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Evaluation of *illicium verum* (star anise) against *Biomphalaria alexandrina* Snails and their Infection with *Schistosoma mansoni*

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ABSTRACT

Schistosomiasis, the second most common parasitic disease worldwide, is caused by trematodes of the *Schistosoma* genus. One of the most promising methods for controlling schistosomiasis in Egypt is the use of plant-based molluscicides to target snails. The application of plant-origin molluscicides offers several benefits, including being safe, inexpensive, and environmentally friendly. The purpose of this study was to evaluate the molluscicidal and larvicidal effects of *Illicium verum* (star anise) on the free larval stages of the *Schistosoma mansoni* parasite and its intermediate host. Additionally, the study assessed various biological, biochemical, and histological characteristics of *Biomphalaria alexandrina* snails treated with *I. verum*. The most promising variety of *I. verum* was identified, and *B. alexandrina* snails were infected with *S. mansoni*. Hemocyte counts, antioxidant enzyme activities, and histological reactions of the snails were analyzed. The cold-dried powder of *I. verum* fruit, suspended in water, demonstrated molluscicidal effects on *B. alexandrina* snails and larvicidal effects on the cercariae and miracidia of *S. mansoni*. The survival rate of *S. mansoni*-infected snails exposed to sublethal concentrations of star anise at the time of first cercarial shedding was significantly lower compared to the control group ($P< 0.05$). Furthermore, infection rates and cercarial production in infected snails were significantly reduced. Antioxidant enzymes, such as superoxide dismutase (SOD), showed a notable increase after the snails were exposed to a sub-lethal concentration (LC25) of *I. verum* (36.60 for the treatment group compared to 5.43 for the control group). The use of a fruit dry powder water suspension of *Illicium verum* proved to be effective in snail management. This treatment interferes with snail biology and infection with *S. mansoni*, potentially halting and limiting the spread of the parasite.

INTRODUCTION

The neglected tropical disease schistosomiasis is prevalent in many developing nations in Asia, Latin America, the Middle East, and tropical Africa; it impacts both livestock and the poor rural areas. An estimated 800 million people in Sub-Saharan

Africa are at risk of acquiring schistosomiasis, despite the fact that several African nations have made notable progress in eradicating the illness **(Aula *et al.*, 2021)**. Bilharzia, also known as schistosomiasis, is a tropical disease that spreads when human water comes into contact with surface water tainted by infected excrement and certain snails from freshwater that serve as intermediate hosts. The three primary species responsible for causing schistosomiasis are *Schistosoma* (S.) haematobium, *S. mansoni*, and *S. japonicum* **(Gryseels *et al.*, 2006)**. The *B. alexandrina* snail is the main African species that act as an intermediate host in Egypt **(Abou-El-Naga *et al.*, 2011)**, therefore, it takes into account the mandatory intermediate host for *S. mansoni* miracidia **(Colley *et al.*, 2014)**. In order to propagate its life cycle, the parasite strives to escape the snail host's weaponry at the same time **(Abou-El-Naga *et al.*, 2015)**. While the internal environment of incompatible snails inhibit schistosome formation, compatible snails enable successful schistosome development **(Nacif-Pimenta *et al.*, 2012)**. Like other invertebrates, mollusks are shielded from external infections by their internal immune systems. The first line of defense in mollusks is their innate immunity, which is a non-specific reaction. Mollusks' innate immune response is composed of humoral mediators, phagocytic cells, physiological and cellular components, and anatomic barriers. Circulating mollusks hemocytes make up the cellular components, which are responsible for elimination of small invaders, while large intruders are encapsulated. Following ingestion, the foreign particles are hemolyzed by specific toxic enzymes that initiate oxidative burst reactions that can eliminate foreign invaders and pathogens. Additionally, molluscan immunity includes humoral components such as lectins, lysozyme activity, nitric oxide, and the phenyloxidase system **(Al-Khalaifah & Al-Nasser, 2019)**. Hemocytes are a key component of the innate immune system in invertebrates, where they play an important role in both cellular cytotoxicity and the release of humoral defense components. Countless killing mechanisms, such as the release of oxidative and degradative enzymes and the generation of highly reactive oxygen metabolites, are used by mollusks to strengthen their immune systems. The first line of defense against oxidative stress, superoxide dismutases (SODs), a type of metal-containing enzymes, are found both within and outside of cells. They catalyze the conversion of superoxide anion into hydrogen peroxide and molecular oxygen **(Soliman *et al.*, 2019)**. The biological cycle of this etiological parasite is disrupted by prevention measures. Molluscicides, for instance, are used to reduce the number of intermediate hosts (mollusks), which are crucial in the prevention of this illness **(Duarte Galhardo de Albuquerque *et al.*, 2020)**. Chemical molluscicides used to eradicate snails may be unnecessary and pose risks to the ecological balance, as well as to water quality for irrigation, drinking, plants, and animals **(Zanardi *et al.*, 2019)**. Numerous infectious and non-infectious disorders are treated worldwide with natural herbal remedies, which constitute an essential component of the traditional medical system. It can be used as antivirus, antibacterial and molluscacide **(Sasidharan *et al.*, 2010)**. Since the majority of these natural components may be derived

from underutilized plant species and food byproducts, interest in them is of ecological worth and economic significance **(Lourenço *et al*., 2019)**. *Illicium verum*, also known as star anise, is a medium-sized, fragrant, evergreen tree in the Illiciaceae family that produces fruit shaped like a star. It is a significant herb that grows widely throughout the southwestern regions of Asia. Although star anise has additional potential benefits, including antibacterial, antifungal, anthelmintic, insecticidal, secretolytic, antinociceptive, anti-inflammatory, gastroprotective, estrogenic, sedative, expectorant, and spasmolytic properties **(Patra *et al*., 2020)**, no studies in the literature have examined its activity as a molluscicidal agent. Therefore, this study investigated the molluscicidal effects of *Illicium verum* on *Biomphalaria alexandrina* snails and their infection with *Schistosoma mansoni*.

MATERIALS AND METHODS

1. Snails breeding

Biomphalaria alexandrina snails (5-6mm) used in the present study were from Medical Malacology Department, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt. Dechlorinated tap water was used to thoroughly wash them, and they were kept in plastic aquaria with dechlorinated aerated water (10 snails/L) and were fed oven dried lettuce leaves.

2. Miracidia and cercariae

S. mansoni ova and cercariae were obtained from medical malacology unit, TBRI.

3. *Illicium verum* plant

Dried star anise fruits were purchased from a local market and ground into a powder using an electric mill. The dry powder was stored in a clean, dry, dark glass bottle until used in biological tests **(Mahmoud *et al*., 2011)**.

Preparation of plant extracts was as follows **(Bakry, 2009)**: Five types of extracts were prepared: cold water extract, hot water extract, methanol extract, ethanol extract, and cold dry powder suspension. To prepare the cold water extract, 100g of plant powder was soaked in 500mL of dechlorinated water at room temperature (25 ± 1°C) for seven days. For the hot water extract, 100g of plant powder was boiled in 500mL of dechlorinated water for 15 minutes, then allowed to cool to room temperature. To create the methanol and ethanol extracts, 250g of plant powder was soaked in 1L of each solvent (methanol and ethanol) at room temperature for seven days. After the filtrate was vacuum distilled, the residue was stored in a clean, dry, dark glass bottle until further

use. The filtrates from each extract and the dry powder of *Illicium verum* were used to create a series of concentrations to determine both fatal and sub-lethal concentrations.

4. Molluscicidal and larvicidal screening

In order to determine the molluscicidial activity of the various star anise extracts against adult *B. alexandrina* snails, a range of concentrations were made from plant powder using water suspensions depending on weight/volume. A further set of concentrations from the extracts of cold water, boiled water, and organic solvents were made **(WHO, 1965)**. Three duplicates were made, each containing ten snails per liter. Three more duplicates in dechlorinated water served as the control. Exposure and recovery periods were 24h each at 25 ± 1°C **(Andrews *et al.*, 1982)**. Regarding the larvicidal activity, Miracidia and cercariae, free larval stages of *Schistosoma mansoni* were exposed for 120 minutes at intervals of 15, 30, 45, 60, 90 and 120min. For estimating the number of dead and living stages in connection to the snails sublethal doses LC10, LC25, LC50, and LC90, hand lens (10×) and a stereomicroscope were used to determine the mortality rates **(Wagner & Ansari, 1974)**.

5. Snails infection

Calculation of the infected *Biomphalaria alexandrina's* infection incidence and survival rate at initial shedding, prepatent time, shedding duration, and cercarial production following exposure to sublethal doses LC10 of star anise, experimental groups were immediately exposed to *S. mansoni*. Ten *S. mansoni* miracidia were added to each snail in a single exposure at 25+1°C. The snails were cleaned and placed in sterile tanks with dechlorinated water after being exposed to miracidia. To maintain their perpatency, they were then kept in a laboratory environment at 25°C. Snails were given oven-dried lettuce leaves during the incubation or prepatency period, when the parasites developed within their tissues, and dead snails were taken out each day. Moreover, the water was replaced once a week **(Mahmoud *et al.*, 2011)**. Three duplicates in a control group were subjected to miracidia at the same time as the experimental snails and received the same care until cercarial emergence.

6. Hemolymph sampling and hemocytes count

In accordance with the method described by **Nduku and Harrison (1980)**, a small portion of the snail's shell, located just above the heart, was removed. A capillary tube was then inserted into the heart to collect hemolymph. After the snails were exposed to the parasite and/or plant powder for 3, 7, 14,

and 21 days, the hemolymph from approximately ten snails in each group (treated and control) was collected. Hemocyte counts were performed using a Bürker-Türk hemocytometer.

7. Antioxidant parameters

Hemolymph was collected from approximately ten snails in each group at 3, 7, 14, and 21 days after exposure to the parasite and/or plant powder. The antioxidant levels of glutathione reductase (GSH), lipid peroxidation (LP), superoxide dismutase (SOD), catalase (CAT), and nitric oxide (NO) were evaluated in the hemolymph. The average volume of hemolymph used in each experiment was 100 µL. Antioxidant tests were performed using the colorimetric method, following the instructions provided by Biodiagnostic Company. Kits for these tests were purchased from Biodiagnostic, Dokki, Giza Governorate, Egypt (website: www.bio-diagnostic.com).

8. Histological study

A group of snails was used to record histological changes in the head-foot tissues after exposure to LC90 of the fruit plant dry powder. The tissue sections were stained with hematoxylin and eosin, and then observed under a light microscope equipped with a digital camera **(El-emam *et al.*, 2015)**.

9. Statistical analysis

In order to analyze the data and to calculate the LC50 and LC90 by probit analysis, the Statistical Program for Social Science (SPSS) version 20.0 was utilized. The t-test, chi-square test of significance, and one-way analysis of variance (F) (ANOVA) were among the analytical statistics used **(Goldstein & Avram, 1964; Southwood, 1978)**. A 95% confidence interval and a 5% acceptable margin of error were established. The following criteria were used to compute the probability (*P*-value): A *P*-value was deemed significant if it was less than 0.05.

RESULTS

1. Molluscicidal and larvicidial activity

As shown in Table (1), the fruit dry powder of *I. verum* plant has a promising molluscicidal effect against *B. alexandrina* snails as a cold water suspension (LC_{50} 389ppm);meanwhile, the hot water extract has no effect up to 500ppm. Additionally, the molluscicidial activity of the star anise plant's fruit water, methanol, and ethanol extracts against *B. alexandrina* snails was less than that of cold water suspension.

Table 1. Molluscicidal activity of the plant *illicium verum* fruit dry powder extracts against *Biomphalaria alexandrina* snails (24-h exposure)

Extract	LC $_{10}$ (ppm)	LC $_{25}$ (ppm)	LC $_{50}$ (ppm)	LC $_{90}$ (ppm)
Water suspension	315	342	389	410
Ethanol	366	395	412	476
Methanol	323	377	392	419
Cold water	333	385	421	436
Hot water	Over 500 ppm			

Therefore, the dry powder of plant's fruit was selected to evaluate its effect on larval stages of *S mansoni*. Fig. (1A, B) shows that *S. mansoni* free larval stages (miracidia and cercariae) were killed (100% death) after 30 and 60min of exposure to snail's LC_{90} (410ppm) and LC_{50} (389ppm), respectively, compared to about 10% death of control specimens.

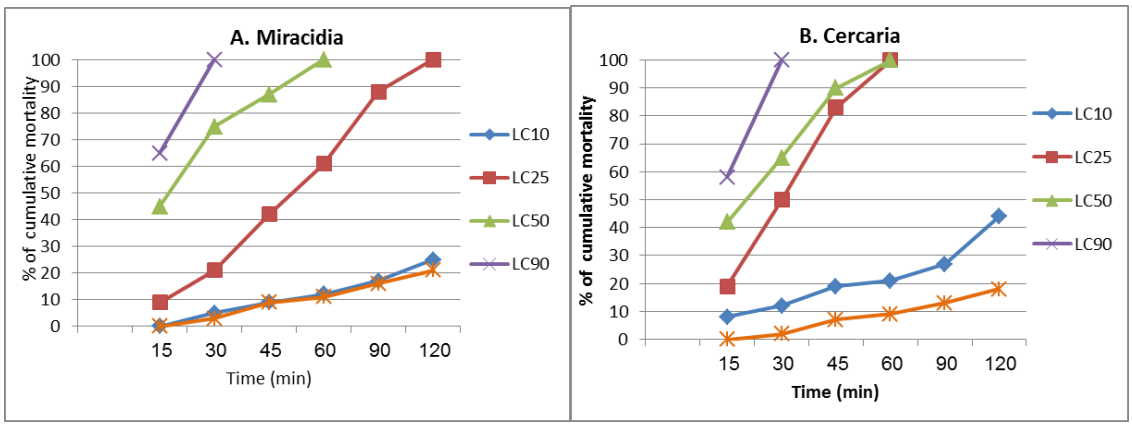

Fig. 1. Effect of snails' sublethal concentrations from the fruit dry powder of the plant on free larval stages of *Schistosoma mansoni* (miracidia & cercariae)

2. Snails infection

Table (2) demonstrates that *B. alexandrina* snails exposed to fruit dry powder of *I. verum* (LC_{10} 3165ppm) exhibited low survival rate at the start of cercarial shedding compared to infected control group. Accordingly, the survival rate was 70% as opposed to 93.3% for the control group ($P< 0.05$). Besides, the infection rate of treated snails was less (57.1%) than that of the control group (89.3%). Furthermore, the prepatent period and cercarial production/treated snail were similar as those of the control snails, but duration of cercarial shedding was noticeably shorter.

Table 2. Effect of LC_{10} (315ppm) of *I. vernum* fruit dry powder on infection of *B. alexandrina* with *S. mansoni* (mean ± SE, *=*P*<0.05)

Parameter		Infected-exposed snails	Control (infected snails)
Survival rate at 1st shedding	**No. of exposed**	30	30
	No. of survived	21	28
	%	70%*	93.33
Infection of snails	**Number**	12	25
	%	57.1*	89.3
Prepatent period (days)	**Range**	21-27	21-24
	Mean	22.5	22.2
	±S.E.	1.8	1.4
Total cercariae/snail	**Range**	15-984	22-1015
	Mean	652.8	783.7
	±S.E.	156	187
Duration of shedding	**Range**	7-21	7-28
	Mean	15.5*	24.9
	±S.E.	4.5	3.1

3. Hemocytes count

Four groups were examined in these experiment: the 1st was the control group; the 2nd was exposed to *I. verum* dry powder only (LC10); the 3rd was exposed to S. *mansoni* miracidia only; and the 4th was exposed to both *I. verum* dry powder and *S. mansoni* miracidia. Total hemocytes counts were recorded for all groups following numerous exposure periods represented in Fig. (2A). The findings revealed a noteworthy rise in the group of infected snails (*P*<0.05) from the 3rd day of exposure (3300±210*) in comparison with that of the control ones (2200± 210). Group 2 showed a non-significant change in total hemocytes count from the 14th day of exposure (2300±230) compared to the control group (2300± 240). However, infected-exposed snails group (group 4) showed highly

significant increase in hemocytes count during all periods of exposure compared to the control group.

With respect to differential hemocytes count, *B. alexandrina* snail hemolymph samples have three morphologically different hemocytes, designated as large granulocytes, small granulocytes and hyalinocytes. The sublethal concentration (LC10) of star anise group only markedly increased the number of tiny granulocytes in the treated infected snail (P<0.05) from the 3rd day of exposure, and the significant increase was continued till the 21st day in contrast to the control group. Conversely, there was a decrease in the percentage of large granulocytes and hyalinocytes in treated infected snail group correspondning to the control group from the 3rd day of exposure to the 21st day. The percentage of large granulocytes and hyalinocytes were reduced in both infected and exposed snail groups compared to the control group (Fig. 2B).

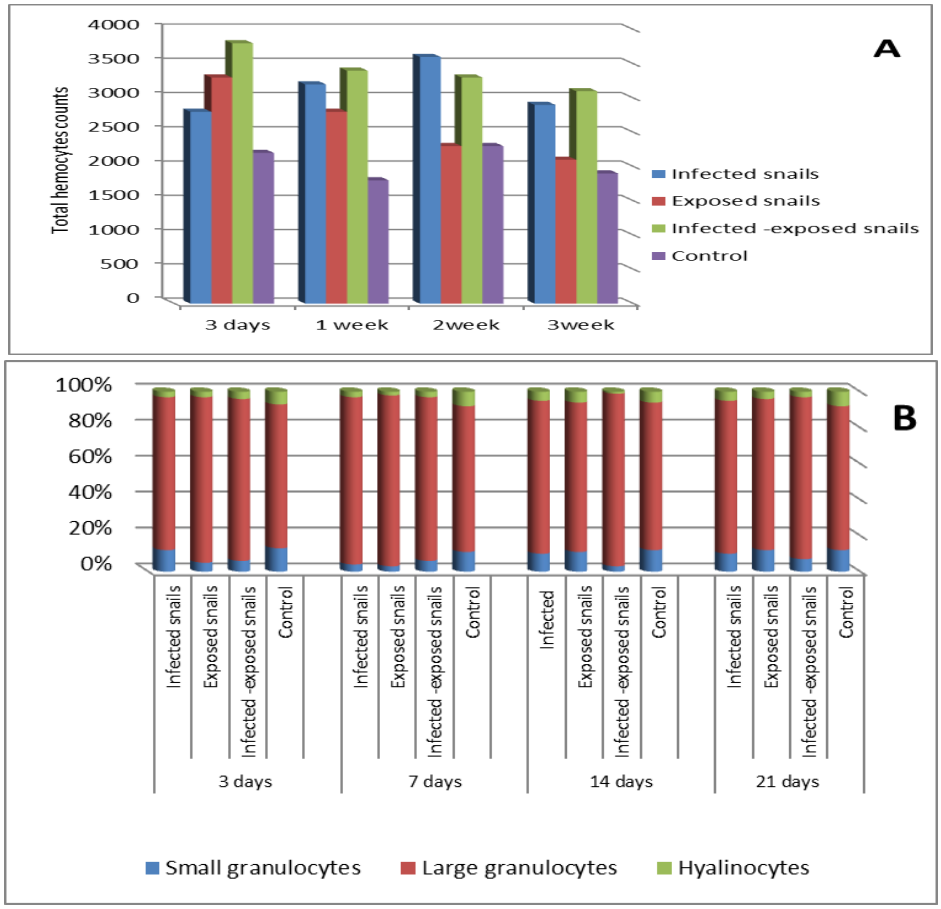

Fig. 2. The total and differential counts of hemocytes in the hemolymph of *Biomphalaria alexandrina* snails following exposure to *Schistosoma mansoni* for a number of durations and/or sublethal level of *Illicium verum* fruit dry powder

4. Antioxidant parameters

As indicated in Table (3), five antioxidant parameters are assessed in the hemolynph of four groups of *B. alexandrina* snails. These parameters contain two non-enzymatic antioxidants: Nitric oxide and reduced glutathione (GSH) in addition to three enzymetic antioxidents: Superoxide dismutase (SOD), catalase, and lipid peroxide. The results in Table (3) indicates that no significant response was recorded for non-enzymatic antioxidants after exposure to sublethal concentration of *I. verum*. However, when compared to the control group, the exposed and/or infected groups' antioxidant enzyme activity was noticeably higher. Infected-exposed snails group had the highest enzymes activity among the groups; SOD activity reached 36.19± 9.94 in infected exposed group compared to 5.43± 0.21 for the control group, while lipid peroxidase activity recorded 19.23±2.33 compared to 6.27± 0.06 in the control group.

Table 3. Antioxidant parameters in hemolymph of *Biomphalaria alexandrina* snails after 7 days of infection with *S. mansoni* miracidia and /or exposure to *Illicium verum* fruit dry powder (mean ± SD, *= $P < 0.05$)

Antioxidant parameter	Infected snails	Exposed snails	Infected - exposed snails	Control
Nitric oxide (µmol/L)	0.29± 0.03*	0.34± 0.05	0.23±0.01**	0.36± 0.02
GSH (nmol/ml)	143.13± 11.2	144.67± 9.07	145.68±7.66	139.67± 8.39
SOD (U/ml)	18.42±0.64**	36.60± 1.55**	36.19± 9.94**	5.43± 0.21
Catalase (nmol/ml)	21.55± 2.18**	27.40± 1.35**	31.33±1.96**	16.60± 2.25
lipid peroxide (µmol/L)	17.81± 2.61**	16.97± 3.03**	19.23±2.33**	6.27± 0.06

5. Histological studies

In *B. alexandrina* snails, the typical head-foot area is encased in a protective outer layer of cuticle. A tall columnar epithelium with cells with basal nuclei is found within this lining. Modified sac-like cells that mimic unicellular glands are seen inside this columnar epithelium. The cuticular layer allows these cells to open up to the outside of the foot surface. The mucus is secreted by these unicellular glands. There are longitudinal muscle fibers, which are transverse muscle fibers, in between. Salivary glands and mouse opening are also clearly represented in Fig. (3A). Significant degradation of the connective tissue of the oblique muscle fibers was discovered by histological analysis of the treated

snails' head-foot area, as well as necrotic alterations in the mucosa secreting unicellular glands. In addition, results showed necrotic changes in salivary glands and mouth opening (Fig. 3B).

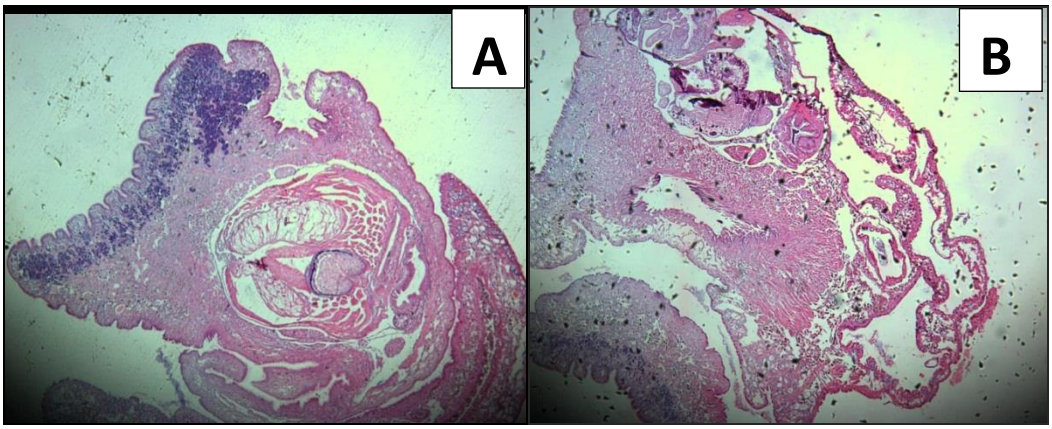

Fig. 3. Transverse light micrographs of the head-foot region of *Biomphalaria alexandrina* snails. **(A)** Displaying a normal slice; **(B)** Exhibiting a section in a treated snail following exposure to LC_{90} of *Illicium verum*

DISCUSSION

The current findings demonstrated that, after 24 hours of exposure, fruit dried star anise powder showed a molluscicidal impact on *B. alexandrina* snails. It was observed that this investigation had a distinct molluscicidial effect. Antibacterial, antifungal, anthelmintic, insecticidal, secretolytic, antinociceptive, anti-inflammatory, gastroprotective, spasmolytic, estrogenic, and sedative properties are only a few of the potential applications for star anise **(Rahman *et al.*, 2016)**. The molluscicidial activity of fruit star anise against *B. alexandrina* in addition to *S. mansoni* larvicidal activity are compatible with the results of **Shobana and Naidu (2000)**. According to their findings, star anise is among the several species that include bioactive substances along with a number of phenolic and flavonoid compounds that have antibacterial, antimicrobial, antioxidant, and preservation qualities.

With respect to the snails infection, exposure to LC10 from the fruit dry powder of the star anise significantly decreased their survival rate at first shedding, infection rate, and cercarial production (total cercariae/snail) of *B. alexandrina* snails. As reported by **El-Ansary *et al.* (2001)**, the survival rate of these snails' initial shedding values was correlated with the amount of phenol oxidase present in the tissues of *B. alexandrina* treated with the plant. After being exposed to various sublethal concentrations of the plant's dry powder for

24 hours, the infection rates of *S. mansoni* miracidia in *B. alexandrina* were significantly reduced. Furthermore, the enzymes in the hemolymph of the treated snails were inhibited, which decreased cercarial secretion and impaired their physiological processes, negatively affecting parasite growth. The duration of cercarial shedding in infected snails was also significantly shortened in the current study after exposure to the LC10 concentration of star anise dry powder. This effect was likely due to disruptions in the snails' enzyme system and a reduction in the total protein content of their hemolymph, both of which adversely impacted the development of the parasite within the snails, as noted by **Badawy (1991)**, **Gawish (2008)**, **Bakry (2009)** and **Mahmoud *et al.* (2011)**.

The internal defense systems of invertebrates are based on the innate immune system, which is composed of both humoral and cellular components **(le Clec'h *et al.*, 2016)**. The buildup of various chemicals, including pesticides, heavy metals, and molluscicides, led to the cell-mediated immune response, as well as the existence of many cell types that are essential for defense and operate as the main barrier against invasive germs and parasites. Snails amoebocytes or hemocytes are amoeboid cells that move and encapsulate the invasive parasites **(Fried, 2016)**. Compared to *S. mansoni*-infected snails and other groups, the hemocytes of the snail group exposed to the sublethal concentration (LC10) of star anise in the current investigation showed a great significant increase. Prior researches demonstrated that parasite infection raised the hemolymph (hemocytes) count in a variety of snail species **(Ibrahim, 2019; Suwannatrai *et al.*, 2019)**. **Esmaeil (2009)** provided an acceptable explanation, speculating that repairing tissue injury could be the cause of the rise in hemocyte counts in *S. mansoni*-infected snail and exposed groups. This study identified three distinct hemocyte types—hyalinocytes, micro granulocytes, and large granulocytes—in the hemolymph of *B. alexandrina*. This observation is in line with the results of previous researchers **(Kamel *et al.*, 2006; Cavalcanti *et al.*, 2012)**. Granulocytes and hyalinocytes are two different cell types, according to some authors, others believed that they were two phases of the evolution of the same cell type **(Oliveira *et al.*, 2010)**. According to a different study, hyalinocytes are smaller, spherical cells without pseudopods, while granulocytes are pseudopods on phagocytic cells that have the ability to enclose big particles **(Yoshino *et al.*, 2008)**. Similarly, the proportion of tiny granulocytes was higher in the infected snail group treated with the sublethal concentration (LC10) of star anise than in the normal uninfected control group, while the percentage of hyalinocytes was lower in the exposed and infected snail groups compared to the control group. Consistent with the present findings, **Barky *et al.* (2012)** discovered that methanol extracts of the *Azadirachta indica* plant markedly enhanced the quantity of granulocytes in the hemolymph of *B. alexandrina* snails. This

suggests that the snails responded favorably to the treatment. A number of studies showed how medication treatment altered the proportion of different hemocyte types in aquatic snails **(Barky *et al.*, 2012)**. Mespiquat chloride was shown to be the most sensitive hemocyte type in *B. alexandrina* snails treated with the plant growth regulator **(Mohamed & Abdel-Gawad, 2005)**. The hematological organ was stimulated to create undifferentiated hemocytes that evolved into granulocytes to make up for their decreased number, which may be why one study revealed that the percentage of small round undifferentiated cells rose **(El-Sayed, 2006)**. Moreover, hyalinocytes are believed to play a central role in wound healing, as they aggregate at the site of injury. As a result, their quantity in the hemolymph decreases **(Barçante *et al.*, 2012)**. In contrast, granulocytes—immunologically active cells—are primarily found in the snail's hemolymph, rather than remaining at the injured tissue to respond to external stimuli **(Oliveira *et al.*, 2010)**. The current research showed a significant increase in the activity of the antioxidant enzyme superoxide dismutase (SOD), catalase, and lipid peroxidase in the hemolymph of infected and/or exposed snails, with the highest increase observed in the infected-exposed group. The combined effects of *Schistosoma mansoni* infection and exposure to *Illicium verum* fruit dry powder may account for this elevation in enzyme activity. These findings align with the observations of **Kiros *et al.* (2014)**, who noted that the molluscicidal effect of *Glinus lotoides* fruits appears to be linked to changes in the antioxidant activity of the snails' hemolymph.

The histological analysis further revealed necrotic changes in the mucous-secreting and salivary glands, along with degeneration in the connective tissue of the oblique muscle fibers. This observation is consistent with **Ibrahim and Bakry (2019)**, who found that the application of chlorophyllin extract as a molluscicide caused histological alterations in the glands of treated snails including the loss of cell nuclei, vacuolization, tissue degeneration, and rupture, as well as lumen expansion of these glands.

CONCLUSION

The use of a fruit dry powder water suspension of *Illicium verum* was found to be effective in snail management, as it disrupts snail biology and their infection with *S. mansoni*, potentially halting or limiting the spread of the parasite.

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