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An Anthelmintic Assessment of *Balanites aegyptiaca* Fruits on Some Multiple Drug Resistant Gastrointestinal Helminthes Affecting Sheep

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THE goal of this study is to evaluate the effect of *Balanites aegyptiaca* methanolic extract fruits as an alternative sustainable source of anthelmintic drugs, on adult gastrointestinal multiple drug resistant helminthes and clarify how *B. aegyptiaca* fruits directly affect helminthes. To fulfill this study, three hundred and fifty sheep reared in a private farm, in Giza Governorate, had been subjected to clinical investigation. Of them, fifty five severely affected sheep had been isolated relying on their clinical history of multiple anthelmintic resistances, to be slaughtered. The post mortem examination revealed that the animals had a mixed infection with *Haemonchus contortus*, *Moniezia expansa* and *Fasciola gigantica*. Light and scanning electron microscopic studies were undertaken to evaluate changes in body wall of these adult worms following 24 h exposure to methanolic extract of *B. aegyptiaca* fruits utilizing concentrations of 10, 50, 100, 200 and 400 µg/ml. The main target organ that was highly affected was the body wall, whose damage was observed by light and scanning electron microscopic studies. These alterations occurred in particular sequences in response to *Balanites* extract concentration, initiated with swelling that was even disrupted, resulting in sloughing off cuticle or tegument, and finally exposure and disruption of basal lamina. This damage would induce disturbance for many of the physiological processes associated with the body wall, especially nutrition and immunoprotection. This plant may offer an opportunity for a new natural anthelmintic and an alternative source for the control of such infectious disease in sheep.

Keywords: Sheep, *Haemonchus contortus, Moniezia expansa, Fasciola gigantica, Balanites aegyptiaca* fruits.

Introduction

The problem of gastrointestinal helminth infections in small ruminants and the development of anthelmintic resistance have been well documented worldwide, inflicting heavy production losses in grazing animals [1]. The control of infections with gastrointestinal nematodes, cestodes and liver flukes in sheep industry still relies on the use of anthelmintics. However, the continuous occurrence of multiple drug resistant helminthes, beside the increased awareness of consumers about drug residues, have directed the attention to medicinal plants as an alternative sustainable source of anthelmintic drugs [2]. Desert date (*Balanitesa egyptiaca* L. Delile) is a tiny tree of the Zygophllaceae family, produces yellow date-like fruit, nearly ten thousands fruits annually [3]. *B. aegyptiaca* is one of the extremely spread woody plants in African, and has been grown in Egypt for more than four thousand years. This fruit is a rich source of beneficial nutrients and is utilized in a variety of ways in Africa [4]. The efficacy of methanolic extract of *B. aegyptiaca* fruits had been reported

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against *Paramphistomum microbothrium*, *Toxocara vitulorum*, *Trichinella spiralis* [5, 6, 7], *Schistosoma japonicum* and *Fasciola gigantica* [8]. It had revealed potent molluscicidal effects against intermediate host of Bilharizia [9], and anti larval effect against larvae of *Aedes aegypti* mosquito [10]. It is commonly used to purge intestinal parasites, yet it is unclear how *B. aegyptiaca* fruits directly affect helminthes. The body wall of helminthes is a main absorptive surface for the drugs' uptake, by the worm and considered an incipient drug target [11]. Thus, light and scanning electronmicroscope (SEM) studies were undertaken to evaluate changes in thebody wall of some multiple drug resistant gastrointestinal helminthes following exposure to methanolic extract of *B. aegyptiaca* fruits in vitro.

Materials and Methods

Ethics approval

The experiments were conducted in compliance with the requirements and recommendations of the Ethical Committee of the National Research Centre and the current Egyptian Law and Regulations that assigned for the protection of the experimental animals to minimize the negative states (harms) and improve feeding and housing conditions under certificate number (16229). Consent was obtained from the owners of sheep included in this study. All authors read and approved the final article.

Animals

This study was conducted on three hundred and fifty sheep reared in a private farm in Giza Governorate. The animals had been subjected to clinical investigation. Fifty five severely diseased animals were isolated, based on their multiple anthelmintics resistances and sacrificed.

Plant extract

B. aegyptiaca fruits were procured from the local markets in Aswan, Upper Egypt and authenticated at the Herbarium of National Research Centre. Soaking of the mesocarps (1 kg) was done in methanol 90% for about 48 h. Followed the extraction utilizing Soxhlet apparatus, concentration of the methanolic extract was performed on a rotatory evaporator under reduced pressure then dissolved in a vehicle mixture of liquid paraffin and Tween 80 (v/v) to gain a 10% liquid extract [5].

Anthelmintic assays

Adult helminthes of *H. contortus*, *M. expansa* and *F.gigantica* were compiled from abomasa, intestines and bile ducts, successively, of naturally infected sheep after slaughtering. After recovery, the worms were washed thoroughly several time in warm (37°C) sterile Ringer solution for nematode and complete RPMI 1640 culture medium for both cestode and trematode having antibiotics (penicillin, 50 IU/ml; streptomycin, 50 mg/ml). Under sterile conditions, five concentrations of *Balanites methanolic* extract were prepared including 10, 50, 100, 200 and 400 µg/ml and added into culture medium, whole fresh motile worms were put, as recommended by Hanser et al. and Ibarra and Jenkins [12, 13] thensubsequently maintained for 24 h at 37°C in 5% CO2 atmosphere.Five worms were examined for each concentration. Thesolvent control worms were put in culture medium having 0.2 % (v/v) mixture of liquid paraffin and Tween 80, for 24 hwhile the normal control worms were fixed immediately after washed.

Light microscopy

After 24h incubation, the adult worms were sectioned into tiny specimens about 5mm, then treated with 10% buffered formol saline for fixation, and processed as described by Bancroft et al. [14]. The adult worms' body wall was studied and illustrated utilizing an Olympus CX41 microscope.

SEM

After 24 h of incubation with 400 µg/ml *Balanites* extract, fixation of the anterior end of adult worms intact was done 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. Then, the samples were prepared for SEM as mentioned by Shalaby et al. [6].

Results

Clinical examination

The animals were suffering from general weakness, pallor mucous membranes, weight loss, and recurrent diarrhea.

Post mortem examination

The examination of the gastrointestinal tract and liver of the isolated and severely affected sheep exposed that the animals were infected with *H. contortus, M. expansa* and *F. gigantica.*

Light microscopic findings

H. contortus

The body wall of normal *H. contortus* anterior end (Fig.1a, b) had three layers; cuticle, hypodermis and an inner layer of muscle cells.

The hypodermis, lying beneath the cuticle, was relatively thin and consisted of a syncytium of cells. Internal to the hypodermis were one or more layers of longitudinally arranged striated muscles. These were exceedingly tied with the hypodermis and also connected to the cuticle through fibers passing from the contractile part of each muscle cell.

The results revealed that the incubation of worms with 100 µg/ml *Balanites* extract for 24 h had induced cuticular changes in form of swelling of the cuticle and vacuolization of the hypodermis (Fig.1c, d). By exceeding the concentration to 200 µg/ml,a disruption of the outer cuticular layer was detected (Fig.1e).While atconcentration of400 µg/ml *Balanites* extract, the cuticular disruption became more pronounced and exhibited small isolated areas of cuticular splitting. Some specimens showed small areas of complete cuticularsloughing accompanied with distortion of the subcuticular region and disorganization of the cuticular musculature (Fig.1f, g).

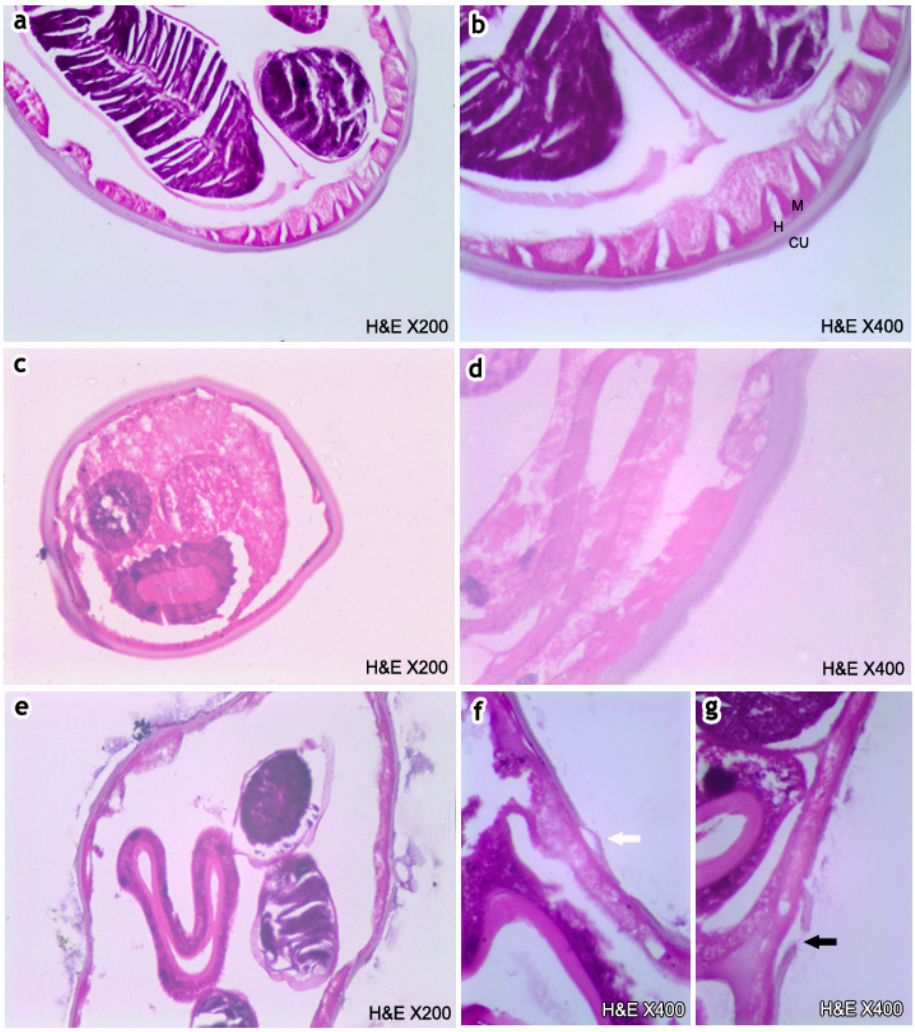

Fig. 1. Light microscopy of the body cover cross section of adult *H. contortus*. (a, b) Normal fresh worm showing three layers of the body wall; cuticle, hypodermis and an inner layer of muscle cells. (c, d) Following 24 h incubation with 100 µg/ml *Balanites* extract. Note swelling of the cuticle and vacuolization of the hypodermis. (e) Following 24 h incubation with 200 µg/ml Balanites extract. The micrograph shows disruption of the outer cuticular layer. (f, g) Following 24 h incubation with 400 µg/ml Balanites extract. Note small isolated areas of cuticular splitting (white arrow) and/or small areas of complete cuticular sloughing accompanied with distortion of subcuticular region and disorganization of the cuticular musculature (black arrow). CU cuticle, H hypodermis, M muscle layer.

M. expansa

The tegument of normal *M. expansa* gravid segment (Fig.2a, b) showed intensively stained syncytial layer with wave-like infoldings on the dorsal and ventral surfaces of the segment. Those infoldings were coated with cilia-like processes called microtriches; which are essential for the nutritive function, and rich in vesicles and rhabdiform organelles; an organelle characteristic of flatworms in general. Beneath the outer tegumentary layer was a sheath of muscle fibers; an extrinsic rounded muscle layer and an intrinsic longitudinal muscle layer. Those layers were not massively developed, and followed by subtegumentary layer of branching parenchymal cells filled the whole space around the uterine branches.

The results showed that exposure of worms to 10 µg/ml *Balanites* extract for 24 h caused swelling of tegument in comparison with the normal, to the extent that fusion of the tegumentalinfoldings had occurred (Fig.2 c,d), with separation of microtriches from some areas of the tegument. These changes became more severe with microtriches entirely separated from tegument, on increasing the concentration to 50 µg/ml *Balanites* extract (Fig.2 e,f). With 100 µg/ml *Balanites* extract (Fig.2 g,h)., severe distortion was distinct in the tapeworm's tegument. Patches of microtriches were entirely removed and severe deterioration had been occurred in muscle bundles and parenchymal tissues. The most potent extract impacts were recorded in both 200µg/ml (Fig.2 i, j) and 400µg/ml (Fig.2 k - m) *Balanites* extract which caused focal areas of huge swelling of the tegument and entire damage of the muscle cells. In some areas, massive tearing out of the tegument was found, with remaining of the basal lamina.

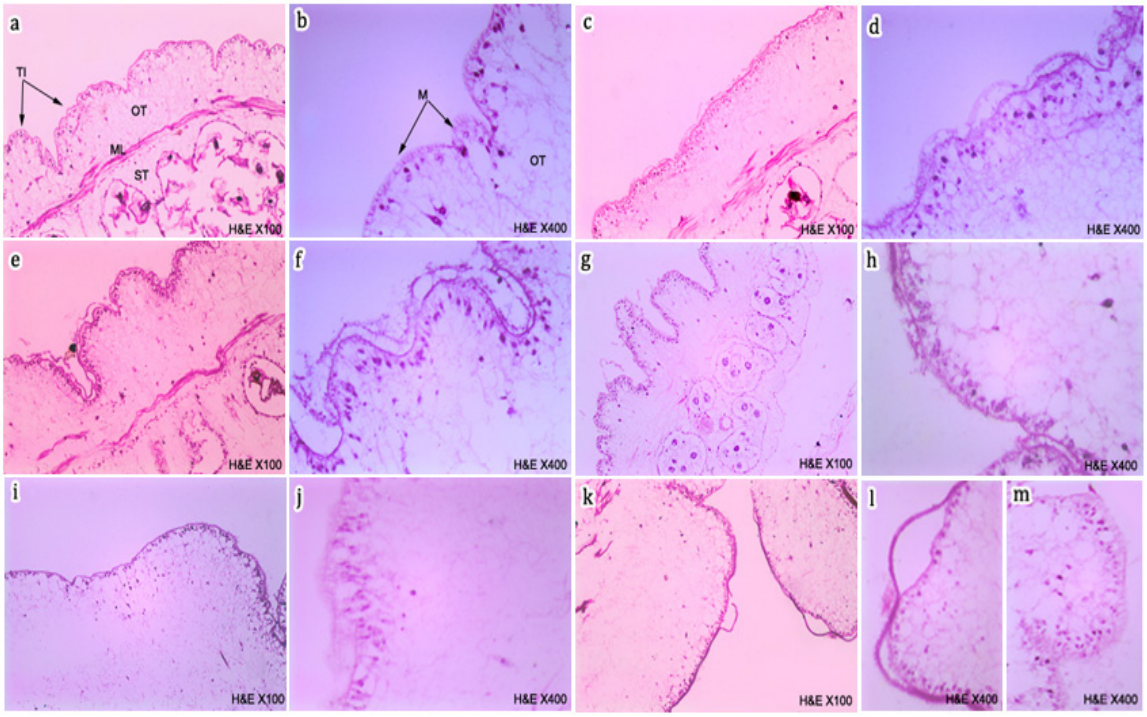

Fig.2. Light micrographs of the tegument cross section of adult M. expansa gravid segment. (a, b) Normal fresh worms. Note cilia-like processes called microtriches coated the tegumental infoldings. (c, d) Following 24 h incubation with 10 µg/ml Balanites extract. Note swollen tegumental infoldings with separation of microtriches from some areas of the tegument. (e, f) Following 24 h incubation with 50 µg/ml Balanites extract. The swelling becomes so severe with microtriches entirely separated from the tegument. (g, h) Following 24 h incubation with 100 µg/ml Balanites extract. Note removal of patches of the microtriches with severe damage of muscle bundles and parenchymal tissues. Following 24 h incubation with both (i, j) 200 µg/ml Balanites extract and (k - m) 400 µg/ml Balanites extract, the micrographs show focal areas of huge swelling of the tegument, complete disruption of the muscle cells and extensive tegumental sloughing. TI tegumental infolding, OT outer tegument, ML muscle layer, ST subtegument, M microtriches.

F. gigantica

The tegument of normal *F. gigantica* mid-body region showed even and intensely stained cytoplasmic syncytial layer present on layer of connective tissue named reticular lamina, which attached the former to the underlying two muscular layers (Fig.3 a, b). The incubation of worms for 24 h with 10 µg/ml *Balanites* extract had induced swelling of the tegument in contrast to the normal while the underlying structures still appeared normal (Fig.3c, d). This swelling turned to be more obvious, by exceeding the concentration to 50 µg/ml *Balanites* extract (Fig.3e, f). With the higher concentration of 100µg/ml *Balanites* extract, swelling and wrinkling of the tegument were observed accompanied with appearance of small vacuoles in the tegumentalsyncytium (Fig.3 g, h). The greatest effect was recorded utilizing *Balanites* extract at 200 and 400 µg/ml (Fig.3 i,j) and (Fig.3 k,l) respectively, where, prominent wrinkles of the tegument and severe damage of the tegumental syncytium were notable. Moreover, the tegument seemed swollen with extensive damage towards its surface and severe vacuolization towards the basal lamina. Severe disruption was detected in the muscle bundles which looked like to be isolated from the surrounding tissue.

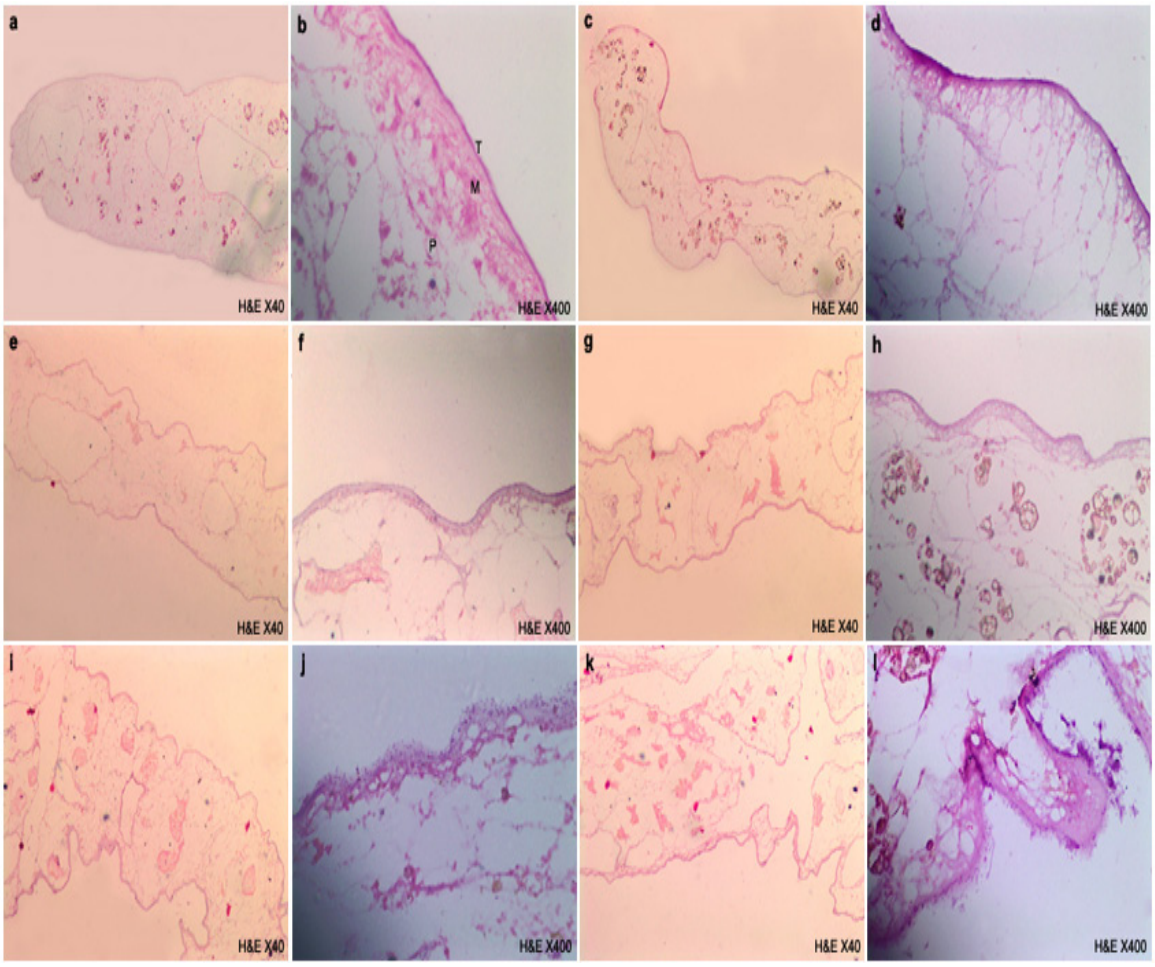

Fig. 3. Light micrographs of the tegument cross section of adult *F. gigantica*. (a, b) Normal fresh flukes. (c, d) Following incubation with 10 µg/ml *Balanites* extract. (e, f) Following incubation with 50 µg/ml *Balanites* extract. Note tegumental swelling. (g, h) Following incubation with 100 µg/ml *Balanites* extract. The tegument appears to be swollen and shows small vacuoles in the tegumental syncytium. (i, j) Following incubation with 200 µg/ml *Balanites* extract. (k, l) Following incubation with 400 µg/ml *Balanites* extract. Note prominent wrinkles of the tegument, extensive damage towards its surface and severe vacuolization towards the basal lamina. *T* tegument, *M* muscular layer, *P* parenchyma.

SEM findings

H. contortus

A typical intact anterior end of adult *H. contortus* showed hexagonal mouth provided with rudimentary lips, papillae, lateral amphids and dorsal buccal lancet (Fig.4a and inset). A pair of spine-like cervical papillae was seen situated on lateral surface of the cuticle (Fig.4a). The surface of the body showed a smooth cuticle characterized by a series of transverse striations with prominent lateral ridges (Fig.4b and inset).

Following incubation of worms with 400 µg/ml Balanites extract for 24h, the cuticularaterations concerned the buccal capsule in most of the examined specimens, in which damage to mouth and papillae was extreme that little recognizable structure remained (Fig.4c and inset). The cuticle which exhibited a smooth surface in control worms lost its normal aspect and appeared to be more swollen than normal showing longitudinal wrinkles (Fig.4d and inset).

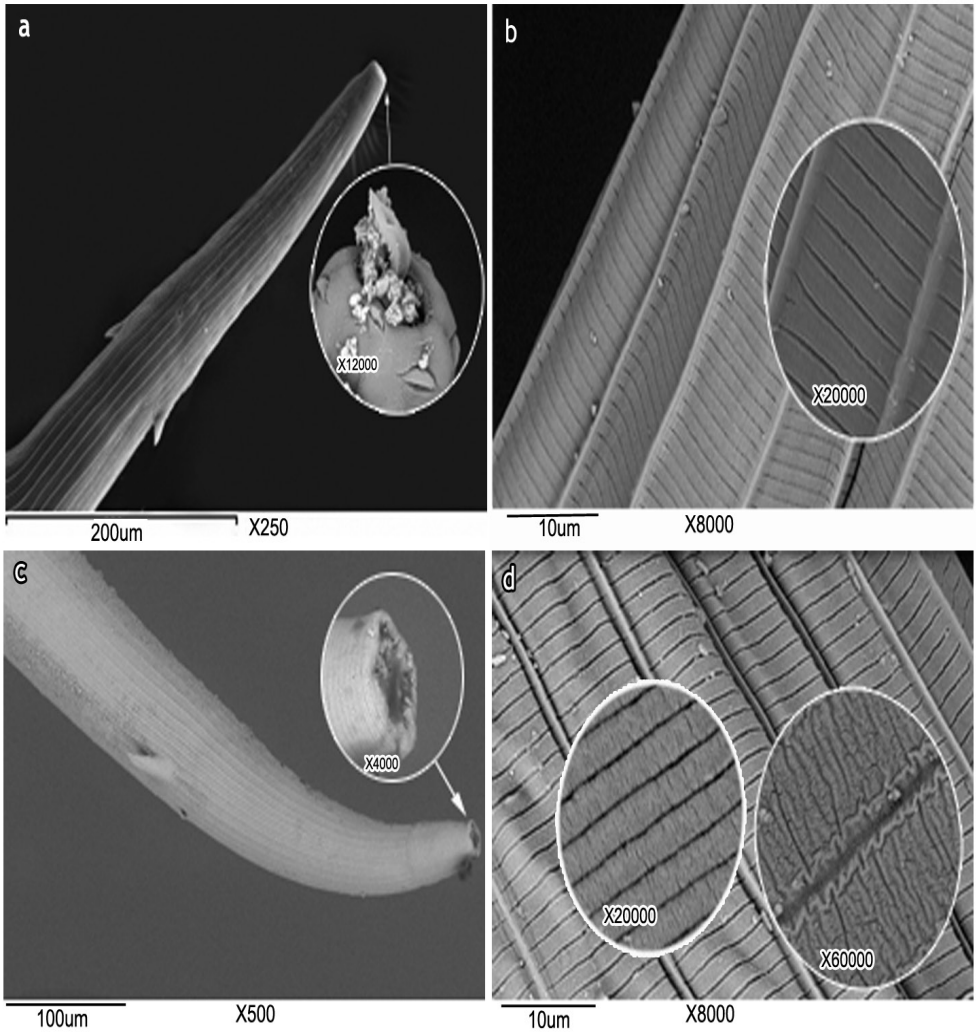

Fig. 4. Scanning electron microscopy (SEMs) of the anterior end of adult *H. contortus*. (a, b) SEMs of normal fresh worm showing the mouth with six semicircular rudimentary lips and a pair of spine-like cervical papillae. The cuticle is transversally striated and with lateral ridges (Inset). (c, d) Following 24 h incubation with 400 µg/ml *Balanites* extract showing severe damage to mouth and cuticle so that little recognizable structure remained as well as swollen cuticle with longitudinal wrinkles (inset).

M. expansa

The typical intact adult *M. expansa* showed a globular scolex provided with four oval suckers (Fig.5a), and strobila constituted of series of proglottids (segments) which were wider than longer (Fig.5b). Each proglottid had a common genital pore on either side (Fig.5c). Following incubation with 400 µg/ml *Balanites* extract for 24h, the tegumental alterations were detected in the proglottides other than the scolex in most of the examined specimens yet, some specimens showed swollen scolex with severely folded tegument and contractions to the sucker's opening (Fig.5d). The proglottides lost their normal aspect showing severely distorted tegumental surface which included rounded areas of prominent swelling found along their border (Fig.5e, f).

F. gigantica

The representative sound tegumental surface of adult *F. gigantica* showed microridges and furrows with a lot of serrated spines that were highly abundant on the apical cone particularly surrounding to both of the oral and the ventral suckers (Fig.6 a, b). The samples exposed to *Balanites* extract, showed distortion to the surface morphology, as illustrated in Fig.6c-f. At low concentration 10 µg/ml, there were spots of apparently flattened tegument throughout the anterior mid-body area. When examined at higher magnification, the flattening was shown to be attributed to a thin layer of syncytial secretion overlying the surface of the tegument which distinguished by a number of pits induced by the loss of spines (Fig.6c, d). In using the highest concentration 400 µg/ml Balanites extract and in nearly all specimens examined, distortion to both the oral and the ventral suckers was the greatest (Fig.6e). Besides, no tegumental surface remained, where the complete syncytial layer had been removed to reveal the underlying basal lamina (Fig.6f).

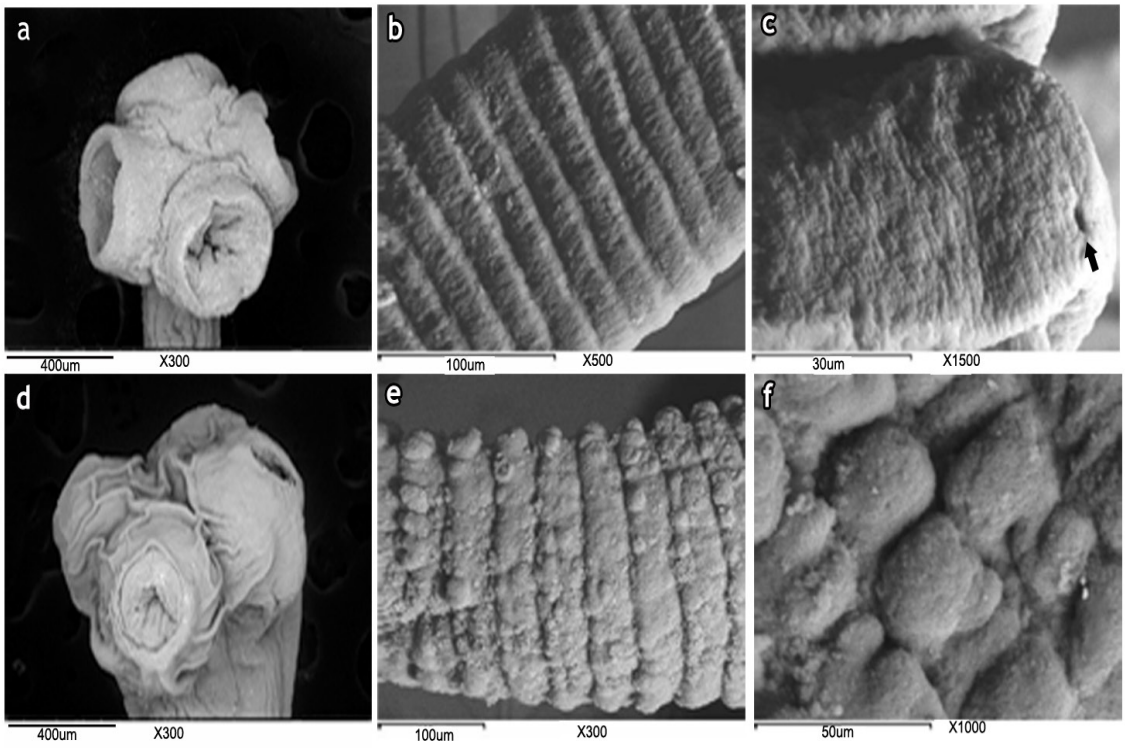

Fig. 5. Scanning electron microscopy (SEMs) of adult *M. expansa*. (a, b) Normal fresh worm showing a globular scolex with four oval suckers and an elongated ribbon-like structure called strobila. (c) SEM shows a genital pore on the lateral side of the gravid segment (arrow). (d, e, f) Following 24 h incubation with 400 µg/ml *Balanites* extract. The changes concern the whole-body surface. The suckers appear slightly swollen with wrinkled surface and the proglottides show severely distorted tegumental surface with rounded areas of prominent swelling present along their borders.

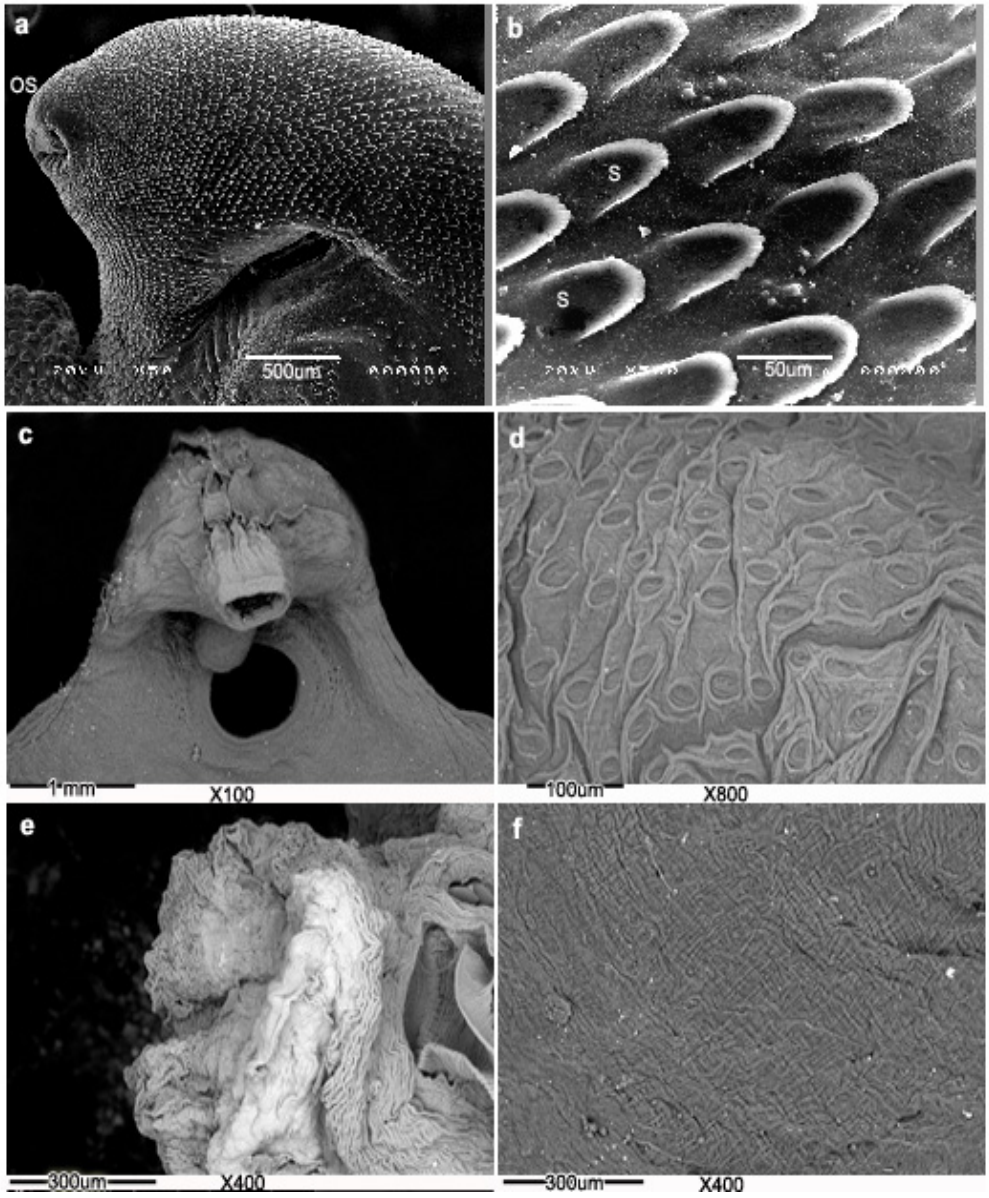

Fig. 6. Scanning electron microscopy (SEMs) of the anterior end of adult *F. gigantica*. (a, b) Normal fresh flukes. (a) SEM of the apical cone surface showing smooth oral sucker at its tip and the ventral sucker. (b) SEM of the tegument at mid-body area revealed ridged tegument with numerous serrated spines. (c, d) Following incubation with 10 µg/ml Balanites extract. SEMs of the tegument reveal a thin layer of syncytial secretion overlying the tegumental surface which marked by a number of pits caused by the loss of spines. (e, f) Following incubation with 400 µg/ml *Balanites* extract. (e) SEM of the apical cone surface showing distortion to both oral and ventral suckers. (f) SEM of the tegument showing massive loss of the entire syncytial layer to expose the underlying basal lamina.

In all experiments, the body wall (cuticle or tegument) of the solvent control worms showed normal morphology resembling to that of the fresh control worms, on examining with light and scanning electron microscopy. Hence, no abnormalities were observed in the control worms.

Discussion

The present study is the first that evaluate the in vitro efficacy of the *B.aegyptiaca* fruits methanolic extract on the adult worms of some gastrointestinal helminthes of naturally infected sheep and clarified how *B. aegyptiaca* fruits

directly affect helminthes. The current clinical and postmortem results confirmed the infection of the examined animals with *H.contortus, M.expansa* and *F.gigantica* which induced sever weight loss, anemia, weakness, mal –nutrition, diarrhea and even death. This goes parallel with the findings obtained by many authors [15-18] who reported that helminthosis is considered a global responsible, for impeding productivity in sheep that caused high morbidity and mortality.

The main target organ that was highly affected was the body wall, whose damage was observed by light and SEM studies. Light microscopy was used to detect alterations in a restricted area of the body wall syncytium while its surface changes, which reflected the changes in the body wall cytoplasm, could be detected over a considerably wider area utilizing SEM. The alterations occurred in particular sequences according to *Balanites* extract concentration, initiated with swelling which then after, was disrupted, resulting in sloughing off the cuticle or the tegument, and finally the basal lamina exposure and disruption occurred. Potent anthelmintic effects were found with a concentration of 400 µg/ml *Balanites* extract, at which damage to the body wall of the helminthes was very much distinct. This damage would certainly obstruct some of the important functions of the helminthes' body wall such as nutrition and immunoprotection [19]. The body wall of gastrointestinal helminthes is metabolically active and morphologically responsible for selective uptake of nutrients. Consequently, passive diffusion of anthelmintic drugs through the worm body wall might be the main reason for damaging alterations and disfiguration of the helminthes' body surface [20, 21]. Parallel to the current observations, the helminthes' body surface was noticed to be a main objective organ for several synthetic and natural anthelmintics as evidenced by previous histomorphological studies [1,22-24]. The swelling and distortion of the body surface of the examined helminthes induced by *Balanites* extract had been previously described for these helminthes by a number of anthelmintics [1,25]. This was might be due to a stress reaction by the helminthes, in a trial to replace the distorted surface membrane [26]. Previous studies on *B. aegyptiaca* fruits suggested that its methanolic extract had anthelmintic action in vitro [6], with a high saponin content that had a high potential of anthelmintic activity as confirmed by Gnoula et al. [27]. Wiesman and Chapagain [10] suggested a strong association between the saponin content of methanolic extracts of *B. aegyptiaca* and mortality among the mosquito *Aedesaegypti* larvae. The images of dead larvae taken in SEM exposed a rupture of the larval body surface that was obviously observed. The reason of this rupturing might be due to the disarrangement of this membrane through the combination of the saponin molecule with the cholesterol molecules existing in the cuticle membranes of the larvae and this might be the most possible cause for death of the larval. Moreover, the antifungal effect of saponins was linked with their capacity to combine with sterols in the fungal membranes and herewith led to damage of membrane integrity [28].

Based on the results presented in the current study, *B. aegyptiaca* fruits extract has a destructive effect on the body wall surface of gastrointestinal multiple drug resistant helminthes affecting sheep. This plant may offer an opportunity for a new natural anthelmintic and an alternative source for the control of such infectious disease in sheep.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions Statement

HAS and HAAA designed the plan of work. SMN, NMFH and TKF carried out the worm collection, and Balanites extract preparation. NMFH, TKF clinically examined the animals. SMN, NMTA, NMFH and TKF conducted the laboratory experiments. HAS performed data analysis and drafted the manuscript. HAS and NMFH prepared the manuscript for publication. HAAA completed the critical revision of the article. All authors read and approved the final version of the article.

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تقييم القدره المضاده للديدان لثمار Balanites aegyptiaca على بعض الديدان المعوية المتعددة المقاومة للأدوية التي تصيب الأغنام

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الهدف من هذه الدراسة هو تقييم تأثير مستخلصات Balanites aegyptiaca الميثانولية المستخلصة كمصدر بديل مستدام للأدوية المضادة للديدان ، على الديدان الطفيلية المتعددة المقاومة للأمعاء لدى البالغين وتوضيح كيف تؤثر ثمار B. aegyptiaca مباشرة على الديدان الطفيلية. لإنجاز هذه الدراسة ، تعرض ثلاثمائة وخمسون خروفًا تم تربيتها في مزرعة خاصة ، بمحافظة الجيزة ، لفحص سريري. من بينها ، تم عزل خمسة وخمسين غنمًا مصابًا بشدة ، اعتمادًا على تاريخهم السريري للمقاومات المضادة للديدان ، ليتم ذبحهم. كشف الفحص اللاحق للوفاة أن الحيوانات كانت مصابة بعدوى مختلطة من ديدان *Haemonchus contortus* و *Moniezia expansa* و *Fasciola gigantica*. أجريت دراسات مجهرية باستخدام الميكرسكوب الضوئي ومسح ب الميكرسكوب الالكترونى لتقييم التغيرات في جدار الجسم لهذه الديدان البالغة بعد التعرض لمدة ٢٤ ساعة لمستخلص الميثانول من ثمار *B. aegyptiaca* باستخدام تركيزات ١٠ و ٥٠ و ١٠٠ و ٢٠٠ و ٤٠٠ ميكروغرام / مل. كان الجسم المستهدف الرئيسي الذي تأثر بشدة هو جدار الجسم. حدثت هذه التغيرات في تسلسلات خاصة استجابة لتركيز خلاصة البلانيت ، الذي بدأ مع تورم تم تعطيله ، مما أدى إلى تلف البشرة أو tegument ، وأخيراً تلف شديدفى الصفيحة القاعدية. هذا الضرر من شأنه أن يؤدي إلى تعطيل العديد من العمليات الفسيولوجية المرتبطة بجدار الجسم ، وخاصة التغذية والوقاية من المناعة. قد يوفر هذا النبات فرصة للحصول على عقارمضاد للديدان طبيعي جديد ومصدر بديل للسيطرة على هذا المرض المعدية في الأغنام.