In vitro evaluation of antihelminthic activity of Praziquantel, Flubendazole and some plant extracts against the adult eye fluke (*Philophthalmus palpebrarum*) transmitted by the freshwater snail *Melanoides tuberculata*.

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**ABSTRACT**

*Philophtalamus* species (the eye flukes) are digenetic trematodes that cause damage to the ocular tissues of birds and humans. Diagnosis of subclinical philophthalmosis and effective therapeutic regimens are lacking, despite the increasing number of cases reported in birds and humans. So, the present study aimed to evaluate in vitro the antihelminthic activity of Praziquantel (PZQ), Flubendazole, and plant leaves extracts (*Jatropha curcas* and *Moringa oleifera*) against the adult eye fluke *Philophthalmus palpebrarum* (*Philophthalmidae*) which extracted from the conjunctival sac of the experimentally infected chickens eyes by excysted metacercariae. The cercariae obtained from the naturally infected freshwater snails *Melanoides tuberculata* collected from watercourses in Giza Governorate, Egypt. The efficacy was determined based on the dead worms percentage after treatment and morphological changes recorded by scanning electron microscopy. The worms were incubated in the culture media with different concentrations tested materials up to 72 h. The calculated LC<sub>50</sub> were 7.857, 9.170, 65.534 & 44.509μg/mL and LC<sub>90</sub> were 10.149, 13.489, 91.474 & 53.004μg/mL, respectively. Worms incubated for 48h with LC<sub>50</sub> of PZQ and *Moringa* revealed deformity, swelling of oral and ventral suckers, erosion, and disorganization of the tegument when examined with scanning electron microscopy. It can be concluded that Praziquantel, Flubendazole, *Jatropha curcas*, and *Moringa oleifera* plant leaves extracts possess in vitro antiphilophthalmus activities; however, studies in vivo should be conducted to examine their antiphilophthalmus effects.

**INTRODUCTION**

*Philophtalamus palpebrarum* are small trematodes that have the freshwater snail *Melanoides tuberculata* (Müller, 1774) as intermediate host and parasitize primarily the eyes (conjunctival and orbital tissues) of wild and domestic birds, commonly associated with freshwater birds and wading birds (Kingston, 1984; Sapp et al., 2019). Transmission to birds occurs by ingestion of encysted metacercariae on substrates...
(crustacean shells, vegetation, etc) or direct ocular contact with cercariae or metacercariae in water (Alicata and Ching, 1960). The freshwater snail *M. tuberculata* is known to be the intermediate host of several species of the eye fluke. Eye flukes originating in *M. tuberculata* have been identified as *Philophthalmus grallli* in the Middle East, specifically the United Arab Emirates (Ismail and Arif, 1993), Jordan (Ismail and Issa, 1987), Saudi Arabia (Kalantan et al., 1998). Also Radev et al. (1999) recorded *Philophthalmus lucipetus* in Israel that was considered an European species in the past. While, in Egypt *Philophthalmus distomatosa* was first known by its larval stage, Cercaria *distomatosa* and its adult worm was experimentally grown and described by Radev et al. (2000). Also, has been found Venezuela (Diaz et al., 2002), Brazil (Verocai et al., 2009) and in Zimbabwe (Mukaratirwa et al., 2005). All these mentioned species have the freshwater snails of the family Thiariidae (*Melanopsis praemosa*, *M. tuberculata*, *Cleopatra bulinoides*, and *Tarebria granifora*) as intermediate hosts. *Philophthalmus hegneri* is the only representative of *Philophthalmus* in Arabia developing in a marine snail (*Cerithium scabridum*) which was found in Kuwait (Abdul-Salam et al., 2004). Moreover, only the adult worm of *P. palpebrarum* recorded in Egypt by Looss (1907 and 1899) from the eyes of Yellow Billed Kite and Little Owl. But, Ayoub et al. (2020) completed and described life cycle of *P. palpebrarum* experimentally and identifying the larval stages originating in *Melanoides tuberculata* snails for the first time in Egypt.

In general a variety of mammals, including humans, occasionally serve as the intermediate as well as the definitive hosts for the genus *Philophthalmus*. Moreover, the zoonotic philophthalmiasis in human cases has been also documented in various parts of the world (Sapp et al., 2019 and Prompetch et al., 2021). Lang et al., 1993; Kanev et al., 1993 & Nollen and Kanev, 1995 reported cases of human infection and conjunctivitis in eyes due to infection with *P. palpebrarum; P. lucipetus* and *P. lachrymosus*, respectively. Recently, a unique pattern of granulomatous anterior uveitis in rural children swimming in El-Fayoum, Egypt, was attributed to a waterborne helminthic infection (Nadar et al., 2017). It is usually minimally pathogenic in natural hosts; heavy burdens and/or infections inaberrant hosts may be associated with conjunctivitis, episphora, hemorrhage, and itching/swelling of the eyelids (Pinto et al., 2005 and Heneberg et al., 2018). Despite the increasing number of cases reported in birds and human, studies related to the diagnosis of subclinical philophthalmosis are lacking, and there are no effective therapeutic regimens available (Assis et al., 2018).

Praziquantel (PZQ), an acetylated quinoline-pyrazine, is a medication used to treat a number of types of parasitic worm infections in mammals, birds, amphibians, reptiles, and fish (WHO, 2009). In humans specifically, it is used to treat schistosomiasis, clonorchiasis, opisthorchiasis, tapeworm infections, cysticercosis, hydatid disease, and other fluke infections (WHO, 2009 and Heneberg et al., 2014). Also, Flubendazole has wide-spectrum activity against various nematodes and cestodes affecting human. It is a
member of the class of mebendazole in which the benzoyl group is replaced by a p-fluorobenzoyl group (William et al., 2003).

Plants represent an important source for drug discovery and have produced some very effective chemotherapeutic treatments for parasites, eg. Jatropha curcas (Euphorbiaceae) and Moringa oleifera (Moringaceae) (Eguale & Giday, 2009). J. curcas is a species of flowering plant in the genus Jatropha, family Euphorbiaceae, originates in Central America and Mexico, and has been naturalized in a number of other tropical and sub-tropical countries in Asia, Africa and Latin America. Its roots, stems, leaves seeds and fruits of the plant have been widely used in traditional folk medicine in many parts of West Africa. The seeds have been used as purgative, anthelmintic and abortifacient, for treating ascites, gout and skin diseases. Its seeds have also been reported to be effective against Strongyloides papillosus infection in goats (Vollesen et al., 1995).

M. oleifera belongs to the family of Moringaceae, originates in the Himalayas in north western India, but this species has been planted in tropical and subtropical climates throughout the tropics (Tilman et al., 2006). M. oleifera is one of the important plants which have bioactive compounds with nutritive and medicinal values present in the leaves, seeds, roots, barks, and flowers (Fahey, 2005). In addition, this plant was utilized in the treatment of malaria, leishmaniasis, trypanosomiasis, schistosomiasis, dracunculiasis, and filariosis thus suggesting its inherent antiparasitic property (Fahey, 2005 and Wang et al., 2016). Therefore, the main aim of the present study is an attempt to evaluate the antihelminthic activity of Praziquantel, Flubendazole and some plant extracts (J.curcas and M. oleifera) against the adult eye fluke P. palpebrarum (Philophthalmidae).

MATERIALS AND METHODS

1. Experimental materials:
Praziquantel (PZQ)(C_{19}H_{24}N_{2}O_{2}), an acetylated quinoline-pyrazine,sold under the brandname Biltricide
Flubendazole, sold under the brandname Fluver, It is a member of benzimidazoles, a carbamate ester, an organofluorine compound and an aromatic ketone(C_{16}H_{12}FN_{3}O_{3}). The Physical and chemical properties of the two compounds are shown in (Table 1).

Table (1): Some physical and chemical properties of Praziquantel (PZQ) and Flubendazole

<table>
<thead>
<tr>
<th>Structure</th>
<th>Praziquantel (PZQ)</th>
<th>Flubendazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C_{19}H_{24}N_{2}O_{2}</td>
<td>C_{16}H_{12}FN_{3}O_{3}</td>
</tr>
<tr>
<td>Synonyms</td>
<td>Biltricide, Droncit, Pyquiton</td>
<td>Flubendazol, Flumoxane, Fluvermal</td>
</tr>
<tr>
<td>Molecular weight (g)</td>
<td>312.4</td>
<td>313.28</td>
</tr>
</tbody>
</table>
Preparation of plant extracts: 2 species of plants used in this study consists of *J. curcas* and *M. oleifera* (Family: *Euphorbiaceae* and *Moringaceae*, respectively). The scientific classification of the two species are shown in Table 2. The leaves of each plant, was collected for preliminary bio-screening. They were collected from EL-Orman Garden, Giza during full growing season (March, 2020). Plants identification was carried according to Boulos (1999 and 2000). At 40°C the leaves were dried in a solar oven and extracted with ethanol at ambient temperature by percolation. Extracts were filtered and methanol was evaporated to dryness under reduced pressure and totally freed from water by freeze drying, and stored under freezing at −20°C until used. The main bioactive compounds of ethanol leaf extract of *J. curcas* and *M. oleifera* were shown in Table 3.

### Table 2: Scientific classification of *Jatropha curcas* and *Moringa oleifera*

<table>
<thead>
<tr>
<th>Kingdom: Plantae</th>
<th>Order: Malpighiales</th>
<th>Order: Brassicales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family: <em>Euphorbiaceae</em></td>
<td>Genus: <em>Jatropha</em></td>
<td>Family: <em>Moringaceae</em></td>
</tr>
<tr>
<td></td>
<td>Species: <em>J. curcas</em></td>
<td>Genus: <em>Moringa</em></td>
</tr>
<tr>
<td></td>
<td>Species: <em>M. oleifera</em></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. The main active compounds of ethanolic leaves extract of *Jatropha curcas* and *Moringa oleifera*.

<table>
<thead>
<tr>
<th>Bioactive compounds</th>
<th><em>Jatropha curcas</em></th>
<th><em>Moringa oleifera</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyanogenic glycosides</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

2. Parasite material

Cercariae of *P. palpebrarum* (Trematoda: *Philophthalmidae* occurring in the birdseye) emerged from naturally infected freshwater snails *M. tuberculata* collected from water courses in Giza Governorate, Egypt (Ayoub et al., 2020). Chicks (1-3 days old) are experimentally infected directly by pipetting 10-15 excysted metacercariae into each eye orbit (Alicata and Ching, 1960; Howell and Bearup, 1967). Infected chicks were housed in animal house of TBRI (Theodor Bilharz Research Institute, Imbaba, Giza, Egypt) according to the internationally valid guidelines regularly followed in the institute, where they were provided feed and water and libitum for 40 days. The chickens were slaughtered after 35-40 days of infection and the adult flukes were carefully tested out with fine forceps from conjuctival sac of eyes and rapidly placed in culture medium Roswell Park Memorial Institute medium (RPMI) 1640 containing 300 mg streptomycin, 300 units penicillin and 160 μg gentamycin/100 ml medium (Ayoub et al., 2020).
3. Philophathalmusidal bioassay and determination of LC$_{50}$ and LC$_{90}$

A stock solution (10 mg/ml) of each tested materials was prepared in dimethyl sulfoxide (DMSO) and diluted with RPMI to produce 3 ml test solution of 100 µg/ml final concentration for the screening (Yousif et al., 2007). The screening were bioassayed at descending concentrations, e.g. 15, 12, 9, 6 & 3µg/ml for Praziquantel and Flubendazole, while 100, 80, 60, 40 &20 µg/ml for J. curcas and M. oleifera plant leaves extracts. For each concentration three replicates were used and three pairs of worms were placed in each vial using sterilized tissue forceps and the incubation was maintained at 37°C. Negative control exposed only to media were similarly used. The mortality of worms was recorded at each concentration, then, treated and control worms were examined every 24 h under a dissecting microscope to record motility, integument damage and dead worms. Treated worms that did not exhibit motility for two minutes were considered dead. Based on the observations, mortality rates, and the LC$_{50}$ and LC$_{90}$ of each compound against adult Philophathalmus were calculated after 72 h using SPSS computer program version 16.

4. Scanning electron microscope examination(SEM)

To report the possible morphological changes, adult worms were treated in triplicate with LC$_{50}$ value of the most effective anthelmintic materials against P. palpebrarum for 48h and another untreated group was maintained as control under the same experimental conditions. 10 living flukes were fixed in 2.5% glutaraldehyde for the examination by SEM, washed with PBS, treated with 1% osmium tetroxide (OsO4) and dehydrated in ascending alcohol concentrations. Then the samples were dried, mounted on SEM stubs, and coated with a thin layer of platinum before examination with a SEM.

5. Statistical analysis of LC$_{50}$ and LC$_{90}$ of tested materials were carried out using one-way ANOVA on SPSS program version 16.

RESULTS

In vitro Philophathalmusidal activity

The adult worm was extracted from the conjunctival sac of the chicken eyes after 35-40 days post experimental infection by pipetting 10-15 excysted metacercariae into each eye orbit of (1-3 days old chicks). The flukes were exposed in triplicate at 15, 12, 9, 6 & 3µg/ml for Praziquantel (PZQ) and Flubendazole, while 100, 80, 60, 40 &20 µg/ml for J. curcas and M. oleifera plant leaves extracts. The efficacy was assessed as the mortality rate based on the number of live and dead flukes after 24, 48 and 72 h post-exposure, then the LC$_{50}$ and LC$_{90}$ of each tested material against adult P. palpebrarum were calculated after 72 h using SPSS computer program version 16. The results in Table (4) claimed that the LC$_{50}$ and LC$_{90}$ values for these tested materials were 7.857&10.149µg/mL for PZQ, 9.170&13.489 µg/mL for Flubendazole , 65.534 &91.474µg/mL for J. curcas, 44.509&53.004 for M. oleifera, respectively.
Table 4. LC$_{50}$ and LC$_{90}$ of the the tested materials against adult eye fluke Philophthalmus palpebrarum (Philophthalmidae).

<table>
<thead>
<tr>
<th>Tested materials</th>
<th>LC$_{50}$ (μg/ml)</th>
<th>LC$_{90}$ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Praziquantel (PZQ)</td>
<td>7.857</td>
<td>10.149</td>
</tr>
<tr>
<td>Flubendazole</td>
<td>9.170</td>
<td>13.489</td>
</tr>
<tr>
<td>Jatropha curcas (Leaves Methyl Extract)</td>
<td>65.534</td>
<td>91.474</td>
</tr>
<tr>
<td>Moringa oleifera (Leaves Methyl Extract)</td>
<td>44.509</td>
<td>53.004</td>
</tr>
<tr>
<td>D DMSO</td>
<td>No effect</td>
<td></td>
</tr>
</tbody>
</table>

Control worms incubated in media for 72h survived; showed normal tegumental appearance and motor activity characterized by wavy movement along the body axis. Treatment of adult P. palpebrarum with different concentrations of the tested materials for 24h resulted in changing the worm behavior in the form of slow locomotion. After 48 h exposure to 9 μg/mL of Praziquantel (PZQ), 66.70% of the worms died (Fig. 1A), with increasing the incubation time for additional 24 h, 83.33% of worms died. At 12μg/mL, 66.70% of worms died after 24 h of incubation and the remaining worms died over the next 24 h (Fig. 1A).

The results presented in Fig. (1B) revealed that 6 μg/mL of Flubendazole exerted only 16.70% mortality of the worms after 72h of exposure, while increasing the concentration to 12 μg/mL resulted in 33.33 % death among the treated worms, with increasing the incubation time for 48h, the death rate reached 83.33% in comparison to the control group. 100% death of worms resulted after 72h of exposure to 15 μg/mL.

The results in Figs. (1C and 1D), indicated no apparent effect on worm survival was seen after 72h exposure to 20 μg/mL of J. curcas and M. oleifera, while increasing the concentrations to 60, 80 and 100 μg/mL exerted (16.70, 33.33 & 66.70%) and (16.70, 83.33 & 100%) mortalities after 48h of exposure, respectively. Increasing the incubation time for 72h, lead to 50, 83.33 & 100% death among worms exposed to J. curcas and 66.70, 100 &100% among worms exposed to M. oleifera, respectively.
Antihelminthic activity of Praziquantel, Flubendazole and plant extracts against the eye fluke

Fig. 1. Mortality percent of the adult eye fluke *Philophthalmus palpebrarum* treated with Praziquantel (PZQ) (A), Flubendazole (B), *Jatropha curcas* (C) and *Moringa oleifera* (D) plant Leaves extracts for 72 h.

**Scanning electron microscopy results**

The characteristics of the tegument of untreated worms were compared with those exposed to LC50 (7.857 and 44.509 µg/mL) of PZQ and leaf extract of *M. oleifera* for 48h, respectively. Control worms exposed to culture media only has normal surface membrane with no apparent destruction and normal body structure (Figs. 2a and b). Generally, all exposed worms to tested materials showed tegumental or surface membrane ultrastructural damage, worms treated with PZQ and *Moringa* revealed deformity of the oral sucker and anterior part of worms, shrunken body with swellings and furrows (Figs. 2c & e). Moreover, disorganization, deformity and shrinkage of the dorsal aspect of the worm with presence of erosion of the tegument in several areas of the body and extreme distortion of the tegumental folds (Figs. 2c & e). Disfigurement of the ventral sucker was also noticed in the form of marked contraction and deformity, with erosion, shrinkage and wrinkling of the area between the oral and ventral suckers (Figs. 2d & f). Moreover, in vitro incubated worms with tested materials showed longitudinal muscle contraction, and the worms were bent, shortened.
Fig. 2. Oral (A) and ventral (B) suckers of untreated eye flukes *Philophthalmus palpebrarum* were compared with those exposed to LC$_{50}$ (7.857 and 44.509 µg/mL) of PZQ and leaf extract of *M. oleifera* for 48h, respectively. Deformity of the oral sucker and anterior part of worms, shrunken body with swellings and erosion (C & E). Disfigurement of the ventral sucker was also noticed in the form of marked contraction and deformity, with erosion and shrinkage (D & F).
A
ntihelminthic activity of Praziquantel, Flubendazole and plant extracts against the eye fluke

DISCUSSION

The trematodes of the genus Philophthalmus are eye flukes that cause damage to ocular structures of animals and humans (Assis et al., 2018). Globally, many studies have scientifically assessed numerous natural and synthetic compounds on the Philophthalmus adult worm, however, a lot of them still remain unused, pending their efficacy on the parasite biology is confirmed. It is realized that the study of parasite biology is fundamental to potential drug discovery and development. In the present investigation, Praziquantel (PZQ), Flubendazole and plant leaves extracts (J. curcas and M. oleifera) showed in vitro antihelminthic activity against the adult eye fluke P. palpebrarum and the mortality percent of the worm is directly proportional to the time and the tested concentrations. The considerable toxic effect of the tested materials might be due to the main constituents of active compounds (Tables 1 & 3), that are known to possess anthelminthic activities (Vollesen et al., 1995; William et al., 2003; WHO, 2009; Wang et al., 2016). Also, the tested materials can deeply penetrate into the internal structures to bind muscles of the parasite or causing disturbance in the normal biochemical and physiological process of the worm (Taman et al., 2020). Similar results have been previously reported by many researchers when testing the in vitro effect, eg. PZQ (Kato et al., 2013; Prompetch et al., 2021), seeds of J. curcas (Kandil et al., 2018), seeds of M. oleifera (Eguale & Giday, 2009) on Schistosoma mansoni, Philophthalmus gralli, Haemonchus contortus, Fasciola hepatica, respectively.

On the basis of LC₉₀ values, PZQ (LC₉₀= 10.149 µg/mL) and M. oleifera (LC₉₀ = 53.004 µg/mL) were the most toxic ones. So, the two compounds were used for detailed studies in this work. The results of electron microscope of the present study demonstrated the LC₅₀ of PZQ and leaf extract of M. oleifera caused tegumental or surface membrane ultrastructural damage if compared control worms. The tegument has a pivotal role in sensation, protection, immunomodulation, nutrient ingestion, osmoregulation, synthesis of some proteins, and excretion (Skelly & Wilson, 2014). It is noteworthy that alterations in the tegument structure deteriorate its functions and lead to worm death (Shaw & Erasmus, 1987). Also, the main changes induced by treatment are related to deformity and damage in oral and ventral suckers. Suckers are muscular organs essential for fixing worms in the conjunctival and orbital tissues. So, if this effect is the same in vivo, both compounds will affect worm nutrition and subsequent interference of worm and worm oviposition (Halton and Maule, 2004). The obtained results can also be supported by those obtained by Chienwichai et al. (2020) who studied the parasiticidal activity of 40 µg/mL concentration of PZQ against Schistosoma mekongi. The parasiticidal activity of prazquantel against cestodes, including Hymenolepis nana (1 g/mL) and Diphyllobothrium latum (0.1 g/mL), was previously reported (Bylund et al., 1977; Becker et al., 1980). Interestingly, Heneberg et al. (2014) studied the effective therapeutic intramuscular medication of PZQ in patient chicken with P. gralli infestation.
Also, the same findings was reported by Prompetch et al. (2021) who evaluated the efficacy of a novel drug delivery system of the modified rice hydrogel containing praziquantel (PZQ) against P. gralli isolated from ostrich eyes and determine the toxicity of the preparation on chicken eye model.

**CONCLUSION**

Finally it can be concluded that Praziquantel, Flubendazole and plant leaves extracts (J. curcas and M. oleifera) possess in vitro antihelminthic activities against adult eye fluke P. palpebrarum (Philophthalmidae); however, studies should be conducted in vivo to examine their antiphilophthalmus effects. As the evaluation of new protocols, routes of administration and anthelmintic drugs are needed for successful pharmacological treatment of Philophthalmosis.

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Praziquantel on Schistosoma mekongi Proteome and Phosphoproteome. Pathogens, 9, 417.


