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Original Article

Triclabendazole (Anthelmintic Drug) Effects on the Excretory-Secretory Proteome of *Fasciola hepatica* in Two Dimension Electrophoresis Gel

Ashkan FARIDI [1], *Ali FARAHNAK [1], Taghi GOLMOHAMMADI [2], Mohammadreza ESHRAGHIAN [3], Yosef SHARIFI [1], Mohammadbagher MOLAEI RAD [1]

1. *Dept. of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran*
2. *Dept. Of Biochemistry, School of Medicine, Tehran University of Medical Sciences, Iran*
3. *Dept. of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Iran*

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***Correspondence**
Email:
farahnak@sina.tums.ac.ir

Abstract

Background: The aim of this study was to evaluate the protein spots of excretory - secretory products of *Fasciola hepatica* using two dimension electrophoresis method in the presence and absence of triclabendazole drug which can be considered to detect the target protein of the drug.

Methods: *F. hepatica* parasites were collected from infected cattle livers, divided in two groups and cultivated in RPMI 1640 medium. First group was treated with triclabendazole (TCBZ) and second group considered as control. The excretory-secretory (ES) products of each group were separated and total protein determined by Bradford method. To provide proteome spots, the ES proteins were precipitated and two dimension electrophoresis (2-DE) gel prepared. Protein amounts of two groups were compared using the statistical *t*-test and protein spots from 2-DE in test and control groups were also statistically analyzed. The protein spots of gels were identified by using protein database.

Results: The t-test showed a significant increase of total proteins in treated group ($P<0.5$). The protein spots count in the control group was less than test group however statistically not significant ($p>0.05$). Cathepsin L- protein (MW 36.7 pH 5.34), 14-3-3 epsilon 2 isoform (MW 28.2 pH 5.36), Cathepsin L1D (MW 36.5 pH 5.8) and Cathepsin L1D (MW 36.6 pH 6.26) were identified in test group.

Conclusion: It seems that, these results can be considered to determine the proteins which the drug acts as a target on them.

Introduction

Fasciola hepatica and *F. gigantica* are Platyhelminthes trematodes that cause fascioliasis (1). The disease has two clinical phases; "immigration phase" occurs in liver parenchyma and correlates to life cycle of the parasite and "emplacement phase" or worm hold in the biliary duct. For reaching maturity and reside in the biliary duct, parasite migrates in liver parenchyma by secreting various excretory-secretory (ES) protein such as protease. Parasite causes liver cirrhosis, inadequacies in liver functions and obstructive disorders of the biliary tracts (2). Triclabendazole, the choice drug for fasciolosis, is given in a single dose of 10 mg/kg (3, 4).

Excretory-secretory proteins could be considered as a marker in the interactions between parasite and host and could be a target in diagnosis, treatment, and even candidates for vaccines production. In vitro studies shows that these proteins have a role in nutrition modes, detoxification of biliary component and hiding the *Fasciola* from immune system escape (5).

Two-dimensional electrophoresis is the combination of two electrophoresis: Isoelectric focusing (IEF) gel and Sodium dodecyl sulphate poly acrylamide electrophoresis (SDS-PAGE), where proteins are first separated according to their isoelectric points and then differentiated on the SDS-PAGE according to their molecular weight. IEF separates proteins according to their isoelectric points (pI), where their charge is neuter, can be drawn to any electrical poles and has the lowest dissolving power. Sodium dodecyl Sulphate is anionic detergent, attached to more molecular proteins in different pH and gives them a negative charge. Since the ionic charge in these polypeptides is almost similar, the separation of proteins is dependent on the difference in molecular weight; finally every protein is placed on the 2-dimensional surface of the gel depending on the two factor pH and molecular weight (6).

This study was designed to evaluate the available protein spots in the excretory - secretory products of *F. hepatica* using 2-DE technique with / without TCBZ drug which may be considered to detect the target protein of drug.

Material and Methods

Parasites isolation and cultivation

Infected cattle livers were collected from local slaughter-house (Poryaie-Shargh Tehran, Iran) and transferred to the laboratory. *Fasciola hepatica* parasites were isolated, washed 6 time in PBS (0.15 M, pH 7.2) and cultivated in RPMI 1640 culture medium (Gibco 51800-019) with 10 µl penestrep for 16h at 37˚C and 5% CO2. To evaluate TCBZ (Eaten 250mg tablet; Novartis; Switzerland) effects on parasite, 20 *F. hepatica* treated with egaten. To achieve this, TCBZ stock solution was prepared with dimethyl sulphoxide (DMSO) and added 0.6 µl (15 microgram/1 adult parasite / 1ml culture medium) to each test culture medium. The same volume of DMSO was used alone to each control medium. ES products were collected, and centrifuged for 10 minutes at 25 ˚C at 13000 g. Supernatant were kept in -20˚C until use them (7).

Protein concentration measurement of ES products

ES proteins were measured with Bradford method. Bradford reagent (100 mg Coomassie Brilliant Blue G-250, 50 ml 95% ethanol, 100 ml 85% phosphoric acid) and Bovine Serum Albumin (BSA) were applied to prepare a standard curve and protein were measured based on observation of the solution absorbance in 595 nm using a spectrophotometer (8).

Two- dimensional electrophoresis gel preparation of ES Products

Proteins of 3 samples of tests and 3 of controls were precipitated with TCA/acetone, pellets resolved in buffer lysate and rune with 4500Vh on IEF gel (Urea 110 mg, acrylamide

7.8 mg, bisacrylamide 210 µg, triton X-100 4µl, ampholines pH 3-10 2 µl, ampholines pH 5-7 8 µl, d.d Water 120 µl, TEMED 0.3 µl, AMPS 0.5 µl), resulting gel differentiated on 10% SDS-PAGE (acrylamide 1.6 gr, bisacrylamide 43 mg, HCl 1M 1cc, tris base 366 mg, SDS 20 mg, d.d Water up to 16ml, temed 5 µl, AMPS 80 µl) with 20mA constant current. The gels were stained with Coomasie Brilliant Blue G250 (8). To determine the molecular weight of protein spots, Rf of ladder bands were calculated, standard curve were prepared in Excel software and finally protein MW were determined. pH of each spots was detected by using the total length of IEF gels. The protein spots of gels were identified by using protein database (http://web.expasy.org/tagident/).

Statistical analysis

The protein amounts of control and test groups were compared (with confidence interval of 95 %) using the statistical t-test method (http://www.evansres.com/ttest.html) and the protein spots resulted from 2-DE gels were compared also precisely.

Results

The protein amounts of control and test groups are presented in table 1.The mean of total protein of control samples (20 parasites) was 396 µg/ml (SE = 64.11) and for the test samples (20 parasites) 718 µg/ml (SE = 63.99). Statistical analysis by the t-test shows a significant difference between two groups [T (18) =3.85, T-value = 2.1] ($P < 0.05$). Randomly 3 test gels (T8, T9, and T10) and 3 control gels (C8, C9 and C10) were compared precisely which are presented in figures 1-6. In all gels, protein spots between pH of 3 to 6 with molecular weight between 25 - 35 kDa are observed. An obvious spot is seen at pH=6.6 with MW 53.2 in gels. 4 couple of spots beside each other are observed at pH 5 - 7 with MW 30-35 kDa, and one protein spot at pH=8.6 with MW 32 kDa are observed.

Table1: Measured protein concentrations in the excretory- secretory products of cultivated *F. hepatica*

Cultivation Sample	Control (µg/ml)	Test (µg/ml)
1	443.63	354.54
2	514.54	456.36
3	360.00	543.63
4	378.18	587.27
5	743.63	670.90
6	327.27	765.45
7	410.90	585.45
8	254.54	750.90
9	807.27	509.09
10	252.72	234.54
11	278.18	387.27
12	161.81	403.63
13	70.90	950.90
14	149.09	776.36
15	361.81	1050.90
16	1252.72	1129.09
17	269.09	954.54
18	145.45	1032.72
19	74.54	1087.27
20	130.90	1130.90
Mean(P<0.05)	396 µg/ml (± 64.11)	718 µg/ml(± 63.99)

Protein spots were identified by using protein database and the results are presented in table 2. In 2-DE, test gels in compare to the control gels shows Cathepsin L- protein (MW 36.7 pH

5.34), 14-3-3 epsilon 2 isoform (MW 28.2 pH 5.36), Cathepsin L1D (MW 36.5 pH 5.8) and Cathepsin L1D (MW 36.6 pH 6.26). The protein spots count in the control group was less than test group, however was not significant (P>0.05).

Table 2: Descripted protein spots from 2-DE of excretory- secretory products of cultivated *F. hepatica* by using expasy database (http://web.expasy.org/tagident/)

Spot Number	Pi	MW	Gel Number	Gel sample Test \ Control	Descripted Proteins
1	6.6	53.2	All	T&C	Phosphopyruvate (Endolase) (MW 49 pH 6.95)
2	5.2	32	6	T	Cathepsin L- protein(MW 36.7 pH 5.34)
3	5.3	30	6	T	14-3-3 epsilon 2 isoform(MW 28.2 pH 5.36)
4	5.8	35	6	T	Cathepsin L1D(MW 36.5 pH 5.8)
5	5.9	31.6	2,3,4,5,6	T&C	Cathepsin L1D(MW 36.5 pH 5.8)
6	5.9	30	1,2,4,5,6	T&C	Cathepsin L-Like(MW 36.7 pH 5.94)
7	6.3	35	4,6	T	Cathepsin L1D Catepsin L1 protease secreteal catepsin L2 (MW 36.6 pH 6.26)
8	6.4	31.6	All	T&C	Cathepsin B (MW 37.6 pH 6.96)
9	6.4	30	All	T&C	Cathepsin B (MW 32.6 pH 6.65)
10	6.5	35	6	T	Cathepsin L1D(MW 36.6 pH 6.26)
11	6.6	33.1	2,3,4,5,6	T&C	Cathepsin B(MW 37.6 pH 6.96)
12	6.7	33.6	All	T&C	Cathepsin B(MW 37.6 pH 6.96)
13	7	33.1	1,2,3,4 ,6	T&C	Cathepsin B(MW 37.6 pH 6.96)
14	7.1	31.6	1,2,3,4 ,6	T&C	GST Ω Class(MW 27.3 pH 7.04)
15	7.6	33.1	1,4,6	T&C	NADH- dehydrogenase Subunit 2 (GST) F.gig(MW 32.3 pH 7.94)
16	7.6	31.6	1,4,6	T&C	NADH- dehydrogenase (GST) (MW 32.3 pH 7.94)
17	8.3	33.1	2,4,6	T&C	NADH- ubquinine oxidoreductase (MW 34.3 pH 8.43)
18	8.6	32	1,5,6	T&C	Vitellin protein B1 or NAD- ubiquinine Oxidoreductase(MW 34.3 pH 8.43)

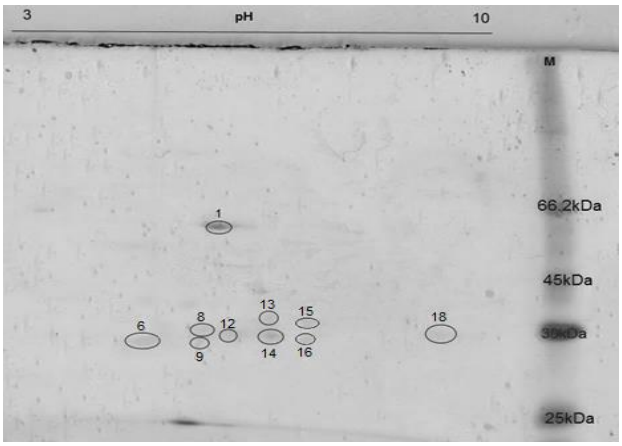

Fig. 1: The Protein spots of *Fasciola hepatica* ES products, control sample (C8), in 2-DE gel. Spot number 1 (MW 53.2 pH 6.6), Spot number 6 (MW 30.0 pH 5.9), , Spot number 8 (MW 31.6 pH 6.4), Spot number 9 (MW 30.0 pH 6.4), Spot number 12 (MW 33.6 pH 6.7), Spot number 13 (MW 33.1 pH 7.0), Spot number 14 (MW 31.6 pH 7.1), Spot number 15 (MW 33.1 pH 7.6), Spot number 16 (MW 31.6 pH 7.6), Spot number 18 (MW 32.0 pH 8.6), M; protein marker

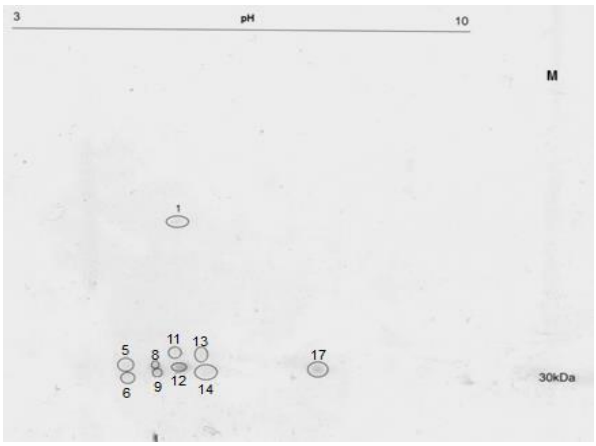

Fig. 2: The Protein spots of *Fasciola hepatica* ES products, test sample (T8), in 2-DE gel. Spot number 1 (MW 53.2 pH 6.6), Spot number 5 (MW 31.6 pH 5.9), Spot number 6 (MW 30.0 pH 5.9), Spot number 8 (MW 31.6 pH 6.4), Spot number 9 (MW 30.0 pH 6.4), Spot number 11 (MW 33.1 pH 6.6), Spot number 12 (MW 33.6 pH 6.7), Spot number 13 (MW 33.1 pH 7.0), Spot number 14 (MW 31.6 pH 7.1), Spot number 17 (MW 33.1 pH 8.3) , M; protein marker

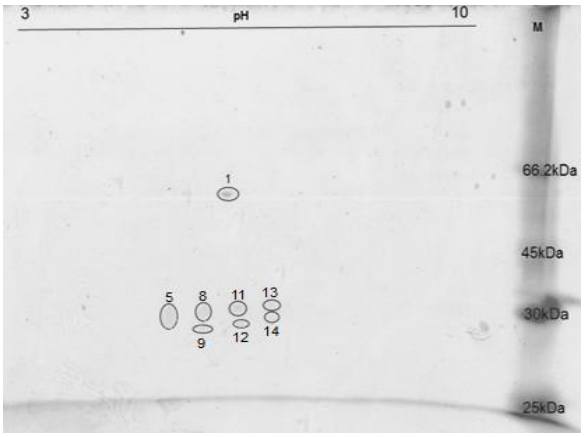

Fig. 3: The Protein spots of *Fasciola hepatica* ES products, control sample (C9), in 2-DE gel. Spot number 1 (MW 53.2 pH 6.6), Spot number 5 (MW 31.6 pH 5.9), Spot number 8 (MW 31.6 pH 6.4), Spot number 9 (MW 30.0 pH 6.4), Spot number 11 (MW 33.1 pH 6.6), Spot number 12 (MW 33.6 pH 6.7), Spot number 13 (MW 33.1 pH 7.0), Spot number 14 (MW 31.6 pH 7.1), M; protein marker

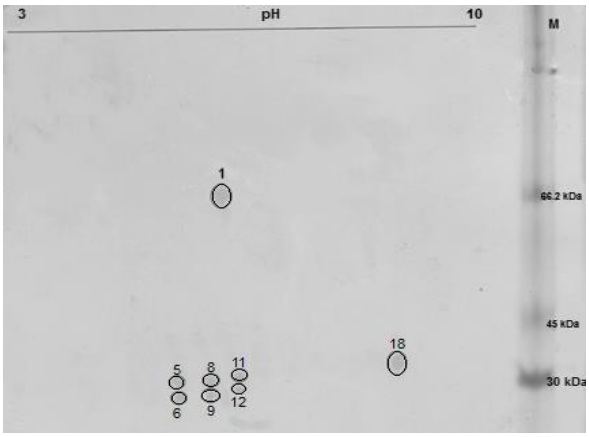

Fig. 5: The Protein spots of *Fasciola hepatica* ES products, control sample (C10), in 2-DE gel. Spot number 1 (MW 53.2 pH 6.6), Spot number 5 (MW 31.6 pH 5.9), Spot number 6 (MW 30.0 pH 5.9), Spot number 8 (MW 31.6 pH 6.4), Spot number 9 (MW 30.0 pH 6.4), Spot number 11 (MW 33.1 pH 6.6), Spot number 12 (MW 33.6 pH 6.7, Spot number 18 (MW 32.0 pH 8.6), M; protein marker

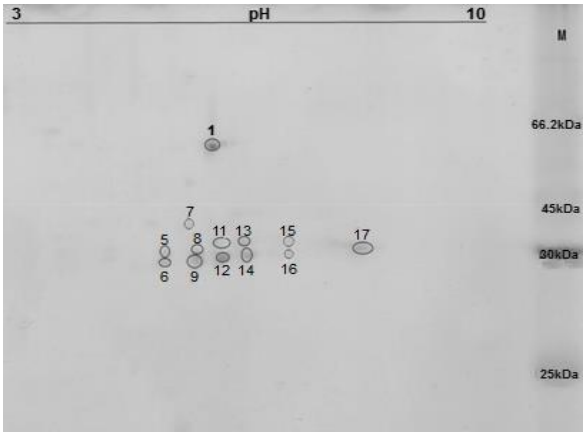

Fig. 4: The Protein spots of *Fasciola hepatica* ES products, test sample (T9), in 2-DE gel. Spot number 1 (MW 53.2 pH 6.6), Spot number 5 (MW 31.6 pH 5.9), Spot number 6 (MW 30.0 pH 5.9), Spot number 7 (MW 35 pH 6.3), Spot number 8 (MW 31.6 pH 6.4), Spot number 9 (MW 30.0 pH 6.4), Spot number 11 (MW 33.1 pH 6.6), Spot number 12 (MW 33.6 pH 6.7), Spot number 13 (MW 33.1 pH 7.0), Spot number 14 (MW 31.6 pH 7.1), Spot number 15 (MW 33.1 pH 7.6), Spot number 16 (MW 31.6 pH 7.6), Spot number 17 (MW 33.1 pH 8.3), M; protein marker

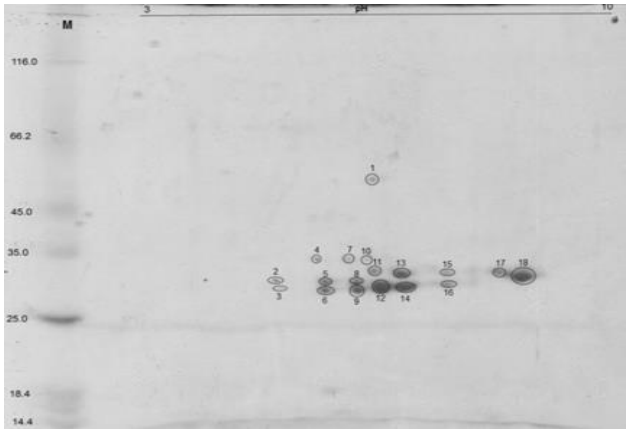

Fig. 6: The Protein spots of *Fasciola hepatica* ES products, test sample (T10), in 2-DE gel. Spot number 1 (MW 53.2 pH 6.6), Spot number 2 (MW 32 pH 5.2), Spot number 3(MW 30.0 pH 5.3), Spot number 4 (MW 35.0 pH 5.8), Spot number 5 (MW 31.6 pH 5.9), Spot number 6 (MW 30.0 pH 5.9), Spot number 7 (MW 35 pH 6.3), Spot number 8 (MW 31.6 pH 6.4), Spot number 9 (MW 30.0 pH 6.4), Spot number 10 (MW 35.0 pH 6.5), Spot number 11 (MW 33.1 pH 6.6), Spot number 12 (MW 33.6 pH 6.7), Spot number 13 (MW 33.1 pH 7.0), Spot number 14 (MW 31.6 pH 7.1), Spot number 15 (MW 33.1 pH 7.6), Spot number 16 (MW 31.6 pH 7.6), Spot number 17 (MW 33.1 pH 8.3), Spot number 18 (MW 32.0 pH 8.6), M; protein marker

Discussion

TCBZ effect on *Fasciola* tegument and is able to change membrane of parasite, causing inflammation, plowing, blebbing (replacing the tegument with a new one via shedding mechanism) and shearing of the tegmental spine (9, 10). Based on these findings we can expect an increasing of total protein in TCBZ treated parasite culture media which correlates our study that shows an average protein of test group is more than control group.

The protein spots count in the control group was less than test group and statistically not significant however the comparison of calculated *P*-value and significant level show the difference between two groups will be significant when choice samples are larger.

In a recent study, 29 to 60 spots of *Fasciola* ES product were identified by 2-DE, including; Cathepsin L-protease, secreted cathepsin L1, Cathepsin L-like proteinase, thiol-specific antioxidant protein, superoxide dismutase (SOD), thioredoxin peroxidase (TPx), glutathione S-transferase (GST), fatty-acid-binding protein (FABP), actin, glycolytic enzyme enolase, glyceraldehyde-3-phosphate dehydrogenase, and all of these enzymes can play a role in migration, detoxification , escape from immune system and survival of the *Fasciola* in the host's body (11). In this study, protein spot with molecular weight 53.2 kDa pH 6.6 (spot number 1 in all gels) in comparing with similar past studies may be related to phosphopyruvate (Endolase), which catalysis the sixth stage of glycolysis and breaks down glucose for conversion into energy and CO_2. Protein spot with MW32 pH 5.2 (spot 2 in Fig. 6) may be related to Cathepsin L- protein that belongs to the peptidase C1 family with cysteine-type peptidase activity. Cathepsin L like protease, passively preserve in lysosomes of the intestinal epithelial cells and are secreted in secretory granules. As digestive enzymes have a critical role in the breakdown of the host extracellular proteins. Protein spot with MW 30 pH 5.3 (spot 3 in Fig. 6) may be related to 14-3-3 epsilon 2 isoform protein which mediate signal transduction. Protein spot with MW 35 pH 5.8 (spot 4 in Fig. 6), protein spot with MW 31.6 pH 5.9 (spot 5 in all figures), protein spot with MW 35 pH 6.3(spot 7 in Fig. 4, 6) and protein spot with MW 35 pH 6.5 (spot 10 in Fig. 6), may be related to Cathepsin L1D that belongs to the peptidase C1 family with cysteine-type peptidase activity. Protein spot with MW 30 pH 5.9 (spot 6 in all Fig. except 3) may be related to Cathepsin L-Like that belongs to the peptidase C1 family with cysteine-type peptidase activity. This proteolytic enzyme is critical for development and survival of organism. Protein spots with MW 31.6 kDa pH 6.4 (spot 8 in all fig), protein spot with MW 30 kDa pH 6.4 (spot 9 in all fig), protein spot with MW 33.1 kDa pH 6.6 (spot 11 in all Fig. except fig1), protein spot with MW 33.6 kDa pH 6.7 (spot 12 in all fig) and protein spot with MW 33.1 pH 7.0 (spot13 in all Fig. except 5), may be related to cathepsin B that belongs to the peptidase C1 family with cysteine-type endopeptidase activity. Protein spot with MW 35 pH 6.5(spot 10 in Fig. 6) may be related to cathepsin L1D catepsin L1 protease secreteal catepsin that belongs to the peptidase C1 family with cysteine-type peptidase activity. Protein spot with MW 31.6 pH 7.1 (spot 14 in all Fig. except 5) may be related to glutathione S-transferase with glutathione transferase activity, a protein that presents in both eukaryotes and prokaryotes. It plays detoxificative role in cells and constitute more than 10% of cytosolic protein. Protein spot with MW 33.1 pH 7.6 (spot 15 in Fig. 1, 4, 6) and protein spot with MW 31.6 pH 7.6 (spot 16 in Fig. 1, 4, 6) may be related to NADH-dehydrogenase Subunit 2 that is a part of cellular component mitochondrion with dehydrogenase activity. Protein spots with MW 33.1 and pH 8.3 (spot 17 in all fig) and protein spot with MW 32 pH 8.6 (spot 18 in Fig. 1, 6) may be related to NADH- ubquinine oxidoreductase belongs to the family with oxidoreductase activity (7, 11, 12).

A comparison between 2-DE patterns of the control and test groups shows appearance spots numbers 2,3,4,7,10 which are equal Ca-

thepsin L- protein(MW 36.7 pH 5.34), 14-3-3 epsilon 2 isoform(MW 28.2 pH 5.36), Cathepsin L1D(MW 36.5 pH 5.8) and Cathepsin L1D (MW 36.6 pH 6.26) in test group respectively.

Based on literature review protein and or enzyme like thiol-specific antioxidant proteins, superoxide dismutase (SOD), thioreduxin peroxidase (TPx), fatty-acid-binding protein (FABP) were not detected in our study (7, 11, 12). Where protein spots were not identified, it seems that parasite isolates acts specifically on choice proteins including the enzymes. It shows that TCBZ and parasite isolates effect on excretory secretory proteome of *F. hepatica*.

Conclusion

TCBZ has effect on the amount of ES protein of treated *F. hepatica* in compared with control and produces Cathepsin L- protein, 14-3-3 epsilon 2 isoform, and Cathepsin L1D isoenzyme in test group as compare to control group. Therefore this drug effects on quantity and probably quality of parasite proteome. The results of this work can be considered in determining the target protein of the drug.

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