Journal of Experimental Zoology Part A: Ecological and Integrative Physiology

WILEY

JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY

RESEARCH ARTICLE

Nematicidal Potentiality of Four Marine Molluscans' Defensive Secretions From the Red Sea Against *Syphacia obvelata* (Nematoda: Oxyuridae) In Vitro

Nahla A. Radwan[1] | Aalaa I. Atlam[1] | Asmaa R. Abdel- Malek[2] | Alaa Y. Moustafa[3]

[1]Department of Zoology, Faculty of Science, Tanta University, Tanta, Egypt | [2]Department of Zoology, Faculty of Science, Assuit University, Asyut, Egypt | [3]Department of Zoology, Faculty of Science, Sohag University, Sohag, Egypt

Correspondence: Nahla A. Radwan (nahla.radwan@science.tanta.edu.eg)

Received: 24 April 2024 | **Revised:** 20 August 2024 | **Accepted:** 1 October 2024

Keywords: acid secretion | cephalopods | in vitro | inking | nematicidal activity | sea hares | sea slugs | *Syphacia obvelata*

ABSTRACT

The continuous requirement to substitute safe and affordable alternatives for helminth medications, as well as address the resistance of some used drug classes, introduced bioactive products derived from marine animals into the field of competition; however, almost all the previous research only focused on their impact on bacterial and protozoal infection. In the present work, we investigated the potential in vitro nematocidal effect of the aqueous extract of defense secretions for four species of marine mollusks: two cephalopods, namely the cuttlefish *Sepia pharaonis* and the common Octopus *Octopus vulagris* and two gastropods, the sea hare *Aplysia argus* and the sea slug *Berthillina citrina*, against the adult murine pinworm *Syphacia obvelata*. Data showed dose and time efficacy in all examined extracts. The sea slug's skin acid secretion has the highest impact, causing death in the cultivated worms, followed by the ink of the sea hare, the common octopus and the cuttlefish, where LC90 after 10 h of exposure were 250, 290, 316, and 391 µg/mL, respectively. Comparatively with the control and albendazole-treated groups, the skin acid secretion of the sea slug caused the highest levels of the antioxidant enzymes SOD, Cat and GSH-PX; however, albendazole prompted the highest level of GSH-PX enzyme in all experimental groups.

1 | Introduction

Given the side effects of synthetic drugs, people are always searching for safe and efficient alternative primary healthcare therapies. Fortunately, nature has provided us with an enormous repository of cures for all human ailments and diseases related to them. Before, all medicines had originated from plants; however, there was a subsequent trend to using herbs instead, which contain compounds like flavonoids, terpenoids, tannins, alkaloids, and steroids and have been demonstrated to have a major physiological impact on human health (Yadav and Agarwala 2011; Roy et al. 2021, 2022).

Among the most common human infections, intestinal helminths influence around 2 billion individuals globally. Even though intestinal helminth infections are not as harmful to humans as other infectious agents, individuals with high worm burdens frequently have malnourishment, anemia, stunted growth, and other intestinal diseases (Sueta et al. 2002). Among the intestinal parasitic helminths, *Syphacia obvelata* (Nematoda: Oxyuridae) is a pinworm parasite infecting the colon and cecum of man and the laboratory animals in high abundance. Intestinal intussusception in children, mucoid enteritis, and rectal prolapse have been linked to this infection (Tjaden & Schropp, 2014). Because of the constant re-exposure to this nematode caused by direct

© 2024 Wiley Periodicals LLC.

Summary

- The study focuses on the potential anthelminthic properties of marine molluscans' bioactive materials.
- Significant in vitro nematocidal activity against pinworms was demonstrated by the aqueous extract of skin acid secretion of the sea slug, *Berthillina citrina* and ink of the sea hare, *Aplysia argus*.

transmission via contaminated food, water, and bedding, and the drug resistance of worm populations, controlling pinworm holdings can be quite challenging.

For the past 15 years, the field of finding new medications that capitalize on marine biodiversity has grown at an exponential rate (Lhullier et al. 2020; Estrella-Parra et al. 2022; Romano et al. 2022). There is a vast array of chemicals that marine organisms naturally create, including many active substances with unique biological characteristics (Zhong et al. 2013). More than 15,000 novel compounds have been discovered from marine origins; the largest source, with about 8000 compounds, comes from sponges and cnidarians (Mehbub et al. 2014; Pati, Sahu, and Panigrahy 2015). However, only a small number of them have undergone in vitro screening (Tejuca et al. 1999; Salas-Sarduy et al. 2013; Mehbub et al. 2014; Tapilatu 2015; Estrella-Parra et al. 2022) and still have not been validated as potential sources of alternative medicines to prevent and treat human diseases.

Mollusks constitute an important group of marine animals, representing 23% of total organisms (Pati, Sahu, and Panigrahy 2015). Due to their habitats, they are capable of overcoming different types of stress and synthesizing secondary metabolites possessing immunological properties and anticancer, anti-inflammatory, and antimicrobial activities (Cimino and Sodano 2006; Schneiderb et al. 2021). Inking behavior is a well-known and fascinating defensive phenomenon within two molluscan' groups: cephalopods and sea hares (Kicklighter et al. 2005; Bush, Robison, and Caldwell 2009; Nair et al. 2011; Derby 2014). Cephalopod inks have yielded several substances, including glycoproteins, peptides, indole alkaloids, and chlorinated acetylenes, with anticancer, antiretroviral, antioxidant, and antimicrobial properties that could be used in chemotherapeutic injuries (Rajaganapathi, Thyagarajan, and Edward 2000; Liu et al. 2011; Vennila et al. 2011; Fahmy, 2013; Senan, Sherief, and Nair 2013; Saleh et al. 2015; Soliman, Fahmy, and El-Abied 2015; Hossain et al. 2018). However, the ink of sea hare had antimicrobial, antioxidant, hemolytic, and haemagglutinin activities (Melo et al. 2000; Yang et al. 2005; Vennila et al. 2011; Cherif et al. 2015).

Some compounds from marine gastropods like Caenogastropoda (Thompson 1969; Wägele, Knezevic, and Moustafa 2022) and Heterobranchidae (Gillette, Saeki, and Huang 1991; Wägele, Knezevic, and Moustafa 2017) contain mainly hydrochloric and sulphoric acids, which have been shown to have potential biological and medical applications and antimicrobial activities, but most of these compounds have not been characterized or even clearly identified (Kaviarasan, Siva, and Yogamoorthi 2012; Turner et al. 2018; Tortorella et al. 2021). Nevertheless, the anthelminthic impact of these bioactive metabolites remains out of focus.

The goal of this work is to evaluate the potential in vitro nematicidal effect of the defense secretions for four species of marine mollusks: two cephalopods, namely the cuttlefish, *Sepia pharaonis* and the common octopus, *Octopus vulagris* and two gastropods, the sea hare, *Aplysia argus* and the sea slug, *Berthillina citrina*. The pinworm *S. obvelata* serves as an experimental nematode model due to its short life cycle and high infection prevalence.

To the best of our knowledge, our study is the first to document the anthelminthic effect of defense secretions from either gastropod or cephalopod on parasitic nematodes, which is considered a fundamental step in validating the potential of employing some of them as therapeutic alternatives against intestinal nematode infections.

2 | Materials and Methods

2.1 | Cephalopods (Figure 1)

The cuttlefish, *S. pharaonis*, and the common octopus, *O. vulgaris* were collected by fishermen at the National Institute of Oceanography in Hurghada, Red Sea Governorate, Egypt.

2.2 | The Sea Hare, *A. argus* (Figure 1)

Specimens were collected from 40 km north of Al-Quseir city, Red Sea governorate, Egypt.

2.3 | The Sea Slug, *B. citrina*

Specimens were collected from the Red Sea coast at two sites: 67 km north of Safaga city and 14 km south of Al-Quseir city, Red Sea governorate, Egypt.

2.4 | Albendazole

Pharmaceutical grade of albendazole- PHR1281, from Sigma-Aldrich company, Saint Louis, MO, United States.

2.5 | *S. obvelata*

Gravid females were collected from the colon and cecum of naturally infected albino mice, *Mus musculus* from El

FIGURE 1 | Collection sites of the examined samples; ● the cuttlefish, *Sepia pharaonis*, and ● the common octopus, *Octopus vulgaris*, ■ the sea hare, *Aplysia argus*, ★ the sea slug, *Berthillina citrina*.

Gharbiyah Governorate, Egypt, and identified according to Hussey (1957).

2.6 | Ink Extraction From the Cephalopods and Sea Hare

Animals were transported to the laboratory in sea water. The ink fluid was obtained by disturbing the animals and extracted with water (Vennila et al. 2011). Animals were stimulated to eject their ink using forcipes. The ink fluid was collected in vials and immediately frozen at −20°C. All ink samples of the sea hare and cephalopods were centrifuged at 15,000 rpm for 15 min. The supernatant was lyophilized to residue using a freeze dryer and stored at −20°C until use (Vennila et al. 2011).

2.7 | Skin Acid Secretion (SAS) Extraction From the Sea Slug

Specimens were washed three times using 3.2% NaCl solution, and gently dried with absorbent paper to remove sea salt traces (Thompson 1983). Animals were stimulated to expel acid secretion using a smooth glass rod; the secretion was collected in vials and immediately frozen at −20°C. Samples were centrifuged at 5000 rpm for 15 min, and the supernatant was lyophilized to an orange-red residue and stored at −20°C until use.

2.8 | Experimental Maintenance of *S. obvelata*

The gravid females of *S. obvelata* were dissected, and the uteri were taken out and sliced open to release the eggs. The recovered eggs were incubated in 0.9% saline solution for 24 h at 20°C, and then concentrated by centrifugation at 1500 rpm for 5 min. One hundred fifty male albino mice, aged 6–8 weeks, were experimentally infected with eggs. 1.5 mL of a 0.9% saline solution containing- 500 eggs was directly placed into the stomach of each mouse by a gavage needle (16 G × 3 in., 7.62 cm). Fourteen days post infection, the worms were recovered from the colon and cecum (40–110 worms/mouse), washed in a 0.9% saline solution, and kept in a 37°C incubator until in vitro cultivation.

2.9 | Mortality Assay

A stock water solution at a concentration of 700 μg/mL was performed for the inks of *S. pharaonis*, *O. vulagris*, and *A. argus* and the skin acid secretion of *B. citrina*, and seven gradual concentrations (10, 50, 100, 200, 300, 400, and 500 μg/mL) of each extract were established.

In vitro cultivation of worms was achieved in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine (Sigma-Aldrich R8758). For each concentration, three replicas have been prepared with a worm sample size of 7–10. The control group contained only RPMI 1640 medium, whereas, albendazole group contained 100 μg/mL albendazole (Chai, Jung, and Hong 2021) in the cultivated medium. The cultivation process has been accomplished in Nu-437-400E class II laminar flow cabinet under normal gas phase at 37°C. Activity and mortality of cultivated worms were detected by mechanical and light stimulations. The number of dead worms was recorded after 2, 4, 6, 8, and 10 h' exposure time. By deducting the dead worms in the control group from the total number of dead worms in the experimental group, the actual number of dead worms in each concentration is determined. The mortality rate of cultivated worms in each tested concentration was calculated according to the following equation:

$$\text{Mortality rate} = \frac{\text{Mean no. of live worm in control group - Mean no.of live worm in treated group}}{\text{Mean no. of live worm in control group}} \times 100$$

Half-lethal concentration (LC50) and sub-lethal concentration (LC90) of the four extracts were determined after 10 h of exposure.

2.10 | Statistical Analysis

Data were presented as the means and standard deviations of the independent experiments (experiments were triplicated). LC50 and LC90 values for the worm's mortality were calculated using probit analysis. Two-way analysis of variance (ANOVA) test was established to understand the dependence of the worm's mortality on the tested concentrations of each molluscan' secretion, on the exposure times, and on both factors. The statistical analysis of the data was conducted using SPSS.

2.11 | Scanning Electron Microscopic Inspection

Adult worms were fixed in 70% ethyl alcohol, dehydrated, and carbon-dioxide critical point dried before being adjusted to aluminum stubs and coated with gold palladium in a gold sputter coating apparatus (Bozzola and Russell 1999). The specimens were viewed and photographed using a JSM-IT200Jeol (Japan) scanning electron microscope.

2.12 | Measurement of Antioxidant Enzyme Activities

Six groups each of 200 worms were cultured for 10 h under optimal in vitro conditions with RPMI medium (control group), 100 μg/mL albendazole (albendazole-treated group), and LC90 of the water extract of the four molluscans' marine secretions (LC90-treated groups). Worms were washed in buffered saline solution and stored at −80°C until used. Worms were homogenized and centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was collected for the evaluation of antioxidant enzyme markers using Elisa kits (Munteanu and Apetrei 2021). Catalase (CAT) (Cat No. MBS2600683), superoxide dismutase (SOD) (Cat No. CSB-E08555r), and glutathione peroxidase (GSH-Px) (Cat No. CSB-E12146r) activities were measured for each specimen.

3 | Results

3.1 | Mortality Assay

Water extracts of the inks of the cuttlefish, *S. pharonis*, common octopus, *O. vulgaris*, sea hare, *A. argus*, and the skin acid secretion of the sea slug, *B. citrina* were tested in vitro on *S. obvelata*, and the results showed material and dose-dependent nematicidal activity (Figures 2–5).

The most effective secretion against the cultivated worms was the acid secretion from *B. citrina* where 33.3 ± 15.2 of the cultivated worms died at a concentration of 100 μg/mL after 8 h, followed by the ink of *A. argus*, which caused only 19 ± 8.2 at the same concentration and exposure time. The mortality increased up to 53.3 ± 5.77 and 28.6 ± 14.28 in the acid secretion of *B. citrina* and the ink of *A. argus*, respectively, at 10 h' exposure time. Increasing concentration resulted in a higher death rate, particularly during the peak of cultivation; for instance, an 83.3 ± 15.3 death rate

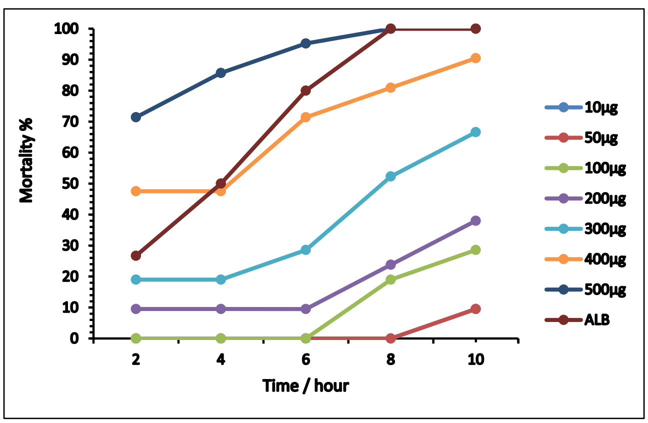

FIGURE 2 | Mortality rate of *Syphacia obvelata* in seven concentrations of the water extract of *Sepia pharaonis*' ink during five-time period intervals. ALB, albendazole-treated group. (Sample size = 10).

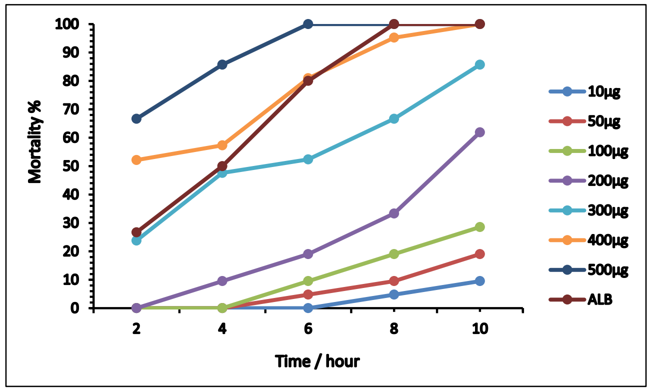

FIGURE 3 | Mortality rate of *Syphacia obvelata* in seven concentrations of the water extract of *Octopus vulagris*' ink during five-time period intervals. ALB, albendazole-treated group. (Sample size = 10).

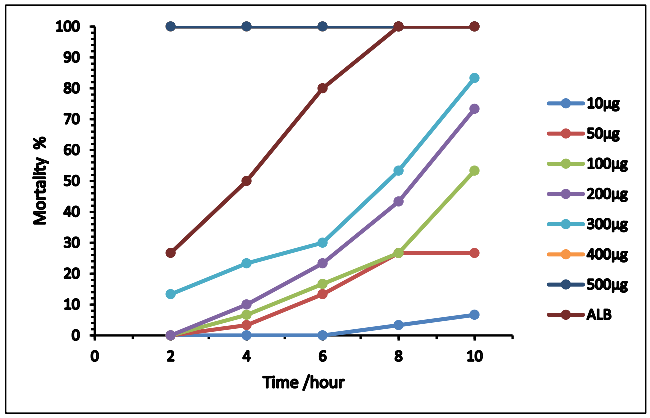

FIGURE 4 | Mortality rate of *Syphacia obvelata* in seven concentrations of the water extract of *Aplysia argus* ink during five-time period intervals. ALB, albendazole-treated group. (Sample size = 10).

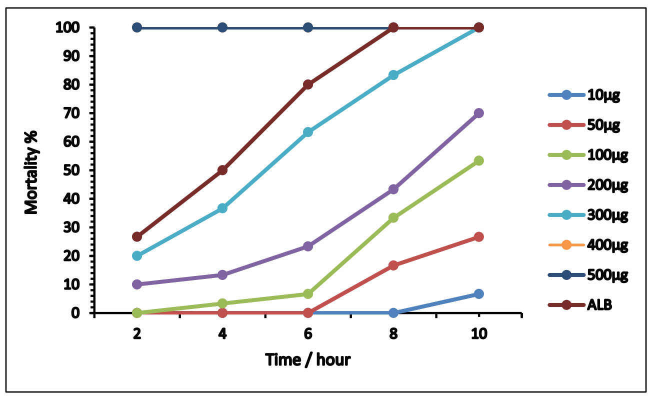

FIGURE 5 | Mortality rate of *Syphacia obvelata* in seven concentrations of the water extract of *Berthillina citrina* skin acid secretion during five-time period intervals. ALB, albendazole-treated group. (Sample size = 10).

TABLE 1 | LC50 and LC90 of the four marine molluscans' secretions against *Syphacia obvelata* at 10 h exposure time.

Conc. μg/mL Molluscans' secretions	LC50	LC90
Ink of *Sepia pharaonis*	251	391
Ink of *Octopus vulagris*	200	316
Ink *of Aplysia argus*	158	290
Skin acid secretion of *Berthillina citrina*	89	250

from *B. citrina'* skin acid secretion at 300 μg/mL occurred within 8 h, whereas *A. argus*' ink caused a 66.7 ± 8.2 death rate, followed by *O. vulagris*' ink (63.3 ± 15.3), then *S. pharaonis*' ink with the lowest mortality rate (52.3 ± 8.2). In contrast to the albendazole group, 100 μg/mL of albendazole resulted in 50 ± 0 mortality after 4 h increased to 100 ± 0 at 8 h.

In general, the mortality rate of *S. obvelata* was at the minimum level within 2–4 h' exposure in low concentrations (10–100 μg/mL) of all Mollusca's secretions, when compared with 8 and 10 h' exposure times.

The LC50 and LC90 values for worm mortality were calculated for the four secretions at 10 h of exposure (Table 1). Skin acid secretion of *B. citrina* was the most effective, with the lowest concentrations, followed by the ink of *A. argus*, the ink of *O. vulagris*, and finally the ink of *S. pharaonis*. Worms treated with LC90 of the four defense secretions have shown physical damage under the bright field.

The dose–response effect has been confirmed by a two-way ANOVA test that demonstrates a highly significant interaction between the concentration of *B. citrina* skin secretion and the death rate of *S. obvelata* ($F(8,90) = 470.324$, $p < 0.000$). Additionally, exposure time has a significant impact on the rate of death ($F(4,90) = 131.370$, $p < 0.000$), and both exposure time and dose concentration had an impact on worm mortality ($F(32,90) = 12.506$, $p < 0.000$). The ink from *O. vulagris*, *S. pharaonis*, and *A. argus* showed a similar relationship, showing a statistically significant impact of exposure time and dose concentration on the *S. obvelata* death rate (p value < 0.000), that is, the toxicity of the nematode increased by increasing the concentration of marine molluscan's secretions and the time of their exposure. There was a statistically significant variation in the mortality rate between the four secretions, as well as with albendazole at 100 μg/mL ($F(16,60) = 3.31$, $p < 0.000$).

3.2 | Ultrastructural Damage of Female *S. obvelata* After LC90 Treatment of the Four Molluscans' Defensive Secretions

Using scanning electron microscopy, we investigated the ultrastructure damage in female *S. obvelata* and followed the in vitro treatment with the LC90 of the water extract of the ink of *S. pharaonis* (representing the cephalopods ink), the ink of A. argus, and the skin acid secretion of *B. citrine* (Figure 6). The data are compared with the worms in both albendazole-treated and control groups. The control group exhibits a normal architecture of the worm, including the transversally annulated body cuticle confined to the level of the crescent-shaped anus; the bulb-like head capsule carries four submedian cephalic papillae; three triangular fleshy lips naked from papillae; two amphids; and a triradiate mouth lined with three thickened chitinized plates. The small, striated cervical alae is followed by a small slit-like vulva with guarded folds. Albendazole-treated worms show shrinking of the body, especially the head structures, which leads to curving inward the mouth opening. Flattening and deformation of the cuticle annulation along the body and the cervical alae were detected with enlargement of the vulva and flattening of its folds. No detected damage on the anal opening.

The treated worms exhibited varied degrees of cuticular changes due to the secretions of the four molluscans; the cuticular defects following treatment with the acid secreted by *B. citrine* were significant, whilst those following treatment with cephalopod ink were rather minor. *B. citrine*'s acid secretion caused a significant swelling in the head capsule and lips, making the amphids hardly detectable. In addition to discernible shrinkage and abrasion in the cuticular alae, there is a disfiguring and deep groove separation between the cuticular annulations. The cuticle around the anus had a deformity with scars formation, and the aperture was disfigured. Cuticular ruffling and slight body shrinkage are the effects of cephalopod inks, particularly in the anterior region around the deformed and shrunken cervical alae. There is a little deformation in the folds of the vulva and small, dispersed bulb-like abnormalities visible in the cuticular annulations surrounding it. With normal appearance of the anus.

3.3 | Alterations in the Activities of CAT, SOD, and GSH-PX Enzymes (Figure 7)

We assessed the activity levels of enzymes implicated in mitigating oxidative stress, such as GSH-PX, CAT, and SOD, in both control and altered worms following treatment with albendazole and LC90 of the four molluscans' secretions (Figure 7). Exposure of the worms to the water extracts of the marine secretions produced an increase in the internal intracellular production of CAT, SOD, and GSH-PX activity, which were significantly different from the control. Sea slug secretion shows the highest enzymatic activities for CAT, SOD, and GSH-PX, indicating strong antioxidant properties; on the other hand, the ink of Octopus exhibits moderate enzymatic activities across all three enzymes, while the ink of Sea hare shows relatively lower enzymatic activities, and the ink of Sepia exhibits the lowest activities, particularly for GSH-PX. There are statistically significant differences between the four molluscan's secretions for all enzymes, as indicated by the p-values (< 0.001 for CAT, GSH-PX and SOD).

4 | Discussion

Pinworm infection is one of the most common types of parasitic diseases that can affect animals and people. Poverty and unhygienic conditions have a direct correlation with the high prevalence of this infection, which may pose a risk to public

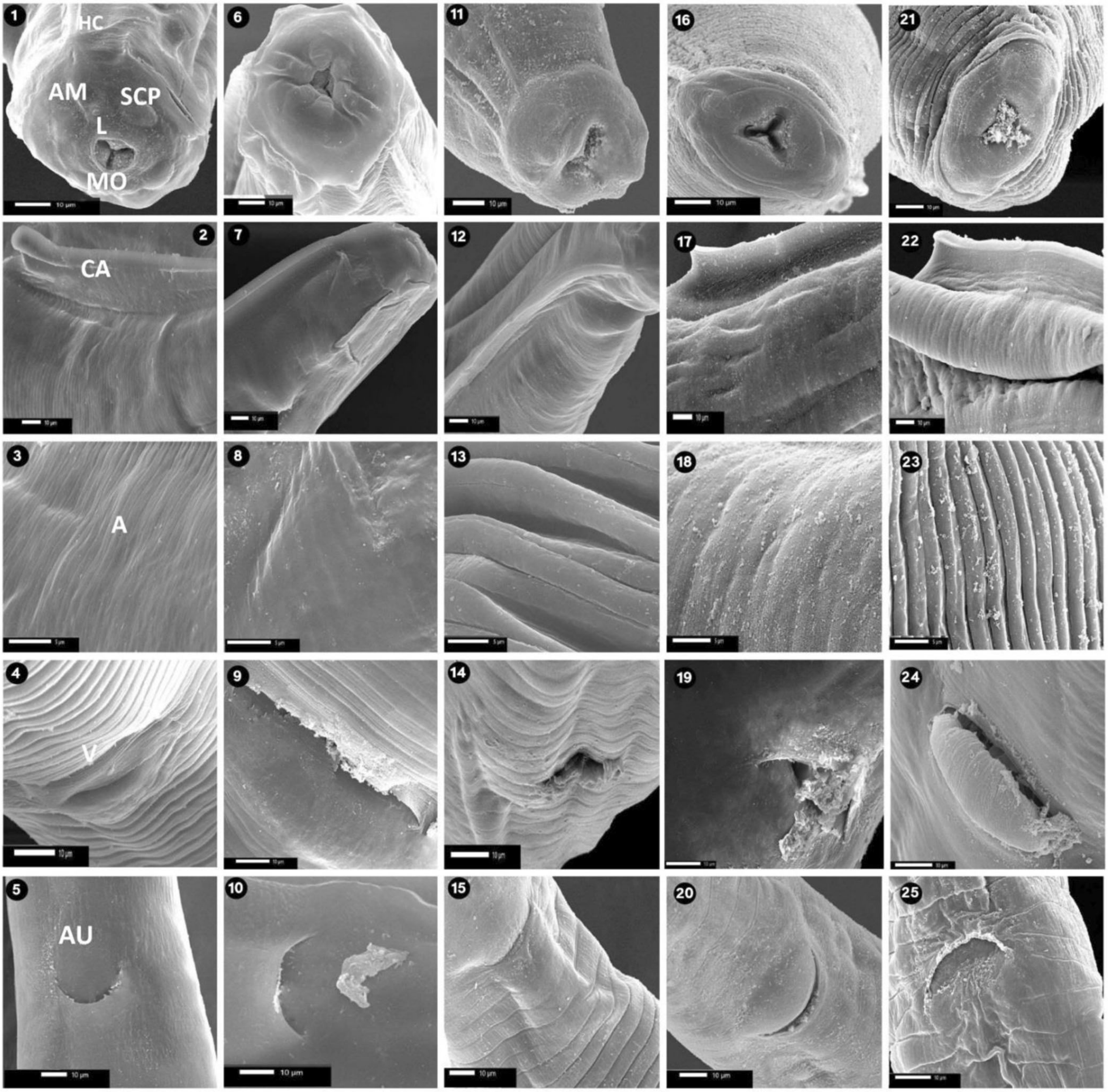

FIGURE 6 | Scanning electron micrographs of female *Syphacia obvelata*. 1–5. Control group; (1) enface view shows the bulb-like head capsule (HC), submedian cephalic papillae (SCP), three triangular lips (L), two amphids (AM), triradiate mouth opening (MO) with three chitinized plates. (2) striated cervical alae (CA). (3) transverse annulations (A). (4) slit-like vulva with guarded folds (V). (5) crescent-shaped anus (Au). (6–10) Albendazole-treated group. (11–15) ink of *Sepia pharaonis*-treated group. (16–20) ink of A. argus-treated group. (21–25) acid secretion of *Berthillina citrina*-treated group.

health. Although symptoms are confined to pruritus or a prickling sensation in the perianal area, diarrhea and acute appendicitis have been detected in heavy infections (Ramezani and Dehghani 2007). Infection could also mimic other disease processes and, however infrequently, can result in significant infection consequences such as ovarian abscesses (Craggs et al. 2009). Endometritis, urinary tract infection, and vulvovaginitis are also complications that have been noted in some clinical cases (Huh 2023).

The present work uses the murine pinworm *S. obvelata* as an experimental model to shed light on potential safe substitutes for widely used chemical medications for pinworm infection by using natural bioactive products. These alternatives are less likely to cause side effects when used in the recommended therapeutic doses and can be found in abundance in the marine environment. We investigate the nematicidal impact of the defense secretions of four species of marine cephalopods and gastropods on *S. obvelata in vitro*.

Cephalopod ink is one of the best sources of bioactive products among various marine defense secretions. Melanin, proteins, peptides, glycosaminoglycans, and certain enzymes, such as tyrosinase, dopamine, and L-DOPA, are the chemical components of this ink.

Furthermore, it includes lipids, heavy metals, and trace levels of taurine, aspartic acid, glutamic acid, alanine, and lysine, among other amino acids (Derby 2014). Cuttlefish, squid, and octopus ink have been the subject of several studies due to their potential medicinal benefits. It is a rich source of anti-inflammatory, antioxidant, antibacterial, anticancer, and antiarthritic compounds (Naraoka et al. 2000; Fahmy, 2013; Hernández-Zazueta et al. 2021; Sumi, Thazeem, and Sunish 2023).

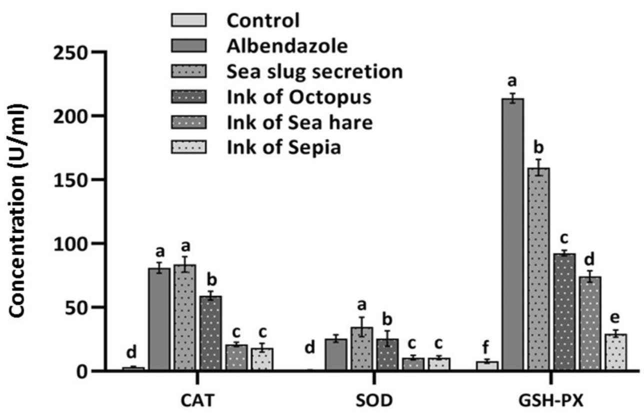

FIGURE 7 | Alterations of the activity level of the oxidative stress-responsive enzymes of S. obvelata followed the treatment with LC90 of different molluscans' secretions. Statistically, different groups are represented with different letters.

In the present work, the lethal effect of *S. pharaonis* and *O. vulagris* ink could be explained by the presence of numerous enzymes, such as tyrosinases, which may lead to the slight body shrinkage and cuticular ruffling with bulb-like abnormalities in the treated worms. These enzymes have been shown to induce cuticular alterations and tissue disruption in adult *Toxocara vitulorum* in vitro (Shalaby et al. 2020). The authors detected folding and wrinkling in the inner cuticular layer with deformation of the underlying muscle cells. However, the current investigation detected that only a high concentration (200 μg/mL) of *S. pharaonis* and *O. vulagris* ink had a moderately lethal impact (63.3 ± 15.3 and 52.3 ± 8.2, respectively) on the cultivated worm, which was achieved after a comparatively lengthy exposure (8 h). This limited effect can be largely attributed, firstly, to the presence of melanin, which constitutes approximately 15% of the total wet weight of the ink (Wang et al. 2014), associated with providing a survival advantage to parasites (Nosanchuk and Casadevall 2006) and reduced cellular susceptibility to cell wall-degrading enzymes (Nosanchuk and Casadevall 2003). Secondly, despite the lack of toxins in the majority of cephalopod ink, tetrodotoxin (TTX) has been detected in the blue-ringed octopus, *Hapalochlaena lunulata* (Huang et al. 2012) and shown to have a major defense role (Williams and Caldwell 2009).

Although it has been observed that a number of molluscan gastropods possess bioactive chemicals that may have hazardous properties (Odeleye, White, and Lu 2019), researchers have focused their efforts mostly on *Conus* species, resulting in the clinical trials of many conopeptides, and the majority of gastropods remain unfocused or poorly studied (Turner et al. 2018). Sea hare toxins have shown considerable activity against many bacterial and fungal strains (Ibrahim et. al., 2020), but the anthelminthic potential has not yet been examined.

In the present work, we evaluated the nematicidal effect of the ink of the sea hare, *Aplysia argus*, for the first time against nematodes. Data showed that the ink has a considerable effect on cultivated *S. obvelata*, where a significant mortality began at a relatively high concentration (300 μg/mL) after 6 h of exposure, and LC_{90} (290 μg/mL) was achieved at 10 h. The effect of the toxin could be explained considering the activities of a series of bioactive peptide/macrolides, known as aplyronines, that have been detected in some sea hare species (*Aplysia* sp. and *D. auricularia*) and shown to be L-amino acid oxidase with possible antibacterial and cytotoxic activity (Iijima, Kisugi, and Yamazaki 2003; Kamiya, Sakai, and Jimbo 2006). Additionally, Ibrahim et al. (2022) detected the presence of benzenesulfonic acid in the ink of *A. fasciata*, which is known to have a nematicidal activity and effect on egg hatching (Ohri and Kaur 2010). However, the current moderate impact of *A. argus* ink may be related to the presence of nitric oxide donor "L-arginine" that has been detected in sea hare's toxins (Derby 2007) and previously improved to enhance resistance to oxidative stress on *Trichinella spiralis* related to albendazole treatment (Abdeltawab et al. 2024).

There are numerous antimicrobial compounds against marine microorganisms described in heterobranch orders such as homarine (Cephalaspidea), iso-naamidine-A (Nudibranchia), pyropheophorbides a and b (Anaspidea), and siphonarienolone and diemenensins A (Pulmonata) (Avila and Angulo-Preckler 2020), however, Pleurobranchoids haven't been researched for this behavior as far as we know. *B. citrina*, like all other Pleurobranchoideans, is recognized for producing acidic secretion with a pH as low as 1–2 that might ward off potential predators (Avila and Angulo-Preckler 2020).

The acid secretion from *B. citrina* shown to be the most efficient secretion against the cultured *S. obvelata* in the current study. The abrasion and disfigurement of the cuticular striations and the scar formation, along with the swelling in certain body regions, are probably alterations that caused a higher death rate of the cultured worm. This effect could be correlated with the activities of abundantly present sulfate and chloride ions and taurine, which is one of the main components of the secretion's free amino acids (Moustafa, Wägele, and El Behairi 2014). It would be more accurate to say that while the acid secretion of *B. citrina* and albendazole both have distinct therapeutic effects on worms, they have different mechanisms of action, and the acid secretion continues to be the most potent.

Oxidative stress greatly affects the ability of parasites to survive and develop. Antiparasitic medications often cause parasites to produce more reactive oxygen species (ROS). This oxidative assault may harm the parasite's essential biomolecules, reducing its ability to survive and reproduce (DeMichele et al. 2023). Parasites have evolved extensive adaptation strategies that comprise the production of small-molecule antioxidants and the expression of antioxidant enzymes to combat OS and increase their chances of survival (Masamba and Kappo 2021).

In this study, we investigated the possibility that the oxidative stress caused by marine secretions in *S. obvelata*'s body has changed the activity levels of enzymes that help reduce oxidative stress, including GSH-PX, CAT, and SOD. After 10 h of exposure to LC90 of the four extracts and albendazole, data showed an increase in the level of the three enzymes, with the sea slug's acid secretion producing the greatest amounts of CAT, SOD, and GSH-PX, comparable to the ink of the other species. However, albendazole produced the highest level of GSH-PX among all treated groups.

5 | Conclusion

Finding novel nematicidal bioactive metabolites from natural sources is crucial, given the dearth of new antinematode drug classes and the difficulties currently associated with treatment resistance. For the first time, the current study examined the potential use of four marine molluscans' defensive secretions against a parasitic nematode that can be relied upon to screen more species of nematodes and combat their infections in vivo.

5.1 | Remarks

In vivo administration of the bioactive compounds derived from invertebrates remains required several research works including structural characterization, recombinant and chemical manufacturing, molecular target identification, drug optimization, and efficient delivery techniques.

Acknowledgments

Authors would like to thank Prof. Mohamed M. Abd El-Monsef, Department of Mathematical Statistics, Faculty of Science, Tanta University, Tanta, Egypt, for his help in the statistical analysis of the data in this research.

Ethics Statement

All animal experiments and protocols will approve by the ethical committee of Faculty of Science, Tanta University, Tanta, Egypt (code no. IACUC-SCI-TU-0420).

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

Abdeltawab, M. S., I. R. Abdel-Shafi, B. E. Aboulhoda, A. M. Mahfoz, and A. M. Hamed. 2024. "The Neuroprotective Potential of Curcumin on *T. spiralis* Infected Mice." *BMC Complementary Medicine and Therapies* 24, no. 1: 99. https://doi.org/10.1186/s12906-024-04399-0.

Avila, C., and C. Angulo-Preckler. 2020. "Bioactive Compounds from Marine Heterobranchs." *Marine Drugs* 18: 657. https://doi.org/10.3390/md18120657.

Bozzola, J. J., and L. D. Russell. 1999. *Electron Microscopy: Microscopy: Principles and Techniques for Biologists*. London: Jones & Bartlett Learning.

Bush, S. L., B. H. Robison, and R. L. Caldwell. 2009. "Behaving in the Dark: Locomotor, Chromatic, Postural, and Bioluminescent Behaviors of the Deep-Sea Squid *Octopoteuthis deletron*." *The Biological Bulletin* 216, no. 1: 7–22. https://doi.org/10.2307/25470719.

Chai, J. Y., B. K. Jung, and S. J. Hong. 2021. "Albendazole and Mebendazole as Anti-Parasitic and Anti-Cancer Agents: An Update." *The Korean Journal of Parasitology* 59, no. 3: 189–225. https://doi.org/10.3347/kjp.2021.59.3.189.

Cherif, E., K. Lahbib, M. Charaf, and M. Yahaia. 2015. "Antioxidant Activity of *Aplysia depilans* Ink Collected from Bizerte Channel (NE Tunisia)." *Algerian Journal Natural Products* 3, no. 1: 138–145.

Cimino, G., and G. Sodano. 2006. "Biosynthesis of Secondary Metabolites in Marine Molluscs." *Marine Natural Products -Diversity and Biosynthesis* 167: 77–115. https://doi.org/10.1007/BFb0034371.

Craggs, B., E. D. Waele, K. D. Vogelaere, et al. 2009. "*Enterobius vermicularis* infection with Tuboovarian Abscess and Peritonitis Occurring During Pregnancy." *Surgical Infections* 10, no. 6: 545–547. https://doi.org/10.1089/sur.2008.090.

DeMichele, E., O. Sosnowski, A. G. Buret, and T. Allain. 2023. "Regulatory Functions of Hypoxia in Host–Parasite Interactions: A Focus on Enteric, Tissue, and Blood Protozoa." *Microorganisms* 11: 1598. https://doi.org/10.3390/microorganisms11061598.

Derby, C. 2014. "Cephalopod Ink: Production, Chemistry, Functions and Applications." *Marine Drugs* 12, no. 5: 2700–2730. https://doi.org/10.3390/md12052700.

Derby, C. D. 2007. "Escape By Inking and Secreting: Marine Molluscs Avoid Predators through a Rich Array of Chemicals and Mechanisms." *The Biological Bulletin* 213: 274–289. https://doi.org/10.2307/25066645.

Estrella-Parra, E. A., R. Arreola, M. E. Álvarez-Sánchez, et al. 2022. "Natural Marine Products As Antiprotozoal Agents Against Amitochondrial Parasites." *International Journal for Parasitology: Drugs and Drug Resistance* 19: 40–46. https://doi.org/10.1016/j.ijpddr.2022.05.003.

Fahmy, R. 2013. "In Vitro Antioxidant, Analgesic and Cytotoxic Activities of *Sepia officinalis* Ink and *Coelatura aegyptiaca* extracts." *African Journal of Pharmacy and Pharmacology* 7: 1512–1522. https://doi.org/10.5897/AJPP2013.3564.

Gillette, R., M. Saeki, and R. C. Huang. 1991. "Defense Mechanisms in Notaspid Snails: Acid Humor and Evasiveness." *Journal of Experimental Biology* 156: 335–347.

Hernández-Zazueta, M. S., I. Luzardo-Ocampo, J. S. García-Romo, et al. 2021. "Bioactive Compounds from *Octopus vulgaris* Ink Extracts Exerted Anti-Proliferative and Anti-Inflammatory Effects *in Vitro*." *Food and Chemical Toxicology* 151: 112119. https://doi.org/10.1016/j.fct.2021.112119.

Hossain, M. P., M. S. Rabeta, and T. Husnul Azan. 2018. "Medicinal and Therapeutic Properties of Cephalopod Ink: A Short Review." *Food Research* 3: 188–198. https://doi.org/10.26656/fr.2017.3(3).201.

Huang, F., Z. Yang, D. Yu, J. Wang, R. Li, and G. Ding. 2012. "Sepia Ink Oligopeptide Induces Apoptosis in Prostate Cancer Cell Lines Via Caspase-3 Activation and Elevation of Bax/Bcl-2 Ratio." *Marine Drugs* 10: 2153–2165. https://doi.org/10.3390/md10102153.

Huh, S. 2023. Pinworm (Enterobiasis): Drugs & Diseases. Infectious Diseases. February 10, 2023. https://emedicine.medscape.com/article/225.

Hussey, K. L. 1957. "*Syphacia muris* vs. *S. obvelata* in Laboratory Rats and Mice." *The Journal of Parasitology* 43: 555–559.

Ibrahim, H. A. H., M. S. Amer, H. O. Ahmed, and N. A. Hassan. 2020. "Antimicrobial Activity of the Sea Hare (*Aplysia Fasciata*) Collected from the Egyptian Mediterranean Sea, Alexandria." *Egyptian Journal of Aquatic Biology and Fisheries* 24, no. 4: 233–248. https://doi.org/10.21608/ejabf.2020.98019.

Iijima, R., J. Kisugi, and M. Yamazaki. 2003. "A Novel Antimicrobial Peptide from the Sea Hare *Dolabella auricularia*." *Developmental &*

Comparative Immunology 27: 305–311. https://doi.org/10.1016/s0145-305x(02)00105-2.

Kamiya, H., R. Sakai, and M. Jimbo. 2006. "The Chemo-Ecological Study to Bbiotechnology Application." In *Molluscs: Bioactive Molecules from Sea Hares*, edited by G. Cimino and M. Gavagnin, 215–239. Berlin-Heidelberg: Springer.

Kaviarasan, T., S. R. Siva, and A. Yogamoorthi. 2012. "Retracted: Antimicrobial Secondary Metabolites from Marine Gastropod Egg Capsules and Egg Masses." *Asian Pacific Journal of Tropical Biomedicine* 2, no. 11: 916–922. https://doi.org/10.1016/S2221-1691(12)60253-6.

Kicklighter, C. E., S. Shabani, P. M. Johnson, and C. D. Derby. 2005. "Sea Hares Use Novel Antipredatory Chemical Defenses." *Current Biology* 15, no. 6: 549–554.

Lhullier, C., M. I. G. Moritz, E. O. Tabalipa, et al. 2020. "Biological Activities of Marine Invertebrates Extracts from the Northeast Brazilian Coast." *Brazilian Journal of Biology* 80, no. 2: 393–404. https://doi.org/10.1590/1519-6984.213678.

Liu, H., P. Luo, S. Chen, and J. Shang. 2011. "Effects of Squid Ink on Growth Erformance, Antioxidant Functions and Immunity in Growing Broiler Chickens. *Asian-Australasian*." *Journal of Animal Sciences* 24, no. 12: 1752–1756. https://doi.org/10.5713/ajas.2011.11128.

Masamba, P., and A. P. Kappo. 2021. "Immunological and Biochemical Interplay between Cytokines, Oxidative Stress and Schistosomiasis." *International Journal of Molecular Sciences* 22: 7216. https://doi.org/10.3390/ijms22137216.

Mehbub, M., J. Lei, C. Franco, and W. Zhang. 2014. "Marine Sponge Derived Natural Products between 2001 and 2010: Trends and Opportunities for Discovery of Bioactives." *Marine Drugs* 12, no. 8: 4539–4577. https://doi.org/10.3390/md12084539.

Melo, V. M. M., A. B. G. Duarte, A. F. F. U. Carvalho, E. A. Siebra, and I. M. Vasconcelos. 2000. "Purification of a Novel Antibacterial and Haemagglutinating Protein from the Purple Gland of the Sea Hare, *Aplysia dactylomela* Rang, 1828." *Toxicon* 38: 1415–1427. https://doi.org/10.1016/s0041-0101(99)00234-2.

Moustafa, A. Y., H. Wägele, and S. I. El Behairi. 2014. "Toxicological and Biological Activities of the Acid Secretion of *Berthellina citrina* (Heterobranchia, Pleurobranchidae) from the Red Sea." *International Journal of Advanced Research* 2: 13–24.

Munteanu, I. G., and C. Apetrei. 2021. "Analytical Methods Used in Determining Antioxidant Activity: A Review." *International Journal of Molecular Sciences* 22, no. 7: 3380. https://doi.org/10.3390/ijms22073380.

Nair, J. R., D. Pillai, S. M. Joseph, P. Gomathi, P. V. Senan, and P. M. Sherief. 2011. "Cephalopod Research and Bioactive Substances." *Indian Journal of Marine Sciences* 40, no. 1: 13–27. http://nopr.niscpr.res.in/handle/123456789/11363.

Naraoka, T., H. S. Chung, H. Uchisawa, J. Sasaki, and H. Matsue. 2000. "Tyrosinase Activity in Antitumor Compounds of Squid Ink." *Food Science and Technology Research* 6: 171–175. https://doi.org/10.3136/fstr.6.171.

Nosanchuk, J. D., and A. Casadevall. 2003. "The Contribution of Melanin to Microbial Pathogenesis." *Cellular Microbiology* 5, no. 4: 203–223. https://doi.org/10.1046/j.1462-5814.2003.00268.x.

Nosanchuk, J. D., and A. Casadevall. 2006. "Impact of Melanin on Microbial Virulence and Clinical Resistance to Antimicrobial Compounds." *Antimicrobial Agents and Chemotherapy* 50, no. 11: 3519–3528. https://doi.org/10.1128/AAC.00545-06.

Odeleye, T., W. L. White, and J. Lu. 2019. "Extraction Techniques and Potential Health Benefits of Bioactive Compounds from Marine Molluscs: A Review." *Food & Function* 10: 2278–2289. https://doi.org/10.1039/c9fo00172g.

Ohri, P., and S. Kaur. 2010. "Effect of Phenolic Compounds on Nematodes. A Review." *International Research Journal of Natural and Applied Sciences* 2: 344–350. https://doi.org/10.31018/jans.v2i2.144.

Pati, P., B. K. Sahu, and R. C. Panigrahy. 2015. "Marine Molluscs As a Potential Drug Cabinet: An Overview." *Indian Journal of Geo-Marine Science* 44, no. 7: 961–970.

Rajaganapathi, J., S. P. Thyagarajan, and J. K. Edward. 2000. "Study on Cephalopod's Ink for Anti-Retroviral Activity." *Indian Journal of Experimental Biology* 38: 519–520.

Ramezani, M. A., and M. R. Dehghani. 2007. "Relationship between *Enterobius vermicularis* and the Incidence of Acute Appendicitis." *The Southeast Asian Journal of Tropical Medicine and Public Health* 38, no. 1: 20–23.

Romano, G., M. Almeida, A. Varela Coelho, et al. 2022. "Biomaterials and Bioactive Natural Products from Marine Invertebrates: From Basic Research to Innovative Applications." *Marine Drugs* 20: 219. https://doi.org/10.3390/md20040219.

Roy, A., S. Datta, K. S. Bhatia, P. Bhumika, and R. Prasad. 2021. "Role of Plant Derived Bioactive Compounds against Cancer." *South African Journal of Botany* 2021: 1017–1028. https://doi.org/10.1016/j.sajb.2021.10.015.

Roy, A., A. Khan, and I. Ahmad, et al. 2022. "Flavonoids a Bioactive Compound from Medicinal Plants and Its Therapeutic Applications." *Biomedical Research International* 2022: 5445291. https://doi.org/10.1155/2022/5445291.

Salas-Sarduy, E., A. Cabrera-Muñoz, A. Cauerhff, et al. 2013. "Antiparasitic Effect of a Fraction Enriched in Tight-Binding Protease Inhibitors Isolated from the Caribbean Coral *Plexaura homomalla*." *Experimental parasitology* 135, no. 3: 611–622. https://doi.org/10.1016/j.exppara.2013.09.013.

Saleh, H., A. M. Soliman, A. S. Mohamed, and M. A. Marie. 2015. "Antioxidant Effect of Sepia Ink Extract on Extrahepatic Cholestasis Induced By Bile Duct Ligation in Rats." *Biomedical and Environmental Sciences: BES* 28, no. 8: 582–594. https://doi.org/10.3967/bes2015.082.

Schneiderb, N. F. Z., M. H. Moraesc, L. Constantinoa, F. H. Reginattoa, and M. Steindelc. 2021. "Natural Marine Products As Antiprotozoal Agents Against Amitochondrial Parasites." *Natural Product Reports* 38: 2214–2235.

Senan, V. P., P. M. Sherief, and J. R. Nair. 2013. "Anticancer Property of Purified Fraction C2 of Cuttlefish (*Sepia Pharaonis*) Ink on Cervical Cancer Cells." *Indo American Journal of Pharmaceutical Research* 3, no. 9: 7444–7454.

Shalaby, H., H. Ashry, M. Saad, and T. Farag. 2020. "In Vitro Effects of Streptomyces Tyrosinase on the Egg and Adult Worm of Toxocara Vitulorum." *Iranian Journal of Parasitology* 15, no. 1: 67–75.

Soliman, A. M., S. R. Fahmy, and S. A. El-Abied. 2015. "Anti-Neoplastic Activities of *Sepia officinalis* Ink and Coelatura Aegyptiaca Extracts against Ehrlich Ascites Carcinoma in Swiss Albino Mice." *International Journal of Clinical and Experimental Pathology* 8, no. 4: 3543–3555.

Sueta, T., I. Miyoshi, T. Okamura, and N. Kasai. 2002. "Experimental Eradication of Pinworms (*Syphacia obvelata* and *Aspiculuris tetraptera*) from Mice Colonies Using Ivermectin." *Experimental animals* 51: 367–373.

Sumi, M. S., B. Thazeem, and K. S. Sunish. 2023. "Bioprospective Studies of Pigmented Ink from *Sepioteuthis lessoniana* and Its Molecular Identification Using CO1 Gene." *The Journal of Basic and Applied Zoology* 84: no. 5: 325. https://doi.org/10.1186/s41936-023-00325-x.

Tapilatu, Y. H. 2015. "Status of Drug Discovery Research Based on Marine Organisms from Eastern Indonesia." *Procedia Chemistry* 14: 484–492. https://doi.org/10.1016/j.proche.2015.03.065.

Tejuca, M., G. Anderluh, P. Maček, et al. 1999. "Antiparasite Activity of Sea-Anemone Cytolysins on *Giardia duodenalis* and Specific Targeting with Anti-Giardia Antibodies." *International Journal for Parasitology* 29, no. 3: 489–498. https://doi.org/10.1016/s0020-7519(98)00220-3.

Thompson, T. 1969. "Acid Secretion in the Pacific Ocean Gastropods." *Australian Journal of Zoology* 17: 755–764.

Thompson, T. E. 1983. "Detection of Epithelial Acid Secretions in Marine Molluscs: Review of Techniques, and New Analytical Methods." *Comparative Biochemistry and Physiology Part A: Physiology* 74: 615–621.

Tjaden, B. L., and K. P Schropp. 2014. "Pediatric Intussusception Associated With Enterobius vermicularis." *Journal of Pediatrics* 165, no 6: 1272. https://doi.org/10.1016/j.jpeds.2014.08.006.

Tortorella, E., R. Giugliano, M. De Troch, B. Vlaeminck, G. C. de Viçose, and D. de Pascale. 2021. "The Ethyl Acetate Extract of the Marine Edible Gastropod *Haliotis tuberculata* Coccinea: A Potential Source of Bioactive Compounds." *Marine Biotechnology* 23: 892–903. https://doi.org/10.1007/s10126-021-10073-0.

Turner, A., D. Craik, Q. Kaas, and C. Schroeder. 2018. "Bioactive Compounds Isolated from Neglected Predatory Marine Gastropods." *Marine Drugs* 16: 118. https://doi.org/10.3390/md16040118.

Vennila, R., R. K. Rajesh, S. Kanchana, M. Arumugam, and T. Balasubramanian. 2011. "Investigation of Antimicrobial and Plasma Coagulation Property of Some Molluscan Ink Extracts: Gastropods and Cephalopods." *African Journal of Biochemstry Research* 5, no. 1: 14–21. https://doi.org/10.5897/AJBR2024.1182.

Wägele, H., K. Knezevic, and A. Y. Moustafa. 2017. "Distribution and Morphology of Defensive Acid-Secreting Glands in Nudipleura (Gastropoda: Heterobranchia), with an Emphasis on Pleurobranchomorpha Distribution and Morphology." *Journal of Molluscan Studies* 83: 422–433. https://doi.org/10.1093/mollus/eyx030.

Wägele, H., K. Knezevic, and A. Y. Moustafa. 2022. "Defensive Acid-Secreting Glands in Cypraeoidea (Caenogastropoda, Mollusca)." *Molluscan Research* 42, no. 4: 320–327. https://doi.org/10.1080/13235818.2022.2124581.

Wang, F. R., Z. G. Xie, X. Q. Ye, et al. 2014. "Effectiveness of Treatment of Iron Deficiency Anemia in Rats with Squid Ink Melanin-Fe." *Food & Function* 5: 123–128. https://doi.org/10.1039/c3fo60383k.

Williams, B. L., and R. L. Caldwell. 2009. "Intra-Organismal Distribution of Tetrodotoxin in Two Species of Blue-Ringed Octopuses (*Hapalochlaena fasciata* and *H. lunulata*)." *Toxicon* 54: 345–353. https://doi.org/10.1016/j.toxicon.2009.05.019.

Yadav, R. N. S., and M. Agarwala. 2011. "Phytochemical Analysis of Some Medicinal Plants." *Journal of Phytology* 3: 10–14.

Yang, H., P. M. Johnson, K. C. Ko, et al. 2005. "Cloning, Characterization and Expression of Escapin, a Broadly Antimicrobial Fad-Containing L-Amino Acid Oxidase from Ink of the Sea Hare *Aplysia californica*." *Journal of Experimental Biology* 208, no. 18: 3609–3622. https://doi.org/10.1242/jeb.01795.

Zhong, J., W. Wang, X. Yang, X. Yan, and R. Liu. 2013. "A Novel Cysteine-Rich Antimicrobial Peptide from the Mucus of the Snail of *Achatina fulica*." *Peptides* 39: 1–5. https://doi.org/10.1016/j.peptides.2012.09.001.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.