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DISCOVERY OF NOVEL ANTI-CANCER COMPOUND FROM THE IDENTIFIED PHYTOCONSTITUENT OF "JABUNG" AN HERBAL MEDICINAL PLANT

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ABSTRACT: The everlasting herbal rich north-east India has many successful stories on herbal medicines towards the treatment of different ailments, which was evidentially proven by many herbal medicinal practitioners. The Pakyntein Indigenous Herbal Medicine and Research clinic is the one among many herbal clinics. The rhizome of *Stephania Hernandifolia* is actively used by Dr. Pakyntein as a traditional medicine for the treatment to cure cancer in the tribal area of Meghalaya, north-east India. Jabung is the local name of this rhizome. The potential curing action of this rhizome for the various types of cancer treatment led us to undertake a thorough study and identified a major phytochemical namely, dl-Tetrahydropalmitine (THP) of molecular mass 355Da and its hydroxyl analogue (Stepholidine) present in the trace level. Cell-line studies showed that THP has anti-cancer activities with respect to colorectal, lung and breast cancer. During the induced-fit docking analysis, the Glide energy score showed that the molecular interactions were found to be in the increasing order, when the stimulation was done with the replacement of methoxy group with hydroxyl group one by one in the identified structure of THP. The results led us to design a novel anti-cancer compound by replacing the entire four methoxy groups in THP with hydroxyl groups. This newly discovered compound with molecular mass 299Da was synthesized from the tetrahydropalmatine isolated from the extract of Jabung through chemical synthesis and completely characterized though LCMS and LCMSMS analyses. The work on cell-line studies is being carried out for this novel compound.

KEYWORDS: Jabung; Stephania Hernandifolia; Phytochemical identification; Herbal medicine; Cancer treatment; Indian herb.

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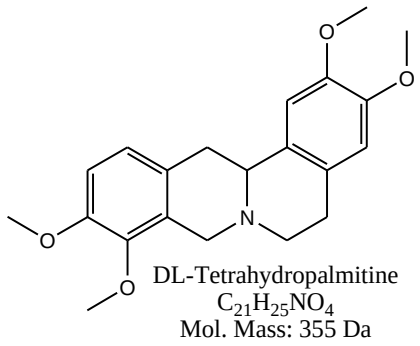
1.INTRODUCTION

The rhizome Jabung was made into small pieces and shade dried. The dried pieces shown in Fig.1, were taken and soaked in the different polar solvents, namely, Water, Ethanol, Methanol, Ethylacetate, Methylenechloride and Hexane to identify the potential phytoconstituents with various polarities. The prepared solvent extracts were subjected to HPLC analysis and we identified one of the major peaks at 32min in all the organic solvent extracts. The highest percentage 86.98% of this major peak is observed in ethylacetate extract along with other smaller peaks (which are also present in the other extracts with different proportions) having total percentage of 13.02%. Since all the peaks are present in ethylacetate extract, further studies have been carried out with the ethylacetate extract of Jabung. Upon GCMS [1] LCMS & LCMSMS analyses of ethylacetate extract, the molecular mass of the major peak is identified as 355 Da, whose structure is reported and confirmed as dl-Tetrahydropalmitine (shown in Fig.1) using single crystal XRD by Suhitha et al 2015.

Picture of Jabung



Structure of DL-Tetrahydropalmitine

Fig.1: The picture of Jabung and the structure of DL-Tetrahydropalmitine

During, further screening of the ethylacetate extract of the rhizome of *Stephania Hernandifolia*, [10-18] we have identified the presence of monohydroxy THP (7.27%) with molecular mass 341Da and di-hydroxy compound of THP (1.85%) with molecular mass 327Da, namely, Stepholidine [2], whose structures are shown in Fig.2.

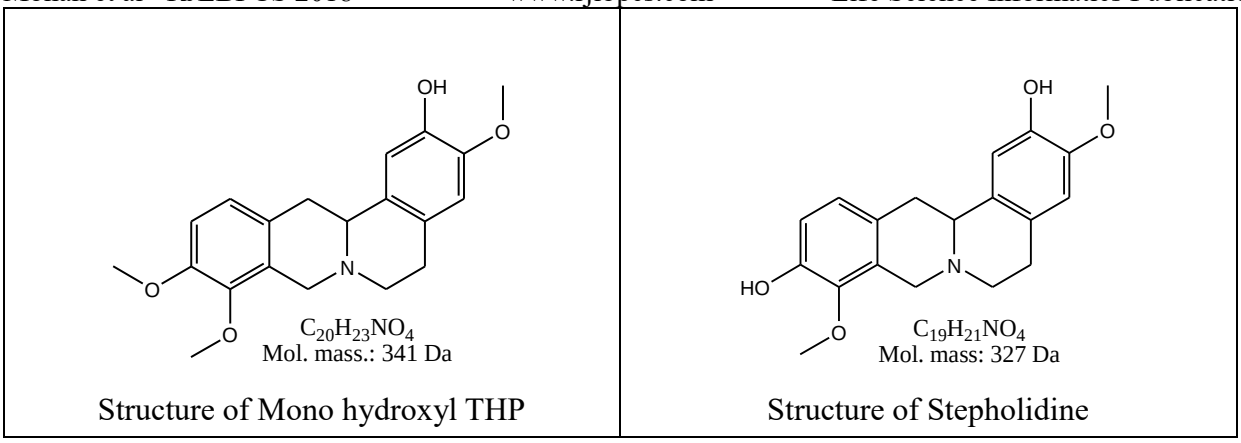


Fig.2: The structure of Monohydroxy-THP and Stepholidine.

The above identified compounds were subjected to *in-silico* analysis [19-21] along with THP and the compound with hydroxyl group (instead of methoxy group in the structure) is found to have more molecular interaction when compared with THP. This induced us to design a novel compound structure by replacing all the methoxy groups in the THP by hydroxyl group with molecular mass 299Da and found that the novel compound has better molecular interaction, which is identified through the observed Glide energy and Docking score. Further, to confirm the existence of this novel compound shown in Fig.3, we have isolated the THP using prep-HPLC from the ethylacetate extract of Jabung and subjected to chemical reaction with Borontribromide to replace the entire four methoxy groups with hydroxyl group. This successful one-pot reaction produced 54% of the required hydroxyl compound with molecular mass 299Da, evidenced through LCMS analysis. Further prep-HPLC isolated fraction gave us with the HPLC purity 94.5% of the final compound, the complete detailed process has been captured in this article. The synthesized compound has been confirmed through LCMS, LCMSMS and NMR characterization.

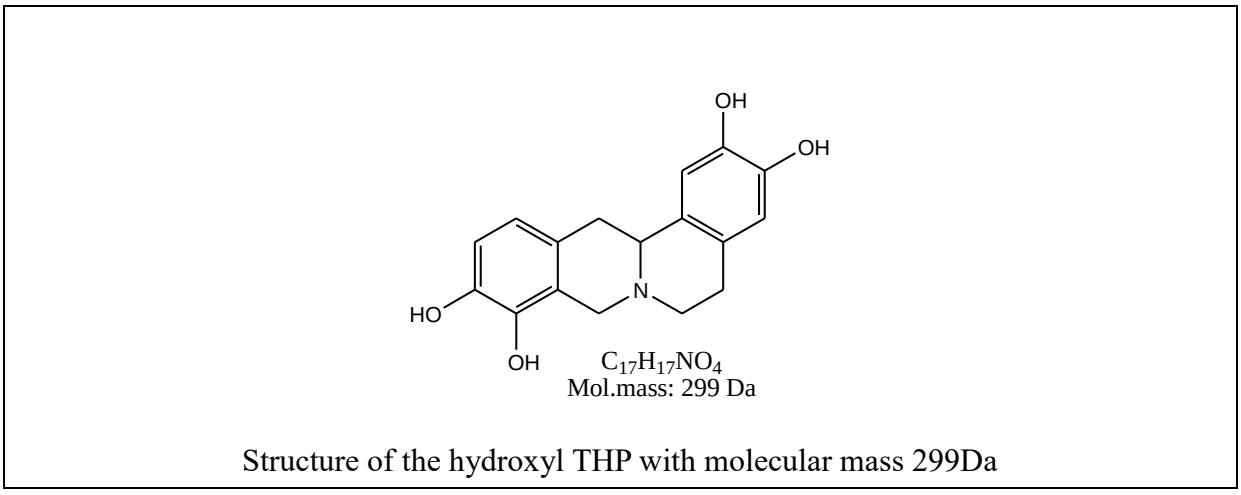
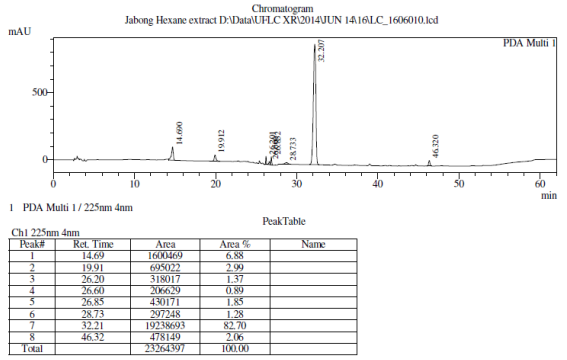


Fig.3: The structure of the newly designed compound from the isolated THP.

2. MATERIALS AND METHODS

Jabung, *Stephania Hernandifolia* was collected from the Pakyntein Indigenous Herbal Medicine and Research, West Jaintia Hills, Jowai, Meghalaya. A sharp knife was used to cut the rhizome into small pieces and shade dried for a week time and used for this study. The organic solvents Ethanol, Methanol, Ethylacetate, Methylenechloride, Hexane and Acetonitrile were used from the make of Merck analytical grade. The Millipore purified water has been used for the aqueous extraction as well as the entire HPLC, LCMS analysis [22-30] and prep-HPLC isolations. The Liquid Chromatographic analysis was carried out using Phenomenex Gemini NX RPC18 column of particle size 5µm (250mm x 4.6mm) with mobile phase consisting of 10mM Ammonium formate (make: Merck) in water :acetonitrile. The outlet of the LC-PDA detector was connected to the SHIMADZU LCMS-8040 ESI interface having the needle voltage of ±4.5kV, desolvation line temperature 250°C, heat block temperature 270°C. Nitrogen gas was used as nebulizer and drying gas with the flow rate of 2.80L/min and 14.00L/min, respectively. The detection mass range was set from 50 Da to 2000 Da. Argon gas was used as a collision gas while carrying out the MSMS operations to obtain characteristic fragment ions of the phytoconstituents of *Jabung*. The HPLC chromatogram and the table of the extracts of Jabung are shown in Fig.4

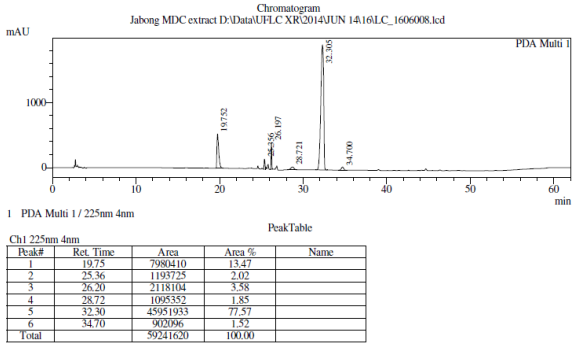


PeakTable

Ch1 225nm 4nm

Peak#	Ret. Time	Area	Area %	Name
1	14.69	1600469	6.88	
2	19.91	695022	2.99	
3	26.20	318017	1.37	
4	26.60	206629	0.89	
5	26.85	430171	1.85	
6	28.73	297248	1.28	
7	32.21	19238693	82.70	
8	46.32	478149	2.06	
Total		23264397	100.00	

Hexane extract

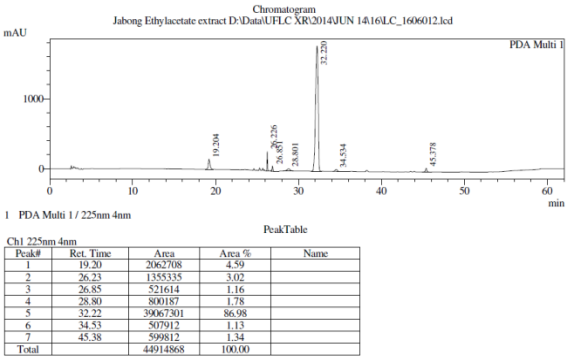


PeakTable

Ch1 225nm 4nm

Peak#	Ret. Time	Area	Area %	Name
1	19.75	7980410	13.47	
2	25.36	1193725	2.02	
3	26.20	2118104	3.58	
4	28.72	1095352	1.85	
5	32.30	45951933	77.57	
6	34.70	902096	1.52	
Total		59241620	100.00	

Dichloromethane Extract

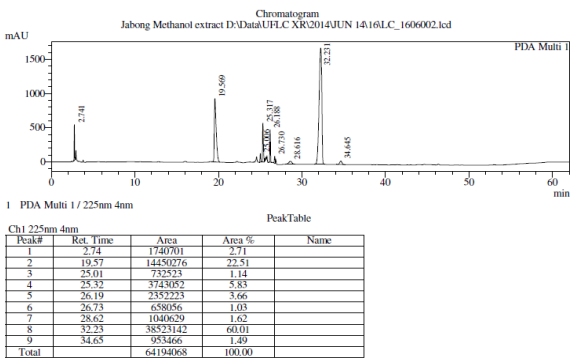


PeakTable

Ch1 225nm 4nm

Peak#	Ret. Time	Area	Area %	Name
1	19.20	2062708	4.59	
2	26.23	1355335	3.02	
3	26.85	521614	1.16	
4	28.80	800187	1.78	
5	32.22	39067301	86.98	
6	34.53	507912	1.13	
7	45.38	599812	1.34	
Total		44914868	100.00	

Ethylacetate – Extract



PeakTable

Ch1 225nm 4nm

Peak#	Ret. Time	Area	Area %	Name
1	2.74	1740701	2.71	
2	19.57	14450276	22.51	
3	25.01	732523	1.14	
4	25.32	3743052	5.83	
5	26.19	2352223	3.66	
6	26.73	658056	1.03	
7	28.62	1040629	1.62	
8	32.23	38523142	60.01	
9	34.65	953466	1.49	
Total		64194068	100.00	

Methanol- Extract

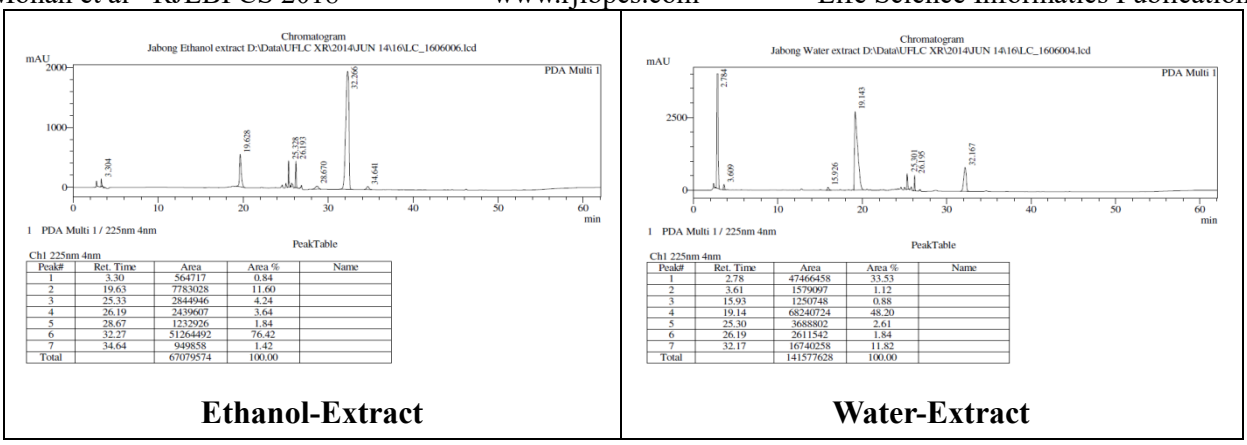


Fig.4: The HPLC Chromatogram and its purity table – Extracts of Jabung

3. RESULTS AND DISCUSSION

3.1 Identification of phytochemicals of Jabung by LCMS

The ethylacetate extract of Jabung has been subjected to LCMS analysis and we identified the molecular masses of the major phytoconstituents present in the extracts. The HPLC (LCMS) chromatogram of the ethylacetate extract is shown in Fig.5.

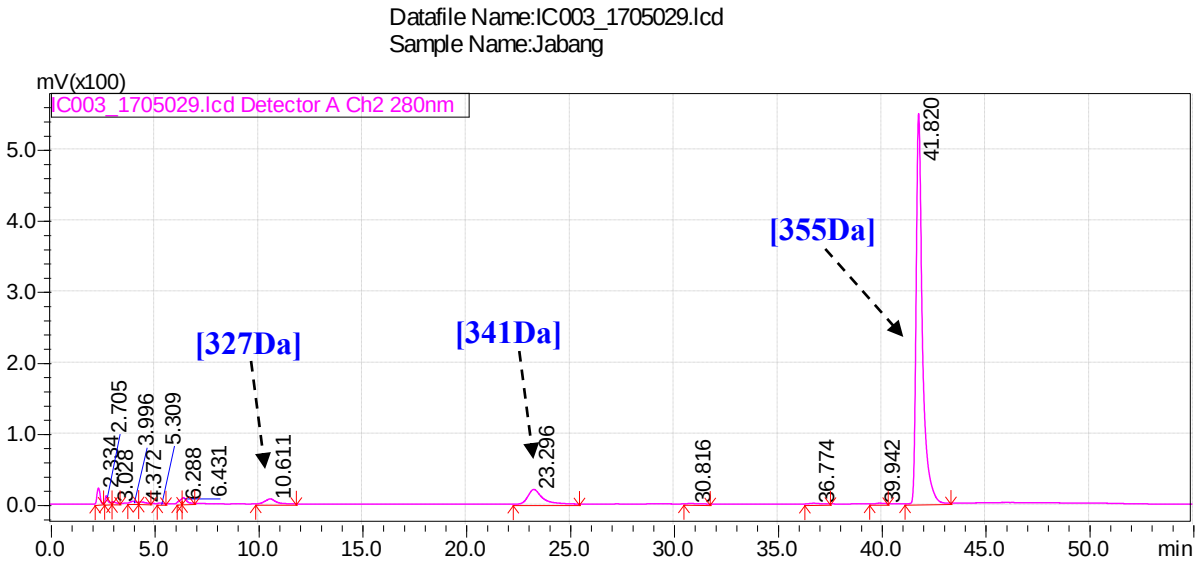


Fig.5: The HPLC Chromatogram from LCMS analysis of Ethylacetate extract of Jabung

LCMS analysis of peak at RT 41.82min (DL-Tetrahydropalmitine)

The quasi molecular ion of the compound at retention time 41.82min was identified as m/z356 using ESI positive ionization. This molecular ion m/z 356[M+1] was confirmed by its acetonitrile adduct formation at m/z 397. Therefore, the molecular ion of the major peak was identified as 355 Da. Further the identified quasi molecular ion m/z 356 was subjected to MSMS fragment analysis using SHIMADZU LCMS-8040 Triple Quadrupole System with ESI positive ionization mode under eight different collision energies ranging from -5V to -60V (-5V, -10V, -15V, -25V, -35V, -40V, -50V & -60V) to understand the structural fragments. It is known that the lower collision energies produce the major fragments and the higher collision energies produce the complete smaller fragments of

the structure. Hence the different collision energies were chosen in the collision cell of mass spectrometer. This approach is called nMS^2 technique in the triple quadrupole mass spectrometry. This will aid us to understand the real MSMS data and its consistency among the electronic ion artefacts. The collision of the molecular ion m/z 356 produced the MSMS product ions of m/z 192, m/z 176, m/z 165, m/z 148, m/z 131, m/z 107, m/z 103 and m/z 77. The mass spectrum and MSMS spectra of the identified DL-Tetrahydropalmitine are shown in Fig.6. The proposed MSMS fragmentation pathway for the identified compound DL-Tetrahydropalmitine is shown in Fig.7.

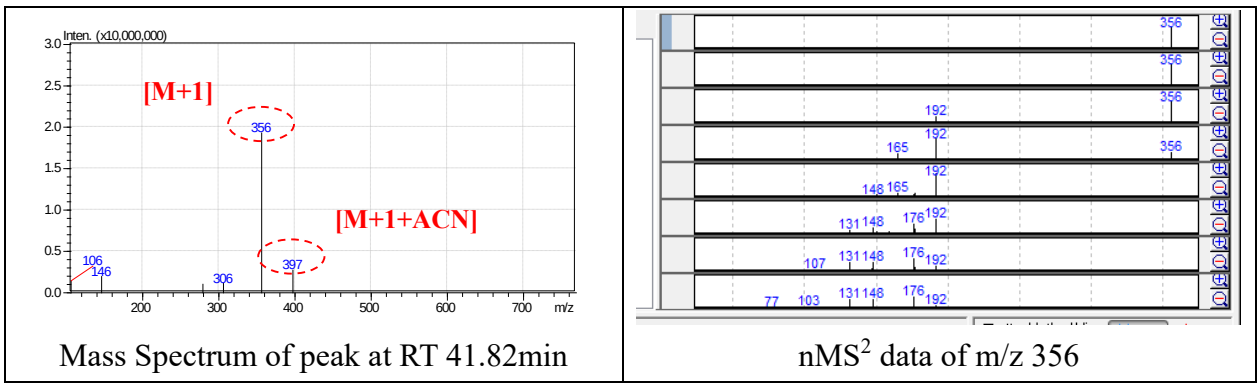


Fig.6: The MS and MSMS spectra of peak at RT 41.82min

The proposed MSMS fragmentation pathway of the identified DL-Tetrahydropalmitine

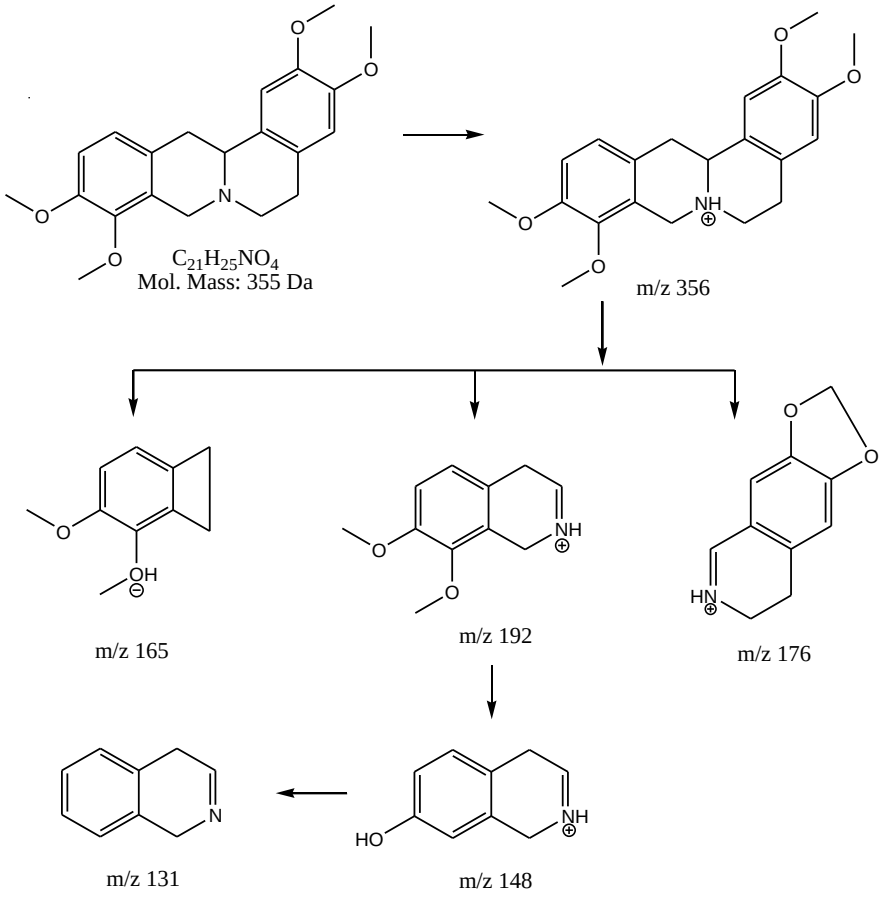


Fig.7: The proposed MSMS fragmentation pathway of DL-Tetrahydropalmitine

LCMS analysis of peak at RT 23.29min (mono hydroxyl-Tetrahydropalmitine)

The identified quasi molecular ion of the peak at 23.29min is m/z 342 [M+1], further this molecular ion is confirmed through the presence of its acetonitrile adduct of m/z 383 [M+1+41]. Therefore the molecular mass of this peak is 341 Da. The ion m/z 342 was subjected to MSMS analysis under eight different collision energies in ESI positive ionization mode and the MSMS spectra were obtained. The MS and MSMS spectra are shown in Fig.8.

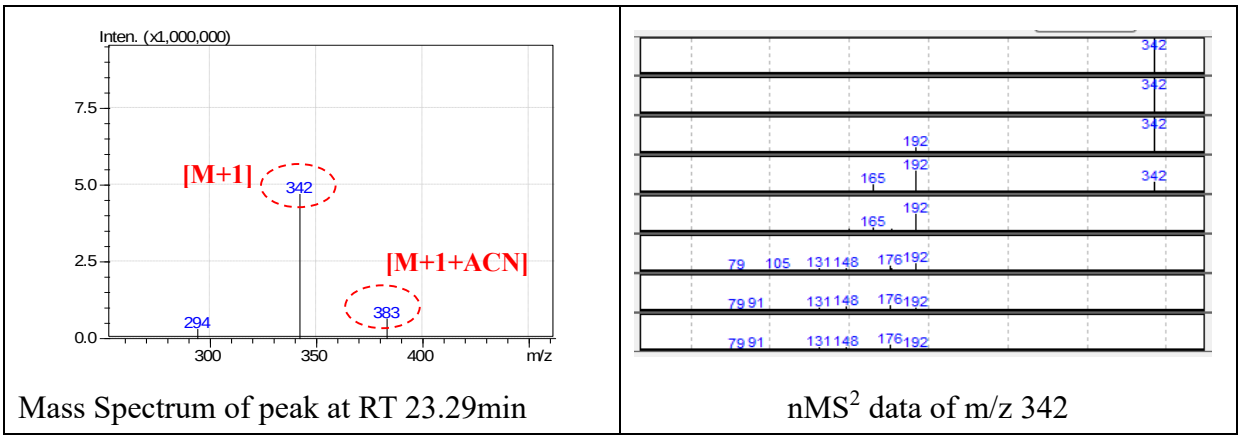


Fig.8: The MS and MSMS spectra of the peak at RT 23.29min

Based on the above mass spectral data, the identified structure of the compound with molecular mass 341 Da is the mono hydroxyl of THP shown in Fig.9. The proposed MSMS fragments confirm the structure of mono hydroxyl of THP shown in Fig.10

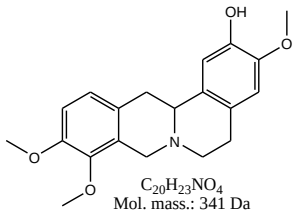


Fig.9: The structure of monohydroxy THP

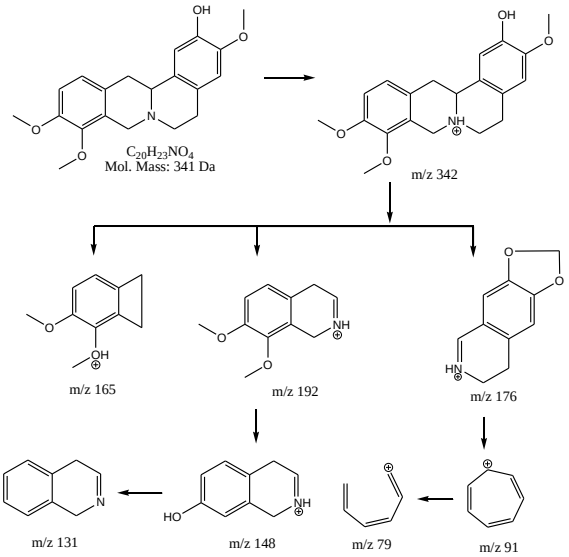


Fig.10: The proposed MSMS fragmentation pathway of mono hydroxyl THP

LCMS analysis of peak at RT 10.61min (Stepholidine)

The identified quasi molecular ion of the peak at 10.61min is m/z 328 [M+1], further this molecular ion is confirmed through the presence of its acetonitrile adduct of m/z 369 [M+1+41]. Therefore the identified molecular mass of this peak is 327 Da. The ion m/z 328 was subjected to MSMS analysis under eight different collision energies in ESI positive ionization mode and the MSMS spectra were obtained. The MS and MSMS spectra are shown in Fig.11.

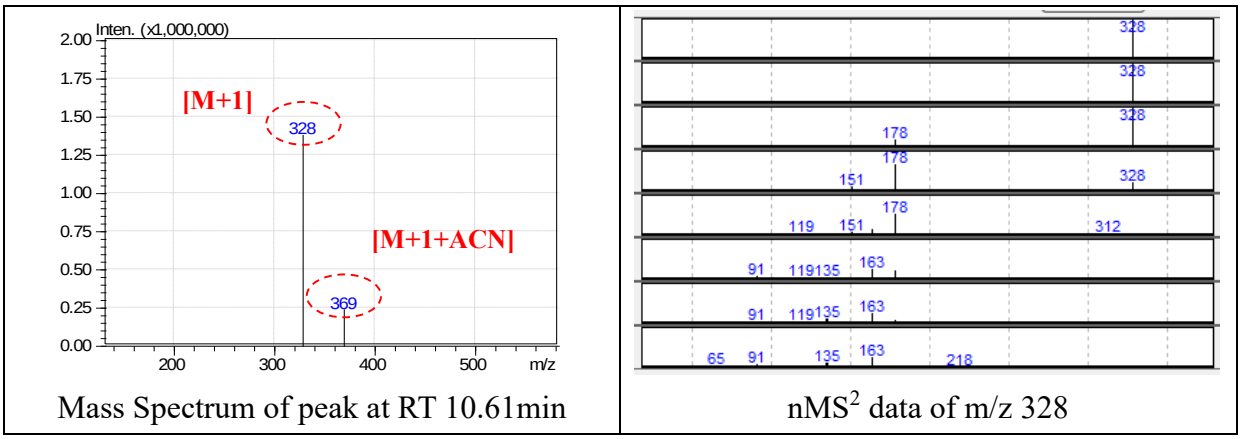


Fig.11: The MS and MSMS spectra of the peak at RT 10.61min

Based on the above mass spectral data, the identified structure of the compound with molecular mass 327 Da is the di hydroxyl of THP, i.e. Stepholidine shown in Fig.12. The proposed MSMS fragments confirm the structure of di hydroxyl of THP shown in Fig.13

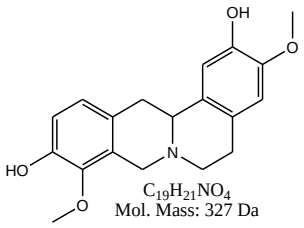


Fig.12: The structure of dihydroxyl THP

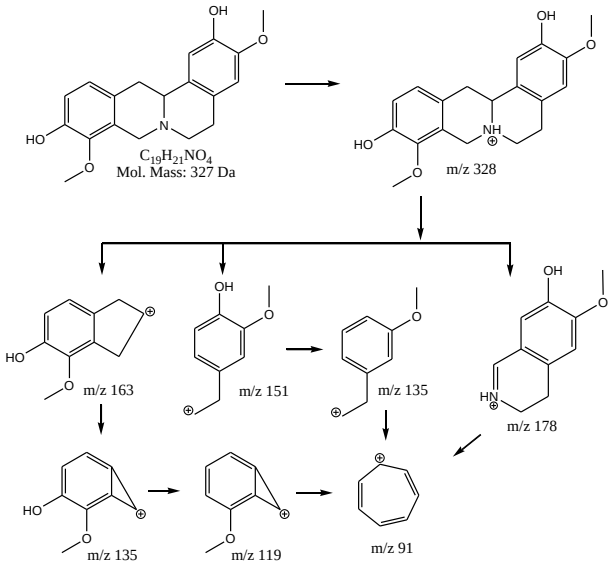
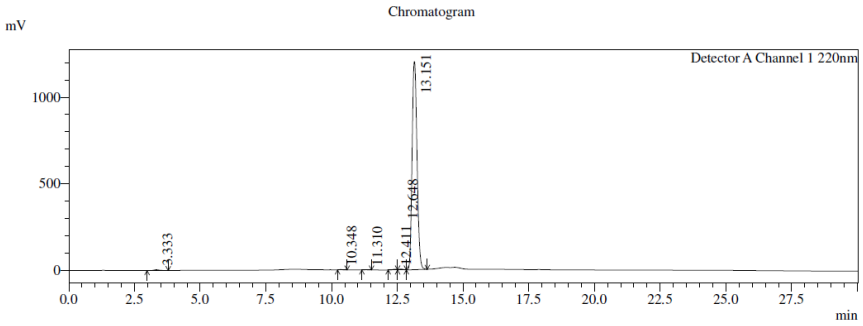


Fig.13: The proposed MSMS fragmentation pathway of di hydroxyl THP

Based on the above identified structures, we have explored the possibility and designed a novel compound by replacing the entire four methoxy groups of the THP by hydroxyl group synthetically. For this, we have isolated the THP from the Jabung using the Shimadzu semi-Prep-HPLC instrument. The ethylacetate extract of Jabung was used for the isolation of THP. The HPLC purity of THP before purification was 86.98% and after purification was 98.71% by area normalization method. The HPLC purity chromatogram (tested in shorter runtime method) of the prep-HPLC purified THP is shown in Fig. 14.



Peak Table

Detector A Channel 1 220nm

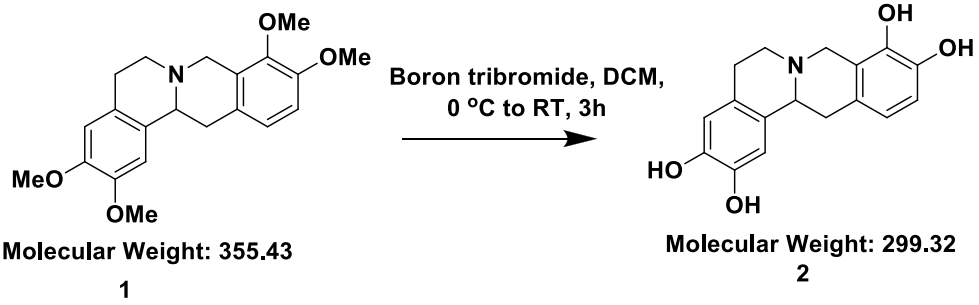
Peak#	Ret. Time	Area	Area%	Name
1	3.33	64535	0.41	
2	10.35	19626	0.13	
3	11.31	13043	0.08	
4	12.41	44060	0.28	
5	12.65	59716	0.38	
6	13.15	15392889	98.71	355Da
Total		15593870	100.00	

Fig.14: The HPLC chromatogram of THP Prep-HPLC isolated

The above purified THP was subjected to the chemical reaction with borontrifluride as per the following procedure.

3.2 Synthesis of a Novel Tetrahydroprotoberberine with MW: 299 Da.

The compound tetrahydropalmatine isolated from the ethylacetate extract of *Stephania Hernandifolia* has been used for the synthesis of Tetrahydroprotoberberine (MW: 299 Da). The reaction scheme (shown in Fig.15) and the process of reaction carried out at the lab have been described below.



Molecular Weight: 355.43

1

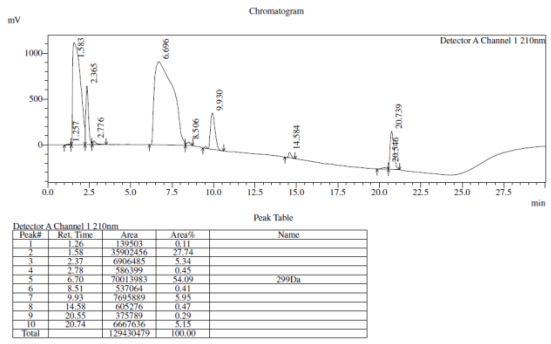
Molecular Weight: 299.32

2

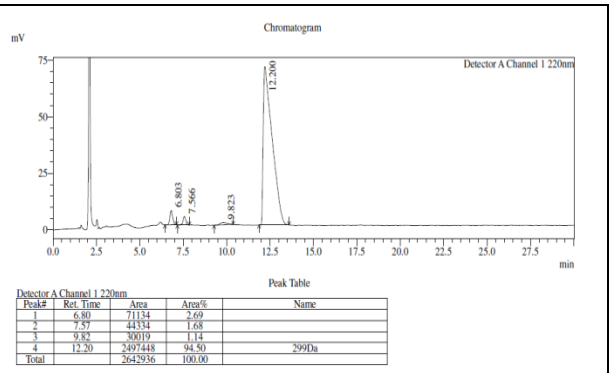
Fig.15: The Synthetic reaction scheme of Novel Tetrahydroprotoberberine (THPB)

To a suspension of 1 (100mg, 0.281mmol) in dichloromethane (10mL) cooled to 0°C, boron tribromide (0.162mL, 1.68mmol, make: Avra chemicals, Hyderabad) dissolved in 3mL of methylenedichloride was added dropwise over 5 minutes such that the internal temperature was maintained below 10°C. The resulting mixture was stirred at 0°C to 10°C for 30 minutes and at 30°C

for 3 hours. Then methanol and water 1:1 (5 mL) was added dropwise to quench the reaction. The progress of the reaction was monitored through LCMS analysis by identifying the molecular mass of the product. The formation of the compound with molecular mass 299 was found to be 54.09% in HPLC. Further, the crude material obtained from the successful chemical reaction was subjected to Prep-HPLC purification and isolated few mg with HPLC purity 94.50% of the novel hydroxyl compound namely, Tetrahydroprotoberberine with MW: 299Da. The HPLC chromatograms of the crude and purified Tetrahydroprotoberberine are shown in Fig.16. The mass spectrum and its nMS2 data are shown in Fig.17. The proposed MSM fragmentation pathway is shown in Fig.18.

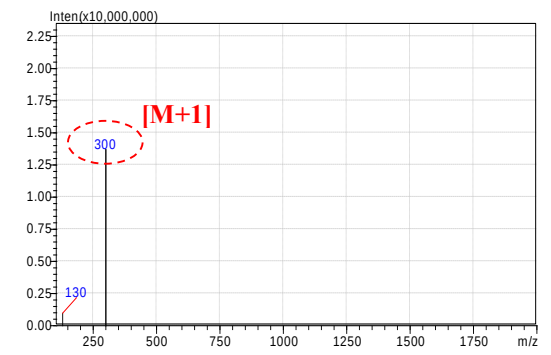


The HPLC chromatogram of the synthesized crude Tetrahydroprotoberberine

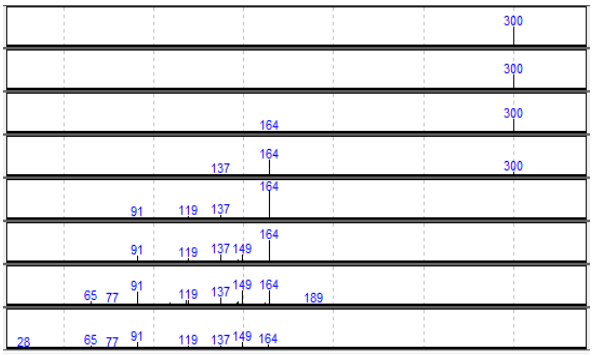


The HPLC chromatogram of the synthesized & purified Tetrahydroprotoberberine

Fig.16: The HPLC chromatograms of the synthesized crude and purified Tetrahydroprotoberberine



Mass Spectrum of peak at RT 10.61min



nMS^2 data of m/z 328

Fig.17: The mass spectrum and its nMS2 data of synthesized Tetrahydroprotoberberine (MW 299Da)

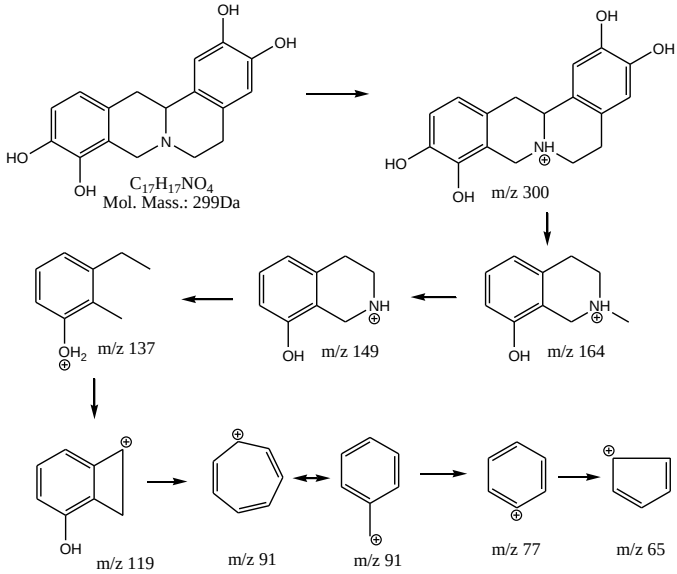
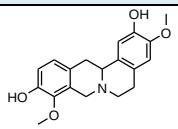
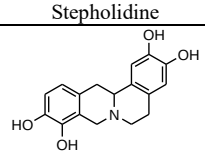
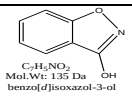


Fig.18: The proposed MSMS fragmentation pathway of Tetrahydroprotoberberine

3.3 *In-silico* studies – Induced Fit Docking

The literature survey revealed the Dopamine and σ receptor affinity of stepholidine, one of the tetrahydroprotoberbarine alkaloids [2]. The authors have made the detailed study on the docking of stepholidine with the two receptors and also carried out molecular dynamics stimulations studies. The Biological evaluation of the affinity of this compound was also reported along with various other substitutions. These authors have used Glide program for validating the docking results. The docking studies [19-21] of THP are already reported from our group for various breast cancer targets like Protein Kinase Cθ, p38alpha MAP Kinase, Abl Tyrosine Kinase and Bcl2 [1]. In the present paper, docking studies of Tetrahydroprotoberberine [THPB] were carried out for the above four targets and the comparison with THP showed the best binding of THPB with p38alpha MAP Kinase, Abl-Tyrosine Kinase and also Protein Kinase Cθ as targets. Further, the identified compounds, namely, Tetrahydropalmitine, Monohydroxy Tetrahydropalmitine, Dihydroxy Tetrahydropalmitine, trihydroxy Tetrahydropalmitine and the designed novel compound Tetrahydroprotoberberine (MW 299Da) from Jabung have also been subjected to molecular docking in induced fit mode (the receptor site and the ligand are made flexible) using the Glide program of commercial Schrodinger suite (Schrodinger LLC, USA 2014) [6]. All permutations for the mono, di and tri-hydroxy substitutions in THP in the place of methoxy groups have been studied in docking. The breast cancer target Phosphoglycerate dehydrogenase (PHGDH) of PDB ID: 5NZP [9], σ1 receptor with PDB ID: 6DK0 [7] and D3 receptor [2] with PDB ID: 3PBL [8] were used for the binding of the ligand to understand the mechanistic pathway of interactions between the target proteins and ligands. The values of docking scores and Glide energies are listed in Table-1. The table listed below shows the results of docking studies for the two better permutations of the hydroxyl substitutions instead of the methoxy groups in THP. The corresponding Ligplots are also shown.

Table 1: Results of docking studies with Breast Cancer Target PDB ID: 5NZP

Ligand	Docking Score	Glide Energy	Interaction	Distance Å	Hydrophobic Interaction
 Stepholidine	-6.724	-38.241	-	-	Leu151, Tyr174, Pro176, Leu193, Trp197, Pro208, Leu210 and Leu216
 Tetrahydroprotoberberine (MW: 299Da)	-6.824	-41.946	(O-H...O)Asp175 Ser212(O-H...O) (O-H...O)Ser212	3.06 2.94 2.98	Leu151, Leu153, Tyr174, Pro176, Ile177, Leu193, Pro208, Leu210 and Leu216
 Native	-6.256	-26.262	Asp175(O-H...O)	3.15	Leu151, Leu153, Tyr174, Pro176, Ile177, Pro208, Leu210 and Leu216

Tetrahydoprotoberberine (MW:299Da) shows better binding than the other combination and also than that of the co-crystal.

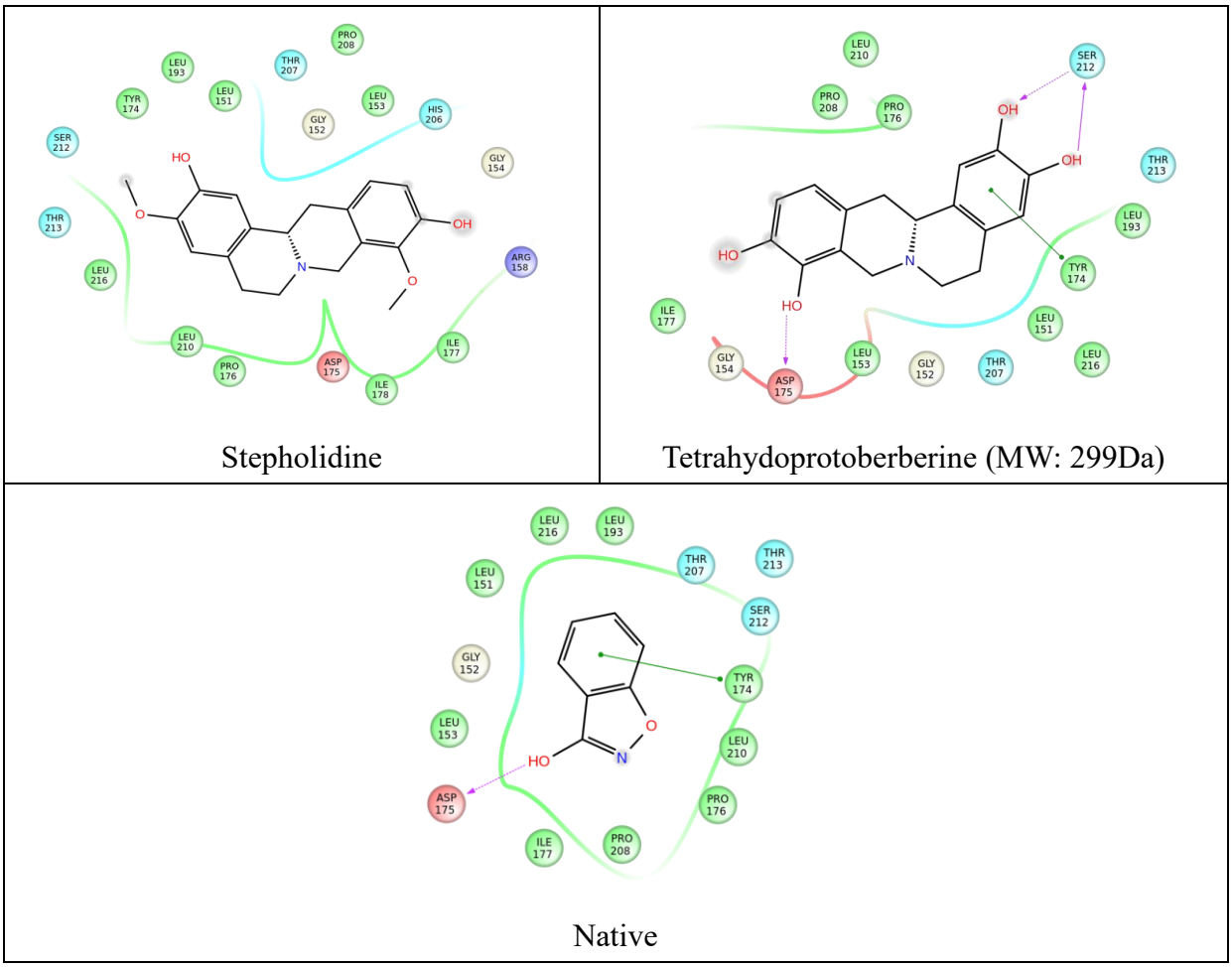
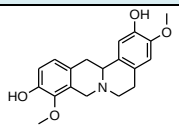
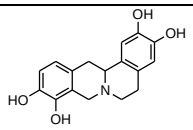
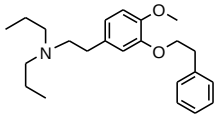


Fig.19: The ligand interaction diagram with Breast Cancer Target PDB ID: 5NZP

Table 2: Results of docking studies of σ1 receptor with PDB ID: 6DK0

Ligand	Docking Score	Glide Energy	Interaction	Distance Å	Hydrophobic Interaction
 Stepholidine	-8.811	-54.819	(O-H...O)Tyr120 (O-H...O)Gln135	3.04 3.18	Val84, Trp89, Met93, Leu105, Phe107, Tyr120, Ile124, Phe133, Val152, Val153, Val162, Trp164, Met170, Phe184 and Ala185.
 Tetrahydroprotoberberine (MW: 299Da)	-7.069	-49.288	(O-H...O)Asp126 (O-H...O)Thr181	3.04 2.94	Val84, Trp89, Tyr103, Phe107, Tyr120, Ile124, Phe133, Val152, Val162, Trp164, Met170, Leu182 and Ala185.
 N-(4-methoxy-3-(phenethyloxy)phenethyl)-N-propylpropan-1-amine Native	-7.834	-55.524	Tyr89(O-H...O) Tyr120(O-H...O)	3.05 3.01	Val84, Ala86, Trp89, Met93, Leu95, Tyr103, Leu105, Phe107, Ile124, Ile128, Phe133, Val152, Val153, Val162, Trp164, Met170, Leu182, Phe184 and Ala185.

The Stepholidine shows better binding when compared with the other combinations and also with that of the co-crystal.

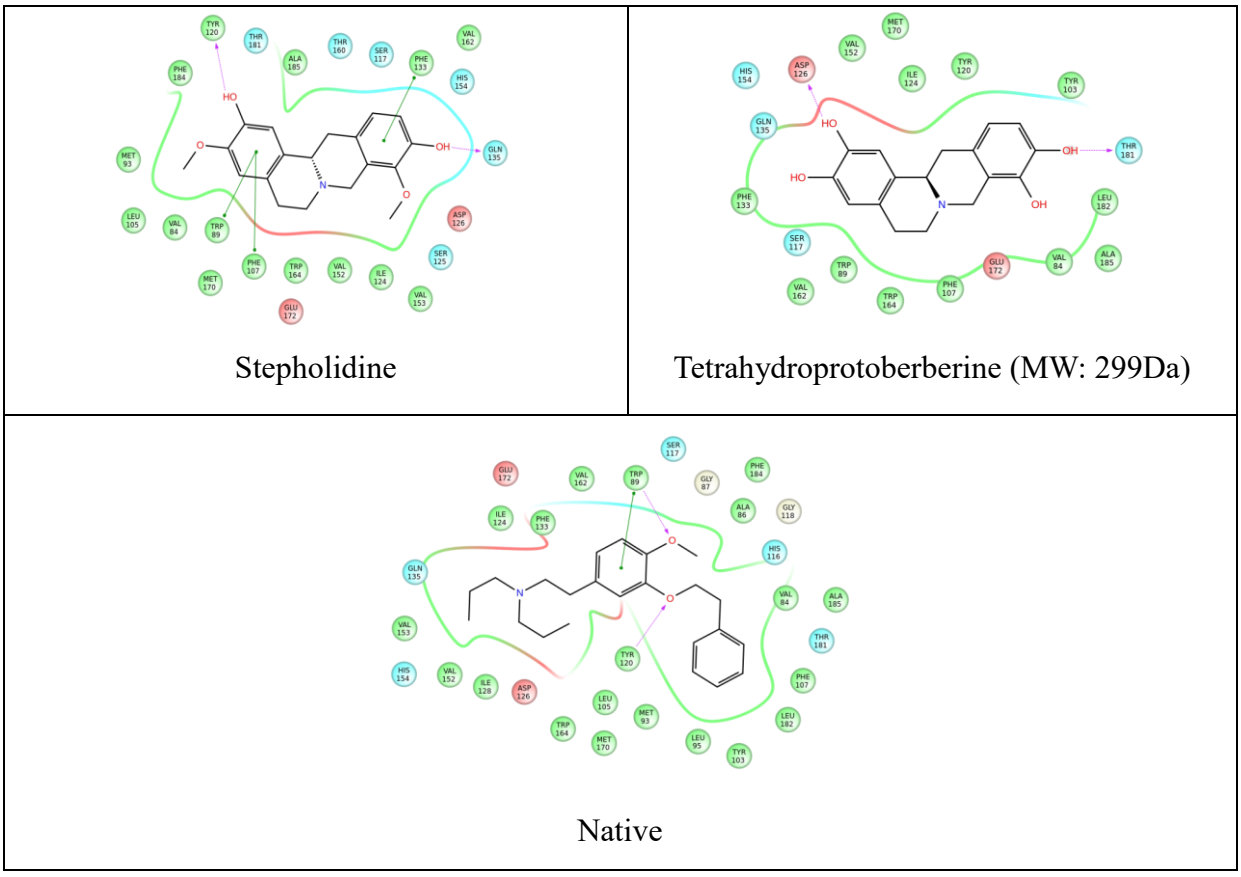
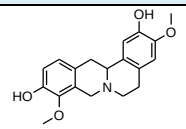
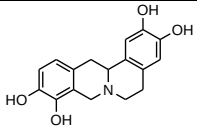
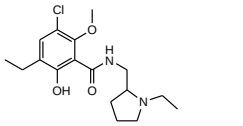


Fig.20: The ligand interaction diagram of σ1 receptor with PDB ID: 6DK0

Table 3: Results of docking studies of D3 receptor with PDB ID: 3PBL

Ligand	Docking Score	Glide Energy	Interaction	Distance Å	Hydrophobic Interaction
 Stepholidine	-7.436	-49.094	Tyr36(O-H...O) (O-H...O)Glu90 (O-H...O)Asp110	3.05 2.89 3.12	Val86, Leu89, Phe106, Val107, Val111, Cys181, Ile183, Phe345, Tyr365 and Tyr373.
 Tetrahydroprotoberberine (MW: 299Da)	-8.636	-52.840	(O-H...O)Cys181 (O-H...O)Ser192 (O-H...O)Ser192 (O-H...O)Tyr365	3.02 2.94 2.98 3.18	Leu89, Phe106, Val107, Val111, Ile183, Phe188, Val189, Phe345, Phe346 and Val350.
 3-chloro-5-ethyl-N-((1-ethylpyrrolidin-2-yl)methyl)-6-hydroxy-2-methoxybenzamide Native	-7.876	-51.950	(O-H...O)Asp110 (N-H...O)Asp110 His349(O-H...O)	3.15 3.06 2.97	Leu89, Phe106, Val107, Val111, Cys114, Cys181, Ile183, Phe188, Val189, Trp342, Phe345, Phe346, Tyr365 and Tyr373.

Tetrahydoprotoberberine (THPB, MW: 299Da) shows better binding than the other combination and also than that of the co-crystal.

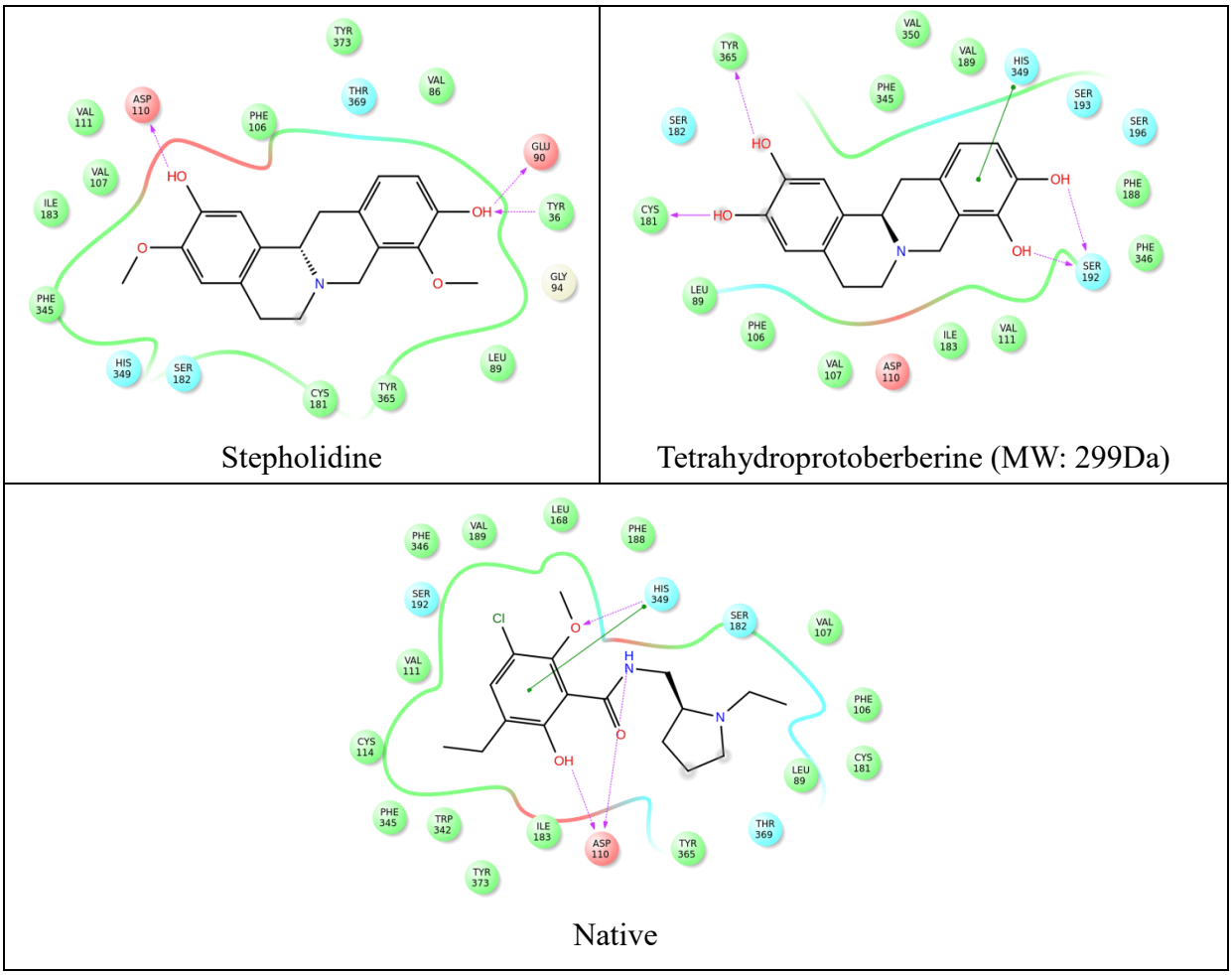


Fig.21: The ligand interaction diagram of D3 receptor with PDB ID: 3PBL

4. CONCLUSION

Our earlier work on docking studies of THP with the four cancers related targets showed good binding with receptor sites. The docking results indicate better binding of newly discovered THPB with D3 and σ receptor than the Stepholidine. THPB binding results with the four anti-cancer targets listed above show better binding of THPB with the receptor sites compared to THP. Cell line studies are under way for THPB with different cancer targets.

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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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