The efficiency of N-heterocyclic analogs against Biomphalaria alexandrina snails infected with Schistosoma mansoni; immune responses, histological structure and protein pattern

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ABSTRACT

Schistosomiasis is one of the most important tropical diseases. Analogs of nitrogen-based heterocycles hold a unique position in medicinal chemistry as a valuable source of therapeutic agents. This study aims to investigate the effects of different concentrations (0.25 and 1 ppm) of quinoline derivative as 7-chloro 4-amino ethyl amino quinoline hydrochloride salt (CAAQ) on survival, growth rates, mortality, immune response, histological structure and protein patterns of Biomphalaria alexandrina snails with/without S. mansoni infection under the effect. Exposure of snails to 0.25 and 1 ppm significantly (P ≤ 0.001) increased mortality and marginally decreased growth rates. In addition, exposure increased hayalinocytes (17.3 and 17.4 %, respectively) when compared to the control (5 %), decreased the granulocytes “spreading and non-spreading” (48.5 %, 51.6 % and 34.2 %, 31 %, respectively) when compared to the control (56.1 and 38.9 %, respectively). Exposure to both concentrations decreased the phagocytic haemocytes and increased the unphagocytic haemocytes percentages compared to the control snails. The present results indicated that the histological effect of CAAQ exposure on non and/or infected snails caused marked changes in the histological architecture of the ovotestis and digestive gland. The sub-lethal concentrations of CAAQ caused disturbance in the tissue total protein content of the ovotestis and digestive gland. SDS-PAGE of total protein pattern in ovotestis and digestive gland of exposed-infected snails showed occasional appearance and/or absence of certain protein bands. In conclusion, CAAQ modulated the immune responses of B. alexandrina with/without S. mansoni infection, histological structure and protein expression (SDS-PAGE). Consequently, CAAQ was recommended as a molluscicide of plant origin for the control program of schistosomiasis.

INTRODUCTION

Schistosomiasis represents a public health problem of the century (Ross et al., 2001). Estimates show that at least 236.6 million people required preventive treatment in 2019, which must be repeated over a number of years to reduce and prevent morbidity (WHO, 2019).
2022). The intestinal schistosomiasis has a great effect on the Egyptians health status. One of the main targets of the Egyptian Ministry of Health is to control schistosomiasis and decrease the intensity and prevalence of the disease. **Ghazy et al. (2022)** recorded the infection in a total of 2259 fecal samples (1113 in summer and 1146 in fall) collected from 861 children for 3 following days (46.9% were males and 31.8% and were aged 6–10 years). The prevalence of *S. mansoni* infection during the summer was higher than during the fall (19.1% and 7.2%, respectively).

*Biomphalaria* snails is the intermediate host of *S. mansoni*. Breaking the life cycle of the parasite by combating its snail host is one of the methods for controlling Schistosomiasis. Control is a key intervention to achieve the WHO goal for Schistosomiasis eradication (**King and Berto**, 2015). The control of the disease was depending entirely on praziquantel as a drug of use. An emerging resistance to the drug is shown by laboratory tests (**Doenhoff et al., 2009**). The high cost and hazards accompanied with synthetic compounds in the endemic areas of the world for snail control have caused a great need for molluscicides from plant origin (**Adewunmi, 1991**). The most promising compounds from plants against infectious diseases that with Alkaloids. Quinoline is one of the pharmaceutical cores used in drug discovery that containing N-heterocyclic with potent biological role as antioxidant, antiviral, anti-inflammatory and anticancerous agents (**Al-Sha’alan, 2007; Gupta and Mishra, 2016; Wilhelm et al., 2017; De la Guardia et al., 2018; Solomon et al., 2019 and Lauria et al., 2021**). Recent literatures reported the potency of quinoline based drugs as anti-schistosomal agent (**Taman et al., 2020**).

This study was aimed to investigate the effect of amino ethyl amino quinoline (CAAQ) on some biological aspects as survival, growth, immune responses, histological and protein patterns in of ovotestis and digestive gland of *B. alexandrina* snails with/without *S. mansoni* infection as a natural molluscicide of plant origin.

**MATERIALS AND METHODS**

**Experimental materials**
N-1-(7-chloroquinolin-4-yl) ethane-1, 2-diamine hydrochloride 4 (CAAQ) was synthesized in the Organic Chemistry Laboratory, Dept. of Chemistry, Faculty of Science, Menoufia University, Egypt.

NMR analyses with the Bruker magnet system 400, 54 Ascend/R (USA) 400MHz and 100MHz for 1H-NMR and 13C-NMR, respectively, were performed with Alpha-Bruker ATR mode (USA) at Zagazig University. All starting materials were purchased from Sigma Aldric such as 4, 7-dichloro quinoline, Diamines and the afforded products were prepared according to **Ruiz et al. (2011); Ahmed et al. (2020)** and **El Sayed et al. (2022).**
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**Experimental Animals**

**Snails**

Adult *B. alexanderina* snails were used in the main experiment. The snails were obtained from Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute, Giza, Egypt.

**Experimental Design**

Sub-lethal concentrations (0.25 and 1 ppm) of CAAQ were used in this study according to LC50 and LC90 calculated in the previous work (Sheir et al., 2023). Total of 540 mature snails (8-11 mm shell diameter) were divided into six groups. The 1st group was kept as the control group (neither exposed nor infected snails). The 2nd group was exposed to 1 ppm of CAAQ. The 3rd group was exposed to *S. mansoni* infection (infected control). The 4th group was exposed to 1 ppm of CAAQ and infected with *S. mansoni* (exposure + infection). Then, the 5th group was exposed to 0.25 ppm of CAAQ. Finally, the 6th group was exposed to both 0.25 ppm of CAAQ and *S. mansoni* infection (exposure + infection). Snails (30 snails/replicate and 90 snails/group) were immersed in 1000 ml of dechlorinated tap water treated with the experimental concentrations. Exposed water was regularly changed with freshly prepared ones twice a week. The experiment was lasted for 2 weeks.

Mortality of snails and growth rate were recorded daily. The mean total numbers of haemocytes/ml and differential number and phagocytic activity were examined after 24 hours of exposure. Histological structure, total protein content and electrophoretic protein pattern (ovotestis and digestive gland) were examined after the first and the second weeks of exposure.

**Bioassay tests**

**Mortality rate**

Mortality rate was determined in the control and exposed/infected groups according to Frank (1963) as the following equation:

\[
\text{Mortality rate} = \frac{\text{Number of dead snails}}{\text{Total number of snails (at the beginning of the experiment)}} \times 100
\]

**Growth rate**

The growth rate was measured by calculating the mean values of the shell diameter of the snail using a caliper from each experimental group. Measurements were taken at zero time and after two weeks in mm according to Chernin and Michelson (1957).

**Haemolymph collection and immune parameters**

Collection of haemolymph of *B. alexandrina* snails was carried out as described by Sminia (1972) after 24 hours of exposure. The snail was collected from each experimental group and the head foot was cleaned with tissue paper. The haemolymph was collected using the Micropipette from the haemocoel. 30 µl of haemolymph were
obtained from each snail. In each specified group the haemolymph of 10 individuals was pooled. Haemolymph samples were kept on ice until further investigation.

The total number of circulating haemocytes per ml of haemolymph was determined in fresh haemolymph diluted with Phosphate Buffer Saline (PBS, 7.4) using a haemocytometer. The number of total haemocytes was counted and the mean number of haemocytes/ml of haemolymph was calculated.

Haemocytes of *B. alexandrina* were challenged with yeast cells as a target for phagocytosis according to *Abdul-Salam and Michelson (1980)*, to determine the phagocytic activity of the haemocytes. Samples were incubated with yeast cells and the fixed monolayers were stained with Giemsa stain, air dried and mounted in DPX. From each slide, 100 cells were randomly examined and counted using a light microscope (Optica, Italy). Data were expressed as a percentage of positive/negative haemocytes from the calculated 100 cells/concentration and triplicate/concentration.

**Histological study**

After the first and the second weeks of exposure, randomly three selected snails were dissected from each group. The ovotestis and digestive gland were dissected out, and immediately fixed in Bouin’s fluid for 24 hours. Tissues were transferred into 70 % ethanol. Dehydration of the tissue was carried out in ascending series of ethanol (70 - 100%). Tissues were impeded in paraffin wax, serial sections were cut at 5 µ and stained with Ehrlich’s haematoxylin and eosin (*Romies, 1989*). Slides were examined under the microscope, and photographs were taken with a digital camera (Optika, Italy).

**Determination of total proteins**

The concentrations of total protein of the ovotestis and digestive gland after the first and the second weeks of exposure were measured by Bradford protein assay (*Bradford, 1976*). Samples of homogenates (0.04 ± 0.02 of ovotestis and 0.07 ± 0.04 of digestive gland) and blanks were prepared together. Proteins concentrations were expressed as mg of total protein per ml.

**Electrophoretic analysis of total protein**

Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed under reducing conditions according to the protocol of *Laemmli (1970)*. Total proteins of each ovotestis and digestive gland homogenates (0.04 ± 0.02 of ovotestis and 0.07 ± 0.04) were separately run on 12 % resolving gel with 3.3 % stacking gel using electrophoresis apparatus (OmniPAGE Mini Vertical Protein Electrophoresis System, Cleaver Scientific and United Kingdom).

**Statistical analysis**

All data sets were analyzed using Statgraphics Centurion XVI (Stat-Point Technologies Inc., Warrenton, VA, USA). The statistical analysis was carried out by one-way analysis of variance (ANOVA) under the effect of treatment. Data were considered significant when $P ≤ 0.05$. 
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RESULTS

Effect of CAAQ on some biological aspects of B. alexandrina snails

1.1. Survival rate

The survival rate of B. alexandrina snails exposed to 0.25 ppm and 1 ppm CAAQ recorded reduction during the experimental period (two weeks) when compared to the control snails (Table 1). The survival rate of S. mansoni infected snails was 93 % (2.0 ± 1.16), whiles the exposed non-infected snails with (0.25 ppm and 1 ppm) were 97 % (1.0 ± 1.73) and 100 % (0.0 ± 0.0), respectively and the treated-infected snails were 77 % (7.0 ± 2.11) and 70 % (9.0 ± 5.19) at the end of the 1st week when compared to the control snails. The mortality rate of S. mansoni infected snails had a significant increase by the effect of CAAQ after one week of exposure and infection (P ≤ 0.001, ANOVA).

The survival rate of S. mansoni infected snails was 96 % (1.33 ± 0.58), whiles the exposed non-infected snails with (0.25 ppm and 1 ppm) were 38 % (18.67 ± 2.08) and 61 % (11.67 ± 0.58), respectively and the treated-infected snails were 61 % (11.67 ± 0.58) and 79 % (6.34 ± 2.11) at the end of the 2nd week when compared to the control snails. The mortality rate of S. mansoni infected snails had a significant increase by the effect of CAAQ after two weeks of exposure and infection (P ≤ 0.001, ANOVA).

Table (1): Effect of sub-lethal concentrations of CAAQ on dead snail’s mean number of B. alexandrina snails

<table>
<thead>
<tr>
<th>Time of exposure</th>
<th>Exposure concentrations</th>
<th>Non-infected snails</th>
<th>Infected snails</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>0.0 ± 0.0</td>
<td>1.15 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1st week</td>
<td>Control</td>
<td>0.67 ± 1.15</td>
<td>2.0 ± 1.16</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>1.0 ± 1.73</td>
<td>7.0 ± 2.11*</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>11.67 ± 0.58*</td>
<td>9.0 ± 5.19*</td>
</tr>
<tr>
<td>2nd week</td>
<td>Control</td>
<td>1.0 ± 0.0</td>
<td>1.33 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>9.0 ± 5.19*</td>
<td>11.67 ± 0.58*</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>11.67 ± 0.58*</td>
<td>6.34 ± 2.11*</td>
</tr>
</tbody>
</table>

Note: Data were expressed as mean ± SD. Significant difference* when P ≤ 0.05 when compared to the control.

1.2. Growth rate

The growth rate (expressed as mean of shell diameter/mm) of B. alexandrina snails in the control and experimental groups is shown in Table 2. The results revealed that there
was a slight decrease in the growth rate in all experimental groups with \( P \) value \( \geq 0.05 \) (ANOVA). The obtained results indicated that there was a non significant \( (P > 0.05) \) change in the shell diameter by the effect of CAAQ after 2 weeks of exposure but not significant for exposed and exposed-infected snails. The shell diameter of snails at the end of the 2\(^{nd}\) week was 1.0 \( \pm \) 0.09 mm, 0.98 \( \pm \) 0.11 mm and 1.02 \( \pm \) 0.13 mm for the exposed 0.25, 1 ppm and the control snails, respectively. At zero time, the shell diameter of snails was 1.02 \( \pm \) 0.09 mm, 1.0 \( \pm \) 0.06 mm and 1.24 \( \pm \) 0.10 mm for the exposed 0.25, 1 ppm-infected snails and infected control snails, respectively.

**Table (2):** Effect of sub-lethal concentrations of CAAQ on growth rate of *B. alexandrina* snails

<table>
<thead>
<tr>
<th>Time of exposure</th>
<th>Exposure concentrations</th>
<th>Non-infected snails</th>
<th>Infected snails</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>Control</td>
<td>1.02 ( \pm ) 0.08</td>
<td>1.21 ( \pm ) 0.11</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>0.99 ( \pm ) 0.07</td>
<td>1.03 ( \pm ) 0.07</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>1.01 ( \pm ) 0.13</td>
<td>1.05 ( \pm ) 0.08</td>
</tr>
<tr>
<td>After 2 weeks</td>
<td>Control</td>
<td>1.02 ( \pm ) 0.13</td>
<td>1.24 ( \pm ) 0.10</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>1.0 ( \pm ) 0.09</td>
<td>1.02 ( \pm ) 0.09</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>0.98 ( \pm ) 0.11</td>
<td>1.0 ( \pm ) 0.06</td>
</tr>
</tbody>
</table>

**Note:** Data were expressed as mean \( \pm \) SD.

1.3. **Effect of CAAQ on *B. alexandrina* snails' immune responses**

The mean total numbers of circulating haemocytes/ml of the control, infected control, CAAQ -treated and CAAQ treated-infected snails after 24 hours are represented in Table (3). The total numbers of haemocytes of exposed snails to 0.25 and 1 ppm were 3.62 \( \pm \) 1.0 and 2.7 \( \pm \) 0.35 when compared to 9.5 \( \pm \) 0.0 of the control, respectively \( (P \leq 0.001) \). The total number of haemocytes of the infected-exposed snails to 0.25 and 1 ppm were 1.23 \( \pm \) 0.12 and 1.23 \( \pm \) 0.13 when compared to 1.15 \( \pm \) 1.0 of the infected control, respectively after 24 hours of exposure \( (P \geq 0.05) \).

Examination of haemocytes obtained from the control *B. alexandrina* revealed that the haemolymph contained three distinct types of haemocytes, designated as one round small (hyalinocytes) and two granulocytes (spreading and non-spreading) presented in Tables 3 & 4. Concerning the effect of CAAQ on the percentage number of hyalinocytes of *B. alexandrina* snails after 24 hours of exposure, there was a significant increase \( (P \leq 0.001) \) in the non-infected snails when compared to control. On the other hand, there was a non significant increase \( (P > 0.05) \) in the infected snails when compared to infected control. After 24 hours of exposure, the percentage number of hyalinocytes of the exposed snails to 0.25 and 1 ppm were 17.3 % and 17.4 %, compared to 5 % of the
control, respectively. The percentage number of hyalinocytes of infected-exposed snails to 0.25 and 1 ppm were 23 % and 27.3 % when compared to 23.2 % of the infected control, respectively. CAAQ caused a significant reduction ($P \leq 0.001$) in the percentage number of spreading granulocytes of the non-infected snails when compared to the control, while CAAQ caused a non significant reduction ($P > 0.05$) in the infected snails when compared to infected control. The percentage number of spreading granulocytes of the exposed snails to 0.25 and 1 ppm were 48.5 % and 51.6 %, respectively when compared to 56.1 % of the control. The percentage number of spreading granulocytes of infected-exposed snails to 0.25 and 1 ppm were 51.5 % and 41.5 % respectively, when compared to 53.3 % of the infected control, respectively after 24 hours of exposure. CAAQ caused a significant reduction ($P \leq 0.001$) in the percentage number of non-spreading granulocytes of the non-infected snails when compared to control, while CAAQ caused an insignificant increase ($P > 0.05$) in the infected snails when compared to infected control, after 24 hours of exposure. The percentage number of non-spreading granulocytes of the exposed snails to 0.25 and 1 ppm were 34.2 % and 31 %, respectively when compared to 38.9 % of the control. On the other hand, the percentage number of non-spreading granulocytes of infected-exposed snails to 0.25 and 1 ppm were 25.5 % and 31.2 % respectively when compared to 23.5 % of the infected control, after 24 hours of exposure.

**Table (3):** Effect of sub-lethal concentrations of CAAQ on the mean number of haemocytes count/ml of *B. alexandrina* snails

<table>
<thead>
<tr>
<th>Time</th>
<th>Exposure concentrations</th>
<th>Non infected snails</th>
<th>Infected snails</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>Control</td>
<td>9.5 ± 0.0</td>
<td>1.15 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>3.62 ± 1.0*</td>
<td>1.23 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>0.35* ±2.7</td>
<td>1.23± 0.13</td>
</tr>
</tbody>
</table>

Note: Data were expressed as mean $\times 10^5 \pm$ SD. Significant difference* when $P \leq 0.05$ when compared to the control.
Table (4): Effect of sub-lethal concentrations of CAAQ on the percentage number of differential haemocytes count of *B. alexandrina* snails

<table>
<thead>
<tr>
<th>Time of exposure</th>
<th>Exposure concentrations</th>
<th>Haemocyte types</th>
<th>Non infected snails</th>
<th>Infected snails</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyalinocytes</td>
<td>Granulocytes</td>
<td>Hyalinocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>spreading</td>
<td>spreading</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>non-spreading</td>
<td>non-spreading</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>5.0</td>
<td>56.1</td>
<td>23.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>53.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23.5</td>
</tr>
<tr>
<td>0.25 ppm</td>
<td></td>
<td>17.3</td>
<td>48.58*</td>
<td>34.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.5</td>
</tr>
<tr>
<td>1 ppm</td>
<td></td>
<td>17.4*</td>
<td>51.6*</td>
<td>31*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>41.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.2</td>
</tr>
</tbody>
</table>

Note; Significant difference* when $P \leq 0.05$ when compared to the control.

The phagocytic activity of *B. alexandrina* snails markedly affected as a result of CAAQ exposure and/or *S. mansoni* infection after 24 hours of exposure (Figure 1 & Table 5). The phagocytic activity of *B. alexandrina* exposed to 0.25 and 1 ppm of CAAQ recorded significant ($P \leq 0.05$) decrease when compared to the control snails after 24 hours of exposure. The percentages of phagocytic and non-phagocytic cells in the control group were 53.7 % and 46.3 %, respectively. The snails exposed to 0.25 ppm of CAAQ recorded 29 % and 71 % phagocytic and non-phagocytic haemocytes, respectively. However, in snails exposed to 1 ppm CAAQ phagocytic and non-phagocytic haemocytes percentages were 35 % and 65 % respectively. The percentages of phagocytic and non-phagocytic cells in the control-infected group were 49.7 % and 50.3 %, respectively. The infected snails exposed to 0.25 ppm of CAAQ recorded 14.3 % and 85.7 % phagocytic and non-phagocytic haemocytes, respectively. However, in infected snails exposed to 1 ppm of CAAQ phagocytic and non-phagocytic haemocytes percentages were 16.7 % and 83.3 % respectively.
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**Table (5):** Effect of sub-lethal concentrations of CAAQ on the phagocytic activity (%) of haemocytes of *B. alexandrina* snails

<table>
<thead>
<tr>
<th>Exposure Time</th>
<th>Exposure concentrations</th>
<th>Phagocytic haemocytes</th>
<th>Non-Phagocytic haemocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>Control</td>
<td>53.7</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm</td>
<td>29*</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>35*</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Infected control</td>
<td>49.7</td>
<td>50.3</td>
</tr>
<tr>
<td></td>
<td>0.25 ppm + infection</td>
<td>14.3*</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>1 ppm + infection</td>
<td>16.7*</td>
<td>83.3</td>
</tr>
</tbody>
</table>

*Note:* Significant difference*\(^*\) when \(P \leq 0.05\) when compared to the control.

**Figure (1):** Light micrographs of haemocytes (stained with Giemsa) showing the phagocytic activity of haemocytes in (A) the control snails; (B) the infected control snails; (C) 0.25 ppm of CAAQ-exposed snails; (D) 0.25 ppm of CAAQ + infection snails; (E) 1 ppm of CAAQ exposed snails; (F) 1 ppm of CAAQ + infection snails. h, haemocytes; y, yeast cells.
Histopathological effect of CAAQ on *B. alexandrina* snails

1.1. The ovotestis
In the control snails, the ovotestis is composed of a number of acini connected together by connective tissue. Each acinus is lined with germinal epithelial layer that give rise to many developmental stages of oogonia and spermatogonia (female and male gametocytes). The histological effect of CAAQ exposure on a non–infected snails caused marked changes in the histological architecture of the ovotestis (Figure 2). Exposure to 0.25 ppm of CAAQ for one week caused loose connective tissue and disappearance of some developmental stages of oocytes and sperms. However, exposure to 0.25 ppm of CAAQ and infection together caused necrosis in connective tissue, disorganization in developmental stages of gametocytes inside the acini and degenerated mature ova. After 2 weeks of exposure to 0.25 ppm of CAAQ, necrosis and degeneration of the acini and absence of oocytes were observed. Meanwhile, after 2 weeks of exposure to 0.25 ppm of CAAQ and infection, disappearance of gamete stages occurred. Exposure to 1 ppm of CAAQ after one week caused fusion and degeneration of the acini when compared to the control snails. On the other hand, exposure to 1 ppm of CAAQ and infection caused disorganization of the acini and disappearance of developmental stages. After 2 weeks of exposure to 1 ppm of CAAQ, the acini lost their normal shape and became scattered (data are not shown). Inflammation in connective tissue and necrosis in epithelium cells were also noticed. After 2 weeks of exposure to both 1 ppm of CAAQ and infection caused disappearance of gamete stages.

1.2. The digestive gland
In the control snails, the digestive gland comprises a tubulo-acinar gland with bundles of the tubules. Each tubule formed from a single layer of epithelial cells differentiated into digestive and secretory cells surrounding a central lumen and connected to each other by connective tissue. As a result of *S. mansoni* infection, some histological alterations in the digestive gland were found such as: the tubules had deformed shape and were connected together with loose connective tissue (Figure 3). As a result of exposure of snails to 0.25 and 1 ppm of CAAQ for 2 weeks, marked histolopathological changes were noticed. After 1 week of exposure to 0.25 ppm of CAAQ, erosion of cilia, fusion of tubules, inflammation in the epithelial cells and decrease in secretory cells were noticed. However, exposure to 0.25 ppm of CAAQ and infection, some pathological changes were detected as vacuolization, increase in secretory cells and complete fusion of digestive tubules. After 2 weeks of exposure to 0.25 ppm of CAAQ deformation of tubules, vacuolization and increase in fat droplets were detected. Moreover, after two weeks of exposure to 0.25 ppm of CAAQ and infection together, an increase in secretory cells, complete clogging of the tubule's lumen, necrosis in epithelium and disappearance of nuclei.
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Figure (2): Light photomicrographs of sections through ovotestis of *B. alexandrina* snails stained with E & H. (A,B): the control snails; (C) the infected snails with *S. mansoni* showing disorganization of acini (arrows); (D) the exposed snails to 0.25 ppm for one week showing disappearance of oocytes and acini became filled with sperms (arrows); (E) the exposed snails to 1 ppm for one week showing disorganization of gametes distribution (*), enlargement in ova, necrosis in gereminal layer (arrows) and sperms; (F) one week- exposed snails to 1 ppm showing inflammation in connective tissue and necrosis in epithelium cells. acini (Ac), connective tissue (Ct), epithelial cells (Ep). sperms (Sp), mature ova (Ov), sperms (Sp), inflammation (Inf), necrosis (Ne).

After 1 week of exposure to 1 ppm of CAAQ necrosis in the epithelial cells, fat droplets formation and increase in the secretory cells. Exposure to both 1 ppm of CAAQ and infection resulted in a hypertrophy in digestive cells, necrosis and fusion in digestive tubules. In addition, hypertrophy and hyperplastic changes of the digestive tubules epithelial (data are not shown). However, exposure to 1 ppm of CAAQ showed complete fusion of tubules, after 2 weeks. Exposure to 1 ppm of CAAQ and infection together caused fusion in tubules and the digestive cells became filled with undigested food/material.
**Figure (3):** Light photomicrographs of sections through the digestive gland of *B. alexandrina* snails and stained with E & H. (A, B) the control snails; (C) the infected snails with *S. mansoni*, showing deformed shape and fusion of tubules with necrotic connective tissue; (D) the exposed snails to 0.25 ppm after one week showing necrosis in epithelial cells of tubules, fat droplets and decrease in of secretory cells; (E) the exposed snails to 0.25 ppm showing increase in secretory cells, complete clogging of tubules lumen, and necrosis in epithelium and connective tissue; (F) the exposed snails to 1 ppm after one week showing complete disorganization of tubules and digestive cells were filled with undigested food. tubule (Tu), connective tissue (Ct), epithelial cells (Ep), secretory cells (Sc), digestive cells (Dc), lumen (L), necrosis (Ne), fat droplets (F), undigested food (Udf).

**Total protein content of *B. alexandrina* snails**

The total protein content in the ovotestis and digestive gland of non-infected snails and infected snails exposed to sub-lethal concentrations (0.25 and 1 ppm) of CAAQ were measured. The total protein content in the ovotestis of non-infected snails were significantly reduced (*P ≤ 0.001*) after 1 week of exposure. The total protein content in the ovotestis of exposed to 0.25 ppm and non-infected snails were 0.24 ± 0.14 and 0.27 ± 0.15, respectively. The total protein content in the ovotestis of the exposed to 0.25 ppm and infected snails were insignificantly increased (*P > 0.05, 0.76 ± 0.35*), while the infected snails exposed to 1 ppm were also insignificantly reduced to 0.17 ± 0.14.

After the second week of exposure, values of the total protein content in the ovotestis of non-infected exposed snails to 0.25 and 1 ppm, were insignificantly increased to reach 0.25 ± 0.14 and 0.22 ± 0.14, respectively. Meanwhile, the total protein
of exposed infected snails to 0.25 and 1 ppm were significantly reduced ($P > 0.05$) to reach $0.18 \pm 0.14$ and $0.22 \pm 0.14$, respectively.

The total protein content in the digestive gland of non-infected snails and infected snails had a significant reduction ($P \leq 0.001$) after the first week of exposure. The total protein content in the digestive gland of non-infected snails exposed to 0.25 and 1 ppm after one week of exposure were $0.22 \pm 0.14$ and $0.71 \pm 0.32$, respectively. While, the infected snails exposed to 0.25 and 1 ppm were $0.02 \pm 0.19$ and $1.97 \pm 1.02$, respectively.

After the second week of exposure, the total protein content in the digestive gland of non-infected snails and infected snails had a reduction but not significant ($P > 0.05$), except the exposed non-infected snails to 1 ppm had an insignificant increase ($P > 0.05$). The total protein content in the digestive gland of non-infected snails exposed to 0.25 and 1 ppm after two weeks of exposure were $0.23 \pm 0.14$ and $0.36 \pm 0.17$, respectively whiles the infected exposed snails to 0.25 and 1 ppm were $0.51 \pm 0.23$ and $0.22 \pm 0.14$, respectively.

**Total protein Electrophoretic pattern of *B. alexandrina* snails` ovotestis and digestive gland**

The obtained results showed significant qualitative and quantitative differences in total protein pattern between exposed and exposed-infected groups when compared to the control and infected control groups. The total molecular weights of the ovotestis proteins of snails exposed to 0.25 ppm were 522 KDa and 502 KDa for the first and second weeks, respectively. The total molecular weights of the ovotestis proteins of infected and exposed snails to 0.25 ppm were 714 KDa and 408 KDa after the first and second weeks of exposure, respectively.

Protein electrophoretic analysis of snails ovotestis exposed to 0.25 ppm of CAAQ showed some different patterns in the protein bands (Figures 4 a, b, c & d and 5). The results showed an increase in proteins molecular weights throughout the whole experimental period except in the exposed-infected group at the second week. The protein band of 180; 57 and 21 KDa were appeared only in exposed- infected group at the first week; the protein band of 63 KDa was appeared only in infected control group of the second week. The protein band of 48 KDa was appeared only in exposed group of the second week, and the protein band of 35 KDa was appeared only in exposed-infected group of the second week and disappeared from other groups. The protein band of 20 KDa was the dominant in all groups. The protein band of 11 KDa was appeared at the first week (control; exposed; infected control and exposed-infected groups) and was disappeared completely at the second week.

The total molecular weights of ovotestis proteins of snails exposed to 1 ppm were 345 KDa and 308 KDa for the first and second weeks, respectively. The total molecular weights of ovotestis proteins of infected and exposed snails were 296 KDa and 290 KDa for the first and second weeks, respectively. The dominant protein bands were 28 and 20 KDa in exposed, exposed-infected and control groups. There was occasional appearance
and absence of certain proteins in different experimental time intervals. The results showed that protein band of 75 KDa was appeared only in exposed group of the first week. The protein band of 57.94 KDa was appeared only in exposed-infected group of the second week. The protein band of 48 KDa was disappeared from exposed groups and appeared only in the control group at the 2 week. The protein band of 25 KDa was appeared only at the second week (control; exposed; infected control and exposed-infected groups) and was disappeared from all of them at the first week.

SDS-PAGE profile of digestive gland proteins extracted from *B. alexandrina* exposed to sub-lethal concentrations of CAAQ during 2 weeks of exposure was illustrated in Figures (4 e, f, g & h and 6). The total molecular weights of digestive gland proteins of snails exposed to 0.25 ppm were 598 KDa and 728 KDa for the first and second weeks, respectively. The total molecular weights of digestive gland proteins of infected snails exposed to 0.25 ppm were 631 KDa and 579 KDa for the first and second weeks, respectively. The protein band of 48 KDa was only appeared at the second week of exposure (control; exposed; infected control and exposed-infected groups). The 40 KDa band appeared only at the exposed-infected group and 19 KDa appeared only at the exposed group at the first week of exposure. The 25 KDa band was a dominant band in all groups.

The total molecular weights of digestive gland proteins of snails exposed to 1 ppm were 401 KDa and 624 KDa for the first and second weeks, respectively. The total molecular weights of digestive gland proteins of infected snails exposed to 1 ppm were 610 KDa and 835 KDa for the first and second weeks, respectively. There was a decrease of total proteins molecular weights during 2 weeks of exposure when compared to control and infected control groups except the exposed-infected group of the second week. There were some protein bands appeared only in exposed infected group of the second week such as the protein band 374, 75 and 19 KDa. The dominant band in all groups was 245 KDa. The protein band of 25 KDa was disappeared from all experimental groups except the control at the first week. The 11 KDa protein band disappeared only in exposed and exposed-infected groups at the second week of exposure.
Figure (4): Electrophoretic pattern of total proteins extracted from *B. alexandrina* snails exposed to CAAQ over 2 weeks interval stained by CBB. (A, C) ovotestis of snails exposed to 0.25 ppm; (B, D) ovotestis of snails exposed to 1 ppm; (E, G) digestive gland of snails exposed to 0.25 ppm and (F, H) digestive gland of snails exposed to 1 ppm. lane 1 high Mol. Wt. marker, lane 2 control non-infected snails 1 week post exposure, lane 3 snails 1 week post exposure, lane 4 control-infected snails 1 week post exposure, lane 5 infected sails 1 week post exposure, lane 6 control non-infected snails 2 weeks post exposure, lane 7 snails 2 weeks post exposure, lane 8 control-infected snails 2 weeks post exposure and lane 9 infected snails 2 weeks post exposure.
Figure (5): Lane charts of total ovotestis intensity of *B. alexandrina* snails with/without *S.mansonii* infection and exposure to (A) 0.25 ppm and (B) 1 ppm of CAAQ for 2 weeks.
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**Figure (6):** Lane charts of total digestive gland intensity of *B. alexandrina* snails with/without *S. mansoni* infection and exposure to (A) 0.25 ppm and (B) 1 ppm of CAAQ for 2 weeks.

**DISCUSSION**

In this study, exposed *B. alexandrina* snails to only *S. mansoni* infection or both infection and sub-lethal concentrations of CAAQ showed reduction in survival rate than the control group. These results agree with that described by several investigators who obtained similar results (Osman *et al.*, 2008; Sheir *et al.*, 2013 and Osman *et al.*, 2014). In this concern, the two isolated alkaloids (lycorine and crinamine) from the fresh bulb and leaf methanolic extracts of *Crinum jagus* showed molluscicidal activities with 40% mortality at 1000 mg/L against adult *B. glabrata* (Elusiyan *et al.*, 2017). Angaye *et al.* (2014) reported that the crude and 70% methanolic extracts of *Jatropha curcas* leaves against *B. pfeifferi* snails showed high mortality within 12 hours, as the phytochemical screening of the crude extract, revealed the presence of alkaloids, flavonoids, tannins and saponins. Saad *et al.* (2017) declared that the two algae *Cystoseira barbata* and *Dictyota dichotoma* caused a gradual reduction in the survival rate of *B. alexandrina* due to the presence of alkaloids and saponins. Also, Mostafa and Gawish (2009) stated that the survival rate of *B. alexandrina* snails exposed to the algal culture filtrate of *Spirulina*
*platensis* (micro-alga) was highly suppressed. This reduction in survival rates could be resulted from the presence of alkaloids, saponins and mineral salts that increase the harmful effects by facilitating the penetration rate of algal byproducts through the epidermal layer of the snails. **Carvalho et al. (2019)** reported that the alkaloid-rich fraction of the plant *Ruta graveolens* (Rutaceae) caused 100% mortality against adult worms of *S. mansoni*.

The current data showed that the growth rates of *B. alexandrina* exposed to the tested compound for 2 weeks were less than that of control ones. This reduction of the growth suggesting that snails may be allocates their energy for maintenance/survival (**Ibrahim, 2006**). In addition, this inhibition may be due to alkaloids, which were considered plant growth inhibitor and have molluscicidal activity (**Vieira and Kubo, 1990**). The current study showed that exposed *B. alexandrina* to both infection and sub-lethal concentrations of CAAQ caused inhibition in growth rate than control. **Sheir et al. (2013)** reported a significant reduction in the growth rate of *B. alexandrina* infected/treated with water extract of *C. limon* peels, due to the presence of limonene. **Mello-Silva et al. (2010)** reported that *Euphorbia splendens* var. *hislopii* latex (plant) caused a variation in glucose content in non-infected and *S. mansoni* infected *B. glabrata* snails treated with this latex. They mentioned that the infection of trematode and the latex treatment could increase the ATP consumption and accelerate the glycolysis process. In addition, changing the mother sporocysts to daughter sporocysts near the digestive gland caused glycogen degradation from this organ. The larvae obtain the glucose required for the process of glycogenesis from the haemolymph.

The present results showed that the exposure of *B. alexandrina* to CAAQ caused reduction in the mean total number of haemocytes when compared to the control individuals. Both concentrations (0.25 and 1 ppm) of CAAQ in exposed and infected-exposed snails caused an increase in hyalinocytes percentage, while, spreading granulocytes were deceased in the exposed than the control groups. The non spreading granulocytes decreased in the exposed snails, while they increased in the infected-exposed snails of both concentrations. This reduction in the total haemocytes count in exposed snails could be due to their participation in the repair of damaged tissues of hermaphrodite and digestive glands (**Esmaeil, 2009; Ibrahim et al., 2018**). **Kamel et al. (2007)** suggested that the inhibition in transaminase enzyme (asparatate and alanine aminotransferase) activity to catalyze protein synthesis; may be the cause of the decrease in the total number of haemocytes of exposed *B. alexandrina* to the aqueous suspension of the plants *Anagallis arvensis* and *Calendula micrantha*. Similarly, **Ibrahim and Abdel-Tawab (2020)** recorded haemocytes abnormalities such as a shrunken nucleus of hyalinocytes and the granulocytes had irregular cell membrane, forming pseudopodia of the exposed *B. alexandrina* snails to ethanolic extract of *Cystoseira barbata* algae. Moreover, **El-Sayed (2006)** reported that the increase in small round undifferentiated cells percentage was thought to be because of the hematopoietic organ was stimulated for
production of more undifferentiated haemocytes that could be differentiated into granulocytes. He concluded that the reduction in hyalinocytes percentage could be due to their aggregation during the wound healing process at the injury site. Furthermore, Ibrahim et al. (2022) stated that the methanolic extract of Nerium oleander and Tecoma stans caused a significant reduction in the mean total haemocyte count in exposed B. alexandrina and disturbance in the percentage of the different haemocytes types. They concluded that the participation of haemocytes in tissue repair of damaged digestive and hermaphrodite glands might be the cause of the decrease in the total haemocyte count (Ibrahim et al., 2018).

The herein data showed that the phagocytic activity of B. alexandrina recorded a significant decrease after 24 hours of exposure to CAAQ and/or S. mansoni infection. The lysosomes of haemocytes can accumulate large amounts of pollutants resulting in membrane instability, which leads to cell damage and incompatibility (Nicholson, 2001; Essawy et al., 2022). The later authors suggested that CuO NPs exposure inhibited the immune responses of mussels’ haemocytes most likely via three pathways: (1) changes of the in vivo content of neurotransmitters; (2) induction of reactive oxygen species, which reduces the cell viability of haemocytes and causes DNA damage; and (3) affecting the expression of immune- and neurotransmitter-related genes. Martins-Souza et al. (2009) recorded that the significant decrease in the number of the different sizes of haemocytes was the cause of the reduced phagocytic activity of infected Biomphalaria species. The response of haemocytes pattern depends on the nature of the stressor type or the alterations in the sensitivity of intercellular adhesion molecules such as parasites, pathogens or xenobiotics (Livingstone et al., 2000).

The effect of the exposure of B. alexandrina to sub-lethal concentrations of CAAQ resulted in several histopathological changes. Cellular damage of the ovotestis and digestive gland were obviously presented in the current work. These results are compatible with Mostafa and Gawish (2009) who reported that total phenolic compounds, alkaloids and saponins may be the reason of the severe damage in the hermaphrodite gland cells of B. alexandrina exposed to sub-lethal concentrations of the microalga Spirulina platensis. Similar results obtained by Odo et al. (2016) who reported histopathological changes such as vacuolization of the crop and reduction in sub-mucosal fat in the intestinal wall of Archachatina marginata snails under the effect of the ethanolic extract of Carica papaya seeds. They explained these results due to the high concentrations of saponins and low concentration of triptertenes and alkaloid in C. papaya seeds. Ibrahim and Abdel-Tawab (2020) showed also that C. barbata algae caused histopathological alterations in B. alexandrina such as rupture, vacuolization and increase in the number of secretory cells in the digestive tubules due to the presence of phenolic compounds, saponins and alkaloids. Saad et al. (2012) reported vacuolization, degeneration of secretory cells and necrotic changes occurred in the cells of digestive gland under the effect of Cestrum diurnum plant against B. alexandrina. That was
explained due to the presence of saponins which have characteristic detergent effect on epithelial tissues of the snails. Similar signs were detected after the treatment with plant derivative, dihydro-artemisinin methyl ether (Artemether) against *B. alexandrina*, the hermaphrodite gland cells became short and irregular sperms, dense mature ova appeared and loose of connective tissues. It might be due to the molluscicidal properties of the tested material as it selectively killed the ova; it prevented their development and thus stopped their normal formation (Mossalem et al., 2013). Ibrahim et al. (2022) reported histopathological alterations in the hermaphrodite and digestive glands of *B. alexandrina* by the methanolic extract of *N. oleander* due to the presence of alkaloids, saponins, steroids, tannins, terpenoids, anthraquinones.

The sub-lethal concentrations of CAAQ and/or *S. mansoni* infection on *B. alexandrina* snails for up to 2 weeks caused disturbance (decrease and sometimes increase) in the tissue total proteins content of the ovotestis and digestive gland. This decrease in the protein content may be due to the interference of the tested compound with protein (structural and functional proteins) synthesis. Ibrahim et al. (2022) found that exposed *B. alexandrina* to the methanolic extract of the plants *T. stans* or *N. oleander* showed a significant reduction in the alkaline phosphatase and acetylcholinesterase levels and the protein content. They suggested that the inhibition in alkaline phosphatase level may cause the reduction in the protein level, as the alkaline phosphatase plays a role in protein synthesis and other secretory activities in gastropods. The increase in protein content may be due to the increase in globulin concentrations, which specific with marked reduction in the activity of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) by the activity of different molluscicides (Ragab et al., 2003 and Sheir et al., 2013). Ibrahim and Bakry (2019) found that Chlorophyllin (*Moringa oleifera* leaves) caused a reduction in protein levels in the nervous tissue of *B. alexandrina*. They suggested that this inhibition in protein levels could be due to the decrease in alkaline phosphatase and may be due to the direct interference of the chlorophyllin with the biosynthesis of protein. The present results indicated that, SDS-PAGE of total protein pattern in ovotestis and digestive gland of exposed and *S. mansoni* infected snails showed occasional appearance and absence of certain protein bands. The results of this study showed that the band 28 KDa was dominant protein band in the ovotestis of treated snails. According to the molecular weight of the separated bans, this band was known as the ETFB protein (Chen et al., 2015). ETFB is an Electron transfer flavoprotein subunit beta. This enzyme is normally active in the mitochondria. The ETFB gene provides instructions for making one part of an enzyme called electron transfer flavoprotein. The protein band of 57.94 KDa appeared only in exposed-infected group of the second week. The protein band of 58 KDa found to be unrelated structurally to similarly size cytoskeleton-associated proteins, like tubulin (Bloom and Brashear, 1989).
CONCLUSION

In conclusion, CAAQ modulated the immune responses of *B. alexandrina* with/without *S. mansoni* infection, histopathological structure and protein expression represented in total protein content and SDS-PAGE protein profile. Consequently, CAAQ was recommended as a molluscicide of plant origin for the control program of schistosomiasis.

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