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Effect of *Ailanthus altissima* and *Zizyphus spina-christi* on Bilharzial Infestation in Mice: Histological and Histopathological Studies

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Abstract: The objective of this study is to test the ability of certain traditional plants for reducing the hazardous effect of schistosomiasis without any side effects. The chloroform extract of *Ailanthus altissima* stem bark and the ethanolic extract of *Zizyphus spina-christi* root were selected and tested through histological and hsitopathological studies. Treatment with both plant extracts reduced number of worm burden, ova count, granuloma size and count as well as improvement in histopathological picture of liver, kidney and spleen. The chloroform extract of *A. altissima* showing a more pronounced improving effect against organs damage caused by parasitic infection.

Key words: *Ailanthus altissima*, *Zizyphus spina- christi*, schistosomiasis, liver, spleen, kidney

INTRODUCTION

Schistosomaisis is a common intravascular trematode infection, mostly prevalent in Africa and affects about 200 million people in the developing countries (Abo-Madyan *et al*., 2004; Wang *et al*., 2004).

Organs typically affected include, the urinary tract, the bowel and the liver (Blanchard, 2004). The ova reaching the liver initiating schistosoma granulomata in variable numbers beside pathological changes ranging from early to advanced stage of fibrosis (Amiri *et al*., 1992; Njenga *et al*., 1998; Silva *et al*., 2003; Blanchard, 2004). Endothelial cells activation within these granulomas may contribute to their development and to increased vascularization in the granuloma periphery (Loeffer *et al*., 2002). Acietes and hepatosplenomegaly could be also demonstrated (Bosshardt *et al*., 1997; Njenga *et al*., 1998).

Chemotherapy with praziquantel (PZQ) provides the ministry for morbidity control of schistosomiasis by preventing chronic liver disease or bladder cancer (WHO, 2002). However, there has been recent discussion about the possibility of development and spread of shistosoma strains that are resistant to PZQ (El Lakkany *et al*., 2004) as well as its induction of lung hemorrhage beside abdominal pain and diarrhea (Flisser and Mclaren, 1989, Kabatereine *et al*., 2003). This necessitate to search for a new safe and effective drug (Abo-Madyan *et al*., 2004).

In recent years, researches have examined the effects of traditional plants for prevention or cure of schistosomiasis (Rizk *et al*., 2000; Ojewole and Adewunmi, 2004; Nergard *et al*., 2005). The potency of the two traditional plants; *Ailanthus altissima* and *Zizyphus spina- christi* as antischistosomiasis have not studied yet, although stem bark and seed extracts of *A. altissima* and *A. excelsa* recorded antifungal (Joshi *et al*., 2003) and antiplasmodial (Okunade *et al*., 2003) activities as well as antitumor promoting action (Tamura *et al*., 2003). Different extracts of leaves, fruits, seeds of *Z. spina-christi* L. showed antiviral, antifungal and antibacterial activities (Shahat *et al*., 2001). Glombitza *et al*. (1994) recorded a remarkable improvement in glucose utilization and insulin level in diabetic rats administered with butanolic extract of *Z. spina-christi* leaves. Moreover, the methanolic extract of the stem bark of the plant recorded antidiarrheal effects in rats (Adzu *et al*., 2003), while aqueous extract of its root bark showed analgesic effect in mice (Adzu *et al*., 2001 and 2002). External use of leaves, Fruits, seeds extract showed anti-inflammatory effect (Ali-Shtayeh *et al*., 1998).

In the present study, the chloroform extract of *Ailanthus altissima* stem bark and ethanolic extract of *Zizyphus spina-christi* roots were evaluated for its protective effect against schistosomiasis through histopathological studies of liver, kidney and spleen of bilharzial infested mice. Number of worm burden and ova count were also demonstrated.

MATERIALS AND METHODS

Chemicals: The chemical used were of analar quality, product of Merck, Germany, Sigma, USA and El-Nasr Pharmaceutical Chemical Company, Egypt.

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Animals: Forty eight male albino mice of similar age and weight (20-25 g) were selected for this study. They were obtained from Theodor Bilharz Research Institute, Cairo, Egypt. Animals were kept in a controlled environment and were allowed free access of food and water during the study.

Plant extracts: One kilogram of *Ailanthus altissima* stem bark and 450 g of *Zizyphus spina-christi* roots were extracted with chloroform and 70% ethanol, respectively. The extract were concentrated under reduced pressure and phytochemically screened. The results showed the presence of alkaloids, sterol and/or triterpenes, while saponins were also present in *Z. spina-christi* extract. Extraction process was carried out at Drugs and Medical Plants Department, National Research Center, Dokki, Cairo, Egypt.

Doses and route of administration: The present study was carried out at Medicinal Chemistry Department, National Research Center, Dokki, Cairo, Egypt.

The LD_{50} of *A. altissima* extract was estimated in mice by oral administration route using the method described by Lorke (1983), where five groups of eight mice each were used. Group one served as a control and received normal saline (10 ml kg^{-1} body weight), while the remaining four groups received graded doses (100-4000 mg kg^{-1}) of the extract. The animals observed for clinical signs of toxicity and death for 24 h. The LD_{50} was estimated from the graph of percentage mortality against the log-dose of the extract. 1/4 LD_{50} (500 mg kg^{-1} body weight) was given orally five times a week for one month.

The LD_{50} of *Z. spina- christi* was 2236.07 mg kg^{-1} body weight (Adzu *et al*., 2001). A dose of 560 mg kg^{-1} body weigh representing 1/4 LD_{50} was given orally five times a week for one month.

Experimental design: Duration of experiment was four months. Animals were divided into six groups each of eight mice. Group I: normal healthy control mice. Groups II and III : normal healthy mice treated orally with five successive weekly doses of *A. altissima* and *Z. spina-christi* at the last month of the experimental period. Group IV: Infected mice with 80 cercariae of Egyptian *S. mansoni* strain by tail immersion technique (Oliver and Stirewalt, 1952) for four months. Groups V and VI: infected mice with *S. mansoni*, left for three months then orally treated with *A. altissima* and *Z. spina-christi* extracts as described above.

Histopathological determination: Representative slices from liver, kidney and spleen tissues were taken from the eviscerated animals and fixed in 10% formaline. Paraffin embedded sections were taken after fixation. Sections (4 μm thick) were stained with haematoxylin and eosin (H and E) (Hirsch *et al*., 1997) and the stained sections were studied by light microscope for demonstration of pathological changes in different organs. Counting of granulomas was carried out in five successive fields (10×10) of serial tissue sections of more than 250 μm a part. Stained section were used in measuring granuloma dimensions using an ocular micrometer for the selected lobular granuloma with central ova.

Parasitological determination

Worm burden: Adult *S. mansoni* worms were recovered from hepatic portal system and the liver by perfusion technique (Smitheres and Terry, 1965). The % of reduction of worms number was calculated by Tendler *et al*. (1986) as follows:

$$P = C - \frac{V}{C} \times 100$$

where p = % of protection, C = mean number of parasites recorded from infected mice and V = mean number of parasites recorded from treated mice.

Ova count: The number of ova/g tissue of liver was carried by the method of Cheever and Anderson (1971).

Statistical analysis: Data are expressed as mean±SD and statistically analyzed using one way analysis of variance (ANOVA) accompanied with post-hoc (SPSS Computer Program).

RESULTS

Histological and histopathological studies: Light microscopic examination of the liver sections of normal mice revealed the hepatic lobules formed of radially arranged cords of normal liver cells that radiated from the central vein to the periphery of the lobule. The cell cords were separated by narrow blood sinusoids lined by endothelial cells and Van Kupffer cells, (Fig. 1A). Similarly, the liver sections of healthy treated mice with *A. altissima* and *Z. spina-christi* plant extracts showing increased lymphoma cells within hepatic sinusoids (Fig. 1B and C). The bilharzial liver (Fig. 1D) showed the full-blown pathological picture of infection. Multiple granulomatous lesions, focal areas of hepatic necrosis, cloudy swelling as well as hydropic degeneration of hepatocytes were seen

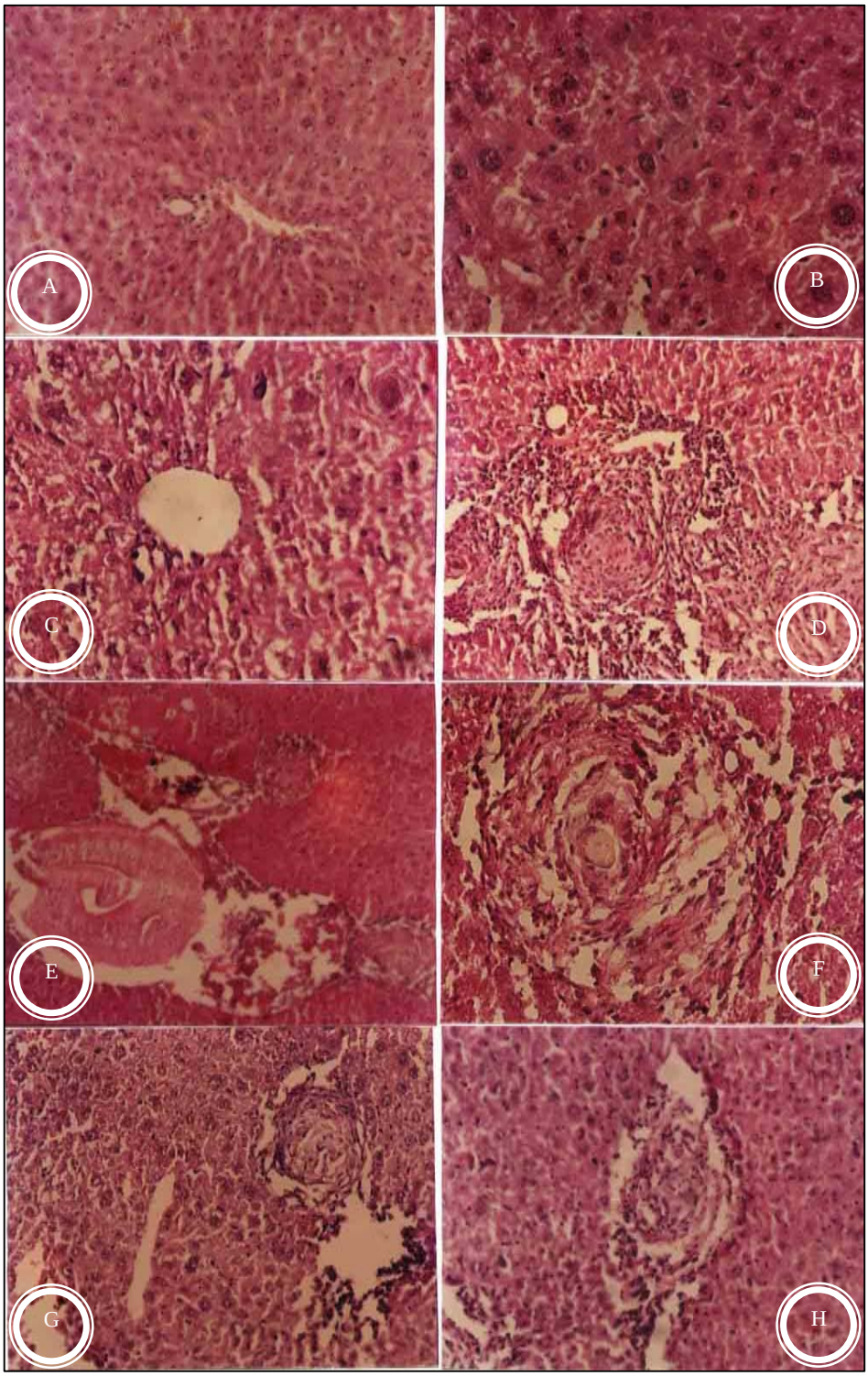

Fig. 1: H and E stained liver sections of: (A) Control (uninfected) (100X). (B) Control treated with chloroform extract (200X). (C) Control treated with ethanolic extract (200X). (D) Infected (*S. mansoni*) (100X). (E) Infected (*S.mansoni*) (40X). (F) Infected (*S. mansoni*) (200X). (G) Infected treated with *Z. spina-chrisiti* extract (100X). (H) Infected treated with *A. altissima* extract (100X)

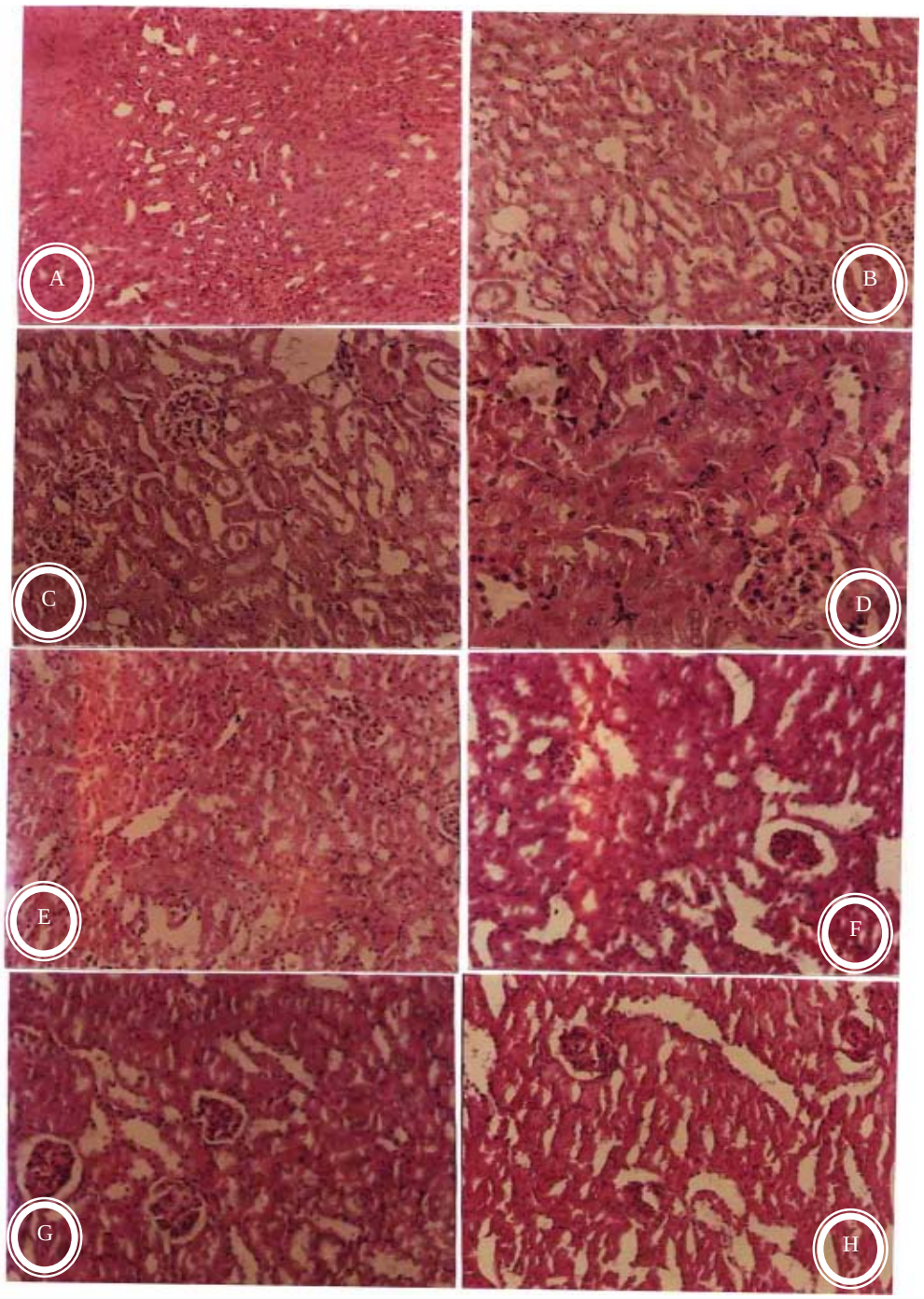

Fig. 2: H and E stained kidney sections of: (A) Control (uninfected) (40 X). (B) Control (uninfected) (100X). (C) Control treated with chloroform extract (100X). (D) Control treated with chloroform extract (200X). (E) control treated with ethanolic extract (100X). (F) Infected (*S. mansoni*) (200X). (G) Infected treated with *Z. spina-chrisiti* extract (100X). (H) Infected treated with *A. altissima* extract (100X)

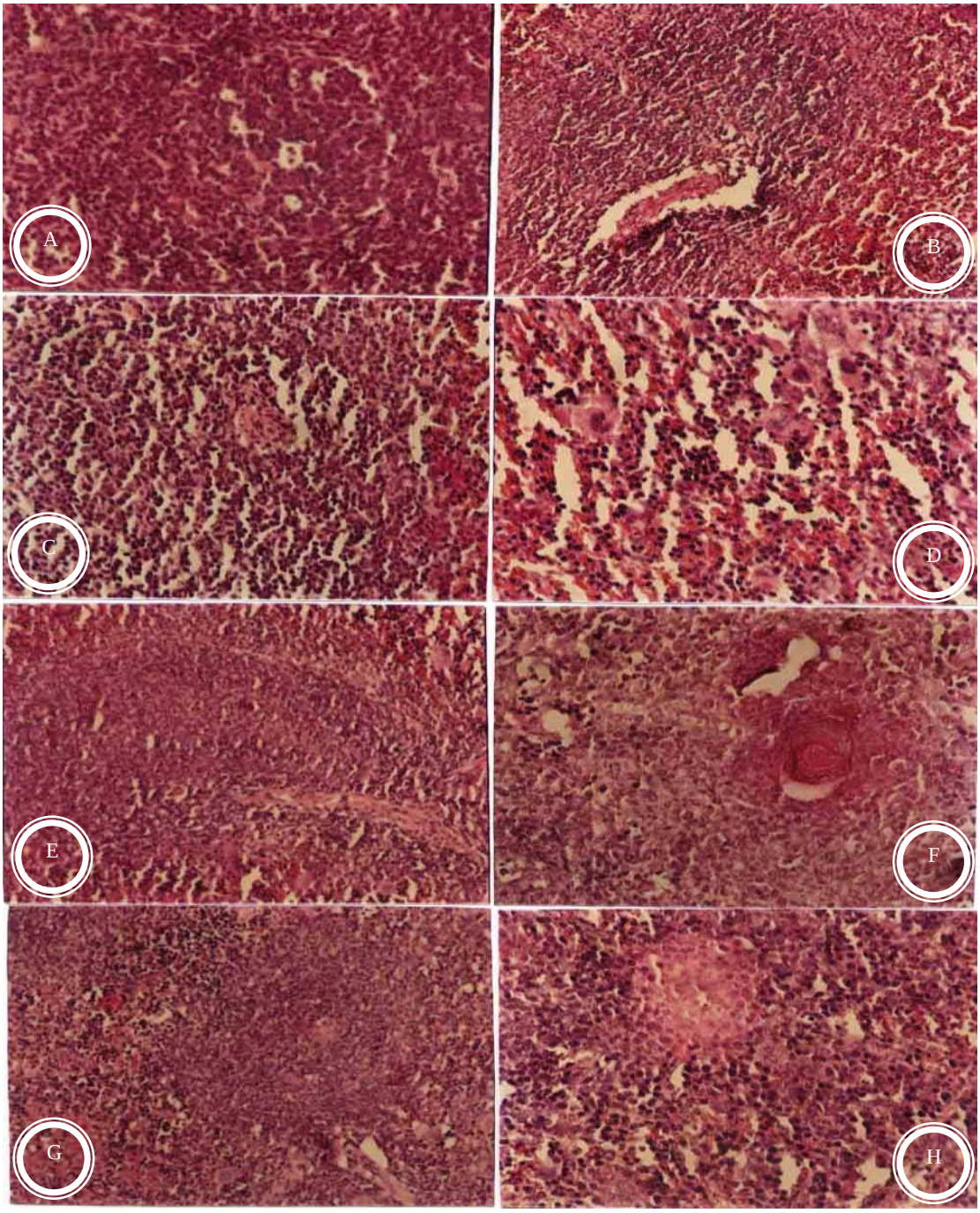

Fig. 3: H and E stained spleen sections of: (A) Control (uninfected) (200X). (B) Control treated with ethanolic extract (100X). (C) Control treated with chloroform extract (200X). (D) Control treated with ethanolic extract (200X). (E) Infected (*S.mansoni*) (100X). (F) Infected (*S.mansoni*) (200X). (G) Infected treated with *Z. spina-chrisiti* extract (100X). (H) Infected treated with *A. altissima* extract (200X)

in some parts. Figure 1E showing worm in the lumen of portal vein branch with the development of cellular granulomas. Figure 1F showing granulomatous lesions consisted of activated macrophages and epitheloid cells and attained the maximum size surrounding ovum at the 4th month. Granuloma present recorded a mean number of 12.40±0.24 with diameter of 170.81±5.60 μm (Table 1). Liver section of infected mice treated with *A. altissima* and *Z. spina-christi* plant extracts, Figure 1G and H showed a noticeable degree of improvements in comparison to infected group represented by fewer granulomas with mean number of 4.6±0.24 and 6.6±0.25 and diameters of 99.23±2.24 and 134.38±3.36 μm, respectively (Table 1). In addition, decreased cellular constituents, degenerative change in the ova and the pigments were minimal.

Light microscopic examination of kidney section of normal animals showing normal glomeruli, tubules and interstitial tissues (Fig. 2A and B). The kidney sections of control treated mice with the two plant extracts showed no abnormal changes (Fig. 2C- E). In *S. mansoni* infected mice, the effected glomeruli exhibited different forms of degeneration. Some glomeruli showed mild dilation of Bowman's space with glomerular atrophy, other exhibited congestion in the capillary loops with an adhesion between visceral and parietal layers of Bowman's capsule. In the renal cortex and in cortico-medullary border, interstitial inflammatory cell infiltrations were clearly apparent. There was an evident increase in the connective tissue cells in these regions of infiltration as an indicator of fibrosis. Regarding blood vessels, there was considerable congestion in the peritubular vessels (Fig. 2F) Treatment of *S. mansoni* infected mice with *A. altissima* and *Z. spina- christi* showed normal appearance of the glomeruli and tubules. The interstitial inflammatory cell infiltrations and interstitial fibrosis were absent. An increase in the connective tissue cells was also not observed, but rare vascular congestions were present in the cortical regions. (Fig. 2G and H).

Microscopically, the normal mice spleen tissue was composed of capsule, trabeculae, white pulp (rich in lymphocytes and active in immune responses), red pulp (antigen trapping and storage of red blood cells). The white pulp consisted of two types of tissues comprising B-or T-cells. B cells formed germinal centers in the lymphatic nodules, usually located near an arteriole, where irregular masses around the central arteries were formed by white pulp T cells (Fig. 3A). The spleen sections of treated mice with *A. altissima* and *Z. spina-christi* plant extracts showed activation of lymphocytes and lymphoblasts of white pulps (Fig. 3B-D). In *S. mansoni* infected group splenic section demonstrate thickening of membrane of the spleen, coarsening of trabeculae, fibrosis round the splenic arteries and stasis of blood and dilatation of the splenic sinus. A nodular replacement of the white pulp, typically with a central core of small lymphocytes with minimal cytoplasm and a peripheral paler (marginal zone) consisting of larger cells with more abundant cytoplasm. Invasion of residual germinal centers is highly characteristic than invasion of the splenic red pulp. Hence, the most characteristic features of splenic schistosomiasis is the infiltration of its two cells type especially the nodular white pulp cells lending to splenomegaly appearance (Fig. 3E). In addition, egg deposition in the splenic tissue were also be demonstrated (Fig. 3F).

Table 1: Effect of *Ailanthus altissima* and *Zizyphus spina-christi* extracts on granuloma diameters and count in *S. mansoni* infected mice liver

Parameters	Infected (1)	Infected + treatment with *A. Altissima* (2)	Infected + treatment with *Z. spina-christi* (3)	p<	Reduction percentages (a)	(b)
Geranuloma diameter (μm)	170.81±5.60 (2,3)	99.23 ±2.24 (1,3)	134.38±3.35 (1,2)	0.0001	41.90	21.32
Granuloma count	12.40±0.24 (2,3)	4.60±0.24 (1,3)	6.60±0.25 (1,2)	0.0001	59.37	43.75

Data are means±SD of eight mice in each group. Number between parentheses indicate the percentage reduction from untreated *S. mansoni* infected group. a and b are reduction percent of *A. altissima and Z. spina-christi* groups, respectively. p is level of significance, where p<0.0001 is significant. Analysis of data is carried and by one way analysis of variance (ANOVA) accompanied by post-hoc (SPSS computer program)

Table 2: Effect of *Ailanthus altissima and Zizyphus spina-christi* extracts on total male, female worms and relative six ratio between ♂ and ♀ worms in infected mice liver

Groups	Total worm (TW) (1)	♂ worm (2)	♀ worm (3)	%R TW	%R♂ worm	%R ♀ worm	RSR	p<
Infected	20.33±2.16	12.22±1.50	8.11±0.31	-	-	-	1	-
Treatment with *A. altissima*	10.50±1.87 (1,2,3)	7.29±0.65 (1,3)	3.21±0.11 (1,2)	48.35	40.34	60.41	1.51	0.0001
Treatment with *Z. spina christi*	14.17±1.41 (1,2,3)	9.06±0.43 (1,3)	5.04±0.14 (1,2)	31.13	25.85	37.85	1.20	0.0001

Data are means±S.D. of eight mice in each group. %R is percentage of reduction of total ♂ and ♀ worms. RSR is relative six ratio between ♂ and ♀ worms. p is level of significance, where p<0.0001 is significant. Analysis of data is carried and by one way analysis of variance (ANOVA) accompanied by post-hoc (SPSS computer program) as compared to infected group

Table 3: Effect of *Ailanthus altissima* and *Zizyphus spina-christi* extracts on ova count in mice liver

Infected (1)	Infected + treatment with *A. altissima* (2) (a)	Infected + treatment with *Z. spina-christi* (3) (b)	p<	Reduction percentages (a)	(b)
9.11±0.53	5.16±0.73	5.45±0.24	0.0001	43.35	40.17
(2,3)	(1,3)	(1,2)			

Data are (means±SD)×10^3 of eight mice in each group a and b are reduction percent of *A. altissima* and *Z. spina-christi*, respectively. p is level of significance, where p<0.0001 is significant. Analysis of data is carried out by one way analysis of variance (ANOVA) accompanied by post-hoc (SPSS computer program)

Treatment of the spleen with both extracts improve the histological picture of the spleen to some what extant. However, there was still nodular white pulp infiltration and a limited feature of splenomegaly (Fig. 3G and H).

Parasitological studies: Table 2 shows significant decrease in total, male and female worms in infected treated mice with either *A. altissima* or *Z. spina-christi* extracts recorded 48.35, 40.34, 60.41 and 31.31, 25.85, 37.85%, respectively. There were a higher mortality rate of female than male worms as shown with the elevated relative sex ratio (RSR) of both treated groups. Ova count recorded significant decrease in mice treated with *A. altissima* and *Z. spina- christi* amounting 43.35 and 40.17%, respectively (Table 3).

DISCUSSION

In this study, we hypothesized that *A. altissima* and *Z. spina-christi* plant extracts would effectively protect liver, kidney and spleen against schistosomiasis. Present results demonstrate that both extracts will be able to reduce the damage of different mice organs by schistosoma toxins which was verified by histopathological observations.

In *S. mansoni* infection, the major pathologic changes are not cause by the adult worms itself but by eggs which do not reach the intestinal lumen, but instead, become trapped in other body tissues. At these sites, areas of local inflammation are produced, cumulating in the formation of granulomus around eggs (Modha *et al*., 1998 and Blanchard, 2004) and seems to be the major cause of pathology in schistosome infections (Modha *et al*., 1998). The lesions represent a localized interface between the infectious agents and the immune system. Macrophages often fuse and form characteristic flattened epithelioid and multinuclear giant cells around the irritation (Sandor *et al*., 2003). Compromised granuloma formation is accompanied by dissemination and often the course of an infectious disease changes from chronic to acute and leads to death of the host (Sandor *et al*., 2003).

The present results demonstrated that the histopathological section of the liver in schistosomiasis revealed an increased number and size of granulomata, live miracidia, extensive fibrous tissue accumulation, widening of the portal tracts as angiomatoid reaction and extensive bile duct proliferation. This is in agreement with the results of Mansy *et al*. (1990) who observed proliferation of bile ductules and bile canaliculi as a result of schistosomiasis. These results are also in accordance with Guangjin *et al*. (2002); Soliman *et al*. (2002) and El-Lakkany *et al*. (2004), who found the same histopathological articuture of liver after *S. mansoni* infection.

Infected mice treated with both plant extracts showed abatement of schistosomal activity, diminution in number and size of granulomata, evidence of increased immune reaction manifested by a lymphocytic cuff surrounding the granuloma, diminution of its fibrotic and collagen content and destruction of schistosoma ova. These results are confirmed by the observed dimension of worm burden, where the mortality of female worms is highly than male as shown by an elevation of relative sex ratio in the infected mice treated with plant extracts. Morphological alterations were also observed in live adult schistosome post treatment with *A. altissima* or *Z. spina-christi*, where both male and female worms appeared to be shrunken and thinner when compared with control worms, however these changes were more apparent in female than in male worms. These observation give an additional support of the effect of both extracts on female worms and its fertility by the observed dimension in ova count in liver tissue.

It is clear that, Schistosoma toxins elaborated by worms activated H_2O_2/myelperoxidase system, which is the cornerstone of the defense mechanism associated with inflammation, is activated on close contact with parasite egg. Although the process contributes to egg killing *in vivo*, it causes accumulation of H_2O_2, superoxide anions and hydroxyl radicals in the host's tissues (Abdallahi *et al*., 1999). The observed improvement in histopathological picture of the

liver, in this study, may also be attributed to the antioxidative properties of *A. altissima* and *Z. spina-christi* extracts (Saba El-Rigal *et al*., 2005) by eliminating the products of oxidative reactions and assists in the immune-mediated destruction of eggs. This finding is also confirmed by the previous results of Saba El-Rigal *et al*. (2005) through measuring alanine aminotransferase (AST), aspartate aminotransferase (ALT) and alkaline phosphatase (ALP) enzyme activities, where liver function enzymes revealed significant amelioration level after treatment with both plant extracts.

In the present study, histopathological section of the kidney in *S. mansoni* infected mice revealed distended hypercellular congested glomeruli and sever degenerative changes of lobules with obliterated lumina. These histologic changes may be responsible for decrease of glomerular filtration rate accompanied with increased glomerular capillary pressure, Yang *et al*. (2004) also found sever tubular degeneration, necrosis and medullary congestion rats suggesting acute renal failure. The cytoplasmic vaculation appeared may be attributed to either hydropic degeneration caused by more embission of water and electrolytes into the metabolically disturbed cells (Bogolepov, 1983; Hafez *et al*., 1999).

Moreover, congestion of veins and vessels may be due to the increase in vascular permeability which should lead to loss of fluid from the blood, so the vessel was engorged with red cells with consequent slowing down of the blood stream which would result in stasis (Cormack, 1987).

The micrographic picture of kidney showed that treatment of mice with both plant extracts, attenuated the deleterious effects in morphologic structure of kidney induced by infection as well as more or less normal architecture.

The morphological examination of the infected spleen, indicating the state of splenomegaly, where the spleen continued to increase in size, became dark red and rubbery in appearance. This enlargement of the spleen may be attributed to the direct deposition of the eggs in that organ or due to inflammatory and fibrotic reactions in the splenic host are the main factors responsible for obstruction to portal venous flow which its major consequence is splenomegaly (Warren, 1975). In histopathological examination, a congestion was evident in sinusoids of red pulp and lymphoid follicles (White pulp) were enlarged. This marked congestion in red pulp showing evidence of hemorrhages.

Treatment with both plant extracts improved the splenomegaly condition, where the spleen returned in part to its normal size with more or less normal architecture.

In conclusion, treatment of *S. mansoni* infected mice with *Ailanthus altissima* or *Zizyphus spina-christi* extracts, have potential effect for reducing the intensity of schistosomal infection by reduction in worm burden, ova count, granuloma size and number leading to improvement in histopathological picture of liver, spleen and kidney as a result of reducing inflammatory and fibrotic reactions of schistosoma toxins.

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