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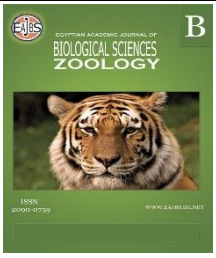

2-*In vitro* Scolecidal Effects of Chitosan (isolatedfrom some scorpions), Chitosan Nanoparticles, Scorpion'S Venom, and Scorpion Venom-Loaded Chitosan Nanoparticles

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ABSTRACT

Hydatid cysts of *Echinococcus granulosus* were recovered from 27.59% (149/540) of camels *Camelus dromedarius* from a slaughterhouse in Cairo. The organ distribution of cysts was 57=38.26% in the lung, 65=43.62 % in the liver, 23=15.44 % in both liver and lungs, and 4=2.68% in the other organs such as the brain, tongue, and spleen, *etc*. The investigation looked at how four compounds from scorpions: chitosan, chitosan nanoparticles (Cs-NPs), venom as well as venom-loaded chitosan nanoparticles (V-CN) affect against *in vitro* hydatid cyst protoscoleces. Aseptically, cyst fluid protoscoleces were aspirated. Each of the aforementioned compounds was always examined in triplicates at doses of 20, 50, and 100 µg/ml for incubation periods of 30, 60, 120, and 240 min to show its scolicidal effects. Using the 0.1% eosin test, the viability of protoscoleces was determined.

According to the degree of deacetylation (DDA), concentration, exposure period, and size, all compounds demonstrated statistically significant differences ($P > 0.05$) against the protoscoleces activity. Chitosan and chitosan nanoparticles had a scolicidal action, directly related to DDA and inversely proportional to size. The most effective scolicidal compound was venom-loaded chitosan nanoparticles, which destroyed all protoscoleces at a concentration of 100 µg/ml, after 240 min of incubation.

INTRODUCTION

One-humped camel *Camelus dromedarius* is the most prevalent species of camel and is potentially important in transporting and producing milk, meat, and wool (El-khabaz *et al.*, 2019). For camels, parasitic diseases are one of the biggest issues (El-khabaz *et al.*, 2019). One of the most common infectious zoonotic illnesses affecting camels is camel echinococcosis (Toaleb *et al.*, 2020), which is considered a major problem in that it affects the productivity and working efficiency of camels and is also a potential danger to public health.

The small intestine of a canine, particularly a dog, serves as the final host for the matured phase of *Echinococcus granulosus* (Thompson, 2017). People, cattle, camels, and

sheep are intermediate hosts by ingesting eggs that hatch and release six-hooked oncospheres that penetrate the intestinal wall and migrate through the circulatory system into various organs especially the liver, lungs, or whatever other organs where the larval type of the parasite creates (Thompson, 2017).

Human hydatid cysts can result in respiratory illness or liver dysfunction, and if cysts rupture, anaphylaxis can develop (Firouzeh *et al.,* 2021). Surgery is still the main treatment for hydatid disease (Firouzeh *et al.*, 2021). However, several drug classes, such as cytostatics, antibiotics, sulphonamides, antiprotozoal substances, and several antihelmintic drugs, have been studied for their effectiveness against the metacestode stages of *Echinococcus* and used before and after surgery to reduce the risk of parasites invading nearby organs and eliminate the protoscoleces (Vidoura *et al.,* 2017 and Firouzeh *et al.,* 2021).

For the transport of polypeptides like insulin, tetanus toxoid, diphtheria toxoid, scorpion's venom, and proteins, chitosan nanoparticles have been extensively studied (Mohammadpour Dounighi *et al.*, 2012).

Studies have shown that several human life-threatening parasites are significantly affected by the venom of snakes and scorpions, including *Plasmodium, Leishmania,* and *Trypanosoma* species (Firouzeh *et al.,* 2021). Numerous peptides found in the venom of scorpions have caught the interest of numerous scientists interested in the potential medicinal applications of these compounds (Perumal Samy *et al.,* 2017). Jafari *et al.,* (2019) were the first who studied the scolicidal effects of the crude venom (and its fractions) of *Mesobuthus eupeus* scorpion against protoscoleces of *E. granulosus*. They found that *M. eupeus* venom peptides can quickly and effectively kill the protoscoleces of hydatid cysts and may be employed as a scolicidal agent in the treatment of hydatidosis. Venom-loaded chitosan nanoparticles combine the effects of chitosan and venom in nano size and provide a variety of benefits and biomedical features that are crucial to the biological properties (Alalawy *et al.,* 2020).

The aim of the study was based on the considerable biological activities of chitosan, chitosan nanoparticles, venom, and venom-loaded chitosan nanoparticles to determine their effects on the protoscoleces of *E. granulosus* parasitizing camels *in vitro*.

MATERIALS AND METHODS

1-Scorpions:

Preparation and characterization of chitosan (from some scorpions), chitosan nanoparticles, scorpion venom, and venom-loaded chitosan nanoparticles have been published **(El-**Sheikh ***et al,*** 2022).

2-Collection and Viability of Protoscoleces of Hydatid Cyst:

The study was performed on a total of 540 one-humped camels *Camelus dromedarius,* slaughtered in Basateen Automated Slaughterhouse in Cairo. Animals recently slaughtered were carefully checked for the presence of hydatid cysts of *E. granulosus*. Hydatid cysts were obtained from the livers as well as lungs of slaughtered camels. Specimens were instantly transported in plastic bags to the Parasitology Lab, Zoology Department, Faculty of Science, Al-Azhar Univ.

For parasitological examination. Three repetitions of meticulous sterile saline washing were performed on protoscoleces. The viability of protoscoleces was tested before the experimental study by observing their motility using the wet mount drop method and by the eosin test as follows: 0.1% eosin solution was added to the protoscoleces solution in a ratio of 1:1. After 15 min, the viability of protoscoleces was microscopically determined by observing the change of their color. In 10 randomly

selected fields, the number of viable and non-viable protoscoleces was estimated. Viable protoscoleces remained colorless, whereas dead protoscoleces absorbed eosin and turned crimson, then placed on slides, covered with coverslips, and counted under a light microscope, viable protoscoleces were primarily motile and displayed flame cell activity (Tawfik, 2018). For the subsequent studies, only protoscoleces with a viability rate of greater than 90% were chosen (Al-Malki *et al.,* 2022).

3-Effect of the Prepared Compounds on Protoscoleces:

Approximately 5×10^3 viable protoscoleces were transferred into a test tube under sterilized conditions and set for 45 min. Three trials of normal saline washing were performed on the sedimental protoscoleces. Then, 2.5 ml of a sterile RPMI 1640 medium (which was prepared by adding 100 mg/ml streptomycin and 100 IU penicillin) was added to the protoscoleces, which were considered as a control group.

Ten treated groups, Cs1, Cs2, Cs3, Cs-NPs1, Cs-NPs2, Cs-NPs3, V, V-CN1, V-CN2, and V-CN3, each with three concentrations: 20, 50 and 100 ug/ml RPMI-1640 were prepared. Then, 2.5 ml of each concentration of the treated groups were separately added to test tubes containing washed viable protoscoleces (approximately 5×10^3), softly mixed, and incubated at 37°C in a 5% CO_2 incubator for 30, 60, 120, and 240 min (Al-Malki *et al.*, 2022 and Firouzeh *et al.,* 2021).

Care was taken to avoid upsetting the settled protoscoleces, and the upper portion of the solution was removed after each incubation period. One milliliter of 0.1% eosin stain was added to the remaining settled protoscoleces and gently mixed. After 5 min, the upper portion of the solution was again discarded. The remaining settling protoscoleces were spread out on a glass slide, protected with a cover glass, and inspected under a light microscope for the viability of protoscoleces (Al-Malki *et al.,* 2022).

Estimates were made of the proportions of stained (dead) and un-stained (living) protoscoleces at each concentration. A minimum of 500 protoscoleces were counted in randomly selected fields to calculate the percentages of dead protoscoleces (Fakhar *et al.* 2015 and Norouzi *et al.,* 2019). The used sample in the experiment's positive control contained a medium devoid of any compounds, while the used sample in the experiment's negative control contained the hydatid fluid (Fakhar *et al.,* 2015). Average values were estimated from all trials carried out in triplicates (Al-Malki *et al.,* 2022).

4-Statistical Analysis:

With the use of the statistical analysis program SPSS, one-way analysis of variance (ANOVA) was used to compare the test results (version 25). The P-value of 0.05 or less was regarded as statistically significant.

RESULTS AND DISCUSSION

1-Prevalence and Intensity of Infection:

Of 540 examined camels, 149 (27.59%) were found infected with hydatid cysts of *Echinococcus granulosus*. The organ distribution of cysts was 57=38.26% in the lungs, 65=43.62 % in the liver, 23=15.44 % in both liver and lungs, and 4=2.68% in the other organs such as the brain, tongue, and spleen. For many infected camels 73.2 % harbored 2–5 cysts each, 18.2 % had 6–10 cysts, 6.1% had 11–15 cysts and 2.5 % had more than 15 cysts. It was noticed that the number of the present hydatid cysts increased with the increase of camel age in agreement with the results recorded by Gareh *et al.* (2021).

2-Viability of Protoscoleces:

Hydroid cyst viability was determined using the wet mount drop method in the cystic fluid by the presence of free protoscoleces (Fig. 1A). Before the trials, protoscoleces viability was examined using a 0.1% aqueous eosin stain. Under light microscopy, on one

hand, live protoscoleces were found colorless, having a distinctive muscle motion, and flame cell activity (Fig. 1 A-E). On the other hand, after exposure to the prepared compounds, live protoscoleces remained colorless when partial death occurred, however**,** dead protoscoleces took up 0.1% eosin stain and turned red (Fig. 1 F and G), when complete death happened, all protoscoleces turned red (Fig. 1H-J).

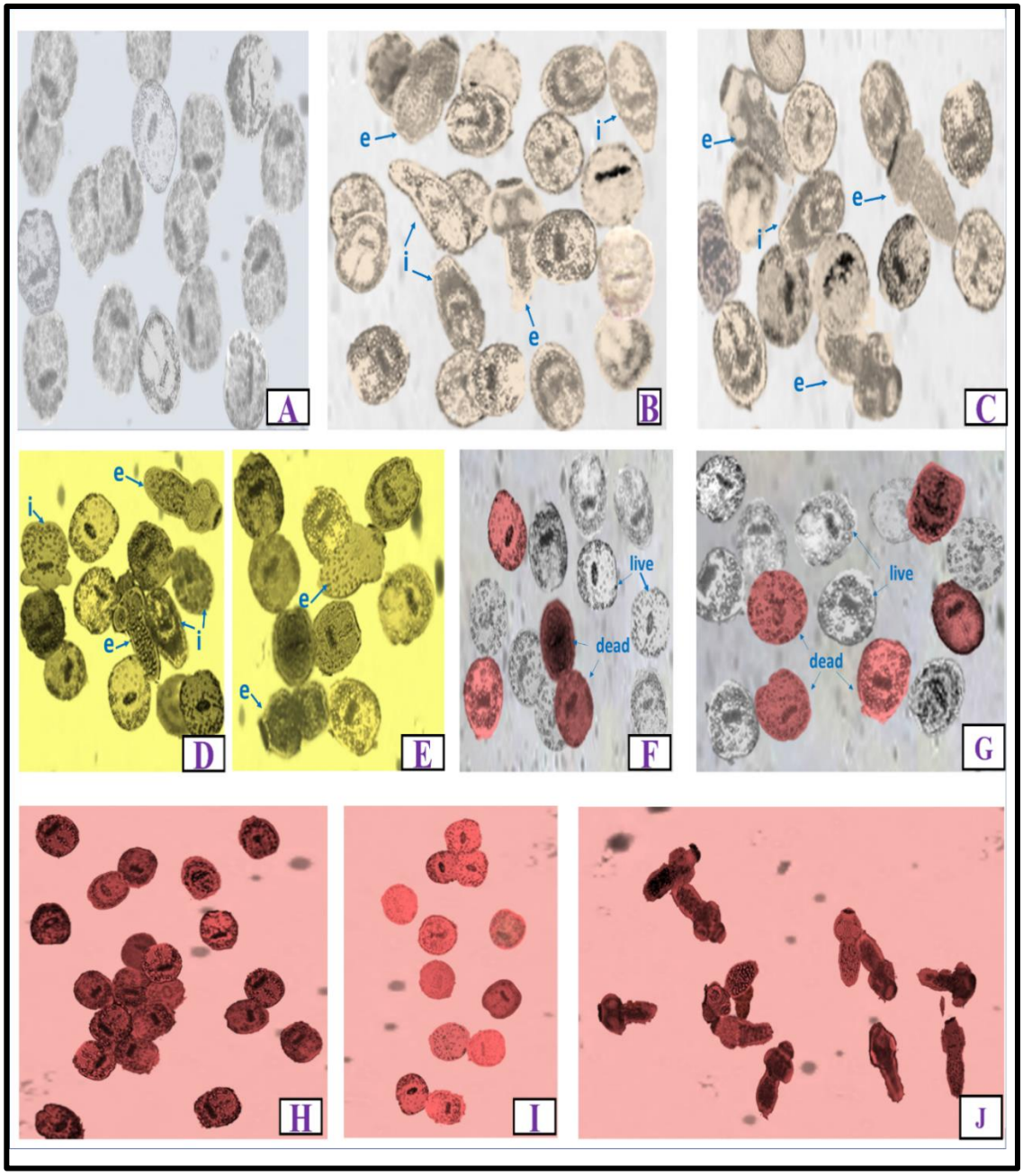

Fig. 1: Photomicrographs showing the protoscoleces of *E. granulosus* cysts collected from naturally infected livers and lungs of camels *Camelus dromedarius***:** (A) Live protoscoleces without staining**;** (B & C) Live invaginated (i) and evaginated (e) Protoscoleces without staining**;** (D & E) Colorless protoscoleces after staining with 0.1% eosin; live evaginated (e) and invaginated (i)**;** (F & G) live and dead colored protoscoleces after exposure to the prepared compounds**;** (H & I) Dead invaginated colored protoscoleces after exposure to the prepared compounds and staining with 0.1% eosin**;** and (J) Dead evaginated protoscoleces (colored) after exposure to the prepared compounds and staining with 0.1% eosin.

3-*In vitro* Solicidal Effect of Chitosan:

The scolicidal effects (mortality rates) of the present chitosan samples (Cs1, Cs2 and Cs3) after various exposure times and different concentrations were depicted in Table (1) and Fig. (2). When protoscoleces of *Echinococcus granulosus* were exposed to chitosan Cs1 with DDA 70.41 % at the concentration of 20 µg/ml after 30, 60, 120, and 240 min, respectively, the mortality rates were 15.26, 18.86, 23.80, and 36.00 %, respectively. At the concentration of 50 µg/ml after the same time, these values were 18,46, 23.80, 27.20, and 37.40 %, respectively. While after the same time and at a concentration of 100 µg/ml, the values were 24.00, 29.46, 37.00, and 53.40%, respectively (Table 1 & Fig. 2). When protoscoleces were exposed to chitosan Cs2 with DDA 82.71% at the concentration of 20 µg/ml after 30, 60, 120, and 240 min, respectively, the mortality rates were 20.86, 26.26, 32.93, and 44.26 %, respectively.

Table 1: The mortality numbers after exposure to the diff. samples of the current chitosan at diff. concent. for diff. times.

Chitosan (Cs)	Concentration	Mortality numbers after different exposure times			
		30 min	60 min	120 min	240 min
Cs1	20 ug/ml	76.33 ± 10.40	94.33 ± 7.02	119.00 ± 6.00	180.00 ± 7.21
	50 ug/ml	92.33 ± 3.51	119.00 ± 3.60	136.00 ± 2.64	187.00 ± 6.24
	100 ug/ml	120.00 ± 12.00	147.33 ± 8.32	185.00 ± 10.81	267.00 ± 10.44
Cs2	20 ug/ml	104.33 ± 5.50	131.33 ± 8.32	164.66 ± 4.72	221.33 ± 7.76
	50 ug/ml	125.66 ± 7.02	155.66 ± 8.08	183.66 ± 7.09	233.00 ± 6.55
	100 ug/ml	144.66 ± 8.32	175.33 ± 8.02	209.00 ± 7.00	293.00 ± 9.16
Cs3	20 ug/ml	137.33 ± 11.59	169.66 ± 8.50	207.00 ± 7.21	291.66 ± 8.50
	50 ug/ml	174.66 ± 22.72	190.00 ± 9.16	225.00 ± 6.24	311.66 ± 7.63
	100 ug/ml	193.00 ± 8.18	212.66 ± 13.05	247.00 ± 13.22	383.66 ± 2.98
Positive control	—	9.88 ± 0.50	14.49 ± 1.73	20.33 ± 0.66	60.11 ± 6.61
Negative control	—	18.22 ± 3.56	25.33 ± 6.35	44 ± 3.51	79.55 ± 7.16

At the concentration of 50 µg/ml after the same times, these values were 25.13, 31.13, 36.73, and 46.60 %, respectively. Whereas, at the concentration of 100 µg/ml after the same time, the results were 28.93, 35.06, 41.80, and 58.60 %, respectively (Table 1 & Fig. 2).

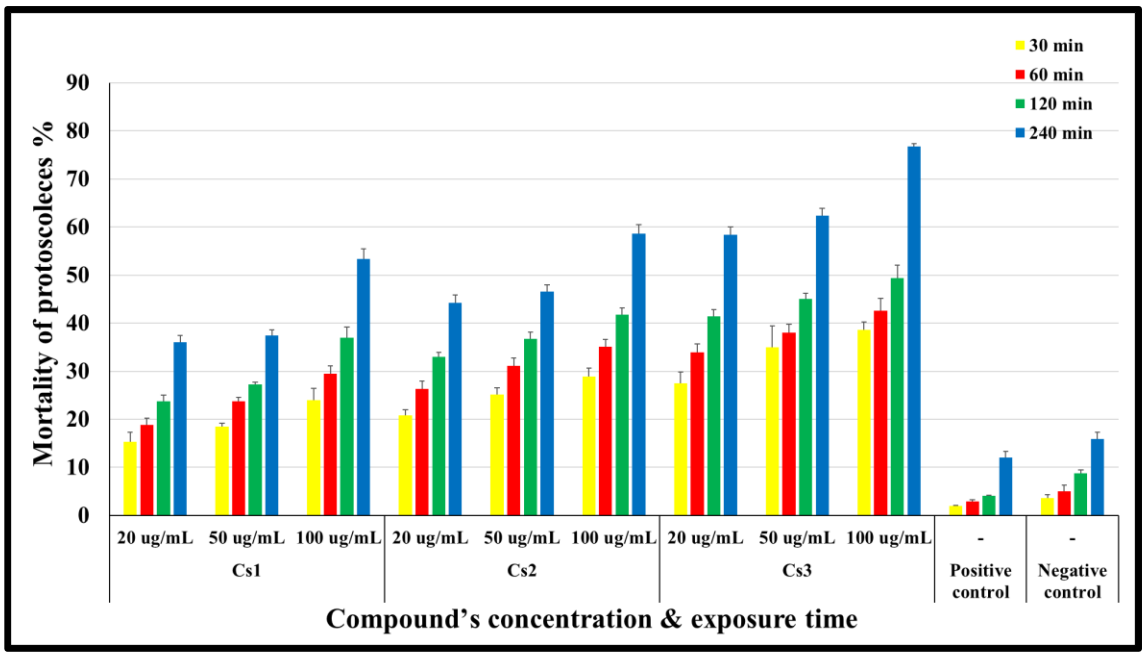

Fig. 2: Graph showing the effect of the present chitosan samples at diff. concent.: 20, 50, and 100 mg/ml on the mortality rates of protoscoleces of *Echinococcus granulosus* from camels after exposure times for 30, 60, 120, and 240 min in comparison with the positive and negative controls.

In chitosan sample Cs3 which had a DDA of 95.10 %, the death rates at the concentration of 20 µg/ml after the same time, were 27.46, 33.93, 41.40, and 58.33 %, respectively. At the concentration of 50 µg/ml after the same time, the death rates were 34.93, 38.00, 45.00, and 62.33 %. Whereas the mortality rates at the concentration of 100 µg/ml were 38.60, 42.53, 49.40 and 76.73 %, respectively at the same time intervals (Table 1 & Fig. 3). In comparison to the positive control group, the differences between the scolicidal effects of various chitosan samples were significant ($P < 0.05$) at all concentrations.

For the same exposure times, the mortality rates in the positive control group were 1.97, 2.86, 4.06, and 12.02%, respectively (Table 1 & Fig. 2). In the current study, it was noticed that formation of a film surrounding some protoscoleces**,** which may be affected on feeding and causing the biomaterial inaccessible for survival indirectly in agreement with the results obtained by Lopez-Moya *et al.* (2019) who found the same effect of chitosan against some fungi**.**

It was noteworthy in the current study that the mortality rates of protoscoleces were considerably significantly increased ($P < 0.05$) when the concentrations of the chitosan sample and exposure periods were increased. These results agreed with the results obtained by Fakhar *et al.* (2015) who stated that increasing the exposure time and concentration of chitosan led to better results in increasing the death rates of protoscoleces. According to Kaya *et al.* (2016), scorpion's chitosan has a greater antibacterial action than commercial chitosan, which made it a target for the current study.

According to the current findings, the mortality rates of protoscoleces were directly correlated with DDA%, which was consistent with the findings of Fakhar *et al.* (2015), who recorded that raising DDA to 65.1% resulted in higher scolicidal activity. Rahimi-Esboei *et al.* (2013) showed encouraging outcomes for the scolicidal effectiveness of both fungus cell walls and commercial chitosan, with 100% scolicidal activity after 180 min exposure time at 400 µg/ml concentration**.** Despite substantial research being done on

chitosan's biological activity, little is known about its antiparasitic abilities (Fakhar *et al.*, 2015).

4-*In vitro* Scolicidal Effect of Chitosan Nanoparticles:

The chitosan nanoparticles: Cs-NPs1, Cs-NPs2, and Cs-NPs3 were physically obtained by the ball-milling method for 12 hrs and had diameters less than 100 nm, as confirmed by TEM and DLS in the prior study **(**El-Sheikh *et al*, 2022). DDA of the present chitosan nanoparticles was calculated using FT-IR, and the results were 72.47, 84.18, and 96.11%, for Cs-NPs1, Cs-NPs2, and Cs-NPs3, respectively. The current results confirmed that the ball-milling process was the way to create chitosan in nano size, without altering the functional group properties, in agreement with the results carried out by Taher *et al.* (2019) and Sari *et al.* (2019). The present chitosan nanoparticles: Cs-NPs that had been physically manufactured were assessed the extent of its effect for the first time all over the world on the viability of protoscoleces of *Echinococcus granulosus*. The current Cs-NPs1 at concentration 20 µg/ml after 30, 60, 120 and 240 min, showed mortality rates of 25.13, 29.73, 34.40 and 45.60 %, respectively. While at the concentration of 50 µg/ml after the same exposure times, the death rates were 30.26, 35.73, 40.73 and 54.13 %, respectively. At the concentration of 100 µg/ml, the unviability rates were 35.86, 41.33, 48.40 and 66.20 %, respectively at the same time intervals (Table 2 and Fig. 3).

Table 2: The mortality numbers after exposure to the diff. samples of the current chitosan nanoparticles samples at diff. concent. for diff. times**.**

Chitosan nanoparticles (Cs-NPs)	Concentration	Mortality numbers after exposure times			
		30 min	60 min	120 min	240 min
Cs-NPs1	20 ug/ml	**125.66** ± 8.14	**148.66** ± 5.03	**172.00** ± 8.88	228.00 ± 6.55
	50 ug/ml	151.33 ± 8.02	178.66 ± 10.69	203.66 ± 4.72	270.66 ± 7.09
	100 ug/ml	179.33 ± 2.51	206.66 ± 3.78	242.00 ± 2.64	331.00 ± 3.00
Cs-NPs2	20 ug/ml	**136.66** ± 3.51	**168.66** ± 6.50	**186.00** ± 5.29	251.00 ± 6.24
	50 ug/ml	166.00 ± 5.56	195.33 ± 6.65	228.33 ± 13.20	288.66 ± 16.44
	100 ug/ml	189.66 ± 2.51	215.33 ± 8.50	247.66 ± 4.72	384.33 ± 6.80
Cs-NPs3	20 ug/ml	**148.66** ± 7.57	**189.66** ± 12.22	**228.33** ± 8.08	310.66 ± 9.01
	50 ug/ml	190.00 ± 14.42	219.33 ± 4.72	260.66 ± 10.26	339.66 ± 5.68
	100 ug/ml	217.33 ± 12.01	254.66 ± 9.86	329.00 ± 13.52	452.00 ± 7.21
Positive control	—	9.88 ± 0.50	14.49 ± 1.73	20.33 ± 0.66	60.11 ± 6.61
Negative control	—	18.22 ± 3.56	25.33 ± 6.35	44 ± 3.51	79.55 ± 7.16

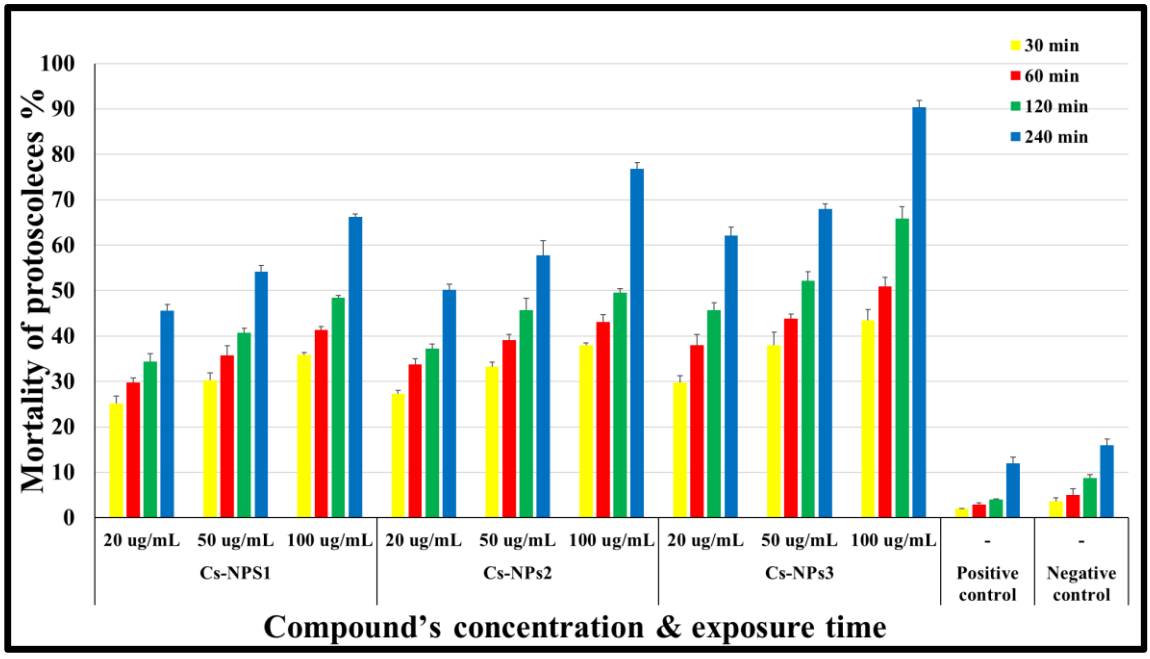

Fig. 3: Graph showing the effect of the present samples of chitosan nanoparticles at diff. concent.:20, 50, and 100 mg/ml on the mortality rates of protoscoleces of *Echinococcus granulosus* cyst recovered from camels after exposure times for 30, 60, 120, and 240 min in comparison with positive and negative controls.

The mortality rates of protoscoleces of *Echinococcus granulosus* after exposure to the present Cs-NPs2 at a concentration of 20 µg/ml after 30, 60, 120, and 240 min were 27.33, 33.73, 37.20 and 50.20 %, respectively. Whereas the mortality rates were 33.20, 39.06, 45.66, and 57.73 %, respectively, at a concentration of 50 µg/ml. At the same exposure times for Cs-NPs2, the mortality rates at the concentration of 100 µg/ml were 37.93, 43.06, 49.53 and 76.86 %, respectively (Table 2 and Fig. 3). The current Cs-NPs3 induced the highest unviability rates of protoscoleces than in all samples of chitosan nanoparticles at the same time intervals. They were 29.73, 37.94, 45.66 and 62.13 %, respectively at the concentration of 20 µg/ml, while at the concentration of 50 µg/ml, the unviability rates were 38.00, 43.86, 52.13 and 67.93 %, respectively. When protoscoleces were exposed to Cs-NPs3 at the concentration of 100 µg/ml, the unviability rates were 43.46, 50.93, 65.80 and 90.40 %, respectively (Table 2 and Fig. 3). The mortality rates of the current protoscoleces were significantly increased by the increase of the concentration and exposure time of chitosan nanoparticles ($P < 0.05$). In this investigation, it was also observed that the effect of chitosan nanoparticles was more significant than that of chitosan with nearly the same DDA%, proving that nano-size had a substantial impact on the effectiveness of different compounds. In this study, the mortality rates of protoscoleces were inversely correlated with size and directly proportionate to DDA%. Taher *et al.* (2019) suggested that Cs-NPs may have an effect on protein or DNA synthesis in breast cancer cell lines.

Torabi *et al.* (2018) and Firouzeh *et al.* (2021) investigated the efficacy of chitosan nanoparticles chemically prepared on protoscoleces of hydatid cysts of *Echinococcus granulosus* and indicated that chitosan nanoparticles of various concentrations had a significant scolicidal effect. These results matched the present results. They added that, after an investigation using scanning electron microscopy, both the length and width of treated protoscoleces were significantly reduced in comparison to that of the control group.

According to Firouzeh *et al.* (2021), chitosan and its forms: nano chitosan, solution, threads, as well as films are thought of as innovative compositions and have been used in a variety of applications because of their special properties. Numerous experimental research has been done employing this naturally occurring linear biopolyaminosaccharide as a bactericidal, fungicidal, and virucidal agent against a variety of infections (Firouzeh *et al.*, 2021). Norouzi (2017) and Norouzi *et al.* (2020) suggested that chitosan nanoparticles chemically created have been shown to have scolecidal effects against the protoscoleces of *E. granulosus* in time-and-dose-dependents. Mikami *et al.* (2013) suggested that the main mechanisms identified by gold nanoparticles in the biological response are the production of oxidative stress and DNA damage induction, cell cycle effect, and potential interference. The current results demonstrated that chitosan nanoparticles had a potent scolicidal action and can be considered an alternate scolicidal agent.

5-*In vitro* Scolicidal Effect of Scorpion's Venom:

The present results demonstrated extremely significant scolecidal effects ($P < 0.05$) against protoscoleces of *E. granulosus* at various concentrations of scorpion's crude venom (V), in comparison to the positive control group having the same concentration and exposure period (Table 3 & Fig. 4). The mortality rates of protoscoleces of *E. granulosus* at concentration 20 µg/ml, after exposure to V, for 30, 60, 120, and 240 min were 32.86, 40.60, 48.26 and 65.60 %, respectively. Whereas, at the concentration of 50 µg/ml, the mortality rates after the same exposure times to V were 40.46, 47.40, 54.06 and 73.26 %, respectively. After the same exposure times to V, at a concentration of 100 µg/ml the mortality rates were 50.13, 53.73, 68.93 and 91.40 %, respectively (Table 3 & Fig. 4).

Table 3: The mortality numbers after exposure to the present crude venom at diff. concent. for diff. times.

Crude venom	Concentration	Mortality numbers after exposure times			
		30 min	60 min	120 min	240 min
V	20 ug/ml	**164.33** ± 9.29	**203.00** ± 9.53	**241.33** ± 6.02	328.00 ± 3.60
	50 ug/ml	202.33 ± 10.26	237.00 ± 7.93	270.33 ± 7.50	366.33 ± 6.65
	100 ug/ml	250.66 ± 9.50	268.66 ± 11.67	344.66 ± 9.45	457.00 ± 8.54
Positive control	—	9.88 ± 0.50	14.49 ± 1.73	20.33 ± 0.66	60.11 ± 6.61
Negative control	—	18.22 ± 3.56	25.33 ± 6.35	44 ± 3.51	79.55 ± 7.16

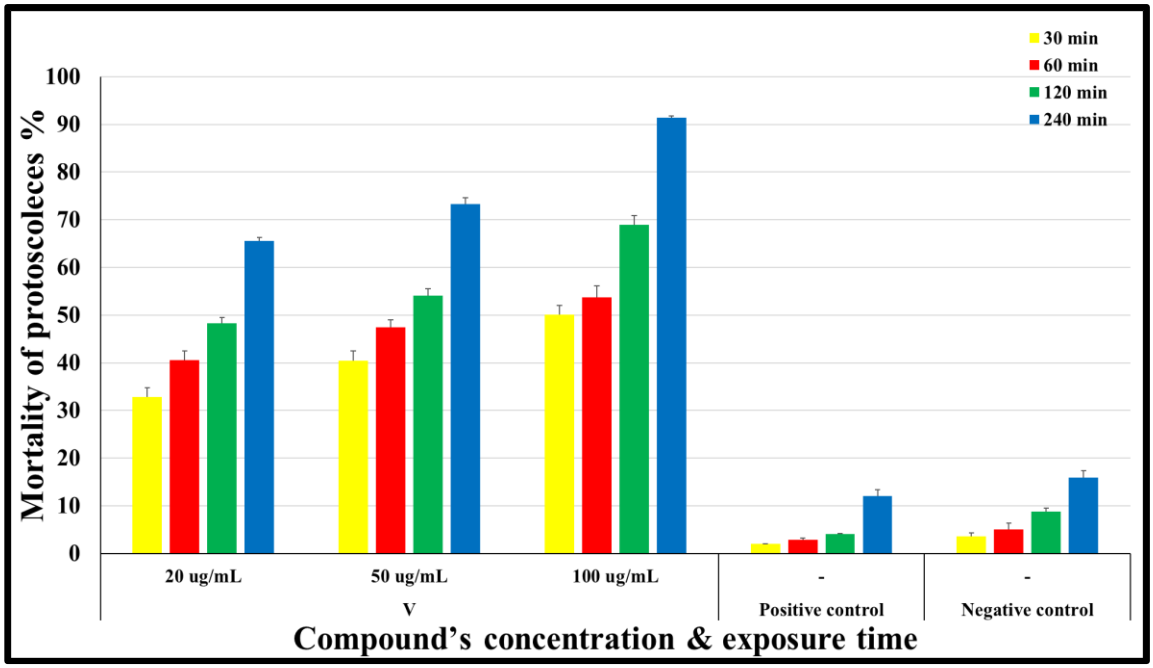

Fig. 4: Graph showing the effect of diff. concent. (20, 50, and 100 mg/ml) of scorpion's venom samples on the mortality rates of protoscoleces of *Echinococcus granulosus* recovered from camels after exposure times for 30, 60, 120, and 240 min in comparison with positive and negative controls.

The present findings showed that the fatality rates significantly increased between 30 and 240 min of exposure ($P < 0.05$).

According to Alajmi *et al.* (2020), scorpion's venom is a valuable source of active chemicals, including several polypeptides, making it crucial in a variety of biological applications. Indeed, polypeptides (which block sodium, potassium, or calcium-gated channels) as well as enzymes are the two most significant substances in scorpion's venom (Al-Malki and Abdelsater, 2020). Additionally, a few compounds such as phospholipase A2, lysozyme C, and hyaluronidase exhibit enzymatic characteristics (Al-Malki and Abdelsater, 2020). Many dangerous diseases have previously been treated by interrupting or blocking gated ion channel activities, and scorpion's venom is also acknowledged as an effective biological antibacterial treatment (Al-Malki *et al.*, 2022). According to Jafari *et al.* (2019), the effect of *Mesobuthus eupeus* scorpion's venom on protoscoleces of *Echinococcus granulosus* considerably increased from 30 to 240 min of exposure ($P < 0.05$), which was consistent with the current findings. Al-Malki and Abdelsater (2020) concluded that the scolicidal activity of crude venom of *Androctonus crassicauda* scorpion at the concentration of 20 µg/ml after application for 30, 60, 120, and 240 min, was 25.1, 31.2, 36.5 and 57.1%, respectively, while at the concentration of 50 µg/ml after application for 30, 60, 120, and 240 min, the venom activity was 28.6, 39.5, 65.4, and 80.6%, respectively, moreover, at the concentration of 100 µg/ml, the scolicidal activity of crude venom was 34.9, 71.4, 95.8 and 100 % of protoscoleces after 30, 60, 120, and 240 min, which was very similar to the present results. Al-Malki *et al.* (2022) found that the crude venom of *Androctonus crassicauda* scorpion caused apoptosis and structural alterations in protoscoleces of *Echinococcus granulosus* and showed that the expression of caspase-3 was significantly higher in the protoscoleces incubated at 100 µg/ml than 50 µg/ml.

6-*In vitro* Scolicidal Effect of Venom-Loaded Chitosan Nanoparticles:

Venom-loaded chitosan nanoparticles: V-CN1, V-CN2 and V-CN3 were previously prepared via the ionic gelation method and had sizes less than 300 nm which

was confirmed by TEM and DLS in the prior study **(**El-Sheikh *et al,* 2022). In the present investigation, venom-loaded chitosan nanoparticles (V-CN) were used as scolecidal agents. The results of V-CN1 at a concentration of 20 µg/ml after 30, 60, 120, and 240 min, showed that the mortality rates in protoscoleces of *Echinococcus granulosus* were 37.33, 45.06, 52.66 and 68.60 %, respectively. Whereas at concentrations of 50 µg/ml after 30, 60, 120, and 240 min, the death rates were 44.86, 52.06, 59.86 and 80.40 %, respectively. The unviability rates at the concentration of 100 µg/ml were 55.00, 64.13, 78.80, and 94.46%, after 30, 60, 120, and 240 min, respectively (Table 4 & Fig. 5).

Table 4: The mortality numbers after exposure to the diff. samples of the current venom-loaded chitosan nanoparticles at diff. concent. for diff. times.

Venom-loaded chitosan nanoparticles (V-CN)	Concentration	Mortality numbers after exposure times			
		30 min	60 min	120 min	240 min
V-CN1	20 ug/ml	186.66 ± 4.93	225.33 ± 9.01	263.33 ± 9.29	343.00 ± 6.24
	50 ug/ml	224.33 ± 10.78	260.33 ± 9.07	299.33 ± 8.50	402.00 ± 9.16
	100 ug/ml	275.00 ± 3.60	320.66 ± 8.50	394.00 ± 7.00	472.33 ± 4.16
V-CN2	20 ug/ml	230.00 ± 10.14	254.33 ± 5.13	280.00 ± 8.18	370.00 ± 11.13
	50 ug/ml	265.66 ± 7.50	295.00 ± 8.54	324.00 ± 11.35	421.00 ± 5.56
	100 ug/ml	331.00 ± 6.55	375.66 ± 8.02	411.66 ± 9.71	482.66 ± 1.52
V-CN3	20 ug/ml	239.66 ± 7.094	271.33 ± 3.78	335.66 ± 8.08	413.33 ± 12.34
	50 ug/ml	341.33 ± 14.18	364.33 ± 16.80	412.33 ± 9.07	485.00 ± 4.35
	100 ug/ml	365.66 ± 11.93	412.33 ± 10.78	444.66 ± 6.02	500.00 ± 0.00
Positive control	—	9.88 ± 0.50	14.49 ± 1.73	20.33 ± 0.66	60.11 ± 6.61
Negative control	—	18.22 ± 3.56	25.33 ± 6.35	44 ± 3.51	79.55 ± 7.16

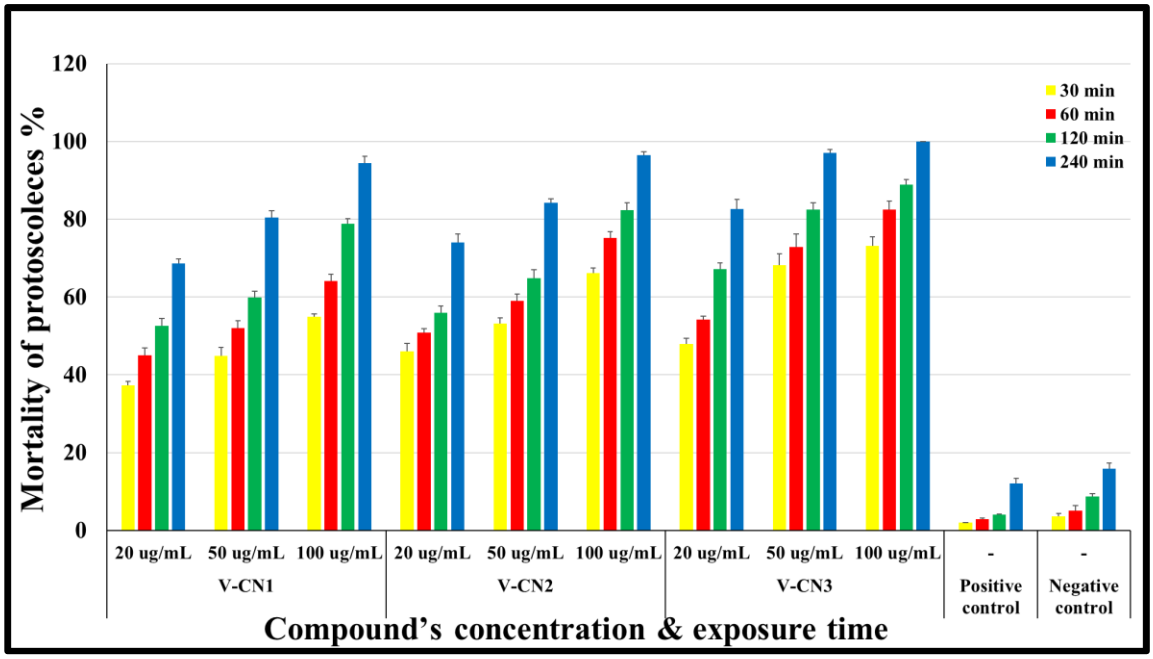

Fig. 5: Graph showing the effect of the present venom-loaded chitosan nanoparticles at diff. concent. (20, 50, and 100 mg/ml) on the mortality rates of protoscoleces of *Echinococcus granulosus* recovered from camels after exposure times for 30, 60, 120, and 240 min in comparison with positive and negative controls.

The mortality rates after exposure to V-CN2 at a concentration of 20 µg/ml after 30, 60, 120, and 240 min were 46.00, 50.86, 56.00 and 74.00 %, respectively. While, at a concentration of 50 µg/ml, the mortality rates were 53.13, 59.00, 64.80 and 84.20 %, respectively. At a concentration of 100 µg/ml after the same exposure times for V-CN2, the mortality rates were 66.20, 75.13, 82.33 and 96.53 %, respectively (Table 4 & Fig. 5).

In this study, the unviability rates of protoscoleces caused by V-CN3 were the highest than that caused by the other samples of venom-loaded chitosan nanoparticles, and accordingly, than that caused by all compounds at the same concentrations, after the same exposure time intervals. The mortality rates were 47.93, 54.26, 67.13 and 82.66 % at the concentration of 20 µg/ml after the same exposure times. While at the concentration of 50 µg/ml, the unviability rates were 68.26, 72.86, 82.46 and 97.00 % after the same exposure times. When protoscoleces were exposed to V-CN3 at the concentration of 100 µg/ml after the same exposure times, the unviability rates were 73.13, 82.46, 88.93 and 100 %, respectively (Table 4 & Fig. 5).

The present results indicated that V-CN had the highest scolicidal activity and was suitable for being used as an antiprotoscoleces compound. The protoscolecidal effects of V-CN compounds seemed to be due to their role in the breakdown of biological activities of protoscoleces through interference with their metabolism. The current chitosan samples used in the preparation of venom-loaded chitosan nanoparticles had a different ascending DDA (V-CN1< V-CN2< V-CN3) which was one of the most important factors for the scolecidal agents. In the present study, it was observed that the effect of V-CN on protoscoleces was directly proportional to the DDA of chitosan samples. Chitosan nanoparticles created a polymeric network that allowed the venom protein to become trapped in the matrix (Mohammadpour Dounighi *et al.,* 2012). Additionally, it appears that the interaction between polymers and venom with opposing charges could result in the formation of a robust matrix polymer network that trapped venom and ultimately reduced venom release in a pH 7.2 condition (Mohammadpour Dounighi *et al.,* 2012).

Venom-loaded chitosan nanoparticles possess numerous benefits and biomedical features that simultaneously integrate the effects of chitosan and venom in nano size and are crucial to the biological properties (Alalawy *et al.,* 2020). Mohammadpour Dounighi *et al.* (2012) concluded that chitosan nanoparticles containing *Hemiscorpius lepturus* scorpion's venom had the potential for antigen delivery. Also, Mohammadpour Dounighi *et al.* (2015) concluded that *Orthochirus iranus* scorpion venom-loaded chitosan nanoparticles could be used for antigen delivery. Whereas Alalawy *et al.* (2020) suggested that the production of bee venom-loaded fungal chitosan nanoparticles is strongly counseled to produce potent natural antitumor agents with augmented activity against cervix carcinoma. Mikami *et al.* (2013) suggested that changes in the microbial tegument are linked to a possible action of the nanoparticle as an inhibitor of protein synthesis.

In this study, mortality rates of protoscoleces for all compounds were significantly increased ($P < 0.05$) by the increase of both concentration of the compounds and exposure time in comparison to the positive and negative control groups (Fig. 6). On one hand, chitosan nanoparticles: Cs-NPs1, Cs-NPs2 and Cs-NPs3 were better in their effect against protoscoleces than chitosan samples: Cs1, Cs2 and Cs3 which had approximately the same DDA, concentration, and exposure time which proved that size was the main and influential factor when comparing them with each other, which made a preference for chitosan nanoparticles side. On the other hand, because of the biological effects as well as the presence of several chemicals in the venom, its scolocidal effects eventually outweighed those generated by chitosan and chitosan nanoparticles. The venom-loaded chitosan nanoparticles, which were considered to simultaneously possess all the influencing properties for all the prepared compounds in DDA, concentration, size, and exposure time, showed the highest protoscoleces fatality rates.

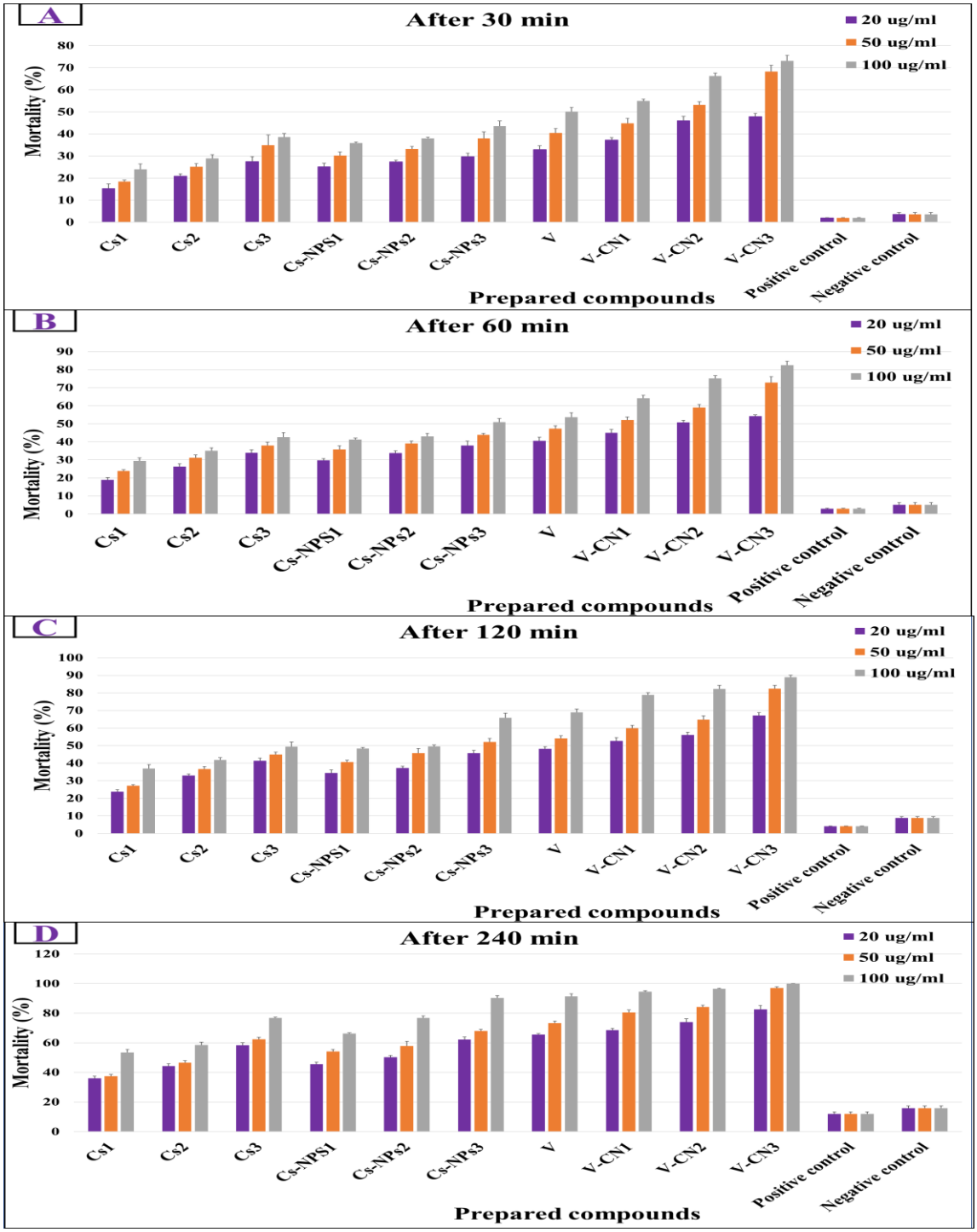

Fig. 6: Graphs showing a comparison between the *in vitro* effect in diff. concent. (20, 50, and 100 mg/ml) of (A) chitosan samples (Cs1, Cs2 and Cs3), (B) chitosan nanoparticles (Cs-NPs1, Cs-NPs2 and Cs-NPs3), (C) crud venom (V) and (D) venom-loaded chitosan nanoparticles (V-CN1, V-CN2 and V-CN3) on the mortality rate of protoscoleces of *Echinococcus granulosus* recovered from camels after exposure times for 30, 60, 120, and 240 min in comparing with positive and negative controls.

Conclusion and Recommendations:

All compounds displayed statistically significant variations in the protoscolecidal efficacy of *Echinococcus granulosus* recovered from camels in various doses ($P > 0.05$). Concentration, DDA, size, and exposure duration were found to be significant

scolecidality factors. With the increase of chitosan deacetylation degree, the scolocidal activity increased. Chitosan nanoparticles had a more scolicidal effect than chitosan for the same features. Venom from *Leiurus quinquestriatus* substantially elevated mortality in protoscoleces of hydatid cysts of *Echinococcus granulosus* at all doses. Venom-loaded chitosan nanoparticles had the highest effect on *E. granulosus* larvae *in vitro* setting.

On one hand, there is a need to identify, study and evaluate the biological activities of each bioactive component of *Leiurus quinquestriatus* venom *in vitro*. On the other hand, before the clinical application, further studies are warranted to explore the biologically safe dose of venom-loaded chitosan nanoparticles and to evaluate their biochemical and biological effects *in vivo*.

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