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THE EFFICACY OF *DIZYGTHECA KERCHOVEANA* AND *AZADIRACHTA INDICA* EXTRACTS AS A MOLLUSCICIDAL AND SCHISTOSOMICIDAL AGENTS IN MICE

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Abstract

In the present study, the effect of methanol extracts of two plant species, *Dizygotheca kerchoveana* (*Maliaceae*) and *Azadirachta indica* (*Araliacaea*) were tested on the activity of *Schistosoma mansoni* worms. Efficacy of two plant extracts compared with praziquantel (PZQ) was evaluated *in vivo*. The results showed that reduction was 90% in case of PZQ treatment compared to 76.91%, 62.64% after treatment with *D. kerchoveana* and *A. indica* extracts respectively. PZQ recorded highest significant number of dead ova into oogram pattern (80±1.80) at 5wk PI. But, good results were obtained by of *D. kerchoveana and A. indica* methanol extracts (65.00±4.05 & 60.60±3.60, respectively) at 3wk PI. Efficacy of the plant extract showed a significant ($P<0.01$) reduction in ova to 91-96.20% in intestine, 83.36-91.24% in hepatic tissues as compared to PZQ (10-66.50% & 1.14-80.64%, respectively). Reduction in hepatic granuloma diameter at 3wk PI was significantly ($P<0.01$) reduced in group treated with *D. kerchoveana* (40.43% & 38.30%) as compared to PZQ (8.70% & 11.7%). Sera were assayed by ELISA for IgM & IgG levels. Highly significant (*P<0.01*) increase in Igs levels in all infected treated or untreated groups was at 3wk & 5wk PI as compared to normal mice.

Key words: *S. mansoni*, PZQ, methanol plant extracts, Hepatic granuloma, IgM, IgG

Introduction

Schistosomiasis continues to rank following malaria at the second position of the world's parasitic diseases in terms of the extent of endemic areas and the number of infected people. Three, significant species of *Schistosoma*, contribute to morbidity and mortality caused in the endemic areas (Abdel-Haadi and Talaat, 2000).

Three distinct syndromes caused by *Schistosoma*. At first, there is a cercarial dermatitis or swimmer's itch, caused by an acute inflammatory reaction against cercariae, which provoke a rash consisting of round unnoticed erythrematous papules. The second syndrome is Katayama fever, often seen in primary infection associated with the maturation of schistosomula and the egg production at 3-9wk PI (Boros, 1989). Fever, loss of appetite, abdominal pain and weakness are frequent symptoms while diarrhea and marked eosinophilia are common signs (Cheever, 1997).

The main impact on public health is due to chronic infection "3rd syndrome" at which half of the number of eggs are not excreted, but are trapped in the liver, lung and intestine. These organs become the target of the granulomatous reaction due to soluble egg antigen (SEA) (Dunne and Doenhoff, 1983; Dunne *et al,* 1992a&b). The size and the cellular composition of the granuloma may vary according to host species (Weinstock, 1999), organ site (Moore, 1977), phase of evolution or involution of the lesion (Botros, 1986), and immunoregulatory influences (sensitization and modulation) (Hillyer, 1969). The cellular composition of schistosomal granuloma are 50% eosinophils, 30% macrophages, 15% lymphocytes of both T & B cell lineage, small members of mast cells and other inflammatory cells (Weinstock, 1988). Antibodies play an essential role in various effector or regulatory mechanisms according to their isotypes. Ghanem *et al.* (1977) and (Silveira *et al,* 2002) detected increased IgG & IgM values in acute and chronic human infections with active *S. ma-*

nsoni, associated mainly with the early stages of infection.

Ismail *et al.* (Ismail, 1994) reported that PZQ is an antischistosomal effective drug which reduces the egg count significantly in *S. mansoni* infected patients. However, they recorded the evaluation of resistant strains which reduce the effectiveness of such drug. Screening and analyzing natural botanical compounds for their potential activity are among these drugs. Therefore, medicinal plants regained their position in the last two decades as a vital remedy for many diseases (Abo-Madyan *et al,* 2004; Massoud *et al,* 2004a; Maryann *et al,* 1998). Numerous plant species were biologically screened for their antischistosomal and antihelmintic activity *in vitro* and *in vivo* (Shuhua *et al,* 2000; Sheir *et al,* 2001; Lyddiard *et al,* 2002; Massoud et al, 2003; Massoud *et al,* 2004b; Diab *et al,* 2005; Koko *et al,* 2005).

This study aimed to evaluate the efficacy of 2 certain medically important plant extracts of *D. kerchoveana* and *A. indica* as schistosomicidal agents and examine their effects on the Igs level in experimental mice.

Materials and Methods

Biomphalaria alexandrina were collected water canal at Abu-Rawash City, and examined for *S. mansoni* infective stages. 100 healthy snails (11-13 mm in size) were kept in glass aquaria (50x30x20 cm) filled with 15 liter of dechlorinated tap water and provided with pieces of *Elodea sp*. plant (15cm long) as a site for egg laying and for oxygenation of water. The snails were fed by fresh *lettuce sativa* leaves. A piece of chalk was added to each container as a calcium source for the snails (Thomas *et al,* 1974).

Male Swiss Albino mice (six to eight wk-old) (weight: 20±2 gm), were provided from the experimental animal unit of Theodore Bilharz Research Institute (TBRI). They were maintained under standard laboratory conditions and fed with high protienic diet (24-25%) and supplied with acidic distilled water (Nessim and Demerdash, 2000). Animal experiments have been carried out according to the internationally valid guideline and ethical conditions.

PZQ was freshly prepared before use as 2% suspension in Cremophor-El. At 3 & 5 wk PI; the drug was administered orally to mice in a dose of 500mg/kg for 2 consecutive days using a blunt stainless steel cannula.

Preparation of plant methanol extract: The whole over ground parts of 2 plants under investigation (*D. kerchoveana*; Family *Maliaceae*), Orman Garden, Giza) and *(A. indica*, Family *Araliacaea*), El Kanater, Egypt) were collected, identified, dried in air, then in an oven at 50°C for 2hr and finally powdered by a mixer. Each plant powder soaked in methanol (70%) 70 ml absolute methanol + 30ml dist. water (Sigma, USA) at 25±1°C for 1wk. The solvent was distilled off under vacuum and the crude extract residues were assayed as aqueous solutions. Plant extracts dose was calculated as 200ppm/mouse for *D. kerchoveana* or 650ppm/mouse for *A. indica*.

Mice infection: Swiss albino CD1 mice were infected with *S. mansoni* cercariae by tail immersion with (100±5) cercariae of the Egyptian strain of *S. mansoni* supplied from the SBSP, TRBI. Glass bottles were filled with dechlorinated water at 25±2°C containing 100 cercariae. The tails of mice were immersed for 2-4hr to ensure maximal cercarial penetration (Fenwick *et al,* 2006).

Experimental design: 165 mice were divided into 11 groups (15 mice each), 4 of them were normal, and the other 7 groups were exposed to cercarial infection. The groups were planned as the following: G1: Normal mice, G2: Normal mice treated with PZQ as 2 consecutive doses (500 mg/kg each), G3: Normal mice treated with *D. kerchoveana* (200 ppm/ mouse) day after day for 2 successive wk., G4: Normal mice treated with *A. indica* (650 ppm) day after day for 2 successive wk., G5: Infected untreated mice, G6: Infected mice treated with PZQ as 2 consecutive doses (500 mg/kg each) at 3wk PI., G7: Infected mice treated with PZQ as 2 consecutive doses (500 mg/kg each) at 5wk

PI, G8: Infected mice treated with *D. kerchoveana* (200ppm) at 3wk PI, day after day for 2 successive wk, G9: Infected mice treated with *D. kerchoveana* (200ppm) at 5wk PI, day after day for 2 successive wk, G10: Infected mice treated with *A. indica* (650ppm) at 3wk PI, day after day for 2 successive wk and G11: Infected mice treated with *A. indica* (650ppm) at 5wk PI, day after day for 2 successive wk. All groups were sacrificed at 8wk PI and assayed for parasitological, histological and immunological assays.

Determination of worm load by hepatic and mesenteric perfusion: Mice were sacrificed by cervical dislocation. Animals were skinned out and their bodies were washed with tap water and fixed to dissecting board. The abdominal muscles and thoracic ribs were removed. For hepatic perfusion, the needle connected to the automatic pipetting machine was inserted through the inferior vena cava and the liver washed with perfusate buffer (145mM NaCl, 25mM Na citrate). After removing, the portal venous ligature, the needle descended through thoracic aorta. Coils of intestine were gently manipulated to relieve twisting and proper washing of the vessels. The collected worms were coming out with the perfusate. Males, females or couples bulk were collected in conical flasks and then were counted either by direct visualization or under a stereomicroscope.

Egg developmental stages percentage (Oo gram pattern): After perfusion, the intestine was removed and its contents were removed. Three parts (1 cm length) from the middle part of small intestine were cut, dried on a filter paper and placed between a slide and cover slide. The preparation was strongly compressed using a thumb (Pellegrino *et al*, 1962). Hundred eggs were counted in each fragment (3fragments/animal). Viable eggs were counted and classified according to their developmental stages. Embryo occupies one-third the egg diameter (Stage I), one-half (Stage II), two-third (Stage III), embryo occupies the entire egg shell (Stage IV), mature egg which contains a fully developed miracidium and the dead ova appeared as semitransparent granular and darkened with retracted embryo.

Tissue egg load (Eggs/gm tissue): At the end of the perfusion, a piece of liver and intestine were taken to find out the number of eggs/gm in both tissues. Pieces were washed with saline and weighed. Each piece was placed in 5ml of 5% KOH solution (Cheever, 1968; Kamel *et al,* 1977) incubated at 37^{o}C for 24hr until the tissues were digested. The digested tissues were well shaken and three samples each of 0.25 ml were pipetted on counting slides. Number of ova was counted in three samples and the average was multip- lied by KOH vol. and divided by the tiss- ue weight to get the number of eggs/gm liver or intestine.

Histopathological study: A lobe of liver was fixed in 10% formalin and embedded in paraffin wax (Sigma, USA). Sections of 5 µm were stained with Ehrlich's hematoxylin and counter stain with eosin. Five slides/ animal and 3sections/slide/group were prepared. Individual granulomas with single egg in the center were selected for measurement using a calibrated ocular micro-meter. The mean granuloma diameter (MGD)/ group were calculated for about 140 lesions. PR of MGD/treated group was calculated according to the formula:

$$\text{Percent reduction (PR)} = \frac{\text{MGD of control group - MGD of treated group}}{\text{MGD of control group}} \times 100$$

Evaluation of serum IgG and IgM levels using ELISA: Sera of normal control, infected untreated and infected treated mice were collected for ELISA (Engvall and Perlman, 1971). Coaster flat bottom high binding plates (Cambridge, MA, USA), were coated with 100µl/well of soluble egg antigen (SEA, given by immunology lab, TBRI, Giza, Egypt) in carbonate/bicarbonate buffer and overnight incubated at 4^{o}C. Plates were

incubated for 1hr at room temperature with 200µl/well of blocking buffer and washed 5 times with PBS/T. 100µl/well of diluted sera (1:250) were added in duplicate and incubated for 1hr at room temperature with 100 µl/well of anti-mouse HRP conjugate diluted 1:1000. Plates were washed with PBS/T and incubated with 100µl/well OPD substrate for 15-30min. at room temperature. Reaction was stopped by stopper buffer 50µl/well. Absorbance was read at 405nm using ELISA reader (Lab. System, Helsinki, Finland).

Statistical analysis: Data was analyzed by ANOVA test to compare the differences between mean values of experimental and control values. Results were expressed as mean ± standard deviation (SD) and values $P<0.05$ were significant.

Results

In the present study, methanol extracts of *D. kerchoveana* and *A. indica* was used on Albino mice infected with 100±50 *S. mansoni* cercariae in comparison to PZQ. (Tab. 1) recorded PR of worm burden. In comparison to infected control group, treatment with PZQ for 5wk PI showed the significant (*P<0.01*) PR (90%), followed by *D. kerchoveana* treated group after only 3wk PI (76.9%). At 5wk post treatment, *D. kerchoveana* has nearly the same PR of *A. indica* extract at both 3 & 5wk PI (66.35%, 67.80% and 62.64%, respectively).

Table 1: Effect of 70% methanol extract of *D. kerchoveana*, *A. indica* plants and PZQ on PR of *S. mansoni* adults.

Mice	Worm Load X±S.E	Worm Load PR±S.E
Infected control	26±3.00	0%±11.54
PZQ (3 wk)	8.20±0.21	68.46%±2.56^{b}
PZQ (5 wk)	2.60±0.23	90.00%±8.85^{c}
D. kerchoveana (3 wk)	6±0.57	76.92%±9.50^{b}
D. kerchoveana (5 wk)	8.75±0.77	66.35%±8.80^{b}
A. indica (3 wk)	8.38±0.65	67.79%±7.76^{b}
A. indica (5 wk)	9.71±1.23	62.64%±12.67^{b}

Significantly different from infected control $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$

Ovicidal activity and maturity of eggs compared to infected control 62.30±5.40, 33.10±3.40 & 4.60± 0.40 for immature, mature & dead ova res-pectively. PZQ showed significant reduction of mature ova ($P<0.01$) and increasing dead ones ($P<0.001$) at 5wk PI (17.40±1.00 & 80±1.80, respectively). *D. kerchoveana* and *A. indica* caused early significant ($P<0.01$) ova reduction at 3wk PI, 17.30±1.73, 22.10± 1.39 & 60.60±3.60 for *D. kerchoveana* and 12±2.68, 23±3.26 & 65± 4.05 for *A. indica*, respectively.

Table 2: Effect of 70% methanol extract of *D. kerchoveana*, *A. indica* plants and PZQ on oogram pattern of *S. mansoni*.

Mice Groups	Oogram pattern (% Ova)		
	Immature ova X±S.E	Mature ova X±S.E	Dead ova X±S.E
Infected control	62.30±5.40	33.10±3.40	4.60±0.40
PZQ (3 wk)	15±3.10^{b}	40±2.90	45±2.50^{b}
PZQ (5 wk)	2.60±1.50^{c}	17.40±1.00^{b}	80±1.80^{c}
D. kerchoveana (3 wk)	17.30±1.73^{b}	22.10±1.39^{a}	60.60±3.60^{c}
D. kerchoveana (5 wk)	25.30±2.73^{b}	22.10±3.36^{a}	52.60±5.50^{c}
A. indica (3 wk)	12±2.68^{b}	23±3.26^{a}	65±4.05^{c}
A. indica (5 wk)	22±3.97^{b}	33±3.73	45±4.84^{b}

In infected control, PZQ treatment at 3 wk PI, recorded 1.14% & 8.30% reduction in ova/gm count in hepatic and intestinal tissues, respectively. However, the PR increased significantly ($P<0.01$) at 5wk PI to 80.64% and 66.50%. Treatment of infected mice with each of two plant extracts attained an early (at 3wk PI) significant ($P<0.001$) PR for hepatic and intestinal tissues (ranged from 83.36%-91.24% & 96.85%-91.10%, respectively, in compared to infected control group (Tab. 3).

Table 3: Effect of methanol extract of *D. kerchoveana*, *A. indica* and PZQ on PR of intestinal and hepatic tissues egg in mice.

Mice	Hepatic (Ova Count)		Intestinal(Ova Count)	
	X±S.E	PR±S.E	X±S.E	PR±S.E
Infected control	22975±186.20	0%±0.81	35106±747.50	0%±2.13
PZQ (3 wk)	22714.70±747.50	1.14%±3.29	32167.90±747.50	8.37%±0.08[a]
PZQ (5 wk)	4450±747.50	80.64%±16.80[c]	11750±747.10	66.53%±6.36[b]
D. kerchoveana (3 wk)	2014.30±234.50	91.23%±11.64[c]	3128±585.40	91.10%±18.71[c]
D. kerchoveana (5 wk)	3825±400.22	83.35%±10.46[c]	3212.50±469.60	90.85%±14.62[c]
A. indica (3 wk)	2442.90±407.44	89.37%±16.68[c]	1357.14±86.90	96.13%±6.40[c]
A. indica (5 wk)	3128.57±713.71	86.38%±23.00[c]	2771.43±489.60	92.11%±17.66[c]

PR in hepatic granuloma diameter of infected treated groups compared to infected controls, PZQ recorded a mild but significant (P<0.05) PR at 3 & 5wk PI (8.70% &11.74%, respectively). *D. kerchoveana* treatment induced highly significant (P<0.01) PR as early as 3wk PI and decreased by only 2% at 5wk PI. Also, treatment of infected mice with *A. indica* extract significantly (P<0.01) reduced the granuloma diameter by 38.26% &21.74% at 3& 5wk PI, respectively (Tab. 4).

Table 4: Effect of 70% methanol extract of *D. kerchoveana*, *A. indica* plants and PZQ on PR of granuloma diameter.

Mice Groups	Granuloma diameter	
	X±S.E	PR±S.E
Infected control	230±5.10	0%±2.21
PZQ (3wk)	210±4.19	8.70%±1.99[a]
PZQ (5wk))	203±3.90	11.74%±1.92[a]
D. kerchoveana (3wk)	137±1.95	40.43%±1.42[b]
D. kerchoveana (5wk)	142±2.50	38.26%±1.76[b]
A. indica (3wk)	150±2.90	34.78%±1.93[b]
A. indica (5wk)	180±3.40	21.74%±1.88[b]

Significantly different from infected control [a]*P<0.05,* [b]*P<0.01,* [c]*P<0.001*

Infection caused a significant (P<0.01) increase in Igs levels (1.40±0.30 & 1.10± 0.25, respectively) compared to normal serum levels (0.30±0.15, 0.30±0.20 for IgM & IgG, respectively). PZQ has no effect on Igs levels of infected mice sera compared to infected control. *D. kerchoveana* and *A. indica* extracts attained a slight modification of infection as increasing IgM level (1.38±0.20 to 1.50±0.20). But, treatment with both plant extracts success to modulate immune system by a significant *(P<0.05)* decrease in IgG level ranged from 0.84±0.19 to 0.99±0.20 at 3 & 4 wk PI, respectively (Tab. 5).

Table 5: Effect of 70% methanol extract of *D. kerchoveana*, *A. indica* plants & PZQ on levels of IgM & IgG in mice.

Mice Groups	IgM X±S.E	IgG X±S.E
Normal control	0.31±0.08	0.30±0.12
PZQ	0.37±0.06	0.38±0.27
D. kerchoveana	0.29±0.02	0.30±0.17
A. indica	0.36±0.16	0.37±0.26
Infected control	1.40±0.31[c]	1.10±0.25[c]
Infec.& PZQ (3 wk)	1.10±0.21[c]	1.10±0.19[c]
Infec.& PZQ (5 wk)	1.50±0.22[c]	1.10±0.21[c]
Infec.*& D. kerchoveana* (3 wk)	1.50±0.20[c]	0.84±0.20[c]
Infec.& *D. kerchoveana* (5 wk)	1.38±0.20[c]	0.86±0.20[c]
Infec.& *A. indica* (3 wk)	1.40±0.23[c]	0.89±0.18[c]
Infec.& *A. indica* (5 wk)	1.50±0.20[c]	1±0.21[c]

Discussion

Schistosomiasis is not only a national health problem, but also a world one calling for international cooperation (Mc-Manus *et al,* 2018). *Araliacaea, Maliaceae, Asteraceae, Compositae and Euphorbiaceae* extracts proved to have molluscicidal, miracicidal, cercaricidal, and killed *S. mansoni* (Abdel Ghaffar *et al,* 2008). In the present study, highly potent extracts were obtained from *D. kerchoveana* and *A. indica.* Ismail *et al.* (1996) reported that PZQ was effective an antischistosomal drug for *S. mansoni* and reduced egg count significantly in patients. In

the present study, treatment of *S. mansoni*-infected mice with either PZQ chemotherapy or methanol extracts of *D. kerchoveana* and *A. indica* showed that potency of plant extracts, more than PZQ with a significant ($P<0.01$) PR of in worm burden (90%) at 5wk PI. *D. kerchoveana* treatment gave PR less than that of PZQ (76.90%), after only 3wk PI which was very important in disease control. Andrews (1978) found that PZQ in *S. mansoni* mice infected delayed hatching of excreted eggs for 24hr. Richard *et al.* (1983) reported that PZQ was lethal to *S. mansoni* eggs, in host tissues in high doses. El Shenawy *et al.* (2006) used crude extract of *Cleome droserifolia* leaves on *S. mansoni* infected mice, found weak reduction in worm burden (32.4%), but increase of dead eggs, and suppressive effect on granulomas. Methanolic leaf extract of *Jatro-pha curcas* caused *S. mansoni* reduction of 8.33%, but PZQ gave 97% (Adamu *et al,* 2006).

In the present study, PZQ gave as high percentage (80%) of dead ova. *D. kerchoveana* and *A. indica* extracts significantly decreased the number of mature ova, increase the percentage of dead ova and reduced granuloma diameter after 3wk PI. But, PZQ did not reduce the ova count in hepatic or intestinal tissues at 3wk PI (1.14 & 10%, respectively), compared to ($P<0.01$) PR of *D. kerchoveana* and *A. indica* extracts (66.5% & 80.64%, respectively), with highly significant ($P<0.001$) increase in PR by 5wk PI (96.20-91% & 83.36-91.24%, respectively). These agreed with Ahmed and Rifaat (2005) who detected that ethanolic extract of *Solanum nigrum* decreased schistosome eggs in mice hepatic tissue. Also, Koko *et al.* (2005) found a significant reduction in egg count/ gm feces, and/count in tissue & recovery of *S. mansoni* adult mice with *Balanites aegyptiaca* fruits. Boros (1989) found the vigorous granulomatous response rather than direct action of egg antigen(s) for pathologic tissue in schistosomiasis. Liver granulomas induce particularly high levels of inflammatory mediators (Selim *et al,* 2014). Reduction in M.G.D. caused by both plant extracts may be due to reducing effects on mediators, macrophages and eosinophils, also to its hepatoprotective effect and inhibitory effect on oogram pattern. PZQ had a moderate effect on granuloma PR after 5wk PI, but PZQ has a week effect on inflammatory mediator's level with a number of worms and eggs at infection time (Botros *et al,* 2000; 2006). This agreed with Massoud *et al.* (2000c; 2005) studied effect of *Myrrh* on *S. mansoni*-infected mice liver, with marked reduction of granulomas number and size and significant reduction in collagen content deposition in portal areas and around central veins proving its efficiency.

The effect of *Daucus carota* extracts on immune responses of *S. mansoni* infected mice was studied. The rate of reduction in worm in mice injected with some fractions indicated a strong protection. Some extracts induced humoral immune response by raising IgG level at 2, 4 & 6wk PI as compared with infected control. Phenotypic analysis of the cellular immune response in spleen and mesenteric lymph nodes was accomplished by direct immunofluorescence. Some extracts stimulated the blastogenesis of $CD4^+$ T splenocytes and mesenteric lymph node cells (Shalaby *et al,* 1999). Total IgE was significantly higher in *Fasciola* and *Schistosoma* patients before treatment compared to control and decreased significantly with *Myrrh* oleo-gum. IL-1 beta & IL-5 were high in fasciolosis and schistosomiasis, but decreased with therapy. Depressed IL-4 production was a parasite immune evasion or host regulatory mechanism & cytokines levels as cure criteria (Massoud *et al,* 2000b).

Immunomodulatory effects of iridoid mixture, iridoid-treated *S. mansoni* homogenate on mice were measured by IgM & IgG levels against soluble *S. mansoni* antigenic preparation (SWAP) antigens by ELISA. Cellular immune responses calculated mean of $CD4^+$, $CD8^+$ T, B-mesenteric lymph node cells (MLNC) & $CD4^+$, $CD8^+$ T thymocytes by direct immunofluorescence staining in

treated mice as compared to untreated homogenate given mice or untreated mice. 1st & 2nd immunizations with iridoid mixture treated homogenate caused significantly elevated ($P<0.05$) IgM & IgG against the antigen compared with sera from control mice. Immunized with homogenate treated with iridoid mixture gave a significant increase in $CD4^+$ T thymocytes, no significant increase in $CD8^+$T thymocytes, a significant increase in $CD4^+$ T lymphocytes (MLNC), without significant increase in $CD8^+$ T & B lymphocytes compared with mice immunized with untreated homogenate or non-treated normal mice (Bahgat *et al,* 2005).

Conclusion

PZQ t showed no effect on Igs picture. *D. kerchoveana* and *A. indica* extracts gave im munomodulating effects by increasing IgM level at 3wk PI. *D. kerchoveana* and *A. indica* extracts caused *S. mansoni* eradication and enhanced reduction in inflammatory granuloma reaction around eggs as a result of the increased diminution in the Igs level. Thus, *D. kerchoveana* and *A. indica* extracts are promising antischistosomal drugs.

Conflicts of interest: The authors declared that they have neither conflict of interest nor received fund.

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