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Salacia fruticosa methanol extract pretreatment attenuates scopolamine-induced cognitive decline and Alzheimer's disease-related pathology in zebrafish

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ABSTRACT

Objective: To examine the effect of the methanolic extract of *Salacia fruticosa* in a zebrafish model of scopolamine-induced Alzheimer's disease.

Methods: High-resolution liquid chromatography-mass spectrometry was used to characterize the phytochemical constituents of *Salacia fruticosa* methanolic extract. The drug-likeness of these compounds was determined *via* the DruLiTo tool, and their acetylcholinesterase (AChE) binding affinities were studied by molecular docking. In *in vivo* studies, adult zebrafish were treated with 3.125, 6.25, and 12.5 mg/L of the extract for seven days and then immersed in scopolamine (100 µM/L) to induce cognitive deficits. T-maze and novel object recognition tests were used for behavioral studies. In addition, the activities of AChE, antioxidant enzymes, and myeloperoxidase were determined in brain tissue of zebrafish.

Results: High-resolution liquid chromatography-mass spectrometry revealed that 40 phytoconstituents were present in the methanolic extract of *Salacia fruticosa*, and 27 compounds met Lipinski's rule of five, indicating good drug-likeness. Some compounds such as stylopine, *p*-coumaroylagmatine, and (-)-heliannuol E, demonstrated high AChE binding affinity. Moreover, pretreatment with the extract significantly mitigated zebrafish cognitive decline, as indicated by increased time spent at the novel object in novel object recognition test, as well as increased time spent and decreased latency in the green arm ($P < 0.001$). The extract also markedly lowered malondialdehyde and myeloperoxidase levels and AChE activity, and enhanced glutathione peroxidase and superoxide dismutase activities ($P < 0.001$) in zebrafish with scopolamine-induced Alzheimer's disease. Histopathological studies revealed that *Salacia fruticosa* extract ameliorated scopolamine-induced abnormalities in neuronal cell morphology.

Conclusions: Pretreatment with the methanolic extract of *Salacia fruticosa* reduces cognitive impairment, enhances antioxidants, and attenuates oxidative stress, highlighting its potential as a preventive agent for Alzheimer's disease.

KEYWORDS: Scopolamine; Acetylcholinesterase inhibitors; *Salacia fruticosa*; Zebrafish; Molecular docking; Antioxidation; Neuroinflammation

Summary

Question: Does the methanolic extract of *Salacia fruticosa* alleviate scopolamine-induced Alzheimer's disease in zebrafish?

Findings: In this study, pretreatment with the methanolic extract of *Salacia fruticosa* attenuated scopolamine-induced cognitive decline, inhibited acetylcholinesterase activity, enhanced antioxidant defense, and reduced inflammatory mediator levels in zebrafish.

Meaning: The methanolic extract of *Salacia fruticosa* shows its potential as a preventive intervention for Alzheimer's disease. However, further studies are needed to elucidate its underlying mechanisms.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the most common type of dementia. It is characterized by deterioration in memory loss, affecting approximately 47.5 million individuals globally, with projections indicating a potential doubling of this figure by 2030[1]. Anxiety and depression are important neuropsychiatric symptoms closely related to the disease process of AD[2]. As AD progresses, acetylcholine is metabolically degraded by acetylcholinesterase (AChE). AChE inhibitors block AChE activity, thereby increasing acetylcholine levels in cholinergic synapses and cholinergic neurotransmission[3]. The administration of AChE inhibitors has demonstrated efficacy in alleviating the behavioral and cognitive symptoms associated with AD[4]. Although there is currently no cure or disease-modifying treatment for AD, cholinesterase inhibitors are commonly used for symptomatic management. Drugs such as donepezil (DNP), galanthamine, tacrine, and huperzine are among the pharmaceutical interventions used for AD treatment. Despite numerous AChE inhibitors that have undergone clinical evaluation over the past two decades, none have successfully advanced to phase III trials, highlighting the necessity of identifying novel compounds with reduced side effects for AD treatment[5]. AD involves multiple pathogenetic mechanisms, including the accumulation of senile plaques and neurofibrillary tangles, and chronic inflammation associated with brain oxidative stress/cholinergic dysfunction[6]. These pathological features interfere with normal brain function, leading to cognitive and behavioral symptoms in patients with AD. Amyloid plaques and neurofibrillary tangles are the main hallmarks of AD. Amyloid plaques are composed mainly of amyloid-beta ($A\beta$) peptides that represent proteolytic fragments originating from amyloid precursor proteins, and neurofibrillary tangles consist mostly of hyperphosphorylated tau proteins. They contribute to synaptic dysfunction and neuronal death, resulting in cognitive loss[7].

The zebrafish (*Danio rerio*) model has been well established as an animal model for studying neurodegenerative diseases such as AD because the genetics of zebrafish are very similar to that of humans, and the zebrafish brain is optically transparent and allows real-time observation of neural processes[8]. As zebrafish are highly amenable to high-throughput formatting and the behavior of this species is also quite tractable, the use of this aquatic vertebrate species is a unique practical platform not only as an *in vivo* immune deficiency model but also for rapid sampling of the efficacy and toxicity assessments of drug candidates[9]. Zebrafish have been used as models to study AD pathology, such as for research on the effects of genetic mutations, including those found in familial AD; the roles of $A\beta$ and tau proteins in aging[10]; and the effects of neurotoxic agents that induce AD-like symptoms and their interactions with impaired memory and learning[11]. The lower cost compared with many other animal models, combined with relatively simple genetics, will also mean that researchers can potentially determine whether a potential

new drug has an impact on a relevant aspect of the disease. This species shares the same neural circuitry as humans and has circuits homologous to humans, which could serve as an animal model *via* molecular mechanisms leading to AD pathophysiology at different stages, even in preclinical validation experiments[12].

Salacia L. (family Celastraceae) is one of the largest genera and includes approximately 196 species in tropical America, Africa, and Asia. Approximately twenty-one species have been reported in India. This genus consists mostly of climbing or creeping shrubs, rarely small trees, but all possess various bioactive compounds, such as steroids, flavonoids, tannins, phenols, saponins, and alkaloids. *Salacia fruticosa* (*S. fruticosa*), a plant of the Celastraceae family endemic to the Western Ghats of India, has been extensively studied for its pharmacological activities. It has antioxidant[13], cytotoxic[14], anti-inflammatory, antiulcer, antihyperglycemic[15], and antidiabetic activities[16] and is widely used in Ayurvedic medicine for the same reason. Studies have shown that *S. fruticosa* possesses a relatively high number of phenolic compounds, which is the reason for its multiple activities. The phenolic compounds present in *S. fruticosa* were identified *via* liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) with an electrospray ionization interface. Various phenolic acids, such as caffeic and ferulic acids, are known for their antioxidant properties. The total phenolic content of *S. fruticosa* has been reported to be 13.50 mg GAE/g[13], which is greater than that of other *Salacia* species. Interestingly, its antidiabetic effects can be attributed to the inhibition of intestinal enzymes, which help to control blood glucose levels. The compound in *S. fruticosa*, 1,5-dicaffeoylquinic acid, inhibits α -glucosidase[17].

S. fruticosa is a rich source of bioactive compounds, including mangiferin, salacinol, kotalanol, and various phenolic acids and flavonoids, which are known for their antioxidant, anti-inflammatory, and potential therapeutic properties[18]. Despite the extensive medicinal importance of these compounds, there remains a significant research gap regarding their specific interactions with AChE and their potential as therapeutic agents for AD. Therefore, in this study, the neuroprotective effects of the methanolic extract of *S. fruticosa* (MESF) in a zebrafish model of AD induced by scopolamine (SCP) were examined.

2. Materials and methods

2.1. Preparation and qualitative analysis of MESF and its characterization via liquid chromatography–mass spectrometry (LC–MS)

Whole *S. fruticosa* plants were obtained from Palayamkottai, Tamil Nadu, India. A voucher specimen was deposited in the herbarium of the Department of Botany, St Xavier College, Palayamkottai, Tamil Nadu, India, where it was subsequently identified and given

the accession number XCH-40551. Whole plants were washed with water and shade-dried before being powdered with an electric grinder. MESF was prepared by dissolving the powder (40 g) in methanol (500 mL), and the mixture was shaken for 3 d at room temperature, followed by centrifugation (2000 rpm). The supernatant was isolated *via* Whatman filter paper, lyophilized, and stored in the dark at 4 °C until further use.

The MESF was analyzed *via* LC-MS (Waters, Synapt-XS #DBA064) at the Sophisticated Analytical Instrumentation Facility, Panjab University, Chandigarh, to identify putative constituents. Chromatographic separations were performed using a C18 Waters Acquity BEH 2.1 mm × 100 mm, 1.7 µm column with a mobile phase of 0.1% formic acid in water (A) and acetonitrile (B). The flow rate, injection volume, and run-time were 0.2 mL/min, 5 µL, and 45 min, respectively. The LC eluate was directly infused into the mass spectrometer, with mass scanning from 0 to 1000 *m/z* *via* electrospray ionization. The MS parameters were as follows: desolvation gas flow, 950 L/h; cone gas flow, 50 L/h; desolvation temperature, 550 °C; source temperature, 120 °C; capillary voltage, 3.22 keV; cone voltage, 50 V; collision energy, 4 eV; source offset, 80 V[19].

2.2. Drug likeliness and ADMET analysis

The drug-likeness tool DruLiTo was used to evaluate the drug-like properties of the phytoconstituents identified in the MESF. These chemicals were further analyzed *via* the methods described by Lipinski's rule of five (LRO5), such as molecular weight, log P, and the number of hydrogen bond donors and acceptors, to determine their properties. This analysis provides insights into the similarity of these phytoconstituents to drug-like molecules.

The pharmacokinetic properties of the prospective compounds present in the identified phytoconstituents were predicted *via* the ADMET prediction tool admetSAR. Computational models have been used to determine five key properties: aqueous solubility,

blood-brain barrier permeability, cytochrome P450 inhibition, hepatotoxicity, and mutagenicity. These observations provide valuable insights into the general drug-likeness and prospective toxicity outcomes of these phytoconstituents. The admetSAR results provide significant insights into the nature and risk details of ADMET, which will be coupled with these ingredients in future investigations involving the development of candidate neuroprotectants[20].

2.3. Molecular docking

The docking study was performed *via* AutoDock Vina, and the input files for this study were created *via* the AutoDock program. In this study, human AChE in complex with DNP (PDB ID:4EY7) was used as the target. The crystallized structures were downloaded from the RSCB Protein Data Bank, and 3D structural information on the ligands was generated *via* PubChem. To be correctly submitted through AutoDock, the files should incorporate polar hydrogen atoms and gesture charges. The center coordinates for AChE in complex with DNP (PDB ID: 4EY7) are −14.108, −43.832, and 27.669, with a size of 12×12×12 and an exhaustiveness of 8. Vina was run *via* a Perl script; the greater the negative score (kcal/mol), the stronger the ligand and receptor bonds. AutoDock Vina returned nine unique positions of ligands, each with a different binding energy for each of the ligands[21].

2.4. Experimental animals and ethical approval

The investigation was conducted on adult zebrafish (3-4 months old) obtained from an approved distributor (WBR Labs) in Chennai, India. We adhered to the directions provided by the CCSEA (878/PO/RE/S/05/CCSEA) in New Delhi. The protocol was approved by the Institutional Animal Ethical Committee [reference number IAEC/IV/05/RIPER/2024 (Dt. 30. 01. 2024)].

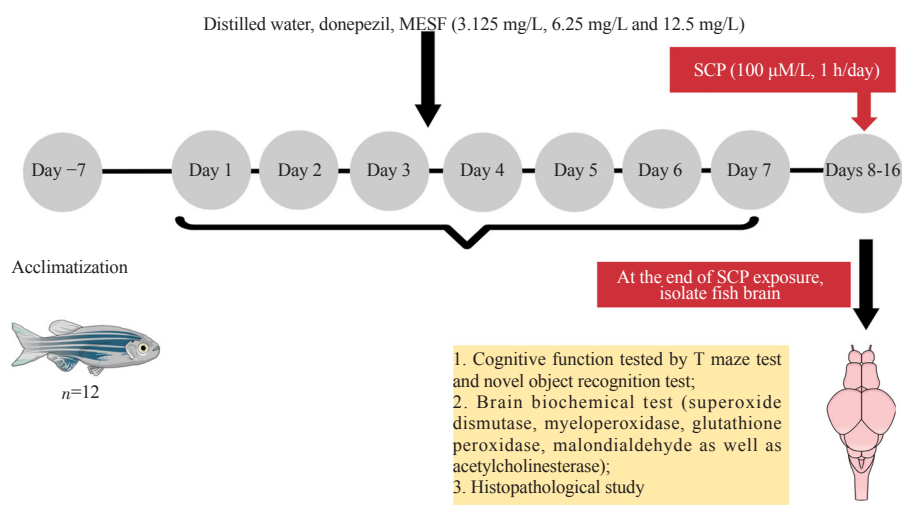


Figure 1. Schematic representation of experiment procedure. MESF: the methanolic extract of *Salacia fruticosa*; SCP: scopolamine.

2.5. Acute toxicity as per OECD 203 and the LD_{50} of MESF

The acute toxicity of MESF was tested in a zebrafish model (*Danio rerio*) according to OECD guidelines 203[22]. The fish were exposed to the sample on the basis of a static exposure regime. For each experiment, seven healthy fish were directly transferred to each prepared concentration. Control groups (7 fish) were also included for each treatment. The fish were considered dead if there was no visible movement, and upon touching, the caudal peduncle produced no reaction. Dead fish were removed when mortality was recorded.

2.6. Experimental procedure for evaluating the neuroprotective activity of MESF in the SCP-induced AD model

The fish were randomly divided into six experimental groups ($n=12$) as follows: group I, normal control; group II, SCP (100 μ M/L); group III, SCP (100 μ M/L) + DNP (1 mg/L); and three treatment groups (IV, V, and VI): SCP (100 μ M/L) + MESF (3.125 mg/L, 6.25 mg/L, and 12.5 mg/L). MESF was given to fish for 7 d before SCP exposure. Treatment doses of 1/8, 1/16, and 1/32 of 100 mg/L were selected (Figure 1). At the end of the treatment, the spatial short-term memory of the zebrafish was tested *via* an offset cross-maze configured as a T-maze, as previously described[23], and recognition memory was tested *via* the novel object recognition test (NOR)[24].

2.7. Brain biochemical analysis

After cognitive function assessment, the zebrafish were euthanized by immersion for 10 min in ice-cold water at 4 °C until gill movement stopped[25]. The brain was isolated and gently crushed with 0.1 mol/

L potassium phosphate buffer and 1.15% KCl in ice at pH 4. The activities of glutathione peroxidase (GPx), malondialdehyde (MDA), superoxide dismutase (SOD), myeloperoxidase (MPO), and AChE were measured *via* ELISA assay[26].

2.8. Histopathological study

The formalin-fixed fish were placed in sodium EDTA solution (0.35 mol/L, pH 7.8) for decalcification for 7 d[27]. The volume of the sodium EDTA solution was 20 times the volume of fish. The decalcified fish were embedded in paraffin, which was used to obtain 5- μ m thick sections *via* a semi-automated rotary microtome. The tissue sections were stained with hematoxylin and eosin (H&E). H&E-stained slides were used to observe microscopic pathological changes in the brain.

2.9. Statistical analysis

The data were expressed as mean $\pm$ SEM. All of the grouped data were statistically analyzed *via* GraphPad Prism V. 8.2. Among the techniques used for hypothesis testing are Dunnett's multiple comparison test and one-way analysis of variance (ANOVA). The threshold for statistical significance was set at a P value of less than 0.05.

3. Results

3.1. High-resolution LC-MS analysis of MESF

High-resolution LC-MS profiling of MESF is presented in Figure

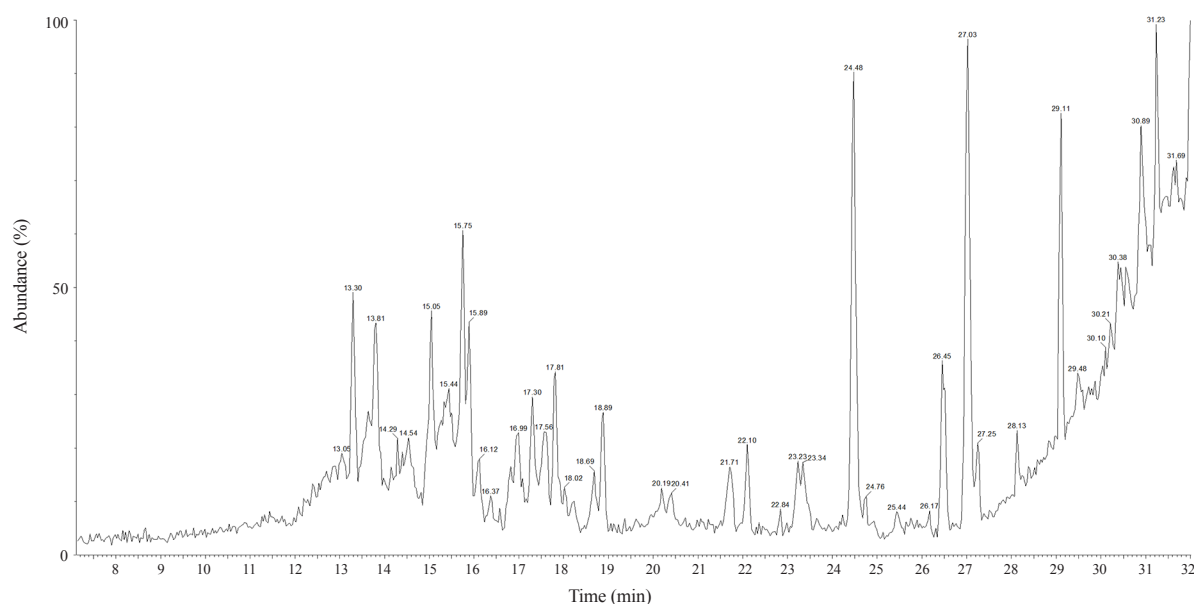


Figure 2. Chromatogram of the identified phytochemical constituents in MESF *via* high resolution LC-MS.

2. The percentage yield of MESF was 8.33% *w/w*; subsequently, the sample was analyzed *via* high-resolution LC-MS to determine its phytoconstituents. A total of 40 compounds were identified on the basis of the retention period, experimental *m/z* values, MS/MS fragments, and NIST database (Supplementary Table 1 and Figure 2). Mass spectrometry analysis was performed in positive ionization mode. The major fraction of the MESF mass-charge values (*m/z*) ranged from 68-957.

3.2. Drug likeliness of phytoconstituents eluted from MESF

High-resolution LC-MS analysis of the MESF yielded a comprehensive dataset of phytoconstituents, each exhibiting unique physicochemical characteristics fundamental to determining drug-likeness. The molecular weights of these compounds ranged from 60 g/mol for isoprene to 899.05 g/mol for ginsenoside Ro, encompassing diverse mass spectra. The logP values varied from highly lipophilic, as observed with hederagenin (LogP 8.349), to hydrophilic compounds such as cyanidin 3-*O*-rutinoside 5-*O*-beta-*D*-glucoside betaine (LogP -1.96). Analysis of hydrogen bond acceptors and donors revealed a significant number of potential interactions with biological targets, from zero donors in several compounds to up to 20 acceptors, as observed for cyanidin 3-*O*-rutinoside 5-*O*-beta-*D*-glucoside betaine. The topological polar surface area, an indicator of drug absorption potential, ranged from nonexistent in compounds without polar atoms to a substantial 89.52 Å² in ginsenoside Ro. These physicochemical parameters provided

the foundational data necessary for further drug-likeness analysis following LRO5 (Supplementary Table 2).

A profiling study of phytoconstituents obtained from MESF revealed rapid variability with respect to physicochemical behavior, especially that of LRO5, an acknowledged critical initiative in drug-likeness interpretation of the 40 compounds analyzed. This revealed that 27 met the LRO5 criterion (67.5%) to a large extent, indicating a sizeable variety of MESF with developability features. Thirteen compounds were filtered out due to the failure of at least one of the LRO5 rules, mainly because of their larger molecular size or higher logP values.

3.3. ADMET analysis of phytoconstituents eluted from MESF

High-resolution LC-MS analysis of MESF revealed that 27 compounds fell into the drug likeliness profile (LRO5). The molecular properties of 27 compounds were analyzed *via* several online web servers, such as admetSAR and Protox-II. Among the compounds listed in Supplementary Table 3, only nine were safe. Further molecular docking studies of these nine compounds were performed to quantify their inhibitory effects on AChE.

3.4. Molecular docking

This study investigated the binding energies and interaction details of various compounds eluted from MESF with AChE, represented by PDB ID: 4EY7. The binding affinities (ΔG) of the ligands varied

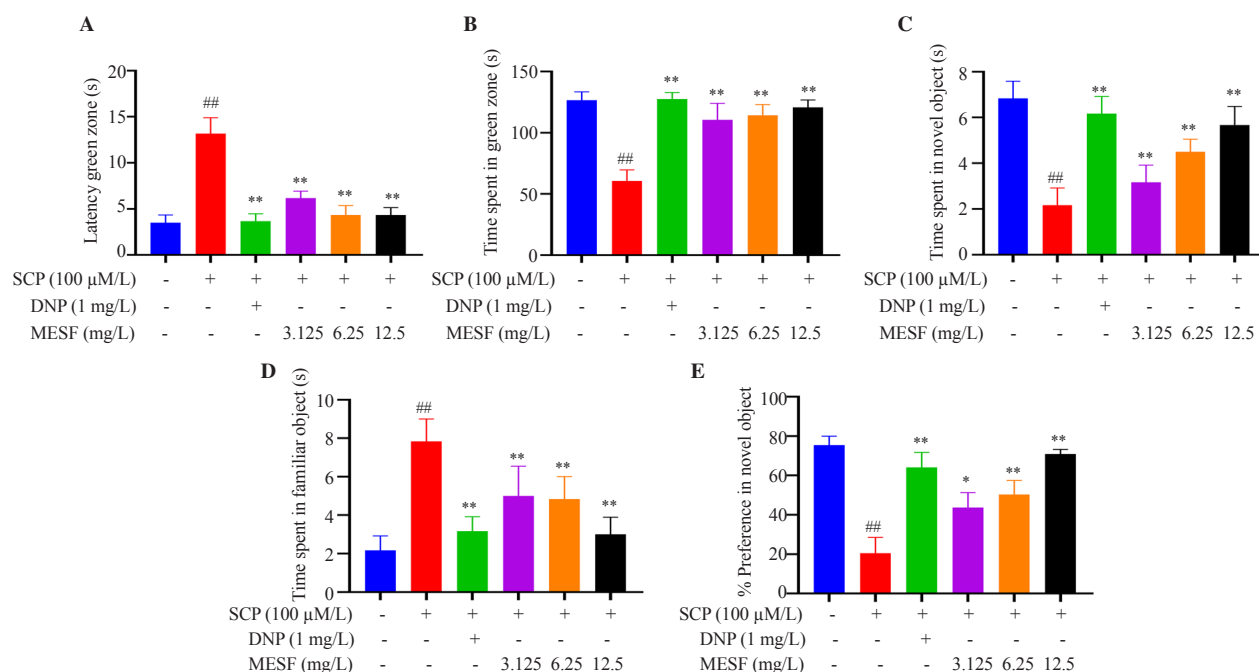


Figure 3. Effects of MESF on zebrafish cognitive function. (A) Latency to enter the green zone and (B) time spent in the green zone in the T-maze test. (C) Time spent in the novel object and (D) the familiar object, as well as (E) percent preference for the novel object in novel object recognition test. Data are expressed as mean $\pm$ SEM ($n=12$) and analyzed by one-way analysis of variance (ANOVA). ^{##} $P<0.001$ versus normal control; ^{*} $P<0.05$, ^{**} $P<0.001$ versus the SCP-treated group. DNP: donepezil.

significantly, as shown in Supplementary Table 4 and Supplementary Figure 1. DNP showed the highest binding affinity, with a ΔG of -10.6 kcal/mol, involving hydrogen-bond interactions with TYR A:124, TYR A:337, and TYR A:341 and hydrophobic interactions with TRP A:86, TYR A:341, and TRP A:386. Stylopine followed with a ΔG of -9 kcal/mol, forming hydrogen bonds with TYR:341 and hydrophobic interactions with THR A:83, PHE A:338, and TYR A:341. *P*-coumaroylagmatine had a binding affinity of -8.9 kcal/mol and interacted with TYR A:124, TYR A:337, and TYR A:341 via hydrogen bonds and hydrophobic interactions. (-)-Heliannuol E and phaseic acid both had a binding affinity of -8.3 kcal/mol. (-)-Heliannuol E formed multiple hydrogen bonds and hydrophobic interactions with several residues, including TYR A:341, TYR A:337, and PHE A:338, whereas phaseic acid primarily interacted with TYR A:124 and TYR A:341. Overall, these findings exhibited the binding interaction profiles of various ligands with AChE, highlighting potential candidates for therapeutic applications.

3.5. Acute toxicity studies

The acute toxicity test was performed as per the guidelines, the limit test was passed, and 100 mg/L was considered a safe dose, as shown in Supplementary Table 5. The observations on loss of equilibrium, abnormal swimming behavior, ventilatory function, skin pigmentation, and mortality parameters revealed no signs. Hence, in this study, 1/8, 1/16, and 1/32 of the safe doses used for treatment were 12.5 mg/L, 6.25 mg/L, and 3.125 mg/L, respectively.

3.6. Effect of the MESF on fish behavior

3.6.1. Latency to enter the green arm

Zebrafish with good memory have a short latency time, whereas those with defective memory have a longer latency time. As shown in Figure 3A, the SCP group showed a significantly greater latency to entry of (13.17 ± 0.40) s ($P < 0.001$) compared with the normal control group $[(3.50 \pm 0.13)$ s]. Pretreatment with MESF significantly ($P < 0.001$) decreased the latency time $[(6.17 \pm 0.23)$ s, (5.67 ± 0.25) s, and (4.33 ± 0.21) s, respectively] in a dose-dependent manner. These findings indicate that pretreatment with MESF provides significant protection against SCP-induced memory loss in the T-maze test.

3.6.2. Time spent in the green arm

Zebrafish with deficient memory spent less time, whereas those with good memory spent more time in the food-containing (green) arm. Figure 3B shows that the SCP group spent significantly less time in the green arm $[(60.07 \pm 0.40)$ s; $P < 0.001$] compared with the normal control group $[(126.67 \pm 0.04)$ s], confirming a significant memory loss. However, MESF pretreatment dose-dependently enhanced the time spent in the green arm $[(110.50 \pm 0.23)$ s, (114.33 ± 0.25) s, and (120.83 ± 0.21) s].

3.6.3. NOR test

In this test, exploratory time and the percentage of preference for familiar and novel objects were measured (Figure 3C-E). SCP administration significantly ($P < 0.001$) increased the preference for familiar objects $[(7.83 \pm 0.48)$ s] over novel objects $[(2.17 \pm 0.48)$

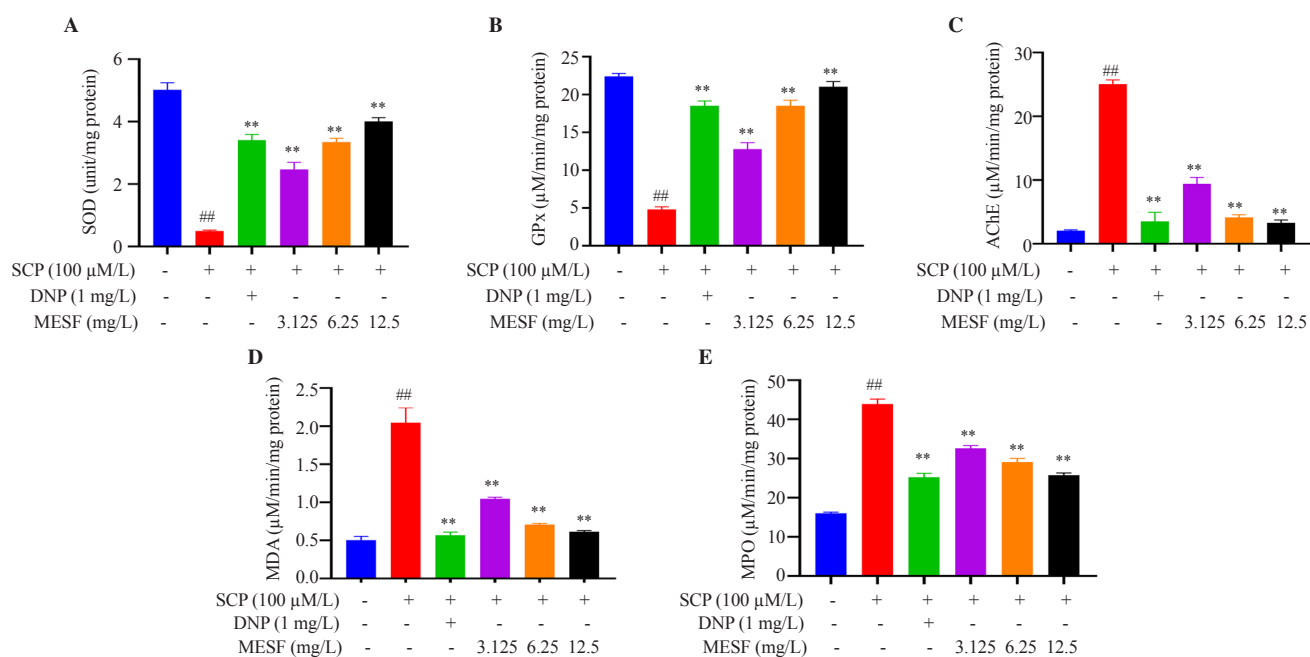


Figure 4. Effects of MESF on biochemical parameters in zebrafish brain. (A) Superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), (C) acetylcholinesterase (AChE), (D) malondialdehyde (MDA), and (E) myeloperoxidase (MPO). Data are expressed as mean $\pm$ SEM ($n=12$) and analyzed by one-way analysis of variance (ANOVA). # $P < 0.001$ versus normal control; ** $P < 0.001$ versus the SCP-treated group.

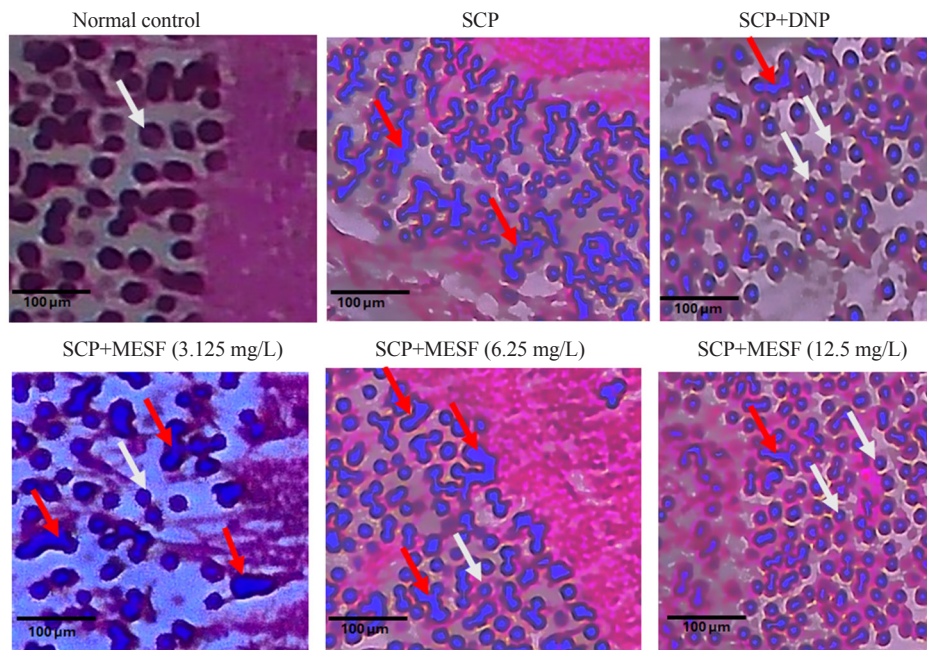


Figure 5. Effect of MESF on the periventricular gray zone of the zebrafish brain (H&E, 400×). The white arrow indicates normal neuronal cells with a rounded shape, while the red arrow shows abnormal neuronal cells with an irregular shape.

s] and reduced the preference percentage for novel objective [(20.49±0.48)%] ($P < 0.001$), implying impaired recognition memory. In contrast, pretreatment with all doses of MESF significantly ($P < 0.001$) reduced the preference for the familiar object [(5.00±0.23) s, (4.83±0.23) s and (3.00±0.20) s, respectively], and significantly ($P < 0.001$) increased the preference for novel object [(3.17±0.23) s, (4.50±0.25) s and (5.67±0.21) s, respectively] and the preference percentage for a novel object [(43.69±0.23)%, (50.27±0.25)% and (70.86±0.20)%, respectively], in SCP-induced zebrafish, implying enhanced memory.

3.7. Effect of MESF on zebrafish brain antioxidant and oxidative stress

Compared with the normal group, MDA level [(2.05±0.48) µM; $P < 0.001$] was significantly increased, and GPx [(4.78±0.48) µM; $P < 0.001$] and SOD [(0.50±0.00) unit/mg protein; $P < 0.001$] activities were significantly decreased in SCP-treated zebrafish. However, MESF pretreatment significantly ($P < 0.001$) reversed SCP-induced changes in MDA level and antioxidants in a dose-dependent manner (Figure 4).

3.8. Effect of MESF on MPO in the brain of zebrafish

As shown in Figure 4E, compared with the normal group, the brain MPO activity of SCP-treated zebrafish was significantly increased [(43.94±0.48) µM; $P < 0.001$]. However, MESF pretreatment dose-dependently decreased MPO ($P < 0.001$) to (32.59±0.23) µM, (29.10±0.25) µM and (25.79±0.20) µM, respectively, in SCP-treated zebrafish.

3.9. Effect of MESF on brain AChE activity

AChE activity in the brains of SCP-treated zebrafish was significantly higher [(25.07±0.40) µM; $P < 0.001$] than that in normal zebrafish [(2.06±0.12) µM]. However, pretreatment with all doses of MESF significantly decreased AChE activity [(9.40±0.20) µM, (4.14±0.25) µM and (3.28±0.21) µM, respectively] (Figure 4C).

3.10. Histopathological examination of the mesencephalon

Histopathological examination of normal neuronal cells showed rounded neuronal cells (white arrow) of the periventricular gray zone (PVG), a region in the optic tectum of the adult zebrafish mesencephalon. In contrast, abnormal shapes and dense neuronal cells were observed in SCP-treated zebrafish (red arrows). Pretreatment with MESF reduced abnormal neuronal cells in SCP-treated zebrafish, especially at 12.5 mg/L (Figure 5).

4. Discussion

AD is characterized by significant cognitive decline, impairing the capacity for logical thinking, behavior and memory, resulting in disturbances in daily life[28]. The neurotransmitter acetylcholine plays a fundamental role in synaptic transmission, but rapid hydrolysis by AChE into acetate and choline at the postsynaptic membrane disrupts cholinergic signaling. This enzymatic degradation has an impact on the cognitive issues observed in AD. AChE inhibition is one of the most important ways of addressing this problem. It also increases the signal strength by preventing the breakdown of

acetylcholine[29,30]. Traditional medicinal plants constitute a rich source of AChE inhibitors of natural origin and contribute to the discovery of drugs against neurodegeneration towards AD. These products have been used for centuries in various forms for treating different diseases in humans and are still reservoirs of the most coveted natural products in terms of pharmaceutical leads[31].

Human AChE plays a key role in the breakdown of acetylcholine, which is important for brain function. The structure comprises a central β -sheet and α -helices that give rise to a structure that enables optimal functioning. There are five important areas of the enzyme that interact with its activity and potential drugs: the catalytic site (Ser203, Glu334, His447), which is involved in the hydrolysis of acetylcholine; the anionic subsite (Trp86, Tyr130, Tyr337, Phe338), which brings acetylcholine closer to the active site; the acyl binding pocket (Phe295, Phe297), which enables firm positioning of the substrate at all time steps; the oxyanion hole (Gly121, Gly122, Ala204), which properly stabilizes the transition state of the reaction; and the peripheral anionic site (Asp74, Tyr124, Trp286, Tyr341), which modulates AChE activity and inhibitory interactions[32–34].

Several compounds were eluted from the LC-MS analysis of MESF, and they interact with the active sites of AChE[12] in different portions of the enzyme. Both stylophine and donepezil are anchored to their catalytic site at high affinity; the side chains of TYR A:341 and TRP A:86 are required for the enzymatic activity of AChE[35]. The substrate specificities of *p*-coumaroylagmatine and (-)-heliannuol E are recognized by binding to the anionic subsite, so the abovementioned motifs are key to substrate binding[36], especially those such as TYR A:124, TYR A:337 and TYR A:341. (E)-Cinnamyl acetate and phaseic acid also interact with the catalyst at a peripheral anionic site, thereby supplementing the inhibitory action. Methyl eugenol and isoprene bind at various peripheral sites and thus modulate enzyme activity. 8-Hydroxygeraniol weakly interacts with isovaleraldehyde, resulting in high binding affinity to the peripheral anionic site. These interactions are consistent with a possible therapeutic target involving the modulation of these compounds on AChE in AD[37].

The SCP is an antagonist of cholinergic receptors that play a role in transmitting learning and memory signals. This interference results in decreased capacity for the performance of memory-related tasks, especially those requiring short-term memory[38]. Zebrafish recognition memory was impaired following SCP treatment, as shown by cognitive deficits in the T-maze and NOR tests. This manipulation directly affects the learning and recognition processes in zebrafish[39,40]. In this study, pretreatment with MESF ameliorated SCP-induced cognitive deficits. This aligns with earlier studies[39–43].

It has been revealed that the patients suffering from AD displays a significant degree of oxidative and inflammatory damage[44]. Moreover, oxidative stress, an inflammatory marker such as MPO, might trigger cellular death in the brain and cognitive impairment[45]. The activation of antioxidant enzymes such as SOD and GPx could scavenge reactive oxygen species and protect cells from oxidative

damage.

In our work, SCP diminished the activity levels of antioxidant enzymes such as SOD and GPx while increasing the levels of MDA and MPO in the zebrafish brain. Consequently, SCP disrupted the equilibrium between antioxidant and oxidant defense mechanisms, potentially accounting for the noted memory impairment in zebrafish. MESF pretreatment enhanced antioxidant defense, as indicated by elevated SOD and GPx activity in SCP-treated zebrafish, which corresponded with a reduction in the MDA and MPO levels in the zebrafish brain. These findings demonstrated that MESF possesses neuroprotective effects against oxidative stress, which is consistent with the findings of previous investigations[42].

AChE is a marker of impaired cholinergic neurons in the brain. Our findings showed a significant increase in AChE activity in SCP-treated zebrafish compared to the normal group. However, MESF pretreatment effectively reduced AChE activity in the SCP-treated group. This suggests that MESF alleviates cholinergic impairments, leading to improved memory performance in zebrafish, as demonstrated by the NOR and T-maze tests.

Histopathological studies have demonstrated that PVG in the zebrafish brain is also a key functional hub for motor and cognitive motility in ocular investigations[27]. Our findings indicate that exposing zebrafish to SCP increased the PVG intensity and caused abnormal cell shape, resulting in brain cell degeneration. However, pretreatment with MESF decreased the cell density and abnormal neural cells in PVG in the mesencephalon.

In this study, MESF ameliorates SCP-induced AD in zebrafish, as evidenced by improved cognitive deficits in the T-maze and NOR tests. MESF pretreatment also increases the activities of antioxidant enzymes (SOD and GPx) and reduced the levels of MDA, MPO, and AChE. Further histopathological examination confirmed its neuroprotective effects. The major phytoconstituents stylophine and *p*-coumaroylagmatine, which were identified *via* molecular docking, are potential AChE inhibitors, supporting the therapeutic potential of MESF for AD. These findings suggest that MESF could be a promising natural source for the development of neuroprotective drugs. However, additional relevant biomarkers, such as Tau and β -amyloid, need to be assessed for further confirmation of the mechanism of action underlying its neuroprotective activity.

Conflict of interest statement

The authors declare no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

PP was responsible for the conceptualization of the study, supervision, design of the animal investigation, and manuscript editing. DP contributed to the computational studies. SP was involved in data acquisition. VM and SB were involved in writing the original manuscript. SA and HM were involved in securing the financial support required for the study.

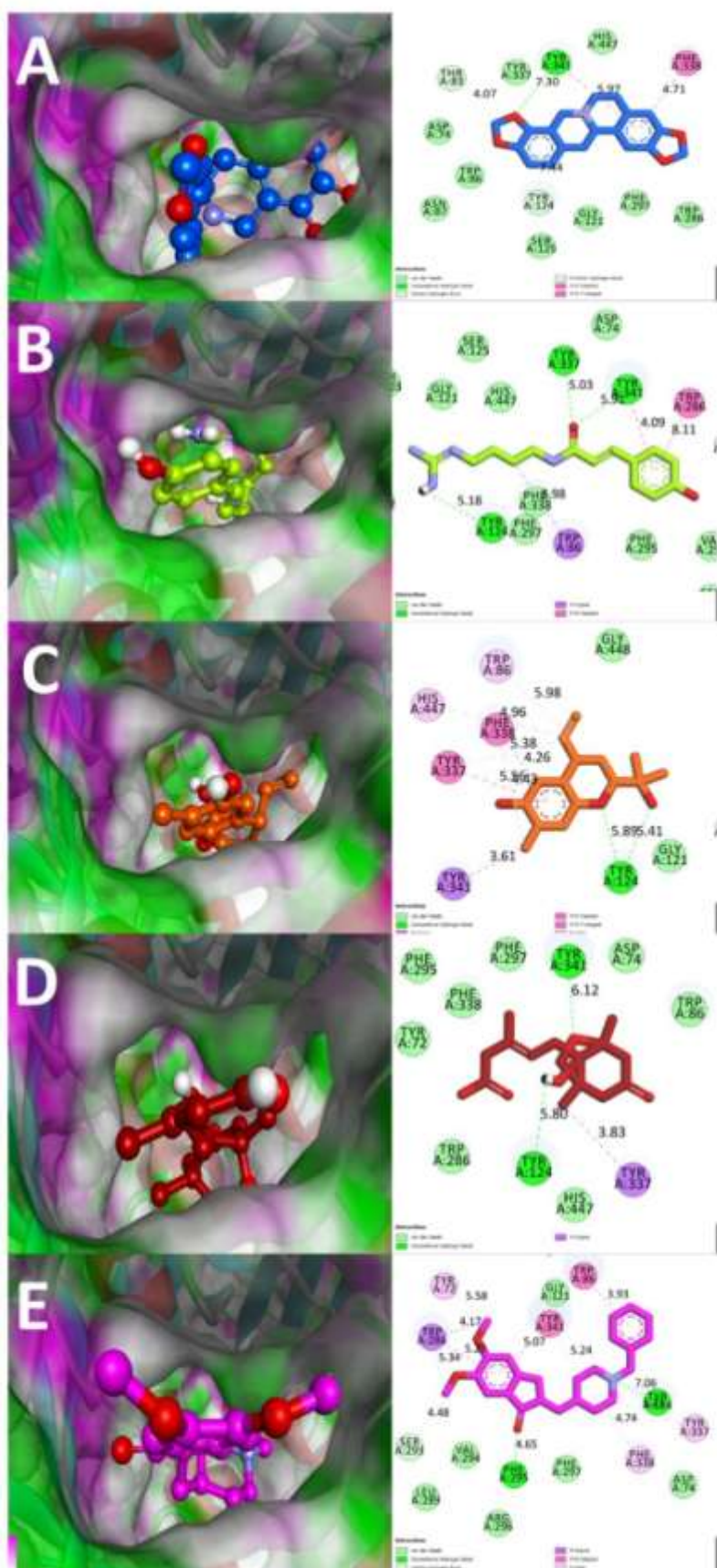
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Supplementary Figure 1. Molecular surface view and 2D representation of selected ligands from MESF with AChE. (A) stylophine, (B) *p*-coumaroylagmatine, (C) (-)-heliannuol E, (D) phaseic acid, and (F) donepezil.

Supplementary Table 1. Phytochemical compounds identified in MESF *via* HR-LC-MS.

S. No	Name	Retention time (min)	Formula	Molecular Weight	m/z	Mass Error (ppm)	Fragmentation Score	Adducts
1.	Isoorientin	13.05	C ₂₁ H ₂₀ O ₁₁	448.379	449.1104966	5.94778005	16.3	M+H
2.	Neohesperidin	13.3	C ₂₈ H ₃₄ O ₁₅	610.563	649.1554125	4.091192363	21.8	M+K
3.	cyanidin 3-O-rutinoside 5-O-beta-D-glucoside betaine	13.81	C ₃₃ H ₄₀ O ₂₀	756.661	795.1701343	-5.722967397	28.5	M+K
4.	Ginsenoside Ro	14.29	C ₄₈ H ₇₆ O ₁₉	957.112	998.5255162	-6.709244145	1.02	M+ACN+H
5.	Vicianin	14.54	C ₁₉ H ₂₅ NO ₁₀	427.405	466.107192	-8.952636344	21.1	M+K
6.	Tricetin	15.05	C ₁₅ H ₁₀ O ₇	302.237	303.0517577	6.0167196	0	M+H
7.	cichoriin	15.44	C ₁₅ H ₁₆ O ₉	340.283	305.0651957	-1.141966393	32.6	M+H-2H ₂ O
8.	Scutellarein	15.75	C ₁₅ H ₁₀ O ₆	286.238	287.0570816	7.258218496	15.5	M+H
9.	Rhamnetin	15.89	C ₁₆ H ₁₂ O ₇	316.264	317.0675469	6.269406286	29.6	M+H
10.	Stylopine	16.12	C ₁₉ H ₁₇ NO ₄	323.347	365.1465487	-9.377028207	0	M+H-2H ₂ O
11.	(-)-pinoresinol	16.37	C ₂₀ H ₂₂ O ₆	358.389	359.1464668	-6.817518517	9.08	M+H
12.	methyl eugenol	16.99	C ₁₁ H ₁₄ O ₂	178.23	143.085631	0.583632637	6.28	M+H-2H ₂ O
13.	S-Allylcysteine	17.3	C ⁶ H ₁₁ NO ₂ S	161.224	203.0854338	3.429156969	4.52	M+ACN+H
14.	(+)-abscisic acid beta-D-glucopyranosyl ester	17.56	C ₂₁ H ₃₀ O ₉	426.46	391.1783986	7.641049942	4.83	M+H-2H ₂ O
15.	vinorine	17.81	C ₂₁ H ₂₂ N ₂ O ₂	334.418	335.1755161	0.355036313	0	M+H
16.	Pterostilbene	18.02	C ₁₆ H ₁₆ O ₃	256.3	221.0979707	7.296412785	6.62	M+H-2H ₂ O
17.	papaverine	18.69	C ₂₀ H ₂₁ NO ₄	339.39	381.1820661	3.469088747	0	M+ACN+H
18.	p-coumaroylagmatine	18.89	C ₁₄ H ₂₀ N ₄ O ₂	276.339	315.1234998	6.198183986	9.74	M+K
19.	miltiradiene	20.19	C ₂₀ H ₃₂	272.474	311.2153542	6.608292496	1.29	M+K

20.	(E)-cinnamyl acetate	20.41	C ₁₁ H ₁₂ O ₂	176.214	141.0710472	6.67399358	8.79	M+H-2H ₂ O
21.	(-)-Heliannuol E	21.71	C ₁₅ H ₂₀ O ₃	248.321	249.1489271	1.61066366	3.46	M+H
22.	(-)-secoisolariciresinol	22.1	C ₂₀ H ₂₆ O ₆	362.42	327.1563188	-7.651641553	2.56	M+H-2H ₂ O
23.	alpha-Bixin	22.84	C ₂₅ H ₃₀ O ₄	394.509	359.1971519	-8.655551534	6.55	M+H-2H ₂ O
24.	p-cymene	23.34	C ₁₀ H ₁₄	134.221	135.1170197	1.493055567	20.7	M+H
25.	Isoprene	24.48	C ₅ H ₈	68.12	69.06944465	-6.329227875	59.4	M+H
26.	Harmaline	24.76	C ₁₃ H ₁₄ N ₂ O	214.267	179.0979017	5.341564602	2.98	M+H-2H ₂ O
27.	Phytosphingosine	25.44	C ₁₈ H ₃₉ NO ₃	317.512	318.29801	-7.139838462	1.4	M+H
28.	Hederagenin	26.17	C ₃₀ H ₄₈ O ₄	472.707	511.3218631	7.303897775	6.94	M+K
29.	deacetylvindoline	26.45	C ₂₃ H ₃₀ N ₂ O ₅	414.5	437.2028577	-4.461318081	8.85	M+Na
30.	Norbelladine	27.03	C ₁₅ H ₁₇ NO ₃	259.304	224.1075848	2.268343424	2.84	M+H-2H ₂ O
31.	Phaseic acid	27.25	C ₁₅ H ₂₀ O ₅	280.319	281.1390759	2.59327881	0	M+H
32.	sphinganine	28.13	C ₁₈ H ₃₉ NO ₂	301.513	302.3069585	5.366975922	0.632	M+H
33.	8-hydroxygeraniol	29.11	C ₁₀ H ₁₈ O ₂	170.251	193.1183022	-9.372322363	13.6	M+Na
34.	curcuquinone	29.48	C ₁₅ H ₂₀ O ₂	232.322	233.1556609	8.841441851	0	M+H
35.	colnelenic acid	30.1	C ₁₈ H ₂₈ O ₃	292.417	293.2095461	-5.432140858	8.97	M+H
36.	Gabaculine	30.21	C ₇ H ₉ NO ₂	139.153	140.0702492	-2.525356965	8.86	M+H
37.	3-ketosphinganine	30.38	C ₁₈ H ₃₇ NO ₂	299.497	322.2710741	-1.95986254	17.2	M+Na
38.	capsaicin	30.89	C ₁₈ H ₂₇ NO ₃	305.416	347.231711	-3.969536962	1.01	M+ACN+H
39.	Isovaleraldehyde	31.23	C ₅ H ₁₀ O	86.1334	128.107328	3.908626376	5.39	M+ACN+H
40.	castasterone	31.69	C ₂₈ H ₄₈ O ₅	464.684	503.3146568	2.820579692	8.38	M+K

Supplementary Table 2. Drug-likeness properties of various compounds eluted from HR-LC-MS of MESF.

Sr. No.	Compound	MW	logp	Alogp	HBA	HBD	TPSA	AMR	nRB	nAtom	LRO5
1	Isoorientin	432.98	-0.359	-3.218	11	0	35.53	114.12	3	37	No
2	Neohesperidin	587.01	-1.11	-3.99	15	0	72.45	148.91	7	54	No
3	cyanidin 3-O-rutinoside 5-O-beta-D-glucoside betaine	731.02	-1.96	-6.323	20	0	81.68	178.12	9	68	No
4	Ginsenoside Ro	899.05	5.258	-3.166	19	0	89.52	231.16	10	86	No
5	Vicianin	412.03	-2.451	-2.358	11	0	60.71	100.69	6	40	No
6	Tricetin	291.96	1.834	-1.35	7	0	26.3	83.36	1	22	Yes
7	cichoriin	328.99	-0.657	-2.186	9	0	44.76	81.51	3	29	Yes
8	Scutellarein	275.97	1.915	-0.787	6	0	26.3	81.76	1	21	Yes
9	Rhamnetin	303.96	2.155	-1.179	7	0	35.53	88.48	2	23	Yes
10	Stylopine	305.98	1.5	-0.064	5	0	40.16	94.32	0	24	Yes
11	(-)-pinoresinol	340	1.316	-0.706	6	0	36.92	101.03	4	30	Yes
12	methyl eugenol	163.99	2.544	1.239	2	0	18.46	57.92	4	13	Yes
13	S-Allylcysteine	150.97	-1.861	0.175	3	0	42.37	42.13	5	11	Yes
14	(+)-abscisic acid beta-D-glucopyranosyl ester	400.99	-0.428	-0.121	9	0	52.6	107.41	6	35	Yes
15	vinorine	316.03	1.244	0.661	4	0	41.9	98.26	2	29	Yes
16	Pterostilbene	239.98	2.69	1.323	3	0	18.46	84.68	4	19	Yes
17	p-coumaroylagmatine	256	0.203	-0.876	6	0	29.43	75.29	8	20	Yes
18	miltiradiene	241.01	7.838	2.909	0	0	0	88.09	1	21	No
19	(E)-cinnamyl acetate	163.99	2.531	1.606	2	0	26.3	56.48	4	13	Yes

20	(-)-Heliannuol E	227.98	2.337	1.502	3	0	9.23	74.47	2	18	Yes
21	(-)-secoisolariciresinol	337.99	0.808	-0.575	6	0	18.46	105.64	9	28	Yes
22	alpha-Bixin	363.98	6.603	5.554	4	0	43.37	129.05	11	29	No
23	p-cymene	120	3.834	2.227	0	0	0	48.29	1	10	Yes
24	harmaline	200	1.351	-0.385	3	0	21.59	68.44	1	16	Yes
25	Phytosphingosine	281.01	5.027	-5.963	4	0	0	70.57	16	25	No
26	Hederagenin	428.01	8.349	1.039	4	0	17.07	135.81	2	38	No
27	deacetylvindoline	387	1.182	-0.641	7	0	42.01	116.6	4	33	Yes
28	Norbelladine	241.99	1.086	0.585	4	0	0	81.46	5	19	Yes
29	Phaseic acid	259.97	0.048	0.38	5	0	43.37	73.86	3	20	Yes
30	sphinganine	264.01	6.268	-5.74	3	0	0	67.52	16	23	No
31	8-hydroxygeraniol	151.99	0.643	1.249	2	0	0	53.07	5	12	Yes
32	curcuquinone	213	3.029	2.646	2	0	34.14	71.78	4	18	Yes
33	colnelenic acid	263.98	5.876	-0.601	3	0	26.3	82.92	13	21	No
34	Gabaculine	129.99	-0.403	-0.238	3	0	17.07	39.12	1	10	Yes
35	3-ketosphinganine	263	6.125	-5.277	3	0	17.07	68.47	16	22	No
36	capsaicin	277.99	3.983	0.278	4	0	26.3	89.55	10	22	Yes
37	Isovaleraldehyde	75.99	1.664	0.415	1	0	17.07	25.11	2	6	Yes
38	castasterone	427.06	5.655	-0.294	5	0	17.07	126.01	5	44	No
39	Isoprene	60	2.348	1.861	0	0	0	24.58	1	5	Yes
40	Papaverine	317.98	1.676	-0.453	5	0	49.28	105.57	6	25	Yes

Supplementary Table 3. ADMET analysis of selected phytoconstituents after screening for drug likeliness from the LC-MS analysis of MESF.

Compound	ADMET LAB							PROTOX-II					Toxicity
	HIA	CaCO2	BBB	CYP1A2	CYP2C19	CYP2C9	CYP2D6	LD50 (mg/kg)	Hepato toxicity	Neuro toxicity	Nephro toxicity	Cardio toxicity	
Scutellarein	0.027	-5.02	0.004	0.964	0.09	0.576	0.569	3919 (Class 5)	Inactive	Inactive	Active	Inactive	Toxic
Tricetin	0.107	-5.255	0.006	0.967	0.04	0.564	0.224	3919 (Class 5)	Inactive	Inactive	Active	Inactive	Toxic
Vinorine	0.159	-4.817	0.965	0.11	0.141	0.229	0.058	370 (Class 4)	Inactive	Active	Inactive	Inactive	Toxic
Papaverine	0.003	-4.859	0.092	0.275	0.839	0.701	0.072	69 (Class 3)	Active	Inactive	Inactive	Inactive	Toxic
Pterostilbene	0.01	-4.929	0.247	0.973	0.808	0.264	0.606	1560 (Class 4)	Inactive	Inactive	Active	Inactive	Toxic
Rhamnetin	0.02	-5.109	0.005	0.949	0.084	0.628	0.482	5000 (Class 5)	Inactive	Inactive	Active	Inactive	Toxic
Norbelladine	0.477	-5.145	0.056	0.264	0.314	0.088	0.775	400 (Class 4)	Inactive	Active	Inactive	Inactive	Toxic
Curcuminone	0.004	-4.774	0.225	0.761	0.189	0.186	0.819	1190 (Class 4)	Active	Active	Inactive	Inactive	Toxic
Stylopine	0.001	-5.21	0.921	0.959	0.969	0.047	0.987	940 (Class 4)	Inactive	Inactive	Inactive	Inactive	Safe
Cichoriin	0.829	-5.943	0.772	0.054	0.014	0.002	0.008	5000 (Class 5)	Inactive	Inactive	Active	Inactive	Toxic

P-Coumaroylagmatine	0.246	-5.852	0.414	0.05	0.085	0.03	0.07	500 (Class 4)	Inactive	Inactive	Inactive	Inactive	Safe
(-)-Pinoresinol	0.008	-4.794	0.101	0.042	0.142	0.427	0.359	1500 (Class 4)	Inactive	Inactive	Active	Active	Toxic
(+)-Absciscic Acid Beta-D-Glucopyranosyl Ester	0.957	-5.637	0.353	0.005	0.018	0.004	0.001	5000 (Class 5)	Inactive	Inactive	Active	Active	Toxic
Capsaicin	0.021	-4.569	0.075	0.707	0.861	0.71	0.837	47 (Class 2)	Inactive	Inactive	Active	Inactive	Toxic
Harmaline	0.003	-5.123	0.976	0.953	0.106	0.026	0.814	550 (Class 4)	Inactive	Active	Inactive	Inactive	Toxic
(-)-Secoisolariciresinol	0.459	-4.557	0.039	0.487	0.132	0.258	0.608	2000 (Class 4)	Inactive	Inactive	Active	Inactive	Toxic
(-)-Heliannulol	0.005	-4.408	0.493	0.152	0.208	0.236	0.286	500 (Class 4)	Inactive	Inactive	Inactive	Inactive	Safe
Phaseic Acid	0.106	-5.39	0.947	0.004	0.026	0.153	0.004	1624 (Class 4)	Inactive	Inactive	Inactive	Inactive	Safe
(E)-Cinnamyl Acetate	0.008	-4.515	0.989	0.988	0.609	0.113	0.094	3300 (Class 5)	Inactive	Inactive	Inactive	Inactive	Safe
Deacetylvindoline	0.918	-5.084	0.962	0.013	0.043	0.018	0.932	325 (Class 4)	Inactive	Inactive	Active	Inactive	Toxic

P-Cymene	0.004	-4.302	0.728	0.941	0.855	0.574	0.778	3 (Class 1)	Inactive	Active	Inactive	Inactive	Toxic
Methyl Eugenol	0.005	-4.338	0.716	0.958	0.862	0.281	0.469	810 (Class 4)	Inactive	Inactive	Inactive	Inactive	Safe
8- Hydroxygeranio l	0.219	-4.299	0.988	0.064	0.022	0.005	0.016	2100 (Class 5)	Inactive	Inactive	Inactive	Inactive	Safe
Gabaculine	0.043	-5.002	0.215	0.033	0.059	0.027	0.016	43 (Class 2)	Inactive	Active	Inactive	Active	Toxic
S-Allylcysteine	0.008	-5.628	0.531	0.016	0.039	0.03	0.012	4000 (Class 5)	Inactive	Inactive	Inactive	Active	Toxic
Isovaleraldehyd e	0.004	-4.223	0.962	0.39	0.043	0.024	0.009	2490 (Class 5)	Inactive	Inactive	Inactive	Inactive	Safe
Isoprene	0.003	-4.274	0.965	0.363	0.05	0.012	0.008	1800 (Class 4)	Inactive	Inactive	Inactive	Inactive	Safe

Supplementary Table 4. Binding energies and interaction details of the compounds eluted from MESF with AChE.

Ligands	Protein	Binding affinity, ΔG (Kcal/mol)	Amino acids involved and distance (Å)		
			Hydrogen-Bond Interactions	Hydrophobic Interactions	Electrostatic Interactions
Stylophine	PDB: 4EY7	-9	TYR :341 (7.30)	THR A: 83 (4.07), PHE A: 338 (4.71), TYR A: 341 (5.97)	-
P-Coumaroylagmatine		-8.9	TYR A:124 (5.18), TYR A:337 (5.03), TYR A:341 (5.91)	TYR A:124 (5.18), TRP A:286 (8.11), TYR A:341 (4.09)	-
(-)-Heliannuol E		-8.3	TYR A:124 (5.41, 5.89)	TYR A:341 (3.61), TYR A:337 (5.38, 5.46), PHE A:338 (4.26, 5.56), HIS A: 447 (4.96), TRP A:86 (5.98)	-
Phaseic Acid		-8.3	TYR A:124 (5.80), TYR A:341 (6.12)	TYR A:337 (3.83)	-
(E)-Cinnamyl acetate		-7.5	TYR A:337 (5.12), TYR A:341 (6.70)	TYR A:86 (4.12), TRP A: 286 (8.14), TYR A:341 (4.19)	-
Methyl eugenol		-7.1	-	TYR A:341 (3.90), PHE A:338 (4.46, 6.75), TYR A:337 (4.84), HIS A:447	-

			(4.82), TRP A:86 (4.40, 4.82, 5.07)	
8-Hydroxygeraniol	-6.7	TYR A:341 (5.99)	TYR A:337 (3.95), PHE A:338 (4.04), TRP A:286 (5.36), TYR A:341 (4.07, 4.66)	-
Isovaleraldehyde	-4.4	TYR A:337 (5.20), TYR A:341 (6.28)	TYR A:337 (3.70, 4.77), PHE A: 338 (3.37)	-
Isoprene	-4.2	-	TYR A:337 (3.87, 4.47, 4.73), TYR A:341 (4.73, 5.50), PHE A:338 (3.43, 5.87), HIS A:447 (4.78)	-
Donepezil	-10.6	TYR A:124 (5.18), TYR A:337 (5.03), TYR A:341 (5.91)	TRP A:86 (3.98), TYR A:341 (4.09), TRP A: 386 (8.11)	-

Supplementary Table 5. Acute toxicity studies MESF on zebrafish according to OECD 203 guidelines.

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