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FACULTY OF VETERINARY MEDICINE

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IN VITRO EVALUATION OF ANTHELMENTIC EFFICACY OF BALANITES EGYPTIACA ON FASCIOLA GIGANTICA

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A B S T R A C T

Fasciolosis is an important disease affecting large and small ruminants and other animals including human. Adult worms of *Fasciola gigantica* (*F.gigantica*) recovered from the bile ducts of sheep slaughtered in a Cairo abattoir. Samples used for determination the effect of ethanolic extract of *Balanites egyptiaca* (*B.egyptiaca*) and triclabendazole (TCBZ) on adult flukes. The *B.egyptiaca* fruits were purchased from Aswan Governorate and ethanolic extract was prepared in Medicinal and Aromatic Plane Research Department at National Research Center. TCBZ used as anthelmentic drug was most effective [8]. The aim of this study was to determine the, in *vitro* effects of plant extracts *B.egyptiaca* in comparing with TCBZ on adult *F.gigantica*, through histopathological and scanning electron microscopy (SEM) of the worms. The results showed that, all flukes treated with ethanolic extract of *B.egyptiaca* show tegumental swelling, blebbing, vacuolization and disappearance of spines compared with intact tegument and spines in control flukes using light microscopic examination. While SEM showed that, tegument of tail region showed furrows embedding of spines and sloughing in which the tegument had been stripped off to expose the basal lamina beneath. Tail region showed furrows with deep folding in the tegument and completely disappearance of spines. Swelling of the tegument led to completely submerged spines leaving spine socket, furrows and deep folding. Some results were obtained for 20 μg/ml TCBZ resulted in, Oral sucker showing sever swollen tegument with sloughing in which the tegument had been stripped off to expose the basal lamina beneath. Swelling around the ventral sucker with smoothness of the tegument. Ventral mid-body region, the tegument showing sloughing and furrows, submerged spines which either lied very flat against the surface or had become submerged in the tegument by the swollen tegument around them leaving deep furrows. Tail region, tegument showed deep furrows and folding. The results concluded that, the *B.aegyptiaca* have a great anthilmintic effect gainst *F.gigantica* in comparing with the effect of a great anthelmintic effect of TCBZ and it very cheaper in its costs than the TCBZ.

KEY WORDS: Fasciola gigantica, Scanning electron microscope, Tricalbendazole

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1. I N T R O D U C T I O N

Anthelmintic treatment is a regular practice in enzootic areas, but fails to eradicate the parasite. Allopathic anthelmintic are neither completely effective against common flukes [7] and have serious disadvantages in some developing countries, cost, risk of misuse leading to drug resistance, environmental pollution and food residues [9]. In addition, almost all adversely affect milk and meat production of animals during the course of their treatment, and even for long after their use [7]. Fasciolosis is distributed all over the world [16], Kenya [24], Romanians and Australian [3], around Lahore, Pakistan was *F. hepatica* with

14.67% prevalence rate [12] and South of the State of Esprito Santo [5]. Fasciolosis is a significant livestock problem, yearly an estimated US$ 2 billion are foregone due to weight loss, reduction in milk yield and fertility in production animals. In Egypt, animal fasciolosis is a dangerous disease leading to huge economic losses in livestock production and causing severe illness in human livers [15, 10]. Prevalence of fasciolosis in Egypt estimated to be 2.17% [19], in acute form of fasciolosis, there was a massive invasion of immature flukes into the liver which cause sudden death. While in chronic form, there was liver cirrhosis caused by the wandering fluke in which mature fluke lodged into the bile ducts, calcification of bile ducts and enlargement of gall bladder has been noticed. Submandibular edema frequently occurs. The parasite may cause loss of production during winter season in milking cows [22], studying the effect of TCBZ on adult fluke in *vitro* revealed that posterior region of the fluke are more severely disrupted than the oral cone[14].

The efficacy of TCBZ on sheep experimentally infected with fasciolosis showed progressive and time dependent increase in disruption of the tegument, culminating in the death of the fluke. Flukes were still active at 48 hrs post treatment and were not severely affected. By 72 hrs, all but one of the flukes was inactive and they showed reliable levels of disruption. After 96 hrs, all the flukes were extremely damaged and dead [8]. The tegumental changes in adult F.hepatica induced by TCBZ were assessed utilizing SEM in vitro by incubation of adult fluke with TCBZ for 24 hrs at a concentration of 10 mg/ml led to sloughing, blebbing and eruptions in the tegument [13].

The fixed oil of *B.egyptiaca* fruits had antimutagenic activity against *F.gigatica* induced mutagenicity besides anthelmentic activity against hepatic worm (*S.mansoni* and *F.gigatica*) [1].

This study aimed to determine the, in vitro effects of ethanolic extract of *B.egyptiaca* on *F.gigantica*, through histopathological and SEM examination.

2. MATERIAL AND METHODS

2.1. *Samples for histopathology and scanning electron microscope examinations*:

Adult worms of *F. gigantica* recovered from the bile ducts of sheep slaughtered in a Cairo abattoir. Samples were used for determination the effect of ethanolic extract of *B. egyptiaca* and TCBZ on adult flukes.

2.2. *Treatment medications*:

2.2.1. *Drugs*:
Triclabendazole (TCBZ) (Fasinex®, Ciba-Geigy Company.
2.2.2. *Plant*:
B. egyptiaca fruits were purchased from Aswan Governorate. Ethanolic extract were prepared at Medicinal and Aromatic Plane Research Dept. at National Research Center. Plant extracts *B. egyptiaca* at five different concentrations 30, 60, 120, 240 and 480 µg/ml. Dilutions were made from a stock solution of plant extracts at 10 µg/ml with 70% (v/v) ethanol.

2.3. *In vitro determination of the efficacy of B. egyptiaca and TCBZ on adult F. gigantica*:

Adult worms of *F. gigantica* recovered from the bile ducts of sheep slaughtered in a Cairo abattoir. Under sterile conditions in a laminar flow cabinet, flukes washed in several changes of warm (37.8˚C), sterile complete RPMI 1640 culture medium containing antibiotics (penicillin, 50 IU/ml, streptomycin, 50 mg/ml). The flukes were subsequently transferred to fresh culture medium containing 50% (v/v) heat denatured rabbit serum, 2% (v/v) rabbit red blood cells [11].

The whole flukes incubated for 24 hrs. at 37.8˚C in an atmosphere of 5% CO2. A positive control group was prepared by incubating whole flukes for 24 hrs. in RPMI culture medium containing 20 µg/ml TCBZ-SX. This level corresponded to maximum blood levels in *vivo*. The TCBZ was initially prepared as a stock solution in Dimethyl Sulphoxide (DMSO) and added to the culture medium to give a maximum solvent concentration of 0.1% (v/v). Solvent control flukes incubated for 24 hrs in RPMI 1640 culture medium containing 0.1% (v/v) DMSO. One fluke examined for each concentration.

2.4. *Specimen preparation for light microscopy analysis*:

2.4.1. *Hematoxylin and Eosin staining*:
Middle parts of flukes from each group were prepared for paraffin embedding. They fixed in 10% buffered formaldehyde for 24 hrs., dehydrated with a series of ethanol, and cleared with xylene. They were embedded in paraffin, sectioned at 5µm using a rotary microtome (HistoSTAT, Reichert, USA), and stained with hematoxylin and eosin. They examined for abnormalities using a Nikon E600 light microscope and photographed using a Nikon DXM 1200 digital camera (Tokyo, Japan) [4].

2.4.2. *Specimen preparation for SEM*:
Following incubation, the oral cone (including ventral sucker) and tail of adult flukes 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. The specimens washed repeatedly in double-distilled water, dehydrated through acetone, critical point dried in carbon dioxide, fixed to aluminum stubs and coated with gold–palladium. The specimens viewed in a Jeol scanning electron microscope (Jeol Corp., Mitaka, Japan) operated at 15 kV.
Measurements of the apical cone and tail of control flukes and the groups of treated flukes were made according to method proposed by, using a computer image analysis system (ELICA QWin 500, Cambridge, England). The measurements included spine characters as well as area of tegumental swelling around the ventral sucker.

3. RESULTS

3.1. *Effect of ethanolic extract of B. egyptiaca on adult F. gigantica using light microscopy examination*:
All treated flukes with different concentrations (30, 60, 120, 240 and 480 µg/ml) showed tegumental swelling, blebbing, vacuolization and disappearance of spines which appear embedded in the swelled tegument compared with intact tegument and spines in control flukes. The severity of tegumental alterations depends up on the concentration of extracts. The highest effect on the tegument and spines appear in high concentration of the extract than the lower (Fig.1).

3.2. *Effect of TCBZ on adult F. gigantica using light microscopy examination*:
Treated flukes with 20µg/ml showed tegumental swelling, blebbing, vacuolization and disappearance of spines which appear surrounded by the tegument compared with intact tegument and spines in control flukes (Fig. 2).

3.3. *Effect of B. egyptiaca using SEM examination*:
After 24 hrs. incubation with 120 µg/ml B.egyptiaca, the oral and ventral suckers appeared to be slightly more swollen than normal (Fig. 3. a & b). Lateral margin and posterior to the ventral sucker, the tegument showing submerged spines by the swollen tegument around them (Fig. 3. c & d). The tegument of the tail region showed furrows, embedding of spines and sloughing in which the tegument had been stripped off to expose the basal lamina beneath (Fig. 3.f).

After 24 hrs incubation with 240 µg/ml *B.egyptiaca*, the oral and ventral suckers appeared to be more swollen tegument than normal (Fig. 4. a & b)). Posterior to ventral sucker, the tegument showing the spines either lied very flat against the surface with swelling (Fig. 4. d). lateral margin, the tegument showing submerged spines by the swollen tegument around them with appearance of spine socket (Fig. 4. c & e). Tail region showed furrows with deep folding in the tegument and completely disappearance of spines. (Fig. 4. f).

After 24 hrs incubation with 480 µg/ml *B.egyptiaca*, the oral sucker showing sloughing apart from swelling of the tegument leaving a basal lamina beneath (Fig. 5. a).The ventral sucker appeared to be severly more swollen than normal (Fig. 5. b). Lateral margin, the tegument showing submerged spines by the swollen tegument around them leaving spine socket (Fig. 5. c). Ventral mid-body region, sever swelling of the tegument showed furrows, submerged spines which appeared sunken with their tips protruding from swollen and blebbed bases (Fig. 5. e). Tail region, swelling of the tegument led to completely submerged spines leaving spine socket, furrows and deep folding (Fig. 5. f).

Tegumental changes depend on the concentrations of extracts. The severity of tegumental alterations observed in 480, 240 and 120 µg/ml, respectively.

Effect of TCBZ using SEM examination:

After 24 hrs incubation with 20 µg/ml TCBZ, the oral sucker showing sever swollen, with sloughing tegument. The tegument had been stripped off to expose the basal lamina beneath (Fig. 6. a). The ventral sucker showing sever swelling with smoothness of the tegument (Fig. 6. b). Ventral mid-body region, the tegument showing sloughing and furrows (Fig. 6. c). The tegument showing submerged spines which either lied very flat against the surface or had become submerged in the tegument by the swollen tegument around them leaving deep furrows (Fig. 6. d & f)). The tegument of the tail region showed deep furrows and folding. (Fig. 6. f).

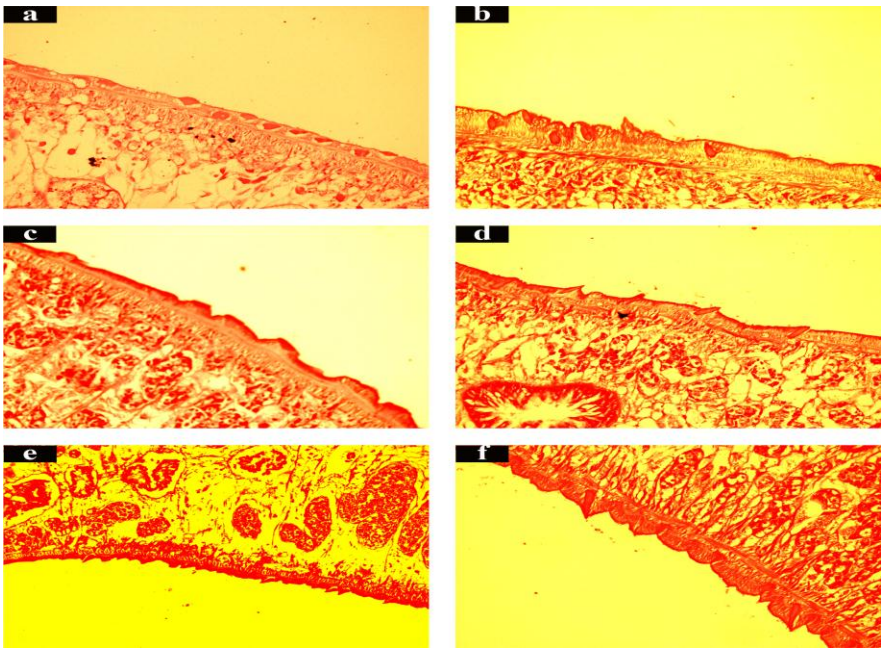


Fig 1. Light microscope of the mid-body part of adults F.gigantica, after 24 hrs of incubation. (a) Control fluke. (b) B.aegyptiaca treated fluke at 30 µg/ml conc. (c) B.aegyptiaca treated fluke at 60 µg/ml conc. (d) B.aegyptiaca treated fluke at 120 µg/ml conc. (e) B.aegyptiaca treated fluke at 240 µg/ml conc. (f) B.aegyptiaca treated fluke at 480 µg/ml conc. All treated flukes show tegumental swelling, blebbing, vacuolization and disappearance of spines compared with intact tegument and spines in control flukes.

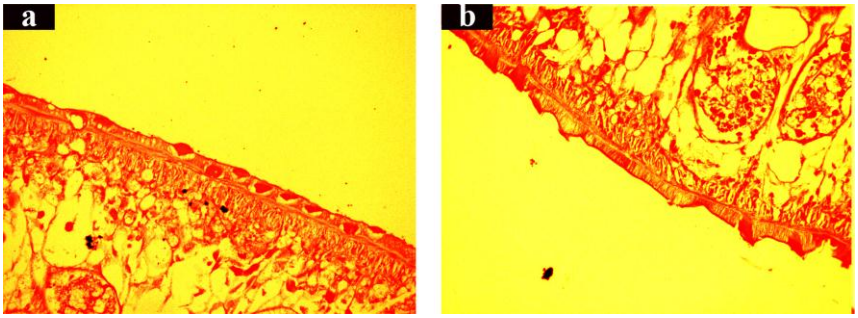
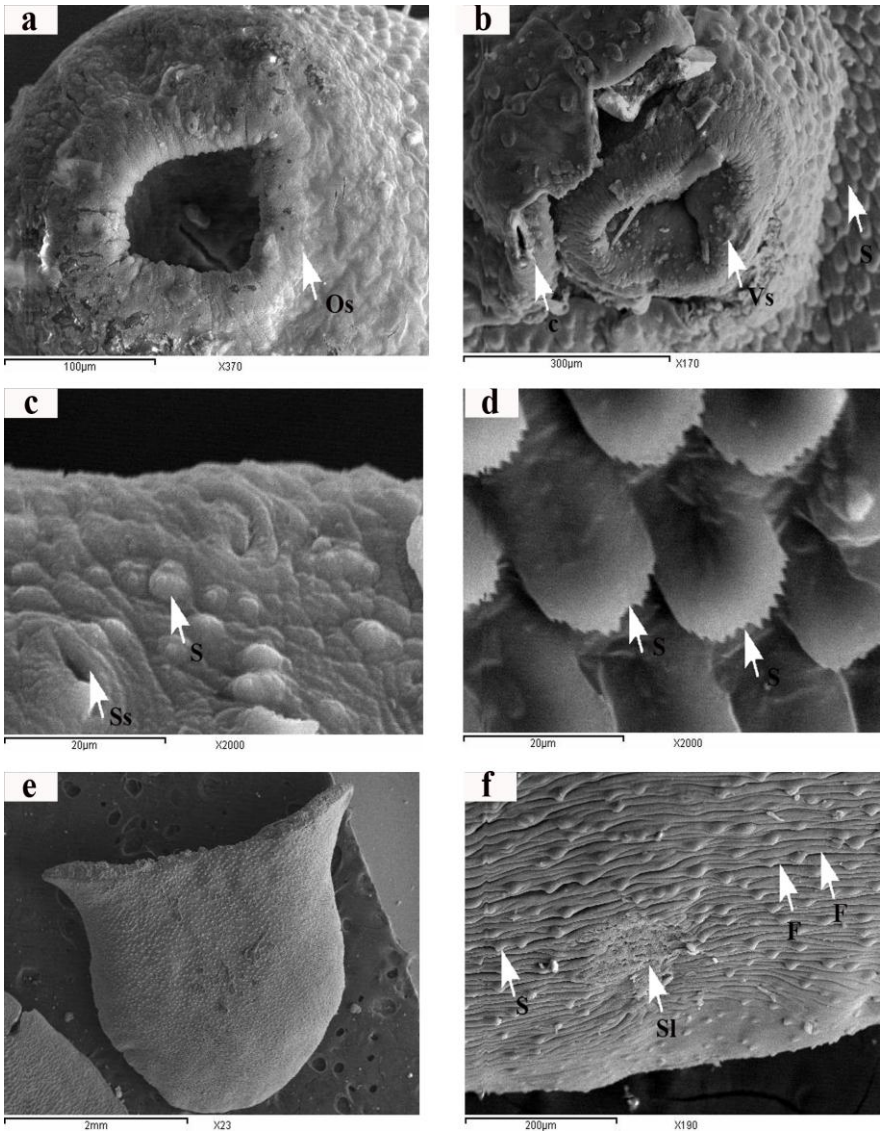
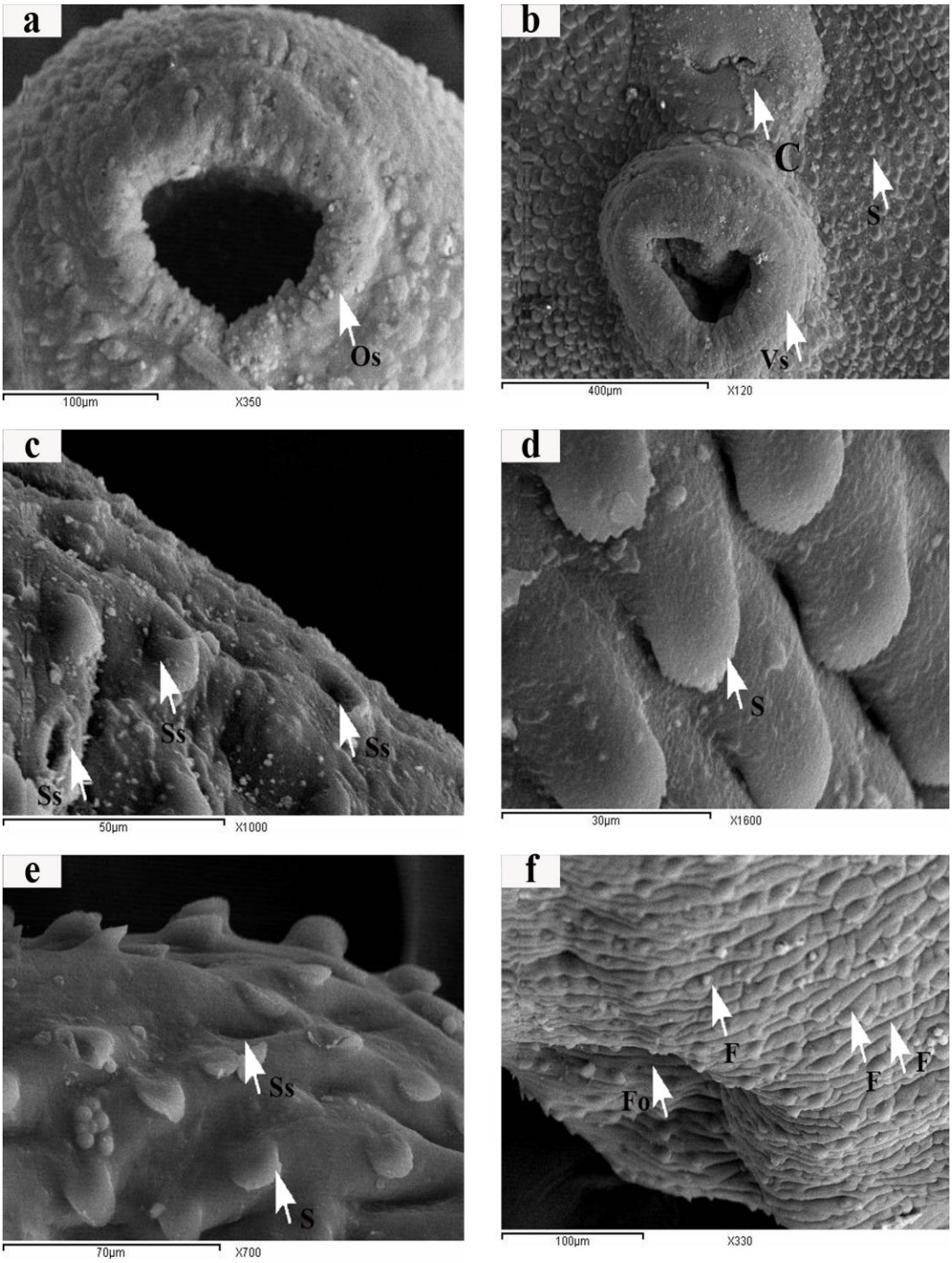


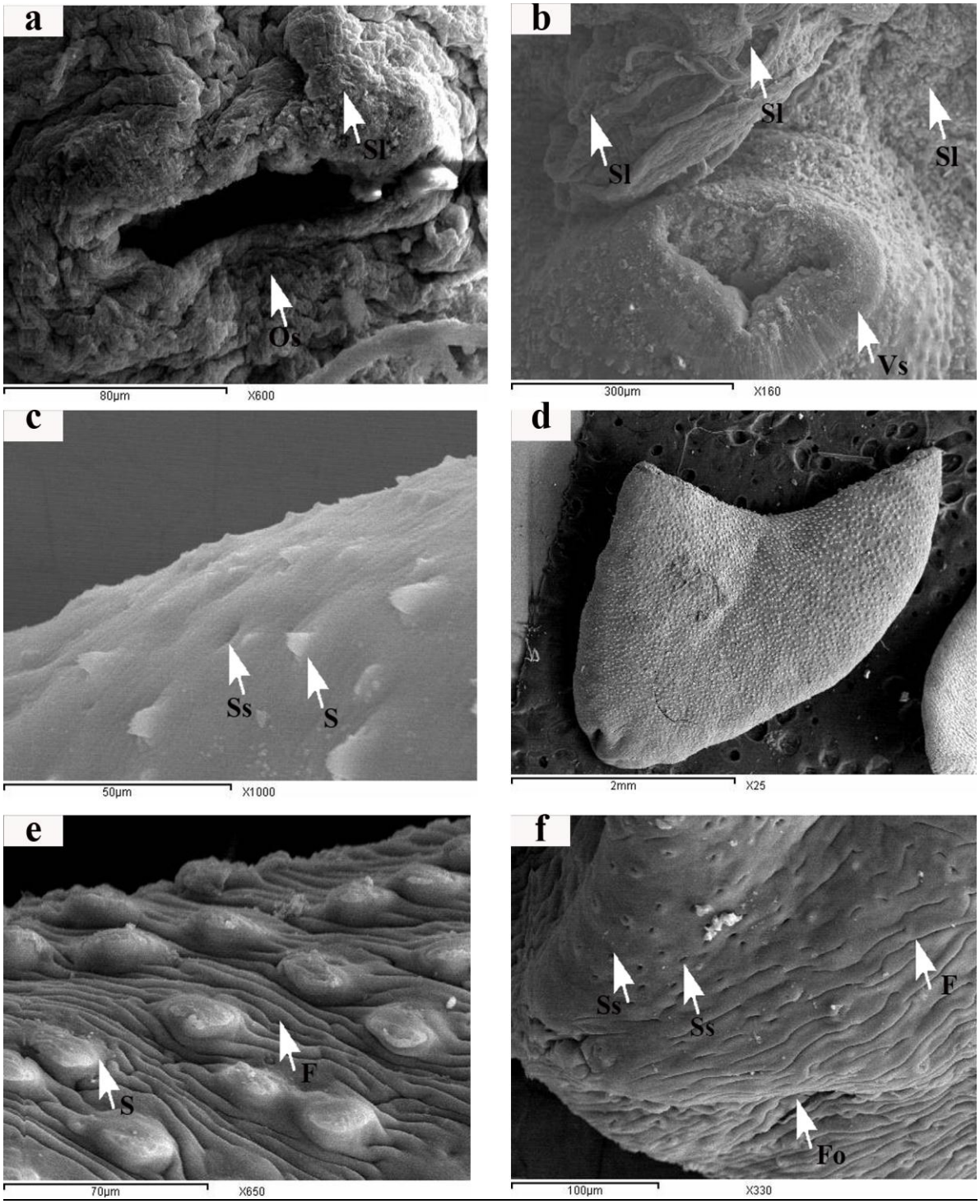
Fig. 2. Light microscope of the mid-body part of adults F.gigantica, after 24 hrs of incubation. (a) Control fluke. (b) Triclabendazole treated fluke at 20 µg/ml conc. treated flukes show tegumental swelling, blebbing, vacuolization and disappearance of spines which surrounded by swelled tegument compared with intact tegument and spines in control flukes.



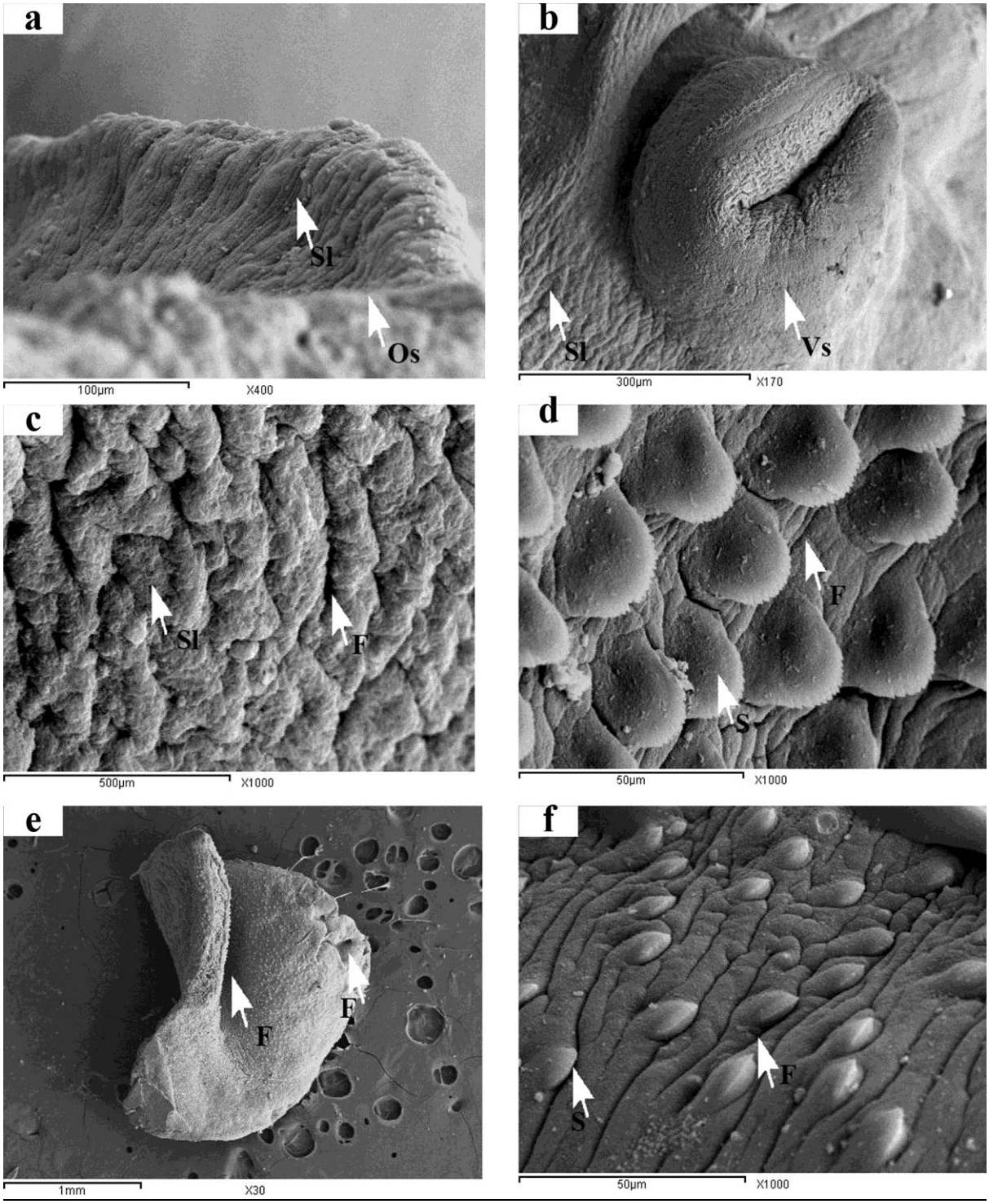
Figs. 3. Scanning electron micrographs SEMs of adult F. gigantica following 24 h incubation in 120 µg/ml B.egyptiaca. (a) Oral sucker (Os) showing slightly more swollen tegument than normal. (b) The ventral sucker (Vs) showing swelling around the ventral sucker and cirrus (c). (c and d) Lateral margin and posterior to the ventral sucker, the tegument showing submerged spines (S) by the swollen tegument around them. (e) Tail region. (f) Tegument of tail region showed furrows (F), embedding of spines (S) and sloughing (Sl) in which The tegument had been stripped off to expose the basal lamina beneath.



Figs. 4. Scanning electron micrographs SEMs of adult F. gigantica following 24 h incubation in 240 µg/ml B.egyptiaca. (a) Oral sucker (Os) showing more swollen tegument than normal. (b) The ventral sucker (Vs) showing swelling around the ventral sucker and cirrus (c). (c) Lateral margin, the tegument showing submerged spines (S) by the swollen tegument around them with appearance of spine socket (Ss). (d) Posterior to ventral sucker,the tegument showing the spines either lied very flat against the surface or had become submerged in the tegument (e) lateral margin, Sever swelling of the tegument led to submerging of spines (S) leaving spine socket (Ss). (f) Tail region showed furrows (F) with deep folding (Fo) in the tegument and completely disappearance of spines.



Figs. 5. Scanning electron micrographs SEMs of adult F. gigantica following 24 h incubation in 480 µg/ml B.egyptiaca. (a) Oral sucker (Os) showing sloughing (Sl) apart from swelling of the tegument leaving a basal lamina beneath. (b) The ventral sucker (Vs) showing swelling around the ventral sucker and sloughing (Sl) of the tegument. (c) Lateral margin, the tegument showing submerged spines (S) by the swollen tegument around them leaving spine socket (Ss). (d) Tail region. (e) Ventral mid-body, sever swelling of the Tegument showed furrows (F), submerged spines (S) which appeared sunken with their tips protruding from swollen and blebbed bases. (f) Tail region, swelling of the tegument led to completely submerged spines leaving spine socket (Ss), furrows (F) and deep folding.



Figs. 6. Scanning electron micrographs SEMs of adult F. gigantica following 24 h incubation in 20 µg/ml trilabendazole. (a) Oral sucker (Os) showing sever swollen tegument with sloughing (Sl) in which The tegument had been stripped off to expose the basal lamina beneath. (b) The ventral sucker (Vs) showing swelling around the ventral sucker with smoothness of the tegument. (c) Ventral mid-body region, the tegument showing sloughing (Sl) and furrows (F). (d and f) The tegument showing submerged spines (S) which either lied very flat against the surface or had become submerged in the tegument by the swollen tegument around them leaving deep furrows (F). (e) Tail region. Tegument showed deep furrows (F) and folding.

4. DISCUSSION

The use of SEM in this study gave an overview of the normal surface architecture of the tegument of *F.gigantica* apical cone, thus establishing any variations. Differences in response to ethanolic extract of *B.egyptiaca* and TCBZ action observed, depending on the used concentration. The swelling and blebbing of the tegument induced by the studied extract had been described for different *schistosome* species as described [21, 1 and 17], moreover [13] studied the efficacy of TCBZ on adult *F.hepatica* in *vitro*.

Concerning the in vitro studying the effect of ethanolic extract of *B.gyptiaca* at different concentration (30, 60, 120, 240 and 480 µg/ml) and TCBZ at 20µg/ml using light microscope, revealed a tegumental swelling, bleebing, disappearance of spines and vacuolization of the tegument compared with control flukes. Sever effect were observed in *B.gyptiaca* and TCBZ depending on the concentration of extract. These results agreed with that obtained by [23, 18] who showed mild to moderate destruction of tegument with presence of several blebs and swollen nodules were observed revealing the underling basal laminar with some intact spines.

The effect of ethanolic extracts of *B.gyptiaca* at different concentrations (120, 240 and 480 µg/ml) and 20 µg/ml of TCBZ using SEM against *F.gigantica* were studied. The obtained results revealed a high efficacy of studied extract and TCBZ on the tegument, which revealed swelling, disappearance of spines, swelling of the oral and ventral suckers, furrowing and folding of the tegument depending on the concentrations of extract. The highest efficacies on adult worm were ethanolic extracts of *B.egyptiaca* and TCBZ. Similar results were obtained by [2, 14, 8 and 13] who found that the effect of TCBZ on adult flukes revealed sloughing, blebbing and eruptions in the tegument. In addition, these results agreed with [1] who found that the fixed oil of *B.aegyptiaca* fruits had anthelmentic activity against hepatic worm (*S.mansoni* and *F.gigatica*).

The effect of *B.egyptiaca* on adult flukes may be attributed to the constituent of alkaloids as mentioned by [20]. In addition *B.egyptiaca* contains 54.53% unsaturated fatty acids and 1.14% sterols which had antimutagenic activity against *F.gigantica* [1]. TCBZ is a benzimidazole that binds to tubulin impairing intracellular transport mechanisms and interfering with protein synthesis [6].

B.egyptiaca (Balantiaceae), mainly the fruit, is used by traditional healers and herbalists for treating many diseases in Africa and Asia. It was investigated that fixed oil composition of fruits and evaluation of its biological activity. Oil content was identified using GC and GC/MS. In vitro examination of the oil biological activity (including cytotoxicity, antimutagenicity, antiparasitic, antiviral and antimicrobial activities) was performed. It was found that, the oil contained 54.53% unsaturated fatty acids and 1.14% sterols. The oil exhibited anticancer activity against lung, liver and brain human carcinoma cell lines. It also had antimutagenic activity against F.gigantica induced mutagenicity besides anthelmintic activity against hepatic worms (*S.mansoni* and *F.gigantica*). Preliminary screening showed that the oil had antiviral activity against Herpes simplex virus. It also had antimicrobial activity against selected strains of Gram-positive bacteria, Gram-negative bacteria and Candida. It was showed remarkable biological activity of *B.egyptiaca* fixed oil and proved its importance as natural bioactive source. [1].

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مجلة بنها للعلوم الطبية البيطرية
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التقييم الخارجى لكفاءة خلاصة نبات بلح الصحراء على الديدان الكبدية

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[2] قسم الطفيليات و أمراض الحيوان- المركز القومى للبحوث، [3] قسم النباتات الطبية والعطرية – المركز القومى للبحوث

الملخص العربى

يعتبر مرض الديادان الكبدية من اهم واخطر الامراض الاقتصادية التى تصيب الابقار و الاغنام كما انه له اهمية صحية فى انه يمكن ان يصيب الانسان ايضا و يسبب مرض الديدان الكبدية خسائر اقتصادية حادة للحيوانات من خلال زيادة الوفيات وخسائر متزايدة فى الحيوانات المذبوحة. تم تصميم هذه الدراسة لدراسة تأثير بعض المستخلصات النباتية على مرض الديدان الكبدية والمقارنة بين المستخلصات النباتية والأدوية التي توجد عادة في الأسواق وشائعة الاستخدام لعلاج المرض. تم تحديد مدى حساسية الديدان البالغة و ذلك بتعريضها فى المعمل لتركيزات مختلفة (30، 60، 120، 240، 480 ميكروغرام/مل) من مستخلصات النباتات بلح الصحراء و الترايكلابندازول 20 ميكروغرام/مل و ذلك لمدة 24 ساعة كفترة حضانة. و بالكشف المجهرى تبين أن جميع التركيزات تؤدي إلى تغييرات فى الجدار و تشكل الفجوات، وتورم في الغلاف واختفاء الاشواك تبعا لمستوى التركيز. و قد لوحظ الفعالية العالية في المستخلصات الأتيه على التوالى بلح الصحراء والترايكلابندازول. و بأستخدام المسح المجهرى الألكترونى فى المختبر و ذلك لتحديد حساسية الديدان الناضجه لتركيزات مختلفة (120، 240، 480 ميكروغرام/مل) و الدواء الترايكلابندازول 20 ميكروغرام/مل لمدة 24 ساعة كفترة حضانة. وكشف الدراسه أن جميع التركيزات تؤدي إلى تغييرات مثل تورم في الفم والمصاص البطني، وتورم في الغلاف والاختفاء فيالاشواك، وقابلة للطي تبعا لمستوى التركيز. لاحظ فعالية عالية في بلح الصحراء والترايكلابندازول و حبة البركة والثوم ، على التوالي. و خلصت هذه الدراسة الى ان استخدام المستخلصات الايثانوليه لنبات بلح الصحراء يمكن أن تكون علاج للحيوانات المصابة بالديدان الكبدية حيث انه يحدث تغيرات فى الديدان الكبدية البالغة مقارنة بتأثير دواء الترای کیلا بندازول. و تساعد على تحسين الحالة الصحية للحيوانات.

(مجلة بنها للعلوم الطبية البيطرية: عدد 22 (2)، ديسمبر 2011: 58-69)