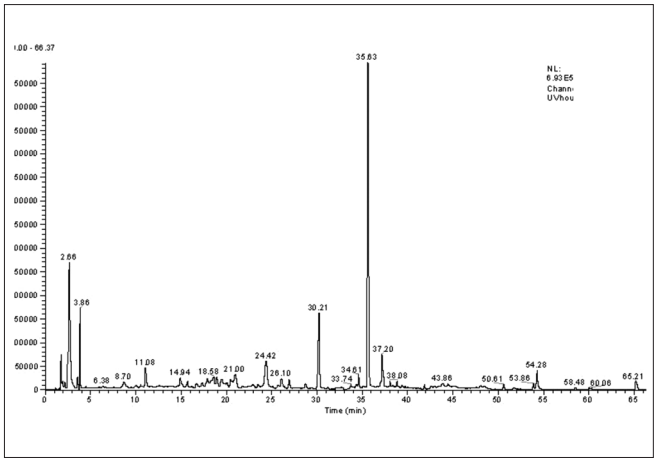

Figure 2: HPLC-DAD chromatogram of the EtOAc extract of *P. fel-terrae*

AChE inhibitory activity of isolated fractions

As shown in Figure 3, the ethanol extract and petroleum ether, ethyl acetate, and water fractions from *P. fel-terrae* showed AChE inhibitory activity ($P< 0.05$) in a dose-dependent manner. In particular, the ethyl acetate fraction exerted the strongest ($P< 0.05$) inhibitory effect on AChE, with inhibition of greater than the ethanol extract, petroleum ether, and water fractions [Figure 3].

LC-MS analysis of AChE inhibitory compounds in the ethyl acetate fraction

The active components in the ethyl acetate fraction of *P. fel-terrae* were investigated using a bioassay-linked LC-MS process. The ethyl acetate fraction was selected because it showed the most effective AChE inhibitory activity. Bioassay-linked LC-MS indicated that the peaks appearing at 24.44, 26.99, 28.12, 28.76, 30.26, 33.76, 35.63, 37.37, and 44.47 min were responsible for the observed activities [Figure 4].

The sample solution was injected in LC-MS system based on above-mentioned chromatographic condition and of condition of Positive-ion MS scanning, and was analyzed by massspectrum. Total ion chromatogram (TIC) was recorded and offered help to detect the desired ions. Six active constituents were analyzed structural characteristic through (+) ESIMS, compared with standard substances, and determined five structural characteristic.

The ethyl acetate extract of *P. fel-terrae* was scanned under the LC-(+) ESIMS conditions. All expected molecular weights of the bioactive constituents were calculated. The reversed-phase HPLC (RP-HPLC) retention times of six pure compounds isolated from *P. fel-terrae* are listed in Table 1.

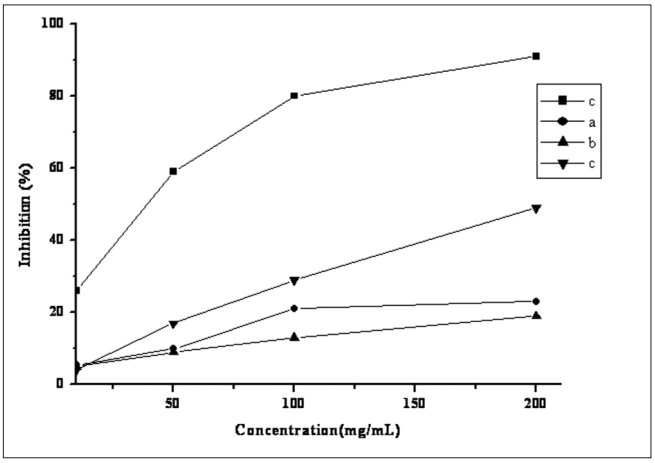

Figure 3: Dose-dependent effects of ethanol extract (**a**) and petroleum ether (**b**), ethyl acetate (**c**), and water (**d**) fractions from *P. fel-terrae* on inhibition of AChE

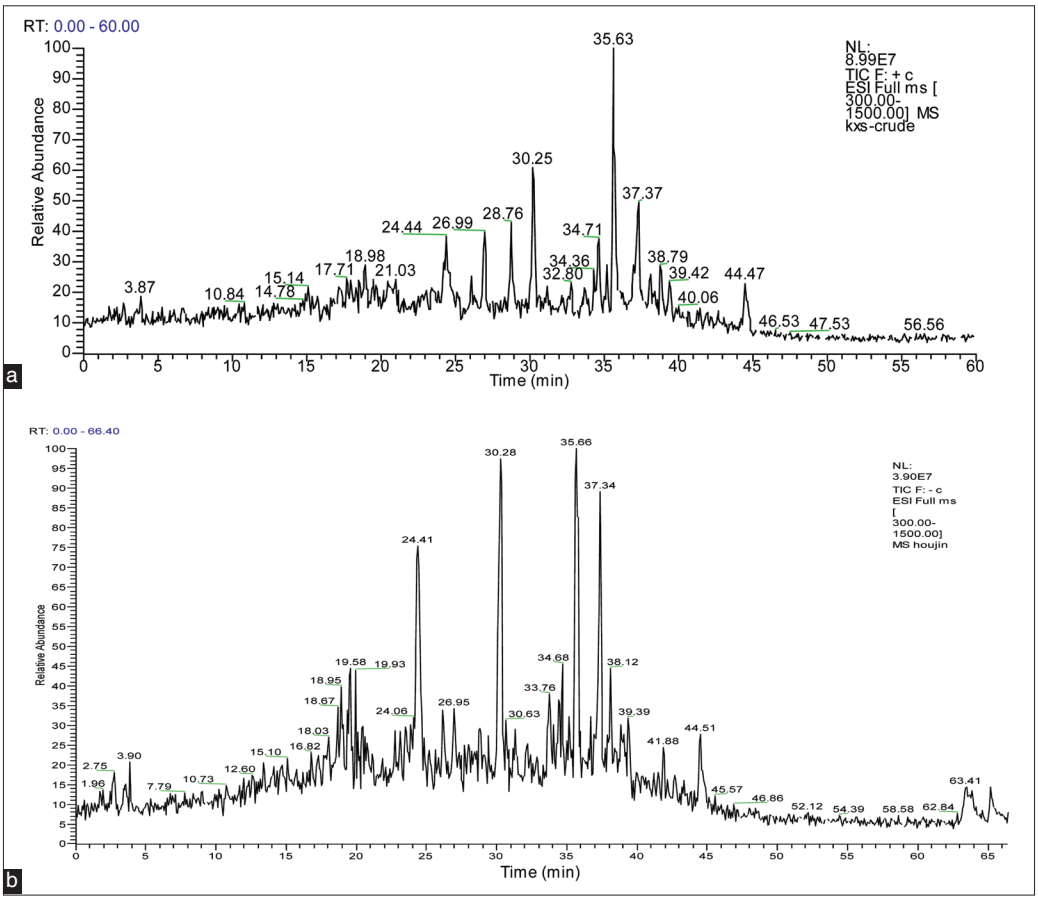

Figure 4: (a and b) Total ion chromatogram of the EtOAc extract of *P. fel-terrae*

Table 1: Compounds, relative molecular weight, HPLC retention time and AChE inhibitory activity of six bioactive peaks from *P. fel-terrae*

No.	Compound	Retention time (min)	Relative molecular weight	Activity (mg/mL)
1	picfeltarraenin IA	35.63	785.6	1.46
2	picfeltarraenin IB	30.25	815.6	0.32
3	picfeltarraeninIV	28.12	947.4	2.19
4	picfeltarraenin X	26.99	685.4	2.69
5	picfeltarraenin XI	28.76	683.5	5.80
7	unknown	37.37	929.6	6.60

Analysis of active compounds

In ESIMS compound **1**showed $[M + Na]^+$ quasi-molecular ions at *m/z* 785, corresponding to a molecular weight of 762 ($C_{41}H_{62}O_{13}$). The protonated aglycon was also revealed by an ion at *m/z* 485. The product ion spectrum of the protonated molecule (*m/z* 762) showed ions at *m/z* 618 [M-146+2H] (loss of 144 μ) and *m/z* 485 [M-146+H-132] (loss of 145 + 132 μ), consistent with the loss of a rhamnosyl-pentoside moiety. Interpretation of the ^{13}C NMR spectral data of **1**led to the identification of **1**as picfeltarraenin IA (picfeltarraegenin I 3-*O*-a-L-rhamnopyranosyl-(1→2)- b-D-xylopyranoside) [Figure 5]. Furthermore, abundant product ions were found at *m/z* 449 (-2 ´ H_2O), which can be considered characteristic of the aglycon [Figure 6].

The structure of compound **2**was elucidated in a similar manner. It showed $[M + Na]^+$ and $[M + H]^+$ quasi-molecular ions in (+) ESIMS at *m/z* 815and 793, respectively, corresponding to a molecular weight of 792 ($C_{42}H_{64}O_{14}$), as well as the protonated aglycon at *m/z* 485. The product ion spectrum of the protonated molecule (*m/z* 793) revealed ions at *m/z* 647 (loss of 146 μ) and *m/z* 485 (loss of 146 + 162 μ), consistent with a rhamnosyl-hexoside moiety [Figure 7]. Compound **2**was identified as picfeltarraenin IB (picfeltarraegenin I 3-*O*-a-L-rhamnopyranosyl-(1→2)-b-D- glucopyranoside) by interpretation of its ^{13}C NMR spectral data [Figure 5].

Compound **3**showed a $[M + Na]^+$ quasi-molecular ion at *m/z* 947, corresponding to a molecular weight of 924 ($C_{47}H_{72}O_{18}$), the product ion spectrum showed ions at *m/z* 863 [M-162+H] (loss of 161 u) and *m/z* 687 [M-162+OH] (loss of 177 u), consistent with the loss of a hexosyl and a deoxyhexosyl moiety. The aglycon part could also be characterized by MS by performing a product analysis

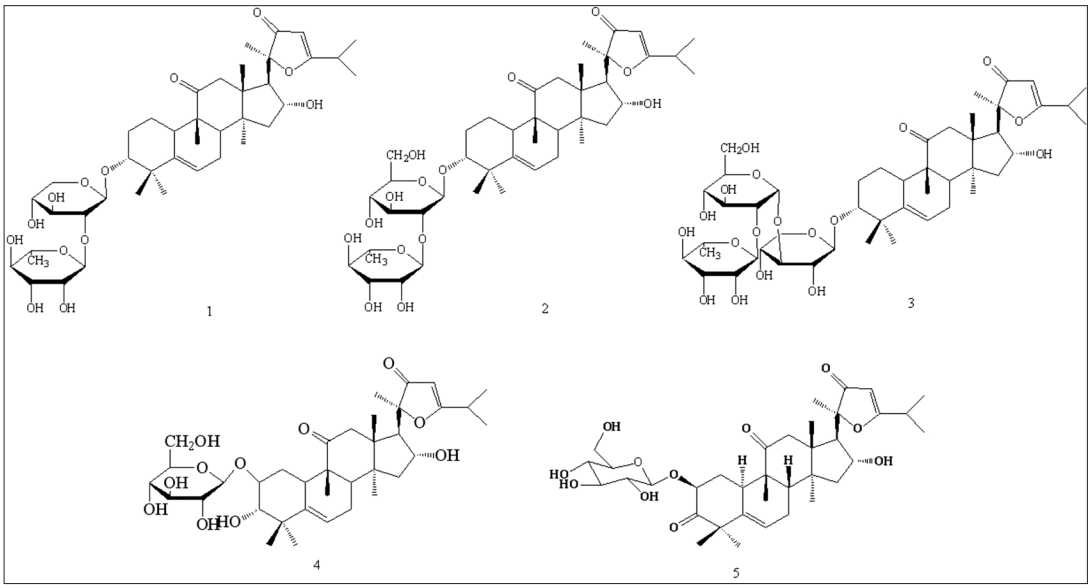

Figure 5: Structures of compounds **1**-**5**

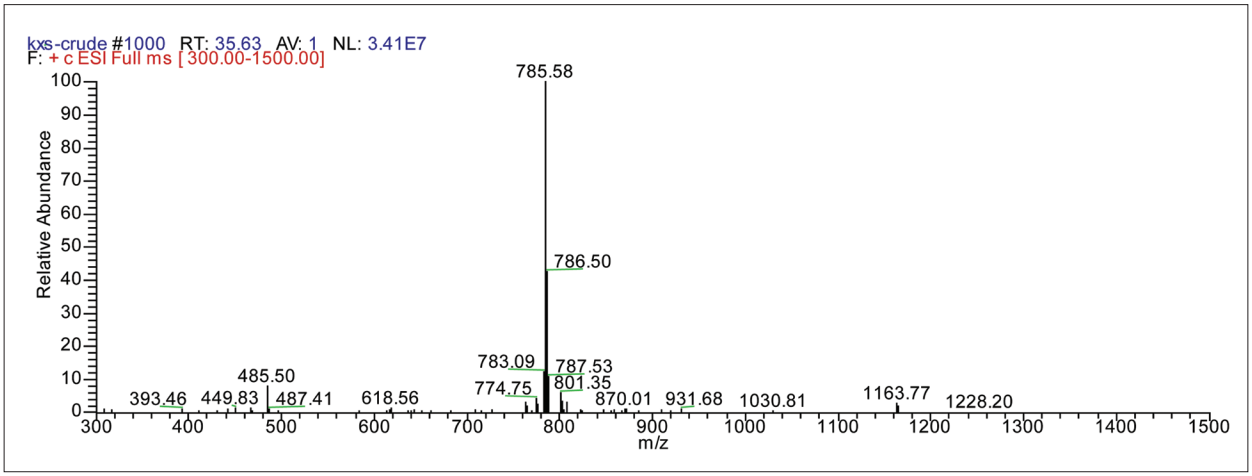

Figure 6: (+) ESIMS of compound **1**

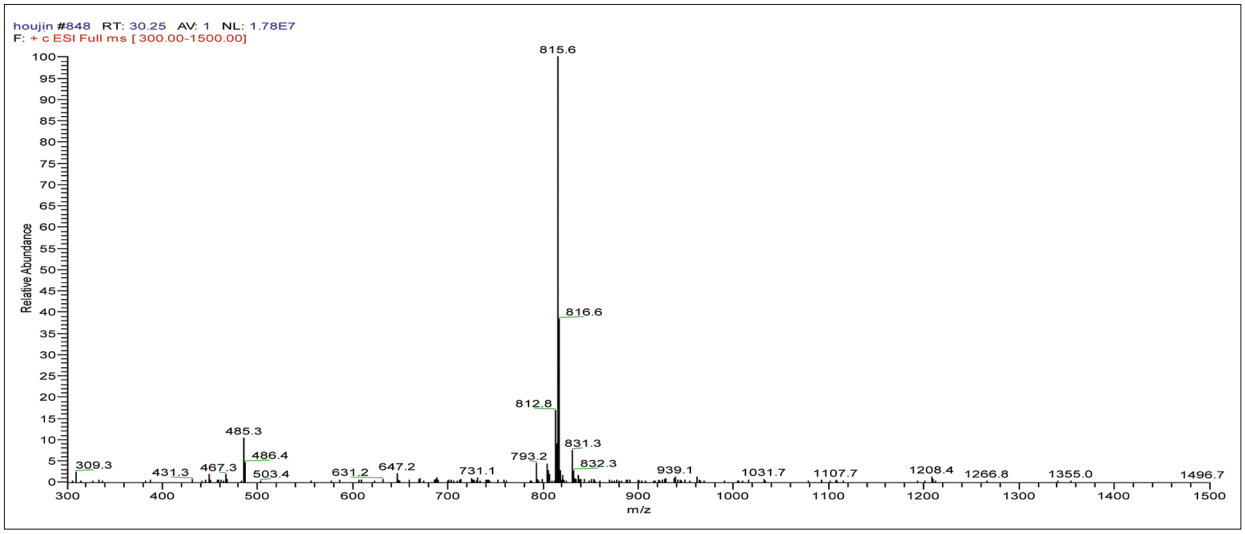

Figure 7: (+) ESIMS of compound **2**

on the protonated aglycon (*m/z* 485) formed during ESI. Thus, abundant product ions were found at *m/z* 449 (-2 × H_2O), which can be considered characteristic of the aglycon [Figure 8]. ^{13}C NMR spectral data were in agreement with those reported for picfeltarraenin IV (picfeltarraegenin I 3-*O*-a-L-rhamnopyranosyl-(1→2)-b-D-glucopyranosyl-(1→3)-b-D- xylopyranoside) [Figure 5].[5]

The (+)ESIMS spectrum exhibited the characteristic molecular positive ions at *m/z* 685 ([M + Na]$^+$), corresponding to a molecular weight of 662 ($C_{36}H_{54}O_{11}$). The product ion spectrum of the protonated molecule (*m/z* 662) revealed ions at *m/z* 501 [M-162+H]$^+$ (loss of 161 u), consistent with a glucopyranosy moiety [Figure 9]. ^{13}C NMR spectral data were in agreement with those reported for picfeltarraenin X [Figure 5].

The (+)ESIMS spectrum exhibited a quasi-molecular ion peak at *m/z* 683 [M+Na]$^+$, and a characteristic fragment ion peak at m/z 499 [M-162+H]$^+$ (loss of 161 u) indicated that there was a glucopyranosyl moiety in **5** [Figure 10]. ^{13}C NMR spectral data were in agreement with those reported for picfeltarraenin XI [Figure 5].

In ESIMS compound **6**showed [M+H]$^+$ quasi-molecular ions at *m/z* 929, corresponding to a molecular weight of 928 ($C_{41}H_{62}O_{13}$). The product ion spectrum of the protonated molecule (*m/z* 928) showed ions at *m/z* 785 (loss of 143 u), consistent with the loss of a glucopyranosyl moiety. Thus, abundant product ions were found at *m/z* 467 (-H_2O) and 309, which can be considered characteristic of the aglycon [Figure 11]. Compared with the MS spectra of compound **1**, the structure was maybe picfeltarraenin IA added a glucopyranosyl moiety.

As discussed previously, all AChE inhibitory compounds produce ions that match this ion pattern. Thus, the actual molecular weights corresponding to the compounds present in a particular fraction can be readily determined. However, to conduct LC-MS experiments, the molecular weight of each component to be dissociated must be previously

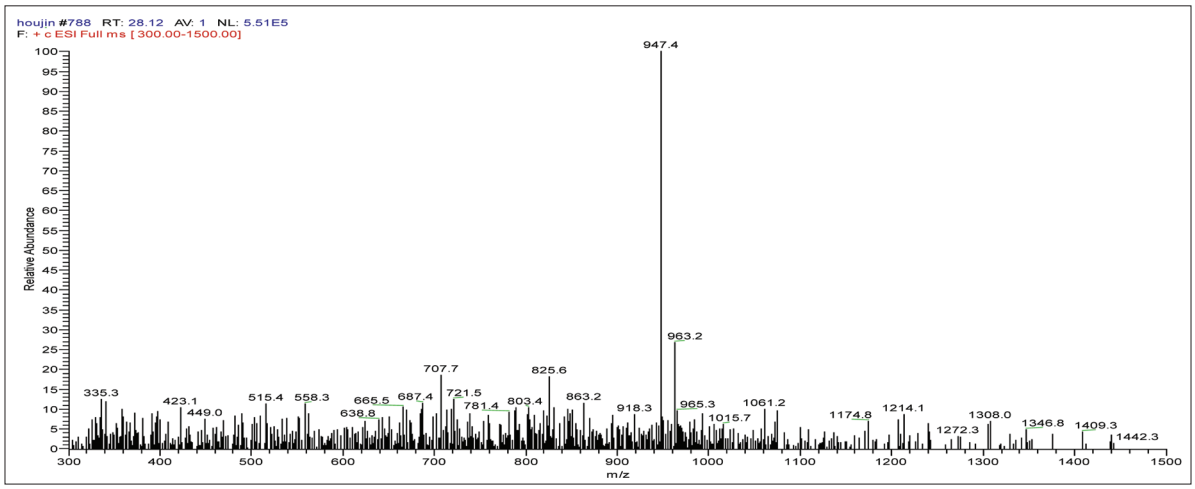

Figure 8: (+) ESIMS of compound **3**

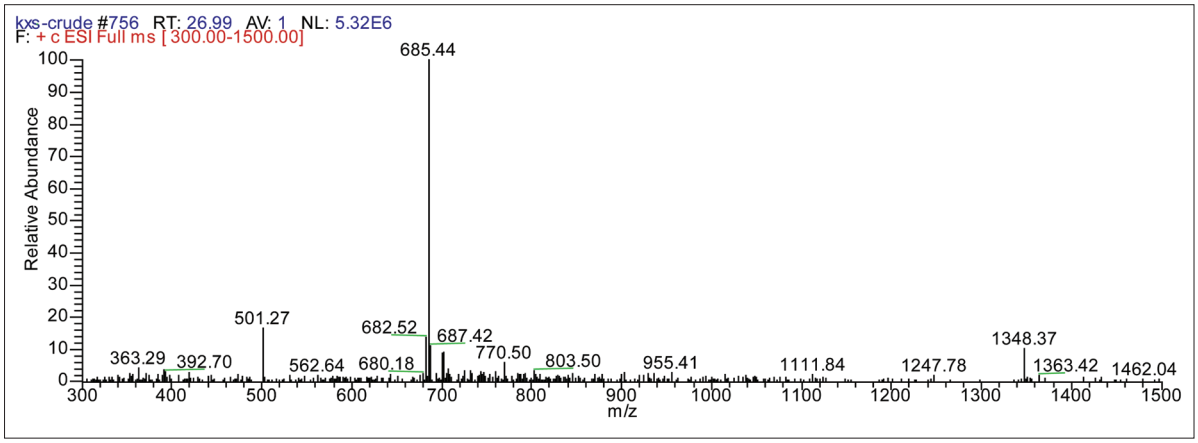

Figure 9: (+) ESIMS of compound **4**

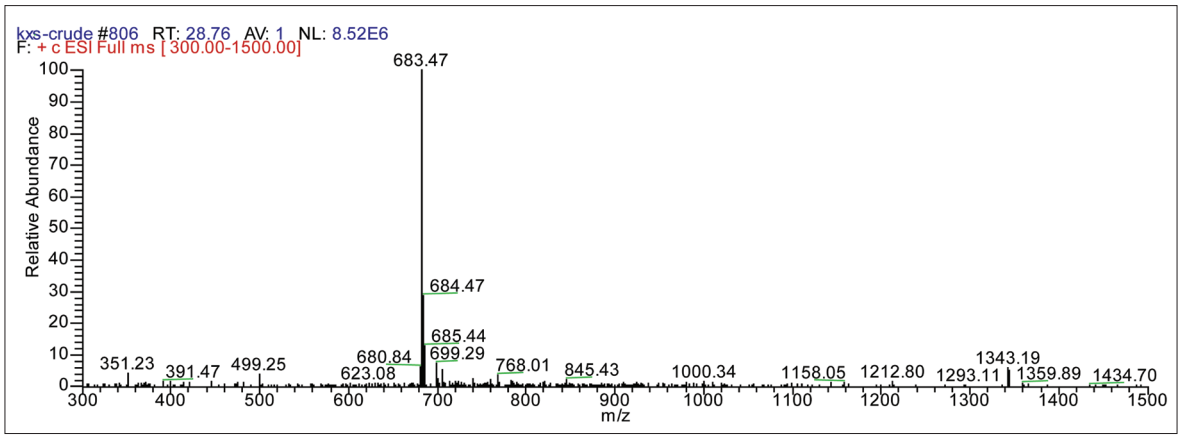

Figure 10: (+) ESIMS of compound **5**

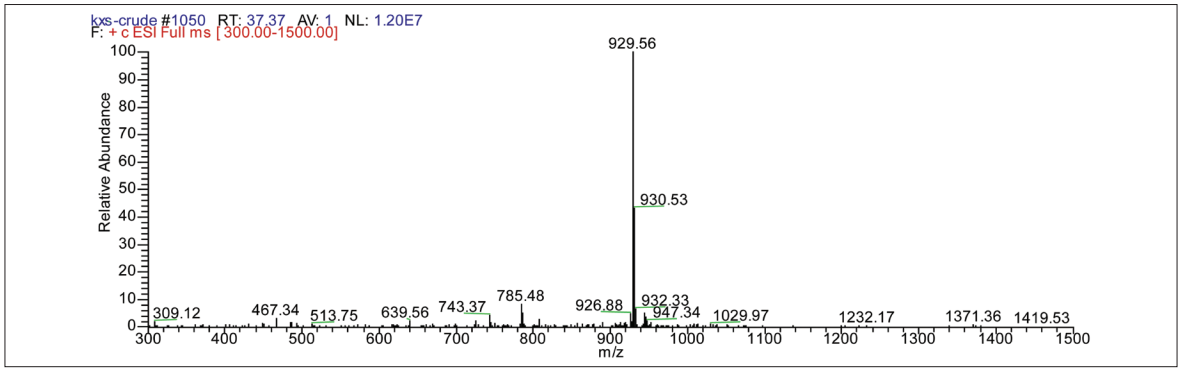

Figure 11: (+) ESIMS of compound **6**

known. This is not possible for a chromatographic fraction containing all unknown compounds. Our experimental results showed that selected ion chromatograms, obtained by carefully analyzing total ion chromatograms, could supply structurally informative ion patterns. Thus, LC-MS was mainly used in this study. The results of the current investigation demonstrate that LC-MS provides a highly sensitive detection method that can assist in: (1) identification of new sources of targeted families of natural products; (2) highlighting specific fractions of extracts as meriting greater or less interest based on the novelty of the components; and (3) specific detection of new derivatives in a series of bioactive plant fractions, as shown by the new bioactive compounds described in this paper.

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