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ORIGINAL RESEARCH

Evaluation of Analgesic Activities of Extracts of Two Marine Molluscs: *Tympanotonus fuscatus* var radula (Linnaeus) and *Pachymelania aurita* (Müller)

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Queensley Eghianruwa[1,2]
Omolaja Osoniyi[3]
Naomi Maina[1,4]
Sabina Wachira[5]
Mabel Imbuga[4]

[1]Department of Molecular Biology and Biotechnology, Pan African University Institute of Science, Technology and Innovation, JKUAT campus, Juja, Kenya; [2]Department of Biochemistry, University of Uyo, Uyo, Nigeria; [3]Department of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile Ife, Nigeria; [4]Biochemistry Department, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya; [5]Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute, Nairobi, Kenya

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Correspondence: Queensley Eghianruwa
Department of Biochemistry, University of Uyo, Uyo, Nigeria
Tel +2348024195501
Email qaeghianruwa@uniuyo.edu.ng

Purpose and Methods: In this study, the analgesic activity of the crude alcohol (acetone-methanol) and aqueous (in PBS, pH 7.2) extracts of the marine molluscs, *Pachymelania aurita* and *Tympanotonus fuscatus,* has been evaluated using the formalin test (for chronic antinociceptive) and the tail-flick (acute antinociceptive) pain models in male swiss albino mice.

Results: The results show that the extracts of *P. aurita* and *T. fuscatus* demonstrated high safety margins as single doses of up to 2000 mg/kg bwt proved to be well tolerated and non-lethal, although the alcohol extract of *P. aurita* caused necrosis in the liver and kidney when administered at a dose level of 2000 mg/kg bwt. In the formalin test, treatment with the aqueous extracts of *P. aurita* and *T. fuscatus* as well as the alcohol extract of *T. fuscatus* 30 min before the subcutaneous injection of 5% formalin to the paw of the mice resulted in a significant time- and dose-dependent reduction in total and phase 2a pain-related behavior and thus nociception. The extracts had no analgesic effect in tail-flick test up to the highest dose tested.

Conclusion: Hence, the results from both models indicate that the site of their analgesic action is probably peripheral.

Keywords: Nigerian periwinkle, formalin test, anti-nociception and analgesia, marine natural products, bioactivity

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) and Narcotic analgesics (Opiates) are extensively used for reducing pain and decreasing fever and inflammation.[1–3] However, Opioids (Opiates) and NSAIDs can only relieve 50% of the pain in about 30% of patients;[3] hence, they are not useful in all cases. Furthermore, NSAIDs have been known to increase the risk of gastrointestinal ulcers and bleeds, heart attack as well as kidney disease.[4–6] Potentially severe side effects of opiates include decreased respiratory ability as well as low blood pressure.[4–6] Furthermore, Narcotics have a high potential for addiction. Hence, research to discover other alternatives to treat pain is critical.

Natural products offer promising sources of new chemical entities that can be used for the treatment of pain. Seventy percent of the world's surface is covered by water bodies; in it are marine natural resources which would be potential sources for metabolites with analgesic/antinociceptive activities. Recent studies have demonstrated that many Secondary metabolites produced by marine life have

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a useful anti-inflammatory and analgesic effect.[7–10] In December 2004, the US Food and Drug Administration (FDA) approved Prialt® (also known as ziconotide), initially isolated from the venom of the predatory marine mollusc, *Conus magnus*, as a painkiller for severe chronic pain in HIV and cancer patients.[11]

Tympanotonus fuscatus var radula (Linnaeus) and *Pachymelania aurita* (Müller), generally called Nigerian Periwinkles, are two of the most commonly found, consumed and utilized marine mud creeper species. They are found in beaches and mangroves bordering the Atlantic Ocean. In folk medicine of people in Delta state, Nigeria, extracts of this mollusc are used for treating ulcers and wounds. Thus, this study aims at evaluating the analgesic potential of extracts of *T. fuscatus* and *P. aurita.*

Materials and Methods

Laboratory Animals

Male inbred swiss albino mice, weighing approximately 25 ± 1.03 g, were bred and housed under the controlled laboratory conditions of the Small Animal Facility for Research and Innovation of the Jomo Kenyatta University of Agriculture and Technology (SAFARI-JKUAT; temperature 27±2°C, relative humidity 60–70%). The animals were fed on a standard commercial diet (whole maize mice pellet; Unga Feeds, Kenya) and water *ad libitum*. All animals were fasted overnight prior to the treatment but allowed free access to water at all times.

Sample Collection

Live *Tympanotonus fuscatus* var radula and *Pachymelania aurita* were purchased during the month of August from the Oron Beach Market, Oron, Akwa Ibom State, Nigeria (GPS coordinates: 4°49′37.6′′N 8°14′04.4′′E). The molluscs were washed thoroughly to remove mud and then deshelled to collect both their flesh and hemolymph.

Preparation of Alcohol Extracts

The alcohol extracts of *T. fuscatus* and *P. aurita* were prepared using the method described by Eghianruwa et al.[12] Briefly, 200 g of mollusc flesh in its hemolymph was macerated using a blender and extracted twice with 1 L acetone for each cycle. Each cycle of extraction with acetone was carried out overnight (14 hrs) at room temperature with constant stirring using a magnetic stirrer and the homogenate was filtered using a muslin cloth. After acetone extraction, the biomass residue of the sample was subjected to two cycles (12 hrs each) of extraction using a total of 1500 mL of methanol. The Acetone and methanol fractions were combined and concentrated by evaporation using a rotary evaporator at 40°C then stored at 4°C.

Preparation of the Aqueous Extracts

Two hundred grams of mollusc flesh in its hemolymph was homogenized with 2 L of Phosphate buffered saline (PBS, pH 7.2 with 0.1 M PMSF) using a blender. The homogenate was left to extract for 48 hrs at 4°C after which it was centrifuged at a speed of 10,000 g using a cold centrifuge, freeze dried and stored at 4°C.

The alcohol and aqueous extracts of *P. aurita* are hereby abbreviated as PAAC and PAAQ, respectively, while the alcohol and aqueous extracts of *T. fuscatus*, as TFAC and TFAQ, respectively.

Acute Toxicity Studies

The Acute toxicity test was important in the determination of drug dosage to be administered and was performed according to the method described by Eghianruwa et al.[13] Male inbred swiss albino mice, weighing approximately 25 ± 1.03 g, were randomly sorted into groups of three animals each (a group for control and four groups for each test material). The animals were each given a single intraperitoneal (ip) dose of the test material, constituted in sterile saline solution, in dosages of 0 (control), 10, 100, 1000 and 2000 mg/kg body weight (bwt) of animal for a range finding test. The animals were first observed for 48 hrs to check for mortality and signs of malaise (including changes in fur colour, posture and response to stimuli) and then twice daily for 14 days, while the body weights are monitored and recorded at the start of the experiment and then at 5 days interval. Representative animals from each group were sacrificed on the 14th day post drug administration and key organs (the brain, the spleen, the kidneys, the liver, the lungs and the heart) were excised and examined macroscopically before the histopathology examination. Histomorphological investigations were carried out by fixing the representative tissues from each group in 10% Neutral buffered formalin by total immersion for 48 hrs after which they were processed via paraffin wax embedding and stained using haematoxylin eosin.[23]

Evaluation of Analgesic Activity Using the Formalin Test Procedure

The formalin test was carried out using the method described by Salama et al.[14] Male inbred mice were

randomly sorted into groups of three animals each (negative control, positive controls and treatment groups). Each animal, in the treatment group, was administered a single dose (ip) of the test material, constituted in sterile saline solution, at dose levels of 10, 100, 1000 and 2000 mg/kg bwt. The positive control drugs used were 100 mg/Kg bwt and 200 mg/kg bwt of Ibuprofen sodium while sterile saline was used as the negative control. Thirty minutes post administration, 10 µL of 5 % formalin solution was injected into the plantar surface of the left hind paw using a Hamilton syringe (31G X 8 mm).

The animals were observed at 5 min intervals for 1 hr and the time spent flinching, licking and biting the injected paw during this period was recorded. After the test period of 1 hr, all animals used were euthanized, preventing further suffering.

The total of pain-related behavior scores was used for comparisons across groups. The sum of the first 15 min was used to express Phase 1, the sum of the next 45 min was used to express phase 2, and the sum of pain-related behavior scores between the 20th and 40th minutes represent phase2a.[14] Dose–response curves were constructed using a scatterplot of the mean responses (pain-related behaviour) to different ip doses of extracts against the logarithm of ip doses. A sigmoid curve was fitted. The equation used was: dose–response; log (inhibitor) vs response—3 parameters: Y=Bottom + (Top-Bottom)/(1 +10^((X-LogIC50))). The equivalent highest ip dose of standard drug used was interpolated from the curves.

Evaluation of Acute Nociception Using the Tail-Flick Test Procedure

The central analgesic activity was determined in swiss albino male mice described by Sayyah et al.[15] Mice in one positive control group were administered 30 mg/kg bwt Morphine subcutaneously while mice in the other control group were administered 200 mg/kg Acetaminophen orally. Mice in the negative control group were given sterile saline (100 µL). One hundred microliters of each dose of crude extracts (10, 100, 1000 and 2000 mg/kg bwt for the extracts of *T. fuscatus* and the aqueous extract of *P. aurita* and 10, 100 and 1000 mg/kg bwt for the alcohol extract of *P. aurita*) in sterile saline was administered intraperitoneally to male mice sorted into groups of three animals each. The mice were restrained in a soft tissue pocket and the distal half of the tail was placed in an analgesia meter set at 50°C. Latency for tail-flick was measured with a 10-s cutoff time to avoid animal injury. Tail withdrawal from the heat (flicking response) was taken as the endpoint. The tail flick latencies were recorded before administration of the drug and then at 30 min intervals for 3 hrs post-treatment.

Statistical analysis: pain-related behaviour and latency times are expressed as mean (±) SEM and analysed using analysis of variance (one-way analysis of variance, ANOVA) followed by Bonferroni comparison Post-test using the GraphPad Prism Program (version 7.0). P values less than 0.05 were considered significantly different.

Ethical Considerations

All animal studies were conducted in accordance with the Small Animal Facility for Research and Innovation of the Jomo Kenyatta University of Agriculture and Technology (SAFARI-JKUAT) and the Kenyan Medical Research Institute (KEMRI) guidelines on animal use and care and the internationally accepted principles for laboratory animal use and care as found in WHO guidelines. All the experimental procedures and protocols used in the study were reviewed and approved by the Animal care and use committee (ACUC) as well as the Scientific and ethics review unit of the Kenyan Medical Research Institute, Nairobi, Kenya. In keeping with requirements for the 3Rs, the statistical allowed minimum of three (3) mice per treatment was adhered to in this study.

Results

Acute Toxicity Study

During the test period of 14 days, no mortality was recorded neither did the animals show any sign of malaise. Animals were active and moving around, their fur was well kempt and smooth, and their response to stimuli, posture and uptake of food and water were all normal during the test period. The animals showed no significant loss of body weight; rather a steady increase in body weight was observed in animals in the treatment group, similar to the saline-treated group (negative control). This is an indication of normal growth and development. The gross necropsy of the organs investigated, including the heart, the brain, liver, lungs and spleen, after sacrificing showed no gross changes in the morphology or any visible pathological lesions. In the histopathological examination, however, single necrosis of hepatocytes as well as patchy tubular epithelial necrosis in the Kidney (Figure 1) was observed in the animals treated with PAAC (the alcohol extract of *P. aurita*) at the highest dose level tested (2000 mg/kg bwt). No organ damage or lesions were observed in the animals treated with the other extracts (TFAQ, TFAC and PAAQ).

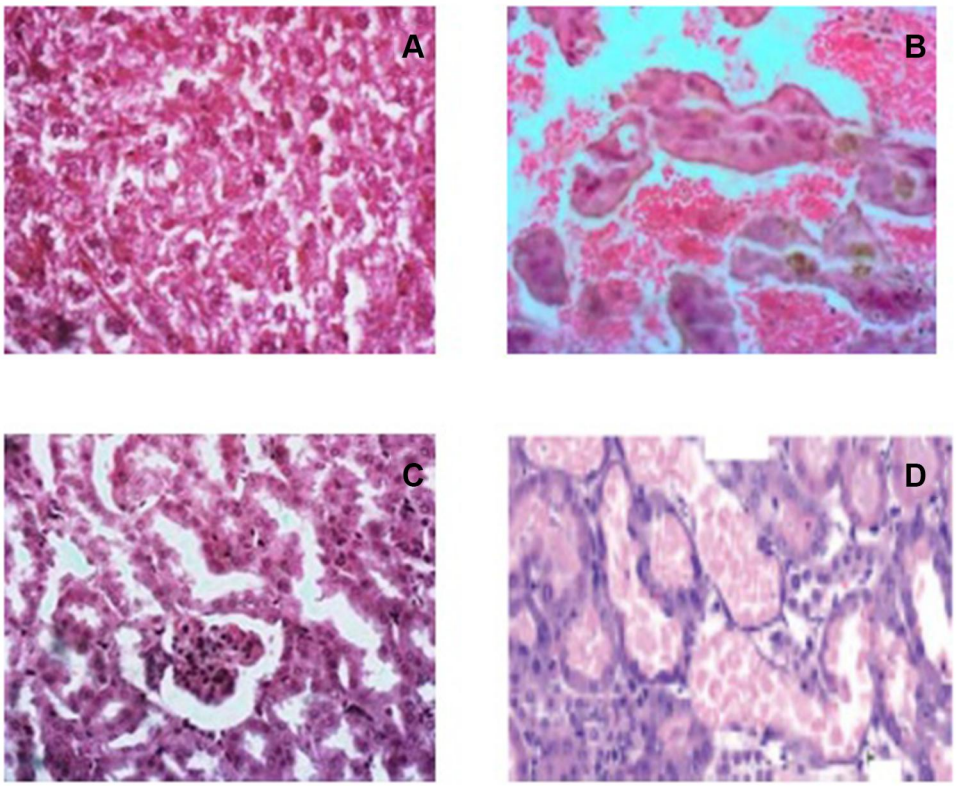

Figure 1 Photomicrographs showing: (**A**). Normal liver from a mice in the control group; (**B**). Liver from a mice with single-cell necrosis of hepatocytes after treatment with PAAC at a dose of 2000 mg/kg bwt; (**C**). Normal Kidney from a mice in the control group; (**D**). Kidney from a mice after treatment with PAAC at 2000 mg/kg bwt showing patchy tubular epithelial necrosis.

Evaluation of Standard Analgesic Effect

In this study, there was no significant difference in formalin-induced pain-related behaviour between the group that received 100 mg/kg bwt of Ibuprofen sodium and the negative control group (Saline only). However, 200 mg/kg of Ibuprofen sodium resulted in a significant reduction in total (70.67%) pain-related behaviour in the mice ($p \leq 0.05$).

Effect of the Alcohol Extract of *P. aurita* (PAAC) on Total Pain-Related Behaviour

Mice administered PAAC, even at the highest dose of 1000 mg/kg bwt, showed no significant reduction ($P>0.05$) in total pain-related behavior when compared to mice in the control group administered saline alone.

Effect of the Aqueous Extract of *P. aurita* (PAAQ) on Total Pain-Related Behaviour

The results show (Figure 2A) that the aqueous extract of *P. aurita* (PAAQ) caused a significant decrease in total pain-related behaviour in mice when compared to the negative control (saline alone) group, but only at a dose of 1000 mg/kg bwt (78.98%) and 2000 mg/kg bwt (86.1%) of the mice. A dose–response curve interpolated showed 198.15 mg/kg of PAAQ was equivalent to 200 mg ibuprofen (Figure 2B).

Effect of the Alcohol Extract of *T. fuscatus* (TFAC) on Total Pain-Related Behaviour

Intraperitoneal injection of different doses (10, 100, 1000 and 2000 mg/kg bwt) of TFAC, 30 min before subcutaneous formalin injection into the ventral surface of the hind paw, resulted in a dose-dependent decrease in total pain-related behaviour when compared to the Negative control (saline only), even at a dose as low at 10 mg/kg of bwt (Figure 3A). Administration of TFAC at a dose level of 10 mg/kg bwt led to a 67.05% reduction in total pain-related behaviour while a dose of 2000 mg/kg bwt TFAC led to 82.45% reduction. This reduction in pain-related behaviour in mice treated with TFAC was not significantly different from Ibuprofen (200 mg/kg bwt). A dose–response curve fit indicated that the equivalent dose of ibuprofen was interpolated to be equivalent to an effective dose of 348.33 mg/kg of TFAC (Figure 3B).

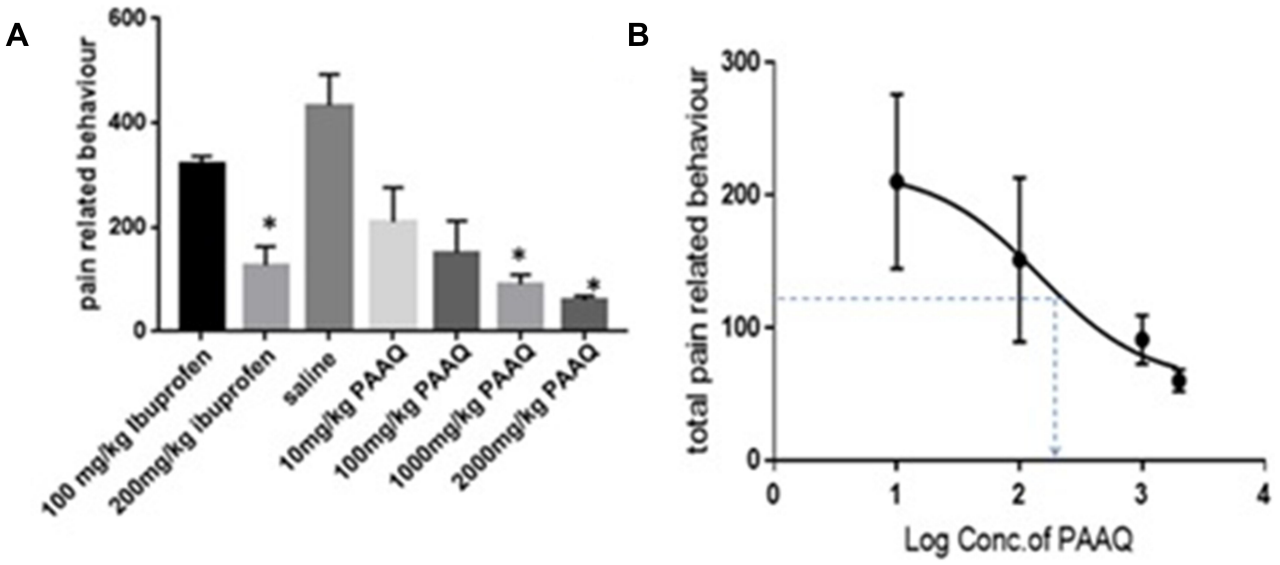

Figure 2 (**A**). Effect of different doses of the aqueous extract of *P. aurita* (PAAQ), administered ip, on formalin-induced pain-related behaviour in mice. Data represented as mean ± S.E.M. with each column representing the sum of pain-related behaviour. * indicates $p < 0.05$ compared to the saline-injected group. (**B**). Dose-response curve of PAAQ administered ip. The curve was constructed using the total pain-related behaviour of the formalin test against logarithmic transformed PAAQ ip doses of 10,100 1000 and 2000 mg/kg bwt. A sigmoid fit was applied and the equivalent ip dose of extract to the administered doses of ibuprofen was interpolated.

Effect of the Aqueous Extract of *T. fuscatus* (TFAQ) on Total Pain-Related Behaviour

Response to TFAQ administered was dose-dependent. The lowest dose of 10mg/kg bwt led to a 70.21% reduction in total pain behaviour while 2000mg/kg bwt of TFAQ led to a 93.8% reduction in total pain-related behavior (Figure 4A). TFAQ at all dose levels showed no significant difference in pain-related behaviour when compared to mice administered with Ibuprofen (200 mg/kg bwt). A dose–response curve fit indicated that the equivalent dose of ibuprofen was interpolated to be equivalent to an effective dose of 8.32 mg/kg of TFAQ (Figure 4B).

Effect of *P. aurita* and *T. fuscatus* Extracts on Phase 1 and Phase 2a Pain-Related Behaviour

There was no significant decrease in phase 1 pain-related behaviour in mice treated with either 200 mg/kg bwt Ibuprofen or any of the extracts of *P. aurita* and *T. fuscatus* when compared to mice administered with saline alone.

However, at phase 2a, 200 mg/kg of Ibuprofen sodium resulted in a significant reduction (83.93%) of pain-related behaviour in the mice (Figure 5). Administration of 1000 mg/kg and 2000 mg/kg PAAQ led to a significant decrease in phase 2a pain-related behavior by 86.45% and 97.09%, respectively (Figure 5). TFAQ showed greater activity than PAAQ. The lowest dose of 10 mg/kg bwt TFAQ resulted in a 97.8% decrease and the highest dose led to a 99.18% decrease (Figure 5). The alcohol extract (TFAC) had slightly lower activity than the aqueous extract. There was no significant decrease in phase 2a pain-related behaviour in mice treated with any of the extracts when compared to mice treated with 200 mg/kg bwt of Ibuprofen (Figure 5).

Effect of *P. aurita* and *T. fuscatus* Extracts on Latency Time in Mice During the Tail Flick

The standard centrally acting analgesic drug, morphine produced a significant analgesic effect in the tail-flick test. However, the extracts of both *P. aurita* and *T. fuscatus*, up to the dose of 2000mg/kg bwt, as well as acetaminophen (200 mg/kg) had no effect on tail-flick latency time when compared to mice administered with saline alone.

Discussion

The extracts of *P. aurita* and *T. fuscatus* demonstrated high safety margins as single doses of up to 2000 mg/kg bwt, proved to be well tolerated and non-lethal. Normal growth and development, as indicated by steady growth weight, was not interfered with and the extracts did not cause the treated animals to display typical signs of malaise. However, the observation of lesions in the liver (single-cell necrosis of hepatocytes) and kidney (acute tubular necrosis) in the mice treated with the highest dose

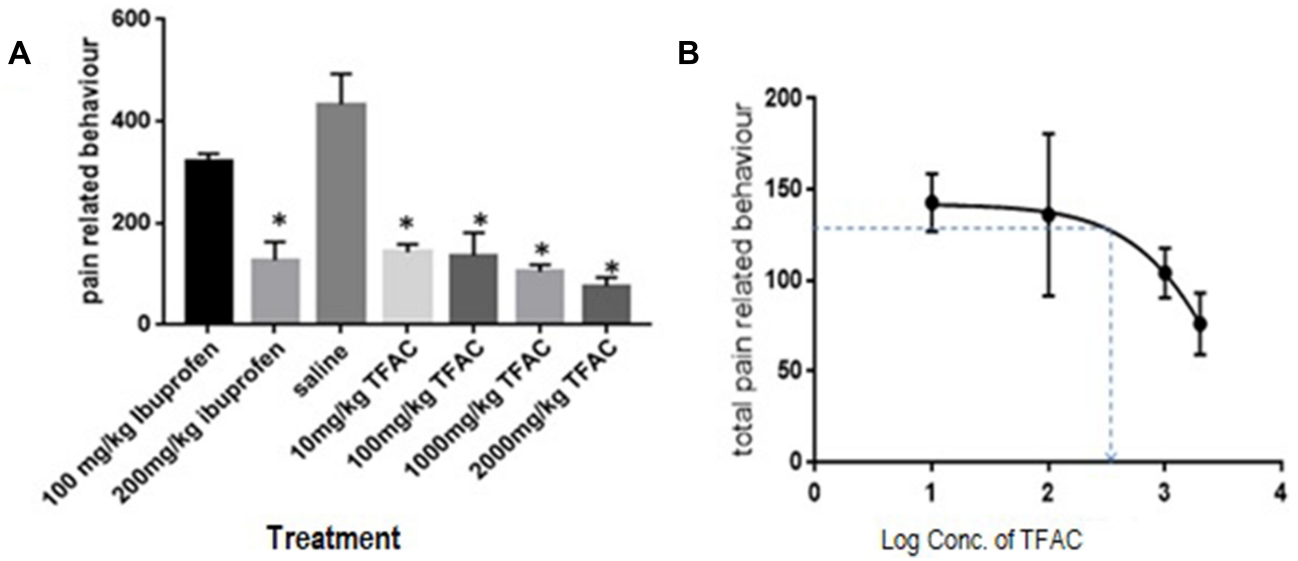

Figure 3 (**A**). Effect of different doses of the alcohol extract of *T. fuscatus* (TFAC), administered ip, on formalin-induced pain-related behaviour in mice. Data are presented as mean ± S.E.M. with each column representing the sum of pain-related behaviour. * indicates $p < 0.05$ compared to the saline-injected group. (**B**). Dose-response curve of TFAC administered ip. The curve was constructed using the total pain-related behaviour of the formalin test against logarithmic transformed TFAC ip doses of 10,100 1000 and 2000 mg/kg bwt. A sigmoid fit was applied and the equivalent ip dose of extract to the administered doses of ibuprofen was interpolated.

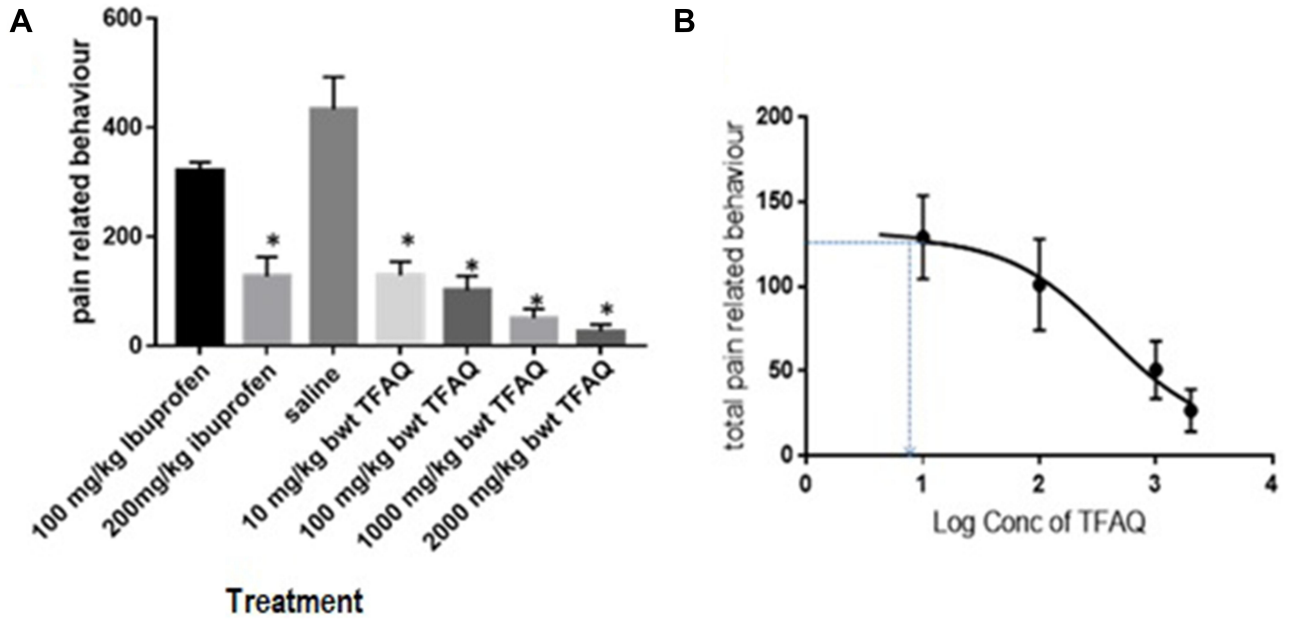

Figure 4 (**A**). Effect of different doses of the aqueous extract of *T. fuscatus* (TFAQ), administered ip, on formalin-induced pain-related behaviour in mice. Data are presented as mean ± S.E.M. with each column representing the sum of pain-related behaviour. * indicates $p < 0.05$ compared to the saline-injected group. (**B**). Dose-response curve of TFAQ administered ip. The curve was constructed using the total pain-related behaviour of the formalin test against logarithmic transformed TFAQ ip doses of 10,100 1000 and 2000 mg/kg bwt. A sigmoid fit was applied and the equivalent ip dose of extract to the administered doses of ibuprofen was interpolated.

(2000 mg/kg) of the alcohol extract of *P. aurita* (PAAC), may be an indication that PAAC is hepatotoxic and nephrotoxic at high doses. The main detoxification organs are the kidney and liver, hence, this toxicity may be linked to the metabolism and clearance of components of the extract. Single-cell necrosis (single, noncontiguous cells that are characterized by cell and nuclear swelling and pale cytoplasm[16] due to uncontrolled apoptosis)[17] is usually due to acute metabolic perturbation as occurs in acute drug-induced hepatoxicity of drugs like acetaminophen among others.[17] Similarly, acute tubular necrosis (which normally has no symptoms and is due to damage to tubule

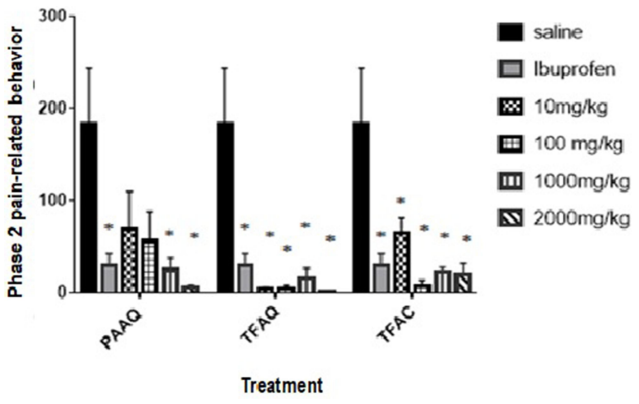

Figure 5 Effect of different doses of PAAQ, TFAC and TFAQ, administered via ip, on the phase 2a formalin-induced pain-related behaviour in mice. Phase 2a represents the time between the 20th and the 40th min of the formalin test. * indicates $p < 0.05$ when compared to the saline-injected group.

cells involved in fluid and mineral reabsorption from the urine as it forms) is caused by low blood flow to the kidneys and nephrotoxic drugs such as NSAIDs.[18] However, before solid deductions and conclusions can be made, further toxicity studies including a sub-acute and subchronic toxicity study would be important in future.

As the acute toxicity study was carried out for dose range finding purposes, for further downstream tests, PAAC was tested using 1000mg/kg bwt as the highest dose administered.

Experimental models of assaying for pain include tests of response thresholds to high-intensity stimuli (acute pain tests) and tests that detect changes in spontaneous or evoked behavioral responses in animals with peripheral injury or inflammation (persistent pain models).[19] In this study, the tail-flick test was the experimental model used for evaluating acute thermal pain while persistent pain was assessed using the formalin test.

The Tail Flick procedure is based on the observation that centrally acting analgesics like morphine are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by temperatures up to 55°C.[15,20] Thermal painful stimuli are known to be selective to centrally acting analgesic drugs while peripherally acting analgesics have little or no influence.[15,20,21] In the present study, morphine, a centrally acting analgesic drug, produced an inhibitory effect on the nociceptive response in the tail-flick test, while the extracts of both *P. aurita* and *T. fuscatus* as well as the peripherally acting analgesic, acetaminophen, failed to affect any significant response. Therefore, it can be inferred that the extracts of *P. aurita* and *T. fuscatus* have no central analgesic activity.

Regarding the formalin test, a dose of 300 mg/kg of ibuprofen administered in mice has been reported to result in high mortality. According to studies conducted by Salama et al, the dose of 75 mg/kg induces a significant decrease in nociception only in phase 2a, while a dose of 50 mg/kg does not exhibit any significant decrease in pain-related behavior.[14] Thus, a dose level of 100 and 200 mg/kg of Ibuprofen was deemed most suitable and was used in this study. A distinct biphasic nociception occurs upon the subcutaneous injection of formalin.[21,22] The first phase begins directly after the formalin is administered and lasts for approximately 10 min, after which nociception appears to diminish, leading to a relatively quiescent period. High levels of nociception return in the second phase (which occurs 15–20 min after the formalin injection) and continue for approximately 60 min.[19,21,22] The two phases of nociception have different properties. The first phase is mediated as a direct result of stimulation of nociceptors and pain fibers (particularly C fibers), while the second phase is mediated by inflammation, and at least to some degree, by so-called central sensitization (the sensitization of central nociceptive neurons).

Both extracts of *T. fuscatus* were observed to be more effective than the extracts of *P. aurita*, with the aqueous extract of *T. fuscatus* being the most effective, in reducing total nociception. Both extracts of *T. fuscatus*, TFAQ and TFAC, were observed to lead to a reduction in nociception at all dose levels tested, even at the lowest dose of 10 mg/kg. However, from the dose-response curves, it was observed that when compared to the control drug (ibuprofen), the aqueous extracts (PAAQ and TFAQ) had much lower equivalent ip dose (effective dose) of extract to the administered doses of ibuprofen, indicating that lower doses extracts are just as effective as Ibuprofen sodium at reducing nociception.

The extracts of *P. aurita* and *T. fuscatus* had no significant inhibitory effect on pain nociception in the first phase of the Formalin test. However, they caused a significant reduction in pain-related behavior during phase two of the test. This indicates that extracts of *P. aurita* and *T. fuscatus* exhibit peripheral-acting analgesic activity, further confirming the findings from the Tail-flick test procedure. Narcotics like morphine and codeine, which primarily act centrally, inhibit both phases of the formalin test equally but peripherally acting drugs such as aspirin only inhibit the second phase of formalin-induced nociception. Peripheral-acting analgesics like the Nonsteroidal anti-inflammatory drugs

(NSAIDs) reduce pain and oedema by suppressing the formation of prostaglandins by inhibiting the activity of the enzyme prostaglandin synthetase (Cyclooxygenase; COX-1 and COX-2) at the site of injury, thus reducing inflammation and decreasing pain sensitivity. They are not habiting-forming and are most times used to treat chronic pain. Our results suggest that these mollusc extracts can be used in instances where peripheral-acting pain relievers are needed.

Conclusion

The aqueous and alcohol extracts of *T. fuscatus* and *P. aurita* display analgesic activity, with extracts of *T. fuscatus* being more effective. This supports the traditional use of these molluscs in pain relief and wound healing of ulcers.

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Disclosure

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