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Efficacy of Miltefosine and Artemether on infected *Biomphalaria alexandrina* snails with *Schistosoma mansoni*: immunological and histological studies

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Summary

Biomphalaria alexandrina snails have received much attention due to their great medical importance as vectors for transmitting *Schistosoma mansoni* infection to humans. The main objective of the present work was to assess the efficacy of miltefosin a synthetic molluscicidal drug and artemether a natural molluscicidal drug. The correlation between immunological and histological observations from light and electron microscopy of the hemocytes of *B. alexandrina* post treatment with both drugs was also evaluated. LC_{50} and LC_{90} values were represented by 13.80 ppm and 24.40 ppm for miltefosine and 16.88 ppm and 27.97 ppm for artemether, respectively. The results showed that the treatment of *S. mansoni*-infected snails and normal snails with sublethal dose of miltefosine ($LC_{25=}$8.20 ppm) and artemether ($LC_{25=}$11.04 ppm) induced morphological abnormalities and a significant reduction in hemocytes count.
Keywords: *Biomphalaria alexandrina*; miltefosine; artemether; hemocytes; immunological and histological studies; light and electron microscope

Introduction

Schistosomiasis represents a global health concern with over 700 million people at risk of contracting this disease (Weber *et al*., 2019). In Egypt eradicating this disease is a top governmental priority (Abou-El-Naga, 2018).
Generally, chemotherapy is still one of the most effective methods for controlling *Schistosoma* infection (Bertão *et al*., 2012a). Since the mid-1970`s, PZQ (praziquantel derivatives) represented the anti-helminthic drug of choice. Despite its efficiency in reducing morbidity and mortality rates in *Schistosoma* infections (Caffrey, 2007), it is not active against early stages of schistosomes and causes species resistance (Bertão *et al*., 2012b). Currently, miltefosine and artemether (antimalarial drugs) have a potential application in schistosomiasis treatment (El Beshbishi *et al*.,2018).
Miltefosine (hexadecylphosphocholine) is an alkyl phospholipid derivative that was developed as a new type of antitumor agent in the 1990s (Eibla and Unger, 1990). Miltefosine has comparative advantage over PZQ as antischistomicidal drug due to its efficacy on the differential developmental stages of *S. mansoni* in infected mice (Eissa *et al*., 2011). Miltefosine used as an schistosomicidal drug increased helminths mortality rate and induced extensive tegumental changes in the adult worms of Egyptian and Brazilian strains of *S. mansoni in vitro* and *in vivo* (Eissa *et al*., 2015; El-Faham *et al*., 2017). In addition, miltefosine presents a molluscicidal activity against both *Biomphalaria alexandrina* and *Bulinus truncatus* snails (Eissa *et al*., 2011). Artemether is a methyl ether derivative of artemisinin, a compound extracted from the leaves of the Chinese wormwood plant (*Artemisia annua*) (Mossalem *et al*., 2013). It was first described as anti-schistosomal agent in

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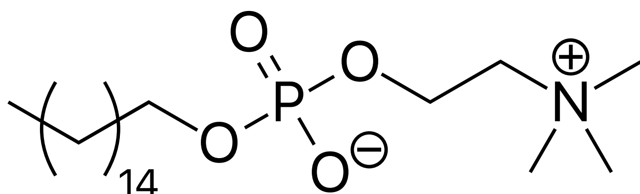
1980s, against juvenile worms (5-21day-old) *S. japonicum* (Liu *et al*., 2012). Artemether efficiency as a schistosomicidal and molluscicidal agent has been previously shown (Al-Kazzaz *et al*., 2014; Madbouly et al.,2015).
The aims of this work were to compare the efficacy of miltefosine, a chemotherapeutic synthetic compound, with artemether, a natural molluscicidal through light and electronic microscopy and to elucidate their effect on normal immune-histological parameters in *S. mansoni*-infected *B. alexandrina* snails.

Materials and Methods

Experimental snails
B. alexandrina snails (8-10 mm in diameter) were obtained from *Schistosoma* Biological Supply Centre at the Medical Malacology Laboratory, Theodor Bilharz Research Institute, Giza, Egypt. The snails were classified as follows:
- Group (1): 50 normal control snails (unexposed snails).
- Group (2): 100 normal snails divided into two subgroups (per 50 snails) exposed to sublethal concentrations LC_{25} of miltefosine and artemether for 2 successive week intervals in two replicates for each drug.
- Group (3):150 snails were exposed to *Schistosoma mansoni* miracida with a dose of 10 per snail (Liang *et al*., 1987). This group was further divided into 3 subgroups;
    - Group (3a): 50 infected snails
    - Group (3b): 50 infected snails treated with a sublethal concentration LC_{25} of miltefosine for 2 successive weeks in two replicates for each drug.
    - Group (3c): 50 infected snails treated with a sublethal concentration LC_{25} of artemether for 2 successive weeks in two replicates for each drug.

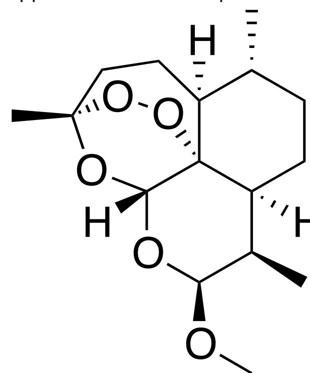
For each drug, the treatment was changed weekly with freshly prepared one to avoid the effect of storage. The snails were exposed to the tested concentrations for 2 successive weeks, then removed from the experimental environment, washed thoroughly with dechlorinated tap water and washed the one time only (after first 24 hours) to recover.

Experimental materials
1 - The drug miltefosine (100 mg) was provided by (Sigma-Aldrich Chemie and GmbH, CA 58066-85-6, MW 407.57, Germany) and its trade name is Impavido (molar mass 407.568 g/mol, chemical formula $C_{21}H_{46}NO_4P$).



Miltefosine structure

2 - The drug artemether was obtained in the form of tablets (Kunming Pharmaceutical Cooperation, PR China) with a documented purity of 99.6%. It is sold under the trade name Riamet and Coartem among others, has a molar mass of 298.374 g/mol and the chemical formula is $C_{16}H_{26}O_5$. The actual concentration was calculated as the percentage of the active material in the used weight. Artemether was applied to snails as aqueous solution of tablets.



Artemether structure

Molluscicidal properties of miltefosine and artemether drugs
A stock solution of 1000 ppm from each drug was prepared on the basis of weight/volume using dechlorinated water. A series of concentrations was prepared for each drug according to the standard procedure recommended by WHO (1965) that allowed us to reach experimental concentrations (LC_0, LC_{10}, LC_{25}, LC_{50} and LC_{90}). The effectiveness of each drug as a molluscicide has been expressed in terms of LC_{50} and LC_{90} according to the procedure of Litchfield & Wilcoxon (1949). Three replicates of gradual concentrations from each stock solution were prepared. The snails were exposed to the tested concentrations for 24 hours, then removed from the experimental environment, washed thoroughly with dechlorinated tap water and transferred to aquaria with fresh dechlorinated tap water for the next 24 hours to recover (25 ± 2 °C). Unexposed snails (controls) were assayed side by side with the treated groups under the same laboratory conditions in dechlorinated tap water (WHO,1965). Dead snails were noticed and removed from the container.

Immunological and histological study of B. alexandrina hemocytes
Hemolymph samples from snails from all studied groups were collected as previously described by Michelson (1966) by removing a small portion of the shell and inserting a capillary tube into the heart. The hemolymph was collected in a vial tube (1.5 ml) and kept in ice-box The collected hemolymph from infected snails and infected treated groups was used to estimate the total hemocytes count using a Bürker- Turk hemocytometer (while the differential haemocyte count was obtained according to a previously published method (Van der knap *et al*., 1981).While the differential haemocyte count was obtained on the light microscopy level, hemolymph samples were placed individually onto a clean glass slide. Hemocytes were fixed in 100 % methanol, then stained

Table 1. Molluscicidal activity of miltefosine and artemether against adult *B. alexandrina* snails (24 hours exposure).

Tested drug	LC_0 Ppm	LC_{10} Ppm	LC_{25} ppm	LC_{50} ppm	LC_{90} ppm	Slope
Miltefosine	1.38	3.16	8.20	13.80	24.40	1.60
Artemether	1.68	5.79	11.04	16.88	27.97	1.75

with Giemsa's stain examined and counted then photographed using Agfa film according to a previously published method (Abdul-Salam & Michelson, 1980). The hemolymph samples from normal control snails (group1) and drug exposed snails (group 2) were used for transmission electron microscopy examinations (Grimaud *et al*., 1980).

Statistical analysis

The results were analysed statistically using the Statistical Package for Social Science (SPSS version 15 package software). Data were expressed as mean (M) ± standard deviation (S. D). The data were statistically analysed statistically significant differences between the treated and the control group using "t" test (Goldstein, 1964).

Results

Molluscicidal activity of miltefosine and artemether against B. alexandrina snails

Bioassay tests results are presented in Table 1, revealing that both compounds has a molluscicidal activity against adult snails. It was noticed that miltefosine showed a marked lethal effect against snails compared to artemether (LC_{50} and LC_{90} of 16.88 and 27.97 ppm, respectively). Its LC_{50} and LC_{90} values were 13.80 ppm and 24.40 ppm with a slope function value of 1.60. Furthermore, these results demonstrated that the sublethal concentrations (LC_0, LC_{10}, & LC_{25}) of miltefosine were lower than the corresponding values of artemether.

Effect of the tested drugs on hemocytes of adult B. alexandrina snails

<u>*Total number of hemocytes of B. alexandrina snails*</u>

The recorded data in Figure 1 denoted that the exposure of snails (groups 3b& 3c) to LC_{25} of miltefosine (8.20 ppm) and artemether (11.04 ppm) for 24 hours for 2 successive weeks exhibited a significant decrease ($P < 0.001$) in hemocytes count compared with the control group (infected snails; group 3a). The reduction in hemocytes was of 65.48% and 47.62% in miltefosine and artemether treated groups, respectively

Effect of the tested drugs on percentage of different types of hemocytes

As for the number of the different haemocyte types; the exposure of adult infected snails (groups 3b& 3c) to the LC_{25} drugs concentrations induced a decrease in hyalinocytes percentage. Miltefosine treated–infected snails had the lowest percentage of hyalinocytes (18.40%) compared to artemether treated (26.60%) and control snails (group3a) (52.3%) (Figure 2). On the contrary, both drugs induced an elevation in the percentage of small round cells and granulocytes in corresponding to the infected-control group (group3a).

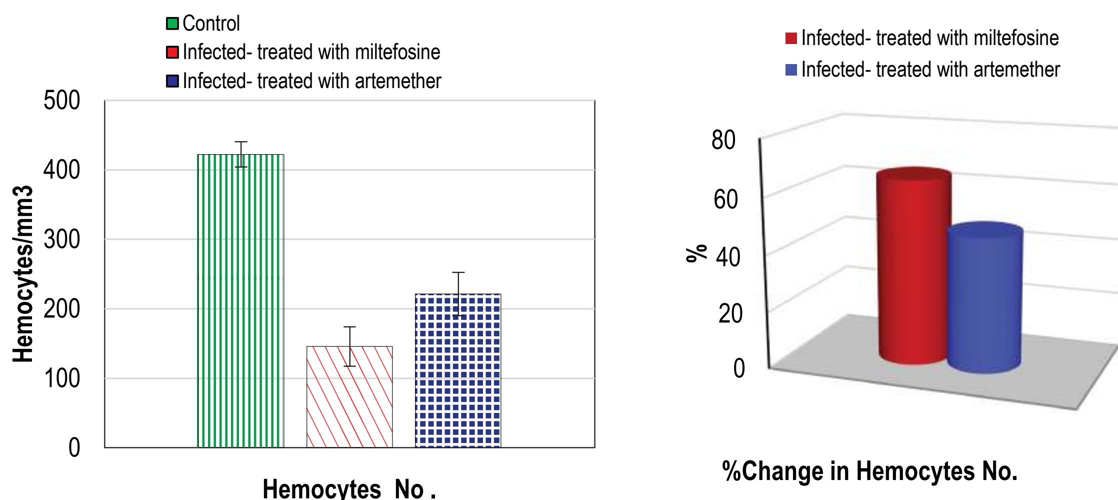


Fig. 1. Effect of LC_{25} of the miltefosine and artemether drugs on hemocytes count adult *B. alexandrina* snails as compared with infected snails (controls)

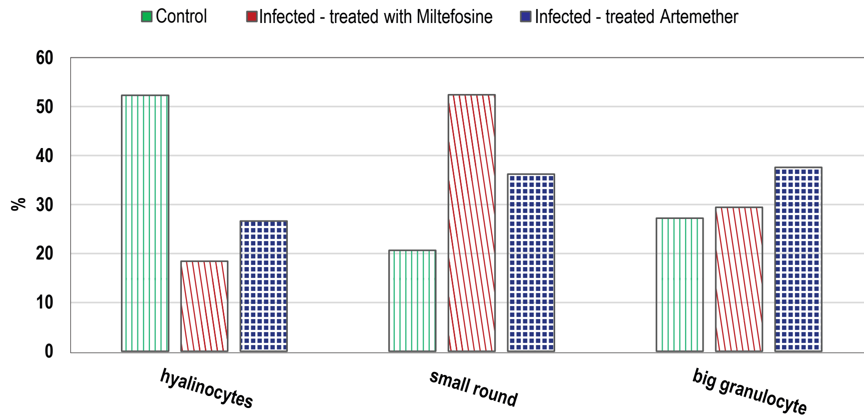


Fig.2: Effect of LC_{25} of the miltefosine and artemether drugs on % different hemocytes types in hemolymph of adult *B. alexandrina* snails as compared with infected snails (controls)

Morphological alterations induced by miltefosine and artemether observed in light and electronic microscopy

Light microscope study

Hemocytes examination of the normal control snails (group1) by light microscopy revealed the presence of three morphologically different cell types (Plate 1 A-C); small undifferentiated cells with a spherical profile (A), large spherical granulocytes with a double membrane and a relatively large cytoplasm filled with a variable number of basophilic granules (B), and polymorphic hyalinocytes with either a large eccentric nucleus or two nuclei, suggesting atypical cell division (C).

The exposure of adult non-infected *B. alexandrina* (group 2) to LC_{25} miltefosine (8.20 ppm) and artemether (11.04 ppm) for 24 hours for 2 successive weeks induced some alterations on the morphology of granulocytes and hyalinocytes, but small undifferentiated cells were not affected. In drug exposed snails noticed by light microscopy that granulocyte appeared under different sizes granules. Hyalinocytes on the other hand, had a large vacuolated cytoplasm and a shrunk nucleus (Plates 2 & 3).

Electron microscope study (TEM)

The ultrastructural examination of normal control hemocytes (group1) showed the presence of three morphologically different cell types (Plate 4 A-C). The exposure of adult snails to sublethal concentrations (LC_{25}) of the examined drugs showed a different type of cells with two types of globules in the cytoplasm that appeared only in the hemolymph of treated snails (group 2) (Plate 5 A-C).

Miltefosine treatment induced the following alterations in hemocytes types of non-infected snails (group 2 a): small undifferentiated cells showed a large nucleus, an intact cell membrane, cytoplasmic extensions (pseudopodia), vacuoles, and phagolysosomes in the cytoplasm (5 A); granulocytes presented nucleus with irregular boundaries and an irregular chromatin distribution, the cytoplasm contained phagolysosomes, granules, and some cell organelles such as the mitochondria and rough endoplasmic reticulum (5 B); hyalinocytes presented a degenerated outer cell membrane, the nucleus had an irregular membrane, and the nucleus shrunk in size (5 C).

Artemether induced the following on different types of hemocytes of snails (group 2 b) (Plate 6 A-C): small undifferentiated cells had an intact cellular membrane with extended pseudopodia, the cytoplasm and the nuclei contained phagolysosomes and vacuoles (6 A); granulocytes showed pseudopodia, cytoplasmic granules,

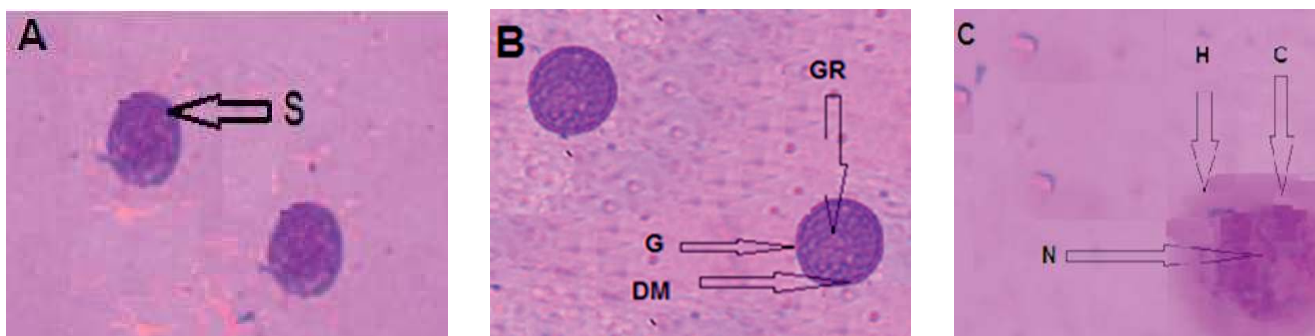


Plate 1. **A)**. Normal round small (non-differentiated) cells **B**). Granulocyte and **C)**. Hyalinocyte of *B. alexandrina* adult snails (×40). S: Round small cell, G: Granulocyte, C: Cytoplasm, DM: Double membrane, GR: Granules, N: Nucleus, (H): Hyalinocyte.

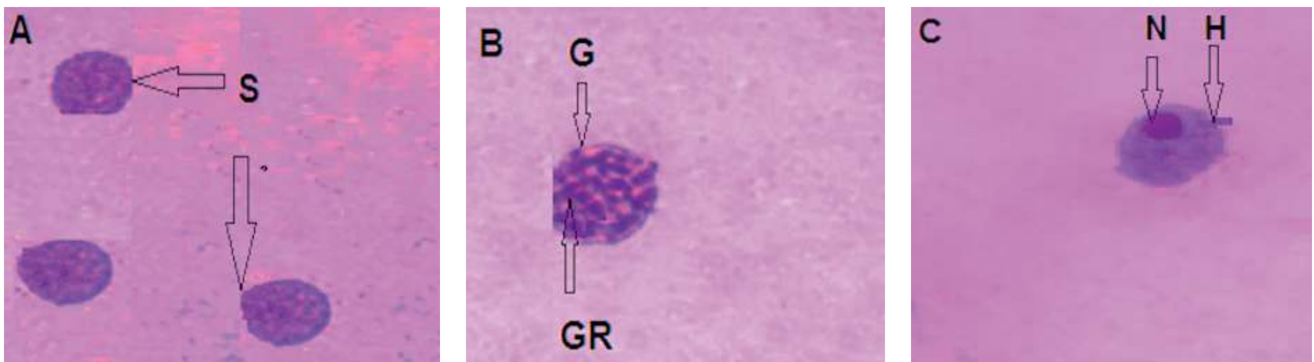


Plate 2. The morphological effect of miltefosine LC_{25} on different types of hemocytes in the haemolymph of treated-infected *B. alexandrina* snails. **A)**: Round small non-differentiated cell, **B)**: Granulocyte and **C)**: Hyalinocyte (x40). S: Round small cell, G: Granulocyte, GR: Granules, N: Nucleus, (H): Hyalinocyte.

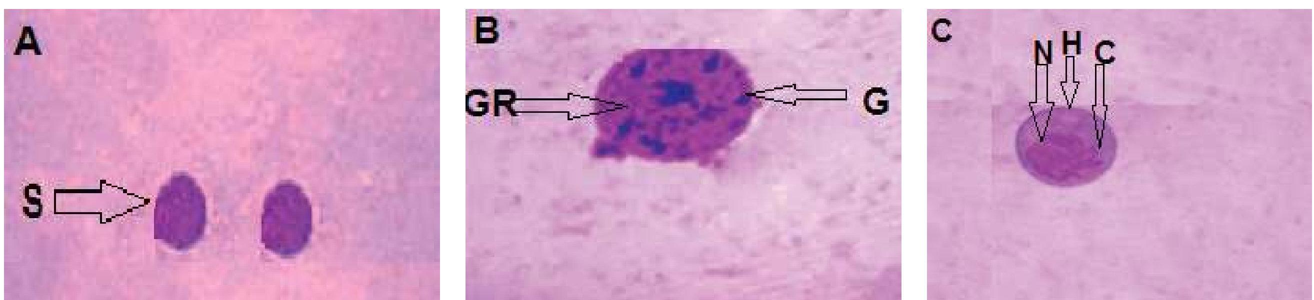


Plate 3. The morphological effect of artemether LC_{25} on different types of hemocytes in the haemolymph of treated *B. alexandrina* snails. A): Round small (non-differentiated), B): Granulocyte and C): Hyalinocyte (x40). S: Round small cell, G: Granulocyte, C: Cytoplasm, GR: Granules, N: Nucleus, (H): Hyalinocyte.

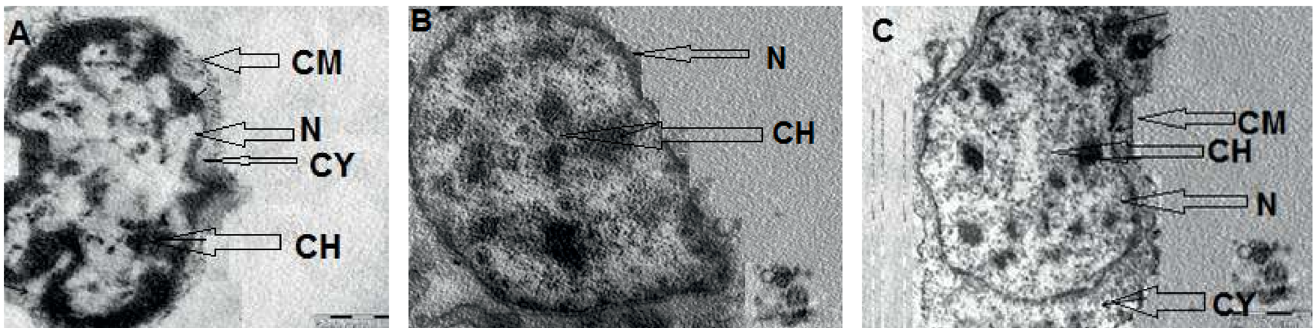


Plate 4. TEM micrographs showing normal cells of *B. alexandrina* snails, **A)**. Round small non- differentiated cell, **B).** Granulocyte and **C**). Hyalinocyte. CH: chromatin, CM: cell membrane, CY: Cytoplasm, N: nucleus.

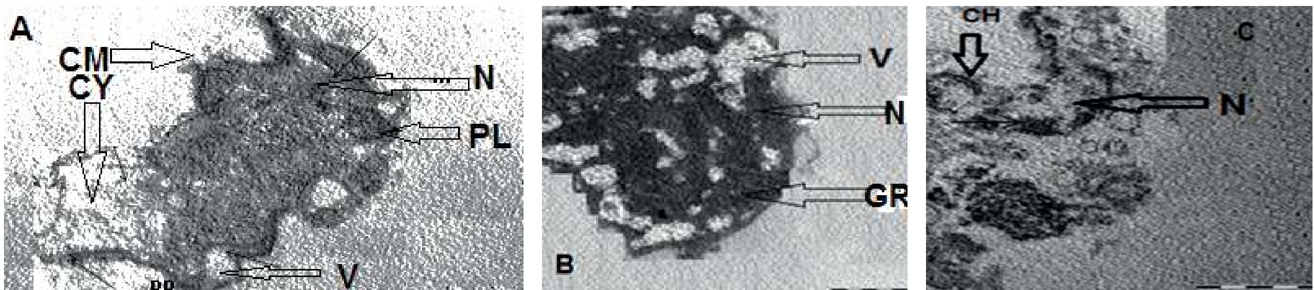


Plate 5. TEM micrographs showing the effect of miltefosine LC_{25} on hemocytes **A)**. Round small (non- differentiated) cell, **B**). Granulocyte and **C)**. Hyalinocyte of treated-infected B. alexandrina snails. CH: chromatin, CM: cell membrane, CY: Cytoplasm, GR: granules, N: nucleus, V: vacuole, PL: phagolysosome.

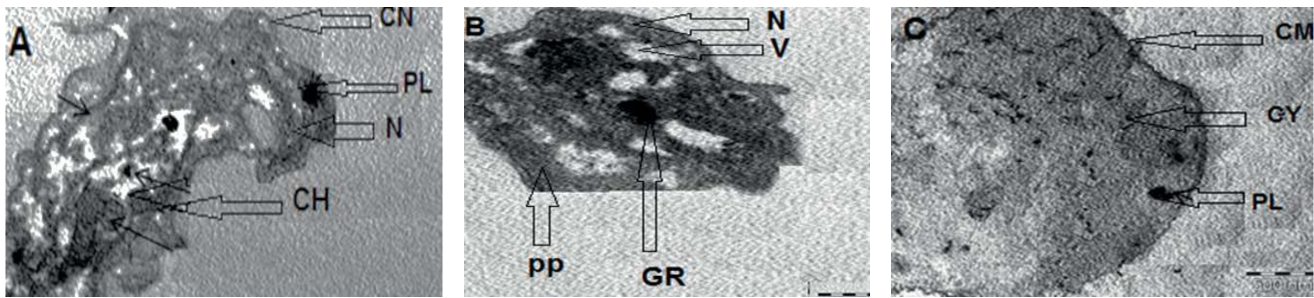


Plate 6. TEM micrographs showing the effect of LC_{25} artemether drug on hemocytes **A).** Round small (non- differentiated) cell, **B).** Granulocyte and **C)**. Hyalinocyte of treated-infected *B. alexandrina* snails, CH: chromatin, CM: cell membrane, CY: cytoplasm, GR: granules, V: vacuole, N: nucleus, PL: phagolysosome, PP: pseudopodia.

and vacuoles in cytoplasm (6 B); while hyalinocytes showed an intact cell membrane, degenerated nuclei, and difficult to identify organelles (6 C).

Discussion

Internal defense mechanisms of invertebrates depend upon an innate immune system including cellular and humoral components (Le Clec'h *et al*., 2016). Cell-mediated immune response resulted from the presence of various cell categories that are vital in defense and constitute the primary barrier against invading parasites and bacteria as well as accumulate different substances such as molluscicides, heavy metals and pesticides. These are mobile amoeboid cells referred to as amoebocytes or hemocytes (Bernard, 2016). Furthermore, hemocytes of the snail are used to determine the prepatency period of infection with schistosomiasis (Kamel *et al*. 2006). Previous experimental studies proved that the parasitic infection induced positive effect upon hemocytes count by increasing their number in the hemolymph of various species of snails (Barcante *et al*., 2012; Mossalem & Ibrahim, 2019; Suwannatraia *et al*., 2019).

In the present study, the hemocytes of *S. mansoni* infected snails (had higher significant increase compared to the treated -infected snail groups with the sublethal concentration (LC_{25}) of miltefosine and artemether as in previous studies (Mossalem *et al*., 2013; Ibrahim *et al*.,2018; Mossalem & Ibrahim, 2019). The decrease in hemocytes count in treated snail groups might be a result from tissue damage in digestive and hermaphrodite glands as they participated in tissue repair (Esmaeil, 2009). Another explanation might be the reduction in transaminase enzyme activities, sensitive tools in physiological alterations detection (Kamel *et al*., 2007**)**, or perhaps, it is the result of hemocyte cells from the hemolymph migrating to connective tissues (Cochennec-laureau, 2003).

In regard to the types of hemocytes, the present study detected 3 types of cells in *B. alexandrina* hemolymph; hyalinocytes, small round cells and granulocytes. This finding agrees with the results of other studies (Mohamed *et al*. ,2006; Cavalcanti *et al*., 2012). Some authors suggested that the hyalinocytes and the granulocytes are different cell types, while others considered that they represent different developmental phases of the same cell type (Oliveira *et al*., 2010). Another study revealed that granulocytes were phagocytic cells with pseudopods capable of encapsulating large particles, while the hyalinocytes were spherical smaller cells without pseudopods (Yoshino *et al*., 2008). Additionally, the present study showed that the percentage of hyalinocytes decreased in both miltefosine and artemether exposed groups, while the percentage of round small cells (undifferentiated cells) and granulocytes increased in exposed groups compared to the normal uninfected control group. The present data are in accordance with Bakry *et al*. (2012) with a study showing that methanol extracts of *Azadirachta indica* plant induced a significant increase in the number of granulocytes in the hemolymph of *B. alexandrina* snails indicating a high response of the snails against the treatment. Several studies explained the fluctuations in percentage of the types of hemocytes of aquatic snails as a result of drug treatment (Bakry *et al*., 2012). A study reported that granular hemocytes were the major responsive hemocytes type in *B. alexandrina* snails treated with a plant growth regulator (Mepiquat chloride) (Mohamed & Abdel-Gawad, 2005). Another study detected an increase in the percentage of small round undifferentiated cells, which may be due to the stimulation of the hematopoietic organ producing undifferentiated hemocytes that differentiated into granulocytes to compensate their reduction in number (El Sayed, 2006). Furthermore, a study stated that hyalinocytes are thought to be responsible primarily for wound repair requiring aggregation at injury site, thus their number decreased in the hemolymph (Barcante *et al*., 2012). It was reported that granulocyte cells are immunological active cells found mainly in the hemolymph of snails, instead of remaining in the damaged tissue to face external stimuli (Oliveira *et al*., 2010)

The ultra-structural observation of hemocytes showed that the tested drugs induced morphological alterations, such as irregular nuclear boundaries, irregular chromatin distribution, degenerated nuclei, and vacuolated cytoplasm with electron-dense phagolysosomes; this is in agreement with the results obtained by others (El Sayed *et al*., 2011; Ibrahim *et al*.,2018). Kamel *et al*. (2006, 2007) revealed the presence of a remarkable activation in hemocytes due to a sublethal concentration of plant treatment and mol-

luscicidal compound. They attributed the degenerative changes of hemocytes cellular organelles to a direct toxic effect of these molluscicidal drugs. Further, the continuous exposure to sub-lethal concentrations of artemether caused an increase in glycogen content in hemocytes causing them to increase in size by increasing the time of exposure as reported by Mossalem *et al*. (2013).
Despite the promising molluscicidal activity of miltefosine, studies show administration results in severe side effects, miltefosine had broad biocide activity as well as it has been shown that miltefosine is a teratogenic agent and its use in the treatment as an anti-Leishmania medication has been associated with severe side effects (Bhattacharya *et al*., 2007; Eissa *et al*., 2011). On the contrary, the present study suggests that artemether can be effectively used as a safe plant origin molluscicide in the national *S. mansoni* control program due to some studies, do not show evidence of harm on non-target organisms. So, further research studies are warranted to evaluate the impact of artemether as safe molluscicidal and schistosomicidal agent (Piola *et al*., 2010; Elmorshedy *et al* et al., 2016).

Conclusion

Miltefosine and artemether have a toxic effect on *B. alexandrina* as they negatively affect immunological processes inducing degenerative changes and fragmentation of hemocytes. Consequently, tested drugs could be ranked as beneficial molluscicidal agents for the control program of schistosomiasis. However, artemether is cheaper to produce and safer for vector control. To further asses artemether in the control of schistosomiasis, it should be evaluated and tested for complete efficacy within the operational research bases for *Schistosoma* infection control.

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Conflict of Interest

The authors declare that there are no conflicts of interest

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