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Research Article
Therapeutic Efficacy of Intropar® and *Artimisia annua* Extract on Treatment of African Catfish Infected with Trypanosomiasis

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Abstract

Background and Objective: Trypanosomiasis affecting fish caused by genus *Trypanosoma* which is considered as one of the most important protozoal disease affecting freshwater fishes. The present study aimed to investigate some trials for treatment of trypanosomiasis in catfish *Clarias gariepinus* with histopathological examination of the naturally infested catfish, *C. gariepinus*. **Materials and Methods:** A total number of 100 alive catfish *(Clarias gariepinus)* with 120±10 g weight were collected from private fish farm at Kafer El-Kheish governorate. The fish subjected to parasitological examination for the trypanosome species. For treatment trials, a total number of 120 catfish were divided into 4 groups each 10 with three replicate were used for treatment trials with Intropar® I/M, bath with *Artimisia annua* leaves ethanol extract (100 and 150 mg L^{-1} for 120 min). **Results:** The main clinical and postmortem lesions of infected *Clarias gariepinus* were paleness of the outer body surface, eroded fins, gulping the atmospheric air. Histopathological investigation revealed degenerative, necrotic and inflammatory changes in skin, gills and all internal organs. Experimental infection of *C. gariepinus*, *Oreochromis niloticus*, gold fish *Carassius auratus* and male white mice to with *Trypanosoma mukasai* were carried out. The prevalence of trypanosomiasis in catfish *C. gariepinus* was 63%. The result of the treatment revealed that 150 mg L^{-1} for 120 min was the treatment of choice for trypanosomiasis in *C. gariepinus*. **Conclusion:** It was concluded that the treatment of choice for trypanosomiasis in *C. gariepius* was bath treatment with *Artimisia annua* leaves ethanol extract (150 mg L^{-1} for 120 min).

Key words: Trypanosomiasis, *Clarias gariepinus*, emaciation, histopathological changes, treatment, Intropar®, *Artimisia annua*

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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

One of the most important problems facing our world is food deficiency. The protein deficiency is one of the major global challenges facing the third world today. In Egypt, the continuous increase in human population requires more food population to meet the consequent increasing demands. With increasing demands for animal protein, fishes were considered to compensate the continuous lack of such element due to its comparatively low price .

Fish diseases have long been considered as a serious problem either in cultured or wild fishes causing enormous economic loses in tropic and sub-tropic countries like Egypt[1]. Parasitic diseases are critical concern in warm water fishes[1,2]. Heavy infestation of fish with Protozoan parasites cause great damages and high mortalities[1]. Blood parasites affecting fish are prevalent in Egypt due to the warm water conditions and availability of the intermediate hosts (Cyclops, mollusca and leeches)[1-4]. Fish trypanosomiasis affects several freshwater fishes[5,6]. Infected fish suffer from anemia with dull appearance, watery blood and cannibalism[7-9,2].

Trypanosomes are parasitic haemoprotozoa that infect both humans and animals, causing morbidity, mortality and economic losses worldwide. Parasites from the variety *Trypanosoma* (Kinetoplastida: Trypanosomatidae) are universal protozoans that taint an extensive variety of creatures, including leeches, bugs, angle, creatures of land and water, reptiles, flying creatures and warm blooded animals and are the causative specialists of probably the most dismissed human and creature diseases[8,9].

Available literatures handling the treatment of trypanosomiasis in fish is scanty so that, the present study was aimed to perform some trials for treatment of trypanosomiasis in catfish *Clarias gariepinus* using commercial product; Intropar® (Sweed Pharma, Egypt) and *Artimisia annua* leaves ethanol extract.

MATERIALS AND METHODS

Fish: From June, 2016-August, 2017 a total number of 100 alive catfish (*Clarias gariepinus*) with an average body weight of 120±10 g were randomly collected from private fish farm at Kafer El-Kheish governorate, Egypt. Fish transferred to the Department of Hydrobiology, National Research Centre, Egypt. Fish was kept in fully prepared glass aquaria (1.5×2.0×1.5 cm) at 20°C for 2 weeks and subjected for parasitological examination for the *Trypanosoma* species. Then subjected to experimental infection. All fish were fed with commercial diet 2.5% b.wt., twice daily. Blood was collected from caudal vein with 1 mL heparinized syringe, examined and the parasitemia was estimated from wet blood preparation[10]. The trypanosomes infected fish were then separated from the trypanosomes free fish. The fish were then subjected to clinical, postmortem, histopathological examinations.

Clinical and postmortem examinations: The infected catfish were subjected to the clinical as well as postmortem examinations using the methods described by Lucky[11] for determination any external and internal abnormalities on the external body surface and internal organs.

Parasitological examination: Fresh blood samples were collected from caudal veins according to Lied *et al.*[12]. Blood films were prepared, air dried, fixed with methanol and stained with freshly prepared and diluted Giemsa stain. The stained blood films were examined with oil immersion lens of light microscope to detect the presence of trypanosomes according to Kabata[13].

Experimental infection: From 5 naturally infected catfish *C. gariepinus*, blood withdrawn with heparinized syringe from the caudal blood vessels, pooled and injected Intra/ Peritoneal (I/P) into 5 *C. gariepinus* and injected Intra/Muscular (I/M) into 5 another *C. gariepinus*, injected also into 10 *Oreochromis niloticus* (5 fish I/P and 5 fish (I/M) and injected also into 10 gold fish *Carassius auratus* (5 fish I/P and 5 fish I/M) finally injected into 10 male white mice (I/P). All injected fishes and mice were examined for trypanosomiasis infection 5 days intervals, making samples from blood film, dried and fixed with methanol and stained with freshly diluted Giemsa stain and examined with oil immersion light microscope.

Drugs and plants used in treatment of trypanosomiasis

Intropar®: Commercial drug produced by Seweed Pharma, Egypt Company, for treatment of trypanosomiasis in animals.

***Artimisia annua* leaves ethanol extract:** *Artimisia annua* plant purchased from National Research Center, the leaves were washed thoroughly in running tap water to remove sand and debris. Thereafter, they were dried by spreading under the sun for 3 days and finally in a hot air oven at 60°C for 8 h. The dried leaves were crushed to powder in a mortar and pestle and subjected to Soxhlet extraction with 70% ethanol as the extracting solvent. The solvent was exhausted from the extract

with the help of a rotary evaporator. The extract was stored in a refrigerator until required for use[14].

Preparation of stock and working solutions of *A. annua*: The ethanolic extract for *A. annua* was used for the preparation of a stock solution from which the working solution used for the efficacy testing was prepared. The stock solutions were obtained by dissolving 1 g of the extract powder in 5 mL of dimethyl sulfoxide (DMSO) and made up to 100 mL with de-ionized water and up to 150 mL with de-ionized water.

Experimental design for treatment of experimentally infected catfish: A total number of 120 *Clarias gariepinus* experimentally infected fish with trypanosome mukasi divided into 4 groups each 10 fish with 3 replicate, first group was injected I/M with Intropar® (Sweed Pharma, Egypt) with a dose 2 mL kg^{-1} b.wt., fish and 2nd group subjected to bath treatment with *Artimisia annua* leaves ethanol extract bath (100 mg L^{-1} for 120 min)[14] and 3rd group was subjected to *Artimisia annua* ethanol extract bath (150 mg L^{-1} for 120 min)[14] and 4th group was set as a control group with no treatment. All groups were subjected for examination 5 days intervals for presence of trypanosome in blood.

Histopathological examination: Infected fish with *Trypanosoma* as well as non infected fish were subjected to histopathological examination. Tissue specimens were rapidly fixed in Davidson's fixative for 24 h then transferred to 70% ethanol till processing proceeds. The fixed specimens were processed through the conventional paraffin embedding technique (dehydration through ascending grades of ethanol, clearing in xylen and embedding in paraffin wax at 60°C). Paraffin blocks were prepared and cutting 3 µm thick tissue sections by using microtome (Leica 2155), then the slides were stained with H and E stain then examined by light microscopy according to Bancroft and Gamble[15].

Ethical cosiderations: The list of committee of ethics of scientific research at National Research Centre, Egypt does not include fish therefore, the committee refused to give us the required certificate.

Statistical analysis: Data were presented as Mean±Standard Error (SE) and the significance of differences was estimated using Student's t-test at $p \leq 0.01$ as described by Snedecor[16].

RESULTS

Clinical picture and postmortem lesions: The present investigation displayed that the inspected catfish *Clarias gariepinus* naturally infected with trypanosomiasis revealed paleness of the outer body surface, emaciation and eroded fins (Fig. 1a) gasping the atmospheric air with dullness, slack appearance and paleness of gills and dendritic organ (Fig. 1b). Internal inspection revealed enlargement of spleen, watery blood with pale internal organs.

Parasitological examination: Morphological features of isolated protozoan were extremely related to *Trypanosoma mukasai* (Fig. 2).

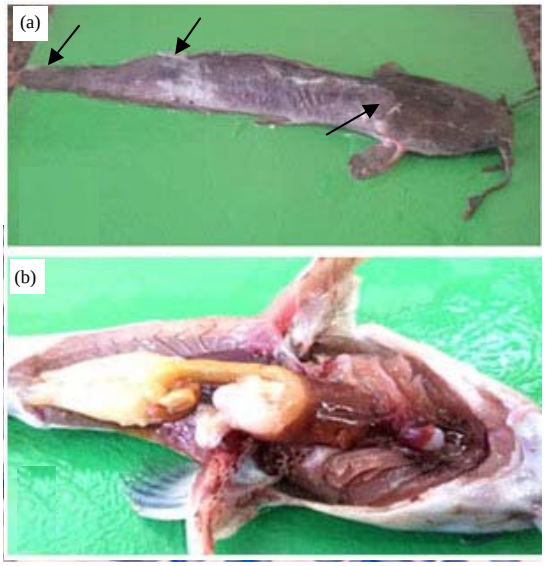

Fig. 1(a-b): (a) Catfish *Clarias gariepinus* infected with trypanosomiasis suffered from emaciation, eroded fins and pallness of external body surface (arrows) and (b) Gill and dendritic organ pallness of infected catfish with *Trypanosoma mukasai*

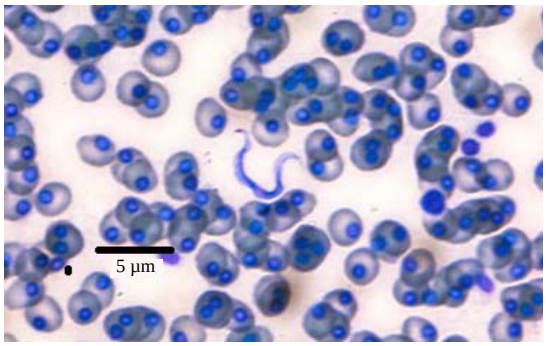

Fig. 2: *Trypanosoma mukasai* in blood film from naturally infected *C. gariepinus* stained with Giemsa stain (arrow)

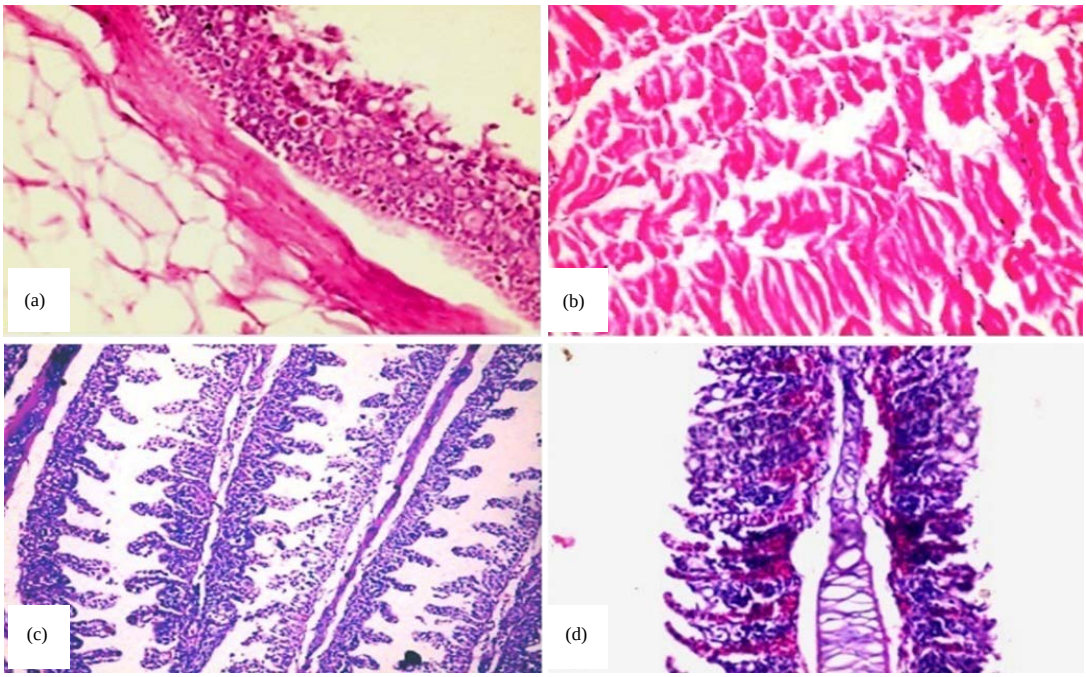

Fig. 3(a-d): Catfish infected with trypanosome, (a) Skin epidermal layer (H and E, X400), (b) Skin muscular H and E, X400) (c) Gills in the epithelial lining (H and E, X200), (d) Gills in the respiratory epithelium (H and E, X400)

Table 1: Cross infection from *C. gariepinus* to other species

Received species	I/P injection	I/M injection	Subcutaneous
Claris gariepinus	+ve	+ve	-ve
Oreochromus niloticus	+ve	+ve	-ve
Goldfish *Carassius auratus*	+ve	-ve	-ve
Albino rat	-ve	-ve	+ve

Table 2: Treatment efficacy in trypanosomiasis in experimentally infected *C. gariepinus*

Groups	Number of fish	Drug	Dose	Treated	Survival	Treated fish (%)
1st	10	Intropar®	2 mL kg^{-1} b.wt.	8*	7	80
2nd	10	*A. annua*	100 mg L^{-1} for 120 min	4	9	40
3rd	10	*A. annua*	150 mg L^{-1} for 120 min	9*	9	90
4th	10	No treatment	Control	0	5	0

*Significant difference by student t-test at $p \leq 0.01$ n = 10

Prevalence of trypanosomiasis in catfish *Clarias gariepinus*: Prevalence of trypanosomiasis in *Clarias gariepinus* was 63.

Experimental infection (Cross infection): Transmission of *Trypanosoma mukasai* from infected *C. gariepinus* to non infected *C. gariepinus* was succeeded through both I/P and I/M routes. The *O. niloticus* also showed +ve infection through both routes I/P and I/M and -ve through subcutaneous while, goldfish *Carassius auratus* showed +ve infection only through I/P route. On the other hand mice showed -ve results through both I/P and I/M while +ve through subcutaneous route Table 1.

Treatment of trypanosomiasis in experimentally infected *C. gariepinus*: Results showed that the efficacy of treatment determined throughout lower number of mortalities in treated fish groups. The highest rate was recorded for *A. annua* in the 3rd group 90% followed by 1st group 80% , 2nd group 40% while the lowest rate of treatment was recorded for 4th group (control) 0 treatment and 5% survival (Table 2).

Histopathological study: The histopathological changes resulted from *Trypanosoma* infection revealed degenerative and necrotic changes in the epidermal cell layer (Fig. 3a) , sub epidermal edema, zenkers necrosis and infiltration of chronic inflammatory cells in between the muscle fibers (Fig. 3b).

The gills showed severe vacuolar degeneration and necrosis in the epithelial linning the secondary lamellae (Fig. 3c) associated with congestion in the lamellar and

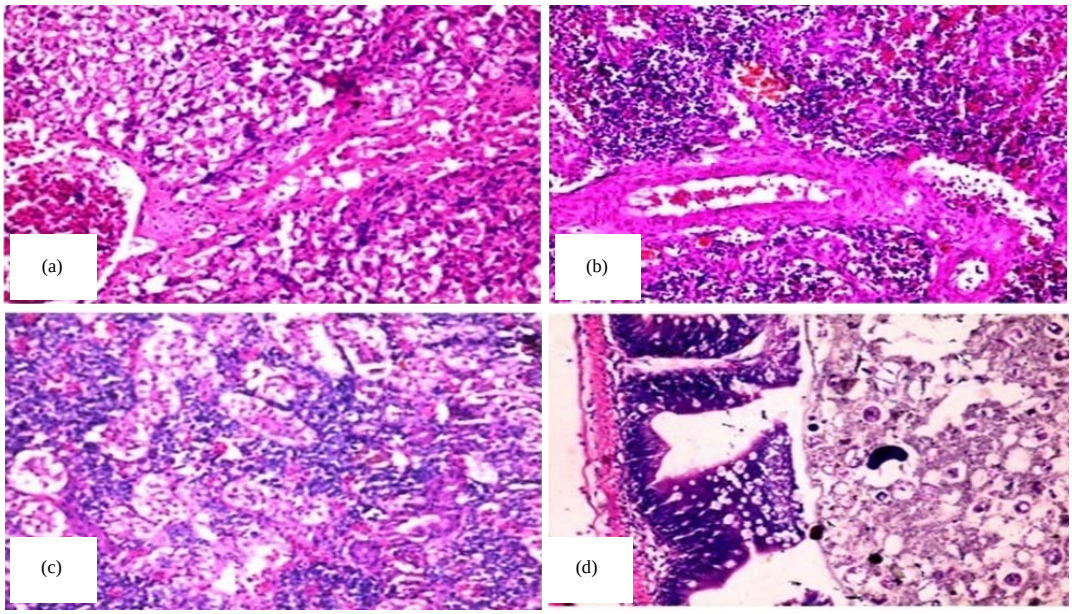

Fig. 4(a-d): Catfish infected with trypanosome, (a) Liver (H and E, X400), (b) Kidneys (H and E, X400), (c) Spleen (H and E, X400) and (d) Intestine (H and E, X400)

branchial blood vessels, hyperplasia also detected in the respiratory epithelium leading to adhesion between the secondary lamellae (Fig. 3d).

The liver showed vacuolation and necrotic changes in the hepatocytes, congestion in the hepatic blood vessels, hyperplasia in the wall of blood vessels and infiltration of chronic inflammatory cells and fibrous connective tissues between the hepatic parenchyma (Fig. 4a).

The kidneys showed severe degenerative and necrotic changes in the tubular epithelium, endothelial linning the glomerular tuft and in the interstitial hemopoietic tissues, congestion in renal blood vessels and peritubular and periglomerular edema also detected (Fig. 4b).

Spleen showed congestion in the splenic blood vessels, depletion in the hemopoietic tissues associated with hyperplasia in the wall of splenic blood vessels (Fig. 4c).

Intestine showed degenerative and necrotic changes in the epithelial linning the intestinal villi also parasitic sections appeared in the intestinal lumen (Fig. 4d).

DISCUSSION

The present study was carried out to determine the therapeutic effect of commercial product Intropar® produced by (Sweed Pharma, Egypt) company and medicinal plant *Artimisia annua* leaves ethanol extract on treatment of trypanosomiasis in catfish *Clarias gariepinus* with histopathological alterations of the naturally infested catfish.

The clinical sings and postmortem lesions noticed in *Clarias gariepinus* infected with trypanosomiasis revealed paleness of the outer body surface, emaciation and eroded fins, gulping the atmospheric air with lethargy and paleness of gills with dendritic organ. Spleen was enlarged, paleness of the internal organs was characteristic with watery blood similar to that obtained by Mariam[17], Essam and El-Khateib[18], El-Khatib and Elias[19] and Adawy and Deeb[20]. The clinical sings and postmortem lesions may be due to that infected fishes suffered from anemia and haemodilution as *Trypanosoma* sp. produce hemolysins that lyse the RBCs by Essam and El-Khateib[18].

Prevalence of trypanosomiasis in catfish *Clarias gariepinus* was 63% in accordance with Overath *et al*.[8], Ahmed[21] and Kidchakan[22]. Catfish are non scaly fish and live at the bottom where leeches (blood sucking vector) are abundant.

The experimental infection of *Trypanosoma mukasai*. The present study revealed that transmission of *Trypanosome mukasai* in *C. gariepinus* was succeeded through out both routes of injection I/P and I/M also, *O. niloticus* showed +ve infection through both I/P and I/M and -ve trough subcutaneous. Infection in goldfish, *Carassius auratus* was only carried out through I/P on the other hand white mice showed -ve results with I/P and I/M and +ve through subcutaneous route. The results agreed with the results of

Woo and Black[23], who reported that trypanosome is not host specific however *Trypanosoma danilweski* Laveran and Mesnil, 1904 causes mortality in experimentally infected goldfish *Carassius auratus*[24,25]. These results also supported also by Nazrul Islam and Woo[5]. Cross infection in the present study was –ve in albino rat due to the physiological difference between two genus in mammals and fishes.

Treatment of trypanosomiasis in catfish *Clarias gariepinus* revealed that the highest rate of treatment was recorded for *A. annua* in the 3rd group 90% treatment followed by 1st group 80%, 2nd group 40% while the lowest rate of treatment was recorded for 4th group (control) 0 treatment 5%.

Present study revealed that all infected fish were successfully treated with *A. annua* leaves ethanol extract (150 mg L^{-1} for 120 min), the obtained results supported by several modern investigations which have also displayed that artemisinin has a therapeutic potential against *Toxoplasma gondii*[26], *Trypanosoma* and *Schistosoma* sp.[27,28] as well as other pathogens responsible for *Cryptosporidiosis*, *Amoebiasis*, *Giardiasis*, *Leishmaniasis*[29].

Artemisinin (active principle of *A. annua*) destroy the cells of parasitic organisms through the production of highly reactive oxygen-based free radicals or electrophilic intermediates, by alkylating and oxidizing proteins and lipids of parasite membranes as well as inactivation of channel proteins[30]. It has been demonstrated that the effect of artemisinin is equally mediated through disruption of membrane potential by interacting with the electron transport chain in the mitochondrial membrane, resulting in free radical damage and dysfunction of mitochondria[31].

Concerning the histopathological changes of naturally infested catfish *C. gariepinus* in the present study revealed degenerative, necrotic and inflammatory changes in all internal organs, skin and gills these changes may be resulted from the eggs produced by adult worms which are carried by the vascular system into the gills of the host fish where they become lodged in the arterioles or epithelium. The eggs hatch in this site and the miracidium exits through the epithelium so the histopathological changes caused by these parasites is primarily due to host reaction to these eggs also the exiting of miracidia through the gills[32]. Also these results confirmed by Bunnajirakul *et al.*[33] and Supamattaya *et al.*[34].

The study is highly valuable for treatment of trypanosomiasis affecting cultured fishes in Egypt. Control of blood protozoa affecting fish can be achieved through eradication of the intermediate hosts like crustaceans and also through adjustment of water quality parameters to avoid stresses on fishes. On the other hand, treatment of fishes by injection (I/P or I/M) is not practical and make some limitations to this method.

CONCLUSION

From the present study it was concluded that the most important method for control of trypanosomiasis in fishes can be achieved through eradication of intermediate hosts (crustacean) like leeches and cyclops. The treatment of choice for trypanosomiasis in fishes was achieved by the ethanolic extract of leaves of *A. annua* as a bath 150 mg L^{-1} for 120 min.

SIGNIFICANCE STATEMENT

This study discover the treatment of choice for trypanosomiasis in *C. gariepius* was bath treatment with *Artimisia annua* leaves ethanol extract that can beneficial for treatment and control trypanosomiasis. This study will help the researcher to uncover the critical areas of control and treatment of trypanosomiasis in *C. gariepius* that many researchers were not able to explore. Thus a new theory on treatment of blood parasitic diseases of fishes may be arrived at using medicinal plants (*Artimisia annua*).

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