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Evaluation of *Carissa spinarum* methanol extracts and flavonoid-rich fractions for pain management: insights into receptor-mediated mechanisms

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ABSTRACT

Background: Herbal remedies have played an essential role in the development of modern medicine. One herbal medicine used to treat pain is *Carissa spinarum*. Pain remains a constant health issue despite the presence of many pain medications, which are sometimes costly, unavailable, and have undesired side effects. This underscores the need to identify affordable and safer alternatives from medicinal plants and their bioactive fractions for pain management. **Objective:** To evaluate the antinociceptive effects of the crude methanol extract and flavonoid-rich fraction of *C. spinarum* and investigate their possible mechanism of action. **Materials and Methods:** Antinociceptive activity was evaluated in Swiss albino mice using the formalin-induced paw-licking test and the hot water tail-flick test. Diclofenac was used as the standard drug. Pain was determined by recording the time mice displayed pain behavior in the formalin and the time it took for mice to flick their tails in the tail-flick test. The mechanism of action was determined by administering blockers (atropine, ketamine, and naloxone), known to target cholinergic, N-methyl-D-aspartate (NMDA), and opioid receptors, before administration of the extracts. **Results:** The crude extracts and flavonoid-rich fractions of *C. spinarum* significantly reduced pain responses in both the formalin and tail-flick tests ($p < 0.05$ and $p < 0.01$). Pretreatment with ketamine and atropine significantly ($p < 0.05$) altered the antinociceptive effects of the extracts. In contrast, pretreatment with naloxone did not, suggesting the possible involvement of cholinergic and NMDA receptor pathways. **Conclusion:** The results demonstrate that the crude extracts and flavonoid-rich fractions of *C. spinarum* possess significant antinociceptive activity. The analgesic effect of the fraction appears to be mediated via cholinergic and NMDA receptor pathways, supporting the plants' traditional use in pain management and highlighting them as a potential source of new analgesic compounds.

Keywords: *C. spinarum*, Antinociceptive, Flavonoids, Pain mechanisms, Receptor-mediated mechanisms, Analgesic activity.

INTRODUCTION

Traditional medicine remains the most cost-effective and readily available treatment within economically disadvantaged communities' primary healthcare systems. One of the plants used to treat various ailments is *C. spinarum*. The plant is a small spiny evergreen shrub that grows in different parts of Africa, including Kenya, Ethiopia, Malawi, Zimbabwe, and Zambia. It contains high levels of bioactive secondary metabolites such as tannins, flavonoids, alkaloids, cardiac glycosides, saponins, terpenoids, anthraquinones, and phenols [1]. Locally, it has been used to treat epilepsy, malaria, rabies, heartburn, gastrointestinal diseases, pneumonia, cough, polio, infertility, and pain [1,2]. Research studies have shown that *C. spinarum* possesses anti-tumor [3], vasorelaxant, cardioprotective, and hepatoprotective activities [4], antidiabetic [5], antimicrobial [6], and anticonvulsant [7]. Although previous studies have reported the analgesic activity of *C. spinarum*, limited information exists regarding the pharmacological activity of the flavonoid-rich fraction and its possible receptor-mediated mechanisms of action. The present study, therefore, addresses this knowledge gap by evaluating both the crude extract and flavonoid-rich fraction of *C. spinarum* using the established nociceptive models together with receptor agonist experiments.

MATERIALS AND METHODS

Preparation of plant materials

Collection and preparation of plant material

C. spinarum's roots were collected from the Juja area in Kiambu County, Kenya, during the dry season. The plant was authenticated and assigned voucher specimen No. VW/2017/02. Fresh roots were transported to the laboratory, cleaned, chopped, and dried under shade for a month. They were ground into powder using an electric grinding mill, weighed, and stored in a clean, airtight glass bottle at room temperature.

Preparation of root extract

Two hundred grams of the root powder was defatted using petroleum ether. The fat-free powder was soaked in methanol, stirred, and allowed to stand for 2 h before decanting. This procedure was repeated after 24 and 48 h. The supernatant was filtered using a Whatman No. 1 filter paper, and the filtrate was concentrated at reduced pressure using a rotary evaporator to obtain the extract.

Preparation of flavonoids-rich fractions

Flavonoids were extracted using the method described by Houghton and Roman [8] and used by Wambugu *et al* [9] as follows: 200 g of extract was defatted with petroleum ether and extracted with methanol through sequential soaking, stirring, and decanting over varying periods of 30 min, 2 h, 24 h, and 72 h. The methanol extract was then filtered and concentrated using a rotary evaporator. The extract was then treated with 150 mL of 1N hydrochloric acid (HCl) and then partitioned with 150 mL of diethyl ether. The ether fraction was left to dry in the open air to obtain the flavonoids. The crude flavonoid fraction was kept at room temperature in a clean, airtight glass bottle until use.

Test Doses

The doses for methanolic crude extract (12.5, 25, and 50 mg/kg) and flavonoid-rich fraction (2.5, 5, and 10 mg/kg) of *C. spinarum* used in this study were selected based on the pilot experiments conducted to determine the appropriate dose range that would produce a measurable pharmacological effect without causing adverse effects following intraperitoneal administration. There were no previous reports available to guide dose selections for intraperitoneal administration of *C. spinarum*.

Experimental Animals

Healthy male and female Swiss Albino mice weighing 18-24 g, 6-8 weeks old, were used for this experiment. The mice were placed in cages in the animal house in a room maintained at room temperature one week before the beginning of the experiment to allow acclimatization. Water and food were provided *ad libitum*. The mice were randomly allocated into six groups, with each group having six animals (n=6). The experiment was conducted per the guidelines for laboratory animal use and care [10], and the permit was granted by the Kenyatta University Animal Care and Use Committee (Ref: PKUA/013/I013) and the National Commission for Science and Technology (NACOSTI).

Drugs and chemicals

This experiment used petroleum ether, methanol, normal saline, formalin, hydrochloric acid, diethyl ether, diclofenac sodium, ketamine, atropine, and naloxone, all purchased from Chemoquip Limited, Kenya.

Antinociceptive Assay

C. spinarum antinociceptive activity was determined by using the formalin-induced paw-licking method described by Hunskaar and Hole [11] and Deuis *et al* [12] in the tail-flick model.

Tail flick test

The tail-flick test involved 30 experimental animals randomly divided into six groups (n=6). Mice were injected intraperitoneally with the vehicle, diclofenac (standard drug) (15 mg/kg), and three experimental doses at (12.5 mg, 25 mg, and 50 mg/kg of root extract and 2.5 mg, 5 mg, and 10 mg of flavonoid-rich fraction). Pain was induced in all groups except group 1, which served as a normal control. Mice were restrained, and their tails were marked 3 cm from the tip, and dipped in water heated to 55 °C up to the marked line. The latency of tail withdrawal was recorded, with a maximum latency of 18 seconds, to avoid tissue damage. Baseline latency was established before testing, and the tail-flick test was conducted 30 min after the administration.

Formalin-induced paw edema test

The study involved five groups of six mice each, which were treated with various test doses (12.5 mg, 25 mg, and 50 mg of root extract, and 2.5 mg, 5 mg, and 10 mg of flavonoid-rich fraction), normal saline, and diclofenac (15 mg/kg) via intraperitoneal injection. Thirty minutes later, 5 µL of 5% formalin was injected into the left hind paw to induce nociceptive behavior. The mice were observed during the early phase (0–5 min) and late phase (20–30 min) post formalin injection. The time mice spent biting, lifting, licking, and flinching the affected paw was recorded.

Antinociceptive mode of action

The antinociceptive mode of action was determined by administering receptor agonists and blockers, as described by Mwangi *et al* [13] and Mwonjoria *et al* [14], as follows: Group One of mice (n = 6) received a vehicle (normal saline). The second group received 10 mg of flavonoid-rich fraction and a receptor blocker, and the third group received 10 mg of flavonoid-rich fraction. The receptor blockers used were (atropine 4 mg/kg, ketamine 2.5 mg/kg, and naloxone 1 mg/kg), and were administered intraperitoneally 15 min prior to administration of the extracts. After 30 min, 5% formalin solution was injected into the left hind paw, and the time spent in nociception in both phases was noted.

Statistical analysis

Data were expressed as the mean and standard error of means. Data were analyzed using IBM SPSS Statistics, Version 28 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was used to assess data normality. Differences among the groups were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Significance level was set at ($p<0.05$ and $p<0.01$).

RESULTS

Antinociceptive effects of *C. spinarum* on formalin-induced pain

In the early phase, the 50 mg dose significantly reduced pain behavior ($p<0.05$), while in the late phase, all doses showed a significant reduction ($p<0.01$) in pain behavior compared to the vehicle, with effects comparable to diclofenac. On the other hand, all doses of flavonoid fractions of the root extract showed significantly higher activity in reducing pain behavior in both phases.

Antinociceptive effects of *C. spinarum* on heat-induced pain

The 50 mg dose of the methanol extract significantly increased tail-flick latency. Similarly, the 5 and 10 mg (P<0.01) doses of the flavonoid fraction also showed significant antinociceptive effects, with the 10 mg dose exceeding the effect of the standard drug, diclofenac.

Antinociceptive Mechanism of Action of Flavonoid-Rich Fractions from *C. spinarum*

The flavonoid-rich fraction of *C. spinarum* significantly reduced nociception time (P<0.05) in both phases compared to the vehicle after pretreatment with atropine and ketamine, but showed no significant difference with the test group when pretreated with naloxone, an opioid antagonist.

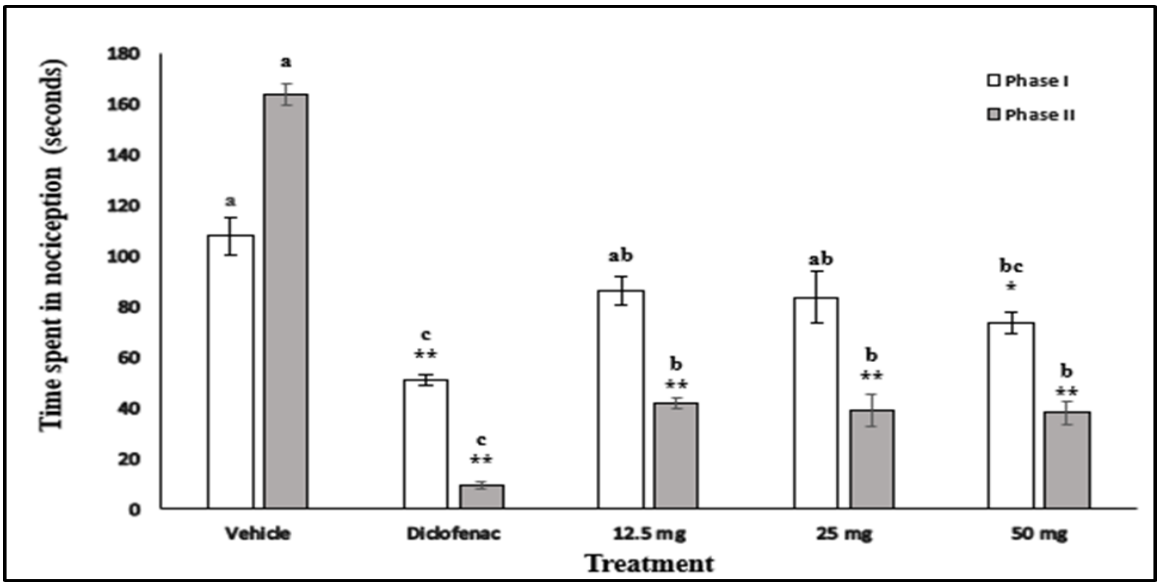

Figure 1: Antinociceptive effects of *C. spinarum* extracts in mice. *(p < 0.05), **(p < 0.01) relative to the vehicle. Means that share superscript letters are not significantly different

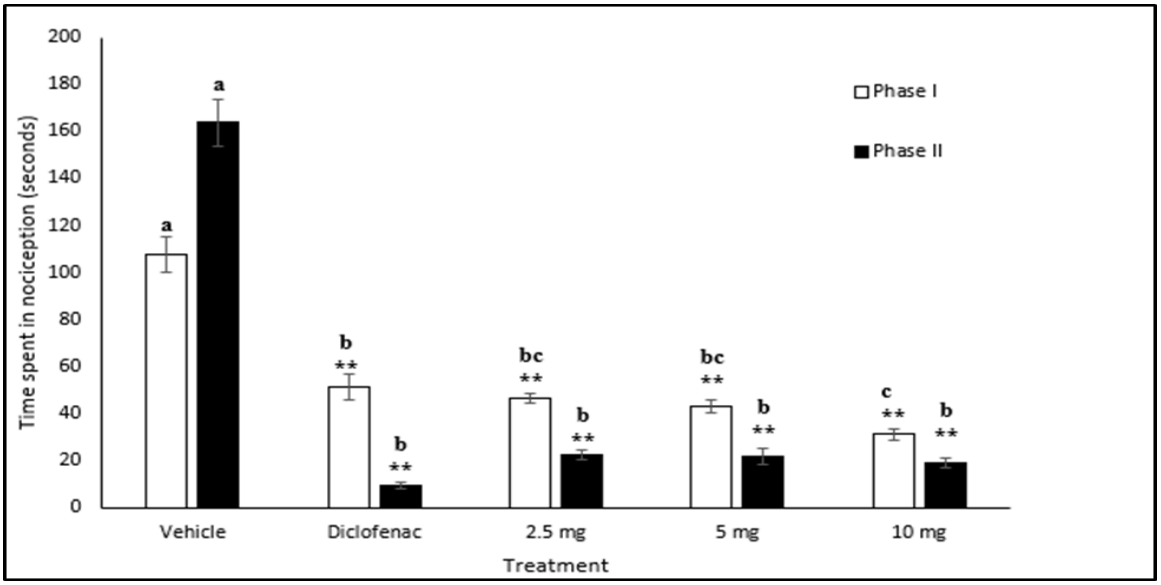

Figure 2: Effects of flavonoid fraction of C. *spinarum* on formalin-induced pain in mice. *P<0.05, **p<0.01 against vehicle. Means that share letter superscripts are not significantly different

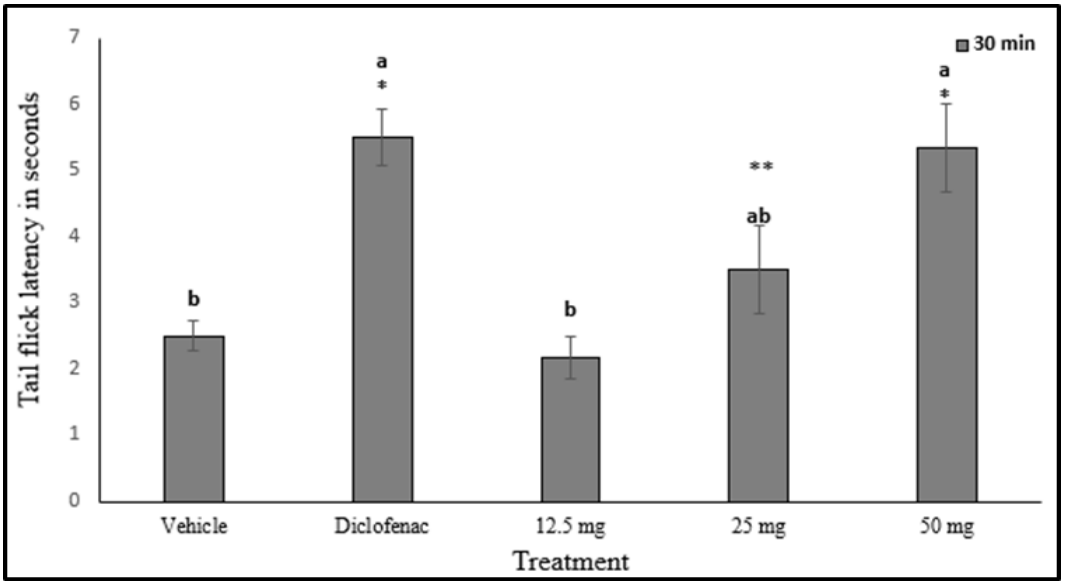

Figure 3: Effects of *C. spinarum* methanol extract on heat-induced pain in mice. *P<0.05 against vehicle. Means that share letter superscripts are not significantly different

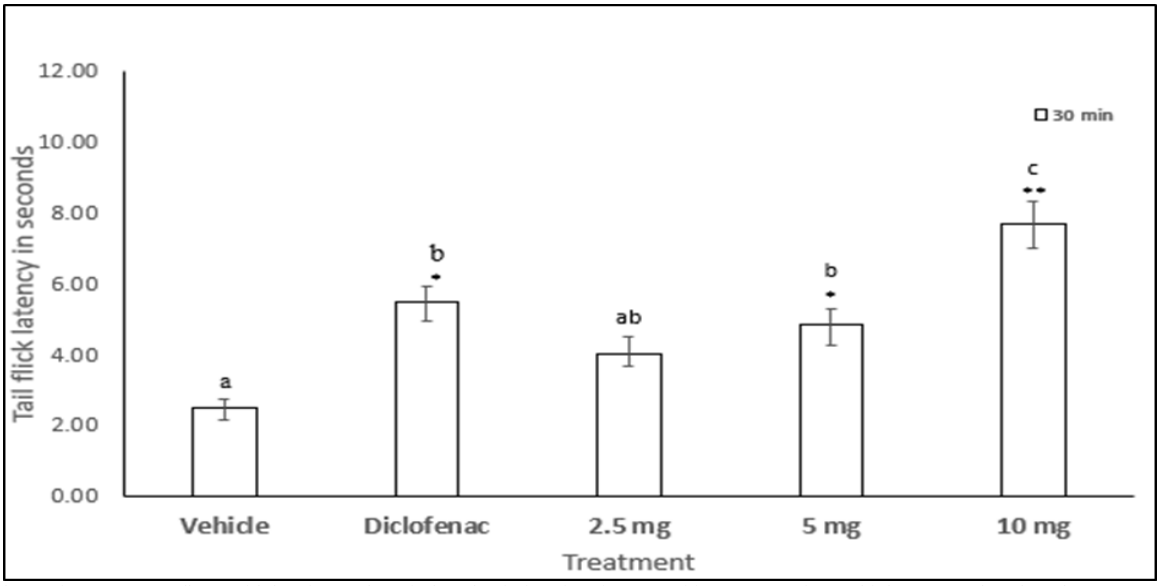

Figure 4: Effects of *C. spinarum* flavonoid-fraction on heat-induced pain in mice. *P<0.05, **P<0.01 against vehicle. Means that share letter superscript are not significantly different

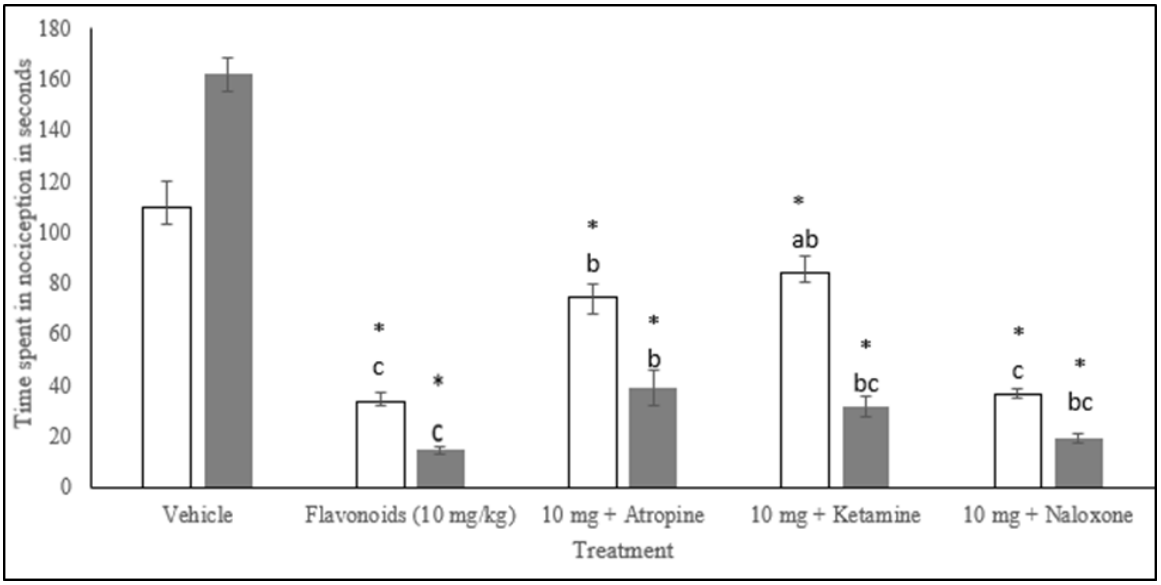

Figure 5: Effects of atropine, ketamine, and naloxone on the antinociceptive effect of *C. spinarum* flavonoid fraction in the formalin test. *($p < 0.05$) relative to the vehicle. Letters with the same superscripts are not significantly different

DISCUSSION

There has been a need to search for effective analgesics, considering the socio-economic impacts of pain and prior knowledge of the herbal medicines used to relieve pain. *C. spinarum* has been identified as one of the herbal medications used by many communities for pain relief. However, there is insufficient scientific data to prove this, particularly when specific plant extract-rich fractions are used. This paper sought to unravel the antinociceptive effects of *C. spinarum* methanol root extracts and its flavonoid-rich fraction. Pain, as a major symptom of many medical conditions, serves to alert an individual that something is wrong and needs attention. The current pain medications target the various pain pathways to either modify their perception or reduce pain sensation.

In the current study, the methanol extract of C. spinarum and its flavonoid fraction abolished pain induced by formalin. The formalin test in this study produced two distinct phases: the neurogenic and inflammatory phases. The acute neurogenic phase involves primary afferent sensory neurons and is commonly accompanied by the release of glutamate, nitric oxide, and substance P. On the other hand, the late phase represents a mix of central nervous system hypersensitivity and peripheral tissue damage. In this stage, tissue injury and mast cell degranulation cause the release of chemical messengers like histamine and serotonin, which excite the local nociceptors and upregulate cyclooxygenase enzymes, leading to the synthesis of prostaglandins, a chemical that directly lowers the activation thresholds of peripheral nerves [15]. Pro-inflammatory cytokines and glutamate released continue to sustain these peripheral thresholds and ultimately induce a state of spinal hypersensitivity. Therefore, the data obtained from this study, particularly in the late phase, cannot be divorced from central sensitization pathways. The overlapping peripheral and central systems that eventually drive the persistence of NMDA receptor activation in the spinal dorsal horn validate the use of the formalin model for capturing nuanced, multi-target drug effects [16]. Therefore, extracts or flavonoid-rich fractions may act like non-steroidal drugs, such as diclofenac, by blocking cyclooxygenase pathways that produce prostaglandins, which sensitize nociceptors and contribute to inflammation. They could also be working like opioids, e.g., morphine, by targeting the opioid receptors, which reduces pain signal transmission by inhibiting neurotransmitter release. Other drug mechanisms known to assist in pain modulation and sensation include blocking voltage-gated sodium channels, as demonstrated by drugs like lidocaine, and modulating calcium channels in the nervous system, which Gabapentin does. Several plants have been identified, and their impacts on pain relief have been thoroughly expounded. For instance, the willow tree *Salix alba* is a perfect example of how traditional herbal treatments can be transformed into an artificially created drug. Its active ingredient, acetylsalicylic acid, is converted to aspirin, a well-known pain management drug [17].

The *C. spinarum* extracts and flavonoid fractions also significantly increased tail-flick latency. The tail flick response indicates a spinally mediated reflex. Heat-induced pain often involves the direct activation of TRPV1 channels, which are often thermosensitive to temperatures below 15 °C or above 43 °C [18]. In this study, the extracts were shown to possess analgesic effects, increasing pain tolerance in mice. This effect may be due to the plant's flavonoid and alkaloid phytochemicals. Alkaloids have been demonstrated to have antinociceptive activity in diverse studies [19, 20]. Similarly, flavonoids have been shown to relieve pain by inhibiting cyclooxygenase, arachidonic acid, phospholipase A_2, and nitric oxide synthase [21]. The presence of these compounds could have been responsible for *C. spinarum* analgesic activity.

The flavonoid-rich fraction used in the study was found to be more potent than the methanol extract, as enrichment of bioactive flavonoids is expected to yield higher pharmacological activity than that of the crude extract. To understand the mechanisms by which they worked to relieve pain, atropine, ketamine, and naloxone were used. The study found that pretreatment with ketamine and atropine significantly reduced time spent in nociception in both phases, whereas naloxone did not show a significant difference between the test groups. These results suggested a similarity between flavonoids from C. spinarum and those that utilize the cholinergic system, which has been found to treat neuropathic pain [22]. Flavonoid-rich fractions also indicate possible participation in NMDA receptors, one of the new molecular targets capable of inhibiting or neutralizing the transmission of painful stimuli [23].

CONCLUSION

In conclusion, *C. spinarum* has demonstrated significant activity, with the flavonoid-rich fraction showing a greater potency than the crude extract. Its effects suggest involvement of cholinergic and NMDA receptor pathways rather than opioid mechanisms, supporting its traditional use and potential as a source of novel pain-relieving agents. However, additional phytochemical characterization, mechanistic investigations, toxicity evaluations, and clinical studies are required before therapeutic applications can be considered.

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Author Contributions

JNS and JKM contributed equally to the conceptualization, methodology, investigation, data curation, formal analysis, and writing of the original draft. SNW and MBW contributed to supervision, guidance, and critical review and editing of the manuscript. All authors reviewed and approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available and will be made available upon request.

Use of AI in Drafting of Manuscript

The authors declare that they have not used any generative AI/AI-assisted technologies in the writing of this manuscript.

Conflict of interest

The authors declared no conflict of interest.

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