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The anti-nociceptive effects of ethanol extract of aerial parts of *Schkuhria pinnata* in mice

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ABSTRACT

Ethnopharmacological relevance: The growing challenge to access conventional analgesics, contraindications, and adverse effects could have led individuals to use *Schkuhria pinnata* (Lam.) Kuntze ex Thell. (Compositae), as an alternative traditional therapeutic strategy for pain. However, evidence of its safety and efficacy is scarce.
Aim of the study: This study evaluated the anti-nociceptive effect of the ethanol extract of the aerial parts of *S. pinnata* in mice.
Methods: The mice were randomly assigned to nine groups: (1) vehicle; (2) acetylsalicylic acid (intraperitoneally 150 mg/kg); (3) pentazocine (intramuscularly 1.0 mg/kg); (4 a & b) orally 100 mg/kg extract; (5 a & b) orally 200 mg/kg extract; (6 a & b) orally 400 mg/kg extract. We used an acetic acid-induced writhing model and a tail-flick test. The number of writhes and time taken for the tail to flick was recorded. A one-way analysis of variance followed by Tamhane T2 post hoc was used for multiple comparisons.
Results: Compared to a vehicle (59.0 ± 2.68), *S. pinnata* ethanol extract at a dose of 200 and 400 mg/kg, p.o reduced writhes to 42.5 ± 1.12 and 27.0 ± 2.62, ($p < 0.05$) respectively. Similarly, the pain threshold of mice increased dose-dependently; doses of 200 and 400 mg/kg, increased time to 5.33 ± 0.42 and 8.67 ± 0.21 min, ($p < 0.05$) respectively. The extract had an EC50 of 348.8 mg/kg and acute toxicity established an LD50 of 1224.8 (95% CI: 952.2–1575.3).
Conclusion: S. pinnata ethanol extract had anti-nociceptive activity by central and peripheral mechanisms that could justify its traditional use in pain management. Further studies could now focus on identifying active fractions and pure isolated compounds responsible for anti-nociceptive activity.

1. Introduction

Pain is a mutually recognizable somatic experience that reflects a person's apprehension of threat to their bodily or existential integrity (Cohen et al., 2018). Although it is primarily protective in function, it often causes discomfort. It is also an important symptom that brings the patient to a physician. The sensation of pain, 'nociception,' is mediated by numerous intracellular and extracellular molecular messengers (Dinakar and Stillman, 2016). These messengers are also important targets for medicines. For instance, prostanoid chemicals produced by peripheral nociceptors in response to pain stimulus are the targets for nonsteroidal anti-inflammatory drugs (NSAIDs) medications (Besson, 1999; Lleo et al., 2007).

Currently, available analgesic drugs such as opiates and NSAIDs contributed significantly to pain control. However, these conventional drugs are increasingly associated with adverse effects such as induced gastric ulcers, drowsiness, dizziness, and hepatotoxicity. Also, several contraindications limit their clinical utility (Benyamin et al., 2008; Yoon et al., 2016). Besides, the challenges of health care systems in most low- and middle-income countries, such as poorly developed medicine supply chains and the soaring cost of conventional therapy, have aggravated the demanding access to such medications. It is therefore critical to develop therapies that could be efficacious, safe, and readily accessible.

Since the earliest human civilization, humankind has used medicinal plants to relieve pain (Almeida et al., 2001). Furthermore, in the recent

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Abbreviations

ANOVA	Analysis of variance
ASA	Acetylsalicylic acid
FCR	Follin-Ciocalteu reagent
GAE	Gallic acid equivalent
HPLC	High-performance liquid chromatography
MPA	Mean possible analgesia
MUST-REC	Mbarara University of Science and Technology-research ethical committee
NCRL	Natural chemotherapeutics research laboratory
NSAIDs	Nonsteroidal anti-inflammatory drugs
OECD	Organization of Economic Co-operation and Development
QE	Quercetin equivalent
SEM	Standard error of the mean
TPC	Total phenolic content
UK	United Kingdom
USA	United States of America

decade, research on the antinociceptive effect of plant species has intensified. Consequently, researchers identified several plant preparations and isolated pure compounds to have promising activities. Essential oils and isolated compounds - eugenol and myrcene from *Ocimum gratissimum* L. (Lamiaceae) had antinociceptive activity in neuropathic pain (Paula-Freire et al., 2016). The *O. gratissimum's* flavonoid-rich fraction of leaves also had anti-inflammatory activity (Ajayi et al., 2017). *Boswellia serrata* resins, *Aloe vera* polysaccharides, and *Matricaria chamomilla* and *Melissa officinalis* polyphenols prevented intestinal damage and visceral pain in male Sprague-Dawley rat (Parisio et al., 2020). Similarly, *Mikania scandens* (L.) Willd. (Compositae) (Hasan et al., 2009), *Mitragyna parvifolia* (Roxb.) Korth (Rubiaceae) (Kaushik et al., 2009), and *Albizia lebbeck* (L.). Benth. (Leguminosae) (Saha and Ahmed, 2009) have demonstrated potential benefits in the management of chronic pain. In line with this, research on plant species traditionally used as pain killers could likely result in potential analgesic and anti-inflammatory drugs.

Schkuhria pinnata (Lam.) Kuntze ex Thell. (Compositae) is a natural weed. It is native to South America and widely spread to other Latin American and African countries such as Uganda (Taylor, 2019). Traditionally, local Ugandan healers used a decoction of leaves and flowers to manage pain and inflammation (Obbo et al., 2019). In Uganda's neighbouring country Kenya, local communities of Machakos and Makuen counties macerate the whole plant in water and drink one glass of infusion three times daily, for two weeks or until one recover from chronic pain (Wambugu et al., 2011). Ethnomedical studies have also shown that traditional healers in Limpopo province, South Africa use the whole plant leaves to treat painful eyes (Semenya and Maroyi, 2018), while northern Peru used the plant as an anti-inflammatory and anti-acne (Bussmann et al., 2008). A recent study identified four sesquiterpene lactones with moderate anti-inflammatory activity (Kudumela et al., 2019). The biological activities demonstrated by the isolated compounds support the traditional uses of *S. pinnata* in inflammatory-related illness. However, scientific evidence to validate the local use of *S. pinnata* in pain management is scarce. This information is essential to inform local communities on the effectiveness of traditional remedies used. Therefore, this study investigated the *in vivo* analgesic properties of ethanol extract of aerial parts of *S. pinnata.*

2. Methods

2.1. Materials

Acetyl-salicylic acid and pentazocine were offered as a kind gift of Kampala Pharmaceutical Industry (Kampala, Uganda); Gallic acid, Aluminium chloride, sodium nitrate, sodium carbonate, and acetic acid were of analytical grade purchased from Toronto research chemicals (Ontario, Canada) while HPLC grade methanol, acetonitrile, and trifluoroacetic acid, as well as quercetin flavonoid standards, were purchased from Sigma-Aldrich, Inc. Germany.

2.2. Plant collection and extraction

The fresh whole aerial parts of *S. pinnata* were collected in mid-July along Bukoto-Kisaasi Rd, Kampala, located at 0° 21′16.9″N 32° 35′ 54.6″E. The plant was identified and authenticated by taxonomist; Mrs. Medius Kyoshabire of the botany section at Natural Chemotherapeutics Research Laboratory (NCRL). A voucher specimen number SD. 01 was then deposited at the herbarium of NCRL.

The plant was dried under shade and then crushed into a coarse powder using an electric grinder. About 50 g of the powder was soaked in 70% v/v ethanol solution (1.0 L) for 72 h, at 27 °C. The extracts were filtered using a muslin cloth and concentrated at 40 °C under reduced pressure in a rotary evaporator. Finally, the concentrated extract was lyophilized. The powder was weighed and stored in an airtight container under 4 °C until use.

2.3. Qualitative phytochemical screening

Qualitative phytochemical screening for reducing compounds, alkaloids, tannins, coumarins, saponins, and flavonoids was performed according to standard methods (Evans, 2009).

2.4. Determination of total phenolic content (TPC)

We determined the TPC of extract according to the Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). About 1 mg/kg sample solution was oxidized with 2 mL of 10% v/v Follin-Ciocalteu reagent (FCR) and 2 mL of 7.5% sodium carbonate. The mixture was incubated for 40 min at 45 °C, and absorbance was measured at 760 nm using a UV/VIS spectrophotometer (JENWAY, 6705; Stone, Staffs, UK). The TPC was calculated as a Gallic acid equivalent.

2.5. Determination of total flavonoid content

According to the aluminum chloride colorimetric method, the total flavonoid content of the extract was determined (Baba and Malik, 2015). Briefly, 1 mg/mL of crude extract was mixed with 4 mL of distilled water and 0.5 mL of 5% $NaNO_2$, then allowed to stand for 5 min. Later, a 0.5 mL of 10% AlCl3 was added and allowed to stand for 6 min before adding 1 mL of 1 M NaOH and made the volume of the mixture to 10 mL with distilled water. The mixture was allowed to stand for 15 min at room temperature, and absorbance was measured at 510 nm using a UV/VIS spectrophotometer (JENWAY, 6705; Stone, Staffs, UK). Based on the standard calibration curve, we calculated the total flavonoid content as quercetin equivalent.

2.6. HPLC fingerprint

The fingerprint of the extract was determined by the HPLC method. The chromatographic system consisted of a Shimadzu LC-10AT equipped with an SPD-20A UV/VIS detector (Tokyo, Japan), communicator CBM-20A (Tokyo, Japan), and degassing unit DGU-$20A_{5R}$ (USA) with an isocratic binary system of the mobile phase. Chromatography separation was performed on a Lunar® C_{18} column (5 μm; 250 × 4.6 mm;

Phenomenex, U.S.A.) maintained at a temperature of 40 °C in a Shimadzu column oven (CTO-20AC, Tokyo Japan). The mobile phase used was methanol: water: acetonitrile (60:30:10 v/v) containing 0.01% trifluoroacetic acid and a flow rate of 1.0 mL/min. The volume injected was 10 μL at a pressure of 2596.17 psi with a detection wavelength of 210 nm. The sample was prepared in triplicate.

2.7. Animals

A 10–12 weeks old Swiss albino mice of either sex weighing 30–35 g were purchased from Natural Chemotherapeutics Research Laboratory. The animals were housed in clean cages under 20–25 °C and a 12 h light/dark cycle. They were fed on standard rat pellets and water *ad libitum* and acclimatized for one week before the experiment. Just before the experiment, animals fasted for 4 h with free access to water. The fasting ensured a more uniform drug absorption by preventing the mixing of test drugs with food and thus prevented variability of measurement that would otherwise mask subtle effects (Ellacott et al., 2010; OECD, 2008). Mbarara University of science and technology research ethics committee approved the study protocol (MUST-REC 29/06–18). We performed the experiments according to the guidelines for the care and use of laboratory animals of the National academy of sciences and the ethical guidelines for experimental pain investigations in conscious animals.

2.8. Acute oral toxicity study

According to the Organization of Economic Co-operation and Development (OECD) guideline 423 (OECD, 2001), the acute oral toxicity was determined. Briefly, female mice aged 8–13 weeks were divided into three groups of 3 mice each. The extract was dissolved in water (40 mg/mL) and was given orally to the animals in each group at one of the defined doses between 100, 1000, and 2000 mg/kg. The extract was studied in a step-wise procedure with each step using three mice. The absence or presence of mortality at one step determined the dose in the next step. The animals were observed continuously for the first 30 min post-administration, then every 1 h for 4 h, then once daily for 48 h. We observed any changes in the skin fur, eyes, and mucous membranes. We carefully observed signs for salivation, tremors, convulsions, diarrhoea, lethargy, sleep, and coma. Also, any other behavioral changes were noted for 14 days. Finally, the LD50 value was determined by probit analysis.

2.9. Nociceptive model and treatment

According to the previous method, peripheral pain (abdominal writhing) was induced by intraperitoneal injection of 0.6% acetic acid (Collier et al., 1968). The acetic acid produced contraction of abdominal muscles and stretching of hind limbs. Briefly, fasted animals were randomly divided into nine groups with 6 animals each; group 1; normal control received normal saline (1 mL/kg), Group 2; positive control received acetylsalicylic acid (150 mg/kg), Group 3; positive control received pentazocine (1 mg/kg, i.m.) Group 4a and 4b; ethanol extract (100 mg/kg, p.o), Group 5a and 5b; ethanol extract (200 mg/kg, p.o), and Group 6a and 6b; ethanol extract (400 mg/kg, p.o). The extract and standard drug were given 30 min before 0.6% acetic acid was administered. Then writhes were induced by an intraperitoneal injection of 0.6% v/v acetic acid (10 mL/kg). Five minutes after injecting acetic acid, the number of abdominal muscular contractions (particularly the hind limbs) were observed and counted for 20 min.

We used Sewell and Spense method to assess central pain (hot tail-flick) (Sewell and Spencer, 1976). The method is based on the observation that opioid analgesic drugs selectively prolong the reaction time of a typical tail withdrawal reflex in mice (Toma et al., 2003). The animals in groups (4b–6b) were treated with the extract with an equivalent dose to those of groups (4a-6a). Group 3 animals were positive control for the central analgesia experiment. About 1–2 cm of each mouse's tail was immersed in warm water kept at a constant temperature of 55 °C. Then the reaction time (time taken by the mouse to deflect its tail) was recorded. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. One minute was allowed in-between two consecutive readings for each mouse. A latency period of 15 s was defined as complete analgesia and the measurement then stopped to avoid injury to the mice. The latent period of the tail-flick response was determined at 0, 15, 30, and 45 min after the administration of the drugs. Immediately after the experiment, the animals were humanely sacrificed.

2.10. Data management and analysis

Data for each animal were recorded in a separate sheet. Two independent individuals were blinded during the assessment and collection of data. Later were checked for discrepancies. Continuous data were summarized into mean ± SEM, and percentage pain inhibition was also calculated. For the acetic acid-induced abdominal writhing test, the percentage of pain inhibition was calculated based on formula (1).

$$\text{Inhibition } (\%) = \left(\frac{[Nc - Nt]}{Nc}\right) \times 100 \quad (1)$$

Nc = average number of writhes by the negative control group and Nt = average number of writhes by test group.

While the mean possible analgesia (MPA) was also calculated for the tail-flick test based on a formula (2)

$$\text{MPA} = \left(\frac{[\textit{Test reaction time} - \textit{Negative control reaction time } (Tc)]}{15}\right) - Tc \quad (2)$$

The effective concentration (EC50) values representing the concentration of extract that exert 50% of its maximal response were determined by regression analysis, using a Quest Graph™ EC50 Calculator (A.A.T. Bioquest Inc, 2019). One-way analysis of variance (ANOVA) was used to test for mean differences between treatment groups, followed by Tamhane T2 post hoc test for multiple comparisons. We considered $p < 0.05$ as statistically significant.

3. Results

3.1. Qualitative phytochemical screening

The ethanol extract was found to have reducing sugars, alkaloids, tannins, glycosides coumarins, saponins, phenolics, and flavonoids. Table 1 illustrates the reagents used and the intensity of reactions.

Table 1
Phytochemical constituents of aerial parts of *S. pinnata* plant.

Test	Reagents	Observations	Ethanol extract
Alkaloid	Dragendrof's	Orange ppt	++
Glycosides	Keller killiani	Formation of brown ring at the junction of the two layer	+
Saponins	Distilled water	Foamy	++
Reducing sugar	Fehling reagent	Brown ppt	++
Phenolics	5% $FeCl_3$	green colour	++
α-amino acids	0.1% Ninhydrin	Purple colour	+
Tannins	5%ferric chloride	Dark green colour	++
Flavonoids	10% lead acetate	Yellow ppt	++

(+) present (−) absent.

3.2. Phenolic and flavonoid contents

Based on the calibration curve (R^2 = 0.9923), the total phenolic content of ethanol extract was calculated as 14.3 ± 1.4 mg GAE/g, and total flavonoid content was calculated from the calibration curve (R^2 = 0.9903) as 12.5 mg Q.E./100g.

3.3. HPLC fingerprint of S. pinnata

The HPLC chromatogram of methanol crude extracts shown 26 characteristic peaks (Fig. 1) and additional materials (S1).

3.4. Acute oral toxicity

Ethanol extract at a dose of 500 mg/kg, p.o., produced neither mortality nor behavioral change. However, a dose of 1500 mg/kg produced maximum mortality (66.7%) on day 5 followed by a dose of 1000 with a 33.3% mortality on day 6. Animals in both doses 1500 and 1000 mg/kg had experienced a drowsy effect, reduced feeding, and piloerection before death. The LD50 was calculated as 1224.8 (95% CI; 952.2–1575.3) based on probit analysis.

3.5. The ethanol extract reduced peripheral and central pain

The oral administration of ethanol extract of *S. pinnata* at doses 200 mg/kg and 400 mg/kg significantly inhibited writhing response compared to vehicle control. The observed effect was in a dose-dependent manner. However, a dose of 100 mg/kg of the extract did not inhibit the acetic acid-induced abdominal writhes.

Similarly, in comparison to vehicle control, the extract significantly increased tail withdrawal reflex time in a dose-dependent manner ($p < 0.05$). Table 2 illustrates the various comparisons.

According to Table 3, the mean writhes observed in the control group was 59.00 ± 2.68 counts. Acetylsalicylic acid (ASA) at a dose of 150 mg/kg, extract (200 mg/kg) and (400 mg/kg) significantly ($p < 0.05$) reduced the number of writhes to 13.17 ± 1.56, 42.50 ± 1.12 and 27.00 ± 2.62 counts respectively. On the other hand, extract group 1 (100 mg/kg) had a non-significant reduction. The ASA produced the highest percentage (77.68%) of protection against writhes, followed by group 3 (54.24%), group 2 (27.97%), and finally group 1 with the least percentage (10.45%) of protection. The EC50 of the extract was calculated as 304 mg/kg, based on the sigmoid dose-response equation.

Table 2
Effects of the ethanol extracts of aerial parts of *S. pinnata* on acetic acid-induced writhing in mice.

Group	Dose (mg/kg)	Mean number of writhes (20 min)	% Inhibition of writhing	P-value
Normal	2 mL	59.00 ± 2.68	0.00	<0.05
Positive	150	13.17 ± 1.56	77.68	
I	100	52.83 ± 1.66	10.45	
II	200	42.50 ± 1.12	27.97	
III	400	27.00 ± 2.62	54.24	

Table 3
Effects of ethanol extract of aerial parts *S. pinnata* on the latency to hot water.

Group	Mean latency (minutes)				MPA (minutes)		
	0	15	30	45	15	30	45
Control	1.83 ± 0.17	1.83 ± 0.17	1.67 ± 0.21	1.83 ± 0.17	–	–	–
Positive	1.83 ± 0.17	5.50 ± 0.22*	9.83 ± 0.17*	12.00 ± 0.26*	27.87	61.21	77.22
I	2.00 ± 0.00	2.17 ± 0.17	2.17 ± 0.17	2.83 ± 0.17*	2.58	3.75	7.59
II	1.83 ± 0.17	2.33 ± 0.21	3.17 ± 0.30*	5.33 ± 0.42*	3.65	11.25	26.58
III	2.00 ± 0.00	4.67 ± 0.21*	6.50 ± 0.22*	8.67 ± 0.21*	21.56	36.23	51.94

Values are mean ± SEM, (n = 5); *: $p < 0.05$.

Throughout the 15-min observation, test groups did not significantly affect the latent period of tail-flick response (Table 3). On the other hand, the antinociceptive effect of standard -pentazocine was evident at 15 min. The mean latency time before a tail-flick for group 3b (400 mg/kg) increased from 6.50 ± 0.22 (30 min) to 8.67 ± 0.21 (45 min), which remained elevated above the basal levels throughout the observation period. Pentazocine also exhibited significant anti-nociception, which began at 15 min following oral administration and the effect remained significantly ($p < 0.05$) high throughout the 45 min of observation. The MPA calculated for group 3b increased significantly to 59.94% compared to pentazocine's 77.22% at 45 min. The antinociceptive effect

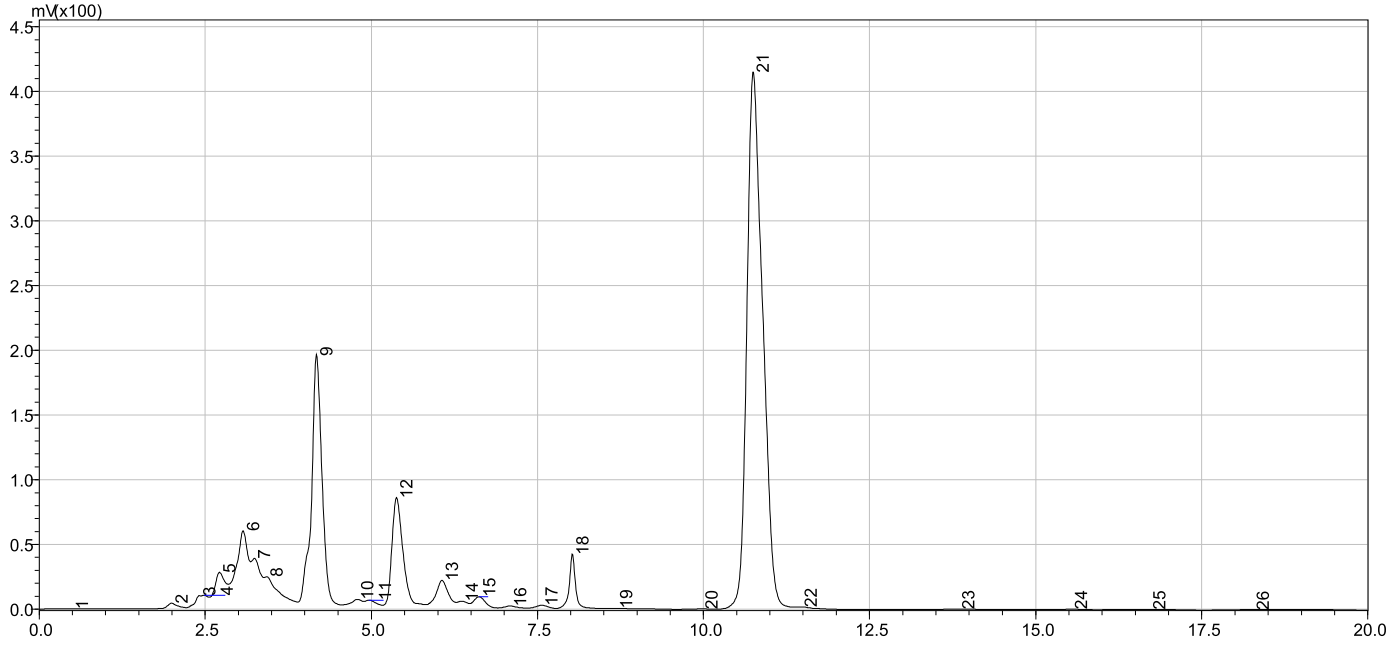

Fig. 1. The HPLC fingerprint of *S. pinnata*.

of *S. pinnata* at a 200 mg/kg dose was only significant at 30 min, and the effect remained significant throughout the 45-min observation period. Pentazocine had the highest mean possible analgesia (77.22%), followed by group 3b (59.94%), group 2b with 26.58%, and finally group 1b with 7.59%.

4. Discussion

Pain represents the sum of reactions that include specialized and non-specialized tissues and psychological and cognitive reactions to painful stimuli. A useful way of categorizing pain for studying analgesics is distinguishing between visceral and somatic types (Pasternak, 1993). Visceral pain is perceived as a diffuse and burning sensation. It also increases aggressive behavior in mice (Khosravi et al., 2021). In contrast, somatic pain is localized and sharp.

In this study, the *S. pinnata* ethanol extract of aerial parts was moderately toxic at doses of 1500 mg/kg following the estimation of LD50 under the acute oral toxicity study. Therefore, relatively lower doses, i.e., 100–500 mg/kg, were chosen to screen the plant for its analgesic activity and determine the therapeutically significant doses.

The acetic acid-induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. The response is thought to be mediated by peritoneal mast cells (Ribeiro et al., 2000), acid-sensing ion channels (Voilley, 2004), and the prostaglandin pathways. Preliminary phytochemical screening reveals flavonoids, steroids, alkaloids, tannins, and saponins in the plant extract. Our findings are incongruent with those reported in another study (Masoko and Masiphephethu, 2019). Further to this, our study found that ethanol extract contained about 15.5 ± 1.4 mg GAE/g of total phenolic content and 0.125 ± 0.61 mg QE/g of total flavonoid content.

Contrary to our findings, Masoko and his colleague reported a higher total phenolic and flavonoid content at 55.33 ± 3.51 mg GAE/g and 4.00 ± 0.35 mg QE/g, respectively (Masoko and Masiphephethu, 2019). Such an observed difference in the amount of both total phenol and flavonoid could be attributed to variation in the plant's geographical distribution and method of extraction. Phenol is the largest group of phytochemicals with diverse bioactivities. Studies showed that phenols have antioxidants, anti-inflammation, and analgesic effects (Boussouf et al., 2017). Similarly, flavonoids exert powerful antioxidant and analgesic activity primarily by targeting prostaglandins (Vinson et al., 1995). The observed analgesic activity in our study could likely be attributed to the presence of these compounds.

For the first time, we reported the analgesic activities of ethanol extract of *S. pinnata* at an oral dose of 400 mg/kg. On the other hand, a dose of 100 mg/kg extract had non-significant inhibition of peripheral pain.

The tail immersion test is considered to be selective to examine compounds acting through the opioid receptor. The extract increased mean basal latency, which indicates that it may act via a centrally mediated analgesic mechanism. Narcotic analgesics inhibit both peripheral and central pain mechanisms, while nonsteroidal anti-inflammatory drugs inhibit only peripheral pain (Elisabetsky et al., 1995). The extract doses (200–400 mg/kg) inhibited the central mechanism of pain, suggesting that the plant extract also acts as a narcotic analgesic.

5. Conclusion

This study's findings have demonstrated that ethanol extract of *S. pinnata* has significant moderate central and peripheral anti-nociceptive properties. The observed antinociceptive effect could justify its use in traditional pain management. Future studies could attempt to identify fractions and pure compounds responsible for pain-relieving activities.

Authors' contribution

CDS designed the study and wrote the initial draft of the manuscript. CDS and ELP performed the experiment; CDS, ELP, and AM analyzed the data and revised the manuscript's drafts. All authors read and approved the final version to be published.

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Declaration of competing interest

Authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.113913.

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