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Original Research Article

Efficacy of Black Shallot Extract in Analgesic and Antipyretic Activities in Experimental Mice

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ABSTRACT

Black shallot (*Allium ascalonicum*) is a variant of the common shallot, characterized by its distinctive dark purple-black skin and a milder, sweeter taste. Within the *Allium* genus, which includes garlic and onions renowned for their medicinal properties, back shallot has garnered attention for its potential health benefits. The current study specifically explores black shallot extract's analgesic and antipyretic effects. The analgesic potential of black shallot extract (EABS) was gauged using the hot plate test, Haffner tail clip test, writhing test induced by acetic acid, and formalin-induced pain test. The antipyretic effects of EABS were investigated using a yeast-induced fever model in mice. Concurrently, antipyretic effects were assessed through a yeast-induced fever model in mice. In pain models, EABS-treated mice demonstrated significantly prolonged reaction times ($P < 0.05$) compared to controls. All EABS doses exhibited maximum pain reduction effects (MPA) after 45 minutes ($P < 0.05$), indicating positive outcomes in the hot plate test. EABS efficiently alleviated pain, reaching peak efficacy within 10 minutes of the Haffner tail clip test ($P < 0.05$). Notably, EABS displayed superior pain reduction in both the acetic acid-induced writhing and formalin-induced pain tests. Furthermore, EABS consistently reduced rectal temperature ($P < 0.05$) throughout treatment, achieving a maximum antipyretic effect (63.24%) after 3 hours with a 200 mg/kg dose ($P < 0.05$). These findings provide robust evidence supporting the analgesic and antipyretic efficacy of EABS, underscoring its potential as a therapeutic option for pain and fever management.

***Keywords*:** Black shallot extract, Analgesic effect, Antipyretic effect, Experimental mice, Herbal remedy, *In vivo* study.

Introduction

Injuries and various illnesses commonly present with symptoms like pain and fever. Prescribed medications for pain management and fever reduction often include paracetamol, aspirin, ibuprofen, and others. Misuse of these drugs can result in adverse effects such as allergies, skin rashes, itching, and liver and kidney toxicity.[1] While selective cyclooxygenase-2 (COX-2) inhibitors address gastrointestinal side effects caused by NSAIDs, these drugs, pose a risk of toxicity to liver cells, kidney glomeruli, the brain's cortex, and cardiac muscles. Opioid analgesics such as morphine present a significant risk of addiction and various adverse effects, including respiratory depression, sedation, reduced gastrointestinal motility, and nausea.[2] The misuse of analgesic and antipyretic medications is currently playing an adverse role in the effective management of pain and fever. The lingering negative consequences of chronic pain further exacerbate, imposing a substantial economic and societal burden on both individuals and communities, adversely affecting overall quality of life and general work productivity.[3] Furthermore, presently available analgesic drugs demonstrate inefficacy in adequately alleviating pain, contributing only a 50% reduction in pain for approximately 30% of patients in certain cases.[4]

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Given the limitations and other associated issues of analgesic and antipyretic drugs, the exploration of novel therapeutic agents for pain and fever control becomes imperative. Natural alternatives emerge as a pivotal option in this context, as approximately 25% of all synthetic drugs have direct or indirect origins from medicinal plants. The desirable characteristics for the development of new medications, such as lower side effects, widespread distribution, and adherence to traditional herbal usage, render natural sources an appealing avenue for drug discovery.[1]

Allium ascalonicum L., commonly referred to as shallot, is a plant with a widespread distribution, though its predominant utilization is observed in tropical regions. Emitting a subtle aroma, this plant belongs to the Amaryllidaceae family. Characterized by short, diminutive, cylindrical, and hollow leaves, it is often colloquially known as green onions or shallots.[5] The primary physiological actions of *A. ascalonicum* are posited to be mediated through redox-dependent mechanisms, manifesting a spectrum of pharmacological attributes encompassing antioxidant, anti-inflammatory, antibacterial, anticancer, antihyperglycemic, and organ-protective effects.[6] Additionally, it finds application as a culinary seasoning and serves as a natural source of Vitamin C.[5] Shallot's plant constituents comprise diverse phytochemicals and plant nutrients, including flavonoids such as quercetin and allicin.[7] Scientific reports substantiate the analgesic and anti-inflammatory properties attributed to the biological characteristics of *A. ascalonicum* (shallot).[5] Black shallot is the result of the fermentation process from fresh bulbs of *A. ascalonicum*, carried out over an appropriate duration and under optimal temperature and humidity conditions. Following fermentation, black shallot presents itself with a dark brown hue, a sweet flavor, and a delicate aroma reminiscent of ripe fruit.[8] The bioactive compounds identified in extracts of black shallot encompass quercetin 3,4′-diglucoside, isorhamnetin 3,4′-diglucoside, quercetin 3′-glucoside, quercetin 4′-glucoside, isorhamnetin 4′-glucoside, quercetin aglycone, and

isorhamnetin. Additionally, extracts of black shallot demonstrate notable antioxidant, anticancer, and anti-inflammatory activities.[9] *Allium ascalonicum*, commonly referred to as shallot, has been documented for its analgesic and anti-inflammatory attributes.[5] Nevertheless, there is a lack of literature regarding the analgesic and antipyretic effects of black shallot, a recently developed derivative obtained from the aging process of fresh shallot bulbs subjected to elevated temperatures and humidity. Consequently, this research endeavors to examine the analgesic and antipyretic properties of black shallot extracts in murine subjects.

Materials and Methods

Collection of plant material

On May 15, 2023, shallots (*Allium ascalonicum* L.) were collected in Vĩnh Châu town, Sóc Trăng province, Vietnam. The botanical specimen with the code AS150523VST has undergone taxonomic classification by a botanical taxonomist, and it is currently archived at the Plant Biotechnology laboratory of the Institute of Biotechnology and Food Technology, Ho Chi Minh City University of Industry.

Preparation of the extract

Shallots were carefully chosen based on specific criteria, including a vibrant and consistent purple color, absence of rot or pests, and firm and uniformly rounded bulbs. After this selection, the outer husk layer was meticulously peeled off to eliminate impurities and damaged sections. Subsequently, a thorough washing process was carried out, and shallots were left to drain excess water. The entire shallot bulbs were then subjected to the fermentation process within an aging chamber (Shellab, USA) using the method elucidated by Tran and Ngo.[10] In particular, the shallots were fermented at 80°C with a relative humidity of 70% for 21 days, resulting in the production of distinctive dark brown or black-colored products. Following the creation of black shallots, they were sliced and submerged in a 98% ethanol solution with a ratio of 1:5 (shallot volume/ethanol volume) for 7 days. This procedure was reiterated twice to enhance the extraction efficiency. The resultant ethanol extracts were blended and filtered through the Whatman No. 4 filter paper. The ultimate filtrate, post-filtration, underwent further concentration using a rotary evaporator R-II apparatus (BUCHI, Switzerland), operating at a speed of 40 revolutions per minute and a temperature of 65°C, yielding the concentrated extract (referred to as EABS). The extract was securely stored in a moisture-sealed container at 4°C, shielded from light, until required for subsequent experimental protocols.

Phytochemical screening

Chemical screening of the black shallot extract aimed to identify the presence of both primary and secondary metabolites. The screening methodology adhered to the procedure outlined by Reveny *et al*.,[11] wherein the presence or absence of secondary metabolites in the extract was denoted by either a positive (+) or negative (-) sign.

Quantitative phytochemical analysis

The quantification of flavonoids, phenolics, alkaloids, and saponins in the black shallot extract was conducted utilizing methodologies established by Ilukho *et al*.[12] The experimental procedures were meticulously followed, ensuring precision and reliability in the assessment of these pivotal secondary metabolites. The adherence to these rigorous experimental processes contributes to the accuracy and credibility of the evaluation of these crucial secondary metabolites.

Experimental animals

Swiss albino mice weighing between 30-32 g and aged 7-8 weeks were sourced from the Pasteur Institute in Ho Chi Minh City, Vietnam, for the research investigation. These mice were housed in the experimental animal facility of the East Agriculture and Food Company, Ho Chi Minh City, maintaining standard environmental conditions with a temperature of 24 ± 2°C, humidity levels at 55 ± 5%, and a 12-hour light-dark cycle within glass cages. These cages, kept in a hygienic state, were equipped with sterile wood shavings as bedding material. The mice were granted ad libitum access to a standard pellet diet and filtered water. A period of 7 days was allotted for the animals to acclimate to the laboratory environment before the initiation of the study. The mice were then randomly assigned to experimental and control treatments, with unrestricted access to water but with food withdrawn 12 hours before and during the experimental sessions. Throughout our animal experiments, we meticulously adhered to the ethical principles that oversee the well-being of animals, as outlined in the Basel Declaration on Animal Research.[13] Additionally, we observed the regulations stipulated in the Livestock Law in 2018 (Number 32/2018/QH14) in Vietnam.[14] Our experimental procedures conformed to the guidelines for clinical and preclinical trials in traditional medicine and traditional medicine (Decision 141/QD-K2DT), which were promulgated in Vietnam in 2015.[15] The utilization of experimental animals followed the national ethical principles established by the Ministry of Health of Vietnam for medical research.[16] The treatment protocols for the animals were under the guidelines provided by the World Health Organization in 2000.[17] All treatment interventions and safety protocols were executed following the experimental procedures established by the Department of Biotechnology at Ho Chi Minh City University of Industry. Competent personnel were trained and held responsible for the welfare and treatment of experimental animals, strictly observing ethical principles associated with animal research. Additionally, they abided by the directives provided by the Ethics Committee for Animal Research at Ho Chi Minh City University of Industry, Vietnam

Experimental design

Assessment of analgesic activity: Mice were randomly assigned into five treatments for each pain model, with each treatment consisting of five individuals. The initial treatment served as the negative control, receiving normal physiological saline solution (NSS, 10 mL/kg). The second treatment acted as the positive control and was administered standard drugs: aspirin (ASA, 10 mg/kg) via oral administration for visceral pain models (acetic acid writhing test, formalin pain test) or tramadol (TRAMA, 10 mg/kg) via oral administration for visceral pain models (formalin pain test), thermal pain models (hot plate test), and mechanical pain models (Haffner tail clip test). The experimental treatments were treated with various doses of black shallot extract ($EABS_{100}$, $EABS_{150}$, and $EABS_{200}$), receiving black shallot extract at doses of 100 mg/kg, 150 mg/kg, and 200 mg/kg, respectively.

The thermal pain model (Hot plate test): In the thermal pain model (hot plate test), we evaluated the centrally mediated analgesic efficacy of EABS in Swiss albino mice, employing the methodology outlined by Hijazi *et al.*[18] The experimental subjects were positioned on a hot plate apparatus (IITC Inc. Model 39) maintained at 55 ± 0.5°C. Latency, denoting the reaction time in seconds, was gauged as the interval within which a mouse responded to thermal pain by either paw licking or jumping behavior, indicative of an effort to alleviate the discomfort. Preceding the experimental procedure, subjects in the treatment cohorts were administered EABS extracts orally at dosages of 100, 150, and 200 mg/kg. The positive control cohort received tramadol (10 mg/kg), while the negative control cohort was given a physiological saline solution (10 mg/kg). Reaction times (licking or jumping latency) were meticulously recorded at intervals of 0, 15, 30, 45, and 60 minutes. A maximum reaction time of 45 seconds was predetermined to avert potential paw tissue damage. Instances, where the recorded time surpassed 45 seconds, were considered indicative of the maximal achievable analgesic effect. The maximum possible analgesia (MPA) was computed utilizing the ensuing formula:

$$\text{MPA}\ (\%) = \frac{(\text{Reaction time post-treatment}) - (\text{Reaction time pre-treatment})}{45\ \text{seconds} - (\text{Reaction time pre-treatment})} \times 100$$

Visceral pain model (formalin test and acetic acid-induced writhing test)

Acetic acid induced writhing test: The visceral pain model of EABS was investigated in the writhing test induced by acetic acid, as outlined by Nhung and Quoc.[19] The $EABS_{100}$, $EABS_{150}$, and $EABS_{200}$ treatments were administered with EABS extract at doses of 100, 150, and 200

mg/kg, respectively, through oral administration. The positive control treatment was given standard aspirin (10 mg/kg), while the negative control treatment received a physiological saline solution (10 mg/kg). The impacts of both EABS and aspirin on acetic acid-induced writhing were scrutinized and compared against the control treatment. To elicit the writhing response in the animals, an intraperitoneal injection of 0.2 mL (0.2% acetic acid v/v in physiological saline) was administered. This induced a pain sensation, manifested by arching of the back, stretching of the hind limbs, and contractions of the abdominal muscles. Subsequently, each mouse was housed in individual glass cages and subjected to monitoring. The tally of writhing episodes commenced 5 minutes after the administration of acetic acid, and counts were conducted thrice (with each count separated by 15 minutes). The percentage inhibition of writhing (PIW) for each treatment was determined using the following formula:

$$\text{PIW (\%)} = \frac{\text{Mean number of writhes (control} - \text{test)}}{\text{Mean number of times of writhing in the control}} \times 100$$

Formalin-induced pain test: The formalin experiment was carried out by the methodology detailed by Nhung and Quoc.[20] Twenty-five mice were subjected to an overnight fasting period and were then randomly allocated into distinct treatments. These treatments included the negative control treatment, receiving treatment with physiological saline solution (10 mL/kg); the positive control treatment, treated with ASA (10 mg/kg) and tramadol (10 mL/kg); and experimental treatments treated with varying doses of EABS (100, 150, and 200 mg/kg). The pain was induced by the intraplantar injection of 20 µL of 1% formalin in physiological saline into the right hind paw of the mice, administered 30 minutes after the administration of ASA, TRAM, and EABS. The duration of time (measured in seconds) spent licking and biting the injected paw, indicative of a pain response, was recorded for each mouse. Mouse behaviors were assessed during both the early phase (0-5 minutes) and the late phase (15-30 minutes) following formalin injection. The reaction times of the animals were then compared to those of the control treatment and expressed as a percentage of pain control (PPC):

$$\text{PPC (\%)} = \frac{\text{Latency (test} - \text{control)}}{\text{Latency test}} \times 100$$

The levels of cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) in the formalin test: During the formalin experiment, blood samples were obtained from mice at 0 and 30 minutes using the eye bleeding method. The serum was isolated by centrifugation at approximately 12,000 revolutions per minute for 5 minutes. COX-2 and PGE2 ELISA kits from Absolute Biotech Co., Ltd., were utilized for individual assessments. Samples and standards were introduced into ELISA wells pre-coated with COX-2 and PGE2 antibodies, followed by the addition of an HRP conjugate at 37°C. After a 1-hour incubation period, each well underwent three washes with a washing solution. The experiments were independently conducted three times. Test substances A and B (crystalline standard substances of COX-2 or PGE2) were introduced into the solution and incubated at 37°C for 15-30 minutes. Finally, the stop solution was added to halt the reaction. The absorbance of the resulting color was measured at a wavelength of 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader. COX-2 and PGE2 concentrations were determined by comparing the optical density values with those of the standard concentration curve.[20]

Mechanical pain model (Haffner tail clip test)
The Haffner tail clamp trial adhered to the experimental protocol devised by Nhung and Quoc.[19] Preceding the experiment, a sensitivity assessment was conducted to select suitable animals, with mice failing to attempt to escape from the tail clamp within a 10-second timeframe being excluded due to inadequate sensitivity as per the test standards. A total of twenty-five mice were randomly selected and assigned to different treatments. The tail clamp was administered 30 minutes after the oral administration of EABS (100, 150, and 200 mg/kg), tramadol (10 mL/kg), and physiological saline solution (10 mL/kg). The response time was meticulously observed and documented at 5, 10, and 15 minutes following the application of the tail clamp. The presence of an analgesic effect was ascertained if there was no attempt to escape the clamp within 10 seconds. The pain inhibition ratio (PIR) was computed utilizing the subsequent formula:

$$\text{PIR (\%)} = \frac{\text{Latency (test)} - \text{Latency (control)}}{\text{Latency (test)}} \times 100$$

Antipyretic activity
The antipyretic activity was evaluated utilizing a yeast-induced fever model in Swiss albino mice, following the procedures outlined by Nhung and Quoc.[20] A total of thirty mice were divided into six treatments (n = 5). The normal treatment (Saline treatment) received oral administration of physiological saline solution (10 mL/kg) without inducing fever. The negative control treatment (Yeast treatment) involved inducing fever in mice by injecting 20% yeast (10 mL/kg) without subsequent treatment. The positive control treatment (Yeast + Paracetamol treatment, positive control) included mice induced with fever by injecting 20% yeast (10 mL/kg) and administered paracetamol (PCM, 150 mg/kg). The experimental treatments with EABS (Yeast + $EABS_{100\ -\ 200}$ treatments) comprised mice induced with fever by injecting 20% yeast (10 mL/kg) and orally receiving EABS at doses of 100, 150, and 200 mg/kg, respectively. Before the experiment, the animals underwent an overnight fast with unrestricted access to water. Rectal temperature measurements of the experimental animals were taken using a TESTO 106 thermometer (Germany). Eighteen hours after subcutaneous yeast injection, animals with an increase in rectal temperature of approximately 0.3 - 0.5°C were selected for antipyretic activity assessment. The rectal temperature of the experimental animals was monitored at 1, 2, and 3 hours after yeast injection, and the percentage fever reduction (PFR) was calculated using the formula:

$$\text{PFR (\%)} = \frac{\text{E} - \text{F}_n}{\text{E} - \text{D}} \times 100$$

with E being the post-fever temperature; F_n being the temperature after 1, 2, and 3 hours and D being the normal body temperature.

Data analysis
The data were analyzed using Statgraphics Centurion version XIX, and the results were presented as mean ± SD. Subsequently, Dunnett's post hoc test following ANOVA was employed to compare the outcomes between the treatments and the control treatment. Results were considered statistically significant when $P < 0.05$.

Results and Discussion

Phytochemical screening and quantitative analysis of plant constituents in the extract
In Table 1, the initial analysis outcomes depict the botanical chemical composition of EABS, revealing the existence of carbohydrates, alkaloids, flavonoids, tannins, phenolic compounds, terpenoids, saponins, and steroids in the extract, with the absence of proteins and cardiac glycosides. The flavonoid content in EABS measured 19.41 ± 1.28 mg/g, the alkaloid content was 20.47 ± 1.16 mg/g, and the saponin content was 39.25 ± 2.05 mg/g (refer to Table 2).
Biologically active constituents present in natural products have the potential to be developed into new pharmaceuticals. The initial screening of plant chemicals plays a crucial role in the identification of bioactive compounds within plants, paving the way for the creation of innovative medications.[3]

Table 1: The chemical composition of black shallot extract

Phytochemicals	EABS	Phytochemicals	EABS
Tannins	+	Alkaloids	+
Flavonoids	+	Saponins	+
Steroids	+	Phenolics	+
Terpenoids	+	Cardiac glycosides	-
Proteins	-	Carbohydates	+

(+): presence, (−): absence of tested phytochemicals.
Analyses of extracts from black shallot have revealed the presence of terpenoids, steroids, flavonoids, tannins, saponins, alkaloids, steroids,

and carbohydrates. These compounds are predominantly accountable for the antioxidant and anti-inflammatory characteristics of the plant extract. Terpenoids and steroids can impede the oxidation process of molecules, thereby reducing free radicals. Flavonoids can hinder pro-inflammatory enzymes and mitigate cell damage. Tannins can create complexes with metals, impede the oxidation process, stabilize cell membranes, and diminish hormone secretion. Saponins can mitigate oxidative stress by curbing the production of reactive oxygen species. Carbohydrates can partake in cell signaling pathways and impact inflammatory responses.[21] The alteration and influence on pain perception also are intricately linked to the functionality of plant compounds present in the extract. Terpenoids and steroids exert an influence on pain perception by interacting with opioid receptors or modulating biochemical pathways associated with pain perception. Flavonoids can alleviate pain by inhibiting pro-inflammatory enzymes, interacting with vanilloid receptors, and modulating pain pathways. Saponins, through their anti-inflammatory effects, inhibit inflammatory mediators and interact with the opioid system to alleviate pain. Alkaloids can influence the nervous system by interacting with opioid receptors or inhibiting pain signaling to diminish pain perception.[22]

Assessment of analgesic activity of extracting black shallot
The thermal pain model (Hot plate test)
Table 3 outlines the results of the EABS analgesic activity assessed through the hot plate method. No notable disparity in thermal stimulation was observed in mice treated with a physiological saline solution (normal control) throughout the experiment. Administration of tramadol (TRAMA) significantly prolonged the animals' response time, peaking at 42.81 ± 0.19 seconds (after 45 minutes). The analgesic efficacy of TRAMA gradually waned over time, reaching 39.48 ± 0.15 seconds (after 60 minutes). All EABS doses exhibited a substantial elevation in mouse response time compared to the control treatment ($P < 0.05$), with the $EABS_{200}$ treatment demonstrating the highest response at 42.35 ± 0.13 seconds ($P < 0.05$). As depicted in Table 3, the peak activity recorded after 45 minutes was 39.24, 40.81, and 42.35 seconds for EABS 100, 150, and 200 mg/kg, respectively.
Figure 1 depicts the maximum possible analgesia (MPA) of EABS in contrast to the negative control treatment (Yeast treatment) during the hot plate experiment. All EABS doses exhibited a notable analgesic effect after 60 minutes. The peak activity of EABS after 45 minutes approximated the efficacy of TRAMA ($P < 0.05$). The peak latency response for TRAMA (after 45 minutes) was 25.18 ± 0.09 seconds, while EABS recorded 24.57 ± 0.07 seconds (200 mg/kg).
The hot plate method is a commonly employed technique for assessing the central analgesic effects of pharmaceuticals. In the hot plate experiment, EABS demonstrated significant analgesic activity in a dose-dependent manner, leading to an increase in the reaction time in mice across all tested concentrations. In comparison to the negative control (Saline treatment), TRAMA, a standard drug, displayed the highest analgesic activity among all tested samples (Figure 1) after 60 minutes of the experiment. TRAMA is acknowledged as a potent analgesic acting through the activation of opioid receptors (μ, δ, and κ). The activation of these receptors is associated with pain reduction in the spinal cord, supraspinal regions, and periphery.[18] The delayed onset and prolonged duration of the extract's analgesic effect suggest the presence of a metabolically active compound, potentially more effective in the extract or bound to serum proteins. The overall analgesic response, primarily mediated by opioid receptors, indicates that, when combined and considering the integrated response on the hot plate, EABS may demonstrate substantial and promising analgesic activity by activating opioid receptors in the central nervous system.

Mechanical pain model (Haffner tail clip test)
Following the application of the Haffner tail clip test, response times were evaluated for all mice at various time intervals. In the control experiment, at 5, 10, and 15 minutes post-tail clip, the average response times were 3.05 ± 0.04, 3.08 ± 0.05, and 2.88 ± 0.05 seconds, respectively. The observed response times indicated an increasing pain tolerance trend in the EABS-treated individuals. At the 10-minute mark, the highest response times were 4.94 ± 0.04, 5.22 ± 0.05, and 7.15 ± 0.04 seconds, recorded at the respective doses of 100, 150, and 200 mg/kg. These values were significantly higher than the response time to saline treatment, which was 3.08 ± 0.05 seconds ($P < 0.05$) (Table 4).

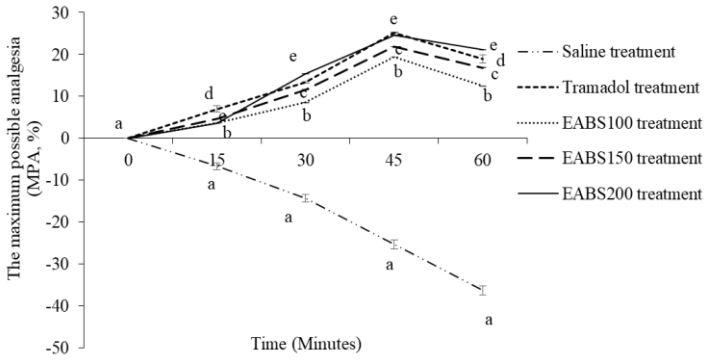

Figure 1: The maximum possible analgesia (MPA) of black shallot extract in mice evaluated through the hot plate test method. The results are presented as Mean ± SD, with letters (a, b, c, d, and e) denoting significant differences between treatments ($P < 0.05$).

Table 2: Quantification of flavonoid, alkaloid, and saponin contents in black shallot extract

Sample	Total saponin content (mg/100 g)	Total alkaloid content (mg/100 g)	Total flavonoid content (mg/100 g)
EABS	19.41 ± 1.28	20.47 ± 1.16	39.25 ± 2.05

Concentration amount (mean ± SD) in mg per 100 g of the extract.

Table 3: The analgesic effect of black shallot extract in mice using the hot plate test method

Treatments	Reaction time (seconds)				
	0 minutes	15 minutes	30 minutes	45 minutes	60 minutes
Saline treatment	31.48 ± 0.19^{a}	29.51 ± 0.16^{a}	27.54 ± 0.14^{a}	25.12 ± 0.15^{a}	23.09 ± 0.12^{a}
Tramadol treatment	32.03 ± 0.18^{a}	34.45 ± 0.19^{e}	36.97 ± 0.17^{d}	42.81 ± 0.19^{e}	39.48 ± 0.15^{d}
$EABS_{100}$ treatment	31.62 ± 0.15^{b}	32.81 ± 0.16^{b}	34.57 ± 4.23^{b}	39.24 ± 0.17^{b}	36.13 ± 0.13^{b}
$EABS_{150}$ treatment	31.87 ± 0.14^{b}	33.42 ± 0.16^{d}	36.05 ± 0.18^{c}	40.81 ± 0.17^{c}	38.31 ± 0.13^{c}
$EABS_{200}$ treatment	31.95 ± 0.13^{b}	33.13 ± 0.15^{c}	37.77 ± 0.12^{e}	42.35 ± 0.13^{d}	40.51 ± 0.16^{d}

The values are expressed as Mean ± SD, where the letters (a, b, c, d, and e) indicate differences between treatments ($P < 0.05$).

Table 4: The analgesic effect of black shallot extract in mice using the Haffner tail clip test method

Reaction time (seconds)	Saline treatment	Aspirin treatment	$EABS_{100}$ treatment	$EABS_{150}$ treatment	$EABS_{200}$ treatment
5 minutes	3.05 ± 0.04^{a}	7.59 ± 0.05^{e}	4.79 ± 0.07^{b}	4.96 ± 0.05^{c}	7.01 ± 0.06^{d}
10 minutes	3.08 ± 0.05^{a}	7.67 ± 0.06^{e}	4.94 ± 0.04^{b}	5.22 ± 0.05^{c}	7.15 ± 0.04^{d}
15 minutes	2.88 ± 0.05^{a}	7.59 ± 0.05^{e}	4.52 ± 0.06^{b}	4.68 ± 0.05^{c}	5.01 ± 0.05^{d}

The values are expressed as Mean ± SD, where the letters (a, b, c, d, and e) indicate differences between treatments ($P < 0.05$).

Table 5: Analgesic effect of black shallot extract on acetic acid-induced writhing pain

Parameters	Saline treatment	Aspirin treatment	$EABS_{100}$ treatment	$EABS_{150}$ treatment	$EABS_{200}$ treatment
Number of times writhing (times)	44.31 ± 0.22^{e}	10.12 ± 0.12^{a}	23.18 ± 0.18^{d}	17.09 ± 0.19^{c}	12.29 ± 0.15^{b}
PIW (%)	0.00 ± 0.00^{a}	77.15 ± 0.31^{e}	47.68 ± 0.48^{b}	61.43 ± 0.47^{c}	72.06 ± 0.35^{d}

Table 6: Analgesic effects of black shallot extract on formalin-induced pain

Experiment phase	Paw licking time (seconds)					
	Saline treatment	Aspirin treatment	Tramadol treatment	$EABS_{100}$ treatment	$EABS_{150}$ treatment	$EABS_{200}$ treatment
Early phase	19.54 ± 0.29^{a}	35.66 ± 0.15^{e}	31.78 ± 0.11^{f}	26.54 ± 0.18^{b}	28.36 ± 0.12^{c}	30.44 ± 0.13^{d}
Later phase	17.43 ± 0.13^{a}	41.07 ± 0.15^{c}	47.87 ± 0.12^{f}	38.45 ± 0.14^{b}	43.75 ± 0.16^{d}	46.59 ± 0.18^{e}

The values are expressed as Mean ± SD, where the letters (a, b, c, d, e, and f) indicate differences between treatments ($P < 0.05$).

The analgesic efficacy of EABS peaked at 10 minutes post-test initiation, with the highest percentage observed in the 200 mg/kg extract treatment (56.94%), significantly differing from the saline treatment (0%) ($P < 0.05$) (Figure 2). However, at 15 minutes post-tail clip, equivalent to 45 minutes after EABS administration, the delayed response times decreased in both the experimental and standard treatment groups ($P < 0.05$) (Table 4).

The central nervous system, comprising the brain and spinal cord, plays a pivotal role in central pain mechanisms. The posterior region of the spinal cord is rich in neurotransmitters, including substance P, endogenous opioids, somatostatin, and other inhibitory hormones, acting as targets for pain modulation and inflammation. The Haffner tail clip test model serves as a validated approach to evaluate the central analgesic properties of drugs, particularly through their interactions with opioid receptors.[19] The Haffner tail clip test unveils central anti-nociceptive mechanisms, with a primary emphasis on alterations in spinal cord activity. The substantial increase in pain threshold observed in response to EABS during the Haffner tail clip test suggests its involvement in central pain pathways. The analgesic effect is attributed to central mechanisms linked to receptor systems or peripheral mechanisms that involve the inhibition of prostaglandins and other endogenous substances crucial to the pain process.[19] Our ongoing study provides empirical evidence supporting the pain-reducing efficacy of black shallot extract at doses of 100, 150, and 200 mg/kg. This observation achieves statistical significance ($P < 0.05$) within the EABS-treated compared to the saline treatment, confirming the analgesic potential of EABS.

Visceral pain model (formalin test and acetic acid-induced writhing test)

Acetic acid-induced writhing test: Concerning the results from the acetic acid-induced writhing test, a significant reduction in the number of writhes ($P < 0.05$) was observed in the treatments compared to the control treatment (Table 5). Aspirin (ASA) demonstrated the highest protective efficacy against acetic acid-induced writhing (77.15%). Administered 30 minutes before acetic acid injection, EABS exhibited noteworthy analgesic properties, achieving peak effectiveness at the EABS dose of 200 mg/kg (72.06%) ($P < 0.05$).

The writhing response induced by acetic acid in experimental settings is a widely adopted model for investigating peripheral analgesic agents. The expression of abdominal writhing resulting from intraperitoneal acetic acid injection is characterized by contractions of abdominal muscles, subsequent extension of hind limbs, and elongation of the body. This constriction is believed to be mediated through local nociceptive reflex pathways. The administration of acetic acid is employed to release endogenous substances that activate pain-sensitive nerve endings, and the exacerbation of pain by acetic acid is attributed to increased capillary permeability.[1] Pain sensitivity arises from the release of endogenous substances and specific analgesic intermediates, such as the arachidonic acid metabolism through cyclooxygenase, exemplified by prostaglandins.[18] The outcomes presented in Table 4 demonstrate a noteworthy reduction in acetic acid-induced abdominal writhing following 30 minutes of EABS administration. This implies that EABS harbors pharmacologically active components associated with peripheral analgesia, which may hinder the release or action of endogenous substances responsible for nerve-ending stimulation. The peripheral analgesic effectiveness of EABS (200 mg/kg) is on par with that of ASA (10 mg/kg), showing no significant difference in protecting against acetic acid-induced writhing. Certain components within EABS exert their actions through the activation of opioid receptors post-metabolism, while others act peripherally by inhibiting endogenous pain-inducing substances without any noticeable onset delay.[18]

Formalin-induced test: Table 6 presents a chronological overview of pain perception responses induced by formalin and the black shallot extract in the formalin test. The subcutaneous injection of formalin into the mouse paw elicits distinct pain perception behaviors in both the early and late phases.

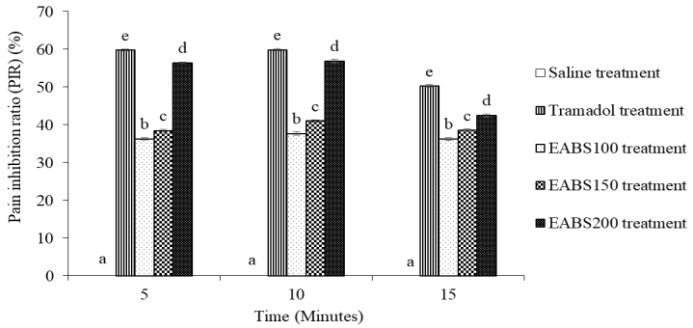

Figure 2: The pain inhibition ratio (PIR) of black shallot extract in mice evaluated through the Haffner tail clip test method. The results are presented as Mean ± SD, with letters (a, b, c, d, and e) denoting significant differences between treatments ($P < 0.05$).

In the negative control treatment, administering an equivalent volume of physiological saline (10 mg/kg) does not result in any observable effects. In contrast, during both the early and late phases of the experiment, volumes equivalent to ASA (10 mg/kg), TRAMA (10 mg/kg), and EABS (100, 150, and 200 mg/kg) demonstrate a noteworthy reduction ($P < 0.05$) in pain-related behaviors and significantly contribute ($P < 0.05$) to the anti-inflammatory effects of the black shallot extract (Figure 3).

The formalin test, implemented in mice, functions as a reliable and validated model for assessing the absorption capacity and responsiveness to various analgesic drugs. The experimental protocol entails the subcutaneous administration of formalin (1% in physiological saline) into the dorsal surface of the right hind paw, and the resultant reaction is quantified by measuring the duration of paw licking by the experimental subjects. Two distinguishable phases in paw-licking behavior emerge, encompassing the initial phase persisting for the initial 5 minutes and the subsequent phase extending from 15 to 30 minutes post-formalin injection. These phases encompass distinct pain perception mechanisms, with the initial phase directly influencing peripheral pain receptors and the subsequent phase involving an inflammatory response associated with centrally induced pain sensation due to inflammation.[23] Agents with central actions (TRAMA) can impede both phases, whereas agents with peripheral actions (ASA) selectively impede the initial phase.[24] Administration of EABS (100, 150, and 200 mg/kg) and ASA (10 mg/kg) has been substantiated to mitigate pain perception responses during the early phase induced by formalin injection. Conversely, treatment with EABS (100, 150, and 200 mg/kg) and TRAMA (10 mg/kg) diminishes pain perception responses during the late phase prompted by formalin injection. The findings suggest that the analgesic efficacy of EABS may be attributed to its anti-inflammatory properties.

The plasma levels of COX-2 and PGE2 in formalin test: The findings in Table 7 reveal a statistically significant reduction ($P < 0.05$) in the expression levels of COX-2 and PGE2 in the serum during both the early and late phases of the formalin test in the EABS treatments (100, 150, and 200 mg/kg), ASA treatment (10 mg/kg), and TRAMA treatment (10 mg/kg) as compared to the physiological saline treatment (10 mg/kg). The experimental results validate that the treatment modalities of EABS, ASA, and TRAMA effectively suppressed COX-2 concentration ($P < 0.05$) and attenuated PGE2 production ($P < 0.05$) to achieve analgesic effects.

Preclinical investigations point to neuroinflammation, especially in the spinal cord, characterized by heightened levels of inflammatory factors like prostaglandin E2 (PGE2) and cyclooxygenase-2 (COX-2), contributing to the progression of diseases. COX-2, one of the two COX isoforms, is specifically involved in the generation of inflammation-induced PGE2. PGE2 serves as the primary mediator of heightened pain sensation in both acute and chronic pain conditions by activating four subtypes of PGE2 receptors (EP1–4). Notably, the EP1 receptor (EP-1R) plays a critical role in facilitating pain perception associated with injury and inflammation, significantly contributing to heightened pain sensitivity related to PGE2.[25] PGE2's predominant influence on pain response is attributed to its interaction with endogenous mediators such as histamine, serotonin, bradykinin, and substance P, thereby increasing the sensitivity of pain receptors to these mediators.[1] In our study, we demonstrated the effectiveness of mitigating pain behaviors in the formalin-induced pain model in mice treated with black shallot extract. Experimental outcomes indicated that EABS assumed a defensive role in reducing pain perception behaviors triggered by formalin. Consistently, the efficacy of EABS alleviated both the acute and tonic phases of the formalin test. In EABS-treated animals, there was a significant decrease in serum COX-2 concentration, leading to a subsequent reduction in PGE2 levels. This observation underscores the potential of EABS to alleviate pain by reducing inflammation. These findings imply that EABS treatment could play a pivotal role in pain regulation both centrally and peripherally.

Antipyretic activity

Table 8 outlines the antipyretic effectiveness of the black shallot extract. Following the administration of the solution, all subjects displayed fever, with rectal temperatures ranging from 38.26 ± 0.16 to 39.45 ± 0.12 °C. EABS, administered at doses of 100, 150, and 200 mg/kg, significantly mitigated yeast-induced fever ($P < 0.05$) when compared to the rectal temperature observed in the saline treatment ($P < 0.05$). Across all EABS doses, there was a notable reduction in rectal temperature relative to the negative control treatment ($P < 0.05$), with the 200 mg/kg extract exhibiting the most prominent fever reduction ($P < 0.05$ at 1, 2, and 3 hours). The percentage decline in rectal temperature gradually increased throughout EABS and standard drug (paracetamol, PCM) administration. The antipyretic effects of the standard drug (PCM) and EABS doses (100, 150, and 200 mg/kg) became evident as early as 1-hour post-administration, reaching their peak in the 3rd hour following treatment. The maximum antipyretic effect (63.24%) was observed after 3 hours of EABS treatment at a dose of 200 mg/kg, equivalent to 66.9% of the standard drug PCM (Figure 4).

Table 7: Effect of black shallot extract on the plasma concentrations of COX-2 and PGE2 in the formalin test

Parameters	Time	Saline treatment	Aspirin treatment	Tramadol treatment	$EABS_{100}$ treatment	$EABS_{150}$ treatment	$EABS_{200}$ treatment
COX-2 levels (ng/mL)	5 min	6.55 ± 0.06^{f}	4.32 ± 0.06^{a}	4.69 ± 0.09^{b}	5.31 ± 0.07^{e}	5.16 ± 0.04^{d}	4.84 ± 0.06^{c}
	30 min	7.56 ± 0.06^{f}	4.01 ± 0.04^{b}	3.74 ± 0.05^{a}	4.77 ± 0.04^{e}	4.43 ± 0.06^{d}	4.19 ± 0.06^{c}
PGE2 levels (ng/mL)	5 min	0.84 ± 0.04^{d}	0.55 ± 0.04^{a}	0.61 ± 0.06^{b}	0.68 ± 0.05^{c}	0.66 ± 0.04^{bc}	0.62 ± 0.05^{bc}
	30 min	0.97 ± 0.04^{d}	0.51 ± 0.07^{ab}	0.48 ± 0.05^{a}	0.61 ± 0.07^{c}	0.57 ± 0.05^{bc}	0.54 ± 0.05^{abc}

The values are expressed as Mean ± SD, where the letters (a, b, c, e, and f) indicate differences between treatments ($P < 0.05$).

Table 8: Effect of black shallot extract at therapeutic doses of 100, 150 and 200 mg/kg on yeast-induced fever

Treatments	Initial (ºC)	Fever (ºC)	1 hour (ºC)	2 hours (ºC)	3 hours (ºC)
Saline treatment	36.51 ± 0.19^{a}	36.64 ± 0.12^{a}	36.78 ± 0.17^{a}	36.75 ± 0.13^{a}	36.77 ± 0.12^{a}
Yeast treatment	36.72 ± 0.16^{b}	39.02 ± 0.12^{d}	39.49 ± 0.12^{d}	39.74 ± 0.13^{f}	40.02 ± 0.13^{f}
Yeast+PCM treatment	36.59 ± 0.19^{ab}	38.26 ± 0.16^{b}	37.95 ± 0.12^{b}	37.57 ± 0.12^{b}	37.14 ± 0.13^{b}
Yeast+$EABS_{100}$ treatment	36.63 ± 0.11^{ab}	38.87 ± 0.12^{d}	38.63 ± 0.15^{d}	38.34 ± 0.14^{e}	37.98 ± 0.12^{e}
Yeast+$EABS_{150}$ treatment	36.55 ± 0.12^{ab}	38.61 ± 0.13^{c}	38.39 ± 0.14^{c}	38.16 ± 0.13^{d}	37.72 ± 0.15^{d}
Yeast+$EABS_{200}$ treatment	36.68 ± 0.12^{ab}	39.45 ± 0.12^{c}	38.21 ± 0.16^{c}	37.81 ± 0.13^{c}	37.33 ± 0.13^{c}

The values are expressed as Mean ± SD, where the letters (a, b, and c) indicate differences between treatments ($P < 0.05$).

In the yeast-induced fever model, the subcutaneous administration of the yeast solution substantially elevates the rectal temperature in mice by triggering the release of pro-inflammatory cytokines, inducing PGE2 synthesis in the surrounding region, and influencing the thermoregulatory centers in the hypothalamus.[26] Treatment with EABS results in a statistically significant decrease in rectal temperature compared to the negative control treatment (Yeast treatment). The mechanism responsible for reducing fever in yeast-induced fever involves the suppression of prostaglandin (PGE) synthesis in the brain, particularly PGE2, followed by the inhibition of enzymes responsible

for PGE production through the suppression of cyclooxygenase (COX) enzymes.[26] Compounds such as steroids, tannins, and flavonoids present in EABS are acknowledged for their capacity to inhibit PGE2 synthetase, cyclooxygenase, and lipoxygenase, thereby exerting antipyretic effects. Flavonoids have been demonstrated to interfere with PGE and possess the ability to inhibit the peroxidation of arachidonic acid, resulting in a reduction in PGE concentrations and, consequently, a decrease in fever. One potential antipyretic mechanism of EABS might involve mediating the dilation of surface blood vessels, facilitating enhanced heat dissipation by resetting the thermoregulatory center in the hypothalamus.[26]

Conclusion

In summary, EABS demonstrates promising efficacy against pain and fever in mice. The thermal pain reduction model (hot plate test) reveals the maximum analgesic effect (MPA) of EABS after 45 minutes, while the mechanical pain model (Haffner tail clip test) demonstrates effective pain reduction, peaking at 10 minutes. Significantly, EABS exhibits noteworthy analgesic effects in the acetic acid-induced writhing and formalin-induced pain test (organ pain model). The consistent reduction in rectal temperature by EABS leads to the maximum antipyretic effect after 3 hours of treatment. This study provides compelling evidence supporting the effectiveness of black shallot extract in pain and fever management.

Conflict of Interest

Authors declare no conflict of interest

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

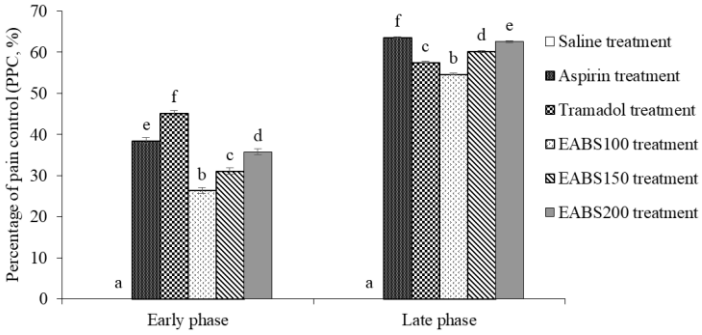

Figure 3: The percentage of pain control (PPC) of black shallot extract in mice was evaluated through the formalin-induced test. The results are presented as Mean ± SD, with letters (a, b, c, d, and e) denoting significant differences between treatments ($P < 0.05$).

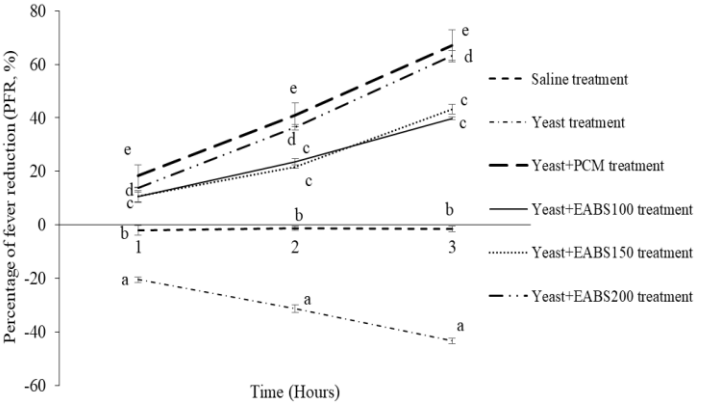

Figure 4: The percentage of fever reduction (PFR) of black shallot extract in anti yeast-induced fever. The results are presented as Mean ± SD, with letters (a, b, c, d, and e) denoting significant differences between treatments ($P < 0.05$).

References

1. Subedi NK, Rahman SMA, Akbar MA. Analgesic and antipyretic activities of methanol extract and its fraction from the root of *Schoenoplectus grossus*. Evid Based Complement Alternat Med. 2016; 2016: 3820704.
2. Tesema S, Makonnen E. In vivo analgesic and antipyretic activities of n-butanol and water fractions of *Ocimum suave* aqueous leaves extract in mice. Ethiop J Health Sci. 2015; 25(2): 139-146.
3. Mohankumar R, Prakash SEL, Irfan N, Mohanraj S, Kumarappan C. Evaluation of analgesic, anti-inflammatory, and antipyretic activities of *Ziziphus mauritania* Lam leaves in animal models. Pharmacol Res Mod Chin Med. 2022; 4: 100153.
4. Mestdagh F, Steyaert A, Lavand P. Cancer pain management: A narrative review of current concepts, strategies, and techniques. Curr Oncol. 2023; 30(7): 6838-6858.
5. Owoyele BV, Abioye AIR, Afinowi NO, Jimoh SA, Soladoye AO. Analgesic and anti-inflammatory effects of *Allium ascalonicum.* Trop J Health Sci. 2006; 13(1): 28-32.
6. Ounjaijean S, Somsak V. Preliminary study on hepatoprotective activity of aqueous crude extract of *Allium ascalonicum* against ethanol-induced liver injury in mice. Walailak J Sci Technol. 2020; 17(10): 1088-1094.
7. Moldovan C, Frumuzachi O, Babotă M, Barros L, Mocan A, Carradori S, Crişan G. Therapeutic uses and pharmacological properties of shallot (*Allium ascalonicum*): A systematic review. Front Nutr. 2022; 9: 903686.
8. Son NH, Duong VB, Giang DT, Hiep NH, Hien PV, Diep NT. Study on preparation of black shallot dried extracts by spray drying method. J Mil Pharm Med. 2022; 5: 167-179.
9. Tran GB, Nguyen NT, Nguyen HN, Pham HH, Ngo TMT. Chemical composition and antioxidant, antiinflammatory, and anticancer effects of ethanol extract of black shallot (*Allium ascalonicum*). Pharmacophore. 2020; 11(3): 30-37.
10. Tran GB, Ngo TMT. The effect of thermal treatment on antioxidant and physicochemical properties of black shallot (*Alliuma scalonicum*). J Teknol. 2023; 85(4): 179-187.
11. Reveny J, Maha HL, Laila L. A comparative study of phytochemical screening and DPPH radical scavenging activity of *Ficus carica* Linn. leaves extracts. Trop J Nat Prod Res. 2023; 7(2): 2337-2340
12. Ilukho FA, Fasipe OJ, Aigbe FR. Evaluating the hepatoprotective, ameliorative and antioxidant potentials of the crude aqueous leafy extracts of *Mangifera indica* plant against acute paracetamol-induced hepatotoxicity in a mouse model. Future Sci OA. 2022; 8(6): 801.
13. Alison A. Basel declaration defends animal research. Nature. 2010; 468: 742.
14. Livestock law in Vietnam, No. 32/2018/QH14. 2018. Ha Noi, Vietnam.
15. Guidelines for Preclinical and Clinical Trials of Oriental Medicines and Herbal Medicines, Decision 141/QD-K2ĐT. 2015. Ministry of Health, Ha Noi, Vietnam.
16. Luyen LT, Quang NN. National Guideline on Ethics in Biomedical Research. Ministry of Health of Vietnam; 2013, 180 p.
17. WHO (World Health Organization). General guidelines for methodologies on research and evaluation of traditional medicine. 2000. Geneva.
18. Hijazi MA, El-Mallah A, Aboul-Ela M, Ellakany A. Evaluation of analgesic activity of *Papaver libanoticum*

extract in mice: involvement of opioids receptors. Evid Based Complement Alternat Med. 2017; 2017: 8935085.
19. Nhung TTP, Quoc LPT. Analgesic and antipyretic activities of ethanol extract of *Gardenia jasminoides* Ellis fruits in mice. Trop J Nat Prod Res. 2023; 7(10): 4902-4907.
20. Nhung TTP, Quoc LPT. Investigation of the Inflammatory, Antipyretic, and analgesic potential of ethanol extract from *Hedyotis capitellata* Wall. ex G. Don leaves in mice. Trop J Nat Prod Res. 2023; 7(12): 5501-5508.
21. Nwozo OS, Effiong EM, Aja PM, Awuchi CG. Antioxidant, phytochemical, and therapeutic properties of medicinal plants: A review. Int J Food Prop. 2023; 26(1): 359-388.
22. Lee JH, Kim N, Park S, Kim SK. Analgesic effects of medicinal plants and phytochemicals on chemotherapy-induced neuropathic pain through glial modulation. Pharmacol Res Perspect. 2021; 9(6): 00819.
23. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain. 1987; 30(1): 103-114.
24. Chang CW, Chang WT, Liao JC, Chiu YJ, Hsieh MT, Peng WH, et al. Analgesic and anti-inflammatory activities of methanol extract of Cissus repens in mice. Evid Based Complement Alternat Med. 2012; 2012: 135379.
25. Li QB, Chang L, Ye F, Luo QH, Tao YX, Shu HH. Role of spinal cyclooxygenase-2 and prostaglandin E2 in fentanyl-induced hyperalgesia in rats. Br J Anaesth. 2018; 120(4): 827-835.
26. Tegegne BA, Alehegn AA. Antipyretic potential of 80% methanol extract and solvent fractions of *Bersama abyssinica* Fresen. (Melianthaceae) leaves against yeast-induced pyrexia in mice. J Exp Pharmacol. 2023; 15: 81-91.