

IN VITRO EVALUATION OF THIOREDOXIN REDUCTASE INHIBITOR (AURANOFIN) ACTIVITY IN COMPARISON WITH TRICLABENDAZOLE ON ADULT *FASCIOLA GIGANTICA*

By

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Abstract

Fasciola gigantica causes a worldwide waterborne/foodborne zoonotic disease in which humans are incidental hosts. Fascioliasis has a major impact on human health and its control mainly depends on triclabendazole (TCBZ). Unfortunately, the effectiveness of this drug is decreased because of indiscriminate use resulting in development of resistance. Therefore, the search for another effective anthelmintic is now compulsory. This work aimed to evaluate the *in vitro* anthelmintic effects of auranofin (a thioredoxin reductase inhibitor) on adult *F. gigantica* in comparison with the drug of choice; TCBZ. This study involved *in vitro* petri dish incubation of seventy-five adult *F. gigantica* worms of nearly equal size with the tested drugs and classified into five groups (fifteen worms each) as follows; G1 served as a control group, G2 was exposed to TCBZ (20µL/mL), G3, G4, & G5 were exposed to auranofin (3, 5, & 10µg/mL, respectively). All adult worms were incubated and observed for three hours and subjected to motility and egg hatchability assays, histopathological and ultrastructural studies, glutathione-S-transferase and superoxide dismutase assay, and cathepsin-L gene expression analysis. Auranofin in all concentrations significantly decreased adult motility and egg hatchability. It induced histopathological and ultrastructural deformities including apoptosis. Auranofin in higher concentrations significantly suppressed the activity of the detoxifying enzyme; glutathione-S-transferase, and significantly stimulated superoxide dismutase enzyme activity reflecting the oxidative stress. At all concentrations, it suppressed the expression of the cathepsin-L gene responsible for *Fasciola* invasive function.

Key words: Auranofin[®], *Fasciola gigantica*; *In-vitro*, Triclabendazole[®].

Introduction

Fasciola hepatica (*F. hepatica*) and *Fasciola gigantica* (*F. gigantica*) are two parasite species that cause a foodborne zoonotic disease named fascioliasis (Rokni *et al*, 2018). Fascioliasis is an emerging/re-emerging trematodal disease in many countries with major impact on human health and development (Mas-Coma *et al*, 2005), including Egypt (Abo-Madyan *et al*, 2004). Animal and human fascioliasis constitute major economic and health problems in tropical and subtropical areas (Sarkari *et al*, 2017). Epidemiological data proved that fascioliasis endemic areas are expanding (Sarkari and Khabisi, 2017). The disease causes hepatic lesions and chronic inflammation as well as fibrosis of bile ducts (Ashrafi, 2015) with the devel-

opment of gall bladder stones (Mas-Coma *et al*, 2019). It is also associated with helminth-induced hepatic cancer (Machicado and Marcos, 2016). Besides, fascioliasis caused ectopic lesions elsewhere with tissue damage and fibrosis (Taghipour *et al*, 2019). The neurological and ocular disorders were recorded in hepatic fascioliasis patients (Gonzalez-Miguel *et al*, 2019).

The control of fascioliasis relies on an anthelmintic drug, triclabendazole (TCBZ), a member of benzimidazole derivatives (Fairweather, 2009). This drug is the most effective treatment for fascioliasis in humans and animals. It penetrates the worm tegument by diffusion, then, becomes metabolized by the worm into an active sulfoxide metabolite that binds to β -tubulin inhibiting the for-

mation of microtubules which are components of the *Fasciola* cytoskeleton. The resistance of TCBZ is recently reported in humans and animals in many regions of the world due to reduced diffusion and metabolism with a change in efflux activity that reduces its efficacy (Merachew and Alemneh, 2020). Therefore, alternative effective fascioliasis treatment became the research targets (Marques *et al*, 2020). Among these targets, cathepsin-L cysteine proteases, glutathione-S-transferases (GST), superoxide dismutases (SOD), and thioredoxin reductases (TrxR) were recommended (Saccoccia *et al*, 2014). These enzymes have different vital functions in *Fasciola* worms including host tissue penetration, worm survival and virulence, detoxification reactions, and antioxidant defense mechanisms (Ullah *et al*, 2017).

Redox enzymes are important in controlling the intracellular levels of reactive oxygen species which are essential for the prevention of DNA damage with subsequent survival of all cell types, including parasites. Auranofin is an FDA-approved treatment for rheumatoid arthritis with a well-studied safety for humans (AbdelKhalek *et al*, 2018). Auranofin acts mainly through the inhibition of reduction/oxidation (Redox) enzymes as thioredoxin reductase. The cells which express redox enzymes show an increase of auranofin affinity towards them (Caroli *et al*, 2011; Tejman-Yarden *et al*, 2013). Inhibition of redox enzymes by auranofin changes the redox state of the cell with increased production of reactive oxygen species, hydrogen peroxide, and oxidative stress causing intrinsic apoptosis of affected cells (Roder and Thomson, 2015).

Fortunately, the auranofin proved effective against different parasites in previous studies (Tejman-Yarden *et al*, 2013; da Silva *et al*, 2015; Capparelli *et al*, 2016). However, to the best of the authors' knowledge, it was not tested before against adult *F. gigantica*.

This study aimed to evaluate *in-vitro* anthelmintic effects of different concentrations of Auranofin[®] on *F. gigantica* adults as co-

mpared with the on drug TCBZ[®].

Materials and Methods

Fasciola worms: *F. gigantica* adult worms were obtained from infected livers and bile ducts of buffaloes slaughtered at El Bagur Governmental Abattoir, Menoufia Governorate. The liver was cut over the bile duct and the flukes were collected in a laminar flow cabinet under sterile conditions using non-traumatic thumb forceps. Worms were put in a warm 0.9% NaCl (37°C). They were cleaned with phosphate buffer saline (PBS) in small sieves & placed in sterile RPMI-1640 medium (supplemented with (100µl/mL) Penicillin[®], (160µ/mL) Gentamycin[®], (100µl/mL) Streptomycin[®] and (30%) fetal calf serum (Diab *et al*, 2010).

Tested drugs: Triclabendazole[®] was provided by Egaten, Novartis Pharma AG (USA). Auranofin[®] was provided by Tocris Bio-Techno Brand (USA) as a stock solution, and was freshly prepared by dissolving 1mg auranofin in 100ml dimethylsulfoxide (DMSO).

Experimental design: Anthelmintic effects of the tested drugs were studied by *in vitro* petri dish incubation method using RPMI-1640 medium (Githiori *et al*, 2006). Seventy-five adult *F. gigantica* worms of nearly equal size were selected and classified into five groups (fifteen worms each) as follows: G1: Non-drug-exposed worms (Control). G2: Worms were exposed to TCBZ at a concentration of 20µL/mL (TCBZ-exposed) after Nassef *et al*. (2014). G3: Worms were exposed to auranofin at a concentration of 3µg/ml (Aur 3µg/ml) after Song *et al*. (2012). G4: Worms were exposed to auranofin at a concentration of 5µg/ml (Aur 5µg/ml). G5: Worms exposed to auranofin at a concentration of 10µg/ml (Aur 10µg/ml). All groups were incubated for 3hrs at room temperature. Tested drugs were evaluated through motility assay, egg hatchability test, histopathological study for pathological and apoptotic changes, ultrastructural scanning and transmission electron microscopic studies, evaluation of the antioxidant state by GST and SOD assays, and finally, determination

of cathepsin-L gene expression by real-time PCR (RT-PCR).

Parasitological study: a- Motility assay: Motility of *Fasciola* adults of all groups was recorded for 3hrs post-incubation at half an hour intervals (Ullah *et al*, 2017). The motility was scored into the following (Jiraungkoorskul *et al*, 2005) score 3: Moving whole body, score 2: Moving only parts of body score 1: Grossly immobile but microscopically alive, and score 0: Microscopically dead. b- Egg hatching study: Eggs of each group were collected, washed five times with dechlorinated water and then incubated in 50ml tap water at 25°C in the darkness for 15 days. Exposure to day light for 2hr were done to test egg hatchability, where percentage of hatched and unhatched eggs were assessed using dissecting microscope (Hanna *et al*, 2006)

Histopathological study: From each group, five worms were randomly picked and assigned for histopathological studies. a- Hematoxylin and Eosin (H&E) staining: Adults from each group were fixed in 10% formaldehyde, dehydrated in ascending grades of ethanol, cleared with xylene, and then embedded in paraffin, for 5µm longitudinal sections to be stained in H&E stained (Carleton *et al*, 1980). They were examined and photographed under an Olympus CX41 light microscope at Pathology Department, Faculty of Medicine, Menoufia University. b- Immunohistochemical (IHC) staining for caspase- 3: Serial sections (4µm) were cut from paraffin-embedded flukes of all groups and mounted on positive slides (Sigma Aldrich, UK), stained by IHC technique according to the datasheet (Cat. #RP-096-05, Diagnostic Biosystems, 6616 Owens drive Pleasanton, CA, 94588). Finally, Mayer's hematoxylin was used as a counterstain (Sigma Aldrich, UK).

Caspase-3 expression was confirmed in the examined cells by cytoplasmic and/or nuclear stain. Pattern of expression was categorized as a patchy pattern in the form of irregular distribution or diffuse pattern in fo-

rm of uniform distribution. While cellular localization was assessed as cytoplasmic or nucleo-cytoplasmic localization. The cells stained intensity was evaluated using Histo-score (H-score) with a score of 0-300 calculated according to the following equation:

$$\text{H-score} = (\% \text{ of mildly stained cells} \times 1 + \% \text{ moderately stained cells} \times 2 + \% \text{ strongly stained cells} \times 3) / 300$$
 and mean \pm SD of H-score was calculated for each group (Smyth *et al*, 2007).

Electron microscopic study: From each group, 5 worms were randomly picked and assigned for the electron microscopic studies. a- SEM study: Following incubation, control and drug-exposed flukes were rinsed in fresh RPMI- 1640 medium, flat fixed for 30 minutes at room temperature in the freshly prepared 4% glutaraldehyde and thereafter free-fixed for another 3.5 hours in fresh fixative at 4°C. Worms were then washed 4 times with sodium cacodylate buffer (pH 7.4), dehydrated in ascending grades of ethanol, critically point dried, mounted on aluminum stubs, and sputter-coated with 20nm gold (Shareef *et al*, 2014). They were examined on JEOLSEM, (Japan) at 5 kV at Electron Microscope Unit, El- Mansoura University. b- TEM study: Flukes from all groups were fixed overnight in 2.5% glutaraldehyde in phosphate buffer (pH 7.4). They were sliced into transverse strips (1-2mm width). Strips were transferred to fresh phosphate buffer for the rest of the fixation period and washed in phosphate buffer (pH 7.4) then again re-fixed in 1.0 % osmium tetroxide, dehydrated in ethanol, and embedded in a low-viscosity resin. Ultrathin sections were cut using an ultra-microtome, mounted, double-stained with aqueous lead citrate, and alcoholic uranyl acetate (Soliman and Taha, 2011), and viewed on JEOLSEM at EMUnit, Mansoura University.

Antioxidant activity assays: From each group, 5 worms were randomly picked and assigned for the antioxidant activity assays and determination of cathepsin-L gene expression by real-time PCR (RT-PCR). a- Glu-

tathione-S-transferase (GST) assay: Glutathione-S-transferase assay kit (Biodiagnostic, Egypt) was used. Five μl of each sample was added to a reagent cocktail [(0.1 M phosphate buffer (pH 6.5) (880 μL), 100 mM reduced glutathione (10 μl) and one-chloro-2, 4-dinitrobenzene (10 μl)] and PBS (5 μl) and mixed well. Absorbance was recorded for 3 minutes at 340 nm using a spectrophotometer (GENESYS 10S UV-Vis, USA) after Habib *et al.* (1974). b- Superoxide dismutase (SOD) assay: It was measured using a superoxide dismutase assay kit (Biodiagnostic, Egypt). Fifty μl of the sample were added to 2.85 mL of Triscacodylate buffer (pH 8.5) followed by the addition of 0.13 mM pyrogallol (100 μl). Change in absorbance was recorded for 3 min at 420 nm (Marklund and Marklund, 1974).

Determination of cathepsin-L gene expression by real-time PCR (RT-PCR): Total RNA from adult *F. gigantica* worms was isolated using DirectzolTM RNA Miniprep Plus (Zymo Research) then reverse transcribed using Quanti-Tect Reverse Transcription Kit (Qiagen, Applied Biosystems, USA). Briefly, 10 μl of extracted RNA were added to the reverse transcription master mix to achieve a reverse-transcription reaction of 20 μl total volume to obtain complementary DNA (cDNA) needed for further amplification. Reverse transcription reactions were stored at -20°C. Amplification of the resulting cDNA was done using the Quanti-Tect SYBR Green PCR Kit (Applied Biosystems, USA). Gene expression of the cathepsin-L gene was determined using forward and reverse primers (Clinilab, Egypt) according to Ullah *et al.* (2017). The forward primer was GATCGTTTGAGCCATGGAGT, and the reverse primer was CACATGTTTCCTCGG TTCCT. Forward and reverse primers were used as an endogenous reference control. Primers were reconstituted before use in the labeled amount of Tris-EDTA buffer. For each reaction, a mixture of 5 μl of cDNA, 1 μl of each primer, 12.5 μl of SYBR Green master mix, and 5.5 μl of RNase-free water

was prepared in an Eppendorf. An initial denaturation step at 95°C for 15 min was done followed by 35 PCR cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. PCR was then terminated by a final extension at 72°C for 15 min with the cooling of samples down to 4°C. Melting curve analysis of the PCR yield was done using software version 2.0.1 incorporated in the cycler (Applied Biosystems, USA).

Statistical analysis: Data were collected, tabulated and analyzed by Statistical Package of Social Science (SPSS) version 20 and Epi Info 2000 programs, where the following statistics were applied. Quantitative data were presented in the form of the mean (x), standard deviation (SD), range, and qualitative data were presented in the form of numbers and percentages (%). Chi-square test, t-test, Mann-Whitney, one-way ANOVA, and Kruskal, Wallis tests were also used. P values ≤ 0.05 were considered statistically significant.

Ethical approval: The study was conducted following the International Animal Ethics Committee, the Ethical Committee of Faculty of Medicine, Menoufia University, and the current Egyptian regulations for dealing with experimental animal.

Results

Worm motility: Adult *F. gigantica* exposed to either TCBZ (G2) or auranofin (G3, G4, & G5) were less motile with time, whereas no major loss of motility was observed in control (G1). Both control (G1) and TCBZ-exposed (G2) showed a non-significant decrease in adult motility with time throughout the study (P= 0.863 & 0.216, respectively) while auranofin in all concentrations (3, 5 & 10 $\mu\text{g}/\text{ml}$) produced a highly significant (P < 0.001) inhibition of the worms motility over the incubation time in time and concentration-dependent manners. After incubation for 2 hrs and at study end (3 hrs), all auranofin-exposed worms died (Fig. 1).

Egg hatchability: There was a significant

decrease in the percentages of hatched *F. gigantica* eggs with the increase in the duration of incubation in control (G1), TCBZ-exposed (G2), auranofin-exposed (G3, G4, & G5) (P= 0.001, 0.003, 0.041, 0.013 & 0.010, respectively). There was a significant difference in *Fasciola* egg hatching between all the studied groups after half an hour, one hour, two hours, and three hours of incubation (P= 0.001, 0.002, 0.012, and 0.032, respectively).

Hematoxylin and eosin staining: The tegumental morphology of *Fasciola* adults of the control specimens (G1) showed intact tegument, spines, muscular layer, and parenchyma. The cytoplasmic syncytial layer of the tegument showed numerous spines embedded throughout its matrix, intact reticular lamina, and deeply stained two muscular layers. Regarding TCBZ-exposed flukes (G2); they showed tegumental swelling with the appearance of numerous vacuoles in the tegument syncytium and mild apoptosis. Some spines were dislodged with empty sockets while others appeared embedded in the swollen tegument. All flukes exposed to different concentrations of auranofin (3, 5, & 10µg/ml) showed tegumental swelling and vacuolations with severe apoptosis in the tegument syncytium with sloughing and detachment of parts of the tegument. Partial separation of some spines was also detected. At highest concentration of auranofin (10µg/ml) showed a complete separation of a large number of spines with severe apoptosis and necrosis. Tegument severity alterations in auranofin-exposed worms (G3, G4, & G5) were concentration-dependent (Fig. 2).

Immunohistochemical staining: Caspase-3 IHC staining of control (G1) showed mild patchy cytoplasmic expression of caspase-3 of the tegumental cells (brown-stained bodies) with a very mild expression in the spermatocytes of the testes. Some worms showed a negative cytoplasmic expression of caspase-3. The tegument of TCBZ-exposed flukes (G2) showed a mild patchy cytoplasmic expression of caspase-3 and mild

expression in the spermatocytes of the testis. All *F. gigantica* worms exposed to low auranofin concentrations (G3) showed a moderate patchy cytoplasmic expression of caspase-3 and moderate expression in spermatocytes of the testis. At higher auranofin concentration (5µg/ml), there was a strong patchy expression of caspase-3 in the cytoplasm in the ductal cells. At 10µg/mL auranofin, there was a strong diffuse expression of caspase-3 in both cytoplasm and ductal cells (Fig. 3). Only higher concentrations of the auranofin (5 & 10µg/ml) induced significant increases in caspase-3 expression H-scores when compared to control (G1) (*p*3 & *p*4: 0.008, respectively) and the TCBZ (G2) (*p*6 & *p*7:0.009, respectively).

SEM: Control (G1) exhibited normal surface morphology, both ventrally and dorsally. Tegument was covered with uniformly distributed, tightly packed, posteriorly directed, broad, and serrated tegumental fungi-form spines. The apical cone spines showed finger-like protrusions at their tips. Oral and ventral suckers were smooth with normal tegumental infoldings with intact and sharply pointed spines. Regarding TCBZ-exposed flukes (G2), oral and ventral suckers were smooth but surrounded by swollen tegumental in-folding. There were disrupted spines in some regions. However, some of the spines showed distortions of the upper surfaces. Some areas showed swelling and furrowing of the tegument. All *F. gigantica* worms exposed to auranofin different concentrations (G3, G4, & G5) showed obvious damages everywhere. At an auranofin concentration of 3µg/ml, tegument was severely swollen. A distorted ventral sucker was seen. Some spines were swollen with a bullous appearance. Others were dislodged from their sockets leaving pits. Exposure to 5µg/ml of auranofin caused swelling of oral and ventral suckers, extensive tegumental damage with complete loss of the spines in some areas. Moreover, other regions appeared sunken in swollen tegument with blebbing. Exposure to 10µg/ml of auranofin resulted in a marked

disruption of tegumental surface with appearance of deep furrows, extensive spine loss on the ventral surface of oral cone with loss of finger-like spines, and sloughing damaged tegument with a widening of anterior ventral sucker's ring (Fig. 4).

TEM: Control (G1) exhibited a normal tegumental syncytium, intact apical plasma membrane, and normal tightly close basal in-folds. Secretory bodies T1 & T2 were normal in shape and numbers of muscle layers beneath the basal lamina were intact. Adult *Fasciola* worms incubated with TCBZ (G2) showed areas with a normal tegumental syncytium and other areas showed swelling of the tegumental cells with accumulation and swelling of T1 secretory bodies. Basal lamina was swollen with swollen basal in-folds. The muscle layers were surrounded by large spaces. Some mitochondria were swollen and rounded. Adults exposed to different concentrations of auranofin (G3, G4, & G5) showed swollen basal in folds with swollen mucopolysaccharide masses in between, swollen rounded mitochondria, numerous T1 & T2 secretory bodies, and electron-lucent vacuoles in syncytium below apical plasma membrane. At higher auranofin concentrations (5 & 10µg/ml), there were severe swelling and disintegration of basal in-folds, small blebs projecting from tegumental surface, some separated blebs, and multiple lar-

ge autophagic vacuoles filling tegumental syncytium (Fig. 5).

Glutathione S-transferase: Exposure to auranofin initially stimulated activity of detoxifying enzyme; GST at low concentration (G3) and inhibited at higher concentrations (G4 & G5). Auranofin at a concentration of 3µg/ml (G3) caused a non-significant increase in GST activity when compared to control (G1) (p_2 : 0.076). Higher auranofin concentrations (G4 & G5), induced a significant decrease in enzyme activity when compared to control (G1) (p_3 and p_4 : 0.009, respectively) and to TCBZ (G2) (p_6 & p_7 : 0.009, respectively).

SOD activity increased significantly in higher concentrations of auranofin (G4 & G5) when compared to control (G1) (p_3 & p_4 : 0.009, respectively) and when compared to TCBZ-exposed worms (G2) (p_6 & p_7 : 0.009, respectively).

Cathepsin-L gene expression by RT-PCR showed a significant inhibition of cathepsin-L gene expression in a concentration dependent manner in auranofin-exposed worms. All concentrations (G3, G4 & G5) were reduced significant when compared to control (G1) (p_2 & p_3 :0.009 & p_4 :0.008, respectively) and to TCBZ-exposed worms (G2) (p_5 & p_6 : 0.009 & p_7 : 0.008, respectively).

Details were given in tables (1, 2, 3, 4, 5, & 6) and figures (1, 2, 3, 4, & 5).

Table 1: Mean worm motility score over the incubation time among the studied groups

Studied groups	Mean motility/hours					
	½ hour	1 hour	1.5 hours	2 hours	2.5 hours	3 hours
G1 (Control)	2.66±0.48	2.66±0.48	2.60±0.50	2.33±1.04	2.20±1.21	2.13±1.18
% of decrease	--	0 %	2.20 %	12.4 %	17.3 %	19.9 %
G2 (TCBZ-exposed)	2.66±0.48	2.53±0.83	2.20±1.21	2.13±1.18	1.93±1.33	1.60±1.29
% of decrease	--	4.88 %	17.3	19.9 %	27.4 %	39.8 %
G3 (Aur 3 µg/mL)	2.13±1.24	1.40±1.24	1.13±1.18	0.40±0.82	0.00±0.00	0.00±0.00
% of decrease	--	34.3 %	46.9 %	81.2 %	100 %	100 %
G4 (Aur 5 µg/mL)	1.60±1.29	0.46±0.63	0.13±0.35	0.00±0.00	0.00±0.00	0.00±0.00
% of decrease	--	71.2 %	91.8 %	100 %	100 %	100 %
G5 (Aur 10 µg/mL)	1.40±1.24	0.27±0.45	0.06±0.25	0.00±0.00	0.00±0.00	0.00±0.00
% of decrease	--	80.7 %	95.7 %	100 %	100 %	100 %

* Significant, **Highly significant, K: Kruskal Wallis test (K1: 1.89, p_1 : 0.863; K2: 7.06, p_2 : 0.216; K3: 39.9, p_3 : <0.001**; K4: 38.4, p_4 : <0.001**; K5: 39.9, p_5 : <0.001**)

Table 2: Comparison between the studied groups regarding egg hatching

Studied groups	Duration of incubation							
	Half an hour		One hour		Two hours		Three hours	
	No.	%	No.	%	No.	%	No.	%
G1 (Control)	27	90.0	18	60.0	15	50.0	12	40.0
G2 (TCBZ-exposed)	21	70.0	11	36.7	10	33.3	8	26.7
G3 (Aur 3 µg/mL)	15	50.0	9	30.0	8	26.7	5	16.7
G4 (Aur 5 µg/mL)	14	46.7	7	23.3	5	16.7	4	13.3
G5 (Aur 10 µg/mL)	12	40.0	4	13.3	4	13.3	3	10.0
χ^2	20.83		16.67		12.76		10.57	
<i>p</i> . value	0.001*		0.002*		0.012*		0.032*	

χ^2 Chi-square, *p* value for each group (χ^2_1 : 17.5, *p*₁: 0.001*; χ^2_2 : 13.85, *p*₂: 0.003*; χ^2_3 : 8.25, *p*₃: 0.041*; χ^2_4 : 10.84, *p*₄: 0.013*; χ^2_5 : 11.35, *p*₅: 0.010*)

Table 3: Comparison between groups regarding H-score of caspase-3 immunohistochemical study

Studied groups (n = 5)	H-score		% of increase	K
	Mean ±SD	Range		
G1 (Control)	10.0±17.3	0 - 40	---	19.9
G2 (TCBZ-exposed)	74.0±42.1	0 - 100	6.40%	
G3 (Aur 3 µg/mL)	84.0±58.5	0 - 140	7.40%	
G4 (Aur 5 µg/mL)	186.0±43.3	140 - 230	17.6%	
G5 (Aur 10 µg/mL)	228.0±52.1	150 - 270	21.8%	

Post hoc test *p*-values: *p*₁: Comparison of G1 Vs G2 (0.052); *p*₂: G1 Vs G3 (0.052); *p*₃: G1 Vs G4 (0.008**); *p*₄: G1 Vs G5 (0.008**); *p*₅: G2 Vs G3 (0.525); *p*₆: G2 Vs G4 (0.009**); *p*₇: G2 Vs G5 (0.009**); *p*₈: G3 Vs G4 (0.015*); *p*₉: G3 Vs G5 (0.009**); *p*₁₀: G4 Vs G5 (0.140)

Table 4: Comparison between groups regarding Glutathione S-transferase activity

Studied groups (n = 5)	Glutathione S-transferase		% of decrease	K
	Mean ±SD	Range		
G1 (Control)	1927.8±484.8	1614.1 - 2775.4	----	21.1
G2 (TCBZ-exposed)	1445.2±161.1	1265.4 - 1625.1	25%	
G3 (Aur 3 µg/mL)	2401.9±593.8	1912.3 - 3388.4	----	
G4 (Aur 5 µg/mL)	976.1±151.0	807.0 - 1209.5	49.4%	
G5 (Aur 10 µg/mL)	930.7±664.4	762.0 - 1100.3	51.7%	

Post hoc test *p*-values: *p*₁(0.016*); *p*₂: (0.076); *p*₃ (0.009**); *p*₄ (0.009**); *p*₅ (0.009**); *p*₆ (0.009**); *p*₇ (0.009**); *p*₈ (0.009**); *p*₉ (0.009**); *p*₁₀ (0.754)

Table 5: Comparison between the studied groups regarding superoxide dismutase activity

Studied groups (n = 5)	SOD		% of increase	K
	Mean ±SD	Range		
G1 (Control)	0.09±0.01	0.08 - 0.11	---	18.9
G2 (TCBZ-exposed)	0.12±0.02	0.11 - 0.15	33.3%	
G3 (Aur 3 µg/mL)	0.11±0.04	0.08 - 0.19	22.2%	
G4 (Aur 5 µg/mL)	0.19±0.02	0.17 - 0.25	111.1%	
G5 (Aur 10 µg/mL)	0.24±0.01	0.23 - 0.28	166.6%	

Post hoc test *p*-values: *p*₁ (0.036*); *p*₂ (0.338); *p*₃ (0.009**); *p*₄ (0.009**); *p*₅ (0.341); *p*₆ (0.009**); *p*₇ (0.009**); *p*₈ (0.028*); *p*₉ (0.009**); *p*₁₀ (0.036*)

Table 6: Comparison between groups regarding Cathepsin-L gene expression

Studied groups (n = 5)	RT-PCR		% of decrease	K
	Mean ±SD	Range		
G1 (Control)	17.6±0.80	16.4 - 18.5	---	22.9
G2 (TCBZ-exposed)	15.7±0.41	14.2 - 16.7	10.8%	
G3 (Aur 3 µg/mL)	11.3±1.56	10.1 - 14.1	35.8%	
G4 (Aur 5 µg/mL)	8.73±1.85	5.59 - 10.0	50.4%	
G5 (Aur 10 µg/mL)	1.94±2.66	0.00 - 5.11	88.9%	

Post hoc test *p*-values: *p*₁ (0.016*); *p*₂ (0.009**); *p*₃ (0.009**); *p*₄ (0.008**); *p*₅ (0.009**); *p*₆ (0.009**); *p*₇ (0.008**); *p*₈ (0.009**); *p*₉ (0.008**); *p*₁₀ (0.008**)

Discussion

Fascioliasis is more common in animals, but also zoonotic worldwide parasite exceeded two million. It causes serious economic loss, with public health consequences

(Yamson *et al*, 2019).

In the current study, auranofin caused a significant reduction in worm motility compared to the control worms. After 2.5 hours and at the end of the experiment (3 hours),

auranofin showed a mortality rate of 100% even at the lowest concentration. The antiparasitic activity of auranofin was directed against the critical cellular enzyme TrxR resulting in its inhibition and subsequent disruption of the cellular redox state of the parasite (Fan *et al*, 2014) and increase in reactive oxygen species (ROS) intracellularly resulting in DNA damage and death of the parasite (Pessetto *et al*, 2013). These results were in accordance with many researchers who studied the effects of auranofin on several parasitic species including *Echinococcus granulosus* (Bonilla *et al*, 2008), *P. falciparum* (Sannella *et al.*, 2008), *Schistosoma mansoni* (Caroli *et al*, 2011), *L. major* (Ilari *et al*, 2012), *Entamoeba histolytica* (Debnath *et al*, 2013), *Giardia lamblia* (Tejman-Yarden *et al*, 2013), *Toxoplasma gondii* (Andrade *et al*, 2014), *Leishmania infantum* (Sharlow *et al*, 2014), and *Naegleria fowleri* (Peroutka-Bigus and Bellaire, 2019) who reported a significant, dose-dependent reduction in the metabolic activity and viability of the parasite.

In the present study, egg hatchability, exposure to auranofin caused time and dose-dependent reduction in percentage of the hatched *Fasciola* eggs with a significant difference between all groups at all incubation periods. The highest reduction of egg hatching was seen in the higher concentrations of auranofin (5 & 10 µg/ml) after 3 hours of incubation (13.3% & 10%, respectively). But, some eggs detected in culture media were deformed, disintegrated and others were collapsed. These effects of auranofin on *Fasciola* eggs could be attributed to the induction of apoptotic changes in spermatocytes of the testes of the exposed worms as revealed in histopathological examination. Lakshmi and Sudhakar (2010) reported antioxidant inhibitory effects on eggs (ovicidal effects) using methanolic extract of ginger (*Zingiber officinale*) on *F. hepatica* eggs, Also, Hegazi *et al.* (2018) and El Shenawany *et al.* (2019) who confirmed the ovicidal effects of *Moringa leifera* aqueous extract on *F. gig-*

antica eggs.

In the present study, H&E histological examination, TCBZ-exposed flukes showed tegumental swelling with the appearance of numerous vacuoles in the tegument syncytium and mild apoptosis in spermatocytes in the testis. This agreed with Hanna (2015) who showed degenerative changes in *Fasciola* testis as multiple pyknotic nuclei, eosinophilic cytoplasm with rounded profiles of the spermatocytes suggesting apoptosis.

In the present study, Auranofin exposure of adults by different concentrations showed tegumental swelling, vacuolations with severe apoptosis in tegument syncytium. At the highest auranofin concentration of 10 µg/ml, there was complete separation of a large number of spines and severe necrosis of the tegument. Also, Sharlow *et al.* (2014) found that anti-*Leishmania* activity of auranofin induced apoptosis of promastigote.

In the present study, caspase-3 activity was assessed as being a marker of apoptosis. It was found that TCBZ-exposed adults *F. gigantica* showed a weak patchy cytoplasmic expression of caspase-3 in the form of brown staining bodies in the cytoplasm and the spermatocytes of the testis. This agreed with Hanna (2015) who reported positive labeling of caspase-3 with the TUNEL method. This proved the occurrence of endonuclease-induced DNA breakdown which is a marker of apoptosis. Regarding auranofin, exposure of adult *Fasciola* to different concentrations resulted in moderate patchy expression of caspase-3 (brown staining bodies) in the cytoplasm and the spermatocyte of the testis. At the highest auranofin concentration of 10 µg/ml, there was a strong caspase-3 expression in the testis and ductal cells. This agreed with Sharlow *et al.* (2014) who found that auranofin possesses an apoptotic activity in the culture of *Leishmania* promastigotes.

No doubt, the tegument of trematodes has an important defensive role in the protection of the parasite and also performs an interface between the parasite and the host. It is con-

sidered a primary drug targeting site (Halton, 2004). SEM has a beneficial application to study and evaluate surface tegumental changes after exposure to anthelmintic agents (Fairweather and Boray, 1999).

In the present study, the effects of auranofin on adult *F. gigantica* were assessed by SEM and TEM. Regarding the SEM results, adult worms exposed to different concentrations of auranofin showed great damage in the form of swollen tegument with bullous appearance and disturbed ventral sucker in addition to dislodgement of some spines from their sockets leaving pits. At the highest concentration of 10µg/ml, extensive spine loss occurred on the ventral surface of the oral cone with loss of finger-like appearance of the spine and sloughing with the appearance of furrows in some areas of the tegument and widening of anterior ventral sucker's ring. The results of tegumental deformity after auranofin exposure coincide with those of Tejman-Yarden *et al.* (2013) who tested the *in vitro* effects of auranofin on *Giardia lamblia* using phase-contrast microscopy. They observed that auranofin-exposed trophozoite had a distorted shape with numerous blebs extending from the cell membrane and slowing of flagellar beating. Auranofin also induced ultrastructure changes in adult worms of *Brugia pahangi* and *Onchocercus ochengi*. The hypodermal area of the exposed worms was highly vacuolated with remnants of swollen mitochondria containing dark bodies (Bulman *et al.*, 2015).

It is known that targeting the antioxidant enzyme system of helminths renders them more vulnerable to the reactive oxygen radicals generated by the host, hence facilitate their elimination. The activity of GST; the major detoxification enzyme in all helminthes was assessed in this work. Auranofin at the low concentration caused a non-significant increase in GST activity when compared to control group. At the higher auranofin concentrations, it induced a significant decrease in GST activity when compared to both control and TCBZ groups.

This effect of the initial increase of GST in low-concentration exposure and subsequent increase in high-concentration exposure was typically recorded by Ullah *et al.* (2017) who recorded the same results by exposing adult *F. gigantica* to curcumin. They stated that this initial increase of GST at the low concentration of treatment could be possibly because of the parasite protective responses that could not be maintained by the adult *Fasciola* worms at the higher treatment concentrations.

In the current study, the SOD activity was also assessed. Auranofin in higher concentrations significantly stimulated SOD levels in *Fasciola* worms when compared to both control and TCBZ-exposed groups. The increase of SOD activity by auranofin reflects increased oxidative stress. Oxidative stress and generated reactive oxygen species cause oxidative DNA damage in flukes (Sluphaug *et al.*, 2003). Similarly, enhancing SOD enzyme activity was obtained after exposing the adult filarial worm to curcumin (Nayak *et al.*, 2012). This agreed with Morawietz *et al.* (2020) who assessed the fasciolicidal activity of a tyrosine kinase inhibitor; imatinib, they recorded an up-regulation of SOD genes and contributed this effect to the anthelmintic activity of imatinib.

Considering the essential involvement of cathepsins in the virulence of *Fasciola* worms, the effect of auranofin on cathepsin-L gene expression of *F. gigantica* was investigated in this work. Auranofin induced a significant inhibition of the cathepsin-L gene expression in a concentration-dependent manner. This clearly indicated the effect of auranofin in the reduction of virulence of *F. gigantica* mainly by interfering with the invasive capacity resulting from inhibiting the cysteine protease activity as cathepsin-L gene silencing studies revealed the loss of invasive potential of the worms (McGonigle *et al.*, 2008). Similar results were obtained by exposing adult *Fasciola* to thymoquinone caused a significant cathepsin-L gene expression inhibition (Ullah *et al.*, 2017).

Conclusion

Auranofin which targeted the TrxR enzyme and plays a central role in controlling the intracellular levels of reactive oxygen species was compared with TCBZ against adult *F. gigantica* *in vitro*. Auranofin significantly inhibited worm motility, decreased egg hatchability, caused apoptosis and disruption of the tegumental surface, marked ultrastructure changes in tegument and testis. It also suppressed free radical scavenging ability and detoxification of worms, and inhibited potential invasive ability by suppressing cathepsin-L gene expression. Thus, Auranofin is a promising fasciolicidal drug.

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Explanation of figures

Fig. 1: Mean worm motility over incubation time among groups.

Fig. 2: H & E stained cut sections of adult *F. gigantica*: (a&b): Adult from control (G1) showed intact tegument (T), spine (s), muscular layer (m), and parenchyma (p) (a:×200,b: ×400). (c&d): Adult exposed to TCBZ (G2) showed numerous vacuoles (red arrows), mild apoptosis (yellow circles), dislodged spines (yellow arrow), and a swollen tegument (green arrow). (c:×400, d:×200). (e&f): Adult from G3 (Aur 3µg/mL) showed vacuolations in tegument syncytium (red arrows) and severe apoptosis (yellow circle) (×400). (g&h): Adult from G4 (Aur 5µg/mL) showed severe vacuolations in tegument syncytium (red arrows) with sloughing and detachment (yellow arrows) and severe apoptosis (yellow circles) (g:×200, h:×400). (i&j): Adult from G5 (Aur 10µg/mL) showed numerous vacuolations in tegument syncytium (red arrows), tegumental sloughing with complete separation of spines (yellow arrows), severe apoptosis (yellow circles), and necrosis (blue circles) (i: ×200, j:×400).

Fig. 3: Caspase-3 immunohistochemically stained cut sections of adult *F. gigantica*: (a): Adult from control (G1) showed mild patchy cytoplasmic expression of caspase -3 in tegumental cells (red arrows) (×200). (b): Adult from G1 showed negative expression in testes spermatozoa (red arrows) (×200). (c): Adult from TCBZ-exposed (G2) showed mild patchy cytoplasmic expression in tegumental cells (red arrows) (×400). (d): Adult from G2 showed mild caspase-3 expression in testes spermatozoa (red arrows) (×400). (e): Adult from G4 (Aur 5µg/mL) showed strong patchy cytoplasmic expression in tegumental cells (red arrows) (×200). (f): Adult from G5 (Aur 10µg/mL) showed strong diffuse cytoplasmic expression in tegumental and ductal cells (×200).

Fig. 4: SEM of adult *F. gigantica*: (a): A control (G1) showed smooth oral sucker (OS) and ventral sucker (VS), intact uniformly distributed tegumental spines (s) with ridged tegument (arrows) (×50). (b): A high power SEM of spines (s) on an apical cone of a control adult worm (G1) showed finger-like protrusions at tips (arrow) (×1000). (c): A SEM of a TCBZ-exposed adult from (G2) showed smooth oral sucker (OS) surrounded by sensory papillae (b), moderately swollen tegumental infoldings (a), disrupted spines in some regions in apical part (s) arrows (×150). (d): A high power of apical cone of adult from G2 showed swelling (a) and furrowing (b) of tegument (×1000). (e): A SEM of auranofin-exposed worm (G3) showed severe tegumental swelling (a,b) around ventral sucker (VS) with dislodgement of some spines from their sockets leaving pits (c) (×200). (f): A high-power of spines on apical cone of auranofin-exposed worm (G3) showed severe tegumental swelling (a), swollen spines with bulbous appearance (s), and some blebs in tegument (c) (×1000).

Fig. 5:TEM of adult *F. gigantica*: (a): Adult from control (G1) showed a full depth of tegumental syncytium, from apical plasma membrane (APM) to basal lamina (BL), normal and tightly closed basal infolds (BI), normal muscle layer (Mu) beneath basal lamina, and normal numbers of secretory bodies (T1 & T2). (b): Adult from TCBZ-exposed (G2) showed basal lamina with swollen basal infolds (BL), normal muscle layer (Mu) surrounded by large spaces (arrow). (c): Adult from G2 showed accumulated swollen type-1 secretory bodies (T1) within a cell with a normal and round nucleus (N) and swollen rounded mitochondria (M). (d): Adult from G3 (Aur 3µg/mL) showed electron-lucent vacuoles (arrows a) in cell syncytium just below apical plasma membrane (APM) apex and swelling of mucopolysaccharide masses (arrow b). (e): Adult from G3 showed numerous T1 & T2 secretory bodies, normal nucleus (N) morphology, and swollen mitochondria (M). (f&g): Adult from G4 (Aur 5µg/mL) showed large electron-lucent vacuoles (Vc) in cell syncytium just below apex of apical plasma membrane (APM) and numerous large autophagic vacuoles (AV). (h): Adult from G5 (Aur 10µg/mL) showed large tegumental defect (D) and separated

bleb (bl), open bodies (ob), multiple electron-lucent vacuoles (VC) and autophagic vacuoles (AV) filling tegumental syncytium. (i): Adult from auranofin (G5) showed swelling and disintegration of basal infolds (BI) above basal lamina (BL) with wide spacing inbetween.



