ORIGINAL ARTICLE

Antinociceptive and anti-inflammatory activity of extract of *Acorus calamus* rhizomes in experimental animal models

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Abstract

Background: Complex biological responses like inflammation are part and parcel of body's immune system to any noxious stimuli. Mediators released during inflammation irritate the free nerve endings which are perceived by the body as pain. Hence, inflammation and pain often coexist and add up significant morbidity in humans. **Aims and Objectives:** The aims and objectives of the study are to evaluate the antinociceptive and anti-inflammatory activity of extract of *Acorus calamus* rhizomes in experimental animal models. **Materials and Methods:** Ethanolic extract of *A. calamus* rhizomes was prepared by Soxhlet extraction. Inflammation, both acute and chronic, was tested using carrageenan-induced paw edema and cotton pellet-induced granuloma model in rats whereas peripheral and central anti-nociceptive activity was tested using acetic acid-induced writhing in mice and tail immersion test in rats. **Results:** Significant reduction in writhing, paw edema volume, granuloma size, and increase in reaction time with tail immersion was observed with the extract treated groups in a dose-dependent manner. standard drug diclofenac was found to be better than the extract but failed to show significant difference with a higher dose of the extract. **Conclusion:** The ethanolic extract of *A. calamus* rhizome possesses significant antinociceptive and anti-inflammatory activity.

Key words: Acetic Acid, *Acorus calamus*, anti-inflammatory, antinociceptive, carrageenan, cotton pellet

INTRODUCTION

Inflammation is a complex process associated with the bodily response to any harmful stimuli. This response evoked by the immune system and through the elaboration of mediators helps in identifying and eliminating substances that are considered noxious and helps the body to reconcile by initiating tissue repair.[1]

The process of inflammation may vary from acute to chronic depending upon the response elicited by the immune cells in identifying the antigen. Rapid onset of symptoms lasting only for a short duration is the hallmark of an acute inflammatory process in which neutrophils and macrophages play a major role. The chronic inflammatory process is a late response that usually takes weeks which induces progressive changes in the affected area characterized by an alternating process of destruction and healing of the affected tissue.[2]

Even though inflammation is considered a defensive stimulus, at times, it becomes disorderly and itself becomes a disease. Pain is the sensory response to any injury with or without associated inflammation. The process of pain perception is complex and is usually associated with the response elicited by the free nerve endings toward the mediators of inflammation. Related to the process, pain may be the first symptom appreciated by the body in case of inflammation associated with tissue injury.

Non-steroidal anti-inflammatory agents (NSAIDs) are pivotal in controlling pain as well as inflammation. Unlike opioids, they do not produce dependence or depress the central

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nervous system. Corticosteroids by inhibiting phospholipase A2 halt the very first step of the formation of arachidonic acid and the down toward events produce a myriad of side effects by interfering with the production of constitutive enzymes necessary to maintain the physiological functions.[3,4]

Although NSAIDs are considered safe compared to opioids and steroids, they are not always without untoward effects. Gastrointestinal problems such as mucosal erosion, bleeding, and peptic ulcers restrict the use of NSAIDs and the search for a substitute is always ongoing.[5]

Traditional medicinal plants are nature's gift to earthly beings and can be considered as an answer for our bodily ailments. Adopted and practiced by systems such as Siddha and Ayurveda, they become a potential candidate for the search for an alternative to modern medicine.

Acorus calamus aka sweet flag is a perineal marshland plant native to central Asia and Eastern Europe and belongs to the *Acoraceae* family.[6] The rhizomes and leaves were valued for their antimicrobial, antioxidant, antispasmodic, insecticidal, anticancer, antiepileptic, and anti-asthmatic properties.[7] Although many of its inherent properties are already explored, scientific data regarding its actions on the inflammatory and nociceptive system are still unconvincing. Hence, the present study was undertaken to validate its anti-inflammatory and antinociceptive potential.

MATERIALS AND METHODS

Chemicals

Tramadol, diclofenac, and ibuprofen were purchased from the hospital pharmacy. Carrageenan, acetic acid, and ethanol were purchased from Sigma Aldrich, Bengaluru.

Ethics Approval

Ethical clearance was taken from the Institutional Animal Ethics Committee (IAEC) before the commencement of the study (Approval no: 10/IAEC/MG/08/2019).

Animals

Following approval from IAEC Swiss albino mice and Wistar albino rats weighing 20 ± 5 g and 180 ± 10 g, respectively, was procured from Tamil Nadu Veterinary and Animal Sciences University, Chennai and housed in the central animal house of Mahatma Gandhi Medical College and Research Institute 1 week before the initiation of the study for proper acclimatization to the laboratory environment. The animals were kept in polypropylene cages covered with steel mesh with dry paddy husk bedding. The rats were allowed for free access to rat chow and water *ad libitum*. The room temperature was maintained at 26 ± 2°C and 12:12 h dark and light cycle (8.00 am–8.00 pm) with a relative humidity of 45–50%. Animals were cared according to guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

Collection of Plant Material and Extraction

The fresh rhizomes of *A. calamus* were purchased from Tanjore district, Tamil Nadu, in the month of August and were authenticated by a botanist from local science college. The rhizomes were then cleaned thoroughly, shade dried, and later pulverized into coarse powder using a mechanical grinder and were stored in sealed containers. Ethanolic extract of *Acorus calamus* (EEAC) rhizome was prepared with 80% ethanol using a Soxhlet apparatus.[8] About 45 g of the coarse powder was subjected for continuous extraction at a temperature of 55–70°C which yielded a dark brown mass which was concentrated by evaporation using a hot oven at 45°C. The final yield was about 17% (~7 g) per cycle.

Acute Toxicity Test and Dose Selection

Before determining the actual dose, a pilot study was conducted to ascertain the non-toxic nature of the extract. Adult Wistar albino rats in a group of 3 with 3 animals in each were tested at a dose of 500, 1000, and 2000 mg/kg. The animals were continuously monitored for any behavioral changes and signs of toxicity for the first 24 h and then occasionally for the next 72 h.

With no changes observed, it is evident that the extract is non-toxic even at 2000 mg/kg. A dose of 150 mg/kg and 250 mg/kg p.o. was fixed for the study.

Anti-Nociceptive Activity

Antinociceptive activity of *A. calamus* extract was evaluated using acetic acid-induced writhing and tail immersion test.

Peripheral Antinociceptive Activity

The peripheral antinociceptive activity of *A. calamus* extract was tested using acetic acid-induced writhing in mice.[9] Writing is a reflexive response exhibited with arching of back, contraction of abdominal muscles, and extension of hind limb when an irritant is injected into the peritoneal cavity. It was induced using acetic acid solution (0.6%) at a dose of 10 mL/kg injected intraperitoneally (i.p.) into all the animals, 30 min after administration of vehicle, extract and the standard drug via oral gavage (p.o.). 24 Swiss albino mice of either sex was randomly divided into 4 groups with 6 mice in each (n = 6). Group 1 which served as normal control received normal saline. Group 2 and Group 3 received the EEAC at a dose of 100 mL/kg and 200 mL/kg, respectively. Group 4 received

diclofenac at a dose of 10 mg/kg served as standard control. In all the groups, the number of contractions of the abdominal muscles together with extension of hind limb was recorded for 30 min following 5 min after administration of acetic acid. Analgesic activity which is expressed as percentage inhibition was calculated using the formula below:

$$\%Protection = \frac{N_c - N_t}{N_c} \times 100$$

Were,
N_c – Writhing's observed in control (normal and standard)
N_t – Writhing's observed in test groups (EEAC 100 and EEAC 200).

Central Anti-Nociceptive Activity

The central antinociceptive activity of *A. calamus* extract was tested using tail immersion test as described by Aydin *et al*.[10] 24 adult Wistar albino rats of either sex were randomly divided into 4 groups with 6 rats in each group ($n = 6$). Group 1 being normal control received normal saline, Groups 2 and 3 received EEAC at 100 and 200 mg/kg, and Group 4 which served as standard control received tramadol at 12.5 mg/kg i.p. Rats were placed in the restrainer in such a way that their tails hang out freely. The distal part of the tail (5 cm) was marked with a permanent marker and was immersed in a beaker containing warm water not exceeding 55°C. The reaction time (i.e., complete withdrawal of distal end of tail following immersion in water) was noted after 60 min following administration of the extract. The maximum cut-off time was limited to 20 s to prevent possible tissue damage.

Anti-Inflammatory Activity

Anti-inflammatory activity of *A. calamus* was evaluated using carrageenan-induced paw edema and cotton pellet-induced granuloma model.

Carrageenan Induced Paw Edema

The acute anti-inflammatory activity of *A. calamus* extract was assessed using carrageenan-induced paw edema model.[11] 24 adult Wistar albino rats of either sex were divided into 4 groups with 6 rats in each. Group 1 which received normal saline served as normal control. Groups 2 and 3 received EEAC at 100 and 200 mg/kg p.o. Group 4 which served as standard control received ibuprofen at 100 mg/kg. 30 min after administration of control, extract, and standard drug orally, the animals were injected with 0.1 mL of 1% carrageenan suspended in 0.9 mL of normal saline into the subplantar region of the left hind paw. The paw volume of all the rats was measured after 1 h and thereafter 2nd h up to 6 h following carrageenan injection using a digital plethysmograph. Anti-inflammatory activity was expressed in terms of percentage inhibition of paw edema between control and test animals which is given by the formula:

$$\frac{Control\ mean - Treated\ mean}{Control\ mean} \times 100$$

Cotton Pellet Induced Granuloma

This method is widely used in studying the exudative and proliferative phases which is characteristics of chronic inflammation. The method of Hicks was adopted wherein under ether anesthesia, sterile cotton pellets weighing 7 ± 1 mg were inserted subcutaneously through a skin incision in the dorsal region of the rat, one in each axilla.[12] Vehicle, EEAC, and standard drug were administered 2 h following recovery from anesthesia and continued for 7 consecutive days. All the groups received the same volume of preparation once a day p.o. Group 1 which received normal saline served as normal control. Groups 2 and 3 received EEAC at 100 and 200 mg/kg. Group 4 which served as standard control received ibuprofen at 100 mg/kg. All the animals were sacrificed on the 8th day using carbon dioxide euthanasia chamber and the granulomatous cotton pellet was removed and dried at 50°C for 24 h. The weight of the granuloma thus formed was calculated by deducting the initial weight from the final dry weight of the cotton pellets. Percentage protection was calculated using the formula:

$$\frac{Control\ mean - Treated\ mean}{Control\ mean} \times 100$$

Statistical Analysis

Data were collected as master chart in Microsoft Excel 2019 and analyzed using the Jeffrey's Amazing Statistics Program software. Results were expressed as Mean ± SEM. One-way analysis of variance followed by *post hoc* Bonferroni test was used to analyze the data. The $P < 0.05$ was considered statistically significant.

Table 1: Effect of *Acorus calamus* extract on acetic acid-induced writing response in mice

Group	Dose	No. of writhes	% writhing inhibition
Normal control	Vehicle	39.50±1.02	0
EEAC 150	150 mg/kg	22.83±1.01**	42.2
EEAC 250	250 mg/kg	20.50±0.43**	48.1
Standard control	Diclofenac 10 mg/kg	17.17±1.47**	56.5

Results were expressed in Mean±SEM. (n=6) *significant P-value (<0.05) with one-way analysis of variance (ANOVA) followed by *post hoc* test Bonferroni when compared with control. **Significant P-value (<0.001) with one-way ANOVA followed by *post hoc* test Bonferroni when compared with control. EEAC: Ethanolic extract of *Acorus calamus*

Table 2: Effect of *Acorus calamus* extract on tail immersion test for nociception in rats

Group	Tail flick latency in seconds (Mean±SEM)			
	0	30 min	60 min	90 min
Normal control	3.82±0.21	4.83±0.28	5.83±0.37	5.50±0.39
EEAC 150	3.93±0.30	6.83±0.55*	9.17±0.28**	7.17±0.28**
EEAC 250	3.93±0.14	8.50±0.39**	13.83±0.55**	12.17±0.55**
Standard control	4.13±0.21	15.83±0.55**	14.33±0.38**	13.50±0.39**

Results were expressed in Mean±SEM. (*n*=6), *significant *P*-value (<0.05) with one-way analysis of variance (ANOVA) followed by *post hoc* test Bonferroni when compared with control. **Significant *P*-value (<0.001) with one-way ANOVA followed by *post hoc* test Bonferroni when compared with control. EEAC: Ethanolic extract of *Acorus calamus*

Table 3: Effect of *Acorus calamus* extract on Cotton pellet-induced granuloma in rats

Group	Dose	Weight of the cotton pellet (mg)	% Inhibition
Normal control	Vehicle	63.50±1.18	-
EEAC 150	150 mg/kg	44.67±1.50**	29.65
EEAC 250	250 mg/kg	32.83±1.01**	48.29
Standard control	Diclofenac 10 mg/kg	29.33±1.31**	53.81

Results were expressed in Mean±SEM. (*n*=6), *significant *P*-value (<0.05) with one-way analysis of variance (ANOVA) followed by *post hoc* test Bonferroni when compared with control. **Significant *P*-value (<0.001) with one-way ANOVA followed by *post hoc* test Bonferroni when compared with control, EEAC: Ethanolic extract of *Acorus calamus*

RESULTS

Effect of *A. calamus* Extract on Acetic Acid-Induced Writing Response in Mice

Ethanolic extract of *A. calamus* rhizome at both doses showed a significant reduction in the writhing response as compared to the control.

EEAC at 150 and 250 mg/kg was able to significantly reduce the writhing response in the test animals in a dose-dependent fashion. The percentage inhibition, 42.2 and 48.1 of EEAC at both doses was found to be statistically significant ($P < 0.05$) when compared to normal control.

Among the extract-treated groups, EEAC at 200 mg/kg exhibited the maximum inhibition. Better than the extracts diclofenac was found to be better in inhibiting the writhing but failed to show a significant difference when compared to EEAC at a higher dose [Table 1].

Effect of *A. calamus* Extract on Tail Immersion Test for Nociception in Rats

Ethanolic extract of *A. calamus* showed a significant increase in the tail flick latency time at the end of 30, 60, and 90 min as compared to the normal control group.

The group which severed as standard control showed a graded increase in the reaction time at the end of 30, 60, and 90 min which was highly significant ($P < 0.001$) when compared to the control group.

The group which received EEAC at 150 mg/kg showed a significant ($P < 0.05$) increase in the latency time at 30 min and showed high significance ($P < 0.001$) at the end of 60 and 90 min when compared to the control.

Group treated with EEAC at 250 mg/kg exhibited increased latency time at the end of 30, 60, and 90 min which was highly significant ($P < 0.001$) as compared to the control.

Maximum tail-flick latency was observed with EEAC at 250 mg/kg among the test groups and the results were comparable with that of the standard [Table 2].

Effect of *A. calamus* Extract on Cotton Pellet-Induced Granuloma in Rats

The mean weight of the cotton pellet-induced granuloma from the vehicle-treated group was 63.5 ± 1.18 mg. The ethanolic extract of the *A. calamus* at both doses showed a significant dose-dependent reduction in the weight of granuloma induced by the cotton pellet as compared to the control.

The reduction in the weight of cotton pellet-induced granuloma at both the doses of the extract was found at 29.65 and 48.29%. The standard drug diclofenac was found to be better in controlling inflammation and producing significant ($P < 0.001$) results when compared to the control (53.81%).

However, the decrease in inflammation by EEAC at 250 mg/kg was comparable to the standard, which reduced the weight of cotton pellet granuloma by 89.75% [Table 3].

Effect of *A. calamus* Extract on Carrageenan-Induced Paw Edema in Rats

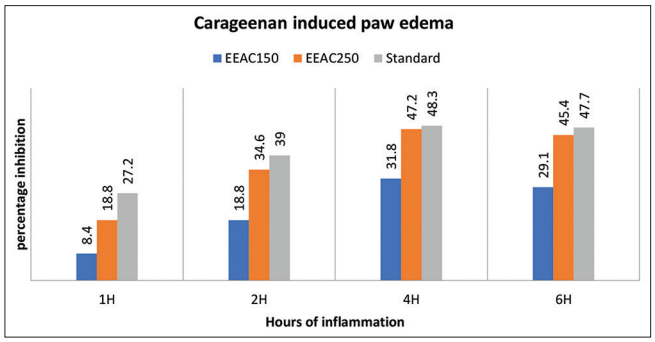
Following plantar injection of carrageenan, there was a progressive increase in the paw volume up to 4 h and started

Table 4: Effect of Acorus calamus extract on carrageenan-induced paw edema in rats

Group	Paw edema at various time intervals in mL			
	1 h	2 h	4 h	6 h
Normal	0.357±0.016	0.382±0.005	0.422±0.008	0.388±0.010
EEAC150	0.327±0.007	0.310±0.010**	0.288±0.010**	0.275±0.007**
EEAC250	0.290±0.006*	0.250±0.008**	0.223±0.008**	0.212±0.010**
Standard	0.260±0.012**	0.233±0.007**	0.218±0.005**	0.203±0.006**

Results were expressed in Mean±SEM. (n=6) *significant P-value (<0.05) with one-way analysis of variance (ANOVA) followed by *post hoc* test Bonferroni when compared with control. **Significant P-value (<0.001) with one-way ANOVA followed by *post hoc* test Bonferroni when compared with control

Group	Percentage inhibition			
	1 h	2 h	4 h	6 h
Normal	-	-	-	-
EEAC150	8.40	18.8	31.8	29.1
EEAC250	18.8	34.6	47.2	45.4
Standard	27.2	39.0	48.3	47.7



to decrease at the end of 6 h. These results clearly show that maximal inflammation will be evident 4 h following administration of carrageenan.

EEAC at 150 mg/kg showed highly significant results only after 2 h following the administration of carrageenan and maintained its action by progressively reducing the paw volume when compared to the control.

EEAC at 250 mg/kg showed a significant ($P < 0.05$) reduction in paw edema volume at the end of 1st h and became highly significant ($P < 0.001$) in the 2nd, 4th, and 6th h when compared with the control. EEAC at 250 mg/kg was found to be better among the extract groups and did not show a significant difference from that of the standard drug ibuprofen.

The anti-inflammatory potential of a drug is generally expressed in terms of percentage inhibition of inflammation. In our study, the percentage inhibition values were highest with the standard drug ibuprofen whereas the EEAC at 150 mg/kg possessed moderate inhibition at all measured hours of inflammation. EEAC at 250 mg/kg produced significant inhibition only after the 2nd h and thereafter exhibited similar results at the 4th and 6th h compared to the standard [Table 4].

DISCUSSION

In the present study, the antinociceptive and anti-inflammatory activity of *A. calamus* extract was evaluated using experimental animal models.

Peripheral and central antinociceptive activity was evaluated using acetic acid-induced writhing and hot water tail immersion methods, respectively.

Acute pain is induced by intraperitoneal injection of acetic acid which causes severe irritation of the peritoneum in mice leading to a stereotypic response called writhing.

Writhing is characterized by episodes of arching of the abdomen with stretching of the hindlimbs which is a result of stimulation of the peripheral nociceptive neurons by mediators like bradykinin, serotonin, prostaglandin (PGE), and capsaicin.[13]

Following the administration of acetic acid, the number of writhes elicited by the mice is noted for 30 min. The percentage inhibition with different doses of the extracts and the standard drugs was calculated. Percentage reduction in the writhing response indicated the level of analgesia produced by the test/standard drug.[14]

In our study, ethanolic extract of *A. calamus* rhizomes at both doses i.e., 150 and 250 mg/kg produced a significant reduction in the writhing response. The higher dose of the extract was found to be better and was comparable to that of the standard.

Central analgesic activity tested using the hot water tail immersion method showed a significant increase in the tail flick latency period following administration of the extract and the standard. A higher dose, i.e., 250 mg/kg was found to be better and produced no significant difference from the standard.

The anti-inflammatory activity of EEAC was tested using the carrageenan-induced paw edema model. Carrageenan is an inflammatory agent widely used to induce acute inflammation. It is usually administered below the plantar aponeurosis of the hind paw and being a phlogistic agent it induces severe inflammation and edema.[9,15]

Ethanolic extract of the *A. calamus* at both doses produced a significant reduction in paw edema volume as measured using a digital plethysmograph. The extract at a higher dose produced a highly significant reduction and a relatively higher percentage of inhibition when compared to its lower dose and the control group.

Inflammation-induced by carrageenan is biphasic[16] wherein the first phase which is observed within the 1st h of administration is due to the release of mediator histamine, serotonin, and kinins. The 2nd phase that follows up is related to the release of prostanoids, interleukins, and tumor necrosis factor-alpha (TNFα).[1,17] The extract at both doses was active only after the 1st h, suggesting that they can inhibit only the 2nd phase of carrageenan-induced inflammation.

The cotton pellet-induced granuloma model induces inflammatory granuloma, which is a typical feature of chronic inflammatory reaction. Subscapular implantation of compressed cotton pellets promotes an inflammatory response which is used to assess the transudative, exudative, and proliferative phases of chronic inflammation.[18] Accumulation of fluids and proteinaceous material along with the infiltration of macrophages, neutrophils, and fibroblasts accounts for the wet weight whereas dry weight represents the amount of granulation tissue.[19-21]

In our study, administration of EEAC significantly reduced inflammation which was evident by the reduction in the dry weight of the cotton pellet. Percentage protection produced by the higher dose of EEAC (48.29%) was comparable to the standard drug ibuprofen (53.81%).

From the above results, it is evident that the ethanolic extract of *A. calamus* possesses significant analgesic action by acting both centrally on the higher centers (opioid-like action) and peripherally by suppressing inflammation.

Previous study findings showed that volatile oil of *A. calamus* rhizome such as elemicin, linalool, and isoeugenol in inhibit transient receptor potential cation channels, especially the voltage-activated calcium channel that is associated with the sensory neurons.[22] A study conducted by Saldanha *et al.* on alpha Asarone (α-Asarone), a prime phytochemical constituent of the rhizome showed significant central antinociceptive action which was abolished by naloxone.[23] The possible mechanism behind it might be partly by elevation of pain threshold and partly by the inhibition of central pain receptors.[9] Other possible mechanism includes facilitation of gamma-aminobutyric acid-mediated attenuation of pain signals at the level of spinal cord or the higher centers.[24]

Inflammation as a result of the release of cytokines, TNFα, interleukin 2, nitric oxide, and PGE is effectively inhibited by the administration of the extract. Previous studies showed similar results wherein it is concluded that *A. calamus* rhizome is effective in controlling both acute and chronic inflammation, and this activity is attributed to the presence of the phytochemical eugenol, p-cymene, and α-asarone.[25,26]

P-cymene and α-asarone are potent anti-inflammatory agents which exert its action by suppressing inflammatory cell infiltration, TNFα production, inducible nitric oxide synthase expression, and release of arachidonic acid metabolites, especially the PGE.[23-27] By controlling inflammation, they indirectly produce analgesic action mainly by obtundation pain signals at the free nerve endings. Furthermore, the presence of antioxidants such as flavonoids, tannins, and triterpenoids augments the anti-inflammatory action by reducing the reactive oxygen species and scavenging the free radicals.[7]

CONCLUSION

From this study, it is evident that the rhizome extract of *A. calamus* showed remarkable analgesic action and protection against inflammation and its sequalae in a dose-dependent fashion wherein the results of the higher dose of 250 mg/kg were comparable with that of the standard. This activity may be attributed to the presence of phytoconstituents such as α-asarone, eugenol, p-cymene, elemicin, and flavonoids in the extract. Further detailed clinical research may be required before potential use in humans.

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