Liver integrity during experimental *Schistosoma mansoni* infection and treatment with RO 15-5458 in C57BL/6 mice

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Abstract: Chemotherapeutic treatment against schistosomiasis is the effective available option for infection control. In an attempt to further investigate the effect of RO 15-5458 in treatment of schistosomiasis, 6 weeks old female C57BL/6 mice were infected three times with 60 *Schistosoma mansoni* cercariae divided into 3 equal doses and given at 6 weeks intervals apart. While, treatment was carried out using 100 mg/kg Ro 15-5458 (Ro) administered orally at day 6 of each infection (treated group; T). Control mice included: naive (N) receive no infection or treatment, infected three times without treatment group (re-infection; R) and a group infected once without treatment(prolonged infection; P). Five mice from each group were sacrificed at week 6, 12 and 18 post initial infections. Sera and Livers were collected at each time point. Livers were subjected to Real time (PCR) of ALT and AST expression. Sera were subjected to Alanine transaminase (ALT), Aspartate transaminase (AST),Lactate dehydrogenase (LDH), bilirubin and total protein investigation. AST activity, ALT and AST gene expression all significantly decreased during week 6, 12 and 18 post initial infections compared to (N).ALT gene expression at week 6 of (R) and week 6, 12 of (P) group are non-significantly changed. On the other hand, ALT activity, bilirubin, LDH, total protein significantly increasedin all tested groups at all-time points. (P) group of total protein and (T) group of LDH, where non-significant increase were noted. In conclusion, RO treatment has reduced liver damage which is the main goal of anti-pathology vaccine.

[Sherif H. Abdeen,Gamal M. Edrees and Noureen Y. El-Ashry. **Liver integrity during experimental *Schistosoma mansoni* infection and treatment with RO 15-5458 in C57BL/6 mice]** *Nat Sci* 2018;16(5):120-129]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 17. doi:10.7537/marsnsj160518.17.

Key words: schistosomiasis, RO-15-5458, liver function tests.

1. Introduction

Schistosomiasis is a parasitic disease occurred as a result of trematodes infection. Schistosome infection is the most killing disease after malaria and helminthiasis of the intestine, being the main cause of morbidity and death in Africa, South America, the Caribbean, the Middle East, and AsiaFerreira *et al.* (2017).In Egypt, after the building of the Aswan High Dam, a remarkable change occurred in the distribution of *S. mansoni* and *S. haematobium. S. haematobium* in Upper Egypt became endemic with 7.8%, while *S. mansoni* became endemic in Lower Egypt with 36.4%Elmorshedy *et al.* (2016). Schistosomiasis caused liver pathology as a result of immune response against schistosome's eggs forming granuloma that finally form collagen fibers giving protection against antigens released from miracidiumNegrão-Corrêa *et al.* (2014).From the schistosomicidal drugs, praziquantel (PZQ) and oxamniquine are the only WHO-approved effective drugsavailable till now. In 2015, it was noted that about 66.5 million people received PZQCao *et al.* (2010). PZQ is most effective against adult worms and an intact immune respond to be fully efficient. Even so efficacy, PZQ does not provide resistance against re-infection especially during childhood and adolescence(Ammar *et al.,* 1994, Utzinger *et al.*, 2001). However, Ro 15-5458, the acridine derivative of class 9-acridanone-hydrazones (Ro) showed anti-schistosomal prophylactic effects schistosome larvae through the inhibited of genes expression of parasiteEshete and Bennett (1991). Thus praziquantel is not efficient at this stage of infectionLai *et al.* (2015). Considering that antigens of larvae stage of schistosomes in particular those of lung stage are potent inducers of protecting immunityGao *et al.* (2017).Several evidences confirmed the existence of anti- schistosome protection in humans who been able to express a combinedrather than polarized TH1/TH2 response(Abdel-Ghaffar *et al.*, 2017, Mbanefo *et al.,* 2014). This has encouraged the research for the development of a vaccine that eliminates the parasite or ameliorates the resulted pathogenesis.

The present work was of interest to carry out experiments investigating the changes of liver associated with Rotreatment against *S. mansoni* infection in C57 BL/6 mice.

2. Material and Methods

A. Mice and S. mansoni cercariae

S. mansoni cercariae, female C57BL/6 mice6 to 7 weeks old were obtained from Biological Production Unit of Theodore Bilhariz Research Institute (TBRI, Cairo, Egypt).

B. Animal grouping and infection

After obtaining an ethical approval from the Department of Zoology, Faculty of Science, Mansoura University, animal treatment and care was performed according to the guideline of the American Society of Mammalogists. Four groups of twenty mice each were divided into; naive group (N) normal group without infection and treatment, treated group(T) with three repetitive infection rounds of twenty *S. mansoni* cercariae each time, followed by treatment with 100 mg / kg RO 15-5458 ([10-(2-diethylamino) ethyl] -9-acridanone (thiazolidin-2-ylidene) hydrazine, CAS 92928974-7) in 10% aqueous glycerol, reinfected group (R)with three repetitive infection rounds of twenty *S. mansoni* cercariae each time, prolonged infected group(P) infected with one round of twenty *S. mansoni* cercariae and tested after 18 week.

Sera were collected at Wk 6, Wk 12 and Wk 18 for investigation of ALT, AST and LDH activity and also for detection of total bilirubin and total protein. In addition, Livers were collected at Wk 6, Wk 12 and Wk 18 and preserved in liquid nitrogen for gene expression of ALT and AST by RT (PCR).

C. Detection of serum total bilirubin

The method ofKaplan *et al.* (1996) was followed. The absorbance of specimen was calculated at wavelength 578 nm against specimen blank.Totalbilirubin concentration in the sample was calculated as follow.

Total bilirubin = $A_{specimen}$ X 10.8 = …… (mg/dl)

D. Detection oftotal serum protein

The Biuret method ofVan Kley and Hale (1977)was followed.

The absorbance for the standard and sample weredetected at 546 nm wavelength against blank. Total protein concentration ofin the sample, was calculated as follow.

Total serum protein = $\frac{A_{Specimen}}{A_{Standard}}$ x 6.0 = ……(g\dl)

E. Detection of LDH activity

Serum LDH was estimated by the method ofLegras *et al.* (1990).The mean absorbance change was calculated per minute (ΔA/min.)

F. Detection of ALT activity

The method of IFCC Bergmeyer *et al.* (1986)was followed. The change of absorbance was measured per minute (ΔA/min.) during 150 seconds at wavelength 340 nm.

G. Detection of AST activity

The method of IFCC Bergmeyer *et al.* (1976) was followed. The change of absorbance was measured per minute (ΔA/min.) during 150 seconds at wavelength 340 nm.

H. Real time (PCR)for ALT and AST

1. RNA isolation

RNA was isolated from different specimen after the method of Handbook (2006).

2. cDNA synthesis

Thermo scientific first strand cDNA synthesis kit was followedNagalakshmi *et al.* (2010).The resulted first strand cDNA synthesis used straight in PCR.

i. Amplification of first strand cDNA

2 µl of the first strand cDNA synthesis reaction mixture act as a template for following PCR in 50 µl final volumeFrohman *et al.* (1988).

ii. First strand cDNA synthesis reaction control

The method of Frohman *et al.* (1988) was followed.

iii. PCR amplification control

The method of Malek *et al.* (2000) was used.

3. RT (PCR) step

Sensi FASTTM SYBTR® No-ROX kit was followed afterLoewe (2013).

Statistical results

Statistics were presented as mean ($\bar{X}$) ± standard deviation (SD). Statistical Program of Social Science (SPSS) software for windows, version 10 was used. Comparisons between all tested groups were carried out using Two-way analysis of variance (ANOVA) except for the protection experiments where comparison between T and R groups was performed by the independent Student's t-test. The statistics were considered significant with $P \leq 0.05$.

3. Results

A. Serumtotal bilirubin concentration

At Wk 6, Wk 12 and Wk 18 post initial infections, all tested groups R group, p group and T group showed significant increase relative to untreated group (N). Table 1 (Fig. 1).

B.Serum total protein concentration

At week 6 and week 12 of infection, R group showed significant increase. P group and T group showed non-significant increase compared to untreated group (N).At week 18 of infection R group and P group showed non-significant increase, while T group showed significant increase relative to untreated group. Table 1 (Fig.2).

C. Serum LDH activity

At Wk 6, Wk 12 and Wk 18post initial infection, R group and P group showed significant increase, while T group showed non-significant increase relative to untreated group (N).Table 2 (Fig. 3).

Table 1: serum bilirubin and total protein in different time points for different groups

Parameter	Group	Time (weeks)		
Bilirubin (mg/dl)		**Week 6**	**Week 12**	**Week 18**
	N	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
	R	0.72 ± 0.04 [a]	0.92 ± 0.06 [a]	1.3 ± 0.05 [a]
	P	1.3 ± 0.03 [a,b]	0.88 ± 0.04 [a,b]	0.7 ± 0.03 [a]
	T	0.9 ± 0.03 [a,b,c]	0.67 ± 0.04 [a,b,c]	0.55 ± 0.04 [a,b,c]
Total protein(g/dl)	**N**	4.6 ± 0.24	4.6 ± 0.24	4.6 ± 0.24
	R	6.2 ± 0.25 [a]	5.2 ± 0.25 [a]	4.5 ± 0.38
	P	4.7 ± 0.22	5.1 ± 0.61 [b]	5.9 ± 0.25 [b]
	T	5.8 ± 0.25 [b]	6.1 ± 0.25 [b]	6.4 ± 0.1 [a,b,c]

Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to p.

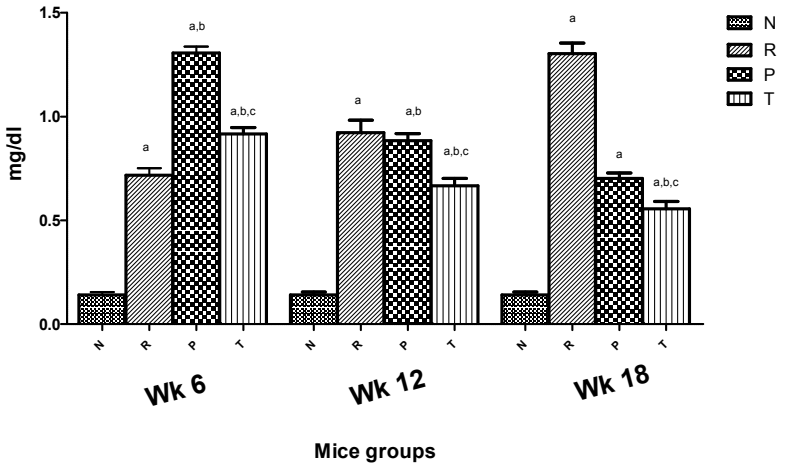

Fig. 1:Serum total bilirubin in mice from different groups (n = 5), $\bar{X} \pm S.D.$
Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to P.

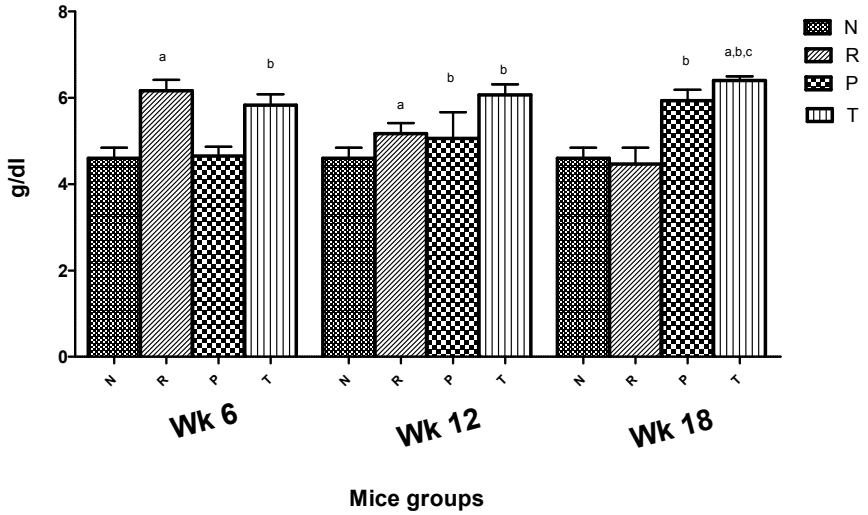

Fig. 2:Serum Total protein in mice from different groups (n = 5), $\bar{X} \pm S.D.$
Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to P.

Table 2: LDH, ALT and AST activities in different time points for different groups

Parameter	Group	Time (weeks)		
LDH (IU)		**Week 6**	**Week 12**	**Week 18**
	N	322.2 ± 25.8	306.7 ± 42.8	334.4 ± 52.5
	R	553.6 ± 32.5[a,c]	619.6 ± 69.8[a,c]	1089.0 ± 159.4[a,c]
	P	780.0 ± 32.9[a,b]	523.8 ± 44.4[a]	475.5 ± 41.9[a,b]
	T	391.9 ± 42.5[b,c]	340.6 ± 50.4[b,c]	313.7 ± 20.9[b,c]
ALT (U/l)	**N**	29.6 ± 3.3	29.6 ± 3.3	29.6 ± 3.3
	R	138.0± 3.6 [a]	67.7 ± 6.7 [a]	78.5 ± 7.9 [a]
	P	47.1 ± 5.8[a,b]	32.3 ± 7.3 [a,b]	103.0± 5.7[a,b]
	T	29.13± 3.7 [b]	45.07 ± 2.5 [a,b,c]	72.37± 6.0[a,b,c]
AST (U/l)	**N**	56.3 ± 6.7	56.3 ± 6.7	56.3 ± 6.7
	R	87.3 ± 3.6 [a]	64.6 ± 3.9 [a]	318.0 ±10.3 [a]
	P	97.8 ± 5.2 [a,b]	57.6 ± 6.2	157.0 ± 8.0 [a.b]
	T	108.0 ± 8.9 [a,b]	168.0 ± 7.0 [a,b,c]	264.0 ±16.3[a,b,c]

Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to p.

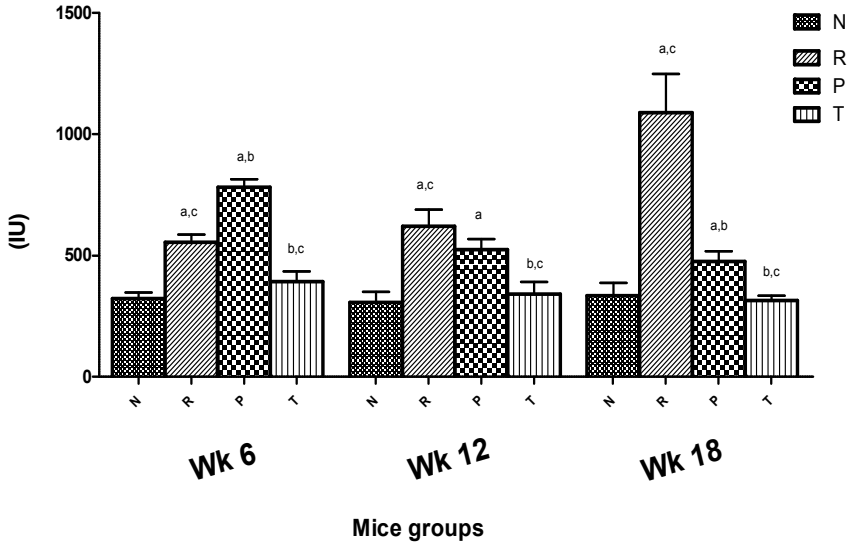

Fig. 3:LDH activity in mice from different groups(n = 5), $\bar{X} \pm S.D$.
Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to P.

D. Serum ALT activity

At Wk 6, Wk 12 and Wk 18 post initial infection, R group, P group and T group showed significant increase relative to untreated group N. T group showed significant decrease compared to P and R group. Table 2 (Fig. 4).

E. Serum AST activity

At WK 6, Wk 12 and wk 18 post-initial infection, R group, P group and T group showed significant increase relative to untreated group N. Table 2(Fig. 5).

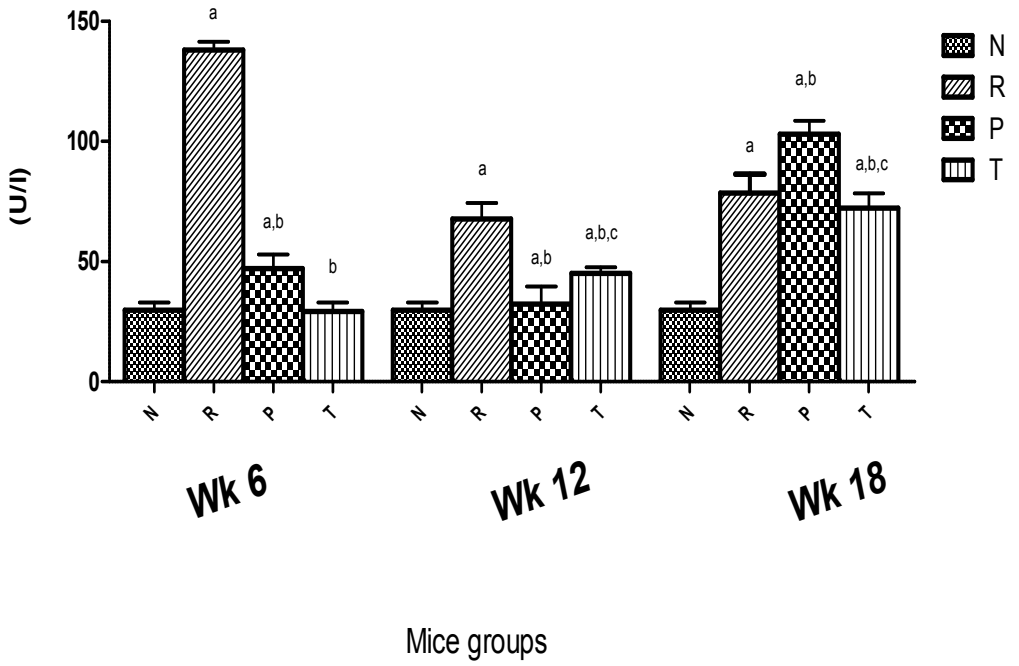

Fig. 4: ALT activity in mice from different groups (n = 5), $\bar{X} \pm S.D$ .Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to P.

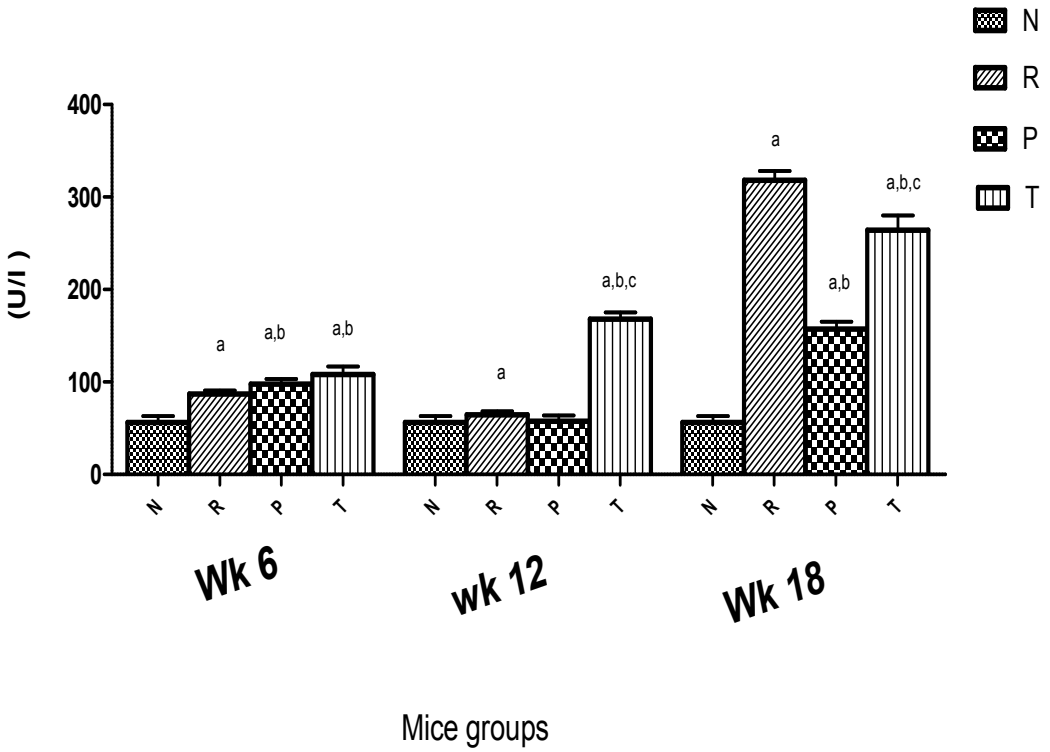

Fig. 5: AST activity in mice from different groups(n = 5), $\bar{X} \pm S.D$.
Note: [a] significant compared to N, [b] significant compared to R and [c] significant compared to P.

F. RT (PCR) of ALT

Livers of treated group (T) showed significant down regulation at week 6, week 12 and week 18. While, reinfected group (R) showed significant down regulation at week 12 and week 18. On the other hand, prolonged infection group (P) showed significant down regulation at week 18, all compared to house-keeping gene. Table 3 (Fig. 6).

Table 3: Geneexpression of ALT and AST by RT (PCR) for different groups:

Parameter	Group	Time (weeks)		
ALT gene expression (%)		**Week 6**	**Week 12**	**Week 18**
	R	1.057 ± 0.05	0.19 ± 0.04 [a]	0.17 ± 0.04 [a]
	P	1.057± 0.05	0.83 ± 0.3 [b]	0.25 ± 0.07 [a]
	T	0.28 ± 0.09 [a,b,c]	0.27 ± 0.05 [a,c]	0.22 ± 0.02 [a]
AST gene expression (%)	**R**	0.09 ± 0.04[a]	0.004 ± 0.001[a]	0.011 ± 0.001[a]
	P	0.09 ± 0.04 [a]	0.17 ± 0.08 [b]	0.2 ± 0.03[a,b]
	T	0.09 ± 0.019 [a]	0.07 ± 0.02 [a,b]	0.06 ± 0.02 [a,b,c]

Note: **[a] significant compared to House-keeping gene, [b] significant compared to R and [c] significant compared to P.**

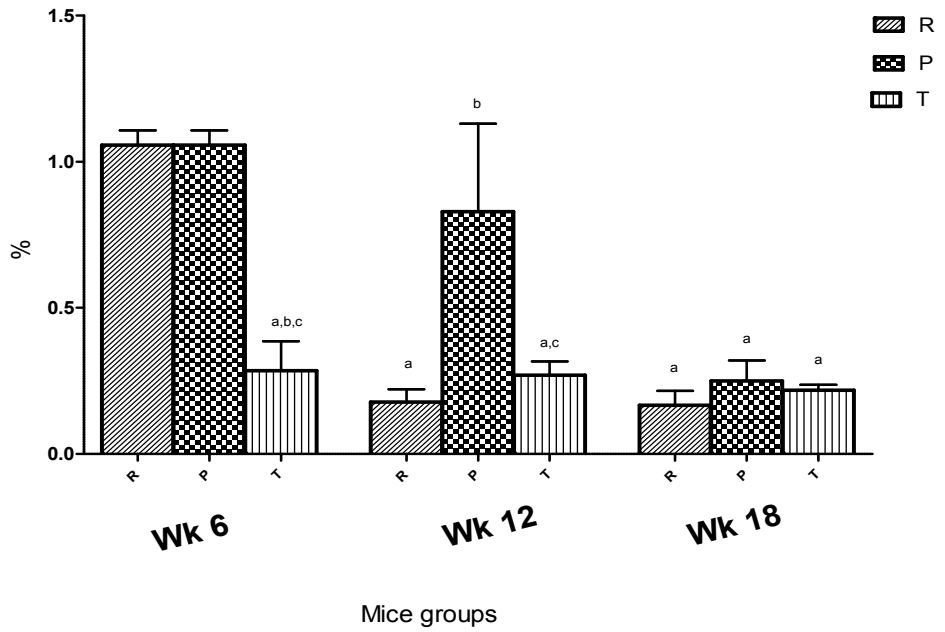

Fig. 6:ALT gene expression in mice from different groups(n = 5),$\bar{X} \pm$ S. D.*Note:* [a] significant compared to House-keeping gene, [b] significant compared to R and [c] significant compared to P.

G. RT (PCR) of AST

At Wk 6, Wk 12 and Wk 18 post-initial infection, R group, P group and T group showed significant decrease compared to house-keeping gene. Table 3(Fig. 7).

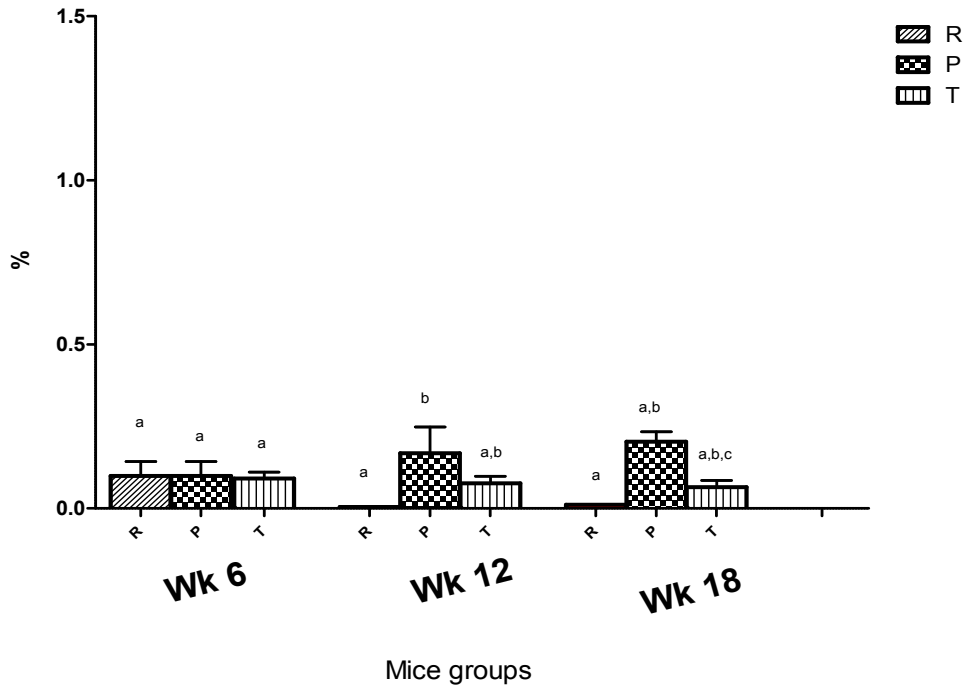

Fig.7:AST gene expression in mice from different groups (n = 5), $\bar{X} \pm S.D.$
Note: **[a] significant compared to House-keeping gene, [b] significant compared to R and [c] significant compared to P.**

4. Discussion

Schistosomiasis is a disease induced by trematode blood flukes of genus *Schistosoma*. After presence of morbidity and death in 74 developing countries, schistosomiasis still causes 200, 000 mortalities every yearKing *et al.* (2005).

Schistosomiasis resulted from immunological reactions against schistosome eggs stuck in several host's tissues. The released egg-antigens stimulate T cells, macrophages and eosinophils to convince granulomatous reactions that cause the clinical form of the diseaseGryseels *et al.* (2006). Indications and symptoms of the infection are dependent on the number and the site of trapped eggs. Generally, the primaryinflammatory reactions are reversible whereas, the last mentionedstage of pathology is associated with collagenous depositions and fibrosis, creating body organinjurewhich may be partially irreversible Colley *et al.* (2014).

Even though chemotherapeutic treatment with praziquantel, the most commonly used schistosomicidal drug, is very efficient for parasite treatment, it does not provide resistance against re-infection. This kind of raises the need for extending research to drugs that possibly eliminate the parasite and induce protecting immunity as wellDoenhoff *et al.* (2008).

Serum total bilirubin levels are significantly increased in each tested groups compared to naive group (N), while (T) group showed significant decrease in all time points relative to (R) and (P) group. These resultsarein agreement with those of Sherlock *et al.,*(1981) and El-Guinidy*et al.(*1997) that reported the release of serum total bilirubin level in patients with active schistosomiasis and liver cirrhosis, thisismainly recognized to the direct part due to reducedsecretion or backward leakage of the bilirubinpigment(El Guiniady *et al.,* 1994, Fahim *et al.,* 2000).

Serum total protein levels are increased in all tested groups in compare with that of naive group (N), while (T) group showed minor increase in all time points relative to (R) and (P) group.These results arein agreement with that of Cioli *et al.* (1995) who reported that treatment with Ro 15-5458 reduced the content of protein as a result of improving the integrity of the liver.

LDH at Wk 6, Wk 12 and Wk 18post-initial infection, R group and P group showed significant increase, while T group showed non-significant increase relative to untreated group (N), while (T) group showed significant decrease in all time points relative to (R) and (P) group. LDH production in hepatic cellsexpresses the degree of intracellular oxygen concentration and increased LDH levels showed that over activation of macrophages and microcirculation disturbance in the liver play a vital

role in development of acute liver failure Lu *et al.* (2006).

Transaminases are accurate indicators of liver cell functionand most useful in identifying acute hepatocellular diseasesThimme *et al.* (2002), In the present study ALT at week 6, 12 and 18 of first infection, R group, P group and T group showed significant increase relative to untreated group N. while, (T) group showed significant decrease in all tested groups relative to (R) and (P) group. AST at Week 6, 12 and 18 of first infection, R group, P group and T group showed significant increase relative to untreated group N, while (T) group showed minor increase in all time points relative to (R) and (P) group. Michielsen *et al.* (1997) reported that, the rise of serum AST activity maybe because this cytosolic and predominantly mitochondrial enzymeis present in higher amounts in the liver compared to the cytosolic ALT and consequently more is released in tissue damage.The heart may be involved subsidiary to liver affection in case of severe liver damage leading to increase serum AST level Fahim *et al.* (2000).

In the present study RT (PCR) of ALT and AST showed down regulation in all tested groups especially (T) group showed significant down regulation relative to (R) and (P) group in all time points. These results showed that there are inverse relationship between ALT, AST gene expression and ALT, AST activity because during hepatocytes injured protein level inside cell decreased and consequently increased in the circulation , an explanation in agreement withAnderson *et al.* (2000) that indicated that ALT is usually used as the only biochemical sign for chronic hepatitis C (CHC), though, its value may be normal in cases with active disease. Lately, AST has been recommended as a suitable sign of liver injury.

In conclusion, RO treatment has reduced liver damage which is the main target for schistosomiasis treatment to keep organ integrity.

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4/24/2018