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Immunological and physiological parameters of *Biomphalaria alexandrina* snails exposed to *Azadirachta indica* plant

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Abstract. – BACKGROUND, Plant molluscicides could be appropriate for snail control measures against schistosomiasis in endemic areas.

OBJECTIVES, The present study was undertaken to evaluate the immunological and physiological responses of *Biomphalaria (B.) alexandrina* snails to the effect of methanol extract of *Azadirachta (A.) indica* plant.

MATERIALS AND METHODS, Haemolymph samples were collected from snails treated with LC25 from methanol extract for 1 month and untreated snails. The collected haemolymph samples from treated and untreated snails with tested plant were used for flow cytometric analysis of cell cycle.

RESULTS, The obtained results indicated that hemolymph samples from *B. alexandrina* snails contained two morphologically distinct types of hemocytes, designated as Hyalinocyte and Granulocyte cells. In addition, the number of both snail's hyalinocytes and granulocytes and the mortality rate was significantly increased with treatment with *A. indica* extract. Phagocytosis in group treated with tested plant was highly significant increased than control one indicating a highly increase response of snail against the treatment. The lipid peroxide and glucose levels in hemolymph of treated snails were elevated while the protein and glycogen contents showed a decrease in soft tissues when compared with the control group. In addition, the activity level of some enzymes representing glycolytic enzymes as hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK), lactate dehydrogenase (LDH),and glucose phosphate isomerase (GPI); glycogenolytic enzymes as glycogen phosphorylase, glucose-6-phosphatase (G-6-Pase); gluconeogenic enzymes as fructose-1-6 diphosphatase (F-D-P ase), phosphoenolpyruvate carboxykinase (PEPCK) was also significantly reduced in response to treatment.

CONCLUSIONS, It was concluded that the application of methanol extracts of *A. indica* plant may be helpful in snail control as it interferes with the snails'immunology and physiology.

Key Words:

Biomphalaria alexandrina snails, *Azadirachta indica* plant, Phagocytosis by flow cytometry, Physiological parameters in snails' hemolymph and tissues.

Introduction

Schistosomiasis is a neglected parasitic disease caused by at least 8 species of blood flukes in the genus *Schistosoma*. Schistosomes infect over 200 million people across Africa, Asia, the Middle East, South America, and parts of the Caribbean[1-3]. *Biomphalaria* snails have a great medical importance as intermediate hosts of *Schistosoma mansoni*[4]. In this respect, interactions between *Biomphalaria* snails and schistosomes have received much attention. Defense system of freshwater snails mainly depends on hemocytes, which are the circulating cells involved in mediating internal defense and immune functions[5].

Molluscan innate immunity comprises humoral and cellular elements, with the cellular component considered to be more important for defense. Mobile cells called hemocytes are functionally analogous to mammalian monocytes and macrophages[6]. The hemocytes may be circulating in hemolymph or fixed in tissues. All molluscan species posses granular or agranular hemocytes[4,7,8]. A second type of hemolymph cells are small cells called hyalinocytes which may be agranular or slightly granular[9-11]. Mohamed[12] indicated that herbicide treatment of snails led to significant increase in total hemocytes count during exposure period. Examination of hemocytes

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monolayers resulted in observation of 3 morphologically different cell types, round small, hyalinocytes and spreading hemocytes.

The defense mechanisms and immunological responses which consist of the immune system have been considered as biomarkers of pollution in aquatic invertebrates[13]. Despite the lack of an adaptive immune system, invertebrates are able to survive among potential pathogens and respond to infection by activation of various defense mechanisms[14]. Phagocytosis is one of the various functions of hemocytes, which is a non-specific immune mechanism against non-self materials[13,15]. The response patterns, density and functions, of hemocytes can be affected by xenobiotics and parasites[16]. Furthermore, the most important metabolic pathways are glycolysis, gluconeogenesis and glycogenolysis. Contribution of these pathways elucidate the metabolic relationship between glucose, glycogen and energy release.

The present study was undertaken to evaluate the immunological and physiological responses of *B. alexandrina* snails to the effect of methanol extract of *Azardiracta indica* plant.

Materials and Methods

Snail

Laboratory bred *Biomphalaria alexandrina* snails (8-10 mm) were obtained from the Schistosoma Biological Supply Program (SBSC) at Theodor Bilharz Research Institute (TBRI), Imbaba Giza, Egypt.

Plant

The plant used in this study *Azadirachta indica* was collected from fields of Jeddah, Saudi Arabia during flowering stages and identified in Botany Department. Faculty of Science, Cairo University. The collected plant leaves[17] were left to dry in air and then in an oven at 50°C then powdered by a mixer.

Plant's Extract

The dry powder of the plant species was extracted by soaking with 95% methanol (0.5 kg/L) for seven days[18]. Then the solvent was filtered and distilled under vacuum and the crude extract residues were stored in clean dry dark vessel till use.

Bioassay Tests: Molluscicidal Screening

For plant extract, a series of concentrations that would permit the computation of LC_{50} and LC_{90} was prepared on the basis of weight/volume. Three replicates, each of 10 snails/L, were prepared. Another 3 replicates in dechlorinated water were used as control. Exposure and recovery periods were 24 hours each at 25±1°C[19,20]. Then, snails' mortality was recorded and corrected according to Abbots' formula[21].

Haematological Studies: Phagocytosis by Flow Cytometry

Hemolymph samples were collected from snails treated with LC_{25} from methanol extract for 2 weeks and untreated snails as outlined by Michelson and Du Bois[22] via removing a small portion of the shell and inserting a capillary tube into the heart. Hemolymph was pooled from 7 snails collected in a vial tube (1.5 ml) and kept in ice-bath. The collected hemolymph from treated and untreated snails with tested plant used for flow cytometric analysis of cell cycle.

Flow cytometry protocols were as described by Gagnaire et al[23,24]. For each sample, 3000 events were counted using an EPICS XL 4 flow cytometer (GMI, Inc., Ramsey, MN, USA, Beckman Coulter) equipped with a compact aircooked low power 15 mW argon ion laser beam (488 nm). Results were depicted as cell cytomgrams indicating cell size in terms of forward scattering cytometer (FSC, size) and side scattering cytometer (SSC, granularity, volume) with the fluorsescence channel(s) corresponding to the marker used. A gate was defined on the basis of FSC value in order to eliminate cell debris. The type of fluorescence recorded depended on the parameter to be monitored: reactive oxygen species (ROS) production, lysosomes and phagocytosis were measured using green fluorescence and cell mortality using red fluorescence. The EPICS XL 4 software allowed differentiation between supposed populations of granulocytes and hyalinocytes based on their FSC and SSC values. Mortality was quantified using 200 μL of hemocytes incubated in the dark for 30 min at 4 °C with 5 μL propidium iodide (PI, 1.0 mg ml^{-1}, Interchim Panjiva's manufacturer, San Pedro, CA USA). Lysosome presence was measured using a commercial Kit (LysoTarcker Green and DND-26, 1 mM in dimethylsulfloxide: DMSO, Molecular Probes, Life Technologies, Carlsbad, CA, USA). A 1 μL aliquot of Lyso Tracker was added to 200 μL hemocyte suspension. Cells were incubated for 2

hours in the dark at room temperature and the reaction was then stopped by placing the tubes on ice for 5 minutes. ROS production was measured using dihydrorhodamine 123 (DHR123, Molecular Probes), specific to the superoxide anion O_2. A 1 μL aliquot of a DHR123 solution (145 μM) was added to 200 μL hemocyte suspension. Cells were incubated for 30 minutes in the dark at room temperature and the reaction was then stopped by placing the tubes on ice for 5 minutes. Phagocytosis was measured by ingestion of fluorescent beads. Two hundred μL of hemolymph was incubated for 1 h in the dark at room temperature with 10 μL of a 1\10 dilution of fluorospheres carboxylate-modified microspheres (diameter 1 μm, Interchim).

Effect on Physiological Parameters in Snails' Hemolymph and Tissues

For studying physiological parameters of *B. alexandrina* snails (8-10 mm) snails were randomly divided into two groups (50 snails each). The 1st group was continuously exposed to LC_{25} of tested plant for 2 weeks. A 2th group of snails was left unexposed under the same laboratory conditions as control. Snails surviving after exposure were used to study the effects of LC_{25} of tested plant on some enzymes representing glycolytic pathway as hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) and glucose phosphate isomerase (GPI); glycogenolytic pathway as glycogen phosphorylase, glucose-6-phosphatase (G-6-Pase); gluconeogenic pathway as fructose-1-6 diphosphatase (F-D-Pase), phosphoenolpyruvate carboxykinase (PEPCK) and glycogen in soft tissue. Also, its effect on glucose, lipid peroxidase, protein in hemolymph.

For preparation of tissue extracts of snails exposed to the tested plant for 2 weeks as well as of the control snails, one g of the soft tissues of the snails was homogenized in 5 ml distilled water at pH 7.5. A glass homogenizer was then used to grind the tissue, and the homogenate was centrifuged for 10 minutes at 3000 rpm and the fresh supernatant was decanted. Hemolymph of the treated and control snails was collected in accordance with techniques described by Michelson and Du Bois[22]. The hemolymph was obtained via small hole made in the shell into which capillary tube was inserted; then, it was drawn into tube by capillary suction. The hemolymph was pooled from 10 snails collected in a vial tube (1.5 ml) and kept in ice-bath.

Enzyme Assays

All physiological parameters in this study were determined spectrophometrically (Milton Roy Company, CA, USA) using reagent kits purchased from BioMerieux Company, Marcy l'Etoile, France. Determination of tissues glycogen was evaluated according to Carrol et al[25]. Determination of glucose concentrations according to the glucose oxidase method of Trinder[26], lipid peroxide according to Buege and Aust[27] and total protein according to Bradford[28] in hemolymph.

Hexokinase (HK) was assayed according to the method of Uyeda and Raker[29]. Pyruvatekinase (PK) was assayed according to McManus and James[30]. phosphofructokinase (PFK) was measured according to the method of Zammit et al[31]. Lactate dehydrogenase (LDH) activity was measured spectrophometrically according to Cabaud and Wroblewski[32]. Glucose phosphate isomerase (GPI) was measured using the method of King[33].

Glycogen phosphorylase was assayed according to Hedrick and Fischer[34]. Glucose-6-phosphatase (G-6-Pase) was assayed according to the method of Swanson[35]. Fructose-1-6 diphosphatase (F-D-P ase) was assayed with the method of Sand et al[36] and phosphoenolpyruvate carboxykinase (PEPCK)according to Suarez et al[37].

Statistical Analysis

Total and differential hemocyte counts and phagocytosis data are presented as mean ± Standard Deviation. The significance of difference between the means was calculated according to the way analysis (ANOVA) followed by Student's *t*-test[38].

Results

The Molluscicidal Activity

The molluscicidal activity of methanol extract of *A. indica* plant on *B. alexandrina* snails after 24 hours of exposure is presented in Table I. The data obtained indicate that LC_{50} and LC_{90} values for *A. indica* plant were 44 and 76 pmm respectively. The sublethal concentrations (LC_0, LC_{10} & LC_{25}) were found to be 4.4, 22 and 36 ppm respectively.

Flow Cytometric Analysis: Number and Mortality of Amoebocytes

Hemolymph samples from *B. alexandrina* snails contained two morphologically distinct

Table I. Molluscicidal activity of methanol extract of *Azadirachta indica* plant against *Biomphalaria alexandrina* snails after 24 hours of exposure under laboratory conditions.

				Sublethal concentration		
LC_{50} ppm	Confidence limits	LC_{90} ppm	Slope	LC_0 ppm	LC_{10} ppm	LC_{25} ppm
44.0	36.6-52.8	76	1.4	4.4	22	36

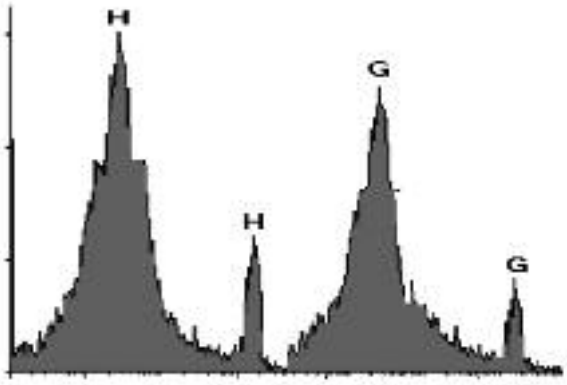
types of hemocytes, designated as round small hyalinocyte cells that are circular shape with clear dense cell membrane, few granules, eccentric nucleus and granulocytes which characterize by small size and are found as groups accumulated together, the nucleus was small and eccentric.

Table II shows that the number of amoebocytes in the hemolymph was significantly increased ($p < 0.05$) in group treated with tested plant compared to control group. Means of amoebocytes in the hyalinocyte cells of 7 snails is 39.1 ± 11.3 for group treated and control group 24.4 ± 6.0. Also, means of amoebocytes in the granulocyte cells of 7 snails is 22.9 ± 6.0 for group treated and control group (14.3 ± 3.7).

In hemolymph study, mortality rates of hyalinocytes and granulocytes in group treated with tested plant were significantly higher ($p < 0.05$) than that of control one. The death rate of hyalinocytes was 15. 3% for treated group and 8.1% for control group. Also, the mortality rate of granulocytes was 17.7%, 7.5% for treated and control group respectively (Table III).

Phagocytosis

Data illustrated in Figures 1 and 2 show that phagocytosis in group treated with tested plant was highly significant than control one. The amount of propidium iodide uptake by hyalinocyte cells was highly significant than granulocyte cells in both treated and control one. The flow cytometric analysis showed that the total number of cells in treated groups (both hyalinocytes and granulocytes) were significantly increase than that of control one, in addition,



Phagocytosis in snails treated with LC_{25} of methanol extract of *Azadirachta indica* plant

Figure 1. Flow cytometer counts histogram of dead hyalinocytes (H) and granulocyte (G) stained by propidium iodide, and unstained living hyalinocytes (H) and granulocytes (G) represented by the large peak to the left.

Table II. Means of amoebocytes in the hemolymph of 7 snails for each group treated with LC_{25} of *Azadirachta indica* plant extract.

Group	Granulocytes (cells/mm^3)	Hyalinocytes (cells/mm^3)
Control	14.3 ± 3.7	24.4 ± 6.0
Snails treated with LC_{25} of *A. indica*	22.9 ± 6.0	39.1 ± 11.3

Table III. Mortality of amoebocytes in hemolymph of 7 snails for each group treated with LC_{25} of *Azadirachta indica* plant extract.

Group	Granulocytes (cells/mm^3)	Hyalinocytes (cells/mm^3)
Control	1 (7. 5%)	2 (8.1%)
Snails treated with LC_{25} of *A. indica*	4 (17.7%)	6 (15.3%)

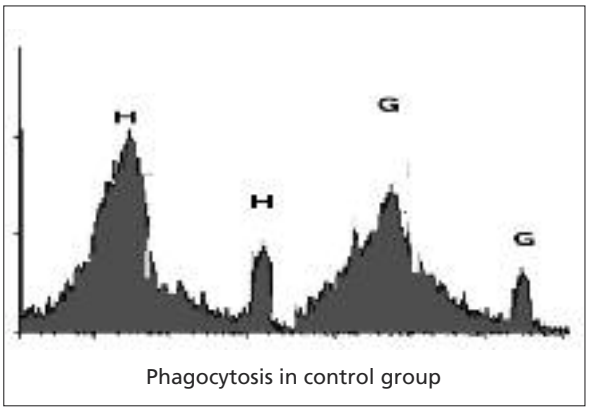


Figure 2. Flow cytometer counts histogram of dead hyalinocytes (H) and granulocyte (G) stained by propidium iodide, and unstained living hyalinocytes (H) and granulocytes (G) represented by the large peak to the left.

the number of hyalinocytes was significantly increase than granulocytes in both treated and control one (Figure 3A and B).

Physiological Parameters

Table IV reveals significant increase ($p < 0.001$) in lipid peroxide and glucose levels accompanied with significant reduction in total protein in hemolymph of snails treated with tested plant. On he other hand, the glycogen contents in tissues of the treated snails with tested plant were significantly ($p < 0.01$) lower than in the control group.

The levels of glycolytic pathway as hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) and glucose phosphate isomerase (GPI) in the soft tissue in normal and treated snails are displayed in Table V. The HK activity in snails exposed to LC_{25} of the tested plant for 2 weeks was 2.24 ± 0.64 mmol/min/g wet (-45.76%). Such reduced values were statistically significant than those of the corresponding controls (4.13 ± 0.64 mmol/min/g). The activity levels of PK, PFK, GPI and LDH were also significantly reduced in response to treatment with the tested plant, the percentage of reduction was -44. 46.1, -31.37 and -56.25% (Table V).

The results in (Table VI) show that the levels of glycogen phosphorylase, glucose-6-phosphatase (G-6-Pase), fructose-1-6 diphosphatase (F-D-P ase) and phosphoenolpyruvate carboxykinase (PEPCK in the soft tissues of normal and treated snails were also significantly reduced in reponse to treatment with the tested plant. The glycogen phosphorylase activity in snails exposed for 2 weeks was 3.21 ± 0.28 u/mg tissue (-44.66%). Such reduced values were significant than those of the corresponding controls (5.8 ± 0.43 u/mg tissue).

Discussion

Flow cytometry is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. In the present work, flow cytometric analysis was carried out for detecting apoptosis of *B. alexandrina* hemocytes isolated from pooled hemolymph samples from 7 snail in each experimental group 2 weeks post-tested plant treatment. The obtained results indicated that hemolymph

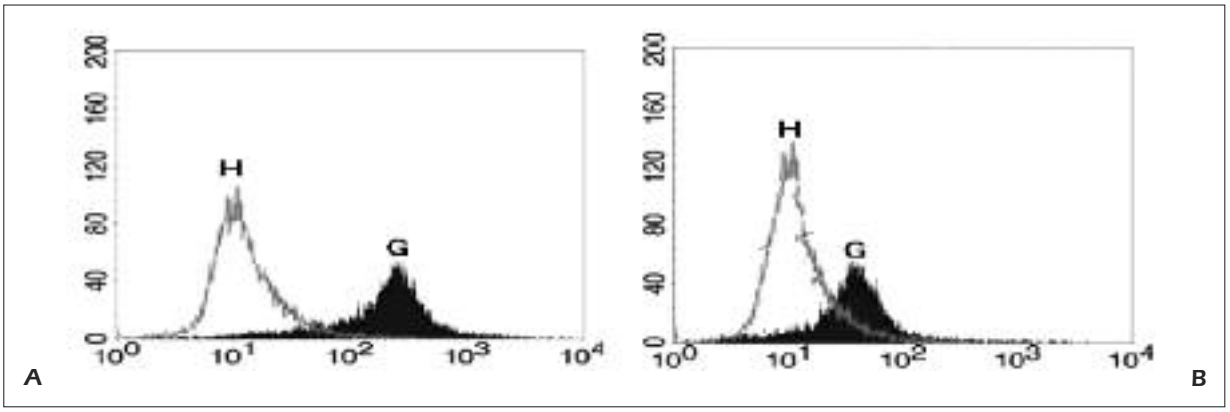


Figure 3. Flow cytometer counts histogram plot of snail' samoebocytes, G: Granulocytes count, H Hyalinocytes count. *A*, Control, *B*, Treated with LC_{25} of *Azadirachta indica* plant extract.

Table IV. Effect of prolonged exposure to LC_{25} of methanol extract *Azadirachta indica* plant for 2 weeks on glucose level, lipid peroxide, total lipid in hemolymph and glycogen content in soft tissues of *Biomphalaria alexandrina* snails.

Plant extract	SOFT TISSUES Glycogen content (mg/g tissue)		HEMOLYMPH Lipid peroxide		Total protein		Glucose level (mg/ml)	
	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%
Control	30.6 ± 1.8		0.62 ± 0.03		41.21 ± 2.03		22.6 ± 2.13	
Snails treated with LC_{25} of *A. indica*	18.2 ± 2.1**	-41.5%	1.11 ± 0.08***	79%	18.31 ± 0.63**	-508%	34.1 ± 1.6**	50.9%

X ± SD mean of 4 experiments ± SD. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

Table V. Effect of prolonged exposure to LC_{25} of methanol extract of *Azadirachta indica* plant for 2 weeks on some glycolytic enzymes in soft tissues of *Biomphalaria alexandrina* snails.

Parameters treatment	Hexokinase (HK)		Pyruvate kinase (PK)		PFK		Enzyme activity µ mol/min/mg protein Lactate dehydrogenase (LDH)		Glucose phosphate isomerase (GPI)	
	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%
Control	4.13 ± 0.64		0.92 ± 1.2		4.1 ± 3.2		5.1 ± 1.6		2.56 ± 0.56	
Snails treated	2.24 ± 0.64***	-45.76	0.48 ± 0.6***	-44%	2.21 ± 1.3***	-46.1%	3.5 ± 0.8***	-31.37%	1.12 ± 0.33***	-56.25

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table VI. Effect of prolonged exposure to LC_{25} of methanol extract of *Azadirachta indica* plant for 2 weeks on some Glycogenolytic and Gluconeogenic enzymes in soft tissues of *Biomphalaria alexandrina* snails.

Treatment	Glycogenolytic enzyme activity µ mol/min/mg protein				Gluconeogenic enzyme activity µ mol/min/mg protein			
	Glycogen phosphorylase		Glucose-6-phosphatase (G-6-Pase)		Fructose-1-6 diphosphatase (F-D-P ase)		Phosphoenolpyruvate carboxykinase (PEPCK)	
	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%
Control	5.8 ± 0.43		0.61 ± 0.07**		6.1 ± 0.8**		1.21 ± 0.32	
Snails treated	3.21 ± 0.28**	-44.66%	0.48 ± 0.0.2*	-21.31%	4.2 ± 0.61**	-31.15	0.71 ± 0.32**	-41.23%

*$p < 0.05$, **$p < 0.01$.

samples from *B. alexandrina* snails contained two morphologically distinct types of hemocytes, designated as round small. Hyalinocyte cells are circular shape with clear dense cell membrane, few granules, eccentric nucleus while granulocytes, are characterized by small size, are found as groups accumulated together, the nucleus was small and eccentric. This result agrees with Bakry[39] who found three main haemocytes categories in *B. alexandrina,* distinguished namely: hyalinocytes, agranulocytes and granulocytes according to the presence or absence of granules. Mohamed[12] indicated that the *B. alexandrina* hemocytes are classified according to cell size and shape into two cell types, designated as small round, hyalinocytes and granular spreading hemocytes. Matricon-Gondran and Letocart[40] identified three subpopulations of hemocytes in hemolymph of *B. glabrata* snails based on their size and ultrastructure aspects. Also, using optical microscopy, Martins-Souza et al[41] identified three circulating hemocytes subsets in *Biomphalaria* species. Recently, cytometric analysis carried out by Martins-Souza et al[42] revealed that *B. glabrata* snails and two strains of *B. tenagophila* have three major circulating hemocytes subsets, referred to as small, medium and large hemocytes. As reported previously, the round small cells have low immunological competence whereas spreading hemocytes displayed immunological activities[43].

The number of amoebocytes in the hemolymph was significantly increased in group treated with tested plant compared to control group. In addition, in hemolymph study, mortality rates of hyalinocytes and granulocytes in group treated with tested plant were significantly higher than that of control one. This finding is supporting by Mohamed[12] who found that short-term exposure of *B. alexandrina* snails to Roundup herbicide in sublethal concentration induced rapid changes in hemocytes responses. Changes in numbers, types and behavior of hemocytes in mollusks experimentally challenged by foreign materials are well documented[44-46].

The results reported up till now suggest that the hemocyte could be the effectors element in the destruction mechanism of trematodes, being directly involved in the death of some encapsulated parasites[47,48] or in the production of soluble factors which could be cytotoxic[49]. The majority of the Authors[48,49] agree that the snails´ defense generally occurs by means of destruction, total or partial, of the primary sporocyst at the first few hours following the penetration of the miracidium.

The flow cytometric analysis is showed that the total number of cells in treated groups (both hyalinocyte and granulocytes) were significantly increased than that of control one. In addition, the numer of hyalinocytes was significantly increased than granulocytes in both treated and control one. Similar findings were obtained by Russo and Lagadic[45] who stated that dramatic increase of the total number of hemocytes in *L. palustris* exposed to atrazin herbicide was recorded. In addition, Russo and Lagadic[46] reported that atrazin was responsible for a significant increase in the number of *Lymnaea stagnalis* hemocytes, mainly granulocytes ''spreading''. The elevation of total hemocytes is the most commonly observed response in different molluscan species exposed to different stressors. Mohamed[12], found that exposure of *B. alexandrina* snails to sublethal concentration of Roundup herbicide led to significant increase in total hemocytes counts during 7 days of exposure. Consequently, the spreading hemocytes were also increased during the whole experimental period.

The defense system is made by cellular and humoral elements. The cellular defense system is operated by hemocytes (moving cells), which have phagocytic capacity. The humoral immunity is measured by lectins and opsonins, which are proteins synthesized by hemocytes with specific affinities to carbohydrates[50]. Phagocytosis is a response helpful to assess the immunological impact of environmental pollutants[13]. In the present investigation, the flow cytometric analysis showed that phagocytosis in group treated with tested plant was highly significant than control one indicating a highly increased response of snail against the treatment. This result agrees with Mohamed[12] who revealed that the phagocytic activity of *B. alexandrina* hemocytes was increased during 7 days of exposure. Sublethal concentration (10 mg/L) of herbicide Roundup may lead to cytotoxic effects. Matricon-Gondran and Letocart[40] explained that these cytotoxic effects may be due to the changes in the sensitivity of intercellular adhesion molecules involved in the ingestion of foreign materials, resulting in increase of phagocytic activity. Phagocytic activity of mussel hemocytes was stimulated as a result of exposure to organic compounds at short-term exposure[51,52]. Moreover, Canesi et al[53] reported that phagocytic activity of *Mytilus hemocyes* was enhanced by estrogenic chemicals.

Regarding the sources of energy for snails, LC_{25} of plant extract significantly decreased the glycogen content of soft tissues while the glucose level in hemolymph was increased. This may be attributed to the activity of the tested plants that impedes oxygen consumption of snails, thus inducing anaerobic respiration. To restore its energy requirements, the snail has to increase the rate of glycolysis, thus, bringing about a reduction of the glycogen content and increase glucose level in the hemolymph. This finding agrees with the results of similar experiments applying some pesticides[54] and plant extracts[55-57].

The data obtained in the present study showed that lipid peroxide was elevated in the hemolymph of snail. Lipid peroxidation is known to require the participation of highly reactive oxygen and other reactive metabolites in the chain of biochemical reaction[58]. Such depletion is critical, as shown by the increased cytotoxicity of H_2O_2 in endothelial cells, as a result of inhibition of glutathione reductase, which keeps glutathione in its reduced state[59].

Total protein content showed significant reduction in soft tissues of the snails treated with plant extract This could be attributed to cellular damage caused by toxin[60]. The significant decrease in total protein content is mainly due to increase in messenger RNA degradation which is the possible cause for the hypoalbuminemia[61]. Moreover, there is evidence of conformational changes of serum albumin induced by its interaction with low molecular weight of dyes and drugs, which appears to affect the secondary and tertiary structure of albumins[62].

In the present report, the glycolytic enzymes hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) and glucose phosphate isomerase (GPI) in the snail tissues showed variable decrease between significant and highly significant on applying the tested plant. The depletion in HK activity in the soft tissues causes an alteration of glycolytic mechanism which in turn induces a state of anoxia. A similar effect was detected by Mohamed et al[63] using Abamectin as a molluscicide, and El-Ansary et al[64] using *Ambrosia maritima* (Damsissa) and Bakry et al[55,56,57] using plant extracts.

The present study showed a significant decrease in LDH activity in the whole tissue extract of *B. alexandrina* in response to treatment with LC_{25} of the tested plant. Several Authors have reported significant decline in LDH activi-

ty of tissues of various mollusks in response to some molluscicides[65,66]. The decreased in LDH activity of *B. alexandrina*'s tissue was due to the release of the enzyme from the tissues as a result of cellular damage caused by the toxic effect of molluscicides. Some Authors reported that tissue damage followed the release of cellular enzymes such as LDH[67,68]. Besides in spite of the decrease in LDH activity, there was insignificant change in D-lactate and pyruvate level as compared to untreated snails, as reported by Reddy et al[69].

In the present work, the levels of glycogen phosphorylase, glucose-6-phosphatase (G-6-Pase) and fructose-1-6 diphosphatase (F-D-P ase) in the soft tissues of normal and treated snails were also significantly reduced in response to treatment with the tested plant. With respect to G-6-Pase as glycogenolytic enzyme, it showed reduced activity in treated snails which was attributed to either synthesis and/or degradation of glycogen[70]. Increasing the glucose concentration stimulated glycogen synthesis and decreased the activity of glycogen phosphorylase glucose was incorporated into glycogen during period of net glycogen breakdown, and vice versa; glycogen degradation occurred during periods of net glycogen synthesis which depends on glucose concentration[71].

Conclusions

The number of both hyalinocytes and granulocytes and the mortality rate was significantly increased in treated group with tested plant. The phagocytosis was increased in treated group than that of control group indicating a highly increase response of snail against the treatment. Also, the glucose level in hemolymph of exposed snails was elevated, while the protein content showed a decrease in soft tissues when compared with the control group. In addition, the activity level of some enzymes representing glycolytic enzymes as hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) and glucose phosphate isomerase (GPI); glycogenolytic enzymes as glycogen phosphorylase, glucose-6-phosphatase (G-6-Pase); gluconeogenic enzymes as fructose-1-6 diphosphatase (F-D-P ase), phosphoenolpyruvate carboxykinase (PEPCK) was also significantly reduced in response to treatment.

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