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RESEARCH

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Assessment of *Verbesina alternifolia* and *Mentha piperita* oil extracts on *Clinostomum phalacrocoracis* metacercariae from *Tilapia zillii*

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Abstract

Background: Clinostomiasis (yellow grub disease) is a disease of freshwater fish caused by the encysted metacercariae (EMC) of *Clinostomum* spp. showing retarded growth, unusual host behavior, and even death in fishes. Thus, the purpose of this study was to conduct an assessment of two selected plant extract: *V. alternifolia* and *M. piperita* oil extracts on tegument surface of *C. phalacrocoracis* metacercariae (MC) from *T.zillii* as utilisation of biodegradable, eco-friendly plant extracts in environmental remediation to avoid utilization of chemotherapy to control of parasitic diseases leading to potential long-term health risks on the environment and humans.

Results: The results of evaluation efficacy of plant oil extracts, namely *V. alternifolia* and *M. piperita* on *C. phalacrocoracis* MC infecting *T. zillii*, were dependent on dose and exposure time. The lethal concentrations caused by *V. alternifolia* extract were determined LC50 at (400 ppm/24 h), and contrarily, the worms from gp2 exposed to *M. piperita* extract at LC50 (1000 ppm/48 h) and did not cause complete mortality among the exposed worms. Statistically, mortality of *C. phalacrocoracis* caused by *V. alternifolia* was found to be a stronger effect significantly higher than that caused by *M. piperita*. The fine integument structures observed suffered stronger effect that appeared as severe damage and desquamation of worm's teguments after exposure of *V. alternifolia*. In contrast, the *M. piperita* treatment exhibited edematous, swollen teguments, and blebs. Therefore, *C. phalacrocoracis* was an adequate model for evaluation of in vitro anthelmintic effects, contributing to the endeavors to identify suitable plant extracts, *V. alternifolia* and *M. piperita*.

Conclusions: This study highlights on assessment of selected two plant extracts; *V. alternifolia* and *M. piperita* revealed a stronger effect of *V. alternifolia* than *M. piperita* on tegumental surface of *C. phalacrocoracis* worms and, also, recommended the successful utilization of *V. alternifolia* on investigated worms as anthelmintic efficacy.

Keywords: *C. phalacrocoracis*, *V. alternifolia*, *M. piperita*, Micromorphological appraisal, *T. zillii*

1 Background

Clinostomiasis is a disease of freshwater fish caused by digenetic trematodes in the genus *Clinostomum*. *Clinostomum* spp. are digenetic trematodes with heteroxenous life cycles, involving both definitive and intermediate hosts [1]. The metacercariae of cosmopolitan species of *Clinostomum* "yellow grub" produce severe damages in their freshwater fish intermediate hosts [2] and [3] and cause the zoonotic disease Halzoun [1]. Kabunda and Sommerville [4] reported the parasitic worm of *Clinostomum* species causing the rejection of tilapia (*Oreochromis* species) in Zaire. A fish heavily infected with *Clinostomum* spp. exhibits hindered development, retarded growth, unusual host behavior, and even death [5, 6]. To eliminate parasitic diseases attacks in the aquaculture industry, different synthetic antibiotics, chemical drugs,

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vaccines, and chemotherapeutics are being used at high rates from recent years [7]. Using of these chemical substances causes mass killing of beneficial aquatic bacteria [8] and produces multi-drugs resistant pathogens [9], leaving residues in fish which can be passed on to human [10]. The infectious parasitic diseases treatment with natural substances/compounds are the demanding sustainable aquaculture features [11]. Concerned, Dawood et al. [12] demonstrated essential oils (EOs) extract from herbal plants effectiveness against *Ichthyophthirius multifiliis, Gyrodactylus sp., Euclinostomum heterostomum,* in vivo and in vitro. Terpens, terpenoids, phenylpropenes, and isothiocyanates are the key chemical groups identified in EOs [13]. Furthermore, EOs comprise different compounds that have no specific cellular target in parasites. EOs cause leakage of potassium ions and cytoplasmic content of parasitic cells due to hydrophobicity and cell permeability, which cause cell morphology alteration and cessation of parasitic activity [14]. Different microbial and parasitic diseases are the major threats to the aquaculture industry. Application of herbal products to combat microbial and parasitic diseases is considered a new alternative approach for sustainable aquaculture [15–17]. Extensive research activities were performed for the identification and characterization of EOs effects for the fish preservation. Several health benefits have been attributed to members of the *Verbesina* genus, reported to exhibit antibacterial, antiparasitic, and antioxidant activities [18]. Several chemotherapeutants, such as ivermectin, praziquantel, and trichlorfon, have been studied for their effects against *C. marginatum* [19]. Furthermore, an alternative strategy implies the use of nonchemical compounds such as medicinal plants [20, 21]. The significant impact of *Verbesina* plants is associated with the high levels of nitrates, galegine, and phyto-constituents, for example terpenoids, flavonoids, and fragrant mixes [22]. Among plant extracts, *V. alternifolia* (crown beard) have been considered due to their potentially lethal effects on parasites [23]. Rosato et al. [24] demonstrated EO,*Mentha piperita* L. is employed for significant antiviral, antifungal, and antibacterial properties. Furthermore, *M. piperita* L. (*Lamiaceae*) is used as raw material in several different applications in foods and cosmetics; leaves and flowers are used for medicinal preparations according to McKay and Blumberg [25]. Thus, the purpose of this study was to conduct an assessment of selected eco-friendly plant extracts; *V. alternifolia* and *M. piperita* oil extracts on tegument surface of *C. phalacrocoracis* MC from *Tilapia zillii* and histopathological alternations in infected tissues before and after were performed.

2 Methods

2.1 Collection of encysted metacercariae (EMC)

Tilapia zillii ($n = 477$) specimens were caught by anglers from January to December 2017, from the Nile River at Giza governorate, Egypt. These specimens were kept alive until examined at the parasitology laboratory, Faculty of veterinary medicine, Cairo University, for the detection of encysted metacercariae. The observed cysts were collected from the buccal cavities and gill chambers from investigated fish. Five cysts with their surrounding tissues were preserved in neutral buffered formalin (10%) for the histopathology study. Ten specimens of the isolated parasites were excysted by a sharp needle, preserved in 70% ethanol, clarified with Amman's lactophenol, and stained with Semichon's acetocarmine [26]. The other MC were assigned into four groups (Table 1) as described in Experimental design section.

2.2 Plant material

Verbesina alternifolia (Crown beard plant) were collected from different areas and carefully washed with tap water and then dried at room temperature. The air-dried powdered parts were exhaustively extracted with 90% ethanol by maceration. The total alcoholic extract was combined and evaporated under reduced pressure by vacuum distillation at a temperature not exceeding 40°C to yield a semisolid residue. Essential oil extract was obtained by hydro-distillation for 2 hours using a Clevenger-type

Table 1 Anthelmintic efficacy [Mean mortality % ± standard error of the mean (SEM)] of different concentrations of *Verbesina alternifolia* and *Mentha piperita* oil extract on excysted metacercariae of *Clinostomum phalacrocoracis* as compared to phosphate buffer saline (PBS) control and solvent control

Group	*N*	Concentration (ppm)	Mean mortality % ± SEM	
			Per concentration	Overall
Verbesina alternifolia	10	100	10.0 ± 7.07	34.38[a] ± 7.24
	10	200	17.5 ± 8.54	
	10	400	50.0 ± 10.80	
	10	600	60.0 ± 14.72	
Mentha piperita	10	400	0.0 ± 0.0	6.25[b] ± 3.40
	10	600	0.0 ± 0.0	
	10	800	7.50 ± 4.79	
	10	1000	17.50 ± 11.81	
PBS control	20	–	0.0	0.0[b] ± 0.0
Solvent control	20	–	0.0	0.0[b] ± 0.0

Different letters within the overall mortality column indicate significantly different means at $p < 0.05$ (Tukey HSD test)

apparatus using the methods of Cetin and Yanikoglu [27]. Investigation of the prepared oils was carried out on an Agilent (USA) GC-MS system. The extracts and oil were concentrated and stored in dark glass tubes under refrigeration at (4°C) until evaluation according to Araújo et al. [28].

2.3 Experimental design

Active live newly excysted MC of *C. phalacrocoracis* were collected and washed three times in phosphate buffer saline (PBS). Assigned to four groups (GP), GP (1) was consisted of four Petri dishes (each contain $n = 10$ MC), exposed to different dose and duration time of *V. alternifolia*. GP (2) exposed to different dose and duration time of *M. piperita* extract, while GP (3 & 4) were kept on PBS and solvent solution (negative control). All tested concentrations of plant extracts were prepared according to Salama et al. [29]. All groups were checked for estimation and calculated the mortality rate at lethal concentrations and were determined (LC50 and LC100) according to Taher et al. [23]. The bioassay was performed according to the World Health Organization [30] guidelines as given in Table 1.

The morphological descriptions of obtained *C. phalacrocoracis* MC were identified according to Caffara et al. [31]. Images of the morphological features were obtained by using a digital camera (Sony, 3.0 MP, Japan) attached to an inverted microscope (Olympus, Japan).

2.4 Scanning electron microscopy (SEM)

Ten specimens from each exposed/unexposed group of worms were prepared for SEM by serial washing in PBS and fixed with 2.5% glutaraldehyde according to Ibrahim and Mahdy [32] and Mahdy et al. [20]. Specimens were dehydrated with an ascending ethanol series, then dried in a carbon dioxide critical point drier (Autosamdri-815, Germany), glued over stubs, and coated with 20-nm gold particles in a sputter coater (Spi-Module Sputter Coater, UK). The worms were examined and imaged by SEM at magnifications from 35× to 500× (JSM 5200 electron probe microanalyzer, JEOL, Japan) at the Faculty of Agriculture, Cairo University, Egypt.

2.5 Histopathological studies

The EMC with the surrounding tissue were collected before and after treated with *V. alternifolia* plant extract at concentration of LC50 (400 ppm/24 h) from the infected fishes. The collected specimens were fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethyl alcohol, cleared in two changes of xylene, and blocked in paraffin. Five-micron-thick paraffin sections were stained by hematoxylin and eosin (H&E; [33]).

2.6 Statistical analyses

A one-way repeated-measures analysis of covariance (ANCOVA) was used to test differences in mortality of MC among treatment groups (*V. alternifolia*, *M. piperita*, PBS control, and solvent control). Data were blocked by concentration and repeated over exposure times (6, 12, 24, and 48 h). Exposure times were involved in the analysis as a continuous covariate. The Shapiro–Wilk test was utilized for normality analysis of the variables, and if there were significant differences, the Tukey's studentized range (HSD) test was used for post hoc analysis. Otherwise, the nonparametric tests were used for data, which were not normally distributed. Statistical significance was set at $p < 0.05$, and all data were presented as the mean ± standard error of the mean (*SEM*). Analyses were performed with SAS® version 9.4 [34].

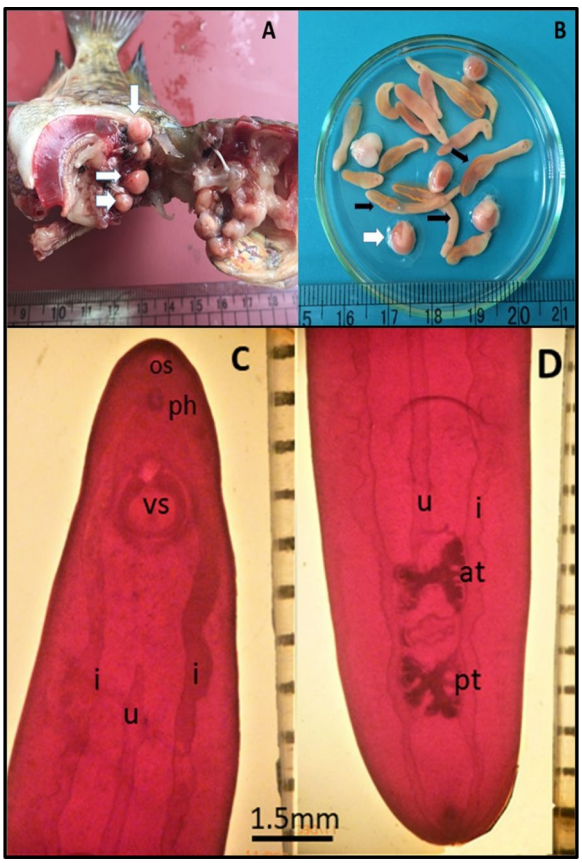

Fig. 1 *Clinostomum phalacrocoracis* EMC infected *T. zillii*. **a** Infected buccal cavity with EMC (white arrows) and ExMC (black arrow). **b** Fresh specimens of encysted MC (white arrows), ExMC (black arrow). **c, d** Stained specimens of *C. phalacrocoracis*; oral sucker (os), Pharynx (ph), ventral sucker (vs), intestinal ceca (i), uterine tube (u), anterior testis (at), posterior testis (pt), intestinal ceca (i)) scale bar 2 mm

3 Results

3.1 Clinostomum phalacrocoracis (Fig. 1a–c).

EMC were heavily distributed in buccal cavities and gills tissues of the investigated *T. zillii* with prevalence 40.7%. The MC encysted and excysted were yellowish in color with intensity of infection ranged from 3 to 12 cysts/fish (mean = 7cysts/fish). The C. *phalacrocoracis* MC is illustrated in Fig. 1.

The MC exposed to various concentrations of *V. alternifolia* extract exhibited degenerative effect increased with dose and exposure time vs control worms were viable, highly active movement. There was a significantly higher effect of *Verbasina* exposed MC on mortality percentage than that caused by *M. piperita.*

The ultrastructure observation of MC from control unexposed groups is shown in Figs. 2a, b and 3a–c. Worms appeared to have distinct smooth transverse annulations and ridges. The oral sucker consisted of two distinct collar-like ring was in a semicircular and flat shape, which was provided with sensory papillae (Figs. 2, 3a). On the ventral surface, a large ventral sucker was closed located near the oral sucker (Fig. 2b). A distinct ventral fold with a sponge-like character surrounded the ventral sucker, and dome-like papillae were present around the fold margins (Fig. 3b).The worms had a distinct, marked smooth and normal tegument structure at the hind body Fig. Fig. 3c.

The ultrastructure observation of worm from exposed group to *V. alternifolia* extract, at dose (400 ppm/24 h), is shown in Figs. 2c, d and 3d–f.

Worm exhibited disappearance of the transverse striations and dislocation of suckers. The oral sucker exhibited marked desquamated teguments, disfiguration of two collar-like rings, and disappearance of the sensory papillae (Fig. 3d). The worms showed a thickened ventral sucker and elevated ventral folds with the disappearance of sensory papillae around the margins. The tegument surface that surrounded the sucker was a

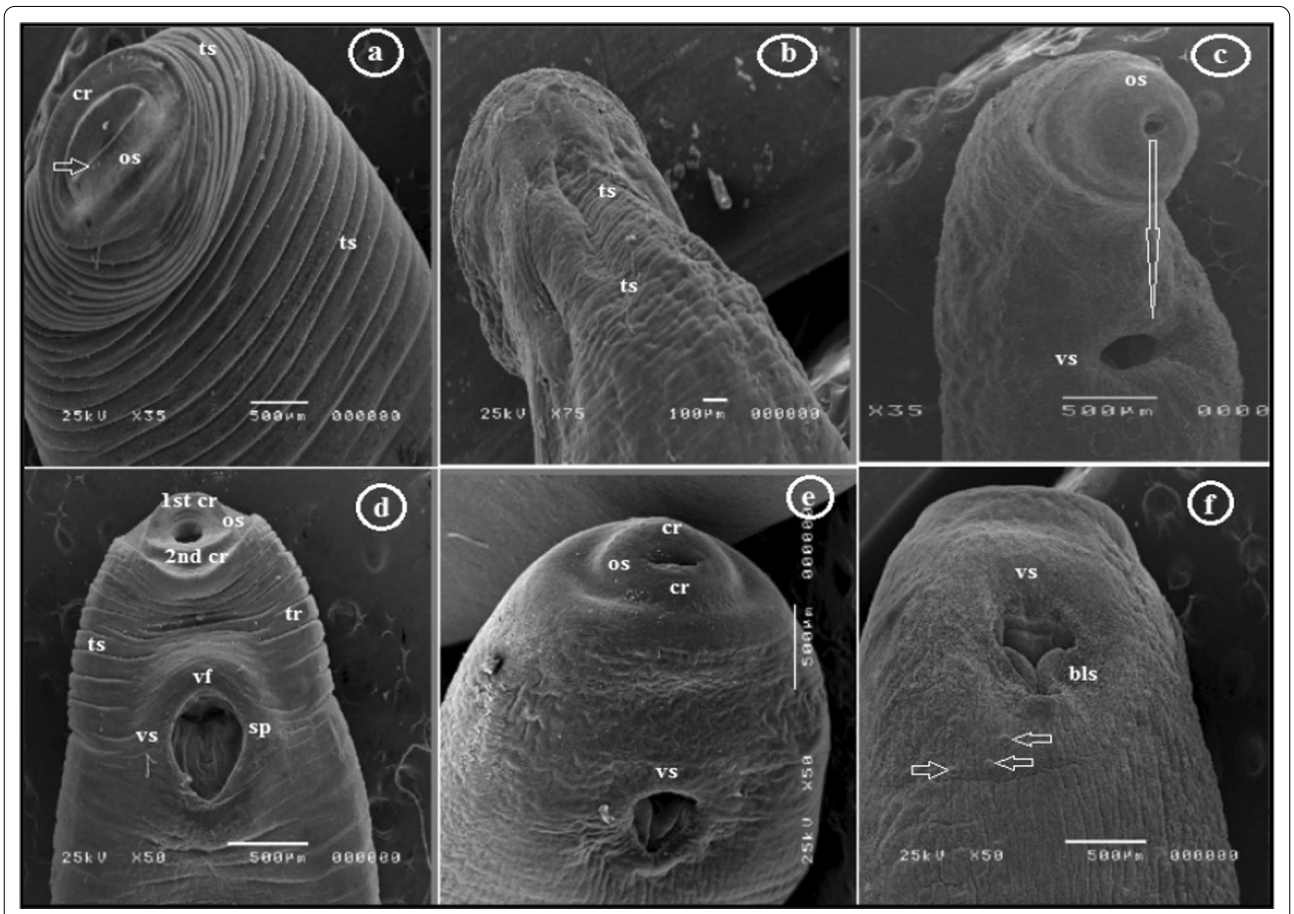

Fig. 2 SEM micrograph of *C. phalacrocoracis*; **a**, **b** A control unexposed worm exhibited distinct circular collar-like rings (1st and 2nd cr), oral sucker (os), transverse striation (ts) **c**, **d** Exposed worms to *V. alternifolia* extract dorsal and ventral view exhibited ill distinct transverse striation (tr).; disappeared of ventral transverse striation, dislocated suckers (os-vs with arrow). **e**–**f** Exposed worms to *M. piperita* extract; ventral view of worm exhibited edematous tegument with completely disappeared (tr) on the lateral sides, ejection of numerus blebs (bls) around the vs (arrow)

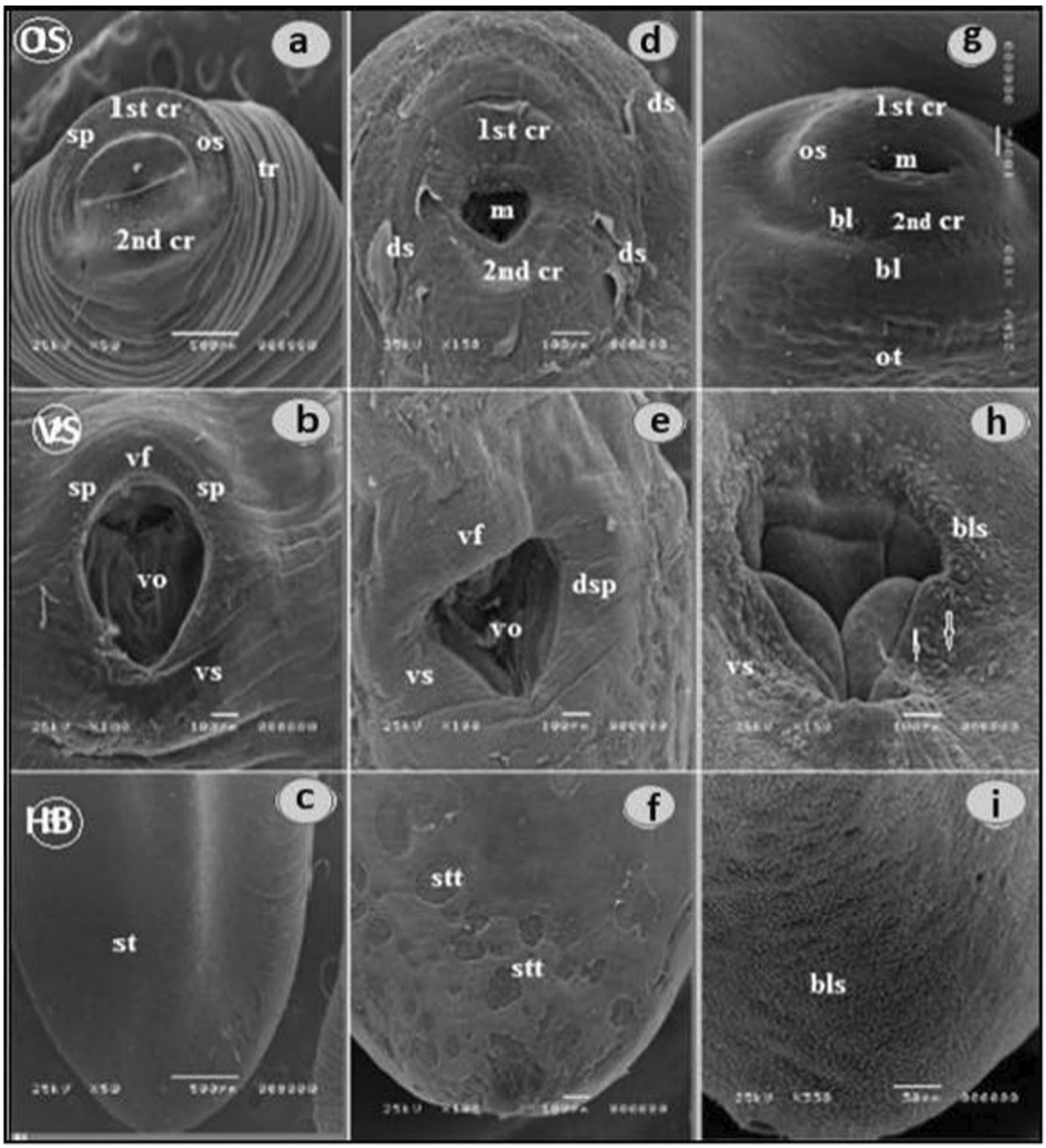

Fig. 3 SEM micrograph of *C. phalacrocoracis* metacercaria. **a–c** Unexposed control group; **a** fore body tegumental surface exhibited; distinct transverse annulation (ts), first collar-like ring; semicircular shape (1st cr) and second flat shaped (2nd cr), sensory papillae (sp). **b** A large ventral sucker (vs), ventral spongy fold (vf), sensory dome like papillae (sp). **c** Hind body exhibited smooth normal tegument without spines (st). **d–f** Exposed worm to *V. alternifolia* extract; **d** oral sucker showed disappearance (ts), desquamated tegument (ds), and disfigured collar-like rings **e** ventral sucker exhibited thicker vf, disappeared of dome like papillae (dsp) surrounded the ventral sucker. **f** Hind body exhibited detached and starched tegument (stt). **g–i** Exposed worm to *M. piperita* extract; **g** apical view of oral sucker exhibited; edematous tegument with completely disappeared (tr), collar-like ring (cr) and ejection of blebs (bl). **h** Ventral sucker showed a thinner ventral fold and ejection of blebs (bl). **i** Hind body exhibited ejection blebs (bls)

distinctly stretched tegument (Fig. 3e) and also showed stretched and detached tegument surfaces at the hind body (Fig. 3f).

In contrarily, the worms from gp2 exposed to *M. piperita* extract at LC50 (1000 ppm/48 h) are shown in Figs. 2e–f and 3g–i, of marked disappearance of the transverse ridges' striations and dislocation of the two suckers of the parasite, edematous, swollen and numerous blebs on the tegument surface as compared to the control specimen's forebody. The oral sucker exhibited

disfiguration of collars-like ring (Fig. 3g) and ejections of blebs around the margins the of ventral sucker fold (Fig. 3h) and also showed ejections of numerous blebs on hind body tegument of worms (Fig. 3i).

The histopathologic examination of the control group revealed various alterations in pseudobranch, and gill arch was showed presence of large EMC of *Clinostomum* species that enclosed by dense fibrous connective tissue capsule, the capsule attached to fish tissue structures (Fig. 4a) . Massive inflammatory and necrotic reactions were evident in gill arch started from gill rakers and extended deep to be severe in deeper tissue adjacent to parasitic cyst (Fig. 4b). Lesions included heavy infiltration of eosinophilic granular cells (EGCs) mixed with mononuclear cells into the adipose tissue of gill arch associated with extensive edema (Fig. 4c). The muscular structures showed necrosis with infiltration of inflammatory cells. The microscopic examination of gill arch from exposed group showed the presence of EMC, the tissue reaction adjacent to cyst wall was relatively mild compared with the control in exposed group (Fig. 4d), the reaction composed of few aggregations of EGCs (Fig. 4e), and mild edema with minimal necrotic reaction of muscles was evident (Fig. 4f). The

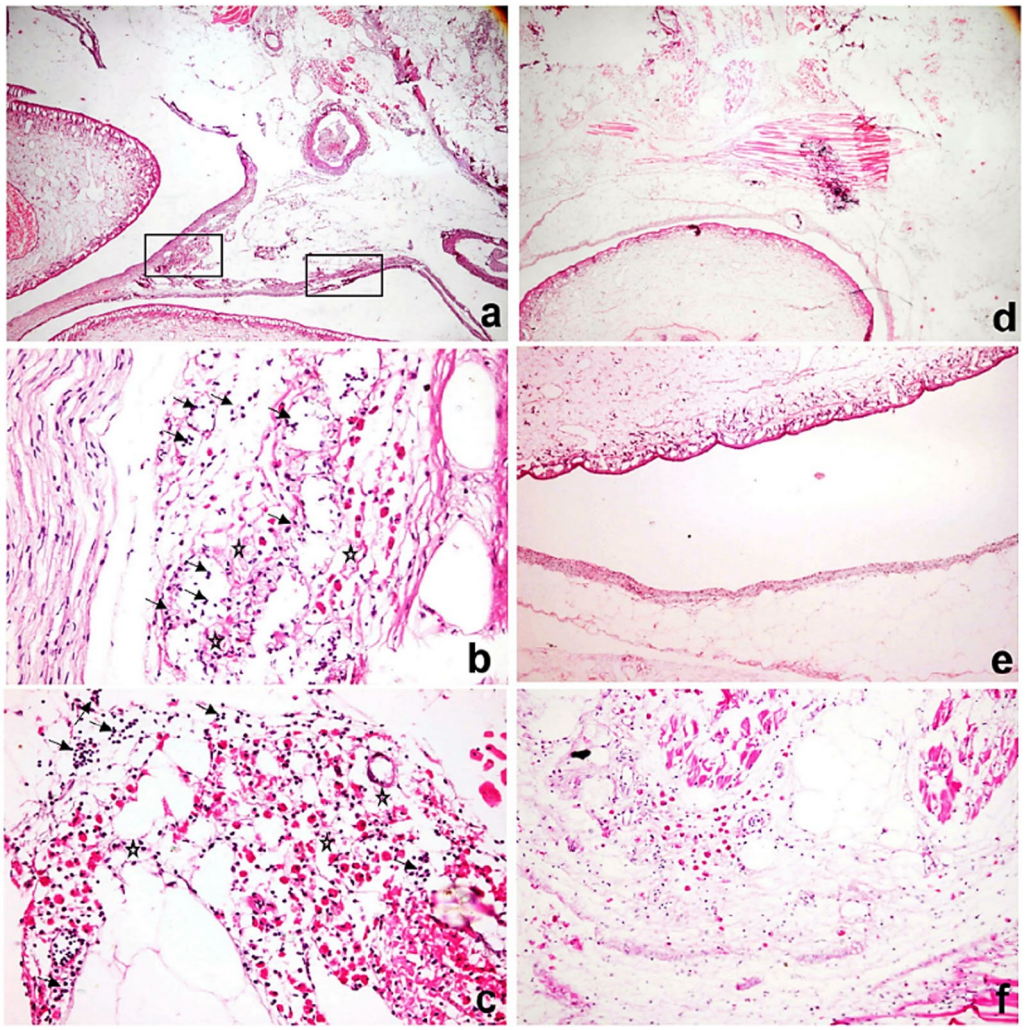

Fig. 4 **a**–**c** Histological section of fish gill arch and pseudobranch of control untreated group showing; **a** Presence of large EMC enclosed by dense fibrous capsule with destruction of the adjacent tissue structures with intense inflammatory cells infiltration (insert box) (×40) **b**. The cyst wall firm and incorporated in gill arch adipose tissue with intense inflammatory cells infiltration mainly EGCs admixed with mononuclear cells (arrows) and edema (asterisks) (×200). **c** Intense EGCs infiltration with myocytolysis and necrosis of muscle fibers (×200). **d**–**f** Histological section of fish gill arch of treated group showing, **d** presence of large encysted metacercaria enclosed by dense fibrous capsule with no destruction of the adjacent tissue structures (×40). **e** Intact adipose tissue with no inflammatory reaction (×100). **f** Few EGCs infiltration the gill arch with minimal necrosis of myocytes (×100)

pseudobranch of the control group EMC showed massive destruction of osseous tissue and muscle fibers with intense inflammatory cells infiltration (Fig. 5a). The cyst wall was dense composed of compact bundles and was closely adhered to the host adipose tissue with EGCs infiltration mixed with other mononuclear cells (Fig. 5b, c) in the exposed group and the tissue reaction comprising the pseudobranch was minimal (Fig. 5d). The cyst wall of the treated group showed dispersion and edema of individual fibers with degranulation of EGCs (Fig. 5e, f). The microscopic examination of pseudobranch and gill arch showed presence of large EMC of *Clinostomum* species with histologically normal internal structures (cuticle, suckers) (Fig. 6a). The worms was enclosed by dense thick fibrous connective tissue capsule that firmly attached and incorporated into to fish tissue structures with EGCs infiltration (Fig. 6b) . While MC of the treated group showed degeneration of internal structures (Fig. 6c), the cyst wall of treated group was thin and dispersed fibrous tissue with few EGCs infiltration that showing degranulation (Fig. 6d).

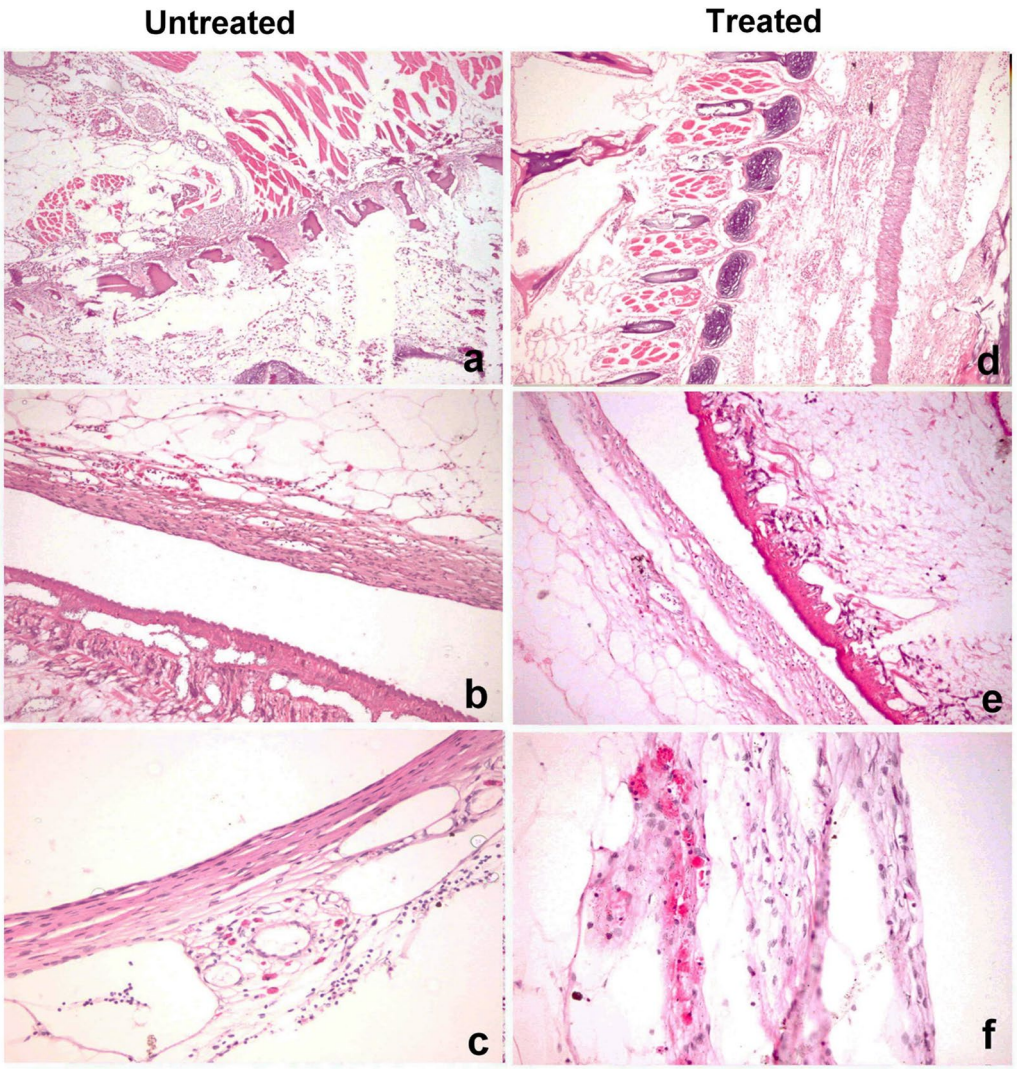

Fig. 5 **a**–**c** Histological section of fish pseudobranch of control untreated group showing; **a** Massive destruction of bony tissue and muscle fiber with intense inflammatory cells infiltration (×100). **b** Compact fibrous bundles of the cyst wall (×200) **c** Intense EGCs infiltration mixed with mononuclear cells in the adipose tissue that incorporated in the structure of cyst wall (×200). **d**–**f** Histological section of fish pseudobranch of treated group showing, **d** histologically normal of pseudobranch bony structures and muscle bundles with few EGCs infiltrating the underlying adipose tissue (×100). **e** Depression of fibrous bundles of cyst wall with minim al reaction od of the adjacent host adipose tissue(×100).**f** massive dispersions and edema of the cyst wall fibrous structure with degranulation of EGCs (×400)

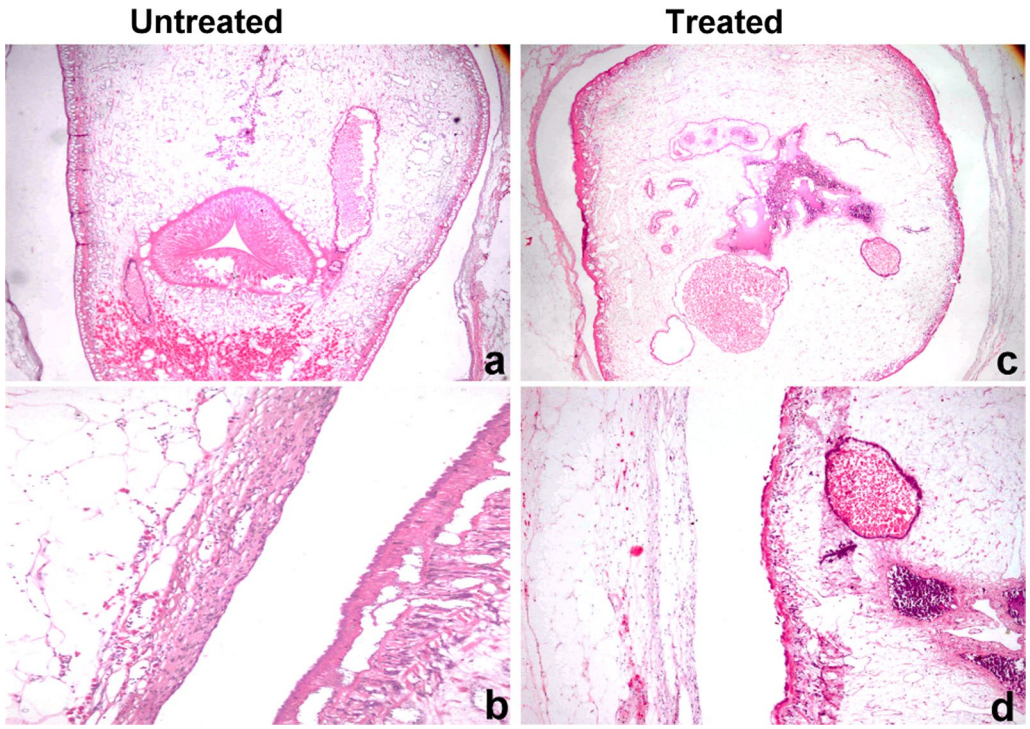

Fig. 6 **a–c** Histological section of *clinostomum* spp. In untreated control group showing normal intact internal structures. **d–f** In treated control group showing degeneration and edema of the internal structures of metacercaria

4 Discussion

Food-borne zoonotic trematode (FBZT) infections are caused by accidental consumption of encysted metacercariae of trematodes: heterophyid, prohemistomid, clinostomid by consumers via improperly cooked fish [3], 35. FBZT infections in fishes were increased, and chemotherapeutic control or cure is used for preventive measures [36]. Despite the advancements in disease treatment and control in recent decades, praziquantel and triclabendazole are currently the main accessible medications that exhibit activity against adult worms (Chai 2013). In the present study, *Verbesina alternifolia* determined LC50 at (400 ppm/24 h) and LC100 at (600 ppm/48 h) on the treated worms. This result agrees with the result by Amer and Mehlhorn [37] who reported 100% mortality after 48h with higher concentration on culex Larvae. In addition, the medicinal uses of *Verbesina alternifolia* appeared to be limited and not widely documented although it was reported that it has an anti-inflammatory action. The major phyto-constituents are terpenoids, flavonoids, and aromatic compounds [22]. Poisoning caused by *Verbesina* may be corresponded to high levels of nitrates and galegine. In the present study, *M. piperita* extract is not determined that cause complete mortality among the exposed worms. This result may conceivably depend on the different compositions: monoterpenes, menthone, menthol and their derivatives (Rosato et al., [24]).

Several strategies have been proposed for screening plant extracts; Souza et al. [38] assessed artesunate as an anthelmintic medication that had in vitro effects on MC of *Echinostoma paraensei.* Concerning, our previous studies for assessment of anther plant extract was determined by Abou Shady et al. [39], Ibrahim and Mahdy [32], who described *Carica papaya* extract *in vivo*, as a potential anthelmintic agent on *Hymenolepis nana* and *Prohemistomum vivax,*, respectively, using SEM from experimentally infected mice in Egypt.

Statistically, mortality of *C. phalacrocoracis* caused by *V. alternifolia* was found to be significantly higher than that caused by *M. piperita*. A stronger effect of *V. alternifolia* has been confirmed due to consist of menthol, which is the fundamental substance found in this extract to have anthelmintic activity [40]. The current result is consistent with Souza et al. [38], while it was inconsistent with the findings reported by Xiao and Catto [41], who reported there was no effect on worms with lowest concentrations of artemether.

In the present study, it was found that the main ultrastructures of the untreated C. *phalacrocoracis* MC tegument surfaces were consistent by Marwan and Mohammed [42] who found that no spines were covered

the body of *C. phalacrocoracis* in Egypt. The present ultrastructure observation was consistent with the fundamental morphological effects (ejection, blebs, sloughing, swelling, and disfiguration) as described by Jiraungkoorskul et al., [43] and Keiser et al. [44].

Histopathological observation of the un-treated control cyst revealed various histological alterations: intact structure of worm surrounded. The cyst was enclosed by a dense, thick, fibrous, and connective tissue capsule that was firmly attached to and incorporated into fish tissue structures with EGC infiltration. This result is consistent with the findings of Adeyemo and Agbede [45], Purivirojkul [46], Hamouda and Younis [47] and Mahdy et al. [3]**.** In addition, the infected tissues with helminth related EGCs infiltration and the related tissue reaction because of degranulation of EGCs, tissue reaction included vasodilatation, and neutrophils infiltration. This finding also showed that the defence mechanism of the host was functioning adequately. Concerning, Colwell et al. [48] observed no serious pathology of fish infected by Clinostomatids except that the fish were unsightly infected. However, massive metacercarial infections have sometimes resulted in the death of young fish [49]. On the other hand, the group of parasites exposed to *V. alternifolia* extract exhibited degeneration of the internal structures of worms such as the suckers, gut structures, and teguments. The cyst walls of the parasites were thin with dispersed fibrous tissue and little EGC infiltration. Additionally, the pseudobranchia and gill arches exhibited minimal tissue histopathological alterations, as demonstrated by the low level of EGCs and minimal necrotic reactions of the muscles. This result indicated improved in the treated tissue with extract indication. The effect of *V. alternifolia* extract on the parasites with reduction in tissue reaction is induced by EGCs infiltration and reduction in their related tissue damage. These observations require further study in the future.

5 Conclusions

This study highlights on assessment of selected two plant extract revealed that a stronger effect of *V. alternifolia* than *M. piperita* on tegumental surface of *C. phalacrocoracis* worms. The lethal concentrations caused by *V. alternifolia* extract were determined LC50 at (400 ppm/24 h), while in worms that exposed to *M. piperita* extract at LC50 (1000 ppm/48 h) and did not cause complete mortality among the exposed worms. The ultrastructural observation showed that *V. alternifolia* has stronger medicinal effects than *M. piperita* which causes severe damage and desquamation of the worm tegument. In contrast, the *M. piperita* treatment exhibited edematous, swollen teguments, and blebs. Therefore, *C. phalacrocoracis* was an adequate model for evaluation of in vitro anthelmintic effect of *V. alternifolia* and *M. piperita*. Also, recommended the successful utilization of *V. alternifolia* on investigated worms as anthelmintic efficacy.

Abbreviations

EMC: Encysted metacercariae; MC: Metacercariae; *T. zillii*: *Tilapia zillii*; *C. phalacrocoracis*: *Clinostomum phalacrocoracis*; *V. alternifolia*: *Verbesina alternifolia*; *M. piperita*: *Mentha piperita*.

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Authors' contributions

All authors contributed to the aim of works; OAM, SZAM and MAS involved in samples collection and parasite identification FFM contributed to the histopathological analysis of the fish tissues; HAR performed the statistical analysis of this study. All authors involved in manuscript writing and revision. All authors read and approved the final manuscript.

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None.

Availability of data and materials

All data generated or analysed during this study are included in this paper.

Declarations

Ethics approval and consent to participate

All institutional, ethical, and animal welfare guidelines were followed in accordance with the Laboratory Animal Care and Use Guide (Vet-CU-11112018014).

Consent for publication

Applicable.

Competing of interests

The authors declare that they have no competing interests.

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