Tehran University of Medical Sciences Publication
http:// tums.ac.ir

Iran J Parasitol

Open access Journal at
http:// ijpa.tums.ac.ir

Iranian Society of Parasitology
http:// isp.tums.ac.ir

Short Communication

Tegumental Effects of Methanolic Extract of *Balanites aegyptiaca* Fruits on Adult *Paramphistomum microbothrium* (Fischoeder 1901) under Laboratory Conditions

***Hatem SHALABY, Soad NASR, Tarek FARAG**

Dept. of Parasitology and Animal Diseases, National Research Centre, Giza, Egypt

Received 22 Dec 2015
Accepted 08 Mar 2016

Keywords:
Paramphistomum microbothrium,
Balanites aegyptiaca,
In vitro effect,
Tegument

***Correspondence Email:**
shalaby85@gmail.com

Abstract

Background: Weak efficacy of different fasciolicidal compounds used for treatment of paramphistomosis has drawn the attention of many authors to alternative drugs. The purpose of this study was to assess, for the first time, the effect of the methanolic extract of *Balanites aegyptiaca* fruits (BAE) on adult *Paramphistomum microbothrium*.

***Methods*:** The effect of BAE on adult *P. microbothrium* after 24 h incubating the parasites in RPMI 1640 culture medium containing 10, 50, 100 and 200 μg/ml BAE was determined by light and scanning electron microscopic studies.

***Results*:** Differences in response to BAE action were concentration dependent.The major target organ that was highly affected was the tegument. Maximum anthelmintic activity was found with a dose of 200 μg/ml BAE, at which distinct damage to the whole body surface of the trematodes was very much distinct. Shape and structure of both suckers were deformed due to BAE. This damage would undoubtedly disrupt many of the physiological processes associated with the tegument. Besides, the damage of the tegumental folds of the acetabular region might disrupt its function in drawing the rumen wall tissue of the host into the acetabular cavity.

***Conclusion*:** the use of methanolic extract of *B. aegyptiaca* fruits offers a new dimension and potential for control of such a neglected infectious disease in ruminants, at a time when paramphistomosis has emerged as an important cause of productivity loss.

Introduction

Paramphistomosis has been an ignored trematode infectious disease in ruminants however, it is distributed cosmopolitan and has appeared as an important cause of productivity loss (1). Its economic loss may be greater than those caused by many other parasites (2). Death due to immature paramphistomes is very high, and may be as high as 80-90% in domesticated ruminants (3, 4). "Paramphistomosisis caused by specific species of the parasite depending on the regions" (1). In Egypt, *Paramphistomum microbothrium* is a standout amongst the vast majority species of paramphistomes (5). Adult paramphistomes are the main parasites in the rumen and reticulum of sheep, goats, cattle and water buffaloes. Although treatment for adult fluke has no direct benefit to the animal, it may reduce the source of infection for the snail intermediate host. This then reduces the size of the next generation of infective fluke larvae on pasture.

Herbs have been used as food and for medicinal purposes for centuries. However, the use of medicinal herbs has increased over the past few years. *Balanites aegyptiaca* Del., also known as 'desert date', a member of the family *Zygophyllaceae*, is one of the most common but neglected wild plant species of the dry land areas of Africa and South Asia (6). *B. aegyptiaca* fruits are commonly used to purge intestinal parasites, and have been found to be effective against *Fasciola gigantica*, *Schistosoma japonicum* (7), *Trichinella spiralis* (8) and *Toxocara vitulorum* (9). The fruit mesocarps are used for control of fresh water snails that act as intermediate host of Bilharzia (10) and of water flea that acts as an alternate host of the guinea worm (6). The methanolic extract of *B. aegyptiaca* revealed potent larvicidal effects against *Aedes aegypti* mosquito larvae (11). These findings motivated this study in order to assess the in vitro effects of methanolic extract of *B. aegyptiaca* fruits on attachment organs and tegument of adult *P. microbothrium*; as the integrity of the trematode tegument is essential for nutritive, immunoprotective and osmoregulatory functions (12).

Materials and Methods

Plant extract

The methanolic extract of *B. aegyptiaca* fruits was obtained as described previously (8). It was concentrated under reduced pressure on a Rotatory evaporator, and then dissolved in a vehicle mixture of liquid paraffin and Tween 80 (v/v) to obtain a 10% liquid extract.

Effect of B. aegyptiaca extract (BAE) on adult P. microbothrium

Adult worms of *P. microbothrium* were collected from the rumen of cattle slaughtered in a Cairo abattoir, and were identified according to the method of Sey and Abdel-Rahman (13). Under sterile conditions in a laminar flow cabinet, worms were washed in several changes of warm (37°C), sterile complete RPMI 1640 culture medium containing antibiotics (penicillin, 50 IU/ml; streptomycin, 50 mg/ml). They were subsequently transferred to fresh culture medium containing 50% (v/v) heat denatured rabbit serum; 2% (v/v) rabbit red blood cells, as recommended by Ibarra and Jenkins (14); and BAE at four different concentrations; 10, 50, 100 and 200 μg/ml. Then the whole worms were incubated for 24 h at 37°C in an atmosphere of 5% CO_2. Solvent control worms were incubated for 24 h in RPMI1640 culture medium containing 0.2 % (v/v) mixture of liquid paraffin and Tween 80. Normal control worms were fixed immediately following the initial washing. Six worms were examined for each concentration.

Light microscopy

Following incubation, adult worms of *P. microbothrium* were cut into small, 5-mm pieces before being fixed in 10% buffered formol saline. After dehydration, samples were embedded in paraffin and sectioned at 4-6 µm. Sections were stained with hematoxylin and eosin according to the method of Bancroft et al. (15). The tegument of adult flukes was studied and photographed using an Olympus CX41 microscope.

Scanning electron microscopy (SEM)

Following incubation, adult worms of *P. microbothrium* were fixed intact for 12 h in a 3:1 mixture of 4% (w/v) glutaraldehyde in 0.12 M Millonig's buffer, pH 7.4 and 1% aqueous osmium tetroxide. After this, the specimens were processed for SEM following a method previously reported (16).

Results

Light microscopic observations of the tegument cross section of adult P. microbothrium

Normal fresh and control flukes

The tegument of normal adult flukes showed even cytoplasmic syncytial layer. It rested on layer of connective tissue called basal lamina, which connected the former to the underlying and deeply stained two muscular layers. The tegumental cells were located deep among the parenchymal tissues. The interior of the parasite was filled with the parenchyma composed of loose connective tissues and parenchymal cells (Fig.1a, b).No significant differences were observed between fresh and control flukes incubated for 24 h in solvent (0.2 % (v/v) mixture of liquid paraffin and Tween 80).

Treated flukes

Flukes incubated in 10 µg/ml BAE for 24 h revealed only relatively slight swelling of tegument accompanied with corrugated appearance of its surface (Fig.1c). This swelling became pronounced, on increasing the concentration to 50 µg/ml (Fig.1d), and appeared so severe, with folded tegumental surface and blebbing on increasing the concentration to 100 µg/ml (Fig.1e). The strongest BAE effects were reached with concentration of 200 µg/ml (Fig.1f), where the treated flukes showed severe tegumental disruption, patches of tegument had been completely removed, exposing the basal lamina and severe damage was observed in muscle bundles and parenchymal tissues.

Scanning electron microscopic observations of the adult flukes

Normal fresh and control flukes

Adult *P. microbothrium* is pear-shaped with a broadly rounded posterior end and narrower anterior end. The oral aperture is terminally positioned surrounded by the oral sucker, and the acetabulum is subterminally positioned (Fig.2a). The anterior third of the body has clearly visible concentric aggregations of dome-shaped papillae (Fig.2b, inset). These papillae are very densely arranged towards the oral end, but decrease gradually toward the middle of the body before disappearance. Around the acetabular aperture, the tegument has folds irregularly directed with prominent domed papillae arranged singly or in groups of a few between the folds. The tegument covering the body is transversely folded or ridged (Fig.2a, inset), the folds being closer and more numerous near the anterior end, decreasing in number posteriorly. No significant differences were observed between fresh and control flukes incubated for 24 h in solvent (0.2 % (v/v) mixture of liquid paraffin and Tween 80).

Treated flukes

After 24 h of incubation with 10 µg/ml BAE, the changes in adult flukes concerned the anterior end other than the posterior one, which retained its normal morphology (Fig.2c). At the anterior third of the fluke's body, the tegument appeared to be slightly more swollen than normal with contractions to sucker's opening (Fig.2d). The papillae surrounding the oral aperture were slightly swollen with a few blebs covering their surfaces (Fig.2e). Swelling was also present towards the mid-body region of the fluke (Fig.2f).

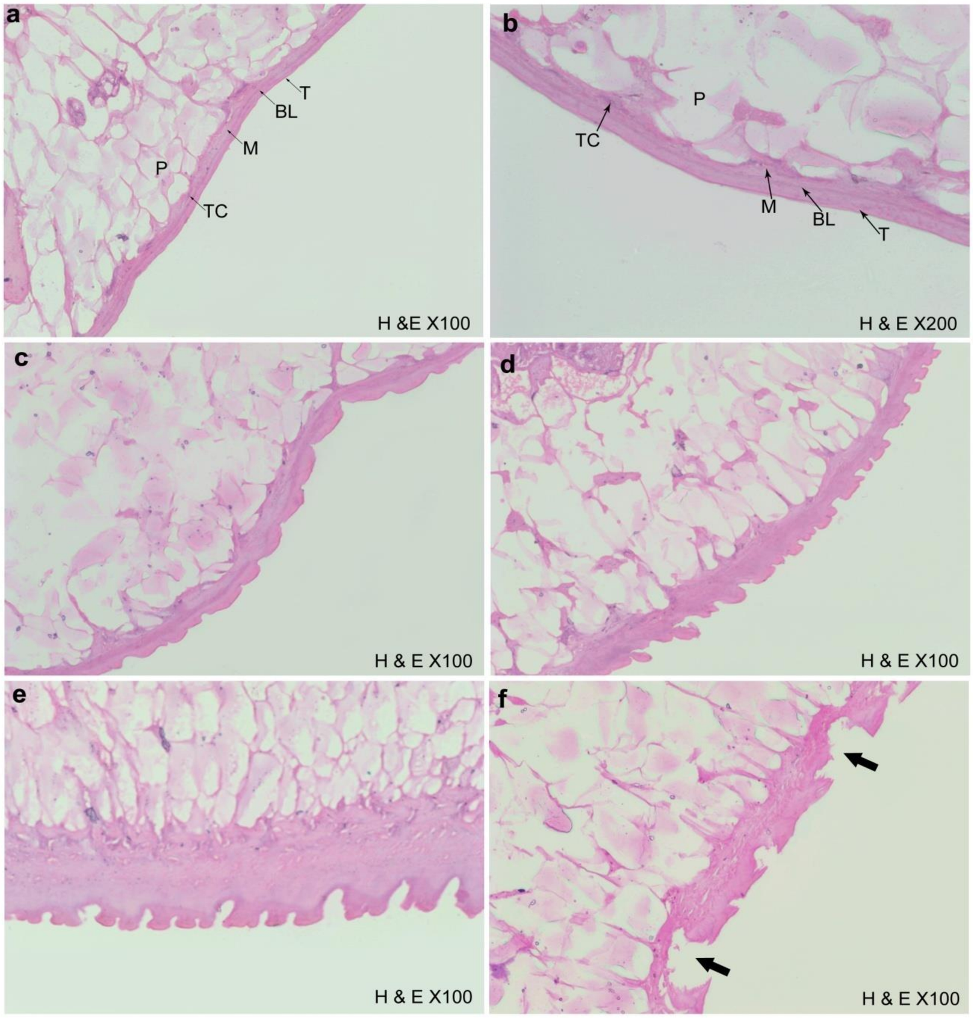

Fig. 1: Light micrographs of tegument cross section of adult *P. microbothrium*. (**a, b**) Normal fresh fluke. (**c**) Following 24 h incubation with 10 µg/ml *B. aegyptiaca* extract (BAE). Note a slightly swollen and corrugated tegument. (**d**) Following 24 h incubation with 50 µg/ml BAE. The swelling becomes pronounced. (**e**) Following 24 h incubation with 100 µg/ml BAE. The swelling becomes so severe; besides appearance of folded tegumental surface and blebbing. (**f**) Following 24 h incubation with 200 µg/ml BAE. Note severe tegumental disruption and complete removal of tegumental patches (*arrow*). *T* tegument, *BL* basal lamina, *M* muscular layer, *TC* tegumental cell, *P* parenchyma

After 24 h of incubation with 50 µg/ml BAE, the tegumental alterations were similar to that described for 10 µg/ml concentration, except that the blebbing became more pronounced (Fig.3a-d). The papillae surrounding the oral aperture exhibited severe blebbing in the form of small bulbous structures at their surfaces; some of them were disrupted causing a number of pits on the tegumental surface (Fig.3c). Besides, a few large blebs extended towards the mid-body region of the fluke (Fig.3 d). The degrees of tegumental changes became more severe, on increasing the concentration of BAE to 100 µg/ml. Both oral sucker and acetabulum were distorted in most of examined specimens (Fig.4a, b). The tegumental surface around the oral sucker exhibited severe blebbing on top of papillae (Fig.4c). There were wide and deep furrows between the transverse folds; because of tegumental swelling (Fig.4 d).

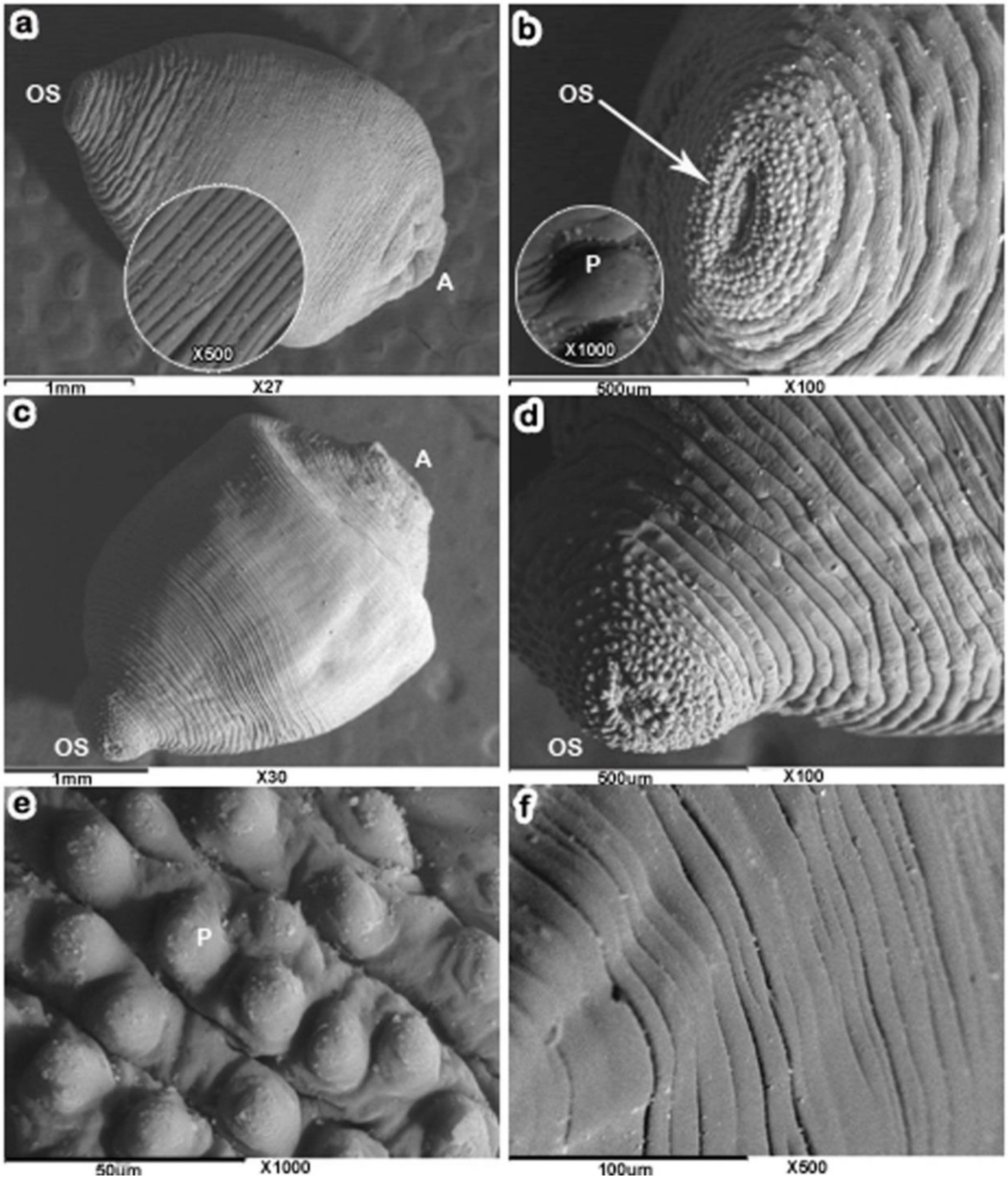

Fig. 2: Scanning electron micrographs (SEMs) of adult *P. microbothrium*. (**a, b)** Normal fresh fluke. (**a)** SEM of entire fluke showing broadly rounded posterior end and narrower anterior end. The tegument shows folds or ridges, closer to each other near the anterior end of the fluke (*inset*). (**b)** SEM of anterior third of the body showing concentric aggregations of dome-shaped papillae (*inset*) arranged towards the oral end. (**c- f)** Following 24 h incubation with 10 µg/ml BAE. The anterior third and the mid-body regions of the fluke's body show slightly swollen tegument and papillae, with a few blebs covering their surfaces. *OS* oral sucker, *A* acetabulum, *P* papilla

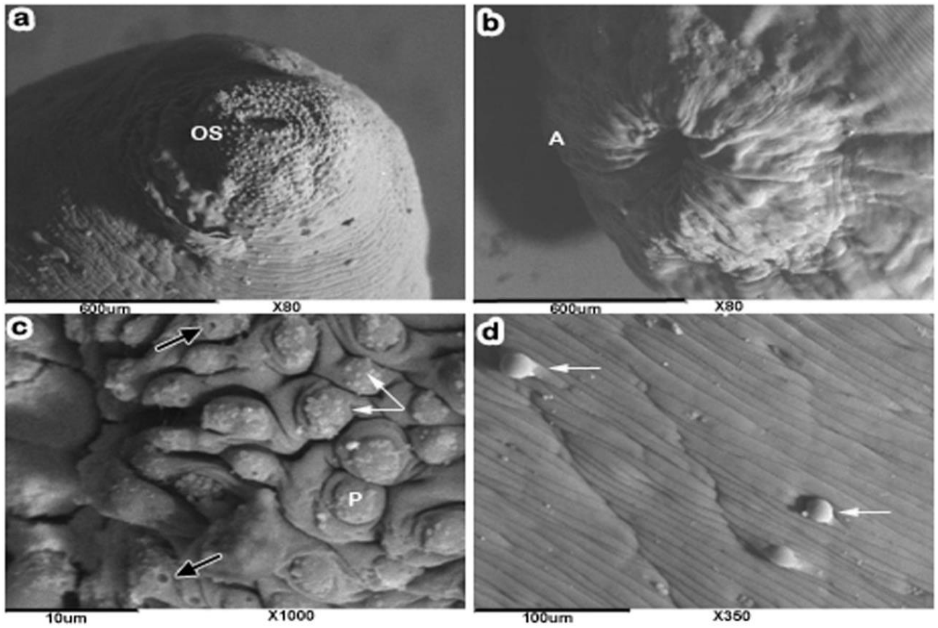

Fig. 3: SEMs of adult *P. microbothrium* following 24 h incubation with 50 µg/ml BAE. (**a- c)** SEMs of anterior and posterior ends reveal severe blebbing surrounding the oral aperture (*white arrow*), and a number of pits resulted from disruption of some blebs (*black arrow*). (**d)** SEM of the mid-body region showing a few large blebs (*white arrow*). *OS* oral sucker, *A* acetabulum, *P* papilla

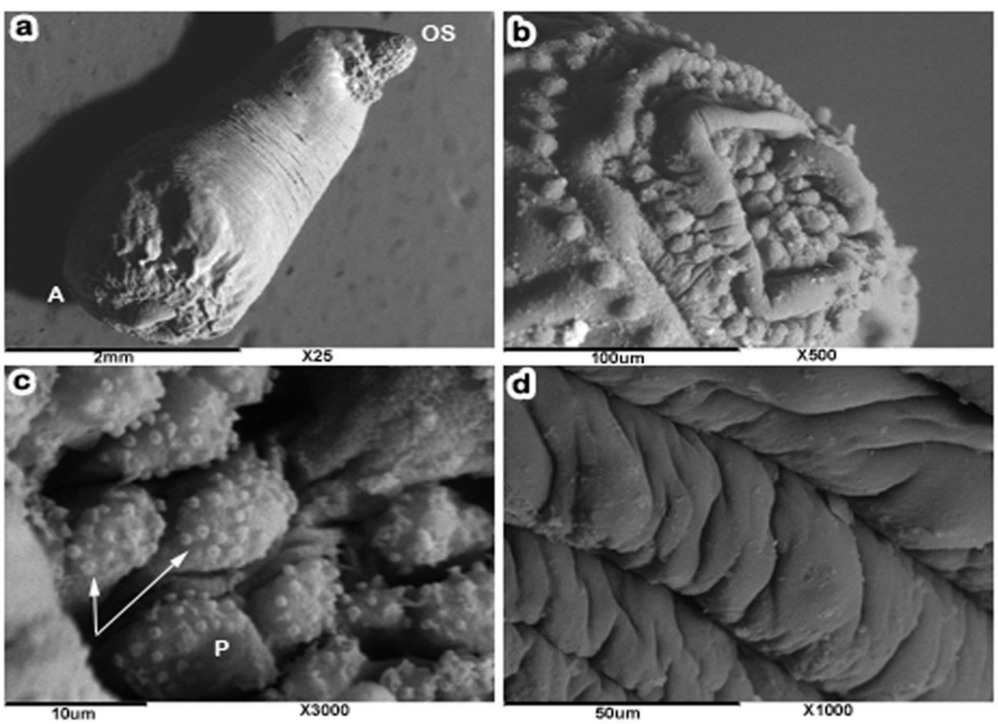

Fig. 4: SEMs of adult *P. microbothrium* following 24 h incubation with 100 µg/ml BAE. (**a, b)** SEMs revealing distortion of both oral sucker and acetabulum. (**c**) SEM of the tegumental surface showing severe blebbing on top of papillae (*arrow*). (**d)** SEM of the tegument showing wide and deep furrows between the transverse folds. *OS* oral sucker, *A* acetabulum, *P* papilla

With 200 μg/ml BAE, severe tegumental disruption was evident in all regions of the fluke (Fig.5a) in the majority of examined specimens. The oral sucker was retracted and deformed, and the concentric tegumental rings encircling the oral aperture were no longer seen, but were substituted by tegumental ridges (Fig.5b). The tegument at the anterior third of the fluke's body was more swollen so that the papillae appeared to be submerged by the tegument, which gave the surface a smooth appearance. Some areas of the tegument were characterized by a number of pits caused by rupture of papillae (Fig.5b, inset). In the mid-body region, severe damage was apparent in the form of focal erosions of the surface; resulted from rupture of blebs (Fig.5 c). This disruption was more severe in the acetabular region, with large areas of tegument were completely removed; exposing the basal lamina (Fig.5d). In this region, several flukes displayed a large swelling that was clearly visible to the naked eye. One fluke, however, displayed extreme disruption with a large hole that had penetrated completely through the tegument (Fig.5 a).

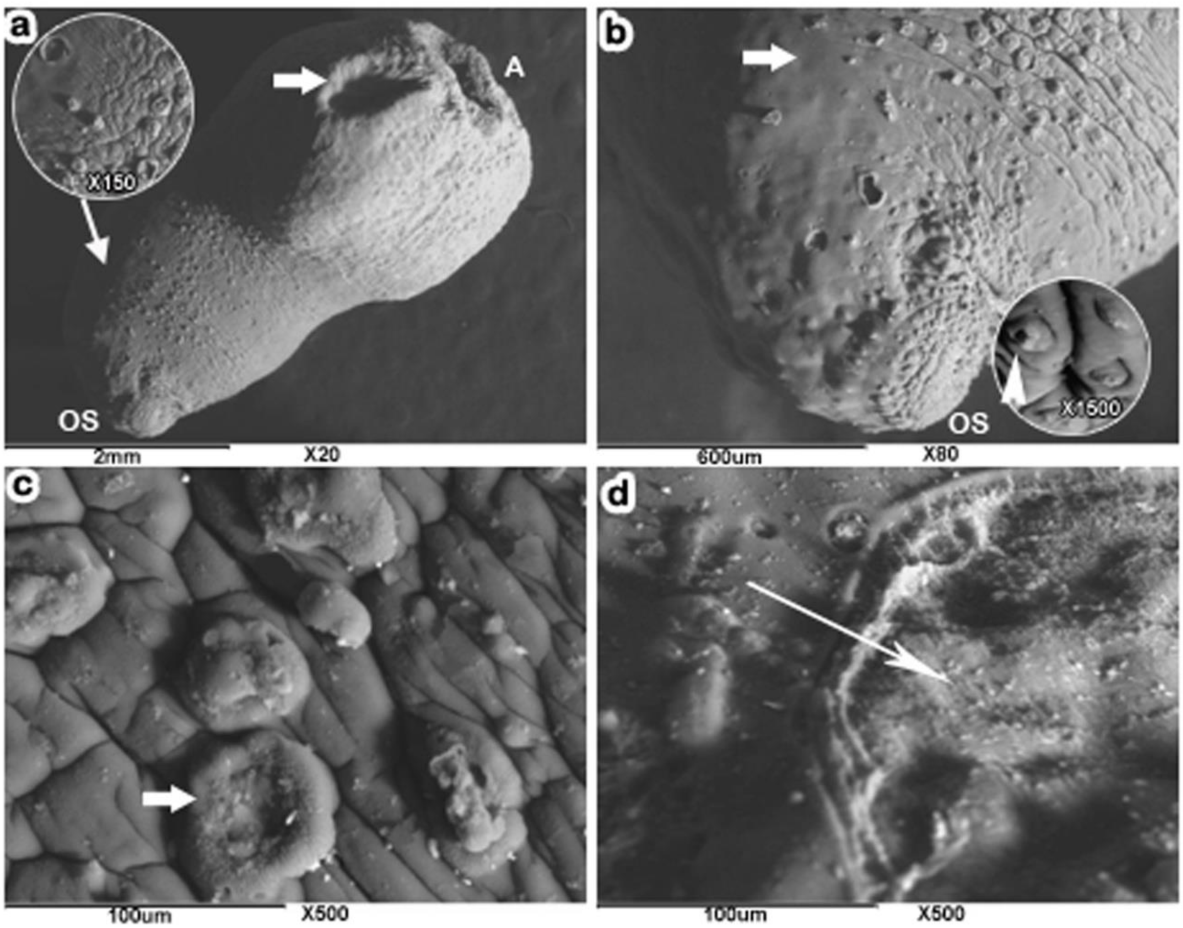

Fig. 5: SEMs of adult *P. microbothrium* following 24 h incubation with 200 μg/ml BAE. (**a**) Entire worm revealing tegumental disruption in all regions of the fluke, with a large hole penetrated completely through the tegument (*arrow*). (**b**) Tegumental swelling at the anterior third of the fluke's body so that the papillae appeared to be submerged by the tegument (*arrow*). Inset shows a number of pits caused by rupture of papillae (*head arrow*). (**c**) Focal erosions of the surface resulted from rupture of blebs (*arrow*). (**d**) Large area of the tegument, at the acetabular region, is completely removed exposing the basal lamina (*arrow*). *OS* oral sucker, *A* acetabulum

Discussion

In this study, we have assessed, for the first time, the effect of BAE on adult worms of *P. microbothrium*. Differences in response to BAE action were concentration dependent. The major target organ that was highly affected was the tegument, whose damages were observed by LM and SEM studies. LM was used to observe changes in a limited area of the tegumental syncytium while tegumental surface changes, which reflected the changes in the tegument cytoplasm, could be observed over a much wider area by SEM. These changes occurred in definite sequences in response to BAE concentration, consisted of swelling, blebbing that was later disrupted, leading to erosion and desquamation of the tegument, resulting in the lesion, and finally the exposure and disruption of basal lamina. Maximum anthelmintic activity was found with a dose of 200 μg/ml BAE, at which distinct damage to the whole body surface of the trematodes was very much distinct. Shape and structure of both suckers were deformed due to BAE. This damage would undoubtedly disrupt many of the physiological processes associated with the tegument, including osmoregulation, protection, secretion and synthesis (17). Besides, the damage of the tegumental folds of the acetabular region might disrupt its function in drawing the rumen wall tissue of the host into the acetabular cavity (18) resulting in a weak hold for the parasite. Surface changes observed in this study resembled that demonstrated on *P. explanatum* treated with methanolic extracts of leaves of *Bombax malabricum* (19) and *Dregea volubilis* (20). Besides, similar sequence of tegumental changes occurred in *P. microbothrium* treated with artemether; which was obtained from the leaves of qinghao (16) as well as *P. cervi* treated with plumbagin; a compound that was rich in the roots of *Plumbago indica/rosea* (21). Moreover, the tegumental surface alterations induced by BAE, in the present study, had also been observed in specimens of a biologically related trematode, *Fasciola gigantica*, following incubation with a number of anthelmintics (22).

Surface blebbing was a common feature of drug-treated parasites and had been described for other trematodes and flatworm parasites after exposure to anthelmintics (23). It had been suggested that the blebbing occurred because of increased efforts on the part of the parasite to shed and replace outer tegumental membrane damaged by drug action (24). It might be significant, then, that the process of blebbing appeared to start at the surfaces of papillae leading to their rupture. The rupture of sensory papillae induced by BAE, in the present study, had also been observed following treatment with artemether (16) and in specimens of a closely related trematode, *Cotylophoron cotylophorum*, treated *in vitro* with praziquantel, after 30 h of exposure (25). The damage that BAE caused to the papillae would undoubtedly disrupt many of their sensory functions. The disruption of the tegument became more severe, on increasing BAE concentration and then more widespread sloughing occurred. In the highest BAE concentration (200 μg/ml), damage to one fluke was so severe that a hole had penetrated through its entire tegument. Such damage had only been observed in specimens of *Fasciola gigantica* treated *in vitro* with the sulphoxide metabolite of triclabendazole (23).The literature survey on *B. aegyptiaca* suggested that methanolic extracts of the plant's tissues had anthelmintic activity (9), with saponins being one of the major secondary metabolites in plant tissues (26). Wiesman and Chapagain (11) reported a strong correlation between the saponin content of methanolic extracts of *B. aegyptiaca* and mortality of larvae of the mosquito *Aedes aegypti*. This activity was confirmed by Gnoula et al. (27) who isolated a steroidal saponin with a high potential of anthelmintic activity, Balanitin-7. Saponins to which balanitins belong were widely distributed in plants. Their surface-active properties were what distinguished these compounds from other glycosides (28).

Saponins had a broad range of biological properties. Most were haemolytic and toxic to cold-blooded animals, while certain saponins also displayed molluscicidal, anti-inflammatory, anti-fungal, anti-bacterial, anti-parasitic and anti-viral activities. Numerous reports had also highlighted the highly cytotoxic properties of many saponins (28). Therefore, it might be speculated that phytochemicals, most probably saponins, found in BAE might be responsible for its potent anthelmintic activity.

Conclusion

BAE has a potent and progressive effect on the tegument of *P. microbothrium*. The surface changes induced by this plant *in vitro* may serve to illustrate why it is so effective against a biologically related trematode, *F. gigantica* in vivo. The use of methanolic extract of *B. aegyptiaca* fruits offers a new dimension and potential for control of such a neglected infectious disease in ruminants, at a time when paramphistomosis has emerged as an important cause of productivity loss.

Acknowledgements

The authors declare that there is no conflict of interest.

References

1. Anuracpreeda P, Wanichanon C, Sobhon P. *Paramphistomum cervi*: Antigenic profile of adults as recognized by infected cattle sera. Exp Parasitol. 2008; 118: 203-207.
2. Hanna REB, Williamson DS, Mattison RG, Nizami WA. Seasonal reproduction in *Paramphistomum epiclitum* and *Gastrothylax crumenifer*, rumen paramphistomes of the Indian water buffalo, and comparison with the biliary paramphistome *Gigantocotyle explanatum*. Int J Parasitol. 1988; 18: 513-521.
3. Ilha MR, Loretti AP, Reis AC. Wasting and mortality in beef cattle parasitized by *Eurytrema coelamaticum* in the state of Parana, southern Brazil. Vet Parasitol. 2005; 133: 49-60.
4. Khani UJ, Tanveerl A, Maqbool A, Masood S. Epidemiological studies of paramphistomosis in cattle. Vet Arc. 2008; 78: 243-251.
5. Hiekal F, Hilali M. Scanning electron microscopy of the tegument of *Paramphistomum microbothrium* Fischoeder, 1901 and *Cotylophoron cotylophorum* (Fischoeder 1901) in Egypt. Arab Gulf J Sci Res. 1993; 11: 105-113.
6. Hall JB, Walker DH. *Balanites aegyptiaca* Del.- A Monograph. School of Agricultural and Forest Science, University of Wales, Banger, 1991, pp. 1-12
7. Koko WS, Galal M, Khalid HS. Fasciolicidal efficacy of *Albizia anthelmintica* and *Balanites aegyptiaca* compared with albendazole. J Ethnopharmacol. 2000; 71: 247-252.
8. Shalaby MA, Moghazy FM, Shalaby HA, Nasr SM. Effect of methanolic extract of *Balanites aegyptiaca* fruits on enteral and parenteral stages of *Trichinella spiralis* in rats. Parasitol Res. 2010; 107: 17-25.
9. Shalaby HA, El Namaky AH, Khalil FA, Kandil OM. Efficacy of methanolic extract of *Balanites aegyptiaca* fruit on *Toxocara vitulorum*. Vet Parasitol. 2012; 183: 386-392.
10. Kloos H, McCullough FS. Plant molluscicides. Planta Med. 1982; 46: 195-209.
11. Wiesman Z, Chapagain BP. Larvicidal activity of saponin containing extracts and fractions of fruit mesocarp of *Balanites aegyptiaca*. Fitoterapia. 2006; 77: 420-424.
12. Skuce PJ, Fairweather I. *Fasciola hepatica*: the effect of the sodium ionophore monensin on the adult tegument. Parasitol Res. 1989; 75: 223-232.
13. Sey O, Abdel-Rahman MS. Studies on *Paramphistomum* species of cattle and sheep in Egypt. Assiut Vet Med J. 1975; 2: 145-149.
14. Ibarra OF, Jenkins DC. An in vitro screen for new fasciolicidal agents. Zeitschrift für Parasitenkunde.1984; 70: 655-661.
15. Bancroft JD, Stevens A, Wiley-Liss. Theory and Practice of Histological Techniques, 4th ed. Churchill Livingstone, New York/London/San Francisco/Tokyo, 1996.
16. Shalaby HA, El Namaky AH, Kamel ROA, Derbala AA. Tegumental surface changes in adult *Paramphistomum microbothrium* (Fischoeder

1901) following in vitro administration of artemether. J Helminthol. 2010; 84: 115-122.
17. Halton DW. Microscopy and the helminth parasite. Micron. 2004; 35: 361-390.
18. Nollen PM, Nadakavukaren MJ. *Megalodiscus temperatus*: scanning electron microscopy of the tegumental surfaces. Exp Parasitol. 1974; 36: 123-130.
19. Hossain E, Chandra G, Nandy AP, Mandal SC, Gupta JK. Anthelmintic effect of a methanol extract of *Bombax malabaricum* leaves on *Paramphistomum explanatum*. Parasitol Res. 2012a; 110: 1097-1102.
20. Hossain E, Chandra G, Nandy AP, Mandal SC, Gupta JK. Anthelmintic effect of a methanol extract of leaves of *Dregea volubilis* on *Paramphistomum explanatum*. Parasitol Res. 2012b; 110: 809-814.
21. Saowakon N, Lorsuwannarat N, Changklungmoa N, Wanichanon C, Sobhon P. *Paramphistomum cervi*: The in vitro effect of plumbagin on motility, survival and tegument structure. Exp Parasitol. 2013; 133: 179-186.
22. Massoud AM, Shalaby HA, El Khateeb RM, Mahmoud MS, Kutkat MA. Effects of Mirazid® and myrrh volatile oil on adult *Fasciola gigantica* under laboratory conditions. Asian Pac J Trop Biomed. 2012; 2: 875-884.
23. Meansy M, Fairweather I, Brennan GP, Ramasamy P, Subramanian PB. *Fasciola gigantica*: tegumental surface alterations following treatment in vitro with the sulphoxide metabolite of triclabendazole. Parasitol Res. 2002; 88: 315-325.
24. Stitt AW, Fairweather I (1993) *Fasciola hepatica*: tegumental surface changes in adult and juvenile flukes following treatment *in vitro* with the sulphoxide metabolite of triclabendazole (Fasinex). Parasitol Res. 1993; 79:529-536.
25. Veerakumari L, Munuswamy N. *In vitro* studies on the effects of some anthelmintics on *Cotylophoron cotylophorum* (Digenea, *Paramphistomidae*): a structural analysis.Cytobios.1999; 98: 39-57.
26. Chapagain BP, Wiesman Z. Metabolite profiling of saponins in *Balanites aegyptiaca* plant tissues using LC (RI)–ESI/MS and MALDITOF/MS. Metabolomics. 2008; 4: 357-366.
27. Gnoula C, Guissou P, Duez P, Frederich M, Dubois J. Nematocidal compounds from the seeds of *Balanites aegyptiaca* isolation and structure elucidation. Int J Pharmacol. 2007; 3: 280-284.
28. Sparg SG, Light ME, van Staden J. Biological activities and distribution of plant saponins. J Ethnopharmacol. 2004; 94: 219-243.