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Comparative Screening of the Possible Activity of *Rhopilema nomadica* Jellyfish Venom Against Different Protista and Invertebrates

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ABSTRACT

Marine-derived components from various aquatic invertebrates have become a diverse source of structures utilized in scientific research. In this study, different unicellular and multicellular organisms were exposed to *R. nomadica* venom at various concentrations, and their activity and movement were monitored three times within 24 hours. Organisms that displayed complete retardation of movement were classified as dead and subjected to the same venom concentrations for three consecutive days, with their mortality rates calculated. Among the exposed organisms, only protists (Ciliphora and Rhizopoda) exhibited a complete delay in their activities when exposed to the highest venom concentrations. By the third day, both protists displayed 100% mortality at the highest concentrations, accompanied by morphological malformations. In summary, *R. nomadica* venom significantly affected the movement of protists compared to control samples, resulting in their death at the highest concentrations. Furthermore, the study recommended further investigations to test the venom's impact on a wider range of species using varying concentrations.

INTRODUCTION

Water covers more than 70% of the planet's surface. The oceans are the most unique region on earth and have a rich supply of marine and freshwater creatures with a more extensive variety of natural and biochemical elements **(Kiuru *et al*., 2014)**. Marine-derived small particles obtained from different invertebrates represent a highly diverse and potential source of numerous chemicals from different structural classes (**Hrckova & Velebny, 2013)**. The study of marine bioactive substances, or "marine drugs," has significantly developed in recent years. In 2022, seventeen marine-derived medications were authorized for clinical use **(Dyshlovoy & Honecker, 2022)**.

In the recent years, there is a worldwide trend about using drugs made of natural sources, including animals venoms and their components **(Keyhani *et al*., 2021; Rashed e*t al*., 2021; Tawfik *et al*., 2021)**. There are some data regarding the effects of

snake venoms on protists and protozoans, such as *Naja nigricollis* cobra venom, which has a great mortal effects against *Trypanosoma brucei* **(Adade *et al.*, 2011; Esteban *et al.*, 2018)**.

Jellyfish tentacles trigger and stimulate the explosive release of nematocysts that lead to delivering potent and rapid-acting venom to the victim or prey **(Badre, 2014)**. Various studies have shown that coelenterates' venoms contain neuroactive components like quaternary ammonium compounds, vasoactive ingredients such as histamine, catecholamines, and 5-hydroxytryptamine (5-HT), as well as proteins, including hemolytic and cytolytic elements, proteases, and phospholipases. There is still much to find out about the components of jellyfish venoms as well as their mode of action. **(Lee *et al.*, 2016)**.

The nomad jellyfish *Rhopilema nomadica* is a tropical schyphozoaan creature that blooms off along the Midterranean Sea close to Port Said Governerate in northern Egypt. The nomad jellyfish venom has shown promising selective anticancer and antioxidant activities (**Tawfik *et al.*, 2021**). Consequently, the present study aimed to evaluate the potential efficiency of *R. nomadica* venom against different organisms. Moreover, the efficiency was evaluated by studying the movement pattern and mortality rates after exposure to the venom.

MATERIALS AND METHODS

1. Ethics statement

All studies were confirmed by the research animal care ethical committee of the Faculty of Science, Suez Canal University under protocol REC224/2023.

2. *R. nomadica* collection

Animals were collected throughout the burgeoning summer season from the Port Said coast of the eastern Egyptian Mediterranean in 2021. Tentacles were put into fresh seawater-filled tanks and brought instantaneously to the Toxicology research laboratory at the Faculty of Science, Port Said University for further preparations.

3. Venom extraction

Nematocysts were isolated from the enucleated tentacles as described with minor change **(Bloom *et al.*, 1998)**. In order to facilitate the autolysis of the tissues, dissected tentacles were submerged in cold saltwater at a mass volume ratio of 1:3 at 4°C, with gentle swirling for 30min every two hours. Over three days, this step was repeated.

The nematocyst suspension was filtered through a plankton net to remove tissue fragments followed by centrifuging at a speed of 10,000rpm for 30 minutes at 4°C. The

supernatant was then gathered, lyophilized, and finally stored at -20°C. The nematocysts' freeze-dried venom was removed to get the crude venom. In accordance with **Marino *et al.* (2008)**, 10mg of nematocyst powder was suspended in 1mL of phosphate-buffered saline solution (PBS, pH 7.4, 4°C) and centrifuged for a duration of 15 minutes at 15,000rpm using a cooling centrifuge. The recovered *R. nomadica* venom stock solution from the supernatant was obtained and employed in the current study. To prevent the freezing and rethawing process, the stock solution was aliquoted and kept at 20°C.

4. Design of the experiment

The evaluation was conducted initially across various species, both unicellular and multicellular for three times within a 24-hour period to detect the efficiency of the venom against the varied organisms' activities, represented by a reduction in their movement. Only the species that were recorded with complete retardation in their movement or died, underwent the next step of the evaluation within three days to calculate their mortality rates, as summarized in Fig. (1). The degree of movement was symbolized as (+++), which means that there were no changes in the movement. (++) indicates moderate movement or slowing in the pattern. (+) reflects the lowest grade of the movement. Finally, a (-) is recorded for the dead organisms without any movement pattern.

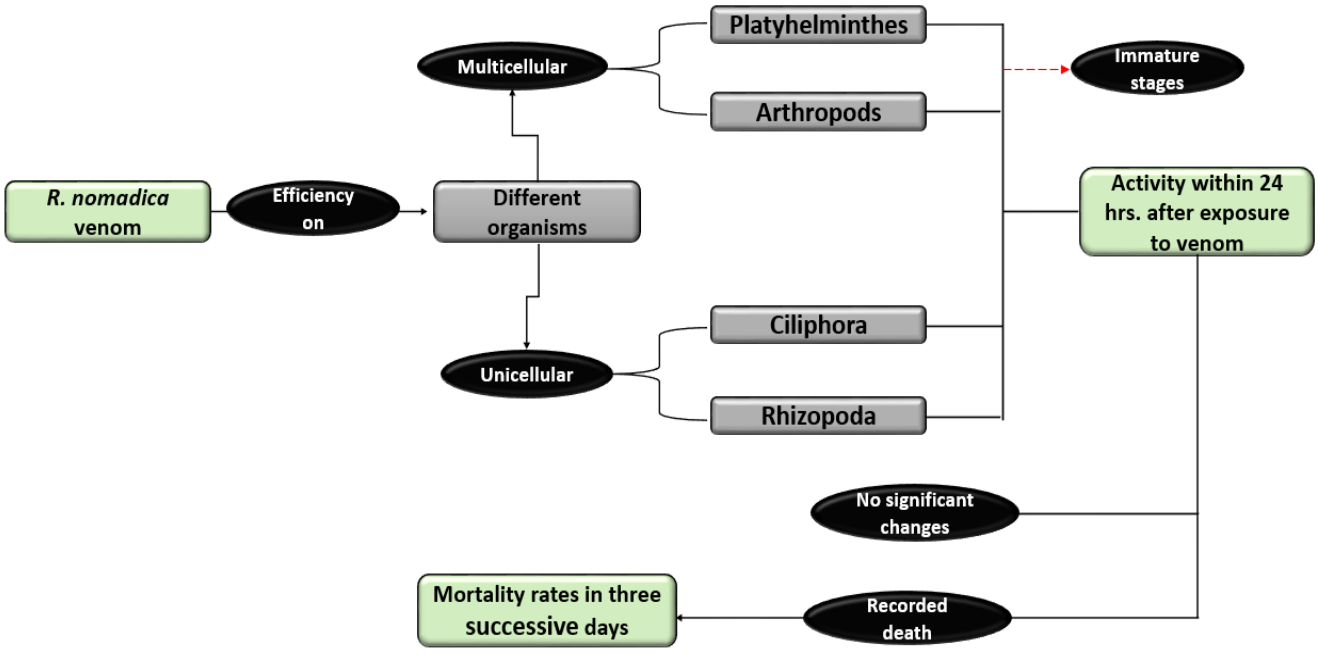

Fig. 1. Experiemental design

5. *In vitro* preparation of different organisms

5.1. Schistosomula

By exposing infected *Biomphalaria alexandrina* snails to direct light for two hours at 37°C, cercariae of *S. mansoni* were shed from the snails. According to **Ramalho-Pinto *et al*. (1974)**, the cercariae were mechanically transformed into schistosomula using a vortex mixer. Schistosomula were grown at 37°C in 5% CO_2 in 169 medium supplemented with 10% fetal serum of bovines and antibiotics **(Basch, 1981)**. The schistosomula of *S. mansoni* were cultivated for seven successive days, representing the skin and lung stages of the worm **(Moraes, 2012; Veras *et al*., 2012)**.

The schistosomula (±30 parasite/well) were cultured in 24-well plates. The final concentrations of the venom against the parasites ranged from 120 to 450µL/ mL. Each assessment was carried out three times, and the sum of the wells for each concentration was used for the final calculation. The plates were then incubated for 24hrs at 37°C and 5% CO_2. Control schistosomula were incubated in RPMI-1640 medium. The activity of the schistosomula was monitored after every 1, 2, and 24hrs using an inverted microscope in comparison with the control group.

5.2. *Trichinella spiralis larvae*

The *T. spiralis* strain was introduced to the parasitology lab at Theodor Bilharz Research Institute in Giza, Egypt, through repeated passages in mice from infected hog flesh. Ten male Swiss albino mice (25±2 g) were utilized. The mice were kept in appropriate cages, given commercial rodent food. Each mouse received 200 of *T. spiralis* larvae orally **(Abou Rayia e*t al*., 2017)**. Thirty five days post-infection, the skeletal muscles were dissolved in pepsin-HCL in accordance with the method described by **Jiang *et al.* (2012)** to exrract mature larvae from the muscles.

T. spiralis mature larvae were placed in 24-well plates (±30 larvae/well) that were previously filled with RPMI-1640 media (which contains 20% fetal bovine serum, 200U/ ml penicillin, and 200g/ ml streptomycin). *R. nomadica* venom was added to RPMI-1640 medium. The final concentrations of the venom against mature larvae ranged from 120 to 450µL/ mL. Each estimation was performed three times, and the total sum of wells for each concentration was determined. The plates were kept in incubation for 24hrs at 37°C and 5% CO_2. Control larvae were incubated in RPMI-1640 medium. The activity of the larvae was monitored after every 1, 2 and 24 hrs in comparison with the control group.

5.3. Fresh water samples

Samples of fresh water from the El-Salam canal were obtained in clean, sterilized glass jars and transported directly to the Parasitology Research Lab in the Faculty of

Science, Port Said University, Egypt. The samples were examined using an inverted stereomicroscope to detect the most abundant organisms. One ciliate and one rhizopod were chosen as examples of protista, while the most prevalent fly larva represented the arthropods. In general, protists are included in the food chain of insect larvae, which may lead to a faked result of protist death due to the venom. Hence, the larvae were separated by using thin forceps with an appropriate quantity of water.

The samples were kept in the lab for three days, and the organisms' activity was sequentially monitored to be sure that the natural organic supplements in the media were sufficient. After three days, protists and larvae were still alive, and there were no changes in their activity. Consequently, there was no need to add any supplement. Furthermore, 1mL of water samples with venom concentrations ranging from 120 to 450L/ mL was added to the well plates. Their activity was monitored three times within 24 hours, similar to schistosomula and *Trichinella* larvae, using control samples as an ideal model for movement

6. Venom efficiency

The venom efficiency depending on movement pattern activity was statistically represented according to the following theory:

The highly active movent pattern represented by $(+++) \rightarrow 0$
The mild movent pattern represented by $(+) \rightarrow 2$
No movement/dead $(-) \rightarrow 3$

7. Mortality rates

Organisms exhibiting complete retardation in their movement within the first 24 hours of the survey were exposed to the same venom concentrations (120 to 450µL/ mL) over three days. The mortality rates were calculated as percentages using the following formula:

$$\boldsymbol{P = (T_c - A) \times 100}$$

As *P* means the percent of dead organisms; T_C indicates the total count of the organism, and A represents the alive organisms after exposure to the venom.

8. Statistical analysis

The mean and standard error (S.E.) of the values were calculated using the Statistic Program Sigma Stat (SPSS), version 20.

RESULTS

Following exposure to *R. nomadica* venom for 1, 2, and 24 hours, the movement patterns of the investigated organisms were summarized in Table (1), and the average efficiency was statistically expressed in Fig. (2).

In general, there was no lethal impact on the developmental stage of *S. mansoni* as a result of exposure to *R. nomadica* venom. The effect of the venom against schistosomula began after 2hrs. at 300µL/ mL causing moderate movement and an average efficiency of (1±0.50). At a concentration of 450µL/ mL, the shistomulae showed the least movement pattern and the greatest effect of the venom over the course of 24hrs. (Fig. 2). In the case of *T. spiralis*, movement was slowed after 2hrs. of exposure to *R. nomadica* venom at 150µL/ mL, but with time, the larvae resumed full activity after 24hrs., and the venom's efficiency against the larvae was zero. *R. nomadica* venom was the most effective against the larvae at concentrations of 300 and 450µL/ mL (1.33±0.29, and 2), respectively (Table 1 & Fig. 2).

In freshwater samples, *Amoeba* sp. and *Protoopalina* sp. were chosen as the most prevalent protists, while red larvae of midges represented the arthropods. Midge larvae were the most tolerant to the *R. nomadica* venom among the different species, displaying high to moderate degrees of movement, with mild movement only recorded at 300 and 450/mL after 24hrs. and with a venom's average efficiency of 0.67±0.58 and 1.33±0.29, respectively (Table 1 & Fig. 2).

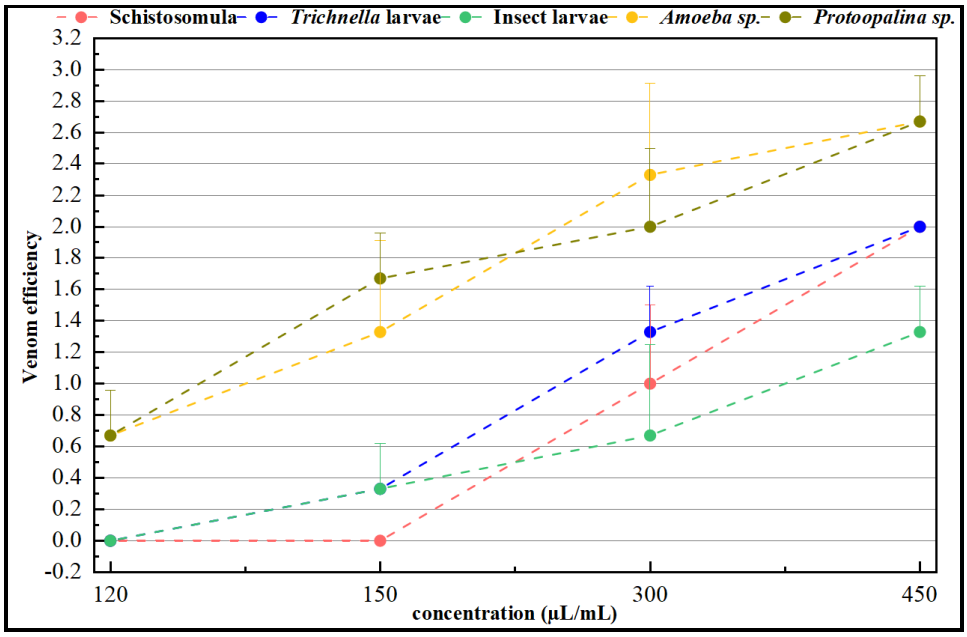

Fig. 2. The average efficiency of the *R. nomadica* venom against the different organisms within 24hrs

Table 1. Movement pattern of different organisms after exposure to *R. nomadica* venom

Concentration (µL/mL)	Activity within 24hrs														
	Schistosomula			Trichnella larvae			Insect larvae				*Amoeba* sp.		*Protoopalina* sp.		
	1hr	2hrs	24hrs	1hr	2hrs	24hrs	1hr	2hrs	24hrs	1hr	2hrs	24hrs	1hr	2hrs	24hrs
120	+++[a]	+++[a]	+++[a]	+++[a]	+++[a]	+++[a]	+++[a]	+++[a]	+++[a]	+++[a]	++[b]	++[b]	+++[a]	++[b]	++[b]
150	+++[a]	+++[a]	+++[a]	+++[a]	++[b]	+++[a]	+++[a]	+++[a]	++[b]	+++[a]	+[c]	+[c]	++[b]	+[c]	+[c]
300	+++[a]	++[b]	+[c]	++[b]	++[b]	+[c]	+++[a]	++[b]	+[c]	++[b]	-[d]	-[d]	++[b]	+[c]	-[d]
450	+[c]	+[c]	+[c]	+[c]	+[c]	+[c]	++[b]	++[b]	+[c]	+[c]	-[d]	-[d]	+[c]	-[d]	-[d]

[a] High [b] Moderate [c] Mild [d] Null/dead

Based on the previous results, only *Amoeba* sp. and *Protoopalina* sp. among the five studied organisms showed mortality after exposure to *R. nomadica* venom. When compared to the control samples (Fig. 3A, E), the death of *Amoeba* sp. was characterized by irregular movement of pseudopodia, followed by the organism becoming rounded (retracted), as illustrated in Fig. (3F). Subsequently, there was complete retardation of movement, indicating the organism's death.

Regarding *Protoopalina* sp., the movement pattern in control samples was characterized by the very rapid model in the examination field with a fast rotating mechanism in a vertical position (Fig. 4A, C). Once they were exposed to the highest concentration of *R. nomadica* venom 450µL/ mL, they showed highly vigorous motion in the beginning for a few seconds and slowed down gradually. The ciliate then retracted on itself (Fig. 4D) and finally lost its complete activity (dies).

The mortality rates of *Amoeba* sp. caused by different concentrations of *R. nomadica* venom for three consecutive days were slightly higher than those of *Protoopalina* sp. *Amoeba* sp., which were severely affected by venom exposure, particularly on the third day of the experiment, with mortality rates ranging from 15.15 to 100% at concentrations of 120 to 450µL/ mL, respectively. On the second day, *Amoeba* sp. showed the same mortality rate of 12.12% when exposed to concentrations of 120 and 150µL/ mL (Fig. 5).

The mortality rates of *Protoopalina* sp. as a result of exposure to different concentrations of *R. nomadica* venom for three successive days are shown in Fig. (5). The mortality rates ranged from 9.09 to 100% on the third day at concentrations of 120

and 450µL/ mL, respectively. At 150µL/ mL of venom, the mortality rate 12.12% remained constant from the second to the third day at this concentration of venom (Fig. 5).

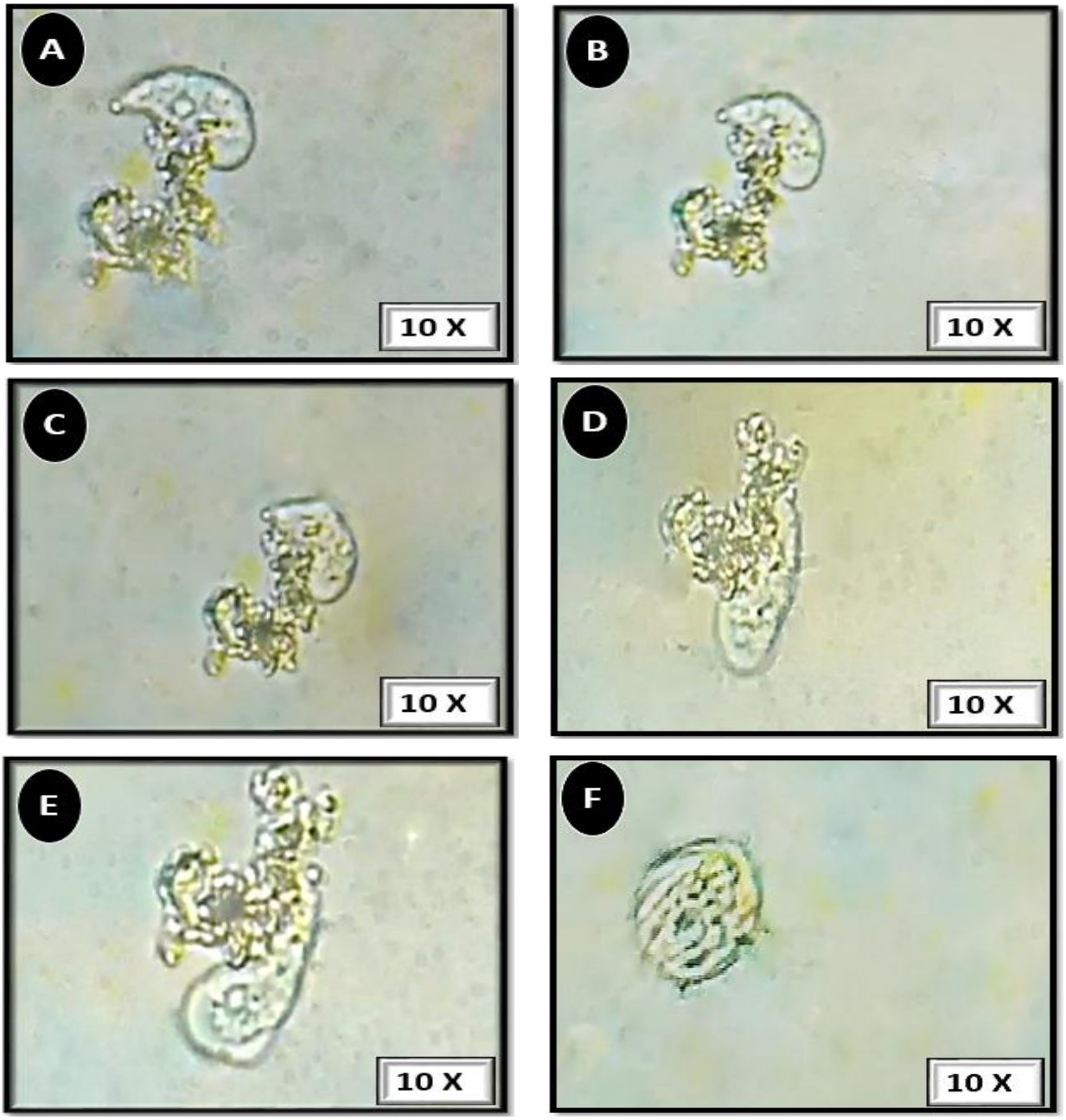

Fig. 3. *A*moeba sp. movement pattern showing: (**A-E**) Control samples moving around food particles, and (**F**) Dead *Amoeba* sp. after exposure to *R. nomadica* venom

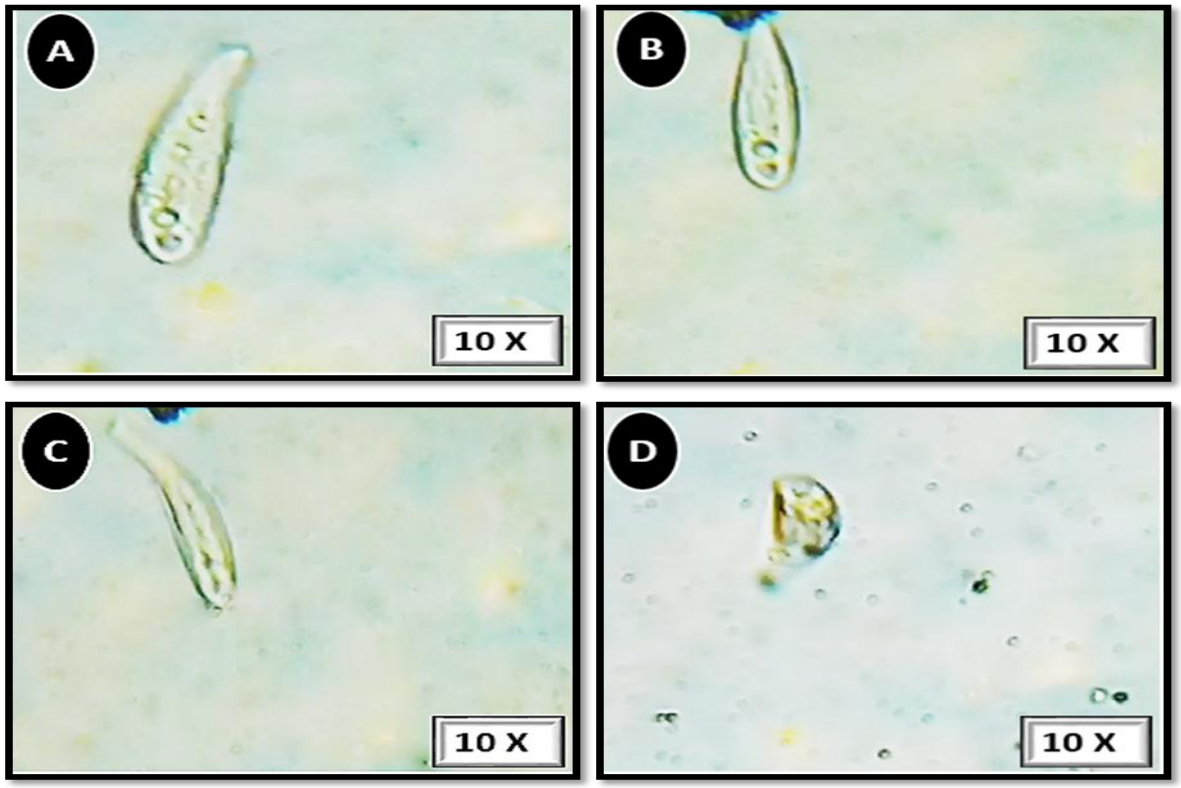

Fig. 4. *Protoopalina* sp. movement pattern showing: (**A-C**) Control samples, and (**D**) Dead *Protoopalina* sp. after exposure to *R. nomadica* venom

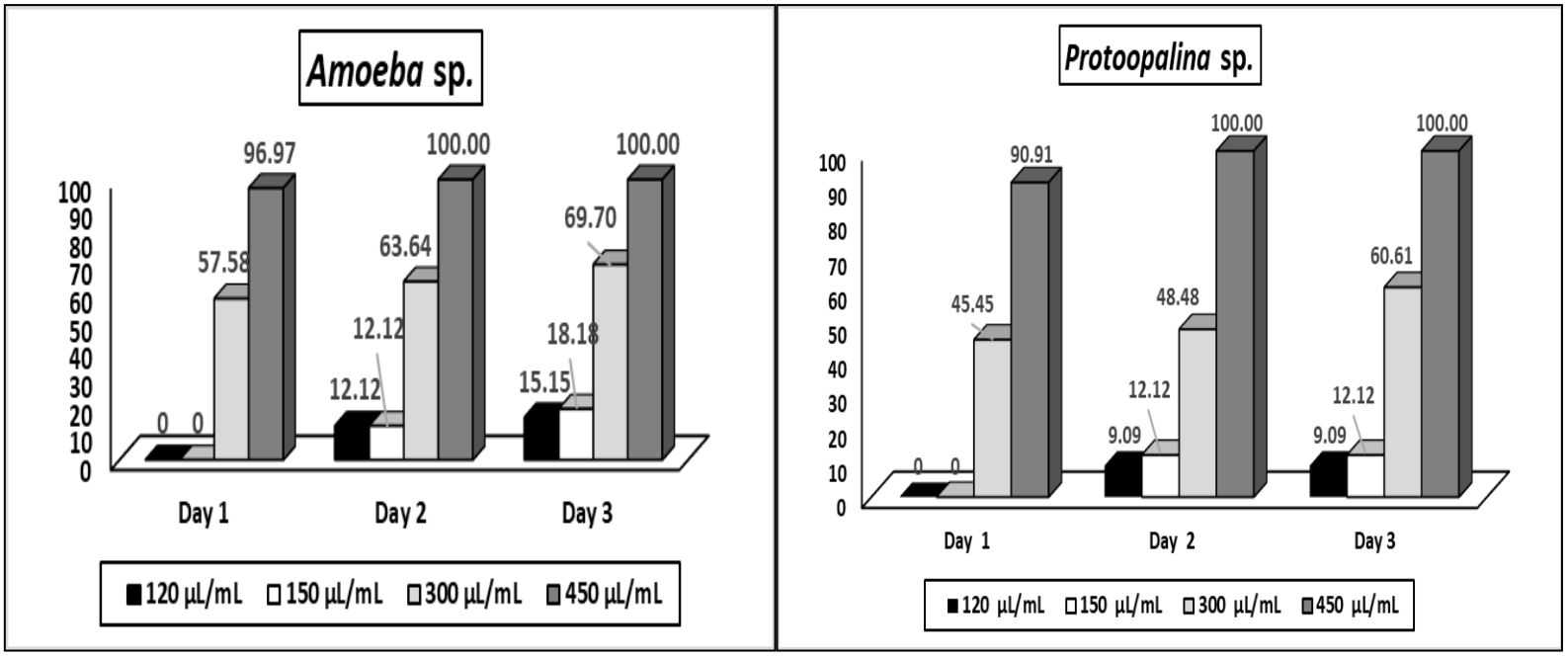

Fig. 5. Mortality rate (**%**) of *Amoeba* sp. and *Protoopalina* sp. exposed to different concentrations of *R. nomadica* venom for three consecutive days

DISCUSSION

R. nomadica showed a mild anti-parasitic effect against the developmental stages of two helminths, *S. mansoni* schistosomulae and *T. spiralis* larvae. The highest recorded efficiency was after 24hrs at a concentration of 450µL/ mL. The effect of the venom was only represented in the form of movement retardation, without recorded deaths. This can be regarded as a promising point for further concentrations used or even using the venom as a complementary drug against other stages of the parasites. Interestingly, the praziquantel (the antischistosomal medication of preference) has potent efficiency against adults with minor or no effect on the lung stage *Schistosoma* larvae, as reported in several studies **(de Oliveira *et al*., 2017; Kovač, *et al*., 2017; Abu Almaaty *et al*., 2021)**. From this latter point, there is a need to testify *R. nomadica* venom against the adult worms.

R. nomadica venom showed the lowest efficiency against the midge larvae among all tested organisms. At the highest used concentration of *R. nomadica* venom (450µL/ mL), the midge larvae exhibited moderate activity. The efficacy of jellyfish venom against insects was previously confirmed; *Rhopilema esculentum* venom was tested and found effective against *Tephanitis pyri* Fabriciusa, *Aphis medicaginis* Koch, and *Myzus persicae* Sulzer **(Yu *et al*., 2005)**.

There were noticeable changes in the movement of *Amoeba* sp. when exposed to *R. nomadica* venom, especially at higher concentrations. Pseudopodia moved rapidly in different directions followed by encystement appearance without any movement. These observations agree with those of **Hashim *et al*. (2015)** who found that, the acanthopodia of *Acanthamoeba* sp. were greatly affected and reduced, while the cell itself became rounded upon exposure to toxic material. Based on this previous observations, it can be concluded that the main actions of the toxins against rhizopods are their effects on the cellular shape and the pseudopodia.

After exposure to *R. nomadica* venom, the ciliated *Protoopalina* sp. was recorded as dead after more vigorous movement than normal, which quickly turned into slow motion a few seconds later and finally stopped. Changes in the movement pattern was correlated with morphological changes since the cell as a whole became smaller in size and rounded. Similar effects were recorded for other animal venoms, such as cobra cytotoxins, which were previously displayed robust antiprotozoal against ciliated *Tetrahymena pyriformis*. These cytotoxins resulted in great changes in the morphology, from a teardrop-like appearance to an almost spherical appearance, with a leakage of cell contents **(Kuleshina *et al*., 2020)**. Such deformities of *T. pyriformis* when exposed to cobra venom are similar to the effect of the jellyfish venom against *Protoopalina* sp. Both venoms affect the cell's morphology, leading to rounded shape rather than straight.

The exposure to *R. nomadica* venom had the greatest effect on the protozoa of all the samples studied. They not only recorded changes in their movement pattern but also recorded mortality rates at the higher concentrations of the venom (300 and 450µL/ mL) after 24hrs. By the second day, the mortality rates increased, and finally, on the third day, 100% of the tested protozoa died at 300 and 450µL/ mL of venom. The efficiency of the *R. nomadica* venom against *Amoeba* sp. and *Protoopalina* sp. may be attributed to the hypothesis that the small plankton is one of the main food sources for jellyfish **(Guo *et al*., 2014)**.

CONCLUSION

R. nomadica venom showed considerable antiprotozoal effects against both *Amoeba* sp. and *Protoopalina* sp., which finally resulted in high mortality rates. On the other hand, the effect of the venom as antihelminthic and pesticide was moderate. The study also recommended additional research for investigating the venom against a broader spectrum of species at various concentrations.

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