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Research Article
In vitro and *In vivo* Effects of *Carica papaya* Seed Extract on the Ultrastructure of the Tegument of *Prohemistomum vivax* (Sonsino, 1892) (Trematoda: Prohemistomatidae)

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Abstract
Background: In the present study, aqueous extract of dried papaya seeds (*Carica papaya* Linn.) (AEP) tended to have trematodes anthelmintic activity on encysted metacercariae (EMC) and adult flukes according to the method of preparation of AEP and the concentration of the active principles. **Materials and Methods:** Prohemistomatidae EMC were isolated from muscles of *Clarias gariepinus* and divided into three groups, two of them were *in vitro* treated with AEP, boiled (BAEP) and soaked (SAEP) then used for experimental infection of mice. The 3rd group of EMC (without treatment) were used for experimental infection of mice which then were *in vivo* treated with BAEP and SAEP after development of adult flukes. Scanning Electron Microscopy (SEM) was performed to study the ultrastructure changes of the tegument surface of adult *Prohemistomum vivax* treated with AEP. **Results:** *Carica papaya,* aqueous seed extracts resulted in a significant reduction of total count of egg output and morphological changes. Scanning electron microscopy revealed *P. vivax* showed edema and wrinkle on the body surface. Deformities of the anterior and middle spines, bending at the border of the spines, blistering and rupturing at the body surface were noticed in the worms. **Conclusion:** The present study confirmed the promising anthelmintic property of the *C. papaya* seed aqueous extract where it create tegumental surface changes of *Prohemistomum vivax* flukes demonstrated by Scanning Electron Microscope (SEM). The boiled aqueous extracts of *C. papaya* seeds have strong anthelmintic activity that encourages its future application in aquaculture.

Key words: *Prohemistomum vivax, Carica papaya*, scanning electron microscope, morphological changes, spines, infection

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Catfish, *Clarias gariepinus* is one of most common fishes in Egypt which act as 2nd intermediate host for larval stages of digenetic trematodes as encysted metacercariae (EMC) of different species. Trematodes and their metacercariae take a major attention in most countries for the human protection against the transmissible diseases[1,2]. They were estimated as one of the most prevalent parasites infecting fish leading to low weight gain, high mortality, less marketability and may be of zoonotic importance[3]. The World Health Organization (WHO)[4] had estimated people actually infected with fish-borne trematodes to exceed 18 million and many more are at risk. *Prohemistomum vivax* was listed to infect human and may lead to death[5]. In addition, *P. vivax* recovered from experimentally infected rats fed on EMC from muscles of *C. gariepinus*[6,7].

Previous studies have shown various *in vitro* preparations of *C. papaya* (*Carica papaya* Linn.) seeds were effective against trematode infecting chicks[8,9]. It has potent anticestodal properties and its seed extract could be used as alternative to piperazine against *Hymenolepis nana, in vivo*[10].

Bioactive components accountable for the anthelmintic properties of papaya seeds were assigned to carpaine, carpasemine and benzyl isothiocyanate[11]. Most importantly, these seeds are inexpensive and readily available in tropical countries.

However, there are no reports regarding the effect of the papaya seed extract on the tegumental surface of digenetic trematode*, P. vivax* as well as morphological changes of its egg output. Thus, the aim of this study was to determine the effect of *C. papaya* dried seed aqueous extracts as potent anthelmintic on adult *P. vivax* by observation under Scanning Electron Microscope (SEM) and egg output from experimentally inoculated mice with EMC.

MATERIALS AND METHODS

Isolation of EMC from infected muscles of *C. gariepinus*: Thirteen *C. gariepinus* were collected from a private farm at Kafr El-Sheikh governorate, Egypt in April, 2016 and inspected for the presence of EMC. Present study was carried out by squeezing a piece (1 g) of muscle between two glass slides and the examination under a dissecting microscope. The EMC were examined, mechanically removed (minced in a blender) and isolated, counted according to Marzouk *et al.*[12]. With the aid of a dissecting microscope, they were then withdrawn by a Pasteur pipette and kept in 0.75% saline solution for the infection of laboratory mice either two ways with or without treatment by *C. papaya* aqueous extract. Identifying the collected EMC was based on important characteristic features, such as the shape of the cyst, suckers and excretory bladder, according to Mahdy[13].

Experimental animals: The experiments were performed on 36 Albino mice weighing 20-25±5 g and of 6-8 week's age. They were obtained from small breeding animal house in the National Research Centre. The mice were kept in the laboratory for 1 week before the experimental study and maintained on a standard rodent diet and water available *ad libitum*. The experimental protocol was approved by the Local Ethics Committee and Animals Research.

Experimental infection of laboratory animals: Encysted metacercariae were isolated, collected and feeding orally inoculation by the use of rubber catheter and a mouth gag in accordance with protocols approved by the Cairo University Institutional Animal Care and use committee (stomach tube). Each mouse in group (1-3) was inoculated with 300±50 EMC. Each inoculated mouse was sacrificed from 4th day post infection (dpi) till the end of experiment (8th dpi).

Preparation of the *C. papaya* aqueous extract from seeds: The seeds were collected freshly from ripe papaya fruits and washed with clean water to remove dirt. The seeds were sun dried and later grinded into a fine powder. For preparation of aqueous extract of *C. papaya* (AEP) seeds, two methods were used.

Extract I: The aqueous extract of papaya seeds was prepared according to the Thai Pharmacopoeia. Papaya seeds powder, 0.75 g were boiled in 200 mL distilled water until the volume was 150 mL and then filtered through a sterile filter paper to obtain the study extract I. One milliliter of the filtrate was expected to contain 0.005 g, i.e., 5 mg mL^{-1} of AEP[9].

Extract II: The papaya seed powders were weighed (7.5 g) and soaked in 150 mL of distilled water for 24 h at room temperature (30°C). The mixture was then centrifuged at 1500 rpm, the supernatant was filtered through sterile filter papers into a conical flask as the study extract II. One milliliter of the filtrate was expected to contain 0.05 g, i.e., 50 mg mL^{-1} of AEP[14].

Experimental design: Thirty six mice were assigned into four groups, each group or subgroup contains 6 mice (Table 1). All

Table 1: Groups of experimentally inoculated mice treated with *C. papaya* seed extracts

Groups	Inoculated mice	No. of mice	Aqueous extracts of papaya seed AEP	Duration AEP
1	Positive control inoculated	6	Without exposing of EMC to AEP	-
2A	Inoculated	6	*In vitro* exposed EMC to BAEP (Extract I)	4-8
2B	Inoculated	6	*In vitro* exposed EMC to SAEP (Extract II)	4-8
3A	Inoculated	6	*In vivo* treated mice with BAEP after developed adult *P. vivax* (5 dpi)	6-8
3B	Inoculated	6	*In vivo* treated mice with SAEP after developed adult *P. vivax* (5 dpi)	6-8
4	Non inoculated negative control	6	-	-

AEP: Aqueous extract of papaya, BAEP: Boiled aqueous extract of papaya, SAEP: Soaked aqueous extract of papaya

mice were inoculated with 300 EMC per mouse except the mice in group 4 left as negative control group (without inoculation), while the mice in group 1 were inoculated with (300 EMC per mouse) as positive control group. *In vitro,* group 2 was grouped into two subgroups A and B. The mice in subgroup 2A and 2B were inoculated with (300 EMC per mouse) after exposing the collected EMC to 20 mL of boiled and soaked AEP (extract I and II). The EMC were exposed on each extract at room temperature (30°C) for an hour (till the movement of EMC stopped). The movement of EMC was examined under a light microscope (Olympus CX21, Japan) according to Buddhachat *et al.*[9].

Each mouse was orally infected with 300 EMC per mouse, except group 4. Mice in group 1 and 2 were sacrificed starting from the 4th dpi till the end of experiment on 8th dpi. Mice from each subgroup 3A and 3B were sacrificed on the 1st Day Post Treatment (DPT), 2nd DPT and 3rd DPT. Mice in group 4 were sacrificed at the end of experiment (8th dpi).

Egg output: Daily fecal samples from each inoculated mice were examined by direct floatation and sedimentation examination techniques according to Faust *et al.*[15] for the demonstration of adult fluke's eggs. Egg count per gram of feces was done using the Mc-Master slide[16].

After shedding the eggs in feces, the mice in group 3 were grouped into two subgroup 3A and 3B, then were treated with AEC (I and II) at a dose level of 1.2 g kg^{-1} b. wt., according to Ameen *et al.*[14].

Parasitological methods

Worm burden: In the sacrificed inoculated and controlled mice, the small intestine of each mouse was removed, left in a petri dish containing saline for half hour, dissected and adult *P. vivax* were obtained from the mucosa, washed with 0.85% NaCl solution and counted.

Preparing samples for SEM: *Prohemistomum vivax* was fixed in 2.5% glutaraldehyde for 24 h. Glutaraldehyde was then replaced with 0.1 M phosphate buffer pH 7.2 for an hour or overnight at 4°C. Fixed worms in 0.1 M phosphate buffer pH 7.2 were stained in 1% osmium tetroxide solution just enough to submerge the whole worm for an hour. Then, coating with gold using S150A Sputter Coater-Edwards-England was performed and the specimens were then ready to be examined by SEM (QUANTA FEG 250, Holland SEM at the electron microscope unit at NRC and JSM 5200, Electron prob Microanalyzer, Jeol, Japan at Faculty of Agriculture, Cairo University).

Statistical analysis: Results are presented as Means±SE. Significant differences in the measured values between the control and experimental groups were determined by one-way ANOVA test followed by Duncan's Multiple Range Test (MRT)[17]. All statistical analysis was performed using a computer program of SPSS Inc. (version 17.0 for windows) at the 5% level of significance.

RESULTS

The total length and weight of the all inspected catfish (*C. gariepinus*) ranged from 22-28 cm total length and 65-130 g weight. The prevalence rate of prohemistomatid EMC infection was 100% and there was high intensity of EMC (21-40 EMC per microscopic field) in different organs and tissues (Fig. 1a, b). The number of EMC per gram of muscles ranged from 100-150 EMC g^{-1}. The present experiment showed that only one species of adult digenetic; *P. vivax* were obtained from experimentally inoculated mice with EMC that were isolated from the skeletal muscles of naturally infected *C. gariepinus* after 4 dpi.

Worm burden: In positive controlled group 1, the total percentage of recovered adult fluke was 68.89% after 4 dpi of inoculation with 900 EMC. The recovery rate significantly decreased after treating mice with the boiled AEP (Extract I) which was more effective than extract II. *In vivo*, studies

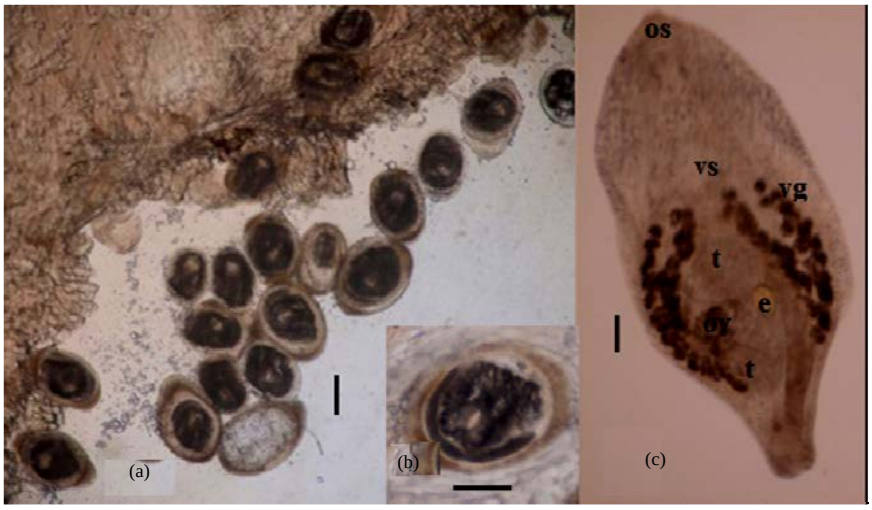

Fig. 1(a-c): (a, b) Prohemistomatidae EMC collected from skeletal muscles of *C. gariepinus* and (c) *Prohemistomum vivax* isolated from experimentally infected mice
os: Oral sucker, vs: Ventral sucker, vg: Vitelline glands, t: Testis, o: Ovary, e: Egg, Scale bar 100 µm

Table 2: Egg output and number of *P. vivax* worms recovered from the intestine of experimentally inoculated mice treated with *C. papaya* seed extracts and control groups

			Mean±SE		
Groups	TEMI	NM	NRW	RR (%)	NE/Mf
1	4th DEI	3	206.67±2.19^{h}	68.89±0.73^{h}	40.67±1.20^{f}
	8th DEI	3	188.67±4.67^{g}	62.89±1.55^{g}	23.67±2.33^{e}
2					
2A	4th DEI	3	13.00±0.58^{d}	4.33±0.19^{d}	4.00±0.58^{b}
	8th DEI	3	6.67±0.67^{bc}	2.20±0.20^{bc}	2.00±0.00^{ab}
2B	4th DEI	3	30.00±0.58^{f}	10.00±0.19^{f}	12.00±0.58^{d}
	8th DEI	3	18.67±1.20^{e}	6.22±0.40^{e}	7.67±0.67^{c}
3					
3A	1st DAT	2	3.00±2.00^{ab}	1.00±0.67^{ab}	1.00±1.00^{ab}
	2nd DAT	2	1.00±1.00^{ab}	0.33±0.33^{ab}	0.00±0.00^{a}
	3rd DAT	2	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
3B	1st DAT	2	9.00±3.00^{cd}	3.00±1.00^{cd}	2.00±1.00^{ab}
	2nd DAT	2	5.50±1.50^{abc}	1.83±0.50^{abc}	0.50±0.50^{a}
	3rd DAT	2	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
4	8th DEI	6	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Means with the same letter within the same column are not significantly different (p>0.05). TEMI: Time of examination of mice intestine, DEI: Day of experimental infection, DAT: Day after treatment, NM: No. of mice, NRW: No. of recovered worms, RR: Recovery rate, NE/Mf: No. of eggs in feces per microscopic field (X4), SE: Standard error

showed treatment of mice by the AEP (I and II) after development of the adult worms (group 3) was more effective than exposing of EMC in AEP (I and II) before inoculation of mice in group 2.

Determination of the egg output changes: *Prohemistomum vivax* eggs were collected from fecal samples of positive control group 1 and corresponding mice in groups 2 and 3. Examination was done by using the sedimentation technique before mice sacrifice. The difference in egg output between experimentally inoculated mice with AEP (I and II) *in vitro* and *in vivo* and the control positive group are presented in Table 2 and 3. *In vitro*, mice inoculated with EMC after being soaked in AEP (group 2) showed a significant reduction in the egg output starting from the 4th dpi. While *in vivo* in group 3, there was a significant decrease in egg output from the 1st day after treatment. There were deformities of eggs contour and ill distinct embryo and yolk cells in groups treated by extract I more than those treated with extract II in comparing with that of control group (Fig. 2a-d).

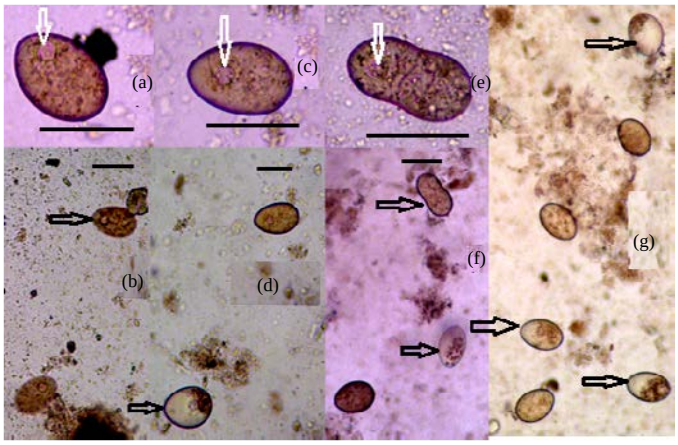

Fig. 2(a-g): Eggs of *P. vivax* isolated from stools of experimental infected mice (a, b) Egg with distinct embryo (white arrow) isolated from control positive group, (c, d) Egg with ill distinct embryo (white arrow) isolated from the infected group with extract I (boiled AEP) and (e-g) Egg with ill distinct embryo and deformity in shape (white arrow) isolated from the infected group with extract II (soaked AEP). All scale bar 100 µm

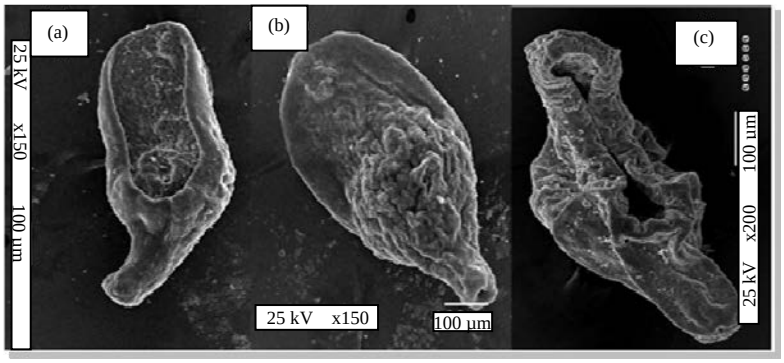

Fig. 3(a-c): SEM micrographs of the tegumental surface of adult *P. vivax* recovered from experimental infected mice. (a) Untreated adult, (b) Treated *P. vivax* with boiled AEP after 1st DPT (24 h) showing edema and wrinkle on the hind body surface and (c)Treated *P. vivax* with boiled AEP 2nd DPT (48 h) showed shrinkage on the all body surface. All scale bar 100 µm

Table 3: One-way ANOVA test of egg output and adult *P. vivax* worms recovered from intestine of experimentally infected mice treated with *C. papaya* seed extracts and control groups

Parameters	Source of variation	Sum of squares	Df	Mean square	F-value	Sig.
NRW	Between groups	182902.8	12	15241.9	1692.182	0.000**
	Within groups	207.1667	23	9.007246		
	Total	183110	35			
RR (%)	Between groups	20323.5	12	1693.625	1698.54	0.000**
	Within groups	22.93345	23	0.997107		
	Total	20346.44	35			
NE/Mf	Between groups	5189.139	12	432.4282	189.4448	0.000**
	Within groups	52.5	23	2.282609		
	Total	5241.639	35			

F-value: ANOVA F-test, Sig.: Significance level, **ANOVA (highly significant difference, p<0.01)

Effect of *C. papaya* seed aqueous extracts on *P. vivax* by using Scanning Electron Microscope (SEM): The SEM micrograph of the tegumental surface of the positive control group showed normal whole body surface of *P. vivax* (Fig. 3a). The spines on the dorsal surface were differing in shape and size along the body. It was covered with dense large scale-like

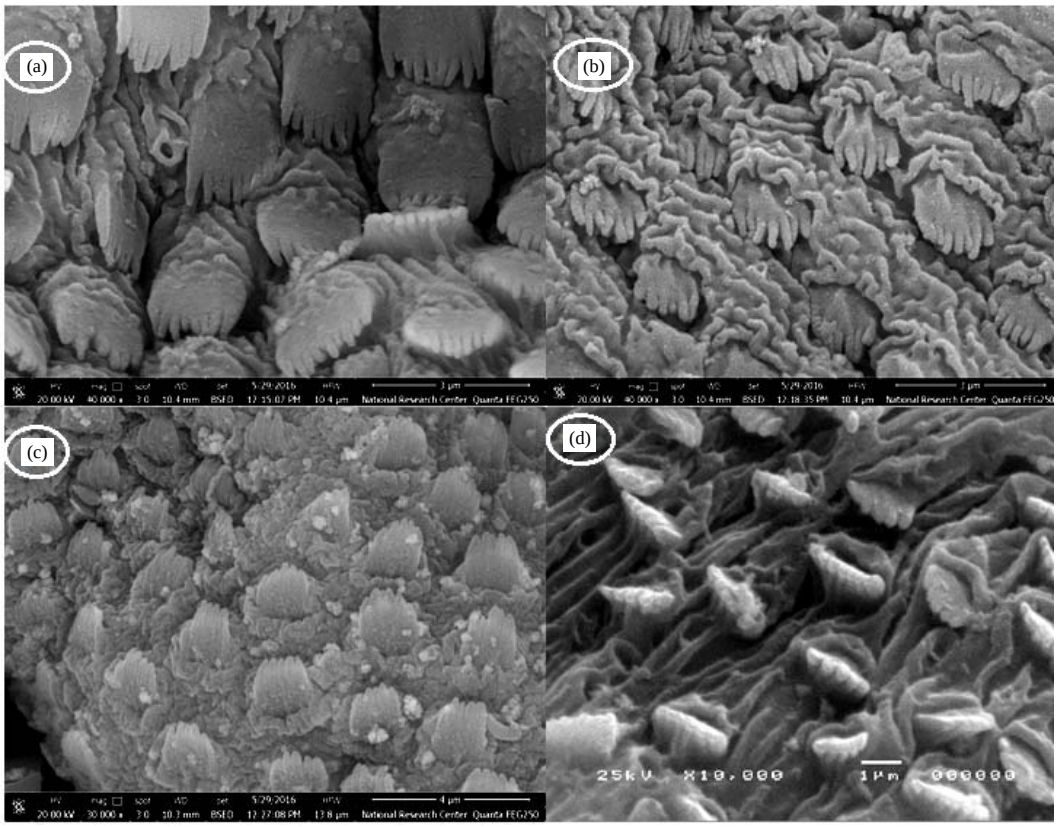

Fig. 4(a-d): SEM micrograph of the tegumental surface of *P. vivax* after *in vivo* treatment by boiled AEP. (a) Untreated *P. vivax* showing normal spines (scale bar) 3 µm, (b) Treated *P. vivax* showing edematous spines (scale bar 3 µm), (c) Untreated *P. vivax* showing normal spines (scale bar 4 µm) and (d) Treated *P. vivax*, the spines showing edema and bending at its edges (scale bar 1 µm)

(a, b) Anterior spines, each spine ends with 8 edges, (c, d) Middle spines; each spine ends with 5 edges

spines in the anterior part. Each spine ends with 8 pointed edges (Fig. 4a-c). These spines decreased in number towards posterior part of the fluke body. The middle part of the body showed relatively small size spines. Each spine ends with 5 pointed edges and carried at the base (Fig. 4). The oral sucker and posterior appendage showed normal smooth tegumental surface without any abnormalities (Fig. 4a-c).

In vivo, adult *P. vivax* recovered after treatment of mice with boiled AEP (extract II) at a concentration of 4.6 mL per mouse and after 1st DPT showed a lot of damages on the worms' body surface. The tegumental surface appeared edema, wrinkle body (Fig. 3b, c) and changes in all parts of body. A number of irregular swellings or blobs were present over the body surface but particularly concentrated in the anterior regions. Also, the oral sucker tegumental surface showed edema and a number of blebs or vesicles. Anterior and middle spines and sensory organ showed severe edema and bending at the edge of the spines (Fig. 4b, d). The posterior part of fluke showed edema, rupturing of the body surface on the appendix and the tegumental surface (Fig. 5b) with vesicles on the body surface (Fig. 5c, d). The same morphological changes were observed in the treated mice after 2nd and 3rd DPT.

DISCUSSION

Digenetic trematodes and their metacercariae considered as one of the most common parasites infecting fish, inducing a negative impact on fish production, causing low weight gain, high mortality, unmarketability and may have zoonotic importance[1,3].

In the present study, the prevalence of Prohemistomatidae EMC infection found 100% of all inspected *C. gariepinus* with high intensity (21-40 EMC per field) in different organs and tissues. The obtained results supported those reported by Al-Bassel[18] and Shaapan[19], recorded an incidence rate of 92 and 95.07%, respectively and nearly agree with Saleh *et al.*[20] and Saba[21] reported 87.1 and 85.3% incidence rate, respectively. However, it came at variance with

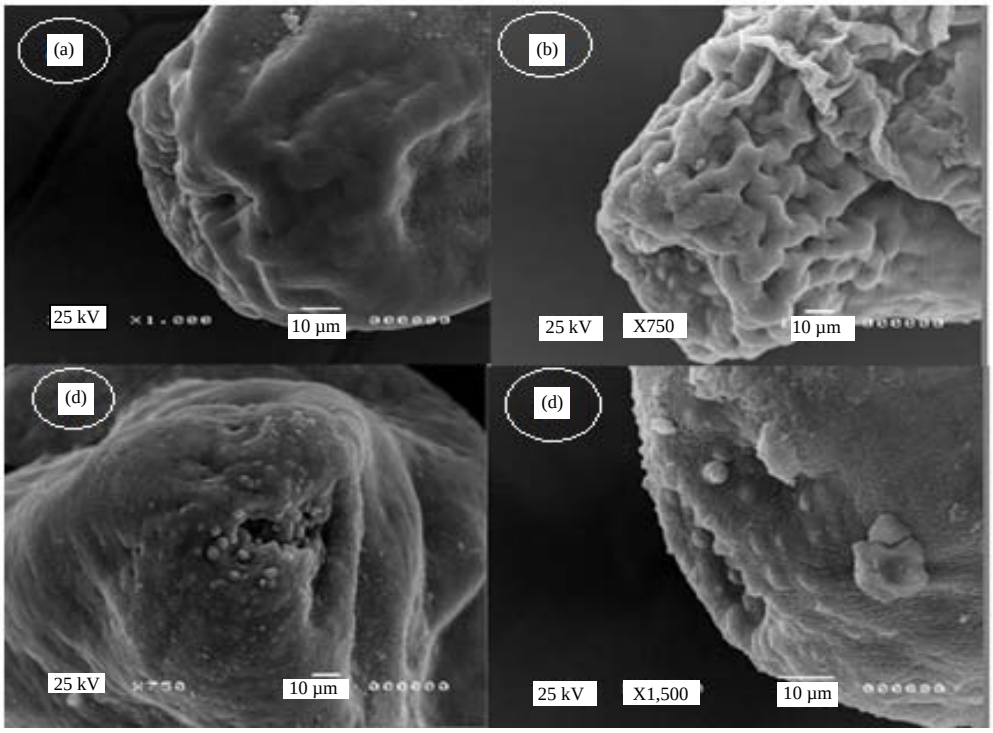

Fig. 5(a-d): SEM micrograph of tegumental surface of adult *P. vivax* (a) Untreated adult fluke on the posterior part, (b) Adult fluke after treatment with boiled AEP showing high edema in posterior part, (c) Treated fluke showing blebs on the anterior tegumental surface and (d) Treated fluke showing blebs on the posterior tegumental surface
All scale bar 10 µm

27.85% incidence rate of EMC in cultured African catfish in Egypt[22]. The present study showed only one species of adult digenetic trematode, *P. vivax* was obtained from experimentally infected mice with EMC isolated from the skeletal muscles of naturally infected *C. gariepinus* 4 dpi. The morphological characters of this trematode are consistent with that were described by Alghabban[7] and Marzouk *et al.*[12].

The high incidence of EMC infection in the examined *C. gariepinus* mainly related to the prevalence of the first intermediate host snails (*Cleopatra bulimoides*) of *P. vivax*[23] and the disability of their exact control in the natural water resources in Egypt. Moreover, the high organic load in waters of Nile Delta and the suitable water temperature constitute the main survival factors for its snail. Also, the spring season during which the final host (birds) of this digenetic trematodes increased in the cultivated Nile Delta regions with the escape of the parasitic eggs and miracidia through agricultural drainage water to the water resources[24].

Many studies have confirmed that the aqueous seed extract of *C. papaya* have anthelmintic efficacy which can be used in the treatment of digenetic trematode infection[8,9,11]. However, there is no *in vivo* report study concerning the influence of the papaya seed extract on the tegumetal surface of trematodes. The current study supports and validates anthelmintic activity of *C. papaya* aqueous seed extract (AEP).

In this study, different egg output counts were reported after treatment of experimentally infected mice with AEP (boiled and soaked) in comparing with the positive control group. There were a significant reduction in the egg output counts started on the 4th dpi in group 2 and a 1st DAT in group 3. Beside the marked decrease in the egg number, deformities were noticed in eggs contour and ill distinct embryo and yolk cells from groups treated by boiling extract more than that treated by soaked one in comparison with control positive group.

Egg count is an indicator showing the intensity of infection and as a parameter for cure[25]. It can be applied both experimentally and clinically to determine the validation of the treatment by egg counting before and after drugs have been given[16].

Concerning worm count (WRR) recovered from the small intestine, there were significantly lower WRR in each group treated with AEP than positive control one. In all results, the boiled AEP showed higher efficacy than soaked one. The

present results, nearly agree with that recorded by Chantima *et al*.[8] and Buddhachat *et al*.[9] that proved the *in vitro* effect of *C. papaya* seed aqueous extract on death and tegumental surface alteration of *Stellantchasmus falcatus* (Heterophyidae). The anthelmintic properties of *C. papaya* seeds may be due to its contents of bioactive compounds which were assigned to carpaine, carpasemine and benzyl isothiocyanate[11].

Micrograph by SEM of the dorsal tegumental surface of *P. vivax* from the non-treated group showed that it was covered with scale-like spines, each spine with 8 edges in the anterior surface and 5 edges in the middle one. This result agreed with the same observation recorded by Wongsawad *et al*.[11] detected the same finding in *Haplorchis taichui* from Thailand.

In the present study, the treated group of mice *in vivo* with boiled AEP 1st DPT, showed edematous spines and curving at its edges. This result agreed with the same observation recorded by Wongsawad *et al*.[11] described the damage to other parts of the fluke, roughened, flaky appearance surface and swollen spines; that their tips ill distinct and protruded out of the whole body of *H. taichui* after exposure to aqueous extract of *Artocarpus lakoocha* at different concentrations *in vitro.* Concerning, the tugmental surface of posterior part of *P. vivax* in the present study, edema and rupturing of the body surface on the appendix with vesicles on the body surface were observed. This result was nearly similar to that recorded by Chantima *et al*.[8] and Buddhachat *et al*.[9] recorded *S. falcatus* treated with *C. papaya* seed extracts appearing blebbing and rupturing at the body surface, curving at the edge of the spines and may be destruction of them mainly around the oral sucker and posterior region.

The functions of the tegumental surface of the worm are for attachment and nutrient uptake from the host[26]. Worms were released from the epithelial tissue of host small intestine when the tegument was deteriorated by anthelmintic medication. Also, the deterioration of the worm tegument could weaken and destroy the worm defense system, making it susceptible to the host immune system as the main function of the tegument is against immune invasion[27]. Hence, their tegument is an important object for anthelmintic drugs. The active principals in AEP may be associated with different physiological properties of the tegument of *P. vivax*.

A comparison of the effectiveness of the two *C. papaya* seed aqueous extracts (boiled and soaked) as anthelmintic agent, the result of the present study suggested the boiled AEP has higher anthelmintic activity than soaked one. This result may be referred to the variable concentration of chemical constituents of examining *C. papaya* seeds as the preparation by boiling method, concentrate the active components of the seeds than soaking it in water. This result agree with that reported by Kumar *et al*.[28], found that *in vitro* anthelmintic efficacy of boiled AEP extract were more potent than cold one against *Trichostrongylus, Bunostomum, Oesophagostomum* and *Trichuris* spp. of parasites.

The results of this study proved the anthelminthic property of *C. papaya* seed aqueous extract against *Prohemistomum vivax* (Trematoda: Prohemistomatidae). Details regarding the preparation methods of dried seed aqueous extract of *C. papaya* of either boiling or soaking method, its concentrations and the exposure time of EMC to these extracts (*in vitro*) need further studies.

CONCLUSION

The present study confirmed the promising anthelminthic property of the *C. papaya* seed aqueous extract where it create tegumental surface changes of *Prohemistomum vivax* flukes demonstrated by Scanning Electron Microscope (SEM) showed the patterns of damage.

SIGNIFICANCE STATEMENT

- Trematodes and their metacercariae take a major attention in most countries, they were estimated as one of the most prevalent parasites infecting fish leading to low weight gain, high mortality, less marketability and may be of zoonotic importance.
- Natural plant-derived products have been known for many decades to possess anthelmintic properties and more preferable than chemical therapy. Their use could reduce costs of treatment and be more environmentally friendly and they are less likely to produce drug resistance in parasites
- *Carica papaya* seeds are cheap and available in tropical countries. The aqueous extract of papaya seeds has anthelmintic activity which can be used in the treatment of digenetic trematodes infection in fish

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