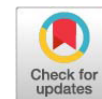
Pakistan J. Zool., pp 1-12, 2024. DOI: https://dx.doi.org/10.17582/journal.pjz/20240506024014



Phytochemical Analysis and Investigation of Anti-Inflammatory, Anti-Nociceptive, and Anti-Pyretic Activities of *Acorus calamus* Linn. Rhizome Extracts in Animal Models

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Article Information
Received 06 May 2024
Revised 25 May 2024
Accepted 04 June 2024
Available online 26 September 2024 (early access)

Authors' Contribution
MUB, BA, and MNF: Study design. MUB, BA and WM: Data collection and analysis. MUB: Manuscript-writing. BA and WM: Manuscript-proofreading. All authors have read and agreed to the published version of the manuscript.

Key words
***Acorus calamus*, Polyphenols, Antioxidants, Inflammation, Pain, Fever**

ABSTRACT

Acorus calamus Linn. (family Acoraceae) is a perennial plant that has traditionally been used to alleviate pain, inflammation, and other ailments. This study was aimed to assess the phytochemical analysis, antioxidant, anti-inflammatory, anti-nociceptive, and anti-pyretic properties of this plant's rhizome *in vitro* and *in vivo*. Crude methanol and subsequent *n*-hexane, ethyl-acetate, *n*-butanol, and aqueous extracts were prepared. Phytochemical analysis of all plant extracts revealed the presence of phenols, flavonoids, alkaloids, glycosides, terpenoids, saponins, and tannins. Among all extracts, methanol and *n*-hexane showed higher total phenol and flavonoid contents, as well as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) inhibition activities, and were selected for *in vivo* studies. In rat models, methanol and *n*-hexane extracts of *A. calamus* at doses of 125, 250, and 500 mg/kg demonstrated anti-inflammatory, analgesic, and anti-pyretic effects in a dose-dependent manner. Both extracts dose-dependently inhibited ear edema induced by xylene as well as significantly decreased paw inflammation in carrageenan model. Furthermore, *A. calamus* methanol and *n*-hexane showed substantial analgesic effects in both phases of formalin test and a dose-dependent and time-dependent nociception inhibition was observed in hot-plate test, with the highest inhibitory effects found at 500 mg/kg dose of each plant extract. In pyretic rats, plant extracts notably reduced rectal temperature after 4 h of study. These findings support the use of *A. calamus* rhizome to treat inflammation, pain, and fever and could be beneficial in a variety of pathological conditions.

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INTRODUCTION

Inflammation is a defensive physiological process in which the body responds in a nonspecific immunological protective manner to tissue harm caused by chemical, physical, or thermal damage or infections (Gusev and Zhuravleva, 2022). Inflamed tissues produce hydroxyl radicals, which stimulate the production of pro-inflammatory cytokines such (TNF-α, IL-1β, and IL-6), nitric oxide, chemokines, and prostaglandins (PGE2). The release of prostaglandins promotes vasodilation. Also, cytokines act as endogenous pyrogens that induce pyrexia and have a detrimental effect on the development of metabolic dysfunction. Chemokines activate leukocytes and are an important stimulus for directing them to regions of damage or inflammation. These mediators together produce inflammation and fever and serve an important role in guaranteeing host survival by repairing tissue damage (Abdulkhaleq *et al*., 2018).

It is regarded as a risk factor for a variety of pathophysiological problems. If it lasts a few days, it might be acute. Acute inflammation is typically good since it provides the body with an important protective response. However, this discomfort is very transitory and goes away after the inflammatory reaction has finished its job (Ptaschinski and Lukacs, 2018; Raziyeva *et al*., 2021). In chronic form, an improper immune response to particular environmental chemicals or hypersensitivity to foreign antigens can prolong inflammation, resulting in substantial tissue damage and organ dysfunction. Inflammation

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0030-9923/2024/0001-0001 $ 9.00/0

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manifests as swelling, loss of function, heat, and pain in the afflicted region (Zhang and Kurashima, 2021).

Pain is an unpleasant sensation caused by tissue injury or noxious stimuli. Its protective mechanism leads individual consciousness to remove the damaged area from stimuli that cause discomfort (Ellison, 2017). Toll-like receptors (TLRs) are immunological pattern recognition receptors that detect and identify harmful signals. TLR activation stimulates the production of pro-inflammatory mediators *via* NF-κB transcription (Lacagnina *et al*., 2018). The existence of these mediators can determine the degree of inflammation as well as activate and sensitize nociceptors (Coghill, 2020). Although pain is a protective reaction to an organ's dysfunction or impaired activities in response to potentially harmful stimuli, is required for survival. The continuous pain can induce anxiety, depression, and a decrease in quality of life (Roughan *et al*., 2021). Moreover, pyrexia involves a cytokine-mediated increase in core temperature as well as activation of the endocrinological and immune systems. Prostaglandin (PGE2) in areas of the brain causes abnormal neuronal firing in the hypothalamus and raises body temperature (Blomqvist and Engblom, 2018).

Nonsteroidal anti-inflammatory medications (NSAIDs), opioids, glucocorticoids, immunosuppressants, and biologics are all being used to treat inflammation, pain, and fever (Kamel-Escalante *et al*., 2019; El-Tallawy *et al*., 2021). However, their potential side effects, including gastrointestinal issues, respiratory depression, renal damage, and possibly opioid dependency, continue to be a source of concern (Bindu *et al*., 2020; Kichloo *et al*., 2021; Stone *et al*., 2021). As a result, alternative therapies with few side effects are required, particularly those derived from natural sources. Plant-derived compounds are considered a valuable source for the formulation of new anti-pyretic, analgesic, and anti-inflammatory drugs (Parvin *et al*., 2023).

Acorus calamus (Linn.), also known as sweet flag or calamus, is a member of the Acoraceae family. This perennial, semiaquatic, and scented plant can be found in the temperate and subtropical areas of Asia, Europe, and North America. *A. calamus* has grass-like, long, and thin leaves that radiate out from a pinkish base. Damaged or cut leaves emit a pleasant fragrance. The flower stalk grows from the base of outer leaves. The plant produces greenish, angular berries with one to three seeds. The most important part of *A. calamus* is the rhizomes. The rhizomes have a strong, distinct odor and are bitter to taste (Khwairakpam *et al*., 2018). *A. calamus* rhizomes have a variety of therapeutic uses in the traditional medicine system. They are used in the forms of powder, balms, enemas, and tablets to treat intermittent fevers, otitis media, cough, asthma, gastrointestinal disorders like dysentery, colic, chronic diarrhea, and persistent dyspepsia, mental issues such as schizophrenia, epilepsy, and memory impairments, as well as glandular and abdominal tumors. Rhizomes are also used to treat kidney and liver issues, rheumatism, and eczema. The skin of the rhizomes is thought to be hemostatic (Umamaheshwari and Rekha, 2018). Furthermore, several studies have reported pharmacological properties of *A. calamus* rhizome, such as sedative, anticonvulsant, memory enhancer, antioxidant, anti-inflammatory, anti-spasmodic, hypolipidemic, immunosuppressive, cytoprotective, anti-diarrheal, anti-microbial, anthelmintic, and insecticidal, associated with its phytoconstituents, particularly a- and b-asarone (Loying *et al*., 2019; Sharma *et al*., 2020). Based on the reported traditional therapeutic benefits of *A. calamus* rhizomes, this research was planned to evaluate its antioxidant, anti-inflammatory, analgesic, and anti-pyretic activities in experimental models of rats.

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MATERIALS AND METHODS

Reagents and drugs

Analytical grade reagents, carrageenan from Sigma-Aldrich®, USA, and formalin from Merck®, Pakistan, were purchased. Standard compounds such as gallic acid and catechin were procured from Sigma-Aldrich®, USA. Drugs used as standard treatments in animal studies, including aspirin from Unichem®, Pakistan, paracetamol from GSK®, Pakistan, diclofenac sodium from Searle Pharmaceutical®, Pakistan, indomethacin from Wilshire Laboratories®, Pakistan, and meloxicam from Sigma-Aldrich®, USA, were acquired.

Plant material

The rhizomes of *Acorus calamus* were procured from the Northern areas of Pakistan. Plant specimen was identified by a plant taxonomist and deposited in the herbarium of Department of Basic Sciences, University of Agriculture, Faisalabad, Pakistan, with herbarium number 172-1-22 for future reference.

Preparation of extracts

Shade dried rhizomes of *A. calamus* were pulverized with a mechanical grinder and sieved. The coarsely powdered plant material was soaked in 70% methanol for 48 h at room temperature. Then, it was filtered with muslin cloth and through Whatman no. 1 filter paper afterwards. Maceration was done three times to get maximum yield and concentrated using a rotary evaporator from Heidolph , Germany (Subhan *et al*., 2021). The obtained concentrated methanol extract was reconstituted with deionized water

and used to prepare subsequent *n*-hexane, ethyl-acetate, *n*-butanol, and aqueous extracts. The extraction yields were calculated and extracts were stored in airtight containers at 4ºC.

Preliminary phytochemical screening

Several secondary phytocompounds in all extracts of *A. calamus* were detected utilizing various phytochemical tests, according to previously described methods (Shaikh and Patil, 2020).

Quantifications of phenolic and flavonoid content

The total phenolic contents of *A. calamus* extracts were estimated by adopting the Folin-Ciocalteu method (Sultana *et al*., 2009). Extract samples were separately combined with 500 µL of Folin-Ciocalteu reagent and 7.5 mL of deionized water. Mixtures were incubated for 10 min, mixed with 1.5 mL of a 20% Na_2CO_3 solution, and heated in a water bath at 40°C for 20 min. The blue-colored complex appearance was detected in the reaction mixture, and absorbance was checked at 765 nm. Quantification of total phenols was done using a calibration curve of gallic acid (y = 0.0116x + 0.0927, R^2 = 0.9955) and contents were presented as mg GAE/g extract.

The total flavonoid content of extracts of *A. calamus* was quantified by employing the aluminum chloride ($AlCl_3$) colorimetric method (Sultana *et al*., 2009). For this purpose, each extract was diluted with 4 mL of deionized water, combined with 300 µL of 5% $NaNO_2$ solution, incubated for 5 min, and then mixed with 300 µL of 10% $AlCl_3$ solution. After 5 min, the mixtures were treated with 2 mL of 1M NaOH. The mixtures were further diluted with 2.4 mL of deionized water, and absorbance was taken at 510 nm. The contents were calculated using a catechin calibration curve (y = 0.0027x + 0.1609, R^2 = 0.9938). Results were represented as mg CE/g extract.

DPPH scavenging assay

The antioxidant capacity of all plant extracts was measured through 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging test, as previously described (Hussain *et al*., 2022). Different dilutions of extracts ranging from 100 to 500 µg/mL were prepared. A blank solution containing all reagents, without plant extract, was used as a control. A UV/visible spectrophotometer was used to analyze the absorbance of samples at 517 nm. The percentage of DPPH inhibition was calculated using the given formula, and IC50 values were determined.

$$\text{DPPH inhibition } (\%) = \left[\frac{(Ac - As)}{Ac}\right] \times 100$$

Ac, absorbance of control; As, absorbance of sample.

Experimental animals

All experiments were conducted on Wistar albino rats of body weight ranging from 150 to 210 g. Rats were housed at the animal house of the Institute of Microbiology, UAF, Punjab, Pakistan. Experimental conditions such as room temperature (25±2°C), humidity (40-50%), and a 12 h light and 12 h dark period were set. A standard pellet diet and water *ad libitum* were provided. Animals were acclimatized for one week.

Toxicity study

In vitro test results indicated that methanol and *n*-hexane extracts demonstrated higher polyphenol contents as well as antioxidant potential than the others. As a result, these plant extracts were chosen and assessed for their *in vivo* pharmacological activities. The safe dose of *A. calamus* methanol and *n*-hexane extracts was assessed per OECD-425 criteria (OECD, 2008). Animals were allocated to control and test groups, with 3 male and 3 female rats in each group. Each extract was given orally in increasing quantities: 300, 500, 1000, and 2000 mg/kg, while 3 mL/kg of normal saline was administered in control group. All animals were closely monitored for toxicity symptoms for the first 6 h and then any behavioral changes or mortality was noted for three days.

Xylene-induced ear edema model

A previously proposed approach of Hosseinzadeh *et al*. (2000) was used to investigate the anti-inflammatory effects of plant extracts in xylene test. Eight groups of rats (n = 6) were given oral treatments of normal saline (3 mL/kg), 10 mg/kg of indomethacin (standard), and different doses (125, 250, and 500 mg/kg) of each plant extract. A single injection of xylene (30 µL) was given into the inner area of right ear of all animals after 30 min of treatments. After 120 min, animals were decapitated, and spherical portions of both ears measuring 7 mm in diameter were recovered using a cork borer. The treatment effect was established by determining the weight difference between both ears and percentages of inhibition were computed using the following formula:

$$\text{Inhibition } (\%) = \left[\frac{(\Delta W.\text{Control} - \Delta W.\text{Treatment})}{\Delta W.\text{Control}}\right] \times 100$$

ΔW.Control: mean weight difference in control group, ΔW.Treatment: mean weight difference in treatment group.

Carrageenan-induced paw inflammation model

To induce paw inflammation with carrageenan, an injection (100 µL) of 1% carrageenan suspension in normal saline was given into the sub-plantar area of right hind paw of all rats (Aslam *et al*., 2023). Paw thickness (mm) was noted with the help of a digital vernier caliper. Animals

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received normal saline (3 mL/kg), meloxicam as standard (3 mg/kg), and plant extracts (125, 250, and 500 mg/kg) *via* oral route. Then, paw thickness (mm) of all groups was determined at 0, 1, 2, 4, and 6 h following carrageenan injection. The edema inhibition (%) was estimated using the formula given below:

$$\text{Inhibition (\%)} = \left[\frac{\text{(Control - Treatment)}}{\text{Control}}\right] \times 100$$

Formalin-induced nociception model

Eight groups of rats (n = 6) were orally given 3 mL/kg of normal saline (vehicle), 200 mg/kg of aspirin (standard), and graded doses of both plant extracts (125, 250, and 500 mg/kg). After 60 min of treatments, each rat received a 20 µL injection of 1% v/v formalin solution into the right paw (Hussain *et al*., 2021). Paw licking and biting reflexes of rats were noted in early (0-10 min) and late phases (10-60 min) following formalin injection.

$$\text{Latency (\%)} = \left[\frac{\text{(Control - Treatment)}}{\text{Control}}\right] \times 100$$

Heat-induced nociception model

A heat test was adopted to evaluate the analgesic effect of plant extracts, as described by Akindele *et al*. (2012), with a few modifications. Rats underwent a sensitivity test at 55 ± 1°C for 15 s. Animals that demonstrated jumping, paw licking, or biting were chosen and put into eight groups (n = 6). After 1 h of oral treatments with normal saline (vehicle; 3 mL/kg), diclofenac (standard; 50 mg/kg), and each plant extract (125, 250, and 500 mg/kg), the pain reaction time for each rat was noticed by placing it on a hot-plate at 30, 60, 90, and 120 min of therapy. To avoid paw tissue damage, a 30 s cut-off time was employed, and the increase in latency time (%) was calculated.

Yeast-induced pyrexia model

Rats were fasted and given water *ad libitum* before being separated into eight groups of six rats each (n = 6). A digital thermometer was used to note the rectal temperature of rats. To induce pyrexia, rats were injected with a 10 mL/kg of 15% Brewer's yeast suspension in back of neck. Temperatures were noted again after 18 h, and rats with at least a 0.5°C increase in temperature were selected (Lino *et al*., 2017). The pyretic rats were administered orally with each plant extract (125, 250, and 500 mg/kg), paracetamol as a conventional antipyretic drug (150 mg/kg), and 3 mL/kg normal saline in control group. The rectal temperatures of all groups were recorded during the 1st, 2nd, 3rd, and 4th h following treatment administration and the percentage change in temperature was calculated as:

$$\text{Reduction (\%)} = \left[\frac{\text{(Temp. at 0 h - Temp at different times)}}{\text{Temp. at 0 h - Temp. at -18 h}}\right] \times 100$$

Statistical analysis

In vitro experiments were done in triplicates, while *in vivo* investigations were conducted with six animals per group. All obtained results were provided as mean±SEM. GraphPad Prism® (USA) was used to perform one-way and two-way ANOVA tests followed by post-hoc Dunnett's test or Bonferroni test (wherever applicable). A *p* value less than 0.05 was considered statistically significant.

RESULTS

Phytochemical screening

The preliminary phytochemical assessment detected alkaloids, flavonoids, phenols, glycosides, saponins, tannins, and terpenoids in all *A. calamus* extracts, as summarized in Table I. Results indicated that methanol extract had the highest amount of phenolic content, followed by *n*-hexane, ethyl-acetate, aqueous, and *n*-butanol, with contents ranging from 24.31±0.60 mg GAE/g to 198.73±1.68 mg GAE/g (Table II). Maximum flavonoid content was found in *n*-hexane, followed by methanol, *n*-butanol, ethyl-acetate, and aqueous extracts. The contents ranged from 8.32±0.44 mg CE/g to 56.01±0.79 mg CE/g.

Table I. Phytochemicals detected in different extracts of *A. calamus* rhizomes.

Phytocompounds	Methanol	n-Hexane	Ethyl-acetate	n Butanol	Aqueous
Alkaloids	+	-	-	+	-
Flavonoids	+	+	+	+	+
Glycosides	+	+	+	+	+
Phenols	+	+	+	+	+
Saponins	+	-	+	-	+
Tannins	+	-	-	+	+
Terpenoids	+	+	+	+	+

(+) Present; (-) Absent.

Table II. Total phenolic and flavonoid contents quantified in *A. calamus* extracts.

Extracts	Total phenols (mg GAE/g)	Total flavonoids (mg CE/g)
Methanol	198.73±1.68	49.98±1.48
n-Hexane	120.61±1.03	56.01±0.79
Ethyl-acetate	55.87±0.97	10.82±0.86
n-Butanol	24.31±0.60	25.36±1.07
Aqueous	34.86±1.09	8.36±0.44

Triplicate values are mentioned as mean±SEM.

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Table III. DPPH scavenging potential of *A. calamus* extracts.

Concentration (µg/mL)	Methanol	n-Hexane	Ethyl-acetate	n Butanol	Aqueous
100	21.38±0.11	19.57±0.16	20.77±0.28	11.21±0.07	15.18±0.09
200	30.60±0.08	28.56±0.09	26.69±0.26	22.51±0.18	16.52±0.05
300	42.26±0.14	38.49±0.08	28.63±0.11	32.67±0.12	18.66±0.12
400	50.52±0.24	46.59±0.14	39.24±0.09	42.20±0.07	26.36±0.03
500	68.49±0.05	72.38±0.06	61.62±0.27	54.43±0.15	48.31±0.09
IC50	346.70	359.80	475.70	472.20	676.40

Triplicate values are mentioned as mean±SEM.

Antioxidant potential of A. calamus *rhizome extract*

Table III shows the dose-dependent DPPH inhibitory activity of methanol, *n*-hexane, ethyl-acetate, *n*-butanol, and aqueous extracts. Among all extracts, methanol showed the highest antioxidant activity (IC50: 346.70 µg/mL) following *n*-hexane (IC50: 359.80 µg/mL), *n*-butanol (IC50: 472.20 µg/mL), ethyl-acetate (IC50: 475.70 µg/mL), and aqueous (IC50: 676.40 µg/mL).

Acute oral toxicity of extracts

Rats did not show any sign of physical or behavioral toxicity after oral administration of methanol and *n*-hexane extracts of *A. calamus*, and no mortality was recorded. This suggests that methanol and *n*-hexane extracts have lethal doses (LD50) above 2000 mg/kg.

Table IV. Ear edema inhibitory effects of *A. calamus* extracts observed in xylene test.

Groups	Dose (mg/kg)	Ear edema (mg)	Inhibition (%)
Control	-	7.91±0.45	-
Indomethacin	10	2.76±0.23[####]	65.11
Methanol	125	5.64±0.28[###]	28.69
	250	3.82±0.26[####]	51.70
	500	3.41±0.21[####]	56.89
n-Hexane	125	6.93±0.47[#]	12.38
	250	4.86±0.36[####]	38.56
	500	4.22±0.34[####]	46.64

Results are mentioned as mean±SEM, n = 6. [#]*P*<0.05, [###]*P*<0.001, [####]*P*<0.0001, significance from control group.

Anti-inflammatory effects of A. calamus *methanol and n-hexane extracts*

Table IV shows the dose-dependent anti-inflammatory effects of *A. calamus* methanol and *n*-hexane extracts against xylene-induced ear edema. The *A. calamus* methanol extract treatment of rats at doses of 250 and 500 mg/kg significantly (*P*<0.0001) reduced ear edema by 51.70% and 56.89% after 2 h, respectively, as compared to control group. Meanwhile, *n*-hexane extract at 250 and 500 mg/kg inhibited ear edema by 38.56% and 46.64%, respectively (*P*<0.0001). Indomethacin administration resulted in a 65.11% reduction 2 h after ear edema induction.

Table V showed that administration of methanol and *n*-hexane extracts of *A. calamus* at graded doses (125, 250, and 500 mg/kg) demonstrated dose-dependent anti-inflammatory effects on carrageenan-induced paw inflammation and maximum effects were observed at 6 h. Significant anti-inflammatory effects of both extracts appeared after 4 to 6 h of experiment. At 6 h of study, methanol extract significantly inhibited 24.26% paw inflammation as compared to 19.72% observed with *n*-hexane extract at 500 mg/kg dose. Whereas meloxicam showed 29.59% inflammation induced by carrageenan.

Anti-nociceptive effects of methanol and n-hexane extracts of A. calamus

Table VI shows the noteworthy anti-nociceptive potential against formalin-induced pain. Both extracts exhibited dose-related analgesia in early neurogenic and late inflammatory phases, particularly at 250 and 500 mg/kg doses. While aspirin demonstrated significant inhibition in both the early (14.58%) and late (55.02%) phases.

Table VII shows that the plant extracts at 125, 250, and 500 mg/kg doses showed significant dose-dependent and time-dependent increase in latency at different time intervals (30, 60, 90, and 120 min) in hot-plate test. Whereas diclofenac significantly inhibited nociception at all given time intervals. Moreover, the highest analgesic effects were observed after 60 min in rats administered methanol (111.71%) and *n*-hexane extracts (91.96%) at 500 mg/kg compared to diclofenac (131.80%).

Hypothermic activity of A. calamus

The anti-pyretic activities of *A. calamus* methanol and *n*-hexane extracts were determined by noticing the progressive decrease in the rectal temperature of all

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Table V. Paw inflammation inhibitory effects of *A. calamus* extracts assessed in carrageenan test.

Groups	Dose (mg/kg)	Paw inflammation (mm)				
		0 h	1 h	2 h	4 h	6 h
Control	-	5.46±0.21	6.81±0.29	7.64±0.36	8.38±0.33	8.82±0.21
Meloxicam	3	5.08±0.14	5.97±0.10 (12.33%)	6.39±0.27$^{\#}$ (16.36%)	6.20±0.31$^{\#\#\#\#}$ (26.01%)	6.21±0.23$^{\#\#\#\#}$ (29.59%)
Methanol	125	5.25±0.17	6.54±0.29 (3.96%)	7.11±0.24 (6.94%)	7.78±0.49 (7.16%)	7.61±0.32 (13.72%)
	250	5.16±0.18	6.22±0.20 (8.66%)	6.89±0.37 (9.82%)	6.91±0.13$^{\#}$ (17.54%)	7.32±0.42$^{\#\#}$ (17.01%)
	500	5.21±0.20	6.09±0.22 (10.57%)	6.42±0.18 (15.97%)	6.87±0.53$^{\#\#}$ (18.02%)	6.68±0.40$^{\#\#\#\#}$ (24.26%)
n-Hexane	125	5.65±0.14	6.41±0.25 (5.87%)	7.36±0.49 (3.66%)	7.80±0.44 (6.92%)	7.94±0.28 (9.98%)
	250	5.38±0.11	6.43±0.26 (5.58%)	7.13±0.24 (6.68%)	7.18±0.39 (14.32%)	7.72±0.23 (12.50%)
	500	5.39±0.15	6.27±0.21 (7.93%)	6.72±0.42 (12.04%)	7.07±0.23$^{\#}$ (15.63%)	7.08±0.54$^{\#\#}$ (19.72%)

Results are mentioned as mean±SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$, significance from control group.

Table VI. Pain inhibition activities of *A. calamus* extracts in rats determined in formalin test.

Groups	Dose (mg/kg)	Early phase (0-10 min)		Late phase (10-60 min)	
		Response (s)	Inhibition (%)	Response (s)	Inhibition (%)
Control	-	57.32±4.52	-	194.07±8.64	-
Aspirin	200	48.96±3.71$^{\#\#}$	14.58	87.30±6.48$^{\#\#\#\#}$	55.02
Methanol	125	57.11±2.87	0.00	160.53±8.70$^{\#\#}$	17.28
	250	55.63±2.76	2.94	149.94±5.24$^{\#\#\#\#}$	22.74
	500	51.80±2.09$^{\#}$	9.63	118.01±6.42$^{\#\#\#\#}$	39.19
n-Hexane	125	58.23±3.89	0.00	166.31±5.26$^{\#\#}$	14.30
	250	56.86±3.13	0.80	154.87±6.08$^{\#\#\#\#}$	20.18
	500	55.72±2.91	2.79	131.16±8.77$^{\#\#\#\#}$	32.41

Results are mentioned as mean±SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$, significance from control group.

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treated rats (Table VIII). Paracetamol used as standard drug exhibited a substantial reduction in temperature from the 1st to 4th h, while both extracts of *A. calamus* significantly reduced the temperature in a dose-dependent manner. The 500 mg/kg dose of methanol (75.08%) and *n*-hexane (69.98%) extracts showed remarkable decrease in rectal temperature of pyretic rats at 4 h of experiment, as compared to paracetamol (80.26%).

DISCUSSION

Allopathic medicines have shown incomplete efficacy in their actions, in addition to causing several side effects (Bindu *et al*., 2020; Kichloo *et al*., 2021; Stone *et al*., 2021). In this regard, medicinal plants are a popular option. Generally, they have been reported to be effective in the management of inflammatory disorders while exhibiting minimal toxicity (Parvin *et al*., 2023). Hence, different extracts of *A. calamus* rhizome were prepared and tested for their anti-inflammatory, anti-nociceptive, and anti-pyretic activities in this study to scientifically validate its folkloric usage.

The pharmacological activities of plants are mostly owing to the existence of phytochemicals, which are mainly derived from their secondary metabolism (Akpinar *et al*., 2023; Velázquez-Antunez *et al*., 2023). We observed that methanol, *n*-hexane, ethyl-acetate, *n*-butanol, and aqueous extracts of *A. calamus* rhizomes possess secondary

Table VII. Analgesic potential of *A. calamus* extracts against heat-induced nociception.

Groups	Dose (mg/kg)	Latency (s)				
		0 min	30 min	60 min	90 min	120 min
Control	-	9.31±0.44	9.57±0.64	8.71±0.40	9.07±0.39	8.36±0.64
Diclofenac	50	9.30±0.51	19.06±0.80$^{\#\#\#\#}$ (99.16%)	20.19±0.83$^{\#\#\#\#}$ (131.80%)	18.99±0.68$^{\#\#\#\#}$ (109.37%)	16.20±0.62$^{\#\#\#\#}$ (93.77%)
Methanol	125	8.59±0.30	12.12±0.67 (26.64%)	13.81±0.99$^{\#\#\#\#}$ (58.55%)	13.23±0.67$^{\#\#\#}$ (45.86%)	11.14±0.36$^{\#}$ (33.25%)
	250	9.03±0.38	14.77±0.70$^{\#\#\#\#}$ (54.33%)	16.09±0.85$^{\#\#\#\#}$ (84.73%)	15.38±0.71$^{\#\#\#\#}$ (69.57%)	12.77±0.89$^{\#\#\#\#}$ (52.75%)
	500	8.88±0.51	16.19±0.54$^{\#\#\#\#}$ (69.17%)	18.44±0.86$^{\#\#\#\#}$ (111.71%)	16.92±0.96$^{\#\#\#\#}$ (86.54%)	14.04±0.78$^{\#\#\#\#}$ (67.94%)
n-Hexane	125	9.17±0.40	11.64±0.72 (21.63%)	11.91±0.84$^{\#\#}$ (36.74%)	10.32±0.40 (13.78%)	10.70±0.72 (27.99%)
	250	8.61±0.30	12.43±0.71$^{\#}$ (29.88%)	15.39±0.89$^{\#\#\#\#}$ (76.69%)	14.19±0.56$^{\#\#\#\#}$ (56.44%)	12.19±1.21$^{\#\#\#}$ (45.81%)
	500	9.12±0.62	14.18±0.87$^{\#\#\#\#}$ (48.17%)	16.72±0.92$^{\#\#\#\#}$ (91.96%)	16.29±1.02$^{\#\#\#\#}$ (79.60%)	13.28±0.66$^{\#\#\#\#}$ (58.85%)

Results are mentioned as mean±SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$, significance from control group.

Table VIII. Anti-pyretic effects of *A. calamus* extracts in experimental hyperthermic rats.

Groups	Dose (mg/kg)	Temperature (°C)					
		-18 h	0 h	1 h	2 h	3 h	4 h
Control	-	37.23±0.10	39.31±0.09	39.34±0.12	39.39±0.16	39.33±0.17	39.34±0.10
Paracetamol	150	37.07±0.18	39.36±0.12	38.01±0.18$^{\#\#\#\#}$ (60.28%)	37.68±0.19$^{\#\#\#\#}$ (76.65%)	37.69±0.14$^{\#\#\#\#}$ (74.16%)	37.58±0.21$^{\#\#\#\#}$ (80.26%)
Methanol	125	37.32±0.14	39.27±0.11	38.70±0.19 (24.97%)	38.53±0.32$^{\#}$ (42.66%)	38.34±0.15$^{\#\#}$ (45.33%)	38.17±0.16$^{\#\#\#}$ (57.71%)
	250	37.11±0.11	39.36±0.08	38.81±0.25 (20.34%)	37.94±0.31$^{\#\#\#\#}$ (62.50%)	38.01±0.26$^{\#\#\#\#}$ (57.72%)	37.92±0.36$^{\#\#\#\#}$ (69.07%)
	500	37.13±0.15	39.44±0.12	38.43±0.35$^{\#}$ (45.79%)	37.90±0.10$^{\#\#\#\#}$ (67.83%)	37.79±0.29$^{\#\#\#\#}$ (70.85%)	37.76±0.23$^{\#\#\#\#}$ (75.08%)
n-Hexane	125	37.38±0.09	39.31±0.11	38.95±0.16 (17.78%)	38.81±0.22$^{\#}$ (25.40%)	38.68±0.24$^{\#}$ (31.35%)	38.64±0.17$^{\#}$ (30.58%)
	250	37.51±0.17	39.18±0.08	38.72±0.25 (25.31%)	38.18±0.27$^{\#\#\#}$ (61.54%)	37.93±0.26$^{\#\#\#\#}$ (73.32%)	38.04±0.13$^{\#\#\#}$ (68.84%)
	500	37.19±0.11	39.40±0.11	38.67±0.07 (34.69%)	38.15±0.19$^{\#\#\#}$ (57.60%)	38.11±0.09$^{\#\#\#}$ (59.99%)	37.91±0.24$^{\#\#\#\#}$ (69.98%)

Results are mentioned as mean±SEM, n = 6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$, significance from control group.

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metabolites including alkaloids, phenols, flavonoids, glycosides, tannins, and terpenoids. These phytochemicals are widely known for their ability to reduce inflammation. Also, the pharmacological actions are often attributable to the presence of antioxidants such as phenols and flavonoids (Ong *et al*., 2022). Our findings indicated that methanol and *n*-hexane extracts contained considerably high contents of phenols and flavonoids than the other extracts. These anti-oxidative phytochemicals found in *A. calamus* may be involved in pharmacological activities by various mechanisms, such as regulating nuclear factor kappa (NF-κB) signaling or inhibiting the COX2 enzyme (Laurindo *et al*., 2023).

Plants have a natural capacity to produce various antioxidants that can reduce ROS-induced oxidative stress. The antioxidant activity of plants might be attributed

to free radical scavenging, most likely *via* oxidation by peroxy radicals or hydrogen-holding ability (Maheshwari *et al*., 2022; Sindi *et al*., 2023). The DPPH assay findings in this study revealed that *A. calamus* methanol (IC50: 346.70 µg/mL) and *n*-hexane (IC50: 359.80 µg/mL) extracts had noteworthy antioxidant capacity, which could be correlated to the presence of phenolic and flavonoid components, indicating that these plant extracts might reduce inflammation linked oxidative stress (Aslam and Hussain, 2021; Wu *et al*., 2022).

Edema is the main indicator of the inflammatory state; hence, animal models of xylene-induced ear edema and carrageenan-induced paw inflammation are commonly employed for the assessment of anti-inflammatory potential of synthetic or natural agents (Patil *et al*., 2019). In xylene test, an injection of xylene into the ear of rat is thought to cause the induction of neurogenic edema. Substance-P is a peptide that is associated with both the central and peripheral neurological systems and plays a major role in neurogenic edema induction after xylene injection. Its release, along with other neuropeptides, in sensory neurons causes immune cells to produce cytokines, prostaglandins, and nitric oxide, consequently causing vasodilation and plasma extravasation. It establishes neurogenic ear edema (Soliman *et al*., 2023). The current investigation found that *A. calamus* extracts prevented xylene injection-induced ear edema in rats in a dose-dependent manner, with the maximum effects shown at 500 mg/kg of methanol (56.89%) and *n*-hexane (46.64%) extracts after 2 h of xylene administration.

Carrageenan induces paw inflammation in a biphasic process along with inflammatory cell infiltration and enhanced capillary permeability at the injection site. In early phase of edema induction, chemicals such as bradykinins, histamine, serotonin, and PGE2 are released. During the later phase, neutrophils infiltrate paw tissue and release PGE2, nitric oxide, and pro-inflammatory cytokines. Also, edematogenic chemicals, such as carrageenan, produce hyperalgesia in the affected tissue (Morris, 2003). Results revealed that carrageenan caused significant paw inflammation in rats, and peak inflammation was observed at the 6th h of experiment. *A. calamus* extracts demonstrated considerable edema inhibition in a dose-dependent manner. The maximum dose of methanol and *n*-hexane extracts at 500 mg/kg inhibited paw inflammation by 24.26% and 19.72%, respectively, compared to 29.59% inhibition of meloxicam. The substantial amounts of polyphenols found in plant extracts may be responsible for the anti-inflammatory action of *A. calamus* (Hussain *et al*., 2022; Zhao *et al*., 2023).

Pain is described as an unpleasant sensory and emotional response to chemical, mechanical, or thermal harm. Pain can be caused by neuropathic or inflammatory pathways. Formalin and heat (hot-plate) tests are commonly used to determine whether a test substance works *via* an inflammatory or neuropathic mechanism (Mogil, 2009). In current study, anti-nociceptive properties were assessed using formalin and heat nociception models.

In early neurogenic phase, formalin influences the production of bradykinins and tachykinins, which stimulate primary afferent nerve fibers and pain receptors. In late inflammatory phase, tissue harm is caused by the production of prostaglandins, serotonin, histamine, and other excitatory amino acids (Fischer *et al*., 2014; Hong *et al*., 2020). Results showed that only *A. calamus* methanol extract at 500 mg/kg dose reduced pain by 9.63% in early phase. In late phase, both plant extracts effectively decreased formalin-induced pain responses. Furthermore, methanol (39.19%) and *n*-hexane (32.41%) extracts showed considerable pain inhibition at high doses when compared to aspirin (55.02%). The hot-plate test gives useful information on the central analgesic efficacy of test drugs against partial tissue injury, as well as sensitivity to prospective analgesic compounds (Chy *et al*., 2021). In present study, it was found that *A. calamus* methanol and *n*-hexane extracts have dose-dependent analgesic efficacy. Our findings showed that methanol and *n*-hexane extracts of *A. calamus* had a significant dose-dependent pain-relieving effect in both tests, which is useful in demonstrating the anti-nociceptive responses of both extracts acting *via* central and anti-inflammatory mechanisms. The phytochemical profile might explain the discrepancies observed in the formalin test between methanol and *n*-hexane extracts, as alkaloids are the primary secondary metabolites associated with analgesia (Zhu *et al*., 2020) and were not present in *n*-hexane extract.

Fever is regarded as a physiological signal of the presence of an infectious entity and one of the most common symptoms of inflammation (Walter *et al*., 2016). In hypothalamus, cytokines (TNF-α, IL-1β, IL-6, and IL-8) stimulate the COX-2 enzyme to produce PGE2, which is considered a pyrexia regulatory factor. Microvascular endothelial cells in CNS are mainly responsible for the generation of PGE2 following stress. Moreover, stimulated endothelial cells, macrophages, and leukocytes in inflammation site also contribute to pyrexia (Blomqvist and Engblom, 2018). In pyrexia model, yeast induces pathogenic fever by producing and accumulating PGE2 in hypothalamus (Tomazetti *et al*., 2005). Our findings demonstrated that pyretic rats administered with methanol and *n*-hexane extracts at 500 mg/kg dose exhibited 75.08% and 69.98%, respectively, reduction in rectal temperature in comparison to 80.26% of paracetamol after 4 h of treatment. Both plant extracts of *A. calamus* rhizomes

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showed anti-pyretic efficacy in a dose-dependent manner, possibly *via* inhibiting COX-2 and subsequently lowering PGE2 levels in hypothalamus, which is the primary mode of action of most anti-pyretic medicines (Ahmad *et al*., 2017).

CONCLUSION

The findings provide information about the phytochemical composition and pharmacological properties of *A. calamus* rhizome. Phytochemical analysis revealed the existence of notable polyphenol contents in methanol and *n*-hexane extracts. Both plant extracts demonstrated potent *in vitro* antioxidant activities. Toxicity assessment confirmed the safe nature of each extract for oral use. Methanol and *n*-hexane extracts showed significant inhibition of edema in rat models of xylene and carrageenan, demonstrating dose-dependent anti-inflammatory activities. Similarly, *A. calamus* methanol extract exhibited considerable anti-nociceptive activity against nociception induced by formalin and heat when compared to *n*-hexane extract. Furthermore, a dose-dependent reduction in yeast-induced pyrexia was noticed in rats treated with plant extracts. Overall, results support the utilization of *A. calamus* rhizomes as an alternative option for the treatment of inflammation, pain, and fever.

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DECLARATIONS

Acknowledgments

The authors are thankful to the Higher Education Commission (HEC) of Pakistan for providing funds to conduct this study (Project ID: NRPU/R&D/7510).

IRB approval and ethical statement

All experimental methods were evaluated and approved by the Institutional Biosafety/Bioethical Committee, UAF Punjab, Pakistan, under letter no. 8166/ORIC.

Statement of conflict of interest

The authors have declared no conflict of interest.

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