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COMPARISON OF ANALGESIC EFFECTS OF 2-BENZOXAZOLINONE AND STANDARDIZED SUPERCRITICAL FLUID EXTRACT OF *ACANTHUS ILICIFOLIUS* ON ANIMAL MODELS OF PAIN AND MUSCULAR HYPERALGESIA

AR. Saranya [*1], T. Ramanathan [2], KS. Kesavanarayanan [3] and D. Deepika [1]

Department of Biomedical Sciences [1], Alagappa University, Karaikudi - 630003, Tamil Nadu, India.
Centre of Advanced Study in Marine Biology [2], Faculty of Marine Sciences, Annamalai University, Parangipettai - 608502, Tamil Nadu, India.
Department of Pharmacology [3], College of Pharmacy, Hail University, Hail, Saudi Arabia.

Keywords:

Acanthus ilicifolius, 2-benzoxazolinone, Alkaloid, musculoskeletal pain, Hyperalgesia

Correspondence to Author:
Dr. A. R. Saranya

Teaching Assistant,
Department of Biomedical Sciences,
Alagappa University, Karaikudi - 630003, Tamil Nadu, India.

E-mail: drarsaranya@gmail.com

ABSTRACT: The objective of this study was to evaluate the analgesic properties of supercritical fluid extracts of *Acanthus ilicifoliu*s leaves (SCFE-AI) and Benzoxazolinone (BOA) using the acetic acid-induced writhing method, formalin-induced paw lick assay, and carrageenan-induced muscle hyperalgesia. Results demonstrated that SCFE-AI and BOA exhibited potent dose-dependent analgesic activity in all tested models of analgesia. Administration of BOA (30 mg/kg) and SCFE-AI (200 mg/kg) at their maximum dose showed significant peripheral analgesic activity ($p<0.05$). Oral administration of BOA or SCFE-AI dose-dependently decreased the number of formalin-induced first-phase and second-phase paw licking times in mice. BOA inhibited cold allodynia and mechanical hyperalgesia in a dose-dependent manner, and it was even better than the standard drug. Our results justify the traditional use of *A. ilicifolius* in the treatment of various diseases associated with pain, such as fibromyalgia.

INTRODUCTION: Fibromyalgia syndrome (FMS) is one of the most common disorders affecting the muscles, manifesting with pain, stiffness, and tenderness of the muscles, tendons, and joints [1]. Patients with FMS commonly experience chronic, widespread musculoskeletal pain associated with fatigue, nonrestorative sleep, cognitive dysfunction, and mood disturbances [2]. It is the second most common rheumatologic disorder, behind osteoarthritis.

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Up to 8% of the general global population has been estimated to be affected by FMS, with a higher prevalence in women [3]. The precise etiology and pathophysiology of FMS remain unknown, although some evidence suggests that FMS involves abnormal levels of serotonin (5-HT) and norepinephrine (NE), which are key neurotransmitters in endogenous pain inhibitory pathways [4].

Altered 5-HT and NE levels result in aberrant neuro-chemical processing of sensory signals in the CNS, thus lowering the threshold of pain and amplification of normal sensory signals, causing constant pain [5]. A balance in regulating human NE and 5-HT reuptake inhibition is required for pain management [3]. Current treatment for FMS is focused on reducing the disease symptoms while

improving overall well-being. Therefore, a combination of anti-inflammatory compounds, antidepressants, and antiseizure medications is used for FMS treatment [6]. Milnacipran, a serotonin-norepinephrine reuptake inhibitor (SNRI), is approved by the US FDA for treating FMS. However, chronic use of this drug has been linked to adverse side effects such as nausea, insomnia, headaches, and dizziness [7]. Therefore, new and improved therapeutics are needed to improve patient compliance to treat FMS. So, it is worth searching for novel analgesic compounds from plant sources to treat FMS.

Acanthus ilicifolius Linn. (Acanthaceae) is a true mangrove species that belongs to the family of Acanthaceae. It is commonly known as holy leaf *Acanthus*, sea holly, and holy mangrove. It is a small shrub with spiny leaves and is widely distributed on the south-east coast of India. It is popularly recognized for its secondary metabolites and its traditional usage in Indian and Chinese systems of medicine. Different parts of the plant have been used in folkloric medicine for the treatment of asthma, diabetes, dyspepsia, hepatitis, leprosy, neuralgia, paralysis, ringworm, rheumatism, skin diseases, snakebite, stomach pains, leucorrhoea, and leukemia [8]. Tea brewed from the leaves relieves pain and purifies blood [5]. Extracts of *A. ilicifolius* were reported to have various biological activities, including anti-inflammatory [9] and anti-nociceptive [10].

The traditional medicinal uses, chemical constituents, and biological activities of *A. ilicifolius* were previously reviewed by our team [11]. The leaves of *A. ilicifolius* contain 2-benzoxazolinone (BOA) as a major bioactive principle [12]. BOA and its structural analogs have been widely investigated for their analgesic, anticonvulsant, hypnotic, skeletal muscle relaxant, and CNS depressant activities [13, 14]. Uses of benzoxazinoids-containing cereals were reported to possess anti-inflammatory, and analgesic properties and to get relief from fibromyalgia [15]. Since *A. ilicifolius* contains benzoxazinoids- like bioactive principles, we hypothesize that it may have a possible beneficial effect in ameliorating musculoskeletal pain. Interestingly, our *in-silico* reports indicate that *A. ilicifolius* derived BOA and a few of its derivatives possess a dual inhibitory effect on hSERT/hNET activity [16]. The results obtained from computational studies may trigger interest in unravelling the therapeutic effect of a mangrove plant, *A. ilicifolius* Linn., for the management of musculoskeletal pain using suitable preclinical animal models. To the best of our knowledge, there are no reports concerning the use of standardized supercritical extracts of *A. ilicifolius* and BOA for muscular hyperalgesia. The present study was therefore undertaken to investigate the analgesic effects of BOA and SCFE-AI using acetic acid-induced writhing responses, formalin-induced paw lick assays, and Carrageenan-induced muscular hyperalgesia.

MATERIALS AND METHODS:

Collection of Plant and Supercritical Fluid Extraction: The fresh leaves of *A. ilicifolius* were collected from Pichavaram Mangrove Forest, Tamil Nadu, India. After cleaning, the leaves were shade-dried and made into a coarse powder using a pulverizer. Dried leaf powder of *A. ilicifolius* was extracted by supercritical fluid extraction (SCFE) under optimized CO_2 conditions [17].

Experimental Animals: Male Swiss albino mice (20-25g) were purchased from the Central Animal House, Raja Sir Muthiah Medical College and Hospital, Faculty of Medicine, Annamalai University, India. The animals were housed under standard laboratory conditions and fed a standard diet and water ad libitum. All studies were performed according to the Ethical Guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA). A prior approval (Reg.No.160/1999/CPCSEA; Proposal No. 985) was obtained from the Animal Ethics Committee of Raja Sir Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamil Nadu, India.

Experimental Design: The three doses (50, 100, and 200 mg/kg, *p.o.*) of SCFE-AI and BOA (10, 15, and 30 mg/kg, *p.o.*) were selected for *in vivo* studies. The animals were randomly divided into nine groups of six mice in each group. In the group, I was the normal control and received Saline + 0.5% CMC (10 ml/kg, *p.o.*). Group II was vehicle control and received 0.5% CMC (10 ml/kg, *p.o.*). Group III is a positive control, receiving the

standard drug Milnacipran (40 mg/kg, *p.o.*). Groups IV, V, and VI received plant extract SCFE-AI (50, 100, and 200 mg/kg, *p.o.*, respectively). Groups VII, VIII, and IX received BOA (10, 15, and 30 mg/kg, *p.o.*, respectively).

Anti-Nociceptive Activity:

Formalin-induced Paw Lick Test: The formalin-induced paw licking assay was performed according to the method reported previously [18] with slight modifications. The animals received their treatments orally, as mentioned in the above experimental design. After 45 min of oral treatment, 25μL of 0.5% formalin was injected subcutaneously under the dorsal surface of the left hind paw. Observations started immediately, and nociception was measured based on the time spent licking in periods of 0-5 minutes (early phase) and 15-30 minutes (late phase). Percent inhibition was calculated by using the formula

$$\text{Percent inhibition} = (1-T_t/T_c) \times 100$$

Where, T_c and T_t represent the average time spent licking by the control and treated groups, respectively.

Acetic Acid-induced Writhing Test: This test was conducted as per the method described by Koster *et al* in 1959 [19]. Separate groups of animals were pretreated with respective treatments as described in the experimental design orally for 30 mins before intraperitoneal administration of 0.6% acetic acid. After 5 minutes, the analgesic activity of SCFE-AI and BOA was assessed by counting the number of muscular contractions (writhing) for 10 mins. The percent reduction in the writhing experiment was calculated using the formula.

$$\text{Percent inhibition of writhing} = (1-W_t/W_c) \times 100$$

Where, W_c and W_t represent the average number of writhings produced by the control and treated groups, respectively.

Muscular Hyperalgesia:

Carrageenan-induced Muscular Hyperalgesia:

Experimental Design: As mentioned above, nine groups of animals were used similarly for the muscular hyperalgesia behavioural testing.

Induction of Muscular Hyperalgesia: The hairs on the skin covering the right lateral gastrocnemius muscle were clipped using a hair depilatory a day before injection. Except for group I, all other groups were injected with 1% carrageenan (50 μL) into the right lateral belly of the gastrocnemius muscle [20]. Injections were made with a 1 ml syringe with 10μL increments attached to a 28-G hypodermic needle.

All the above procedures were performed under light ether anaesthesia. Carrageenan was injected when the animal no longer reacted to a toe pinch. The animals received their respective treatments orally, immediately after carrageenan administration.

Behavioural Test: The behavioural testing was performed 45 min after their respective treatments. Each animal was given a brief resting period of 15 mins before subjecting them to the next behavioural test.

Acetone Drop Test: The cold allodynic pain sensitivity of the right hind paw was assessed using the acetone drop method for assessing the reactivity to non-noxious cold chemical stimuli [21]. The mice were placed on top of a wire mesh grid, allowing access to the hind paws. Acetone (0.1 ml) was sprayed onto the plantar surface of the right hind paw of mice without touching the skin. A cold stimuli-sensitive reaction concerning paw licking, shaking, or rubbing the injected hind paw was observed and recorded as paw lifting duration for 30 seconds as the maximum cut-off period.

Pinprick Test: Mechanical hyperalgesia was assessed by the pinprick test [22]. Mice were placed under plastic chambers on a mesh-top table and allowed to acclimate for 2-3 minutes. A 27-gauge needle was gently applied to the plantar surface of the hind paw without breaking the skin, with 30 seconds as the maximum cut-off period. A response was defined as lifting, shaking, or licking the hind paw. The duration of the withdrawal response was recorded with a stopwatch.

Statistical Analysis: Values were expressed as the Mean ± Standard Error of the Mean (SEM). The differences between the groups were calculated using either One-way analysis of variance (ANOVA) or two-way ANOVA with Tukey's multiple tests. Values of $p<0.05$ were considered statistically significant.

RESULTS:

Formalin-induced Paw Lick Test: The antinociceptive profile of SCFE-AI and BOA at different concentrations was assessed using the formalin test in mice. The results were shown in **Fig. 1.** The initial nociceptive scores normally peaked at 0-5 min (early phase) and 15-30 min (late phase) after formalin injection, representing the neurogenic and inflammatory pain responses, respectively. The SCFE-AI and BOA significantly and in a dose-dependent manner exhibited noticeable inhibition of the time spent on licking in both the first and second phases of formalin-induced nociception in mice. Interestingly, higher doses of BOA (30mg/kg) showed complete abolishment of the early phase, as indicated by the absence of paw licking after 15 mins of the formalin injection. However, the standard drug Milnacipran (40mg/kg) exhibited a complete reduction of paw licking at 25 mins. The effect of BOA at 30 mg/kg was better than that of the standard drug and SCFE-AI.

At the maximum oral dose of SCFE-AI (200 mg/kg) and BOA (30 mg/kg), the percent reduction of paw lick at 20 mins was 87 % and 100 %, respectively, as compared to the control group, whereas the standard drug Milnacipran (10mg/kg) showed a reduction of 95 % when compared to vehicle-treated animals.

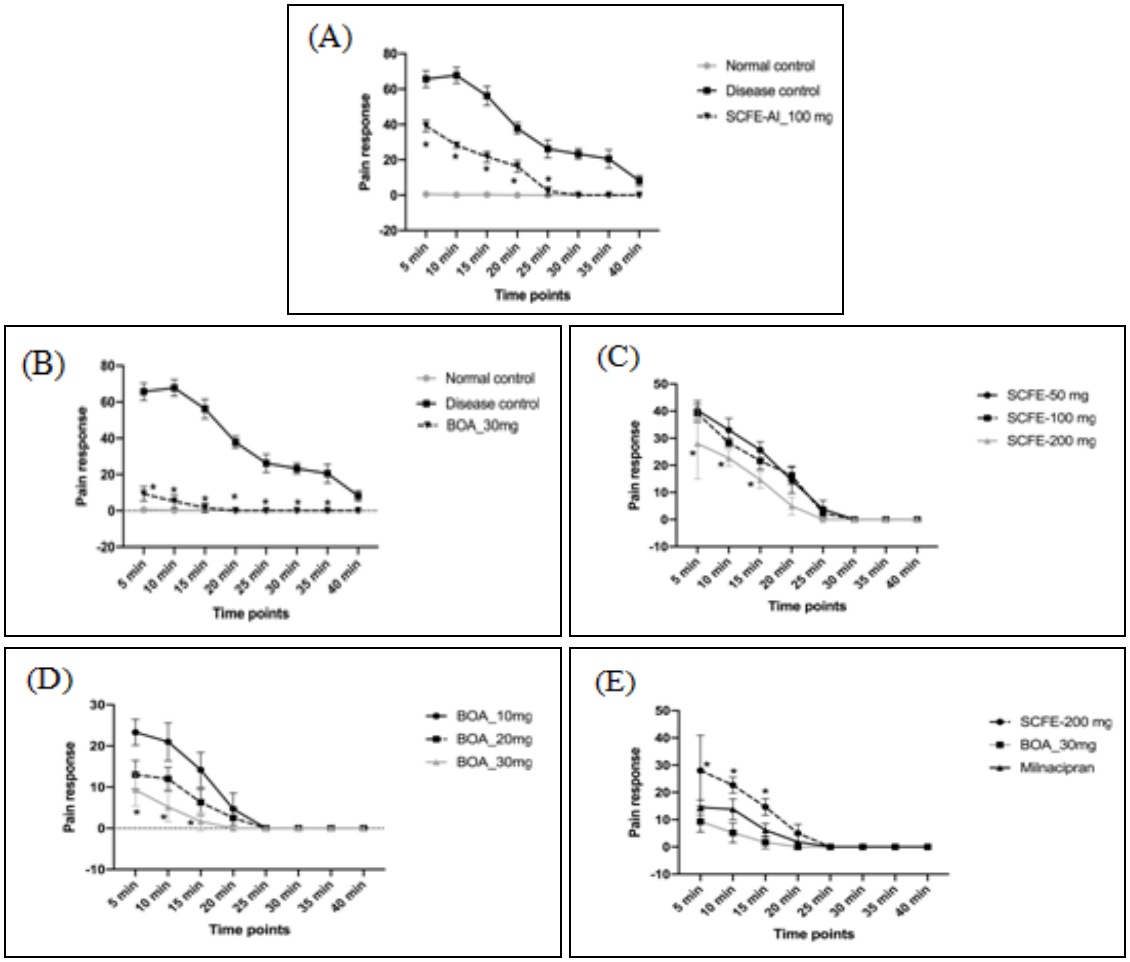

FIG. 1: ANALGESIC EFFECT OF SCFE-AI AND BOA ON EARLY AND LATE PHASE OF THE FORMALIN TEST

SCFE-AI at 100 mg/kg (a) and BOA at 15mg/kg (b) show inhibition against the early and late phases of formalin-induced paw edema (c). SCFE-AI (200 mg/kg) is better than the other two concentrations (50 mg and 100 mg) at 5-, 10-, 15-, and 20-min time points (d). BOA 30mg/kg is better than the other two concentrations (10 and 15 mg) at 5, 10, and 15 min time points (e). 30 mg/kg concentration of BOA significantly ($P<0.05$) inhibited the early and late phases of formalin-induced paw edema in mice compared with milnacipran. Each point indicates the Mean ± SD of the pain response to formalin in animals. *represents $p<0.05$.

Acetic Acid-induced Writhing Test: The observations are shown in **Fig. 2**. The SCFE-AI (200 mg/kg) and BOA (30 mg/kg) exhibited a significant (P<0.05) reduction in the number of abdominal writhes due to acetic acid in mice in a dose-dependent manner compared to vehicle-treated animals. The above treatments showed a percent reduction of writhing was 81.6 and 97.4, respectively, as compared to the control group, Milnacipran (10mg/kg) treatment showed a reduction of 91.1% compared to vehicle treatment. The BOA treatment at 30mg/kg showed a significant (P<0.05) decrease in the number of writhing events when compared to the standard drug.

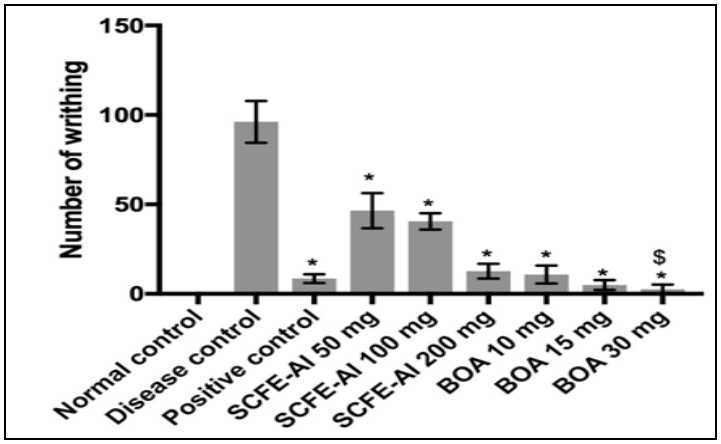

FIG. 2: ANALGESIC ACTIVITY OF SCFE-AI AND BOA IN ALBINO MICE IN ACETIC ACID-INDUCED WRITHING METHOD (MEAN ± SEM, 6 REPLICATES). *p <0.05, when compared to the control group. * indicates significantly different from the standard drug. $ indicates significant differences from all other treatment groups.

Cold Allodynia: The cold allodynic responses of mice to the acetone spray induced quick withdrawal of the paw, flicking or stamping of the paw, prolonged withdrawal or repeated flicking of the paw, and repeated flicking of the paw with licking directed at the ventral side of the paw. BOA (15mg/kg, 30mg/kg) exhibited an anti-allodynic effect by significantly decreasing (P <0.05) the paw withdrawal behaviors compared to those of the control. The observations are shown in **Fig. 3**.

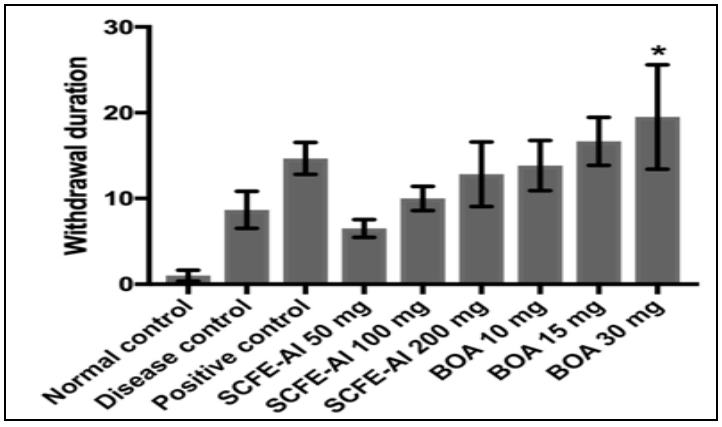

FIG. 3: EFFECT OF SCFE-AI AND BOA ON CARRAGEENAN-INDUCED PAW COLD ALLODYNIA ASSESSED BY ACETONE DROP TEST IN ALBINO MICE. Values are expressed mean ± SEM, (n=6), *P<0.05 compared to the control group.

Mechanical Hyperalgesia: When exposed to a noxious mechanical stimulus, the pinprick test was performed to assess the behavioral responses of mice treated with SCFE-AI and BOA.

Oral administration of SCFE-AI and BOA increased the paw-withdrawal duration. Compared with all the groups, BOA (30mg/kg) significantly increased paw withdrawal duration and significantly increased paw withdrawal threshold of the ipsilateral hind paw in response to a noxious pinprick stimulus compared with the control group **Fig. 4**.

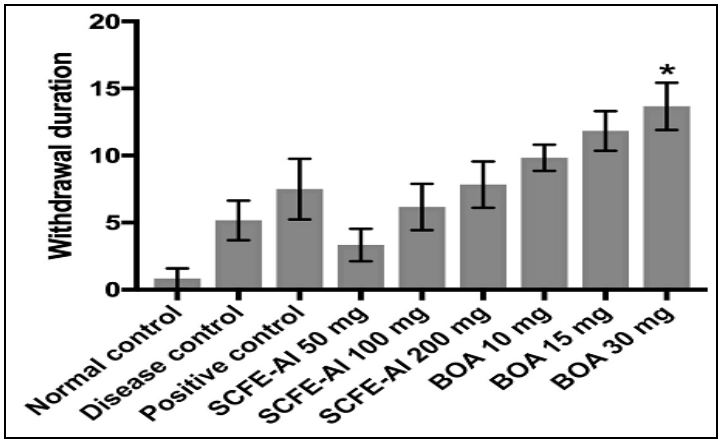

FIG. 4: EFFECT OF SCFE-AI AND BOA ON CARRAGEENAN INDUCED MECHANICAL HYPERALGESIA ASSESSED BY PINPRICK TEST IN ALBINO MICE. Values are expressed Mean ± SEM, (n=6), *P<0.05 compared to the control group.

DISCUSSION: The clinical management of chronic pain in FMS remains a challenge. Despite many years of intense research, developing safe and effective therapeutics for treating pain in FMS remains an unmet medical need. *A. ilicifolius* is a true mangrove species, and its traditional medicinal uses, chemical constituents, and biological activities have been reviewed [11]. The literature review revealed that the plant is rich in many bioactive compounds.

2-Benzoxazolinone, an alkaloid in *A. ilicifolius,* has been extensively investigated as an analgesic and anti-inflammatory agent and has become a promising group to prevent analgesia [23, 24, 25, 26]. 5-Chloro-2-benzoxazolinone (chlorzoxazone) is a centrally acting agent for painful musculoskeletal conditions [27]. This is the first study to report the effect of a standardized extract of *A. ilicifolius* and BOA on muscular hyperalgesia. The formalin test is a reliable nociception model sensitive to various analgesic drug classes. It produced a distinct biphasic response, and different analgesics may act differentially in this test's early and late phases. Substance P and bradykinin participate in the manifestation of the first-phase response and are inhibited by opioid analgesics. Histamine, serotonin, prostaglandin, and bradykinin are involved in the second phase (inflammatory phase), and they are inhibited by non-steroidal, anti-inflammatory drugs (NSAIDs) and opioid analgesics [18]. Oral administration of BOA (30mg/kg) or SCFE-AI (200mg/kg) dose-dependently decreased the number of formalin-induced first-phase and second-phase paw-licking times in mice. However, the effect of the extract was more pronounced in the inflammatory (late) phase. The analgesic effect of BOA (30mg/kg) and SCFE-AI (200mg/kg) on the late phase in this model may be attributed to their peripheral action, which might also suggest their anti-inflammatory action. We already documented the *in-vitro* anti-inflammatory effects of *A. ilicifolius* and BOA by inhibiting TNF-α production in lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophage cells [28].

An acetic acid-induced writhing test further evaluated the analgesic effect of SCFE-AI and BOA. This method was found to be effective in evaluating peripherally active analgesics. The treatment of animals with BOA (30mg/kg) and SCFE-AI (200mg/kg) produced a statistically significant inhibition of the writhes compared to the control. This result indicates that the peripheral analgesic effect of BOA might be mediated by inhibiting prostaglandin synthesis, a peripheral mechanism of pain inhibition. This finding is consistent with the data reported by Islam *et al*., 2012 which show that oral administration of the methanolic extract of *A. ilicifolius* showed significant and dose-dependent antinociceptive activity in acetic acid-induced writhing and formalin test [29]. The acetone spray test incorporates a multimodal stimulus different from direct cold stimulation. In this test, animals reliably showed enhancement of withdrawal reflexes in response to acetone. Administration of BOA (30mg/kg) resulted in tactile mechanical hyperalgesia, as reflected by a significant increase in the hind paw lifting duration in the pinprick test. However, the vehicle administration did not modulate mechanical

hyperalgesia. Moreover, Milnacipran did not show any significant effect on the above-mentioned behavior. Orally administered SCFE-AI and BOA dose-dependently inhibited cold allodynia and mechanical hyperalgesia. This is the first experimental study reporting the potential of SCFE-AI and BOA in attenuating carrageenan-induced Muscular Hyperalgesia. In this study, BOA and SCFE-AI effectively prevented pain in the acetic acid-induced writhing test through their peripheral antinociception. Similarly, it exhibited a potent inhibitory effect in the late phase of the formalin test, which explains its inflammatory nociception. The administration of BOA and SCFE-AI significantly attenuated carrageenan-induced behavioral alterations, including paw cold allodynia and mechanical hyperalgesia. The results obtained from the rodent model of pain and Muscular Hyperalgesia suggest their antinociceptive and anti-hyperalgesic effects, respectively. However, BOA is essential for the antinociceptive effects observed with *A. ilicifolius.* SCFE-AI and BOA showed a beneficial effect on musculoskeletal pain, which suggests that these can be useful in treating chronic musculoskeletal pain syndromes such as fibromyalgia.

CONCLUSION: In conclusion, this is the first scientific evidence for using standardized supercritical fluid extracts of *A. ilicifolius* and 2-benzoxazolinone for treating fibromyalgia. The research revealed that SCFE-AI and BOA can be developed into effective drugs for treating fibromyalgia. This study justified the traditional use of the leaf part of this plant. Our scientific validation expresses that *A. ilicifolius* is a potent species with enormous biological activities. Further clinical trials will explore the application of natural biomolecules from *A. ilicifolius* to human beings.

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CONFLICTS OF INTEREST: Authors declare that there is no conflict of interest.

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