

**SUSCEPTIBILITY OF *BIOMPHALARIA ALEXANDRINA* SNAILS TO INFECTION WITH
SCHISTOSOMA MANSONI MIRACIDIA UNDER THE EFFECT OF SODIUM
ALGINATES AS IMMUNOSTIMULANT**

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ABSTRACT

Molluscs have an effective innate defense system consisting of cellular and humoral defense factors which can be modulated by immunostimulation. The effect of sodium alginates (as immunostimulant) on the immune response and susceptibility of *Biomphalaria alexandrina* snails and their infection with *Schistosoma mansoni* miracidia was studied. The snails were immersed in three different concentrations of sodium alginates (0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml) for three time intervals (1 Day, 3 Days and 7 Days). Exposure of snails to sodium alginates led to a significant increase in the total number of hemocytes in all experimental groups. The most significant increase was recorded after exposure to 0.5 mg/ml of sodium alginates for 7 days being 6650 hemocyte/ml compared to 1950 hemocyte/ml of control snails. Examination of hemocytes revealed that hemolymph contained three morphological different types of hemocytes, designated as round small, granular (spreading) hemocytes and haylinocytes, their average percentage was 20%, 62% and 18% of total cells respectively. Spreading hemocytes were the most affected cell type after treatment with sodium alginates. The most obvious effect on spreading hemocytes was after treatment with 1.0 mg/ml of sodium alginates for 7 days being 80% of total hemocytes. Infection of snails with *Schistosoma mansoni* miracidia was suppressed by exposure to sodium alginates and this was associated with significant reduction of cercarial production. It is concluded that exposure of *Biomphalaria alexandrina* snails to sodium alginates could enhance their immune ability to overcome infection with *Schistosoma mansoni* miracidia.

KEYWORDS: Sodium alginates, *Biomphalaria alexandrina*, *Schistosoma mansoni*, Hemocytes, Susceptibility and immunostimulant.

INTRODUCTION

Schistosomiasis remains as one of the most prevalent infections in the world (King and Dangerfield-Cha, 2008). At least 258 million people required prevention treatment for schistosomiasis (WHO, 2016). During the last two decades increased attention has focused on immunostimulants and nucleotides to reduce susceptibility to various stressors and diseases, as well as enhance the overall health of invertebrates. The immune response can be modulated by many effectors like bacteria, yeast, glucans, peptidoglucans, lipopolysaccharides and other polysaccharides which have been widely studied in fish and crustaceans (Fujiki and Yano, 1997; Song *et al.*, 2006).

Sodium alginate is a natural polysaccharide product extracted from brown seaweed that grows in cold water regions. It is soluble in cold and hot water with strong agitation and can thicken and bind. In presence of calcium, sodium alginate forms a gel without the need of heat (Cheng *et al.*, 2005). Sodium alginate can be used as

immune-modulator for shrimp and modify immune genes expression of shrimp (Chun-Hung *et al.*, 2006). It can also be supplemented in the diet as an immunostimulant to increase the immune ability of shrimp (Winton *et al.*, 2005).

Molluscs have an effective internal defense system consisting of cellular (Balan *et al.*, 1993; Reis *et al.*, 1995; Boehmler *et al.*, 1996 and Winka Le Clec'h *et al.*, 2016) and humoral (Loker *et al.*, 1994; Johnston and Yoshino, 1996; Kofta, 1997) defense factors. Circulating hemocytes represent the primary mediator of cellular defense reactions in molluscs. Several studies examining the composition of hemocyte populations of gastropods suggested that hemocytes are composed of a mixture of different types of cells. In *B. alexandrina* hemolymph samples, granulocytes, rounded cells and hylinocytes were observed (Negm *et al.*, 1995; and El-Sayed, 2006). Several functions have been attributed to the hemocytes in gastropods (Adema *et al.*, 1992) including an important role defense reactions such as phagocytosis

(Furuta *et al.*, 1990), encapsulation and wound healing (Yamaguchi *et al.*, 1989). Phagocytosis is a well-known internal immune defense mechanism in all animals. Phagocytosis is considered to be the primary clearance mechanisms in gastropods (Furuta and Yamaguchi, 2001). The last species from the invertebrates used in immunostimulant researches was a pulmonate snail, *Biomphalaria glabrata* (Mary Jane *et al.*, 2015).

The relationships between larval trematodes and their molluscan hosts are very close (Gibson, 1987). The dynamic interaction between them leads to either to a state of co-existence, in which the trematode survives and produces subsequent stages of its life cycle, or to incompatibility, where the trematode is either destroyed and eliminated by the host's internal defense response or fail to develop because the host is physiologically unsuitable (Bayen and Yoshino, 1989). The aim of the present work was to evaluate effect of immunostimulant sodium alginates on modulating the immune system of *Biomphalaria alexandrina* snails and reducing their susceptibility to infection with *Schistosoma mansoni* miracidia.

MATERIALS AND METHODS

Snails and *Schistosoma mansoni* ova

Biomphalaria alexandrina snails (5-6mm in diameter) used in the present studies were laboratory produced and obtained from colonies maintained in the Medical Malacology Department, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt. They were maintained in plastic aquaria (16 x 23 x 9 cm), provided with dechlorinated aerated tap water (10 snails / L) and covered with glass. Oven dried lettuce leaves were used for feeding. Water in the aquaria was continuously changed weekly, a photoperiodicity of 12hr. light/ 12hr. dark and water temperature of 25±1°C were kept. Dead snails were removed daily. *Schistosoma mansoni* ova used were obtained from CD1 mice livers previously infected with *Schistosoma mansoni* cercariae. The ova were allowed to hatch in small amount of dechlorinated water (25±1°C) for about 15 minutes under a direct light (desk lamp). Then, the fresh hatched miracidia were collected by Pasteur pipette under a stereomicroscope and used in the experimental tests.

Experimental material

A commercial form of Sodium alginates from Sigma-aldrish Company was provided by Egyptian International Center for Import.

Experimental groups for hemocytes examination

Ten experimental groups each of 30 snails as three replicates (10 snails/L dechlorinated water), were prepared as follows: group 1 was non-treated control. Group 2, group 3 and group 4 were treated with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml sodium alginates respectively for 1 day. Group 5, group 6 and group 7 were treated with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml immunostimulant material respectively for 3 days. Group

8, group 9 and group 10 were treated with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml immunostimulant material respectively for 7 days.

Hemolymph sampling for hemocytic counts

Collection of hemolymph of *Biomphalaria alexandrina* snails from different groups was performed as described by Negm *et al.* (1995). Water adhering to the snail was removed and the head foot was cleaned with tissue paper. By touching the foot with the point of the micropipette, the snail was forced to retract deeply into its shell and extruded hemolymph. Thus, as much as 30 µl of hemolymph were obtained from each individual snail.

Total hemocytes count

For total hemocytes count, 20 µl of hemolymph were individually collected from at least 5-10 snails/group after different intervals (1, 3 and 7 days) for each experimental material. The number of cells in every experimental and control groups was counted by diluting freshly collected hemolymph in leucocytes count solution in (1:20) ratio. Using a Bürker- Turk hemocytometer, the hemocytes were counted for 3 replicates and the mean number of circulating hemocytes was calculated.

Differential hemocytes count

Hemocytes monolayers were prepared by placing 10 µl of hemolymph on a glass slide and hemocytes were allowed to adhere the glass in a moist chamber for 15 min. at room temperature, then rinsed with snail Winger buffer/10 mM Ca⁺⁺ (SR) pH 7.3, and incubated in the same buffer for 10 min. Hemocytes were dehydrated with methanol for 5 min. at room temperature, rinsed several times with SR, stained with 10% Giemsa stain (Aldrich) in buffered distilled water (0.021M Na₂HPO₄/0.015M KH₂PO₄) pH 7.2 for 30 min. Differential hemocyte counts were recorded in every experimental and control groups and the mean ± standard deviation for each hemocyte population was calculated.

Experimental groups for infection experiment

Ten experimental groups each of 30 snails as three replicates (10 snails/L dechlorinated water), were prepared as follows: group 1 was only infected snails (control). Group 2, group 3 and group 4 were treated with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml immunostimulant material respectively for 1 day then exposed to *Schistosoma mansoni* miracidia. Group 5, group 6 and group 7 were treated with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml immunostimulant material respectively for 3 days then exposed to *Schistosoma mansoni* miracidia. Group 8, group 9 and group 10 were treated with 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml immunostimulant material respectively for 7 days then exposed to *Schistosoma mansoni* miracidia.

Exposure of snails to *Schistosoma mansoni* miracidia

Adult snails (5-6 mm in shell diameter) were exposed individually to 8-10 *Schistosoma mansoni* miracidia per snail in multidish plates filled with 2 ml dechlorinated tap water for 24 hrs as described by Anderson *et al.* (1982). After 24 hrs from exposure to miracidia snails were washed and transferred to clean aquaria with dechlorinated water and maintained under the same laboratory conditions mentioned above for maintaining stock colonies.

Examination of snails for *Schistosoma mansoni* cercarial shedding

After 21 days post miracidial exposure, surviving snails were individually examined for cercarial shedding in multidish plates. Post 3 hrs of exposure to light (desk lamp) using 2 ml dechlorinated water for each snail/well, positive snails were removed, marked and transferred to clean aquaria with dechlorinated water and maintained in the dark under laboratory conditions. Few drops of iodine solution were added to each well containing cercariae which were counted under stereomicroscope and recoded for each snail. This examination was carried out once weekly to avoid exhausting of snails.

Statistical analysis

Results were expressed as mean \pm SD and the obtained data were statistically analyzed using "t" test (Spiegel, 1981) 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 the SPSS computer program (version 20 for windows).

RESULTS

Total and differential counts of the hemocytes

The results in (Table 1), (Fig. 1) revealed that exposure of *Biomphalaria alexandrina* snails to different concentrations of sodium alginates for different times led to a significant increase in the number of hemocytes in experimental groups in comparison with control groups. Exposure of *Biomphalaria alexandrina* snails to 0.1, 0.5 and 1.0 mg/ml of sodium alginates for 1, 3 and 7 days resulted in increase in the number of hemocytes, being ((3750, 3300 and 4350), (3425, 4875 and 6650) and (4525, 3375 and 4800)) hemocyte/ml respectively compared to 1950 hemocyte/ml of control snails.

Table 1: Total hemocytes count/ml of *Biomphalaria alexandrina* snail's hemolymph exposed to sodium alginates.

Period of exposure	0.1 mg/ml	0.5 mg/ml	1.0 mg/ml	Control (unexposed)
1 day	3750 \pm 87**	3425 \pm 78**	4525 \pm 90***	1950 \pm 43
3 days	3300 \pm 176*	4875 \pm 120***	3375 \pm 101**	
7 days	4350 \pm 59***	6650 \pm 177***	4800 \pm 93***	

Data expressed as mean \pm SE, *, ** & *** = significantly different from control at $p < 0.05$, $p < 0.01$ & $p < 0.001$.

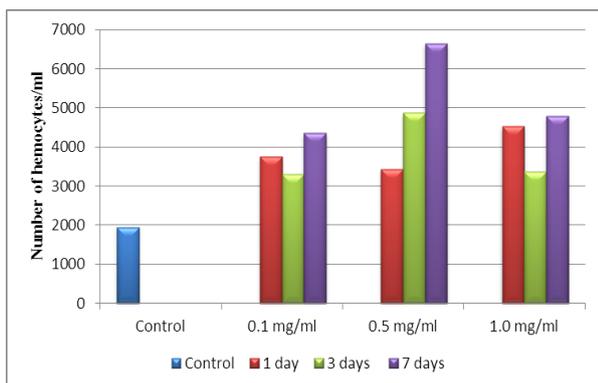


Fig. 1: Total hemocytes count/ml of *Biomphalaria alexandrina* snail's hemolymph exposed to sodium alginates.

The classification scheme of *Biomphalaria alexandrina* hemocytes presented in this study based on the cell size and shape. Fig.2 shows that examination of hemocytes monolayer obtained from control and treated snails using light microscope resulted in observation of three cell types. The first type was measuring 20-25 μ m in diameter, had plentiful cytoplasm with numerous pseudopodia, an irregular nucleus and were adhere to glass, their name granular (spreading) hemocytes denotes their active role in phagocytosis and other defense mechanisms. The second type was designated as round

small hemocytes; which was not fully differentiated, measuring 8-10 μ m in diameter; having a high cytoplasm-nucleus ratio; few cytoplasmic granules and do not adhere to glass or emit pseudopodia. The third type, haylinocytes was morphologically intermediate between round and granular (spreading) hemocytes, resemble round cells but differ in that they have lower nuclear-cytoplasmic ratio; measuring about 12-15 μ m in diameter; markedly basophilic cytoplasm with abundant dense granules and, in addition to their larger size, can form few and short pseudopodia.

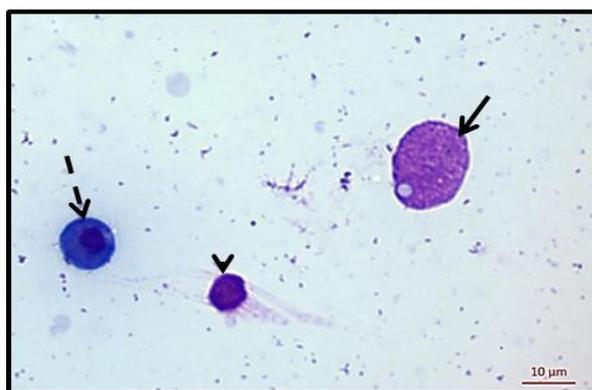


Fig. 2: Light photomicrograph of hemocytes from control *Biomphalaria alexandrina* snail showing

granular (spreading) hemocytes (arrow), round small (arrow head) and haylinocytes (dashed arrow).

The results in table (2) indicate that the three cell types were observed in all experimental groups but varied in their relative proportions according to the stressor. In normal conditions (control groups) the round small hemocytes recorded 20% of total hemocytes. Granular (spreading) hemocytes presented the dominant cell type being 62% of total hemocytes. While haylinocytes did not exceed 18% of total hemocytes.

Granular (spreading) hemocytes were the most affected cell type after treatment with sodium alginates recording 79%, 71% and 74% of total hemocytes after treatment

with 0.1, 0.5 and 1.0 mg/ml of sodium alginates for 1 day compared to 62% of total hemocytes of control snails respectively.

The most obvious effect on granular (spreading) hemocytes was after treatment with 1.0 mg/ml of sodium alginates for 7 days being 80% of total hemocytes compared to 62% of total hemocytes of control snails. The significant increase in granular (spreading) hemocytes proportion was associated with significant decrease in the proportions of the other cell types; recording 12% and 8% of total hemocytes for round small hemocytes and haylinocytes compared to 20% and 18% of total hemocytes of control snails respectively.

Table 2: Differential hemocytes count of *Biomphalaria alexandrina* snail's hemolymph exposed to sodium alginates.

Period of exposure	type of cell	0.1 mg/ml	0.5 mg/ml	1.0 mg/ml	control
1 day	Granular (spreading) hemocytes	79**%	71%	74*%	62%
	Round small hemocytes	12*%	18%	14%	20%
	Hyalinocytes	9*%	11%	12%	18%
3 days	Granular (spreading) hemocytes	69%	63%	68%	62%
	Round small hemocytes	20%	20%	23%	20%
	Hyalinocytes	11%	17%	9%	18%
7 days	Granular (spreading) hemocytes	68%	65%	80***%	62%
	Round small hemocytes	18%	20%	12*%	20%
	Hyalinocytes	14%	15%	8*%	18%

* & *** = significantly different from control at $p < 0.05$ & $p < 0.001$.

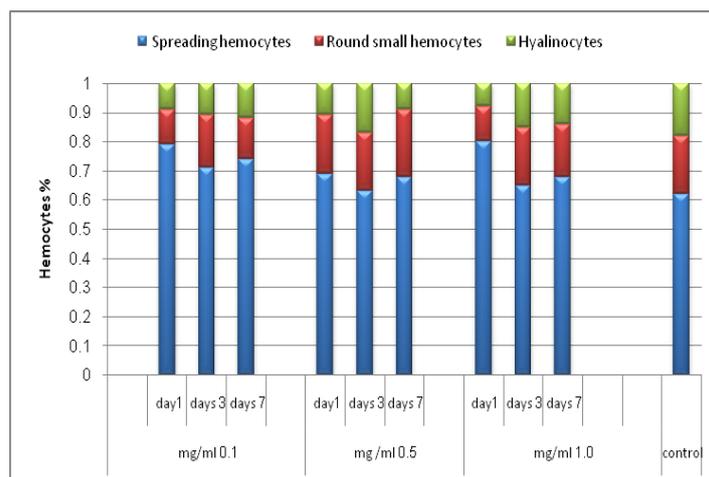


Fig. 3: Differential hemocytes count/ml of *Biomphalaria alexandrina* snail's hemolymph treated with sodium alginates.

Effect of sodium alginates on susceptibility of *Biomphalaria alexandrina* snails to infection with *Schistosoma mansoni* miracidia

As shown in table 3 and fig. 4, exposure of snails to different concentrations of sodium alginates for different times led to a significant reduction in the infection rate with *Schistosoma mansoni* miracidia in all experimental groups compared to control ones ($P > 0.001$).

The results of the study indicated that exposure of *Biomphalaria alexandrina* snails to 0.1 mg/ml of sodium alginates for (1 day, 3 days and 7 days) results a high suppression in the infection rates of the snails being (60%, 42.9% and 40.7% respectively) compared to 92.9% of control groups with a percentage of reduction 35.4%, 53.8% and 56.2% respectively. It was also noticed that raising the concentration of sodium alginates

to 0.5 mg/ml led to significant decrease in the infection rates recording (50%, 43.5% and 27.6%) after exposure for (1 day, 3 days and 7 days) respectively, compared to 88% of control group with a percentage reduction 43.2%, 50.6% and 68.6% respectively. Also, exposure of snails to 1.0 mg/ml of sodium alginates for (1 day, 3 days and 7 days) led to a significant reduction in the infection rates recording (44%, 44.8% and 53.6% respectively) compared to 96.3% of control snails with a percentage of reduction 54.3%, 53.5% and 44.3% respectively.

It is clear that exposure of snails to sodium alginates results a significant suppression in the cercarial production/infected snail in all experimental groups compared to control ones. (Table) 3 and (fig.5 a,b) revealed that exposure of snails to 0.1 mg/ml of sodium alginates for (1 day, 3 days and 7 days) results a significant decrease in cercarial production/infected snail recording (380.4, 444.7 and 347.5 cercariae/snail, respectively) compared to 628.9 cercariae/snail of control snails. The same conclusion was recorded for snails exposed to 0.5 mg/ml of sodium alginates for (1 day, 3 days and 7 days) being (280.7, 205.9 and 291.9 cercariae/snail, respectively) compared to 599.4 cercariae/snail of control snails.

The least number of cercariae/snail was recorded when the snails were treated with 1.0 mg/ml of sodium alginates for 3 days being 186.5 cercariae/snail versus 667.3 cercariae/snail in control groups with a percentage reduction 72.1%.

Table 3: Infection rate with *Schistosoma mansoni* miracidia and cercarial production of *Biomphalaria alexandrina* snails exposed to sodium alginates.

Parameter		0.1 mg/ml				0.5 mg/ml				1.0 mg/ml			
		Control	1 day	3 days	7 days	Control	1 day	3 days	7 days	Control	1 day	3 days	7 days
Infection of snails	Number	26	15	12	11	22	13	10	8	26	11	13	15
	%	92.9	60.0***	42.9***	40.7***	88.0	50.0***	43.5***	27.6***	96.3	44.0***	44.8***	53.6***
	% of reduction	—	35.4	53.8	56.2	—	43.2	50.6	68.6	—	54.3	53.5	44.3
Total cercariae/snail	Range	33-1015	83-620	55-865	31-720	42-983	22-567	25-476	8-532	32-1220	39-810	18-513	66-396
	Mean	628.9	380.4**	444.7**	347.5**	599.4	280.7***	205.9***	291.9***	667.3	308.9***	186.5***	192.6***
	±S.E.	28.5	22.3	42.8	29.4	27.6	20.9	21.5	28.4	29.7	34.7	17.2	15.3
	% of reduction	—	39.5	29.3	44.7	—	53.2	65.6	51.3	—	53.7	72.1	71.1

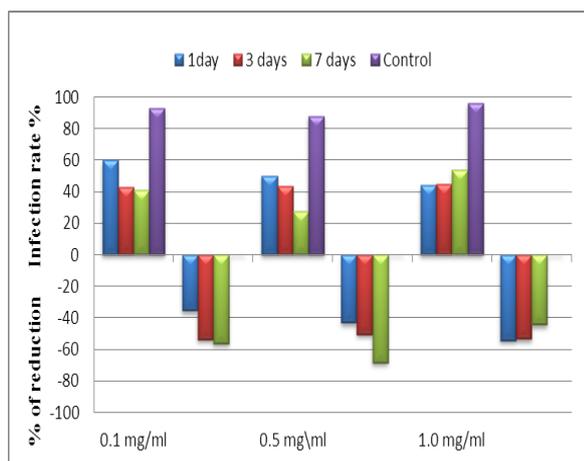


Fig. 4: Infection rate and percentage reduction with *Schistosoma mansoni* miracidia and cercarial production of *Biomphalaria alexandrina* snails exposed to sodium alginates.

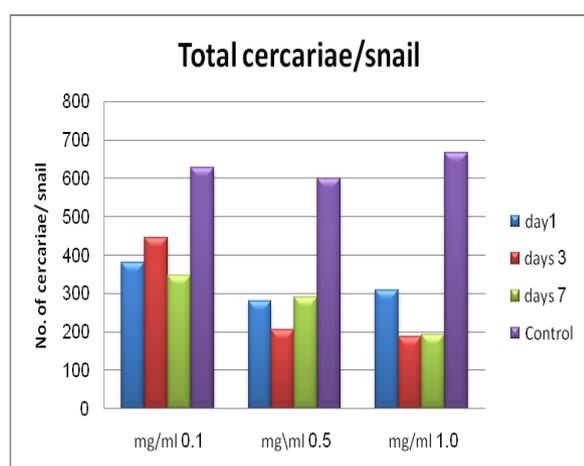


Fig. 5a: Cercarial production of *Biomphalaria alexandrina* snails exposed to sodium alginates.

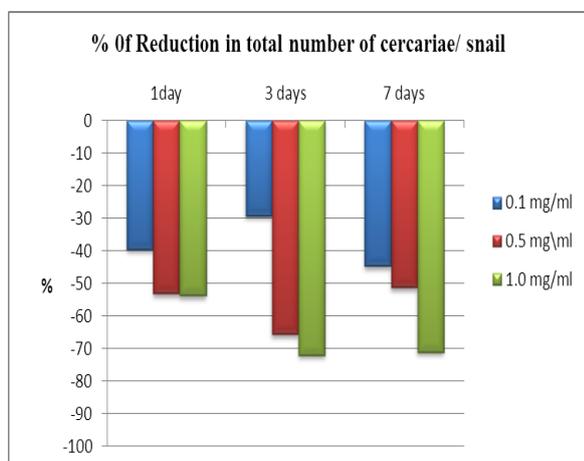


Fig. 5b: % of reduction in the number of cercariae/snail of *Biomphalaria alexandrina* exposed to sodium alginates.

DISCUSSION

Control of schistosomiasis could be achieved by chemotherapy, molluscicides and proper sanitation. The

use of alternative biological control methods targeting the snail intermediate hosts needs to be exploited as an avenue for disease management. Based on these facts and in view of extending problem of schistosomiasis in terms of morbidity, mortality, treatment cost and side effects, it was decided to study the effect of sodium alginates as immunostimulant material on *Biomphalaria alexandrina* snails. Natural immunostimulants like sodium alginates are biocompatible, biodegradable, cost-effective, safe for the environment as well as human health and enhancing disease and stress resistance (Ortuno *et al.*, 2002; Kadowaki *et al.*, 2013; Mohamed, *et al.*, 2015).

The present results show that exposure of *Biomphalaria alexandrina* snails to different concentrations of sodium alginates for different times led to a significant increase in the number of hemocytes in experimental groups in comparison with control groups, these results are in harmony with (Tingjun, 2009) who reported that cell number counting results of the Crab *Charybdis japonica* indicated that the total number of hemocytes increased after treatment with sodium alginates and inactivated vibrios.

In the present study light microscopic investigation recorded that, there are three types of hemocytes distributed in the hemolymph of *Biomphalaria alexandrina* snails. These cells are named granular (spreading) hemocytes, round small and haylinocytes. These results agree with several studies examining the composition of hemocyte populations of gastropods suggested that hemocytes are composed of a mixture of different subpopulations of cells. Negm *et al.*, (1995) and El-Sayed, (2006) found that *B. alexandrina* hemolymph samples were composed of granulocytes, rounded cells (undifferentiated cells) and haylinocytes. Also, Mohamed (1991); Mohamed & Abdel-Gawad (2005) classified the hemocytes of *B. alexandrina* snails to three main types with different morphological and functional characteristics, round hemocytes, granular (spreading) hemocytes and haylinocytes. On the other hand, Cheng (1975) and Sminia *et al.* (1979) reported two categories of hemocytes are found in *B. glabrata* snails, granulocytes and hyalinocytes. In addition, flow cytometric analyses of hemocytes from the freshwater snails *B. glabrata* demonstrated that circulating hemocyte populations could be divided into two main subtypes differing in their granularity and size (Johnston & Yoshino, 2001). El-Sayed, *et al.*, (2011) recorded that there are three types of hemocytes in the hemolymph of *B. alexandrina* and *B. glabrata* snails, these cells are granulocytes, haylinocytes and amoebocytes.

It is well known that granulocytes have a critical role in defense mechanism within the immune system of snails against invading foreign biotic and abiotic agents (Cavalcanti MG *et al.*, 2012). This was supported by the present data as granular (spreading) hemocytes were the most affected cell type after treatment with sodium

alginates for 1 day and 7 days causing a high significant increase in mean number of granular (spreading) hemocytes being 79% and 82% respectively.

The capacity of molluscs to respond strongly to stimuli depends on hemocyte viability and functional capacity. Studies carried out on *Biomphalaria tenagophila* demonstrate that the temporary increase in the number of circulating granulocytes results in decreased susceptibility to infection by *Schistosoma mansoni* (Pereira CAJ, *et al.*, 2006), this phenomenon was established in this study as the infection rate of snails with *Schistosoma mansoni* miracidia was suppressed by exposure to sodium alginates and this was associated with significant reduction of cercarial production. The present study shows that, the susceptibility of *Biomphalaria alexandrina* snails, exposed to 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml of sodium alginates for 1 day, 3 days and 7 days, to infection with *Schistosoma mansoni* miracidia was greatly reduced with a percentage of reduction (35.5%, 53.8%, 56.2%), (43.2%, 50.6%, 68.6%) and (54.3%, 53.5%, 44.3%) respectively. Similar results were stated by (Maftuch *et al.*, 2012) as administration of marine algae (*Gracilaria verrucosa*) enhanced some innate immune parameters in black tiger shrimp (*Penaeus monodon Fabricius*) and increased their resistance against *Vibrio harveyi* infection. El-Sayed, *et al.*, (2011) stated that, maintaining of *B. alexandrina* and *B. glabrata* snails at sublethal concentration LC₁₀ of latex aqueous solution of *cryptostegia grandiflora* for 3 days prior to miracidial exposure led to a significant reduction in the infection rate, this reduction was 55.5% and 58.9% respectively. This may be related to high increase in total hemocytic count and increased proportion of granular (spreading) hemocytes.

It is concluded from this study that exposure of *Biomphalaria alexandrina* snails to sodium alginates could enhance their immune ability to overcome infection with *Schistosoma mansoni* miracidia, as the total count of hemocytes was increased also the percent of granular (spreading) hemocytes was significantly elevated and this was accomplished by reduction in the infection rate of the snails and suppression of the cercarial production.

It is evident that, sodium alginates may be used as biocontrol agent for *Biomphalaria alexandrina*, the snail host of *Schistosoma mansoni* in Egypt and in evaluating their effect on schistosomiasis transmission.

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