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ORIGINAL ARTICLE

Anti-inflammatory and Analgesic Potential of *Amorphophallus commutatus* var. *wayanadensis* and its Inhibitory Effect on Inflammatory Mediators in Lipopolysaccharide-Stimulated Macrophages

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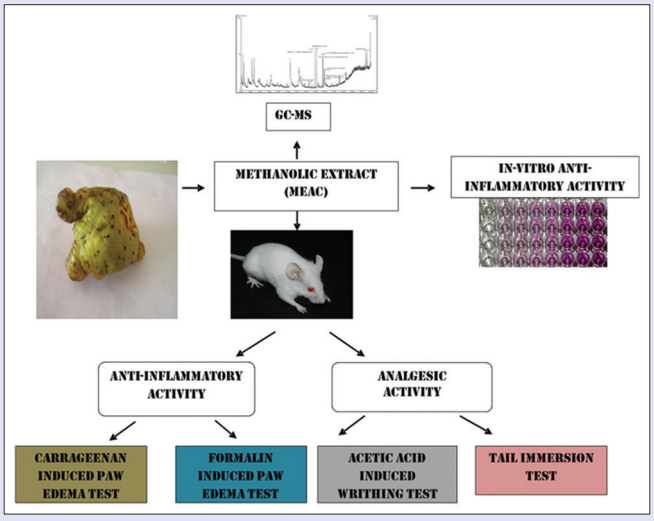
ABSTRACT

Background: An edible tuber named *Amorphophallus commutatus* var. *wayanadensis* (ACW) is used by the local ethnic communities of Wayanad, India, for hemorrhoids while the health benefits remain unexplored to the scientific community. **Objectives:** Hence, our study was performed to screen the anti-inflammatory and analgesic activities of ACW. **Materials and Methods:** Screening for the *in vitro* anti-inflammatory potential on isolated peritoneal macrophages was performed using nitrite assay, nitroblue tetrazolium assay, tumor necrosis factor (TNF)-α production, and cyclooxygenase (COX) enzyme activity. Carrageenan- and formalin-induced paw edema assays were performed to determine anti-inflammatory potential, while tail immersion assays and acetic acid-induced writhing assays were carried out to explore antinociceptive activity in animal models. **Results:** Bio-molecular mechanistic investigation to evaluate the *in-vitro* anti-inflammatory activity confirmed the suppressive effect of MEAC on TNF-α, nitric oxide and COX-2 on lipopolysaccharide stimulated peritoneal macrophages, which benchmarked ACW as a potent medicinal plant against inflammation. Further, the efficacy of methanolic extract of *A. commutatus* var. *wayanadensis* (MEAC) as an anti-inflammatory agent in murine anti-inflammatory models was demonstrated by formalin- and carrageenan-induced paw edema assays. Administration of MEAC significantly increased the tail flicking latency in mice and also showed prominent reduction in the number of writhes induced by acetic acid, which establishes the real time application of ACW as an analgesic agent. **Conclusion:** The vital information regarding *in vitro* and *in vivo* action of MEAC provided scientific evidence for its traditional usage for hemorrhoids. Thus, this herbal medicine of ethnic tribes can be translated toward modern medicine for health maintenance and disease prevention.

Key words: *Amorphophallus commutatus var. wayanadensis,* analgesic activity, anti-inflammatory activity, cyclooxygenase inhibitor, phagocytic modulation, tumor necrosis factor-α inhibitor

SUMMARY

- *Amorphophallus commutatus* var. *wayanadensis* (ACW) (family: Araceae) is traditionally used by local medical practitioners for treating external inflammation and inflammation-related diseases like hemorrhoids. Studies on plants collected from unexplored geographical regions are a key to understand the biochemistry of them and also provide a great impact on discovery of novel drug and nutraceuticals. These tubers are the least explored class for their bioactivity. The results of our study confirmed that ACW has significant anti-inflammatory and analgesic activity providing scientific evidence for its traditional usage for hemorrhoids. This herbal medicine of ethnic tribes can be translated toward modern medicine for health maintenance and disease prevention. It is always need of the hour to bring the pharmaceutical properties of these unexplored plants to the science world.



Abbreviations used: ACW: *Amorphophallus commutatus* var. *wayanadensis;* MEAC: Methanolic extract of *Amorphophallus commutatus* var. *wayanadensis*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NIST: National Institute of Standards and Technology; NO: Nitric oxide; NBT: Nitroblue tetrazolium; TNF-α: Tumor necrosis factor-α; LPS: Lipopolysaccharide; COX: Cyclooxygenase; ELISA: Enzyme-linked immunosorbent assay; PBS: Phosphate-buffered saline; RPMI 1640: Roswell Park Memorial Institute 1640; GC-MS: Gas chromatography–mass spectrometry.

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INTRODUCTION

Inflammation is the first line of defensive mechanism of the body against invading microorganisms, tissue injury, or foreign substances. However, prolonged acute inflammation often leads to a chronic condition of tissue damage. Chronic inflammation results in various inflammation-mediated diseases, including rheumatoid arthritis,[1] atherosclerosis,[2] cancer,[3] and diabetics.[4] Infiltration of immune cells is the major characteristic of chronic inflammation. Pro-inflammatory mediators and the reactive species generated by activated immune cells play a vital role in inflammatory pathogenesis. Inflammatory macrophages and their mediators have a substantial role in the progression of numerous inflammatory ailments.[5] Nonsteroidal anti-inflammatory drugs used as medication for inflammatory diseases are associated with side effects, including gastrointestinal complications, high blood pressure, ulceration, and bleeding, but their long-term use leads to cardiovascular and renal complications.[6] Hence, there is always a need for novel anti-inflammatory compounds with lesser side effects from natural sources.[7]

Amorphophallus, a typical tuberous plant with high nutritive value and health benefits, is distributed evenly in the tropical and subtropical regions.[8] Tuber extracts of *Amorphophallus* have been commonly used as food source and drug in traditional medicinal systems for treating various diseases.[9] Tubers of *Amorphophallus commutatus* var. *wayanadensis* (ACW) are among the major herb used by the local folks of Wayanad district, India. ACW, commonly known as "kattuchena," is an endemic tuberous plant of Western Ghats, India, and has been extensively used by local communities for hemorrhoids.[10] Even though 170 species of *Amorphophallus* have been distributed in tropical countries, still now most of the species are unexplored for their pharmaceutical properties and many are in the verge of extinction due to natural calamities and encroachments. This wild edible plant has significant relevance in traditional therapies and food security of the local communities. Edible plants are widely explored for their therapeutic as well as nutritional properties. Hence, it is literally impractical to discriminate between food and medicine. Previously, we reported the antibacterial,[11] hepatoprotective,[9] and immunomodulatory activity[12] of ACW. Toxicity studies of methanolic extract of *A. commutatus* var. *wayanadensis* (MEAC) confirmed the safety profile to be used in animals.[13] Even though ACW has been used by traditional medicinal practitioners for treating various inflammatory disorders such as hemorrhoids, till date, there is no scientific validation for these ethnobotanical claims. By taking the abovementioned facts into consideration, the objectives of our study were to explore the anti-inflammatory and antinociceptive potential of ACW and evaluate the mechanism underlining the observed activity.

MATERIALS AND METHODS

Materials and chemicals

Collection of ACW tubers from Wayanad, Kerala, India, and its processing was carried out based on the protocols mentioned in our previous study[9,12] and deposited at the Calicut University Herbarium, Kozhikode, India, with the sample code RIA 62. Indomethacin was obtained from Micro Labs Ltd., Bangalore.

Extraction

A methanol-based soxhlet-extracted dried tuber of ACW[9] was filtered using Whatman filter paper (No. 1) and evaporated in vacuum pressure using a rotary flask evaporator. The methanolic extract in the form of a dark blackish-brown extract was stored until use in an air-tight container at 4°C. The dried tuber methanolic extract was redissolved in dimethyl sulfoxide (0.1%) used for *in vitro* activity assays.

Animals

For the present study, Swiss albino mice were procured and acclimatized as previously described.[12] *In vivo* experiments were performed according to the VIT University Animal Ethical Committee (Reference No. VIT/IAEC/VII/15)-approved protocols. Mice in Groups II and III were treated with MEAC at 200 mg/kg and 400 mg/kg, respectively. Control group (Group I) mice were administered with 0.9% saline (25 ml/kg). Positive control group (Group IV) received indomethacin (20 mg/kg).

In vitro anti-inflammatory activity

Peritoneal macrophage isolation and culture

Elicited peritoneal macrophages were isolated from mice by intraperitoneal injection of thioglycollate medium. Mice were intraperitoneally (ip) injected with 3% Brewer's thioglycollate medium. After 5 days, the elicited peritoneal macrophages were separated by ravaging with Roswell Park Memorial Institute-1640 medium. The macrophages were washed and the erythrocytes were separated by centrifugation and lysed using erythrocyte lysis buffer. The peritoneal macrophages thus obtained were cultured as described previously by Lu and Varley.[14]

Nitrite assay

Griess assay was conducted to estimate the inhibitory action of MEAC on nitric oxide (NO) produced by the lipopolysaccharide (LPS)-stimulated peritoneal macrophages.[15] Less stable NO is rapidly oxidized to nitrite in the culture medium, and concentration of nitrite was used to determine the levels of NO produced by the macrophages. LPS (2 μg/ml)-stimulated macrophage cells were seeded in a microtiter plate and treated with various concentrations of MEAC and incubated for 24 h. After incubation, 100 μl of Griess reagent was added to 100 μl of the supernatant, and after 10 min incubation, the reading was taken at 540 nm in a microtiter plate reader (BioTek, USA). Calibration curve was generated using sodium nitrite to determine the amount of NO produced by the macrophage cells.

Cell viability

Viability of the macrophages was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.[16] Briefly, MTT reagent (10 μl) was added to the wells after removing the supernatant for nitrite assay and incubated for 4 h. After incubation, 100 μl of solubilizing solution was added to the wells and mixed gently. A microtiter plate reader (BioTek, USA) was used to read the absorbance at 517 nm.

Nitroblue tetrazolium assay

Nitroblue tetrazolium (NBT) reduction assay evaluated the phagocytic modulation of the isolated macrophages as described by Rainard.[17] In brief, 20 μl of peritoneal macrophage cells (1×10^6 cells/ml) was added to a microtiter plate and treated with various concentrations of MEAC in phosphate-buffered saline (PBS), and the wells treated with PBS alone served as control and the microtiter plates were incubated in a CO_2 incubator. The absorbance was measured at 570 nm using a microplate reader. Phagocytic modulation was expressed as percentage NBT reduction from the given formula: NBT reduction (%) = (Optical Density (OD)$_{Control}$ − OD$_{Sample}$)/OD$_{Control}$ × 100.

Tumor necrosis factor-α production

TNF-α production was determined by treating LPS (2 μg/ml) stimulated peritoneal macrophages with or without the extract. The supernatant of the cells was used to screen the inhibition potential of MEAC on the TNF-α production using Cayman TNF-α human enzyme-linked immunosorbent assay (ELISA) kit.

Cyclooxygenase enzyme activity assay

The potential of MEAC to inhibit the enzymatic activity of cyclooxygenase (COX)-2 and COX-1 was evaluated using Cayman COX colorimetric activity assay kit. Briefly, peritoneal macrophage cells (1×10^6 cells/ml) were added to microtiter plate with various concentrations of MEAC (50–200 μg/ml) in PBS. The peroxidase activity of COX enzyme was used to determine the COX activity of the cell lysate. The peroxidase activity of the enzyme was estimated by measuring the formation of N, N, N′, N′-tetramethyl-p-phenylenediamine. Selective inhibitors were used to estimate the specific COX inhibition activity. Percentage COX inhibition was calculated according to the absorbance at 590 nm using ELISA plate reader (BioTek, USA).

In vivo anti-inflammatory activity

Carrageenan-induced paw edema method

Carrageenan-induced paw edema assay was performed to evaluate the acute anti-inflammatory activity as previously described by Gong *et al.*[18] with slight modifications. The experimental animals were grouped as described earlier. One percent carrageenan (0.05 ml) was subcutaneously injected into the hind paw of the mice after 30 min of drug treatment. The paw volumes were determined plethysmographically at 0, 1, 2, 3, and 4 h after the injection.[19] The percentage inhibition of paw edema was determined to estimate the anti-inflammatory potential of MEAC using the formula:

% Inhibition = (myocardial infarction [MI] [control]– MI [treated])/MI (control) ×100

where MI (control) was the mean increase of paw size of control and MI (treated) was the mean increase of paw size of treated group.

Formalin-induced paw edema method

Formalin-induced anti-inflammatory activities of MEAC were determined according to the protocol previously described by Firdous *et al.*[20] The experimental animals were grouped as described earlier. Thirty minutes after the respective treatment, 0.05 ml of 1% formalin solution was injected into the subplantar region of the right hind paw. The paw edema was measured using plethysmometer at 0, 1, 2, 3, and 4 h. The anti-inflammatory potential of MEAC was estimated by calculating the percentage inhibition (%I) of paw edema using the formula:

% Inhibition = (MI [control]–MI [treated])/MI (control) ×100

where MI (control) was the mean increase of paw size of control and MI (treated) was the mean increase of paw size of treated group.

In vivo analgesic activity/antinociceptive activity

Acetic acid-induced writhing assay

Peripheral antinociceptive activity of MEAC was determined by performing acetic acid-induced writhing assay as described by Wang *et al.*[21] The experimental animals were grouped as described earlier. Thirty minutes after the treatments, animals were ip injected with acetic acid solution (0.6%) at a dosage of 10 ml/kg to induce writhes. The number of muscular cramps or writhes was computed for 30 min and analgesic activity was represented as the total number of writhes in 30 min, and the values were compared with standard drug indomethacin.[22]

Tail immersion assay

The central analgesic activity was evaluated as described by Boakye-Gyasi *et al.*[23] The animals were screened and chosen based on the flicking of tail within 10 s after dipping the tail in hot water (55°C ± 0.5°C). The experimental animals were grouped as described earlier. The response time was calculated as the time taken to flick the tail when immersed in hot water. The analgesic activity of MEAC was evaluated by measuring the tail-flicking latency at 30, 60, 90, and 120 min after treatment, and the results were expressed as the response time in seconds.

Phytochemical analysis

Preliminary phytochemical screening of MEAC was done to determine the major phytoconstituents using simple quantitative tests by Trease and Evans.[24] Gas chromatography–mass spectrometry (GC-MS) analysis of MEAC was performed with Shimadzu SH-Rxi-5Sil MS using Elite-WAX column to determine the accurate mass and ion fragments. The compounds were separated using column (30.0 m, 0.25 mmID, 0.25 μm df). The column was held at initial temperature 70°C and ramped to 300°C at 10°C per min and was held for 10 min. Transfer line and GC-MS were kept at 280°C. The samples were injected at 250°C with a split ratio of 10:1 using helium as carrier gas with a flow rate of 1.20 ml/min. The spectrums of the eluted compounds were compared to the National Institute of Standards and Technology library to identify the compounds present in the extract.

Statistical calculation

All the experimental data were expressed as mean ± standard deviation. statistically significant differences were considered if $P < 0.05$, and one-way analysis of variance was calculated using GraphPad Prism software (GraphPad, USA).

RESULTS

In vitro anti-inflammatory activity

Nitrite assay

When macrophages were stimulated with LPS, higher NO production (23.4 ± 0.75 μM) was observed compared to the unstimulated cells (7.19 ± 2.09 μM). MEAC exhibited dose-dependent inhibition on NO production by the activated peritoneal macrophages [Figure 1a] which was determined by Griess method. To determine the impact of MEAC on cell viability, cytotoxicity assay was performed. As shown in Figure 1b, cells treated with different concentrations of MEAC had no effect on the viability. Therefore, it was confirmed that the cytotoxicity of MEAC has no role in the MEAC-induced inhibitory effect on NO production.

Nitroblue tetrazolium assay

Phagocytic modulation of MEAC was determined by NBT assay using murine peritoneal macrophages stimulated by LPS. Macrophage cells treated with MEAC showed a dose-dependent decrease in the NBT reduction [Figure 2a]. MEAC treatment significantly inhibited the phagocytic activity in murine peritoneal macrophages.

Tumor necrosis factor-α production

The effect of MEAC on the macrophage TNF-α secretion was determined by ELISA method. Low levels of TNF-α were secreted by the unstimulated macrophages. As shown in Figure 2b, cells stimulated with LPS showed a drastic increase in the TNF-α production. TNF-α production of stimulated macrophages was significantly inhibited with MEAC treatment.

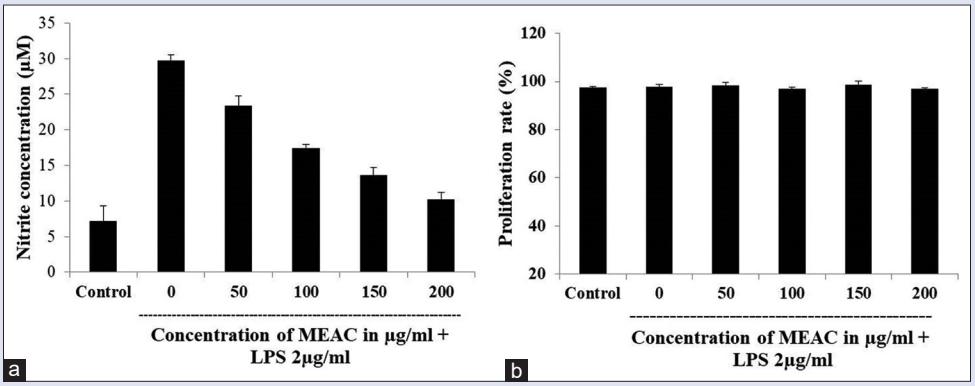

Figure 1: Anti-inflammatory activity of methanolic extract of *Amorphophallus commutatus* var. *wayanadensis* on (a) Nitric oxide production and (b) cell viability in lipopolysaccharide activated peritoneal macrophages. All values are represented as Mean ± Standard deviation (*n*=3)

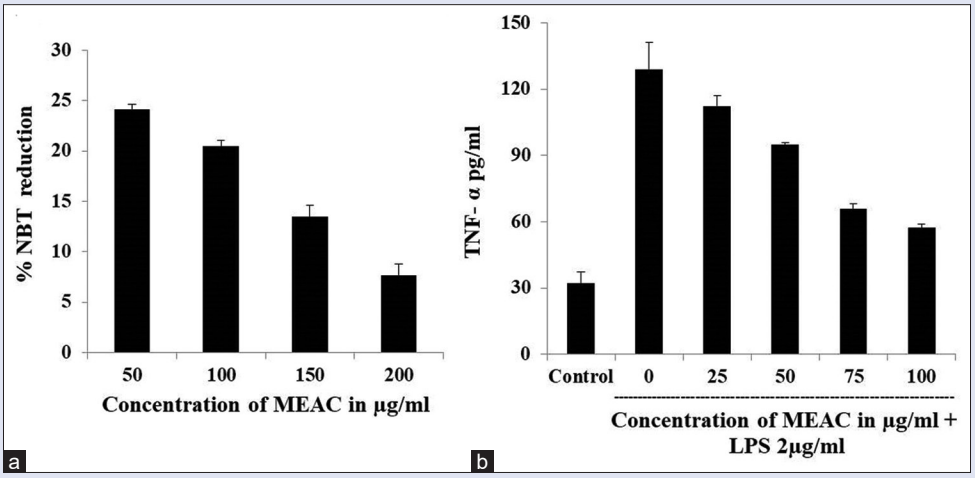

Figure 2: Anti-inflammatory activity of methanolic extract of *Amorphophallus commutatus* var. *wayanadensis* on (a) phagocytic modulation and (b) the release of tumor necrosis factor-α in lipopolysaccharide activated peritoneal macrophages. All values are represented as Mean ± Standard deviation (*n*=3)

Cyclooxygenase enzyme activity assay

Interestingly, the treatment with MEAC significantly inhibited the COX-2 enzyme activity without inhibiting COX-1 enzyme activity. Treatment with MEAC (200 µg/ml) showed 88% inhibition on COX-2 enzyme [Figure 3a]. As shown in Figure 3b, MEAC even at higher concentrations did not exhibit any inhibition on COX-1 activity.

In vivo anti-inflammatory activity

Carrageenan-induced paw edema method

Maximum edema was observed after 3 h of carrageenan injection, while pretreatment with the MEAC and indomethacin significantly decreased the paw edema [Figure 4a]. After 4 h of carrageenan injection, MEAC showed inhibition of 41.39% and 69.21% at 200 and 400 mg/kg, respectively [Table 1]. Similarly, indomethacin (20 mg/kg) showed 64.03% inhibition of paw edema. Better anti-inflammatory activity was exhibited by MEAC at 400 mg/kg.

Formalin-induced paw edema method

As shown in Figure 4b, formalin-induced paw edema was significantly reduced in the animal models pretreated with MEAC. MEAC at 400 mg/kg exhibited 42.11% and 60.48% inhibition of paw edema at later (1 h) and delay (3 h) phases of formalin-induced inflammation [Table 2].

Table 1: Carrageenan-induced anti-inflammatory activity of methanolic extract of *Amorphophallus commutatus* in mouse models

Groups (mg/kg)	Percentage inhibition (h)			
	1	2	3	4
MEAC (200)	27.54±1.22*	34.91±3.58*	41.04±2.49*	41±3.53*
MEAC (400)	46.20±2.44*	58.67±1.19*	63.37±1.65*	69.20±2.31*
Indomethacin (20)	47.04±2.83*	46.96±2.87*	61.80±2.22*	64.03±1.80*

All values are represented as Mean ± Standard deviation, (n=6). * $P < 0.05$, Values compared to control group. MEAC: Methanolic extract of *Amorphophallus commutatus*; SD: Standard deviation

In vivo analgesic activity

Acetic acid-induced writhing assay

Significant reduction in the acetic acid-induced writhes was observed when the mice were administered with MEAC at 400 mg/kg compared with control group. MEAC showed analgesic activity with a maximum inhibitory effect of 52.85% at the dose of 400 mg/kg [Figure 5a]. Higher inhibition percentage

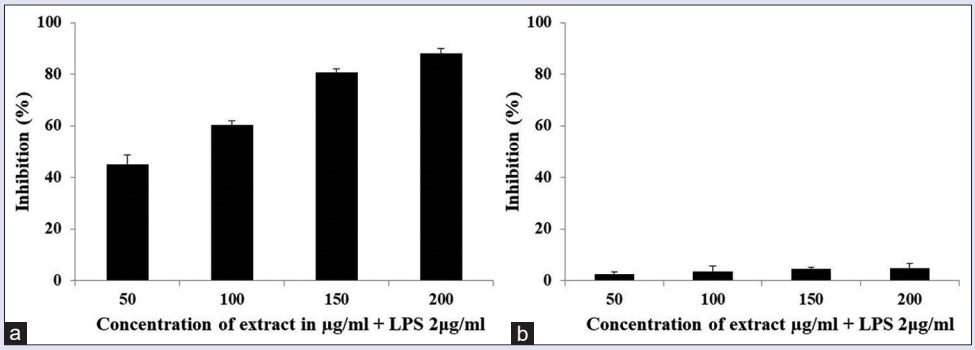

Figure 3: Anti-inflammatory activity of methanolic extract of *Amorphophallus commutatus* var. *wayanadensis* on (a) cyclooxygenase-2 and (b) cyclooxygenase-1 activity in lipopolysaccharide activated peritoneal macrophages. All values are represented as Mean ± Standard deviation (*n*=3)

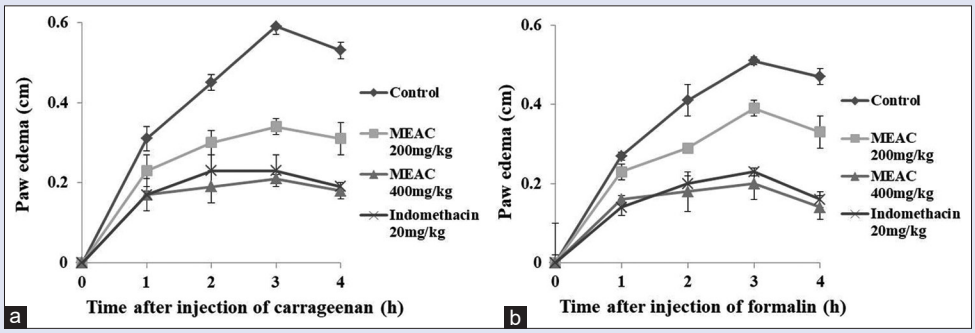

Figure 4: Anti-inflammatory activity of methanolic extract of *Amorphophallus commutatus* var. *wayanadensis* on induced paw edema models (a) carrageenan and (b) formalin. Data are the mean ± standard deviation (*n* = 6). All values are represented as Mean ± Standard deviation, (*n* = 6)

was observed in MEAC (400 mg/kg) pretreated group than indomethacin (20 mg/kg)-treated group.

Tail immersion assay

The central antinociceptive activity of MEAC was evaluated by tail immersion assay. Oral administration of MEAC increased the latency to flick tail compared to indomethacin group [Figure 5b]. Pretreatment with MEAC showed a reduction in the pain sensation caused by immersion of tail in warm water. MEAC exhibited maximum analgesic activity at 90 min, and a gradual decrease was observed at 120 min after administration.

Phytochemical characterization

Phytosterols, lipids, polyphenols, terpenoids, tannins, and glycosides were identified as the major phytocompounds in MEAC. Spectroscopic analysis of MEAC confirmed that the presence of phytosterols and fatty acids has the major bioactive compound in the extract [Table 3] and the chromatogram is shown in Figure 6.

DISCUSSION

ACW has been traditionally used by local medical practitioners for hemorrhoids and inflammation.[10,25] Corns of ACW are considered as a boon for peoples suffering from hemorrhoids and are reported to be effective and safe for reducing the symptoms of inflammation and pain associated with hemorrhoids. Although the corns of ACW have potential ethnopharmacological claims, there is no scientific validation of the traditional claims. The findings of this study provide the first scientific evidence for the antinociceptive and anti-inflammatory activities of ACW and the possible mechanism underlying the bioactivities.

Macrophages are key components in the initiation, promotion, and resolution of inflammatory responses. They are activated by various

Table 2: Formalin-induced anti-inflammatory activity of methanolic extract of *Amorphophallus commutatus* in mouse models

Groups (mg/kg)	Percentage inhibition (h)			
	1	2	3	4
MEAC (200)	15.31±3.81*	28.14±1.02*	35.59±1.24*	40.3±1.22*
MEAC (400)	42.11±1.35*	57.83±2.56*	60.48±1.41*	5.98±3.16*
Indomethacin (20)	47.51±1.21*	52.64±1.05*	56.66±1.15*	54.99±1.38*

All values are represented as Mean ± Standard deviation, (*n* = 6). * $P < 0.05$, Values compared to control group. MEAC: Methanolic extract of *Amorphophallus commutatus*; SD: Standard deviation

Table 3: Phytochemical characterization of methanolic extract of *Amorphophallus commutatus* by gas chromatography- mass spectrometry analysis

Compound name	RT	Area percentage
Lauric acid	17.97	13.03
Palmitic acid	18.31	17.60
Methyl 9,12-hexadecadienoate	19.66	12.14
Linoleic acid	19.95	05.79
Campesterol	28.87	12.21
Stigmasterol	29.14	14.66
Sitosterol	29.86	40.86

RT: Retention time

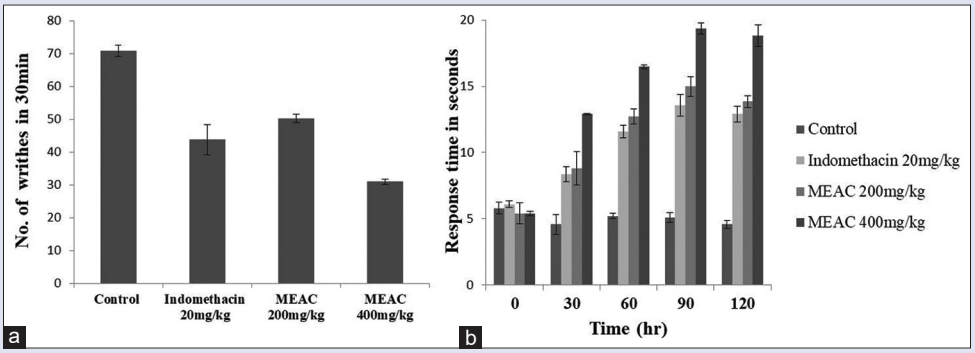

Figure 5: Analgesic activity of methanolic extract of *Amorphophallus commutatus* var. *wayanadensis* on animal models (a) writhing test (acetic acid) and (b) tail immersion test. All values are represented as Mean ± Standard deviation, ($n = 6$)

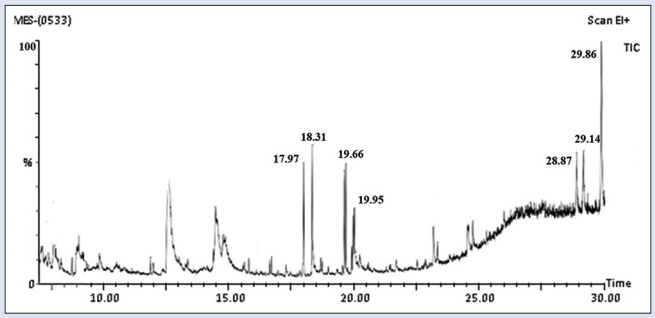

Figure 6: Gas chromatography–mass spectrometry spectrum of methanolic extract of *Amorphophallus commutatus* var. *wayanadensis*

cytokines, LPS, and chemical mediators leading to inflammatory responses and are deactivated by anti-inflammatory cytokines produced by the macrophages. Hence, macrophages and their mediators play a crucial role in the autoregulated inflammatory responses.[26] Therefore, bioactive compounds targeting macrophages and their mediators can be used for treating various inflammatory disorders. NO is an important mediator involved in the defensive mechanism of the body against tumor cells and infections caused by bacteria, viruses, and parasites.[27] Excessive production of NO is considered as an implication of inflammatory responses.[28] Our study confirmed that MEAC acts as a NO inhibitor on peritoneal macrophages. Cell viability studies on the peritoneal macrophages confirmed that cytotoxicity of MEAC has no role in the MEAC-induced inhibitory effect on NO production. The inhibitory effect of ACW on NO production reflects the significant role of MEAC in modulating NO signal transduction pathway. Effect of MEAC on the production of superoxide anion was determined by NBT assay on activated macrophages. NBT assay is a simple quantitative method for screening anti-inflammatory compounds by measuring the respiratory burst activation. A decrease in the NBT reduction represents lesser production of superoxide anion, reflecting the inhibitory effect on phagocytic modulation.[17] COX enzyme catalyzes prostaglandin biosynthesis pathways in which they exist as isozyme, COX-1, and COX-2. COX-2 is an inducible enzyme associated with inflammation, while COX-1 is a housekeeping enzyme which is involved in various biological functions. COX-2 is a vital enzyme in inflammation that mediates the formation of prostaglandins from arachidonic acid. Novel anti-inflammatory drugs with selective COX-2 inhibition provide the most effective therapeutic approach against inflammation-mediated disorders.[29] The results demonstrated that MEAC selectively inhibited the COX-2 enzyme without inhibiting the COX-1 activity indicating MEAC as a selective COX-2 inhibitor, which might be attributed to the anti-inflammatory and antinociceptive activity of ACW. The involvement of COX pathway in the anti-inflammatory activity of MEAC was clearly demonstrated in our study. TNF-α is a major cytokine synthesized by activated macrophages in response to inflammatory stimuli and has a significant role in the pathogenesis of various inflammatory disorders.[30] Inhibiting activity of MEAC on the TNF-α production confirmed its substantial role in the observed anti-inflammatory activity of ACW. ACW exhibited dose-dependent phagocytic modulation and significant reduction in the levels of NO and TNF-α and inhibited the COX-2 activity which can be considered as a benchmark for treating inflammatory diseases.

Paw edema induced by carrageenan is considered as a typical experiment to evaluate acute inflammation in animal models.[31] Inflammation induced by carrageenan is considered as biphasic, inflammatory mediators such as kinins, histamine, and serotonin are released during the early phase of inflammation. Pro-inflammatory cytokines such as interleukins, TNF-α and prostaglandins are mainly involved in the later phase of inflammatory response.[32] Administration of MEAC showed extensive anti-inflammatory activity against carrageenan-induced inflammation. Our study has demonstrated the inhibitory activity of MEAC on COX enzyme, thus leading to the inhibition of prostaglandin synthesis, which might be attributing to anti-inflammatory activity of MEAC on carrageenan-induced acute inflammatory models. Formalin induces peripheral tissue inflammation, which is also a biphasic response with an earlier neurogenic phase followed by delayed phase releasing pro-inflammatory factors, including prostaglandins leading to inflammation and pain.[33] According to Abubakar *et al.*,[34] endogenous mediators such as histamine and bradykinins are released during cellular damage induced by formalin in acute inflammation models. Phlogistic agent-induced edema is a widely established animal experimental setup to screen the anti-inflammatory activity of drugs.[35] MEAC exhibited better anti-inflammatory activity in later phases in formalin-induced inflammatory models. Our studies demonstrated that MEAC has potential anti-inflammatory activity in animal models, suggesting that the inhibitory action of MEAC on COX and other inflammatory mediators might be attributing to the activity which was supported by the *in vitro* anti-inflammatory results.

Inflammation is a complex mechanism often associated with pain. The MEAC showed significant analgesic activity on both central (tail immersion) and peripheral (writhing) analgesic assays in mouse models. The acetic acid-injected abdominal constriction is considered as classic model to evaluate the peripheral antinociceptive activity of drug. Intraperitoneal injection of irritants like acetic acid releases arachidonic acid which mediates the biosynthesis of prostaglandins,

effector molecules for inflammatory pain. Prostaglandins sensitize the local nociceptive receptors in the peritoneal cavity causing abdominal contractions and stereotypical behavior in the animals.[33] Our study validated that MEAC significantly reduced acetic acid-induced writhes. The tail immersion assay is a classical method used to demonstrate the central analgesic activity mediated by spinal reflexes involving opioid receptors.[36] The results of the tail immersion test indicated that the opioid receptor-mediated mechanism might be also involved in the analgesic activity of the MEAC. From the results, it can be established that MEAC is endowed with significant peripheral and central analgesic activity validating the traditional use of tubers of ACW for pain-related diseases. The inhibitory effect of MEAC on COX-2 activity might be responsible for the observed analgesic activity. Prostaglandin biosynthesis is a major regulator of nociception and inflammation. Inhibitory activity of phytocompounds on prostaglandin biosynthesis pathway might be accountable for the observed anti-inflammatory and analgesic activity of MEAC.

Phytosterols and fatty acids were identified as the primary bioactive compounds present in MEAC. Several studies have reported the anti-inflammatory and antinociceptive potential of fatty acids and phytosterols. Phytosterols extracted from plants exhibit significant anti-inflammatory activity on inflammation models.[37] Fatty acids are considered as essential nutrients with significant pharmacological properties.[38] Our results demonstrated that the MEAC rich in fatty acids and phytosterols exhibited substantial anti-inflammatory and antinociceptive activity, thus correlating the traditional use of this tuber for hemorrhoids.

CONCLUSION

We have scientifically illustrated the biomedical benefits of ACW. MEAC exhibited dose-dependent phagocytic modulation without affecting the viability of macrophages while inhibiting the levels of TNF-α and COX-2 enzyme activity without effecting COX-1 activity. ACW exhibited anti-inflammatory and antinociceptive activity which is comparable to the standard drug indomethacin in murine models. The attracting data with the pharmacological studies re-enforced the traditional claim of this tuberous plant for hemorrhoids. The anti-inflammatory and antinociceptive activity through inhibiting COX enzyme to avert the production of prostaglandins provided a promising platform for treating inflammatory disorders. It is to be noted that our study is the prior effort taken to screen the analgesic and anti-inflammatory activity of ACW, validating its ethnopharmacological claims.

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Conflicts of interest

There are no conflicts of interest.

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