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Efficiency of *Citrus limon* extract on biological and molecular activities of *Biomphalaria alexandrina* snails

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ABSTRACT

Molluscicides have gained a great attention in recent years and proved to be a good way of snail control. *C. limon* was tested as a molluscicide against adult *Biomophalaria alexandrina* snails and a larvicide against *Schistosoma mansoni* larvae (miracidia and cercariae). Lethal concentrations, LC_{50} and LC_{90} values were detected for both tests (744.17 and 1821.25 ppm, respectively). *B. alexandrina* snails, non-infected and infected with *S. mansoni* were exposed to *Citrus limon* peel extract concentration of 150 ppm for 4 weeks compared to non expoed with *C. limon* and/or infected controls. The meracidea, cercariae, egg production, protein electrophoresis and DNA fragmentation analysis and organ histology of *B. alexandrina* were assessed. *C. limon* exposure stimulated the growth rate, increased the number of eggs/snail/week (59.31 ± 5.71), inhibited reproductive rate by 20.6 %, hatchability rate (57.7 %) and stimulation of cercarial production. Histological investigation of *C. limon* extract exhibited necrosis and deformation of hermaphrodite and digestive glands of infected and non-infected *B. alexandrina* snails. *C. limon* exposure led to increase in total protein intensity of the hermaphrodite-digestive glands in both exposed/exposed-infected snails. RNA intensity was increased in *C. limon* exposed and exposed-infected snails during the experimental period. Necrotic DNA form was a characteristic of *C. limon* and/or infection.

Key wards: *Citrus limon*, *Biomphalaria alexandrina*, molluscicide, proteins, DNA damage

INTRODUCTION

Schistosomiasis is an important disease that has the same significant as malaria as a major source of morbidity affecting approximately 210 million people in 76 countries of the world, in spite of the continuous control efforts (**Steinmann et al., 2006**). The molluscicidal and cercaricidal potencies of thousands plants were extensively studied all over the world, but a little attention was given to citrus plants **(Luna et al., 2005; Goel et al., 2007)**. *Citrus limon* peels oil contains 51 constituents where limonene is the major constituent followed by geranial, neral and others such as gamma-terpinene, beta-pinene and myrcene **(Ojeda et al., 1998; Vekiari et al., 2002)** and antibacterial oils **(Nannapaneni et al., 2008).** Citrus plants (Family: Rutaceae) have a pharmacological and economic importance because of their beneficial health effects such as anti-inflammatory, cardio-protective and anticancer activities. This pharmacological effect is mainly as they are rich in flavonoids which possess an antioxidant activity **(Moein et al., 2008; Hirata et al., 2009, Shin 2012)**. A comparative study between the antioxidant properties of peel and juice of some commercially grown citrus fruit (Family: Rutaceae), lemon (*Citrus limon*), lime (*Citrus aurantiifolia*), grapefruit (*Citrus paradisi*) and sweet orange (*Citrus sinensis*) was performed by **Guimarães et al. (2010)**. They revealed that the peels of polar fractions exhibited the highest contents in phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars which certainly contribute to the highest antioxidant potential.

The impact of the trematode parasite on the fresh water snails varies according to the trematode-snail compatibility, the tissues analyzed and the analytical method used **(Mostafa and Dajem, 2010)**. Trematode-parasitized molluscan hosts often show a temporary increase in growth rate compared to non-infected snails, but they did not reach the definitive sizes of healthy snails **(Pan, 1965; Coop and Holmes, 1996).** Histological studies on the hermaphrodite and digestive glands of snails were performed by many workers to investigate the molluscicidal effect of plants on *Biomphalaria* species. The

effects of plant molluscicides on the histology were investigated by many researchers. Exposure of *B. alexandrina* snails to *Sesbania sesban* plant extract (Family: Fabaceae) produced noticeable injurious changes in its hermaphrodite gland **(Rizk, 1998)**. **Bakry et al. (2007)** studied the histological changes in the hermaphrodite gland of *S. mansoni* infected *B. alexandrina* snails and exposed to sublethal concentrations of neem extract (Family: Meliaceae).

Every organisms studied to date has been shown to express a set of highly conserved proteins in response to external stresses association with cellular development, differentiation and stimulation **(Schlesinger, 1990)**. **El-Rigal and Hetta (2006)** proved that administration of both Mirazid and *C. reticulate* extracts to *S. mansoni* infected mice showed improved level of protein fraction concentrations. In more recent study, ethanol extract of *Artemisia judaica* L. (Family: Asteraceae) and *Solanum siniacum* have a molluscicidal effect against *B. alexandrina* snails, as they decreased snails fecundity, cercarial production from *S. mansoni* exposed-infected snails was suppressed and glycogen, total protein, pyruvate and lipids levels of exposed snails **(Bakry et al., 2011)**. **Ittiprasert et al. (2009)** revealed that RNA analysis from resistant, non-susceptible and susceptible juvenile *B. glabrata* snails to *S. mansoni* infection elicited that stress-related genes, heat shock protein 70 (Hsp 70) and reverse transcriptase (RT) were co-induced early in susceptible snails and the last two were elicited by infection and not injury. RNA plays an important role in protein synthesis, so the quantity of protein depends on the rate of protein synthesis and its degradation **(Singh et al. 2004)**. Moreover, the amount of RNA in the cells reflects the activity of gene expression and the copying of DNA into RNA with mRNA as an intermediate transcription, with more conversion into protein creature termed translation **(Nicholl, 1996)**.

The aim of the present study is to investigate the effect of *Citrus limon* on some biological, histological and molecular parameters of *B. alexandrina* snails infected with *S. mansoni* as a safe molluscicide of plant origin.

Materials and Methods

1. Experimental animals

The experimental snails used in the present study were adult *Biomphalaria alexandrina* (shell diameter ranged between 9 - 10 mm) and albino CD1 mice. The snails and mice were obtained from Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute, Giza, Egypt. *B. alexandrina* snails were maintained under standard laboratory conditions according to **El-Emam and Ebeid (1989)**.

2. Experimental materials

2.1. Yellow Lemon peels

Fresh yellow lemon (*Citrus limon*), were collected from the local trees found in Shebeen El-koom, washed and peeled. Then, stock solution was freshly prepared according to weigh/volume as one gram of the peels used freshly with 1000 ml of dechlorinated tap water using an electrical blinder and filtered with filter paper to get a concentration of 1000 ppm.

3. Experimental infection

3.1. Mice infection

Male CD1 mice were infected individually by paddling method in dechlorinated tap water contains 80-100 freshly emerged *S. mansoni* cercariae for 2 h according to **(Liang et al., 1987)**.

3.2 Snail Infection

After 6 - 8 weeks post mice infection liver and intestine's homogenate were mixed with dechlorinated tap water. The miracidia were gathered and snails were exposed individually to 2 ml dechlorinated tap water contains 6 - 8 miracidia for 3h **(Anderson et al., 1982)**.

3.3. Molluscicidal activity of Yellow Lemon peels

For tested concentration, triplicates (10 snails / tank) were used. The exposure period was 48h at room temperature (25 ºC ± 2). Triplicates of control snails were kept under the same laboratory conditions in dechlorinated tap water. Dead snails were recorded and removed from the container.

3.4. Miracidicidal and cercaricidal activity

For miracidicidal or cercaricidal activity, 40-50 freshly hutched miracidia or cercaria in 10 ml of declorinated tap water were mixed with another10 ml of double concentrations for each experimental one. Another 20 ml of clean declorinated tap water with 40 - 50 freshly hatched miracidia or cercaria were used as control. For tested and control groups, three replicates were prepared. The activity of *S. mansoni* aquatic larval stage was monitored with a dissecting microscope at time 5, 10, 15, 20, 30, 45 and 60 min. Dead miracidia or cercaria were recorded. Probit Proban analysis (Ver.1.1, **Finney, 1971**) was applied to determine the lethal concentrations, LC_{50} and LC_{90}.

4. Survival rate, growth rate and egg laying capacity of *B. alexandrina* snails

Ninety adults of *B. alexandrina* snails were continuously exposed to 150 ppm of *C. limon* (LC_{12}) for 4 weeks as a sublethal concentration in plastic containers. The exposure was changed weekly with freshly prepared one. Triplicates of control, *C. limon* and/or *S. mansoni* infected groups (10 snails each) were used. Snails were fed daily with fresh lettuce leaves and provided with foam pieces for oviposition. Mortality of snails, egg laying capacity and growth rate were recorded daily. Survival rate was calculated according to **Frank (1963)**.

The growth rate was calculated by calculating the mean values of the shell diameter of the snail form each experimental group weekly using a caliper according to **Chernin and Michelson (1957)**.

The egg masses laid on foam pieces or on the walls of the aquaria of all experimental groups were daily removed and counted by a hand lens (x 10). The egg-lying capacity was calculated according to **El-Gindy and Radhawy, (1965).**

The effect of sublethal concentrations of *C. limon* on hatchability of *B. alexandrina* eggs was investigated. 5 control egg masses (each contains 10 ± 2 eggs) aged 24 h were exposed to 20 ml of the tested solutions in triplicates, alongside with a control group. All groups were maintained at 25 ± 2 ºC till hatching **(Oteifa et al., 1975)**. Eggs were examined daily under a stereomicroscope and the number of normal viable eggs and hatched embryos were recorded **(Oliver et al., 1962)**. At the end of experiment (15 days), the percentage of hatchability was calculated by dividing the mean number of the hatched embryo by the mean number of total eggs at the beginning of the experiment.

The survived infected and exposed-infected snails were individually examined for cercarial shedding to detect the infection rate starting from the 3rd week post miracidial exposure till the end of the experiment (4th week, **Coles, 1973**). For each positive shedding snail, cercariae were

counted in 100 µl (3 replicates) as the mean number of cercariae/snail.

5. Histological study

For histological investigation**,** 5 snails were selected randomly from each experimental group. Shells were crushed and fragments were removed. Hermaphrodite and digestive glands were separated and immediately fixed in aqueous Bouin's fluid for 24 h. Then specimens were dehydrated in an ascending series of ethyl alcohol 15 min each. Then specimens were processed for histological examination according to the method described by **Romeis (1989)**.

6. Molecular study

6.1. Sodium Dodecyle Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS- PAGE was performed under reducing conditions according to the protocol of **Laemmli (1970)** to separate *B. alexandrina* tissue proteins. Total tissue proteins of hermaphrodite-digestive gland complex were separated on 8% resolving gel with 3.75% stacking gel using electrophoresis apparatus (Bio-Rad USA vertical minigel, double side). Hermaphrodite-digestive glands were dissected out from 3-5 snails and pooled with tissue-extracting buffer in a ratio of 1:10 w/v **(Bradford, 1976)**. The protein marker (205 - 29 kDa) obtained from Sigma Chemical Company. Protein bands were visualized by staining the gel with Coomassie Brilliant Blue (CBB) stain **(De-Moreno et al., 1985)** and was analyzed using Gel pro analyzer software (Ver. 3.0) cypermedica USA and gel densitometer Bio-rad G-70, USA.

6.2. Electrophoretic patterns of Nucleic acids

6.2.1. RNA and DNA analysis

Electrophoretic patterns of ribonucleic acid RNA of hermaphrodite - digestive glands from all groups were detected in tissue lysate according to **Hassab El-Nabi et al. (2001)**. DNA extraction from hermaphrodite-digestive glands of the snails was done according to "salting out extraction method" of **Aljanabi and Martinez (1997)** and modification introduced by **Hassab El-Nabi (2004)**. From each experimental group 20 mg of tissue were taken at time intervals of 24h, 48 h and 1st, 4th weeks post exposure. The optical density of apoptotic/necrotic bands were measured by software Gel program as maximum optical density values as the apoptosis bands located at 200 bp and its multiples.

7. Statical analysis

Data were analyzed using Statgraphics 5.1 Plus software. All data are expressed as means ± S.D. for the biological parameters in different groups. One-way ANOVA were conducted for exposure/infection effects. Where ANOVA could not be applied, a non-parametric ranking test was used (Kruskal Wallis test). The level of significance was accepted when $P < 0.05$..

RESULTS

1. Biological activity

1.1. The molluscicidal and larvicidal activity of *C. limon*

Molluscicidal activity of *C. limon* against adult *B. alexandrina* snails after 48h of exposure under laboratory conditions was investigated. The results revealed that *C. limon* peels had a molluscicidal activity against adult *B. alexandrina* snails. The results showed that LC_{50} and LC_{90} values after 48h of exposure to *C. limon* were 744.17 and 1821.25 ppm, respectively with slope function value 1.65.

C. limon had a larvicidal activity against *S. mansoni* miracidia and cercariae after 60 min of exposure. For miracidia, LC_{50} and LC_{90} values were 336.24 and 655.07 ppm, respectively with slope function 1.75. Concerning cercariae, LC_{50} and LC_{90} values were 252.28 and 510.48 ppm, respectively with slope function 2.37.

1.2. Effect of *C. limon* on biological activities of *B. alexandrina* snails

1.2.1. Survival rate

The survival rate of *B. alexandrina* snails exposed with *C. limon* recorded reduction throughout the experimental period when compared to control. The mean number of survived snails were 6.7 ± 1.6, 7.7 ± 1.9, and 6.33 ± 0.58 for *C. limon* exposed, infected and exposed-infected snails, respectively compared to 9.33 ± 2.3 for control at the end of the 3rd week of exposure. Concerning the effect of *C. limon* on infected and non-infected *B. alexandrina* snails during 4 weeks of exposure, there was a reduction in survival rate of exposed/infected snails but not significant ($P \geq 0.4$, ANOVA). The mean number was 6.6 ± 1.6, 5.7 ± 1.42, and 4.3 ± 0.08, respectively compared to 9 ± 2 for control at the end of the 4th week of exposure (Table 1).

1.2.2. Growth rate

The results illustrated in Table (1) revealed that there was a gradual decrease in growth rate indicated by the gradual decrease in shell diameter in all experimental groups. The obtained results indicated that there was a significant change on shell diameter by the effect of *C. limon* after 4 weeks of exposure (kruskal wallis, $P = 0.01$).

2.3. Egg laying capacity

The egg laying capacity of B. alexandrina snails markedly affected as a result of C. limon exposure and/or S. mansoni infection (Fig.1). C. limon caused stimulation in egg laying capacity particularly at the 4th week of exposure ($P \leq 0.01$, Kruskal wallis). The egg masses/snail/week and eggs/snails/week were 2.82 ± 0.23 and 59.31 ± 5.71 for exposed snails when compared to 1.84 ± 0.24 and 33.6 ± 3.04 of control group, respectively at the 4th week of exposure. The egg masses/snail/week in the exposed-infected group was 1.82 ± 0.24 and eggs/snail/week was 28.66 ± 2.63. S. mansoni infection led to significant reduction ($P \leq 0.007$, Kruskal wallis) in egg laying capacity of B. alexandrina snails in comparison with control snails where egg masses/snail/week was 0.9 ± 0.17 and eggs/snail/week was 18.39 ± 1.58 ($P \leq 0.003$, Kruskal wallis, Fig.1).

The net reproductive rate (R0) of B. alexandrina snails under continuous exposure to C. limon and/or S. mansoni infection was recorded in Table (2). The results indicated that R0 was greatly inhibited in all experimental groups when compared to the control group. R0 of C. limon exposed snails and exposed-infected snails were reduced by 20.6 and 53.54 % when compared to the control group of snails.

2.4. Effect of C. limon on B. alexandrina eggs hatchability

The results indicated that C .limon exposure decreased the mean number of hatched egg when compared to the control group. The exposed eggs hatched after 10 days and the mean number of hatched eggs was 83.7 ± 8.07 with hatchability percentage 57.7 %, while control hatched after 7 days of exposure and the mean number was 123 ± 11.5 and hatchability percentages 91.11 %.

2.5. Cercarial production

The results recorded great increase in the cercarial production with 89.5% in snails infected with S. mansoni and exposed with sublethal concentration of C. limon than the infected snails only. The mean number of shedding cercariae/snail of exposed-infected snails was 550.3 ± 33.5 when compared to 290.4 ± 17.3 of the infected group at the 4th week post infection with infection rates 69.23 and 62.5 %, respectively. However, there was no difference in the prepatent period length between infected and exposed-infected group.

3. Histological alterations

3.1. Hermaphrodite gland

Hermaphrodite gland of control B. alexandrina snails consists of acini connected to each other by connective tissue. Each acinus lined with germinal epithelial layer that differentiate into successive developmental stages of spermatogenesis and oogenesis "male and female gametocytes". Each acinus contains groups of primary and secondary oocytes and 1-2 mature ova which are arranged along the periphery of the acinus. Large numbers of developed sperms are in the lumen of the acinus (Fig. 2A).

The examination of infected snails revealed obvious histological alterations in whole architecture of hermaphrodite gland. The acini have a deformed shape separated by loose connective tissue. Degeneration in epithelial cells and ova observed beside the presence of sporocysts (Fig. 2B). The effect of C. limon on B. alexandrina hermaphrodite gland architecture showed severe injuries in all gland structures. In addition to deformed acini with scattered enlarged gametocytes, degenerated ova and destroyed epithelium layer observed (Fig. 2C). Both exposure and infection had a great effect on hermaphrodite gland of B. alexandrina snails (Fig. 2D). Acini lost their normal shape and condensed irregular sperms also observed, in addition to presence of sporocysts (Fig. 2D).

3.2. Digestive gland

The control B. alexandrina digestive gland composed of bundles of tubules. Each tubule lined by a single layer of columnar epithelial cells and differentiated into digestive and secretory cells surrounding a central lumen. The later cells are settled on their basal portion. The tubules connected to each other by connective tissue (Fig. 3A).

At the end of infection, the gland structure deformed, necrosis occurred to the epithelial cells and connective tissue. The acini deformed in shape with appearance of large number of sporocysts (Fig. 3B). Exposure with C. limon revealed some effects on the gland architecture. Part of connective tissue destroyed and the acini shape changed. Degeneration of some secretory and digestive cells and cellular vacuoles observed (Fig. 3C). C. limon exposure combined with S. mansoni infection dramatically affected the digestive gland of B. alexandrina snails. Completely necrotic acini and connective tissue with cellular vaculation and sporocysts observed (Fig. 3D).

4. Molecular study

4.1. Effect of C. limon and/or S. mansoni infection on total proteins in hermaphrodite-digestive glands of B. alexandrina snails

SDS-PAGE profile of tissue proteins extracted from B. alexandrina snails exposed with C. limon during 4 weeks of exposure is illustrated in Fig. (4A). The yielded protein bands in exposed group ranged in molecular weight between 8.23 and 485.3 kDa. The dominant protein bands between control and exposed group were 315.45, 121.18 and 35.06 kDa. The number of protein fractions decreased at 24 h, 48 h and (1st and 2nd) weeks to be 12, 14 and 11 fractions, respectively, and increased to 13 fractions in (3rd and 4th) weeks of exposure when compared to 17 fractions of the control group. Also, there is an occasional appearance of certain protein bands at different time intervals (24h and 4 weeks) as a result of exposure such as 243.04, 205, 54.62 and 6 kDa.

The electrophoretic patterns of tissue proteins separated from B. alexandrina snails infected with S. mansoni and exposed with C. limon during 4 weeks of exposure were illustrated in Fig. (4B). The dominant protein bands in both control and exposed-infected groups were 97.4, 74.65 and 36.06 kDa. Infection combined with exposure led to induction of new proteins at different time intervals. Protein bands of molecular weights 153.38 and 126.62 kDa appeared only at 4th week, 116 kDa appeared at 1st and 2nd weeks and 143.67 kDa appeared at all time intervals except after 24 h and 4th week.

The effect of S. mansoni infection on hermaphrodite-digestive glands proteins of B. alexandrina snails during 4 weeks of exposure was presented in Fig. (4C). There was a decrease in total protein intensity throughout the whole experimental period, 11 protein bands compared to 17 protein bands in control group at the 3rd week. The dominant band of molecular weight 42.35 kDa appeared at all time intervals of infection. Infection caused appearance of a new protein band such as 83.2 kDa. The protein band 129.19 kDa appeared at control and different time intervals of infected group but disappeared at 4th week only. On the other hand, 20.74 kDa and 48.07 kDa protein bands in infected group appeared at different time intervals as a result of S. mansoni infection.

5. Electrophoretic pattern of nucleic acids (RNA and DNA) of B. alexandrina snails exposed with C. limon and/or S. mansoni infection

5.1. RNA analysis

The results of C. limon exposed and exposed-infected snails showed observable increase in RNA expression of hermaphrodite-digestive glands (Fig. 5A). After 48 h of exposure and infection, an increase in RNA intensity in hermaphrodite-digestive glands observed when compared to control snails. On contrast, intensity of RNA decreased in hermaphrodite-digestive glands of C. limon exposed-infected group when compared to control and infected groups (Fig. 5B). After one week of exposure to C. limon exposure/infection, RNA intensity decreased when compared to control (Fig. 5C). After four weeks of exposure to C. limon exposure and/or S. mansoni infection, an increases in RNA intensity of hermaphrodite-digestive glands when compared with control and infected groups observed (Fig. 5D).

5.2 DNA fragmentation analysis

Concerning DNA, two types of fragments were recorded. The first one is apoptotic fragmentation and the second one is necrotic fragmentation. In the apoptotic fragmentation, apoptotic bands appeared at 200 bp and its multiples while the necrotic fragmentation appears as a smear shape.

C. limon exposure increased the amount of intact DNA in hermaphrodite-digestive glands when compared to control snails after 24, 48 h, 1 and 4 weeks of exposure (Fig. 6, Table 3). However, there was decease in the intact DNA amount in infected snails the intact DNA increased at the 4th week groups than control. After 48 h, the intact DNA increased in *S.*

mansoni infected, exposed and exposed-infected groups as 70, 131.4 and 160.2 maximal optical density, respectively when compared to the control 61.31 (Fig. 6B and Table 3). After one week of *C. limon* exposure, the amount of intact DNA in *C. limon* exposed-infected group was decreased with value 90.9 and infected snails with value 199.2 when compared to control 200.7. There was a general increase in the values of necrotic DNA fragmentations at 800, 600, 400 and 200 bp in all infected and/or exposed groups throughout the experiment when compared to control snails (Fig. 6C and D and Table 3).

Discussion

The present study demonstrated that *C. limon* had molluscicidal activity against adult *B. alexandrina* snails and larvicidal activity against *S. mansoni* larval stages (Miracidia and Cercariae). The tested sublethal concentration of *C. limon* (150 ppm) caused reduction in survival rates among adult *B. alexandrina* snails during the experimental period. These results are in agreement with those obtained by **Attia et al. (2009)** who recorded attenuation of cercarial ability to infect the final host and finally death due to the presence of flavonoids in air-dried mandarin peel extract (*C. reticulate*) which caused mitochondrial electron transport inhibition. Similar results obtained by **Mansour et al. (2004)** who proved that *C. limon* peel oil is an adulticide and larvicidal of the mosquito, *Culex pipiens* which may be due to the presence of the limonene as 90.06 % in *C. limon* peel oil.

In the present study, *C. limon* exhibited increase in cercarial production with a percentage 89.5% than the infected snails. Such finding is similar to the results of several previous studies on different molluscicides. **Badawy (1991)** who found that the sublethal concentrations of plant powder of *Agave filifera* (Family: Agavaceae) increased the infection rate of *Biomphalaria* snails and increase the mean number of cercariae/snail/week reaching 53.4% more than control. Similar results were obtained by **Bakry et al. (2007)** under the effect of neem plant, *Azadirachta indica* (Family: Meliaceae).

The present results recorded a significant reduction of the growth rate of *B. alexandrina* snails infected/treated with *C. limon*. Similar results have been reported by several investigators, that reduction of host growth rate during trematode infection is a common phenomenon in the long-term of infection **(Ibrahim, 2006)**. This inhibition in growth explained as the snails may be allocates their energy to maintenance **(Ibrahim, 2006)**. Or by **Becker (1980)** and **Pinheiro and Amato, (1994)** who stated that in trematode-molluscan systems, the physiological changes of infected snails has often been interpreted as being due to nutritional deprivation of the host imposed by the parasite. **Mello-Silva et al. (2010)** analysed the variation in glucose content in non-infected and *S. mansoni* infected *B. glabrata* snails exposed to a sublethal dose of *Euphorbia splendens* var. *hislopii* latex (as a natural selective molluscicide) for 24 h. They stated that the energy expenditure caused by the trematode infection and the latex exposure can cause an increase in the ATP consumption and acceleration of glycolysis. Also, when the mother sporocysts change to daughter sporocysts near the digestive gland, glycogen degradation from this organ occurs. The larvae are located in the interfollicular tissue and are bathed by the haemolymph, from which they obtain the glucose needed for the glycogenesis process.

In the present study, prolonged exposure of the snails to *C. limon* led to a remarkable increase in egg laying capacity but impaired hatchability of *B. alexandrina* eggs. This result may be due to the presence of flavonoids with antioxidant activity that stimulating snail's ova maturation, so increased egg production. Moreover, **Lien et al. (2008)** and **Ting et al. (2011)** studied the effect of flavonoids in laying hens. Those authors reported that feeding diets with added flavonoids extracted from citrus and grapefruit peels caused an increase in the ratio of yolk weight/egg weight and the blood serum superoxide dismutase activity but reduced serum and yolk cholesterols contents. **Abdel-Kader et al. (2005)** studied the effect of *Agave filifera* and *A. attenuate* (Family: Agavaceae) on *B. alexandrina* snails. They mentioned that there is inhibition of egg production, marked increase in the percentage of abnormal eggs and reduction in their hatchability.

The present study showed obviously severe damage of prolonged exposure of non-infected and/or infected snails to *C. limon* on the histological structure of the digestive and hermaphrodite glands. These results are in accordance with other investigators such as **Ragab et al. (2003)** mentioned that the digestive gland of *B. glabrata* snails treated with plant molluscicides was badly affected. **Rawi et al (2011)** investigated the effect of some natural plants and recorded congested tubules with foci necrosis, destructed nucleus/nucleolus and irregularity of the nuclear membrane and a reduction/disappearance in the size of the follicular cavity, destruction of connective tissue and ova. They added that the presence of saponin, catechin and tannins in all these tested plant extracts, were responsible for the molluscicidal activity of the investigated plants.

Exposure to *C. limon*/infection with *S. mansoni* led to an obvious increase in protein content of snails tissues after exposure. This may be due to the increase in globulin concentrations, which indicated with marked inhibition in the activity of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) under the effect of different molluscicides **(Ragab et al., 2003)**. The amelioration of protein fractions concentrations in *S. mansoni* infected mice treated with *C. reticulate* roots extracts is due to the presence of flavones, a class of flavinoides **(El-Rigal and Hetta, 2006)**. The flavonoids have antioxidant properties which protect protein against oxidative damage of free radicals **(Hara et al., 2004)**. Moreover, **Sharaf El-Din and El-Sayed (2001)** explained that such reduction based on protein depletion, movement restriction and castration as a physiological behavior induced in snails post infection to save energy for growth and development of schistosome sporocysts. In addition, **Coustau et al. (2003)** reported that *S. mansoni* produce proteinaceous excretory- secretory products might reflect a stimulatory effect on metabolism and responses due to the presence of toxic compounds, which direct the protein transcription of *Biomphalaria* snails. This reduction could be due to the proteolysis of tissue protein external to the parasite which then absorbed as micromolecules by developmental stages of the parasite **(El-Sheikh and Nagi, 1991)**.

In the present study, the amount of RNA and DNA damage and apoptotic bands in the tissue treated with *C. limon* and/or infected with *S. mansoni* snails were increased. **Hassab El-Nabi et al. (2001)** stated that the amount of RNA was increased in *B. alexandrina* snail's tissue treated with gibberellic acid and cycocel (plant growth regulators) as pollutants or any stress on cells may activate some silent genes to transcript more RNA. **Lockyer et al. (2008)** compared gene expression of hemocytes from resistant and susceptible snails within 24 h after exposure to schistosomes. The microarray data showed the upregulation of numerous transcripts in resistant snails which displayed homology to ornithin decarboxylase I, ADP/ATP carrier, lactate/malate dehydrogenase, glutamyl-prolyl-tRNAsynthase, histidyl-tRNA synthetase and tyrosyl-tRNA synthetase. Moreover, most of these transcripts are associated with protein synthesis

and metabolism and perhaps are involved in the increased egg laying observed in exposed and uninfected (resistant) *B. glabrata* snails **(Blair and Webster, 2007)**. Otherwise, **Wang et al. (1984)** stated that *Schistosoma* parasites unable for synthesis purines by itself and rely on host supplies of bases or nucleosides. A network of reactions converts these into the nucleotides required for DNA and RNA synthesis and other processes for the parasite.

In conclusion, *C. limon* peels water extract had molluscicidal and larvicidal activities against both adult *B. alexandrina* snails and *S. mansoni* larvae, respectively. Exposure with sublethal concentration of the tested material affected protein, DNA and RNA content of *S. mansoni* infected snails tissue. Consequently, the tested material is recommended as safe molluscicides for good control program of schistosomiasis.

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Table 1: Effect of the sublethal concentration of *C. limon* on biological activities of non-infected and *S. mansoni* infected *B. alexandrina* snails during 4 weeks of continuous exposure

Exposure period (week)	Experimental groups / Biological parameters	Control snails		*C. limon* -exposed snails	
		Non-infected	Infected	Non-infected	Infected
1	Survived snails	10 ± 0.0	9.7 ± 2.2	9 ± 2.2	8.66 ± 2.2
	Shell diameter (mm)	8.66 ± 0.42	8.4 ± 0.42	8.83 ± 0.41	8.44 ± 0.44
2	Survived snails	9.7 ± 2.41	8.7 ± 2.1	8 ± 0.98	7.33 ± 1.83
	Shell diameter (mm)	9.00 ± 0.45	8.58 ± 0.37	8.82 ± 0.44	8.74 ± 0.25
3	Survived snails	9.33 ± 2.3	7.7 ± 1.9	6.7 ± 1.6	6.33 ± 0.58
	Shell diameter (mm)	9.66 ± 0.41	9.28 ± 0.31	9.6 ± 0.42	9.50 ± 0.40
4	Survived snails	9 ± 2	6.6 ± 1.6	5.7 ± 1.42	4.3 ± 0.08
	Shell diameter (mm)	10.53 ± 0.44	9.3 ± 0.45	10 ± 0.42 *	9.74 ±0.25

Data are expressed as mean ± S.D., $n = 30$. * indicates significant difference (ANOVA/Kruskal wallis when $p < 0.05$) compared to the control snails.

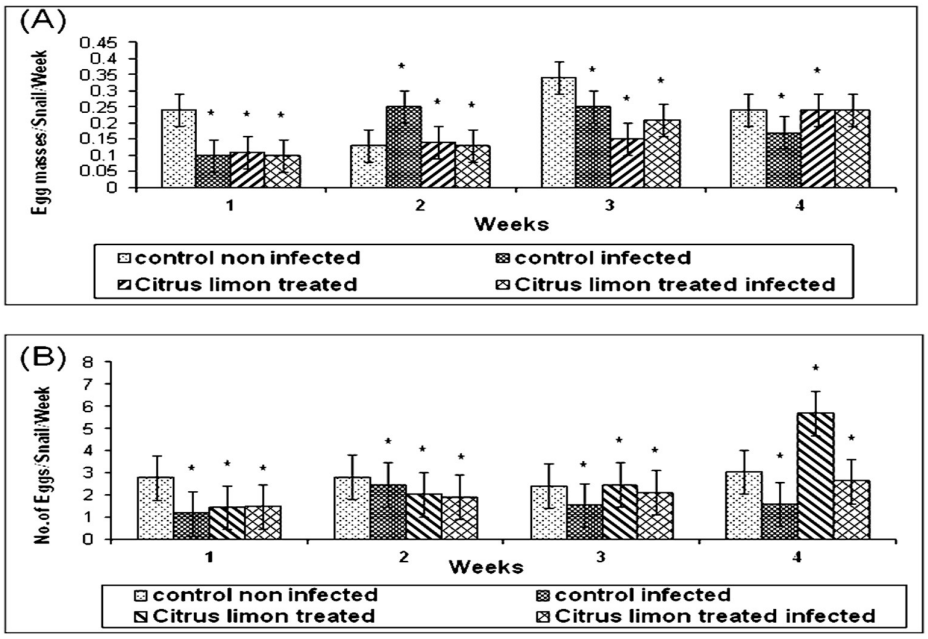

Fig. 1 Effect of the sublethal concentration of *C. limon* on egg laying capacity of non-infected and *S. mansoni* infected *B. alexandrina* snails during 4 weeks of continuous exposure, (A) The egg masses/snails/week and (B) No. of eggs/snail/week. * indicates significant difference (ANOVA/Kruskal wallis when $p < 0.05$) compared to the control snails.

Table 2: Effect of *C. limon* on reproduction of non-infected and *S. mansoni* infected *B. alexandrina* snails after 4 weeks of continuous exposure

Exposure period (week)	Control						*C. limon*-treated					
	Non-infected			*S. mansoni*-infected			Non-infected			*S. mansoni*-infected		
	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx
1	1	25.8	25.8	0.96	15.18	14.6	0.9	15.45	13.9	0.86	16.37	14.1
2	0.96	33.6	32.25	0.86	14.32	13.32	0.8	26.06	20.84	0.73	20.40	14.9
3	0.93	31.9	29.66	0.76	15.12	15.9	0.66	38.9	25.7	0.63	21.44	13.5
4	0.9	33.6	30.24	0.67	18.39	12.32	0.56	59.31	33.21	0.43	28.66	12.32
R_0	118			56.14			93.7			54.82		
Reduction %	-			52.4 %			20.6 %			53.54 %		

n = 10 snails/group. Time of exposure per week (x), Survival rate (L_X), Fecundity (M_X) the mean number of eggs/ snail/ week and the net reproductive rate (R_0) at any given week was represented by L_XM_X.

Table 3: Optical density of DNA fragments in hermaphrodite-digestive glands of non-infected and *S. mansoni* infected *B. alexandrina* snails treated with *C. limon* during 24, 48 h, 1 and 4 weeks of exposure

Visualized DNA (O.D)	After 24 h				After 48 h				After 1 week				After 4 weeks			
	Control	Infected	Treated	Treated infected	**Control**	Infected	Treated	Treated infected	**Control**	Infected	Treated	Treated infected	**Control**	Infected	Treated	Treated infected
Intact DNA	**10 2.9**	7. 71	17 8.4	16 6.3	**61. 31**	70	13 1.4	16 0.2	**20 0.7**	19 9.2	75	90. 9	**32. 71**	13 0.6	60. 4	46
800 bp	**2.2 4**	6. 42	18. 3	18. 11	**23. 1**	3. 45	90	10 0.8	**21. 20**	10 7.3	14 5.6	12 2.6	**35. 73**	61. 7	12 2.2	10 5.2
600 bp	**3.9**	9. 2	15. 84	4.8 3	**8.7**	3. 6	46. 3	66. 82	**15. 41**	44. 71	10 3.6	20 0	**40. 24**	65. 44	12 1.2	10 6.3
400 bp	**2.1 3**	6. 15	13. 1	0.2	**1.1 4**	3. 75	43. 81	65. 32	**4.9 1**	19. 19	11 0	17 5.4	**35. 61**	51. 4	10 7.3	99. 5
200 bp	**1**	6. 35	6.4 5	0.5 1	**7.1 1**	22 .4	80	42. 3	**17. 14**	39. 5	39. 65	41. 7	**8.4 5**	4.2 4	31. 2	26. 1

n = 10 snails/group. O. D; optical density.

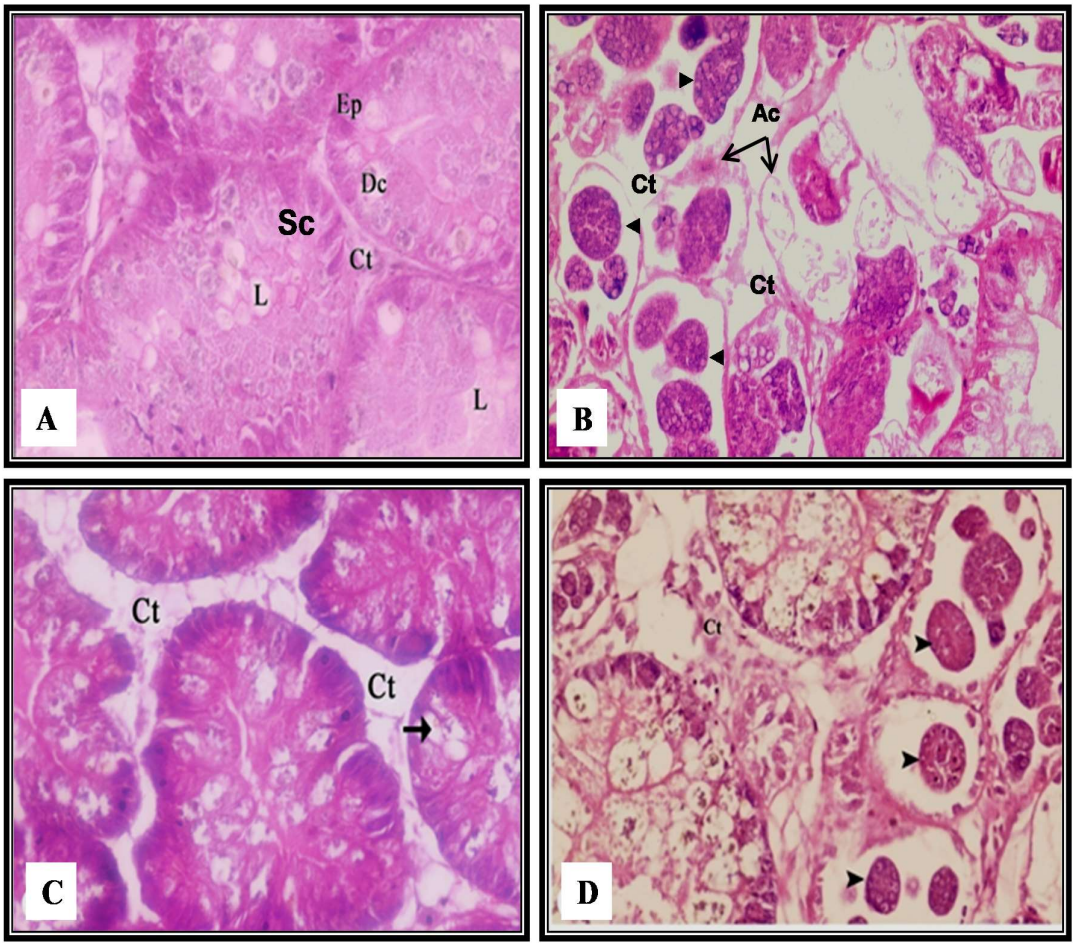

Fig. 2 Light photomicrographs of hermaphrodite gland transverse sections stained with E and H of *B. alexandrina* snails (A) control showing normal gland structure, acini (arrows), connective tissue (Ct), epithelial cells (Ep), sperms (Sp) and mature ova (Ov). (B) *B. alexandrina* snails infected with *S. mansoni* showing deformed acini separated by loose connective tissue, severe degeneration and atrophy (At) presence of sporocysts (arrow heads). (C) snails treated with *C. limon* showing complete destruction in connective tissue, degenerated ova and destroyed epithelial cells. (D) infected and treated snails showing dense connective tissue, irregular and condensed sperms (Isp) and degenerated epithelial layer and Sporocysts, (× 400).

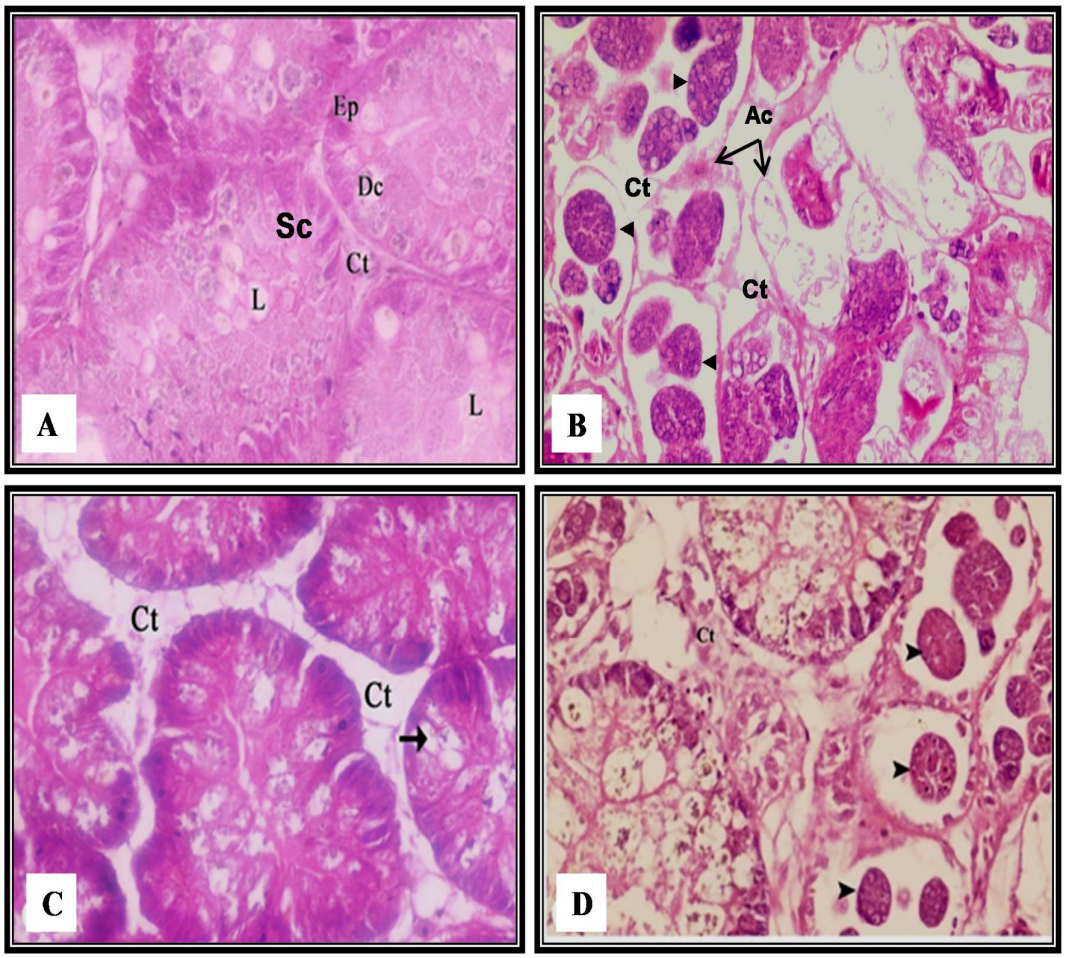

Fig. 3 Light photomicrographs of digestive gland stained with E and H of *B. alexandrina* snails (A) control, secretory cells (Sc), connective tissue (Ct), epithelial cells (Ep), lumen (L) and digestive cells (Dc), (B) Infected snails showing remarkable histological damage, acini (Ac) had deformed shape connected together with loose connective tissue and presence of sporocysts (arrows heads) were observed (B). (C) treated snails showing alterations ranging from degeneration of connective tissue to deformation of gland structure and presence of large vacuoles (arrow). (D) infected treated snails showing necrosis in connective tissue, deformed acini, sporocysts were observed, (× 400).

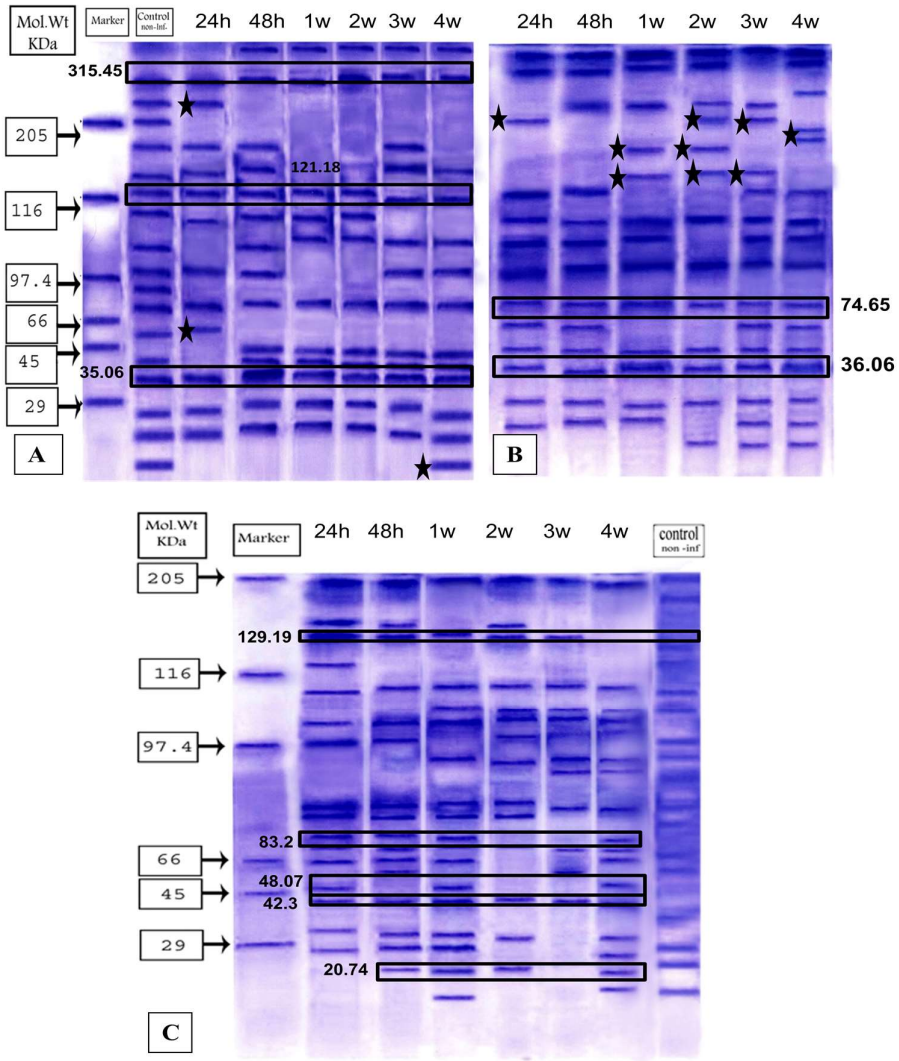

Fig. 4 SDS-PAGE profiles of proteins stained with CBB stain extracted from hermaphrodite digestive glands of: (A) *B. alexandrina* snails treated with sublethal concentration of *C. limon* (150 ppm) during 4 weeks of exposure. (B) *B. alexandrina* snails infected with *S. mansoni* and treated with *C. limon* during 4 weeks of exposure. (C) *B. alexandrina* snails infected with *S. mansoni* during 4 weeks post infection.

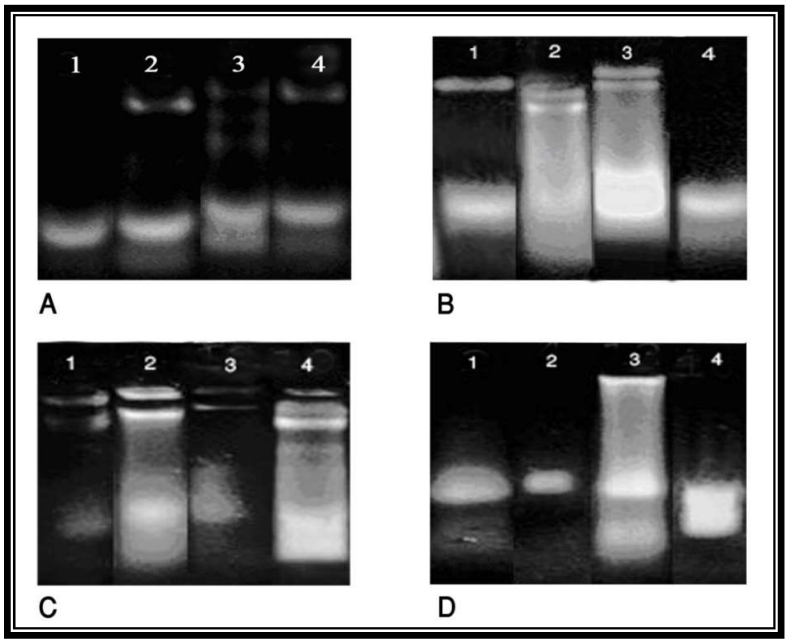

Fig. 5 RNA electrophoretic patterns of hermaphrodite-digestive glands (A, B, C and D) of non-infected and infected-treated snails with *C. limon* during experimental periods 24 h **(A)**, 48 h **(B)**, 1 week **(C)** and 4 weeks **(D)**. **lane (1)** Control snails, **lane (2)** Infected snails, **lane (3)** treated snails with *C. limon* and **lane (4)** Infected-treated snails with *C. limon.*

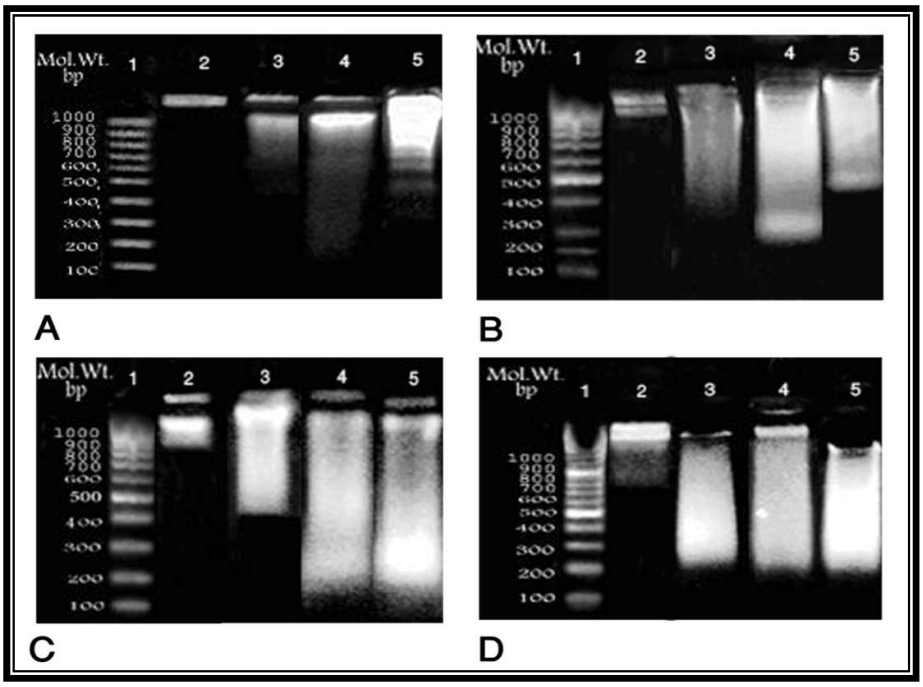

Fig. 6 DNA electrophoretic patterns of hermaphrodite-digestive glands of non-infected and *S. mansoni* infected *B. alexandrina* snails treated with *C. limon* during experimental periods 24 h (A), 48 h (B), 1 week (C) and 4 weeks (D). Lane (1) DNA ladder, lane (2) Control snails, lane (3) Infected snails, lane (4) Non-infected-treated snails, and lane (5) Infected-treated snails.

الملخص العربى

كفاءة مستخلص الليمون علي الانشطة البيولوجية و الجزيئة لقوقع بيموفلاريا الكسندرينا العائل الوسيط لبلهارسيا المستقيم.

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اكتسبت المبيدات الرخوية من اصل نباتي اهتماما كبيرا في السنوات الأخيرة وأثبتت أنها وسيلة جيدة لمكافحة القواقع. تم اختبار مستخلص قشر الليمون باعتباره مبيد الرخويات ضد قواقع بيموفلاريا الكسندرينا البالغة و يرقات بلهارسيا المستقيم (الميراسيديا والسركاريا). أوضحت النتائج أن مستخلص الليمون أثبت كفاءة متوسطة على القواقع حيث كانت قيم $LC90,LC_{50}$ لكل الاختبارات هى 744.17 و1821.25 جزء في المليون على التوالي. تم تعريض القواقع غير المصابة والمصابة بالبلهارسيا لمستخلص قشر الليمون بتركيز 150 جزء في المليون لمدة 4 أسابيع مقارنة مع المجموعة الضابطة. وكذا تم تقييم إنتاج الميراسيديا ، السركاريا، إنتاج البيض، والبروتينات تحليل الدنا, الرنا وعلى أنسجة القواقع. أظهرت النتائج أن القواقع المعرضة لمستخلص قشر الليمون حفز من معدل النمو، وزيادة عدد البيض / القواقع / الأسبوع (59.31 ± 5.71)، مع التأخير فى معدل الإنجاب بنسبة 20.6٪، نسبة الفقس ;كانت (57.7٪) وتحفز فى إنتاج السركاريا. أدي التعرض لمستخلص قشر لليمون إلي تشوه في الغدد الخنثوية و الهضمية في القواقع المصابة وغير المصابة. أدت المعاملة بمستخلص الليمون زيادة في كثافة البروتين الكلي للغدد الخنثوية و الهضمية في كل من القواقع المصابه وغير المصابه. تم زيادة كثافة الرنا في القواقع المصابة وغير المصابة خلال فترة التجربة. كذلك كان التحلل سمة من سمات الدنا للقواقع المعرضة للمستخلص أو المعدية بالسركاريا.