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Evaluation of antinociceptive and anti-inflammatory activities of clove (Syzygium aromaticum) in vivo

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Abstract

Syzgium aromaticum, commonly known as clove, is a type of spice. It has an active substance called eugenol, which has significant pharmacological characteristics such as antipyretic, anesthetic, antioxidant, antibacterial, neuroprotective, and hypolipidemic properties. This study aimed to extract the eugenol oil from clove and evaluate its potential antinociceptive and anti-inflammatory activities. Eugenol was extracted from clove using direct steam distillation and chemical methods. Thin-layer chromatography and 1H NMR spectroscopy were used to identify the extract as eugenol. Eugenol was evaluated in vivo in mice using a hot plate test and carrageenan-induced paw edema for analgesic and anti-inflammatory activities, respectively. The percentages of maximal possible effect (MPE) in the hot plate test were 4.69±0.7, 3.35±0.5, and 3.34±0.6 seconds for G1, G2, and G3, respectively. Significant results were observed at $P<0.05$ and $p<0.001$ between G2 & G3, and G1, respectively, using repeated ANOVA test analysis. The extracted eugenol oil also revealed a reduction of 51.8% ($p<0.05$) in the mouse paw edema induced by carrageenan in comparison to indomethacin, which showed a reduction of 73.5% ($p<0.01$). In conclusion, the results of the current study support the potential analgesic and anti-inflammatory effects of eugenol oil and reinforce the traditional concept regarding various pharmacological activities of clove.

Keywords: Anti-inflammatory, Antinociceptive, Carrageenan, Eugenol oil, Hot plate test.

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1. Introduction

Clove (*Syzygium aromaticum*) commonly grows as an evergreen shrub in South Asia and India. It is also grown in other countries, such as Tanzania and Sri Lanka [1]. It has small white flowers [2]. It needs exceptional nursing before planting in the field [3] and requires humid and warm weather, well-draining soil, sunlight, and air circulation [4]. *Syzygium aromaticum* are known for their biologically active components, contributing to their anti-inflammatory, analgesic, and other medicinal properties [5].

Eugenol is the clove primary bioactive element, comprising 70-90% of its composition. It inhibits inflammatory mediators such as COX-2, TNF-α, and IL-1ß and modulates sensory nerve conduction [6, 7]. According to El Asbahani, et al. [8] clove essential oil (CEO) consists mainly of phenylpropanoids, like eugenol and their derivatives, accompanied by negligible levels of the chemical components- humulene and caryophyllene [8]. Various pharmaceutical benefits of CEO are reported in publications, such as antiseptic, antibacterial, antioxidant, anticarcinogenic, pesticide, and analgesic action. It is also used as food preservative, spice, and natural colorant [9]. Clove contains various constituents (Figure 1), characterized by different chemical structures and properties with various uses and applications. These include eugenol [10] , β caryophyllene [11] α humelene [8], Eugenol acetate [6] Methyl salicylate, 4-Allylanisole and Benzyl acetate [12]. Eugenol possesses good health benefits, making it a valuable natural element.

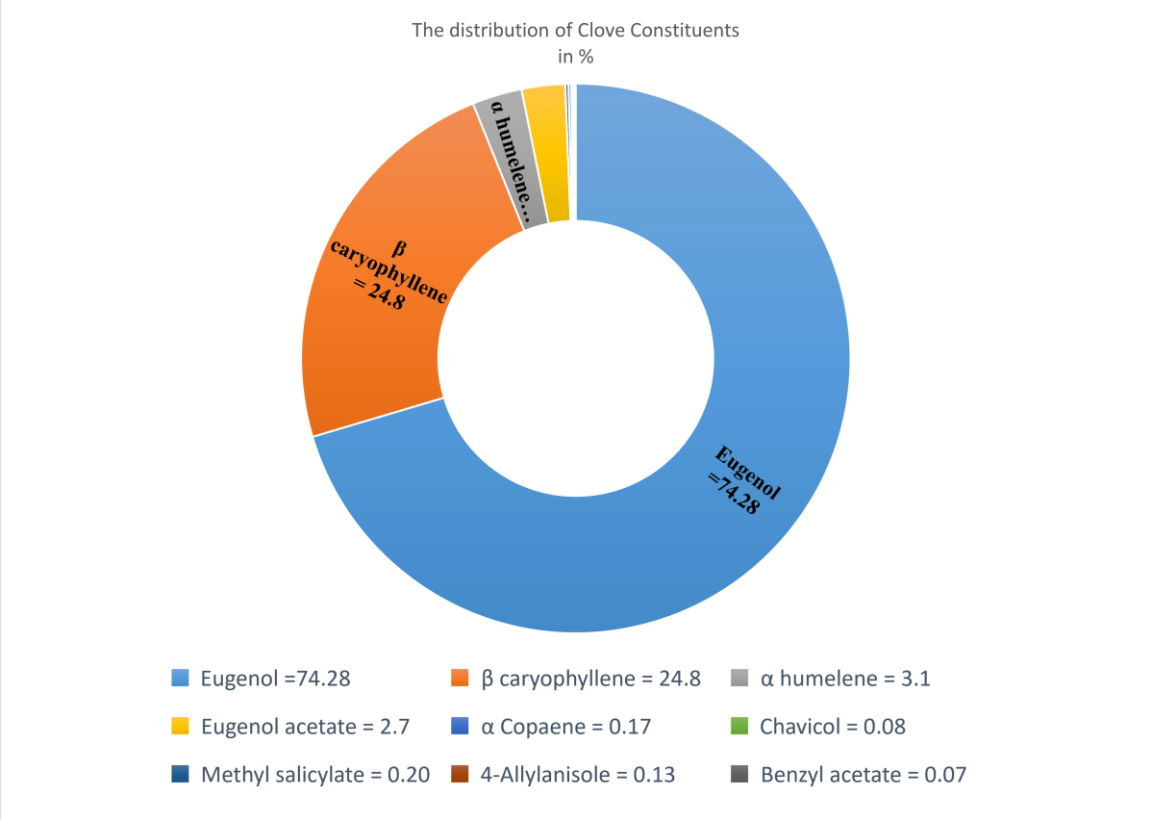

Figure 1.
The distribution of Clove Constituents (%).

Eugenol was isolated from clove leaves and buds and showed antioxidant, antibacterial [13] neuroprotective, hypolipidemic, anti-inflammatory action, and antidiabetic activities [8]. A literature review revealed scarce publications regarding the pharmaceutical effects of eugenol in Iraq. Consequently, this study intends to extract eugenol oil from clove and evaluate its potential antinociceptive and anti-inflammatory activities in vivo.

2. Materials and Methods

2.1. Ethical Approval

This study has been approved by Animal Ethical Research Committee/ College of Dentistry / Al-Iraqia University number (AERC-05-29-075-2024).

*2.2. Extraction of Active Clove Substance (**Eugenol**)*

Cloves were purchased from a local herbal shop in AlSadria / Baghdad. The clove flowering bud was clean and dried (Figure 2 A&B). A grinder-prepared powder was kept in the refrigerator in sterile plastic bags at 4 o C until further processing.

Figure 2.
The A) whole and B) ground clove.

Eugenol was extracted from ground cloves using method described previously by DeFrancesco [14]. The extraction was completed by chemical method. The extracted oil solution felt solid and spicy. Finally, the identification of eugenol from clove was done by ^{1}H-NMR spectroscopy according to the procedure described by Bisergaeva, et al. [15].

2.3. In Vivo Experimentation / Nociceptive Test and Anti-Inflammatory Activity

Thirty-three Swiss male mice (aged 10-12 weeks) were purchased from the Ministry of Health/ The Central Diagnostic Laboratory/ Baghdad / Iraq. The mice adapted to the standard animal house conditions for two weeks. All mice accessed food freely and water ad libitum. All efforts were made to limit distress and minimize the number of animals. All experimental procedures followed the protocol and declaration of the animal ethical committee / College of Dentistry/ Al-Iraqia University. These mice were divided into two categories, 15 and 18 for the first and second, respectively (Figure 3)

2.4. Experimental Design

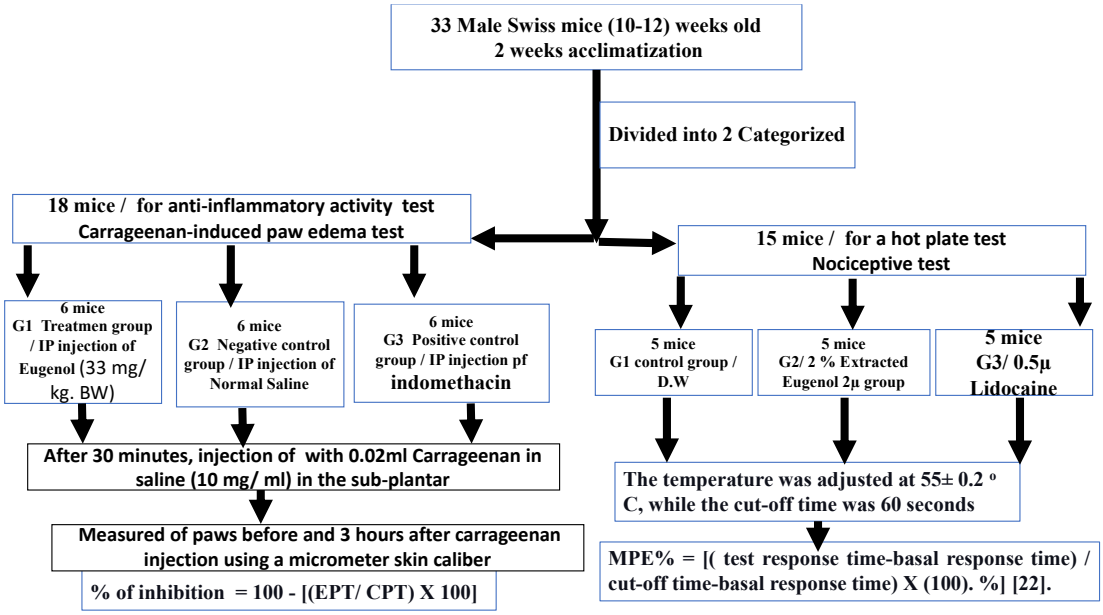

Figure 3.
Flow chart for experimental design to evaluate the antinociceptive and anti-inflammatory activities of clove (Syzgium aromaticum) in Swiss male mice

The first category, with fifteen mice, was used for **the nociceptive test.** The experimental groups were randomly divided into 3 groups (5 each): Group 1(G1), the control group (which received distal water); Group 2 (which received 2% eugenol-extracted oil 20 µL); and Group 3 (which received lidocaine 0.5% 20 µL). In this test, **the mice were** positioned on a hot plate that was used to measure the reaction of the nociceptive test. The temperature was adjusted at 55± 0.2 ° C, while the cut-off time was 60 seconds according to the method described by Hosseini, et al. [16]. The nociceptive test is recognized by licking the forepaws or moving the hind paws. The time interval between placing the animals on a hot plate and licking forepaws or moving hind paws was called the reaction time. The test was done as a three-base registration (10 min interval), 15 minutes before injection of the test materials (intraperitoneal) and thus was repeated five times, each 10 min after injection.

The analgesic effect of placebo, extracted eugenol oil, and lidocaine was calculated as a percentage of the maximal possible effect (MPE). The equation of calculation of MPE % was as follows:

MPE% = [(test response time-basal response time) / cut-off time-basal response time) X (100). %] [17].

All measurements were displayed as mean ± SEM of MPE%. Analysis of the variance (One-way) (ANOVA) and post hoc and Turkey's HSD test were used to compare the base reaction latency time between groups. Moreover, the repeated measure ANOVA followed by post hoc Turkey's HSD test was applied to compare MEP % after the injection of the test extract. Statistically, the significant result was considered at $p<0.05$.

The second category, with eighteen mice, was used to evaluate the anti-inflammatory activity of CEO according to the method previously described by Taher [18] using Carrageenan-induced paw edema test. The 18 mice were divided equally into three groups, each with 6 mice. The mice were injected intraperitoneally with extracted clove oil, which is 100% natural and in a dose of (33 mg/ kg. Body weight based on the previous study [19] normal saline, and indomethacin for first (treatment), second (negative control), and third (positive control), respectively. After thirty minutes of injection, all mice were injected with 0.02ml of the suspension of Carrageenan in saline (10 mg/ ml) in the sub-plantar into the right hind paw. The thickness of the paw was measured before and 3 hours after carrageenan injection using a micrometer skin caliber (Figure 4). The degree of acute inflammation and edema were determined by increasing the thickness of the paw. The inhibition of inflammatory response was reported for each mouse in experimental groups and displayed as percentages for comparison, using the following equation:

% of inhibition = 100 - [(EPT/ CPT) X 100]

(EPT = experimental paw thickness and CPT =Control paw thickness)

Figure 4.
The digital caliber for measuring the skin thickness.

All measurements were displayed as mean ± SEM of MPE%. Analysis of the variance (One-way) (ANOVA) and post hoc and Turkey's HSD test were used to compare the base reaction latency time between groups. Moreover, the repeated measure ANOVA followed by post hoc Turkey's HSD test was applied to compare MEP % after the injection of the test extract. Statistically, the significant result was considered at $p<0.05$.

3. Results

The Clove oil extract was obtained during extraction procedures. About (5.05 g, 5% recovery) eugenol was extracted as a bright yellow oil with a solid particular clove smell from 100 g of clove (Table 1) show the yield result.

Table 1.
Percentage of yield extracted of the clove (*Syzgium aromaticum*).

Concentration	**Extract %**
Yield	
Mean ± SD	5.05 ± 0.038

The ^{1}H-NMR spectroscopy prepared in CDCl3 showed various analysis data / Peaks at Part per Million (PPM), including carbohydrates, phenolic compounds, amino acids, and organic acids. Table 2 shows the concentration of identified eugenol and Figure 5 shows the chemical structures of isolated eugenol.

Table 2.
The metabolite identified in clove (Syzgium aromaticum) with the relative concentration (mmol/mL)

No	Metabolites	^{1}H-NMR Characteristic signals	Concentration
1	Eugenol	δ 3.87 (s)	0.1028

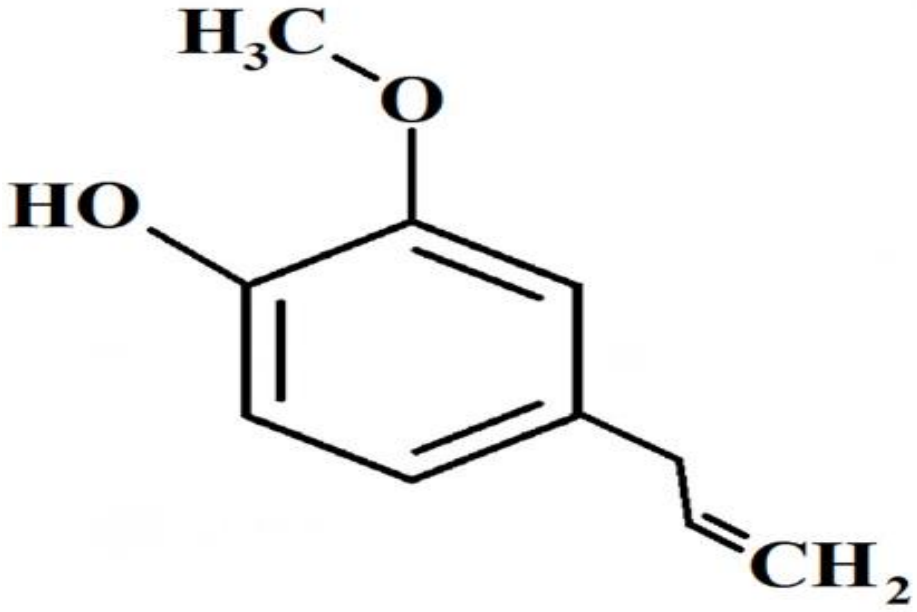

Figure 5.
Chemical structures of isolated eugenol

The reactions of the nociceptive test are displayed in (Figure 6). The percentage of maximal possible effect (MPE) was 4.69±0.7 sec, 3.35±0.5, and 3.34±0.6 for the control group (G1) (received distal water), Treatment Group 2 (G 2) (2 % received eugenol extracted oil 20 µL), and Treatment Group 3 (G3) (received lidocaine 0.5% 20 µL) respectively. Comparison between treated groups G2 and G3 using repeated ANOVA test analysis revealed a significantly higher than control group that received distal water ($P<0.05$ and $p<0.001$ respectively).

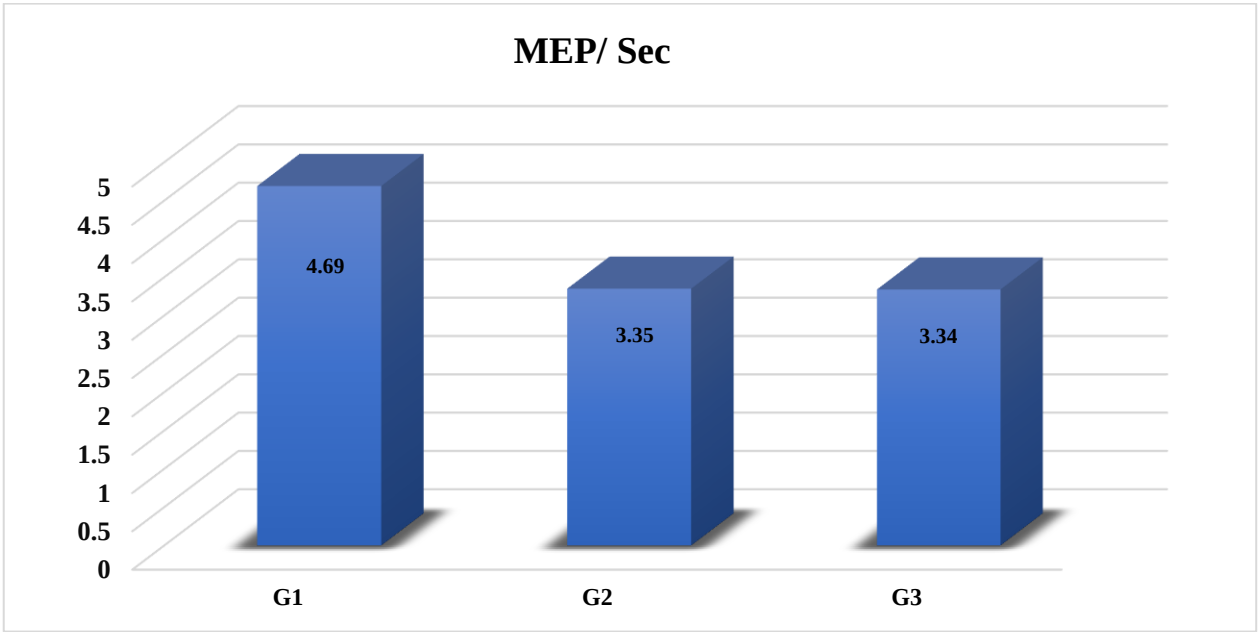

Figure 6.
The percentage of maximal possible effect (MPE) in all treatment groups of mice (control group (G1) (received distal water), Group 2 (G 2) (2 % received eugenol extracted oil 20 µL), and Group 3 (G3) (received lidocaine 0.5% 20 µL).

The results of carrageenan injection revealed a significant inflammation and enlargement in the mouse paw in the second) group (negative control.) The maximum thickness was 21.4 (SEM±4.8) mm in 3 hours (87.2% increase comparison to before carrageenan injection, ($p<0.01$), (Figure 7), however, the enlargement declined progressively over the next hours. A considerable inhibition of induced inflammation and enlargement was seen in the first treatment group, which was injected intraperitoneally by extracted clove oil 30 minutes before carrageenan injection. The extracted clove oil showed apparent

significant suppression in the increased paw thickness by 48.6% (p<0.05) in comparison to the second negative control group. The third (positive control) group, which was injected with indomethacin, produced more obvious edema inhibition (68.4%) suppression (p<0.01) than that of the CEO compared with the control group. The indomethacin's effect was not significantly different (p>0.05) from that of the CEO within the same period.

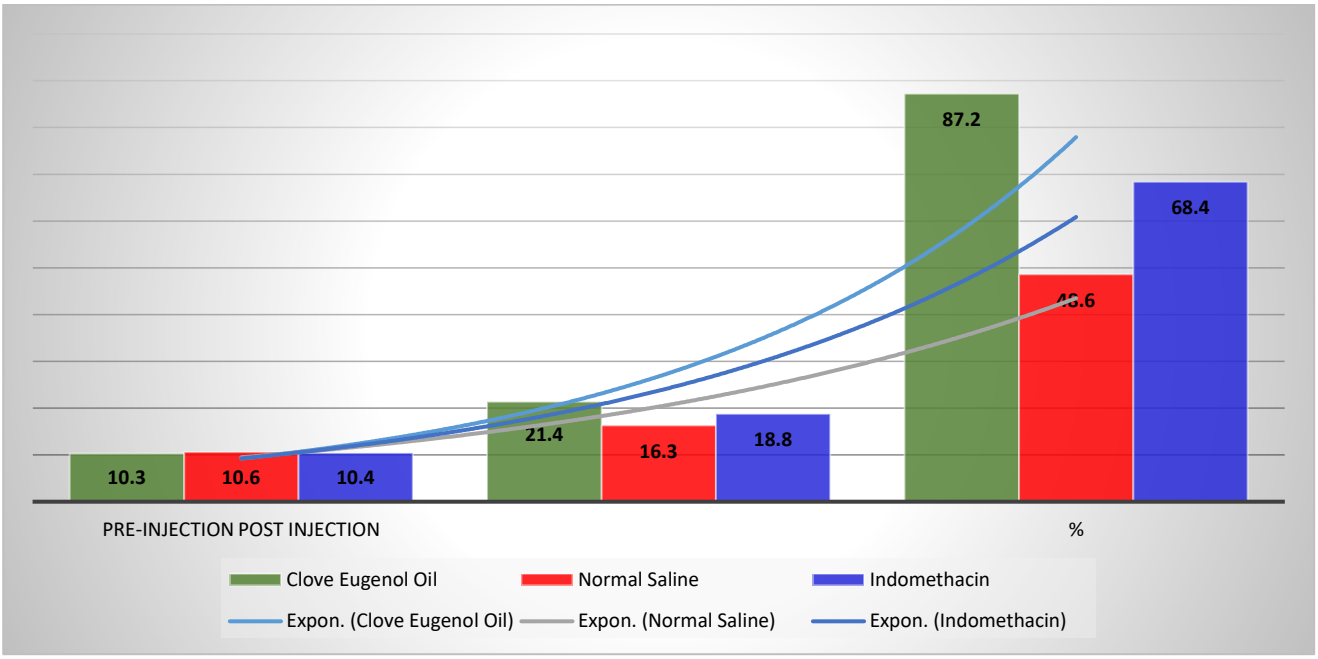

Figure 7.
The results of **Carrageenan-induced paw edema test in different groups.**

4. Discussion

Clove has multiple therapeutic properties. It has been used traditionally as an antibacterial and analgesic herb. Usually, clove is mixed with food seasoning and used as a food preservative [20]. Previous extraction studies of clove obtained eugenol, the main phenolic compound, at 70.8 to 14.6 mg per 100 g of fresh clove material [21].

The current study's extraction procedure yielded eugenol as a bright yellow oil with a clove smell from the clove-dried flowering bud at a concentration of 5% recovery (2.02 g from 40 gm) of clove. This result agrees with previous studies, which extracted the eugenol as a light yellow oil with a strong clove smell. The clove-extracted substance value spots range between 0.1 to 0.9 with various peaks at Part per Million (PPM) in ^{1}H NMR spectroscopy. This peak was eugenol in δ 3.87 (s) and was concentration 0.1028 (mmol/mL). These results are compatible with previously reported studies [22, 23]. In the current study, the nociceptive test was done in three groups of mice. The results of this study showed variation in the percentages of MEP between G1(4.69±0.7 sec), G2 (3.35±0.5), and G3 (3.34±0.6). Moreover, significant (P<0.05 and p<0.001) results appeared during the comparison between treated groups G2 and G3 and G1 (control group). These results approved the anesthetic effects of the clove product that was prepared in the current study. These results are well-matched with previously reported published studies [16, 17, 24, 25] all these studies approved that the clove essential oil has an analgesic effect. Hosseini, et al. [16] found that essential oil extracted from clove had analgesic effects that tested in the hot plate in mice. The maximum anesthetic effect was at 10% concentration, which showed 70 min after injection. Furthermore, the clove oil extract was used regularly to anesthetize the fish during the transferring process, which was analogous to lidocaine [17, 26-28]. These studies approved clove oil's efficacy as an anesthetic material for tropical fish. They showed that the responses of small-bodied tropical fish were species-specific. However, all three species were sedated and recovered in less than 1 and 7 minutes. Another researcher also approved that Eugenia caryophyllata (Clove) buds act as topical anesthetics and analgesics to relieve acute corneal pain via opioidergic and cholinergic pathways [29]. The analgesic effects in the current study on experimental mice were due to the clove-extracted eugenol. It is the active components of clove responsible for the analgesic effects or antinociceptive activity, which was proven by Kurian, et al. [30] and Daniel, et al. [31] against acetic acid tests and thermal pain mechanism. The analgesic effect of clove eugenol oil extract may be due to its ability to suppress prostaglandins and other inflammatory mediators like leukotrienes [32]. Research also suggested that eugenol depresses the sensory receptors related to pain perception [33]. The results of the current study are also compatible with a previously reported study by Taher [18] and Taher, et al. [34] who demonstrated the valuable pharmacological properties of clove oil in mice. They approved the amelioration of pain, fever, and acute inflammation induced experimentally in mice. Eugenol is CEO's primary bioactive compound, and previous studies have broadly used it to confirm its anti-inflammatory therapeutic properties in regulating the inflammatory pathways, alleviating symptoms of inflammation-associated disorders, and lowering oxidative stress [35].

The mechanism of eugenol as an anti-inflammatory is inhibition and downregulates the production of pro-inflammatory mediators (TNF- α, IL-1ß, and IL-6) via suppressing the activation of a nuclear factor kappa B (NF-$_k$B), which is the principle transcriptor factor involved in inflammatory responses [36]. It also diminishes the expression of cyclooxygenase-2 and

inducible oxide synthase (iNOS), which are essential in producing prostaglandins and nitric oxide, respectively, which play in inflammation.

In the current study, Carrageenan-paw edema is an experimental model used to evaluate medications used to relieve acute inflammation, such as anti-edematogenic compounds acting to interfere with the inflammatory mediators [18]. According to previous studies, the injection of Carrageenan, the phlogistic agent, produced acute edematous inflammation by biphasic upshots; the histamine, and serotonin trigger the first phase. The release of prostaglandins is responsible for the later phase of inflammation, which is the continuity of edema to reach its highest degree and encourages the formation of inflammatory tenderness and exudation [37]. The prostaglandin's demagogic effects are usually diminished by non-steroidal anti-inflammatory drugs (NSAID) like diclofenac free acid (Voltaren) [38]. The observations of the current studies showed amelioration of inflammatory reaction in CEO injected group in compare to negative and positive control groups. These results approved the CEO's anti-edematogenic activity due to reducing the inflammatory edema and swelling formation during the late phase, 3 h after carrageenan injection. This reduction might mediated via prostaglandin interference. These results are compatible with previously reported studies [34] who approved the valuable pharmacological properties of clove oil in mice. The administration of clove oil significantly ameliorated the experimental-induced pain, fever, and acute inflammation.

In conclusion, the current study, approved the analgesic and anti-inflammatory effects of eugenol, make it a promising candidate for regulating a broad range of inflammatory reactions. However, further studies, including clinical trials, are needed to establish its safety, toxicity, and efficacy.

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