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Alterations in the Tegument Surface of Adult *Fasciola gigantica*, After *in vitro* Treatment with the Crude Venom of *Vespa orientalis*

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Abstract

The present study aimed to investigate the antiparasitic activity of the wasp venom (WV), *Vespa orientalis*, against the adult *Fasciola gigantica* (*F. gigantica*) worm, as WV has known pharmacological properties. We assessed its anthelmintic efficacy using 40 adult *F. gigantica* flukes collected from slaughtered cattle divided into four groups (10 each). Worms in three groups were treated with 50, 100, and 200 µg/ml of WV, while a fourth untreated one was used as control. After WV treatment, the tegument area of the fluke's body was assessed using a scanning electron microscope. This revealed several tegumental alterations all over the fluke's body, including sunken spines due to local swelling, complete disappearance of spines from their sockets, furrowing and sloughing of the basal lamina, splitting off some spines to resemble an "open jaw," broken oral and ventral sucker teguments, increased swelling of the ventral sucker with loss of its ridges, severe swelling and smoothening of the ventral sucker due to loss of normal transverse ridges, deformed cirrus, and swollen sensory papillae with blebs. The sensory papillae were completely disrupted and dislodged, leaving pits and a series of holes throughout the basal lamina and severely eroded, turning into lesions exposing the basement membrane. These findings indicate that WV destructs the tegument surface of the adult flukes. However, further *in vivo* studies on the activity of WV treatment are recommended.

KEYWORDS
Fasciola gigantica, Tegument surface alteration, Venom, *Vespa orientalis*

INTRODUCTION

The venom of the oriental hornet, *Vespa orientalis*, has several physical and pharmacological properties as it contains bioactive compounds, including small peptides, amines, and low/high molecular weight proteins (enzymes and toxins) (Nakajima, 1986). The biological effects of this venom can be attributed to the presence of serotonin, acetylcholine, kinins, adrenaline, noradrenaline, dopamine, and high molecular weight compounds such as hyaluronidase, phospholipase-A2, histidine decarboxylase, poly-, and disaccharidases, neutral DNAse, and several polycationic peptides (Piek, 1986; Abd El-Wahed *et al*., 2021).

Fascioliasis is an important helminthic disease caused by the parasitic liver fluke species from the genus *Fasciola* (Digenea: Fasciolidae) (Waikagul *et al*., 2015). *F. gigantica* and *F. hepatica* significantly impact the growth rate, development, and productivity of ruminants and thus are economically important (Beesley *et al.* 2018). Fascioliasis is treated exclusively using various anthelminthic drugs (Ullah *et al*., 2017). Since 1983, the benzamide compound "Triclabendazole" has been the only approved drug for veterinary and human use. It was recommended by the WHO in 1997, especially for endemic countries with a high fascioliasis burden (McCarthy and Moore, 2015). Triclabendazole is the only known drug effective against the pre-adult and adult worms of *F. gigantica* in the hepatic parenchyma and bile ducts, respectively. However, several studies have reported resistance to this drug (Cabada *et al*., 2016; Gandhi *et al*., 2019). Currently, novel, effective alternative treatments for fascioliasis are urgently needed due to the increased prevalence of drug resistance. Natural alternative therapies are being explored due to their higher biosafety and fewer side effects than synthetic drugs (Ullah *et al*., 2017).

In this study, we used scanning electron microscopy (SEM) to investigate the *in vitro* effect of the wasp venom on the adult *Fasciola gigantica* worms and to assess its therapeutic potential.

MATERIALS AND METHODS

Collection and preparation of parasites

The adult liver flukes, *F. gigantica*, were either collected from the bile ducts or the gallbladders of the animals slaughtered at the Drunka altar in the Assiut governorate, Egypt, then transported immediately to the parasitology laboratory within thermos flasks containing RPMI-1640. The worms were washed several times with 0.85% NaCl solution, and only intact and actively mobile flukes were selected for the study.

Collection of wasps' crude venom

The wasps were collected by placing traps among the honeybee hives at the fields of the Faculty of Agriculture, Al-Azhar University in Assiut, from October to December 2021. To obtain

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the venom, the wasps were immobilized by rapid freezing at −20°C. Then, the stinging apparatus were dissected at 4°C, and the venom reservoirs were extracted and stored at −20°C until further use. The venom sacs were resuspended in Milli-Q deionized water. The whole wasp venom extract was obtained by disrupting the reservoirs using a glass rod under rapid defrosting and light pressure. The samples were centrifuged at 10,000 rpm for 5 minutes at 4°C, and the supernatants were used as protein and enzyme sources after lyophilization by (Biotran, Speed vaccum concentrator, Model: Ecospin 3180C). The lyophilized crude wasp venom was dissolved in Milli-Q water and filtered through a 0.22 µm syringe filter (Surendra *et al*., 2011). The worms were incubated *in vitro* in sterile RPMI-1640 medium (Caisson Laboratories Inc. Smithfield, UT, USA) supplemented with L-glutamine, penicillin (50 IU/ml), streptomycin (50 µg/ml), and gentamycin (30 IU/ml) (Sigma-Aldrich, St. Louis, Missouri, USA). The working solutions of wasp venom (WV) in concentrations of 50, 100, and 200 µg/ml were prepared by dissolving it in RPMI-1640 complete medium as previously described (Nair *et al*., 2016).

Study design

The *F. gigantica* adults were kept under sterile conditions inside a laminar flow cabinet in a petri dish containing RPMI-1640 complete medium. The worms were divided into four groups (10 flukes/group) as follows; Group 1 (control), the worms were incubated in the medium without WV; Group 2, 3, and 4 were incubated in the medium with 50, 100, and 200 µg/ml of WV, respectively for 24 hours at 37°C (Sumsakul *et al*., 2014).

Scanning electron microscope (SEM)

The *F. gigantica* adult liver flukes were examined using SEM as previously described (Shalaby *et al*., 2016). Briefly, the samples were fixed in 2.5% buffered glutaraldehyde in 0.1 M PBS (pH 7.4) at 4°C for 24 hours, washed with PBS, and re-fixed with 1% osmium tetroxide for one hour. The samples were rewashed with PBS, dehydrated using a serial dilution of ethyl alcohol (30, 50, 70, 90, and 100%), and infiltrated with acetone. The samples were dried with SPI Critical Point Dryer and mounted on gold-coated aluminum stubs in an SPI Module ™ Vac/Sputter coater (SPI Supplies, West Chester, PA, USA). Finally, the specimens were examined by the JSM-52500 LV SEM (JEOL Ltd. Inc., Tokyo, Japan) at the Electron Microscope Unit of Assiut University.

RESULTS

Scanning electron microscope of the control flukes

The morphology of the control group (Figs. 1a, 2a, 3a, 4a, 4b, 5a, and 6a) showed leaf-like bodies with tapered anterior and posterior ends. The apical cone was located anteriorly with an oral sucker at its tip. A ventral sucker was present on the ventral surface at the apical cone-main body junction with the gonopore just anterior to it (Fig. 1a). The typical intact tegument surface exhibited micro ridges and grooves with numerous serrated spines, which were deficient at the rims of both suckers (Fig. 2a). A cluster of fungiform-shaped sensory papillae surrounded the ventral sucker. Also, these papillae were scattered throughout the body's surface between the spines (Fig. 3a and 4b). Each papilla has a small dome with a smooth top and a highly pitted base. The spines were mainly located on the anterior part, especially around the oral and ventral suckers, and were fewer on the posterior part of the body. The size and shape of these spines depended on their location on the fluke's body. The spines on the apical cone were broad, tightly packed, and posteriorly directed, with several fingers-like protrusions at their tips (Figs. 4a and b). The spines on the mid-body were not as closely arranged

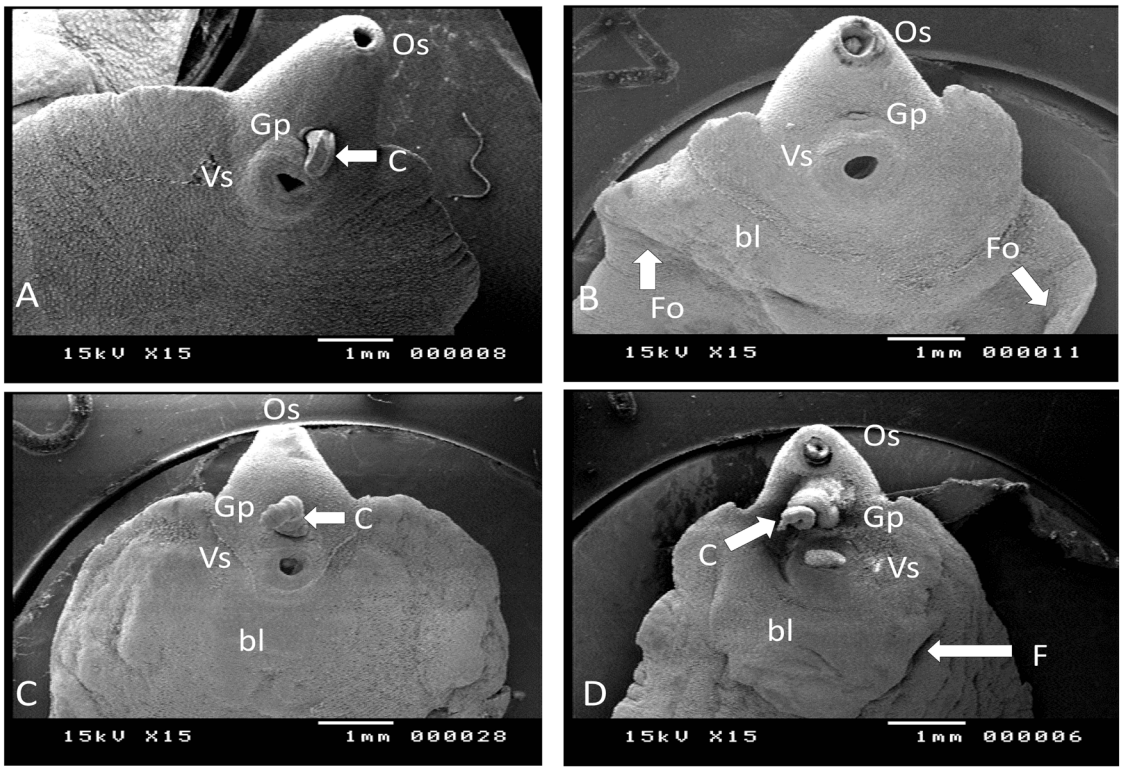

Fig. 1. Scanning electron micrograph (SEM) for the apical region of *Fasciola gigantica* showing, (A) control worms displaying smooth oral (Os) and ventral (Vs) suckers and gonopore (Gp); (B) Worms treated with 50 µg/ml *Vespa orientalis* venom for 24 hr showed severely swollen tegumental surface with exposed basal lamina (bl) and deep folding (Fo); (C) Worms treated with 100 µg/ml *Vespa orientalis* venom after 24 hr showed damaged tegument of oral sucker (Os), deformed cirrus (C), and severely swollen tegumental surface with exposed basal lamina (bl); (D) Worms treated with 200 µg/ml *Vespa orientalis* venom for 24 hr showed damaged tegument of oral and ventral suckers, deformed cirrus (C), severely swollen tegumental surface with exposed basal lamina (bl), and furrows (F).

as those on the apical cone, and the protrusions along their tips were not well-defined (Fig.5a). The spines on the tail surface were smaller than the rest (Fig. 6a).

The effects of wasp venom on adult Fasciola gigantica

After incubation with 50 µg/ml WV for 24 h

The tegumental surface of the apical cone was severely swollen with exposed basal lamina and deep folds (Fig. 1b). The ventral sucker showed increased swelling and loss of ridges (Fig. 2b). The sensory papillae were swollen and covered with blebs, including a few disrupted ones (Fig. 3b). The tegument was sloughed, and few spines were split, appearing like "open jaws" (Fig. 4c). The mid-body region showed tegumental swelling in the interspinal region with the split, "open-jawed" spines (Fig. 5c). The spines in the tail region often appeared sunken due to the swelling in the surrounding tegument (Fig. 6b).

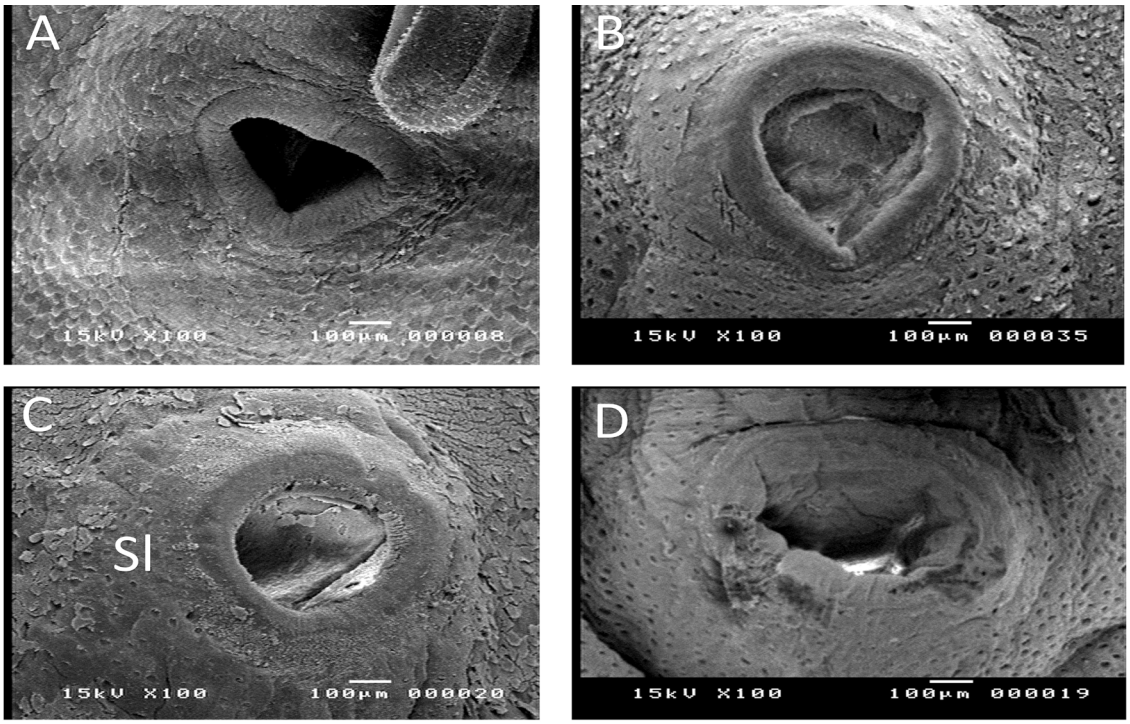

Fig. 2. Scanning electron micrograph (SEM) for the apical region of *Fasciola gigantica* showing, (A) Control worms possessed smooth ventral suckers with thick rims covered with transverse folds. The entire tegumental surface was covered with spines, except the sucker rims; (B) Worms treated with 50 µg/ml *Vespa orientalis* venom for 24 hr displayed a swollen ventral sucker with loss of ridges; (C) Worms treated with 100 µg/ml *Vespa orientalis* venom for 24 hr displayed severely swollen ventral sucker, which appeared smooth due to the loss of normal transverse ridges covering its rim and sloughing of the tegument (Sl); (D) Worms treated with 200 µg/ml *Vespa orientalis* venom for 24 hr displayed apparent damage of ventral sucker with loss of the covering tegument.

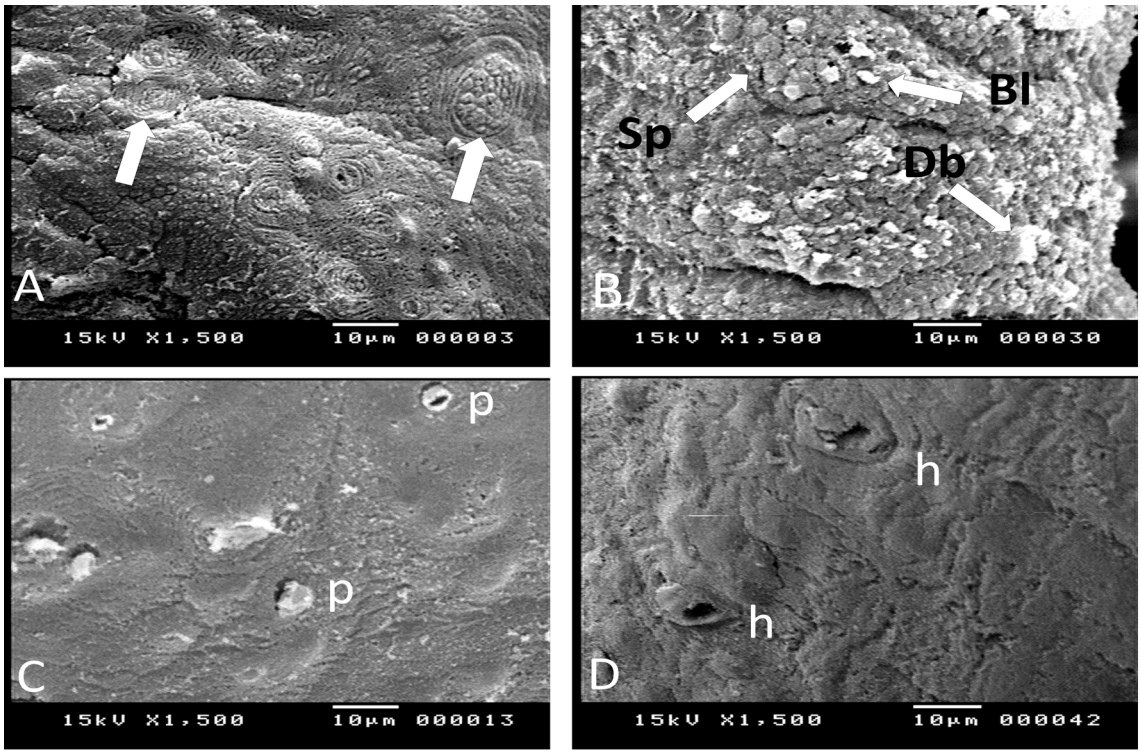

Fig. 3. Scanning electron micrograph (SEM) for the apical region of *Fasciola gigantica* showing, (A) Control worms possessed sensory papillae clusters around the ventral sucker (white arrows); (B) Worms treated with 50 µg/ml *Vespa orientalis* venom for 24 hr revealed swollen sensory papillae (Sp) covered with blebs (Bl), some of which were disrupted (Db); (C) Worms treated with 100 µg/ml *Vespa orientalis* venom for 24 hr revealed that all the sensory papillae were completely disrupted and dislodged, leaving pits (p); (D) Worms treated with 200 µg/ml *Vespa orientalis* venom for 24 hr displayed a series of holes (h) in the basal lamina with sloughed-off tegument around it. The sensory papillae clusters completely disappeared.

After incubation with 100 µg/ml WV for 24 h

The oral suckers in the apical cone region showed damaged teguments with deformed cirrus. The tegumental surface was severely swollen with exposed basal lamina (Fig. 1c). The ventral sucker was severely swollen and appeared smooth due to the loss of the normal transverse ridges around its rim and sloughing of the tegument, exposing basal lamina underneath (Fig. 2c). All the sensory papillae were disrupted and dislodged from their places, leaving pits (Fig. 3c). The spines were also completely damaged and dislodged, leaving behind empty spine sockets (Fig. 4d). The mid-body region showed submerged spines, which were either completely flattened against the surface or submerged in the swollen tegument, leaving deep furrows around them (Fig. 5c and d). In the tail region, the spines disappeared, leaving empty spine sockets (Fig. 6c).

After incubation with 200 µg/ml WV for 24 h

The apical cone region showed damaged tegument of the oral and ventral suckers, deformed cirrus, severely swollen tegumental surface with exposed basal lamina and furrows (Fig. 1d and Fig. 2d). A series of holes were observed around the basal lamina with the sloughed-off surrounding tegument and complete disappearance of the sensory papillae clusters (Fig. 3d). The tegument of the anterior region was severely eroded, eventually turning into lesions, exposing a large area of the basement membrane and complete loss of spines (Fig. 4e). The mid-body region displayed furrowed tegument, complete loss of spines and sloughed tegument with exposed underlying basal lamina (Fig. 5e). The tail region was also furrowed with complete disappearance of spines, leaving spine sockets and sloughed tegument, exposing the basal lamina (Fig. 6d).

DISCUSSION

Fascioliasis treatment is dependent on the use of chemical drugs (Saowakon *et al*., 2009). However, excessive, and continuous usage of these drugs has given rise to drug resistance, which has been reported in many endemic regions globally (Alvarez-Mercado *et al*., 2015; McCarthy and Moore, 2015). Currently, medical, and pharmacological research is focused on investigating novel, natural treatments because of their higher biosafety levels and lower side effects than synthetic drugs (Alvarez-Mercado *et al*., 2015; Toner *et al*., 2010). Animal venoms are abundant in natural bioactive molecules with excellent pharmaceutical and therapeutic properties. Thus, they can be potentially used for drug discovery (Rivero *et al*., 2011; Vigerelli *et al*., 2014; Neto *et al*., 2015). Hassan *et al*. (2016) studied the potential anti-schistosomal activity of the Egyptian snake, Cerastes cerastes venom, *in vitro*. They found that it causes mild to severe tegumental damage to Schistosoma worms. Al-Malki and Abdelsater (2020) reported that 100 µg/mL of crude venom from the scorpion, Androctonus crassicauda (ACCV), can destroy all Echinococcus granulosus protoscolices after 240 min incubation. Al-Malki *et al*. (2022) also reported that ACCV induces severe ultrastructural changes and apoptosis of Echinococcus granulosus protoscolices. Kellershohn *et al*. (2019) assessed the antischistosomal capacity of harmonine, an

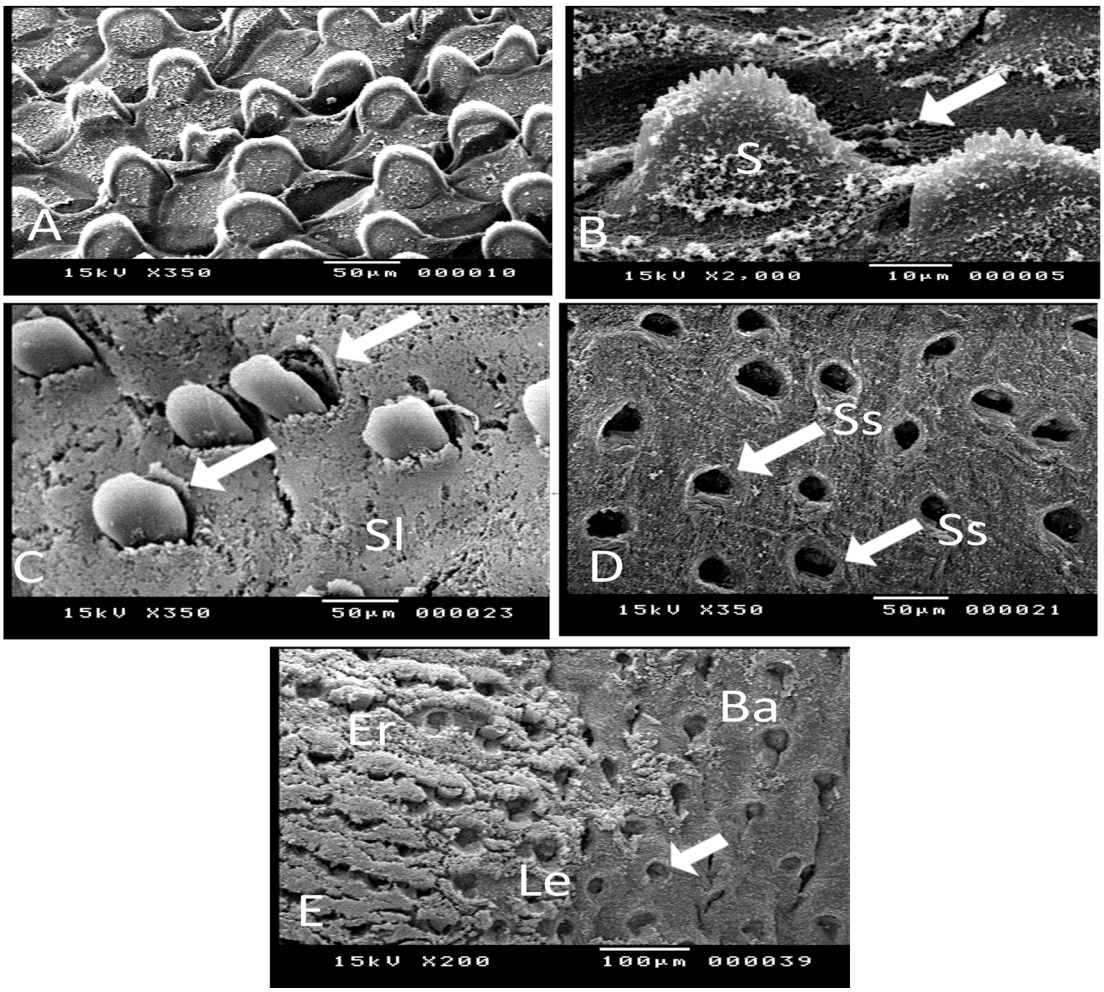

Fig. 4. Scanning electron micrograph (SEM) of the apical region of *Fasciola gigantica* showing, (A, B) Control worms show a tightly packed apical cone, posteriorly directed broad spines with several finger-like protrusions on their tips, and scattered sensory papillae clusters throughout the body between spines (S) (arrow); (C) Worms treated 50 µg/ml *Vespa orientalis* venom for 24 hr showing sloughing (Sl) of tegument and a few split spines resembling "opened jaws" (white arrows); (D) Worms treated with 100 µg/ml *Vespa orientalis* venom for 24 hr display completely destructed and dislodged spines, exposing their empty sockets (Ss); (E) Worms treated with 200 µg/ml *Vespa orientalis* venom for 24 hr revealed the tegument on the anterior region with severe erosion (Er), which eventually formed lesions (Le), exposing a large area of the basement membrane (Ba) with complete loss of spines (white arrow).

antimicrobial alkaloid from the harlequin ladybird Harmonia axyridis. They observed that it had remarkable pleiotropic effects on the physiological, cellular, and molecular processes in adult male and female Schistosoma mansoni. Tonk *et al.* (2020) investigated the antischistosomal activity of the venom from the European predatory assassin bug, Rhynocoris iracundus. The components of wasp venom are commonly categorized as (i) bioactive molecules such as serotonin, histamine, catecholamines, tyramine, acetylcholine, flavonoids, and biologically active amines, (ii) high molecular weight proteins such as hyaluronidases, phospholipases, and antigen 5, and (iii) low molecular weight peptides such as mastoparan (Nakajima, 1986). Mastoparan, a wasp kinin and a chemotactic peptide is a known tetra decapeptide present in wasp venom (Hoffman and Jacobson, 1984) that can create pores in the lipid bilayers of erythrocytes, bacteria, and mast cells (Hoffman and Jacobson, 1984). Moreover, it can also induce pore formation and disruption of the viral lipid envelope (Sample *et al*., 2013). Vespoid wasp phospholipases can disrupt the phospholipid layer of several biological membranes, leading to pore formation and/or cell lysis (Santos *et al*., 2007; Costa and Palma, 2000). The development of electron microscopy has facilitated the morphological analysis of parasites, which helps understand the complex morphological changes due to the parasite and drug-parasite interactions (Sachanonta *et al*., 2011). Scanning electron microscope is used to evaluate the efficacy of many fasciolicides (Anderson and Fairweather, 1998; Buchanan *et al*., 2002; Meaney *et al*., 2002, 2003) because it enables observation of the damaged surface of the fluke tegument, which has different mechanical and metabolic functions in the parasite (Dalton, 1999). To the best of the authors' knowledge, this study is the first to investigate the effect of wasp venom on the tegument surface of adult *F. gigantica* in a dose-dependent manner. The obtained SEM data demonstrated various tegumental, including tegument swelling, the disappearance of spines, damaged tegument of the oral and ventral suckers, deformed cirrus, destroyed and displaced sensory papillae from their places, a series of holes in the basal lamina, splitting of spines (opened jaw), severe erosion, the complete loss of spines, furrowing, folding, sloughing, and blebbing of the tegument. The severity increased with an increase in doses of wasp venom. The tegumental damage might induce extensive alterations in the underlying tissues and internal organs, causing more damage to the entire parasite (Shalaby *et al*., 2016). The wasp venom also disrupts several physiological processes associated with the tegument, including osmoregulation, nutrient uptake, secretion, synthesis, and immune protection (Massoud *et al*., 2013). The destruction of the spines and sensory papillae will undoubtedly deprive the parasite of its vital functions and facilitate its clearance. Consistent with findings from this study, several studies used various plant and animal sources to reveal the same effects. For instance, Keiser and Morson (2008) and Shalaby *et al*. (2009) studied the effects of some plant extracts on the adult *Fasciola* tegument ultrastructure. Saowakon *et al*. (2009) studied the impact of *Artocarpus lakoocha* aqueous extract on the tegument of adult *F. gigantica*. Shehab *et al*. (2009) studied the *in vitro* fasciolicidal activity of *Meryta denhamii* plant extracts against adult *Fasciola*. Ebeid *et al*. (2011) determined the *in vitro* effects of plant extracts from *B. egyptiaca* compared with TCBZ in adult *F. gigantica*. Shoheiba and Darb (2011) evaluated both tegument alterations in adult *F. gigantica* and egg hatchability after *in vitro* incubation with either *Commiphora molmol* (*C. molmol)* or *Nigella sativa* (N. sativa) extract. Ullah *et al*. (2017) studied the anthelmintic and fasciolicidal activity of both curcumin and thy-

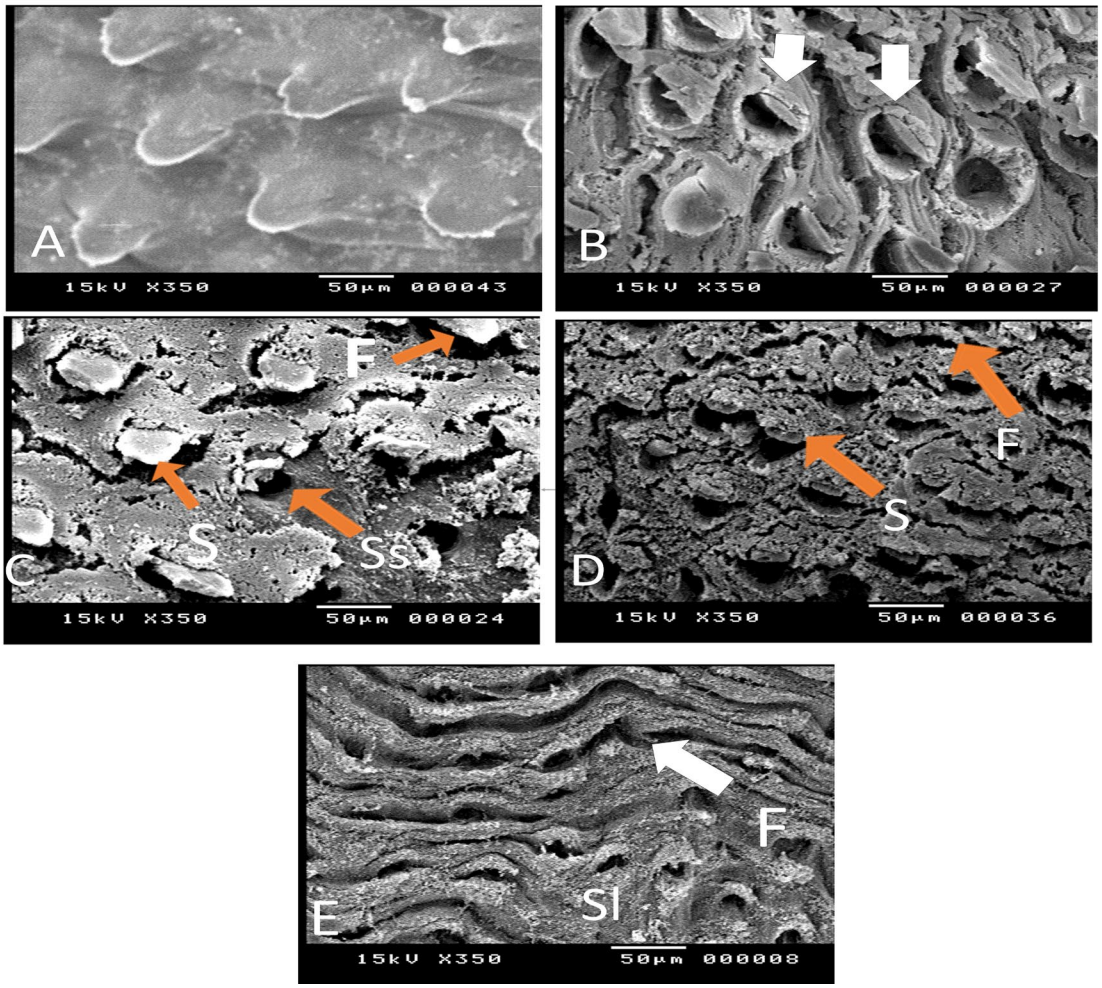

Fig. 5. Scanning electron micrograph (SEM) of *Fasciola gigantica* mid-body region showing, (A) Control or untreated worms are revealing the arrangement of spines; (B) Worms treated with 50 µg/ml *Vespa orientalis* venom for 24 hr displayed tegumental swelling in the interspinal region. Some spines had split and taken the form of "opened jaws" (arrows); (C, D) Worms treated with 100 µg/ml *Vespa orientalis* venom for 24 hr showed submerged spines (S), either flattened against the surface or had become submerged in the swollen tegument around them, leaving deep furrows (F). The spines were partially lost and dislodged from their places, exposing their empty sockets (Ss); (E) Worms treated with 200 µg/ml *Vespa orientalis* venom for 24 hr displayed furrows in tegument (F), complete loss of spines (S), and sloughing (Sl) of the tegument to expose the basal lamina beneath.

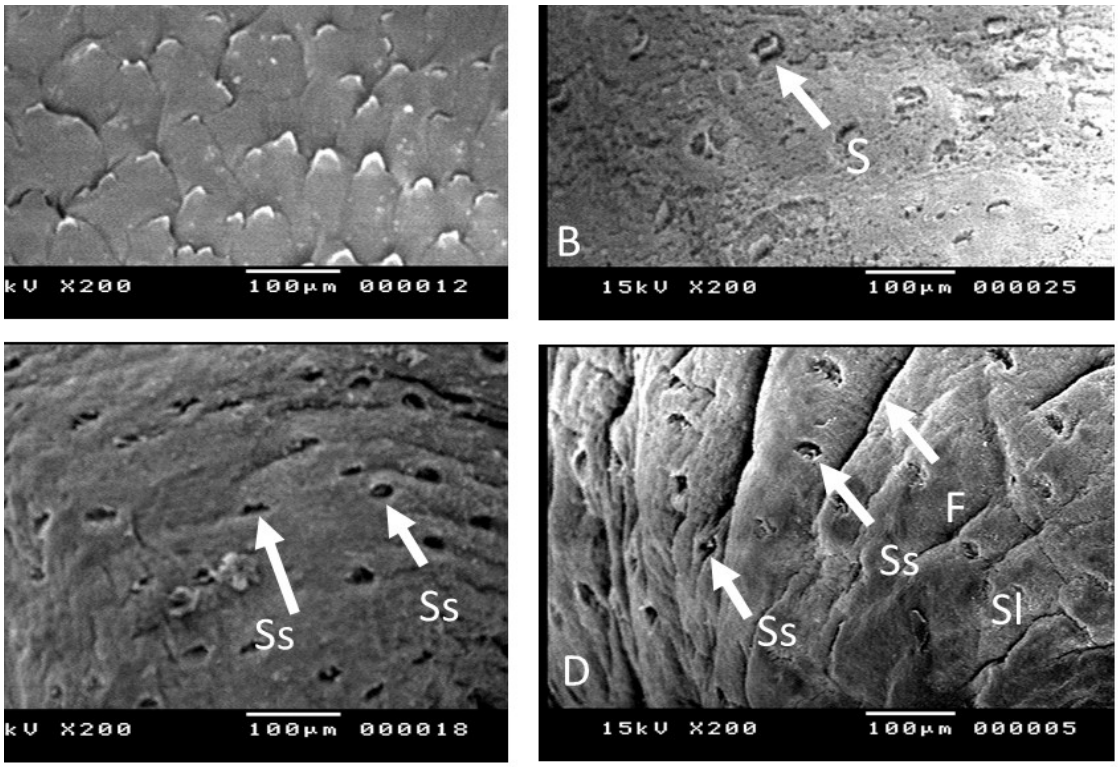

Fig. 6. Scanning electron micrograph (SEM) of *Fasciola gigantica* posterior region showing, (A) Control or untreated worms revealing the smaller spines which cover the fluke's surface; (B) Worms treated with 50 µg/ml *Vespa orientalis* venom for 24 hr displayed sunken spines (S) due to the swollen tegument around them; (C) Worms treated with 100 µg/ml *Vespa orientalis* venom for 24 hr revealed complete disappearance of the spines, leaving empty sockets (Ss); (D) Worms treated with 200 µg/ml *Vespa orientalis* venom for 24 hr displayed furrows (F), complete loss of spines, leaving spine socket (Ss), and sloughing (Sl) of the tegument, exposing the basal lamina beneath.

moquinone on the tegument of the adult *F. gigantica*. Lorsuwannarat *et al.* (2014) and Abdel Fattah *et al.* (2021) suggested that a Plumbagin compound has a fasciolicidal activity on *F. gigantica*.

CONCLUSION

It could be concluded that *Vespa orientalis* venom (WV) has a promising fasciolicidal activity as it can significantly alter the teguments of the adult *F. gigantica*. These results facilitate the development of novel approaches toward controlling and treating fascioliasis using novel trematocidal agents. However, further studies need to be conducted to characterize and identify this venom and its active compound (s) responsible for the fasciolicidal activity and to understand its mechanism of action.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related.

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