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Original Article

Effect of clove flower extract (*Syzygium aromaticum*) administration timing on skeletal muscle damage induced by eccentric exercise: An in vivo study

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Abstract

Eccentric exercise often leads to oxidative stress, inflammation, and muscle damage that impair athletic performance. To counter these adverse effects, clove flower extract (*Syzygium aromaticum*) offers promising potential as a natural remedy to promote muscle repair with its potent antioxidant and anti-inflammatory properties. The aim of this study was to assess the effects of clove flower extract administration timing on oxidative stress and inflammatory responses in skeletal muscle damage induced by acute eccentric exercise in mice. This study used a post-test-only control group design, involving 35 male mice (*Mus musculus*, Balb/c) randomly divided into five groups: a healthy control group (HG) with no exercise and no treatment, a negative control group (NG) with exercise but no treatment, T1 group (receiving clove flower extract 24 hours before exercise), T2 group (receiving clove flower extract immediately after exercise), and T3 group (receiving clove flower extract 24 hours after exercise). The treatment groups received a single dose of clove flower extract (500 mg/kg body weight (BW)). The skeletal muscle damage in mice was collected by measuring NADPH oxidase (NOX) and superoxide dismutase (SOD) activities using spectrophotometry, as well as toll-like receptor 4 (TLR4) and interleukin-8 (IL-8) levels using an enzyme-linked immunosorbent assay (ELISA). Moreover, the skeletal muscle damage was analyzed through the histopathological method. Data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) tests as a post hoc test. The result showed that clove flower extract significantly reduced NOX (p=0.049) and IL-8 (p=0.032) levels, increased SOD activity (p=0.001), and did not significantly affect TLR4 levels (p=0.532). Moreover, the results showed a significant reduction in muscle damage (p=0.001). The study highlights that the administration of clove flower extract (500 mg/kg BW) 24 hours before exercise, immediately after exercise, or 24 hours after exercise can help prevent muscle damage.

Keywords: Clove flower extract, NOX, SOD, TLR4, IL8

Introduction

Eccentric exercise has the potential to induce severe injuries not only in the musculoskeletal system but also in various physiological systems [1,2]. This risk is especially high in certain

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individual sports, low-impact activities, sports that mostly involve female participants, and professional athletes [3]. A study reported that eccentric exercise affects biological systems within seven days [4], whereas a study reported an increase in reactive oxygen species (ROS) 1 to 2 days after intensive exercise [5]. This oxidative response is mediated by the NADPH oxidase (NOX) pathway, which inhibits the antioxidant enzyme superoxide dismutase (SOD) [6]. These findings suggest a connection between inflammation, oxidative stress, and muscle damage resulting from mechanical stress [7].

Prolonged high-intensity exercise increases muscle inflammatory markers and activates toll-like receptor 4 (TLR4) [8]. During cellular stress or injury, muscle damage-associated molecules are released into the extracellular environment and blood circulation, where they are recognized by TLR4 pattern recognition receptors [9]. This receptor recognizes muscle cell damage and then activates downstream signaling pathways, including mitogen-activated protein kinases (MAPKs) and IκB kinase (IKK), which result in the elevated production of inflammatory cytokines [10]. The transcription factors activator protein 1 (AP-1) and nuclear factor kappa B (NFκB) then stimulate the release of proinflammatory cytokine interleukin-8 (IL-8) [11].

Muscle damage caused by eccentric training is frequently overlooked because the effects are not immediately noticeable [12]. However, over time this damage weakens muscles, reduces strength, and interferes with daily activities as tissue injury worsens [13]. Clove flowers (*Syzygium aromaticum*) have been identified as a potential herbal alternative with therapeutic properties comparable to traditional pharmaceutical medicines [14]. Another study reported that cinnamaldehyde and clove flowers had anti-inflammatory characteristics [15]. In addition, a study reported that eugenol, a key compound in cloves, exhibits both anti-inflammatory and antioxidant activities [16]. These findings provide evidence for the use of clove flower extract as a natural source of anti-inflammatory and antioxidant effects, with few side effects [17].

Most studies focus on long-term treatment, often neglecting the potential short-term effects of clove flower extract on muscle damage. Furthermore, standardized dose for short-term use is unclear. As a result, the preventive and therapeutic potential of clove flower extract remains unknown, because limited clinical data validate its effectiveness based on the timing of administration. This short-term study serves as an initial step for developing herbal medicine that promotes rapid muscle healing. The ultimate goal is to help individuals rapidly restore optimal muscle performance after experiencing muscle injury.

Short-term treatment is crucial for maintaining optimal muscle function, particularly in athletes. Despite several studies on clove flower extract and its derivatives, its potential to repair muscle injury has not been fully explored. Determining the optimal timing for clove flower extract as a preventive or therapeutic intervention is essential for increasing its effectiveness in muscle repair. Therefore, the aim of this study was to investigate the effects of clove flower extract administration timing on oxidative stress and inflammation responses in mice with skeletal muscle injury induced by acute eccentric exercise.

Methods

Study design and setting

An experimental study was conducted at the Laboratory of Pharmacy and Biochemistry, Universitas Airlangga, Surabaya, East Java, Indonesia, from November 2023 to January 2024. This study used a post-test-only control group design, involving 35 male mice (*Mus musculus*, Balb/c) randomly divided into five groups: healthy control group (HG) with no exercise and no treatment, negative control group (NG) with exercise but no treatment, T1 group (receiving clove flower extract 24 hours before exercise), T2 group (receiving clove flower extract immediately after exercise), and T3 group (receiving clove flower extract 24 hours after exercise), as presented in **Table 1**. The treatment groups received a single dose of clove flower extract (500 mg/kg BW).

Muscle damage was induced by eccentric exercise through eccentric treadmill running based on previous studies that have been proven to cause muscle damage [5,18-20]. After 24 hours of eccentric exercise, the quadriceps muscles were collected for analysis. TLR4 and IL-8 levels were measured using enzyme-linked immunosorbent assay (ELISA), while NOX and SOD activities

were assessed using spectrophotometry (Elabscience Corporation, Texas, USA) and hematoxylin-eosin staining.

Table 1. Description of experimental groups

Experimental group	Description
Healthy control (HG)	No exercise and no treatment of clove flower extract
Negative control (NG)	Exercise without treatment of clover flower extract
Treatment group 1 (T1)	Administration of clove flower extract 24 hours before exercise
Treatment group 2 (T2)	Administration of clove flower extract immediately after exercise
Treatment group 3 (T3)	Administration of clove flower extract 24 hours after exercise

Sample criteria and size determination

This present study used mice that exhibited normal movement, clean fur, and healthy extremities as inclusion criteria, ensuring that all experimental animals were in good health at the start of the study. Any mice that did not survive during the experiment were excluded, ensuring data integrity by eliminating incomplete observations. The sample size was calculated using the OpenEpi website (https://www.openepi.com/SampleSize/SSMean.htm), which employs widely accepted formulas such as Cochran's Formula for experiments and epidemiological studies [21].

The sample size calculation determined that six mice per group were required. To account for potential losses, a 10% reserve was added, yielding 6+(0.6)=6.6, which was rounded up to seven mice per group. This meets the minimum requirement for in-vivo experimental study [22]. Thirty-five male mice (*Mus musculus*, Balb/c) were divided into five groups, with seven mice per group, as previously described in **Table 1**. However, one mouse did not survive during the experiment.

Clove flower extract preparation

Clove flowers were obtained from Magelang, Central Java, Indonesia, and subsequently air-dried, finely ground, and sieved using a 40-mesh filter. Extraction was carried out using a 1:10 maceration ratio in 90% ethanol, followed by six hours of reflux. The extract was filtered using Whatman No. 41 paper and washed with 50 mL of ethanol. The filtrate was then concentrated to 6 mL using a rotary evaporator (Marshall Scientific, London, UK) at 45°C. The pure extract was then stored at -10°C until further use [23]. For this study, the extract was prepared by maceration in 70% ethanol and diluted in carboxymethylcellulose sodium (CMC Na) to achieve a final concentration of 0.1 mg per gram of mouse body weight. The extract was administered at doses of 500 mg/kg BW, dissolved in 0.2 mL of CMC Na. This dosage was established based on a pilot study involving five doses (100, 150, 250, 350, and 500 mg/kg BW)[24–26] . The 500 mg/kg BW dose resulted in the highest reduction in NOX activity and the number of inflammatory cells in mice with muscle injury, showing its effectiveness in mitigating muscle damage.

Phytochemical screening

The clove flower extract was subjected to phytochemical analysis using gas chromatography-mass spectrometry (GC-MS) at the Central Laboratory of Universitas Gadjah Mada, Yogyakarta, Indonesia. GC-MS is a powerful tool for analyzing mixtures of substances, allowing for the identification of secondary metabolites at low concentrations [27]. The GC-MS results provided mass spectra and chromatograms, enabling the identification of chemical components with antioxidant and anti-inflammatory properties. The analysis of the crude extract revealed a wide composition of chemicals, with chromatographic and spectral data serving as crucial instruments for detecting secondary metabolites with precision, even at trace levels.

Induction of muscle damage

Prior to inducing muscle damage, the mice were subjected to a 7-day acclimatization phase during which they were fed a regular diet and water *ad libitum*. They were kept at room temperature (25°C) with appropriate ventilation, and the bedding was changed every two days. The light cycle was set to provide brilliant light throughout the day and darkness at night. Following the acclimation phase, the muscular damage was induced using a treadmill with a 15° drop and a maximum speed of 25 m/min. The mice were forced to run for 30 to 60 minutes or until they became exhausted, which was defined as their inability to continue running in the face of minor

motivating stimuli. This exercise model was developed based on prior studies that found eccentric exercise causes muscle damage and an increase in ROS levels [4,5]. A pilot study was performed to confirm that this method caused muscle injury, as demonstrated by the analysis of NOX activity and hematoxylin-eosin of the muscle tissue [26]. This verified the treadmill methodology used in the study to induce muscle damage.

Clove flower extract administration and skeletal muscle collection

The clove flower extract was administered at doses of 500 mg/kg BW, dissolved in 0.2 mL of CMC-Na, on three different schedules: 24 hours before exercise, immediately after exercise, and 24 hours after activity (**Table 2**). The extract was administered orally, and skeletal muscles were collected after sacrifice for further analysis. Muscle damage was assessed via biopsy which was conducted either as an open biopsy under general anesthesia or as a needle biopsy under mild sedation. The quadriceps muscle was collected after sacrifice by incising the muscle origin (iliac spine) and the tendon insertion. The excised muscle was placed into a storage container. The excised muscle was separated into two portions: the left portion was immersed in formalin buffer for histological preparation to assess muscle cell condition, whereas the right portion was frozen at -80°C in a sealed dry container for protein measurements (NOX, SOD, TLR4, and IL-8).

Table 2. Study schedule of each treatment group

Day	Healthy control	Negative control	Treatment group 1	Treatment group 2	Treatment group 3
1	-	-	Clove flower extract	-	Exercise
2	-	Exercise	Exercise	Exercise Clove flower extract	Clove flower extract
3	Sacrifice	Sacrifice	Sacrifice	Sacrifice	Sacrifice

Protein measurements and histopathological analysis

The protein measurements and histopathological analysis were carried out at the Laboratory of Biomedicine, Universitas Airlangga, Surabaya, Indonesia. To ensure data integrity, all assessments were conducted by a team of blinded laboratory technicians. Levels of NOX and SOD were measured using spectrophotometry, whereas levels of TLR4 and IL-8 were measured using ELISA (Korain Biotech Corporation, Shanghai, China). The direct ELISA kit (E0080Ra Korain Biotech Corporation, Shanghai, China) was used, pre-coated with antibodies specific to mice.

In the examination of TLR4 and IL-8 using the ELISA method, 100 mg of muscle tissues per sample was required. The tissue was homogenized using a sonicator (Fisher Scientific, UK) until fully disrupted. Measurements were performed using an ELISA kit from Korain Biotech Corporation, Shanghai, China, following the manufacturer's instructions. Briefly, 100 mg of muscle tissue, standards, controls, and blanks were added to a 96-well plate pre-coated with specific antibodies and incubated for two hours at room temperature (25°C). The wells were then washed three times with wash buffer, followed by the addition of 100 µL of biotin-conjugated detection antibody and incubated for one hour at room temperature (25°C). Subsequently, the plate was washed again, followed by the addition of 100 µL of streptavidin-horseradish peroxidase (SA-HRP) conjugate and a 30-minute incubation in the dark at room temperature (25°C). After a final washing step, substrate solution was added and incubated in the dark for 10–15 minutes to allow color development. The reaction was stopped by adding 100 µL of stop solution. Absorbance was measured at 450 nm using a microplate reader (BMG LABTECH, Offenburg, Jerman).

NOX and SOD activity was assessed via spectrophotometry using the E-BC-K806-M kit for NOX and the E-BC-K019-S kit for SOD (Elabscience Corporation, Texas, USA). Skeletal muscle tissue was homogenized with 0.3 mL of 9% saline per 10 g of tissue, followed by 600 ×g centrifugation for 5 minutes to remove insoluble material, then 12000 ×g for 15 minutes to obtain the supernatant [28]. The procedure for spectrophotometric analysis of muscle samples involved several key steps. First, approximately 100 mg of muscle tissue was homogenized in an ice-cold buffer (phosphate buffer, pH 7.4) containing protease inhibitors to preserve enzyme activity. The homogenate was then centrifuged at a high speed of 10,000 g at 4°C to separate the supernatant, which serves as the enzyme extract. In the assay, the enzyme extract was mixed with a reaction

buffer containing the substrate, and the reaction was initiated at 37°C. The decrease in absorbance, indicative of NOX and SOD activity, was monitored using a spectrophotometer centrifuge and microplate reader at 600 nm. Histopathological analysis of longitudinal muscle sections was performed using hematoxylin-eosin staining, and observations were made under a light microscope at 1000× magnification to determine cellular morphology and structural integrity of muscle damage.

Statistical analysis

Data analysis was performed using SPSS version 26.0 (IBM, New York, USA), with a significance threshold set at $p \leq 0.05$. The Shapiro-Wilk test assessed the normality of the data, while Levene's test evaluated the homogeneity of variances ($p > 0.05$), supporting the application of parametric statistical methods. An analysis of variance (ANOVA) test was then performed, followed by post hoc test using Fisher's least significant difference (LSD) to examine the effects of clove flower extract administration timing on the levels of NOX, SOD, TLR4, IL-8 and muscle damage.

Results

Phytochemical screening results

The GC-MS analysis of clove flower extract identified 56 active compounds, with eugenol as the predominant component. The results highlight the complexity and therapeutic potential properties of clove flower extract, as presented in **Figure 2** and **Table 3**. Eugenol was identified as the major active compound, comprising 75.33% of the extract, followed by 3-Allyl-6-methoxyphenyl acetate (9.92%), Bicyclo (7.2.0) undec-4-ene, 4,11,11-trimethyl-8-methylene-, (1R-(1R*, 4Z, 9S*)) (6.73%), Trimethoxyacetophenone (3.40%), β-Longipinene (0.94%), and 3.68% of other active substances.

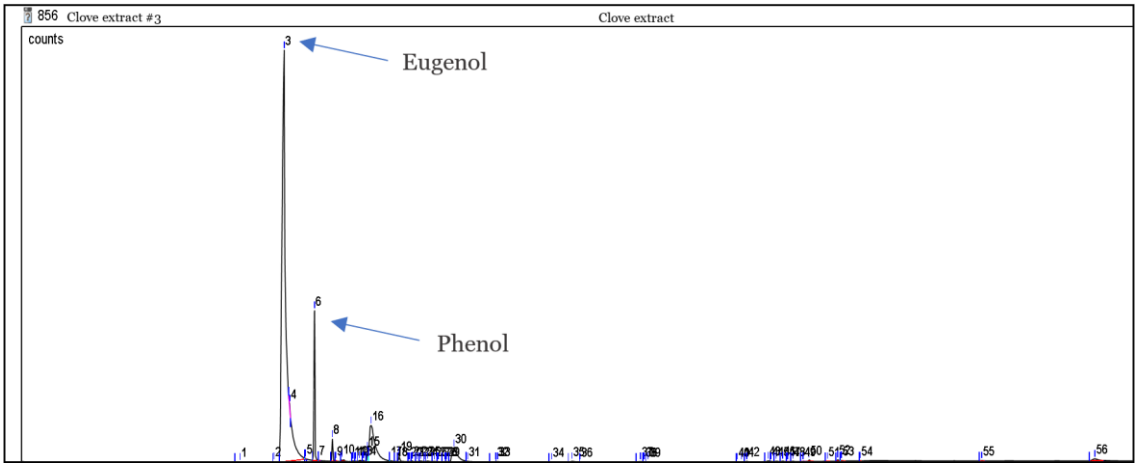

Figure 2. GC-MS chromatogram of chemical compounds from clove flower extract using ethanol solvent.

Effects of clove flower extract on NOX, SOD, TLR4, and IL-8

The levels of each protein per group were presented as mean±SD. The ANOVA test revealed significant differences in three of the four proteins examined (NOX, SOD, and IL-8) ($p < 0.05$), as presented in **Table 4**. These results indicated that the timing of clove flower extract administration had a notable impact on these protein levels.

NOX activity

Administration of clove flower extract significantly reduced NOX activity compared to NG in mice model with eccentric exercise ($p = 0.049$). The mean NOX levels in the HG, T1, T2, and T3 groups were significantly lower than in the NG. Moreover, the mean NOX levels in groups of T1, T2, and T3 were not significantly different from the HG. Among the treatment groups, the T3 group was significantly lower in NOX levels compared to the NG, indicating that T3 was more effective in reducing the NOX levels. Detailed post hoc Fisher LSD results are presented in **Figure 3**.

Table 3. Active compounds of clove flower extract based on gas chromatography and mass spectrometry (GC-MS)

Retention time (min)	Active compound	Chemical formula	Relative area (%)
9.24	2-Allylphenol	C9H10O	0.02
10.05	Alfa Copaene	C15H24	0.04
10.30	Eugenol	C10H12O2	75.33
10.43	3-Allyl-6-methoxy phenol	C10H12O2	0.16
10.81	Phenol, 2-methoxy-3-(2-propenyl)-	C10H12O2	0.04
11.02	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,(1R-(1R*,4Z,9S*))-	C15H24	6.73
11.12	Phenol, 2-methoxy-5-(1-propenyl)-, (E)-	C10H12O2	0.03
11.45	1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z, Z,Z-	C15H24	0.94
11.54	β-Longipinene	C15H24	0.03
11.68	alfa-Copaene	C15H24	0.18
11.95	a-ylangene	C15H24	0.05
12.01	β-copaene	C15H24	0.03
12.10	Naphthalene, 1,2,3,5,6,7,8,8a-octahedron-1,8a-dimethyl-7-(1-methyl ethenyl)-,(1S-(1a,7a,8aa))-	C15H24	0.13
12.19	(S,1Z,6Z)-8-Isopropyl-1-methyl-5-methylenecyclodeca-1,6-diene	C15H24	0.07
12.29	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methyl ethyl)-, (1S-cis)-	C15H24	0.50
12.38	3-Allyl-6-methoxyphenyl acetate	C12H14O3	9.92
12.82	Phenol, 2-methoxy-3-(2-propenyl)-	C10H12O2	0.09
12.95	Phenol, 2-methoxy-6-(1-propenyl)-	C10H12O2	0.01
13.05	Alloaromadendrene oxide-(1)	C15H24O	0.29
13.30	2-((4aS,8R,8aR)-4a,8-dimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalen-2-yl)propane-2-ol	C15H26O	0.02
13.36	1,4-methanoazulen-7-ol, decahydro-1,5,5,8a-tetramethyl-, (1s-(1a,3aβ,4a,7β,8aβ))-	C15H26O	0.04
13.48	5-benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-a-methylene-2-oxo-, methyl ester	C16H20O4	0.01
13.56	a-acorenol	C15H26O	0.07
13.67	a-acorenol	C15H26O	0.02
13.73	Epiglobulol	C15H26O	0.08
13.89	1,4-methanoazulen-7-ol, decahydro-1,5,5,8a-tetramethyl-, (1s-(1a,3aβ,4a,7β,8aβ))-	C15H26O	0.03
13.99	11,13-dihydroxy-tetradic-5-yonic acid, methyl ester	C15H26O4	0.00
14.15	11,13-dihydroxy-tetradic-5-yonic acid, methyl ester	C15H26O4	0.02
14.19	11,13-dihydroxy-tetradic-5-yonic acid, methyl ester	C15H26O4	0.02
14.37	2,4,6-trimethoxyacetophenone	C11H14O4	3.40
14.69	1-Propyl-3,6-diazahomoadamantan-9-ol	C12H22N2O	0.08
15.37	2',3',4' trimethoxyacetophenone	C11H14O4	0.04
15.41	1-propyl-3,6-diazahomoadamantan-9-ol	C12H22N2O	0.05
16.71	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	C24H46O2	0.02
17.20	n-hexadecanoic acid	C16H32O2	0.15
17.39	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediol ester	C35H68O5	0.00
18.83	6,9,12,15-docosatetraenoic acid, methyl ester	C23H38O2	0.09
18.92	6,9,12,15-docosatetraenoic acid, methyl ester	C23H38O2	0.03
19.02	2-bromotetradecanoic acid	C14H27BrO2	0.04
21.15	1-heptatriacotanol	C37H76O	0.00
21.26	2-bromotetradecanoic acid	C14H27BrO2	0.02

Retention time (min)	Active compound	Chemical formula	Relative area (%)
21.38	5aH-3a,12-Methano-1H-cyclopropa[5',6']cyclodeca(1',2':1,5)cyclopenta(1,2-d)[1,3]dioxol-13-one, 1a,2,3,9,12,12a-hexahydro-9-hydroxy-10-(hydroxymethyl)-1,1,3,5,7,7-hexamethyl-, (1aR-(1aa,3a,3aa,5aa,8aR*,9β,12a,12aa))-	C23H32O5	0.03
21.89	Pregnan-20-one, 3-(acetyloxy)-5-hydroxy-6,16-dimethyl-, (3β,5a,6β,16a)-	C25H40O4	0.08
21.97	Podocarpa-1,12-diene-d14,a-acetic acid, 7-hydroxy-8,13-dimethyl-3-oxo-, d-lactone	C21H26O3	0.03
22.09	Hexa-t-butylselenatrisiletane	C24H54SeSi3	0.05
22.25	Ethyl iso-allocholate	C26H44O5	0.05
22.37	1-Phenanthrenecarboxylic acid, tetradecahydro-7-(2-methoxy-2-oxoethylidene)-1,4a,8-trimethyl-9-oxo-, methyl ester, (1S-(1a,4aa,4bβ,8β,8aa,10aβ))-	C22H32O5	0.01
22.49	6,9,12,15-docosatetraenoic acid, methyl ester	C23H38O2	0.03
22.73	5-(3,4-dimethoxyphenyl)-3-(2-hydroxyphenyl)-4,5-dihydro pyrazole-1-carbaldehyde	C18H18N2O4	0.02
22.88	6,9,12,15-docosatetraenoic acid, methyl ester	C23H38O2	0.14
23.32	Hexadecanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a-methanocyclopenta(a)cyclopropa(e)cyclodecen-6-yl ester, (1aR-(1aa,2a,5β,5aβ,6β,8aa,9a,10aa))-	C36H58O6	0.01
23.56	3',8,8'-trimethoxy-3-piperidinyl-2,2'-binaphthalene-1,1',4,4'-tetrone	C28H25NO7	0.13
23.65	3-isopropyl-6a,10b-dimethyl-8-(2-oxo-2-phenyl-ethyl)-dodecahydro-benzo (f) chromen-7-one	C26H36O3	0.07
24.10	8,14-Seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3β-methoxy-4,4-dimethyl-	C24H36O6	0.02
27.02	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	C27H42O4	0.04
29.73	β-Sitosterol	C29H50O	0.48

Table 4. One-way ANOVA results for NOX, SOD, TLR4, and IL-8 levels across different clove flower extract administration times

Variables	Groups					*p*-value
	Healthy control	Negative control	Treatment group 1	Treatment group 2	Treatment group 3	
NOX (U/g protein)	2.17±0.04	5.09±2.78	3.07±1.20	3.09±2.04	2.52±0.69	0.049
SOD (U/mL)	798.90±97.45	449.10±129.31	561.77±61.99	625.05±56.43	634.60±32.79	0.001
TLR4 (ng/mL)	1.70±0.13	1.85±0.22	1.89±0.21	1.78±0.43	1.73±0.36	0.532
IL-8 (ng/L)	147.01±14.11	179.10±17.43	165.59±11.67	153.29±31.62	154.78±10.66	0.032
Muscle damage	167.33±8.36	403.33±19.74	304.33±14.22	214.66±13.89	175.66±15.62	0.001

SOD activity

Administration of clove flower extract significantly increased SOD levels compared to the NG in mice subjected to eccentric exercise. The ANOVA test results showed a *p*-value of 0.001, indicating a significant difference in the effect of clove flower extract administrated 24 hours before exercise, immediately after exercise, and 24 hours after exercise on SOD levels in mice with skeletal muscle damage. According to the post hoc Fisher LSD test, administering clove flower extract immediately after exercise (T2 group) and 24 hours after exercise (T3 group) had the most pronounced effect in enhancing SOD levels compared to 24 hours before exercise (T1 group). Detailed post hoc Fisher LSD results are presented in **Figure 3**.

TLR4 levels

The mean TLR4 levels showed that the T1 had the highest levels, while the HG had the lowest. Among the treatment groups, T1 group exhibited levels similar to the NG, whereas groups T2 and T3 demonstrated lower levels comopared to the NG. However, statistical analysis using the ANOVA test revealed that the administration of clove flower extract did not significantly reduce TLR4 levels, as indicated by a *p*-value of 0.532 (**Figure 3**).

IL-8 levels

The administration of clove flower extract significantly decreased IL-8 levels compared to the NG in the eccentric exercise mouse model, as indicated by the ANOVA test with a *p*-value of 0.032. The post hoc Fisher LSD test showed IL-8 levels in T1 (p=0.223), T2 (p=0.025), and T3 (p=0.033). T2 and T3 groups were found significantly lower compared to the NG. This supports the hypothesis that administering clove flower extract immediately and 24 hours after eccentric exercise effectively reduced IL-8 levels associated with muscle damage. Moreover, no significant differences were observed among treatment groups (T1, T2, and T3), suggesting that clove flower extract administration before, immediately, or 24 hours after exercise provides comparable benefits in reducing IL-8 levels, as presented in **Figure 3**.

Histopathological analysis

The results of the HE preparation reading show the number of inflammatory cells in each group, with varying amounts (**Figure 4**). The histopathological results show that the NG has the most inflammatory cells compared to the HG, T1, T2, and T3. In the treatment group, the T1 group showed the most inflammatory cells compared to the T2 and T3 groups. The histopathological differences in skeletal muscle damage based on the timing of clove flower extract administration are presented in **Figure 4**.

The administration of clove flower extract significantly reduced muscle damage compared to the NG in the eccentric exercise mouse model, as indicated by the one-way ANOVA test (p=0.001) (**Figure 5**). Post hoc Fisher's LSD test revealed significant differences in muscle damage between the NG and both the HG and treatment groups (p=0.001). Furthermore, significant differences were observed between the HG and treatment groups (T1, T2) (p=0.001), as well as among the treatment groups (T1, T2, T3) (p=0.001). However, no significant difference was found between the HG and T3 groups (p=0.339). These findings support the hypothesis that administering clove flower extract 24 hours before, immediately after, and 24 hours after eccentric exercise effectively reduced muscle damage.

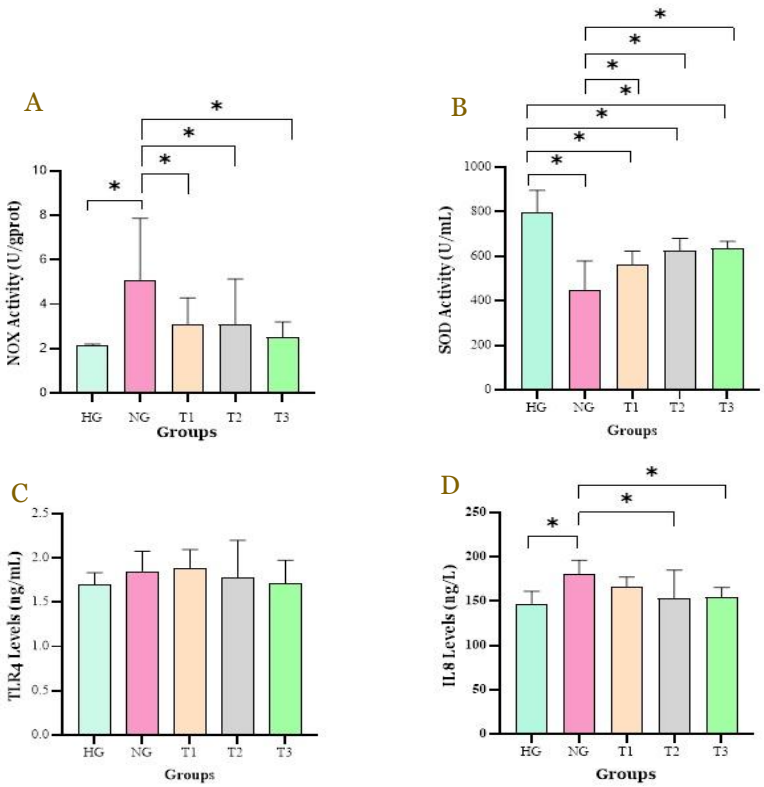

Figure 3. (A) Mean±SD of NOX activity among groups; (B) Mean±SD of SOD levels among groups; (C) Mean±SD TLR4 levels among groups; (D) Mean±SD of IL-8 levels among groups. Asterisk indicate significantly different results from the negative control (NG) group. HG: positive control group; NG: negative control group; T1: receiving clove flower extract 24 hours before exercise; T2: receiving clove flower extract immediately after exercise; T3: receiving clove flower extract 24 hours after exercise.

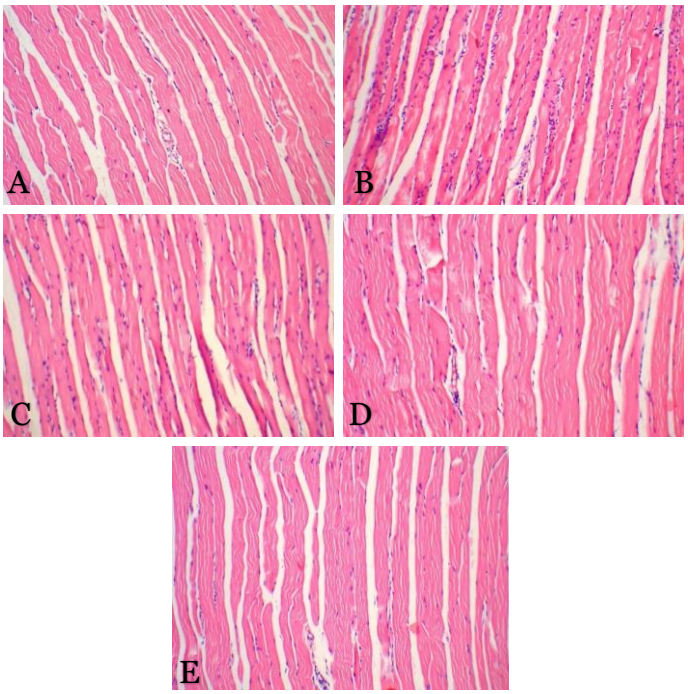

Figure 4. Inflammatory cells and sarcolemma disruption; (A) healthy control group (HG) shows intact sarcolemma and few inflammatory cells; (B) negative control group (NG) displays severe damage with disrupted sarcolemma and many inflammatory cells; (C) T1 group shows partial recovery with improved sarcolemma integrity and fewer inflammatory cells; (D) T2 group shows muscle improvement with fewer inflammatory cells; (E) T3 group shows the most recovery, with firm sarcolemma integrity and fewest inflammatory cells.

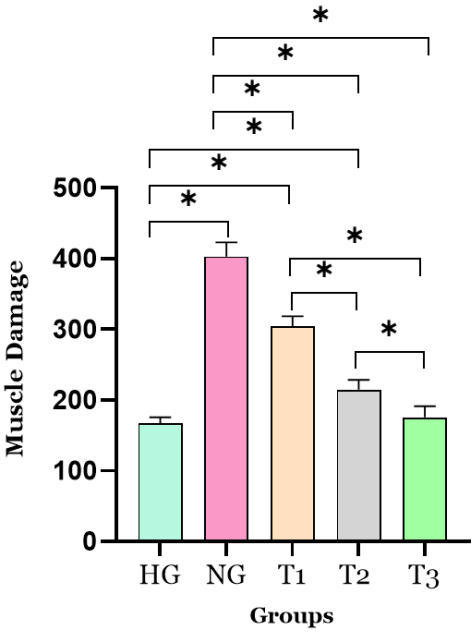

Figure 5. ANOVA test results of muscle damage among groups. Asterisk indicate significantly different results from the negative control group (NG). HG: positive control group; NG: negative control group; T1: receiving clove flower extract 24 hours before exercise; T2: receiving clove flower extract immediately after exercise; T3: receiving clove flower extract 24 hours after exercise.

Discussion

Eccentric exercise can cause muscle damage through oxidative stress and inflammation. The main pathway involved is the TLR4 pathway, which responds to physical stress and tissue damage [29]. The present study found that TLR4 has not decreased significantly. These results indicate that TLR4 is still active when NOX activity begins to decrease. TLR4 activation increases the expression of NOX, which produces ROS [30]. The accumulation of ROS not only damages proteins and cell membranes but also triggers the expression of pro-inflammatory cytokines such as IL-8 [31]. However, studies have shown that although clove flower extract is unable to significantly reduce TLR4 expression, its bioactive compounds, such as eugenol, still provide protective effects through other mechanisms[32–36]. Clove flower extract has been shown to reduce NOX activity, although TLR4 remains active [37]. These results indicate that the benefits of eugenol can only affect the level of NOX activity, but not the level of the TLR4 enzyme. This decrease is due to the direct effect of eugenol in cloves on the NOX enzyme, inhibiting excess ROS production [38]. With ROS level decreased, the level of oxidative damage to muscle tissue is also reduced, helping prevent further degradation of proteins and cellular structures [11].

In addition, clove flower extract can increase the activity of endogenous antioxidant enzymes, such as SOD [39]. SOD activation helps neutralize ROS that has already been formed, converting superoxide into less reactive forms such as hydrogen peroxide [40]. A study shows that eugenol in clove flower extract increases SOD gene expression, providing a protective effect against oxidative stress that occurs after eccentric exercise [41]. Eugenol in clove flower extract is known to interact with regulatory elements at the genetic level, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. Activation of Nrf2 by eugenol induces the expression of antioxidant genes, including genes encoding SOD [38]. This mechanism allows the body to increase endogenous antioxidant capacity to counteract ROS produced during normal metabolic processes or stress conditions such as eccentric exercise [42]. Eccentric exercise often causes ROS accumulation that can damage cell membranes and proteins [43]. Administration of clove flower extract helps increase SOD activity, thereby accelerating ROS detoxification and preventing further oxidative damage [43]. With increased SOD activity, the redox balance in cells can be maintained, which ultimately improves cellular function and accelerates the recovery of damaged tissues [44].

In terms of inflammation, eugenol is also able to reduce IL-8 levels, although it does not directly affect TLR4. This decrease in IL-8 is thought to be related to the reduction of ROS through NOX inhibition, which reduces the activation of downstream inflammatory pathways, such as the NF-κB pathway [45]. Eccentric exercise is known to increase IL-8 expression in skeletal muscle, which contributes to inflammation and muscle damage [46].However, eugenol counteracts this effect by inhibiting pro-inflammatory eicosanoid synthesis and reducing ROS levels, ultimately suppressing the activation of transcription factors such as NF-κB, which regulates *IL-8* gene expression. Thus, eugenol can reduce IL-8 levels, reducing inflammation and muscle damage induced by eccentric exercise. With reduced IL-8, the inflammatory process that usually worsens muscle damage can be minimized, accelerating tissue recovery.

Overall, although clove flower extract has not been able to reduce TLR4 expression, its effects on inhibiting NOX, increasing SOD, and decreasing IL-8 provide significant protection against muscle damage due to eccentric exercise. These mechanisms suggest that clove flower extract has the potential as a therapeutic agent in preventing or reducing the impact of physical stress on muscles.

Administration of clove flower extract maximally reduced NOX activity in skeletal muscle damage 24 hours after eccentric exercise. NOX activity will increase along with the increasing number of free radicals in the body that can arise due to excessive exercise. This repair effect of clove flower extract is demonstrated by a remarkable reduction in DNA damage levels and ROS mediators along with a significant increase in antioxidant enzyme activity and may be explained by the high content of anti-inflammatory and antioxidants [47].

Administration of clove flower extract 24 hours after eccentric exercise may be more effective than administration immediately after exercise and before exercise in reducing NOX, increasing SOD, and reducing IL8, this may occur because at this time, the inflammatory process and oxidative stress have reached their peak [48]. Intervention at this phase can help reduce ongoing inflammation and oxidative stress, accelerate the muscle recovery process, and prevent further tissue damage [49].

Data analysis revealed a decrease in NOX and IL-8 was observed, but not TLR4. This happens because TLR4 activation can trigger a wide range of signaling pathways, including activation of NF-kB and production of inflammatory cytokines [50]. However, although inflammation and NOX are reduced, this does not necessarily mean that TLR4 expression is directly reduced [51]. TLR4 may still be regulated at the transcriptional or protein processing level even after the effects of inflammation have disappeared [52]. This is because the body may maintain TLR4 expression as a "defense" mechanism against possible infection or further damage, or because other mechanisms have not been completely disrupted. While clove flower extract may be effective in reducing levels of oxidative stress and inflammation (including NOX and IL-8), it may not be strong enough or direct enough to reduce TLR4 expression within a relatively short time [53]. Although reducing ROS and inflammation plays a crucial role in muscle recovery, downregulating TLR4 may require a longer time or a stronger inhibitory mechanism. This is because TLR4 is affected by various signaling pathways other than those related to NOX and IL-8 [54]. In accordance with this study, TLR4 can be stimulated by damage-associated molecular patterns (DAMPs) caused by maximal eccentric exercise. The increase in TLR4 levels observed in the HG and NG shows a slight elevation. This result is in accordance with previous studies reported [50,55,56]. Other studies have shown that a significant increase in TLR4 occurs in severe muscle damage due to ischemia in skeletal muscle [57,58].

Administration of clove flower extract containing eugenol on skeletal muscle damage provides antioxidants that donate hydrogen radicals as chain-breaking antioxidants, known as primary antioxidants. These antioxidants will react with peroxide radicals which are then converted into more stable radicals or non-radicals, which are indicated by increasing SOD levels in blood serum [59]. A previous study reported that eugenol, an active compound found in cloves, can reduce levels of inflammatory mediators at the level of gene and protein expression and pro-inflammatory proteins such as cytokines, prostaglandin synthesis, neutrophil chemotaxis, and inhibit the cyclooxygenase (COX) [60].

Histopathological analysis showed varying numbers of inflammatory cells across each group (**Figure 4**). The preparation figure shows the negative control group has the most inflammatory

cells compared to the healthy control group. The T1, T2, and T3 groups showed a tendency to have fewer inflammatory cells than the negative control group, particularly in the perivascular region. In detail, the negative control group had the highest number of inflammatory cells, indicating that muscle damage was most severe in this group due to the lack of clove extract treatment and only receiving rest. In contrast, the healthy control group had the lowest number of inflammatory cells, suggesting optimal muscle condition. Among the treatment groups, the group that received clove extract treatment before eccentric exercise showed the highest number of inflammatory cells, compared to the groups that received clove extract immediately after exercise and 24 hours after eccentric exercise.

Clove flower extract inhibits antiproliferative biomarkers, with its activity depending on their concentration, thereby reducing levels of pro-inflammatory biomarkers [61]. Its application has been shown to reduce the number of inflammatory cells [62]. The mechanism of eugenol, as an anti-inflammatory, inhibits COX expression and reduces inflammatory mediator production, thereby reducing NOX activity as a ROS mediator. The decrease in NOX activity will inhibit the NF-kB factor, thereby reducing the pro-inflammatory cytokine IL-8 [63]. A decrease in ROS which is indicated by a decrease in NOX and an increase in SOD activity, as well as a decrease in the pro-inflammatory cytokine IL-8, contributed to the improvement in the structure of muscle cells that are damaged by excessive eccentric exercise. These results indicate that animals given clove flower extract reduced inflammation and improved sarcolemma integrity [64].

Limitations of this study include its focus solely on male rats, thus limiting the generalization of the findings to female rats. Additionally, the acute muscle damage conditions used in this study revealed that the receptor mechanism was not affected by clove extract, suggesting that future research is needed to investigate the effect of clove extract on chronic muscle damage. A single administration of clove extract may not be sufficient to achieve the optimal effect, and a longer treatment duration may be necessary to produce a better outcome. Since this study was conducted in rats, the findings may not be directly applicable to human physiology, and additional studies are necessary to determine the appropriate dosing for humans.

Conclusion

Clove flower extract, with eugenol as its major metabolite, exhibits strong antioxidant and anti-inflammatory properties. It significantly reduces NOX activity and IL-8 levels, while increasing SOD activity. However, it is unable to reduce TLR4 levels in muscle damage. These results indicate that clove flower extract is beneficial as an antioxidant and anti-inflammatory agent for skeletal muscle damage resulting from eccentric exercise. Further research is needed to evaluate its long-term effects, optimal dosing strategies, and potential applications in chronic muscle damage and human studies.

Ethics approval

The protocol of the present study was reviewed and approved by the Ethical Committee of Research, Universitas Respati Yogyakarta, Yogyakarta, Indonesia (Approval number: 0231.3/FIKES/PL/X/2023).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available as part of the study from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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