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Original article

# Scorpion crude venom induced apoptosis and structural changes of *Echinococcus granulosus* protoscolices

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## ABSTRACT

*Echinococcus granulosus* parasitic infection in humans causes hydatidosis (also known as echinococcosis), which is a severe zoonotic illness that puts public health at risk.

This research was designed to assess the status of morphological, ultrastructure, and cell apoptosis of *E. granulosus* protoscolices. For this purpose, the protoscolices were isolated and collected from the hydatid cysts in the lungs and livers of camels, then incubated for 4 h in *Androctonus crassicauda* crude venom (ACCV) (at the concentrations of 50 and 100 µg/ml). The changes of *E. granulosus* protoscolices were monitored using light, transmission, and scanning electron microscopy, and the caspase-3 expression was detected by immunohistochemistry. The current study results have demonstrated the caspase-3 expression was substantially higher in the protoscolices incubated at 100 µg/ml than 50 µg/ml. Also, ACCV caused distinct changes in the morphology and ultrastructure of protoscolices detected by light, TEM, and SEM microscopy. In conclusion, *Androctonus crassicauda* crude venom (ACCV) may provide an alternative and nonsurgical treatment for hydatidosis. Hence, further studies are recommended to explore the impact of ACCV on inducing apoptosis and structural changes for this parasitic disease.

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## 1. Introduction

Zoonotic parasitic illness is known as CE (“Cystic Echinococcosis”) produced by the larval stage of *E. granulosus*. It is endemic and has significant economic and health impacts in various regions of the world, such as Egypt and the Middle East (El-Shazly et al., 2007; Sadjjadi, 2006; Al-Malki et al., 2017; Abdel-Baki et al., 2018; El-Meleh et al., 2019; Abdelbaset et al., 2021). Definitive hosts are dogs and other canids, while herbivores act as intermediate hosts and infecting them by ingesting the parasite's eggs excreted in the feces of the definitive host. Humans get infected when they ingest *E. granulosus* eggs by accident (Sarkari and Rezaei, 2015).

Hydatid cysts are primarily found in the liver (50% to 80%) and lungs (5% to 30%). The cysts also were identified in the central nervous system, bones, heart, kidney, spleen, and other organs, although with less frequency (Sayek et al., 1980). Protoscolices are microscopic larvae inside the cysts that may grow into adult worms in the final host intestine or secondary hydatid cysts in the intermediate host viscera (Sharafi et al., 2017).

There were four treatment choices for hydatid disease: percutaneous aspiration, medical treatment, surgery, and watch & wait (Brunetti et al., 2010). Surgery remains the most efficient and widely used treatment modality. However, recurrence is often caused by the spread of protoscolices-rich fluid during surgery. Secondary disseminated intraperitoneal hydatidosis may result from operative spillage (Rajabi, 2009). The most common method of preventing this severe consequence is to instill a scolicidal agent into a hepatic hydatid cyst (Tozar et al., 2005). Till now, various scolicidal agents, including plant extracts, Albendazole, hypertonic saline, hypertonic glucose, ethyl alcohol, silver, and selenium nanoparticles were tried with various successes (Rahimi et al., 2015; Mahmoudvand et al., 2014; Moazeni and Roozitalab, 2012; Moazeni and Nazer, 2010; Hosseini et al., 2006; Kayaalp et al., 2001; Besim et al., 1998; Pérez-Serrano et al., 1994). Unfortunately,

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most of these scolical agents may cause undesirable side effects. Thus, their usage is restricted (Moazeni and Alipour-Chaharmahali, 2011). Thus, finding new scolical agents with fewer side effects, low cost, and high efficacy urgently needed surgeons (Adas et al., 2009).

Venomous animals, including scorpions, are new candidates for future drugs and can play as new approaches with fewer side effects. Literature showed that the snake and scorpion venoms have significant effects on some life-threatening human parasites, including *Plasmodium*, *Leishmania*, and *Trypanosoma* (Jafari et al., 2019). Many pharmacological peptides are found in scorpion venoms, attracting the interest of scientists in the domain of drug development. (Perumal et al., 2017). The scorpion species *A. crassicauda* (Olivier, 1807) (the Arabian fat-tailed scorpion) is one of the most dangerous species of the family Buthidae with medical significance in Saudi Arabia and the Middle East. Although *A. crassicauda* causes the most human mortalities, its venom components are well known among many other scorpion species. The composition and function of *A. crassicauda* venom are still relatively limited; however, the first molecular and biochemical analysis of the venom components was reported by (Caliskan et al., 2006). The Antimicrobial Peptides extracted from *A. crassicauda* venom revealed an inhibitory effect against gram-negative as well as gram-positive bacteria (Du et al., 2014). Apoptosis, as a kind of cell death initiated with external aspects and eventually results in the cell's self-destruction offers an effective method to killing the parasitic *E. granulosus*. Protoscolices are important component of the parasite and share many identical or similar cell structures (Wang et al., 1994; Morsth, 1967).

The main objective of current research is to detect the possible effects of ACCV on the structure and ultrastructure of *E. granulosus* protoscolices. This study also was planned to reveal the possibility of ACCV impact in inducing apoptosis for the *Echinococcus granulosus* protoscolices.

## 2. Materials & methods

### 2.1. Scorpion crude venom collection

One hundred *A. crassicauda* individuals (Olivier, 1807) scorpion species were collected from Wadi Feiran, southern Sinai, Egypt. The gathered scorpions were maintained individually in plastic containers at 25 °C in the Parasitology Lab, Zoology Department, Faculty of Science, "Al-Azhar University" Assuit branch. Electrical stimulation (20 V) in the telson articulation collected venom as reported by (Sarhan et al., 2012; Alajmi et al., 2020). Purified venom droplets were collected in an Eppendorf tube as well as centrifuged for 15 min at 14,000 rpm at 4 °C. The supernatant was collected, freeze-dried, and kept at -20 °C. The lyophilized specimens were dissolved in purified water and centrifuged at 4 °C for 15 min at 15,000 rpm.

### 2.2. Protoscolices selection and viability analysis

*E. granulosus* Hydatid cysts were obtained from livers and lungs of camels killed at a governmental slaughterhouse in Assiut. The obtained specimens were instantly sent to the Parasitology Lab, Zoology Department, Faculty of Science, "Al-Azhar University", Assiut branch, Egypt. The hydatid fluid was extracted using a sterile syringe and set aside for 45 min before precipitating in a sterile falcon tube. The gathered protoscolices were cleaned thrice with normal saline, and the feasibility of protoscolices was evaluated by 0.1 percent eosin stain. The motility features and muscular motions were observed by light microscopy. In subsequent exper-

iments, protoscolices with viability of more than 90 percent were chosen (Smyth and Barret, 1980) Fig. 1.

### 2.3. Crude venom impact on protoscolices

The viable protoscolices specimens were washed by sterile saline, cultured in sterile RPMI 1640 medium, and split into three groups; one negative control group and two (50 and 100 µg/mL) scorpion venom treated groups (The sterile RPMI 1640 medium was prepared by adding 100 mg/mL streptomycin and 100 IU penicillin). For each, 2.5 ml of the medium was added to a test tube, around  $5 \times 10^3$  protoscolices was then placed to the tube and gently mixed and incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 4hrs (Al-Malki and Abdelsater, 2020).

### 2.4. Light microscopy

Both control and ACCV incubated protoscolices were fixed in 10 percent formalin, treated to paraffin blocks, sectioned, dehydrated, stained by H&E ("Hematoxylin and Eosin"), and assessed by light microscope (Alam-Eldin and Badawy, 2015).

### 2.5. Transmission electron microscopy

TEM was utilized to analyze the ultrastructural changes in both control and ACCV-incubated protoscolices. Parasite specimens were kept in a 0.1 cacodylate solution containing 2.5 percent (v/v) glutaraldehyde for 1 h at pH 7.2. PBS was used to clean the fixed specimens two times before using 1 percent (v/v) osmium tetroxide in 0.1 M cacodylate buffer for 30mins at 7.2pH and room temperature post-fix the samples. They were dehydrated with rising ethanol levels. Propylene oxide was used to dehydrate the samples entirely before they were embedded in Epoxy Resin 812. An ultra-microtome was used to cut thin sections, then put on copper grids and stained using uranyl acetate and lead citrate. Sections were studied under the TEM JEOL JEM-1200 EX II. (Tawfik, 2018).

### 2.6. Scanning electron microscopy

Specimens were fixed for 24 hrs at 4 °C with 3 percent glutaraldehyde in sodium cacodylate buffer, washed several times in cacodylate buffer, and then dehydrated with rising concentrations of ethanol (50 to 100%) before being immersed in hexamethyldisilazane for a 1 h and overnight. The samples were sputter-covered with gold (100 Å thick) and examined on a JEOL JSM-6460LV scanning electron microscopy at 15KV (Elisondo et al., 2006).

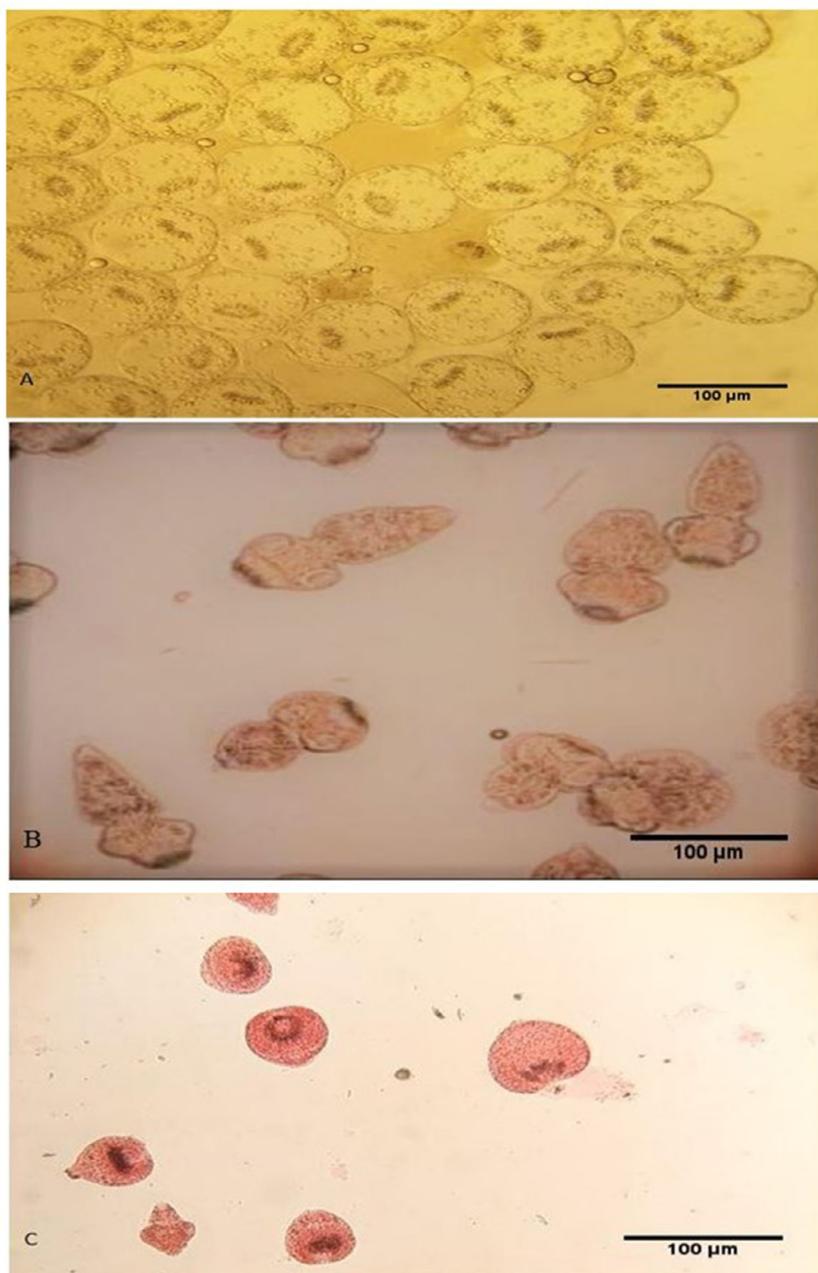
### 2.7. Immunohistochemistry

Caspase-3 antibodies were diluted 1:300, and Sections were exposed to a high temperature, high-pressure antigen extraction, and staining procedure according to the kit instructions (Dako). The slides were colored with DAB, counterstained with hematoxylin, and examined under a light microscope. Cytoplasm brownish staining (may comprise nuclei) was seen as caspase-3 positive (Hu et al., 2011).

## 3. Results

### 3.1. Morphological changes by light microscopy

The calcareous corpuscles were bright and clear with clear hooks and suction cups, and the control protoscolices were clear and undamaged. The specimens incubated in 50 µg/ml ACCV



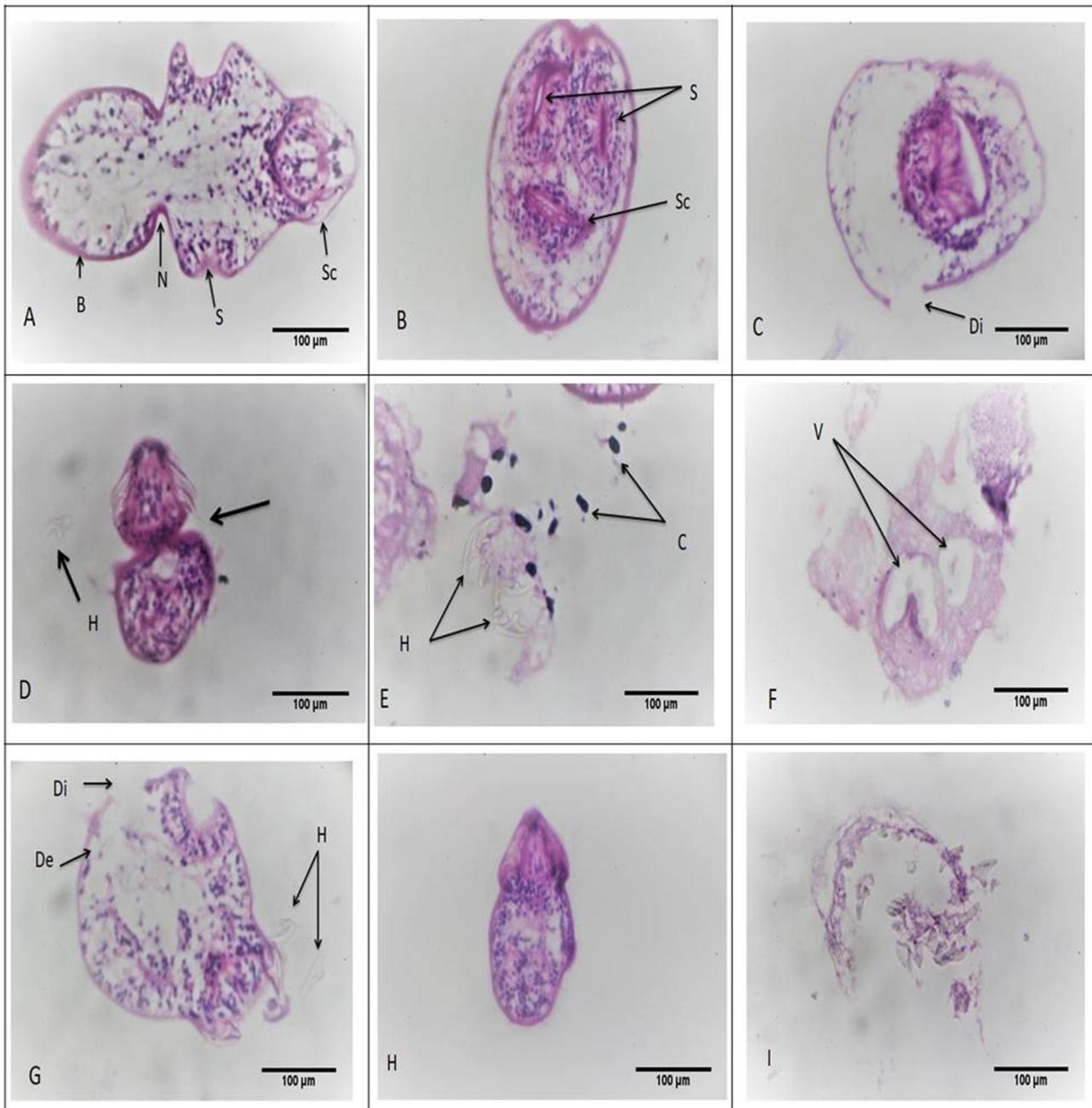
**Fig. 1.** Live (control) unstained protoscolices (A), live (control) protoscolices after staining with 0.1 percent eosin (B), dead protoscolices after treatment with ACCV and staining with 0.1 percent eosin (C). Scale-bar = 100  $\mu\text{m}$ .

showed: cuticle discontinuation, loss of hooks & suction cups, free hooks and calcareous corpuscles, and vacuole formation with deformity. Whereas those incubated in 100  $\mu\text{g}/\text{ml}$  ACCV showed: cuticle detachment and discontinuation, hooks loss, shrunken with deformity, and some were completely lost the integrity and shape (Fig. 2).

### 3.2. Ultrastructural changes by TEM

Control protoscolices contain distinctive germinal layer (GL) with numerous cell types (e.g., round or oval internal parenchymal cells with clear along with large nuclei and nucleoli at the center). The nuclei chromatin were fine & smooth and contained a small quantity of heterochromatin. Also, the control protoscolices showed a tegument syncytium with aligned microtriches coated with a thick layer of PAS ("Periodic Acid-Schiff") positive

material. Protoscolices incubated with ACCV showed apoptotic changes; these changes were patchy in protoscolices incubated with 50  $\mu\text{g}/\text{ml}$  ACCV and generalized in those incubated with 100  $\mu\text{g}/\text{ml}$  ACCV. Moreover, protoscolices incubated with 50  $\mu\text{g}/\text{ml}$  ACCV showed a start loss of the PAS-positive substance outside the tegument microtriches and vacuolation and the occurrence of lipid droplets in their cytoplasm. Whereas the protoscolices incubated with 100  $\mu\text{g}/\text{ml}$  ACCV showed a start loss of the PAS-positive substance and shedding of microtriches, some showed complete loss of PAS, microtriches, and the presence of lipid droplets. There was also increased vacuolation of the distal cytoplasm, compressed cytoplasm in the inside parenchymal cells, apoptotic bodies, heterochromatin mass in the form of the crescent on the nuclear membrane of parenchyma cell, residual lamellar bodies, and the extensive damage of the internal tissue (Fig. 3).



**Fig. 2.** Morphological alterations of protoscolices stained with H&E. (a-b) showed normal intact protoscolices with hooks and suction cups. Sc, scolex region; B, body region; N, neck region; S, sucker region. (c-f) showed protoscolices incubated with 50 µg/ml ACCV; (c) showed cuticle discontinuation (di). (d) showed loss of suction cups (arrow) and loss of hooks (h). (e) showed free hooks (h) and calcareous corpuscles (c). (f) showed vacuoles (v) with deformity. (g-i) showed protoscolices incubated with 100 µg/ml ACCV; (g) revealed cuticle detachment (de), cuticle discontinuation (di) and hooks loss (h). (h) showed shrunken protoscolice with deformity. (i) showed complete loss of integrity and shape (x400).

### 3.3. Ultrastructural changes by SEM

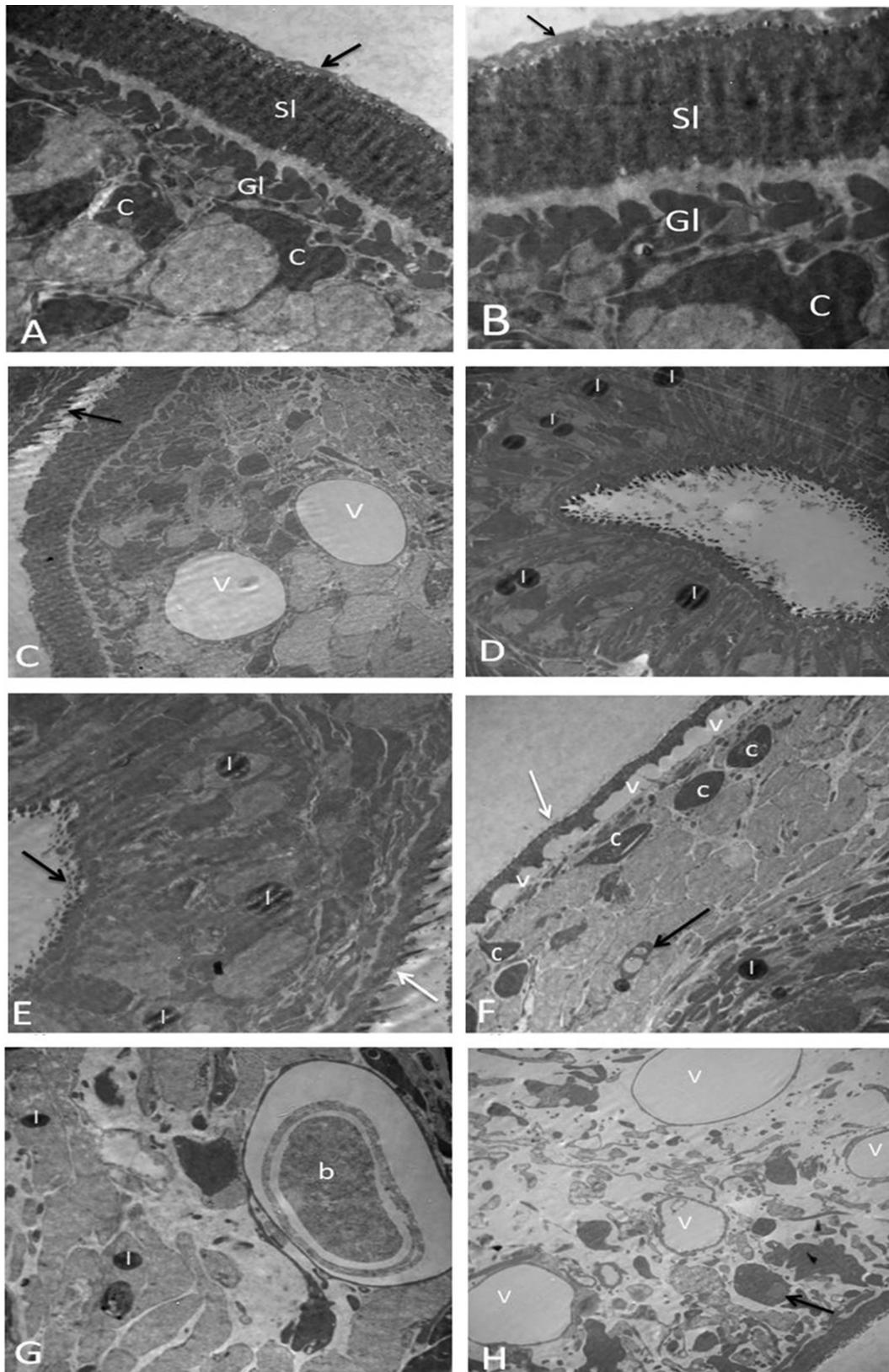
Control protoscolices exhibited no ultrastructural changes during the whole period of incubation. In contrast, the treated protoscolices had ultrastructural damage, with the tegument of the parasite being the main source of damage.

After the 4th hour of treatment with ACCV, protoscolices incubated with 50 µg/ml ACCV showed the existence of tegumental changes and soma region contraction. The ultrastructural changes included rostellar disorganization, hooks loss, and shedding of scolex regions microtriches. Protoscolices incubated with 100 µg/ml

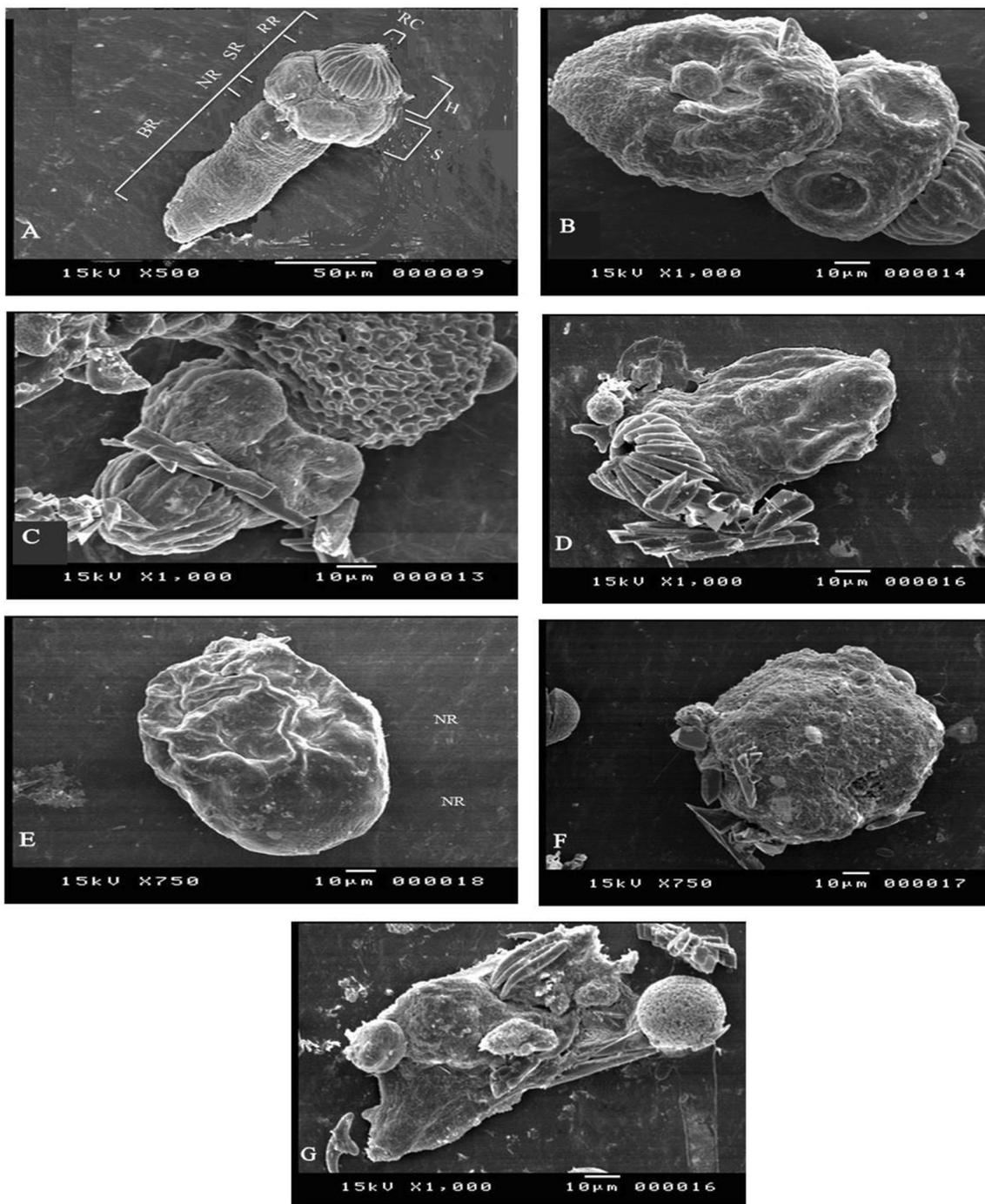
ACCV showed the production of tegumental vesicles. Morphology loss was seen in certain protoscolices, and the bursting of the soma area shows a loss of tegumental integrity, resulting in osmoregulatory damage (Fig. 4).

### 3.4. Immunohistochemistry

Control protoscolices did not show any caspase-3 expression, whereas caspase-3 expression in the protoscolices incubated with ACCV was significantly high. Protoscolices incubated with 50 µg/ml ACCV revealed large areas of brownish staining, while those incu-



**Fig. 3.** Protoscolices by TEM. (A-B) Normal protoscolices with distinctive features like; GI (Germinal layer), which contains numerous cell forms, such as parenchymal cells (c), a syncytial layer of tegument (SI), and PAS-positive material (arrow) (A) (X5810) and (B) (X10000). (C-D) Protoscolices incubated with 50 µg/ml ACCV (C) showed start loss of PAS-positive material (arrow) and vacuolation of cytoplasm (v) (X2900). (D) showed the presence of lipid droplets (L) (X3600). (E-H) Protoscolices incubated with 100 µg/ml ACCV (E) showed start loss of PAS-positive material (white arrow), shedding of microtriches (black arrow), and the presence of lipid droplets (L) (X4810). (F) showed vacuolation of distal cytoplasm (v), condensed cytoplasm of parenchymal cells (c), apoptotic bodies (black arrow), lipid droplets (L), and complete loss of PAS-positive material and microtriches (white arrow) (X3600). (G) showed residual lamellar bodies (b) and the presence of lipid droplets (L) (X4810). (H) showed cytoplasmic vacuolation (v), The nuclear membrane of a parenchyma cell has a crescent-shaped heterochromatin mass (arrow), and extensive damage of the internal tissue (X2900).



**Fig. 4.** Protoscolices by SEM: (A) Evaginated control protoscolices; Body Region (BR); Neck Region (NR); Sucker Region (SR); Rostellar Region (RR); Suckers (S); Hooks (H), Rostellar Cone (RC). (B-D) Protoscolices incubated with ACCV (50 µg/mL) during 4 h of treatment; (B) Presence of tegumental changes, contraction of the soma region and shedding of microtriches (C) Presence of tegumental changes (D) Rostellar disorganization, hooks loss, and microtriches shedding of the scolex region. (E-G) Protoscolices incubated with 100 µg/ml ACCV during 4 h of treatment; (E) Formation of tegumental vesicles, (F) Morphological deformation of protoscolices (G) The loss of tegumental integrity, which leads to osmoregulatory impairment, is shown by the bursting of the soma region.

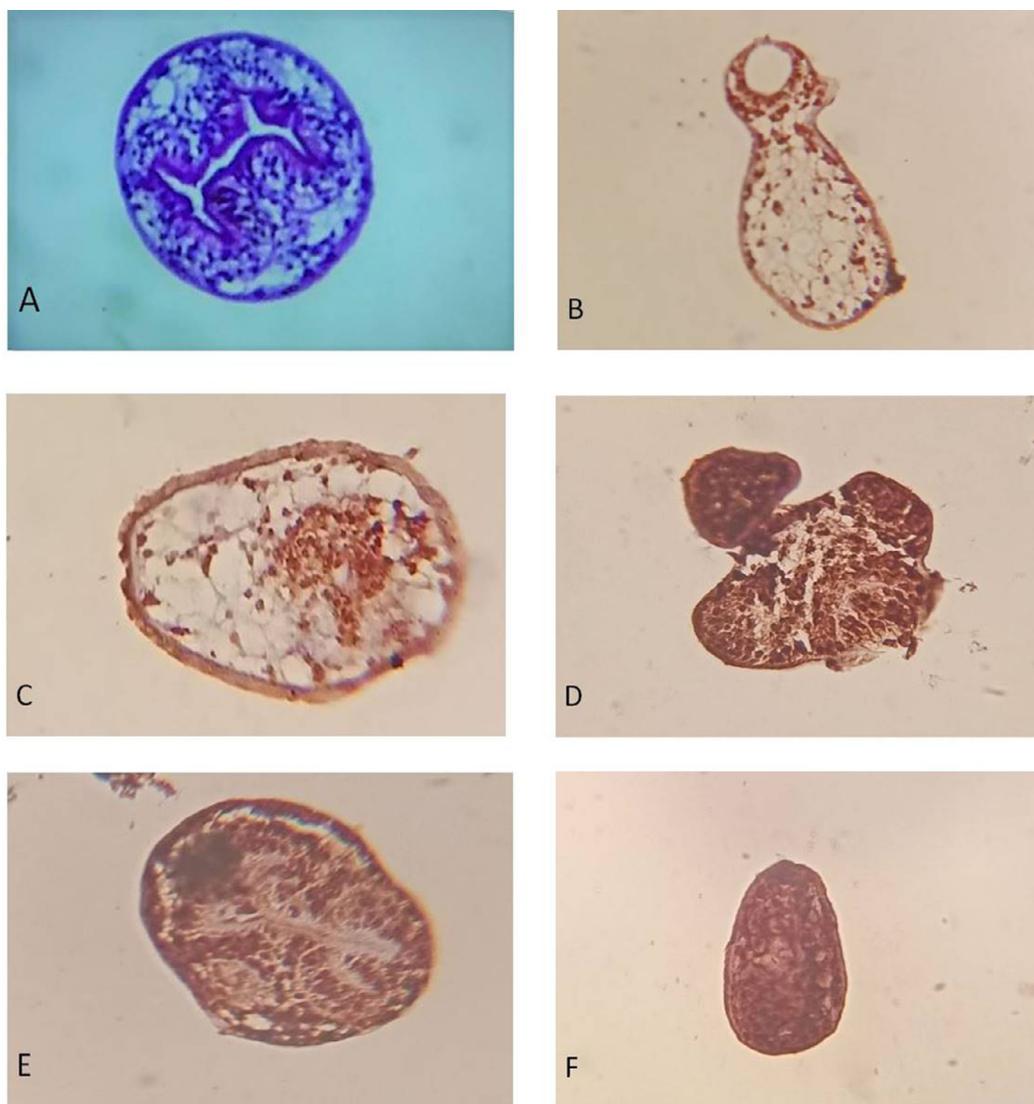
bated in 100 µg/ml ACCV showed complete brownish staining (Fig. 5). However, when the PBS replaced the primary antibodies in the staining procedure, all slides did not show any brownish staining, excluding false-positive.

#### 4. Discussion

Protoscolices are essential targets for therapeutic agents to stop the development of hydatid cysts since they can develop into adult

worms in a definitive host or into new hydatid cysts in an intermediate host (Zou et al., 2009). Numerous scolicalid agents were used to inactivate hydatid cyst contents. However, the majority of these scolicalid agents have many side effects. The optimal scolicalid agent would have fast and comprehensive scolicalid actions, with minimum local or systemic side consequences (Altindis et al., 2004).

Natural venoms have a broad variety of antimicrobial actions against viruses, fungi, bacteria, and parasites, (Bahar and Ren,



**Fig. 5.** Caspase-3 expression in protoscolices using immunohistochemistry. (A) control protoscolices; (B-C) Protoscolices incubated with 50 µg/ml ACCV for 4 h, the results showed few spots stained brownish; (D-F) Protoscolices incubated with 100 µg/ml ACCV for 4 h, the results showed whole protoscolices stained brown (x400).

2013). Biologically, the venoms of scorpions are diverse and have activity because of their defensive and predatory usage in nature (Rodríguez de la Vega and Possani, 2005; Martin-Eauclaire and Couraud, 1995). Borges et al. (2006) found that *Tityus discrepans* crude venom and its components inhibited the development of promastigote forms of *Leishmania spp.* and ultimately leads to parasite mortality. Xu et al. (2008) and El-Asmar et al. (1980) reported that scorpion venom has a cytotoxicity effect on “*Ancylostoma caninum*” as well as *Schistosoma mansoni* cercariae. Some in vitro analyses had demonstrated that some scorpion venoms have anti-Trypanosomal and anti-malarial activities (Perumal et al., 2017). The venom of scorpions contains multiple compounds such as a peptide, proteins, hyaluronidases, lipolysis activating peptides (LVPs), metalloproteases, serine proteases, and phospholipases A2 (Abdel-Rahman et al., 2016; Pessini et al., 2001). Phospholipase A2 degrades the integrity of the cell membrane via hydrolyzing the two-acyl bonds in membrane phospholipids, producing fatty acids and lysophospholipids that induce secondary membrane damage (Habermann, 1972). This enzyme displayed bactericidal activity towards gram-negative enterobacteria and was able to lyse *Trypanosoma brucei brucei* in vitro at a 1 mg/ml concentration (Boutrín et al., 2008). Flores-Solis et al. (2016) examined the impact of two peptides, *Hoffmannihadrurus gertschi* Hge36 and

HgeD, on *Entamoeba histolytica* trophozoites and *Taenia crassiceps* cysticerci. They found that these parasites had considerably decreased while both peptides had little impact on lymphocytes. The ACCV was first revealed experimentally versus *E. granulosus* protoscolices by (Al-Malki and Abdelsater, 2020). They found that *A. crassicauda* crude venom could kill all protoscolices during 240 min.

The findings of the current study revealed that ACCV is not only affected the morphology and ultrastructure of protoscolices but also induces apoptosis. The visible changes of morphology and ultrastructure of protoscolices by light microscopy, SEM, and TEM were consistent with those reported in previous investigations using drugs or other compounds (Zahran et al., 2020; Tawfik, 2018; Pensel et al., 2017; Verma et al., 2013; Hu et al., 2011; Eliassondo et al., 2006, 2008, 2009; Hosseini et al., 2006; Walker et al., 2004). The studies mentioned above confirmed the occurrence of apoptosis and indicated the presence of a CED-3 like apoptotic gene in protoscolices.

Apoptosis was the most common form of eukaryotic cell death (Kerr et al., 1972). Caspase-3 is a cysteine aspartic acid protease that plays a central role in the execution-phase of apoptosis. It is the major terminal proteolytic enzyme in the process of apoptosis (Hu et al., 2011). The detection of caspase-3 was used to distin-

guish cell death pathways between apoptosis and necrosis, however the exact mechanism of apoptosis in *E. granulosus* stages was not quite clear (Van Cruchten and Van Den Broeck, 2002).

Nevertheless, understanding the exact mechanisms of apoptosis in *E. granulosus* stages will help the development of new treatments in the future. In the current study, the incubation of protoscolices with 50 µg/ml and 100 µg/ml of ACCV led to caspase activation at 37 °C after 4hrs. This finding was in line with the study of (Al-Asmari et al., 2018), who had proven the inhibitory effect of scorpion venoms on the proliferation of breast and colorectal cell lines through induction of apoptosis. Also, it was in accordance with the studies of Hu et al. (2011), who found that dexamethasone along with H<sub>2</sub>O<sub>2</sub> can induces the cell apoptosis of protoscolices and that of Tawfik (2018), who had illustrated that the cyst protoscolices incubation with 50 ppm and 100 ppm of bee venom after 30 min at 37 °C leading to caspase activation. Naseri et al. (2016) showed the pro-apoptotic impact of “albendazole sulfoxide” as well as soluble albendazole nano polymeric particles on protoscolices. The impact of bile acids on protoscolices viability in vitro was investigated by Shi et al. (2016), they reported that using chenodeoxycholic acid in culture for six days at a concentration of 3 mM led to a 100 percent death rate of protoscolices due to increased caspase-3 activity. Shahnazi et al. (2017) found incubating cyst protoscolices with extracts of 50 to 100 mg/mL of *Myrtus communis* led to activated caspases for 4hrs at 37 °C. Zahran et al. (2020) exposed protoscolices and metacystode layers to different concentrations of fluralaner, which induced apoptosis in treated protoscolices due to Caspase-3 activity.

## 5. Conclusion

Protoscolices are the essential targets for therapeutic agents to stop the development of hydatid cysts since they can develop into adult worms in a definitive host or into new hydatid cysts in an intermediate host. Numerous scolicidal agents were used to inactivate hydatid cyst contents, however, the majority of these have many side effects. The findings of the current study revealed that *Androctonus crassicauda* crude venom (ACCV) has prominent and promising effects on the structure and / or ultrastructure of the protoscolices and apoptosis of their cells. However, further in vivo studies are recommended to find out the impacts of ACCV on both *E. granulosus* protoscolices and their hosting animals. Also, to conclude the possible use of it as therapeutic agent for this parasite and its similar ones.

## 6. Compliance with ethical standards

All methods used in the present study were approved and allowed by the ethical Committee of scientific research at the Faculty of Science, “Al-Azhar University” (Assiut Branch), Egypt, under the ethical norms of the relevant national guidelines on the care and usage of laboratory animals.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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