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DETERIORATIONS IN BIOLOGICAL AND BIOCHEMICAL ASPECTS OF *BIOMPHALARIA ALEXANDRINA* SNAILS EXPOSED TO METHANOL EXTRACT OF *CALLISTEMON CITRINUS* LEAVES

By

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Abstract

Schistosomiasis is one of the deleterious parasitic diseases in many developing countries. One important approach to control the disease is to eliminate its intermediate hosts. Therefore, methanol extract from *Callistemon citrinus* leaves was evaluated against biological & biochemical aspects of *Schistosoma mansoni* infected and uninfected *B. alexandrina* snails, in addition to examination the histological changes in tissues of the snails' digestive gland. The plant extract exhibited considerable molluscicidal potency against *B. alexandrina* snails. The egg-laying capacity and reproductive rate (R_0) of *S. mansoni* infected and uninfected snails were suppressed post exposure to LC_{25} of methanol extract for 24h/week for 4 successive weeks. Reduction rate of R_0 for snails infected only and those infected treated with LC_{25} were 65.8% and 70.9%, respectively. Infection rates of *B. alexandrina* snails with *S. mansoni* and cercarial production/infected snail were reduced as a reflection of their exposure to LC_{10} of the plant extract either pre-, during or post their exposure to miracidia. The total number of hemocytes of *S. mansoni* infected and uninfected snails was elevated post 24h of exposure to the plant extract. However, it was reduced after 4 weeks of successive exposure to the extract, but the large granulocytes were elevated. Also, exposure of *B. alexandrina* snails (infected and uninfected) to the plant extract caused deleterious morphological changes in their hemocytes. The activities of antioxidant enzymes LPO, CAT & SOD were elevated in tissue homogenate of uninfected snails after one week of exposure to the extract, while that of GSH was decreased. Moreover, the plant extract deteriorated the histological characters of digestive gland cells in treated snails ranged from degeneration and necrotic of its tubules, atrophy of different cell types and hyaline substance in enlarged digestive lumen..

Key words: *Biomphalaria alexandrina* snails, *Callistemon citrinus, Schistosoma mansoni,* hemocytes, antioxidant enzymes, histology.

Introduction

Schistosomiasis, an acute and chronic parasitic disease, is a major health problem in tropical and subtropical countries (WHO, 2016). One of the methods for controlling schistosomiasis is to delink the life cycle of the parasite by combating its snail host(s). Control is a key intervention to achieve the World Health Assembly's goal for schistosomiasis elimination (King and Bertsch, 2015). There is an increase demand to develop new molluscicides of medicinal plant origin, as alternative to chemical ones, which proved to have several advantages over the synthetic compounds (Belet, 2015). The body cavity of fresh water snails (Gastropod) is filled with hemolymph which contains dissolved hemoproteins and several kinds of hemocytes which are the principal line of cellular defense involved in destruction of *S. mansoni* larvae inside the snail intermediate host (Larson *et al*, 2014). The response patterns, density and functions of hemocytes, can be affected by pathogens, xenobiotics and parasites (Livingstone *et al*, 2000).

Immune functions are complemented by an array of killing mechanisms release of degradative and oxidative enzymes (Renwrantz *et al*, 1996) and high reactive oxygen metabolites generation (Arumugam *et al*, 2000). Antioxidant enzymes activity proved

important to clarify immune response of organisms exposed to different stresses (Dhawan, 2005).

Plants contain a variety of antioxidant and free radical scavenging activities (Sampath *et al*, 2016). *Callistemon citrinus* known as Bottle brush of family Myrtaceae has a great medicinal importance; molluscicidal potency (Gawish *et al*, 2008), antioxidant and hepatoprotective activities (El-Dib and El-Shenawy, 2008) and antibacterial and antifungal properties (Dongmo *et al*, 2010).

This study aimed to evaluate the impact of methanol extract of *Callistemon citrinus* leaves on biological, histological and immunological parameters of *Biomphalaria alexandrina*.

Materials and Methods

Biomphalaria alexandrina snails (7-9mm) were collected from irrigation system in Giza Governorate. They were examined for natural trematode infections, then negative and healthy specimens were maintained at Department of Medical Malacology, Theodor Bilharz Research Institute (TBRI), before usage; snails were kept for 4 weeks (10snails/L dechlorinated water, 25±1^{o}C).

Miracidia: *S. mansoni* ova were obtained from Schistosomiasis Biological Supply Center (SPSC), TBRI, left in clean dechlorinated water for hatching under a desk lamp, fresh hatched miracidia were used in bioassay and infection tests.

Plant species: *Callistemon citrinus* (Myrtaceae) was collected from the Giza Zoo, and was kindly identified by Dr. Zaki Turky, Prof of Plant Taxonomy, Department of Botany, Faculty of Science, Menoufia University. The leaves were washed, shade dried, powdered by an electric mill and stored in dry clean dark glass bottle.

Plants' extract: Leaves dry powder was extracted by soaking of 25g/250ml 95% methanol for 3 days. Solvent was filtered, distilled off under vacuum and residues were stored in clean dry dark bottles (Kamel *et al*, 2011).

Bioassay: A series of concentrations was prepared from methanol extract of *C. citrinus* leaves on the basis of weight/volume. Three, each of 10snails/L were prepared for each concentration. Another three replicates in dechlorinated water were used as control (25±1^{o} C). Exposure and recovery periods were 24 h for each. Mortality rates (LC_{50} &LC_{90}) were calculated using the Probit Prob ananslysis (ver 20 for Windows).

Effect of methanol extract on mature *B. alexandrina*: The sublethal effects (LC_{10} & LC_{25}) of methanol extract on reproductive rate, histological features of digestive gland, morphology of hemocytes and types and some antioxidant enzymes of normal and *S. mansoni* infected snails was evaluated 4 weeks post exposure. Snails were divided into 4 groups: **G**1 was maintained as untreated and uninfected control group, G2nd was exposed to *S. mansoni* miracidia representing infected group, **G**3 was exposed to sublethal concentrations of methanol extract as treated uninfected group, and G4 was exposed to both methanol extract and *S. mansoni* miracidia representing treated-infected group.

Egg-laying capacity: Six groups of adult snails were prepared. Two were untreated with methanol extract and kept as control uninfected and control infected. Others were exposed to LC_{10} & LC_{25} of methanol extract for 24h weekly for 4 successive weeks as infected treated and uninfected treated. Three replicates, each of 10 snails as 7-9 mm/L, were used for each snail group. Concentrations were weekly changed by fresh ones. Snails were daily fed oven dried lettuce leaves. Aquaria of treated and untreated snails were provided with foam sheets for oviposition. Eggs laid on these sheets and on the aquaria walls were weekly counted using a stereomicroscope and hand lens (10x). Dead snails were removed daily and the following parameters were weekly recorded (Mohamed *et al*, 2012). Lx (survival rate as a proportion of corrected one), Mx (number

of eggs/snail/week) and R_0 (reproductive rate: summation of Lx Mx).

Infection rate effect on *B. alexandrina*: exposed to miracidia as 10miracidia/snail, for 24h illumination. They were maintained in clean dechlorinated water (25±1˚C) for prepatency. Snails were exposed to LC_{10} & LC_{25} of methanol extract at 1day pre-, during and 15 days post-exposure. Three replicates, each of 10snails/L, were prepared for each concentration. Another three replicates were exposed to miracidia and similarly treated as the experimental snails to cercarial emergence.

Effect on histology of *B. alexandrina*: Four groups of snails were used. Two were untreated with *C. citrinus* methanol extract; one was as clean control and 2nd was exposed to *S. mansoni* miracidia (untreated infected). The other two were treated with LC_{50} of the extract for 24h, one was as treated only and the second group was exposed to *S. mansoni* (treated infected). Digestive gland of survived snails of each group was dissected out and fixed in a Boun's solution. i.e., digestive gland of snails exposed to the plant extract only were prepared after 24h of treatment, but infected treated and infected untreated groups were done after 1 & 3 weeks of miracidial exposure, respectively. Thin sections (5µm) were prepared and stained with Delafield's hematoxyline and eosin (Ragheb *et al*, 2013). Sections were examined and photographed by the Carl Zeiss, Germany microscopy.

Effect on snails' hemolymph and antioxidant enzymes: For total hemocytes number and types, **s**ix groups of *B. alexandrina* were used. G1 & G2 were exposed to LC_{10} & LC_{25} of plant extract only for 24h weekly for 4 successive weeks (treated normal). G3 & $G4^t$ were individually exposed to *S. mansoni* miracidia (5miracidia/snail) for 24h, followed by exposure to LC_{10} & LC_{25} as in G1. G5 was exposed to miracidia only (untreated infected or control infected), and G6 was kept as clean control (untreated normal). Hemolymph samples were collected from survived snails in each group after 24h, 1, 2 & 4 weeks post-exposure (Nduka and Harrison, 1980). Snails' soft tissues were centrifuged and supernatant was used for antioxidant enzymes determination. Total number of hemocytes, types and morphological alterations were recorded (Abou-El-nour *et al*, 2015).

Antioxidant enzymes: Antioxidant enzymes Catalase (CAT, Aebi, 1984), Glutathione reduced (GSH, Beutler *et al*, 1963), Lipid peroxide (LPO, Ohkawa *et al*, 1979) and Superoxide dismutase (SOD, Nishikimi *et al*, 1972) were determined in supernatant previously prepared from tissue homogenate of the experimental and control groups.

Statistical analysis**: D**ata were analyzed for significant differences between mean values of the test and control groups by "t" test (Spiegel, 1981), and between percenttages by Chi-square (Southwood, 1978), using the SPSS computer program (version 20.0 Windows).

Results

Toxicity of *C. citrinus* methanol extract to *B. alexandrina* snails was 5.9 times that of water extract on basis of LC_{90} after 24h of exposure (37.8 & 223.6ppm, respectively). Methanol extract impact on biological, histological and immunological aspects of *B. alexandrina* was tested (Tab. 1).

Survival rate of snails at 1st shedding: Exposure of *B. alexandrina* snails to LC_{10} (9.3 ppm) and LC_{25} (17.2 ppm) from *C. citrinus* methanol extract for 24h either at 1 day pre-, during or at 15 days post-miracidial exposure reduced their survival rate at 1st shedding compared to their corresponding control groups (Tab. 2). Reduction rates of groups exposed to LC_{10} at 1day pre, during and at 15 days post- exposure were 18.7%, 26.7% and 64.7%, respectively (P<0.05 & 0.001). Raising concentration to LC_{25} caused more reduction for groups at during- & 15 days post-exposure were 40% & 70.6%, respectively (P<0.01&0.001).

The 24h of snails' exposure to LC_{10} of *C. citrinus* extract at 1 day pre-, during or at 15

days post-exposure reduced infection with *S. mansoni*. Infection rate during miracidial exposure was 72.73% compared to 86.67% for control group (P<0.05). More reduction was at 15 days post-exposure, as infection rate was 33.33% compared to 82.35% for control snails (P<0.001). Also, infection rate of snails treated with LC_{25} at 15 days post-exposure (40%) was significantly lower than corresponded controls (82.35%, P<0.001).

Cercarial shedding (cercariae/snail): Cercarial production/snail from the groups exposed to LC_{10} and LC_{25} at the three modes of miracidial exposure was less than corresponding control groups. Highest reduction rate was in snails exposed to LC_{25} at 15 days post-exposure, recording 63.1% compared to 18.4% &45.8% for groups treated at 1 day pre- and during exposure, respectively.

Snails' survival rate (L_X): Exposure of *S. mansoni* uninfected snails to LC_{10} & LC_{25} of extract for 24h weekly for 4 successive weeks reduced survival rate (L_X) to be 0.56 &0.50 at the 4th week, respectively, compared to 0.90 in control group (Tab. 3). Infected snails' L_X values were sharply reduced; 0.43 & 0.38 at 2nd week post exposure to these concentrations, respectively, as compared with 0.6 for infected untreated control group. Few snails survived only at LC_{25} within three weeks, dead at 4th week (L_X=0.0) compared to 0.23 for infected untreated control snails. Snails' fecundity (M_X) and reproductive rate (R_0): *S. mansoni* uninfected snails exposed to LC_{10} & LC_{25} of *C. citrinus* extract laid few number of eggs/ snail/week throughout the experimental period (4 weeks) compared to control group. M_X values at 2nd week were 2.0 & 2.4 eggs/ snail compared to 4.5eggs/control. At 4th week, treated snails suffered much as few survived at LC_{25} laid very few eggs (M_X=0.01eggs/snail), but at LC_{10} failed to lay eggs (M_X=0.0).

Exposure of *S. mansoni* infected snails to the concentrations decreased M_X values compared to infected untreated controls (Tab. 3). At 3rd week, M_X values for snails exposed to LC_{10} & LC_{25} were 0.02 & 0.1 eggs/snail compared to 1.1eggs/infected untreated controls. By 4th week, snails survived at LC_{10} failed to lay eggs (M_X=0.0) compared to 0.8eggs/infected untreated controls.

As to reproductive rate (R_0) of uninfected snails exposed to extract, was reduced as compared with controls. Similar observations were recorded for *S. mansoni* infected snails treated with the concentrations. So, R_0 was reduced by 16% in each concentration in proportion to infected untreated controls.

Histology of treated snails: Exposure of snails to LC_{50} of methanol extract for 24h revealed certain histopathological changes in digestive gland. Normal histological structure of *B. alexandrina* digestive gland includes glandular tubules interspersed within connective tissues. The epithelium is rested on thin basement membrane; at least 3-4 types of cells can be recognized in the snail, digestive, calcium and excretory cells (Fig. 1A). Exposure to *C. citrinus* caused wall thickness of digestive gland cells, losing their shape and hyaline substance appeared in enlarged digestive lumen (Fig. 1B). *B. alexandrina* exposed to *S. mansoni* miracidia only for 24h (infected untreated) different developmental stages of sporocycts in the digestive gland tubules and cercariae after 3 weeks of miracidial exposure were detected (Fig.1C). In case of *B. alexandrina* snails exposed for 24h to *S. mansoni* miracidia and treated with LC_{50} of *C. citrinus* (infected treated) atrophied shrunk sporocycts (S), big vacuoles (V) and thickened degenerated cells and connective tissue between the digestive gland tubules were observed (Fig. 1D).

Total hemocytes for *S. mansoni* uninfected and infected snails: A significant increase in total hemocytes of uninfected snails post 24h of exposure to *C. citrinus* extract was recorded (Tab. 4). Recorded number at LC_{25} was 3250 cells/ml compared to 2340 cells/ ml for control group (P<0.05). After one week of exposure, this parameter was significantly decreased to 750cells/ml for snails at

LC_{10} compared to 2150 cells/ml for controls (P<0.001). After 2 &4 weeks of exposure total number of hemocytes for snails treated with LC_{10} & LC_{25} was not significant from corresponding controls. After 4 weeks exposure, total number of hemocytes of snails at LC_{25} was 3050 cells/ml compared to 2700 cells/ ml for controls (P>0.05). As to *S. mansoni* infected *B. alexandrina* total number of hemocytes post 24h of exposure to LC_{10} & LC_{25} of extract was significantly increased compared to controls. At LC_{25} was 3000 cells/ml compared to 1600 cells/ml for controls (P<0.001). This was significant decreased for snails at 1, 2 & 4 weeks post exposure compared to corresponding controls. Number of hemocytes after 4 weeks of snails exposure to LC_{25} was 1950 cells/ml compared to 2950cells/ml for controls (P<0.01).

Hemocytes' types and percentages were affected by snails' exposure to extract concentrations. Hyalinocytes values after 24h, 1 & 2 weeks of exposure to LC_{25} were significantly higher than controls, but large granulocytes were significantly lower. Long period to 4 weeks significantly increased granulocytes for snails exposed to LC_{10} recording 65% compared to 41% for controls (P<0.001). Snails treated with *C. citrinus* concentrations post exposure to *S. mansoni* miracidia, types of hemocytes were deteriorated and large granulocytes at 4^{th} week of treatment were significantly higher (51%) than controls (21%, P<0.001).

The light microscopy photographs of uninfected untreated (control) *B. alexandrina* hemocytes (Fig. 2A) showed two distinct types of hemocytes, granulocytes and hyalinocytes. Granulocytes showed two types, large and small cells. Large ones were variable in size with small nucleus and dense cytoplasmic contents due to refractile granules. Hyalinocytes were small circular in shape with a clear dense cell membrane and a large nucleus. For *S. mansoni* unin-fected treated snails (Fig. 2B&C) hyaline-cytes emit fine long extension (Fp) and a degeneration of large granulocyte cytoplasm with condensed vacuoles (Cv), irregular cell walls and large eccentric nucleus. In *S. mansoni* infected snails (Fig. 2D) large granulocytes (LG) exhibited deformed cell walls (Dw) and condensed granules (CGR). Moreover, large granulocytes of infected treated snails showed incomplete cell division with large granules and having two nuclei (Fig. 2E).

The activities of the enzymes CAT and SOD increased in tissue homogenate of *S. mansoni* uninfected snails post one week of exposure to LC_{10} & LC_{25} of extract compared to controls (Tab. 5). Enzymes activeties after 2 and 4 weeks of snail exposure to concentrations were less than controls. After 4 weeks of snails exposure to LC_{25} CAT activity was 9.9U/g tissue compared to 27.3U /g tissue of controls (reduction rate= 63.7%, P<0.001).

For lipid peroxide (LPO) concentration in snails' tissue homogenate after one week exposure to LC_{25} of extract showed few elevation compared to controls (119.4nm/g tissue compared to 107.2nm/g tissue) After 2 & 4 weeks, enzyme concentrations decreased than controls. Glutathione (GSH) concentration was reduced after exposure compared to controls. As to *S. mansoni* infected snails, activity of CAT decreased by exposure to extract compared to untreated infected groups. SOD & GSH activities were increased after 24h and one week of treating infected snails with tested concentrations compared infected controls, after 4 weeks of exposure to LC_{25} GSH concentrations did not significantly different from infected untreated controls.

Table 1: Molluscicidial activity of water and methanol extracts of *C. citrinus* against *B. alexandrina* (24 hrs exposure):

Extract	LC_{10} ppm	LC_{25} ppm	LC_{50} ppm	LC_{90} ppm	Slope
methanol	9.3	17.2	22.3	37.8	1.6
water	114.1	140.0	168.9	223.6	1.3

Table 2: Effect of methanol extract of *C. citrinus* leaves on survival rate at 1st shedding, infection rate, prepatent period, duration of shedding and cercarial production of *B. alexandrina* exposed to *S. mansoni* miracidia (reduction%):

Miracidial exposure	Conc.	Survival%	Infection%	Prepatent period	duration of shedding	No. of cercariae / snail
1 day pre-exposure	Control	80.00	81.25	23.15±3.36	39.31±14.16	4402.54±496.44
	LC_{10}	65.00 (18.7%)*	76.92	24.50±3.69	31.50±7.56	3951.00±891.13 (10.2%)
	LC_{25}	65.00 (18.7%)*	92.31**	27.42±4.68**	29.17±13.95*	3590.67±590.72 (18.4%) ***
15 days post exposure	Control	75.00	86.67	22.08±2.63	47.92±14.81	3549.77±841.87
	LC_{10}	55.00 (26.7%)*	72.73 *	23.63±3.62	38.50±9.90*	2169.67±1067.04 (38.9%) **
	LC_{25}	45.00 (40.0%) **	88.89	22.75±3.24	36.75±10.42 *	1923.00±237.03 (45.8%) ***
During exposure	Control	85.00	82.35	22.50±4.05	36.00±15.25	2907.0±735.44
	LC_{10}	30.00 (64.7%) ***	33.33 ***	21.00±0	31.50±14.85	2896.5±965.50 (0.38%)
	LC_{25}	25.00 (70.6%)***	40.00 ***	24.50±4.95	24.50±14.85	1072.5±950.50 (63.1%) **

Table 3: Survival rate (L_X) and fecundity (M_X) of *S. mansoni* uninfected and infected *B. alexandrina* treated with sublethal concentrations of methanol extract of *C. citrinus* (24h weekly for 4 successive weeks):

Exposure period (weeks)	Control (untreated)						Treated											
	Uninfected			Infected			Uninfected						Infected					
							LC_{10} (9.3 ppm)			LC_{25} (17.2 ppm)			LC_{10} (9.3 ppm)			LC_{25} (17.2 ppm)		
	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx
0	1.00	5.30	5.30	1.00	5.30	5.30	1.00	5.30	5.30	1.00	5.30	5.30	1	5.30	5.30	1	5.30	5.30
1	1.00	3.70	3.70	0.76	1.6	1.22	0.95	1.40	1.33	0.73	3.10	2.26	0.7	1.4	0.98	0.65	1.1	0.72
2	1.00	4.50	4.50	0.6	0.2	0.12	0.85	2.00	1.70	0.70	2.40	1.68	0.43	0.04	0.018	0.38	0.8	0.3
3	0.90	2.70	2.43	0.56	1.1	0.62	0.73	0.01	0.01	0.65	1.10	0.72	0.23	0.02	0.005	0.06	0.1	0.006
4	0.90	6.50	5.85	0.23	0.8	0.184	0.56	0.00	0.00	0.50	0.01	0.01	0.07	0.0	0.0	0.0	0.0	0.0
R0 (Σ Lx Mx)			21.78			7.44**			8.34**			9.97*			6.30**			6.33**
Reduction R_0%						65.8			61.7			54.2			71.1			70.9

*, **& *** = Significantly different from control at $p< 0.05$, $p<0.01$ & $p<0.001$, respectively

Table 4: Total number & types (%) of hemocytes in *S. mansoni* uninfected and infected *B. alexandrina* treated with methanol extract of *C. citrinus*: Hyalinocytes (Hy), Small granulocytes (SG) and Large granulocytes (LG).

Exposure period	Hemocytes (No.) & Types (%)	Control		Treated			
		Uninfected	Infected	Uninfected		Infected	
				LC_{10}	LC_{25}	LC_{10}	LC_{25}
24h	Total no.	2340±295	1600±155	3170±320*	3250±370*	2050±250*	3000±265***
	Hy	21	8	25	31*	12	8
	SG	26	38	29	35	42	20**
	LG	53	54	46	34*	46	72**
1week	Total no.	2150±306	1150±128	720±140***	850±90***	250±48***	750±52***
	Hy	18	54	21	30*	33**	48
	SG	32	28	56***	55***	54***	33
	LG	50	18	23***	15***	13	19
2week	Total no.	2500±290	3500±344	2350±203	3100±380	2900±380	2450±205**
	Hy	17	51	46***	49***	50	44
	SG	33	21	33	22	29	37**
	LG	50	28	21***	29**	21	19
4week	Total no.	2700±307	2950±325	2600±230	3050±270	2250±223*	1950±134**
	Hy	19	34	13	18	28	28
	SG	40	45	22**	29	37	21***
	LG	41	21	65***	53*	35***	51***

*, **& *** =Significantly different from control at $p< 0.05$, $p<0.01$ & $p<0.001$, respectively

Table 5: Antioxidants in tissue homogenate of *S. mansoni* uninfected and infected *B. alexandrina* exposed to methanol extract of *C. citrinus* for 24h weekly for 4 successive weeks:

Exposure period	Enzymes	Control		Treated			
		Uninfected	Infected	Uninfected		Infected	
				LC_{10}	LC_{25}	LC_{10}	LC_{25}
24h	SOD	257.3±14.3	345.4±13.2	274.4±8.7	290.7±9.4*	407.8±18.2**	512.5±15.6***
	CAT	29.6±2.7	69.4±7.7	27.6±2.0	34.2±1.8*	63.7±8.4	41.5±7.2**
	GSH	4.1±0.4	2.6±0.7	3.6±0.25	2.9±0.3**	3.3±0.85	2.6±0.76
	LPO	103.3±3.2	123.7±5.6	102.0±1.9	94.7±2.4*	109.8±7.5*	117.4±6.0
1week	SOD	241.8±15	312.6±12.8	292.3±7.8**	302.5±9.7**	368.3±11.4**	427.2±13.5***
	CAT	26.8±3.5	51.7±6.8	43.6±6.6*	47.9±6.3**	47.9±6.3	38.7±4.7*
	GSH	3.7±0.27	4.2±0.6	2.7±0.31**	2.3±0.28**	5.2±0.6*	5.8±0.55*
	LPO	107.8±2.6	142.4±6.9	112.3±3.4	119.4±2.8**	106.5±4.1***	117.7±3.9**
2 week	SOD	248.5±9.8	322.9±9.8	200.7±7.4**	217.6±9.6*	199.4±10.6***	204.7±13.2***
	CAT	24.7±2.2	27.4±8.7	14.7±5.4*	12.6±3.9**	21.6±2.7	14.5±3.8*
	GSH	4.2±0.25	6.4±0.73	3.0±0.27**	2.5±0.18***	5.9±0.75	7.5±0.63
	LPO	111.3±4.1	113.6±4.5	101.7±2.2*	98.3±3.4**	123.7±4.7*	134.5±9.2*
4 week	SOD	249.2±11.2	199.6±8.8	168.8±10.7***	194.7±14.2**	118.6±17.3**	106.3±15***
	CAT	27.3±2.4	13.7±3.4	11.8±2.2***	9.9±1.5***	11.6±7.2	8.9±7.7
	GSH	3.6±0.3	7.8±0.5	2.8±0.1**	2.2±0.21**	6.4±0.45*	7.9±0.48
	LPO	106.2±2.2	99.4±5.8	96.5±4.7*	81.7±7.8**	134.8±6.9**	151.6±11.7**

*, **& *** = Significantly different from control at $p< 0.05$, $p<0.01$ & $p<0.001$, respectively

Discussion

Although chemotherapy is one of the most important methods for schistosomiasis control, there is still a need for more selective and efficient molluscicides for controlling the snail intermediate hosts of this parasite (WHO, 2010). The current data showed high toxicity of the plant methanol extract to the snails in comparison with water extract. This was supported by the pronounced negative impact of low con-centrations from this extract on survival rates of *S. mansoni* infected and uninfected snails compared to uninfected untreated (clean control) snails recorded in this work. This agrees with Kamel *et al.* (2010) on the high molluscicidal activity of methanol extract from the plant *Draceana draco* in comparison with the water suspension of its dry powder against *B. alexandrina* snails.

The present results showed a marked reduction in survival rates of *S. mansoni* uninfected *B. alexandrina* exposed for 24h/week for 4 successive weeks to sublethal concentrations from methanol extract of the plant *C. citrinus* in comparison with uninfected untreated control snails. This wass in agreement with previous records on survival rate of *B. alexandrina* snails post exposure to plants *Syzygium jambos* (Gawish, 2008) and *H. canariensis* (Abdel Rahman and Hassan, 2008). Similar observations were recorded post exposure of *B. alexandrina* snails to the plants *D. stramonium* and *S. sesban* (Mahmoud *et al*., 2011) and *Z. officinale* (Bakry *et al*, 2013). The toxic effect of *C. citrinus* to the snails may be due to the presence of the active ingredients cyanidine, delphinine, flavonoids, ursolic acid and saponins (Watson and Dallwitz, 1992). Molluscicidal activities of the plants *Cestrum purpureum* and *Yucca filamentose marginata* (Hamid *et al*, 2015 a; b) against *B. alexandrina* snails were due to the presence of the active chemical groups saponins, sterols and flavonoids. Undoubtedly, there was a high correlation between plants containing saponins and their molluscicidal activity (Lemma, 1970). The survival rate (L_X) of snails exposed to *S. mansoni* miracidia (infected untreated snails) was less than that of normal treated control snails, which indicated that infection was considerably harmful to snails. This coincided with Abdel Hamid *et al*. (2007) and El-Emam *et al.* (2015) on the low survival rate of *B. alexandrina* post infection with *S. mansoni* and/or *E. liei*.

The results revealed a suppression of *S. mansoni* cercarial production from *B. alexandrina* exposed to low concentrations of *C. citrinus* methanol extract for 24h either pre-, during or post-miracidial exposure, in addition to a decrease in snails' survival rates and in duration of cercarial shedding.

This was supported by the disturbances in the activities of the oxidative enzymes and

total number and types of hemocytes of snails infected and treated with this extract that could be a reflection of the interruption in their physiological and defence mechanisms which rendering them to be unsuitable for development of *S. mansoni* larval stages within their tissues. These findings agreed with using different plant species against infectivity of *S. mansoni* miracidia to *B. alexandrina* and on cercarial production from infected ones (Sharaf El-Din *et al*, 2001; Mahmoud *et al*, 2011).

The results elicited few snails' eggs were laid by *B. alexandrina* snails post exposure to methanol extract which reflected on suppression of their net reproductive rate (R_0) compared to control group. Also, treated snails stopped oviposition at the 4th week of the experiment. This agreed with Hasheesh *et al*. (2011) on egg lying of *B. truncatus* exposed to methanol extract of the plant *S. sesban*. Similar observations were recorded on suppressing egg laying of *B. alexandrina* post continuous exposure to ethanol extract of the plant *Z. officinale* (Bakry *et al*, 2013). This was supported by the present adverse effects of the tested extract on the histological structure of their digestive gland and on morphological features of their hemocytes. Ibrahim (2006) found that snail's reproduction is an energy demanding process and any energy diverted by snails treated with molluscicides to detoxificate and repair the damage in their tissues exerted by these compounds could potentially reduce their fecundity and reproductive rates. Similarly, the fecundity and reproductive rates of *B. alexandrina* were highly suppressed post exposure to sublethal concentrations of fungicide Topas (Mohamed *et al*, 2010) and the dry powder of *D. stramonium* and *S. sesban* (Mahmoud *et al*, 2011). This reduction in fecundity could be due to lipophilic pattern of botanical extracts which affect the lipid layers of the cell membrane of treated snails that destroy their specific permeability properties and this may lead to water loss causing dehydration that might lead to abnormalities of egg-laying capacity of these snails (Rashed, 2002).

Histological examination of digestive gland of *S. mansoni* infected and uninfected *B. alexandrina* snails treated with *C. citrinus* methanol extract revealed degeneration of gland tubules in addition to atrophied shrunken sporocysts, big vacuoles and degeneration of connective tissue between the gland tubules. This was in line with El-Deeb and El-Nahas (2005) who recorded severe damage in the digestive and hermaphrodite glands of *B. alexandrina* treated with *Euphorbia nubica* extract. Also, Bode *et al*. (1996) noticed an increase in secretory cells and decreased in digestive ones besides an intense autolysis of cell membrane structure in the digestive gland of *B. glabrata* snails treated with *T. tetraptera* extract. Similar, chloroform extract of the plant *H. tuberculatum* (Rizk *et al*, 2012) and methanol extract of *Callistemon viminalis* leaves (Gohar *et al*, 2014) destruct the epithelial layer and degenerate the secretory cells of *B. alexandrina* digestive gland.

The present increment of total number of hemocytes in *B. alexandrina* treated with *C. citrinus* post 24h of exposure to *S. mansoni* miracidia as well as in normal treated snails could be an immediate stimulation of their internal defense system to attack and encapsulate the invading miracidia and to take a part in repairing the tissue damages caused by the parasite penetration, also an attempt to decrease and minimize the harmful effects of the plant extract. These results agreed with Araque *et al*. (2003) observations on migration of *B. glabrata* hemocytes towards the invasive *S. mansoni* miracidia and surrounding them completely. Similar observations were found in *Oncomelonia nosophora* snails infected with *S. japonicum* (Sasaki *et al*, 2005) and in *B. alexandrina* infected with *S. mansoni* and treated with *M. azadrach* fruit water extract (Mohamed *et al*, 2006). Sharaf El-Din (2003) reported that double infection of *B. alexandrina* with *S. mansoni* and *E. liei* increased the hemocytes in their

hemolymph and added that treatment could stimulate hematopoietic organs to produce a number of new undifferentiated hemocytes through the first three weeks post exposure to miracidia.

Hemolymph granulocytes and hyalinocytes of *B. alexandrina* infected with *S. mansoni* only and of those infected and treated with plant extract suffered from deleterious morphological deteriorations, due to their important role in defense mechanism against these stresses (infection and plant extract). Similar results were observed by Eissa *et al*. (2002) who noticed deformation and hemolysis of hemocytes with ruptured cell membranes and degenerated nuclei of *B. alexandrina* snails exposed to *E. peplus* water suspension. Also, *B. alexandrina* hemocytes showed obvious abnormalities and irregular aggregation especially in snails exposed continuously for 21 days to LC_{25} of Artemether (Mossalem *et al*, 2013). Granulocytes and hyalinocytes showed cell apoptosis with degenerated cytoplasm, released granules and shrinked nucleus in *B. alexandrina* treated with LC_{10} & LC_{25} of the herbicides Herphosate and Stomp (Abdel-Ghaffar *et al*, 2016) and silver nanoparticles of the fungi *P. variotii* and *A. niger* (Abdel-Hamid and Mekawy, 2014).

In the present results, the activities of the antioxidant enzymes CAT & SOD increased in tissue homogenate of uninfected snails one week post exposure to LC_{10} & LC_{25}. The activities after 2 &4 weeks exposure were less than in control groups. This deterioration in may be due to snails immune system activation. This agreed with Hamed *et al*. (2006) who found that exposure of *B. alexandrina* to *A. attenuate* increased the activities of CAT and SOD. Enhancement of oxygen free radicals production post snails exposure stimulated antioxidant activities to cope with increased oxidative stress and protect snails' cells from damage (Torres *et al*., 2002).

The current data exhibited no changes in the activity of the antioxidant enzyme GSH in tissue homogenate of *S. mansoni* infected *B. alexandrina* snails treated with LC_{10} and LC_{25} of the plant *C. citrinus* methanol extract. This is in agreement with Kristoff *et al.* (2008) who reported that there is no change in GSH activity in *B. glabrata* snails exposed to some organophosphate pesticides. But, El-Emam *et al*. (2015) found an elevation in GSH activity in hemolymph of *B. alexandrina* snails exposed to the nematode *P. hermaphrodita* and/or *S.mansoni* miracidia. It was stated that the increase in GSH activity was associated by a depletion of lipid peroxide in snails exposed to S.*mansoni* miracidia and/or the larvae of the nematode *P. hermaphrodita* (Abou-Elnour *et al*, 2015). This was previously documented by Fahmy *et al*. (2014) who observed a correlation between enhancement of lipid perioxidation and consequence depletion of GSH in *B. alexandrina* snails exposed to zinc oxide nanoparticles.

Conclusion

The outcome results showed that *C. citrinus* methanol extract has a molluscicidal potency against *B. alexandrina* snails and reduce *S. mansoni* cercarial production from infected snails which could reduce schistosomiasis transmission. But, isolation of the plant active ingredients needs more studies against other snails of medical importance.

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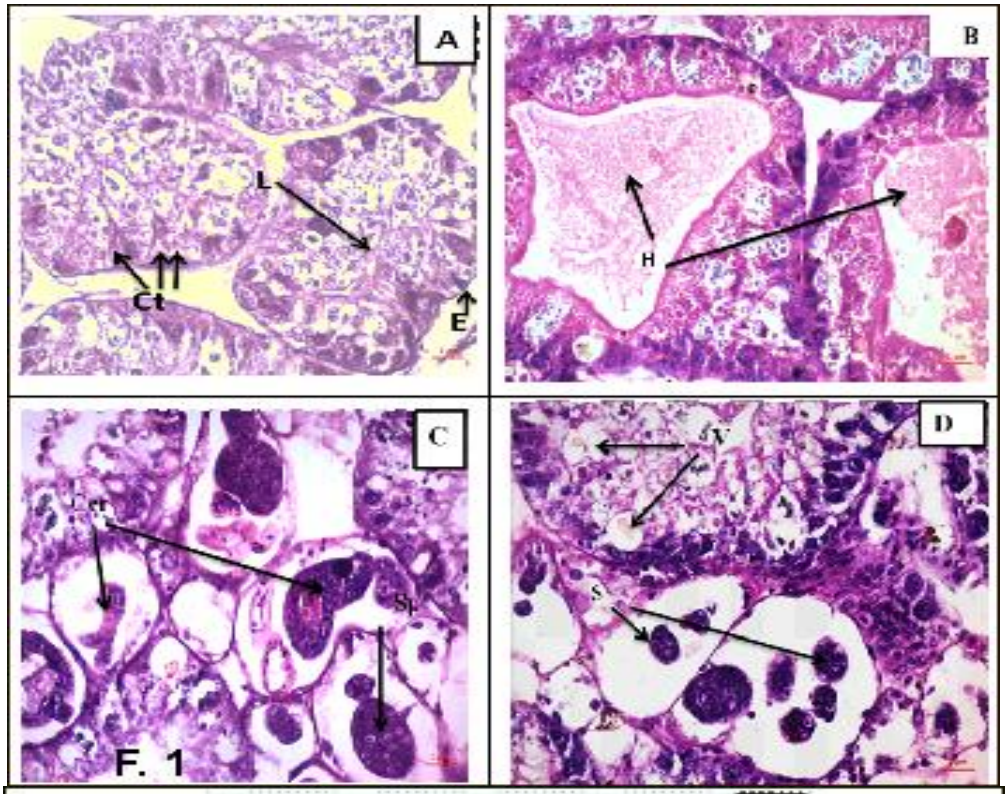
Explanation of figures

Fig. 1: Light photomicrographs showing transverse section in digestive gland of *B. alexandrina*. (A) untreated controls, (B) exposed for 24h to LC_{50} of extract of *C. citrinus*, (C) exposed to S. *mansoni* miracidia only showed cercariae and sporocysts in digestive gland after cercarial shedding, (D) exposed for 24h to S. *mansoni* and treated with LC_{50} stained with H and E. (At) atrophied cells, (Cer) cercariae, (Ct) connective tissues, (E) epithelial cell, (H) hyaline substance, (L) lumen, shrunk sporocycts (S), (Sp) sporocyst, (V) vacuoles.

Fig. 2: Light photomicrograph of hemocytes from *B. alexandrina* snail. (A) control, (B&C) morphological abnormalities in hemocytes post 24h of exposure to *C. citrius* extract, (D) *S. mansoni* infected *B. alexandrina*, (E) infected treated with extract of *C. citrius* stained with Giemsa stain (1000x). (CGR) condensed granules, (Cy) cytoplasm, (Dw), deformed cell wall, (Fp) filopodia, (GR) granules, (Hy) haylinocytes, (LG, large and SG, small) granulocytes, (N) nucleus, (Ir) irregular cell wall.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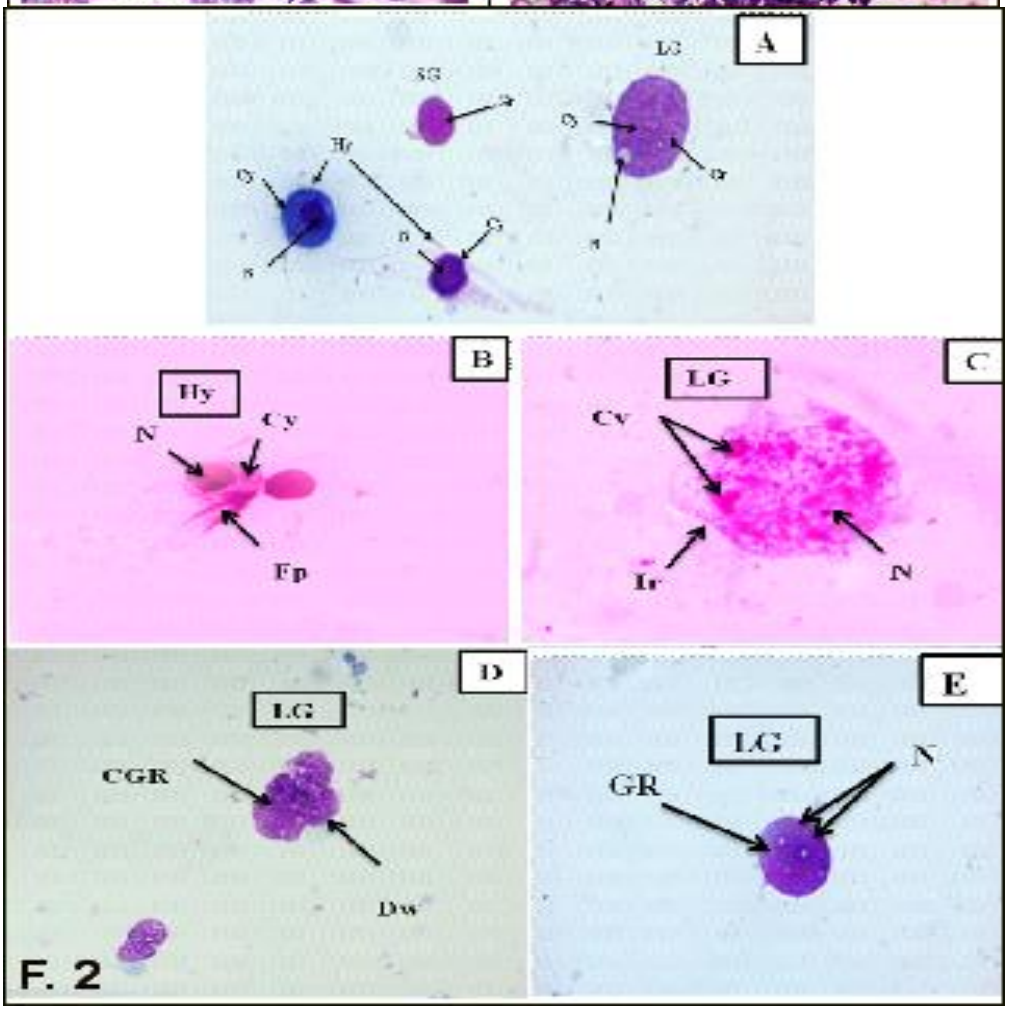
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