Al-Azhar Bulletin of Science, Section C, Vol. 33, No. 2 (December) 2022, pp. 111-119
http://doi.10.21608/ABSB.2022.140291.1187

Al-Azhar Bulletin of Science: Section C

IN VITRO SCHISTOSOMICIDAL ACTIVITY OF FIVE COMMON SPECIES OF RED SEA CONE SNAIL MUSCLE EXTRACTS

Mahmoud Abdallah [a], Mohammed Abdelwahab [a], Nagwa S. M. Aly [b], Omar S. O. Amer [a], Moustafa M. Sarhan [a,*]

[a] Zoology Department, Faculty of Science, Al-Azhar University, Asyut, Egypt.
[b] Parasitology Department, Faculty of medicine, Benha University, Egypt.
* Corresponding Author: msarhan@azhar.edu.eg

Received: 23 May 2022; Revised: 18 July 2022; Accepted: 19 July 2022; Published: 01 Dec 2022

ABSTRACT

The potential antischistosomal activity of *Conus* muscle extract on the adult worms of *Schistosoma mansoni* was studied *In vitro.* Live specimens of five common species of cone snails were collected from several locations on the Egyptian Red Sea coast. The muscles of these snails had been collected after crushing their shells to prepare the muscle extracts. The adult worms of *S. mansoni* were isolated from the blood circulation by perfusion technique using phosphate buffer. After 1- 4 days of exposure to cone snails muscle extracts at varying doses, the worm's motility and mortality rates were examined. The results showed that muscle extracts of 3 species of cone snails; *Conus vexillum, Conus fulgetrum, Conus flavidus*, with no significant effect on the survival and motility of *S. mansoni* worms. In contrast, th**e** muscle extract of *Conus textile* showed significant effect on the survival and motility of *S. mansoni* worms and the LD_{50} was 43.85 µg/ml. However, the muscle extract of *Conus lividus* showed a weak effect (mortality rate 13%) on the viability and motility of *S. mansoni* worms at high concentration only (100µg/ml) after 96 hours of incubation. The probable tegumental alterations of worms after exposure to the muscle extracts were investigated using scanning electron microscopy (SEM). *C. textile* muscle extract could induce tegumental damage in *S. mansoni* worms, including the loss and damage of surface tubercles, as well as the destruction of oral sucker. It can also cause the development of protuberances and shortening of tegumental spines especially around the gynaecophoric canal. In conclusion, the current study revealed the schistosomicidal effect of *C. textile* muscle extract and might lead to novel antischistosomal drugs.

Keywords: Cone snail; Muscle extracts; Red Sea; Schistosoma

1. Introduction

Human schistosomiasis is a chronic, severe disease that caused by *Schistosoma spp* [1]. Currently, Praziquantel (acetylated quinoline-pyrazine) is the only anti-helminthic medicine approved by the World Health Organization for use in schistosomiasis prevention chemotherapy [2]. Praziquantel represents the first medicine of choice for most endemic regions due to its greater effect [3]. Few cases of resistance to treatment by praziquantel were recorded, and they pose a big barrier in the global effort for eliminating the schistosomiasis [4]. In Kenya, researchers discovered that schistosomes from individuals who had previously been treated with Praziquantel were much less susceptible than those who had not. Furthermore, schistosomes taken from a single patient who had been treated with Praziquantel 18 times shown significant resistance [5]. Such resistance, as well as the risk of future resistance, highlights the need for new schistosomicidal medicines to be developed [3,4,6]. Previous research has looked into the application of bioactive molecules as potential therapeutic agents for diseases such hypertension [7,8], thrombosis [9,10] and cancer [11–16]. *Conus* species (family Conidae) are a medically important species due to their production of bioactive molecules. The genus *Conus* has become a valuable genetic resource for conotoxin identification and therapeutic development. The challenges of developing drugs from cone snails venoms have been discussed by [17]. The gastropods (*Conus betulinus* and *Conus inscriptus*) body tissues extracts are considered valuable medication because they contain many bioactive compounds and have a well-balanced antibacterial activity. The active portions of ethanolic, methanolic, and acetonic tissue extraction from *C. betulinus* and *C. inscriptus* may be used as substitute bioactive components to

address the problem of unknown infections spreading through poultry-borne food products [18]. The purpose of this study is to investigate the antischistosomal activity of some *Conus* species muscle extracts on the mature worms of *S. mansoni In vitro*.

2. Materials and Methods

2.1. Collection of *Conus* specimens

Live *Conus* specimens from five different species (figure1) were collected from four different places in Egypt's Red Sea; National Institute of Oceanography and Fisheries (NIOF) coast, 5 km north of Hurghada (27 ° 17' 3" N and 33 ° 46' 21" E), mangrove 17 km south of Safaga (33° 58′ 21″E. 26° 38′ 49″N), 50 km south of Al-Quseir (34° 19′ 06″E. 26° 02′ 33″N) and Marsa Alam (34° 54′ 05″E).

2.2. Muscle extracts preparation

The shell was cracked with a vise, then the shell pieces were removed, and the interior body was left entirely intact. Once the interior body was removed, the muscles were carefully dissected, cut into small pieces 10-15 mm, suspended in different solvents and centrifuged (500 g, 5 min, 4 °C). The pellet was extracted three times with solvents. The collected supernatant was lyophilized and kept at -80 °C prior to use.

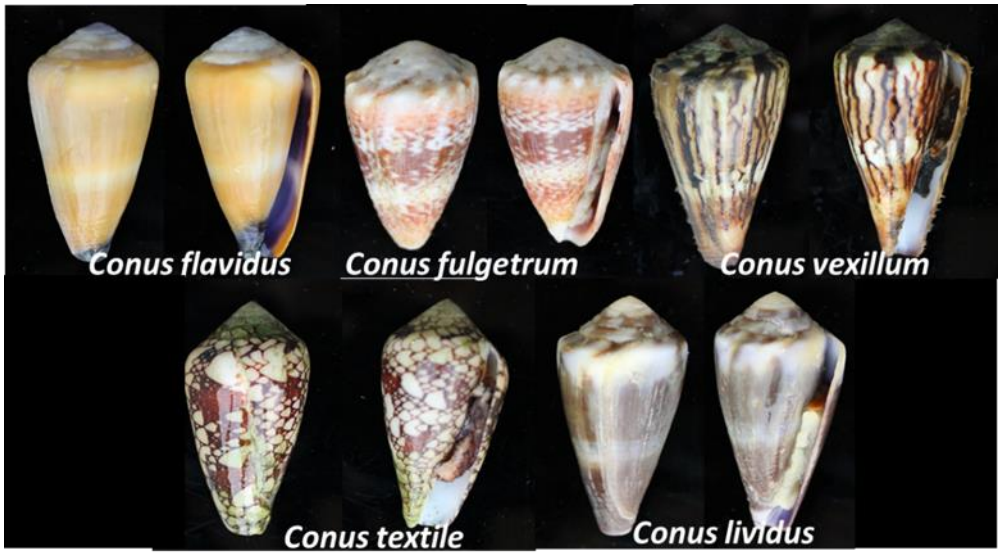

Figure 1. Shows the five species of Conus under study: Cconus flavidus, Conus fulgetrum, Conus vexillum, Conus. textile and Conus lividus.

2.3. Cell culture and parasite reagents preparation

GIBCO provided RPMI-1640 culture media containing 13.3 M (molar) phenol red and 2.05 mM L-glutamine, as well as fetal calf serum (FCS). Before usage, FCS was inactivated by heating for 30 minutes at 56°C. Sigma provided penicillin G, streptomycin sulphate, heparin sodium salt, L-arginine, and glucose [19].

2.3.1 Obtaining the parasites (Schistosoma mansoni)

Schistosome Biological Supply Center (SBSC) supplied Syrian golden hamsters (*Mesocricetus auratus*) weighing 100-120 g each, after that, they were kept in an environmentally - controlled facility with a humidity of 70%, a 12-hour light/12-hour dark cycle and were fully adapted for one week before infection at a temperature of 25°C. Cercariae *of S. mansoni* (Egyptian strain) gained from SBSC were used to infect the hamsters through abdominal skin contact. Cercariae were shed from infected *Biomphalaria alexandrina* snails and utilized within one hour of being shed. Schistosomes were removed from portal and mesenteric veins of mature animals after 90 days [20], then sexed, and counted according to [21].

2.3.2. Application of muscle extracts:

Muscle extracts were used to achieve a final concentration of 2.5 to 100 µg/ml (2.5, 5.0, 10.0, 50, and 100 µg/ml) and applied to 12-well plates filled with a total volume of 3 ml RPMI culture medium. To ensure viability, 6 worms per well were cultured at 37°C and 5% CO2 just after the animals were perfused. Adult worms were incubated with 0.5 percent DMSO and culture media in negative control wells, while worms were incubated with 1 g/ml PZQ and culture media in positive control wells. All the experiments were carried out in quadruplicate and the results are evaluated at four separate time points: 24, 48, 72, and 96 hours.

2.4. Evaluation of the effects of muscle extracts

Worms' viability was evaluated using an optical microscope for four successive days of treatment to determine sub lethal concentrations. Worms that didn't show signs of movement for one

minute, as well as those with abnormalities such as blackening and wrinkling were considered dead.

2.5. Determination of lethal concentration (LD50) of *Conus textile* muscle extracts

Control and test solutions; 2.5, 5, 10, 50, and 100 µg/ml of *conus textile* muscle extract were placed in two replicates, each containing the suitable number of worms at 37°C and 5% CO2 incubator to determine the LD50 of muscle extracts on the *S. mansoni* worms [22].

2.6. Determination of CC50 (cytotoxicity concentration) of *Conus textile* muscle extracts:

Hepatocytes (Huh 7.5) were cultured with varied doses (1-500 g/ml) of *conus textile* muscle extracts for 24 hours to investigate the possible cytotoxic effects on cell proliferation and viability. Cytotoxic concentration was determined using the MTT test.

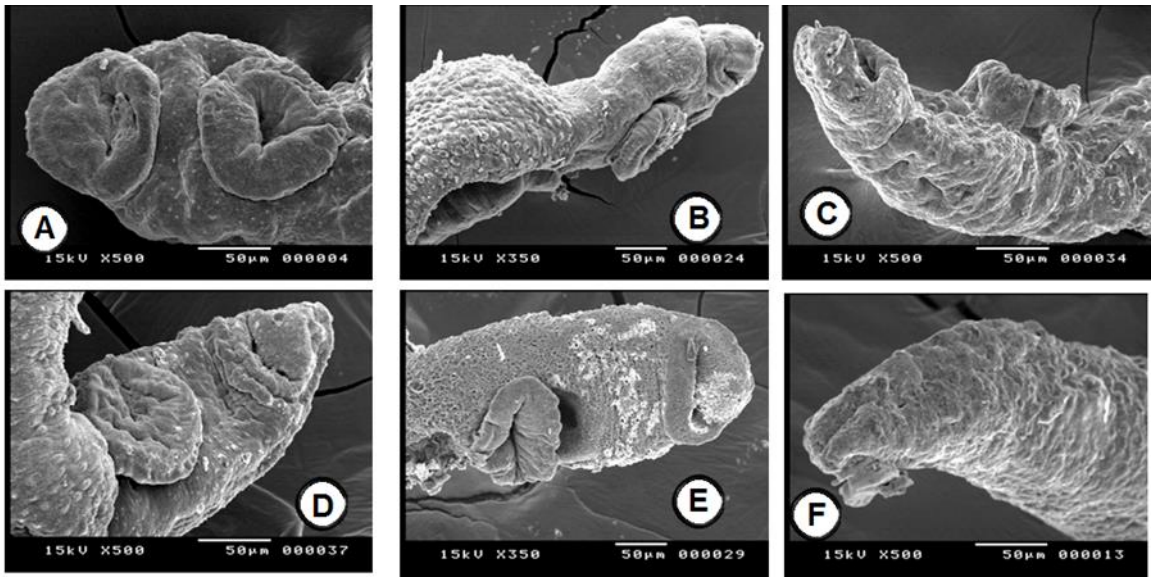

Figure 2. Adult male of Schistosoma mansoni showing the surface ultrastructure alterations after 96 hours incubation with Conus textile muscle extracts: (A) The negative control (RPMI 1640Media) shows intact sucker and tegument. (B), (C), (D), (E) and (F) illustrating loss of the oral sucker and tegument.

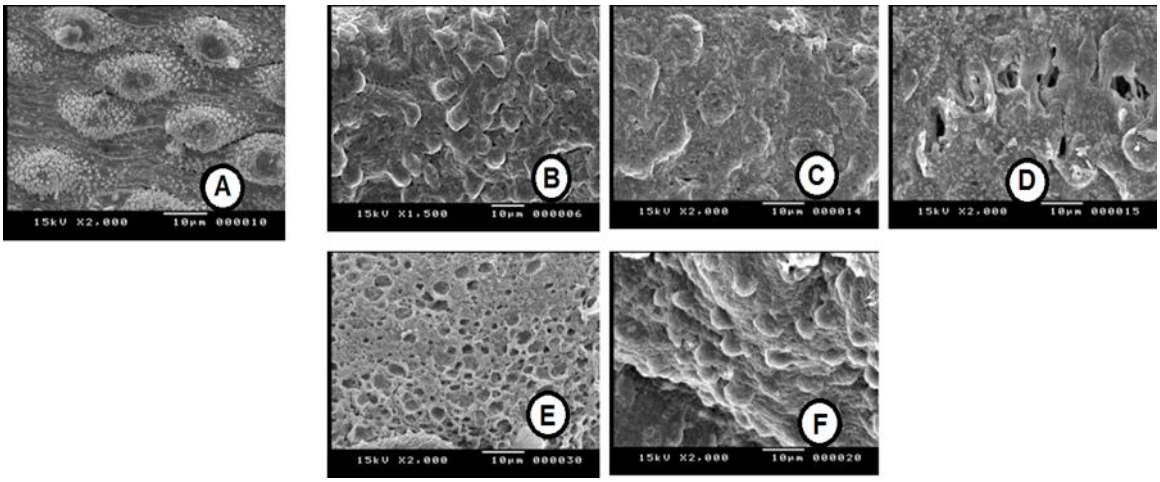

Figure 3. Adult male of S. mansoni showing surface ultrastructure alterations after 96 hours incubation with Conus textile muscle extracts showing: (A) The negative control (RPMI 1640 media) shows intact tubercles spines in the tegumental area around the gynaecophoric canal. (B), (C), (D), (E) and (F) illustrating formation of protuberances and shorten of spines in the tegumental area around the gynaecophoric canal, loss and damage of tubercle spines, erosion, and perforation of tegument.

2.7. Evaluation of the muscle extracts effect on the tegument system of *S. mansoni* worms by Scanning Electron Microscopy (SEM)

Worms were fixed in 4% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.3) for 4 hours, followed by 2 hours of post-fixation in osmium tetroxide (OSO4). The worms were then washed repeatedly three times in the same buffer before being dehydrated with a series of progressive ethanol concentrations ranging from 10-100 % for 10 minutes in each concentration, except for the final concentration (100 %) (30 min,

10 min per change). Critical point drying in aqueous carbon dioxide was used to complete the dehydration process. Finally, worms were placed on copper stubs with two-sided sticky tape, covered with gold using an Edwards S150A sputter coater, and examined with a scanning electron microscopy (JXA-840A Electron Probe Microanalyzer; JEOL, Tokyo, Japan).

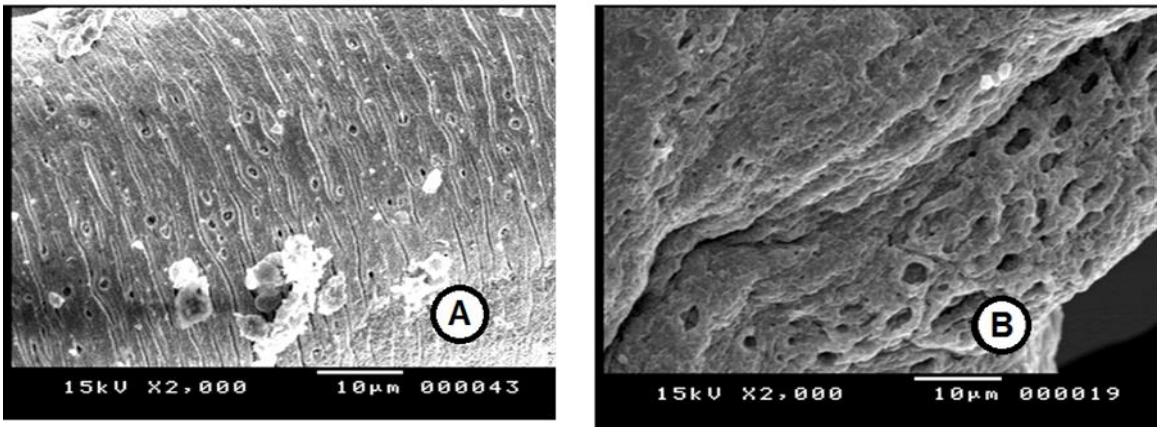

Figure 4. After 96 hours of incubation with Conus textile muscle extracts, SEM analysis of adult male of S. mansoni worms showing: (A) The negative control (RPMI 1640 media) shows intact tubercles on the dorsal region of the tegument. (B) 50µg/ml Conus textile muscle extract shows severe damage, shrinking, and erosion of the female worms' tegument.

2.8. Ethical considerations

Hamsters were purchased from the Theodor Bilharz Institute in Giza, Egypt, and kept in a controlled environment with a 12-hour light/dark cycle and free access to food and water. The National Academy of Sciences' Guide for the Care and Use of Laboratory Animals was followed in all animal experiments.

3. Results

3.1. Antischistosomal effects of *Conus spp* muscle extracts

Our results revealed that muscle extracts of *C. vexillum, C. fulgetrum, C. flavidu*s have no effects on the mobility and vitality of *S. mansoni* worms. The muscle extract of *C. lividus* showed moderate effect on the viability of *S. mansoni* at high concentration only (100µg/ml). However, those of *C. textile* showed obvious influences on the vitality of the same worms at all concentrations (10.0 - 100µg/ml). Reduction of motility was observed with the different concentrations of *C. textile* muscle extracts. At the initial incubation time, the mobility took the form of sluggish motion and a low mortality rate. Moreover, the motility changes progressed with increasing concentration and time, and were associated with total lack of sucking capability of male worms, deformation, and separating of female worms from the gynaecophoric canal of its coupled male. The lethal effect of *C. textile* muscle extract on adult worms was first observed at a dosage of 10.0µg/ml after 96hr (13.0%) and increased to 17% after 24hr of incubation at 100 µg/ml concentration (Table 1). The mortality rate was increased gradually until it reached 20%, 33%, and 67% after 48hr, 72hr, and 96hr at 100.0µg/ml concentration, respectively.

Table 1: Mortality rate (%) of adult S. mansoni worms by different concentrations of different Conus spp muscle extracts for 4 consecutive days in vitro:

Group	Treatment		Mortality rate (hours)			
		Dose (µg/ml)	24	48	72	96
Control	Positive	10.0	100%	100%	100%	100%
		10.0	100%	100%	100%	100%
	Negative		0%	0%	0%	0%
			0%	0%	0%	0%
	Species	Dose (µg/ml)				
Muscle Extract	*C. vexillum*	100	0%	0%	0%	0%
		200	0%	0%	0%	0%
	C. texile	10	0%	0%	0%	13%
		100	17%	20%	33%	67%
	C. lividus	100	0%	0%	0%	0%
		200	0%	0%	0%	13%
	C. fulgetrum	100	0%	0%	0%	0%
		200	0%	0%	0%	0%
	C. flavidus	100	0%	0%	0%	0%
		200	0%	0%	0%	0%

3.2. Determination of the best solvent for *conus textile* muscle extracts.

To determine the best solvents for preparation of *Conus* muscle extracts, 5 different solvents were used: acetic acid, acetonitrile, ethanol, methanol and water. Based on our results, the best solvent was acetic acid which induced the highest mortality rate for *S. mansoni* worms (Table 2).

Table 2: Mortality rate (%) of adult S. mansoni worms by different concentrations of Conus textile muscle extract using different solvents for 4 consecutive days in vitro

	Extraction solvent	Dose (µg/ml)	Mortality rate (hours)			
			24	48	72	96
*C.textile*Muscle Extract	Acetic Acid	10	0%	0%	0%	13%
		100	17%	20%	33%	67%
	Acetonitrile	10	0%	0%	0%	17%
		100	0%	0%	0%	30%
	Ethanol	10	0%	0%	0%	0%
		100	0%	0%	17%	25%
	Methanol	10	0%	0%	0%	20%
		100	0%	0%	17%	50%
	Water	10	0%	0%	0%	0%
		100	0%	0%	0%	33%

3.3. Antischistosomal effects of *Conus textile* muscle extracts.

Mortality rate of adult S. mansoni worms by different concentrations of Conus textile muscle extract showed great effect depending on ascending time and concentration series.The mortality rate of adult S. mansoni worms after 96 hours of incubation were 0, 17, 28, 57, 67% at 2.5, 5.0, 10.0, 50.0, 100 µg/ml respectively (Table 3). The mortality rate of adult *S. mansoni* by different concentrations of *C. textile* muscle extract (2.5: 100 µg/ml) after 24 to 96 hrs exposure show great effect depending on time and concentration.

Table 3: Mortality rate (%) of adult S. mansoni worms by different concentrations of Conus textile muscle extract (2.5: 100 µg/ml) after 24 to 96 hrs exposure.

Concentration (µg/ml)	Mortality rate (hours)			
	24 H	48 H	72 H	96 H
2.5	0%	0%	0%	0%
5.0	0%	0%	14%	17%
10.0	14%	14%	17%	28%
50.0	14%	14%	29%	57%
100.0	17%	20%	33%	67%

3.4. Determination of LD50 and CC50 of *Conus spp* muscle extracts

The 50% cytotoxic values (CC50) of the studied *Conus spp* muscle extracts were determined. Hepatocytes (Huh 7.5) treated with muscle extracts at tenfold ascending doses of 1-100 g/ml showed no significant changes in cell count when stained with MTT. While concentrations of the muscle extract 2.5, 5, 10, 50, and 100 µg/ml were used in two replicates with suitable number of worms and incubated at 37°C were used to detect its LD50. Worms viability was evaluated for four successive days of treatment to determine sub lethal concentrations. Results showed that, the 50% lethal dose or concentrations of *Conus spp* muscle extracts are >100µg/ml for *C. vexillum, C. lividus, C. fulgetrum,* and *C. flavidus,* and 43.85µg/ml for *C. textile* (Table 4).

Table 4: LD50 and CC50 values for 5 different species of Conus muscle extracts

	Species	LD_{50} (µg/ml)	CC50 (µg/ml)
Muscle Extract	*C. vexillum*	>100	>100
	C. lividus	>100	>100
	C. fulgetrum	>100	>100
	C. flavidus	>100	>100
	C. textile (Acetic acid)	43.85	>100

3.5. Influences of *Conus textile* muscle extracts on adult worms of *S. mansoni*

After the incubation with ascending concentrations (2.5–100 µg/ml) of muscle extracts, adult stages of *S. mansoni* were examined using SEM. Results showed the destruction of the oral sucker, tegument loss, damage of tubercle spines, forming of protuberances and shortening of spines in the tegumental area around the gynaecophoric canal. In addition, severe destruction, shrinkage, and erosion of the tegument of female worms was observed (Fig. 2, 3 and 4).

4. Discussion

Schistosomiasis is a parasitic infection caused by blood helminths of the genus *Schistosoma*. *S. mansoni, S. haematobium, and S. japonicum* are the three species which cause most of the human infections [23]. In the absence of an effective vaccination, schistosomiasis is treated and controlled almost entirely using praziquantel (PZQ), a medicine that has been applied in mass drug administration programs since the 1970s [24]. The urgent need for novel anti-schistosomal chemicals has been highlighted [25], due to the development of praziquantel resistant strains in therapeutic practice [26]. Natural product groups have risen to prominence in recent years as potential sources of novel schistosomiasis treatments [27]. Marine environment contains a varied range of biologically active natural compounds, many of which are not available in terrestrial sources [28]. In the realm of bioactive product development, the biodiversity of marine environment and its associated chemical diversity represent a nearly unlimited source of novel active compounds. Primary products obtained from the sea creatures have been act as a source of pharmaceuticals and as initial source for drug development. Furthermore, due to changes in environmental conditions, marine species might generate new or unexpected molecular entities with a biological activity. As a result, it is expected that

search for novel and distinctive substances produced from aquatic species will continue to advance our basic understanding of pharmacology and medicine [29]. In this regard, marine molluscs have been discovered to produce a great range of unique bioactive secondary metabolites, suggesting that they could be a source for new medicine development [30,31]. The genus *Conus* belongs to the group Conidae which is one of the largest groups of gastropods. Cone snails are a rich source for a natural and biologically active components and many of which are discovered from *conus sp*. [32]. *C. betulinus* and *C. inscriptus* were found to have antimicrobial activity for poultry bacterial pathogen strains [29]. They attributed that to the presence of specific proteins in the muscle extracts of those snails. Previous trials were made to find antischistosomal agents from different sources. For example, one study had evaluated the cutaneous secretion of frogs from genus *Phyllomedus* and showed an antimicrobial peptide (Dermaseptin)found in the frog skin, which at 100µg/ml for 48hrs killed all the adult worms of *S. mansoni* [33]. Similarly, adult *S. mansoni* worms were effectively killed by incubation with CO-ArNp (combination of artemisinin-naphthoquine phosphate, antimalarial drug first synthesized in China in 1986 but was not developed for clinical use until late 1990s.) at 20-40 µg/ml for 48-72 hour. In another study, (-)-6,6'-dinitrohinokinin (DNK)(Derivative of the essential oil of *P. cubeba*) at 200 M killed 100% of adult worms in 24 hours [34]. The findings of the present study revealed that *C. textile* muscle extract could induce dose dependent mortality rates of *S. mansoni* adult worms at different concentrations after 24-96h of treatment. The antischistosomal activity *C. textile* muscle extract might be attributed to the presence of specific compound(s) that can disrupt biological processes in Schistosoma cells, such as protein production. The tegument of *Schistosoma* has numerous vital roles for the parasite, such as food absorption and maintenance of host immune response or damage healing. The tegument also is essential for infection success and the survival of the worm in the host. Therefore, it has long been a target for antischistosomal medicines [35]. Several investigators used the alterations and damage of surface topography of Schistosoma worms to assess the schistosomicidal effect in both vitro and vivo studies [36–38]. Antischistosomal medications cause tegmental alterations, which are a necessary mechanism for the worms to die [39]. The characteristics of tegument destruction that shown in the current study, such as tegument disintegration, erosion, and loss of spines, were almost similar to those previously reported following *In vitro* treatment with various schistomocidal drugs as hypericin [40], allicin [41], nerolidol [42], phthalylthiazole (LpQM-45) [43], β-lapachone [44], and DNK [45]. The exact mechanism of *C. textile* muscle extracts as antischistosomal compound is still unclear. However, the current authors suggested that, the active components in muscle extracts might be peptides because several antimicrobial peptides are thought to kill target cells by breaking cell membranes [46–48].

5. Conclusions

The current study revealed that *C. textile* muscle extracts have great promise schistosomicidal characteristics on the adult worm of *S. mansoni*, as proven by morphological and tegumental alterations, as well as a direct killing effect that is both concentration and time related. This also might introduce effective source for controlling human schistosomes and minimizing the health burden of their infection. Additional research is required, however, to explain the mode of action and to address *C. textile* muscle extracts composition. Moreover, further studies are required for the characterization of potential antischistosomal medicines which derived from *Conus* bioactive compounds.

Conflict of interest

There are no conflicts of interest declared by the authors.

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دراسة تأثير مستخلص عضلات خمسة أنواع شائعة من القواقع الحلزونية في البحر الأحمر على ديدان البلهاريسيا

محمود عبدالله [1] ، محمد عبدالوهاب [1] ، نجوى على [2] ، عمر عامر [1] ، مصطفى سرحان [1]

1. قسم علم الحيوان - كلية العلوم - جامعة الأزهر-أسيوط- مصر
2. قسم الطفيليات- كلية الطب – جامعة بنها- مصر

الملخص:

تمت دراسة النشاط المضاد لديدان البهارسيا المحتمل لمستخلص عضلات الحلزونيات المخروطيةعلى الديدان البالغة من البلهارسيا المنسوني في المختبر. تم جمع عينات حية لخمسة أنواع شائعة من الحلزون المخروطي من عدة مواقع على ساحل البحر الأحمر المصري. ثم جمع عضلات هذه القواقع بعد سحق قشرتها لتحضير مستخلصات العضلات. تم عزل الديدان البالغة من النوع المانسونى من الدورة الدموية بتقنية التروية باستخدام محلول الفوسفات. بعد 1-4 أيام من التعرض لمستخلصات عضلات الحلزون المخروطي بجرعات مختلفة ، تم فحص حركة الديدان ومعدلات الوفيات. وأظهرت النتائج أن ثلاثة أنواع من الحلزون المخروطي مستخلص عضلي*Conus vexillum* .، *Conus fulgetrum*، *Conus flavidus*، ليس لهم تأثير معنوي على بقاء وحركة الديدان على النقيض من ذلك ، أظهر المستخلص العضلي لنسيج *Conus textile*تأثيرًا معنويًا على بقاء وحركة الديدان عند تركيز LD50 43.8 ميكروغرام / مل. لكن المستخلص العضلي لنسيج *Conus lividusg*أظهر تأثيراً ضعيفاً (معدل الوفيات 13٪) على حيوية وحركة الديدان بتركيز عالٍ فقط (100 µg / ml)بعد 96 ساعة من الحضانة. تم التحقيق من التغيرات المحتملة للديدان بعد التعرض لمستخلصات العضلات باستخدام الفحص المجهري الإلكتروني الماسح (SEM) تسبب مستخلص عضلات *Conus textile* في فقدان وتلف الدرنات السطحية ، وكذلك تدمير ممص الفم. يمكن أن يسبب أيضًا تغير النتوءات وقصر العمود السداسي خاصة حول القناةالحاضنة. في الختام ، كشفت الدراسة الحالية عن التأثير المضاد للبلهارسيا لمستخلص عضلات نسيج الحلزونات المخروطية وقد يؤدي إلى عقاقير جديدة مضادة للشيستوسومات في المستقبل.