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HPLC-PDA isolation and LC-MS/MS detection of an acetylcholinesterase inhibitory flavonoid from *Tephrosia purpurea* (L.) Pers. in zebrafish brain

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Separation of bioactive compounds or therapeutic small molecules from medicinal herbs is challenging due to the complexity of the phytochemicals. *Tephrosia purpurea* (L.) Pers. (Fam. Fabaceae) is rich in therapeutic compounds, used forisolation of an acetylcholinesterase inhibitor by HPLC coupled with Photo diode array (PDA) and mass spectrometric techniques. The separation was achieved through analytical HPLC by development of gradient mobile phase using methanol and acetonitrile along with 0.1 M ammonium acetate in Milli-Q water by two modes of mobile phase separations to yield the maximum purity, 99.13%. Based on the above-developed strategies, the preparative isolation of the acetylcholinesterase inhibitory flavonoid was purified at 269 nm with a retention time of 13. 9 min. The isolated compound from *T. purpurea* was confirmed as a flavonoid by phytochemical screening tests. The molecular mass was identified as 366.1467 Da by UPLC/Q-ToF-MS for the purified flavonoid.. Further, the molecular formula was found to be $C_{22}H_{22}O_5$ by HR-MS/MS fragmentation pattern analysis through ChemSpider database search. The fragmentation pattern favoured the purified compound for similarity to Cyqualon. The isolated compound showed acetylcholinesterase (AchE) inhibition with the IC_{50} value of 54 μM in the zebrafish brain. However, the comparative study on the commercial cyqualon compound and the isolated compound showed different UV spectrum with the values at 269 nm and 257.5 nm, respectively. These findings concluded that the compound might be a novel flavonoid from *Tephrosia purpurea* which could be used as a therapeutic compound for neurodegenerative diseases after structural characterization.

Keywords: Acetylcholinesterase inhibition, Alzheimer's disease, HPLC-PDA, HPLC purification, Wild indigo

The potential of natural product based drugs have been successfully demonstrated in the treatment of Alzheimer's disease (AD)[1,2]. The acetylcholinesterase (AChE) inhibition is one of the promising treatments of AD[3] and also the treatment of senile dementia, ataxia, myasthenia gravis and Parkinson's disease. The known AChE inhibitors such as physostigmine and tacrine have limitations like short half-life or side-effects such as hepatotoxicity[4]. Currently, there are only two AChE inhibitors, galantamine and rivastigmine, that are derived from natural products, used to treat cognitive symptoms in dementia[5,6]. Galantamine[5] and donepezil have shown toxicity towards urinary incontinence[7]. We have recently reported rapid locomotor behavior in zebrafish embryo based on the neuroactive potential of *Tephrosia purpurea*[8].

Tephrosia purpurea (L.) Pers. (Fam. Fabaceae) is a medicinal herb widely distributed in the South Western Ghats of India amongst the five botanical families richest in therapeutic properties. Their leaves, stem and root are reported to have numerous medicinal values. The previous reports of *T. Purpurea* have demonstrated the presence of flavones, flavanones, prenylated flavonoids, chalcones and rotenoids as major phytochemicals[9,10]. Chemical constituents and extracts of the *T. purpurea* exhibited diverse bioactivities such as wound healing[11], immunomodulatory, anticancer and anti-inflammatory potential[12-14]. These biologically active compounds are generally extracellular and their isolation in highest purity from the complex mixture needs performance of a combination of various separation steps such as solvent extraction, chemical precipitation, ion exchange chromatography, HPLC purification, etc. As part of our ongoing research on finding novel compound against AD from plant based natural products, here, we report the

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Abbreviations: AchE, Acetylcholinesterase; AD, Alzheimer's disease; LC-MS/MS, Liquid chromatography tandem mass spectrometry; PDA, Photo diode array; RP-HPLC, Reverse phase high performance liquid chromatography

HPLC-PDA method for purification of AChE inhibitor from *Tephrosia purpurea*.

Materials and Methods

Reagents and Apparatus

HPLC modules had quaternary gradient pump (0.1-150 mL/min, Waters 2545), PDA detector (Waters 2996), Sunfire C_{18} analytical column (5µm, 4.6×250 mm), OBD Prep Column (100Å, 10 µm, 50 mm×250 mm) and Waters fraction collector III. Sample monitoring and fraction collection were analyzed using Empower 2 chromatography data software. UPLC-MS-MS analysis wascarried out using an ACQUITY UPLC with Xevo G2-S QT of mass spectrometer (Waters) and HR-MS/MS analyses were carried out using Orbitrap Elite hybrid ion-trap mass spectrometer (Thermo Scientific™). Multi mode Plate reader (Perkin Elmer, USA), Bath Sonicator (PCI analytics), Soxhlet apparatus (Borosil), vacuum concentrator (Eppendorf 5305, Germany) were used. Silica gel G 60-120 mesh size, galantamine, rutin, ethyl acetate, chloroform, hexane, HPLC grade acetonitrile, ammonium acetate, TLC silica gel 60 F_{254} and aluminium sheets were purchased from Merck, Germany. HPLC grade methanol was purchased from Sigma-Aldrich. Water was purified using a Milli-Q system (Millipore®, USA). All HPLC samples were prepared and filtered through a 0.22 µm nylon membrane before use. The mobile phase was prepared daily and degassed by sonication before usage in HPLC.

Extraction

The shade dried plant leaves from *Tephrosia purpurea* were ground to fine powder and 50 g of powder, was extracted with 200 mL hexane in Soxhlet apparatus for 6 h. The extract was allowed to evaporate and dried under vacuum. The dried hexane extracts (5 g) was subjected to silica gel column (60-120 mesh) chromatography with 120 mL bed volume and eluted with chloroform:ethyl acetate:methanol (7:2:1) in isocratic condition to get 20 fractions with 5 mL each. The active fractions were pooled, concentrated and re-dissolved in methanol. Phytochemicals in the extracts were detected by standard qualitative phytochemical analysis as described previously[15].

Method development by HPLC-PDA

The preparatory HPLC separation was carried out by injecting 5 mL of column purified extract containing 500 mg of extract into the C_{18} column. The separation was achieved using (A) HPLC grade water (B) methanol as mobile phase with a gradient condition (Table 1). The flow rate was fixed at 10 mL/min, and individual peak fractions were collected separately using automatic fraction collector. About 20 µL of an active fraction from prep-HPLC was subjected to analytical phase separation at 200-800 nm wavelengths. The mobile phase for active compound separation was achieved through the following two modes of mobile phases (Separation mode A) 0.1 M ammonium acetate: methanol (100) (Separation mode B) 0.1 M ammonium acetate w/v acetonitrile/methanol (20:80) with different gradient conditions (Table 1). The column was equilibrated with the mobile phase for 20 min at 1 mL/min. The gradient was started with isocratic (A35: B65) from 0-2 min and then gradually increased the mobile phase (B) to 100%.

Purification by preparatory RP-HPLC

The analytically developed mobile phase was selected for the preparative purification. Prepcalc program was used to optimize the gradient schedule (Table 1). The active sample from silica gel column fraction was injected into the preparative column, and

Table 1—Gradient schedule of flavonoid purification in analytical and preparative HPLC column

Mobile phase solvents	0.1 M ammonium acetate with methanol				0.1 M ammonium acetate w/v acetonitrile/methanol (20:80)			
	Time	Flow	% A	% B	Time	Flow	% A	% B
Analytical HPLC	0	1	35	65	0	1	35	65
	2	1	35	65	2	1	35	65
	5	1	25	75	5	1	25	75
	10	1	15	85	10	1	15	85
	15	1	10	90	15	1	10	90
	18	1	8.0	92	18	1	8.0	92
	25	1	7.0	93	25	1	7.0	93
	27	1	5.0	95.0	27	1	5.0	95.0
	30	1	15	85	30	1	15	85
	35	1	25	75	35	1	25	75
	40	1	35	65	40	1	35	65
	50	1	35	65	50	1	35	65
Preparative HPLC	-	-	-	-	0	10	35	65
	-	-	-	-	5	10	35	65
	-	-	-	-	12	10	20	80
	-	-	-	-	18	10	10	90
	-	-	-	-	25	10	5	95
	-	-	-	-	32	10	10	90
	-	-	-	-	40	10	20	80
	-	-	-	-	50	10	35	65
	-	-	-	-	55	10	35	65

the targeted peak was eluted and collected using automated fraction collector. The Prep-HPLC elution was subjected to Thin Layer Chromatographic (TLC) analysis (Merck) with the mobile phase 9.5:0.5 chloroform and methanol. The eluted single spot was analyzed by analytical HPLC using a gradient solvent system 0.1 M ammonium acetate w/v acetonitrile/methanol (20:80).

Molecular formula identification by UPLC/MS/MS

The accurate mass and element of the HPLC purified flavonoid compound were determined by UPLC-MS/MS operated in positive mode electro spray ionization (ESI) with the resolution 22500 FWHM. The capillary and cone voltage was set to 3 KV, source temperature and desolvation temperature was set to 140 and 400°C. The gas flow of cone was 50 L/h and desolvation gas flow 1000L/h was set to analyze mass and fragmentation of the HPLC purified flavonoid compound. High resolution mass spectrometer equipped with electro spray ionization with both positive and negative mode was scanned in the mass range from m/z 200-2000. The ESI data were processed by Thermo Qual browser, and the collision energy was applied for fragmentation. Molecular formula was identified using elemental composition parameters such as 5 ppm tolerance and elements C, H, N and O for the identified mass.

Acetylcholinesterase inhibition assay in zebrafish brain

The acetyl cholinesterase inhibitory assay was performed by the Elman's method in the 96-well plate in room temperature. 40 µL of enzyme extract incubated with 50 µL of inhibitor in 180 µL PB buffer (pH 7) for 45 min. After incubation, the reaction was stopped by addition of Tris-HCl (pH 8). Then 10 µL of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added to each well and absorbance was taken at 412 nm. 2 µL of acetyl thiocholine iodide was added to each well to measure the hydrolysis of ATCI by formation of yellow reaction of DTNP with thiocholine and the reaction was measured at 412 nm in multi mode plate reader (PerkinElmer) for 10 min with 1 min gap. The percentage of inhibition was calculated by the following formula.

$$\% \text{ of inhibition} = \frac{\text{Enzyme activity of control} - \text{Enzyme activity of HPLC purified compound}}{\text{Enzyme activity of control}} \times 100$$

Different concentration of HPLC purified compound was incubated with the enzyme extract of zebrafish brain, and the IC_{50} value was calculated from dose-dependent inhibition curves using the Graph Pad Prism version 5.00. The commercial compound galantamine which is a plant-derived drug for Alzheimer's disease was used as a positive control inhibitor.

Results and Discussion

Analytical HPLC method development of bioactive compound purification

HPLC coupled with ultraviolet (UV) or diode array detection (DAD) and mass spectrometric (MS) detector have proven to be the method of choice for isolation and identification of small organic compounds[16-20]. Among several chromatographic techniques, HPLC–PDA is most widely used due to its rapidity, simplicity, and convenience for the isolation of small molecules. The present study demonstrated the isolation of AChE inhibitor compound from *Tephrosia purpurea* using HPLC-PDA detector with three-dimensional detection including light intensity, time and entire wavelength. With this strategy, the compound was isolated with most accuracy within a limited duration of time from the complex extract. We initiated the primary purifications with silica gel column and TLC from the crude hexane extract which supports the previous method[21]. The *T. purpurea* sub-fractions from silica gel column were purified in preparative and analytical HPLC. There are several factors involved in the HPLC purification of small organic compounds such as mobile phase, column types and detectors[22]. Thus, the mobile phase 0.1 M ammonium acetate: 100% methanol (Separation mode-A) was selected by considering the above factors for isolation of single active compound, which also supports similar mobile phase strategy reported for the isolation of an acetyl cholinesterase inhibitor from *Narcissus*[23]. The active peak was eluted at the retention time of 19.4 min g. 1A) with 269 nm PDA absorption by bioactivity guided screening with 14.31 % of purity (Fig. 1B). Further, the active peak was re-injected to check the purity with the same mobile phase and shown minimal impurities in the HPLC chromatogram (Fig. 2). To improve the quality of purification another mobile phase was developed using 0.1 M ammonium acetate: acetonitrile/methanol (20/80) (Separation mode-B) with same gradient condition for further separation (Fig. 3) and five major fractions were obtained with 72.43% (Fig. 3B). Thus, the change of mobile phase has reduced the retention time of the active fraction at 16.56 min with more accuracy. The quality of the active compound was achieved and confirmed with 99. 13 % of purity in HPLC chromatogram (Fig. 4 A & B)

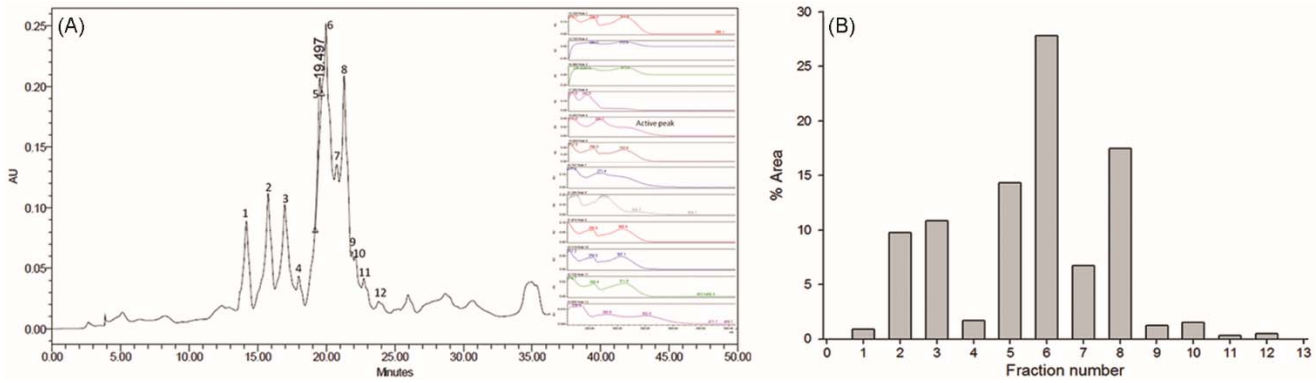

Fig. 1—(A) HPLC chromatogram and UV spectrum of column separated active elution with the gradient mobile phase of ammonium acetate and methanol. The elution were collected in the automated fraction collector and shown as numerical in the chromatogram. The active flavonoid was eluted and detected at the retention time of 19.49 with 269 nm; and (B) The histogram showing the the active fraction with 14. 31 % of purity (peak no. 5) from the total chemical constituents of the elute.

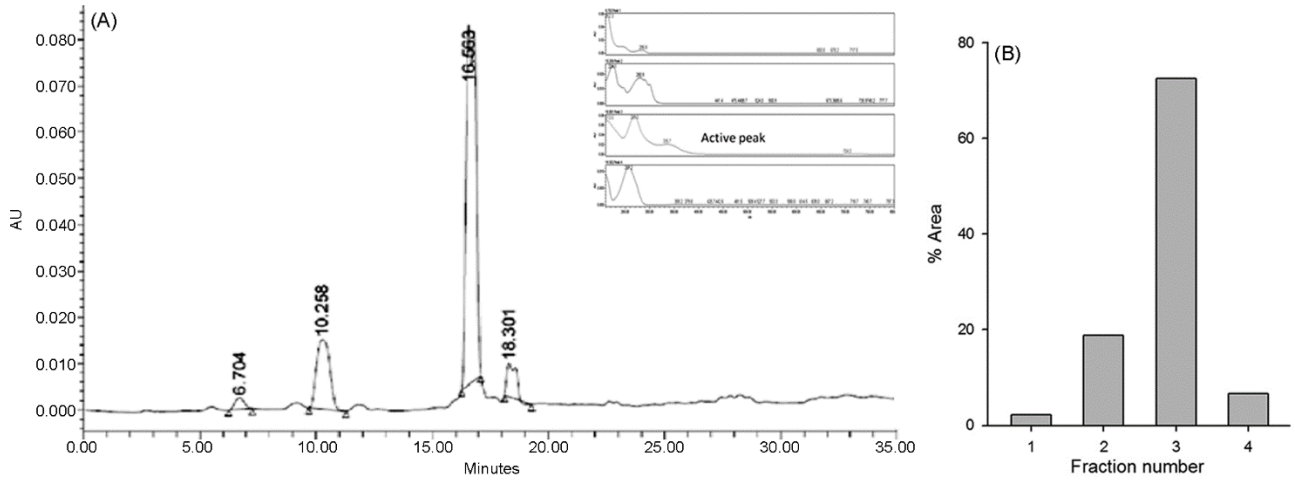

Fig. 3—(A) HPLC separation of ammonium acetate and methanol eluted active peak was run with the modified mobile phase 0.1M ammonium acetate with 20% acetonitrile in methanol with gradient condition. Separation of four major chromatogram peaks were detected and separated. The active flavonoid compound was separated at the retention time of 16.563 with 269 nm. The other three peaks were considered as impurities as they do not have any acetylcholine esterase inhibitory activity; and (B) The histogram showing the active fraction with 72.43 % of purity (peak no. 3) from the first mode of mobile phase separation of elutes.

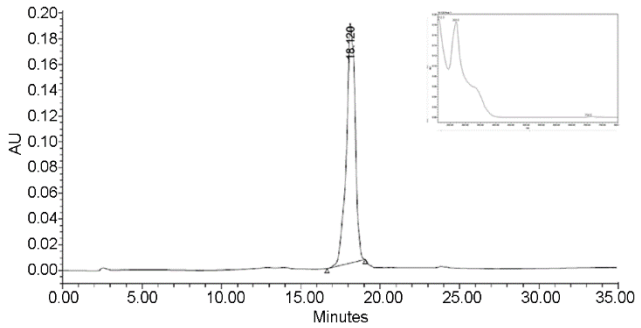

Fig. 2—HPLC chromatogram for the active peak was detected at a retention time of 18.12 at 269 nm using the same gradient mobile phase of ammonium acetate and methanol. Inner figure shows the UV spectrum.

and a single spot was obtained in TLC with Rf value of 0.5 (Fig. 4C). Further, the mobile phase was optimized with preparative fractions for elution of the active compound with the retention time of 13.8 min at 269 nm with maximum purity. The phytochemical analysis of the purified compound favoured for flavonoids with the positive control rutin[24,25]. Phenolics and flavonoids are known to be purified using HPLC in a reversed phase C_{18} column with PDA detector and polar acidified organic solvents[26,27]. However, in our study, we simplified the purification of flavonoid from *Tephrosia purpurea* by developing two mobile phase separation modes to achieve maximum quality (99.13%) with limited duration for isolation using HPLC-PDA detector.

UPLC-MS-MS identification of acetylcholinesterase inhibitor

Systematic identification of compounds in a complex mixture is typically carried out using ultra-performance liquid chromatography/quadruple-time-

of-flight mass spectrometry (UPLC/Q-TOF-MS). Hence in the present study, the mass spectrometric scans were employed to evaluate the accuracy of the molecular mass using UPLC/Q-ToF-MS,and possible fragmentations were obtained to enable the probable structural characterization. Prior to identification of the compound, the exact molecular weight should be defined through adduct recognition[28]. Further to this, the molecular ions are chosen for determination of the molecular formula (MF) based on the accuracy of mass and spectrum[29].Thus, in UPLC/Q-ToF-MS analysis, the compound gets separated and ionized into the formation of charged gaseous species by the electro spray ionization (ESI) and detected using Q-ToF.The protonated molecular mass of the purified AChE inhibitor is shown in Fig. 5. The mass spectrum revealed the presence of m/z at 367.1548 [M+H$^+$], 389.1353 [M+Na$^+$] and 755.2826 [2M+Na$^+$]. The fragmentation pattern of protonated mass fragments of AChE inhibitor was found to be m/z at 337.1058, 295.0954 and 281.0795. Thus, the molecular mass of the AChE inhibitor flavonoid was identified as

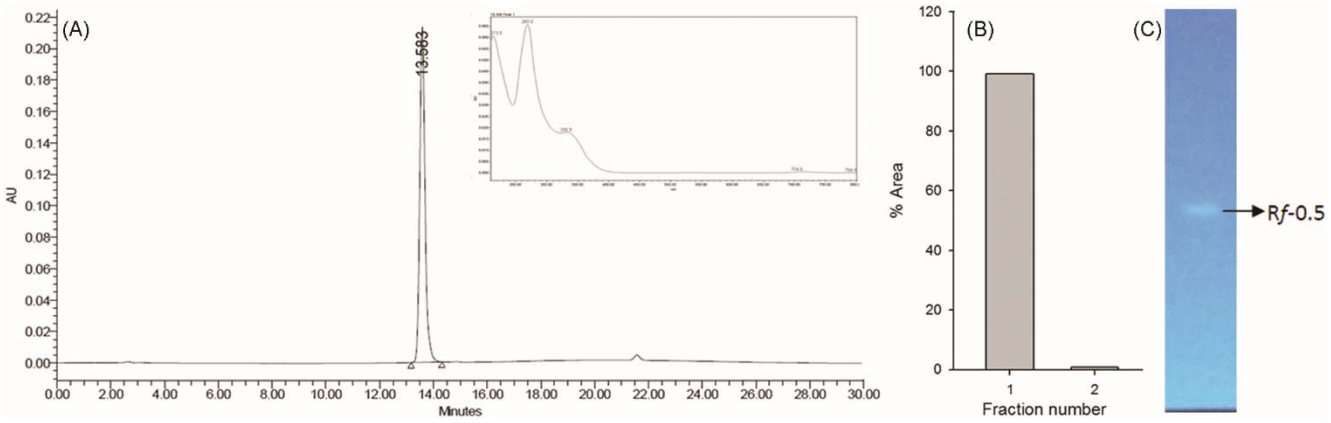

Fig. 4—(A). HPLC spectrum showing purity of active flavonoid with the gradient mobile phase conditions of 0.1M ammonium acetate with 20% acetonitrile in methanol at the retention time of 13.583 with 269 nm; (B) The histogram showing active fraction with 99.13 % of purity (peak no. 1) of the elute; and (C) TLC profile of HPLC purified flavonoid has the R*f* value at 0.5 in the mobile phase of chloroform: methanol (9.5: 0.5).

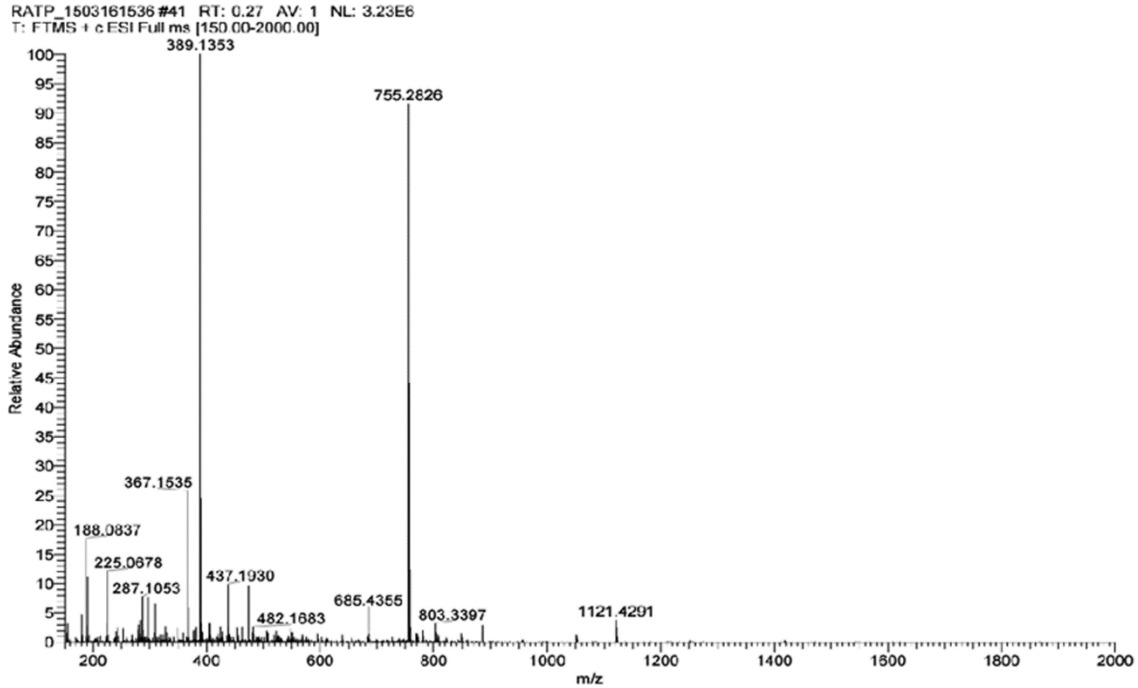

Fig. 5—Mass spectrum showing the ESIPositive ion mode scan of HPLC purified peak at 13.583 m/z at [M+H$^+$]367.1548, [M+Na$^+$] 389.1353, [2M+Na$^+$] 755.2826.

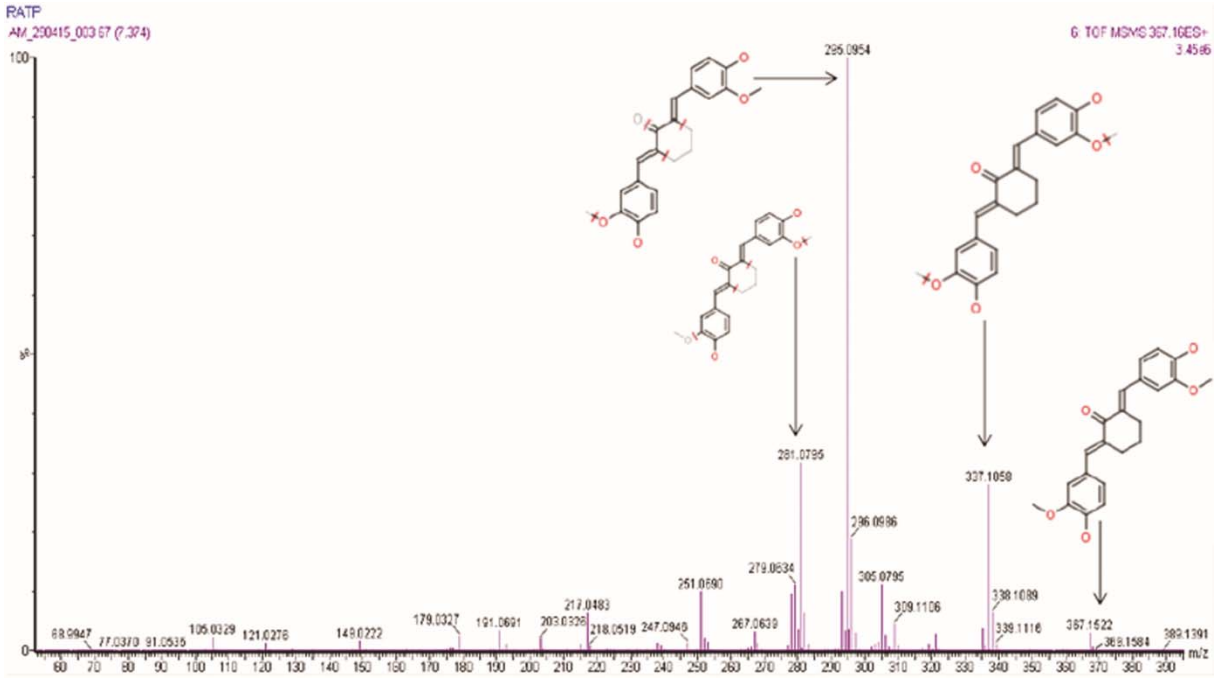

Fig. 6—The spectrum shows the fragmentation pattern of flavonoid by UPLC-MS-MS analysis. The fragmented flavonoid shows the *m/z* at 337.1058, 295.0954 and 281.0795 respectively with close relation to cyqualon.

366.1467 Da. The general strategy for natural product identification is based on the database searches. Hence, the HR-MS and MS/MS spectrum (UPLC/Q-ToF-MS) of the purified compound was compared to a large number of existing databases[30].

Identification of the eluted compounds becomes a challenging task after acquiring a quality separation of the chemical constituents with the highest possible resolution. Kind and Fiehn[31] recommended seven golden rules for heuristic filtering of molecular formulae (MFs), and thus the metabolites can be identified by the putative MFs through natural product database search. However, this strategy typically leads to multiple putative identities for the detected compounds[28]. The fragmentation pattern obtained in high collision energy through MS/MS mode as a '.mol' file was imported to MassFragment (Waters) database and used to assign potential structures for each fragment ion. Based on the heuristic filtering and elemental analysis, the molecular formula was found to be $C_{22}H_{22}O_5$ for the isolated flavanoid. The characteristic of mass fragment ions was observed as cyqualon and shown in Fig. 6. Since the structure have been proposed using a ChemSpider database, there is a possibility that there could be other structure that may also fit into similar fragmentation pattern. Comparison of the commercial cyqualon compound and the isolated flavonoid showed different UV spectrum (Fig. 7) with

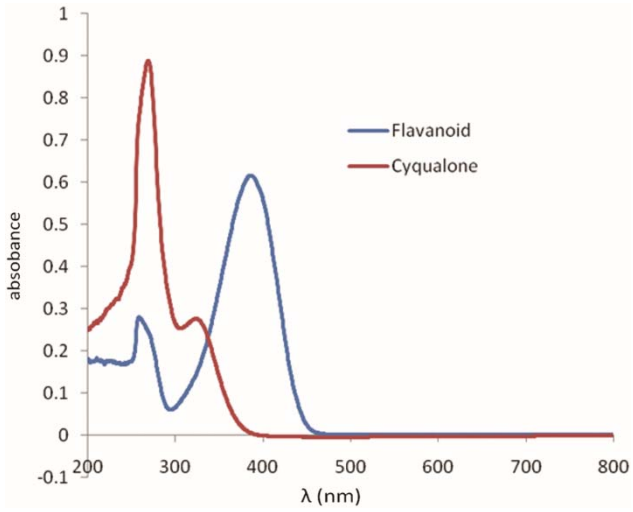

Fig. 7—Comparative UV spectrum of AChE inhibitor flavonoid (λ maxvalues: 269 nm) and Cyqualon (λ maxvalues: 257.5 nm) (Sigma) showing the structural dissimilarity of the isolated flavonoid with the fragmentation pattern based detection of Cyqualon.

the values at 257.5 nm and 269 nm, respectively and therebyrevealed that the identified compound might be a compound related to the structure of cyqualon.

Determination of acetylcholinesterase inhibition and IC_{50} value

The purified flavonoid extract was evaluated for AChE inhibitory activity against the enzyme obtained from zebrafish brain homogenate. Previous works have

also reported that the phenolics and flavonoids isolated from different plants used in medicine for Alzheimer's disease, showed strong inhibitory activity of these enzymes substantiating the potential of these compounds as a flavonoid[32-34]. The IC_{50} value of the isolated flavonoid in zebrafish brain was calculated with different concentration of HPLC purified compound (10-100 µM) and was obtained as 54.286 µM. The IC_{50} value of the positive control galantamine was obtained as 10 µM in zebrafish brain extract. Similar AChE inhibitory activities have been reported from polyphenol-rich plants *Morus lhou* and *Sophora flavescens* (Fabaceae) with IC_{50} value of of 10.95 µM[35] and 3-8 µM[36], respectively.

Conclusion

The present study has demonstrated HPLC-PDA based isolation and LC-MS-MS detection of a flavonoid compound from *T. purpurea*, a possible potential candidate drug for the treatment of neurodegenerative diseases. It needs further characterization for its therapeutic potential and structural novelty.

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