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Original Research Article

Exploring the Analgesic, Antipyretic, and Anti-Inflammatory Properties of *Annona squamosa* Linnaeus Fruit Peel Extract in A Mouse Model

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ABSTRACT

Annona squamosa L. has been reported for its antioxidant, antiarthritic, antidiabetic, hypotensive, and hepatoprotective effects. This study evaluates the analgesic, antipyretic, and anti-inflammatory effects of ethanol extract of *A. squamosa* fruit peel (ASEE) in Swiss albino mice models. ASEE was administered to the animals at doses of 100, 200, and 300 mg/kg, while tramadol (10 mg/kg), aspirin (150 mg/kg), paracetamol (50 mg/kg), and indomethacin (20 mg/kg) were used as reference drugs in algesia, pyrexia, and inflammation models, respectively. Parameters such as tail withdrawal latency, paw licking latency, writhing responses, rectal temperature, paw edema diameter, cytokine levels, pain inhibition percentage (PIT, PIH, and PRW), fever reduction percentage (PFR), and paw edema inhibition percentage (PPE) were assessed. Results indicate significant analgesic effects of ASEE at all doses ($p < 0.05$) within 30, 60, 90, and 120 min post-administration. ASEE significantly inhibited tail flick latency, paw-licking response, and writhing episodes induced by thermal stimuli and acetic acid ($p < 0.05$) and effectively reduced rectal temperature post yeast suspension ($p < 0.05$). Moreover, ASEE demonstrated notable anti-inflammatory activity against carrageenan-induced paw edema, with the highest efficacy observed at 5 h post-induction ($p < 0.05$). Simultaneously, ASEE significantly suppressed pro-inflammatory cytokines; TNF-α, IL-1β, and IL-6 ($p < 0.05$). The findings suggest that ASEE holds promise as a natural therapeutic agent for managing pain, fever, and inflammatory conditions.

***Keywords*:** *Annona squamosa* L., Analgesic, Antipyretic, Anti-inflammatory, Peel extract.

Introduction

Traditional medicinal plants possess therapeutic properties and have been utilized since ancient times. Natural medicinal plants have served as remedies for various ailments throughout history. Despite advancements in modern technologies such as combinatorial chemistry, biotechnology, and drug design aided by computational tools, medicinal plants continue to contribute significantly to the arsenal of clinical therapeutics.[1]*Annona squamosa* L., belonging to the Annonaceae family, is found in tropical and subtropical regions and is traditionally used for diverse medicinal purposes. Studies on the chemical composition of *A. squamosa* have identified several bioactive metabolites including flavonoids, terpenoids, coumarins, anthraquinones, and phytosterols.[2]*A. squamosa* has been reported to exhibit anticancer, antioxidant, anti-inflammatory, antidiabetic, hypotensive, hepatoprotective, antihyperglycemic, and antiparasitic activities.[3] Extracts from the fruit peel of *A. squamosa* have been studied for their protective effects against CFA-induced joint damage in mice.[4] In Indonesia, leaves of *A. squamosa* are used as anti-inflammatory, anticonvulsant, and analgesic agents; root decoctions are employed for toothache relief and fever reduction.[5] Caryophyllene oxide isolated from non-saponifiable ether extract of *A. squamosa* bark also exhibits analgesic and anti-inflammatory activities.[6]

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A. muricata, a species related to *A. squamosa*, has been reported to have anti-inflammatory, anticancer, analgesic, antipyretic, and therapeutic properties for respiratory and dermatological conditions.[7]

In this study, we conducted experiments to evaluate the analgesic, antipyretic, and anti-inflammatory effects of ethanol extract of A. squamosa fruit peel. Peripheral analgesic activity was assessed using the acetic acid-induced writhing test, a recognized method for evaluating peripheral pain reduction. Central analgesic activity was evaluated using the tail immersion and hot plate methods, measuring tail withdrawal latency and hind paw licking latency upon contact with a heat source, directly related to central nervous system pain control. Anti-inflammatory activity was determined by the carrageenan-induced paw edema method. The antipyretic effect was evaluated using Brewer's yeast-induced pyrexia, where a reduction in rectal temperature signifies antipyretic properties.[8] The analgesic, antipyretic, and anti-inflammatory activities of ethanol extract of A. squamosa fruit peel have been previously mentioned in folk medicine. Yet, detailed scientific studies are necessary to provide evidence and elucidate the mechanisms of action of its active constituents. This research may enhance understanding of the medicinal potential of *A. squamosa* and pave the way for novel treatments for conditions related to pain, fever, and inflammation.

Materials and Methods

Chemicals and reagents

The experiment utilized a variety of chemicals and reagents including Carrageenan (Sigma Aldrich, USA), Xylazine and Ketamine (Troy, Australia), Tramadol, and Indomethacin (Windlas Biotech Ltd., India), as well as Ethanol, Chloroform, Ethyl acetate, Acetic acid, and Yeast (Nam Khoa, Vietnam). Additionally, Sodium phosphate, Sodium hydroxide, Sodium carbonate, Distilled Water (DW), Normal saline,

Physiological saline, Aspirin, Paracetamol, and Indomethacin (Sapharco, Ho Chi Minh City, Vietnam) were employed in the study.

Collection of material and preparation of the extract

The fruit peels of *Annona squamosa* were collected in January 2024 in Tan Hung commune, Tan Chau district, Tay Ninh province, Vietnam. The reference sample (code AS150124VST) was meticulously preserved at the Department of Biotechnology, Ho Chi Minh City University of Industry. After sorting and selecting fresh peels with a light yellow-green color and soft large spines, the peels were dried using a Memmert oven (Germany) at 60°C and then ground into a fine powder using an MN300B herbal grinder (Dong Nam Company, Vietnam). For subsequent experiments, the *A. squamosa* powder was stored in moisture-proof bags at room temperature, protected from light.

A. squamosa powder (200 g) was macerated in 1000 mL of 95% ethanol for 24 h, followed by ultrasonic extraction for 45 min. After filtration with Whatman No.4 filter paper and concentration using a rotary evaporator RE301B-T at 50°C (Yamato, Japan), 40 g of extract (designated as ASEE) was obtained. The extract was packaged in moisture-proof containers and stored at 4°C for future experiments.

Phytochemical screening of ASEE

A variety of methods and techniques were utilized to identify the chemical constituents of the ethanol extract of *A. squamosa* fruit peel (Table 1), as described by Tran *et al*.[9]

Determination of total polyphenol content: The sample was boiled with 50 mL of ether for 15 min. Subsequently, 5 mL of the extract was placed in a 50 mL volumetric flask and 10 mL of distilled water was added. After boiling with 50 mL of ether for another 15 min, 5 mL of the extract was again transferred to a 50 mL volumetric flask with an additional 10 mL of distilled water. Then, 2 mL of ammonium hydroxide solution and 5 mL of concentrated amyl alcohol were added. The samples were allowed to react for 30 minutes to develop color, and the absorbance was measured at 505 nm.[10]

The total flavonoid content was determined following the procedure outlined by Nhung and Quoc.[10] In separate test tubes, 0.5 mL of the extract solution and 0.5 mL of the standard solution were added. Each test tube contained 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of 80% methanol, and 2.8 mL of distilled water, and the mixtures were thoroughly mixed. All test tubes were incubated at room temperature for 30 min. The absorbance was measured at a wavelength of 415 nm.

Table 1: Qualitative analysis of phytochemicals and quantitative determination of flavonoids and polyphenols in the ethanol extract of *A. squamosa* fruit peel

Phytoconstituents	Test	Observation	Present in ASEE	Quantification of phytochemicals
Tannins	2 mL extract + 2 mL H_2O + 2-3 drops $FeCl_3$ (5%)	Green precipitate	+	NT
Flavonoids	1 mL extract + 1mL $Pb(OAc)_4$ (10%)	Yellow coloration	+	40.23 ± 1.25 (mg QE/g)
Terpenoids	2 mL extract + 2 mL $(CH_3CO)_2O$ + 2-3 drops conc. H_2SO_4	Deep red coloration	+	NT
Saponins	5 mL extract + 5 mL H_2O + heat	Froth appears	+	NT
Steroids	2 mL extract + 2 mL $CHCl_3$ + 2 mL H_2SO_4 (conc.)	The reddish-brown ring at the junction	+	NT
Cardiac glycosides	2 mL extract + 2 mL $CHCl_3$ + 2 mL CH_3COOH	Violet to Blue to Green coloration	-	NT
Alkaloids	2 mL extract + a few drops of Hager's reagent	Yellow precipitate	+	NT
Polyphenol	2 mL extract + 2 mL $FeCl_3$	Bluish-green appearance	+	65.77 ± 2.08 (mg GAE/g)

Presence of phytochemicals in ASEE: (+) present and (-) absent, and GAE: Gallic acid equivalents, QE: Quercetin equivalents, NT: not tested.

Experimental animals

The animals used for the experiments were adult Swiss albino mice (30-32 g), provided by the Pasteur Institute in Ho Chi Minh City, Vietnam. The animals were housed individually in glass cages and maintained at the experimental animal breeding facility of the Eastern Agriculture and Food Company in Ho Chi Minh City. They were kept under standard environmental conditions, fed with rodent-specific food, and given free access to drinking water. All animals were kept at room temperature in cross-ventilated rooms, with no lighting at night to maintain a 12-hour light/dark cycle. The animals were acclimatized to the laboratory conditions for 14 days before the experiments, during which they had free access to food and water. All experimental procedures and animal handling strictly adhered to the standards and guidelines outlined in the Basel Declaration on Animal Research [11]

Evaluation of the analgesic activity of the extract

*Tail immersion test:*The mice's cold water tail immersion test followed the method described by Ullah *et al*.[12] Each mouse was securely restrained in a polyethylene cage with its tail immersed halfway into a beaker of cold water (1-4°C). The time in seconds taken for the mouse to withdraw its tail from the water was recorded as the reaction time. Baseline measurements were taken before and 30 min after administration of 0.9 % normal saline (negative control, 10 mL/kg), ASEE (100, 200, and 300 mg/kg), and tramadol (positive control, 10 mg/kg) across different groups (n = 5). The percentage inhibition (PIT) of nociceptive response was calculated using the formula:

$$\text{PIT } (\%) = \frac{\text{Latensy (test}-\text{control)}}{\text{Latensy (test)}} \times 100 \text{ (Eq. 1)}$$

Hot plate test

The hot plate test was conducted to evaluate the analgesic activity of the ethanol extract of A. squamosa fruit peel (ASEE). Mice were placed in an open-ended cylindrical chamber with a metal floor heated to a constant temperature of 55°C ± 1°C, as described by Nhung and Quoc.[13] The heated plate induced two behavioral responses: paw licking and

jumping, indicative of nociceptive threshold. The mice were administered three different doses of ASEE (100, 200, and 300 mg/kg), 0.9 % normal saline (negative control, 10 mg/kg), and standard tramadol (positive control, 10 mg/kg) according to their respective groups. Each mouse was placed individually on the hot plate with a cutoff time of 15 sec to prevent paw injury. The latency to paw licking or jumping off the hot plate was recorded at 0, 30, 60, 90, and 120 min to determine reaction times. The percentage inhibition (PIH) of nociceptive response was calculated using the formula:

$$\text{PIH (\%)} = \frac{\text{Latensy (test)} - \text{Latensy (control)}}{\text{Latensy (test)}} \times 100 \text{ (Eq. 2)}$$

Acetic acid writhing test

The method reported by Nhung and Quoc demonstrated the peripheral analgesic effect of ethanol extract from plants.[14] This procedure involved randomly grouping overnight-fasted mice with access to free water. The mice were administered three different doses of ASEE (100, 200, and 300 mg/kg), saline (negative control, 10 mg/kg), and standard aspirin 150 mg/kg (positive control) one hour before acetic acid administration (0.6%, v/v, 10 mL/kg, ip), based on their specific groups. The analgesic effect of ASEE was assessed 5 min after acetic acid injection by counting abdominal writhes, which included abdominal muscle contractions and hind limb stretching over 30 min. The percentage reduction in writhing (PRW) responses compared to the control group was considered the pain reduction index and was determined using the formula:

$$\text{PRW (\%)} = \frac{\text{Mean writhing count (control group} - \text{treated group)}}{\text{Mean writhing count of control group}} \times 100 \text{ (Eq. 3)}$$

Evaluation of the antipyretic activity of the extract

The antipyretic effect of the ethanol extract of A. squamosa fruit peel (ASEE) was assessed using Brewer's yeast-induced pyrexia, following the standard procedure established by Sherif *et al*.[15] Thirty Swiss albino mice were randomly assigned to six groups (n = 5) and were fasted while being allowed free access to water. The baseline body temperature of all animals was measured with a digital thermometer. Pyrexia was induced by subcutaneous injection of a 20% w/v Brewer's yeast suspension (10 mL/kg) into the dorsal region of the rats. After inducing fever, the animals fasted overnight, and their rectal temperatures were measured again. Animals with a temperature increase of at least +0.5°C were included in the study. These febrile animals were then orally administered ASEE at doses of 100, 200, and 300 mg/kg, the standard drug (paracetamol, 50 mg/kg), and the vehicle (saline, 5 mg/kg) for the control groups. The rectal temperature of each animal in each group was recorded at 1, 2, and 3 h post-administration. The percentage fever reduction (PFR) was calculated using the following formula:

$$\text{PFR(\%)} = \frac{\text{Post-fever temperature} - \text{Temperature after 1,2 and 3 h}}{\text{Post-fever temperature} - \text{Normal body temperature}} \times 100 \text{ (Eq. 4)}$$

Evaluation of anti-inflammatory activity

The anti-inflammatory activity was assessed following the methodology outlined by Ashagrie *et al*.[16] Acute inflammation was induced by injecting carrageenan (CAR) (1% w/v carrageenan in normal saline, 100 µL) into the right hind paw of the mice. Before inducing inflammation, the paws were marked with ink at the ankle. Carrageenan was administered to each group of mice 1 h after the oral administration of the ethanol extract of *A. squamosa* fruit peel (ASEE) at doses of 100, 200, and 300 mg/kg, the vehicle (saline, 5 mg/kg), and the standard drug (indomethacin - IND, 20 mg/kg). The degree of inflammation was measured in millimeters, reflecting paw diameter, using a Mitutoyo digital caliper (Japan) at 0, 1, 2, 3, 4, and 5 h after the carrageenan injection. The percentage of protection against edema (PPE) was calculated using the formula:

$$\text{PPE (\%)} = \frac{\text{Swollen leg diameter} - \text{Leg diameter 1,2,3,4 and 5 h}}{\text{Swollen leg diameter} - \text{Normal leg diameter}} \times 100 \text{ (Eq. 5)}$$

Quantification of TNF-α, IL-1β, and IL-6

IL-1β levels were quantified using an enzyme-linked immunosorbent assay (ELISA) combined with an immunoadsorption assay, as described by Nhung and Quoc.[14] In this method, IL-1β capture antibodies were first immobilized in the wells of a 96-well plate and incubated overnight. On the following day, a biotin-labeled antibody was added. This was followed by the addition of streptavidin, resulting in a color change from purple to yellow, which was measured at a wavelength of 450 nm. The quantification of TNF-α and IL-6 was conducted using the same procedure.

Statistical analysis

Data were analyzed using Stagraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, Virginia, USA). The results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to determine statistical significance ($p < 0.05$) between the control and treatment groups.

Results and Discussion

Phytochemical constituents of the extract

Qualitative analysis of phytochemicals in the ethanol extract of *A. squamosa* fruit peel (ASEE) confirmed the presence of tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols, Cardiac glycosides were absent (Table 1). The quantitative analysis shows that flavonoid content in ASEE was 40.23 ± 1.25 mg QE/g, and polyphenol content was 65.77 ± 2.08 mg GAE/g.

Throughout history, nature has provided mankind with a wealth of medicinal plants to address various diseases. Plants are rich in phytochemicals, which are categorized into primary and secondary metabolites. Secondary metabolites, in particular, are vital due to their therapeutic properties for both plants and humans.[17] Our exploration of phytochemicals revealed that the ASEE extract is abundant in diverse plant chemicals, potentially offering significant biological benefits. ASEE contains numerous antioxidant compounds due to their array of secondary metabolites, including tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols. Phenolic compounds and flavonoids are notably significant for their antioxidant properties. Phenolics, as the most diverse group of secondary metabolites, act as antioxidants by scavenging free radicals and exhibit anti-inflammatory effects, with the potential to prevent chronic diseases such as cancer, diabetes, and cardiovascular conditions. Flavonoids provide anti-spasmodic, anti-allergic, and anti-inflammatory benefits, and offer protection against vascular and liver disorders.[18] Tannins help reduce lipid peroxidation, and DNA mutations, and inhibit inflammatory mediators like prostaglandins and histamine, alleviating pain and fever.[19] Terpenoids enhance antioxidant activity, support liver health, and improve inflammatory symptoms.[20] Flavonoids inhibit the enzyme cyclooxygenase (COX), thereby reducing prostaglandin production. Saponins act on nervous system pathways and the immune system, reducing inflammatory environments and pain-causing agents. Terpenoids inhibit ion channels and neurotransmitters such as bradykinin and serotonin, while alkaloids interact with opioid receptors in the central nervous system.[21] In this study, the identification of components such as tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols in the ethanol extract of *A. squamosa* fruit peel (ASEE) demonstrates that ASEE contains a complex of essential plant compounds. These components offer various biological benefits such as antioxidant and anti-inflammatory properties and play roles in pain relief and fever reduction. This highlights the potential of ASEE in medical and pharmaceutical applications, particularly in treating conditions associated with inflammation and oxidative damage.

Evaluation of the analgesic activity of the extract

Effect on heat-induced nociception in mice in the tail immersion test

The results of screening the analgesic activity of the ethanol extract of A. squamosa fruit peel (ASEE) using the tail immersion method are presented in Table 2. The experiment was conducted at doses of 100, 200, and 300 mg/kg body weight. Animals treated in the control group (saline group) exhibited significant differences in reaction time and percentage inhibition (PIT) compared to the test groups within 30 min ($p < 0.05$). ASEE and the standard drug TRM significantly increased reaction times to 53.51 ± 1.15, 35.97 ± 0.72, 41.91 ± 0.95, and 48.78 ± 1.05 s, respectively, within 30 min ($p < 0.05$) (Table 2). PIT for the standard drug TRM and the ethanol extract at a dose of 300 mg/kg body weight was higher than that for ASEE at lower doses (100 and 200 mg/kg) ($p < 0.05$) (Table 2).

Table 2: The analgesic effects of the ethanol extract of *A. squamosa* fruit peel in the tail immersion test

Group	The reaction time (sec)		The percentage inhibition (PIT, %)	
	0 min	30 min	0 min	30 min
Saline group	18.84 ± 0.44[a]	17.22± 0.31[a]	0.00 ± 0.00[a]	-9.41 ± 0.61[a]
TRM group	19.11 ± 0.38[a]	53.51± 1.15[e]	0.00 ± 0.00[a]	64.28 ± 0.06[e]
ASEE100 group	18.93 ± 0.17[a]	35.97± 0.72[b]	0.00 ± 0.00[a]	47.37± 0.58[b]
ASEE200 group	19.05 ± 0.22[a]	41.91± 0.95[c]	0.00 ± 0.00[a]	54.54 ± 0.51[c]
ASEE300 group	18.76 ± 0.21[a]	48.78± 1.05[d]	0.00 ± 0.00[a]	61.54 ± 0.41[d]

Values are expressed as Mean ± SD, and letters (a, b, c, d, and e) represent significant difference between groups ($p < 0.05$).

The brain and spinal cord play crucial roles in central pain mechanisms. The dorsal part of the spinal cord is enriched with substances such as prostaglandins, somatostatin, bradykinin, and various other pain-inhibiting biomolecules. To assess central analgesic efficacy, tail immersion models have been well-established.[1] In the tail immersion experiment, the primary mechanism of pain response involves stimulation and signal transmission from thermal receptors on the skin to the spinal cord and central nervous system structures associated with pain sensation. When a mouse or animal's tail is immersed in cold water, thermal receptors on the skin are stimulated, sending signals to the spinal cord. Here, these signals are relayed to brain regions involved in pain sensation and the body's pain-relieving response such as the thalamus and sensory cortex. Pain reduction responses are measured by monitoring the time the animal withdraws its tail from the water, providing a rapid assessment of the degree of pain reduction induced by active compounds.[22] Tramadol (TRM) acts by binding to opioid receptors in the central nervous system of experimental animals. Upon administration, TRM binds to mu and kappa opioid receptors on nerve neurons, particularly in the thalamus and pain-related areas of the brain, inducing analgesic effects by gradually reducing pain signals transmitted from the skin and spinal cord to central nervous system structures. This results in decreased sensitivity of neurons to pain signals and inhibition of nerve responses associated with pain sensation, thereby reducing the tail withdrawal response time in cold water experiments.[23] In the current study, treatment groups with ASEE100, ASEE200, and ASEE300 demonstrated significant pain reduction ($p < 0.05$) compared to the control group. The inhibitory rates of ASEE were also significantly different from saline ($p < 0.05$) (Table 2), indicating notable analgesic activity of ASEE. The ethanol extract of A. squamosa fruit peel, containing tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols, interacts with mu and kappa opioid receptors on nerve neurons, particularly in the thalamus and pain-related brain regions, and the thermal nociception pathways. These interactions affect neurotransmitter release, receptor activity, or signal transduction pathways related to pain perception and modulation in the central nervous system, thereby regulating pain responses.

Effect on heat-induced nociception in mice in the hot plate test

All three groups ASEE100, ASEE200, and ASEE300 demonstrated significant analgesic effects ($p < 0.05$) at 30, 60, 90, and 120 min compared to the control group (saline group). Similar pain response delays were observed in the TRM group ($p < 0.05$) (Table 3). Maximum pain relief efficacy was observed at 90 min, with percentage inhibition rates for ASEE100, ASEE200, and ASEE300 being 71.48 ± 0.65, 75.01 ± 0.61, and 80.04 ± 0.56, respectively, at this time point (Figure 1). Among all extract groups, the ASEE300 group consistently showed a higher percentage inhibition rate ($p <0.05$) throughout the experiment compared to the saline group, ASEE100 group, and ASEE200 group (Figure 1).

The hot plate test was conducted to investigate the central analgesic activity of the ethanol extract of A. squamosa fruit peel (ASEE). The procedure involved placing mice on a heated plate and observing responses such as jumping and paw licking. The primary mechanism of pain response in the hot plate test involves the activation and transmission of sensory nerve fibers from the skin and surrounding muscle tissue to central nervous system structures in the brain. Upon exposure to high temperatures from the hot plate, thermal receptors on the skin send pain signals to the spinal cord. These signals are then relayed to brain regions associated with pain sensation and the body's pain-relieving response, such as the thalamus and sensory cortex, where pain processing predominantly occurs.[22] For this experiment, tramadol (TRM) was used as a positive control. It is a centrally acting analgesic drug that binds to opioid receptors, specifically mu, kappa, and delta receptors. TRM intervenes in pain signals from the periphery to the spinal cord and activates the gray matter around the aqueduct of Sylvius to release endogenous peptides, which play a significant role in the descending inhibitory pathway.[24] In the hot plate test, all experimental groups exhibited maximum pain-relieving effects after 90 min. This delay may reflect the time required for the drug to reach the central compartment and distribute to the target site. Groups treated with ASEE100, ASEE200, and ASEE300 demonstrated significant pain reduction ($p < 0.05$) compared to the control group (Table 3). The percentage inhibition of ASEE and TRM significantly differed from saline ($p < 0.05$) (Figure 1), indicating substantial pain-relieving activity of ASEE. ASEE exerted its analgesic effects in the hot plate test through a possible interaction with opioid receptors in the central nervous system, mediated by alkaloids and flavonoids present in the extract, thereby reducing pain perception. Tannins, flavonoids, and polyphenols in ASEE inhibit enzymes such as cyclooxygenase (COX) and reduce prostaglandin production, substances associated with pain and inflammation. Concurrently, alkaloids and saponins stimulate the release or action of endogenous opioids, such as endorphins, natural pain-relieving agents in the body. The maximum pain-relieving efficacy was observed at 90 min after administration of the extract, indicating that following oral ingestion, active components such as tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols were absorbed into the bloodstream from the gastrointestinal tract. Upon absorption, these compounds were distributed to organs and tissues throughout the body. In this context, the analgesic effect of ASEE may peak during this period when the highest concentrations of these active ingredients reach their target sites, such as pain-reducing structures in the brain. At 120 min post-extract administration, the pain-relieving effect of ASEE gradually diminished, suggesting that the active components in ASEE had decreased in the body after reaching peak distribution levels, thereby reducing their impact on pain-related structures in the nervous system. Additionally, these compounds may undergo metabolism or excretion over time, and other factors within the body could influence the mechanism of action of ASEE, contributing to the gradual decline in its pain-relieving effect at 120 min.

Effect on acetic acid-induced pain in mice in the acetic acid writhing test

The effect of the ethanol extract of A. squamosa fruit peel (ASEE) on acetic acid-induced writhing response is presented in Table 4. ASEE at doses of 100, 200, and 300 mg/kg significantly reduced ($p < 0.05$) the number of writhing episodes induced by acetic acid compared to the untreated group (saline group). ASEE at these treatment doses exhibited writhing inhibition effects of 33.56 ± 2.63, 49.95 ± 2.73, and 67.13 ± 1.32%, respectively.

This method of writhing induced by acetic acid is popular for assessing peripheral pain-relieving activities of drugs or herbal preparations.[22] It is reported that acetic acid increases the levels of PGE2, PGE2α, and lipoxygenase products in the peritoneal fluid. Additionally, it activates the production of unpleasant endogenous mediators including histamine, serotonin, and bradykinin, responsible for the characteristic signs of inflammation. The acetic acid-induced writhing test simulates visceral pain and writhing behavior and is characterized by abdominal muscle contractions, stretching of the anterior limbs, and elongation of the body. This elongation is induced by the activation of peritoneal nociceptors and prostaglandin pathways in the animal model. Peritoneal nociceptors may contribute to the abdominal writhing response.[25] ASEE significantly reduces writhing responses ($p < 0.05$) at administered doses (100, 200, 300 mg/kg) in mice. The decrease in writhing episodes due to the extract is dose-dependent compared to the control group ($p < 0.05$) (Table 4). The components including tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols in ASEE exert analgesic effects in the acetic acid-induced writhing test by intervening in multiple mechanisms related to inflammation and pain signal transmission in the nervous system. They act on inflammatory and pain mediators such as prostaglandins, histamine, and serotonin, inhibiting enzymes like cyclooxygenase (COX), thereby reducing prostaglandin production. These compounds also interfere with pain signal transmission pathways from skin receptors and sensory nerve fibers to central nervous system structures in the brain, inhibiting pain signal transmission from the skin and surrounding tissues to pain perception regions like the thalamus and sensory cortex. Additionally, certain compounds like alkaloids directly affect opioid receptors in the central nervous system, contributing to pain signal reduction.

Table 3: The analgesic effects of the ethanol extract of *A. squamosa* fruit peel in the hot plate test

Time (sec)	Saline group	TRM group	ASEE100 group	ASEE200 group	ASEE300 group
0 min	2.28 ± 0.12^a	2.31± 0.12^a	2.26± 0.14^a	2.27± 0.13^a	2.29± 0.12^a
30 min	2.24 ± 0.12^a	8.08± 0.25^e	3.39± 0.16^b	4.54± 0.25^c	6.87± 0.28^d
60 min	2.19 ± 0.13^a	10.39± 0.29^e	5.65 ± 0.25^b	6.81± 0.23^c	9.16± 0.36^d
90 min	2.15 ± 0.11^a	12.71± 0.37^e	7.91± 0.33^b	9.08± 0.37^c	11.45± 0.39^d
120 min	2.11± 0.10^a	11.55± 0.24^e	6.78± 0.29^b	7.95± 0.29^c	10.31± 0.29^d

Values are expressed as Mean ± SD, and letters (a, b, c, d, and e) represent significant difference between groups ($p < 0.05$).

Table 4: The analgesic effects of the ethanol extract of *A. squamosa* fruit peel in the acetic acid-induced writhing test

Parameters	Saline group	ASA group	ASEE100 group	ASEE200 group	ASEE300 group
No. of times writhing (times)	29.20 ± 0.84^e	8.40± 0.89^a	19.40± 0.89^d	14.60± 0.55^c	9.60± 0.55^b
% pain inhibition of ASEE (%)	0.00 ± 0.00^a	71.23± 3.05^e	33.56± 2.63^b	49.95± 2.73^c	67.13± 1.32^d

Values are expressed as Mean ± SD, and letters (a, b, c, d and e) represent significant difference between groups ($p < 0.05$).

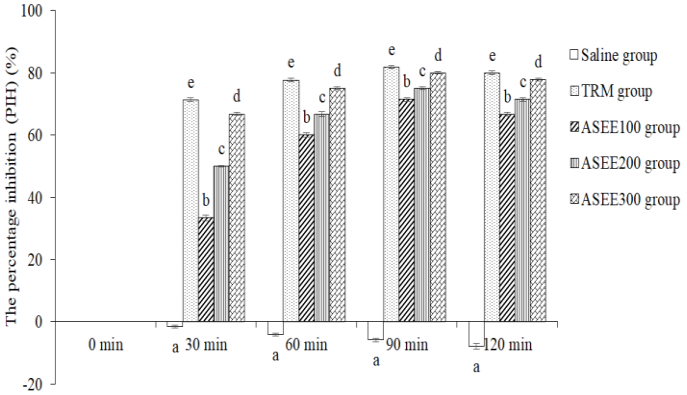

Figure 1: Percentage inhibition of pain of the ethanol extract of A. squamosa fruit peel of tail clip test. Dataare expressed as Mean ± SD, with letters (a, b, c, d, and e) indicating statistically significant differences among the groups ($p < 0.05$)

Evaluation of the antipyretic activity of the extract

The results in Table 5 and Figure 2 demonstrate a significant elevation in rectal temperature following yeast suspension injection compared to the control group ($p < 0.05$). The administration of paracetamol (PCM) and the ethanol extract of A. squamosa fruit peel (ASEE) at different doses resulted in a substantial reduction in rectal temperature for up to 3 h compared to the control group ($p < 0.05$) (Table 5). Specifically, treatment with ASEE at doses of 100, 200, and 300 mg/kg significantly increased the percentage fever reduction (PFR) after 1 h to 26.55 ± 0.04, 28.47 ± 0.21, and 30.82 ± 0.21%, respectively ($p < 0.05$). This increase continued to improve, reaching 66.81 ± 0.03, 71.46 ± 0.11, and 77.02 ± 0.07% after 3 h ($p < 0.05$), in contrast to the yeast group which exhibited PFRs of -24.87 ± 0.28 and -40.16 ± 0.37% (Figure 2).

When yeast cells are introduced into the body, the immune system recognizes them through pattern recognition receptors (PRRs) on immune cells. These receptors detect pathogen-associated molecular patterns (PAMPs) in yeast cells. The interaction between PRRs and PAMPs activates immune cells to produce and release pro-inflammatory cytokines. These cytokines enter the bloodstream and travel to the hypothalamus, the body's thermoregulatory center, where they interact with receptors to stimulate the production of prostaglandin E2 (PGE2). PGE2 acts on hypothalamic neurons, raising the body's set point temperature. This increase in the hypothalamic set point triggers physiological responses such as vasoconstriction to reduce heat loss, an increased metabolic rate to generate more heat, and shivering to produce heat through muscle activity.[26]Paracetamol (PCM) reduces fever by acting on the central nervous system and inhibiting prostaglandin synthesis. PCM inhibits the enzyme cyclooxygenase-2 (COX-2), thereby reducing PGE2 production. This lowers the hypothalamic set point temperature, directly acting on the hypothalamus to effectively

reduce fever.[27] The antipyretic activity of ASEE was studied in mice with yeast-induced fever. Significant temperature reduction was dose-dependent with ASEE and PCM. Among the treatment groups, ASEE300 exhibited the highest antipyretic activity ($p < 0.05$). These results confirm the enhanced antipyretic effect of ASEE. ASEE exerts its antipyretic effect by inhibiting the production of pro-inflammatory cytokines and reducing prostaglandin synthesis.[28] The bioactive compounds in ASEE, including tannins, flavonoids, terpenoids, and polyphenols, inhibit the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Tannins, flavonoids, terpenoids, and polyphenols also inhibit COX-2, thereby reducing PGE2 synthesis. Flavonoids and polyphenols possess strong antioxidant effects, reducing oxidative stress and inflammation, and protecting cells from damage. Moreover, ASEE directly affects the hypothalamus, the body's temperature control center, by inhibiting PGE2 activity, thereby lowering the thermoregulatory set point and reducing fever. The increase in % PFR after 3 h of ASEE administration ($p < 0.05$) compared to 1 and 2 h earlier is a result of effective absorption, distribution, metabolism, and excretion (ADME) processes, along with accumulation and interaction of active compounds with the immune system and intracellular signaling pathways in the body.[29] Upon entering the body, ASEE undergoes ADME processes that reach peak effectiveness after 3 h of use. Compounds in ASEE, such as tannins, flavonoids, terpenoids, saponins, steroids, alkaloids, and polyphenols, need to accumulate to a certain concentration in blood and tissues to achieve optimal effectiveness. After 3 h, these compound concentrations are sufficient to strongly impact fever-causing mechanisms. Additionally, the immune system and inflammatory pathways often respond slowly to anti-inflammatory and antipyretic agents. After 3 h, pro-inflammatory cytokines and other mediators are significantly inhibited, leading to more pronounced antipyretic effects. ASEE contains multiple active compounds with different mechanisms of action, and their synergistic effectiveness requires time to reach maximum efficacy, particularly as they interact with each other and target cells over an extended period.

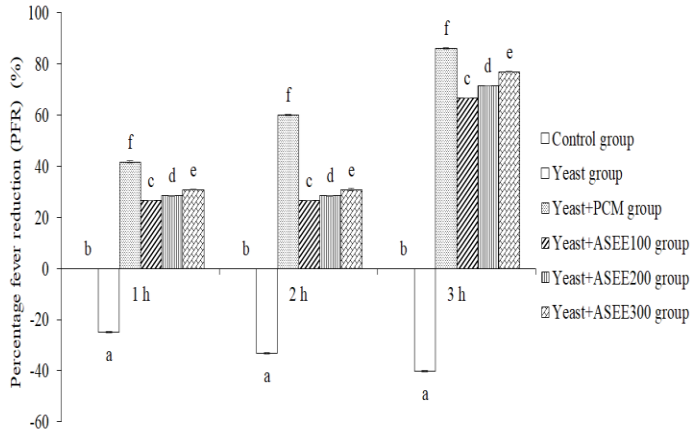

Figure 2: Percentage fever reduction (PFR) of the ethanol extract of A. squamosa fruit peel in the yeast-induced fever test. Results are expressed as Mean ± SD, with letters (a, b, c, d, e, and f) indicating statistically significant group differences ($p < 0.05$)

Assessment of anti-inflammatory activity

Effect on carrageenan-induced paw inflammation in mice

In the case of carrageenan (CAR)-induced paw edema, the ethanol extract of A. squamosa fruit peel (ASEE) at all tested doses demonstrated a significant reduction in paw edema beginning at 1 h and continuing for up to 5 h post-induction ($p < 0.05$) compared to the control (Table 6). A comparison among the ASEE dose groups revealed statistically significant effects between the 100, 200, and 300 mg/kg doses ($p < 0.05$). The standard drug indomethacin (IND) at 10 mg/kg showed significant anti-inflammatory activity ($p < 0.05$) compared to the low and medium doses of ASEE. The maximum anti-inflammatory effects of 100, 200, and 300 mg/kg ASEE were observed at 5 h post-induction, with values of 73.33 ± 1.68, 84.69 ± 5.07, and 90.75 ± 4.01%, respectively, and the effectiveness increased with the dose ($p < 0.05$) (Figure 3). The higher dose of ASEE (300 mg/kg) exhibited anti-inflammatory activity comparable to that of 10 mg/kg IND throughout the observation period (Table 6 and Figure 3).

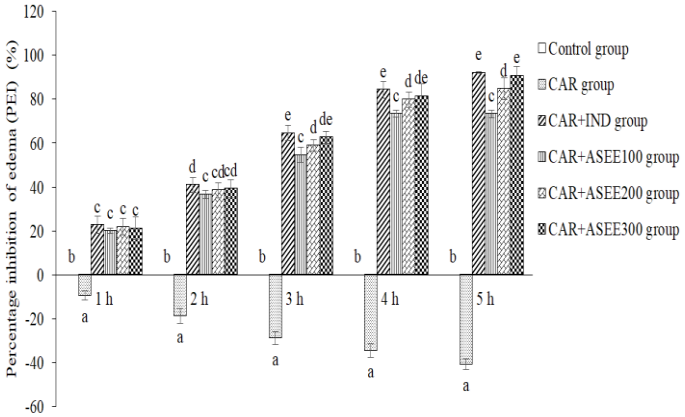

Figure 3: The percentage of protection against edema (PPE) of the ethanol extract of A. squamosa fruit peel in the carrageenan-induced paw edema test model in mice. Data are expressed as Mean ± SD, with letters (a, b, c, d, and e) indicating statistically significant group differences ($p < 0.05$)

Carrageenan (CAR)-induced inflammation follows a biphasic process involving leukocyte infiltration and increased vascular permeability at the injection site. Initially, carrageenan stimulates inflammation by activating early immune cells such as macrophages and mast cells, leading to the release of active compounds including histamine, serotonin, bradykinin, and prostaglandin (PGE2) from immune cells and transporting tissues. In the later phase, CAR triggers immune cells to produce pro-inflammatory cytokines like TNF-α and IL-1β, facilitating neutrophil recruitment and inflammatory mediator transport to the inflamed area. Concurrently, CAR activates transport enzymes and oxidative reactions, enhancing the production of inflammatory mediators such as prostaglandins and leukotrienes, which promote vasodilation and immune cell recruitment to sustain inflammation at the site of contact with CAR.[30] Indomethacin (IND) exerts anti-inflammatory effects by inhibiting COX-1 and COX-2, thereby reducing PGE synthesis and attenuating inflammation, including thrombus formation, pain, and swelling.[31]Current research investigates the anti-inflammatory activities of ASEE and IND in carrageenan-induced paw edema in mice. Results indicate significant paw inflammation due to carrageenan ($p < 0.05$). This inflammation was attenuated ($p < 0.05$) dose-dependently by ASEE, with a notable increase in PPE ($p < 0.05$) in ASEE-treated groups. ASEE exhibits multifaceted effects on carrageenan-induced inflammatory mechanisms, including inhibition of early immune responses, reduction in inflammatory mediator synthesis, oxidative stress reduction, and modulation of intracellular signaling pathways. Active compounds in ASEE stimulate macrophages and mast cells, reducing the production of pro-inflammatory cytokines such as TNF-α and IL-1β. Tannins, flavonoids, and polyphenols in ASEE inhibit COX enzymes, reducing prostaglandin and leukotriene synthesis, key inflammatory mediators. Saponins, steroids, and alkaloids impact intracellular signaling pathways in immune cells and carrageenan-stimulated tissues, reducing inflammation and swelling.However, a higher dose of ASEE (300 mg/kg) shows comparable anti-inflammatory activity to 10 mg/kg IND ($p > 0.05$) throughout the observation period. The equivalence in anti-inflammatory activity between ASEE300 and IND is attributed to their COX inhibition capability, reduction in inflammatory mediator synthesis, and other properties such as antioxidant effects and cell protection during inflammation.

Table 5: The antipyretic effects of the ethanol extract of *A. squamosa* fruit peel in yeast-induced fever test

Experimental group	Initial (ºC)	Fever (ºC)	1 h (ºC)	2 h (ºC)	3 h (ºC)
Control group	36.78 ± 0.03^{a}	36.84 ± 0.05^{a}	36.82 ± 0.04^{a}	36.79 ± 0.03^{a}	36.81 ± 0.02^{a}
Yeast group	36.79 ± 0.02^{a}	41.21 ± 0.05^{b}	42.31 ± 0.07^{f}	42.68 ± 0.06^{f}	42.99 ± 0.05^{f}
Yeast+PCM group	36.81 ± 0.03^{a}	41.23 ± 0.04^{b}	39.39 ± 0.05^{b}	38.58 ± 0.04^{b}	37.43 ± 0.02^{b}
Yeast+ASEE100 group	36.78 ± 0.02^{a}	42.29 ± 0.04^{e}	40.83 ± 0.04^{e}	39.72 ± 0.03^{e}	38.61 ± 0.03^{e}
Yeast+ASEE200 group	36.77 ± 0.02^{a}	42.29 ± 0.04^{d}	40.45 ± 0.04^{d}	39.34 ± 0.03^{d}	38.24 ± 0.02^{d}
Yeast+ASEE300 group	36.79 ± 0.03^{a}	41.92 ± 0.04^{c}	40.09 ± 0.06^{c}	38.99 ± 0.05^{c}	37.89 ± 0.03^{c}

Values are expressed as Mean ± SD, and letters (a, b, c, d, e, and f) represent the difference between groups ($p < 0.05$).

Effect on expression levels of inflammatory cytokines

To elucidate the molecular mechanism underlying the anti-inflammatory effects of the ethanol extract of *A. squamosa* fruit peel (ASEE), its ability to inhibit pro-inflammatory cytokines (TNF-*α*, IL-1*β*, and IL-6) in the paw was investigated (Figure 4). Carrageenan (CAR) significantly upregulated TNF-*α*, IL-1*β*, and IL-6 expressions ($p < 0.05$) compared to the control group. In contrast, ASEE at doses of 100, 200, and 300 mg/kg markedly reduced ($p < 0.05$) the levels of pro-inflammatory cytokines (TNF-*α*, IL-1*β*, and IL-6) compared to the yeast-induced inflammation group. Particularly, ASEE at 300 mg/kg exhibited the highest anti-inflammatory activity among all treatment methods ($p < 0.05$).

The increase of pro-inflammatory cytokines in the inflamed area results from a series of complex immune and tissue reactions activated by CAR. Immune cells such as macrophages and neutrophils are stimulated by inflammatory agents like CAR. CAR triggers the synthesis and release of inflammatory mediators such as histamine, serotonin, and bradykinin from immune cells and tissues, thereby stimulating immune cells to release cytokines such as TNF-*α*, IL-1*β*, and IL-6.[32] TNF-*α*, a pro-inflammatory cytokine, promotes leukocyte adhesion to endothelial tissue through upregulation of adhesion molecules, vasodilation, and edema formation. IL-1*β*, another pro-inflammatory cytokine, plays a crucial role in acute and chronic inflammatory pathologies. The use of inflammatory agents like CAR induces mechanical pain sensitivity or tissue fever in inflamed tissues through the induction of pro-inflammatory cytokines, including IL-1*β* and IL-6.[27] The current study findings demonstrate significant foot inflammation induced by CAR ($p < 0.05$) and elevated serum levels of TNF-*α*, IL-1*β*, and IL-6 ($p < 0.05$). Treatment with ASEE significantly reduces ($p < 0.05$) the concentrations of TNF-*α*, IL-1*β*, and IL-6. ASEE inhibits cytokine production, thereby reducing the synthesis of inflammatory mediators and oxidative stress, resulting in decreased serum levels of TNF-*α*, IL-1*β*, and IL-6. Compounds within ASEE effectively suppress immune cell production of pro-inflammatory cytokines such as TNF-*α*, IL-1*β*, and IL-6, inhibit intracellular signaling pathways and other inflammatory mediators, decrease PGE2 synthesis and other inflammatory mediators, and attenuate TNF-*α*, IL-1*β*, and IL-6 production. Flavonoids and polyphenols in ASEE exhibit potent antioxidant properties, thereby mitigating inflammation and reducing the production of pro-inflammatory cytokines.

Table 6: The anti-inflammatory effects of the ethanol extract of *A. squamosa* fruit peel in the carrageenan-induced paw edema test model in mice

Experimental group	Paw diameter (mm)						
	0 h	Foot swelling	1 h	2 h	3 h	4 h	5 h
Control group	0.55 ± 0.03^{a}	0.56 ± 0.02^{a}	0.55 ± 0.02^{a}	0.54 ± 0.03^{a}	0.56 ± 0.02^{a}	0.55 ± 0.03^{a}	0.56 ± 0.01^{a}
CAR group	0.54 ± 0.02^{a}	0.86 ± 0.03^{d}	0.89 ± 0.03^{d}	0.92 ± 0.04^{d}	0.95 ± 0.03^{e}	0.97 ± 0.02^{d}	0.99 ± 0.03^{d}
CAR + IND group	0.54 ± 0.03^{a}	0.79 ± 0.03^{b}	0.74 ± 0.02^{b}	0.69 ± 0.04^{b}	0.63 ± 0.04^{b}	0.58 ± 0.04^{ab}	0.56 ± 0.03^{a}
CAR + ASEE100 group	0.55 ± 0.02^{a}	0.85 ± 0.04^{d}	0.79 ± 0.04^{c}	0.74 ± 0.03^{c}	0.69 ± 0.02^{d}	0.63 ± 0.03^{c}	0.63 ± 0.02^{c}
CAR + ASEE200 group	0.56 ± 0.01^{a}	0.84 ± 0.03^{cd}	0.78 ± 0.03^{c}	0.73 ± 0.03^{c}	0.68 ± 0.02^{cd}	0.62 ± 0.02^{c}	0.61 ± 0.01^{b}
CAR + ASEE300 group	0.55 ± 0.01^{a}	0.81 ± 0.02^{bc}	0.76 ± 0.03^{bc}	0.71 ± 0.02^{bc}	0.65 ± 0.02^{bc}	0.60 ± 0.02^{bc}	0.58 ± 0.01^{a}

Values are expressed as Mean ± SD, and letters (a, b, c, and d) represent the difference between groups ($p < 0.05$).

Conclusion

The ethanol extract of *Annona squamosa* L. fruit peel (ASEE) shows notable analgesic, antipyretic, and anti-inflammatory effects in mouse models. ASEE significantly reduced pain in tail immersion and acetic acid-induced writhing tests. The maximum analgesic effect of ASEE was observed at 90 minutes post-administration, reflecting the time required for its active compounds to reach peak concentrations and interact with central pain pathways. The analgesic effect diminished at 120 minutes, likely due to decreased active compound levels. In yeast-induced fever, ASEE lowered body temperature effectively, with the highest efficacy at 300 mg/kg. This antipyretic effect may be due to the inhibition of pro-inflammatory cytokines and COX-2, leading to reduced synthesis of PGE2. The peak effect was observed 3 hours after administration, indicating optimal absorption and distribution of ASEE's active compounds. Additionally, ASEE demonstrated anti-inflammatory activity comparable to indomethacin (IND) in carrageenan-induced paw edema, through reduction of inflammatory mediators. ASEE's diverse components, including tannins, flavonoids, and polyphenols, contribute to its efficacy. Overall, ASEE exhibits significant therapeutic potential for managing pain, fever, and inflammation, warranting further investigation for clinical applications.

Conflict of Interest

The 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.

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