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Biological, Parasitological and Biochemical Parameters of *Biomphalaria alexandrina* Snails Post Exposure to *Bacillus subtilis* Metabolites

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ABSTRACT

Background: Schistosomiasis is a parasitic trematode-borne disease that can be acute or persistent. Its control focuses on reducing the disease through two main parallel roles: one of which is the curative role, such as using praziquantel in the periodic treatment of patients on a large population scale, and the other is the preventive role, such as applying biological control of snails that transmit the disease.

Aim: This study aims to evaluate the effect of bacterial metabolites from *Bacillus subtilis* bacteria on *Biomphalaria alexandrina* snails as biological control agent as well as studying its effect on fecundity, sex hormones, histological changes and release of *Schistosoma mansoni* cercariea.

Material and Methods: *B. alexandrina* were exposed to secondary metabolites from *B. subtilis* (SMBS) at stated concentrations under suitable laboratory conditions.

Results: The results revealed that sublehal concentrations (ppm) were 93.66, 105.30, 118.23 and 142.79 for LC_{10}, LC_{25}, LC_{50} and LC_{90} respectively. Meanwhile, upon exposure for four successive weeks, significant reduction in the rate of egg laying capacity (fecundity) was observed according to the reduced levels of estradiol and testosterone. Also, significant reduction was observed in both infection rate and cercarial production. Eventually, lesion patterns were observed in the hermaphrodite glands upon investigating their histological sections at gradual bacterial metabolites concentrations.

Conclusion: metabolites of *Bacillus subtilis* has molluscicidal properties against *Biomphlaria alexandrina* snails. Consequently, it can aid at controlling of schistosomisis transmission.

Keywords: Shistosomiasis, *Biomphlaria alexandrina* snails, *Bacillus subtilis* bacteria, snails' fecundity and sex hormones

INTRODUCTION

Schistosomiasis, a chronic and acute disease caused by *Schistosoma spp*., is the principal freshwater gastropod borne disease worldwide. It has the second most significant socioeconomic impact of any parasite disease (next to malaria). However, it seems to be a neglected tropical disease [(1)].

Schistosomiasis prevalence has been recorded in eighty-seven nations and estimating 240 billion sufferers [(2)]. Humans become infected when released cercarieae from freshwater snails penetrate their skin through direct contact with contaminated water [(2)]. Humans infected with schistosomiasis transmit the disease by polluting freshwater sources with their faces or urine, which contain eggs of parasitic organisms that develop in water. Parasitic larvae mature into adult schistosomes in the blood arteries where females lay their eggs. Some of these eggs are discharged from the body via faeces or urine, allowing the parasite to complete its life cycle via snail vectors. While the remaining eggs become stuck in body tissues, producing immunological responses and progressive organ damage [(2)]. Controlling these snail vectors is now one of the World Health Organization's disease prevention initiatives [(1)]. The World Health Organization (WHO)'s World Health Roadmap identified schistosomiasis elimination as a priority public health goal [(1)]. The primary step of schistosomiasis control strategies includes preventive therapy, individual diagnostics, praziquantel administration, snail population management, and health education [(3)]. However, beyond to the expenses related to receiving medical care, other key aspects of society and the environment should be considered when designing a complete One Health-based disease management strategy[(4)] the environment solutions for controlling schistosomiasis include reducing contaminants in the environment and minimizing infections in both hosts.

Stauffer and Madsen [(5)] recommended regulating the snail vector community using chemically or natural techniques, minimizing cercariae produced by snail intermediate hosts with no harming the snails, and lowering the prevalence of infection in propagation zones. However, chemical control has several significant drawbacks, including the killing of organisms that are not targets and contaminating things, the inability to use the water resource for an extended period of time, and cost [(6)]. Controlling carrier populations of snails constitutes one of the WHO's advised schistosomiasis prevention methods [(7)]. **Eilenberg *et al.*** [(8)] described biological control

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as "the application of living organisms in order to decrease the population frequency or impact of a specific particular organism, thus rendering it less widespread or harmful than it would normally be." In this context, *Biomphalaria alexandrina*, a species of fresh-water snail that serves as an intermediate host for *Schistosoma mansoni* [3], is an organism that acts as a transmitter of such a neglected disease and, rather than being considered a pest in and of itself, should be regarded as a target that should be controlled using the **Eilenberg *et al***. concept [8]. The primary goal of biological control in this scenario is to reduce schistosomiasis transmission by controlling the intermediate host [1]. Over the last few years, a variety of strategies have been used to control *Biomphalaria spp.* snail populations, including direct (predation) or indirect (competition to obtain nourishment or habitat) processes or ecosystem interactions [9] each of these agents can be extra categorized as microbial agents (viruses, fungi, protozoa, and bacteria), predators, or competitors [10]. Many arthropod pests, such as *Deois flavopicta, Mahanarva fimbriolata,* and *Cornitermes cumulans*, have been effectively managed through application of entomopathogenic micro-organisms [11], [12]. Recently, these drugs also employed to manage freshwater snails microbiologically [13]. One important reason for using entomo-pathogenic fungi, bacteria, and nematodes as pesticides is that they are environmentally friendly agents, making them a green method for controlling farming pests like insects, with an abundance of commercial procedures already available [14]. Several microorganisms have also been demonstrated to exhibit molluscicidal action, **Duval *et al***. [13] discover a new *Paeni bacillus* strain that infects both adult and new-hatched *B. glabrata*, resulting in considerable morbidity. As a result, biocontrol agents, notably fungi and bacteria, have received increased attention over the last decade [13], [15]. Some of these bacteria are native to the target animal's biome or aquatic habitat, have the potential to disturb physiological systems or microbiological diversity. Based on this standpoint, the gut bacteria of snails and other freshwater species could serve a vital part in control, affecting their propensity to spread illness [16].

Bacillus subtilis is a Gram-positive, aerobic bacteria, it's rod-shaped and catalase-positive. It can be found in the soil and guts of both animals and humans [17]. It can generate endospores that help it survive in harsh settings, as well as secrete compounds that stimulate plant development and health [18]. *B. subtilis* has been identified as a biofertilizer, phytostimulator, and biopesticide [19]. Biocontrol of fungal and bacterial phytopathogens entails competition for resources or colonization sites, cytolytic effects, and antibiotic synthesis [20], [21]. *B. subtilis* produces antimicrobial metabolites such as antibiotics the lipopeptides representing various antifungal and antibacterial antibiotics, which include fengycins, iturins, and surfactins [21]; secretes hydrolytic enzymes with cell lysis effect; produces endospores; and alters the microenvironment conducive for plant growth, which favors its use as a biocontrol agent. B. subtilis can emit antibiotics and hydrolytic enzymes as secondary metabolites; it can use the environment to its advantage, and it also creates resistant endospores to survive in harsh settings, *B. subtilis* produces lipopeptides, which act as a strong pesticide [22].

In this context, the present study aims at detecting the molluscicidal potency of *B. subtilis* secondary metabolites against *B. alexendrina* snails via determining the lethal and sub-lethal concentrations of such bacterial metabolites for the snails, determining the effect of sub-lethal concentrations on the fecundity of snails (egg laying capacity), investigating the effect of sub-lethal concentrations on levels of sex hormones Estradiol (Estrogen) and Androgen (testosterone), studying the histological changes in hermaphrodite gland after exposure to sub-lethal concentrations) and, finally, investigating the effect of sub-lethal concentrations on the infectivity of snails and their cercarial production.

MATERIALS AND METHODS

1. Snails & Miracidia: Laboratory-bred *B. alexandrina* (8–10 mm) and *S. mansoni* miracida acquired from the Medical Malacology Department, Theodor Bilharz Research Institute. The snails were kept in water (chlorine free), temperature adjust from 24 to 26°C and fed dried lettuce leaves.

2. *Bacillus subtilis* Bacteria and its Metabolites: *B. subtilis* bacteria were isolated, identified and extracted its metabolites in the Microbiology lab., Agricultural Genetic Engineering Research Institute, Giza, Egypt. Cultivation of *B. subtilis* by Luria-Bertani agar medium [23] and identified by Bruker standard reference database [24]. The secondary metabolites of *B. subtilis* were extracted using the following procedure: A *B. subtilis* colony was injected into a test tube with Luria-Bertani media and incubated for 24 hours at 37°C, 180r/min. The seed liquid was injected into LB medium at a 1:10 ratio for 24 hours. Bacteria were precipitated from cultures via

centrifugation. The secondary metabolites of *B. subtilis* (SMBS) were collected, filtered, and kept at -20°C for future use. The supernatants were chromatographed on a silica gel column. Eluent samples were collected and identified using blotting on thin layer chromatography (TLC) plates to mix identical components. The samples were then evaporated, freeze-dried, and redissolved in distilled water [25].

3. Molluscicidal screening: Molluscicidial activity of adult *B. alexandrina* snails was determined by preparing a series of concentrations SMBS suspension based on weight/volume. For both the exposed and control groups, three replicates (10 snails/L) were prepared. Exposure periods were 24h, followed by the same time for recovery period at 25±1°C, the number of dead and life snails were counted and recorded [26].

4. Snails' Infectivity: Each of the two groups contained 30 snails: one for snails exposed to SMBS and the other as a control. Snails in each group were individually exposed to S. *mansoni miracidia* (7-10 miracidia per snail); snails in the first group were treated to SMBS water suspension during miracidial exposure. Then, they were transported to clean dechlorinated water in a dark room for preparation, while control groups ran parallel to the exposed group. During the prepatency stage, the snails were fed every day and chlorine free water was changed once a week. The snails' survival rate was recorded on day 21 following miracidial exposure, and the infection rate and average cercarial shedding were estimated at the end of the experiment [27].

5. Snails' Fecundity: *B. alexandrina* snails capable of laying eggs were selected and arranged (8-10 mm) as 3 replicates, each containing ten snails/L, were kept in LC_{25} SMBS for 24 hours then get picked up from bacterial suspension for recovery and observation over four weeks. The control groups were operated in tandem with the exposed groups. The control snails were maintained only in clean water. The survival rate and total number of deposited eggs were recorded every 7 days, and the reproductive rate (Ro) was computed at the last day of fourth week of the experiment (final week). Throughout the experiment, the snails were given plastic sheets to deposit eggs on, and the water was changed every week [28].

6. Steroid Sex Hormones: *B. alexandrina* (three repetitions, 20 snails/L each) were subjected to LC_{10} and LC_{25} of SMBS, and control groups were situated as previously described. The surviving snails in the exposed and control groups had their hemolymph collected in eppendorf tubes in an ice container. The obtained hemolymph was subjected to ELISA tests to determine the of sex hormones levels (testosterone and estradiol). The absorbance of the calibrators, controls, and treated samples was evaluated using an ELISA reader in accordance with Diagnostics Biochem corporate protocols in Canada. www.dbc-labs.com.

7. Histological Studies: Two groups of snails was performed by exposure to LC50 and LC90 of SMBS as water suspension for monitor the histological alternation in snails` hermaphrodite gland after exposure. The sections were stained with haematoxylin and eosin and inspected using a light microscope equipped with a digital camera [29].

8. Statistical Analysis: The Student t test was performed to assess the significance of differences between the treatment and control groups. Snails' Infectivity values were presented as mean ± standard error. The statistical software Graph Pad Prism and SPSS version 22.0 were used to determine data significance using the t-test and one-way ANOVA.

RESULTS:

1. Molluscicidal activity:

The data in **Table 1** showed that mortality rate of *B alexandrina* snails was a directly proportional to the concentrations of SMBS .The snails was placing in series concentrations of *B. subtilis* metabolites for 24 hrs, then replacing in clean dechlorinated water for the same period. The sublethal concentrations chosen to perform the following experiments are represented in **Table 1**.

Table 1: Gradual sublethal concentrations of metabolites extracted from *B. subtilis* for adult *Biomphalaria alexandrina* snails:

	Concentration (ppm)
LC_{10}	**93.66**
LC_{25}	**105.30**
LC_{50}	**118.23**
LC_{90}	**142.79**

2. Snails' infectivity:

Infection of *B. alexandrina* snails with miracidia of intestinal schistosomiasis was affected by the addition of bacterial metabolites SMBS as shown in table 3. The survival rate showed a significant decline after 21 days of treatment recording 76.7 % compared to 90 of control. Likewise, the infection rate decreased to 60.9% in opposite to 89.3% in infected –non-exposed group (control group). A regression was also observed in cercarial production rate. No significant alternation was observed in prepatent or cercarial shedding periods **(Table 2)**.

Table 2: the effect of SMBS on survival rate at 1st shedding, Infection rate, Prepatent period, Duration of shedding and Cercarial production of *Biomphalaria alexandrina* exposed to *Schistosoma mansoni* miracidia:

Parameter		Infected-exposed snails	Control (infected snails)
Survival rate at 1st shedding	**No. of exposed**	30	30
	No. of survived	23	27
	%	76.7*	90
Infection of snails	**Number**	14	25
	%	60.9*	89.3
Prepatent period (days)	**Range**	21-28	21-28
	Mean	23.7	24.1
	±S.E.	1.5	1.3
Total cercariae/snail	**Range**	9-844	10-1108
	Mean	592.8*	777.6
	±S.E.	164	193
Duration of shedding	**Range**	7-28	7-28
	Mean	21.7	23.8
	±S.E.	3.9	2.8

3. Snails' Fecundity:

The data in table 2 indicate that there is a significant reduction in snails' egg laying capacity (Mx) was observed after exposure to LC_{10} and LC_{25} , at the 2nd week the values of Mx were recoded 2.01, 1.46 eggs/ snails/ week for LC_{10} and LC_{25} respectively, opposite to 2.62 for control group at the same week. This decline in numbers increased until it reached 1.11 and 0.97 eggs/ snails/ week for LC_{10} and LC_{25} respectively at the 4th week. Consequently, the reproduction rate decreased significantly with increasing duration as well as increasing concentration, recording 4.34 at LC_{25} compared to 9.28 for the control group. By the end of the 4th week, the reduction rate was 53.2% for the groups exposed to LC_{25} of SMBS **(Table 3)**.

Table 3: survival rate (Lx) oviposit rate (Mx) and reproductive rate (R_o) of adul *B. alexandrina* snails during 4 successive weeks of exposure to *B. subtilis metabolites:*

Week	Control			LC10			LC25		
	Lx	Mx	LxMx	Lx	Mx	LxMx	Lx	Mx	LxMx
Zero week	1.00	2.84		1.00	2.92		1.00	2.71	
1st week	0.79	2.84	2.24	0.90	2.53	2.28	0.87	2.2	1.91
2nd week	0.93	2.62	2.44	0.83	2.01	1.67	0.77	1.46	1.12
3rd week	0.93	2.57	2.39	0.77	1.63	1.26	0.67	1.08	0.72
4th week	0.93	2.38	2.21	070	1.11	0.78	0.60	0.97	0.58
R_o ∑ LxMx			9.28			5.98			4.34
Reduction %						**36.5**			**53.2**

4 Steroid Sex Hormones:

As shown in Fig 3 Sub-lethal concentrations of bacterial metabolites disrupted the levels of two tested steroid sex hormones in the hemolymph of *B. alexandrina* snails (Estradiol & testosterone). The level of estradiol hormone (E2) gradually decreases with increasing concentration of SMBS, the levels of hormone in control group was 191.88 pg/mL, The level fell considerably ($p < 0.05$) after exposure to LC_{10} to 157.64 pg/mL and then continued to decrease until reached 139.57 by increasing concentration to LC_{25} **(Fig. 1A)**. The same effect also noticed in testosterone hormone, a noticeable and significant decrease was observed after exposing the snails to sublethal concentration LC_{25}, as the hormone level fell to 0.13 ng/dL, while the hormone level in the unexposed group was 0.19 ng/dL **(Fig. 1B)**.

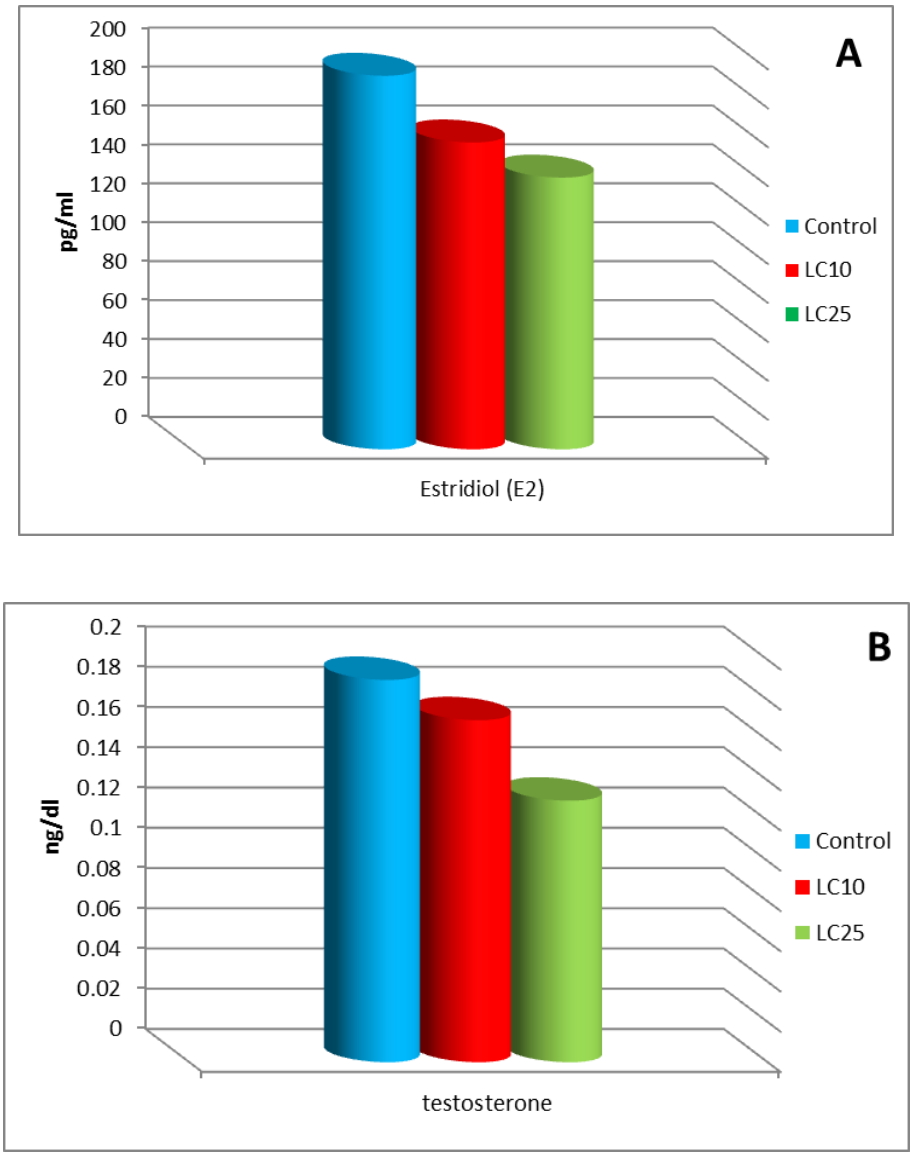

Fig 1: Levels of sex hormones of control and exposed snails A: Estradiol (E2) hormone ; B: testosterone hormone.

5. Histological studies:

Normal hermaphrodite gland of *B. alexandrina* snail consist of a group of acini connected to each other by connective tissue, each acinus lined by germinal epithelial layer that gives rise to oogenesis and spermatogenesis. In oogenesis, oocytes differentiated into ova which grow in few numbers (1-2/ acinus) and arranged peripherally however, in spermatogenesis, spermatocytes differentiated into sperms, grow in large number and aggregated in the lumen (**Fig. 2A)**.

Exposure of snails to the LC_{50} of SMBS showed noticeable damage to the gonads cell. Mature ova almost disappeared **(Fig. 2B)**, degenerated sperms and germ cells **(Fig. 2C)** were present, many acini lost their membranes and the lumen expanded widely. As the concentration increased to LC_{90}, the destruction of the cells of the hermaphrodite gland and its components worsened, as surface protrusions in membrane surrounding the gland appeared and losing its attachment with acini **(Fig 2D)**.

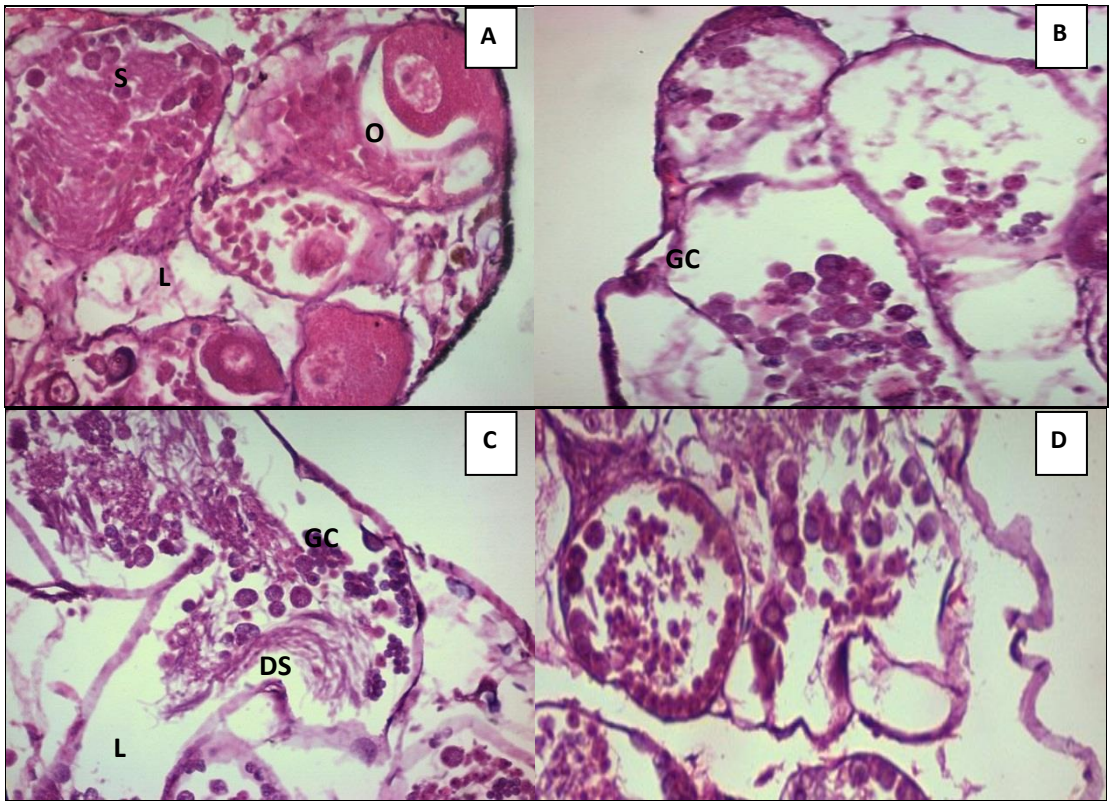

Fig 2: Light micrographs showing transverse sections in hermaphrodite gland of *Biomphalaria alexandrina*, plate A: normal OTG; plates B&C: OTG after acute exposure to LC_{50}; and plate D: after acute exposure to LC_{90} of SMBS. O: ova; S: sperms; L: lumen; GC: germ cells; and DS: degenerated sperms.

DISCUSSION

The tendency to use molluscicides of biological origin in combating intermediate hosts of parasitic diseases is one of the recommendations of the World Health Organization [(6)]. This recommendation is due to the fact that these molluscicides are often less harmful or safer to humans, living organisms, and surrounding environment. In this context, the present study was designed to evaluate the metabolites *B. subtilis* bacteria as a biological agent that combats intermediate host of intestinal schistosomiasis.

The present results revealed that the *B. subtilis* metabolites have a molluscicidal activity *against B. alexandrina*, so, SMBS recorded as a novel in molluscicidial effect. This result is considered a distinctive and important addition to the effectiveness of the previously observed biocide activity of these bacterial metabolites, as antimicrobial, antifungal and insecticidal effects due to their production of lipopeptides [(18), (21)].

Determination of the molluscicidal activity was followed by evaluating the effect of the SMBS on the susceptibility of snails to infection of snails with miracidia intestinal schistosomiasis. The study demonstrated that there is a significant reduction in snails` survival rate, infection rate and its production of cercariae, which consider the most effective measurements in reducing infection. The survival rate fell to 76.7%, a difference of 13.3% compared to the infected control group, while the reduction was greater in the infection rate, as it recorded 60.960.9%, a difference of 24.4 from the control group. The survival rate dropped to and the infection rate also dropped to 60.9, a difference of and 28.4 compared to the infected control group. Numerous investigations have been undertaken on the effect of molluscicides on *B. alexandrina* snail infections by using chemical molluscicides [(28)] or plant origin molluscicides [(27), (30)] which have produced consistent results with the present study.

Regarding the snails fecundity, the results indicate a noticeable gradual decrease in the survivorship (Lx) of B. alexandrina post-exposed to LC_{10} and LC_{25} for 4 weeks in. Keeping *B. alexandrina* with torpedo grass, blackberry, and ginger roots yielded similar results [(31), (32)]. The oviposit capacity (Mx) and reproductive rate (R_0) of B. alexandrina showed a marked regression after exposure to previous experimental concentrations from SMBS. This observation in harmony with agrees with **Bakry *et al.*** [(30)] and **El-Emam *et al.*** [(32)] on the oviposit capacity of snails after exposure to methanol extracts of *Z. officinale* and *C. citrinus plants*,

respectively. Same results also obtained by using *Haplophyllum tuberculatum* chloroform extract by **Rizk *et al.* [33]**.

In an attempt to explain this restriction in the rate of egg lying, the levels of some sex hormones which in snails were determined. The results agreed with expectations as they showed a preservation of the levels of both estradiol (E2) and testosterone in the snails' hemolymph. the imbalance in sex hormones levels of the exposed *B. alexandrina* are agree with **Rageb *et al.* [34]** who discovered that exposing the snails to copper and magnesium chlorophyllin caused a Limits in their sex hormones levels (testosterone, estradiol and progesterone). The results partially agreed with what was recorded by **El-Emam *et al.* [30]**, where he observed an decrease in the hormones progesterone and testosterone by exposure to the methanolic extract of ginger and turmeric, while the level of the hormone 17β-estradiol elevate in snails` hemolymph by exposure to these extracts.

The observed restriction in the reproductive rate and levels of sex hormones led to studying the histological changes in hermaphroditic gland of *B. alexandrins* snails to clarify the reason for this restriction. The defect in oogenesis and the deterioration of both oocytes and sperms at high concentrations (LC_{50} and LC_{90}) of the bacterial metabolites explain the pervious restriction resulting from exposure to lower concentrations (LC_{10} and LC_{25}). These results in compatible with those of **Song *et al.* [35]**, who discovered that exposing the snail *Oncomelania hupensis* to *Camellia sinensis* seed extraction, caused spermatogonia degeneration **Ibrahim *et al.* [31]** also investigated the influence of the plant molluscicide *Solanum elaeagnifoljum* on *B. alexandrina* gonads and discovered that the hermaphrodite gland was significantly damaged, particularly in snails subjected to an ethanolic extract of *S. Elaeagnifolium.*

CONCLUSION

This study pointed out to the crucial role of *B. subtilis* bacteria against *B. alexendrina* snails' mortality, fertility and cercarial production. Obtained results revealed the molluscicidal potency of *B. subtilis* metabolites for this aspect confirmed by biochemical and histological observed alterations in the treated snails. Hence, *B. subtilis* metabolites could be considered as molluscicidal agent in the control programs of their prevalence.

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