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Impact of clove oil on behavioral and biochemical parameters in restrained rats by using docking and experimental approaches

Sana Sadaf[1,4]*, Shazia Dawood[2], Shaista Emad[3,4], Sarwat Yousuf[4], Sara Qadeer[4,5], Yousra Sarfaraz[4], Sheeza Sheikh[4] and Tahira Perveen[4]

[1]Department of Biochemistry, Faculty of Health & Medical Sciences, Hamdard University, Karachi, Pakistan
[2]Department of Allied Health Sciences, Iqra University, North Campus, Karachi, Pakistan
[3]Department of Biochemistry, Jinnah Medical and Dental College, Sohail University, Karachi, Pakistan
[4]Neurochemistry and Biochemical Neuropharmacology Research Unit, Department of Biochemistry, University of Karachi, Karachi, Pakistan
[5]Department of Biochemistry, Sir Syed College of Medical Sciences for Girls (SSCMS), Karachi, Pakistan

Abstract: **Background**: Acute restraint stress activates the (HPA) axis, elevating corticosterone and influencing cognitive function. Clove oil (*Syzygium aromaticum*), known for its antioxidant and neuroprotective properties, may counteract stress-induced biochemical and behavioral alterations. **Objective**: This study evaluated the effects of clove oil pretreatment on stress-induced memory changes and biochemical responses in rats, supported by molecular docking of its active constituents. **Methods**: Rats were divided into stressed and unstressed groups. Memory performance was assessed using the Morris water maze (MWM) for long-term memory and an elevated plus maze (EPM) for short-term memory. Plasma corticosterone levels and acetyl cholinesterase (AChE) activity were measured. Molecular docking was performed to assess interactions between clove oil constituents and AChE. **Results**: Acute restraint stress (2 hours) significantly enhanced long-term and short term memory ($p < 0.001$). Clove oil pretreatment reduced escape latency, transfer latency ($p=0.000$), corticosterone levels ($p=0.000$), and AChE activity ($p= 0.000$), indicating attenuation of stress-related effects. Docking analysis showed strong binding affinity of isoeugenol to AChE, with a docking score of –74.2657 kcal/mol. **Conclusion**: Clove oil exhibits neuroprotective and cognition-enhancing effects in stress-exposed rats, suggesting its potential therapeutic value for managing stress-related cognitive impairments.

Keywords: Acetylcholinesterase; Clove oil; Memory; Molecular docking; Stress

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INTRODUCTION

Stress, a ubiquitous aspect of modern life, profoundly impacts the coordinated physiological systems of the human body, influencing various brain functions and behavioral activities (Shchaslyvyi *et al*., 2024). Acute stress has been shown to enhance learning and memory ability, predominantly through the action of corticosteroid stress hormones (Ye *et al*., 2018). Stress triggers the activation of a hypothalamic pituitary adrenal (HPA- axis) that prompts secretion of glucocorticoids (rodents' corticosterone) hormone. (McEwen and Akil, 2020). The (HPA) axis is the central stress response system that regulates the release of glucocorticoids during stressful experiences. Upon exposure to a stressor, the hypothalamus secretes corticotropin-releasing hormone (CRH), stimulating the anterior pituitary to release adrenocorticotropic hormone (ACTH), which, in turn, prompts the adrenal cortex to secrete corticosterone in rodents (de Kloet and Joels, 2024). The primary glucocorticoid in rats, corticosterone is released by the adrenal cortex and is commonly referred to as the stress response hormone. Its blood level can be utilized as a stress indicator (Leistner and Menke, 2020). An instant synthesis of glucocorticoids is directly related to a wholesome response to an extreme stressor as well as an operative cessation of stress reaction to restrict glucocorticoids. Nevertheless, comparable intermediaries that were linked to encouraging temporary changes to improve survival may ultimately result in irreversible harm if stress becomes chronic. (McEwen and Akil, 2020). Under the impact of stress hormones and neurotransmitters, stressful events may turn into a crucial modulator to encode new *memories* (Sherman *et al.*, 2023; Tomar and McHugh, 2022) through the activation of sympathetic nervous system during a flight or fight response (Nackley and Friedman, 2021). In contrast to chronic or persistent stress, which alters neural architecture and may inhibit memory formation, multiple types of remedies are extensively employed to aid stress. The most abundant source of medication and a fundamental component of customary remedies worldwide are plants (Chaachouay *et al*., 2023). Currently practice of phytomedicine as a substitute remedy has grown and has provided foundation to substitute chemical drugs. Clove (of *Syzygium aromaticum*), a member of the Myrtraceae family, has significant therapeutic value and used broadly in orthodox medicine for its reputed healing importance (Batiha *et al*., 2019).

**Corresponding author:* e-mail: sana.sadaf@hamdard.edu.pk

Sesquiterpenes, specifically α-, β-caryophyllenes, eugenol, acetyleugenol, are bioactive compounds found in clove plant. Along these Sesquiterpenes some trace alcohol, ketones, esters make up to 14–20% of clove's volatile oil. (Winska *et al*., 2019; Batiha *et al.,* 2019). Anxiety, sadness and sleeplessness are commonly treated with the essential oil extracted from cloves (Sadeh *et al*., 2019). Other compounds found in clove oil comprise flavonoids and other alkaloids, which have robust possessions on the central nervous system, antioxidant and anti-anxiety assets. (Grabadu and Sharma, 2019; Hadidi *et al*., 2019). One of clove oil's active ingredients, eugenol, has anti-inflammatory, anti-microbial, anesthetic, anticonvulsant and muscle-relaxing qualities (Veerapandi *et al.*, 2022; Kim *et al*., 2023). Clove oil's various benefits have been deliberated in the previous years, but few studies have looked at how stress from constraint affects rats' memory.

Therefore, the present study was designed to investigate the effects of clove oil pretreatment on memory changes induced by two hours of acute restraint stress in an animal model. We hypothesized that clove oil would mitigate the adverse effects of stress on memory and associated biochemical parameters and that its active components, particularly isoeugenol, would demonstrate significant binding affinity to acetylcholinesterase, a key enzyme in cognitive function. This research aims to provide further insights into the neuroprotective potential of clove oil and its constituents in the context of stress-related cognitive dysfunction.

Experiment protocol

Animals

The investigation was carried out on 24 adult albino Wistar male rats (150–200 g) purchased from ICCBS, University of Karachi. One week before the experiment, animals were accommodated individually in their cages for the purpose of acclimatization with 22 ± 2 °C temperature, 12-h light/dark cycle, permitted for eating rodent diet and water.

Clove oil and chemical reagents

Clove oil (Roghan-e-Long) obtained from Hamdard Laboratories Waqf Pakistan, Karachi, while all other chemicals were sourced from Sigma-Aldrich.

Experimental procedure

Animals were randomly assigned as control (water,0.2ml/kg/day) and test group, (clove oil, 0.2ml/kg/day) for three weeks. Afterward rats of both groups were divided into unrestrained and restrained group (n=6). An acute stress was given to restrained animals for 2h /day for 2 days while unrestrained animals were positioned in their cages. After an acute stress of 2h animals were shifted from restraining tubes to their home enclosures. Both restrained and unrestrained rats were subjected to the EPM and MWM to assess short-term and long-term memory performance, respectively. were subsequently euthanized via guillotine decapitation, followed by the collection of plasma and brain tissue samples and stored at -70ºC until explored for biochemical estimations.

Restraint stress

A restraint stress protocol is a widely employed and empirically validated model of stress in rodents, allowing minimal caudal movement while remaining non-nociceptive. The rodents were confined within sealed, ventilated polymeric enclosures (length: 20 cm; diameter: 6.5 cm), which permitted only controlled tangential mobility within the containment apparatus (Sadaf *et a*l., 2017).

Behavioral analysis

For the comprehensive behavioral evaluation, both experimental groups (control and clove oil-treated, under unrestrained and restrained conditions) underwent assessment using two established paradigms. Short-term memory was evaluated using the Elevated Plus Maze (EPM), a widely accepted test for anxiety-like behavior and memory in rodents. Long-term memory assessment was conducted using the Morris Water Maze (MWM), a spatial learning and memory task (d'Isa *et al*., 2021). The comprehensive procedure followed by (Sadaf *et al*., 2017).

Biochemical analysis

Corticosterone estimation

Plasma corticosterone levels were precisely quantified using a fluorimetric assay, strictly adhering to the methodology previously outlined by (Qadeer *et al*., 2018). This method provides a reliable measure of the physiological stress response in the animals.

Acetylcholinesterase (AChE) quantification

Acetylcholinesterase (AChE) activity in brain tissue samples was determined following the established protocol described by (Sadaf *et al.*, 2017). This enzymatic assay provides insights into cholinergic system function, which is critically involved in memory and cognitive processes.

Molecular docking

Protein of interest

The three-dimensional crystal structure of acetylcholinesterase (AChE) was retrieved from the Protein Data Bank (PDB) database (https://www.rcsb.org/pdb). Structures with a resolution of ≤ 2.8 Å and intricate non-mutated forms were prioritized. The specific AChE enzyme (PDB ID: 4EY7) was selected for this investigation, as it is characteristically bound to donepezil, a known inhibitor used for exploration.

Docking parameters

Molegro Virtual Docker (MVD) software was employed for all molecular docking simulations. The 3D crystal structure of the acetylcholinesterase protein (PDB ID: 4EY7) was imported into the MVD workspace. Prior to docking, the imported protein was prepared by addressing

any missing bonds and charges to ensure an accurate representation for the simulations.

Ligands

The chemical structure of the ligand, isoeugenol, was obtained in SDF format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and subsequently imported into the MVD workspace for docking studies. Donepezil, the native ligand, was also included for comparative analysis.

Docking simulations

Docking simulations commenced once both the protein and all ligands were appropriately prepared. Co-factors, where relevant, were also incorporated into the docking process. Each ligand was systematically docked onto the active site of acetylcholinesterase by the MVD software. For each ligand, ten independent docking runs were performed, yielding ten distinct docking solutions (poses). These poses were then ranked based on their increasing interaction energies. The pose exhibiting the lowest energy was selected as the most favorable binding conformation. Furthermore, the hydrogen bonds formed between the ligand and the amino acid residues within the active site of the selected protein were meticulously examined for the highest-scoring poses. MVD utilizes a scoring function derived from the Piecewise Linear Potential (PLP) to evaluate ligand conformations and quantify the energy of interaction between each docked conformation and the protein, thereby predicting the optimal binding pose of a ligand (drug candidate) (Badaway and Dawood., 2024).

Statistical analysis

Statistical evaluation of the experimental data was performed using SPSS version 20.0. Independent sample T-tests were employed to determine significant differences between groups. All results are presented as mean ± standard deviation (SD). Leven's test for equality of variances was conducted and a p-value > 0.05 was considered non-significant, indicating that equal variances could be assumed for the t- tests. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Impact of clove oil on plasma corticosterone level in unrestraint and 2-hour restraint rats

The study exposed a notable significant effect of stress, $t_{(10)}$ = −7.17,p=0.000, clove oil, $t_{(10)}$=3.946,p<0.003 and interaction between stress and clove oil, $t_{(10)}$=22.845,p=0.000. Mean corticosterone levels differed significantly, with the restrained control group showing a mean of 229.7155 compared to 139.9320 for the restrained group treated with clove oil. Similarly, in the unrestrained groups, the control mean was 197.4553, while the clove oil group mean was 176.450 (Table 1). Markedly, the difference in means was more pronounced in the restrained groups compared to the unrestrained groups. Fig. 1: (a).

Effect of clove oil on elevated plus maze test (STM) in unrestraint and 2-hour restraint rats.

The study exposed a notable significant effect of stress, $t_{(10)}$= 4.452, p<0.001, clove oil, $t_{(10)}$=4.844,p<0.001 and interaction between stress and clove oil, $t_{(10)}$=6.789, p=0.000. Mean transfer latency differed significantly, with the restrained control group showing a mean of 25.00 compared to 11.833 for the restrained group treated with clove oil. Similarly, in the unrestrained groups, the control mean was 43.66, while the clove oil group mean was 18.500 (Table 1). Remarkably, the difference in means was more pronounced in the restrained groups compared to the unrestrained groups. Fig. 1: (b).

Impact of clove oil on Morris water maze test (LTM) in unrestraint and 2-hour restraint rats.

The study exposed a notable significant effect of stress, $t_{(10)}$= 7.152, p=0.000, clove oil, $t_{(10)}$=4.710,p<0.001 and interaction between stress and clove oil, $t_{(10)}$=5.064, p=0.000. Mean escape latency differed significantly, with the restrained control group showing a mean of 23.500 compared to 10.333 for the restrained group treated with clove oil (Table 1). Similarly, in the unrestrained groups, the control mean was 42.1667 while the clove oil group mean was 18.000. Fig. 1: (c).

Impact of clove oil on acetylcholinesterase activity in unrestraint and 2-hour restraint rats

The study revealed a notable significant effect of stress, $t_{(10)}$ =9.790, p=0.000, clove oil, $t_{(10)}$=14.692,p=0.000 and interaction between stress and clove oil, $t_{(10)}$=9.233, p=0.000. Mean escape latency differed significantly, with the restrained control group showing a mean of 3770.0600 compared to 2046.7133 for the restrained group treated with clove oil. Fig. 1: (d). Similarly, in the unrestrained groups, the control mean was 5471.1727 while the clove oil group mean was 2774.3885. (Table 1).

Docking result of clove active component (isoeugenol)

The binding energies obtained from the molecular interactions of the selected ligands are presented in (Table 2). Fig. 2 presents the structure of isoeugenol. Donepezil (1-benzyl-4-[(5,6-dimethoxy-1-indanon-2-yl)methyl]piperidine), used as the native ligand, showed a significantly stronger binding affinity, with a MolDock score of −119.647 kcal/mol and a Rerank score of −91.1668 (Table 2). Donepezil interacted with amino acid residues Phe295, Phe297, Tyr337, His447, Gly121, Ser203, and Gly126, table 3 and figs. 3(a) and 3(b). In contrast, isoeugenol, a key ligand, exhibited a MolDock score of −74.2657 kcal/mol and a Rerank score of −43.8498 (Table 2). Isoeugenol interacted with amino acid residues Tyr72, Asp74, Asp87, Trp86, and Ser125, and formed hydrogen bonds with Gly126 and Gln71 within the active site of acetylcholinesterase, table 3 and figs. 4(a) and 4(b).

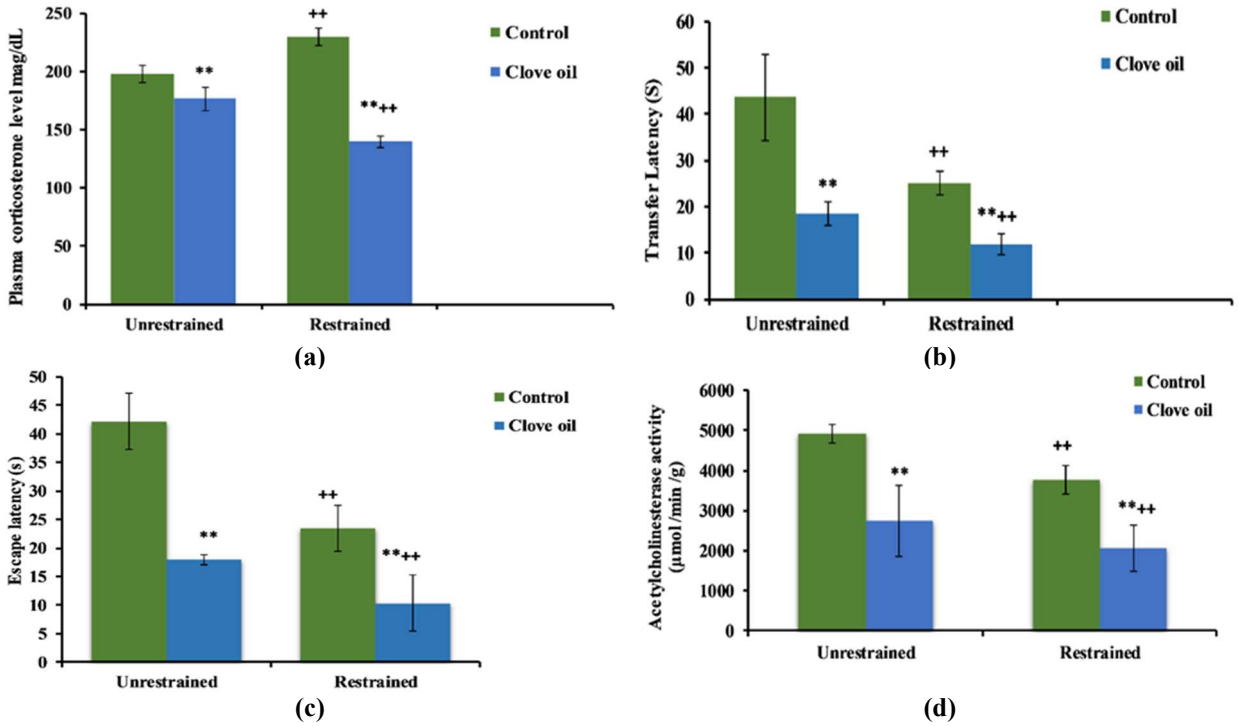


Fig. 1: Impact of clove oil on behavioral and biochemical parameters.

(a) Effect of clove oil on plasma corticosterone level, (b) Effect of clove oil on short term memory, (c) Effect of clove oil on long term memory (d) Effect of clove oil on brain AChE level.
Significant differences by independent sample T test. **p<0.000 from respective untreated controls, ++p <0.001 from their respective unrestrained control.

Table 1: Effects of Clove oil on behavioral and biochemical parameters in unrestrained and restrained animal.

	Unrestrained Control	Unrestrained Clove oil	Restrained Control	Restrained Clove oil	Independent sample T test
CORT.	197.45 ±7.58	176.54 a** ±9.61	229.71[b]** ±7.97	139.93 c** ±4.92	[a] $t_{(10)}$ =3.946,p<0.003 [b] $t_{(10)}$=22.845,p=0.000. [c]$t_{(10)}$= −7.17,p=0.000
STM	38.33 ±9.68	18.5 a** ±2.58	25[b]** ±4.14	11.83 c** ±2.31	[a] $t_{(10)}$=4.844,p<0.001 [b] $t_{(10)}$= 4.452, p<0.001 [c] $t_{(10)}$=6.789, p=0.000.
LTM	42.16 ±4.95	18 a** ±8.07	23.5[b]** ±4.03	10.33 c** ±4.92	[a] $t_{(10)}$ =4.710,p<0.001 [b] $t_{(10)}$ = 7.152, p=0.000 [c] $t_{(10)}$=5.064, p=0.000.
AChE	5471.27 ±229.59	a**27774.38 ±388.86	[b]**3770.06 ±358.39	c ** 2046.71 ±283.90	[a] $t_{(10)}$ =14.692,p=0.000 [b] $t_{(10)}$ =9.790, p=0.000 [c] t(10) =9.233, p=0.000

All values are stated as mean ±SD (n=6). Significant differences by independent sample T test. ***p*<0.000. [a]Comparison between unrestrained controls with clove oil, ** [b]Comparison between unrestrained control with restrained control. ** [c]Comparison between restrained control with restrained clove oil. **

Table 2: MolDock score; Rerank score and the hydrogen bond energy of the docked compounds

Ligand	MolDoc score Kcal/mol	Rerank score	HBOND	MW
OWN ligand(1-benzyl-4-[(5,6 dimethoxy-1-indanon-2-yl)methyl]piperidine)	-119.647	-91.1668	-6.84917	380.5
isoeugenol	-74.2657	-43.8498	-3.7266	164.201

Molecular interaction diagram showing ligand binding within the active site of the target protein. Key residues involved include Tyr124,72, Asp74,Asn87, Ser125,Trp86 exhibiting π–π stacking, and Gln71,Gly126 forming a hydrogen bond. Green sticks represent the ligand, while red and blue indicate oxygen and nitrogen atoms, respectively.

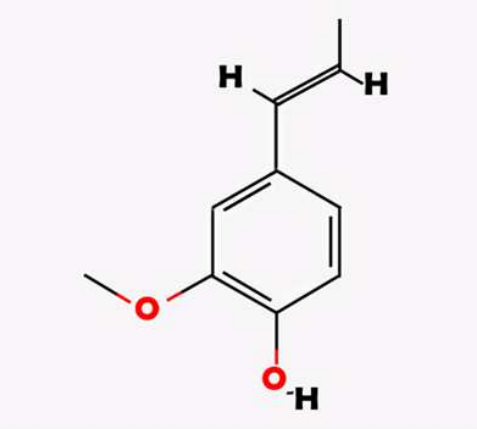


Fig. 2: Isoeugenol

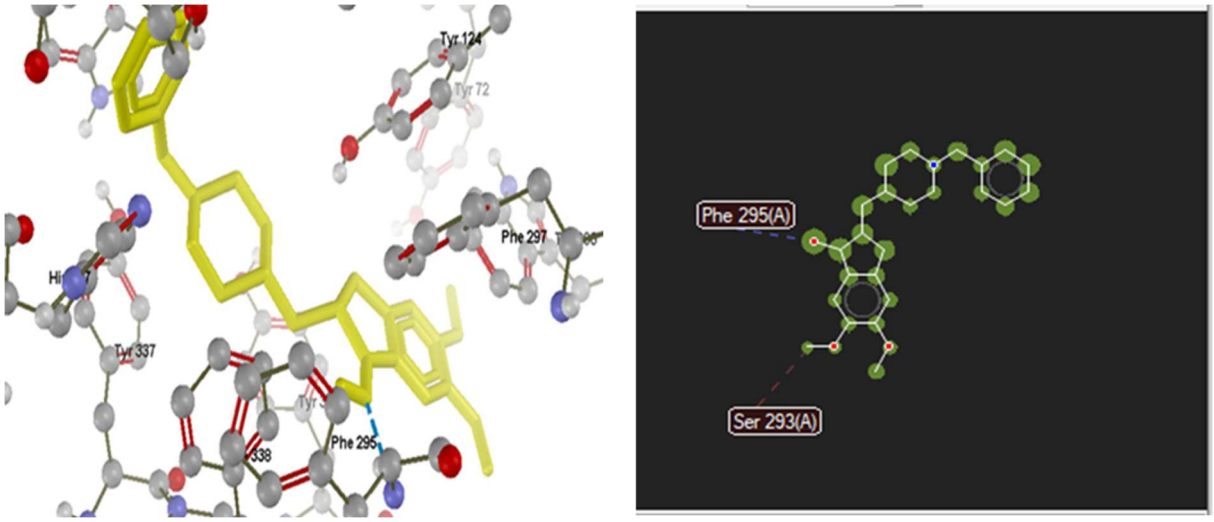
DISCUSSION

Our findings demonstrate that acute restraint stress significantly impacts memory and biochemical parameters in rats and importantly, that clove oil pretreatment can effectively mitigate these stress-induced alterations. The observed increase in plasma corticosterone levels following 2-hour restraint stress is consistent with the well-established activation of the HPA axis in response to acute stressors, which is consistent with the findings of (Shoji and Miyakawa, 2020). Glucocorticoids, such as corticosterone, play a crucial role in mediating the body's response to stress, influencing various physiological processes, including those related to memory. Our results show that clove oil pretreatment significantly reduced these stress-induced increases in plasma corticosterone, aligning with previous research indicating the ability of clove components, particularly eugenol, to modulate HPA-axis activation and reduce stress-induced corticosterone levels (Siyall *et al*., 2020). This suggests a direct physiological mechanism by which clove oil exerts its anti-stress effects.

Chronic stress has detrimental impacts on health, and acute stress is necessary for homeostasis maintenance and adaptability. Acute stress can enhance learning and memory ((Ballan and Gabay, 2020). According to the data from the current study, both the unrestrained and restrained groups' STM and LTM were improved by a 2-hour restraint stress and a chronic pretreatment of clove oil, as evidenced by respective decreases in transfer latency in EPM and escape latency in MWM. It provokes the thought that stress is not always to cause individual's memory impairment, it may function in a positive way to increase memory. Our study was further supported by (Wang *et al*., 2022), Scholars reported that 2 h acute restraint stress but not 3.5h acute restraint stress improves synaptic plasticity and genes transcription associated with learning and memory in the hippocampus of male mice (Wang *et al*., 2022; Surget and Belzung, 2022). Moreover, rats that were restrained and given clove oil before treatment showed a greater improvement in memory. Since oxidative stress impairs memory, using antioxidants may be an effective way to address memory problems (Jomova e*t al*., 2023). Clove oil extract boosts memory function by exerting potent antioxidant effects. Neuroprotective and antioxidant activities of eugenol play a role in modulating memory processes (Sargsyan *et al*., 2025). The existing study showed that a 2-hour restraint stress notably suppressed AChE activity in the brains of restrained rats. An acute stress provokes a fleeting rise and release of acetylcholine, which initiates a phase of heightened neuronal excitability.

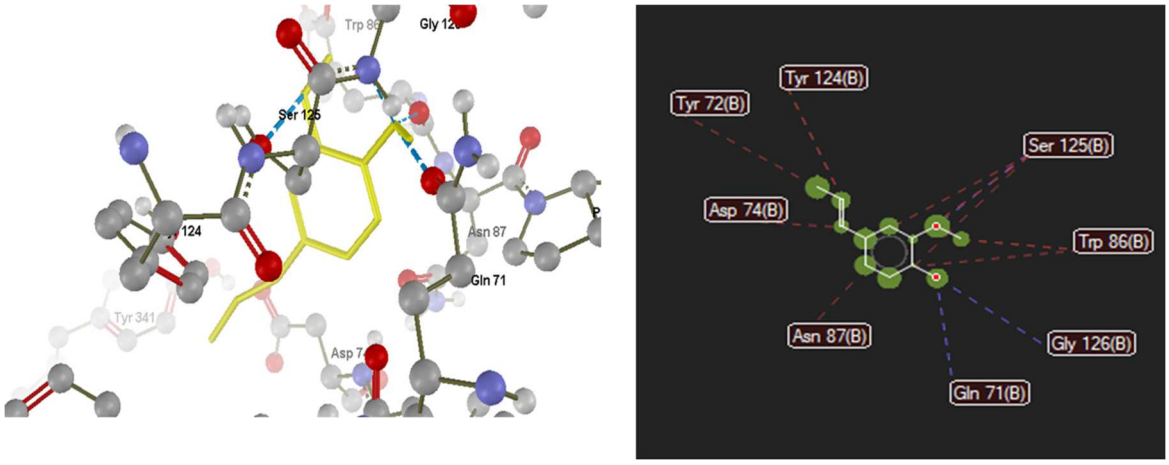
Acetylcholinesterase inhibitors are believed to amplify electrical brain activity by prolonging the availability of acetylcholine at synaptic junctions (Topal, 2019). Our study validated that chronic consumption of clove oil inhibited AChE in both groups, which is a key mechanism for enhancing cognitive function, particularly in neurodegenerative diseases. The significant decrease in corticosterone (CORT) levels in clove oil-treated animals compared to controls further supports the neuroprotective role of clove oil.

The observed improvement in both STM and LTM performance in clove oil-treated groups strongly indicates enhanced cognitive functions. Inhibition of AChE is an important target for the treatment of neurodegenerative diseases (Walczak-Nowicka and Herbet, 2021). A reduced level of acetylcholine (ACh) in the brain is strongly associated with cognitive decline and behavioral changes in individuals with Alzheimer's disease (Chen *et al*., 2022). STM and LTM performance improved significantly in clove oil-treated groups, indicating enhanced cognitive functions. Acetyl cholinesterase (AChE) activity was greater in unrestrained animals treated with clove oil compared to controls. It has been previously documented that clove extract can suppress the activity of acetyl cholinesterase (AChE). Eugenol and isoeugenol have also been identified as important compounds involved in enhancing learning and memory functions by hampering AChE activity that hydrolyzes ACh and augmenting neuroprotective role (Akbar *et al*., 2021; Alyami *et al*., 2025). Our results are consistent with these previous findings, demonstrating that clove oil enhances memory by inhibiting AChE activity. To further elucidate the potential mechanism of action, we performed molecular docking simulations to investigate the interaction of isoeugenol with acetylcholinesterase. The selection of AChE as a target protein is based on its critical role in cognitive function and its established relevance in therapeutic strategies for neurodegenerative diseases (Zhang *et al*., 2024).



(a) **(b)**

Fig. 3: (a) Own ligand and acetylcholine esterase, and (b) ligand map (donepezil)



(a) **(b)**

Fig. 4: (a) Isoeugenol acetylcholine esterase (b) ligand map (Isoeugenol)

Table 3: Amino acids residue around active site docked against acetylcholinesterase

Ligand	Active site amino acid	H bond amino acid residue
Own ligand(1-benzyl-4-[(5,6-dimethoxy-1-indanon-2-yl)methyl]piperidine)	Phe295,297,338,Tyr337,His447, Gly121,Ser203.Gly126	Phe295,Ser293
Isoeugenol	Val73,Asp74,Asn87,Tyr124,Ser125, Gln71,Pro88,Gly121	Ser 125,Gly126,Gln71

Our docking results showed that isoeugenol exhibited a MolDock score of -74.2657 kcal/mol, indicating a favorable binding affinity to AChE. This interaction involves key amino acid residues such as Ser125, Gly126 and Gln71 within the active site (Table 3). For comparison, Donepezil, a well-known AChE inhibitor used as a standard, demonstrated a significantly stronger binding affinity with a MolDock score of -119.647 kcal/mol. While isoeugenol's binding affinity is weaker than that of Donepezil, its ability to interact with the active site of AChE and form hydrogen bonds with residues like Gly121 and Gly126, which are also involved in Donepezil binding, suggests a plausible mechanism for its observed cognitive enhancing effects. This computational finding supports the experimental results demonstrating AChE inhibition by clove oil and its components. The outcomes of the contemporary study collectively suggest that clove oil offers significant protection against stress-induced oxidative damage and cognitive impairment, largely attributed to the powerful antioxidant activity and AChE inhibitory properties of its bioactive compounds, particularly isoeugenol.

CONCLUSION

This study highlights the significant therapeutic potential of clove oil in mitigating stress-induced biochemical changes and improving cognitive function in a rat model. Our findings demonstrate that clove oil pretreatment effectively reduces elevated corticosterone levels and inhibits acetylcholinesterase activity, both of which are crucial for maintaining cognitive health under stress. The molecular docking analysis further supports these observations by revealing a favorable binding interaction between isoeugenol, a key component of clove oil and acetylcholinesterase, suggesting a direct mechanism for its neuroprotective properties and cognitive enhancement. These results advocate for the continued exploration of clove oil and its active constituents as natural therapeutic agents for stress-related cognitive dysfunctions, paving the way for potential pharmacological applications.

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Author's contributions
Sana Sadaf and Shazia Dawood were involved in the study design and manuscript writing. Shaista Emad and Sarwat Yousuf were involved in the acquisition of data and manuscript revision. Sara Qadeer, Yousra Sarfaraz and Sheeza Sheikh were involved in making figures and literature survey. Tahira Perveen supervised and critically evaluated the research.

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Data availability statement
Original data in this study are available from the corresponding author on reasonable requests.

Ethical approval
The experimentation was accomplished after formal approval of the Institutional Review Board (IRB) of University of Karachi with reference No.03363/SC-2015.

Conflict of interest
The authors assert that they have no conflicts of interest.

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