Effects of *Syzygium aromaticum* (clove) Extract on the Acquisition and Expression of Morphine-Induced Conditioned Place Preference in Swiss Albino Mice: an *In vivo*, *In Silico* Approach

Sarmin Akther Tithy[1], Md Monir Hossain[1], Diponkor Kumar Shill[1], Md Siam Hossain[2], Arifin Islam[3], Md Islam Molla[1] and Sayeda Jahan[1]

[1]Department of Pharmacy, Jagannath University, 9-10, Chittaranjan Avenue, Dhaka-1100, Bangladesh
[2]Department of Pharmacy, Noakhali Science and Technology University, Sonapur, Noakhali 3814, Bangladesh
[3]Department of Accounting & Information Systems (Statistics), Jagannath University, 9-10, Chittaranjan Avenue, Dhaka-1100, Bangladesh

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ABSTRACT: Empirical findings have demonstrated that the application of an N-Methyl-D-aspartate (NMDA) receptor antagonist effectively prevents the formation of morphine-induced location preference. Eugenol, the primary constituent found in the extract of *S. aromaticum* (SA), exhibits notable efficacy in traversing the blood-brain barrier and possesses inhibitory properties against the metabotropic NMDA receptor. This study aimed to evaluate the actions of the hydro-alcoholic extract of *S. aromaticum* on the acquisition and expression of morphine-induced conditioned place preference (CPP) in mice, followed by *in silico* studies. The conditioned place preference (CPP) test was used for investigating addictive-seeking behavior. Morphine (5 mg/kg) was used to produce CPP, and saline was used as a control. Morphine significantly increased the preference scores on the drug-paired side ($p<0.001$), whereas both doses of *S. aromaticum* extract (200 and 400 mg/kg) did not reveal any liking compared to the control group. Higher doses of *S. aromaticum* extract (400 mg/kg) reduced the acquisition of MOR-induced CPP ($p<0.001$) but had no significant effect on the expression of morphine CPP. Molecular docking analysis showed that the binding affinities of eugenol to NMDA receptor are -5.1 kcal/mol, comparable to the reference NMDA antagonist memantine which has -5.6 kcal/mol binding affinity to NMDA receptor. Eugenol formed hydrophobic bonds with NMDA receptors at VAL644, PHE554, TRP563 and TYR647 residues, comparable to the binding affinity of NMDA antagonist memantine. ADMET analysis showed that eugenol has high intestinal absorption and good bioavailability (>90%) and can cross the BBB easily (logBB> 0.3). On the basis of our *in vivo* trials and *in silico* report, we settled that the acquisition effect of *S. aromaticum* might be due to the antioxidant and antagonistic properties of eugenol to NMDA receptor. It would be reasonable to conduct mechanistic research in the forthcoming days to elucidate the underlying mechanisms utilizing various methodologies.

Key words: Conditioned place preference, *Syzygium aromaticum*, morphine (MOR), NMDA, mice.

INTRODUCTION

Opioids are presently considered the conventional approach for managing moderate to severe pain, despite the associated occurrence of undesirable consequences such as addiction, tolerance and physical reliance. The problem of drug addiction poses a substantial challenge to worldwide public health. According to research conducted by the United Nations (UN)[1], the global population of individuals suffering from drug addiction exceeds 180 million. Bangladesh is situated in a prominent transportation corridor that is frequently utilized for the illicit transportation of marijuana, heroin, opium and morphine. Psychosocial interventions and pharmacological treatments have been consistently employed in the management of substance use

Correspondence to: Sayeda Jahan
Email: sayedajahan2013@pharm.jnu.ac.bd

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disorders. These remedies typically diminish drug cravings and the chance of relapsing into addictive drug use. Thus far, most of these therapeutic techniques have shown unsatisfactory outcomes.[2]

The development of physiological dependence resulting from the repeated administration of opioids is primarily due to down-regulation and/or desensitization of opioid receptors. The mesolimbic dopamine (DA) system, which regulates the central reward system, has been found to be associated with opioid dependency.[3-5] Opioids block local GABAergic inhibitory interneurons which in the ventral tegmental area (VTA) activate DA neurons and enhance DA transmission to nucleus accumbens (NAc).[6] The VTA and NAc acquire glutamatergic inputs from the limbic and prefrontal cortex (PFC). Moreover, glutamate regulates the release of DA. Memantine, an antagonist for the NMDA receptor, has been established to prevent the formation of MOR-induced place preference.[7] Furthermore, the brain-derived neurotrophic factor (BDNF) facilitates the behavioral processes and neuronal plasticity by exerting its effects on the mesolimbic dopamine system, which is a crucial reward circuit within the brain.[8] Through tropomyosin-related kinase B (TrkB), BDNF may control dopamine issues as well as interactive reactions to drug usage, such as seeking, relapse, dependence and sensitization.[9,10]

Previous studies reported that oxidative stresses are significantly associated with the foundation of MOR-induced location preference. Furthermore, it has been observed that inhibiting these stresses can mitigate the adverse effects associated with morphine use. [11-12] The repetitive use of morphine leads to the initiation of microglia in the spinal cord, which subsequently triggers the formation of a diverse range of substances like- cytokines, nitric oxide (NO) and excitatory amino acids. These mediators are responsible for the emergence of morphine withdrawal syndrome and the development of morphine dependency.[13-15]

Clove (*Syzygium aromaticum* L.) (SA), a plant under Myrtaceae family, is used extensively in traditional medicine due to its wide range of biological activity. SA, also recognized as *Eugenia caryophyllata*, goes to the Myrtaceae family and is renowned for its dried flower buds that are commonly utilized as spices.[16] The plant components mentioned encompass glycosides, kaempferol, hydroxyphenyl propels, salicylic acids, hydroxyphenyl benzoic acid, hydrolyzable tannins, essential oil, ellagic, ferulic, flavonoids, farnesol, 2-heptanone, α-humulene, β-pinene, benzaldehyde, ethyl hexanoate, limonene and others.[17,18] According to phytochemical research conducted on clove essential oil, eugenol is identified as the primary constituent responsible for the powerful and characteristic aroma exhibited by this spice. Eugenol constitutes around 15% of the dry mass of clove buds and accounts for a significant proportion, ranging from 70% to 90%, of the essential oils present.[19] The antioxidant and antibacterial qualities of clove make it one of the spices that may be utilized as preservatives in a variety of dishes.[20]

Eugenol (EUG), the predominant chemical, is efficacious in permeating the BBB to penetrate the brain and perform its function *in vivo* due to its hydrophobic nature.[21] The neuroprotective effects of EUG against excitotoxic and oxidative damage caused by NMDA have been observed in neuronal cells.[22] EUG exhibits a neuroprotective potential on hippocampus tissues because it attenuates the aberrant blockage of Ca^{2+} resulting from amyloid-β peptide (A-β) and lowers BDNF.[23] *In vitro*, EUG boosts the actions of several glutathione-related proteins and defenses important brain cells from oxidative and excitotoxic damage.[24] Numerous studies have shown the effects of EUG on the symptoms of parkinson disease, motor neuron disease (MND), and Alzheimer's disease. [25] EUG has also shown anti-stress and anticonvulsant qualities. The short- and long-term memory impairments caused by scopolamine-treated mice can be reversed by clove essential oil.[26] According to earlier research, EUG presents neuroprotective properties through the inhibition of glutamate metabotropic receptors. It has the potential to fend off oxidative stress-related damage.[27] Additionally, EUG increases the availability of antioxidant enzymes like- glutathione

peroxidase (GPX) and superoxide dismutase (SOD) etc.[28] The existing body of literature pertaining to the pharmacological activity of EUG demonstrates notable anti-inflammatory, antioxidant, analgesic, antibacterial, and neuroprotective effects, which have substantial implications for human health.[28]

Molecular docking represents a widely utilized and fundamental approach within drug development initiatives. The computational model has the capability to anticipate the ligand-protein interactions while they are in a bound state. Furthermore, it provides insights into the ideal affinity, direction and binding position.[29] Current study focused on the NMDA receptor, a glutamate-gated ion channel, which is primarily responsible for synaptic plasticity and also important for both the rewarding benefits of morphine and its adverse effects, such as addiction and tolerance.[30] Plants have long been regarded as alternate sources of medicinal compounds from ancient times in human society. In contemporary times, more than half of pharmaceutical substances are derived from natural sources, specifically plants and animals. Natural herbal medicines' putative anti-addictive and anti-tolerance properties are currently generating a lot of interest.[31] However, these benefits require accurate scientific experimental testing and clinical studies before they can be widely utilized to manage the side effects of opioids.

Given that SA antagonizes metabotropic glutamate receptors, has antioxidant and anti-inflammatory properties, and diminishes BDNF levels, it was postulated that SA may potentially mitigate the inclination towards morphine dependence in animal subjects. The current investigation was conducted to assess the potential impacts of a hydro-alcoholic extract of SA on MOR induced CPP in mice.

MATERIALS AND METHODS

In vivo experimental procedures

Animals. The experimental subjects consisted of Swiss-albino mice weighing between 25-30 g, obtained from ICDDR, B in Dhaka, Bangladesh. Each experiment involved a total of 8 mice per group. The animals were accommodated in cages with a capacity of six individuals each. The cages were exposed to a 12-hour cycle of light and dark. The animals were delivered with unrestricted access to food and water.

Drug. In this investigation, morphine sulfate (Renata Limited, Bangladesh) was utilized. Before being used, morphine sulfate and SA (clove) extract were prepared as follows: they were dissolved in regular saline and given at 5 mg/kg (morphine) and 200 400 mg/kg (SA) respectively.

Preparation of the extract. The collection of clove buds (SA) was conducted at Karwan Bazar, a local market located in Dhaka, Bangladesh. The samples underwent verification by an herbalist affiliated with the Department of Botany at Jagannath University. In order to produce clove extract, a quantity of two kilograms of clove buds was subjected to a drying process at a temperature of 25°C. The drying process was conducted in a manner that ensured protection from direct sunlight. Subsequently, the dried clove buds were crushed into a fine powder using a grinder. The dried buds were subjected to grinding prior to extraction. Subsequently, they were dissolved in a solution consisting of 2 liters of methanol (70% v/v). Then, the mixture was stored at ambient temperature for a duration of seven days. During this time frame, following repeated agitation, the solution underwent filtration using filter paper (Whatman No. 1). A rotary evaporator (BÜCHI-water bath-480) at a temperature below 40°C was used to evaporate the methanol filtrate. The resultant extracts underwent freeze-drying using a Thermo-Electron Corporation Heto Power Dry LL300 freeze drier. Subsequently, the dried extract was stored at a temperature of 20°C for subsequent studies. In order to attain the desired concentration, the extract was dissolved in a solution of normal saline.

Ethical approval. The research conducted in this study received permission from the Ethical Review Committee of Jagannath University (JnU) with the reference number JnU/ERC/01/2023.

CPP apparatus. The studies utilized a three compartment CPP apparatus with dimensions of 15

cm × 30 cm × 15 cm. The apparatus consisted of two primary compartments (A and B), which were equal in size but exhibited variations in texture and shading. Compartment A was adorned with black dots against a white backdrop on its wall, while its floor maintained a smooth surface. In contrast, compartment B exhibited a pattern of black and white stripes on its painted surface, also accompanied by a smooth floor. The third, relatively smaller container was coated with a layer of white paint, thus demarcating and creating a physical separation between the two primary chambers. At the conditioning stage, the compartments were detached by a partition that could be easily removed.[32]

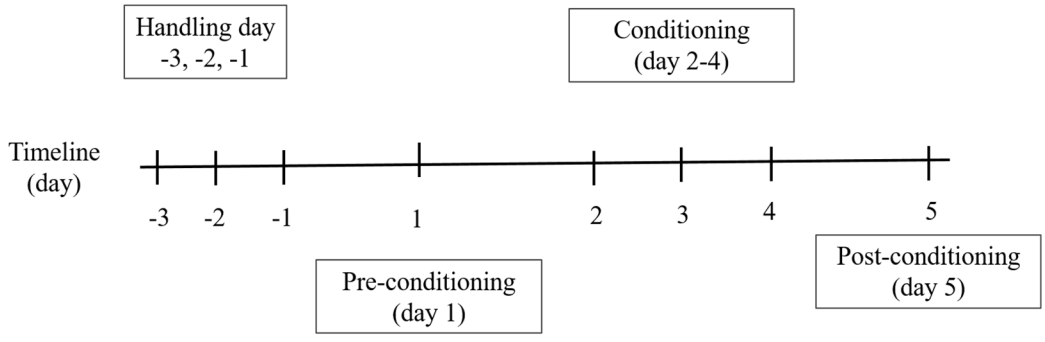
Behavioral procedures

Measurement of conditioned place preference. This study consists of three phases, namely-preconditioning, conditioning, and post-conditioning.

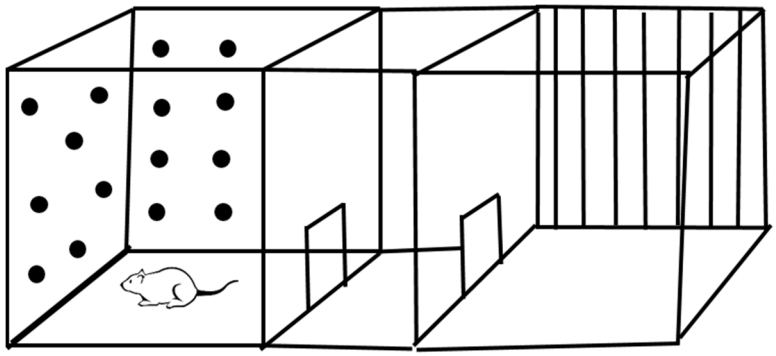
Pre-conditioning phase. Prior to exposure (referred to as day-1), each rodent was individually introduced into the device for a duration of 45 minutes, during which they had unrestricted access to all compartments. During the pre-conditioning chapter, the mice remained positioned in the central region of the neutral compartment and given unrestricted movement between the three compartments. The duration spent by the experimental animal in every compartment was observed and documented over a period of 15 minutes.

Conditioning phase. A three-day regimen of conditioning activities made up this segment. Animals were given three trials in which they were kept in one compartment for 45 minutes and exposed to the effects of a medicine called clove extract and three trials in which they were confined in the other compartment and exposed to the effects of saline. Day 2: The mice were given one dose of morphine in the 09:00 am – 12:00 pm (morning) session and were then put in compartment-A for forty-five minutes. The animals were given a single saline injection and kept in compartment B for forty-five minutes in the afternoon (16:00–18:00 h). Day 3: In the morning session (compartment B) and afternoon session (compartment A), the animals were given injections of saline. Day 2's protocol applied to the same following days. The animals in the saline group were given saline in the compartments A and B. On these days, entry to the compartments was restricted.

Post-conditioning phase. The separation was eliminated on the fifth day, granting the mice unrestricted entry to the whole instrument during the preference test. The preference criteria were calculated by calculating the mean period each mouse spent in each chamber over 15 minutes. No administration of injections occurred during the acquisition tests. The modification in preference was determined by calculating the discrepancy in seconds between the duration of time spent in compartment A on the pre-conditioning day and the duration of time passed in compartment A on the post-conditioning day. This quantity of time signifies the comparative rewarding characteristics of morphine. The experimental methodology is depicted in figure 1.



(a)



(b)

Figure 1**.** Experimental procedures. (a) Experimental procedures of conditioning plan of the acquisition and expression program for MOR-induced condition place preference study. (b) Conditioning place preference apparatus.

Experimental design

Experiment 1. Evaluation of the conditioning effects of morphine. To assess the capacity of morphine to condition place preference or place aversion, morphine (5 mg/kg) and saline were administered to two groups of mice in the conditioning phase.

Experiment 2. Assessment of the effects of *Syzygium aromaticum* (clove) extract on the acquisition of CPP. Three groups of animals, in the conditioning phase of the CPP procedure, one hour before the treatment with the dose of morphine received saline and different doses of SA extract (200, 400 mg/kg, p.o.) respectively. On the test day, the mice were not subjected to any form of therapy.

Experiment 3. Assessment of the effects of *Syzygium aromaticum* (clove) extract on the expression of CPP. Three sets of animals were habituated with morphine (5 mg/kg). On the test day (post-conditioning day) of the investigations, one hour prior to the test, the mice sets got saline and different doses of SA extract (200 and 400 mg/kg, p.o.), respectively.

Experiment 4. Assessment of the conditioning effects of *Syzygium aromaticum* (clove) extract. To weigh the capacity of clove extract to yield place preference or place aversion, in the conditioning phase, different doses of SA extract (200 and 400 mg/kg, p.o.) and saline were dispensed orally to three groups of mice.

***In silico* procedures**

Structure preparation of protein and energy minimization of protein and ligands. The optimization of both protein and ligand structures was conducted to attain a state of maximum stability in their respective geometries. The crystal assembly of the NMDA target protein (PDB ID: 7SAD) was obtained from the Protein Data Bank (PDB) of the Research Collaborator for Structural Bioinformatics (RCSB). Prior to reduction, the protein structure underwent cleaning using Discovery Studio Client 2020. The protein's energy was minimized using the Swiss-PDB Viewer, and calculations were accomplished in a vacuum using the GROMOS 96 43B1 parameter set. The protein was ultimately purified and condensed, resulting in a refined sample that was then stored in the Protein Data Bank (PDB) format.

The 3-dimensional assemblies of eugenol and memantine were found from the PubChem database. The utilization of memantine, an anti-Alzheimer's medicine that acts as an NMDA receptor antagonist, was employed to enhance the understanding of docking trials. The optimization of ligands was performed using the Gaussian09 W quantum chemistry program, using the B3LYP hybrid density functional theory and the 6-31 G basis set.

Molecular docking. The technique of molecular docking is widely utilized in the field of bioinformatics to forecast the potential binding

interactions that may occur between tiny molecules and proteins. The PyRx program was employed to execute molecular docking by the application of a virtual screening technique. The output of this molecular docking analysis can be observed in the form of a pdbqt file using the Auto Dock Vina Software, which was developed by Morris *et al.*[33] The PyRx software is a streamlined virtual screening tool that offers a minimalistic workflow, enabling users to obtain the docking output file in the most efficient manner possible. The protein and ligands were subjected to docking assessment using Autodock Vina, while the conversion of the pdb file to pdbqt format was performed using Auto Dock Tools (ADT) from the PyRx software package. The grid box sizes for X, Y, and Z in Auto Dock Vina were consistently maintained at 16.54, 17.66, and 33.68, respectively. Through the process of computing the energy value associated with each ligand, the present technology is able to predict the potential consequence of docking.

ADMET prediction of target compound. The canonical Simplified Molecular Input Line Entry System (SMILES) representation of EUG was obtained from the PubChem database for the purpose of calculating its absorption, distribution, metabolism, and excretion-toxicity (ADME-T). To predict the ADME-T, both the PKCSM and SWISSADME online servers were utilized. These two servers were utilized to forecast crucial parameters like- human intestine absorption, skin permeability, caco-2 cell permeability, CYP450 metabolism, blood-brain barrier infiltration, VDss, organic cation transporter 2 (OCT2) substrate and toxicity characteristics.

Statistical analysis. The analysis raw data was accomplished by using the software- GraphPad Prism (version 5). A one-way analysis of variance (ANOVA) followed by a Tukey post hoc test was performed in order to ascertain whether there were any statistically significant differences across the various treatment groups. Changes in preference were presented as mean ± SD.

RESULTS AND DISCUSSION

In vivo experimental results

The actions of morphine on the initiation of CPP. Based on the findings presented in figure 2, the outcomes of two independent sample t-tests demonstrated that the recurrent administration of morphine (5 mg/kg) over a span of four consecutive days resulted in the development of a preference for the compartment associated with morphine ($p < 0.001$). The administration of saline solution did not demonstrate any statistically significant inherent positive or negative effects. In this experiment, the assumption of equal variance was made based on the results of Levene's test, (Table S1) which indicated that there was no significant change in the variances of the two groups (p-value > 0.05). Additionally, in order to corroborate the findings of the t-test, a nonparametric Mann-Whitney U test was also performed (Table S2). A comparable outcome was seen. The outcomes of this investigation demonstrated that the use of morphine at 5 mg/kg dose resulted in the beginning of conditioned location preference.

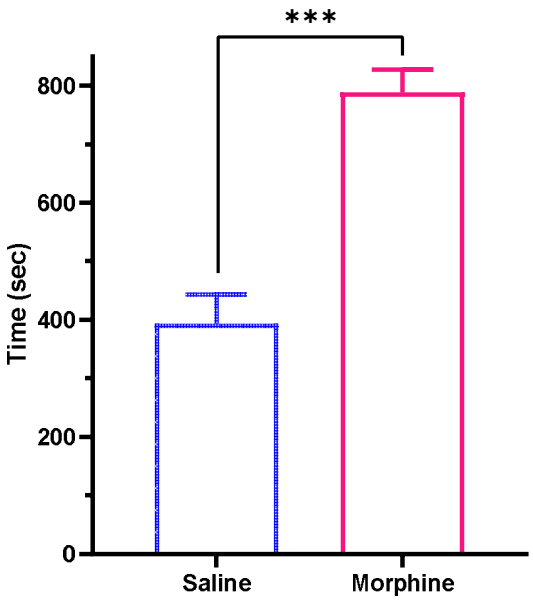


Figure 2. The effects of morphine (5 mg/kg) on the induction of condition place preference.

During the conditioning sessions, two sets of mice were treated with saline and morphine, respectively. The duration of drug exposure was measured for each animal during the 15-minute test sessions. The calculation of preference change involved subtracting the time passed in the drug-paired compartment on the test day from the time passed in the same compartment at the preconditioning session. Values are portrayed as mean ± SD and *** indicated $p<0.001$, compared with saline (n=8 in each group).

The effect of *Syzygium aromaticum* (clove) extract on the acquisition of MOR-induced CPP. In order to assess the impact of SA (clove) extract on the acquisition of MOR-induced CPP, a one-way analysis of variance (ANOVA) was performed, followed by the post hoc Tukey's honestly significant difference (HSD) test. Nonparametric statistical tests, such as the Kruskal-Wallis H test and the Mann-Whitney U test, have been used in the analysis to provide further support for the findings obtained from the analysis of variance (ANOVA). The analysis of variance (ANOVA) yielded statistically significant results, showing a notable variation in the treatment means ($F(3,8) = 79.158$, p-value <.05). Further analysis using Tukey's honestly significant difference (HSD) test discovered (Table S3) that pretreatment with hydroalcoholic extracts of SA at a dosage of 400 mg/kg, administered 60 minutes prior to the injection of morphine (5mg/kg) at the conditioning stage, had a significant impact on the difference in occupancy time in compartment A between the pre-conditioning and post-conditioning periods when compared to the morphine group. These detections are visually represented in figure 3. The aforementioned result has been reinforced by the non-parametric equivalent. There was no statistically significant distinction observed between SA 200 and morphine.

Different doses of saline, SA 200 mg/kg and SA 400 mg/kg were given to four separate sets of mice one hour before getting a morphine 5 mg/kg dose during the conditioning periods. At the experimental session, the rodents were not subjected to any form of intervention or therapy. The calculation of preference change involved determining the discrepancy between the duration of time spent in the compartment associated with the medication at the test phase and the duration of time spent in the same compartment during the preconditioning phase. Values are portrayed as mean ± SD and *** indicated $p<0.001$; * indicated $p<0.05$ (n=8 in each group). SA: *Syzygium aromaticum;* Mor: Morphine.

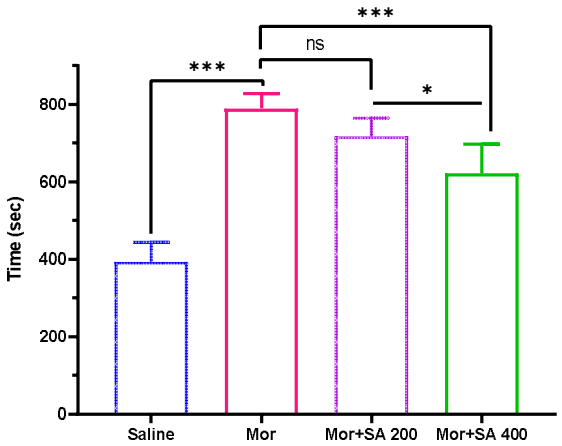


Figure 3. The effect of *Syzygium aromaticum* (clove) extract on the acquisition of MOR-induced CPP.

The effect of *Syzygium aromaticum* (clove) extract on the expression of MOR-induced CPP. Throughout the pre- and post-conditioning days, morphine administration enhanced and saline decreased the variation in compartment A's occupancy time. The observed disparity between the morphine set and the saline set was found to be statistically significant ($p<0.001$). The results of the one-way ANOVA analysis directed that the administration of clove extract (SA 200 and SA 400 mg/kg) on the test day, specifically one hour prior to the test, did not exhibit a statistically significant inhibitory outcome ($p>0.05$) on the expression of MOR-induced CPP, as illustrated in figure 4. The non-parametric counterpart has also proposed a similar result (Table S4).

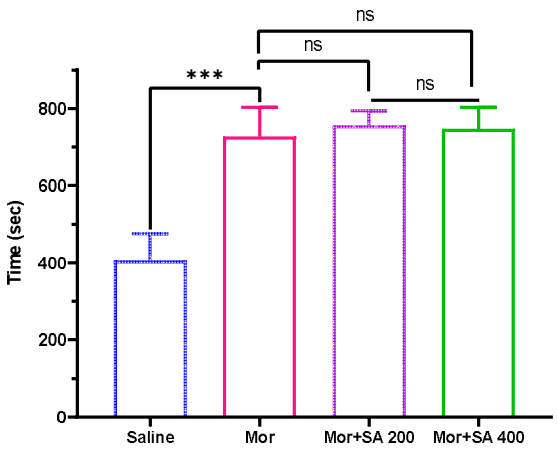


Figure 4. The effect of SA (clove) extract on expression of MOR-induced CPP.

Four groups of mice received morphine (5 mg/kg) during conditioning sessions of CPP. The animals, on the test day, got SA (200 and 400 mg/kg) and saline one hour prior to the test sessions. The alteration in preference was calculated as the difference between the time passed in the drug-paired compartment in the test stage and the time passed in the same compartment in the preconditioning stage. Values are portrayed as mean ± SD and ***indicated $p<0.001$ (n=8 in each group). SA: *Syzygium aromaticum;* Mor: Morphine.

Conditioning effects of *Syzygium aromaticum* (clove) extract. The outcomes of the one-way analysis of variance (ANOVA) specified that there was no statistically significant change seen among the means of the three treatments (saline, SA-200, and SA-400) ($F(2,21) = 1.248$, $p > .05$). This suggests that the administration of SA (clove) extract did not have a conditioning effect on the animals, as depicted in figure 5. Moreover, the Kruskal-Wallis test (Table S5) further supports the conclusion derived from the analysis of variance (ANOVA). Neither the SA extracts at 200 mg/kg nor at 400 mg/kg dose were found to elicit any conditioned location preference.

During the conditioning phase, the animals were administered SA extract at 200 mg/kg and 400 mg/kg dose, as well as saline. The calculation of preference change involved determining the disparity between the duration of time spent in the drug-associated compartment at the test phase and the duration of time spent in the same compartment at the preconditioning phase. Values are portrayed as mean ± SD and *** indicated $p<0.001$ (n=8 in each group). SA: *Syzygium aromaticum.*

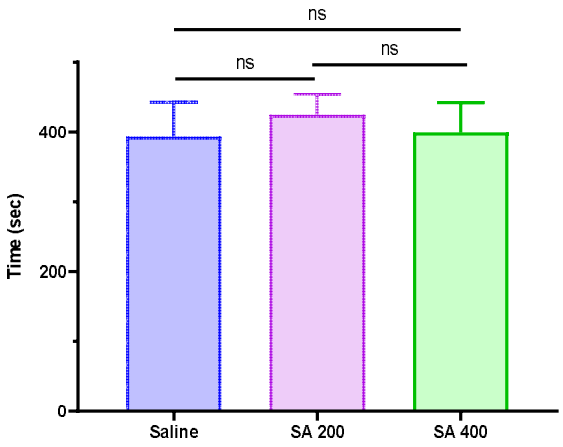


Figure 5. Conditioning effects of SA (clove) extract.

***In silico* study assessment**

Optimization of ligands. The conformational characteristics of a molecule exert a substantial influence on its physical and chemical properties. Hence, the present study involved the thorough optimization of memantine and EUG by the application of Density Functional Theory (DFT) for geometry analysis. The compounds' stoichiometry, enthalpy, Gibbs free energy, electronic energy and dipole moment are presented in table 1. The best configurations of these chemicals can be viewed in figure 6.

Molecular docking analysis. The binding mechanisms of selected chemicals were investigated by the utilization of Autodock Vina for molecular docking calculations. EUG and memantine were subjected to docking simulations under identical optimized conditions, targeting the same binding site pocket on the NMDA receptor. The subsequent investigation after docking simulations indicated that eugenol exhibited a binding affinity of -5.1 kcal/mol, while memantine displayed a binding affinity of -5.6 kcal/mol towards the NMDA receptor (Table S6). EUG exhibited the formation of six Pi-alkyl bonds

with specific amino acid residues, namely PHE554, PHE558, TRP563, VAL644 and TYR647 inside the NMDA receptor. Additionally, EUG displayed two alkyl interactions with VAL566 and ILE643 residues, as illustrated in figure 7. In figure 7, it can be observed that memantine established two alkyl bonds with the VAL644 residue, and also demonstrated five pi-alkyl interactions with the PHE554, TRP563 and TYR647 residues.

Table 1. The stoichiometry, enthalpy, Gibbs free energy, electronic energy and dipole moment (Debye) of memantine and EUG.

Name	Stoichiometry	Electronic energy	Enthalpy	Gibbs free energy	Dipole moment (Debye)
Memantine	C12H21N	-524.41	-524.41	-524.46	1.239
EUG	C10H12O2	-538.49	-538.49	-538.55	2.211

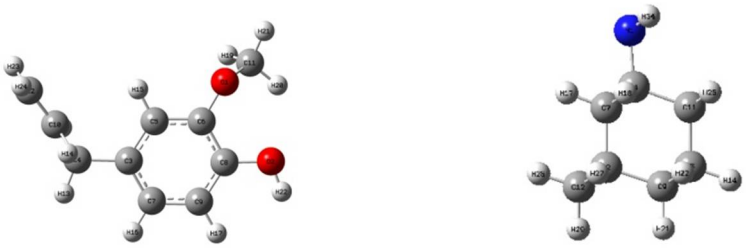


Figure 6. Stable optimized structure of Memantine and Eugenol.

Table 2. ADME/T properties of eugenol.

	Parameter	Eugenol
Absorption	Water solubility (log mol/L)	-2.25
	Caco-2 permeability (log Papp, cm/s)	1.559
	HIA(%)	92.041
	Bios (from swissADME) (Bioavailability score)	0.55
	Skin permeability (log Kp cm/s)	-2.207
Distribution	VDss (human) (logL/kg)	0.24
	BBB permeability (logBB)	0.251
	BBB perm. (swissADME)	Yes
Metabolism	CYP2D6 substrate	No
	CYP3A4 substrate	No
	CYP1A2 inhibitor	Yes
	CYP2C19 inhibitor	No
	CYP 2C9 inhibitor	No
	CYP2D6 inhibitor	No
	CYP3A4 inhibitor	No
Excretion	Total clearance	0.282
	Renal OCT2 substrate	No
Toxicity assays	Hepatotoxicity	No
	Oral rat acute toxicity (LD_{50}, in mol/kg)	2.118

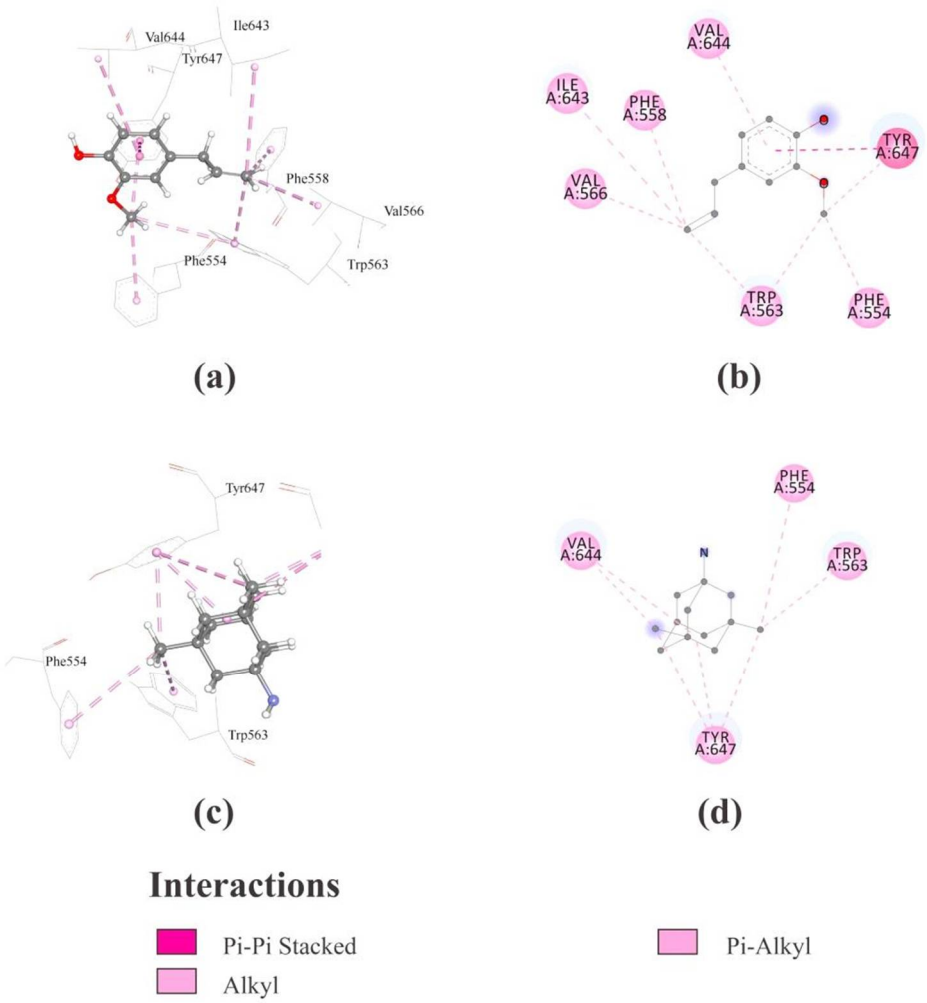


Figure 7. a) Possible 3D interaction of EUG and NMDA-R. b) Possible 2D interaction of EUG and NMDA-R. c) Possible 3D interaction of Memantine and NMDA-R. d) Possible 2D interaction of Memantine and NMDA-R.

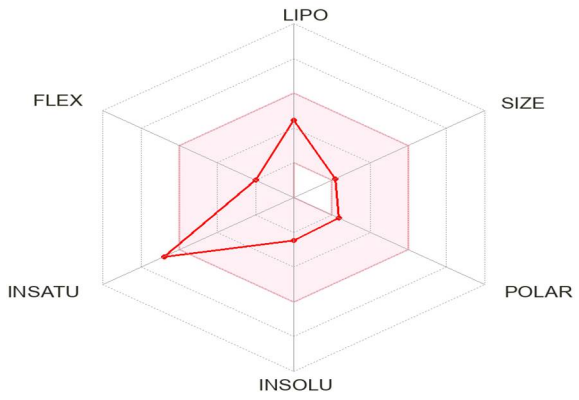


Figure 8. Bioavailability radar representation.

ADMET prediction

The ADME-Tox characteristics of EUG are predicted in table 2. The bioavailability values of EUG are depicted in figure 8 according to the prediction of physicochemical properties.

DISCUSSION

Drug addiction and dependence have exerted a substantial impact on various aspects of social harmony, economic development and government health both in historical and contemporary contexts. Morphine and other opioid use disorders linked to opioid prescriptions affect about 16 million people

globally, contributing to an epidemic of drug abuse.[34] Morphine, a widely utilized opioid analgesic exhibits a range of behavioral and molecular consequences. The addiction to morphine poses a substantial concern within the realm of public health.[34]

Present study systematically explored the impact of clove extract on the rewarding properties of morphine, as assessed during different stages of CPP, namely acquisition/development and extinction. Based on the results found from our study, it was observed that the hydro-alcoholic extract of SA exhibited a statistically significant effect ($p < 0.05$) on the developmental stage of CPP. On the other hand, a lower dosage has been observed to decrease the duration of preference during the developmental phase, but this decrease does not reach statistical significance ($p > 0.05$). Furthermore, there was no statistically significant difference in the reduction of MOR-induced CPP expression by the SA extract. Lastly, no conditioning effect was observed with SA extract at any of the doses.

The phenomenon of MOR-conditioned place preference (CPP) is a complex process that entails modifications in various brain regions, including the nucleus accumbens, VTA, prefrontal cortex, hippocampus, and others.[35] In VTA, chronic morphine release of neurotransmitters causes a gradual alteration in signal transduction cascades. The ventral tegmental area (VTA) plays a crucial role in the process of neuronal remodeling that is triggered by addictive substances. The rewarding action of morphine requires the activation of NMDA receptors in the nucleus accumbens and ventral tegmental area.[36] The glutamatergic system is known to have significant involvement in the worthwhile properties of morphine as well as its associated negative effects, such as the development of tolerance and addiction.[37,38] Abuse-related drugs modify glutamatergic synaptic plasticity through modulating dopamine release as well as more straight effects on glutamate receptors.[39] According to earlier research, NMDA antagonists have inhibited the morphine propensity.[40] The use of memantine, an NMDA receptor antagonist, has been shown to mitigate the worthwhile effects of morphine in the CPP paradigm.[41] Moreover, BDNF, which acts on the brain's mesolimbic dopamine system, a crucial reward circuit, fosters neuronal and behavioral plasticity.[8] EUG, the primary constituent found in cloves, demonstrates efficacy in traversing the BBB, enabling its entrance to the brain and subsequent *in vivo* activity.[21] EUG prevents oxidative and excitotoxic damage caused by NDMA in neuronal cells.[22] EUG has been shown in earlier research to possess neuroprotective properties through the inhibition of glutamate metabotropic receptors. It is probable that one of the mechanisms through which SA extract exercised its effects is the antagonistic modulation of NMDA receptors. According to past and present docking studies, the active ingredient in cloves, EUG, significantly inhibited the molecular targets of neurological diseases by binding to ten different metabotropic and inotropic glutamate receptors.[24] In addition, EUG has been seen to reduce the levels of BDNF and inhibit the cell death caused by amyloid-β peptide (A-β) through the aberrant inhibition of calcium ions (Ca^{2+}) caused by A-β. Nevertheless, additional research is necessary to determine the specific underlying mechanism and potential active component responsible for the CPP effect of the extract derived from SA.

Previous research revealed that oxidative stresses are significantly associated with the initiation of MOR-induced location preference. Furthermore, it has been observed that inhibiting these oxidative stresses can effectively mitigate the adverse effects associated with morphine usage. [12,13] The repeated administration of morphine leads to microglial activation in the spinal cord, which subsequently triggers the creation and release of a variety of substances like- nitric oxide (NO), inflammatory cytokines and excitatory amino acids. These mediators play a vital role in the development of morphine dependency.[14-16] Additionally, NO is thought to have a role in the action of opioids, and NOS inhibitors can decrease the development of morphine tolerance[42] and lessen the expression and development of abstinence syndrome.[43] EUG, the primary chemical in cloves, has been shown in

multiple publications to exhibit high levels of antioxidant activity.[44] Furthermore, the upregulation of antioxidant enzymes such as GPX and SOD is markedly increased by EUG.[34] According to earlier research, EUG could considerably reduce the generation of NO via downregulating NF-kB signaling pathways. Further work is essential to illuminate the molecular approach underlying the site of action of EUG.

According to Brooijmans *et al* (2003), *in silico* molecular docking is a very efficient approach in the identification of novel ligands for receptors with established structures. Consequently, it plays a decisive role in the advancement of structure-based pharmaceuticals.[45] The docking approach is utilized to forecast the binding affinity and ligand orientation (posing) within the intended binding site. The amino acid residues (VAL644, PHE554, TRP563, and TYR647) necessary for the hydrophobic interaction with memantine are also present in EUG, according to this *in silico* analysis. Thus, based on the docking result analysis, we may infer that EUG inhibits the NMDA receptor and demonstrates its activity by blocking NMDA-R. EUG displayed a greater dipole moment of 2.211 Debye than memantine, indicating higher polarity in the natural world, as seen in table 1.

The determination of the ADMET profile is a key process for any substance that is not native to the human body. EUG exhibited significant intestinal absorption and favorable bioavailability, with a high intestinal absorption value of over 90%. The penetration of the BBB is a crucial factor in allowing chemicals to effectively exert their activity within the central nervous system (CNS). Table 2 presented empirical evidence indicating that EUG has a high permeability across the blood-brain barrier. The toxicity profile suggests that EUG exhibits a favorable safety profile. The pharmacokinetic parameters included in table 2 indicate that EUG has characteristics that make it appropriate for human use.

Empirical findings have demonstrated that the administration of an NMDA receptor antagonist effectively inhibits the formation of MOR-induced location preference. Extracts from SA, or cloves, include a variety of chemicals that have varying degrees of effect on the various components. Because of this, it is difficult to pinpoint the precise mechanism behind SA's beneficial modulating effect something that might have been done with ease in a trial involving only one molecule. EUG, the primary constituent found in the extract of SA, exhibits notable efficacy in traversing the blood-brain barrier. Additionally, it has demonstrated considerable anti - inflammatory, anti-oxidant and neuroprotective characteristics. The acquisition effect of the hydro-alcoholic extract of SA is believed to be attributed to the antagonistic activities of EUG towards the NMDA receptor, as indicated by an *in silico* investigation. Additional research involving *in vivo* and *in vitro* electrophysiological and imaging techniques is necessary to validate the effects of EUG on NMDA receptors.

CONCLUSION

In summary, the administration of morphine presented a notable elevation in the preference ratings on the side associated with the medication. Conversely, the two different doses of SA extract (200 and 400 mg/kg) did not demonstrate any preference when compared to the control group. The results of the current study indicate that the observed acquisition effect of the hydro-alcoholic extract of SA may be attributed to the antagonistic activities of EUG towards the NMDA receptor, as demonstrated by an *in silico* investigation. The administration of the SA extract did not yield any statistically significant impact on the expression of morphine conditioned place preference (CPP). Mechanistic investigations should be carried out in the future to provide other approaches for explaining the underlying mechanisms.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPLEMENTARY MATERIALS

Table S1: Levene's test of equality of variances. Table S2: Independent samples Maan-Whitney *U* test. Table S3: Multiple comparisons: Tukey's HSD of four different groups of treatment. Table S4: results of the Maan-Whitney *U* test for the four different groups of treatment. Table S5: Kruskall Wallis-H test. Table S6: molecular docking analysis of Memantine and Eugenol with NMDA receptor.

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SUPPLEMENTARY DATA

Table S1. Levene's test result.

Hypothesis	F	P- value	Decision
H_0: Equal variance assumed	0.283	0.603	Equal variance assumed
H_0: Equal variance not assumed			

Table S2. Mann-Whitney *U* test.

Null hypothesis	Sig	Decision
The distribution of time is the same across the treatments (saline and morphine)	0.000	Reject the null hypothesis

Table S3. Multiple comparisons: Tukey's HSD.

Treatment (i)	Treatment (J)	Mean Differences	Std. Error	P-value
Saline	Morphine	-395.00000	27.33922	0.000
	Morphine+ SA-200	-323.75000	27.33922	0.000
	Morphine +SA-400	-227.50000	27.33922	0.000
Morphine	Saline	395.00000	27.33922	0.000
	Morphine + SA-200	71.25000	27.33922	0.065
	Morphine + SA-400	167.50000	27.33922	0.000
Morphine + SA-200	Saline	323.75000	27.33922	0.000
	Morphine	-71.25000	27.33922	0.065
	Morphine + SA-400	96.25000	27.33922	0.008
Morphine +SA-400	Saline	227.50000	27.33922	0.000
	Morphine	-167.50000	27.33922	0.000
	Morphine + SA 200	-96.25000	27.33922	0.008

Table S4. Mann- Whitney -U test results.

Null hypothesis	P-value	Decision
Distribution of time is same across the treatments (Saline and Morphine)	0.000	Reject the null hypothesis
Distribution of time is same across the treatments (Saline and SA-200)	0.000	Reject the null hypothesis
Distribution of time is same across the treatments (Saline and SA-400)	0.000	Reject the null hypothesis

Distribution of time is same across the treatments (Morphine + SA-200)	0.113	Accept the null hypothesis
Distribution of time is same across the treatments (Morphine and SA-400)	0.140	Accept the null hypothesis
Distribution of time is same across the treatments (SA-400 and SA-200)	0.874	Accept the null hypothesis

Table S5. Kruskall Wallis-H.

	Kruskal-Wallis H	df	P-value	Decision
H_0: Distribution of time is same across the four treatments	2.229	2	.328	Accept H0

Table S6. molecular docking analysis of Memantine and Eugenol with NMDA receptor.

Compound name	Binding affinity (kcal/mol)	Hydrophobic		
		Binding type	protein Interacting amino acid	Distance (Angstrom)
Memantine	-5.6	Pi-Alkyl	PHE554	4.833
			TRP563	4.883
			TYR647	4.014
			TYR647	3.975
			TYR647	5.229
		Alkyl	VAL644	5.440
			VAL644	3.721
Eugenol	-5.1	Pi-Alkyl	PHE554	4.496
			PHE558	5.142
			TRP563	4.391
			TYR647	4.108
			VAL644	5.216
			TRP563	5.058
		Alkyl	VAL566	4.202
			ILE643	4.965
		Pi-Pi T-shaped	TYR647	3.976