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CLOVE OIL OR COMBINED WITH PRAZIQUANTEL DOWN REGULATED TGF-*B1* AND REVERSED FIBROGENIC DYNAMIC PROGRESSION CAUSED BY CHRONIC SCHISTOSOMIASIS *MANSONI* IN MICE

By
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Abstract

The most serious form of chronic schistosomiasis is the life-threatening hepatosplenic disease, accompanied by severe periportal fibrosis, a permanent condition once established. Reversion of such wound healing process "fibrolysis" is possible if a balance between hepatic cellular regeneration and the process of scar formation is ensued. In the current work, clove oil alone and combined with PZQ were used to investigate their anti-fibrotic effect on hepatic fibrosis resulted from acute and chronic schistosomiasis *mansoni* in mice. To determine the state of hepatic fibroblasts, local expression of TGF-β1, a chief profibrogenic molecule was quantified using digital real-time image analysis and compared with the conventional parasitological and histopathological analysis. PZQ monotherapy caused a significant reduction in ova count, granuloma number and size. Local expression of TGF-β*1* gave better data as compared to acute and chronic infected treated mice (11.91±2.53- 20.34±3.05 vs. 7.51±2.11-11.23±2.23) with clove alone treatment & 4.95±1.95-7.51±1.92 in combined therapy (p=0.0000)), indicated low potential in achieving liver tissue repair. Significant drop in TGF-β1 expression with clove oil treatment, especially when combined with PZQ, indicated anti-fibrotic potentiality and good impact on liver cells proceeded towards regeneration supported by such drop. A significant positive correlation between mean TGF-β1 values and mean granuloma parameters in number and size of different infected groups (R^2 = 0.680 & 0.988, respectively in acute infection, 0.363 & 0.505 respectively, in chronic phase of infection, *P* value <0.05).

Key words: Mice, *Schistosoma mansoni,* Clove oil, Praziquantel, Hepatic fibrosis, TGF-β1

Introduction

Schistosomiasis is reported globally to affect about 236.6 millions of people in more than 70 countries, particularly in Africa, Asia and South America with a worldwide mortality more than 200,000 deaths annually (WHO, 2017). The risk of chronic schistosomiasis is the life-threatening hepatosplenic disease, which was usually accompanied by sever periportal fibrosis, portal hypertension and porto-systemic shunting of venous blood (Dunne and Pearce, 1999). Tissue fibrosis is an exquisitely complicated multicellular process thought to be irreversible once established in most of the affected patients (Trautwein *et al*, 2015).

PZQ has been used as drug of choice for all types of schistosomiasis for several decades. However, evolving of drug resistance to PZQ imposes great fear, as being available, effective, and cheap as there was no effective vaccination (Botros and Bennett, 2007). Destruction of mature eggs by PZQ eliminated the antigenic stimulation source, and so treatment resulted in stabilization of collagen deposition causing the hepatic fibrotic scar. PZQ has undoubtedly been widely examined, either as a monotherapeutic anti-schistosomal treatment or combined with other medicinal agents, using the standard param-

eters of tissue egg load, oogram study, number and diameter of hepatic granulomas, performing H & E (Dupré *et al,* 1999) and Masson Trichrome staining methods (Hussein *et al,* 2017). However, PZQ disrupts immune regulation that occurs naturally during a persistent infection and thus the prognosis of fibrotic scar resorption was poor (Whiteland *et al,* 2020**).** Thus, anti-fibrotic agent used as PZQ adjuvant proved useful treating choice (Kresina *et al*, 1994).

The cumulative success of antiviral therapies in hampering or even reversing the fibro genic dynamic progression within hepatic tissues encouraged the interested researches in treatment of chronic schistosomiasis to adopt the same principles of targeting antifibrotic agents to support treatment of chronic schistosomiasis (Hayasaka *et al*, 1996).

Among specific antifibrotic therapies used in chronic liver disease of different etiologies, Clove oil induced autophagy and apoptosis in many abnormal and tumor cells (Abdullah *et al,* 2021)**.** Eugenol (clove oil) and its derivatives protected against oxidative injury and tissue da-mage (Shin *et al*, 2007) by inhibition of thioacetamide (TAA) induced hepatocyte prolife-ration (Ali *et al,* 2014).

The present study evaluated the clove oil alone or combined with PZQ in treating chronic schistosomiasis *mansoni* infected mice as to anti-fibrotic effect on hepatic fibrosis, and to determine hepatic fibroblasts post-treatment, local expression of TGF-*β1* using the digital real-time image analysis compared to the parasitological and histopathological examinations.

Material and Methods

Experimental animals and infection: Fifty five laboratory-bred Swiss albino mice of 18-20gm weight were used. They were maintained in the biological unit of Schistosome Biology Supply Center, Theodor Bilharz Research Institute (TBRI), Giza, in well-ventilated plastic cages in conditioned rooms and away from direct sunlight. They were kept on a standard diet and water. The protocol was approved by the ethical committees of Kasr Alainy School of Medicine, Theodor Bilharz Research Institute (TBRI) and the Institutional Animal Care and Use Committee (IACUC) of Cairo University.

An Egyptian strain of *S. mansoni* cercariae was used, which was maintained by laboratory passage in an Egyptian strain of *Biomphalaria alexandrina* provided by TBRI. Cercarial suspension was prepared from at least 50 shedding snails. Infection of mice was carried out by subcutaneous injection with 60±10 *S. mansoni* cercariae suspended in 0.2ml of dechlorinated water (Peters and Warren, 1969).

Drugs: Praziquantel (E.I.P.I.Co. Pharmaceuticals, Cairo) was prepared in 2% (v/v) Cremophore-EL (Sigma-Aldrich Chemical Co, St. Louis, MO) as a suspension and given orally as a single oral dose of 250 mg/kg (S.O.D PZQ monotherapy). Natural clove oil (100% pure) was purchased from the local market [Maharishi Ayurveda Company, India. the used dose of clove oil was 33 mg/kg body weight (Sharma *et al*, 1994), given orally diluted in dimethyl sulfoxide as a solvent for *in-vitro* and *in-vivo* drug testing. Clove oil was administered for 60 days as a single oral dose daily (Han & Parker, 2017)

Experimental design: Mice were divided into 7 groups, each of 5 where groups I, II, & III (non-infected control groups) while each of the infection groups IV, V, VI & VII were divided into two subgroups (5 mice each) representing acute and chronic infections, where treatment started 6 weeks and 12 weeks post infection (P.I.), respectively. Mice were randomly distributed under the 7 groups designed as follows: GI: uninfected untreated, GII: uninfected, PZQ treated control, GIII: non infected clove oil treated control, GIV: infected untreated, GV: infected treated with S.O.D PZQ, GVI: infected treated with clove oil, & GVII: infected treated with combined clove oil & S.O.D PZQ

Parasitological examination: Mice were sacrificed at the end of the15th week P.I for

the acute group and 21th week P.I for chronic one. Anti-schistosomal effect was assessed parasitological by counting ova in both hepatic and intestinal tissue (Cheever, 1969).

Biochemical analysis: Collected blood samples were put at room temperature for 30 minutes before centrifugation at 3000rpm for 15min. Sera were separated to measure liver alanine transaminase (ALT) and aspartate transaminase (AST) enzyme levels (liver function tests), and serum creatinin and urea (kidney function tests).

Histopathological examination: Right lobe of liver was dissected out, preserved in a 10% formalin solution and processed paraffin blocks and stained with hematoxylin and eosin (H & E) and with Masson's Trichome to study and to measure mean granuloma diameter (µm) using an ocular micrometer (von Lichtenberg's *et al,* 1973).

Immunohistochemical analysis for TGF-*β1*expression: Tissue fixation, cutting, embedding, and tissue sectioning on a microtome were all performed for prepared liver samples. Tissue sections were deparaffinized, rehydrated, incubated for 5 minutes in 3% hydrogen peroxide and after that washed twice in PBS. For antigen retrieval, the slides were immersed in citrate buffer in a water bath, then incubated for one hour at room temperature with the primary antibody, murine anti-human TGF-*β1* monoclonal antibodies (Dako, USA) and after that washed in PBS. Biotinylated goat anti-polyvalent secondary antibody was added followed by streptavidin peroxidase enzyme for 10min. After washing in PBS, diaminobenzidine chromogen was added for 5min to visualize peroxidase activity. Haematoxylin was used as a counterstain then the slides were mounted by DPX. Positive control slides were provided within the test kits and used according to the manufacturer's recommendation. Negative controls were made according to the previous procedure, except for the step related to the primary antibody. Positive results for TGF-*β1* were evident if there was expression of membranous or cytoplasmic brownish immuno-staining in fibroblasts or hepatic cells. Expression was automatically quantified in 10 real time representative high-power fields HPFs (×400), in which the brownish color turned to navy blue during quantitative image analysis using the Owin 500 Image analyzer (LEICA Image System Ltd, Cambridge, England).

Statistical analysis: Data were analyzed by statistical package for the social sciences (SPSS) version 25 to code and data entery (IBM Corp., Armonk, NY, USA). For quantitative variables, and frequencies (cases nu mber) and relative frequencies (%) for categorical variables data were summarized using mean and standard deviation. Comparison between groups was done by using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables, while Mann-Whitney test and non-parametric Kruskal-Wallis test were used for non-normality

Results

There was no significant difference in liver enzymes (ALT & SGOT) values or in renal function tests (serum creatinine and urea) post-treatment with combined or any therapy alone as compared to normal untreated group. Histopathological examination of liver sections was normal.

Parasitological: There was a significant reduction in ova count in hepatic and intestinal tissues of all infected treated mice when compared with positive controls ($P< 0.05$). Clove, PZQ, and combined PZQ-clove in acute infected treated mice showed 15.24%, 87.56 % & 87.29% reduction, respectively in hepatic tissue with 34.99%, 86.75% & 88.53% reduction, respectively in intestinal tissue. In chronic infected treated mice, clove, PZQ and combined PZQ-clove showed 65.77%, 76.33% & 62.67% reduction in hepatic tissue, with 64.74%, 75.70% & 64.67% in intestinal tissues respectively.

Histopathology of infected treated mice: In acute infection subgroup of positive control mice, mean liver granuloma diameter was 354.25±15.92µm, and mean granuloma nu-

mber was 15.3± 2.9. In chronic infection sub group, parameters were 345.16± 19.45µm & 17.28±3.24, respectively with an increase in fibrous tissue density and a decrease in inflammatory granulomas outer. Clove oil when given alone reduced number and size of hepatic granulomas without significant, but in chronic infection subgroup as reduction in granuloma diameters was significant. Clove oil combined with a single dose of PZQ caused a significant reduction in granuloma size and number ($P < 0.05$).

Immunohistochemistry (IHC): **M**ean levels of TGF-*β1* expression in liver sections of acute and chronic infected mice were analyzed by software image analysis of 10 low power fields in each section. There was a significant reduction in TGF-*β1* expression in all infected treated mice. The best reduction in immune modulator was in infected mice treated with clove combined with PZQ, which minimized fibro-sclerotic lesions in liver in acute and chronic phases.

A significant positive correlation was found between the mean granuloma parameters, both number and size, and the TGF-*β1* expression levels (R^2 =0.680 & 0.988, respectively in acute infection, 0.363 & 0.505, respectively in chronic phase of infection with P value <0.05).

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

Table 1: Tissue egg count and reduction% in liver and intestine of mice with acute and chronic *S. mansoni* infection, received clove with/without PZQ and S.O.D PZQ, compared to positive controls.

S. mansoni infected group		ova count / gm liver	% reduction	ova count/gm intestine	% reduction
Acute infection	Infected control	6856.5±1110.86	--	9097±455.37	--
	Clove	5061.33±586.90 *	15.24	5913.55±1112.46 *	34.99
	S.O.D PZQ	853.05±137.43 *	87.56	1205.75±241.93*	86.75
	PZQ + Clove	871.35±77.92 *	87.29	1043.43±88.64 *	88.53
Chronic infection	Infected control	55599.2±20166	--	59830.21±19298.9	--
	Clove	19030±4181.69 *	65.77	21093.25±5019.5 *	64.74
	S.O.D PZQ	13160±6284.09 *	76.33	14537.67±6332.8 *	75.70
	PZQ + Clove	20753.67±3627.28 *	62.67	21139.33±3711.4 *	64.67

Values = mean ±SD, *Significant compared to positive control mice (P<0.05)

Table 2: Hepatic granuloma number & diameter in *S. mansoni* infected mice, treated 6 (acute stage) and 12 weeks (chronic stage) P.I.

S. mansoni group	variants	Number #	% reduction	Diameter #[in µm]	% reduction
acute infection	Infected control	15.3± 2.9		354.25±15.92	
	Clove	14.23 ±0.89	6.99	337.28.28±18.2	4.79
	S.O.D PZQ	8.2±3.02 *	46.41	285±18.74 *	19.55
	PZQ + Clove	6.16±1.47 *	59.74	253±19.74 *	28.58
chronic infection	Infected control	17.28±3.24		345.16±19.45	
	Clove	16.1±3.4	6.8	314±14.22 *	9.03
	S.O.D PZQ	15.24±4.21	11.81	257.72±16.52 *	25.33
	PZQ + Clove	12.08±3.4 *	30.09	279±18.84 *	19.17

Mean values of 10 successive low power fields in each slide, *Significant compared to positive control mice (P<0.05)

Table 3: TGF-*β1* expression % in mice liver tissue in acute and chronic stages of *S. mansoni* infected mice after treatment.

Treatment of *S. mansoni* infected groups	TGF-*β1* expression#	
	Acute stage Treated initiated 6 weeks p.i.	Chronic stage Treated initiated 12 weeks p.i.
Infection control	16.11 ± 3.41	25.99 ± 3.61
Clove only	7.51 ± 2.11*	11.23 ± 2.23*
S.O.D PZQ	11.91 ± 2.53**	20.34 ± 3.05***
Clove +PZQ	4.95 ± 1.95*	7.51 ± 1.92*

Mean area of TGF-*β1* expression % in 10 low power fields, *Significant difference (p=0.0000) compared positive control, **Significant difference (p=0.0131), ***Significant difference (p=0.0014)

Discussion

Liver fibrosis either due to acute or chronic cellular injury is considered a reversible wound healing process if there is a balance between hepatic cellular regeneration and process of scar formation (Lee *et al,* 2015). Therefore, for the schistosomiasis, choosing the medication that can eliminate the infection and achieve this balance is something that calls for careful thought.

In fact, PZQ has been widely used for more than 40 years in treating all types of schistosomiasis (Silva *et al*, 2005). Despite the benefit of this drug, yet there was an urg ent need for new therapeutic protocol against this endemic parasitic infection whose control exclusively depends on this therapeutic agent whose failure unfortunately was repeatedly reported (Praticò *et al*, 2014).

In the present work, PZQ monotherapy caused a significant reduction in ova count, granuloma number and size. The local expression of TGF-*β1*, although significantly lower than that in the positive control, yet it was higher than that in the other groups, received clove alone or together with PZQ. Thus, despite that PZQ initiated a substantial decline in tissue egg burden; it limited potential in repairing *Schistosoma*-induced liver lesions. Homeida *et al,* (1991) reported that PZQ altered the early stages of periportal fibrosis. This was due to collagen constitution that didn't pass via process of cross-linking stabilized the tissue against fibrolysis (Pellegrino and Katz, 1968). PZQ prevented the production of more fibrous tissue, but, it was unclear whether it affected the established fibrous granulomas or not (Rahoud *et al*, 2010). Besides, Nono *et al*. (2020) reported that fibrosis failure within existing granulomas was recorded among some medications, including PZQ.

In the present study, clove oil caused a significant drop in TGF-*β1* expression, indicated an anti-fibrotic potential. Combining clove with PZQ potentiated the down regulation of TGF-*β1* affected by each of them separately, thus augmenting their immunomodulatory effects.

Clove oil is a natural product rich in Eugenol that reaches concentrations up to 90% (Cortés-Rojas *et al,* 2014)**.** In fact, Eugenol has a powerful anti-inflammatory and immunomodulatory effect (Bachiega *et al,* 2012). It is also reported to protect tissues against oxidative injury and cellular damage (Batiha *et al,* 2020), in addition to its apoptotic capability towards human osteosarcoma cells as recorded by Shin *et al,* (2007)**.** All properties of the clove oil influenced liver fibrosis where hepatic stellate cells (HSCs), usually activated (Hernández *et al,* 2012). Apoptotic changes of the activated HSC might be a mechanism for protecting the liver tissues against fibrosis (Ali *et al,* 2014). The results of the study done by the previous authors concerning Eugenol product of clove oil is going with ours as they reported an improvement in the signs of liver cirrhosis, as verified by histopathology and biochemical markers of hepatic injury. Eugenol reduced elevated levels of alkaline phosphatase, γ-glutamyl transferase and other markers of liver cirrhosis. Also**,** Eugenol protected against carbon tetrachloride (CCl_4) induced liver injury at a low dose of 5- 25mg/kg, particularly when given simultaneously or soon after CCl_4 (Nagababu *et al,* 1995). Besides, low eugenol dose protected liver against ischemic injury, by decreasing levels of lipid peroxidation, down-regulating inflammatory mediators and inhibiting apoptosis, but larger doses amplified liver injury via oxidant and inflammatory effects (Abd El Motteleb *et al*, 2014).

In the present work, clove oil reduced tissue egg load mainly in chronic phase, compared to positive control with antifibrotic character. Anti-parasitic property was attributed to immune- mediated parasitic stages' destruction by clove oil rather than antioxidant (Gülçin *et al,* 2012)**. A**nti-schistosomal activity of herbal plants to anti-inflammatory and immunomodulatory effect was not due to direct action on the parasite (Metwally *et al.* (2018). Loverde *et al.* (2007) found that clove oil lowered expression of TGF-*β1* affected parasitic stages and embryogenesis that subsequently reflected on the egg number. This explained the reduction in egg count in mice treated with clove oil alone, although not registered as an anti-parasitic drug yet, where the TGF-*β1* local expression level was significantly lower win this group than that of the control as well as mice group that received PZQ monotherapy.

The present study showed positive correlation between hepatic granuloma number/size and liver TGF-*β1* level of expression, denoted that any decrease in the granuloma number and size observed in any of the medicated groups was associated with a more or less equivalent drop in TGF-*β1*expression level. But, there was a difference in appearance between fibrotic granulomas with Masson staining and the IHC that showed a drop in TGF-*β1* levels indicated liver healing or regeneration. This agreed with El Hawary *et al.* (2016) who reported that in limited resources and large number of chronic granulomatous disease patients, analysis of defective proteins by flow cytometry was an optimum solution for confirming the diagnosis and was a step for targeted sequencing in families seeking prenatal diagnosis.

Besides, clove oil succeeded in lowering the level of TGF-*β,* thus increased the possibility of healing without scarring resembling foetal wound (Liu *et al,* 2004). This work hoped that by preceding clove by praziquantel administration would be possible not only to eliminate infection, but also to heal the wound without scarring and ensure successful hepatocyte regeneration in patients with this endemic parasitic disease.

Conclusion

This study proved that clove oil-PZQ combined regimen not only to treat schistosomiasis, but also to heal patient' wound without scar successful hepatocytes regeneration.

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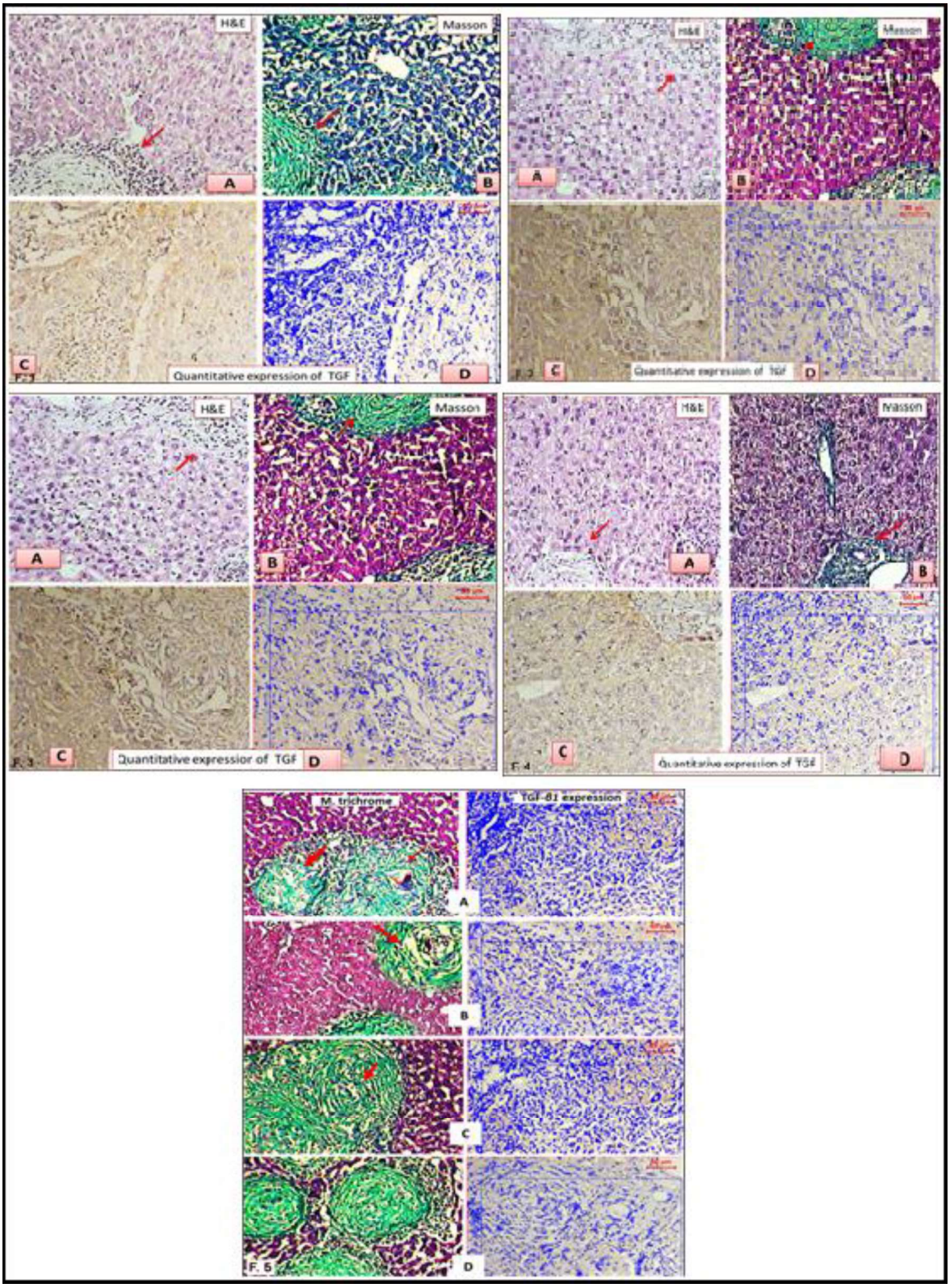
Explanation of figures

Fig. 1: Liver sections with different stains in infection control (= non-treated). A: With granuloma formation (red arrow, H&E, ×100); B. well-formed fibrous tissue granuloma (red arrow) [Masson trichrome, ×100]; C,D: TGF-*β1* expression and localization in liver after IHC staining, showed positive expression for TGF-*β1* in fibroblasts (arrow head) and mild focal expression in hepatocytes (black arrow, ×100).
Fig. 2: Liver sections of *Schistosoma mansoni* infected mice received S.O.D PZQ from week 6. A. H&E stained (×100) liver tissue with granuloma formation (red arrow); B. Masson trichrome (×100) showed well-formed fibrous tissue granuloma (red arrow); C, D. IHC (×100) shows positive expression for TGF-*β1* in fibroblasts and in hepatocytes.
Fig. 3: Liver sections of *S. mansoni* infected mice received clove oil only, from week 6. Granulomas (red arrows) within both Hx&E & Masson Trichrome stained sections (×100).
Fig. 4: Liver sections of *S. mansoni* infected mice received clove oil together with PZQ, from week 6 as compared group treated with clove only, TGF-*β1* expression appeared to be at a lower level when clove combined with PZQ.
Fig. 5: Liver sections of chronic *S. mansoni* infected mice and untreated (A), received clove oil only (B), received S.O.D of PZQ (C) and last one received clove oil and PZQ. Right ones level of TGF-*β1* expression at a lower level in clove oil combined with PZQ (D, right).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C
D
Quantitative expression of TGF
H&E
Masson
A
B
C
D
Quantitative expression of TGF
H&E
Masson
A
B
C
D
Quantitative expression of TGF
M. trichrome
TGF-B1 expression
A
B
C
D