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Phytochemical profiling and biological evaluation of *Piper schmidtii* leaf extract: Insights from *in vitro* bioassays and molecular docking

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Abstract
Background: Since ancient times, medicinal plants have been used for their medicinal purposes and it continues to have significance in present healthcare due to the various bioactive compounds present in medicinal plants.
Objectives: The leaf extract of *Piper schmidtii* was evaluated for its antioxidant, neuroprotective and anti-ulcer activities.
Materials & Methods: The phytochemical constituents were identified by GC–MS the inhibitory activity of acetylcholinesterase (AChE) was determined by spectrophotometric method, the antioxidant capability was evaluated byDPPH, ABTS, FRAP, & nitric oxide scavenging assays and the anti-ulcer activity was determined by evaluating the inhibition of protein denaturation.
Results: The phytochemicals identified were 29 compounds, with 9,12-octadecadienoic acid methyl ester being the most prevalent (10.85%). The extract exhibited dose-dependent inhibition of (AChE) enzyme in the range of 6.76% (50ug/mL) to 73.68% (250ug/mL) and significant DPPHIC$_{50}$ value of 56.47 µg/mL, The TEAC value was 80,416.67 µM TE/g, ferric reducing capacity of 275.55 mM Fe (II)E/mg extract, nitric oxide scavenging activity (41.66 ± 0.57%) and IC50 value of 57.68ug/mL demonstrated a significant ability of the extract to inhibit denaturation of proteins. The docking study highlighted that 17, GC–MS identified phytocompounds displayed substantial binding affinities towards the ulcer associated target protein (PDB ID: 5YLU) with docking score ranging from −3.9 to −9.9 kcal/mol.
Conclusion: These findings suggest that *Piper schmidtii* has significant pharmacological potential and further studies on gene expression are needed for further development as an option for treatment of ulcer care and diseases.

Keywords: Enzyme inhibition, gastro-protective potential, molecular interaction, secondary metabolites

Introduction

The idea of going back to the "roots" of medicine is now starting to gain attraction in the present era[1]. Before the ancient era, humans have been using plants for medical purposes. The plant-based products market is predicted to reach $83 billion USD and is still growing. These plant-based products are still relevant to the medical field. As the public becomes aware of the impact of these plants on human health, the popularity of these products is increasing, and the public is now using plant-based medicines instead of artificial medicines [2]. Due to the advancements of science, the study of various plants of ancient cultures is now possible by using modern technology. Using modern technology to analyse the pharmacological properties of the plants used for centuries as food, medicine, or spiritual purposes has been verified [1]. Many pharmaceutical companies have recently updated the ways of studying natural products in an attempt to discover new compounds and possible sources of new medicines. Due to their importance in human medicine or their vast pool of bioactive chemical diversity, natural products are valuable sources of potential therapeutic leads in drug development. Ethnobotanical fieldwork, which is of major importance in understanding the role of plants in society, is still an academic pursuit rather than an industrial pursuit. Random collection and automated *in vitro* screening are the bases of the industrial approach to bioactive chemical research. Some of the bioactive compounds of medicinal herbs, such as morphine, emetine, strychnine, quinine, colchicine, atropine, papaverine, and salicin, were discovered during the early nineteenth century [3]. The use of medicinal herbs has been a popular practice owing to various cultural and historical reasons. According to the documented researches, the medicinal plants are an important economic and health component of biodiversity, conservation, and sustainable use [4]. Medicinal plants possess a unique microbiome owing to their bioactive secondary metabolites, which are structurally divergent and are probably responsible for the high degree of specificity of the associated microbiota [5].

The Piperaceae has been deemed one of the largest families of basal dicots, occurring in tropical and subtropical regions. There are five genera in the family Piperaceae: Piper, Peperomia, Manekia, Zippelia and Verhuellia. The genus Piper, the largest genus of the family Piperaceae, contains approximately 4000 species. They also find applications in folk medicine of treating numerous diseases in various countries, such as Brazil, China, India, Jamaica and Mexico [6]. Some Piper spp. in India, southeast Asia and Africa are also economically important as they are exploited as spices and traditional medicine. Piperaceae is an example of many plant families with broad world distribution, however, with a rich ethnobotanical and ethnopharmaceutical history [7]. Some of them are biologically active, and have antitumor, antimicrobial, antioxidant, insecticidal, antidiabetic, hepatoprotective property [8]. In this work *Piper schmidtii* leaf extract was analysed for its antioxidant, neuro toxic and anti ulcer properties.

Material and Method

Sample collection and preparation

Piper schmidtii leaves that were robust and healthy were collected from their native habitat and examined by an experienced taxonomist. After carefully rinsing the collected leaves with distilled water to remove any contaminants, they were dried for 10 to 14 days at room temperature in the shade before being processed into a powder using a rotary blender. For further analysis, the powdered material was stored in airtight containers. Soxhlet extraction was performed on about 100 g of the powdered material using methanol as the solvent for six to eight hours. A rotary evaporator was used to concentrate the filtered extract under vacuum at a temperature of no more than 45°C until the crude extract was achieved. The saturated extract was kept at 4°C in refrigerators for use in subsequent experiments. [8, 9]

Phytochemical Characterization by GC–MS

The analysis was done using a Thermo Fisher Scientific GC–MS machine with a DB-35MS -polar capillary column. This column was 30 meters long and 0.25 millimeters thick. The carrier gas was a steady 1.0 mL/min flow of helium. The injector, interface, and ion source temperatures had been adjusted to 270°C, 280°C, and 200°C, respectively. The oven was heated to 120°C at a rate of 4°C min. after being kept at 70°C for three minutes. After that it was increased to 290°C at 7°C/min. The GC–MS machine looked at the mass spectra in a range of 50 to 650 Da. The ionization energy was set at 70 electron volts. The compounds that were found were identified by matching the spectra with reference spectra from the NIST4 and WILEY9 libraries. The GC–MS analysis considered compounds with match values of 700 or more, for identification of the GC–MS analysis [10, 9]

Acetylcholinesterase (AChE) enzyme activity by spectrophotometric method

A colorimetric test based on the interaction of thiocholine with Ellman's reagent was adopted to determine the inhibitory activity of acetylcholinesterase. AChE hydrolyses acetylthiocholine in the course of the experiment to form thiocholine, which then interacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce the yellow-colored 5-thio-2-nitrobenzoate anion. The rise in absorbance was measured at 412 nm. 250 µL of plant extract (25–400 µg/mL), 1710 µL of 50 mM Tris–HCl buffer (pH 8.0), 10 µL of AChE solution (6.67 U/mL), and 20 µL of 10 mM DTNB dissolved in the same buffer comprising the reaction mixture. The reference inhibitor, galantamine, had been prepared at quantities that complemented the test samples in 50 mM Tris–HCl buffer (pH 8.0). The enzymatic reaction was started by adding 10 µL of a 200 mM acetylthiocholine iodide solution after the reaction mixture had been incubated at 37°C for 15 minutes. Using a UV-visible spectrophotometer, standard absorbance values were acquired at 412 nm for an average of three minutes at intervals of ten seconds. The blank was a control reaction that did not contain the enzyme Using the following formula, the percentage inhibition was determined by comparing the sample's reaction rate to the blank's Every experiment was carried out three times. Using Microsoft Excel, a linear regression evaluation of percentage inhibition against extract concentration was used to calculate the concentration needed to inhibit 50% of AChE activity (IC50) [11]

Inhibition (%) = $100 - \Delta A \text{ sample} / \Delta A \text{ blank} \times 100$

ΔA = Change in absorbance over time

In vitro Antioxidant Activity

DPPH radical scavenging activity

Bioflavonoid content (free radical scavenging activity) was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with some modifications of the standard procedure. Several test tubes were filled with different volumes of the test extract (20, 50, 75 and 100 µL) and the final volume adjusted to 100 µL with methanol. Three mL of the freshly prepared 0.004% DPPH solution in methanol was then added to each tube of the antioxidants (BHT and rutin) extract. In the control 100 µL of methanol was used instead of extract and the volume of DPPH solution was kept constant. All reaction mixtures were well mixed, and incubated in the dark at 27°C for 30 minutes until the reaction was completed. The absorbance was then measured at 517 nm using methanol as blank. The value that is obtained when the concentration of the sample is reduced to half of the initial DPPH radical level is called IC_{50} value, and was expressed as the antioxidant potential. [12]

ABTS radical cation scavenging activity

The ability of the extracts to act as radical cations scavengers was evaluated by using the published protocol. The ABTS working solution was prepared by reacting the aqueous ABTS stock solution with the 2.4 mM potassium persulfate, and then stored in the dark for 12–16 hours. The radical solution was diluted with ethanol (1:89, v/v) before use to obtain an absorbance of 0.700 ± 0.02 at 734 nm. The assay combination consisted of 1.0 mL of the diluted ABTS solution, 30 µL of plant extract and 10 µL of Trolox solution (0–15 µM) formulated as a standard. The negative control was a reaction mixture that included 30 µL of ethanol and 1.0 mL of diluted ABTS solution. After all the reaction formulations were fully whisked and left to settle at room temperature for 30 minutes, the absorbance was measured at 734 nm using ethanol as blank. The Trolox calibration curve was used to measure the radical scavenging capacity, which was then reported as Trolox equivalent antioxidant capacity (TEAC; µM Trolox equivalents/g extract). [13]

Ferric reducing antioxidant power (FRAP) assay

The extracts' antioxidant potential has been estimated based on the earlier studies. For the assay the FRAP reagent (25 mL 0.3 M acetate buffer (pH 3.6) + 2.5 mL 20 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl + 2.5 mL

$FeCl_3 \cdot 6H_2O$) was pre-incubated for 37°C, and the test extract was admixed with 900 µL of the FRAP reagent, 90 µL of distilled water and 30 µL of methanol as the blank. The standard indices were rutin and BHT. Following a 30-minute incubation period at 37°C in a water bath, the reaction mixtures' absorbance at 593 nm was measured in comparison to the reagent blank. To create the calibration curve, $FeSO_4 \cdot 7H_2O$ standards (500–4000 µM) were used. The reducing capacity of the extracts was determined as ferric reducing antioxidant power (FRAP), and is presented in the equivalent concentration of antioxidant that has similar reducing power to 1 mM $FeSO_4 \cdot 7H_2O$. [14]

Nitric oxide scavenging activity

The assay based on the previous research was carried out to assess the nitric oxide radical scavenging activity. To assist in the production of nitric oxide, plant extracts were combined with 10 mM sodium nitroprusside derived from 0.2 M phosphate-buffered saline (pH 7.4) and Conditioned at ambient temperature for 150 min. The antioxidant controls used were butylated hydroxytoluene (BHT) and rutin. After incubation, 0.5 mL of freshly prepared Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2% phosphoric acid) was added to each reaction mixture. The intensity of the coloured azo product was determined spectrophotometrically at 546 nm against a blank (phosphate buffer). The following formula was used to calculate nitric oxide scavenging activity from the decrease in nitrite production and represent it as percentage inhibition:

Scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$

where A_1 stands for the absorbance of the test sample or standard of comparison and A_0 for the absorbance of the control reaction. The findings are interpreted as mean ± standard deviation, and each experiment was executed in triplicate. [15]

***In vitro* Anti-ulcer Activity**

The protein denaturation method was used to assess the sample's *in vitro* antiulcer activity. This method relies on the test compound's capacity to prevent denaturation of proteins under stress. One of the main causes of stomach ulcers is protein denaturation. The model protein employed in this investigation was bovine serum albumin. A reaction mixture was made with 0.5 ml of 1% bovine serum albumin and 0.5 ml of the sample extract at graded concentrations (20–200 µg/ml). Using hydrochloric acid, the mixture's pH was brought to 6.5. The samples were heated to 70°C for five minutes in a water bath after maintained at 37°C for 20 minto cause protein denaturation. The absorbance was recorded at 660 nm, after it had cooled to ambient temperature. Omeprazole was utilized as the standard reference medication, and a control was kept using distilled water in place of the sample extract. The formula used to determine the percentage inhibition of protein denaturation was % inhibition = [(A_control − A_sample)/A_control] × 100. Significant anti-ulcer activity of the test sample is indicated by greater prevention of protein denaturation. [16]

Protein and Ligand Data Acquisition

The 3D shape of the target protein was taken from the Protein Data Bank. This is a database that stores information on the structure of proteins and other biological molecules. The protein structure was downloaded in PDB format. The chosen protein structure was obtained then employed for molecular docking evaluation [17]. PubChem provided the Structural depiction of bioactive phytoconstituents. The National Center for Biotechnology Information is in possession of this database. The compounds were downloaded in 3D SDF file format. Used for analyzing how well they bind to the protein [18, 19].

ADME/Toxicity

The ADME/Toxicity characteristics of the chosen compounds were looked at using the FAF-DRUGS 2 software that can be found on the Mobyle platform. This was done to see how the compounds are absorbed and what happens to them in the body. The ADME/Toxicity characteristics include absorption, distribution, metabolism, excretion and toxicity. The compounds were also checked to see if they are like drugs. This was done using Lipinski's Rule of Five. This rule helps figure out if a compound can be easily absorbed by the body when taken orally. According to this rule a compound is considered to be like a drug when it meets conditions. The compound should have a weight of less than 500 Da. It should also have a LogP value of than 5. The compound should not have many donors of hydrogen bonds. Fewer than 5 is the limit. It should also not have many acceptors of hydrogen bonds. Fewer than 10 is the limit. The molar refractivity of the compound should be, between 40 and 130 [20].

Molecular Docking Study

Blind docking computations between the ligands and protein were set up and carried out using MGL tools with AutoGrid and AutoDock vina. The Protein Data Bank (PDB) provided the crystallized three-dimensional structure. PyRx (0.8) Tools were used to prepare ligand (complex) and receptor (protein) files. A box containing a number of grid points in the x, y, and z axes contained the protein. Docking calculations were carried out using Lamarckian genetic methods, as implemented in AutoDock. Every other parameter was set to its default value. The binding mode for each docking scenario was determined by selecting the lowest energy docked conformation based on the AutoDock scoring algorithm and amount of hydrogen bonds. PyMol was used to render the AutoDock output [21].

Pymol

PYMOL is a tool that helps people see molecules in a better way. This tool is made by a company that wants to help scientists and students. PYMOL can make good 3D pictures of small molecules and big molecules, like proteins. It is one of the free tools that scientists use to look at the structure of things. The name PYMOL says it all. It works with the Python programming language and people can even add things to it using Python [22].

A **B**

Fig 1: (A&B) *Piper schmidtii*The plant has been collected from the Coonor hill range and authenticated in Botanical Survey of India (BSI), Southern Regional Centre, TNAU Campus, Coimbatore. The auntheticated number BSI/SRC/5/23/2022/Tech/404.

Results and Discussion

Phytoconstituent analysis of the extract

The study of plant material is very important for making medicines. It helps in creating, changing and ensuring the quality of these medicines. [23] The GC-MS profile of the extract revealed an extensive mixture of bioactive components; the sample's GC-MS analysis detected 29 phytochemical components exhibiting varying retention times and relative abundances (Table 1, & Figure 2). 9,12-octadecadienoic acid methyl ester (10.85%) was the most common of them, followed by dodecanoic acid (3.21%), hexadecanoic acid ethyl ester (3.21%), and a derivative of pyrimidine (5.61%). The chromatogram also revealed moderate concentrations of 2,6-dimethyl cyclohexanol, palmitic acid, and n-hexadecanoic acid. Terpenoids, hydrocarbons, and nitrogen-containing compounds made little contributions to the chemical profile, which was dominated by fatty acids and their esters. Substances with low spectral match values were thoroughly assessed in order to lower compound identification uncertainty.GC–MS studies of *Piper betle* have identified n-hexadecanoic acid, linoleic acid derivatives, methyl esters of unsaturated fatty acids, and other long-chain lipids, along with phenylpropanoids such as eugenol and chavicol, as important constituents [24]. Likewise, *Piper nigrum* extracts have been reported to contain palmitic acid, linoleic acid, oleic acid derivatives, phytol, and various fatty acid esters in addition to the characteristic alkaloid piperine [25]. Similar observations have also been reported for *Piper longum*, in which fatty acids, fatty acid esters, terpenoids, and nitrogen-containing compounds constitute a substantial proportion of the volatile fraction [26]. The occurrence of these common lipid-derived metabolites across different *Piper* species suggests that fatty acids represent conserved chemotaxonomic markers within the genus. Dodecanoic acid and hexadecanoic acid derivatives and long-chain fatty acids were found to be very important after 9,12- acid methyl ester. These compounds are recognized for their diverse pharmacological effects, encompassing their antioxidant and antibacterial characteristics. Reports say that fatty acids, like Dodecanoic acid and hexadecanoic acid derivatives and long-chain fatty acids can kill bacteria by stopping them from making their own fatty acids and by damaging their cell walls [27, 28]**.**

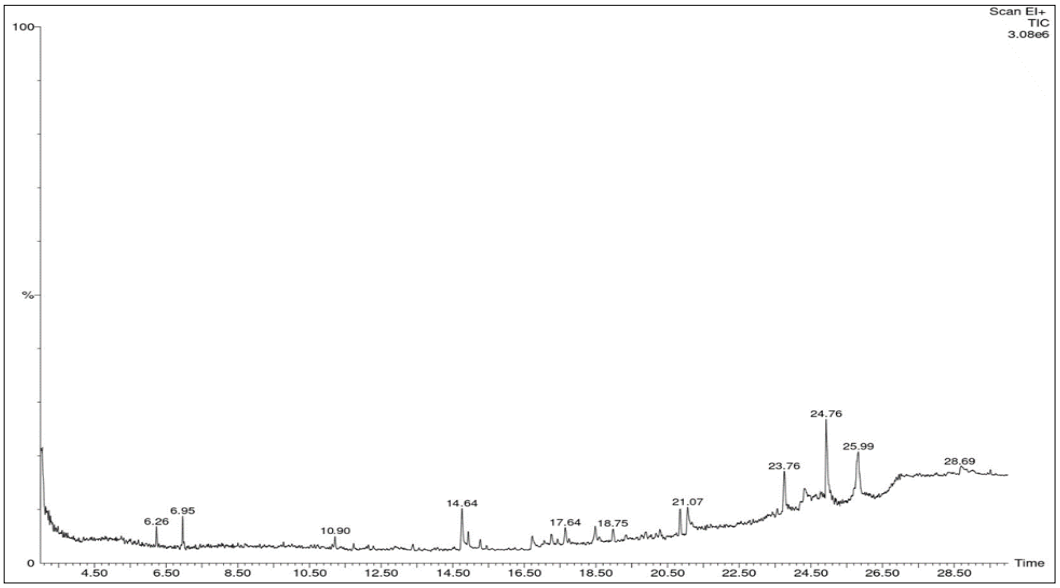


Fig 2: Chromatographic representation of compounds identified in the extract by GC–MS analysis.

Tables and Illustrations

Table 1: Phyto-medicinal components identified through GC-MS analysis of the ethanolic leaf extract of *P. schmidti*

SL. No	Rt	Name of the Compound	Molecular Formula	MW	Component Area	Match Factor
1	4.53	4-Ethoxy-2-butanone	C6H12O2	116	0.01	6.48
2	6.26	Propanoic acid, 2-hydroxy-, ethyl ester	C5H10O3	118	2.06	41.23
3	6.95	2,6-dimethyl-cyclohexanol	C8H16O	128	2.53	37.45
4	7.95	beta-Myrcene	C10H16	136	0.01	7.63
5	9.65	Undecane	C11H24	156	0.01	8.46
6	10.90	1,3-Pentadiene,5(2,2- dimethylcyclopropyl)-2,4- dimethyl-, (Z or E)-	C12H20	164	1.03	23.18
7	11.61	Benzoic acid, 2-methyl-3- nitro-	C8H7NO4	181	0.07	11.25
8	12.92	Tridecane	C13H28	184	0.05	14.45
9	14.64	Dodecanoic acid	C12H24O2	200	3.21	46.23
10	14.72	Copaene	C15H24	204	0.67	22.37
11	14.87	Cedrene	C15H24	204	0.56	18.46
12	16.65	Cyclohexene,1-methyl-4-(5-methyl-1-methyl-4-hexenyl)	C15H24	204	1.23	24.56
13	16.87	Piperidine,1-(1-oxo-3-phenyl-2-propenyl)-	C14H17NO	215	0.79	17.48
14	17.64	1-Piperonyl-3-amino-1,2,4- triazine	C10H10N4O2	218	1.15	29.48
15	18.49	Tetradecanoic acid	C14H28O2	228	1.12	30.42
16	18.75	(2S) -(-)-2-(3,4-Dimethoxyphenyl)-N-methylbutylamine	C13H21NO2	223	0.85	25.63
17	19.75	Octadecanoic acid	C18H36O2	284	0.01	13.02
18	20.82	n-Hexadecanoic acid	C16H32O2	256	2.15	40.18
19	21.07	Palmitic acid	C16H32O2	256	2.35	37.48
20	22.43	Oleic acid	C18H34O2	282	0.01	9.15
21	23.76	Hexadecanoic acid, ethyl ester	C18H36O2	284	3.21	41.19
22	24.45	11-Octadecenoic acid, methyl ester	C19H36O2	296	1.52	32.15
23	24.76	9,12-Octadecadienoic acid, methyl ester, (E, E)-	C19H34O2	294	10.85	66.37
24	24.85	Ethyl2-methyl-4-(4-fluorophenyl)-6- trifluoromethylpyridine-3carboxylate	C16H13F4NO2	327	0.01	7.45
25	24.92	2-(3',5'-Ditrifluoromethylphenyl)-1,1,3,3-tetramethylguanidine	C13H15F6N3	327	0.01	8.56
26	25.99	5-Allyl-4-[1-(p-aminophenyl) ethylidenehydra zono]-6-methyl-2-phenylpyrimidine	C22H23N5	357	5.61	32.15
27	26.72	11-[(3-Hexyl-5-propyl)-2-furyl] undecanal	C24H42O2	362	0.01	10.25
28	28.69	4-(p-Chlorophenyl)-3-methyl-5,6dihydrobenzo(h)thiochroman-2,3-dicarboxylic anhydride	C22H17ClO3S	396	1.03	15.63
29	29.51	Cyclononasiloxane, octadecamethyl	C18H54O9Si9	666	0.03	12.03

Acetylcholinesterase (AChE) enzyme analysis

The *Piper schmidtii* ethanolic extract was dose dependently inhibitory of acetylcholinesterase with 6.76% (50 µg/mL) to 73.68% (250 µg/mL) **(**Figure3**).** The inhibitory effect was better on increasing doses with IC 50 of 176.35 µg/mL. The normal galantamine had enhanced inhibition rate at lower concentration (IC50 274 -1g/mL, 27.06, 71.05 between 20 and 100 -1g/mL respectively). Phytochemicals which modulate cholinergic neurotransmission were detected in ethanolic leaf extract of *Piper schmidtii* with concentration dependent inhibitory activity of acetyl cholinesterase (AChE) which was 176.35 µg/mL. It can be concluded that *P. schmidtii* is a promising source to isolate natural cholinesterase inhibitors based on the observed activity. Some other members of the genus *Piper* have been reported with similar neuropharmacological properties. A recent extensive review showed the methanolic extracts of *Piper nigrum*and *Piper retrofractum* had AChE inhibitory activities with IC_{50} values of 11.13 µg/mL and 14.08 µg/mL respectively, whereas the ethyl acetate extract of *P. nigrum* showed 61.5% inhibition at 100 µg/mL. Moreover, piperine, the main alkaloid of *P. nigrum*, was seen to inhibit AChE with an IC_{50} value of 63.16µg/mL, indicating the significant role of piperamide alkaloids in the neuroprotective activity of the genus [29]**.** Further, bioassay guided phytochemical studies have indicated that the AChE inhibitory activity of *Piper* species is mainly due to the presence of amide alkaloids. The isolated piperine, methyl piperate, guineensine, pipercide and pellitorine from fruit extract of *Piper longum* in dichloromethane and reported that piperine was the potent inhibitor with IC_{50} 0.30 mM while the other compounds showed comparatively weak activity [30] the same AChE inhibitory activity was found in other species of the genus *Piper*. *Piper sarmentosum* stem (IC_{50} = 13.59 µg/mL), *Piper nigrum* fruit (IC_{50} = 11.13 µg/mL) and *Piper retrofractum* fruit (IC_{50} = 14.08 µg/mL) showed the highest inhibitory activity, followed by *Piper nigrum* leaf (IC_{50} = 36.25 µg/mL) and *Piper betle* leaf (40.28% at 100 µg/mL) while the IC_{50} value of *Piper betle* leaf was not obtained [31]**.** Similarly, Du *et al*. (2024) reported the isolation of nineteen amide alkaloids from *Piper nigrum*, which showed several amide alkaloids with remarkable inhibitory activities against acetylcholinesterase and butyrylcholinesterase, indicating that amide alkaloids are the main neuroactive constituents of the genus. [32]

In vitro Antioxidant Assays

DPPH radical scavenging activity

The assay demonstrated that the ethanol leaf extract of *Piper wightii* exhibited hydrogen-donating ability with an IC_{50} value of 56.47 µg/mL (Figure 4a). *Piper schmidtii*'s DPPH radical scavenging action is consistent with earlier findings on other *Piper* species. The prevailing outcomes are consistent with the previous reports which determined that the phenolic and flavonoid components of *P. mullesua* and *P. wightii* extracts corresponded to their antioxidant

scavenging activity. These elements enable free radical reduction through electron donors and hydrogen atom donors. The ethanolic extract of *P. longum* has an IC_{50} value of 10 µg/mL, according to earlier research [33], but *P. guineense* had IC_{50} values between 69.05 and 74.0 µg/mL [34, 35]. The ethyl acetate fraction of *P. nigrum* showed higher scavenging activity than the corresponding hexane and methanolic fractions [36], the results identified *P. nigrum* and *P. refractum* as having strong DPPH radical scavenging activity [37].

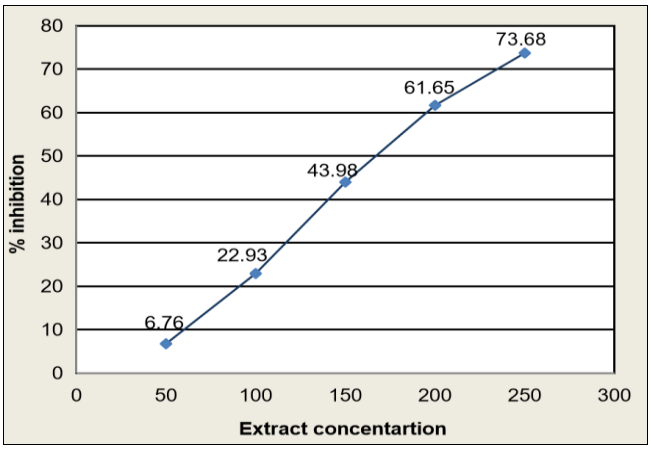


Fig 3: Inhibition of Acetylcholinesterase enzyme by the extract

ABTS radical cation scavenging activity

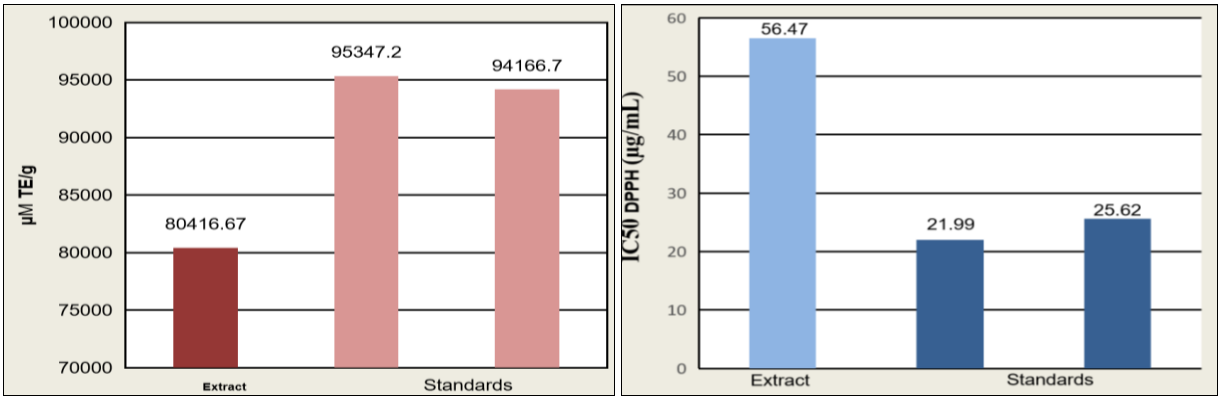
The ABTS-Trolox Equivalent Antioxidant Capacity test was used to assess the ethanol leaf extract of *Piper wightii's* antioxidant capability. The TEAC value of the extract was 80,416.67 µM TE/g (Figure 4b). The scavenging potential of hydrophilic and lipophilic antioxidant substances is determined by the ABTS radical cation assay, which is widely used to assess antioxidant activity. This is primarily associated with the ability of phenolic and other bioactive components found in plant extracts to donate electrons or hydrogen [38, 39]. A wide range of *Piper* species have been reported to have comparable antioxidant properties. The ethyl acetate extract of *Piper longum* exhibited concentration-dependent ABTS radical scavenging activity [40]. Similarly, ethanolic extracts of *P. Nigrum* &*P. longum* showed notable radical scavenging action, highlighting the significance of bioactive secondary metabolites extracted by polar solvents [41]. Moreover, radical quenching ability in *P. nigrum* fruit extracts ranged from 0.68 to 77.05 µg/mL [42]. These results support the antioxidant capability found in the existing research and indicate that *Piper* extracts' bioactive components may enhance their ability to combat free radicals.

Ferric reducing antioxidant power (FRAP) assay

The findings of the assay substantiated that the ethanol leaf extract of *Piper wightii* exhibited a ferric reducing capacity of 275.55 mM Fe (II)E/mg extract (figure 4c). The result reveals that there are compounds in the ethanol extract might have better affinity to the ferrous ions and thereby quench/scavenge them through redox reactions. *Piper schmidtii's* ferric reducing antioxidant power is in line with earlier findings on other *Piper* species. The past study reported significant reductive ability in the ethanolic extracts of *P. nigrum* and *P. longum*, with *P. longum* displaying better reducing power than *P. Nigrum* [43, 44], the phenolic derivatives of both low and high molecular weight have been significantly responsible for plant extracts' capacity to reduce ferric acid by donating electrons to neutralise reactive free radicals [45]. Antioxidants' capability to donate electrons and their possible biological antioxidant effects can be inferred from their ferric reducing capacity as determined by the *in vitro* FRAP assay [46].

Nitric oxide scavenging analysis

The ethanol leaf extract of *Piper schmidtii* showed 41.66 ± 0.57% suppression of nitric oxide radicals. (Figure 4d). Nitric oxide is a crucial nitrogen reactive species that could cause oxidative stress and cell damage in the presence of excessively oxygen and in reaction with the superoxide radical to form peroxynitrite. This effect of the nitric oxide radical protecting effect is a necessary process to minimize oxidative and inflammatory conditions [47]. The methanolic leaf extract of *Piper betle* exhibited 68.4% nitric oxide inhibition at 100 µg/mL, while the corresponding IC_{50} value was reported as 73.5 µg/mL, indicating strong antioxidant activity attributed to hydroxychavicol, eugenol, chavibetol, and other phenolic constituents [48]. Likewise, the ethanolic fruit extract of *Piper nigrum* showed approximately 60–70% activity at 100 µg/mL, with an IC_{50} of 61.8 µg/mL, which has been associated with the presence of piperine, flavonoids, and phenolic acids [49]. Similarly, *Piper longum* fruit extract demonstrated 63.2% inhibition of nitric oxide radicals at 100 µg/mL, with an IC_{50} value of 67.4 µg/mL, reflecting the contribution of alkaloids, lignans, and other antioxidant phytochemicals [50]. The potential of the extract to neutralise reactive nitrogen species is demonstrated in this investigation.



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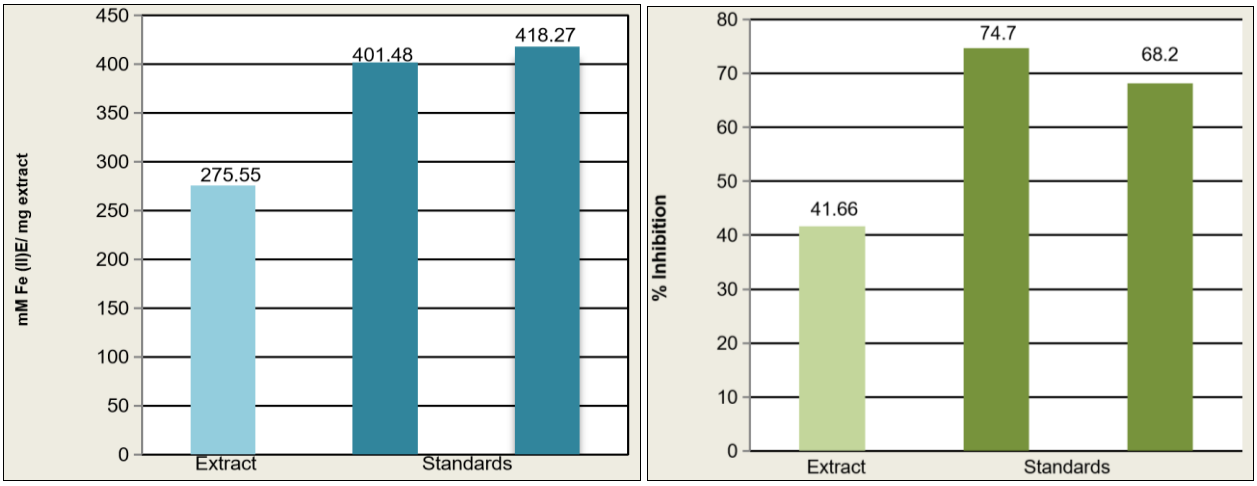

C **D**

Fig 4: (A, B, C, D) -The ethanolic leaf extract of *P. schmidtii* exhibits antioxidant properties with the subsequent tests: (A) DPPH, (B) ABTS, (C) FRAP, and (D) nitric oxide (NO) scavenging. Rutin and BHT served as comparison positive control standards. The data are displayed as mean ± SD (n = 3)

Protein denaturation inhibitory activity

The *Piper schmidtii* extract in ethanol showed high *in vitro* antiulcer activities that inhibited the denaturation of proteins in an inhibitory manner and concentration-effectively. The IC50 value of the plant extract (57.68 µg/mL), on significant increase in dosage increases protein stabilization in the face of stress, shows appreciable effect in inhibiting the protein damage (Figure 5), the percentage inhibition ranged between 81.18% at 250 µg/mL and 16.80% at 50 µg/ml. the ethanolic leaf extract of *Piper betle* has been subjected to many investigations which have demonstrated its gastroprotective effect against stress, ethanol and indomethacin induced ulcer formation. A 78.3% reduction in the ulcer index and significant reduction of lipid peroxidation by 54.9% was observed when the extract was given at 200mg/kg. The same review also found that *P. betle* accelerated ulcer healing, resulting in 93.4% ulcer protection, along with decreases in protein oxidation and malondialdehyde levels and an increase in mucin content and endogenous antioxidant defences [51]**.** Results similar to the present are also reported for *Piper nigrum*, the principle alkaloid of which, piperine, was found to be highly protective of the stomach mucosa. The anti-inflammatory and anti-oxidative stress activity of Piperine was found by mechanistically modulating the Nrf2/HO-1 antioxidant signalling pathway and inhibiting MAPK (ERK, JNK and p38) signalling, which are implicated in stomach damage. [52]**.** In a similar vein, *Piper longum* has been identified as a prospective medicinal species with notable anti-inflammatory and gastroprotective qualities. According to a thorough analysis, piperamide alkaloids, along with phenolic and flavonoid components, protect the gastric mucosa by lowering oxidative stress, inhibiting inflammatory mediators, boosting endogenous antioxidant enzymes, and preserving mucosal integrity [53]**.** The strong protein denaturation inhibitory activity seen for *P. schmidtii* in the current study is consistent with these pharmacological mechanismsfindings, which show that maintaining protein integrity and boosting antioxidant defence constitute significant mechanisms underlying the gastroprotective activity of *Piper* species. The extract's GC-MS profile showed the presence of terpenoid compounds and fatty acid esters, which may work in concert to stabilize proteins and shield stomach tissues from oxidative and inflammatory damage. Thus, *P. schmidtii* appears to be a promising source of natural gastroprotective chemicals, according to the current investigation.

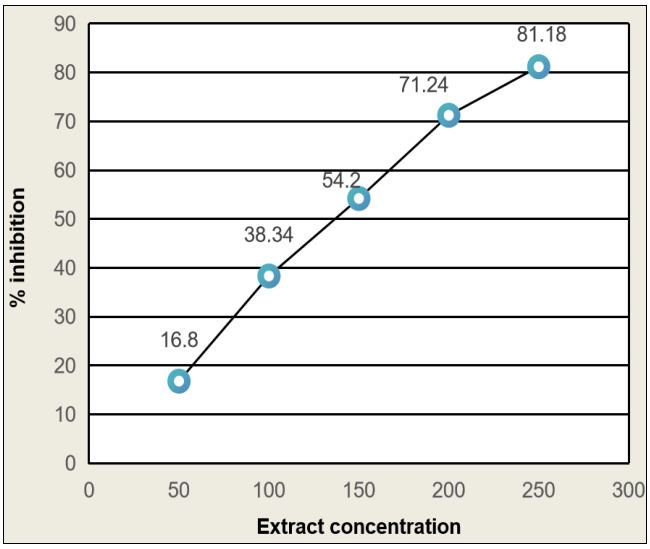

Fig 5: Inhibition percentage of *Piper schmidtii* leaf extract on *in vitro* protein denaturation assay

Molecular docking and binding affinities

A broad spectrum of binding affinities Were found in the molecular docking investigation of seventeen drugs against the ulcer-associated protein (PDB ID: 5YLU) (Table 2 & Figure 6). The range of binding scores was -3.9 to-9.9 kcal/mol. With a score of -9.9 kcal/mol, benzoic acid, 2-methyl-3-nitro-, showed the highest binding affinity. It was closely followed by 5-Allyl-4-[1-(paminophenyl) ethylidenehydrazono]-6-methyl-2 phenylpyrimidine (-8.6 kcal/mol) and cedrene (-8.5 kcal/mol). On the other hand, with scores of -3.9 and -4.1 kcal/mol, respectively, 4-Ethoxy-2-butanone and Propanoic acid, 2-hydroxy-, ethyl ester showed the weakest interactions. The dataset's root-mean-square deviation (RMSD) values varied greatly; notably, oleic acid shown the greatest structural variation

(RMSD/ub 6.823), while the 1,3-pentadiene derivative demonstrated good stability with a low RMSD/ub of 1.367.

The current molecular docking analysis revealed that various compounds identified by GC–MS displayed significant affinity for the gastric proton pump H+/K+-ATPase (PDB ID: 5YLU), a recognized therapeutic target for treating gastric ulcers and acid-related conditions. The crystal structure of 5YLU showcases the gastric proton pump bound to vonoprazan, validating its effectiveness for studies on inhibitor-binding and the discovery of anti-ulcer medications [54]. Among the evaluated compounds, Benzoic acid, 2-methyl-3-nitro- showed the highest binding affinity (-9.9 kcal/mol), suggesting a very stable ligand–protein complex. Earlier research has indicated that compounds showing docking scores around -8.0 to -9.3 kcal/mol against H+/K+-ATPase demonstrate significant anti-ulcer activity and could function as effective proton pump inhibitors [55]. The increased binding affinity noted for Benzoic acid, 2-methyl-3-nitro- could be due to the establishment of several hydrogen bonds and advantageous hydrophobic interactions in the receptor's catalytic pocket. Hydrogen bonds with residues ASP 137, ALA 339, GLU 343, ASN 792, and GLU 795 indicate significant stabilization of the ligand in the enzyme's active site. Comparable results have been observed in earlier molecular modeling research, which identified hydrogen bonding and hydrophobic interactions as the primary factors influencing H+/K+-ATPase inhibition [56].

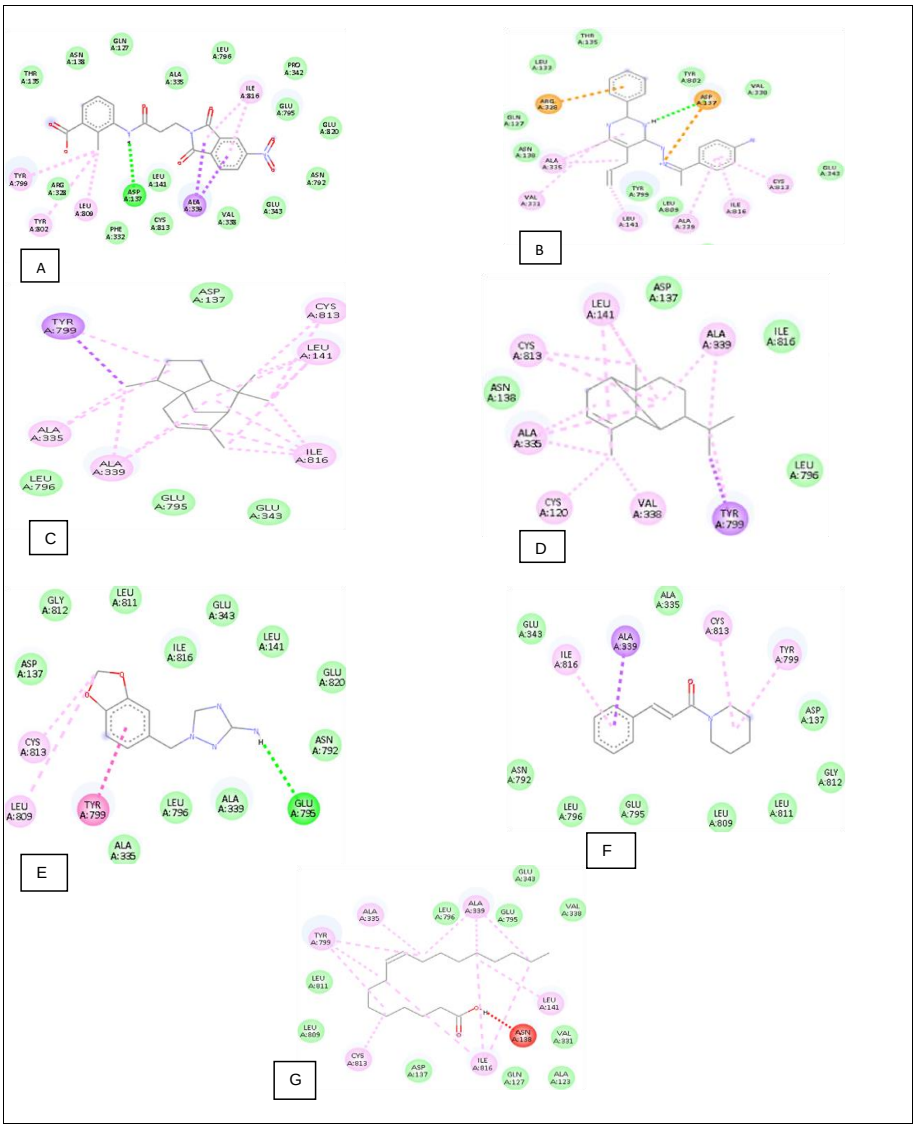

Fig 6: Two-dimensional receptor–ligand interaction diagrams of the highest-affinity phytocompounds identified from molecular docking against the ulcer-associated protein (PDB ID: 5YLU), generated using BIOVIA Discovery Studio Visualizer, A- Benzoic acid,2-methyl-3-nitro-(-9.9), B- 5-Allyl-4-[1-(paminophenyl)ethylidenehydrazono]-6-methyl-2- phenylpyrimidine (-8.6), C- Cedrene (-8.5), D- Copaene (-7.7), E- 1-Piperonyl-3-amino-1,2,4- triazine (-7.6), F - Piperidine,1-(1-oxo-3-phenyl-2-propenyl)- (-7.5), G- Oleic acid (-6.3)

Table 2: Interaction of Compounds with the Ulcer-Associated Protein (PDB ID: 5YLU)

Sl.no	Compound id	Binding score	rmsd/ub	rmsd/lb
1.	n-Hexadecanoic acid	-6.2	2.022	1.432
2.	Dodecanoic acid	-5.6	2.239	1.271
3.	Propanoic acid, 2-hydroxy-, ethyl ester	-4.1	2.931	2.189
4.	Tridecane	-5.2	4.035	0.867
5.	Undecane	-5.1	4.018	0.633

6.	beta-Myrcene	-5.2	2.761	1.74
7.	2,6-dimethyl-cyclohexanol	-5.7	2.775	1.825
8.	Piperidine,1-(1-oxo-3-phenyl-2-propenyl)-	-7.5	3.437	2.68
9.	Oleic acid	-6.3	6.823	4.179
10.	Cedrene	-8.5	3.759	1.008
11.	4-Ethoxy-2-butanone	-3.9	4.616	1.536
12.	1-Piperonyl-3-amino-1,2,4- triazine	-7.6	5.609	3.759
13.	9,12-Octadecadienoic acid, methyl ester, (E, E)-	-6.0	5.523	2.387
14.	1,3-Pentadiene,5(2,2- dimethylcyclopropyl)-2,4- dimethyl-, (Z or E)-	-6.2	1.367	1.062
15.	5-Allyl-4-[1-(p-aminophenyl) ethylidenehydrazono]-6-methyl-2- phenylpyrimidine	-8.6	1.982	1.219
16.	Copaene	-7.7	4.453	1.464
17.	Benzoic acid, 2-methyl-3- nitro-	-9.9	2.36	1.843

Intermolecular interaction analysis

The strong binding of the lead compounds to the H+/K+-ATPase protein was studied by looking at the interactions in its site. Benzoic acid, 2-methyl-3-nitro- made hydrogen bonds with the protein. It formed six bonds with ASP 137 at 2.61 Å, ALA 339 at 2.67 Å, GLU 343 at 2.49 Å, ASN 792 at 3.17 Å. It also made two bonds with GLU 795 at 2.33 Å and 2.17 Å. These polar bonds were helped by hydrophobic interactions with LEU 141 TYR 799 and LEU 809. 1-Piperonyl-3-amino-1,2,4-triazine was stable with five hydrogen bonds. Compounds like Cedrene, Undecane and Copaene relied on hydrophobic interactions. They mainly interacted with TYR 799 LEU 141 and ILE 816. n-Hexadecanoic acid made one H-bond with GLU 343 at 2.10 Å. The lead compounds showed binding affinity to the H+/K+-ATPase protein. Benzoic acid, 2-methyl-3-nitro-, 1-Piperonyl-3-amino-1,2,4-triazine had an interaction and alsoshowed stability with the H+/K+-ATPase protein. The interactions with the H+/K+-ATPase protein were crucial, for the binding of these compounds.

The heterocyclic compound 1-Piperonyl-3-amino-1,2,4-triazine has a binding stability because it forms several hydrogen bonds with residues like ALA 339 ASN 792 GLU 795 and LEU 811. Compounds with nitrogen and rings can make bonds with targets, which helps them work better. This is why they are good at being inhibitors [3]. On the otherhand compounds like Cedrene, Copaene and Undecane mainly make non-polar bonds. They interact with TYR 799 LEU 141 and ILE 816 in a -polar way. These non-polar interactions are important. They help keep the receptor stable inside the proton pump. Some non-polar anti-ulcer medicines work in a way. They interact with H+/K+-ATPase in a -polar way. This has been reported in studies [55].

Drug-likeness and Physicochemical Properties

Physicochemical profiling using SwissADME (Table 3) showed that the best inhibitors usually follow Lipinskis Rule of Five for being like a drug. 2-Methyl-3-nitro-benzoic acid is an example. It does not break any of Lipinskis rules. This compound has properties include 2 H-bond donors (NHD), 7 H-bond acceptors (NHA), 7 rotatable bonds (NRB). Its surface area is 149.6 Å². This is a bit higher than the limit of 140 Å². It is still okay for many gastroprotective drugs. On the hand some long-chain hydrocarbons and fatty acids have problems. Examples include Undecane, Tridecane and n-Hexadecanoic acid. They break least one rule because they are too fatty or have too many rotatable bonds. This can make it hard for them to work well in the body when taken orally. These compounds have many rotatable bonds, more than 10. that is why they are not as good as 2-methyl-3-nitro-benzoic acid. The rule of five helps find compounds. Lipinskis Rule of Five is important, for finding drugs. It checks if a compound can be a drug or not. 2-methyl-3-nitro-benzoic acid is a compound, follows Lipinskis rules which has good properties. It can be a gastroprotective drug. the SwissADME assessment of drug-likeness showed that most of the compounds followed Lipinskis Rule of Five. This rule is a standard that helps predict how well a drug will be absorbed by the body when taken orally. The compound 2-methyl-3-nitro-benzoic acid did not break any of Lipinskis rules which's a good sign for its pharmacokinetic properties. Compounds that follow Lipinskis rules usually get absorbed by the body better. Can easily pass through cell membranes, which is great for drugs that are taken orally. According to even though the TPSA value of the compound was a bit high other studies have found that compounds with TPSA values can still work well as drugs. On the handsome compounds like n-Hexadecanoic acid and Tridecane have long chains of carbon and hydrogen atoms, which makes them more lipophilic. They also have parts that can rotate which can make it harder for them to be absorbed by the body and work, as drugs [57].

Table 3: Drug-likeness prediction of compounds computed by Swiss ADME, NHD: Number of Hydrogen Bond Donors, NHA: Number of Hydrogen Bond Acceptors, NRB: Number of Rotatable Bonds

S. No	Name of the compound	NHD	NHA	NRB	TPSA A^{o2}	Lipinski' rule of five Violation
1.	4-Ethoxy-2-butanone	0	2	4	26.3	0
2.	Propanoic acid, 2-hydroxy-, ethyl ester	1	3	3	46.53	0
3.	2,6-dimethyl-cyclohexanol	1	1	0	20.23	0
4.	beta-Myrcene	0	0	4	0	0
5.	Undecane	0	0	8	0	1
6.	1,3-Pentadiene,5(2,2- dimethylcyclopropyl)-2,4- dimethyl-, (Z or E)-	0	0	3	0	0
7.	Benzoic acid, 2-methyl-3- nitro-	2	7	7	149.6	0
8.	Tridecane	0	0	10	0	1

9.	Dodecanoic acid	1	2	10	37.3	0
10.	Copaene	0	0	1	0	1
11.	Cedrene	0	0	0	0	1
12.	Piperidine,1-(1-oxo-3-phenyl-2-propenyl)-	0	1	3	20.31	0
13.	1-Piperonyl-3-amino-1,2,4- triazine	1	4	2	75.19	0
14.	n-Hexadecanoic acid	1	2	14	37.3	1
15.	Oleic acid	1	2	15	37.3	1
16.	9,12-Octadecadienoic acid, methyl ester, (E, E)-	0	2	15	26.3	1
17.	Cyclononasiloxane, octadecamethyl	0	9	0	83.07	1

Pharmacokinetic Profile and Cytochrome Interaction:

Pharmacokinetic tests showed differences in how well the gut absorbs and breaks down these compounds (Table 4). Many small aliphatic compounds were absorbed well in the gut. They also crossed the Blood-Brain Barrier, which could mean side effects on the brain. The main candidate, acid, 2-methyl-3-nitro and 1-Piperonyl-3-amino-1,2,4-triazine did not cross the Blood-Brain Barrier (BBB). This is good for -ulcer treatments because it means they work only in the gut. 2-Methyl-3-nitro-benzoic acid was broken down by the body in a way. It did not stop enzymes, like Cytochrome P450(CYP) from working. These enzymes are important for breaking down drugs. This means 2-methyl-3-nitro-benzoic acid is unlikely to cause problems when taken with medicines. Some compounds, like Copaene and n-Hexadecanoic acid stopped some of these enzymes from working. This could cause problems when taking these compounds with medicines.

The lead compounds also showed favorable ADME properties, according to pharmacokinetic profiling. Significantly, it was projected that benzoic acid, 2-methyl-3-nitro-, and 1-Piperonyl-3-amino-1,2,4-triazine wouldn't make it through the blood–brain barrier, which could lessen the possibility of negative consequences related to the central nervous system. Additionally, the lack of cytochrome P450 inhibition by 2-methyl-3-nitro- and benzoic acid suggests a minimal likelihood of drug-drug interactions and metabolic interference. The significance of attractive ADME profiles and little CYP interference in the development of safe and effective proton pump inhibitors has also been highlighted by earlier in silico anti-ulcer investigations [58].

Table 4: Pharmacokinetics predictions of compounds compute by Swiss ADME, GI – Gastro intestinal Absorption, BBB - Blood-brain Barrier Permeability, P-gp = P glycoprotein, CYP = Cytochrome-P

Sl. No	Name of the compound	GI	BBB	P-gp	Inhibitor Interaction				
					CYP 1A2	CYP 2C19	CYP 2C9	CYP 2D6	CYP 3A4
1.	4-Ethoxy-2-butanone	High	Yes	No	No	No	No	No	No
2.	Propanoic acid, 2-hydroxy-, ethyl ester	High	No	No	No	No	No	No	No
3.	2,6-dimethyl-cyclohexanol	High	Yes	No	No	No	No	No	No
4.	beta-Myrcene	Low	Yes	No	No	No	No	No	No
5.	Undecane	Low	No	No	Yes	No	No	No	No
6.	1,3-Pentadiene,5(2,2- dimethylcyclopropyl)-2,4- dimethyl-,(Z or E)-	Low	Yes	No	No	No	Yes	No	No
7.	Benzoic acid, 2-methyl-3- nitro-	Low	No	No	No	No	No	No	No
8.	Tridecane	Low	No	No	Yes	No	No	No	No
9.	Dodecanoic acid	High	Yes	No	No	No	No	No	No
10	Copaene	Low	Yes	No	Yes	Yes	Yes	No	No
11	Cedrene	Low	No	No	No	Yes	Yes	No	No
12	Piperidine,1-(1-oxo-3-phenyl-2-propenyl)-	High	Yes	No	No	Yes	No	No	No
13	1-Piperonyl-3-amino-1,2,4- triazine	High	No	Yes	Yes	No	No	No	No
14	n-Hexadecanoic acid	High	Yes	No	Yes	No	Yes	No	No
15	Oleic acid	High	No	No	Yes	No	Yes	No	No
16	9,12-Octadecadienoic acid, methyl ester, (E, E)-	High	No	No	Yes	No	Yes	No	No
17	Cyclononasiloxane, octadecamethyl	Low	No	Yes	No	No	No	No	No

Conclusion

A member of the Piperaceae family, *Piper schmidtii* is known to be rich in a wide variety of secondary metabolites, underlining the possibility of its use as a useful source of medicinal compounds. The acetylcholinesterase (AChE) activity was inhibited by the leaf extract in a concentration-dependent manner. Additionally, the radical scavenging showed significant antioxidant activity. In addition, the extract also showed the ability to prevent protein denaturation supported by docking analysis and therefore, the use of the extract in the treatment of ulcers is considered. All these results indicate *Piper schmidtii's* potential pharmacological value, which warrants further comprehensive research to establish its efficacy as a potential treatment for a variety of illnesses.

Authorship Contribution Statement

Ramprasath, & Srinivasan, were responsible for conceptualization, data curation, and formal analysis, drafting communication, reviewing and editing the manuscript. Shanmugapriya was responsible for conceptualization, data curation, formal analysis, drafting, supervising, validating, reviewing and editing the manuscript.

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Data Availability

All datasets generated or analyzed during this study are are available from the Corresponding author on reasonable request.

Manuscript Metrics: Word count (abstract): 235 | Word count (main text): 5419 | Number of tables: 4 | Number of figures: 6 | Number of references: 58

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