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EFFECT OF *VERBESINA ALTERNIFOLIA* AND *MENTHA PIPERITA* OIL EXTRACTS ON NEWLY EXCYSTED METACERCARIA OF *EUCLINOSTOMUM HETEROSTOMUM* (RUDOLPHI, 1809) (DIGENEA: *CLINOSTOMATIDAE*) FROM NATURALLY INFECTED KIDNEYS OF *TILAPIA ZILLII* IN EGYPT

By

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Abstract

Encysted metacercariae of *Euclinostomum heterostomum* (EEMC) is parasitic trematodes infected kidneys of the freshwater fish (*Tilapia zillii)* in Egypt. The EMC causes severe pathological changes as kidney failure and even death of fish. Development a novel non-chemical approach that decreases the need of anthelmintic drugs, proved to be the only realistic strategy to avoid drug resistance especially with biodegradable eco-friendly plant extracts.

In the present study, *T. zillii* infected with EEMC was 14.5%. Histopathology of the tissue showed marked glomerular congestion with interstitial congestion, hemorrhages, vacuolization and necrosis of tubular epithelium with activation of melanomacrophage center.

The anthelmintic activity of *Verbesina alternifolia*, *Mentha piperita* oil extracts on newly excysted metacercariae (EExMC) of *Euclinostomum heterostomum* was evaluated in vitro. The LC_{50}% & LC_{100}% for *V. alternifolia* was 400ppm/12hrs and 600ppm/24h, respectively. LC_{50}% of *M. piperita* was 1000ppm/24hrs. Doses lower did not cause mortality of exposed EExMC. Exposure to both plants caused marked irreversible alteration in tegumental ultrastructure of the exposed EExMC led to mortality. The effect increased with increase in concentration and exposure time. SEM examination of *V. alternifolia* died EExMC showed ill distinct collar like rings, and lack of transverse ridges which appear highly corrugated edematous tegument. Collar ring in *M. piperita* exposed group was ill distinct with several blebs around ventral sucker and body tegument edematous.

Keywords: Egypt, *E. heretostomum, Verbesina alternifolia, Mentha piperita,* SEM, Histo-pathology, Kidney, *T. zillii.*

Introduction

Among the parasites, trematodes are the dominant group that causes retarded growth, morbidity, and mortality especially in juvenile fishes. It has been estimated that about 30000 species of helminthes are parasites of fishes (Williams and Jones 1994). In addition to the huge economic loss they cause, at least 50 species of these helminths are potentially zoonosis (Deardorff, 1991). The hemophagic clinostomid trematode *Euclinostomum heterostomum* (Rudolphi, 1809) is a common parasite of piscivorous birds in many regions of Africa (Yamaguti, 1971; Kannev *et al,* 2002a; Wang *et al,* 2017). In Egypt, the larval stage of this parasite infected the kidney of its second intermediate host (*Tilapia* sp.) as encysted progenetic metacercariae (Taher, 2009).

The major effect caused by *Verbesina* may be due to high levels of nitrates and galegine (Taher et al., 2012) and due to phyto-constituents of *Verbesina* as terpenoids, flavonoids and aromatic compounds (Song *et al,* 2009, Sindhu *et al,* 2010). Menthol and menthone are the main components of *Mentha piperita* (Mahboubi and Kazempour, 2014).

Histological studies of various pathogenic agents in different fish species have been reported and proposed as an efficient method to asses fish health, such as chemicals and pesticides (Troncoso-Ponce *et al,* 2011) and helminthes parasites (Shareef and Abidi 2012). Apart from the life cycle

and morphometry elucidated by some researchers, no study has been found that reports on the effects of plant oil extract alternation on the ultra structure tegument on the parasite and the pathogenecity and damage exerted on the tissues of fish during infection with *E. heterostomum.*

The present work aimed to study the effect of two plant oil extract; *Verbesina alternifolia*, and *Mentha piperita* on newly EExMC infection by using SEM for alternation of tegumental and histopathological changes caused by EEMC in infected *T. zillii*.

Materials and Methods

Sampling: Forty hundred and fifty freshwater fishes, mainly *Tilapia zillii* were caught by fisherman from natural Nile resources at Giza governorate, Egypt during the period from January-December 2016.

The collected fish were kept alive until examination at the parasitology laboratory, Faculty of Veterinary Medicine, Cairo University, where weight, total length and visual inspection to detect macroscopically visible parasites. The *Euclinostomum* metacercariae, mostly located in the peritoneum covering the kidney surface, were excysted and fixed in 70% ethanol for morphological study. Other number of fresh newly excysted metacercariae of collected parasites exposure to plants oil extracts.

Morphological identification: Whole mounts were made of 20 specimens clarified with Amman's lacto phenol, among which 10 were also stained by *Semichons acetocarmine* (Pritchard and Kruse, 1982). Parasites were identified based international keys for the family Clinostomidae (Kannev *et al*, 2002a, b). The EExMC was identified as *Euclinosto-mum heterostomum* after Fischthal and Kuntz (1963) and Ukoli (1966). The morphological features were taken by a digital camera (Sony, 3.0 MP Japan) attached to an inverted microscope (Olymbus, Japan).

Plant oil extract: Two plants oil extracts; *Verbesina alternifolia* and *Mentha piperita* were prepared (Salama *et al*, 2012).

Fresh active EExMC were washed three times in PBS solution. The EExMC (n=10/well) were exposed to a series of upgrading concentrations of each plant extract oil. Each experiment sets contained two replicates each of 10 EExMC. Control group in solvent as well as in PBS was associated with each exposure time. The bioassay was done according to WHO (1996) guidelines. All experiments were done under the same laboratory conditions. At the end of each exposure time, tested solution was removed; EExMC were washed for five times in PBS, left for another 3 hours, mortality % were evaluated by counting number of dead EExMC from exposed one (Taher *et al*, 2012).

The effect of plant extract on EExMC tegument on motor activity was classified as: live movement, slow movement, slow movement with damaged tegument, and/or dead (Panic *et al*, 2013). Efficacy of plant extracts activity was assessed by comparing the number of affected EExMC in each exposed group with that corresponding to control group.

Scanning electron microscopy (SEM): For SEM, EExMC were obtained from vitro assays study and control prepared by serial washing in saline solution and fixed in 2.5% glutaraldehyde (Colwell *et al*, 2007). Specimens were then dehydrated in ascending ethanol, dried in CO2 critical point drier (Autosamdri-815, Germany), glued over stubs and coated with 20nm (Gold in a sputter coater; Spi-Module Sputter Coater, UK). Specimens were examined and photographed with SEM at magnifications of 35 X to 500 X (JSM 5200, Electron prob Microanalyzer, Jeol, Japan) at Faculty of Agriculture, Cairo University.

Histopathological study: Five specimens from each exposed and control groups of EEMC infected fish were fixed in 10%

buffered formalin solution, processed for histological sectioning, stained with haematoxylin and eosin (Humason, 1979) and examined by light microscope for pathological changes kidney.

Results

Of 447 of *T. zillii* specimens collected, 65 were infected with *Euclinostomum* meta-cer-cariae, with a rate of 14.5%. Cysts were encapsulated in body cavity and kidneys. The peritoneum of the kidney was most favorable site of infection whereas least one was the body cavity with metacercariae varied from1-17cyst/fish (Pl. 1a).

Infection caused severs destruction in the fish tissue. Kidney with EMC of *E. heterostomum* showed parasite with intact normal internal structure enclosed by thick dense fibrous capsule frimalt attached to renal tissue. Also, cyst wall was incorporated with renal interstium and glomerular structures with intense inflammatory cell infiltration and extending into the adjacent renal tissue. Marked glomerular congestion with interstitial congestion, hemorrhages, vacuolization and necrosis of tubular epithelium with active melanomacrophage center (Fig. 1 & 2a, b,c).

Exposure of EExMC to different concentration of *V. alternifolia* oil extract caused mortality associated with irreversible degenerative changes in cuticle, oral and ventral suckers. The degenerative effect increased with the increase of dose and exposure time. At 200- 400ppm/4-6hrs, anterior region showed slow movement, while the posterior one was paralyzed. At 400ppm/10hr, reduced movement and tissue damages were observed. Died worms showed opaque grayish color with opaque appearance and confirmed shape. The mean LC_{50} and LC_{100} of the oil extract reached to 400 ppm/12hrs 600ppm/24hrs and died EExMC with shrinkage and corrugated cuticle.

Exposure of EExMC under the same conditions to different concentrations of *M. piperita* oil extract had no effect at concentration from 200 to 600 ppm and 6-24 hrs exposure periods. Mortality started as 10% in those exposed to 800 ppm/12hr increased to 30% after 24hrs, 50% mortality (LC50%) when exposure to 1000 ppm/ 24 hrs. Tested concentrations failed to induce more mortality to the experimental end. Died worms showed no movement associated with tegumental damage.

Control group: Neither changes in movement nor in tegumental of the EExMC in PBS were noticed nor they remained viable to the experimental end.

SEM: EExMC in control showed tegument (after various incubation times) to be normal (Pl. 2-5a). Oral sucker was smooth collar-like rings, covered by ridges and tegumental surface was distinct ventral transverse striation at ventral sucker posteriorly (Pl. 2b). Ventral sucker exhibited was sponge-like with normal tegument margins (Pl. 4b). Body posteriorly showed normal tegument (Pl. 5b).

SEM in EExMC exposed to *Verbesina* oil for 400ppm/12hrs showed marked edematous tegument at body anterior ventral surface (Pl. 2b), and, distortion of oral and ventral suckers with disappearance of collar like rings around oral sucker (Pl. 3b). Ventral sucker showed deformed, more edematous and thick (Pl. 4b) and posterior tegument surface was clearly edematous (Pl. 5b).

SEM in EExMC exposed to *M. piperita* oil for 1000ppm/24hrs was high swelled, edematous with disorganization of suckers and tegument deformity. Dead EExMC showed complete tegumental changes (Pl. 2c). Oral sucker showed disappeared of the two collar-like rings with highly edematous tegument (Pl. 3c). Tegumental surface was ill distinct ventral transverse striation. Irregular swellings, or blebs were particularly concentrated around ventral sucker (Pl. 4c) and edematous posterior body tegument (Pl. 5c).

Verbesina oil extract gave marked effect on parasites inside the kideny (Fig. 1& 2d,

e, f). kidney showed edema of internal structures and loose attachement between cyst wall and renal tissue. Edema of outer fibrous layer toward renal tissue resulted in loose attachement of cyst to renal tissue degeneration and edema of fibrous tissue cyst wall and interstitial edema with EGCs infiltration with minimal necrosis of tubular epithelium.

Discussion

Parasitic digeneans of family Clinostomidae are widely distributed, with trematode flukes of the genus *Euclinostomum* found as common parasites of piscivorous birds in many regions of world. Metacercariae may affect the growth and survival of fish, or may disfigure fish so that they lose their market value as either food or an ornamental product (Paperna, 1991). Several strategies have been proposed as screening methods for new drugs with anthelmintic potential based on *in vivo* models (Gonçalves *et al*, 2014) in which obtaining the worms requires the use of infected animals. In this context, the most recent research indicates that species of the genus *Echinostoma* satisfy this need (Fried and People, 2009; Keiser, 2010). Previous results showed that *E. paraensei* is a useful *in vitro* and in *vivo* model for both biological and therapeutic studies (Maldonado et al., 2005; Souza et al., 2013). Our previous results showed that *Prohemistomum vivax* (adult trematode) is a useful *in vitro* and in *vivo* model for both biological and therapeutic studies (Ibrahim and Mahdy, 2017). In the present study, compared with exposure the EExMC to both plants oil extract, at high concentrations (400ppm/12hrs) during this exposure periods, causes decreased movement and tegument change and lethal dose fifty LC50% was occur. The similar result to those recorded by Souza et al. (2017) when exposed newly excysted metacercariae of *Echinostoma paraensei in vitro* to artesunate in emphasize that these effects are both time- and dose-independent.

In order to evaluate the *in vitro* use of EExMC, the present study compared ultra-structure morphological alterations caused by *V. alternifolia* and *M. piperita*, with those observed in excysted metacercariae, reporting new qualitative and quantitative morphological changes.

In the present results, body tegument damage and mortality was higher concentrations after 12 hours' incubation. The severe damage was at anterior region around the oral sucker, with high swelling, including disorganization of the suckers and ultra-structure tegument deformity. This agreed with Souza *et al*. (2017). Also, the present results went others who reported tegument morphological alterations especially EExMC exposed to Mint. Also, blebs, furrowing, swelling, disruption and deformity resulted by artemisinin derivatives *in vitro* on *Schistosoma mekongi* (Jiraungkoorskul *et al*, 2006), *Fasciola gigantica* (Diab *et al*, 2010) and *Echinostoma caproni* (Keiser *et al*, 2006).

In the present study, the integrity and function of the tegument were critical and related to survival of EExMC. In Egypt, *Verbesina* alcoholic extracts as molluscicidal and/or mosquito larvicidal activities by *Verbesina* corresponded to high levels of nitrates and galegine (Taher *et al*, 2012). The physiological processes might be impaired, although the mechanism of action of the present plants extracts, actedagainst *C. phalacrocoracis* was elucidated, the possible mechanism and *V. alternifolia* was strong and highlighted the presence of EExMC than the effect of *M. piperita* due to Menthol was the main substance of composition and confirmed to display anthelmintic activities (Girme *et al*, 2006).

Eskes *et al.* (2009) stated that research must be carried out with alternative techniques to provide the same level of information as that in experiments on animals. The present study went in line with Eskes *et al.* (2009) as the effects were the same

as those of Ultrastructure tegumental changes with EExMC.

The present histopathological study, revealed histozoic parasite stages in fish kidney. Infection of *T. zillii* by EEMC decreased fish immunity, allowing other pathogens infections. *E. heterostomum* infections were reported in many fish species, including *Tilapia* (Dönges, 1974, Britz *et al*, 1985, Olurin and Somorin 2006). Jhansilakshmibai and Madhavi (1997) reported that *E. heterostomum* infected the liver and kidney of *C. punctata*. But, in the present study, parasite was encysted only in the peritoneal of kidney. The histo-architectural alterations were induced by *E. heterostomum* with intact normal internal structures and marked glomerular congestion, vacuolization and necrosis of tubular epithelium with activation of melanomacrophage center. The agreed with Shareef and Abidi (2015) in *C. punctata* infected kidney. Also, in exposed to *Verbesina* at 400ppm/12hrs showed edema of internal structures of *E. heterostomum*.

Conclusion

This could be the first time to show the *in vitro* anthelmintic activity of *V. alternifolia* and *M. piperita* against EExMC. The LC_{50}% for *V. alternifolia* and *M. piperita* was 400ppm/12hrs and 1000 ppm/24hrs, respectively. The *V. alternifolia* showed anthelmintic activity higher than *M. piperita* for the exposed EExMC.

The outcome data showed that the newly excysted metacercariae of *Euclinostomum heterostomum* constituted a good model to study *in vitro* contribution and to find out natural plant extracts efficiecy as anthelmintic agents

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Explanation of plates

Plate 2: SEM of ventral tegument of *E. heterostomum* a) A control non exposed EExMC showing distinct normal collar-like rings, oral sucker (os), ventral sucker (vs). b) Exposed EExMC to *V. alternifolia* extract showed corrugated tegument with disfigured and distortion of oral &ventral suckers c) Exposed EExMC to *Mint oil* extract showed edematous tegument with disfigured and distortion of both suckers.

Plate 3: SEM of oral sucker of *E. heterostomum* a) A control non exposed EExMC showing normal collar-like rings (CL) distinct smooth tegument (st). b) Exposed EExMC to *V. alternifolia* extract showed disappearance of CL and sever corrugated tegument (ct) around the oral sucker c) Exposed EExMC to *Mint* oil extract showed highly edematous oral sucker (ot) and shrinkage of the oral sucker (os).

Plate 4: SEM of ventral sucker of *E. heterostomum* a) A control non exposed EExMC showing normal sucker. b) Exposed EExMC to *V. alternifolia* extract showed thicker folds and edematus c) Exposed EExMC to *Mint* extract and appear of numerous blebs around ventral sucker (arrows).

Plate 5: SEM of posterior tegumental surface of *E.hetrostomum* a) A control non exposed EExMC showing normal smooth cuticle. b) Exposed EExMC to *V. alternifolia* extract showed edematous tegument. c) Exposed EExMC to *Mint* extract showed edematous tegument.

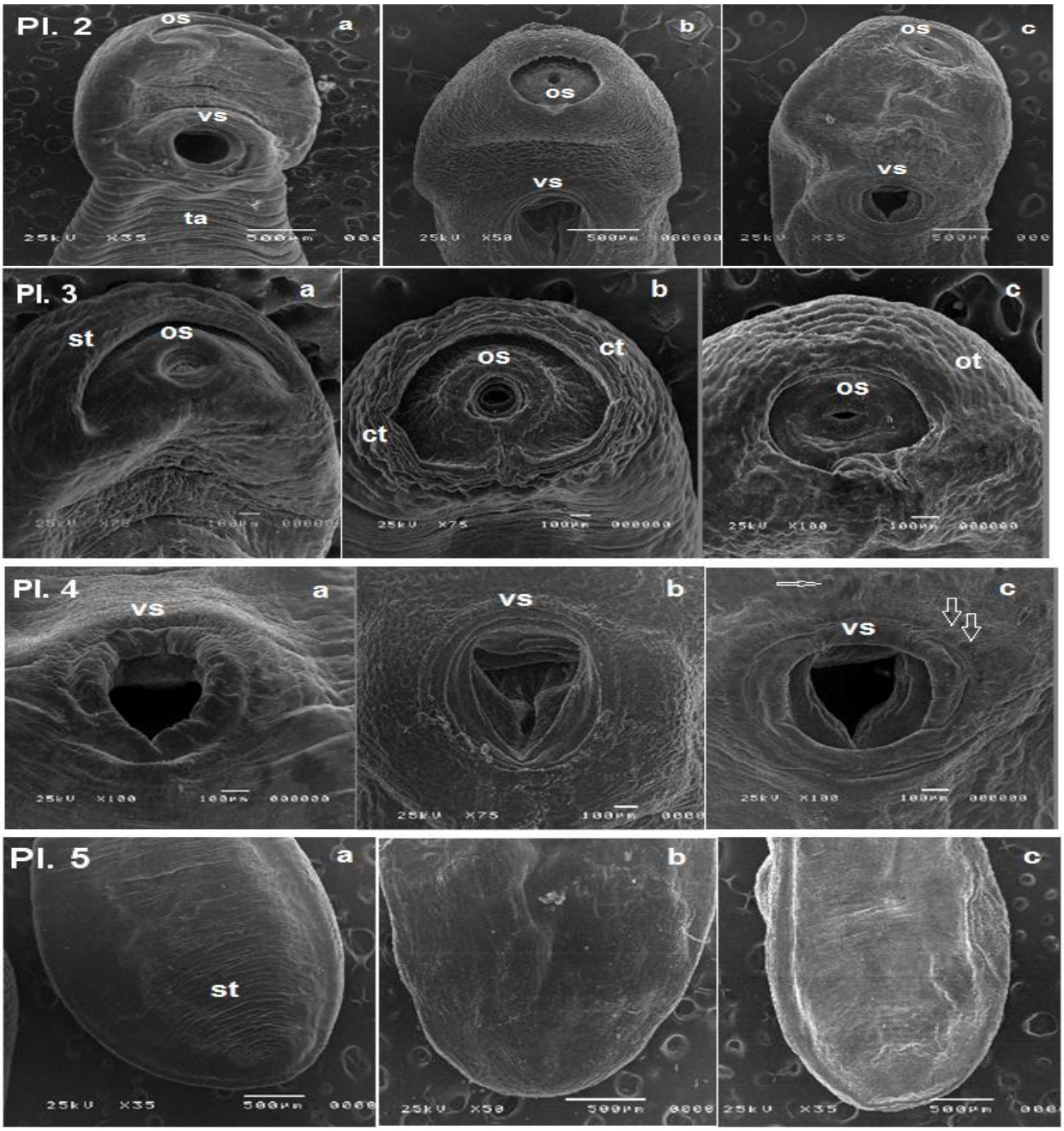

Plate 1; a) Infected kidney *of T.zillii* with encysted metacercaria of *E. heterostomum* showing 17 EEMC in the peritoneum covering kidney and tissues (thick arrow in kidney and thin arrow in other tissues). B-c). Newly excysted metacercariae of *E. heterostomum* fresh specimens, showing distinct oral andventral sucker (os & vs) and branched intestinal cecae (ic). d) Stained *E. heterostomum*, showing distinct; oral sucker (os),ventral sucker (vs), testes; anterior testis (at) and posterior testis(pt) and (o).

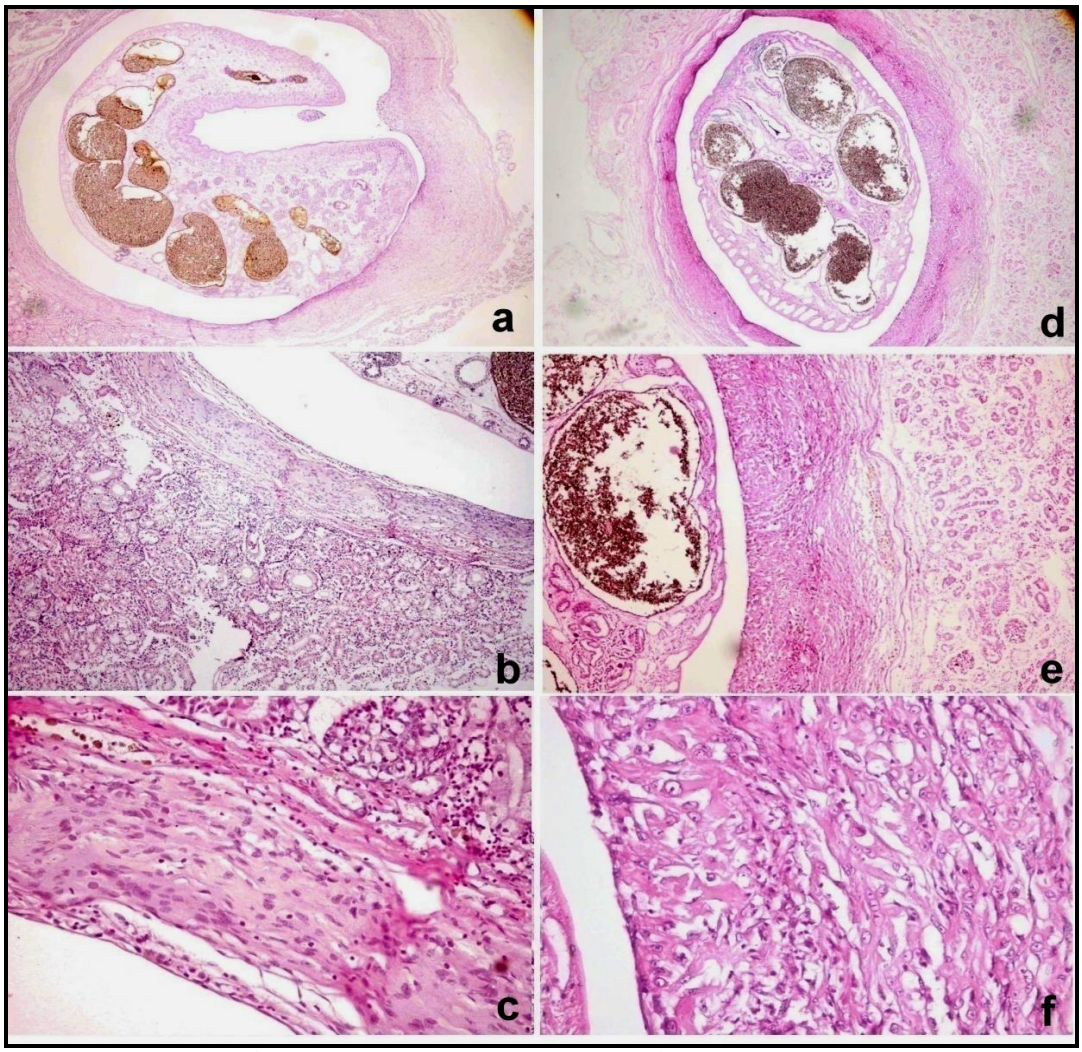

Fig.1: (a-c) Histological section of kidney control group showing; a) presence of large encysted metacercaria enclosed by thick dense fibrous capsule frimalt attached to renal tissue and incorportaed into renal tissure structures (X40). b) control group showing dense fibrous cyst wall incorporated with renal interstium (X100).c) incorporation of glomerular structures in structure of connective tissue of cyst wall associated with intense inflammatory cell infiltration in cyst wall and extending into adjacent renal tissue, (X400). (d-f)Histological section of kidney exposed group to Verbesina oil plant extract showing d)presence of large encysted metacercaria enclosed by fibrous capsule with loose attachement between cyst wall and renal tissue (X40). e) edema of outer fibrous layer to renal tissue resulting in loose attachement of parasitic cyst to renal tissue (X100). f) degeneration and edema of fibrous tissue wall (X400).

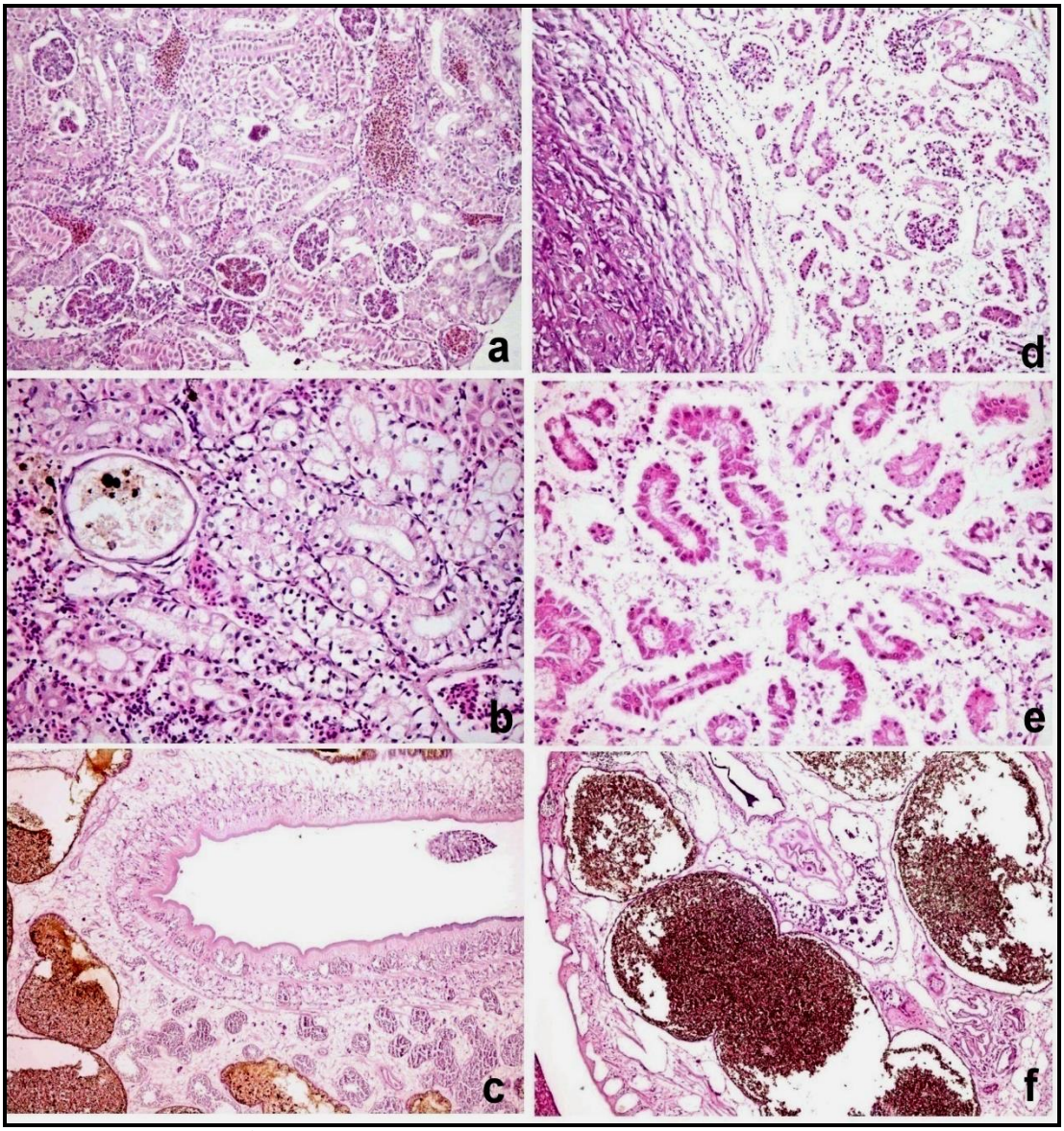

Fig.2: (a-c) Histological section of kidney control non exposed group showing;
a)marked glomerular congestion with interstitial congestion and hemorrhage (X200). b) vacuolization and necrosis of tubular epithelium with activation of melanomacrophage center (X200). c) *Euclinostomum heterostomum* with intact normal internal structures (X100). (d-f) Histological section of kidney exposed group showing d) Exposed group showing massive edema of cyst wall that was extended into adjacent renal interstitium (X100). e) interstitial edema with EGCs infiltration with minimal necrosis of tubular epithelium (X200). f) edema of internal structures (X100).