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ANTIPROTOZOAL AND MOLLUSCICIDAL ACTIVITIES OF *PELARGONIUM GRAVEOLENS*

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ABSTRACT

In this study, the ethanol extract of *Pelargonium graveolens* (PGE) exhibited a strong molluscicidal against *Biomphalaria alexandrina* snails, as well as a strong biocidal activity against the larval stages of *Schistosoma mansoni* (miracidia and cercariae). Treatment of the snails with the LC_{25} (59.7 ppm) of PGE induced a remarkable decrease in the protein levels in the snail tissues and hemolymph by 18 and 29%, respectively. A significant increase in the levels of the transaminases ALT (143%) and AST (161%) in the hemolymph was observed. Alkaline phosphatase was significantly increased by 124% in the snail tissues, while ACP was significantly increased by 76% in the hemolymph. PGE conferred the same inhibitory activity as metronidazole on *Blastocystis hominis* at a concentration of 200 µg/ml. The % mortality was 50, 60 and 70% after 24, 72 and 144 h incubation with PGE. The present study is the first report to provide evidence that PGE has a potent molluscicidal, biocidal and anti-*Blastocystis* effects.

Keywords: *Blastocystis hominis*, snails, molluscicidal, *Schistosoma mansoni, Pelargonium graveolens*

INTRODUCTION

Blastocystis hominis is a common protozoan parasite in the human intestine. Infection rates of up to 10% in developed countries and 50% in developing countries [1]. *Blastocystis* is the most prevalent parasite found in human stool samples. Transmission to humans is through consumption of contaminated food or water [2]. Several studies have reported that *B. hominis* can cause severe intestinal complications, such as severe abdominal pain, alternating diarrhea and constipation, fatigue, enteritis and bloating [3]. A growing body of evidence also suggests that irritable bowel syndrome (IBS) may be caused by *Blastocystis* [1, 3]. A number of antimicrobial agents have been used to treat *Blastocystis* infection. This includes metronidazole, nitazoxanide, trimethoprim-sulfamethoxazole. Metronidazole is the most commonly prescribed drug

for treatment of *B. hominis* [3]. However, metronidazole can cause undesirable adverse effects and may be ineffective in achieving complete eradication of *Blastocystis* infection. Besides, potential carcinogenic and teratogenic effects of metronidazole have been reported [4]. Resistance to metronidazole is also common and metronidazole may no longer be useful as a first line treatment [3]. Therefore, the need for new and safe candidates with anti-*Blastocystis* effect is quite apparent. Natural remedies have been used for centuries for the treatment of several ailments. Natural products remain as important sources of structural diversity and lead structures in drug discovery [5]. Recently, there has been a growing interest in the use of natural products because of their reduced side effects when compared to synthetic drugs.

Schistosomiasis is a major parasitic disease caused by *Schistosoma*, it is a devastating disease second only to malaria in its prevalence, deleterious effects and socioeconomic impact on human populations [6]. Chemotherapy, hygienic practices and snail control are among the available measures to control schistosomiasis. The disadvantages of chemotherapy are that it does not eliminate the infection entirely, there is a risk of re-infection, the cost of recurrent treatment is prohibitive in low-income countries and drug resistance is a major problem [7]. A better method to control schistosomiasis is to destroy the snail host [7]. Plant-derived molluscicides have a wide range of ideal properties, including low cost, availability, solubility in water and biodegradability, which makes them suitable for wide application in endemic areas for control of the snail vector. Furthermore, plant-derived molluscicides have low toxicity to non-target organisms compared to synthetic molluscicides, such as niclosamide, which cause serious environmental pollution [7]. Phenolic compounds are particularly suitable for snail control because their plants are widely distributed. Previous studies showed that phenolic compounds have a strong molluscicidal activity against the snail *Biomphalaria glabrata* and *B. alexandrina* [8, 9]. Phenolic compounds are also less toxic than saponins towards non-target organisms. These attributes prompted us to investigate the molluscicidal activity of a phenolic-rich plant belonging to the family Geraniaceae, namely, *Pelargonium graveolens* L'Her. *P. graveolens*, commonly known as the rose geranium, is a shrub that grows widely throughout the world and is cultivated in many countries for ornamentation [10]. The essential oil of this plant is widely used as a flavoring agent for various food products due to its unique rose-like aroma [10]. The essential oil of *P. graveolens* showed nematicidal [11], insecticidal [12] and mite-control activities [13]. However, no study has been traced on the molluscicidal activity of the non-volatile constituents of *P. graveolens* and their inhibitory effect against the intestinal protozoan parasite *B. hominis.*

In this study, we report the molluscicidal activity of the total ethanol extract *P. graveolens* on *B. alexandrina* snails, along with its activity against the larval stages of *S. mansoni* (miracidia and cercariae). Moreover, the biochemical parameters of the snail tissues treated with *P. graveolens* ethanol extract (PGE) were measured to determine its possible toxicity mechanisms in the treated snails. The second objective of this study is to test the inhibitory effect of PGE on *B. hominis* viability *in vitro,* with the aim to develop a new and safe candidate with anti-*Blastocystis* effect. The individual constituents of PGE were previously identified in an earlier report using the HPLC–PDA–ESI/MS/MS technique. The identified compounds include hydroxybenzoic acid derivatives, hydroxycinnamic acid derivatives, flavonoid aglycones, flavonoid glycosides, (epi) gallocatechin, dimeric and trimeric (epi) gallocatechin [14].

MATERIAL AND METHODS

Plant material: The leaves of *P. graveolens* were collected in July 2011 from El-Maryland Botanical Garden, Cairo, Egypt. The plant was botanically identified by Eng. Anour Ezeldein, the taxonomy specialist at the herbarium of El-Maryland Botanical Garden, Cairo, Egypt. A voucher specimen of *P. graveolens* was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (ASU PGG2011).

Extract preparation: Air-dried powdered leaves of *P. graveolens* (1400 g) were extracted three times with 80% EtOH. The total extract was concentrated to remove the organic solvent and freeze-dried to obtain a dry powder, which was dissolved in absolute EtOH. The EtOH-soluble portion was concentrated and freeze-dried to obtain a dry powder of PGE (130 g).

Isolation and culture of B. hominis: *B. hominis* cells were isolated from patients' fecal samples submitted to the Parasitology Laboratory, in the outpatient clinic of Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The stool samples were subjected to: 1- Direct microscopy using unstained and iodine-stained wet-mount smears, as well as merthiolate iodine formaldehyde concentration technique [15]. 2- Stool culture to exclude bacterial pathogens. 3- *In vitro* culture of *B. hominis*-positive stool samples: Approximately 50 mg of each fecal specimen was inoculated into 2 ml of Jones' medium (0.01% yeast extract in buffer saline), supplemented with 20% horse serum in a screw cap tube [16]. The tubes were then incubated at 37 ° C for 48 h and a drop of the cultured solution was examined by a light microscope at 10 x and 40 x magnifications. The detection of *B. hominis* vacuolar form indicates that the culture is positive. The culture is regarded as negative if *B. hominis* is not detected after 72 h of culture [17].

Experimental groups: PGE and the standard drug metronidazole were dissolved in DMSO and diluted to the desired concentrations with the culture medium. The final DMSO concentration was less than 1%. Following *in vitro* cultivation, the trophozoites of *B. hominis* were counted by microscopy in sterile 10 ml tubes and divided into

different groups. Three replicates were used for each group. Group (I) was treated with the culture media only and served as the negative control group. Group (II) was treated with 1% DMSO. Groups (III-VI) were treated with different concentrations of PGE (100, 200, 500, 1000 and 2000 µg/ml, respectively). Group VI were treated with the standard drug metroindazole (Sanofi-Aventis, Egypt) at a dose of 120 µg/ml. All the tubes of the different experimental groups were incubated in a humidified CO_2 at 37 ° C for different time intervals (24 h, 72 h and 144 h). The parasites in each group were counted using a Neubauer haemocytometer (Weber, England). The living cell count (LCC) of *B. hominis* (total trophozoite numbers per ml) was calculated from the mean of three haemocytometer counts and was compared with those of metronidazole for the same time intervals [18]. The trophoziote viability was assessed using eosin brilliant cresyl blue vital stain. The possible effect of PGE on *B. hominnis* living cell rate (LCR) was assessed after 24 h, 72 h and 144 h and the % mortality was recorded [18].

Snails and s. mansoni miracidia, cercariae: Adult *B. alexandrina* snails were obtained from the laboratory of Medical Malacology Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The snails were maintained at 25°C in dechlorinated water before use. Active snails with a shell diameter of 8-10 mm were chosen for the study. The miracidia and cercariae of *S. mansoni* were obtained from the Schistosome Biological Supply Center at TBRI. The parasite eggs were extracted from the intestines of hamsters infected 45 days earlier. Clean eggs were hatched in small amounts of dechlorinated water at 25°C. The cercariae were obtained from experimentally infected *B. alexandrina* snails. The miracidia and cercariae were maintained at 25 ± 0.5°C.

Molluscicidal activity: The efficacy of PGE against the snails was determined using previously reported methods [19]. PGE was dissolved in DMSO and diluted to the desired concentrations with dechlorinated water. The final DMSO concentration was less than 0.1%. Niclosamide was used as a positive control. For each concentration, three replicates of ten snails each were used. Three replicates of control snails were maintained under the same experimental conditions. The exposure and recovery periods were 24 h for each test. The efficacy of PGE was expressed as the LC_{50} and LC_{90} values [20]. The sub-lethal concentrations LC_{10} and LC_{25} were calculated using the SPSS Statistics program, version 20 for Windows with probit analysis [21].

Miracidicidal and cercaricidal activity: Ten milliliters of dechlorinated water containing approximately 100 freshly hatched miracidia and freshly shed cercariae was mixed with different concentrations of PGE [22]. Approximately equal numbers of miracidia and cercariae were used as the negative control. The exposure period was 1 h for each group. Three replicates were used for each test. Microscopic observation of the movement and mortality of the miracidia and cercariae was performed every 15 min. The organisms were considered dead when their motion ceased completely for one minute; the dead organisms were then counted [22]. The efficacy of PGE against the miracidia and cercariae was expressed as the LC_{50} and LC_{90} values [20].

Biochemical assay: Approximately 50 snails were exposed to a concentration corresponding to the LC_{25} of PGE for three weeks [19]. The control group was left unexposed under the same conditions. The PGE solution was renewed twice weekly. Hemolymph samples were collected according to the reported method (Abdul-Salam & Michelson, 1983). Tissue homogenates were prepared by dissecting the soft parts of the snail, then 1 g from each experimental group was homogenized in 5 ml of distilled water, and the resulting supernatant was used for the assays. All biochemical parameters were determined using reagent kits obtained from the bio-Merieux Company (Marcy l'Etoile, France). The results were expressed as the means ± SEM of the three replicates. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated according to a previously reported method [23]. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were determined as described previously [24]. All enzymes are expressed as units per gram of tissue. The total protein level (µg/mg tissue) was assayed using the Folin-phenol method [25].

Statistical analysis: Multiple comparisons were performed using one-way ANOVA test followed by Tukey-Kramer test for *post hoc* analysis. *P*–values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics program version 20 for Windows. The molluscicidal activity was calculated by probit analysis [20, 21].

RESULTS AND DISCUSSION

In recent years, medicinal plants have received considerable attention for their potential health benefits. There is growing interest in the use of medicinal plants because they are safe and widely

available at low cost [2]. Some plant extracts exhibited potential activity against *B. hominis* including galls [2], asafoetida [4], thyme and grape seed [4]. Furthermore, daily supplementation with oregano emulsified oil lead to complete eradication of *B. hominis* [4]. Many studies suggest that *Blastocystis* infection is difficult to be eradicated. Recent data also indicate that many currently used drugs are not effective in achieving complete eradication of the parasite [3]. Therefore, the investigation of new, natural alternatives against *Blastocystis* is highly warranted.

In the present study the effect of PGE on *B. hominis* was studied at different concentration (100, 200, 500, 1000 and 2000 µg/ml) at different time intervals (24, 72 and 144 h). PGE, at all the tested concentrations, induced a significant reduction in the number of living *Blastocystis* cells at the different incubation intervals. The LCC markedly declined in the groups treated with 2000 µg/ml by 4, 6.7 and 18-fold after 24, 72 and 144 h incubation periods, respectively compared to the control group (Table 1). Previous reports indicated that active drugs should inhibit the growth of *B. hominis* at least more than 2-fold [18]. Notably, PGE was more effective than metronidazole in suppressing the growth of *B. hominis*, which indicates that it could be a promising alternative to metronidazole.

Table 2 shows the effect of PGE on *B. hominis* trophozoites mortality using eosin-brilliant cresyl blue supravital staining method. This staining method is the most sensitive and reliable method for indicating the true effective drug concentration in inhibition of *B. hominis* growth [18]. Notably PGE conferred the same inhibitory activity as metronidazole at a concentration of 200 µg/ml. The % mortality was 50, 60 and 70% after 24, 72 and 144 h incubation periods. Higher concentrations of PGE (500- 2000 µg/ml) were more effective than metronidazole in inducing *B. hominis* mortality. These results clearly indicated the potent effect of PGE against *Blastocystis*. *P. graveolens* is extensively used in folk medicine and many food products. These attributes make *P. graveolens* particularly suitable as a therapeutic agent to control *B. hominis*.

In this study, the biocidal effect of PGE against *B. alexandrina* snails and different stages of the life cycle of *S. mansoni* was tested with the aim of developing a new, safe and readily available antischistosomal drug. The molluscicidal activity of PGE is listed in Table 3. PGE exhibited a potent molluscicidal activity (LC_{50} = 92.8, LC_{90} = 157.6 ppm), which was in the range of the WHO standard

for toxicity, which requires an LC_{50} of less than 100 ppm for plant molluscicides [8, 19]. The probit mortality analysis showed a linear relationship between the molluscicidal activity and the concentrations (ppm) of PGE. The slope value was steep, indicating a large increase in snail mortality with a small increase in the concentration of PGE.

The potent molluscicidal activity of PGE was confirmed by the decreased protein content and the modulation in the activities of vital enzymes in the snail hemolymph and tissues (Table 4), which indicated damage to the snail tissue and disturbance of the physiological activities required for parasite development and cercarial production [26]. Treatment of the snails with the LC_{25} of PGE induced a remarkable decrease in the concentration of the total proteins in the snail tissues and hemolymph by 18 and 29%, respectively, compared to the control snails. The decreased protein content could be attributed either to the destruction of the snail tissue and impairment in protein synthesis or to the stimulation of gluconeogenesis to use proteins as a source of energy under the stressful conditions of intoxication with PGE [26].

The results of the present study also indicated that there was a significant increase in the levels of the transaminases ALT (143%) and AST (161%) in the hemolymph at the end of the third week of PGE exposure as compared to the control levels. In contrast, their tissue levels were decreased. The increased levels of transaminases in the hemolymph may be attributed to their release from the damaged tissue into the hemolymph [26], and this would also explain the decreased levels in the snail tissue. AST and ALT are vital enzymes in the metabolism and generation of energy from amino acids [27]. Therefore, the elevated transaminases may also indicate the high energy demand of the snail under stressful conditions of intoxication. Alterations in the levels of alkaline phosphatase and acid phosphatase were also observed. ALP has a crucial role in shell formation, and ACP plays an important role in catabolism and induction of pathological necrosis [28].

ALP was significantly increased by 124% in the snail tissues. ACP was also significantly increased by 76% in the hemolymph. Previous reports indicated that snail enzymes can be used as specific targets for the development of molluscicidal drugs [29]. Based on the results of this study, it is clear that PGE at a sub-lethal concentration (LC_{25}) disturbs important enzymes that are vital for the physiological activity of the snail; therefore, it can be concluded that PGE can be used as a potent molluscicidal agent that

targets these enzymes. The toxicity and costs of synthetic molluscicides have resulted in a growing interest in the use of locally growing plants as molluscicides. PGE also conferred strong miracidicidal activity (LC_{50} = 11.9, LC_{90} = 15.9 ppm), and cercaricidal activity (LC_{50} = 30.3, LC_{90} = 55.9 ppm), which suggested that PGE can be used as a potent biocidal agent to control the different stages of *S. mansoni* (Table 3). *P. graveolens* is widely planted all over the world; therefore, *P. graveolens* can be regarded as a potential natural alternative suitable for biological application as plant molluscicides to control the intermediate snail host

and as a biocidal agent against cercariae and miracidia in endemic areas.

CONCLUSION
The present study is the first report, to the best of our knowledge, to provide evidence that PGE has a potent molluscicidal, biocidal and anti- *Blastocystis* effects.

CONFLICT OF INTERESTS
The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 1:**Effect of PGE on *B. hominis* living cell count (LCC).**

	Mean count (24 h)	Mean count (72 h)	Mean count (144 h)
Control	100 ± 4.08	100 ± 4.08	100 ± 4.08
1% DMSO	75.75 ± 1.65	75.75 ± 1.65	75.75 ± 1.65
Metronidazole 120 µg/ml	$60.72 \pm 1.93*$	$65.75 \pm 1.70*$	$52.75 \pm 1.90*$
PGE 100 µg/ml	$56.57 \pm 0.85*$	$34.25 \pm 1.31*$	$30.25 \pm 1.37*$
PGE 200 µg/ml	$55.5 \pm 3.06*$	$29.25 \pm 1.25*$	$20.25 \pm 1.10*$
PGE 500 µg/ml	$48.33 \pm 0.77*$	$25.55 \pm 1.73*$	$15.1 \pm 1.01*$
PGE 1000 µg/ml	$35.22 \pm 1.11*$	$20.22 \pm 1.11*$	$10.02 \pm 0.10*$
PGE 2000 µg/ml	$25.33 \pm 1.11*$	$15.02 \pm 1.00*$	$5.55 \pm 0.05*$

*Data represent the mean of living cell count of B. hominis (x 10^4) ± SEM (the mean of three haemocytometer counts) after treatment with different concentrations of PGE and metronidazole for different time intervals (24 h, 72 h and 144 h). * Significant from the control group at P < 0.05*

Table 2: Effect of PGE on *B. hominis* trophozoites mortality using eosin-brilliant cresyl blue supravital stain.

	Control	1% DMSO	PGE 100 µg/ml	PGE 200 µg/ml	PGE 500 µg/ml	PGE 1000 µg/ml	PGE 2000 µg/ml	Metronidazole (120 µg/ml)
24 h	0	0	0	50	75	80	85	50
72 h	0	0	10	60	65	85	90	60
144 h	0	0	15	70	80	90	98	70

Data represent the % mortality after treatment with different concentrations of PGE and metronidazole for different time intervals (24 h, 72 h and 144 h)

Table 3: Effect of PGE against *B. alexandrina* snails and *S. mansoni* miracidia and cercariae

	LC_{10} (ppm)	LC_{25} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)
Molluscicidial activity of PGE	28.005	59.695	92.794	157.583
Molluscicidial activity of Niclosamide	0.1	0.2	0.3	0.4
Miracidicidial activity of PGE			11.93	15.86
Cercaricidal activity of PGE			30.29	55.89

Table 4: Changes in the biochemical parameters in haemolymph and soft tissues of *B. alexandrina* snails after 3 weeks exposure by the LC$_{25}$ of PGE

	Haemolymph			Tissue		
	Control	Exposed	% Change	Control	Exposed	% Change
ALP	74.1 ± 2.0	17.9 ±3.7**	-75.8	26.3 ± 2.3	58.9 ± 18.1*	124.1
ACP	546.0 ± 18.0	960.4 ± 25.5***	75.9	681.3 ± 107.9	660.0 ± 90.2	3.1
AST	61.3 ± 0.7	160.0 ± 30***	161	350.0 ± 36.9	170.0 ± 17.3***	- 51.4
ALT	50. 7 ± 0.7	123.3 ± 45.1***	143	180.0 ± 10.0	173.3 ± 15.3	- 3.7
Total protein	3.1 ± 0.2	2.2 ± 0.2***	-29.0	2.2 ± 0.0	1.8 ± 0.2**	- 18.2

Data represent the mean ± SEM of three separate experiments. Test concentration = 59.7 ppm   * Significant at P < 0. 05   ** Significant at P < 0. 01   *** Significant at P < 0.001. Enzymes were expressed as units/g. Total protein as µg/mg

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