

In vitro Effects of Triclabendazole (TCBZ) on the Excretory-Secretory Products (ESP) of *Fasciola spp* Parasites

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Abstract- *Fascioliasis* is an endemic disease in Iran and triclabendazole (TCBZ) is using for treatment of domestic animals and infected people. Excretory-secretory products (ESP) play an important role in the host biochemical defense by means of activities of detoxifying and antioxidant glutathione S-transferase (GST) and superoxide dismutase (SOD) enzymes respectively. Therefore, the aim of this comparative study was to evaluate fasciola protection against TCBZ drug by detection of enzymatic activities, GST and SOD, in TCBZ treated *Fasciola hepatica* / *Fasciola gigantica* and control ESP samples. *F. gigantica* and *F. hepatica* helminthes were collected and cultured within buffer media (TCBZ treated and untreated or control) for 4 h at 37 °C. Three TCBZ treated and 1 control ESP samples for each species were collected, centrifuged and supernatants were stored at -20°C. ESP samples protein concentrations were measured by Bradford method. SOD and GST enzymes activities of ESP samples were estimated photometrically. To determine the statistically significant difference between ESP of treated and control samples, t-test was conducted. ESP protein bands were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations in treated *F. hepatica* and *F. gigantica* ESP samples were estimated 204.88, 428, 130.4 and 288.2, 488.2, 308.2 µg/ml respectively. Protein concentrations in control samples were estimated 488.18 and 124.8 ug/ml respectively. SOD enzyme specific activities level in treated *F.hepatica* and *F. gigantica* ESP samples were determined 0.14, 0.31, 3.96 and 11.11, 13.54, 19.95 U/mg/protein respectively. SOD activities level in control samples were detected 70.69 and 10.92 U/mg/protein. GST specific activities level in treated *F.hepatica* and *F. gigantica* ESP samples were calculated 25.3, 85.5, 37.3 and 1823, 1314.3, 1320.8 U/mg respectively. GST activities levels in control samples were detected 98.6 and 1083.9 U/mg/protein respectively. Statistical analysis reveal the significant different between proteins concentrations, GST and SOD enzyme specific activities of TCBZ treated ESP samples of *F. gigantica* in comparison to the control samples ($P<0.05$), however, is not significant for treated *F. hepatica* and control ESP samples ($P>0.05$). There is no difference between SDS-PAGE results of treated and control samples. Based on the results of the present work, significant increase of enzymatic activities of GST and SOD in TCBZ treated *F. gigantica* ESP, it seems, the protection of this species against drug is higher than *F. hepatica*.

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Introduction

Fascioliasis is caused by trematodes belonging to the genus *Fasciola* (*Fasciola hepatica* and *Fasciola gigantica*). In the past, infection was limited to specific and typical geographical areas, but is now widespread throughout the world. Human cases are increasingly reported from Europe, the Americas and Australia, New

Zealand (where only *F. hepatica* is transmitted), and from Africa and Asia (where the two species overlap). As a consequence, human fascioliasis should be considered a disease of major global public health importance. Fascioliasis is a serious medical condition due to the size of the parasite which reflects its origin as a livestock worm (adult *F. hepatica* measure 20-30 mm×13 mm wide, while adult *F. gigantica*

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measure 25-75 mm×12 mm wide) (1). In the past years, praziquantel (PZQ) was used to treatment of fascioliasis (2). Due to ineffectiveness of PZQ, in Iran, the drug of choice to treat human cases of fascioliasis is now triclabendazole (TCBZ) (3,4). TCBZ, a benzimidazole compound, is the drug of choice recommended by the world health organization (WHO) for the treatment of fascioliasis. It is active against the adult parasites in the bile ducts and the immature flukes migrating through the liver. A single-dose of 10 mg/kg body weight is the recommended treatment. The dose can be doubled in the case of treatment failure and given in two divided doses 12–24 hours apart (5).

ESP of fasciola has been reported in the many studies (6,7). Glutathione S-transferase (GST) and superoxide dismutase (SOD) enzymes are common in the ESP of fasciola parasite (7). GSTs catalyse the conjugation of reduced glutathione on a wide variety of substrates. This activity detoxifies endogenous compounds such as peroxidised lipids, as well as breakdown of xenobiotics. GSTs may also bind toxins and function as carrier proteins (8). In the other hand, SOD enzymes are an important antioxidant defense in nearly all cells exposed to oxygen. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. These radicals can start chain reactions that damage cells. Antioxidants to end these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions (9). Although, there are many studies on the effects of drugs on the whole bodies of parasite, however, there are few reports on their effects on fasciola products (10,11).

Therefore, in order to evaluate Fasciola spp protection against TCBZ drug, the present study was designed. In this project, GST and SOD enzyme activities in TCBZ treated ESP samples of *Fasciola hepatica* and *F.gigantica* were compared with control samples.

Materials and Methods

Collection of Parasite ESP samples

Mature *F. gigantica* and *F. hepatica* helminthes were collected from abattoir and identified, based on morphologic and morphometric characters. Collected alive Fasciola parasites were washed for a minimum of three times in PBS, pH 7.4, to remove host material. Meanwhile, to prepare of TCBZ dilutions (20, 40, 100

µg/ml), the TCBZ bolus (Tolide Darouhay Dami Iran Co.) was homogenized within ethanol as a solvent. The parasites were cultured within the autoclaved buffer media (treated by TCBZ or untreated as control) contain glucose 1%, for 4 h at 37°C and (2 parasites in 2 ml of culture media for each sample). The total of 8 ESP samples of *F. gigantica* and *F. hepatica* (3 TCBZ treated and 1 control ESP samples for each species) were collected and centrifuged at 4000g for 15 minutes and supernatants were stored at -20°C (12).

Measurement of ESP samples protein concentrations

The Concentrations of total proteins of ESP samples were measured by Bradford assay method which involves reacting the ESP sample with a dye that binds protein. To measure of protein concentration, standard solutions (Bovine Serum Albumin, Merk Product) and ESP samples were prepared and Bradford reagent was added. The absorbance of ESP samples and standard solutions were measured at 595 nm after 5 min incubation at room temperature. A standard curve was prepared by using the standard solutions absorbance and excels software and the protein concentrations of samples were estimated (12).

SDS-PAGE analysis of ESP samples

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and coomassie blue staining were used to separate the components of ESP samples protein. Samples were added to each wells of gel, 7.5 %, and were run for 6 hours at 15 mA and finally the gels were stained by coomassie blue staining. Molecular weights of sample proteins were detected by using the protein marker within the gel (12).

SOD Enzyme activity assay of ESP samples

SOD activity of ESP samples were determined by using RANSOD kit (Randox Labs, crumlin, UK), which is based on the method of McCord and Fridovich (13). The absorbances of samples were measured 30 s after the addition of xanthine oxidase as start reagent at 505 nm on a Cecil 1021UV/Visible spectrophotometer (Cecil Instruments Ltd Milton Technical Centre Cambridge ENGLAND) and 3 minute after reaction, in 37°C. A standard curve was prepared by using the standard provided in the kit and the SOD total activity value, U/ml, for each ESP sample was read from this curve by using the Microsoft-Excel software. One unit enzyme activity is the amount of SOD that inhibits the rate of formazan dye formation by 50% (14). To calculate the specific activity of SOD

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enzyme, total activity or rate value divided by the protein concentration.

GST enzyme activity assay of ESP samples

To activity assay of GST enzyme in ESP samples, reagent cocktail including, potassium phosphate buffer, reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) substrates were prepared in the cuvette. To each assay, from the mentioned mixture, 200 µl sample solution was removed and then the same volume of TCBZ treated *F. hepatica*/*F. gigantica* ESP or control samples was placed into cuvette and mixed well. Meanwhile, the UV spec was set up at 340 nm to assay of GST activity. Finally, the cuvette was placed into the barrel of the UV/Visible spectrophotometer and absorbances recorded for 5 minutes. Finally, Total GST enzyme activity, U/ml, samples was calculated. One unit will conjugate 1.0 µmole of 1-chloro-2, 4-dinitrobenzene with reduced glutathione per minute at pH 6.5 at 25°C (15). To calculate the specific activity of GST enzyme, the rate of enzyme divided by the protein concentration.

Statistical analysis of ESP samples

To detect the statistical difference between protein concentrations, GST and SOD enzyme activities of TCBZ treated and control ESP samples, two sample independent t-tests was conducted.

Results

Protein concentration in treated and control ESP samples

The concentrations of protein in the ESP samples based on Bradford method were estimated and are presented in table 1. Protein concentrations in treated *F. hepatica* and

F. gigantica ESP samples were estimated 204.88, 428, 130.4 and 288.2, 488.2, 308.2 µg/ml respectively. Protein concentrations in control ESP samples were estimated 488.18 and 124.8 µg/ml, respectively.

SOD enzyme activity in treated and control ESP samples

SOD activity was detected, and the results are presented in Table 2. SOD enzyme activities level in treated *F. hepatica* and *F. gigantica* ESP samples determined 0.14, 0.31, 3.96 and 11.11, 13.54, 19.95 U/mg/protein respectively. SOD enzyme activity level in control ESP samples were detected 70.69 and 10.92 U/mg/protein, respectively.

GST enzyme activity in treated and control ESP samples

The results of GST enzyme activity assay are presented in table 3. GST specific activities level in treated *F. hepatica* and *F. gigantica* ESP samples were calculated 25.3, 85.5, 37.3 and 1823, 1314.3, 1320.8 U/mg/protein respectively. GST enzyme specific activity level of control ESP samples were detected 98.6 and 1083.9 U/mg/protein, respectively.

Statistical analysis results of treated and control ESP samples

Two sample t- test results reveal that there is a significant difference between protein concentrations, SOD and GST specific activities of treated *F. gigantica* ESP samples in comparison to the control ESP samples ($P < 0.05$). However, there is no significant difference between mentioned parameters for treated *F. hepatica* and control ESP samples ($P > 0.05$).

Table 1. Protein concentrations of TCBZ treated ESP samples of *F. hepatica*, *F. gigantica* and control samples.

ESP Samples	Protein Concentrations (µg/ml) of <i>F. gigantica</i>	Protein Concentrations (µg/ml) of <i>F. hepatica</i>	Considerations
S1	124.8	444.18	Control ESP samples
S2	204.88	288.2	ESP contains 20 microgram TCBZ
S3	428	488.2	ESP contains 40 microgram TCBZ
S4	130.4	308.2	ESP contains 100 microgram TCBZ

Table 2. SOD enzyme activities level of TCBZ treated ESP samples of *F. hepatica*, *F. gigantica* and control samples.

ESP Samples	SOD specific activities (U/mg protein) of <i>F. gigantica</i>	SOD specific activities (U/mg protein) of <i>F. hepatica</i>	Considerations
S1	10.92	70.69	Control ESP samples
S2	11.11	0.14	ESP contains 20 microgram TCBZ
S3	13.54	0.31	ESP contains 40 microgram TCBZ
S4	19.95	3.96	ESP contains 100 microgram TCBZ

Table 3. GST enzyme activities level of TCBZ treated ESP samples of *F. hepatica*, *F. gigantica* and control samples.

ESP Samples	GST specific activities (U/mg protein) of <i>F. gigantica</i>	GST specific activities (U/mg protein) of <i>F. hepatica</i>	Considerations
S1	1083.9	98.6	Control ESP samples
S2	1823	25.3	ESP contains 20 microgram TCBZ
S3	1314.3	85.5	ESP contains 40 microgram TCBZ
S4	1320.8	37.3	ESP contains 100 microgram TCBZ

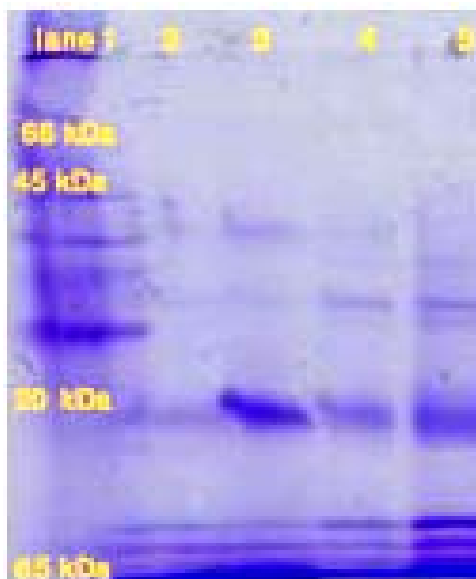


Figure 1. SDS- PAGE analysis of four ESP samples from *F.hepatica*. Each lane represents an ESP sample; the first lane corresponds to the protein marker. The 2, 3, 4 lanes correspond to treated ESP samples by TCBZ dilutions of 20, 40, 100 ug/ml. The lane 5 indicates control ESP sample.

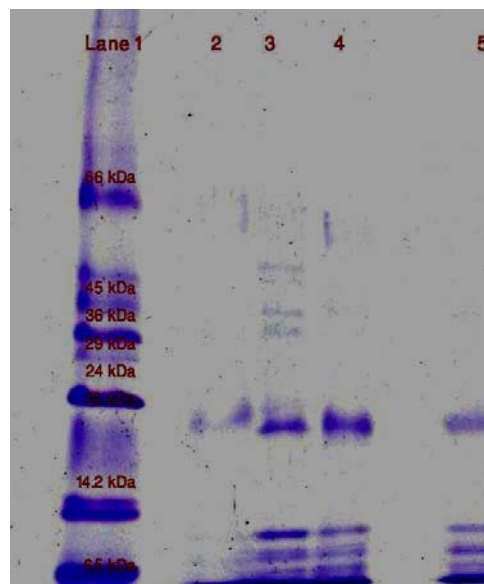


Figure 2. SDS- PAGE analysis of four ESP samples from *F.gigantica*. Each lane represents an ESP sample; the first lane corresponds to the protein marker. The 2, 3, 4 lanes correspond to treated ESP samples by TCBZ dilutions of 20, 40, 100 ug/ml. The lane 5 indicates control ESP sample.

SDS-PAGE results of treated and control ESP samples

Protein bands of ESP samples were detected by SDS electrophoresis and are shown in figure 1, 2. There is no difference between SDS-PAGE results of treated and control ESP samples.

Discussion

Various studies have been recorded on characterization of fasciola parasite ESP (16-18). In this study, significant increase of GST enzyme specific activity in TCBZ treated *F.gigantica* ESP samples may be due to more ability of this species to detoxifies endogenous compounds as well as breakdown of xenobiotics in comparison to the control samples. This phenomenon helps to protect of parasite in the liver tissue of man and animals. Egyptian researchers have demonstrated an

increase in GST level in both juvenile and adult worms of *F.gigantica* (19). The recent research has been demonstrated that GST secreted by the parasite could be involved in evasion of the parasite from the host immune response (20).

At the present research, significant increase of SOD enzyme specific activity of treated *F.gigantica* ESP cause to increase of molecular oxidation inhibition and consequently to assist the parasite protection and establishment in the liver tissue. Indian workers have reported, SOD activity have a role in protecting the *F. gigantica* from Reactive oxygen species (ROS) generated by metabolic process involving O_2 and protection from ROS released by the host immune effector cells (21).

The results of SOD and GST enzyme specific activities of TCBZ treated *F.hepatica* ESP samples in comparison to the control sample indicate, this species has less capability to protect against xenobiotics and free

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radicals than *F.gigantica*. Recently, the results of a study have been indicated that triclabendazole severely disrupts spermatogenesis in the liver fluke, *F.hepatica* (22).

In conclusion, the significant increase of GST and SOD enzyme activities in TCBZ treated *F.gigantica* ESP samples indicate, this species may be able to protect against TCBZ drug higher than *F. hepatica*.

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