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Original Article

Anti-Alzheimer's Potential of Different Varieties of *Piper betle* Leaves and Molecular Docking Analyses of Metabolites

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ABSTRACT

Introduction: Acetylcholinesterase inhibitors are used to prevent symptoms of Alzheimer's disease which is initiated due to oxidative stress. *Piper betle* L. is a tropical evergreen perennial vine whose leaves are widely consumed as masticator in Asia and has medicinal properties. **Objectives:** The present study is aimed to investigate acetylcholinesterase inhibitory property of methanolic extracts of different varieties of *Piper betle* leaves and chemometrically identify different bioactive ingredients *in vitro* and *in silico*. **Materials and Methods:** Methanol extracts of the leaves collected in February and October from eight varieties of *P. betle* (Chhanchi, Bagerhati, Manikdanga, Kalibangla, Bangla, Ghanagete, Meetha and Haldi) were studied for acetylcholinesterase inhibitory properties. Chemical components were analyzed by Gas Chromatography – Mass spectrometry and High Performance Thin Layer Chromatography. Active metabolites were identified chemometrically. The activities were proved *in vitro* and *in silico*. **Results:** All the extracts inhibited acetylcholinesterase. Statistical analysis suggested that several phenolic compounds were correlated to anti-cholinesterase activity. Piceatannol, hydroxychavicol, benzene-1,2,4-triol, and 4-methylcatechol are reported here to have such enzyme inhibitory properties. These four small molecules were further subjected to molecular docking analysis to explore their binding mechanism with the acetylcholinesterase enzyme. All the four small molecules are found to interact with the targeted enzyme in similar fashion like the molecular interactions observed for the standard inhibitor, Donepezil, at the active site of acetylcholiesterase. **Conclusion:** Thus, consumption of *P. betle* leaves may have a beneficial effect in the prevention and treatment of this neurodegenerative disease.

Key words: *Piper betle*, Acetylcholinesterase, GC-MS, Molecular docking, HPTLC, Phenol.

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INTRODUCTION

Alzheimer's Disease (AD), a multifactorial neurodegenerative disorder, is a growing health challenge globally.[1] The symptoms include dementia, apraxia, aphasia, depression, a short attention span, visuospatial navigation deficits, anxiety, and delusions.[2] The disease is caused due to formation of pigmentation (aggregation of amyloid-β peptide) known as senile plaque, perturbed copper, iron, and zinc homoeostasis, metal induced oxidative stress, neuroinflammation, and abnormal activity of acetylcholinesterase.[3] Different hypotheses proposes that formation of amyloid-β peptide is linked with the formation of Reactive Oxygen Species (ROS). Various markers of oxidative stress have been found in the brains of the patients of AD.[3] The senile plaque destroys nerve cells in the brain impairing neurotransmission.[4] According to cholinergic hypothesis, the neuropsychological impairments occurring in AD are consequences of cholinergic disturbances.[5] Hence, it is necessary to develop strategies to boost cholinergic neurotransmission.[6] Inhibition of the enzyme, acetylcholinesterase (AChE) which causes hydrolysis of the neurotransmitter acetylcholine is one of the possible approaches towards improvement of cholinergic function in the brain.[7] Therefore, inhibitors of the enzyme form the principal category of drugs that are presently used in the treatment of AD. There are a few naturally occurring acetylcholinesterase inhibitors (AChEi). Tacrine, rivastigmine, galantamine, donepezil, memantine, mainly cholinergic drugs, have a brief duration of action and are associated with adverse effects.[8] There is a continuous effort throughout the world to explore different plant species for assessment of acetylcholinesterase inhibitory potential and to generate new drug candidates.[9]

Piper betle L. (Piperaceae) is a shade loving tropical evergreen perennial vine. The leaves are edible and largely used for mastication in South Asian countries. The leaves are medicinally important and have experimentally been shown to possess several bioactivities.[10,11] Acetylcholinesterase inhibitory property of the aqueous extracts of three varieties of *P. betle* leaves were reported earlier by Das and De.[12] *P. betle* leaf extract improved the learning and memory functions in aluminium chloride induced AD in rat.[13] The purpose of the present study was to explore *in vitro* acetylcholinesterase inhibitory properties of methanolic extracts of eight varieties of *Piper betle*

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leaves and identify different bioactive ingredients by GC-MS based metabolomics and chemometric studies. Further, molecular docking approach was utilized for elucidating the predictive binding interactions mode for all four small molecules, which revealed strong binding potentiality of all four small molecules towards AChE as compared to its known standard inhibitor or marketed drug donepezil.

MATERIALS AND METHODS

Plant Material

Leaves of *P. betle* belonging to eight varieties namely Chhaanchi (CH), Meetha (ME), Bangla (BA), Manikdanga (MA), Kalibangla (KA), Bagerhati (BG), Ghanagete (GH) and Haldi (HA) were collected from cultivation areas of West Bengal, India, in the months of October 2014 and February 2016. The voucher specimens are available in the Department.

Extraction

Methanolic extracts of eight varieties of *P. betle* leaves were prepared by boiling leaves crushed in liquid nitrogen, at 80°C for 30 min with methanol followed by evaporation of the filtrates to dryness.

In-vitro Acetylcholinesterase Inhibitory Assay

Acetylcholinesterase (AChE) inhibitory property was measured following the modified method of Ellman *et al.*[14] AChE from electric eel was used as enzyme source for the AChE assay. Different concentrations of methanolic plant extract (10 µl) were added to 20 µl AChE (19.93 unit/ml in phosphate buffer, pH 8) and 1 ml of phosphate buffer. The reaction was started by adding 10 µl of 0.5 mM 5,5-dithiobis 2-nitrobenzoic acid and 20 µl of 0.6 mM acetylthiocholine iodide solution. The reaction mixture was incubated at 37°C for 20 min. The optical density was measured at 412 nm immediately against a blank devoid of the enzyme.

Gas Chromatography – Mass Spectrometry Analysis

Agilent 7890 A GC [soft-ware driver version 4.01 (054)] interfaced with 5795C inert MSD with Triple Axis Detector was employed for Gas Chromatography – Mass Spectrometry (GC-MS) analysis. HP-5MS capillary column [Agilent J & W; GC Columns (USA)] of dimensions 30 m × 0.25 mm × 0.25 µm was used in the analytical arrangement. The method of Kind *et al.* was followed, after some modifications by Das *et al.*[15,16] The oven temperature programme for analysis was set as oven ramp 60°C (initial 1 min hold), to 325°C with an increasing rate of 10°C per min. The oven temperature was held for 10 min before cooling down producing a total run time of 37.5 min. The injection temperature was set at 250°C, the MSD transfer line at 290°C and the ion source at 230°C. Helium was used as the carrier gas with a flow rate of 0.723 ml/min (carrier linear velocity of 31.141 cm/s). Adonitol was added to the dried crude extract and was followed by derivatization using methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl) trifluoroacetamide. Fatty acid methyl ester markers prepared in chloroform was added to the sample before injection. Derivatized samples were injected via splitless mode on to the column. The fragmentation patterns of the mass spectra, retention times (RT) of samples were compared with entries of mass spectra, RT in Agilent GC-MS Metabolomics RTL Library (2008). Normalization of peak areas of different metabolites were done by dividing the peak area by dry weight of crude extract followed by area of adonitol (the internal standard used). The relative response ratios obtained by this process was further used for data interpretation.

High-Performance Thin Layer Chromatographic Analysis

Thin layer chromatography was carried out on 20 cm × 10cm precoated silica gel 60 F_{254} plates of 0.25 mm thickness and the operating software used was winCATS. 5 µl methanolic extracts and hydroxychavicol (standard) were applied on silica using Linomat 5 sample applicator (Camag, Switzerland) with the help of a 100 µl micro syringe. 6 mm wide bands were sprayed at a delivery speed of 150 nl/s utilizing the pressure of nitrogen flow. The 20 cm x 10 cm twin trough glass chamber (Camag) was pre-saturated with solvent system of chloroform and methanol mixed in the ratio of 19:1 by volume for 15 min.[17] Camag TLC scanner 4 helped in scanning all the tracks at λ = 280 nm. Spectra from 190 to 400 nm were achieved by Deuterium (D_2) and Tungsten (W) lamp sources. Slit dimension was set to 6.00 × 0.45 mm, micro. The speed of scanning was 20 mm/s. Presence of hydroxychavicol was confirmed by superimposition of spectra of all the tracks on spectra of standard.

Molecular Docking Study

Molecular docking was carried out to explore possible binding mechanism analyses of four small molecules with AChE. For this purpose, three-dimensional (3D) crystal structure of AChE was retrieved from Protein Data Bank (PDB) deposited under PDB ID: 6O4W.[18] On the other hand, all small molecules were collected from PubChem database.[19] Prior to docking execution, the protein and small molecules were prepared using AutoDock Tool (ADT) following the standard protocol and saved in pdbqt format.[20] Precisely, during protein preparation, all H_2O molecules were removed from the protein structure, whereas hydrogen atoms were added to the crystal structure. Any missing atoms were repaired in the crystal structure. Charges were calculated and adjusted to the protein structure. Minimization of the prepared protein structure was carried out in UCSF Chimera v 1.11.2 tool. On the other hand, all small molecules were prepared by adjusting Gasteiger charges and adding of hydrogens through selection of respective options in the ligand panel of ADT v1.5.6. Thereafter, configuration file was created putting the information on grid center (as center_x = 90.69 Å, center_y = 86.29 Å, center_z = -4.69 Å) and dimension or size of the grid box (as size_x = 50 Å, size_y = 50 Å, size_z = 50 Å). Other parameters were kept as default. Finally, the docking was performed employing AutoDock Vina v2.0.[21] In addition, molecular docking protocol was also validated accomplishing by re-docking of donepezil inside the active site of AChE following the same procedure. Upon successful execution of docking for all small molecules with AChE, interaction analysis was explored through a highly accessible protein–ligand interaction profiler (PLIP) tool by browsing the best posed protein-ligand complexes and hence several intermolecular interactions were detected between each molecule and AChE.[22]

Statistical Analysis

Each experiment was performed in triplicates. Percentage inhibition of enzyme activity is presented as mean ± standard deviation (SD). Regression equations were prepared from the concentrations of the extracts and percentage inhibition of enzyme activity. IC_{50} (measure of concentration of sample required to inhibit the enzyme activity by 50 percent) values were calculated from the regression equations. Multivariate analysis including PLS-DA (Partial Least Squares - Discriminant Analysis), orthogonal PLS-DA and heatmap of the identified metabolites from two groups of eight varieties of *P. betle* leaves were carried out with the help of Metaboanalyst 4.0 using log values of the relative response ratios.

RESULTS

Methanol extracts of eight varieties of *Piper betle* leaves, collected in the month of October (O) and February (F) were assayed for acetylcholinesterase inhibitory properties. All the extracts inhibited the enzyme in dose dependent manner. Based on the IC_{50} values of each extract as compared in Figure 1, it was observed that the activities showed seasonal and varietal variations. The activities of the different varieties of *P. betle* leaves in decreasing order are MA(F) > GH(F) > BG(F) > KA(F) > BA(F) > BG(O) > GH(O) > KA(O) > CH(O) > CH(F) > HA(F) > MA(O) > BA(O) > HA(O) > ME(F) > ME(O). Leaves of Manikdanga variety from February collection showed the highest activity against acetylcholinesterase enzyme and Meetha variety from both October and February collections showed the lowest activities. The activities of the leaves collected in February and October were different from each other. The activities of the extracts were also compared to that of the commercially available acetylcholinesterase inhibitor galantamine (IC_{50} value 8.243 ± 0.016 µg/ml or 22.38 ± 0.44 µM), an alkaloid extracted from different plants, and licensed for the treatment of mild to moderate AD.[23] Although the activities of the crude extracts of the leaves were less than that of galantamine, the February extracts of Manikdanga, Kalibangla, Ghanagete and Bagerhati were high being close to the standard drug.

GC-MS based metabolite profiling enabled identification of a total of 125 metabolites with the presence of several sugars and polyols, organic acids, amino acids, fatty acids, phenols, and other metabolites from the different varieties of leaf extracts. In addition to the metabolites reported earlier, a few new and important compounds were identified such as 4-isopropylbenzoic acid, 4-methylcatechol, salicylic acid, pyrogallol, etc. Hydroxychavicol content was measured by HPTLC.[11] Orthogonal PLS-DA (Figure not shown) segregated the varieties of betel leaves into two groups, namely February and October, based on their metabolite profiles. This further explains that the metabolites were highly different for each group of leaves collected at different times. PLS-DA differentiated the leaf extracts into three groups based on IC_{50} values i.e., high activity (18 to 35 µg/ml), medium activity (55 to 107 µg/ml), and low activity (123 to 360 µg/ml) (Figure not shown).

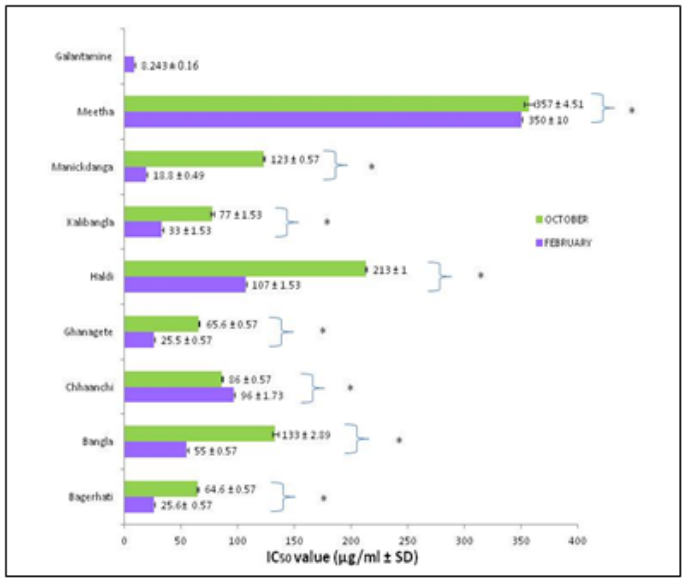

Figure 1: Comparative AChE inhibitory activity of different varieties of *P. betle* leaves.
*Significant difference in activity.

Some of the compounds were found to have significant correlation ($p<0.05$) to the anticholinesterase activity. The list includes the phenolic compounds benzene-1,2,4-triol, caffeic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, ferulic acid, 4-hydroxycinnamic acid, 4-methylcatechol, quinic acid, piceatannol, hydroxychavicol and pyrogallol. A comparative account of hydroxychavicol content in different varieties and at different collection time is shown in Table 1. The hydroxychavicol content differed in different varieties. The content also differed in each variety with time of collection (except for Meetha and Chhanchi varieties). The phenolic constituents also showed differences in quantity of constituents based on variety and time of collection (Figure 2). It was interesting to note that Meetha variety having lowest AChEi activity also contained lowest amount of hydroxychavicol. For other varieties, February collections showed higher activity than those of October collections. Hydroxychavicol content was also higher in February collections than in October collections in each variety. In this study we have evaluated acetylcholinesterase inhibitory activities of 1,2,4-benzenetriol, 4-methylcatechol, piceatannol and hydroxychavicol. The comparative activity of these compounds with galantamine is presented in Table 2.

Molecular Docking Based Binding Interaction Analyses

Molecular docking is a cost-effective computational method which is increasingly being employed in drug discovery process and is considered as fast complementary tool to many experimental biophysical techniques. Herein, molecular docking has been performed to evaluate the probable binding modes and atomic level interactions between all four small molecules and AChE. The molecular docking analyses have deduced several numbers of intermolecular interactions, such as hydrogen bond (H-bond), hydrophobic, and π-stacking between studied small molecules and AChE, and their intermolecular interactions are depicted in Table 3. Particularly, intermolecular interactions between AChE and all small molecules have shown energetically strong binding affinity score ranging from -7.4 to -6.8 Kcal/mol. Moreover, all the studied four small molecules have critically interacted at the several distinguished active sub-sites (such as esteratic sub-site comprised of catalytic triad (Ser203, His447, Glu334), anionic sub-site, acyl binding pocket, peripheral anionic sub-site and oxyanion hole) of AChE, and any interactions association with these sub-sites possibly can exert a controlled inhibition mechanism for AChE.[24] In Figure 3, 3D intermolecular interactions of all four small molecules including the standard known inhibitor donepezil have been displayed. From the molecular docking study, it has been observed that Benzene-1,2,4-triol participated to form H-bond interactions with two amino acid residues Gly120 and His447 of AChE. In addition, amino acid residue Trp86 has been found to

Table 1: Hydroxychavicol content (Mean±SD; n=3) in mg/g in different varieties of *P. betle* leaf.

Variety	October	February	
Bagerhati	64.94 ± 1.80	127.54 ± 22.37	*
Bangla	12.07 ± 4.29	110.28 ± 11.32	*
Chaanchi	63.03 ± 7.47	60.74 ± 5.93	#
Ghanagete	40.50 ± 4.89	105.98 ± 9.11	*
Haldi	82.25 ± 14.13	113.01 ± 9.69	*
Kalibangla	32.64 ± 9.08	128.19 ± 10.12	*
Manikdanga	14.63 ± 3.76	101.50 ± 10.96	*
Meetha	10.85 ± 8.39	7.00 ± 2.50	#

*Content significantly different in October and February; #Content not significantly different in October and February

Table 2: IC_{50} values of phenolic compounds.

Metabolites	IC_{50} value (μM ± SD; n=3)
4-Methyl catechol	108 ± 1.66
Hydroxychavicol	75.75 ± 0.83
Piceatannol	42.72 ± 0.77
Benzene 1,2,4 triol	32.79 ± 0.93
Galantamine hydrobromide	22.38 ± 0.44

SD: Standard deviation.

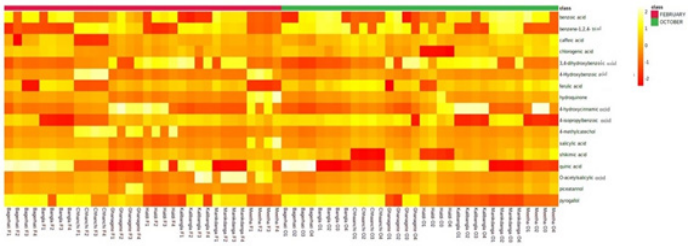

Figure 2: Heat map showing phenolic content in *P. betle* leaf extracts in different varieties at different time of collections.

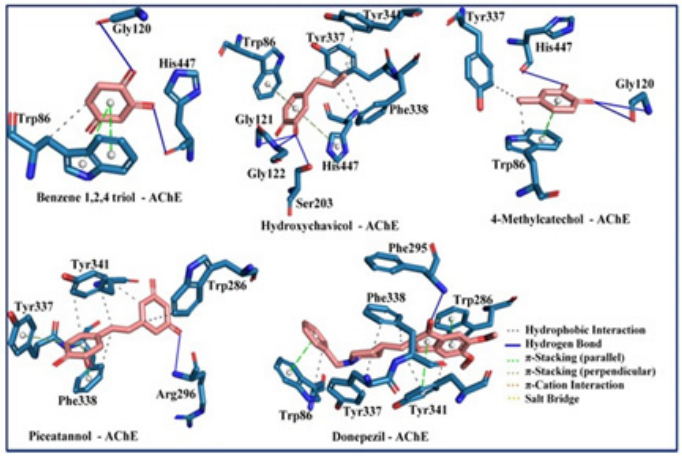

Figure 3: Molecular docking predicted binding interactions profile and associated interacting amino acid residues of AChE with studied four small molecules and standard inhibitor Donepezil.

form hydrophobic and π-stacking interactions with Benzene-1,2,4-triol. Docking analysis of Hydroxychavicol revealed the association of three amino acids (Gly121, Gly122 and Ser203) of AChE in the formation of H-bond interaction. Apart from H-bond interactions, several hydrophobic interactions also have been identified between atoms of Hydroxychavicol and three amino acid residues (Tyr337, Phe338 and Tyr341) of AChE. In addition, two π-stacking interactions have been also found with residues Trp86 and His447 of AChE and Hydroxychavicol. The small molecule, 4-Methylcatechol explicated the similar types of intermolecular interactions as Hydroxychavicol. Particularly, residues Gly120 and His447 accounted to form H-bond interactions, residues Trp86 and Tyr337 mediated hydrophobic contacts and residue Trp86 also created a π-stacking interaction with 4-Methylcatechol. With comparatively higher binding affinity score of -7.4 Kcal/mol than other studied small molecules, the Piceatannol has participated to form H-bond interaction with residue Arg296 of AChE. Many other sub-sites residues, such as Trp286, Phe338 and Tyr341 involved in the formation

Table 3: Binding energy scores and interacting residues at AChE of studied small molecules and standard inhibitor donepezil.

Cpds	Binding affinity (Kcal/mol)	Interacting residues in H-bond interaction	Residues involvement in other type of interactions
Benzene 1,2,4 triol	-6.8	Gly120, His447	Trp86 (Hydrophobic) / Trp86 (π-Stacking)
Hydroxychavicol	-6.9	Gly121, Gly122, Ser203	Tyr337, Phe338, Tyr341 (Hydrophobic) / Trp86, His447 (π-Stacking)
4-Methylcatechol	-6.9	Gly120, His447	Trp86, Tyr337 (Hydrophobic) / Trp86 (π-Stacking)
Piceatannol	-7.4	Arg296	Trp286, Phe338, Tyr341 (Hydrophobic) / Tyr337, Phe338 (π-Stacking)
Donepezil (Control / Standard)	-6.4	Phe295	Trp86, Tyr337, Phe338, Tyr341 (Hydrophobic) / Trp86, Trp286, Tyr341 (π-Stacking)

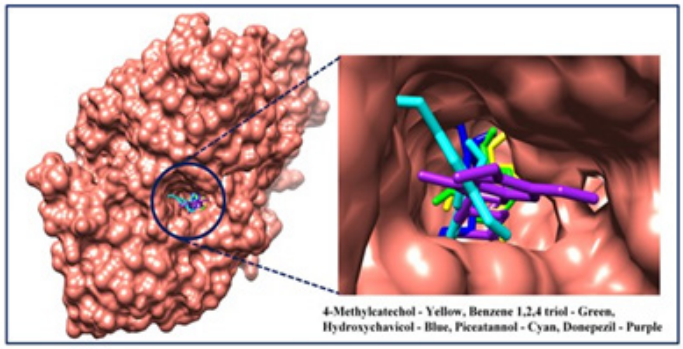

Figure 4: Docked orientation or binding mode of all small molecules including Donepezil into the AChE active site or binding cavity. All small molecules displayed in different colours for better visualization (AChE in surface view and all small molecules are in ball and stick representation).

of hydrophobic contacts and two consecutive residues Tyr337 and Phe338 formed π-stacking interactions with Piceatannol. The standard inhibitor donepezil has been found to form H-bond interaction with residue Phe295 of AChE. Several other important sub-site residues, such as Trp86, Tyr337, Phe338 and Tyr341 have been participated to form hydrophobic interactions with Donepezil. The π-stacking has been also found with residues Trp86, Trp286 and Tyr341 of AChE and atoms of Donepezil. Interestingly, it has been observed that residues Trp86 and Tyr337 of AChE have participated either in hydrophobic or π-stacking interactions with all the studied small molecules including the standard inhibitor Donepezil. Particularly, aromatic rings of these residues have been mediated to form such types of intermolecular interactions, as residue Trp86 and Tyr337 formed a nearly equidistant π orbital sandwich that helps to create π-stacking interactions with bound ligands. Overall conformational binding orientation is depicted in Figure 4 for all four small molecules including the known standard inhibitor Donepezil. Figure 4 suggested that all four small molecules are found to interact with the targeted enzymes in similar fashion at the same active site of AChE, where the standard inhibitor Donepezil bound to it. Undoubtedly, the displayed alike interactions profiles also indicate a well fitted surface

occupancy for all studied four molecules inside the active site cavity, which can promote binding specificity of AChE and hence can implicate desired catalytic activity.

DISCUSSION

Cholinesterase inhibitory properties of caffeic acid and chlorogenic acid, 3,4-dihydroxybenzoic acid derivatives, some hydroxycinnamic acids, pyrogallol were reported.[25-28] So, in this study we have evaluated acetylcholinesterase inhibitory activities of 1,2,4-benzenetriol, 4-methylcatechol, piceatannol and hydroxychavicol. Hydroxychavicol is an abundant phenolic compound detected in the betel leaves. Piceatannol, although could not be detected in all the varieties by GC-MS, showed AChEi activity. Combined effect of the phenolic compounds in *P. betle* leaves could also be responsible for the AChEi activity of the leaf extracts. Thus, the phenolic compounds of *P. betle* leaves may be the sources of acetylcholinesterase inhibitors which could be effective as drug after further *in vivo* studies. However, variety and time of collection is a matter of concern for the use of *P. betle* leaves as acetylcholinesterase inhibitors.

CONCLUSION

Among the eight varieties of *P. betle* methanolic leaf extracts from collections in February and October, Manikdanga variety from February showed the highest potential towards *in vitro* acetylcholinesterase inhibition and Meetha from October being the lowest. GC-MS based metabolite profiling, chemometric studies and correlation of the metabolites profiled from the extract aided in identification of 1,2,4-benzenetriol, 4-methylcatechol, hydroxychavicol and piceatannol that have inhibiting action against AChE *in vitro*. These four small molecules were further employed to molecular docking-based interaction analyses. Docking analyses showed similar binding interaction profiles for all molecules at the active site of the studied enzyme, as revealed by the standard inhibitor, Donepezil of AChE. Certainly, presence of numerous functional groups in the four bioactive constituents in the leaves of *P. betle* makes it an immensely important medicinal plant. This is highly corroborated with the outcomes of molecular docking study of the four bioactivity correlated metabolites that provides an insight to the drug discovery process. Further study is suggested to substantiate the activity of these bioactive phytoconstituents in *in vivo* models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

GC-MS: Gas Chromatography-Mass Spectrometry; **AD:** Alzheimer's Disease; **AChE:** Acetylcholinesterase; **AChEi:** Acetylcholinesterase inhibitor; **CH:** Chhanchi; **ME:** Meetha; **BA:** Bangla; **MA:** Manikdanga; **KA:** Kalibangla; **BG:** Bagerhati; **GH:** Ghanagete; **HA:** Haldi; **GC:** Gas Chromatography; **MSD:** Mass Selective Detector; **HP5-MS:** (5%-phenyl)-methylpolysiloxane; **RT:** Retention Time; **PDB:** Protein Data Bank; **ADT:** AutoDock Tool; **PLIP:** Protein-Ligand Interaction Profiler; **SD:** Standard Deviation; **PLS-DA:** Partial Least Squares-Discriminant Analysis; **O:** October; **F:** February; **HPTLC:** High Performance Thin Layer Chromatography; **DST-FIST:** Department of Science and Technology- Fund for Improvement of S&T Infrastructure.

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