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ASTRAGALUS MEMBRANACEUS AND *LONICERA JAPONICA*: PROMISING FOR NOVEL *SCHISTOSOMA MANSONI* CONTROL APPROACH

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Abstract

Immunostimulants have the ability to reduce susceptibility to different infections and to enhance the overall health. This study assessed the effect of *Astragalus membranaceus* and *Lonicera japonica*, Chinese herbs, on *Biomphalaria alexandrina* snails' susceptibility to *Schistosoma mansoni* infection and their consequences on certain biochemical parameters of these snails, promising for novel *S. mansoni* control approach. Four snails groups each included 50 snails; G1: was infected control, G2: was fed on 0.1% Astragalus extract, G3: was fed on 0.1% Lonicera extract and G4: was fed 0.1% *Astragalus* extract and 0.1% *Lonicera* extract. Snails were fed on immunostimmulants for 7 days then exposed to *S. mansoni* miracidia. The snails' survival rate significantly increased with decreased infection rate in groups exposed to a combination of both herbs with significant suppression in the cercarial production/infected snail in all treated groups compared to control group. A significant increase in the total hemocytic count, glucose and total protein content in soft tissues in all treated groups compared to the control group. Moreover, there was a significant increase of hemolymph total lipid and a significant decrease in aspartate transaminase (AST) and alanine transaminase (ALT) in snails groups treated with herbs compared to control group. *A. membranaceus* and *L. japonica* improved *B. alexandrina*' resistance to schistosomiasis *mansoni* as a novel control.

Key words: Immune response, Chinese herbs, *Biomphalaria alexandrina, Schistosoma mansoni,* infection rate, biochemical parameters.

Introduction

Schistosoma mansoni is the most serious tropical disease after malaria in terms of mortality and morbidity. There is no vaccine accessible against *S. mansoni* and the available chemotherapy is a single drug, praziquantel, for which resistant cases were detected (Melman *et al,* 2009). *S. mansoni* life cycle needs specific freshwater snails as intermediate hosts and human to water contact. *Biomphalaria alexandrina* have their medical and epidemiological importance as intermediate hosts for *S. mansoni* and they should gather considerable research attention. A well understanding of the immunobiological interactions between *B. alexandrina* intermediate host and its parasite *S. mansoni* could be helpful in developing new strategies for preventing and/or controlling schistosomiasis (Galinier *et al,* 2013).

Immunostimulants have gain more attention during the last two decades because of its ability to reduce susceptibility to different infections and diseases and their role in enhancing the overall health by modulating the immune responses (Song *et al*, 2000).

Certain Chinese herbs are one of the immunostimulants used as a traditional medicine for thousands of years (Tan and Vanitha, 2004). They contain many active components which were studied in human cell lines, mice, chickens, fish and snails (Jian and Wu, 2004; Lin and Zhang, 2004; Liu *et al,* 2004; Shao *et al,* 2004; Mary Jane *et al,* 2015). Abd El-Aal *et al*. (2017) reported that Paeoniflorin, Chinese herbal decreased and reversed schistos- omiasis *mansoni* fibrosis. *Astragalus* root extracts have been used in Chinese traditional medicine, as immunostimulants (Lee *et al,* 2003). *Lonicera japonica* is also used as traditional medicine for fever, headache and as an anti-inflammatory and immunomodulatory agents (Lee *et al,* 2001; Wu *et al,* 2004; Kumar *et al,* 2005).

The current trial was to assess the effect of both Chinese medicinal herbs; *A. membranaceus* and *L. japonica* on *B. alexandrina* snails' susceptibility to *Schistosoma* *mansoni* miracidial infection and their consequences on certain biochemical parameters of these snails, promising for novel *S. mansoni* control approach.

Materials and Methods

Herbal extracts: Powdered *Astragalus* extract containing 90% of *Astragalus* polysaccharide and powdered *Lonicera* extract containing 25% of chlorogenic acid were commercial products of Nantong Sihai Plant Extracts Co., Ltd., China.

Snails and *S. mansoni* ova: Clean *B. alexandrina* snails (5-6mm in diameter) were obtained from the Medical Malacology Department, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza and were maintained in the laboratory of Parasitology Department, Faculty of Medicine, Zagazig University in well aerated water under controlled conditions with free access to food (Oven dried lettuce ad libitum). Dead snails were removed daily and the survival rate was calculated. *S. mansoni* ova were obtained from livers of previously infected mice and were allowed to hatch in small amount of dechlorinated water under a direct light for about 15 min. Hatched miracidia were collected using Pasteur pipette under a stereomicroscope and used for snails infection.

Experimental design: Snails were allocated into 4 groups (50 snails/ group) and fed on diets containing different medicinal herbs; group 1; was infected control, group 2; was fed on 0.1% *Astragalus* extract, group 3; was fed on 0.1% *Lonicera* extract and group 4; was fed on 0.1% *Astragalus* extract and 0.1% *Lonicera* extract. Snails were fed on immuno-stimmulants for 7 days then exposed to *S. mansoni* miracidia.

Hemolymph pooling & total hemocytic counts: Hemolymph of *B. alexandrina* was collected from different snails groups according to Borges *et al*. (2006). Snails were cleaned with 70% ethanol to remove debris then the shell was perforated with an insulin needle at the digestive gland level. The hemolymph obtained from each individual snail was aspirated and collected in a tube in an ice-bath. Total number of hemocytes in each experimental group was counted by diluting freshly collected hemolymph in leucocytes count solution in (1:20) ratio using a hemocytometer. Hemocytes were counted for 3 replicates and the mean number of circulating hemocytes was calculated.

Differential hemocytes count: Hemolymph (10µl) from each group were placed on slides and they were allowed to dry at room temperature for 20min. Cells were then fixed with methanol for 10min. and the hemocytes were stained with 10% Giemsa for 15 min. Slides were rapidly washed with distilled water and were examined with oil immersion lens (Brayner *et al*, 2005).

Exposure of *B. alexandrina* to *S. mansoni* miracidia*: B. alexandrina* were exposed individually to *S. mansoni* infection (8-10 miracidia/snail) in multidish plates filled with 2ml dechlorinated tap water for 24 hours (Anderson *et al*, 1982).

Cercarial shedding: Snails were examined for cercarial shedding individually, 21 days post miracidial exposure (Haroun, 1996). Few drops of iodine solution were added to cercarial suspension and cercariae were counted and recoded for each snail.

Estimation of some biochemical parameters of *B. alexandrina:* One gram of snails' soft tissues from each group was homogenized in 5ml distilled water at pH 7.5. A glass homogenizer was used and the homogenate was centrifuged for 10 minutes at 3000 rpm, then the fresh supernatant was used. Protein and glycogen contents were determined spectrophotometrically. Tissues total protein content was determined (Lowry *et al*, 1951) and glycogen was evaluated (Carroll *et al*, 1956).

Hemolymph glucose concentration was determined by glucose oxidase (Trinder, 1969), total protein was determined (Gornall *et al*, 1949) using kits from Bio-diagnostic, total lipid was determined (Knight *et al*, 1972) using kits from Biodiagnostic, Egypt and transaminases (AST & ALT) activities were determined using kits from Biocon Chemical Co., Germany (Reitman and Frankel, 1957).

Animal ethics: All experimented with animals met the international guidelines approved by the Ethics Committee for Animal Exp- erimentation, Faculty of Medicine, Zagazig University

Statistical analysis: Data were expressed as mean ± SD and the obtained data were statistically analyzed using Mantel Hanzel test, ANOVA test and "chi-square" values of contingency tables to determine the significant differences in means between the control and the experimental groups, Statistical analysis was performed by SPSS computer program (version 20 for windows).

$$\% \text{ R} = \frac{100\ (C - E)}{C}$$

Where C: control group, E: experimental snail groups (Penido *et al*, 1994).

Results

There was an increase in the snails survival rates at 1st shedding in groups exposed to *A. membranaceus* and *L. japonica* compared to the control group with a significant difference between the snails group exposed to a combination of both herbs compared to the control group (P<0.05).The snails survival rates were 86%, 88% &92% in group 2, 3, and 4 respectively, compared to 76% for control group (Tab. 1). Exposure of *B. alexandrina* snails to the two herbs for 7 days results in a high significant suppression in their infection rates in all experimental groups compared to control group (P< 0.001) with a percentage of reduction (30.43%, 36.96% & 58.7% for groups 2, 3 & 4 respectively).

The cercarial shedding began 34 days post-miracidial exposure in all groups. Total cercarial production/infected snail groups treated with the tested herbs was 787±238, 758±216 & 416±134 cercariae/snail in groups exposed to *A. membranaceus, L. japonica* and both herbs respectively, compared to 2221± cercariae/snail of the control group (P<0.001). These reduction rates were 64.57%, 65.87% & 51.27% respectively.

The results revealed that treatment of *B. alexandrina* of both immunostimulants for 7 days led to a significant increase in the total hemocytic count in the treated combined groups as compared to the control group. Being 3.72±0.8, 3.81±0.98 & $4.33\pm1.4\times10^5$ hemocyte/ml in snails exposed to *A. membranece- us, L. japonica* and both herbs respectively compared to $2.12\pm0.7\times10^5$ hemocyte/ml in controls (Tab. 2)

Size and shape of hemocytes in *B. alexandrina* from control and treated snails was examined by light microscopie. Three cell types were detected; 1st type was granulocytes; round in shape, 10µm in diameter, cytoplasm filled with granules and nucleus eccentric.

The 2nd type was haylinocytes; 5-10um in size, polymorphic with an eccentric nucleus, and a homogeneous non-granular cytoplasm.

The 3rd type was lymphocyte-like cells with variable size (4-8) and shape (rounded to oval) and a granular cytoplasm without nucleus (Fig. 1.A). All these cells were detected in all groups with varied percentage and most dominant granulocytes.

There was a significant increase in glycogen content and in total protein content in soft tissues in herbs treated snails with compared to controls. Glycogen concentration in treated snails were 34.2±6.5, 33.7±7.8 & 38.91±7.2mg/g tissue in snails exposed to *A. membranaceus, L. japonica* and both herbs respectively compared to 30.3±8.9 mg/g tissue in controls. Total protein concentration in treated snails increased to 53.19±-13.6, 53.87±12.3 & 55.99±11.8mg/g tissue, and in both herbs respectively compared to 48.1±11.9 mg/g tissue in controls (Fig. 1). The glucose, protein and lipid concentrations in snails' haemolymph increased significantly in snails treated with both herbs as compared to controls. But, snails' infection raised the activities of the enzymes AST & ALT in comparison with the snail groups treated with herbs (Fig.2).

Table 1: Survival rate at 1st shedding, infection rate with *S. mansoni* miracidia & cercarial production of *B. alexandrina* in groups

Group / Parameter	Infected control	*Astragalus*	*Lonicera*	*Astragalus + Lonicera*	Test	P
Survival rate (%)	76	86	88	92*	MH 4.76	0.03
No. of infected snails Reduction%	46 -	32** 30.43	29** 36.96	19** 58.7	$\chi 2$ 32	<0.001
Total cercariae/snail Reduction%	2221 ± 523 -	787 ± 238** 64.57	758 ± 216** 65.87	416 ± 134** 51.27	F 325.5	<0.001

*: significant (P < 0.05) and **: Highly significant (P<0.01) means a compared with control infected group.

Table 2: Total and differential hemocytic count among snail groups

Group / Parameter	Infected control	*Astragalus*	*Lonicera*	*Astragalus + Lonicera*	F	P
Granulocytes (%)	1.31±0.4 (62)	1.9±0.5* (51)	1.94 ±0.6* (50.9)	2.2±0.7** (51)	22.43	<0.001
Hyalinocytes (%)	0.382±0.1 (18)	1.09±0.3* (29.3)	1.11 ±0.3* (29.1)	1.26±0.4** (29)	88.72	<0.001
Lymphocyte like cells (%)	0.424±0.1 (20)	0.73±0.24* (19.7)	0.76±0.25* (20)	0.87±0.28** (20)	23.48	<0.001
Total hemocytic count (x105 /mm3)	2.12±0.7	3.72± 0.8*	3.81±0.98*	4.33±1.4 **	45.06	<0.001

*: significant (P < 0.05) and **: Highly significant (P<0.01) means a compared with control infected group

Discussion

Understanding of *B. alexandrina* innate immune mechanisms involved in the defense against *S. mansoni* is a promising novel control approach.

Long time and till 2009 according to the WHO, herbs and traditional plants have been used in the developing countries to fortify the body and its immune system and to combat against diseases. 80% of people in these countries use these plants in order to control and treatment of many diseases as synthetic drugs have higher cost, many adverse effects and toxicity (Mahima *et al*, 2012).

In the present study, there was an increase at 1st shedding in snails groups exposed to *A. membranaceus* and *L. japonica* compared to control group with a significant difference between the snails group exposed to a combination of both herbs compared to control one (P<0.05). This result differed from that of Shaldoum *et al.* (2016) who exposed snails to Cu_2O nanoparticles. Increase the snail survival rate after treatment of herbs may be due to the decrease in the intensity of infection due to stimulation of the snail's immune system and the increase in the number of hemocytes. Moreover, increase the levels of glucose, protein and lipids in the treated snails may play a role in enhancing the vitality of the snails and hence increase their survival.

Regarding rate of snails' infection by *S. ma-nsoni* miracidia, the lowest infection rate was in the group exposed to a combination of both herbs with a statistical high significant reduction (58.7%) as compared to control group (P< 0.001). This reduction in infection rate agreed with Shaldoum *et al*. (2016) who trea-ted *B. alexandrina* with Cu2ONPs and with Mossalem *et al*. (2017) who treated snails with *Punica granatum* extracts, and reported that reduction was due to on hemocytes extraction and external feature that destroyed invading schistosomes larvae.

Dynamics of *S. mansoni* transmission is only by cercariae. In this study, snails treated with the herbs resulted in a high significant suppression in cercarial production/ infected snail in all experimental groups compared to control ones. DFecrease in cercarial number was recorded in snails exposed to cuprous oxide nanoparticles (Cu2O NPs) that was due to stimulation of adaptive immune response and deterioration of cercarial development (Shaldoum *et al*, 2016).

The snails' resistance against any invading organisms depends mainly on the circulating hemocytes activity and its plasma factors (Barbosa *et al*, 2006a, b; Abaza *et al*, 2016).

Treatment of *B. alexandrina* with both immunostimulants for 7 days led to a significant increase in total hemocytic count as compared to control group. These results agreed with Reverter *et al.* (2014) who reported that immunostimulants increased resistance to diseases by stimulating nonspecific and specific immune responses specially phagocytes (Wagner *et al*, 1984). Besides, Hashemi and Davoodi (2012) reported that *A. membranaceus* stimulated the immune system by the macrophages stimulation and production of immunoglobulins. Increase in total hemocytic count was reported by Etewa *et al.* (2011) after using *S. mansoni* whole worm antigen for activation of *B. alexandrina* immunity. But, there were a significant decrease in total hemocytic count and mean number of granulocytes by exposure of *B. alexandrina* to *Cryptostegia grandiflora* LC_{10} (El Sayed *et al*, 2011).

There were a significant increase in the total protein content in soft tissues and hemolymph of snails groups treated with herbs compared to the infected control ones. The decrease in tissue protein in the infected non treated snails resulted from intrusion of protein metabolism by protein synthesis inhibition or by internal organs damages by developing parasites (Tol-ba *et al*, 1997). A decrease in protein content of was reported by Abdel Kader and Tantawy (2000) after snails' exposure to *Agave fififera* and *Agave attenuate*. Bakry *et al.* (2002a) detected decrease in snails’ protein content using *Calotropis procera*, *Euphorbia nubia* and *Atriplex halimus*. This agreed with El-Sayed (2006) used *Ammi majus* flowers and leaves. El Sayed *et al.* (2011) recorded significant reduction in protein content and enzymes activities on exposure of *B. alexandrina* to *Cryptostegia grandiflora* (LC_{25}).

Snails' serum glucose derived mainly from tissue glycogen which is the most important source for anaerobic energy. There was a significant increase in the glycogen content in the soft tissues and the glucose concentrations in the haemolymph in the snails groups treated with herbs compared to the control group. The decrease in tissue glycogen in infected snails may be due to increased glycol- genolysis, to restore energy requirements, so glycogen content decreases and glucose level increases in hemolymph (Gade, 1983). Decrease in tissue glycogen level was reported after exposed to *Euphorbia pseudocactus*, *Yacca alaifolia* and *Portalacca oleracca* methanol extracts (Sakran and Bakry, 2005) and niclosamide (Mohamed *et al*, 2000).

In the present study, there was a significant increase of hemolymph total lipid in snails treated with *A. membranaceus and L. japonica* compared to control group. This data differed from Rajyalakshmi *et al.* (1996) and Abdel-Megeed (1999) who reported a decrease in total lipid content after using different herbs. The decreased lipid contents after infection could be due to reduction of lipids synthesis or due to marked decrease in tissue glycogen content, thus lipid was used as an energy source (El-Wakil and Radwan, 1991).

The raised activities of the AST & ALT in non- treated snails compared to those treated with herbs were due to cellular damage in different organs (Mohamed *et al*, 2012).

Conclusions

The outcome results showed that exposure of *B. alexandrina* to *A. membranaceus* and *L. japonica* herbs improved their immune status, increased their survival rate and decreased susceptibility to infection. The exposure increased glucose, total protein and total lipid contents of snail’s hemolymph and decreased AST & ALT activities.

These give them the power to resist and decrease susceptibility to *S. mansoni* infection. *A. membranaceus* and *L. japonica* herbs could be a biological control of schistosomiasis. Field studies are ongoing and will be published in due time.

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Explanation of figures

Fig. 1: Snails treated with *A. membranaceus* and *L. japonica* increased glycogen and total protein content in soft tissues. , *:significant (P < 0.05), **: Highly significant (P<0.01) means a compared with control infected group.

Fig.2: Effect of *A. membranaceus* and *L. japonica* on some biochemical parameters of *B. alexandrina*.. (A) Herbal treatment of snails increased glucose, protein and lipid concentrations in snails' haemolymph, (B) and activities decreased of enzymes AST & ALT. *significant (P < 0.05), **: Highly significant (P<0.01) means a compared with control infected group.