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Study on Glutathione S-Transferase (GST) Inhibition Assay by Triclabendazole. I: Protoscoleces (Hydatid Cyst; *Echinococcus granulosus*) and Sheep Liver Tissue

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Abstract

Hydatid disease is a term used to refer infection with the methacestode of *Echinococcus granulosus* parasite in humans, and echinococcusis is restricted to infection with the adult stage in carnivores.Glutathione S-Transferase (GST) represents the major class of detoxification enzymes from helminth parasites such as *Echinococcus* protoscoleces (PSC) and it is candidate for chemotherapeutic and vaccine design. Therefore, GST of protoscoleces could be a target for evaluation of drug effect as triclabendazole in hydatid cyst. For this purpose, GST enzymes were purified from protoscoleces of hydatid cyst and sheep liver tissue by glutathione affinity chromatography using a wash-batch method and subsequently detected their SDS-PAGE pattern. Afterward, GST specific activity levels were assayed in the whole extract and purified solutions spectrophotometrically at 30°C with reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzen (CDNB) substrate. Finally, GST inhibition assay was investigated in the solutions by powder and bolus of triclabendazole. GST fraction as a 26 kDa (MW) band was obtained on SDS-PAGE. The level of GST specific activity in purified solutions was detected 10.24 µmol/min/mg proteins for protoscoleces and 37.84 µmol/min/mg protein for liver tissue. Comparison of the effect of powder and bolus of triclabendazole in solutions revealed inhibition concentration (IC_{50}) 8.71 and 11.16 µg/ml for protoscoleces GST and 8.65 and 9.70 µg/ml for liver tissue GSTs, respectively. These findings suggest the possibility of selective inhibition of protoscoleces. GSTs by triclabendazol in vitro and use of these results for understanding of its molecular effect in vivo.

Keywords: *Glutathione S-Transferase,* Echinococcus granulosus *protoscoleces, Triclabendazole*, *Iran*

Introduction

Echinococcus granulosus is a small endoparasitic flatworm belonging to the class cestoda. Infection with *Echinococcus* may be naturally transmitted between humans and animals. It thus claims is one of the most significant groups of zoonoses. The clinical and economic significance of the parasite is almost completely confirmed to infection with the *Echinococcus* protoscoleces. Hydatid disease and hydatidosis are terms used to refer to infection with the metacestode in humans and ungulates and echinococcosis should be restricted to infection with the adult stage in carnivores (1). Measurement of the socio-economic impact of echinococcosis/ hydatidosis represents an important challenge in the involved societies. In humans, the quantifiable items of disease are those connected with preoperative diagnosis, surgical treatment, hospitalization, post surgical examination and drugs (2).

The Glutathione S-transferases (GST, EC 2.5.1. 18) comprise a family of isoenzymes involved in the cellular detoxification of a broad range of chemical substrates. Neutralization of the substrates, through the conjugation of glutathione

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(Phase 2 detoxification), provides the product more water soluble, less toxic and more readily excreted from the host (Phase 3 detoxification). Metaboliztion of substrates by cytochrome P-450 enzymes (Phase 1 detoxification) in mammals has not been identified in helminth parasites (3). In contrast, GSTs are highly abundant and present in all helminth parasites characterized to date (4, 5). Such high levels of GST infer an important role for these enzymes in helminth homeostasis and survival and this may be related to the naked tegument of helminth parasites and their potential exposure to a wide range of xenobiotics. Apart from reaction from their endogenous metabolism,GSTs of helminth parasite may protect against exogenous free radical damage or xenobiotics as a result of immune effector mechanisms from the host directed at the parasite (6). Biochemical studies have shown that certain parasitic helminth glutathione transferase and aldehyde reductase isoenzymes are significantly superior in neutralizing lipid hydroperoxides or cytotoxic carbonyls than are equivalent mammalian enzymes. This, in turn, indicates variation in active site configuration between the host and parasite enzymes (7). Moreover the role of GST as a protective antigen in vaccination against digeneans has been well documented. *Schistosoma mansoni* 28GST vaccine formulations, apart from reducing worm burdens, can also significantly reduce female fecundity and faecal egg output in the primate model (6). Significant reduction in fluke burdens (57%) has been observed in sheep vaccinated with GSTs and the result suggests that the immune response to GST is directed to the juvenile worm (8). Glutathione transferase activity has been detected in cestodes, digeneas and nematodes. Significantly higher activity has been found in intestinal cestodes and digeneas, compared with parasitic nematodes (9). GSTs activity assay has been described in the cytosol of protoscolexes from sheep hydatid cysts in UK (10).

In the present study we have reported the isolation, SDS-PAGE pattern, specific activity assay and specific activity inhibition assay of purified GSTs by triclabendazole from *E. granulosus* protoscoleces and sheep liver tissue.

Materials and Methods

Protoscoleces extract solution preparation Protoscoleces were obtained from liver and lung hydatid cysts of sheep slaughtered at a local abattoir (Puryaye vali, Tehran, Iran). They were washed 3-4 times with washing buffer [phosphate buffer saline (PBS): 0.17 M NaCL, 3.3 mM KCL, 9.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2]. Protoscoleces in 3 volumes of homogenizing buffer [10 mM EDTA, 2 mM PMSF, 0.15 M NaCL, 50 mM Tris (PH 7.5) containing 0.5% V/V Triton X-100 (Sigma)] were freeze-thawed 3 times in liquid nitrogen and were sonicated in a 150W ultrasonic disintegrator, 10 sec ON and 5 sec OFF on ice until no intact PSC were visible microscopically (approximately 15 min). Then suspension was centrifuged (12000g for 30 min at 4°C) and supernatant was stored at -80°C (11- 13).

Liver extract solution prepration Sheep liver sample was obtained at a local abattoir and washed 3-4 times with washing buffer. Then it was homogenized with 3 volums of homogenizing buffer in a glass homogenizer, so the suspension was centrifuged (12000g for 30 min at 4°C) and supernatant stored at -80°C.

Purified GST solution preparation GSTs can be purified by glutathione-affinity chromatography. For most analytical purpose, a relatively rapid glutathione-affinity batch method can be used to isolate a small quantity of GSTs (14). GSTs were purified from protosoleces and sheep liver extract solution as enzyme pool by a glutathione-affinity matrix [glutathione-agarose (Sigma, G4510)] using a wash-batch method. Glutathione-agarose was provided as a lyophilized powder stabilized with lactose. Approximately 70 mg of powder swells to 1 ml of gel. Two hundred µl of glutathione- agarose gel (14 mg of powder in 200 µl of dionized water) was prepared in micro-

tube eppendorf. Typically 90 to 95% swelling occurs within 30 min at room temperature, but it may require overnight at 2 to 8°C for 100% swelling to occur. After swelling, the agarose beads washed thoroughly with 10 volumes of dionized water or equilibration buffer (PBS 10 mM pH 7.4 containing 50 mM NaCL) by centrifugation at low speed (200-500g) for 10 sec in a bench microcentrifuge at 4°C to remove the lactose present in the lyophilized product. The extract, 750 µl (2-3 mg protein), was mixed with the gel for 30 min at 4°C and centrifuged at high speed for 10 sec in a bench microcentrifuge at 4°C. The supernatant was removed and the gel matrix washed with 20 gel volumes of PBS-T (PBS 10 mM, pH 7.4 containing 50 mM NaCL and 1% Triton X-100) by centrifuge at high speed (1000g) for 10 sec at 4°C. Bound GST enzymes were eluted by washing the gels with elution buffer (50 mM Tris-HCL pH 9.6 buffer containing 5 mM reduced glutathione) 3 times, 500 µl each and mixed each elution step gently, then centrifuged at high speed for 10 sec at 4°C and accumulated the supernatant into the clean tubes and stored at- 80°C (15-17).

Protein assay in the solutions The amount of protein in the extract solutions and purified solutions of protoscoleces and sheep liver were estimated by the method of Bradford using purified bovine serum albumin (1mg/ml) as the standard (15).

SDS-PAGE pattern of solutions Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted as was reported previously (18). Briefly about 20 µg of homogenated solutions and purified GSTs from sheep liver tissue and PSC were mixed with sample buffer and were run on 12.5% acrylamide gels. Obtained protein bands were stained with Comassie blue R-250(18).

GSTs specific activity assay in the solutions GSTs activity was assayed spectrophotometrically at 30°C with 1-chloro-2, 4-dinitrobenzene (CDNB) as the standard second substrate and reduced glutathione (GSH) (19). This was done by watching an increase in absorbance at 340 nm. At first PBS 0.1M, pH 6.5 as assay buffer was prepared, autoclaved and stored at 4°C. Then CDNB 100 mM and GSH 100 mM as substrates of assay were prepared and stored in microtube eppendorf (CDNB at 4°C and GSH at -20°C). Switch on spectrophotometer (CECIL 9000) and set water bath temperature to 30°C. Protosoleces and liver extract solutions with purified solutions were removed from -80°C freezer and allowed to thaw on ice. CDNB 100 mM from 4°C and GSH 100 mM from-20°C freezer were removed and allowed to thaw at room temperature, when thawed, incubated at 30°C in water bath. For each assay was prepared one ml of assay cocktail (980 µl PBS pH 6.5, 10 µl of 100 mM CDNB and 10 µl of 100 mM GSH), then removed 100 µl of cocktail and its remaining placed 900 µl of it into 1.5 ml plastic cuvet. To zero spect, was used 1 ml of distilled water and to the blank cuvet added 100 µl PBS to 900 µl of cocktail (because the substrates for the GST can react with glutathione nonenzymatically but at slower rates than the enzyme-catalyzed reaction) and measured absorbance at 340 nm for 5 min. To the test cuvet was added 100 µl of sample to 900 µl cocktail and mixed and measured absorbance at 340 nm for 5 min. For calculation, was subtracted the Δ340/min for the blank reaction from the Δ340/min for each sample reaction. The molar extinction of CDNB is 0.0096 μM^{-1}/cm (20, 21).

Effect of triclabendazole on GSTs activity in the whole extract and purified solutions

In the present study, GST inhibition enzyme assay was investigated in the extract and purified solutions of protoscoleces and sheep liver by powder and bolus of triclabendazole (RAZAK Co).One mM triclabendazole solution was prepared by 3.59 mg of powder and 10.48 mg of bolus, each in 10 ml of ethanol. Inhibition of GST specific activity was measured as a IC_{50}, which is defined as the concentration of antihelminth at which 50% of enzyme specific activity is inhibited. This was determinated by

measuring GSTs specific activity using reduced glutathione and CDNB in the present of different concentration of powder and bolus triclabendazole solutions (3.6, 7.2, 10.8, 14.4, 18, 21.6, 25.2, 36, 43.2, 54 µg/ml) (20, 21).

Results

The amount of protein in the extract solutions (24.5mg/ml for protoscoleces and 33 mg/ml for sheep liver) and in the purified solutions (0.05 mg/ ml for protoscoleces and 0.04 mg/ ml for sheep liver) were obtained. The SDS- PAGE pattern of GSTs in solutions is shown in Fig. 1.The level of GST specific activity in both solutions was detected, and the results presented in Figs. 2 and 3. The effects of triclabendazole (powder and bolus) on GST specific activity of extract and purified solutions were determinated and the results are shown in Figs 4-7. Finally, the inhibitor concentration for remaining specific activity of samples GSTs was calculated graphically and is presented in Table 1 and Fig. 8.

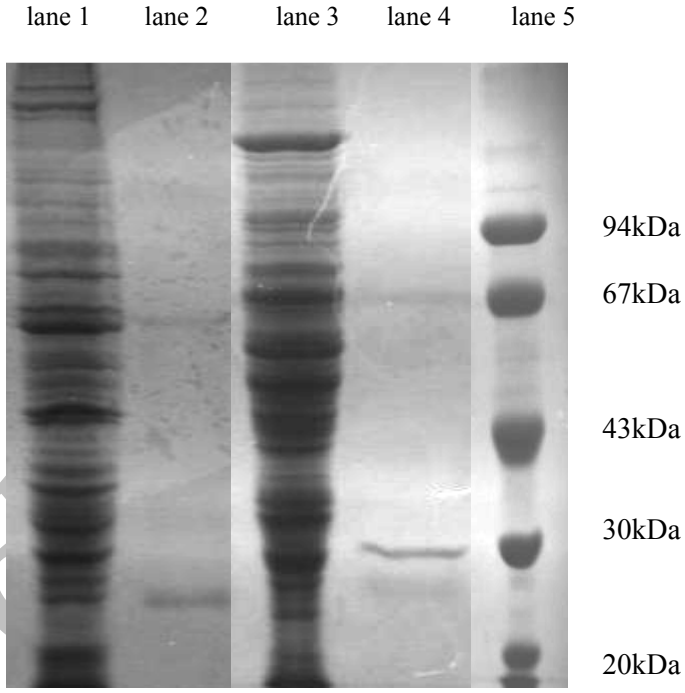

Fig. 1: SDS-PAGE pattern of extract and purified GSTs solutions of protoscoleces (Lane 1, 2) and sheep liver tissue (lane 3, 4) and standard marker (lane 5).

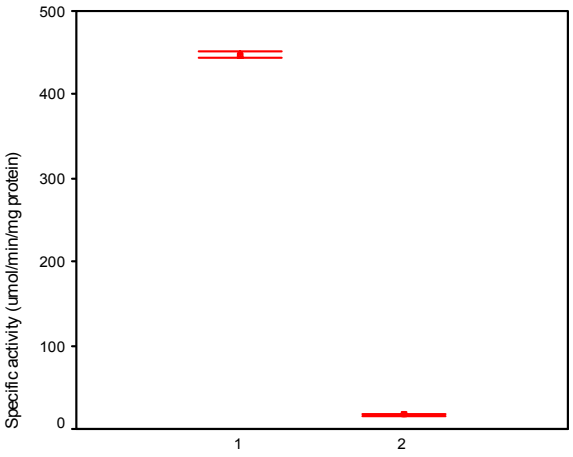

Fig. 2: GST specific activity of extract solutions from sheep liver tissue: 1 and protoscoleces: 2

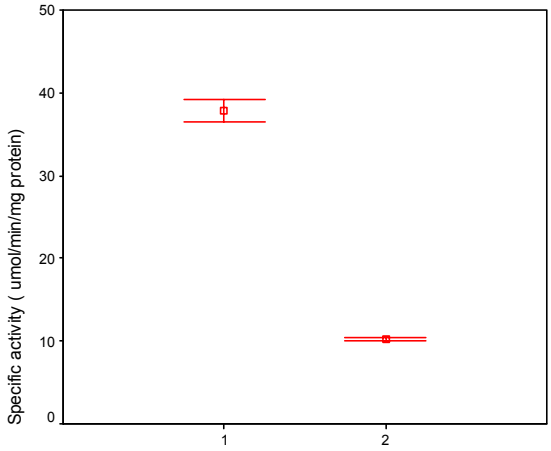

Fig. 3: GSTs specific activity of purified solutions from sheep liver tissue: 1 and Protoscoleces: 2

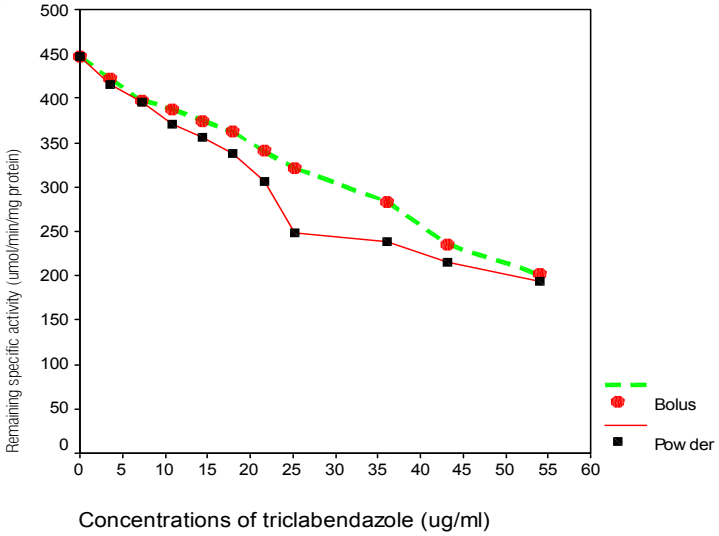

Fig. 4: GSTs remaining specific activity based on triclabendazole concentration in the extract solution of sheep liver tissue.

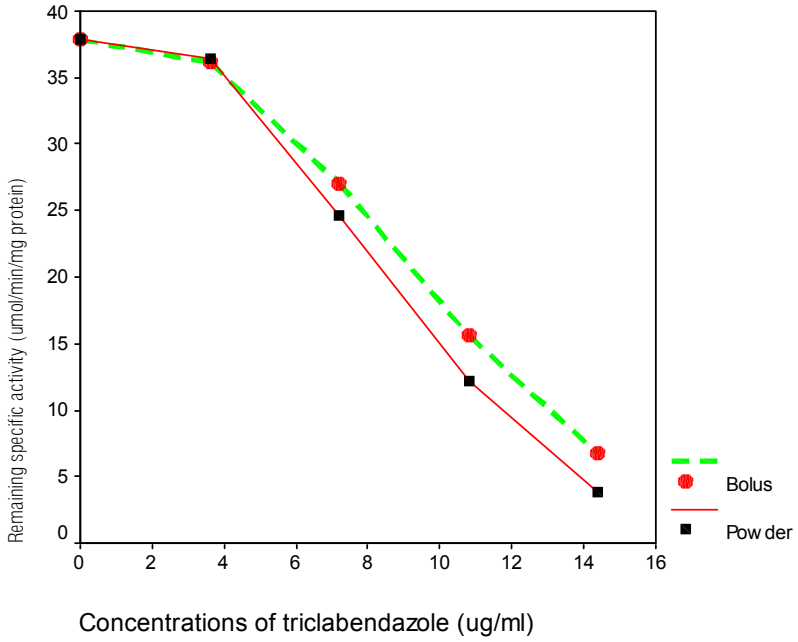

Fig. 5: GST remaining specific activity based on triclabendazole concentration in the purified solution of sheep liver tissue.

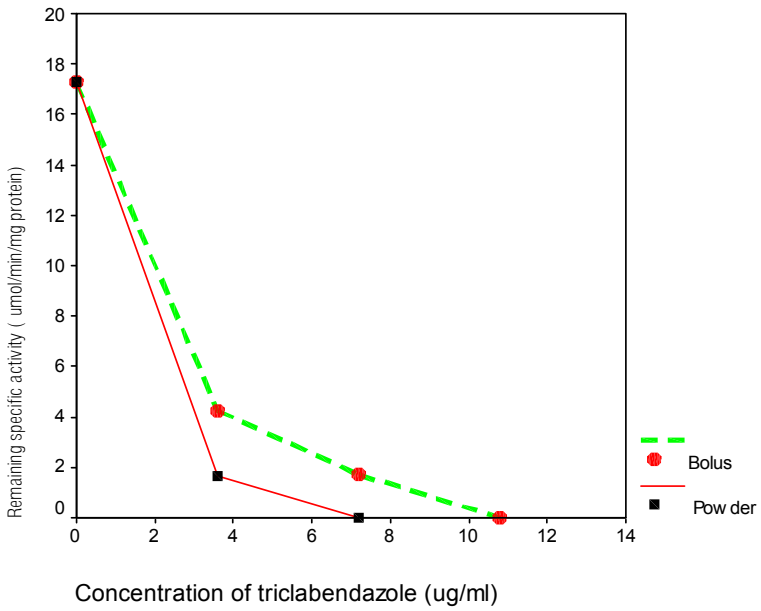

Fig. 6: GST remaining specific activity based on triclabendazole concentration in the extract solution of protoscoleces.

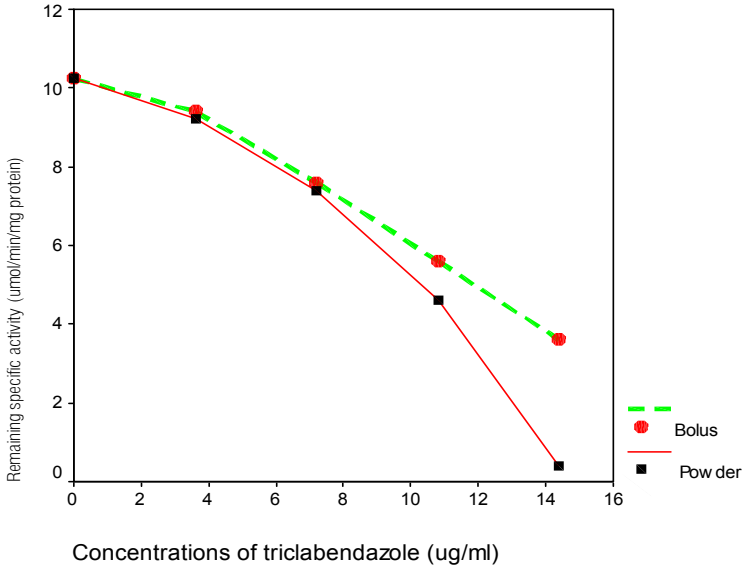

Fig. 7: GST remaining specific activity based on triclabendazole concentration in the purified solution of protoscoleces.

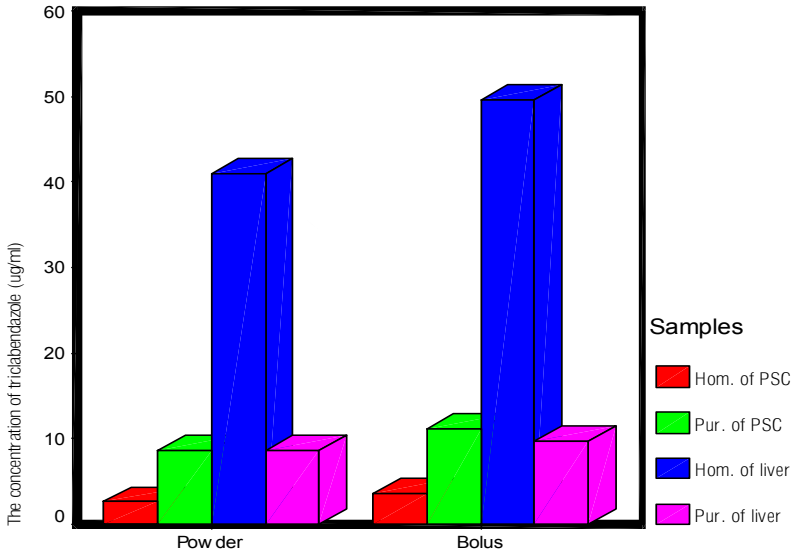

Fig. 8: Inhibition concentrations of 50% of GSTs specific activity (IC_{50}) of protoscoleces and sheep liver tissue by triclabendazole (powder & bolus).

Table 1: Inhibition concentrations of 50% of GSTs specific activity (IC_{50}) of protoscoleces and sheep liver tissue by triclabendazole (powder & bolus).

Samples	Protoscolecs		Liver of sheep	
	Extract solution	**Eluted solution**	**Extract solution**	**Eluted solution**
Powder	2.63	8.71	40.95	8.65
Bolus	3.55	11.16	49.66	9.7

Discussion

Previous studies showed that GSTs participated in detoxifying the exogenous toxins. The protescoleces GSTs, like other cytosolic GSTs of helminthes, may involve in catalyzing the conjugation of glutathione to electrophilic compounds (6). SDS-PAGE of the present study pattern revealed one band protein with 26 kDa that showed purified GST enzymes in the purified solution of protoscoleces, however sheep liver tissue showed two bonds ranging 26-28 kDa, because some isoenzymes of GSTs are present in the liver. GST protein fraction migrating as a 24 kDa band on SDS-PAGE, has been isolated by affinity chromatography on glutathione-agarose from a soluble extract of *E.granulosus* protoscoleces from sheep in UK (10). The results showed that GSTs activity in extract solutions was grater than purified solutions because there were some enzymes and proteins in the homogenate solution that could affect on the reaction. GSTs activity have been described in the cytosol of protoscolexes from sheep hydatid cysts and have had activity of 0.4 µmol/min/mg when was measured using a standard synthetic substrate (10). Comparison of the effect of triclabendazole on GST specific activity revealed that IC_{50} for bolus was grater than powder in both samples. This phenomenon may be due to this fact that the amount of triclabendazole itself in bolus is less than that of issued on its box or may be due to existence of supplementary materials in bolus, so the effectiveness of powder to bolus is a reasonable fact. Comparison of the effect of triclabendazole bolus revealed that the activities of both GSTs were suppressed and the difference between the extents of inhibition was relatively high. It means that the inhibitor can suppress the GST activity in protoscoleces but can not affect the activity of this enzyme in the sheep liver in the same concentrations of triclabendazole. Furthermore, comparison of the effect of triclabendazole powder revealed that activities of both GSTs were suppressed but the difference between of inhibition assay was not significant. The general inhibition of protoscoleces and liver tissue GSTs in the microgram range (as judged by IC_{50} value) by triclabendazole may help explaining the mode of action of this chemotherapeutic agents in vitro. Further characterization of the isolated protein should focus on the elucidation of the role of GST activity in the protoscoleces defense mechanisms against oxidative and toxic damage and efficacy of the other antihelminths on GST activity.

In brief, these findings suggest, possibility of the selective inhibition of the protoscoleces GSTs in vitro and use of these results for understanding of molecular mechanism of triclabendazole in vivo.

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