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Research Article

In Vitro And *In Silico* Cholinesterase Inhibitory And Antioxidant Properties of *Paederia foetida* L.: Potential Implications Against Neurodegenerative Disorders

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Abstract
Objectives: Cholinesterase inhibitors, especially from plant origin, is proved to be the therapeutic choice against neurological diseases. *Paederia foetida* L., a traditional medicinal herb, has been investigated for a number of pharmacological properties. But, its efficacy against neurological conditions has not yet been studied. Therefore, in the present study, anticholinesterase and antioxidant properties of crude methanolic extract of *P. foetida* (MEPF) were investigated both *in vitro* and *in silico*.
Methods: MEPF was investigated for acetylcholinesterase- (AChE) and butyrylcholinesterase (BChE) inhibitory properties. Antioxidant activity of MEPF was investigated by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method. Major bioactive compounds of MEPF were identified using FTIR and GC-MS, and the identified phytochemicals were analysed for molecular docking, drug-likeness properties, and toxicity prediction.
Results: Promising antioxidant, AChE and BChE inhibitory activities were observed with IC_{50} values of 99.26±0.85 μg/mL, 103.55±1.27 μg/mL and 180.45±3.18 μg/mL, respectively. GC-MS analysis revealed nine compounds among which phytol and neophytadiene exhibited strong binding affinities with NAD(P)H oxidase, AChE and BChE, with good pharmacokinetic score and no toxicity.
Conclusions: *P. foetida* may act as an excellent free radical scavenger with potent anticholinesterase properties and may be helpful in preventing the progression of neurodegenerative disorders associated with oxidative stress.

Keywords: Anticholinesterase; Antioxidant; *Paederia foetida*; Molecular docking

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Introduction

Overproduction and inadequate elimination of free radicals result in oxidative stress, which is correlated with the development and progression of neurological disorders like Alzheimer's disease (AD). Moreover, accumulation of amyloid beta (Aβ) leads to degeneration of neurons and liberation of excess free radicals, resulting in mitochondrial dysfunction and inflammation. Furthermore, in AD patients, the reduction in acetylcholine (ACh) by the enzyme acetylcholinesterase (AChE) is a significant factor in the progression of dementia, and therefore, therapeutic strategies involving oxidative stress inhibition, and utilization of acetylcholinesterase inhibitors (AChEI) towards augmenting the amount of ACh in brain are on demand [Alotaibi *et al*., 2024]. The most commonly prescribed ChEIs against AD include tacrine, rivastigmine, donepezil, galantamine, etc. are found to be associated with adverse effects in the gastrointestinal system, causing nausea, vomiting, cramping, and diarrhea [Schneider *et al*, 2007]. Therefore, demand for new drug candidates with lesser side effects is increasing rapidly for the treatment of AD. In this context, ChEIs from plant origin are more preferred over the synthetic drugs.

Paederia foetida L. (Family – Rubiaceae) (Common name: Skunk vine) is native to both temperate and tropical Asia, and popularly known as "Gandha bhadulia" or "Gandha prasarini" in India. To date, 217 phytochemicals have been identified in this species, the most of which belong to glycosides, anthraquinones, phenolics, terpenoids, phytosterols, and other groups. Leaf extract of this plant is reported to possess analgesic, anti-inflammatory, anti-arthritic, antimicrobial, antidiabetic, anthelmintic, antioxidant, cytotoxic, hepatoprotective, gastroprotective, renoprotective, and lipid lowering effects [Dutta *et al*.,2023; Barai *et al*., 2024]. However, the cholinesterase inhibitory activity of *P. foetida* has not yet been investigated. Therefore, the present study was aimed to investigate the anticholinesterase- and antioxidant activities of the methanol extract of *P. foetida in vitro* and *in silico*.

Materials and methods

Collection, identification and preparation of plant extract

Fresh leaves of *P. foetida* were collected from Jalpaiguri town of West Bengal, India in the month of April, 2023 and identified by Dr. Manoranjan Chowdhury, Department of Botany, University of North Bengal, India. A voucher specimen (Accession no. 11207) was deposited at University of North Bengal Herbarium (NBU). Samples were prepared according to a previously described method [Roy et al.,2015]. The lyophilized methanolic extract of *P. foetida* (MEPF) was dissolved in double distilled water for all *in vitro* experiments.

Chemicals and reagents

Acetylthiocholine iodide, Butyrylthiocholine iodide, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), 1,1-diphenyl-2 picrylhydrazyl (DPPH), galantamine procured from Sisco Research Laboratories Pvt. Ltd., Mumbai,India. Solvents, Folin-Ciocalteu (FC) reagent, gallic acid, α-tocopherol, ascorbic acid and other reagents were purchased from HiMedia Laboratories Private Limited, Mumbai, India.

FTIR and GC-MS analysis

FTIR analysis of MEPF was conducted at the sophisticated analytical instrument facility of TCG Lifesciences Private Limited (Kolkata, India). Spectrum Two FT-IR Spectrometer (PerkinElmer, Germany) was used to analyse the sample, with scan range of 400 to 4000 cm^{-1}.GC-MS analysis of MEPF was conducted at the SAIF-IIT Madras, India using Thermo GCTrace Ultra V5.0 equipment and Thermo MS DSQ II (Massachusetts, USA). Compounds were identified using NIST libraries.

Preparation of enzyme source

The enzyme was prepared following a previously established protocol [Ellman *et al*., 1961]. Fresh chicken liver (1g) was obtained from the nearby market and homogenised in 10 mL extraction solution after being rinsed with 50 mM Tris-HCl buffer, pH 7.4 (50 mM Tris HCL buffer, pH 7.4; 1 mM MgCl2; 1 mM CaCl2; and 0.32 M sucrose). To prevent heating, the test tube was put in an ice bucket. Whatman filter paper no. 1 papers were used to filter the contents and centrifuged in a cooling centrifuge at 15,000 rpm for 15 minutes at 4 °C. The supernatant was utilised as an enzyme source. The enzyme source was prepared daily and utilised within a time frame of 4 hours.

Determination of AChE and BChE inhibitory activity

A previously established protocol was followed to assess enzyme activity [Ashraf *et al*., 2011]. The reaction mixture (200 µl) comprised 10 µl of enzyme (40–60 g protein) derived from freshly homogenised chicken liver, combined with 160 µl of 50 mM Tris-HCl buffer, pH 7.4, with or without 30 µl of plant extract addition. The mixture underwent pre-incubation for 10 minutes at 25°C. Plate readers (Bio-Rad, Hercules, CA, USA) were used to pre-read the plates at 412 nm. 10 µl of 1 mM DTNB and 3 mM substrate acetylthiocholine iodide and butyrylthiocholine iodide were introduced to initiate the reaction. Absorbance at 412 nm was measured for a duration of 4 to 7 minutes after the incubation of 15 minutes. Control tests were conducted to rectify nonenzymatic hydrolysis by introducing the enzyme after the addition of DTNB. The control was utilised to deduct the absorbance values, and the resultant data were articulated as the % inhibition of enzymatic activity. The experiments were performed six times, each with corresponding controls.

DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of MEPF was assessed according to a previously described protocol [Roy *et al*., 2013]. Alpha-tocopherol was used as the standard.

Determination of total phenolic and flavonoid content

Total phenolic content (TPC) and total flavonoid content (TFC) were determined according to standard methods [Phuyal et al., 2020]. TPC [gallic acid equivalent (GAE)/g of extract] was determined from a gallic acid standard curve. TFC (quercetin equivalent (QE)/g of extract) was calculated from a quercetin equivalent standard curve.

Molecular Docking Study

Target proteins for docking analysis include nicotinamide adenine dinucleotide phosphate oxidase [NAD(P)H oxidase] (PDB ID: 2CDU) [for antioxidant study], human acetylcholinesterase (PDB ID: 4EY7) and human butyrylcholinesterase (PDB ID: 6EP4) [for anticholinesterase study]. Their crystal structures were acquired from Protein Data Bank (PDB). Docking investigations were performed using AutoDock Vina module of PyRx, a virtual screening tool. The binding energy between ligand and protein was determined using Lamarckian Genetic Algorithm (LGA) programme with an adaptive whole method search in AutoDock Vina.

ADME and toxicity profiling

Physicochemical properties, drug similarity and pharmacokinetic properties of the compounds were investigated using ADME webservers such as SwissADME and pkCSM.

Statistical analysis

Statistical analysis was performed using KyPlot version 6.0. Data are given as mean±SD of six measurements. IC_{50} values were calculated using the formula Y = 100*A1 / (X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. Differences between two groups were determined by paired *t* test. $p< 0.05$ was considered significant.

Results and Discussion

Phytochemical analysis by FTIR and GC-MS

FT-IR is usually used for the qualitative analysis of crude samples to identify biologically and pharmacologically relating phytochemicals**.** FTIR analysis indicated the presence of phenolics (3395.14 cm^{-1}), alkenes (2924.59 cm^{-1}), alkanes (2854.14 cm^{-1}, C-H stretching, and 1446.94 cm^{-1}, C-H bending), esters (1726.21cm^{-1}), amines (1261.62 cm^{-1}), alcohols/ethers (1165.94 cm^{-1}), alcohols (1044.58 cm^{-1}), aromatic C-H (801.39 cm^{-1}), and aliphatic Chloro- (772.02 cm^{-1} and 720.03 cm^{-1}, C-Cl stretching) groups.

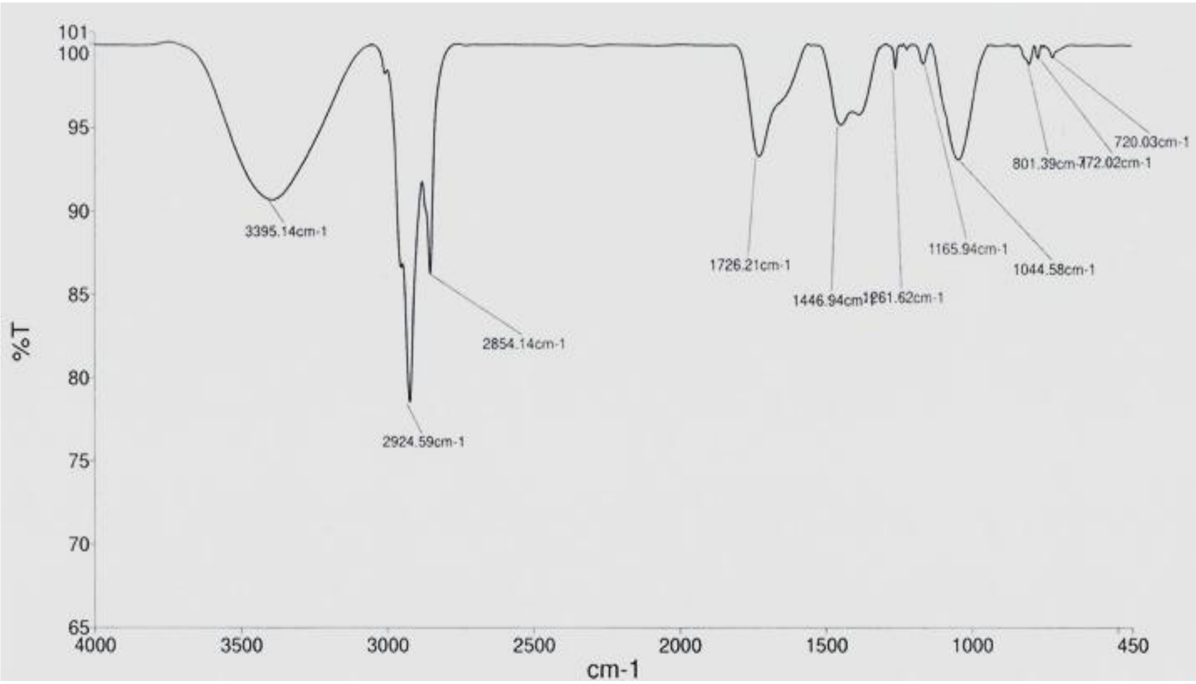

Figure 1: FTIR peaks of MEPF

The GC-MS analysis showed nine distinct peaks at different retention times: 3.288, 8.613, 12.971, 16.969, 18.900, 20.535,22.303, 23.338 and 29.310 minutes. The spectra were matched with the NIST 2017 spectral database.GC-MS analysis revealed the presence of glycerin (10.95%), 2-fluorophenethyl alcohol (2.87%), 1,4,2,5 cyclohexanetetrol (19.43%), 3-hydroxy dodecanoic acid (23.33%), α-L-rhamnopyranose (11.80%), (Z)-Ethyl 3-(4-methoxyphenyl) acrylate (3.98%), neophytadiene (12.09%), 3-Methylene-7,11-dimethyl-1-dodecene (2.18%) and phytol (13.36%).

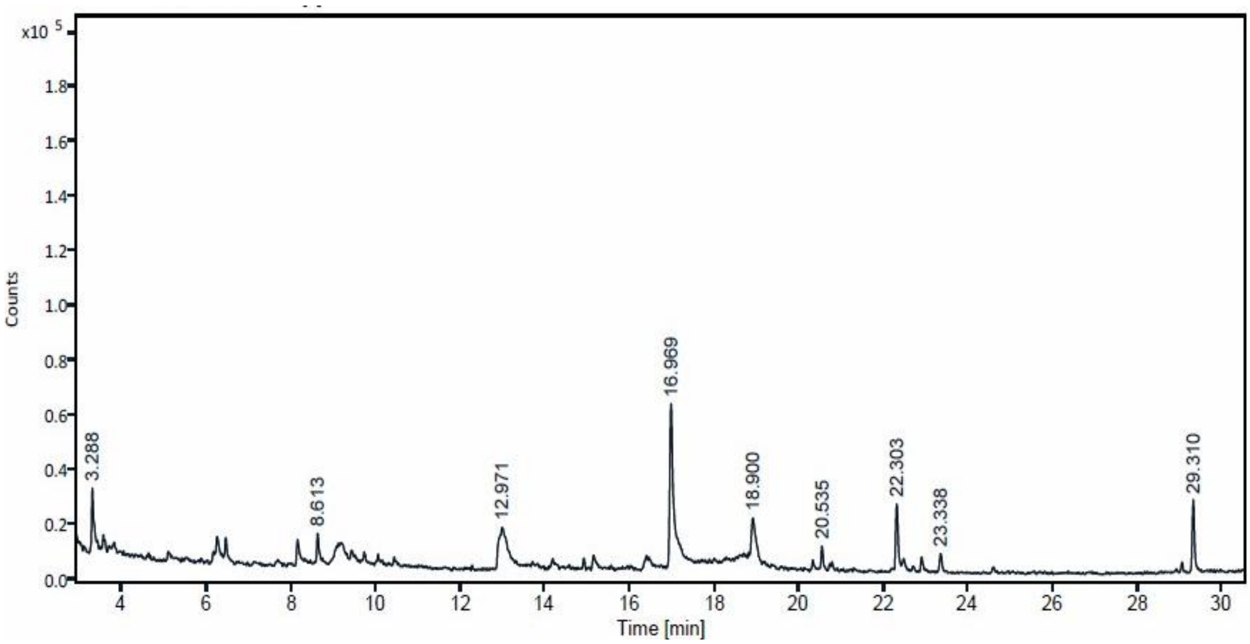

Figure 2: GC-MS chromatogram of MEPF

Table 1: List of compounds identified in MEPF using GC-MS

Sl No.	RT	Compounds	Area %	MW	Molecular formula
1.	3.288	Glycerin	10.95	92.09	$C_3H_8O_3$
2.	8.613	2-FluorophenethylAlcohol	2.87	140.15	C_8H_9FO
3.	12.971	1,4,2,5 Cyclohexanetetrol	19.43	148.15	$C_6H_{12}O_4$
4.	16.969	3-Hydroxy Dodecanoic acid	23.33	236.31	$C_{12}H_{24}O_3$
5.	18.900	α-L-rhamnopyranose	11.80	164.15	$C_6H_{12}O_5$
6.	20.535	(Z)-Ethyl 3-(4-methoxyphenyl) acrylate	3.98	206.23	$C_{12}H_{14}O_3$
7.	22.303	Neophytadiene	12.09	278.51	$C_{20}H_{38}$
8.	23.338	3-Methylene-7,11-dimethyl-1- dodecene	2.18	208.38	$C_{15}H_{28}$
9.	29.310	Phytol	13.36	296.53	$C_{20}H_{40}O$

On best of our knowledge, among these phytochemicals except phytol and neophytadiene, identified for the first time in the genus *Paederia*. Although their presence has been reported in Asteraeceae, Cleomaceae, Theaceae, Lamiaceae and Amaryllidaceae family and found to have diverse biological activities *viz.*, antioxidant, antibacterial, anticholinesterase, anticancer, hepatoprotective, anti-inflammatory, etc (Elmogy *et al*., 2020; Rosatella and Afonso, 2022; Sanaullah *et al*., 2023; Shaikh *et al*., 2023.)

Cholinesterase inhibitory activities of MEPF

Figure 3 showed the acetylcholinesterase- and butyrylcholinesterase inhibitory activities of MEPF, using galantamine as the reference compound. Results indicated dose-dependent increase in AChE inhibitory activity for both MEPF and galantamine, but it is noteworthy to mention that at all the doses (0-200 µg/mL), there is no significant differences in the AChE inhibitory activity between MEPF and the reference compound. At the highest dose, i.e., 200 µg/mL, the percentages of AChE inhibition were 76.92 ± 1.02 and 76.83 ± 0.72 for galantamine and MEPF, respectively. The IC_{50} value of MEPF (103.55 ± 1.27 µg/mL) was also found to be very close to that of the galantamine (103.06 ± 1.18 µg/ml), indicating MEPF as a promising AChE inhibitor.

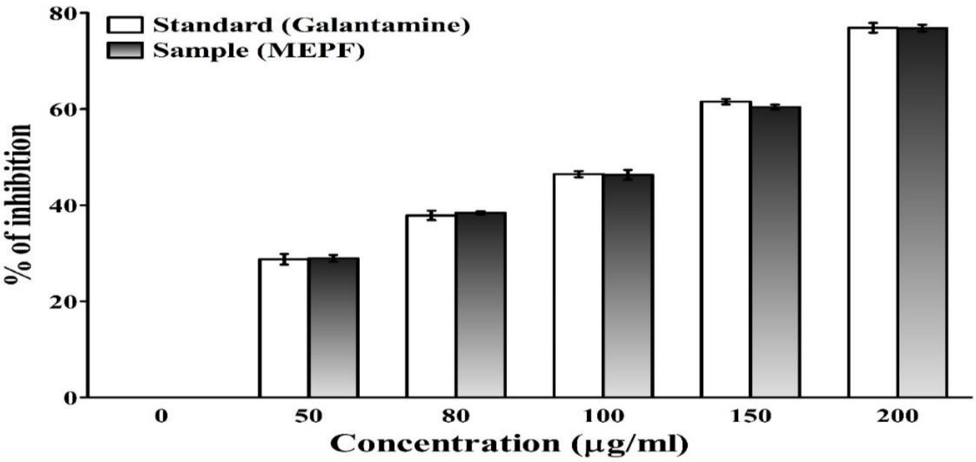

Figure 3: Percentage of inhibition of AChE at different concentration.The results are mean±S.D. of six parallel measurements.

The BChE inhibitory activity of MEPF [Figure 4] also demonstrated dose-dependent increase in BChE inhibition for both galantamine and MEPF. In this case, at lower doses, i.e., 50, 80 and 100 μg/mL, there are significant differences ($p < 0.05$ and $p < 0.01$) in the BChE inhibitory activity between galantamine and MEPF, however, at the higher doses, i.e., 150 and 200 μg/ml, the BChE inhibitory activities of galantamine and MEPF were found to be almost similar, i.e., 49.20 ± 0.52% and 51.06 ± 0.64% (for 150 μg/mL), respectively, and 68.27 ± 0.87% and 66.74 ± 0.57%, respectively; although the IC_{50} value of MEPF (180.45 ± 3.18 μg/mL) was found to be significantly higher (*p* < 0.05) than that of the galantamine (155.32 ± 2.93 μg/mL). To the best of our knowledge, there is no scientific evidence of the anticholinesterase activity of *Paederia foetida* although literature survey revealed that another species of the same genus (*Paederia linearis*) showed 29.31% acetylcholinesterase inhibition [Ingkaninan *et al*., 2003]. Phytol isolated from *Morinda lucida* reported cholinesterase inhibitory activity with an IC_{50} of 12.93 μg/ml (AChE) and 24.90 μg/ml (BChE) (Elufioye *et al*., 2015) and it has also been reported that phytol derivative isolated from *Pycnanthus angolensis* found to inhibit 65.66 ± 1.06% and 49.38 ± 1.66% against acetylcholinesterase and butyrylcholinesterase, respectively (Elufioye *et al*., 2016).

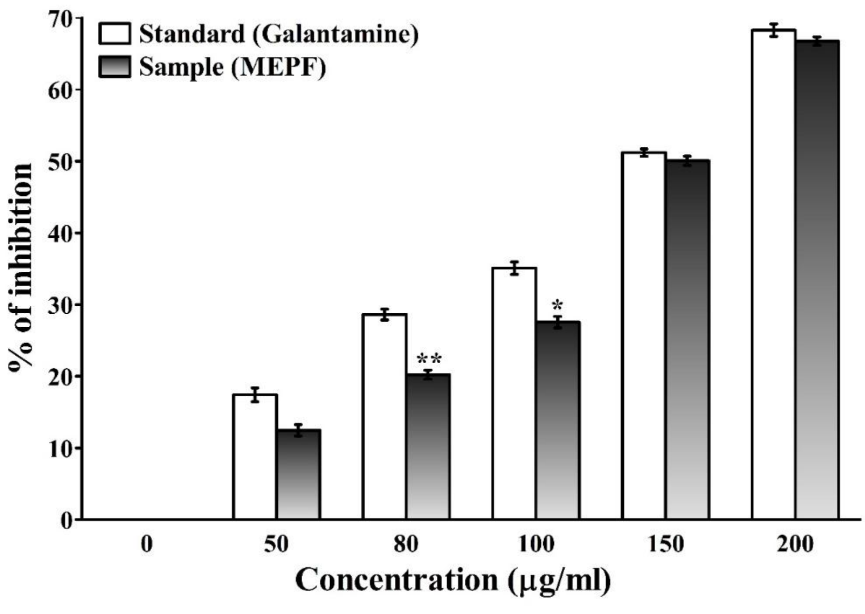

Figure 4: Percentage of inhibition of BChE at different concentration The results are mean±S.D. of six parallel measurements. *p<0.05 and **p<0.01 vs. respective concentrations of the standard.

Antioxidant activity of MEPF

The antioxidant activity of MEPF was evaluated by DPPH radical scavenging assay, using α-tocopherol as the reference compound. Figure 5 indicated dose-dependent DPPH radical scavenging activities of both α-tocopherol and MEPF (0 – 200 μg/mL). It is interesting to note that at higher concentrations (150 and 200 μg/ml), there were no significant differences in the radical scavenging potential between α-tocopherol and MEPF [(68.10 ± 1.15% and 67.09 ± 1.27%, respectively at 150 μg/mL) and (80.21 ± 1.12% and 80.17 ± 0.71%, respectively at 200 μg/mL)]. The results of the antioxidant activities of both α-tocopherol and MEPF were expressed in terms of IC_{50} values which indicated MEPF as a promising antioxidant (IC50= 99.26 ± 0.85 μg/mL) when compared to the reference compound α-tocopherol (IC_{50} = 87.68 ± 0.56 μg/mL). These excellent radical scavenging properties were thought to be provided by phytol, 1,4,2,5 cyclohexanetetrol and neophytadiene, which are detected by GC-MS in the present study, and have already been reported to demonstrate antioxidant activity [El Mihyaoui *et al*., 2023].

Priyanto *et al*. (2023) studied the DPPH and ABTS radical scavenging activities of the leaf extract of *Paederia foetida*, and determined the IC_{50} values, which were 10.94±2.67 μg/mL and 50.04±0.48 μg/mL, for DPPH and ABTS radicals, respectively. These excellent radical scavenging properties were thought to be provided by phytol, 1,4,2,5 cyclohexanetetrol, (Z)-Ethyl 3-(4-methoxyphenyl) acrylate, neophytadiene, which are detected by GC-MS in the present study. These compounds have been reported in different plant extracts and their antioxidant activities could be due the presence of these phytocompounds [El Mihyaoui *et al*., 2023; Swamy *et al.*, 2017]. Thu *et al*., (2020) reported that phytol exhibited antioxidant activity of 59.89 ± 0.73% and 62.79 ± 1.99% scavenging capacity in DPPH and ABTS assay, respectively. The antioxidant action of phytol is attributed to the allylic character of its alcohol group. In the hypothesized mechanism, it is possible that the double bond plays a role in the creation of an intermediate resonance structure. In this configuration, the oxygen atom can establish a double bond with the neighbouring carbon atom, while the double bond shifts to the 3rd and 4th carbon atoms [Islam *et al*., 2018].

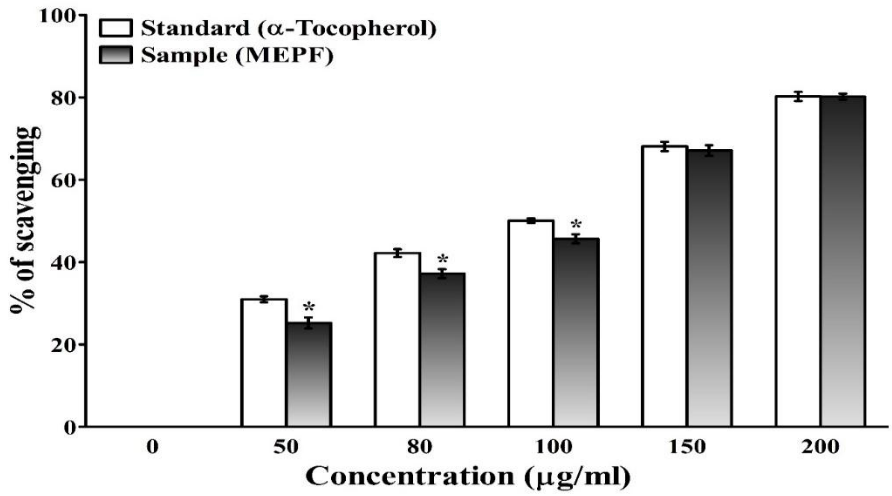

Figure 5: DPPH scavenging activity of MEPF The results are mean±S.D. of six parallel measurements. *$p<0.05$ vs. respective concentrations of the standard

Total phenolic and flavonoid content

MEPF exhibited TPC and TFC values of 142.59±0.011 mg GAE/g of extract and 118.30± 0.013 mg QE/g of extract, respectively. Both of these classes of compounds have already been established as potent antioxidants [Roy *et al*., 2013]. Therefore, MEPF could be used as an excellent alternative in the management of oxidation induced neurodegenerative diseases.

***In silico* antioxidant and anticholinesterase activities**

The present study utilized molecular docking to investigate the binding interactions of the nine of the most significant compounds found in MEPF (by GC-MS) with different proteins, such as NADP oxidase [for antioxidant activity (PDB ID: 2CDU)], and human acetylcholinesterase (PDB ID: 4EY7) and human butyrylcholinesterase (PDB ID: 6EP4), for anticholinesterase activities [Figure 6 (a) – (k)] Phytol exhibited the strongest binding affinities with 2CDU (-6.8 Kcal/mol) and 6EP4 (-6.6 Kcal/mol), whereas Neophytadiene showed the strongest binding affinity with 4EY7 (-7.8 Kcal/mol). Glycerine, on the other hand, showed the weakest binding affinities with 2CDU (-4.1 Kcal/mol), 4EY7 (-4.3 Kcal/mol), and 6EP4 (-3.8 Kcal/mol). ADME/T analysis showed the range of skin permeability for the studied compounds from -1.577 to -4.251. All of the drugs inhibit P-glycoprotein I and II. Blood-brain barrier (BBB) permeability and CNS permeability values were in between -0.079 to +0.983 and -1.299 to -3.846, respectively. Most of them also did not inhibit CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4 inhibitors and they did not interact with renal OCT2 substrate. Moreover, most of the bioactive compounds neither showed AMES toxicity nor inhibited hERG

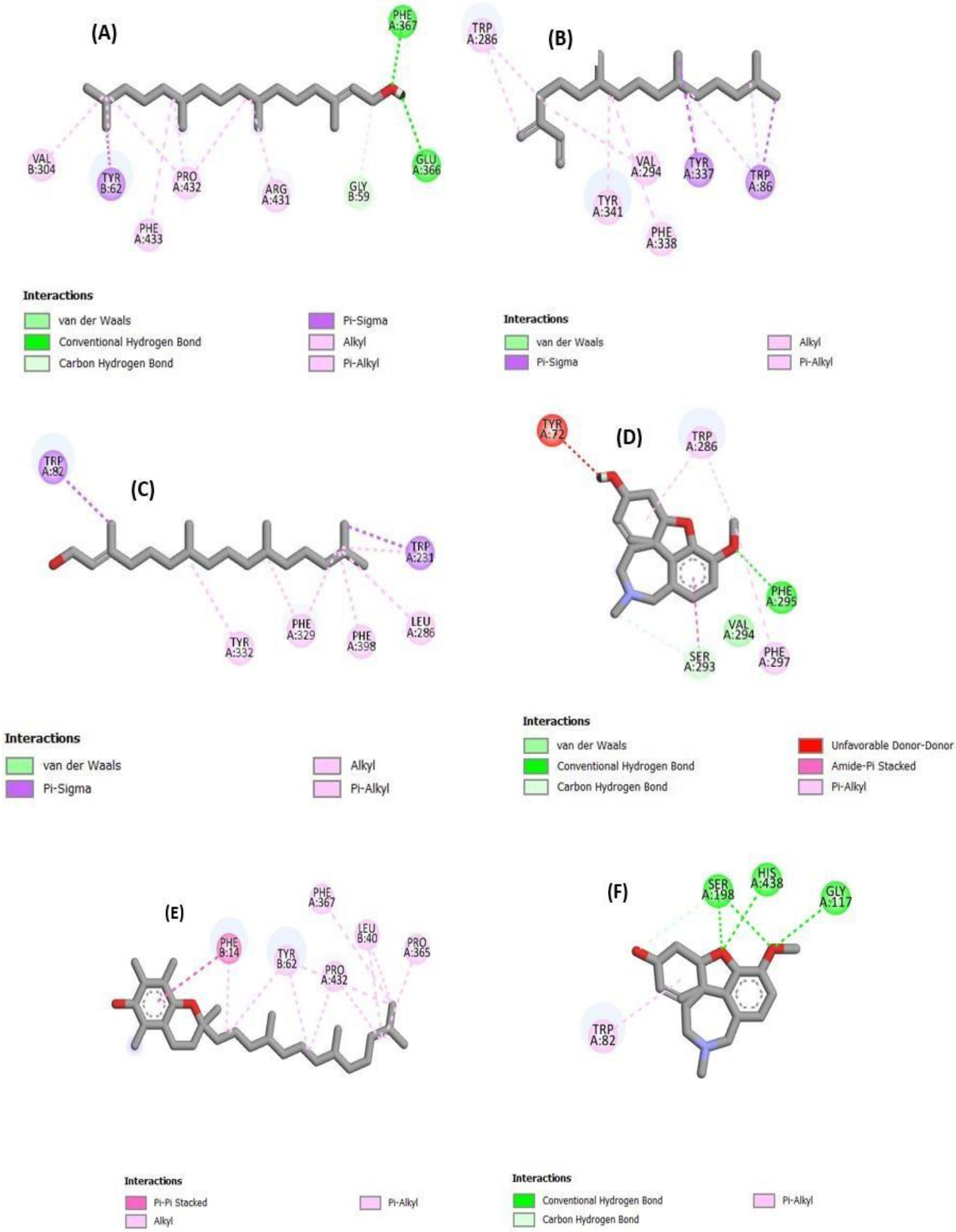

Figure 6: 2D diagram representing the molecular docking interaction of (a) Phytol with human NADP oxidase (PDB ID: 2CDU), (b) Neophytadiene human acetylcholinesterase (PDB ID: 4E7Y), (c) Phytol with human butyrylcholinesterase (PDB ID: 6EP4) (D) Galantamine with 4E7Y (Control) (E) Galantamine with 6EP4 (Control) (F) α tocopherol with 2CDU (Control).

Conclusions

In the present study, the anticholinesterase activity of *P. foetida* was investigated for the very first time both in vitro and in silico, where it showed promising acetyl- and butyrylcholinesterase inhibitory properties, mainly attributed to several bioactive phytochemicals, a few of which was identified for the very first time in this particular species. These phytochemicals along with other previously detected ones are already reported to possess several biological properties including antioxidant and anticholinesterase. Phytochemicals like phytol, neophytadiene, 3-Methylene-7,11-dimethyl-1-dodecene and (z)-ethyl-3-(4-methoxyphenyl) acrylate are shown to possess strong target protein binding affinities with good pharmacokinetic score. These results clearly indicate the effectiveness of *P. foetida* as a potent therapeutic candidate against a number of oxidative stresses induced neurodegenerative diseases. Moreover, this particular plant may be used as an important source of several biologically active compounds towards the development of medication in combating life-threatening diseases.

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Declaration of Interest statement

The authors would like to declare that there are no potential conflicts of interest with respect to the research, authorship and publication of this article.

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