

IMMUNOSTIMULATORY EFFECTS OF EXTRACT OF *PULICARIA CRISPA* BEFORE AND AFTER *SCHISTOSOMA MANSONI* INFECTION

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Abstract: The immunostimulatory effects of methanolic extract from *Pulicaria crispa* were investigated in mice before and after infection with *Schistosoma mansoni*. Mice were subjected for daily intra-peritoneal injection by the extract (33 ng/mouse) for 10 successive days followed by infecting every mouse with 100 *S. mansoni* cercariae. Treatment with the extract induced significant increase ($p < 0.05$) in sera-IL-2 before and after infection. Upon using soluble worm antigen preparation or cancer bladder homogenates as antigens in ELISA, the detected levels of IgG were significantly ($p < 0.05$) higher in sera from treated-infected mice than untreated *P. crispa* infected mice. Using crude *Escherichia coli* lysate as an antigen in ELISA, it was detected a significant ($p < 0.05$) increase in IgG levels in sera from the extract-treated mice before and after infection.

Keywords: *Schistosoma mansoni*, *Pulicaria crispa*, Interlukin-2, crude *E. coli* antigen

Schistosomiasis has been endemic in Egypt at least since the time of the ancient pharaohs, as indicated by presence of calcified ova in the Egyptian mummies (1). It is currently a widespread endemic disease in 75 countries, while 200 million people residing in rural and agricultural areas are infected, between 500 and 600 millions are at risk of exposure to infection (2). Symptoms associated with schistosomiasis include weakness, diarrhea, hepatosplenomegaly, and carcinoma of the intestine, liver, uterus as well as bladder (3). One of the highly implicated etiological factors in urinary bladder carcinoma, the most common malignancy in the Middle East and parts of Africa, is infection with the parasite *Schistosoma haematobium* (4).

Praziquantel still represents the drug of choice for treating human schistosomiasis although the recent reports describe some bilharzial isolates from several African countries resistant to this drug (5). Till present, there is no available effective vaccine that can fully protect against schistosomiasis infection, in spite of extensive work carried out over the last 2 decades to evaluate the protective potential of several candidate parasite molecules (6). Emergence of resistance to praziquantel therapy and absence of

an effective vaccine reflect the very urgent need for alternative effective therapeutic approaches against human schistosomiasis.

Herein, we studied immunomodulatory effect of a methanolic extract from aerial parts of a bushy desert plant belonging to family Compositae, *Pulicaria crispa*. The plant grows in cushion shape and is widely distributed in the Mediterranean basin in Algeria, Libya, France, Syria, Palestine and Egypt. A previous report on phytochemical screening of such plant extract used in the present work revealed the presence of amino acids, carbohydrates, coumarins, flavonoids, glycosides, proteins, sterols, tannins, triterpenes and volatiles (7).

The reason of studying the immunostimulatory effect of such extract is that fractionation of *P. crispa* indicated that it is rich in flavonoids (8), the compounds known to stimulate CD4⁺-T lymphocytes that represent the major source of the IL2 cytokine (9).

Immunomodulatory effects of crude plant extracts or purified flavonoids from plant origin as demonstrated by significant increase in IgG response against several *S. mansoni* antigens were previously reported (10, 11).

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To evaluate the immunomodulatory potential of such extract, we quantified serum IL2 in treated infected and uninfected mice in comparison to control untreated uninfected animals, as such cytokine was previously reported to be involved in protective immunity against both schistosomiasis infection and cancer (12).

In addition, we quantified IgG levels in sera from treated animals with the extract against 3 crude antigens prepared from *S. mansoni* adult worms, cancer bladder tissue and *E. coli*. The reason of including such crude antigens was to study if the induced non specific immune responses by treatment with such plant extract might target such infections.

EXPERIMENTAL

Preparation of plant extract

One hundred grams of air-dried powdered aerial parts of *P. crista* were extracted with 90% methyl alcohol till exhaustion in a continuous extraction apparatus. The solvent was stripped off by distillation under reduced pressure at temperature not exceeding 45°C using rotatory evaporator and dried to constant weight in vacuum desiccators over anhydrous CaCl₂. The experiment was repeated 3 times and the mean percentage of extractives was determined. From 100 g of the air-dried powdered fresh aerial parts, 10.3 g of extract were reproducibly obtained. The final extract was dark brown sticky residue of a stringent taste (7).

Treatment and infection

All the experimental animal work was carried out according to the standard regulations and guide lines of the National Research Center of Egypt. Female Swiss albino mice, weighing 18-20 g were obtained from the animal house of the institute (National Research Centre, Dokki, Egypt). Five mice were injected intraperitoneally with the methanolic extract of *P. crista* at a dose of 33 ng/mouse. Ten days after treatment, individual mice were bled; sera were separated and frozen till being used. On the 11th day after treatment with *P. crista*, 5 mice were infected by the tail immersion method (13) using 100 *S. mansoni* cercariae per mouse. Additionally, an infected untreated group and uninfected untreated group, each of 5 mice, were included. Six weeks after infection, blood was collected from individual mice; sera were separated and frozen till being used.

Assessments of worm recovery

Recovery of adult *S. mansoni* worms from livers of treated *S. mansoni*-infected mice and from

untreated *P. crista* infected ones was done by perfusion through hepatic portal vein (14). The reduction in the percent of the recovered worms upon treatment was calculated (15) as follows:

$$P = C - V/C \times 100$$

where P = % protection, C = mean number of worms recovered from infected untreated mice and V = mean number of worms recovered from treated *S. mansoni*-infected mice.

Antigen preparations

Crude *Escherichia coli* lysate (ECL) from (Bio-Rad Laboratories, Richmond, CA) was reconstituted to the desired concentration in water and stored at -80°C till use. Crude soluble worm antigen preparation (SWAP) from the *S. mansoni*-Egyptian strain was obtained from the Theodore Bilharz Research Institute, Giza, Egypt. Freshly excised cancer bladder tissue from malignant patients with history of *S. haematobium* infection (kindly provided by Prof. Hussein Khaled, the National Cancer Research Institute, Egypt), was homogenized in 0.1 M phosphate buffered saline. The homogenate was centrifuged at 16000 × g for 20 min. The supernatant was carefully collected, divided into aliquots and subjected for protein determination using the BCA reagent kit (Pierce; Rockford, Ill.) before being frozen at -80°C till being used.

Detection of interleukin-2 (IL-2) using sandwich ELISA

A plate was coated with anti-IL2 (raised in goat) at a concentration of 3 µg/mL in 0.1 M phosphate buffered saline containing 0.05% Tween 20 (PBS-0.05%T) containing 1% BSA. The plate was incubated at room temperature overnight then washed 3 times with PBS-0.05%T. The uncoated spaces on the plate wells were blocked against non specific binding using PBS-0.05%T-1% BSA (200 µL/well) then left for 1 h at room temperature. After 3 washes, mice sera (1:100; 100 µL/well) were incubated within the plate wells at 37°C for 2 h. After 3 washes, goat-anti-IL2 (3 µg/mL; 100 µL/well) was incubated within the plate wells for 1 h at 37°C. Peroxidase conjugated monoclonal anti-goat IgG (1:10000; 100 µL) was incubated within the plate wells for 1 h at 37°C. After washing, the substrate, *o*-phenylenediamine (OPD), was diluted in substrate buffer (0.1 M citric acid anhydrous, 0.2 M dibasic sodium phosphate, pH 5.00) containing 0.06% H₂O₂ then applied (100 µL/well) to the plate and left for 15 min at room temperature. The reaction was stopped using sulfuric acid. (4 M; 50 µL/well) and the changes in the optical densities were recorded at

$\lambda_{\max} = 490$ nm using microtiter plate reader (TECAN-SUNRISE, Austria).

Detection of IgG against ECL, SWAP and CBH using enzyme linked immunosorbent assay (ELISA)

The assay was performed according to Bahgat et al. (16). All antigens were diluted in the coating buffer (1 M Na_2CO_3 , 1 M NaHCO_3 , pH 9.6). Plate wells were coated with soluble worm antigenic preparation (SWAP; 5.5 $\mu\text{g/mL}$, 100 $\mu\text{L/well}$), cancer bladder homogenate (CBH; 0.1 mg/mL, 100 $\mu\text{L/well}$) and *E. coli* lysate (ECL; 10 $\mu\text{g/mL}$; 100 $\mu\text{L/well}$) and incubated overnight at room temperature then washed 3 times with PBS-0.05%T. Antigens free sites were blocked against non-specific binding using PBS-0.05%T containing 1% BSA (200 $\mu\text{L/well}$), left 1 h at room temperature and washed 3 times with PBS-0.05%T. A volume of 100 $\mu\text{L/well}$ of diluted mice sera (1:100 in PBS-0.05%T containing 1% BSA) was added to each well and incubated at 37°C for 2 h. After 3 washes, peroxidase conjugated anti-mouse IgG (1:10000 in PBS-0.05%T-1% BSA; 100 $\mu\text{L/well}$) was incubated within the plate wells for 1 h at 37°C. After 3 washes, diluted OPD in substrate buffer (as above mentioned) was applied (100 $\mu\text{L/well}$) and the plate was left for 15 min at room temperature. The reaction was stopped using sulfuric acid (4 M; 50 $\mu\text{L/well}$) and the change in the optical densities were recorded at $\lambda_{\max} = 490$ nm using microtiter plate reader (TECAN-SUNRISE, Austria).

Statistical analysis

The means and standard deviations were deduced using Student's *t*-test.

RESULTS AND DISCUSSION

Anti-schistosomal effect of *P. crispa* extract

The reduction in the mean percentage of the recovered worms from the *P. crispa* extract treated-infected mice (48.48%) in comparison to infected untreated ones is presented in Table 1.

IL-2 levels in sera from mice treated with *P. crispa* extract

Sera from mice treated with the *P. crispa* extract showed significantly higher levels of IL-2 ($p < 0.05$) when compared with sera from untreated mice, however, there was no significant difference in the IL-2 levels between sera from treated-*S. mansoni* infected and untreated *S. mansoni* infected mice (Table 2).

IgG levels in sera from mice treated with the *P. crispa* extract against ECL in ELISA

Sera from treated mice showed significantly higher IgG reactivities ($p < 0.05$) against ECL when compared to sera of untreated mice (Table 3). Also, sera from treated mice that received *S. mansoni* infection showed significantly higher IgG reactivities ($p < 0.05$) against ECL in comparison to sera from infected untreated mice (Table 3).

IgG levels measured by ELISA in sera from mice treated with *P. crispa* extract against SWAP

Sera from mice treated with the *P. crispa* extract followed by *S. mansoni* infection showed significantly higher IgG levels ($p < 0.05$) against SWAP when compared to sera of untreated infected mice (Table 4).

ELISA detection of IgG levels against CBH in sera from mice treated *P. crispa* extract after *S. mansoni* infection

Sera from mice treated with the *P. crispa* extract followed by *S. mansoni* infection showed significantly higher IgG levels ($p < 0.05$) against CBH when compared to infected untreated mice (Table 5). Also, sera from infected mice that were previously treated with the plant extract showed significantly higher IgG levels ($p < 0.05$) against CBH when compared with sera from treated uninfected mice (Table 5).

Our results showed that *P. crispa* extract have an immunomodulatory effect by stimulating significant IL-2 production as well as a significant increase in the IgG levels against SWAP, CBH and ECA in sera of treated mice when compared to control ones. CD4^+ T cells are the major source of IL-2 that is selectively expressed under variety of immunoregulatory controls and can modulate the host immune response (17-19).

Noteworthy, *S. mansoni* infection also results in liver granuloma formation that might also be accompanied by CD4^+ T cells infiltration and IL-2 production (8). Thus, the measured IL2 in treated animals after infection might be not only due to the treatment but also to granuloma resulting from *S. mansoni* infection (8).

The significant increase in the IL-2 and IgG levels in mice after treatment with the *P. crispa* extract might be due to the fact that *P. crispa* extract was previously reported to be rich in flavonoids (17, 19), compounds known to induce a wide range of immunomodulatory effects including induction of IgG (18) and IL-2 both *in vitro* and *in vivo* (19).

In a previous report, it was shown that treating mice with flavones resulted in significant production

Table 1. Anti-bilharzial effect of *P. crista* extract as demonstrated by reduction in *S. mansoni* worm burden.

Group	<i>P. crista</i> treated infected	Untreated <i>P. crista</i> infected
Mean $\pm$ SD	17.00 $\pm$ 1.5	33.00 $\pm$ 1.0
% reduction	48.5	

Table 2. Detection of IL-2 levels in sera from mice treated with *P. crista* extracts before and after infection with *S. mansoni*.

Group	<i>P. crista</i> treated uninfected	Untreated <i>P. crista</i> uninfected	<i>P. crista</i> treated infected	Untreated <i>P. crista</i> infected
Mean $\pm$ SD	0.37 $\pm$ 0.05	0.29 $\pm$ 0.03	0.32 $\pm$ 0.01	0.35 $\pm$ 0.02
p-value	< 0.05		< 0.05	

Table 3. Detection of IgG levels in sera from mice treated with *P. crista* before and after infection with *S. mansoni* by ELISA against *E. coli* lysate as an antigen.

Group	<i>P. crista</i> treated uninfected	Untreated <i>P. crista</i> uninfected	<i>P. crista</i> treated infected	Untreated <i>P. crista</i> infected
Mean $\pm$ SD	0.46 $\pm$ 0.06	0.26 $\pm$ 0.02	0.61 $\pm$ 0.02	0.34 $\pm$ 0.01
p-value	< 0.05		< 0.05	

Table 4. Detection of IgG levels in sera from mice treated with *P. crista* before and after infection with *S. mansoni* by ELISA against soluble *S. mansoni* worm antigen preparation.

Group	<i>P. crista</i> treated infected	Untreated <i>P. crista</i> infected
Mean $\pm$ SD	0.56 $\pm$ 0.02	0.43 $\pm$ 0.01
p-value	< 0.05	

Table 5. Detection of IgG levels in sera from mice treated with *P. crista* before and after infection with *S. mansoni* by ELISA against crude cancer bladder homogenate as an antigen

Group	<i>P. crista</i> treated infected	Untreated <i>P. crista</i> infected
Mean $\pm$ SD	0.55 $\pm$ 0.04	0.37 $\pm$ 0.03
p-value	< 0.05	

of CD4⁺T cells, IgM and IgG as measured by ELISA against several crude *S. mansoni* antigens (10). Also, flavones can modulate both production and levels of several signaling molecules associated with immune function and inflammation, including several cytokines *in vitro* (21-23). Treating mice with flavones purified from *Daucus carota* induced both significant IgG levels and reduction in *S. mansoni* worm burden that reflects both immunomodulatory and anti-bilharzial effects (9). Short term treatment of humans with flavonoid drugs induces IgE antibodies, while long term administration of the same drugs induces IgG antibodies (18). Also, *P. crista*

extract is known to be rich in terpenoids (7, 11, 24, 25), compounds that have immunostimulatory effects as measured by significantly increasing number of antibody producing cells in both spleen and bone marrow as well as antibody production (26).

Cross reactive humoral responses between *S. mansoni* and the ECL used in the present work were preliminary reported by our group (27), followed by a very recent detailed study (28) in which we clearly demonstrated that *E. coli* shares T- and B- epitopes with *S. mansoni*.

A purified flavone from *Cirsium japonicum* known as FLCJ was reported for its activating effect

for the innate immune system and inhibition of tumor growth (29). Previous reports clearly demonstrated that treating mice with *P. crispa* resulted in significant reduction in *S. mansoni* worm burden as well as a significant increase in both IgG and IgM levels against crude cercarial, worm and egg antigens (30).

In conclusion, treating mice with methanolic extract of *P. crispa* reduces the *S. mansoni* worm burden and stimulates significant IL2 production as well as significant IgG response that reacted in ELISA against bilharzial, *E. coli* and cancer bladder antigens. This might represent a new hopeful alternative approach for therapy of infection with such diseases and should need further detailed studies.

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