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Original Research Article

Effect of *Asplenium nidus* ethanolic extract on nociception using a *Caenorhabditis elegans* model

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Abstract

Purpose: *To investigate the antinociceptive effect of Asplenium nidus ethanolic extract (ANEE) using a Caenorhabditis elegans model.*
Methods: *Sublethality assay was performed on ANEE to determine the experimental concentrations to be used for the antinociceptive assays. Antinociceptive effect of ANEE in C. elegans was investigated using mechanosensation assays in four treatment timepoints within 72 hours. Antinociceptive index (AI) was calculated for the cells treated with ANEE cells as well as morphine, paracetamol and control (!% DMSO).*
Results: *The mechanosensation assays revealed that ANEE (10^4, 10^3, 10^2 µg/mL) had a significantly higher antinociceptive index (AI) ($p<0.05$) compared to the vehicle control (1% DMSO). The antinociceptive effects of ANEE, 2.5 µM morphine, and 0.01% mg/mL paracetamol in C. elegans were not significantly different ($p>0.05$). This effect of ANEE continued after four treatments within a 72-hour period.*
Conclusion: *The findings revealed that A. nidus ethanolic extract (ANEE) possesses antinociceptive effect which validates folkloric use of A. nidus and suggest a potential for chronic therapeutic use.*

Keywords: *Analgesics, Asplenium nidus, Antinociceptive effect, Pain, Nociception*

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INTRODUCTION

Acute pain is from an injury or disease and is self-limiting, while chronic pain is a disease state per se due to its persistence even after the primary injury has healed [1-3]. Pain is a complex experience that present differently in different people, even between those with similar injuries and/or illnesses. It can be very mild, almost unnoticeable, or explosive. To manage pain, conventional approaches include the use of non-steroidal anti-inflammatory drugs (NSAIDs), steroids, opioids, and paracetamol while unorthodox approaches range from utilizing medical cannabis to animal venom or various plants with folkloric use [4-7]. Despite the call to explore alternatives with fewer undesirable effects like irritation, bleeding, and addiction as seen in NSAIDs and opioids, respectively [8], there is a lull in pain medication innovation with an expected increase in cost for pain management. This burden is magnified in low- and middle-income countries, as in Southeast Asia where access to pain medication is unreliable [9,10].

Asplenium nidus, is a fern belonging to the Aspleniaceae family. In Philippines, it is known as *Pugad lauin*, while in China, it is called *Tai wan shan su hua* but *Rumah Langsuyar* in Malaysia. Synonymous names include *Asplenium ficifolium, A. antiquum, Neottopteris mauritiana*, and *N. nidus*. *A. nidus* is native to tropical Africa, Australia and tropical Asia. It grows terrestrially on the ground, epiphytically and sometimes on rocks. This ornamental fern has found various uses that include management of labor pains, fever, and inflammation in traditional medicine [6,7]. To the best of our knowledge, experimental validation of the use in nociception has not been reported.

In pain research, *Caenorhabditis elegans* (free-living nematodes) are extensively used. Mammalian models share similar nociceptor dendritic branching and gene expression, and nociceptive behavior with *C. elegans* [11]. In addition, their reproducible behavior, rapid life span, and fecundity provide easily reproducible results [12,13]. *Caenorhabditis elegans* have simple and well-characterized anatomy, and also share physiology similar to vertebrates. This enables the use of this invertebrate model for rapid and efficient screening of antinociceptives with minimal consideration for physiologic complexities [14,15]. In *C. elegans*, pain or nociception is translated as a sensation from noxious stimuli that activate high-threshold mechanosensory neurons called nociceptors. Intact pain detection by nociceptors triggers a rapid reversal, and impairment of nociceptors results in delayed or absent reversal [16]. *Caenorhabditis elegans* nociceptors are primarily PVD and ASH neurons in the midsection and in the head, respectively. PVD neurons are stretch-sensing mechanosensors for mechanical nociception and are stimulated by harsh touch [16]. ASH neurons function for chemosensation, in addition to mechanosensation [16]. They are responsible for only 60% of the response to nose touch as other non-nociceptive sensory neurons are colocalized in the head. Hence, even with an impaired ASH nociceptor, worms may still elicit weak compensatory responses to nose touch [16]. Given these, we can determine if ANEE exhibits antinociceptive effects in *C. elegans* when PVD- and ASH-mediated responses are perturbed.

The use of this model is appealing in studying some substances with mechanisms that are yet to be elucidated. Using this model, this study explored the indication of *A. nidus* ethanolic extract (ANEE) for pain relief. The researchers hypothesized that ANEE has an antinociceptive potential that can be demonstrated in *C. elegans*. To the researchers' knowledge, this is one of the first studies to explore the novel use of local ferns for pain management.

EXPERIMENTAL

Animal cultures

Wild-type N2 strains of *C. elegans* were procured from Caenorhabditis Genetics Center, University of Minnesota. Following standard protocols [16], cultures were maintained in 60 mm Nematode Growth Medium (NGM) agar plates at 20º C and were fed heat-killed *E. coli* (OP50 strain). Cultures were maintained at the Biological Models Laboratory, Department of Biochemistry and Molecular Biology, University of the Philippines, Manila.

Preparation of plant extracts

Asplenium nidus fronds were collected in Lucban, Quezon, Philippines (14°06'43.4"N 121°32'23.9"E). The fronds were air-dried and powdered and 1 kg of the powder was submerged into 1 liter of 95% ethanol (#493511, Sigma-Aldrich, St. Louis, MO, USA) in a covered amber glass jar, stored at room temperature, and filtered after 72 hours. The filtrate underwent rotary evaporation (Buchi® R-200 Rotavapor System, Flawil, Switzerland) and was freeze-dried (Labconco Freezone 2.5L Benchtop Freeze Dry System, Kansas City, MO, USA). The lyophilized extract was reconstituted using dimethyl sulfoxide (DMSO, #D5879, Sigma-Aldrich, St. Louis, MO, USA), and ANEE concentrations from 10^6 to 10^{-1} µg/mL in 1% DMSO were prepared.

Sublethality assay

L4 stage *C. elegans* (n=20 per treatment) were transferred using a worm picker from a stock culture to a fresh 35mm NGM agar plate. As a food source, 100 µL of heat-killed *E. coli* OP50 were seeded at the center of the NGM plate. Then, 100 µL of ANEE concentrations 10^6 to 10^{-1} µg/mL in 1% DMSO were dispensed onto respective 35mm NGM plates. Live worms were counted and transferred to a fresh 35mm NGM plate with heat-killed *E. coli* OP50 after 24, 48, and 72 hours. After 72 hours, the highest concentration of ANEE tested that killed less than 10% of the worm population (LD_{10}) was considered the highest sublethal dose (10^4 µg/mL). This concentration was used as the highest experimental concentration for the study's succeeding steps. Additionally, middle (10^3 µg/mL) and low (10^2 µg/mL) sublethal concentrations were also used in further steps.

Mechanosensation assays

Worms were evaluated for their body movement responses to different touch stimuli (mechanosensation assay) before treatment and 30 minutes post-treatment with ANEE or controls, for 72 hours. L4 stage *C. elegans* (n=15 per treatment) were transferred with a worm picker from a stock culture to a 35mm NGM agar plate. Food administration was done by dropping heat-killed *E. coli* OP50 (100 µL) concentrated solution onto the treatment NGM plate before treatment administration. For the assays, worms were transferred from the treatment plate to the viewing plate without food (35mm NGM). After transfer, a 5-minute acclimatization was observed before the evaluation of mechanosensation. Worms were transferred to a fresh NGM plate with food after evaluation and were re-treated and re-evaluated after 24, 48, and 72 hours. The use of 2.5 µM morphine (MORPH) and 0.01% mg/mL paracetamol (PCM), both in distilled water, as analgesic controls was adapted from the protocols of Nieto-Fernandez et al. and Gorrepati, respectively [13,14].

The mechanosensation assays include harsh touch (to the midbody), gentle touch (to the head and tail), and nose touch. Stimulation of mechanosensory neurons by the said touches elicits a backward initiation of movement (reversal) that is then graded. Reversal grading was adapted from Nieto-Fernandez et al. [13] and was modified by classifying reversals as grades 1 (rapid; unimpaired sensation), 2 (sluggish; impaired sensation), and 3 (no reversal; impaired sensation). The number of worms per reversal grade was recorded.

An antinociceptive effect in *C. elegans* is represented by the antinociceptive index (AI). This is the difference between the number of worms with impaired responses (grades 2 to 3) and the number of worms with unimpaired responses (grade 1), divided by the total number of worms evaluated (AI = (worms with an impaired response - worms with an unimpaired response) / total number of worms evaluated).

The degree of antinociceptive effect is further evaluated in worms with impaired responses using the comparative antinociceptive index (CAI). This is the difference between the number of worms with grade 3 responses and the number of worms with grade 2 responses, divided by the total number of worms with impaired responses (grades 2 to 3) (CAI = (worms with grade 3 responses - worms with grade 2 responses) / total number of worms with grades 2 to 3 responses).

Harsh Touch

Harsh touch mechanosensation assay was done by prodding the worm midsection with a 0.1 mm platinum wire probe [16]. After a harsh touch, a reversal was observed and graded. A rapid (grade 1) reversal indicates unimpaired nociceptive PVD neurons [16]. The number of worms per response grade was recorded.

Nose Touch

In the nose touch assay, the hair attached to the probe was laid in front of the worm. As the worm moves forward, a rapid (grade 1) reversal is expected when polymodal ASH neurons are stimulated via contact with the hair [16]. The previously described response grading was used and recorded.

Gentle Touch

Disinfected eyelash hair (dipped into 70% ethanol) attached to the end of a wooden probe was used to cross-sectionally stroke specific areas on the worm. For anterior gentle touch (to the head), the hair was stroked at the level adjacent to the worm pharynx. Stroking at the level before the anus was done for posterior gentle touch (to the tail). A rapid (grade 1) reversal is expected after a gentle touch stimulates the sensory ALM and PLM neurons located in the head and tail, respectively [16]. The previously described response grading was used and recorded.

Statistical analysis

Statistical analysis and figures were generated using Graphpad 8.0.0. One-way Analysis of Variance (ANOVA) was used to test for statistical significance within treatments, and between controls and treatments. This was followed by multiple pair-wise comparisons using the Holm-Sidak method.

RESULTS

Sublethality

The sublethality assay identified the concentration (LD_{10}) that was not lethal to more than 90% of the treated worm population throughout treatment (
Figure **1**). ANEE was sublethal in *C. elegans* starting at 10^4 µg/mL.

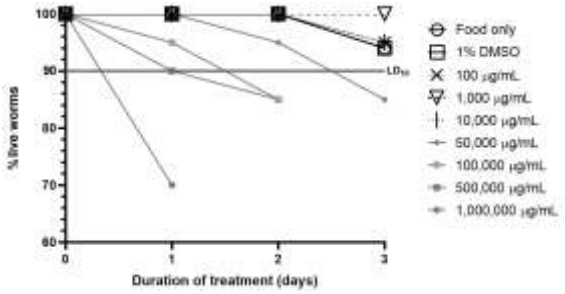

Figure 1: Sublethality of A. nidus
ANEE 10^4 µg/mL is the highest studied concentration with >90% live worms after three days (n=20). Data points of sublethal concentrations are in black

Mechanosensation assays

From four treatment time points in a 72-hour period, the mean number of worms with respective gradings are provided in Table 1. The number of worms with impaired harsh touch responses (grades 2 and 3) was greater in morphine, paracetamol, and ANEE-treated groups when compared to the treatment and control vehicles, 1% DMSO and distilled water, respectively. This observation was similar in nose touch mechanosensation assay. The mean number of worms with impaired mechanosensation to gentle touches was not apparent across treatments.

Harsh touch

The ANEE treatments (10^4, 10^3, and 10^2 µg/mL) displayed statistically higher antinociceptive indices (0.93 ± 0.14, 0.77 ± 0.47, and 0.80 ± 0.40, respectively) from the treatment vehicle 1% DMSO (0.00 ± 0.25) (Figure 2A; ANOVA $p<0.05$). Concurrently, the AI of 2.5 µM morphine (0.80 ± 0.08) and 0.01% mg/mL paracetamol (0.57 ± 0.20) were also significantly higher (ANOVA $p<0.05$) than the treatment vehicle and control vehicle (distilled water, DW) (-0.40 ± 0.42). In comparing the AI of 1% DMSO and DW, unpaired t-test showed no statistical difference ($p>0.05$).

Table 1: *M*ean number of worms in treatments grouped per assay and response grade

	Mean±SEM number of worms						
	MORPH, 2.5 µM	PCM, 0.01 mg/mL	ANEE, 10^4 µg/mL	ANEE, 10^3 µg/mL	ANEE, 10^2 µg/mL	1% DMSO	DW
Harsh Touch							
Worms with Response Grade 3	8.25±1.63	5.00±0.79	3.50±1.03	4.75±1.43	2.25±0.65	0.75±0.41	0.00±0.00
Worms with Response Grade 2	5.25±1.71	6.75±1.24	11.00±1.06	8.50±0.43	11.25±0.65	6.75±0.54	4.50±1.35
Worms with Response Grade 1	1.50±0.25	3.25±0.65	0.50±0.43	1.75±1.52	1.50±1.30	7.50±0.83	10.50±1.35
Nose Touch							
Worms with Response Grade 3	0.75±0.41	0.00±0.00	0.00±0.00	0.0±0.0	0.00±0.00	0.00±0.00	0.00±0.00
Worms with Response Grade 2	5.00±0.94	5.50±0.43	5.00±0.61	5.25±0.0	4.25±0.22	1.00±0.35	0.75±0.41
Worms with Response Grade 1	9.25±0.89	9.50±0.43	10.00±0.61	9.75±0.74	10.75±0.22	14.00±0.35	14.25±0.41
Anterior Gentle Touch							
Worms with Response Grade 3	0.00±0.00	0.00±0.00	0.00±0.00	0.0±0.0	0.00±0.00	0.00±0.00	0.00±0.00
Worms with Response Grade 2	1.75±1.02	1.75±0.74	0.75±0.41	0.50±0.43	0.50±0.43	0.25±0.22	0.25±0.22
Worms with Response Grade 1	13.50±1.02	13.25±0.74	14.25±0.41	14.50±0.43	14.5±0.43	14.75±0.22	14.75±0.22
Posterior Gentle Touch							
Worms with Response Grade 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.25±0.22	0.00±0.00
Worms with Response Grade 2	1.50±1.03	2.00±0.00	1.75±0.74	1.75±0.22	0.75±0.41	1.25±0.65	1.00±0.35
Worms with Response Grade 1	13.50±1.03	13.00±0.00	13.25±0.74	13.25±0.22	14.25±0.41	13.50±0.83	14.00±0.35

n=15 worms evaluated per mechanosensation assay. MORPH, morphine; PCM, paracetamol; ANEE, Asplenium nidus ethanolic extract; DMSO, dimethyl sulfoxide; DW, distilled water

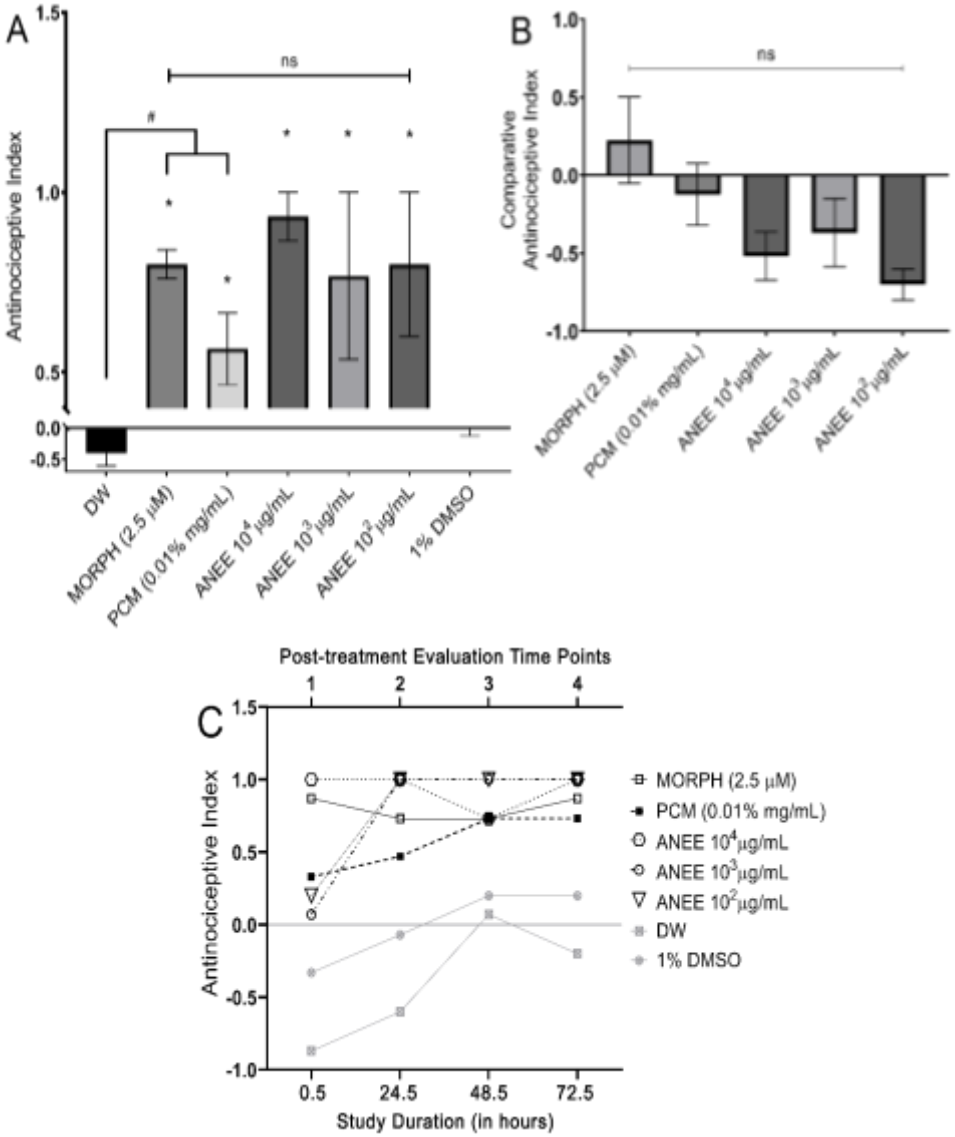

Figure 2: The antinociceptive index (AI) of treatments and controls in the harsh touch assay (n=15). (A) The AI values of ANEE and analgesic controls are not statistically different, and both showed a significant statistical difference versus the 1% DMSO treatment vehicle (*p<0.05). Analgesic controls are statistically different from their distilled water (DW) control vehicle ($^{\#}$p<0.05). (B) The CAI of ANEE (10^4 and 10^3 µg/mL) are not significantly different from controls (p>0.05). Error bars represent standard error. (C) Worms were re-treated at 24, 48, and 72 hours, with re-evaluations before and 30 minutes after each re-treatment. AI values of ANEE and analgesic controls across four time points were consistently above zero and greater than vehicle controls. *MORPH, morphine; PCM, paracetamol; ANEE, Asplenium nidus ethanolic extract; DMSO, dimethyl sulfoxide; DW, distilled water*

For comparing the degree of antinociceptive effect observed among groups with affected nociception, analyses show that the comparative AI (CAI) among ANEE (all concentrations), morphine, and paracetamol were not statistically different from each other (Figure 2B, ANOVA *p*>0.05). Furthermore, the CAIs of all ANEE concentrations were also not significantly different, and dose-dependence was not observed (Figure 2B, ANOVA *p*>0.05).

Data revealed that the analgesic controls and ANEE treatments consistently had AI values above zero (AI>0) across the three re-treatment and re-evaluation time points (Figure 2C).

Nose touch

The administration of controls (morphine, -0.24 ± 0.14; paracetamol, -0.26 ± 0.07) and ANEE, at 10^4 µg/mL (-0.34 ± 0.09), 10^3 µg/mL (-0.3 ± 0.11), and 10^2 µg/mL (-0.44 ± 0.04), affected the worm responses to nose touch statistically

differently from 1% DMSO (-0.87 ± 0.11) (Figure 3A, ANOVA p<0.05). Relative to the DW (-0.90 ± 0.06), morphine and paracetamol AI were also

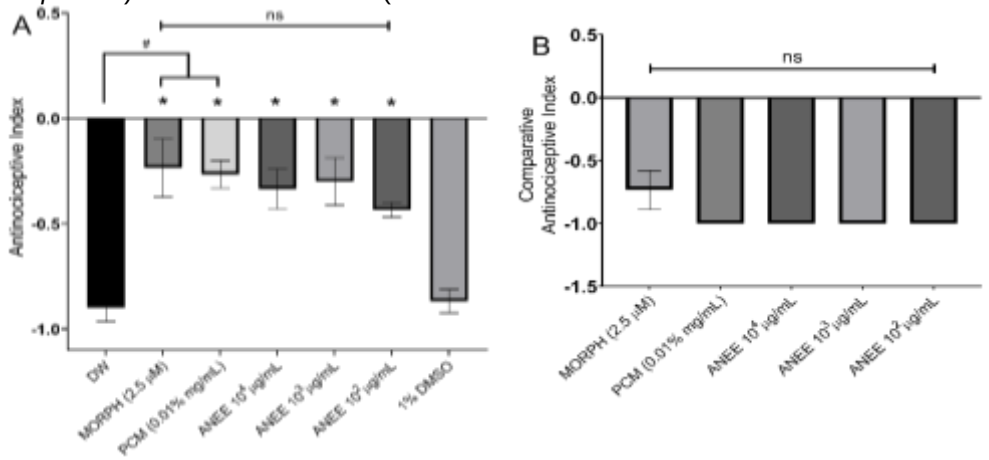

Figure 3: Antinociceptive index (AI) of treatments and controls in the nose touch assay (n=15). (A) The AI of ANEE, morphine, and paracetamol showed significant statistical differences versus the 1% DMSO treatment vehicle (*p<0.05). ANEE and analgesic controls are not statistically different from each other. Morphine and paracetamol AIs are statistically different from the control vehicle (DW) (#p<0.001). (B) The CAIs of all ANEE concentrations are not significantly different from controls (p>0.05). Error bars represent standard error. *MORPH, morphine; PCM, paracetamol; ANEE, Asplenium nidus ethanolic extract; DMSO, dimethyl sulfoxide; DW, distilled water*

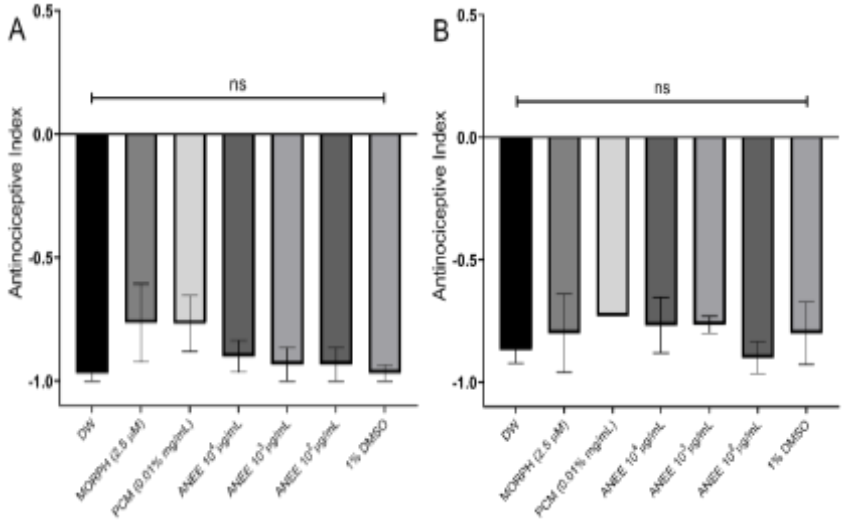

Figure 4: Antinociceptive index (AI) of treatments and controls in the gentle touch assays (n=15). Anterior gentle touch (A) and posterior gentle touch (B) assay data revealed no statistically significant differences in the AI of treatments and controls (p>0.05). Error bars represent standard error. *MORPH, morphine; PCM, paracetamol; ANEE, Asplenium nidus ethanolic extract; DMSO, dimethyl sulfoxide; DW, distilled water*

statistically higher (Figure 3A, ANOVA p<0.001). In comparing the degree of observed antinociceptive effect between controls and ANEE concentrations, CAI of groups was shown to not differ significantly from each other (Figure 3B, ANOVA p<0.05). As represented by AI values less than zero, the number of worms with unimpaired nose touch responses (grade 1) is greater than those with impaired responses (grade 2 to 3). This is observed consistently across all groups.

Gentle touch

No significant AI differences among treatment (10^4 µg/mL, -0.90 ± 0.06; 10^3 µg/mL, -0.93 ± 0.07; and 10^2 µg/mL, 0.93 ± 0.07), controls (morphine, -0.77 ± 0.16; and paracetamol, -0.77 ± 0.11), and vehicles (1% DMSO, -0.97 ± 0.03; and DW, -0.97 ± 0.03) were observed in anterior gentle touch assay (Figure 4A, ANOVA p>0.05). Analysis of posterior gentle touch data also showed no significant differences among treatments (10^4 µg/mL, -0.77 ± 0.11; 10^3 µg/mL, -0.77 ± 0.04; and 10^2 µg/mL, -0.90 ± 0.06), controls (morphine, -0.80 ± 0.16; and paracetamol, 0.73 ± 0.00), and vehicles (1% DMSO, -0.80 ± 0.13; and DW, -0.87 ± 0.06)

(Figure 4B, ANOVA $p>0.05$). Similar to the nose touch assay, AI values for gentle touch in all groups are less than zero.

DISCUSSION

This study showed that sublethal ANEE concentrations significantly affect *C. elegans* nociception, as evaluated by harsh touch and nose touch mechanosensation assays. Additionally, non-nociceptive sensory neurons evaluated by gentle touch were unaffected. These effects are similarly observed in morphine-treated and paracetamol-treated worms. It was also observed that ANEE maintains its antinociceptive effect after three 24-hour interval re-treatments. These data suggest that ANEE shows antinociceptive effects with potential indications for chronic use.

AI in harsh touch of ANEE were greater than zero and are statistically significant from the AI of vehicles (1% DMSO and DW) indicating that in ANEE-treated worms, there is a significantly higher number of worms with impaired PVD nociception compared to the control group. The observed AI > 0 value in known analgesics, morphine and paracetamol agrees with previous investigations on the antinociceptive effects of the said analgesics in *C. elegans* [13,14]. No dose-dependence was observed since the indices of studied concentrations (10^4, 10^3, and 10^2 µg/mL) were not statistically significant. This may be so as the concentration gradient where this can be detected has not been encompassed. The antinociceptive effect of ANEE evaluated by the harsh touch assay, was intact across four treatment time points within 72 hours.
Antinociceptive indices of ANEE, morphine, and paracetamol were consistently greater than zero (AI>0) and showed no overlapping trend with vehicle controls (
Figure **1**C).

Data from the nose touch assay further strengthens the evidence for the antinociceptive effect of ANEE. Although there is a lower number of worms with observable nose touch response impairment in all treatment and analgesic control groups, the AI values of ANEE and analgesic groups were still statistically different from the control. The seemingly minimal but significant AI values in nose touch may be due to the compensation of non-nociceptive sensory neurons when there are perturbations in nociceptors.

Gentle touch assays stimulate six non-nociceptive mechanosensors in the worms. Anterior gentle touch stimulates the ALML/R and AVM neurons, while posterior gentle touch stimulates the PLML/R and PVM neurons [16]. Gentle touch responses are not expected to diminish on analgesic treatment since nociceptors do not mediate the response to both anterior and posterior gentle touch. As anticipated, gentle touch AI values in all groups were less than zero and were not statistically significant from each other. In all groups, there were significantly more worms with no observable response impairment (AI<0). This suggests that ANEE and analgesic controls do not impair non-nociceptive mechanosensors and their detected activities in *C. elegans* are specific to nociception, as far as mechanosensation assays are involved.

The presence of alkaloids, flavonoids, tannins, and anthraquinones in ANEE have been reported previously [18]. These phytochemicals have known anti-inflammatory potentials [19-21] thus providing possible explanations for the folkloric indication of *A. nidus* for pain, wounds, and inflammation, that has been supported by the experimental evidence for the pain relief in this study.

CONCLUSION

This study showed ANEE has antinociceptive potential comparable to morphine (2.5 µM) and paracetamol (0.01% mg/mL) and has low toxicity with a sublethal concentration at 10^4 µg/mL. The ANEE has the potential for chronic antinociceptive effects.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

This work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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