Abdel-Fatah *et al.*
Beni-Suef Univ J Basic Appl Sci (2022) 11:106
https://doi.org/10.1186/s43088-022-00287-z

Beni-Suef University Journal of Basic and Applied Sciences

RESEARCH Open Access

Tegumental alterations and resistance of *Fasciola gigantica* adult worms exposed to flukicides in Egypt

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Abstract

Background: The current study was designed to investigate the in vitro effect of commercially available fasciolicides; albendazole (40 and 400 µg/ml), triclabendazole, rafoxanide and nitroxynil (50 and 100 µg/ml, each) against *Fasciola gigantica* adult worms. For all, worms were incubated for 3 h. Worm's motility was macroscopically and microscopically detected. Reduction of egg deposition was estimated. Alterations of worm's cuticle post-treatments were recorded using scanning electron microscopy (SEM).

Results: Nitroxynil had the most flukicidal effect with mild movement quickly disappeared within 15 min post-treatment. It showed the highest egg reduction (88.3% and 95% at concentrations of 50 and 100 µg/ml, respectively). Findings of SEM showed severe furrowing and destruction of spines. In rafoxanide-treated group, the motility disappeared 75 min post-treatment, and the egg deposition was significantly ($P \leq 0.05$) reduced to 70% and 85% at the same concentrations. Teguments showed thickening, moderate furrowing and destruction of the spines. Albendazole showed the lowest effect: the motility of the worms was observed till 160 min post-treatment and the egg reduction was 43% and 75% at the same concentrations. Interestingly, in albendazole-treated flukes, the tegument had severe furrowing and spines were completely sloughed. Similarly, in triclabendazole-treated flukes, worms motility was observed till 160 min post-treatment and the egg reduction was 76.6% and 88.3%. The tegument showed swelling and mild furrowing with moderately damaged spines.

Conclusions: Nitroxynil was the most potent flukicide inducing evidential cuticular changes. Although albendazole induced the most potent cuticular damage, it showed the lowest flukicidal effect. Further in vivo study to investigate resistance/susceptibility of *Fasciola* species in cattle and buffaloes will be carried out.

Keywords: Flukicides, *Fasciola gigantica*, Motility, Egg reduction, SEM, Cuticular alterations

1 Background

Fascioliasis is a disease of particular concern causing severe economic losses for sheep and cattle production worldwide. It has an estimated annual economic losses approximately $ 2–3 billion worldwide [1, 2] and US $ 29.2 million in Egypt [3]. It causes economic losses comprising reduced fertility and productivity and livers condemnation [4, 5]. Both fasciolids, *Fasciola hepatica* and *F. gigantica*, are common in Egypt [6]. In Egypt, the prevalence of fascioliasis was 50.6% and 32.3% in cattle and buffaloes, respectively [7]. Moreover, *Fasciola* species has zoonotic importance; about 830,000 human infections were recorded in Egypt [8].

Chemotherapy is the practical method that is effective against fascioliasis among infected animals and humans worldwide. Benzimidazoles (BZ) are the most common anthelmintics that are being used in both veterinary and human medication [9]. The primary mode of action

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implies their interaction with the tubulin and secretory processes of the worms [10]. Albendazole (ABZ) is the broadest spectrum member of the benzimidazole group. It acts against nematodes, cestodes and trematodes [11–13]. Triclabendazole (TCBZ) is a benzimidazole derivative that is being massively used against fascioliasis in humans and animals [14, 15]. Triclabendazole has a unique flukicidal activity against mature and immature flukes aging 2 weeks or more [16]. Rafoxanide is a flukicide belonging to salicylanilides, and causes depletion in glycogen, glucose-6-phosphate and ATP in mature flukes [17]. Nitroxynil is one of halogenated phenol flukicides, highly active against adult liver flukes (aged 6–8 weeks), with no effect on the earlier stages [18, 19]. The rapid spastic paralysis and uncoupler of oxidative phosphorylation effects of nitroxynil were documented [20].

The massive use of those compounds results in the developing of the anthelmintic resistance [21, 22]. The first report of *Fasciola* species resistance against flukicides was documented in 1980 [23]. Albendazole failed to treat fascioliasis in a sheep farm in the south-western Sweden [24]. Triclabendazole resistance has been proved in Australia since 1995 [25]. Screening activity of flukicides against *Fasciola* species can be conducted under laboratory and field conditions. In vitro egg hatching assay for screening susceptibility/resistance of *Fasciola* species has been carried out [26, 27]. Moreover, alterations in the tegument of *Fasciola* adult worms exposed to flukicides were recorded in previous literature. The tegument is one of the first tissues to be exposed to anthelmintics, a major pharmacological target. SEM has been proven to be a valuable method for assessing surface changes in flukes treated by anthelmintics. Disruption of the tegument caused by flukicides causes serious effects on flukes, as anthelmintics contact deep tissues and internal organs [28]. The use of SEM to screen activity of flukicides against *Fasciola* species tegument was previously recorded [29, 30].

Therefore, the current study was designed to investigate the in vitro susceptibility and tegument alterations of *Fasciola gigantica* adult worms exposed to high concentrations of commercially used flukicides using SEM in Egypt.

2 Methods

2.1 Sampling and collection of the worms

Adult flukes were collected from a liver of male buffalo aged 2 years, from Beni-Suef (Coordinates: 29°04′ N 31°05′ E) municipal abattoir, Egypt. The infected liver was transported to the department of Parasitology, Faculty of Veterinary Medicine, Beni-Suef University, Egypt, in an ice box within 1 h post slaughtering. All flukes washed several times with saline and then transferred to Petri dishes containing saline. The collected flukes were identified based on their morphological features according to Ashrafi et al. [31]. Also, the collected worms were molecularly identified (Unpublished data). Motility and activity of the adult flukes were assessed before the treatment.

2.2 Screening activity of the used flukicides

Commercially available flukicides: Albendazole® 10%, Pharma Swede, Egypt; Triclabendazole (Triclafluke 10% Pharma Swede, Egypt); Nitroxynil (Dovix 25%, Arabcomed, Egypt); Rafoxanide (Flukanil 7.5%, Pharma Swede, Egypt) were purchased from veterinary drug stores. The effective concentrations of each flukicide selected based on findings obtained from our previous study [27]. Albendazole 10% was dissolved in DMSO 1% at rates of 40 and 400 µg/ml in Petri dishes. Triclabendazole 10%, Nitroxynil 25% and Rafoxanide 7.5% were separately dissolved in DMSO 1% at rates of 50 and 100 µg/ml for each drug in Petri dishes. Five active flukes of the same size were gently added to each dish. Three replicates were evaluated for each concentration per flukicide. Dishes were incubated for 3 h at 37 °C. In the control group, flukes were incubated for 3 h at 37 °C DMSO 1% dissolved in saline. During the incubation, movement of all flukes in the Petri dishes was recorded. Three hours post, flukes were collected and washed again using normal saline 0.9% to remove remnants of the used drugs, then stored in 70% ethanol and they underwent scanning electron microscopy.

2.3 Parasitological parameters

Motility of the treated worms was macroscopically and microscopically observed at intervals of 5, 15, and 30 min throughout 3 h post-treatment. Moreover, eggs which deposited in each dish were counted microscopically to estimate the fecundity of the treated flukes.

2.4 Scanning electron microscopy (SEM) of the treated flukes

Flukes were fixed with 4% (v/v) gluteraldhyde in PBS buffer (pH 7.4) for 24 h at room temperature. Washing, dehydration and fixation of the samples were conducted [32]. Examination of the prepared slides using high resolution SEM was done in Electron Microscope Unit, Faculty of Science, Beni-Suef University.

2.5 Statistical analysis

SPSS computer program v.22 (IBM, Armonk, NY, USA) was used for data analysis. Egg deposition per treatment was expressed as mean ± stander error (SE). Moreover, percent decrease in the mean number of egg deposition was calculated from the equation: mean of egg deposition of control – mean of egg deposition in treated group

/ mean of egg deposition of control × 100. Motility of the flukes was categorized into clear macroscopic (+++), moderate (++), mild (+), and paralysis (–). *P* value less than 0.05 was considered significant.

3 Results

3.1 Effect of flukicides on the motility of the treated worms

In the control untreated groups, *Fasciola gigantica* worms had a clear macroscopic movement (+++) throughout the first hour of the in vitro application. In the second hour, the movement was moderate (++). In the third hour, the movement reduced and scored as mild (+). In nitroxynil-treated groups, mild movement of the worms was observed at the first 5 min post application. The macroscopic and microscopic movement disappeared after 15 min (–). Meanwhile, in rafoxanide-treated groups, the clear macroscopic movement was detected during the first 5–15 min post application. Moderate movement was observed at 15–30 min post-treatment with rafoxanide. Macroscopic and microscopic motility disappeared after 75 min of rafoxanide treatment. In albendazole-treated groups, a clear macroscopic movement of the worms was observed till 45 and 30 min at 40 and 400 µg/ml, respectively. Mild movement of the worms was observed at 90–160 min post-treatment. Macroscopic and microscopic motility disappeared after the third hour of the albendazole treatment. Similarly, in triclabendazole-treated group, flukes had a clear macroscopic movement till 45 and 30 min at 50 and 100 µg/ml, respectively. Mild macroscopic movement of the worms was observed at 75–120 min post-treatment. Macroscopic and microscopic motility disappeared after 160 min of treatment (Table 1).

3.2 Effect of flukicides on the fecundity of the treated worms

The mean of deposited eggs was significantly reduced ($P \leq 0.05$) in all groups except in the group treated with albendazole 40 µg/ml compared to the control untreated worms. In albendazole-treated groups, the egg reduction was 43% and 75% at concentrations of 40 and 400 µg/ml, respectively. Meanwhile, in triclabendazole-treated flukes, the egg deposition was significantly reduced ($p \leq 0.05$) to 76.6% and 88.3% on using concentrations of 50 µg/ml and 100 µg/ml, respectively. Regarding rafoxanide application, the use of 50 µg/ml and 100 µg/ml revealed a significant ($P \leq 0.05$) reduction of the egg deposition to 70% and 85%, respectively. In nitroxynil-treated groups, the use of concentrations of 50 µg/ml and 100 µg/ml revealed a significant ($P \leq 0.05$) reduction of the egg deposition to 88.3% and 95%, respectively (Table 2).

3.3 Scanning electron microscopy (SEM)

The untreated flukes showed a normal tegument with intact spines on both the dorsal and ventral surfaces. No damage was observed on the oral and ventral suckers. Dimensions of the ventral sucker ranged from 238 to 318 µm. The thickness of the tegument ranged from 248 to 297 µm. Dimensions of the spines were 33–40 µm (Fig. 1).

Meanwhile, in nitroxynil-treated flukes, the tegument elucidated severe furrowing and severe destruction in

Table 1 The effect of flukicides on the motility of *Fasciola gigantica* adult worms

Time post-treatment (mins)	Control	Nitroxynil		Rafoxanide		Albendazole		Triclabendazole	
		50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml	40 µg/ml	400 µg/ml	50 µg/ml	100 µg/ml
Zero	+++	+++	+++	+++	+++	+++	+++	+++	+++
Post 5	+++	+	+	+++	+++	+++	+++	+++	+++
Post 15	+++	–	–	+++	++	+++	+++	+++	+++
Post 30	+++	–	–	++	+	+++	+++	+++	+++
Post 45	+++	–	–	++	+	+++	++	+++	++
Post 60	+++	–	–	+	+	++	++	++	++
Post 75	++	–	–	–	–	++	++	++	+
Post 90	++	–	–	–	–	+	+	+	+
Post 120	++	–	–	–	–	+	+	+	+
Post 160	+	–	–	–	–	+	+	–	–
Post 180	+	–	–	–	–	–	–	–	–

+++Denotes clear macroscopic motility,

++Denotes moderate motility

+Denotes mild motility

–Denotes paralysis

Table 2 The effect of flukicides on the fecundity of the treated worms

Drug/conc	Mean of egg deposition ± S.E	Egg reduction % (efficacy %)
Control untreated	1500 ± 346.4[a]	–
Albendazole 40 µg/ml	850.0 ± 28.86[b]	43%
Albendazole 400 µg/ml	375.0 ± 43.30[c]	75%
Rafoxanide 50 µg/ml	450.0 ± 28.86[c]	70%
Rafoxanide 100 µg/ml	225.0 ± 14.43[c]	85%
Triclabendazole 50 µg/ml	350.0 ± 28.86[c]	76.6%
Triclabendazole 100 µg/ml	175.0 ± 14.43[c]	88.3%
Nitroxynil 50 µg/ml	175.0 ± 72.16[c]	88.3%
Nitroxynil 100 µg/ml	75.00 ± 14.43[c]	95%

Superscript letters b and c are significant with the control (a). *P value was significant at* ≤ *0.05*

the spines particularly on middle and the tail of both surfaces. Thickness of the cuticle ranged from 302 to 484 µm. Dimensions of the ventral sucker ranged from 355 to 462 (Fig. 2).

In rafoxanide-treated flukes, the tegument had moderate furrowing and destruction of spines on the anterior, middle, and the tail of both surfaces. Thickness of the cuticle ranged from 328 to 498 µm. Dimensions of ventral sucker were 350–470 µm (Fig. 3).

In triclabendazole-treated flukes, the tegument showed swelling and mild furrowing. Spines were moderately damaged dorsally and mildly damaged on the ventral surface. Thickness of the cuticle ranged from 258 to 270. µm. Dimensions of the ventral sucker were 243–250 (Fig. 4).

In albendazole-treated flukes, the tegument revealed severe furrowing. Spines were sloughed dorsally and damaged on the ventral aspect. At a concentration of 400 mg/ml, the tegument was detached in some areas leaving the basal lamina exposed. Thickness of the cuticle ranged from 250 to 290 µm. Dimensions of ventral sucker were 250–260 (Fig. 5).

4 Discussion

In the current study, the activity of the commercially used flukicides against *Fasciola* species in Egypt was investigated. Interestingly, findings of the used anthelmintics were recorded within a short time, 3 h. Moreover, worms were incubated in normal saline with no specific media. Thus, the present protocol is simple and could be conducted easily to estimate the preliminary screening of flukicide susceptibility prior to the field application. Nitroxynil induced the most potent flukicide effect

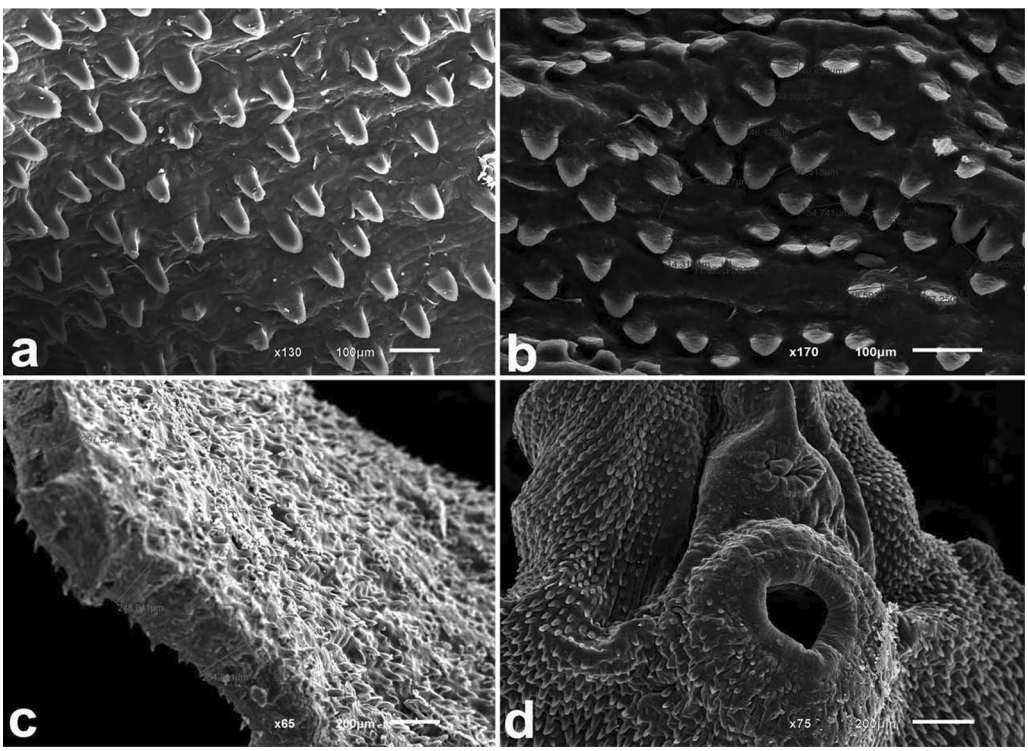

Fig. 1 SEM of control untreated *Fasciola gigantica* adult worms. **a**, **b** Intact spines on the dorsal and ventral surfaces. **c** The tegument thickness 248–297 µm. **d** Ventral sucker ranged from 238–318 µm

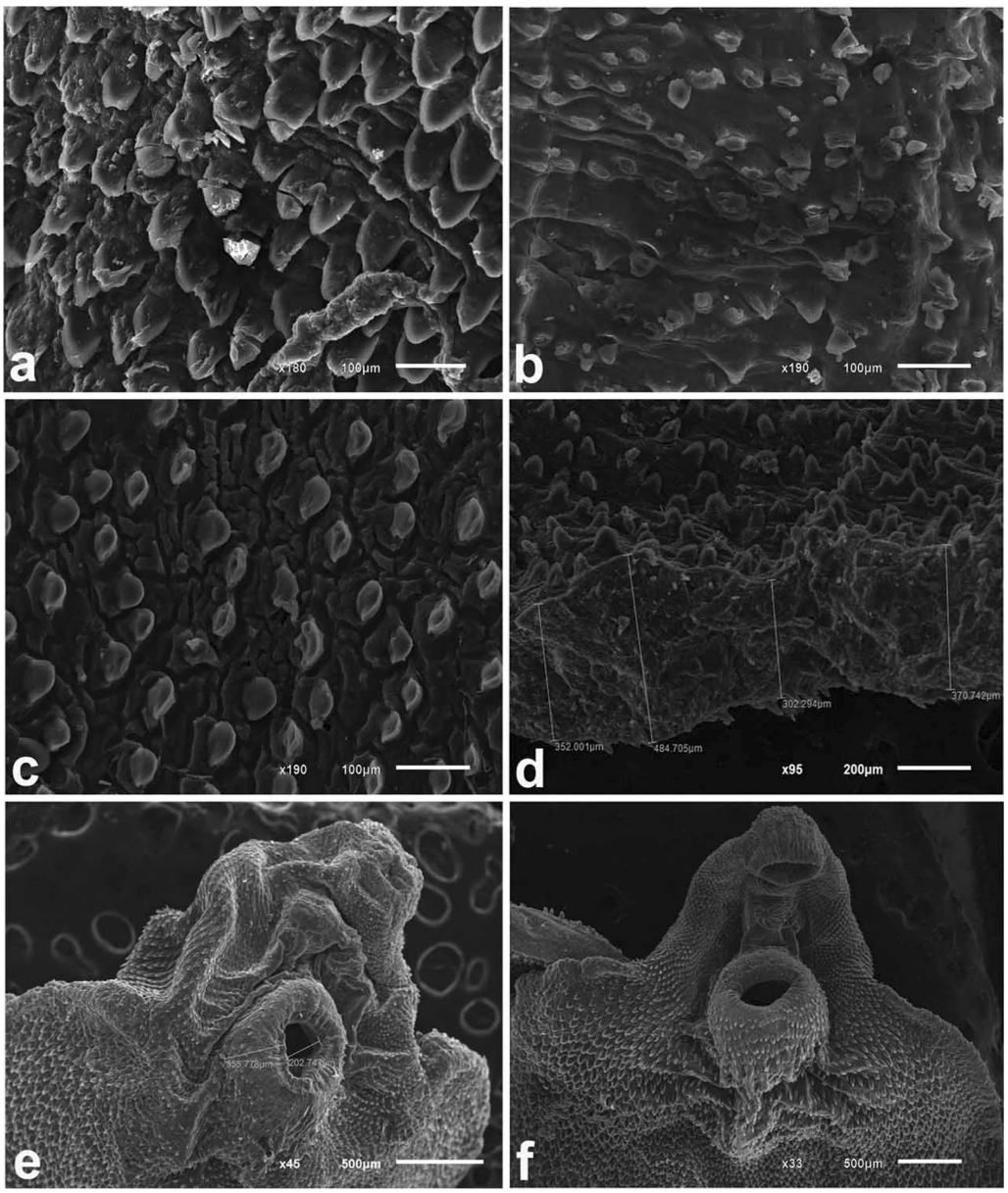

Fig. 2 SEM of nitroxynil-treated *Fasciola gigantica* adult worm. **a** Anterior tegument surface. **b**, **c** Middle and tail tegument surface showing severe furrowing, and severe destruction in the spines. **d**, **e** The thickness of cuticle 302–484 µm. **e**, **f** Ventral sucker 355–462

compared with rafoxanide, triclabendazole and albendazole treatments. Nitroxynil-treated flukes showed a complete paralysis within 15 min post-treatment. Similarly, nitroxynil caused spastic paralysis and uncoupler of oxidative phosphorylation of the treated worms [20]. Nitroxynil disrupt the oxidative phosphorylation process and prevent ATP formation inside flukes, thus, it suppresses their movement [18]. Moreover, a sluggish movement of *Fasciola* species worms treated with nitroxynil was recorded [33]. Currently, the egg deposition was significantly reduced in vitro at a rate of 88.3–95% based on nitroxynil application. The most potential flukicidal activity of nitroxynil was recorded in vivo previously. Nitroxynil efficacy was 99.1% against *F. hepatica* infecting cattle [19]. Furthermore, in *F. hepatica*-naturally infected sheep flock in the west of Ireland, nitroxynil showed 100% reduction in the egg deposition [34]. Moreover, nitroxynil was fully effective against triclabendazole-resistant flukes [35]. Besides, in cattle infected with *F. gigantica* in Tanzania, the fecal egg reduction test

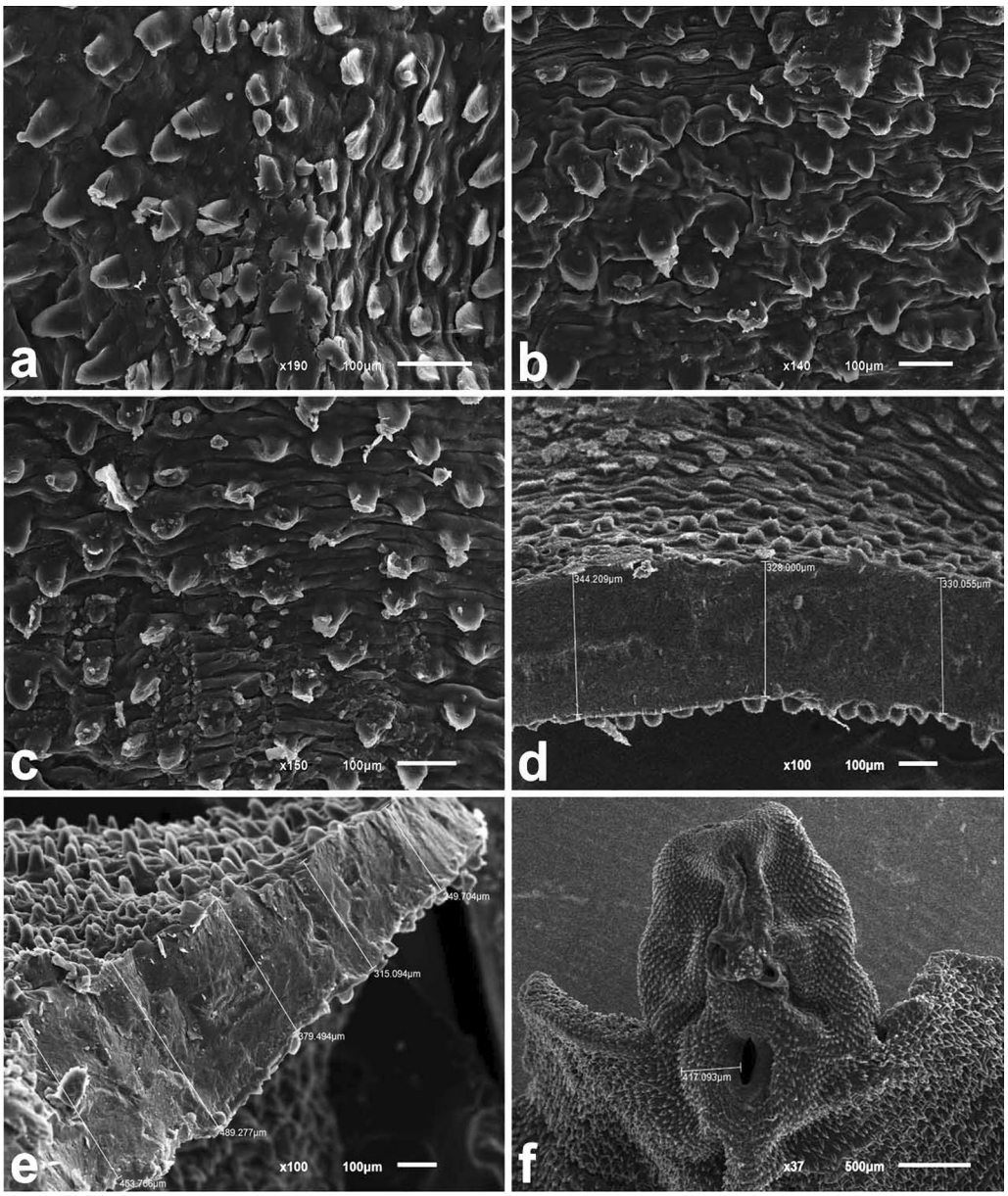

Fig. 3 SEM of rafoxanide-treated *Fasciola gigantica* adult worm. **a** Anterior tegument surface. **b**, **c** Middle and tail tegument surface showing moderate furrowing, and destruction in the spines. **d**, **e** Thickness of the cuticle 328–498 µm. **f** Ventral sucker 350–470 µm

was 100% after nitroxynil treatment [36]. The egg reduction was significantly higher in nitroxynil-treated cattle compared with triclabendazole [37]. In addition, nitroxynil was the most potent flukicide compared with clorsulon, closentel and triclabendazole in Mexico [38]. In the present work, SEM findings of nitroxynil coincided with the flukicidal effect. There was severe furrowing in the tegument together with severe destruction in the spines particularly on the middle and the tail of both surfaces. The potency of nitroxynil to disrupt *Fasciola* species spines was recorded [28]. Causes of spines disruption are unknown, but it might be that the destruction facilitates the entrance of the drug inside flukes [28, 39].

In vivo application showed that flukes collected from nitroxynil-treated rats were less active and their surface was swelled after 48 h, meanwhile, they showed little or no movement 72 h post-treatment [40]. In the authors' opinion, the action of nitroxynil was faster than the later study, because of the in vitro direct contact of the drug with the flukes and the high concentrations used. On the

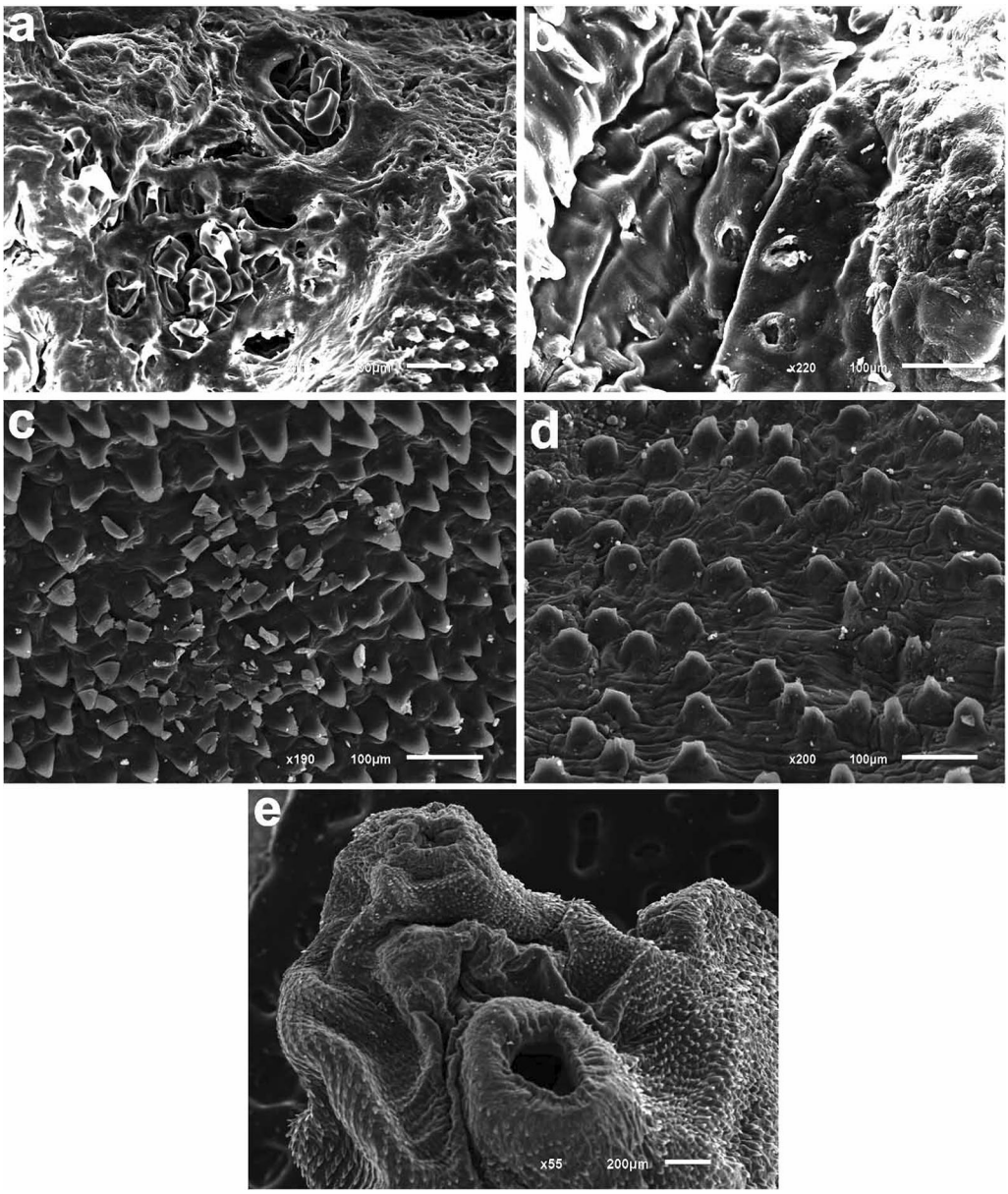

Fig. 4 SEM of triclabendazole-treated *Fasciola gigantica* adult worm. **a** Tegumental surface showing swelling and mild furrowing. **b** Moderate damaged spine dorsally. **c** Mild damaged spines on ventrally. **d** Thickness of the cuticle ranged from 258 to 270. µm. **e** Ventral sucker 243–250 µm

other hand, in vivo application requires a long time for the drug to be in a contact with the worms. Currently, nitroxynil had a potent and rapid flukicidal effect against *F. gigantica* adult worms.

Rafoxanide showed a less flukicidal effect. Paralysis of the treated flukes was recorded 75 min post application. Rafoxanide is a flukicide that causes depletion of glycogen, glucose-6-phosphate and ATP in mature flukes [17]. Salicylanilides hinder energy metabolism through uncoupling oxidative phosphorylation in the worm [20]. Salicylanilide flukicides: rafoxanide, closantel and oxyclozanide cause a quick paralysis in the flukes. For instance, closantel increased the muscle tone of *F. hepatica* followed by spastic paralysis within 2 h [41]. The egg deposition was reduced to 70–85% in rafoxanide-treated groups. Similarly, the efficacy of rafoxanide against *F. hepatica* in naturally infected sheep was 86–88% based on the used concentration [42]. Moreover, the efficacy of rafoxanide against *F. hepatica* in cattle was 90.1% [19]. Furthermore, the egg reduction of *F. hepatica*-infected

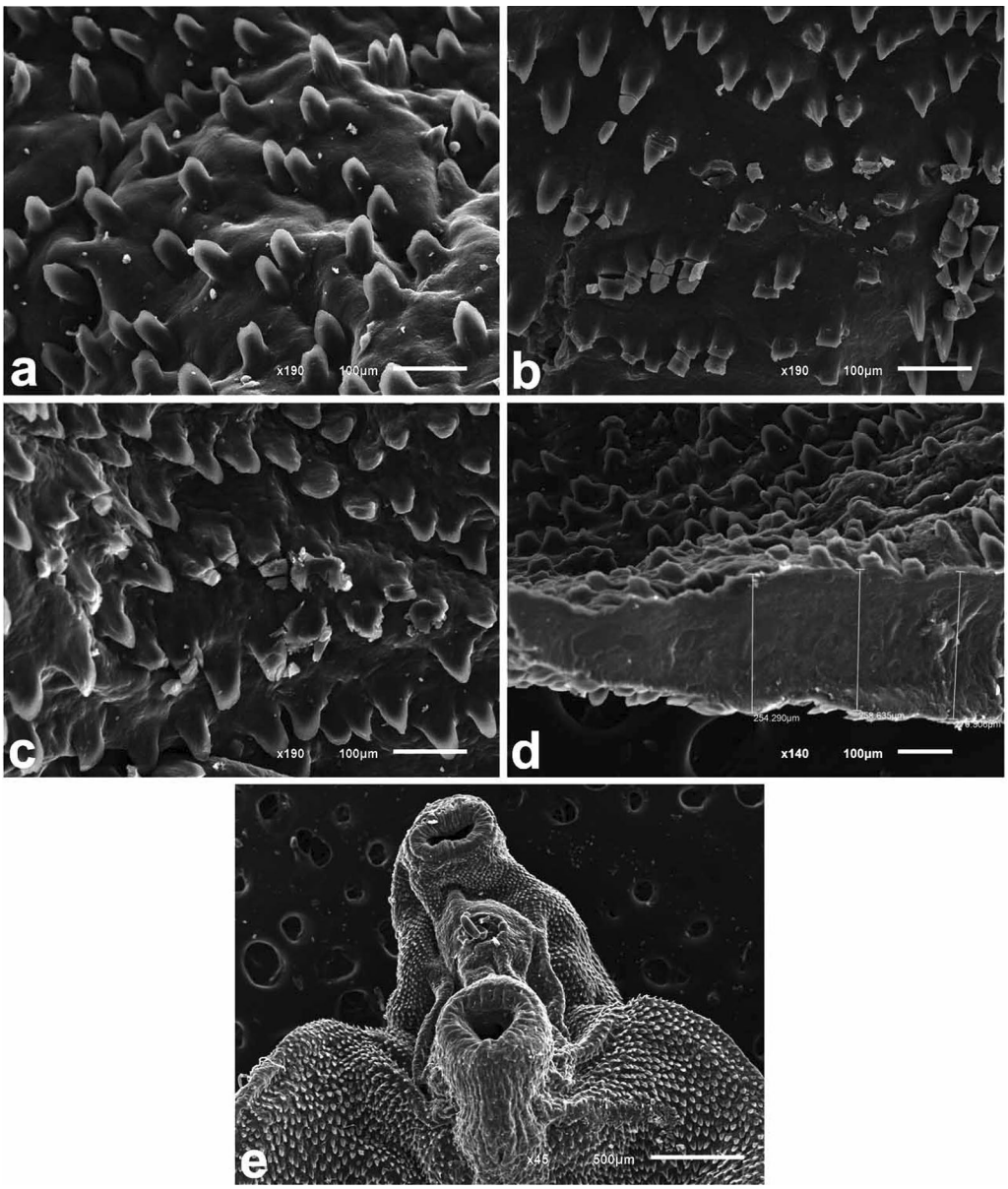

Fig. 5 SEM of albendazole-treated *Fasciola gigantica* adult worm. **a**, **b** Dorsal tegumental surface showing severe furrowing, and sloughed spines. **c**, **d** Ventral tegumental surface. **b** ,**c** Middle and tail tegumental surface showing moderate furrowing, and destruction in the spines. **e** Ventral sucker 250–260 µm

cattle and treated with rafoxanide was 68.2% [43]. In addition, albendazole and rafoxanide showed a fecal egg count reduction of 75–80.58% during 7–84 days post-treatment, respectively [44]. Meanwhile, the efficacy of rafoxanide against *F. gigantica* infected sheep was 98.78% [45]. In the current investigation, SEM findings revealed a moderate furrowing of the tegument, a thickening in the cuticle as well as destruction of spines on the anterior, middle, and the tail of both surfaces. These findings agreed with those reported by Skuce and Fairweather [41] who recorded that salicylanilide flukicide (closantel) caused sloughing of *F. hepatica* tegument post 24 h in vivo using SEM. Therefore, rafoxanide induced a flukicidal effect less than that induced by nitroxynil. Paralysis of the worms was observed but it was later than that caused by nitroxynil.

In triclabendazole-treated flukes, the motility of the worms was observed till 160 min post-treatment. Moreover, the tegument showed a moderate damage, swelling and mild furrowing. Similarly, *F. hepatica* worms

showed normal motility 48 h post-treatment and little morphological changes, after in vivo triclabendazole treatment, were observed [46]. Moreover, 24 h post in vitro treatment with triclabendazole (TCBZ) and its sulfoxide (TCBZ–SO) and sulfone (TCBZ–SO2) metabolites, flukes were actively motile and they had swelling and blebbing of the tegument [30, 46]. In the authors' opinion, the used concentrations of triclabendazole 50–100 µg/ml were higher than of the previous studies, thus, it induced more tegumental alterations. Triclabendazole reduced the egg deposition to 76.6–88.3%. Triclabendazole resistance was detected in Australia [25]. Such flukicide failed to treat sheep infected with *F. hepatica* in Northern Ireland [35]. Furthermore, *F. hepatica* was resistant to triclabendazole for the first time in Chile [37]. On the contrary, findings of the egg hatching assay of a previous work cleared an early embryonic lysis and stopped hatching of *F. gigantica* eggs at various concentrations of triclabendazole [27]. Notably, it was expecting that triclabendazole would be the most potent evaluated flukicide, but it seems that the resistance of such product has emerged in Egypt.

Albendazole had the least flukicidal effect; motility disappeared after the third hour of the treatment. Reduction of egg deposition was 43–75%. Although albendazole induced the most potent tegumental changes, flukes were active and deposited egg masses at the concentration of 400 µg/ml. Conversely, albendazole sulfoxide metabolite 10 µg/ml induced localized blebbing 24 h post-treatment [29]. In the authors' opinion, the used albendazole concentrations were high enough to induce clear tegumental damage but it could not induce a potent flukicidal effect. In our previous literature, albendazole showed 73.7% efficacy against *Fasciola* species in naturally infected cattle [27]. In addition, albendazole achieved 79.17% efficacy against fascioliasis in water buffaloes in the Philippines [47]. Moreover, a lower efficacy of albendazole (67%) against *F. hepatica* in sheep was recorded in Sweden [48]. Furthermore, albendazole showed 77–81.8% efficacy in cattle naturally infected with *F. hepatica* in Slovakia, and the ovicidal activity was 65.40% [49]. Thus, albendazole could not be considered a potent flukicide against fascioliasis among infected livestock.

5 Conclusion

Screening high concentrations of flukicides against *F. gigantica* added useful data that assist to select the suitable drug. Nitroxynil was the most potent flukicide, and the tegument of the treated worms showed severe damage. A lower efficacy of triclabendazole was documented for the first time in Egypt. Albendazole could not be recommended against fascioliasis. Further in vivo study will be conducted for a proper understanding the efficacy and or resistance of the commonly used flukicides against liver flukes in cattle and buffaloes in Egypt.

Abbreviations

ATP: Adenosine tri-phosphate; SEM: Scanning electron microscopy; BZ: Benzimidazoles; TCBZ: Triclabendazole.

Acknowledgements

Authors kindly acknowledge all veterinarians and authorities who facilitated specimens collection.

Author contributions

OR; samples collection, lab work, finding analysis, draft manuscript. WMA; designing, drafting manuscript, revising. ANW; drafting manuscript, KME; Designing, revising. All authors read and approved the final manuscript.

Funding

No funding sources.

Availability of data and materials

Data and materials are available.

Declarations

Ethics approvals and consent to participate

Ethics approvals and consent to participate not applicable, as no specimens were taken from condemned parts in abattoirs with any experiments done for animals or humans.

Consent for publication

All authors give their consent for the publication of this article.

Competing interests

Authors declare that there is no competing of interest.

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Received: 12 May 2022 Accepted: 17 August 2022
Published online: 29 August 2022

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