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In vivo the Immunological Effects of *Fasciola gigantica* Worms Homogenate Mixed with Saponin on Mice Infected with *Schistosoma mansoni*

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Antischistosomal activity, identification and immunoprophylatic effects of a crude *Faciola gigantica* worms homogenate, *F. gigantica* worms homogenate mixed with saponin were studied on *Schistosoma mansoni* infected mice. Mice were divided into four groups (10 animals group). First group was immunized subcutaneously (50 μg/mouse/100 μL/PBS) at 0 and 15 days with a crude *F. gigantica* worms homogenate, second group was immunized subcutaneously with a crude *F. gigantica* worms homogenate mixed with saponin and, third group was immunized with saponin only. The fourth group was used as control. Post second immunization mice were infected by tail immersion with 100 *Schistosoma mansoni* cercariae per each mouse. Relative to electrophoretic analysis and scanning of the standard of known molecular weights, the number of major bands detected from electrophoretic scanning of *Faciola gigantica* worms homogenate were 8 bands, 177.30, 156.81, 110, 105.95, 102.47, 64.084, 56.403 and 45.893 KDa. The electrophoretic scanning of a crude *Faciola gigantica* worms homogenate mixed with saponin showed that 7 bands, 120.49, 112.38, 102, 91.727, 86.203, 69.794 and 66.825 kDa. On the other hand, the number of major bands detected from the electrophoretic scanning of saponin were 9 bands, 167.68, 110, 104.94, 102, 92.620, 79.399, 60, 54.231 and 23.091 KDa. Perfusion and recovery of adult worms were performed at 8 weeks post infection. Immunization with a crude *F. gigantica* worms homogeneta, a crude *F. giganatica* worms homogeneta mixed with saponin and saponin only recorded reduction in total worms by 75.11, 45.24 and 87.33%, respectively. The level of both IgM and IgG from sera of immunized mice with a crude *F. gigantica* worms homogenate, a crude *F. gigantica* worms homogenate mixed with saponin and saponin only showed an increasing against cercarialantigen preparation, soluble worm antigen preparation and soluble egg antigen using Enzyme Linked Immunosorbent Assay (ELISA). Using immunolymphocytes stainingpreparation, the mean number of splenocytes that were prepared from immunized groups with a crude *F. gigantica* worms homogenate, a crude *F. gigantica* worms homogenate mixed with saponin and saponin without homogenate , respectively showed a significant increase ($p<0.05$) as compared with non immunized group.

Key words: *Fasciola gigantica*, Saponin, *Schistosoma mansoni*, Immunoprophylactic, electrophoretic scanning, enzyme linked immunosorbent assay

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INTRODUCTION

Schistosomiasis is an important parasitic disease, which affects more than 200 million people in 74 countries around the world and causes approximately 20,000 death per year (Bergquist, 2002; Julie *et al*., 2007). Currentely, Schistosomiasis control strategy is mainly based on the treatment of infected individuals by chemotherapy with safe and effective drugs (Harder, 2002).

Praziquantel became available for the treatment of schistosomiasis and other trematode inflicted diseases in the 1970s. It was revolutionary because it could be administered orally and had very few unwanted side effects (Doenhoff and Mattoccia, 2006). There is concern that resistance to praziquantel might develop or already exists and could be further facilitated through new control initiatives relying on large-scale administration of praziquantel (Rashida *et al*., 2005). Therefore, monitoring praziquantel efficacy in different epidemiological setting is required. Raso *et al*. (2004) assessed the efficacy and side effects of praziquantel against *Schistosoma mansoni* in a rural community of western cote d"Ivoire. It was found that the most frequent side effects were abdominal pain, dizziness and diarrhea. Therefore, vaccination as a way to control schistosomiasis would contribute enormously to disease eradication, mainly because immunization provides long-lasting immunity to the disease (Fonseca *et al*., 2004)

Several vaccine candidates have been identified in *Schistosoma mansoni* directed against the schistosomulum as well as against other life cycle stages. Some of the more promising antigens have now reached a more advanced stage of development including in the case of glutathione S-transferase, the stage of industrial manufacture and safety testing (Capron *et al*., 1992; Bergquist *et al*., 1994). Another approach has been to study closely related cross-reacting antigens from another trematode, *Fasciola hepatica* (Hillyer, 1995).

The presence of a common antigen between *Schistosoma mansoni* eggs and *Fasciola hepatica* adult worms was demonstrated by utilizing in an anti-*S. mansoni* adult worm antiserum. Although not one of the three major serologic *S. mansoni* egg antigens, its complete cross-reactivity suggests that serologic tests done with these crude antigenic extracts will result in many false-positive cases in areas where both parasites are endemic (Pelley and Hillyer, 1978). Extracts of *Fasciola hepatica* adult worms contain antigens reactive with antisera prepared against *Schistosoma mansoni* adult worms (Hillyer *et al*., 1979; Raina *et al*., 2006).

The adjuvant of the FML-vaccine against mixture was fractionated by ion exchange chromatography on DE AE-cellulose to afford one TLC homogenous, the result confirm in the Riedel de haen saponin extract the presence of deacyl saponin normonoterpene deprivated which are non-toxic and capable of inducing a specific and strong immunoprotective response in vaccination against murine visceral leshmaniasis (Olivera-Freitas *et al*., 2006).

The IgG isotype profile induced during acute infection was obtained by equi merozoite antigen saponin immunization (Cunha *et al*., 2006).

The aim of the present study is to detect the mean percentage of protection as a result of vaccination with *Fasciola gigantica* worms homogenate, *Fasciola gigantica* worms homogenate treated with saponin extracted from *Atriplex nummularia* L. and saponin on *Schistosoma mansoni* infected mice. Study the humoral and cellular immunoprophylactic effects of *Fasciola gigantica* worms homogenate, *Fasciola gigantica* worms homogenate treated with saponin and saponin on *Schistosoma mansoni* infected mice.

MATERIALS AND METHODS

The present study was conducted during (September 2004-October 2006) in our laboratory in the Medicinal Chemistry Department-National Research Center, Dokki, Cairo, Egypt.

Experimental groups and infection: Forty female swiss albino mice, weighing 18-20 g (4 weeks ago) were obtained from the animal house (National Research Center, Dokki, Cairo-Egypt). Mice were divided into four groups (10 mice group). First group was immunized subcutaneously (S.C.) with *Fasciola gigantica* worms homogenate, Second group was immunized with *Fasciola gigantica* worms homogenate mixed with saponin extracted from Atripex nummularia L (the extract was soluble in Tween-80 and dist. water at ratio 7:3 and third group was immunized with saponin (50 μg/mouse/100 microliter at 0 and 15th). The fourth group was used as control. Post 2nd immunization mice were infected by tail immersion method with 100 *Schistosoma mansoni* cercariae per each mouse (Oliver and StireWalt, 1952).

Assessment of worms burden: Perfusion and recovery of adult worms were performed at 8 weeks post infection through hepatic portal vein by the perfusion method. The total tissue worm counts in liver and intestine were determined. Protection was assessed as the percentage reduction in worms counts in liver and intestine (REF) according to the formula:

$P = C\text{-}T/C \times 100$ (Duvall and De Witt, 1961). Where, P: percentage reduction of worms or eggs, C: mean worm burdens in control infected animals, T: mean worm burdens in pre-treated infected animals.

The Relative Sex Ratio (RSR) was used to examine the effect of the immunized infected group compared to infected non-immunized control group as standard on different sexes of worms. RSR was determined according to the formula,

RSR = Male: Female ratio in treated group/Male: Female ratio in untreated group.

The ratio of untreated groups was standardized as 1 (Fallon *et al*., 1995).

SDS-PAGE gel electrophoresis: The *F. gigantica* homogenate, *F. gigantica* worms homogenate mixed with saponin and saponin without a crude homogenate were analyzed by SDS-PAGE gel electrophoresis according to Laemmli (1970). The electrophoresed proteins were scanned after staining with silver stain using spectrophotometer (Beckmann DU 640 USA). The scanning was carried out at the maximum absorbance (500 nm) of the stain. Molecular weights of proteins were determined according to the method of Chrambach (1985).

Enzyme Llinked Immunosorbent Assay (ELISA): The assay was performed according to the Hillyer *et al.* (1979). This assay was used for determination of the levels of IgG and IgM in sera of different experimental groups. Plates were coated with different types of antigens. Cercarial Antigens Preparation (CAP), Soluble Worm Antigens Preparation (SWAP) and egg antigens (SEA). Plate was incubated at room temperature over night. It was washed using PBS-0.05%T20. Plate was blocked for sites free of antigen using blocking buffer (1%BSA -PBS-0.05%T20) then sera at dilution of (1:100) was added and incubated was added and incubated at 37°C for 2 h. Antimouse IgG and IgM peroxidase conjugate were added at dilution of (1:5000,1:10000) in 1% BSA-PBS 0.05 % T20 and was incubated for 1 h at 37°C. Orthophenylene iamin dihydrochloride (OPD) was used as substrate. The reaction was read at 490 nm using ELISA Reader.

Immunostaining of splenocytes: Splenocytes suspension were prepared by teasing the spleens from mice immunized with *F. gigantica* worms homogenate, *Fasciola gigantica* worms homogenate mixed with saponin and saponin only then challenge with *S. mansoni* cercariae. The fourth group was used as control. The supernatant was aspirated and centrifuged at 200 G, 5 min at room temperature. The cell pellet was resuspended in culture medium (RPMI). Red blood cells were lysed with lysis buffer for 3 mins at room temperature. The spleen cells were washed twice by centrifugation in culture medium. Assessing viability was performed by trypan blue dye, exclusion method in which only viable lymphocytes exclude the dye while dead cells appear blue. Equal volume (0.1 mL) of whole suspension and trypan blue were mixed and examined under LEITZ microscope using Neubaur haemocytometer. Viable lymphocytes were counted and viability was calculated. Viable spleen cells = N X Y 2 X 104/mL. Where N: number of viable cell per 16 large squares. Y: the volume of cell suspension (Maghraby, 1989).

Statistical analysis: Statistical significance values between groups are carried out by Student t-test according to Ronald *et al.* (1983), as well as Graph Pad Soft ware, Graph Pad InStat was also used

RESULTS

Identification of molecular weights of antigenic fractions of different antigens preparation: Relative to electrophoretic migration and scanning of the standard of known molecular weights, the number of major bands detected from the electrophoretic scanning of the *Fasciloa gigantica* worms homogenate were 8 bands, 177.30, 156.81, 110, 105.95, 102.47, 64.084, 56.403 and 45.893 KDa (Fig. 1 and Table 1).

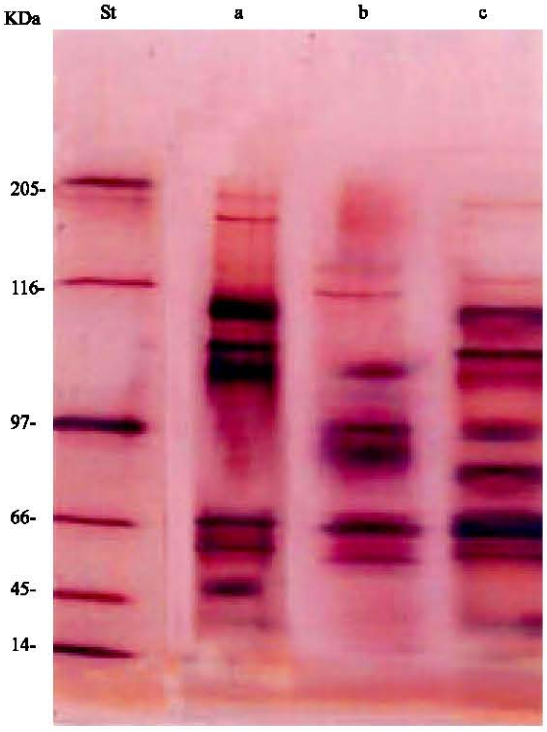

Fig. 1: SDS-PAGE gel electrophoresis for *F. gigantica* worms homogenate, *Faeciola gigantica* worms homogenate mixed with saponin. Kda: Kilo Dalton; St: Standard protein; A: *F. gigantica* worms homogenate; B: *Fasciola gigantica* worms homogenate mixed with saponin; C: Saponin

On the other hand, the number of major bands detected from the electrophoretic scanning of Fasciola worms mixed with saponin showed that 7 bands, 120.49, 112.38, 102, 91.727, 86.203, 69.794 and 66.825 kDa (Fig. 1 and Table 1).

The electrophoretic scanning of the saponin were 9 bands, 167.68, 110, 104.94, 102, 92.620, 79.399, 60.417, 54.231 and 23.091 KDa (Fig. 1 and Table 1).

Notably, two common bands at 110 and 102 KDa were detected between saponine and *Fasciola gigantica* worms homogenate. The electrophoretic analysis showed also one common band at 102 KDa between saponine and *Fasciola gigantica* worms antigen homogenate mixed with saponin (Fig. 1 and Table 1).

Antischistosomal activity: Vaccination with *Fasciola gigantica* worms homogenate recorded reduction in total worms, male and female by 75.11, 86.77 and 92.08%, While, *Faschiola gigantica* worms mixed with saponin recorded diminution by 45.24, 75.96 and 47.78%, saponin showed reduction by 87.33, 90.38 and 93.67%, respectively. There was a high mortality of female worm than male worm as indicated by the higher relative sex ratio in vaccinated mice with Faschiola and saponin antigens (Table 2) .

Immunoprophylactic effect of *F. gigantica* worms homogenate on *S. mansoni* infected mice: Figure 2 and 3 showed the levels of both IgM and IgG induced by Faschiola homogenate against Cercarial Antigens Preparation (CAP) during the acute schistosomiasis phase.

There was an increasing in IgM level against CAP in mice immunized with *Fasciola gigantica* worms homogenate and infected with *S. mansoni* but it was not significant as compared with *S. mansoni* infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected mice as compared with infected unimmunized ones.

Figure 4 and 5 showed the levels of both IgM and IgG, respectively induced by *Faschiola gigantica* worms homogenate against Soluble Worm Antigens Preparation (SWAP). There was a significant increasing ($p<0.05$) in IgM level in immunized infected ones as compared with *S. mansoni* infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected ones as compared with infected unimmunized ones.

Figure 6 and 7 showed the levels of both IgM and IgG, respectively induced by *Faschiola gigantica* worms homogenate against egg antigen (SEA). There was an increasing in IgM level in immunized infected ones but considered non significant as compared with *S. mansoni* infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected ones as compared with *S. mansoni* infected unimmunized ones.

Immunoprophylactic effect of F. gigantica worms homogenate mixed with saponin on *S. mansoni* infected mice: Figure 2 and 3 showed the levels of both IgM and IgG induced by *Fasciola* homogenate mixed with saponin against Cercarial Antigen (CAP) in *S. mansoni* infected mice. There was an increasing in IgM level against CAP in immunized infected mice but it was not significant as compared with infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected mice as compared with infected unimmunized ones. Figure 4 and 5 showed the levels of both IgM and IgG, respectively induced by *Fasciola gigantica* worms homogenate mixed with saponin against Soluble Worm Antigens (SWAP).

Table 1: Electrophoretic scanning of *F. gigantica* worms homogenate, *F. gigantica* worms homogenate mixed with saponin and saponin

	Molecular Weights (KDa)			
No.	Marker	*F. gigantic* worms homogenate	*F. gigantic* worms homogenate mixed with saponin	Saponin
1	205	177.300	120.490	167.680
2	116	156.810	112.380	110.000
3	97	110.330	102.000	104.940
4	66	105.950	91.727	102.000
5	45	102.000	86.203	92.620
6	14	64.084	69.794	79.399
7		56.403	66.825	60.417
8		45.893		54.231
9				23.091

Table 2: Number of worm burden, relative sex ratio and reduction percent of worms in female mice liver vaccinated with *Fasciola gigantica* worms homogenate, *Fasciola gigantica* worms homogenate mixed with saponin and saponin

Groups	Total worm (TW)	Male (M)	Female (F)	%R (TW)	%R (MW)	%R (FW)	RSR
Infected	44.20±5.87	20.8±2.92	15.8±3.35	-	-	-	1.00
F. gigantica worms homogenate	11.00±3.67	2.75±1.08	1.25±0.75	86.77	75.11	92.08	1.60
F. gigantica worms homogenate mixed with saponin	24.20±3.26	5.00±2.22	8.25±4.08	45.24	47.78	47.78	0.45
Saponin	5.60±1.35	2.00±0.74	1.00±0.48	87.33	90.38	93.67	1.50

Data are mean±SD of five mice in each group. %R is percentages of reduction of worm number. RSR is relative sex ratio between male and female worms in immunized groups as compared to infected group

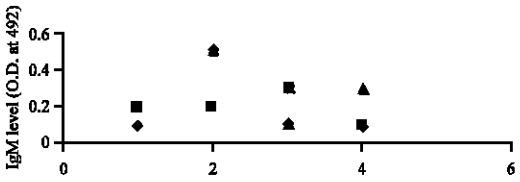

Fig. 2: Detection of IgM levels against Cercarial Antigens Preparation (CAP) in sera from control group (◆), group 2 vaccinated with *Fasciola gigantica* worms mixed with saponin (■), group 3 vaccinated with saponin (▲), group 4 vaccinated with *Fasciola gigantica* worms homogenate (×) and challenged with *Schistosoma mansoni*

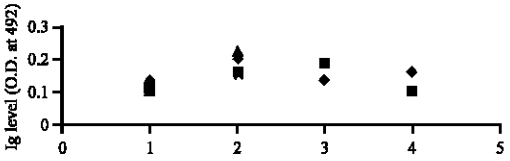

Fig. 3: Detection of IgG level against Cercarial Antigens Preparation (CAP) in sera from control group (◆),vaccinated group with *Fasciola gigantica* worms homogenate mixed with saponin (■), vaccinated group with saponin (▲), vaccinated group with *Fasciola gigantica* worms homogenate (×) and challenged with *Schistosoma mansoni*

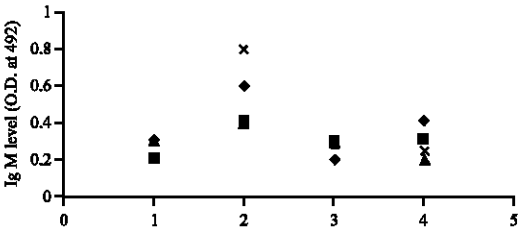

Fig. 4: Detection of IgM levels against Soluble Worms Antigens (SWAP) in sera from control group (◆), group 2 vaccinated with *Fasciola gigantica* worms mixed with saponin (■), group 3 vaccinated with saponin (▲), group 4 vaccinated with *Fasciola gigantica* worms homogenate (×) and challenged with *Schistosoma mansoni*

There was a significant increasing ($p<0.05$) in IgM level in immunized infected ones as compared with *S. mansoni* infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected ones as compared with infected unimmunized ones.

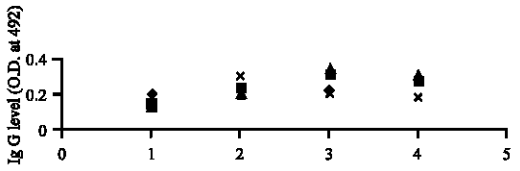

Fig. 5: Detection of IgG level against Soluble Worm Antigens Preparation (SWAP) in sera from control group (◆), group vaccinated with *Fasciola gigantica* worms homogenate mixed with saponin (■), group vaccinated with saponin (▲), group vaccinated with *Fasciola gigantica* worms homogenate (×) and challenged with *Schistosoma mansoni*

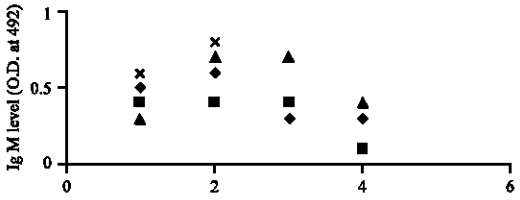

Fig. 6: Detection of IgM level against Soluble Egg Antigens (SEA) in sera from control group (◆), *Fasciola gigantica* worms homogenate mixed with saponin (■), saponin (▲), *Fasciola gigantica* worms homogenate (×) and challenged with *Schistosoma mansoni*

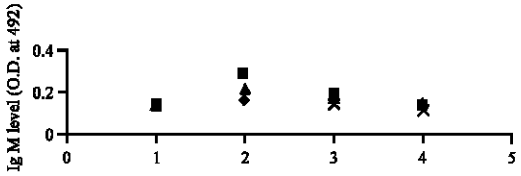

Fig. 7: Detection of IgG level against Soluble Egg Antigens (SEA) in sera from control group (◆), *Fasciola gigantica* worms homogenate mixed with saponin (■), saponin (▲), *Fasciola gigantica* worms homogenate (×) and challenged with *Schistosoma mansoni*

Figure 6 and 7 showed the levels of both IgM and IgG, respectively induced by *Fasciola* homogenate against egg antigen (SEA). There was an increasing in IgM level in immunized infected ones but considered non significant as compared with *S. mansoni* infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected ones as compared with infected unimmunized ones.

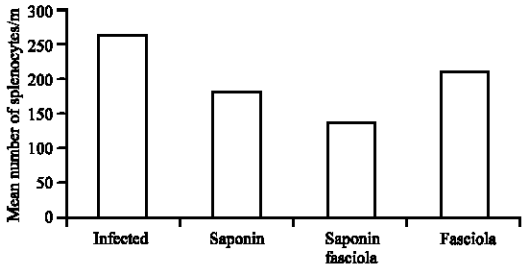

Fig. 8: Mean number of Splenocytes in spleen from *Schistosoma mansoni* infected mice immunized with *Fasciola gigantica* worms homogenate, *Fasciola gigantica* worms homogenate mixed with saponin, saponin or infected unimmunized ones

Immunoprophylactic effect of saponin on *S. mansoni* infected mice: Figure 2 and 3 showed the levels of both IgM and IgG induced by saponin against Cercarial Antigen (CAP) in *S. mansoni* infected mice. There was an increasing in IgM level against CAP in immunized infected mice but it was not significant as compared with infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected mice as compared with infected unimmunized ones.

Figure 4 and 5 showed the levels of both IgM and IgG, respectively induced by Saponin Against Worm Antigen (SWAP). There was an increasing in IgM level in immunized infected ones but considered non significant as compared with infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected ones as compared with infected unimmunized ones.

Figure 6 and 7 showed the levels of both IgM and IgG, respectively induced by Saponin Against Egg Antigen (SEA). There was an increasing in IgM level in immunized infected ones but considered non significant as compared with infected unimmunized group. IgG level showed a significant elevation ($p<0.05$) in immunized infected ones as compared with infected unimmunized ones.

Cellular immune response: The statistical analysis of the observed data using one-way ANOVA test, revealed a high significant increase ($p< 0.05$) between values of the mean number of splenocytes that were prepared from immunized groups by *Fasciola gigantica* worms homogenate, *Fasciola gigantica* worms homogenate mixed with saponin or saponin a and infected with *S. mansoni* as compared with *S. mansoni* infected mice (Fig. 8).

DISCUSSION

Vaccination with *Fasciola gigantica* worms homogenate, saponin and *Fasciola gigantica* worms homogenate mixed with saponin recorded reduction in total worms, by 75.11, 87.33 and 45.24%, respectively. As well as there was a high mortality of female worms than male worms as indicated by the higher relative sex ratio in vaccinated mice with Fasciola and saponin antigens. Present result are agreement with Oliveira-Freitas *et al.* (2006) who assessed that hydrophobic moeties in recombinant proteins are crucial to generate efficient saponin-based vaccine against *Apicomplexan Babesia divergens*. Acylated and deacylated saponins of *Quillaja saponaria* mixture as adjuvants for the FML vaccine against visceral leishmaniasis.

Vaccination studies with purified, native and recombinant Fasciola antigens suggest that this approach to diminished morbidity and mortality and reduced transmission is a realistic goal. Among the major potential vaccine candidates are Fatty Acid Binding Protein (FABP), cysteine (cathepsin) proteases, haemoglobulin, leucine aminopeptidase and a saposin-like protein. In the case of *F. hepatica* FABP, cross-reaction and cross-protection against *S. mansoni* is an important feature. In addition to protective effects with significant worm burden reductions.

Some vaccine candidates also have anti-fecundity (smaller flukes), anti-pathology (less liver lesions) and anti-embryonation effects. Optimism is tempered by the fact that fascioliasis in humans is an orphan disease and in need of governmental and foundation support (Hillyer, 2005).

It has previously been shown that dogs can be vaccinated against heterologous *Babesia canis* infection using a vaccine containing Soluble Parasite Antigens (SPA) from in vitro cultures of *B. canis* and *B. rossi* that are adjuvanted with saponin (Schetters *et al*., 2006) .

Present results showed clearly that the electrophoretic analysis of *F. gigantica* worms homogenate, *F. gigantica* worms homogenate mixed with saponin and saponin, give 8, 7 and 9 bands, respectively. Moreover, the data obtained from the electrophoretic analysis showed the existence of two common bands between *Fasciola gigantica* worms antigen and saponine and one common band between saponin and *Fasciola gigantica* worms homogenate mixed with saponin, respectively.

Present results are in agreement with the data obtained by Tolba *et al.* (1995) who studied the

electrophoresis profile of *Biomphalaria alexandrina* snail antigens after irradiation and chemical treatment. The electrophoretic results are in accordance with the data obtained by Farrag *et al.* (2005) who studied the electrophoresis analysis of crude *B. alexandrina* and *Lymnaea cailliaudi* snail antigens and prove the presence of common bands (shared antigens) between these snail antigens.

The acute schistosomiasis phase is an immunologically active period during which serum levels of IgG significantly elevated. The immune response may be an important determinant of host susceptibility to *S. mansoni* infection (Suda *et al.*, 1997). The present studies showed that homogenate in combination with or without saponine has an immunomodulatory effect by increasing the level of IgM and IgG against CAP, SWAP and SEA.

Present result revealed that saponin had immunological adjuvant activity and elicited induction in splenocyte proliferation by increasing the mean number of splenocytes. As well as *Fasciola gigantica* worms homogenate mixed with saponin or non mixed showed an immunocellular stimulation by increasing the mean number of splenocytes in vaccination against schistosomiasis. Our result are agreement with Oliveira Freitas *et al.* (2006) who showed that the Riedel de Haen saponin extract the presence of deacylsaponins normonoterpene-deprivated which are non-toxic and capable of inducing a specific and strong immunoprotective response and a stronger Leishmania-specific splenocyte proliferation in vaccination against murine visceral leishmaniasis. Ginsenoside Rd (Rd), a saponin isolated from the roots of panax notoginseng had immunological adjuvant activity and elicited a Th1 and Th2 immune response by regulating production and gene -expression of Th1 cytokines and Th2 cytokines (Yang *et al.*, 2006).

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