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Research Paper

Effect of successive applications of the sublethal concentration of *Solanum paniculatum* in *Subulina octona* (Subulinidae)

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ABSTRACT

Land snail *Subulina octona* exposed to lethal concentration (LC_{90}) and sublethal concentration (LC_{50}) of aqueous extracts of *Solanum paniculatum* to assess the changes caused by successive application of the sublethal concentration on the species' fecundity and the number of offspring produced after exposure. Successive application of the CL_{50} of the aqueous extract caused changes in the fecundity, mortality, time to reach maturity and clutch size produced after exposure. The exposed snails' fecundity declined in all applications, but there was no relation observed with the time of exposure. There was a trend for increased fecundity of the unexposed snails that did not occur in the exposed ones. The offspring produced after exposure of the adults took longer to reach maturity and also developed longer shells in this life stage than the young snails produced by the unexposed specimens.

Keywords: Glucose content; Fecundity; Land snail; Molluscicide.

INTRODUCTION

The close relationship between some parasite species and their intermediate snail hosts makes control of snail populations an effective strategy to control parasites (D'Ávila, et al., 2004).

Subulina octona (Brugüère, 1789) is a land snail that is widely distributed in tropical regions (Araújo and Bessa, 1993; D'Ávila and Bessa, 2005), including many regions of Brazil, where it is involved in the transmission of helminths of veterinary importance (Araújo and Bessa, 1993; D'Ávila and Bessa, 2005). The life cycle characteristics of this species, such as fast sexual maturity, short incubation period, high hatchability (Bessa and Araújo, 1995), homogeneous growth (D'Ávila and Bessa, 2005) and reproduction throughout the year make this species excellent for laboratory studies.

Many experiments have been conducted to find molluscicidal substances of plant origin that can be employed instead of the substances currently used to control

snails (Souza and Mendes, 1991; Mello-Silva, et al., 2006; 2010). Although, most of these studies carried out in Brazil have focused on freshwater snail species which transmit diseases that afflict humans (Vasconcelos and Amorim, 2003a; 2003b; Leyton, et al., 2005; Mello-Silva, et al., 2007; 2010). Few studies have been conducted on new methods to control land snails, so these methods are still generally traditional ones, including manual capture.

The effort to find new molluscicidal substances is important because the commercial chemicals currently utilized to control snail populations are harmful to the environment, by killing non-target organisms and accumulating in the food web (Thomas, 1995). Plants naturally produce compounds through secondary metabolism that provide defense against herbivores that biocidal activity and are used in folk medicine as bactericides, fungicides, nematicides, anthelmintics and acaricides, among others (Al-Daihan, 2008). *Solanum paniculatum* Linné (*Solanaceae*) is popularly used to combat hepatic maladies. It contains chemical compounds such as saponins, tannins and glycoalkaloids, all of which have proven activity, including against snails freshwater (Mason, et al., 1994; Treyvaud, et al., 2000; Jorge et al., 2001; Mesia-Vela, et al., 2002; Silva et al., 2005; Souza et al., 2008; El-Sherbini et al., 2009). Researches on the molluscicidal activity of *S. paniculatum* are promising, being this the first study of the effect of this plant on this snail species.

The objective of this study was to verify the changes in the reproductive biology of *Subulina octona* exposed to successive applications of aqueous extracts of *S. paniculatum.* The effects of these extracts were also evaluated on mortality, time to reach maturity, number of young snails reaching maturity and size at maturity of the offspring produced after exposure of the parent snails.

MATERIAL AND METHODS

Snail: The snails used in this study were obtained from a colony maintained at the Snail Biology Laboratory of the Prof. Maury Pinto de Oliveira Malacology Museum at Juiz de Fora Federal University (UFJF).

Plants: *S. paniculatum* leaves were gathered in the São Pedro district of the city of Juiz de Fora (São Pedro borough, Juiz de Fora, Minas Gerais: 21º46.301'S and 43º22.372' W) and identified in Leopoldo Krieger Herbarium, UFJF. Young leaves were selected measuring an average of 18 cm^2, without mechanical injuries or signs of insect attack. The leaves were washed and then dried at room temperature for two weeks, after which they were ground up in a domestic blender and placed in hermetically sealed plastic jars. The aqueous extracts were produced by static soaking of the ground leaf material in distilled water for 48 hours at room temperature.

Phytochemical analysis of aqueous extract of S. paniculatum: The identification of saponins in the aqueous extract and the determination of foaming index were realized according to the World Health Organization protocol (1992). To confirm the presence of tannins in the extracts of *S. paniculatum* and *S. lycocarpum* it was realized a test with a decoction with 5g of the dried plant in 100 ml of distilled water during 30 minutes followed by simple filtration and reserved for the tests. It was made an agar solution (Merck) at 2, 5% in distilled water. To differentiate the class of tannins it was realized the colorimetric test with ferric chloride ($FeCl_3$) at 2%. To the identify and quantify the condensed tannins it was utilized the Stiasny method (Doat, 1978).

Calculation of the lethal concentration (LC_{90}) and sublethal concentration (LC_{50}) of S. paniculatum: To find the lowest concentration that would cause 100% mortality of the snails (maximum concentration). This concentration was established trough

previous tests with the concentrations of 1% a 30% with intervals of 5%. These concentrations were reduced in intervals of 1% until the establishement of the minimal concentration. The predetermined maximum concentration of the aqueous extract of the *S. paniculatum* leaves was 8mg/ml. From this concentration, dilutions were prepared at regular intervals of 1mg/ml of *S. paniculatum*, for a total of eight concentrations.

The LC_{50} and LC_{90} were established by bioassays in which 15 adult snails (mean shell length of 11.0 ± 3.0mm) (5 snails/group) were exposed to each of the concentrations for a period of 24 hours. The exposure was carried out by spraying 8ml of each concentration on the animals using a manual sprayer, in plastic terrariums with capacity of 0.25L, sealed with cotton cloth and rubber bands after application. After the exposure period, the snails were submitted to a recovery period lasting 24 hours. For this purpose, they were transferred to identical terrariums containing 20g of moistened sterilized mulch (120ºC for 1 hour) and fed *ad libitum* with poultry feed enriched with calcium carbonate (3:1 proportion) Bessa and Araújo (1995). The mortality was only recorded after the recovery period. The LC_{90} and LC_{50}, expressed in g/ml, were determined by using the BioStat 2008, version 2.5 programs.

***Mortality and changes in fecundity caused by exposure to the LC_{50} of the aqueous extract of* S. paniculatum:** To assess the reproductive alterations, 30 adult snails (10 snails/group), with mean shell length of 11.0 ± 3.0mm, were exposed to the CL_{50} of the aqueous extract of *S. paniculatum*. The groups were exposed during 24, 48 and 72 hours, followed by evaluation of the mortality and fecundity for 30 days. The snails were exposed to the LC_{50} of *S. paniculatum* 30 and 60 days after the first application and the same parameters were evaluated. Control groups composed of the same number of snails were subjected to the same routine, but only with distilled water.

To assess the mortality and fecundity, the surviving snails were kept in clear plastic terrariums with volume of 1 L (Silva, et al., 2008).

The mortality was evaluated by direct observation of the animals and removal of the dead ones. The fecundity was established by the average production of eggs per snail in each treatment. The eggs produced were removed from the terrariums every two days.

***Alterations in the glucose concentration in the hemolymph caused by exposure to the LC_{50} of* S. paniculatum:** To assess the changes in the glucose concentration in the hemolymph caused by exposure to the LC_{50} of *S. paniculatum*, 120 snails (10 snails/group) were exposed according to the method described above during 24, 48 and 72 hours. Hemolymph samples were collected after each exposure interval by cardiac puncture. The glucose concentration was determined by adding 10 ml of serum to a medium containing 0.05 M of sodium phosphate buffer (pH 7.45), 0.03 mM of aminoantipyrine, 15 mM of sodium p-hydroxybenzoate and at least 12 kU of glucose oxidase and 0.8 KU of peroxidase per liter. The absorbances were then read at 510 nm against a reaction blank, utilizing the D-glucose 100mg/dL standard (Doles®). The spectrophotometric readings were taken with three repetitions and the results were expressed in mg/dL.

Analysis of the young snails after exposure of the parents: To assess the effect of the extracts on the offspring produced after exposure of the adult snails, 30 recently hatched snails (10snails/group) from the treated and control groups were observed from hatching to sexual maturity. The procedure was carried out after each application. The survivals, time to reach maturity and size at maturity were recorded.

***Statistical analysis*:** The results were expressed as mean ± standard deviation and compared by applying the Kruskal-Wallis test, followed by the Student-Newman-Keuls test (significance of 0.05), calculated with the BioEstat 2005 software.

RESULTS

It was confirmed the presence of saponins and tannins in both extracts. To *S. paniculatum* the foaming index was 500. The presence of tannins was confirmada and the Stiasny method detected the presence of condensed tannins, being the mean mass calculated of 4.45mg ± 0.4.

***Mortality*:** The LC_{90} of *S. paniculatum* was 5.04% and the LC_{50} was 2.7%. There was no mortality of *S. octona* after the first application of the LC_{50} of the aqueous extract of *S. paniculatum*. After the second application there was 3.5% mortality due to exposure to the extract for 48 hours, but this figure did not differ significantly from that of the control group (H=1.5; p=0.1). After the third application, the mortality was 3.0% for the snails exposed for 48 hours to the extracts at the LC_{50} of *S. paniculatum* (H=1.4; p=0.1).

***Alterations in the snails' fecundity**:* Important changes in the fecundity of *S. octona* caused by exposure to the LC_{50} of the *S. paniculatum* aqueous extracts were observed. After the first exposure to this concentration, we observed reduction of 12%, 19% and 18% in fecundity among the snails exposed for 24, 48, 72 hours (P<0.05) (Figure 1).

After the second application of the LC_{50}, there was also lower egg production in the three exposure intervals in comparison with the control group being verified reduction de 5%, 54% and 23% in 24, 48 and 72 hours, respectively (P<0.05) (Figure 1). The same result was found after the third application, with reduction of 33%, 28% e 32% in fecundity of the snails exposed to the LC_{50} of *S. paniculatum* in 24, 48 and 72 hours of observations, respectively (P<0.05) (Figure 1).

The fecundity of the snails in the control group increased with the exposure intervals of 24, 48 hours and 72 hours (P<0.03). However, the fecundity of the snails exposed to the LC_{50} of *S. paniculatum* did not increase significantly during exposure of 24, 48 and 72 hours (P>0.05).

Alterations in the glucose content of the hemolymph: There was a reduction of the glucose content reaching 79.5% after 72 hours of exposure to the *S. paniculatum* extract in relation to the control group. The reduction in the glucose concentrations in the hemolymph of the exposed snails was significant after the exposure times of 24 hours (t= 5.3, P=0.02), 48 hours (t=5.4, P=0.04) and 72 hours (t=3.9, P=0.04). The average glucose levels of the snails not exposed and exposed to the LC_{50} of *S. paniculatum* can be seen in Figure 2.

Analysis of the young snails after exposure of the parents: There was no mortality observed among the offspring of the unexposed snails in the period evaluated. The mortality rates of the young snails from the groups exposed to the LC_{50} of *S. paniculatum* for 24, 28 and 72 hours were 16.7%, 13.3% and 23.3%, respectively, with no significant difference observed in comparison to the offspring produced by the control group (H=4.32, P=0.5). The mortality percentage of the young snails from the groups with two and three successive applications of the LC_{50} of *S. paniculatum* also did not differ from the control (H=4.4; P=0.49; H=8.19; P=0.15, respectively). After the second application, the mortality was 36.7% after 24 hours and 13.3% after 48 and 72 hours. After the third application, the mortality rates were 13% and 20% for the snails after 48 and 72 hours, while there was no mortality after 24 hours.

The time to reach maturity of the offspring of the unexposed adults snails did not vary with time (H=4.12; *P*=0.85). However, the young snails obtained after the second and third applications of the LC_{50} of *S. paniculatum* took significantly longer to reach sexual maturity when exposed for 72 hours (H=5.33, *P*=0.02 and H=6.0, *P*=0.007). Table 2 shows the average times to reach maturity.

When the adults were exposed for 24 hours to the *S. paniculatum* extract, the average number of young that reached maturity was significantly less after exposure of 24 hours (H=4.1, *P*=0.04), 48 hours (H=4.4, *P*=0,04) and 72 hours (H=4.4, *P*=0.04). The same result was observed after the third application (24 hours: H=4.5, *P*=0.05; 48 hours: H=3.4; *P*=0.04 and 72 hours: H=4.0, *P*=0.04). However, after the second application, there was no statistical difference between the average number of young snails that reached maturity when compared to the control group (24 hours: H=3.00, *P*=0.08; 48 hours: H=2.60; *P*=0.1 and 72 hours: H=2.50, *P*=0.12).

The young snails produced by the adults exposed to the LC_{50} of the extract reached sexual maturity with significantly larger shell lengths than those produced by adults that had not been exposed to the extracts (Table 3).

DISCUSSION

For a product to be used as a molluscicide, it should be effective at low doses, inexpensive, biodegradable and nontoxic to humans (Singh, et al., 1996; Rao and Singh, 2000). For control of water snails the WHO indicates that the products used should be effective at concentrations lower than 100 ppm (WHO, 1993), but no indicated limits have been established for land snails. Therefore, in this study we considered a maximum concentration up to 10% as the lethal concentration. It is important to consider the differences between the water and land environments in terms of exposure, diffusion of substances and absorption by the target organisms. Besides this, the behavioral strategies of land snails, such as burying themselves (Hyman, 1967), as well as physiological aspects such as estivation (Storey, 2002) hinder the action of molluscicides. Also, the substrate can act as a barrier and reduce the animals' contact with the product.

The tendency for fecundity to increase during growth observed in this study was previously reported for *S. octona* (D'Ávila and Bessa, 2005). According to these authors, for this species there is a strong correlation between increased size and fecundity. When exposed to the *S. paniculatum* extract, the correlation between the size and fecundity of the species was altered.

According to Thompson and Lee (1986), glycemia in snails is very precisely regulated. The upsetting of glycemic homeostasis is an indication of intoxication, and has been observed in other study models (Mello-Silva, et al., 2010). Reduction in the glucose levels in the hemolymph can reflect exhaustion of carbohydrate deposits, such as those in the digestive gland and foot. Intoxication of the snails by exposure to the *S. paniculatum* extract leads to changes in the neuroendocrine cells responsible for regulating the animal's glycemia (light green cells [LG]). The detoxification processes consume high amounts of energy, resulting in exhaustion of these deposits.

To, the use of these energy reserves for detoxification can therefore explain the reduction of fecundity observed in this study. The results found demonstrate that the extract causes physiological changes in the snails, impairing their fecundity through an indirect castration process due to diversion of nutrients that would otherwise be used for reproductive activity.

Similar results were found for freshwater snail *B. glabrata* to a *Solanum malacoxylum* extract during the same intervals as in the present study caused a reduction of the hemolymph glucose levels (Mello-Silva, et al., 2006). The same pattern was observed for this snail species exposed to an extract of *Euphorbia splendens* var. *hislopii* (Mello-Silva, et al., 2007; 2010). These authors also found a reduction of carbohydrate reserves among the exposed snails. Singh and Singh (2004) also demonstrated a reduction in fecundity in *Lymnaea acuminata,* possibly as a result of diminished proteins, amino acids, DNA and RNA in the snails' ovotestis.

Unlike the findings of the present study, Rao and Singh (2000) observed a high mortality percentage of young *A. fulica* produced by snails exposed to the plant extracts tested. However, those authors did not analyze the development of the surviving young to permit comparisons with the results obtained for *S. octona*.

We did not find any works that have evaluated the effects of molluscicidal compounds on the offspring of *S. octona*. Ferreira et al., (2009) assessed the effect of caffeine and thymol on the survival and growth of young *S. octona* of different ages and demonstrated reduced survival, but the authors did not find changes in growth or in time to reach maturity among the exposed snails.

The time to reach maturity of the offspring of exposed adults was longer than 100 days for all the exposure periods. Longer time to sexual maturity implies shorter reproductive life and thus smaller populations in the future. The impaired use of energy for reproduction, evidenced by the smaller number of eggs per snail, implies a greater energy investment in growth, as observed by the larger shell size of these animals. This phenomenon, called gigantism, has also been observed in snails parasitized by trematode larvae. The opposite was found in the present study for the descendants of the unexposed snails.

The negative effects observed for both the adults and descendants produced after exposure can be attributed to the active ingredients present in *S. paniculatum*. We confirmed the presence of tannins and saponins and in this plant. The molluscidal activity of these compounds was previously observerd against freshwater snails such as *Biompharia glabrata* Say, 1818 *Bulinus globosus* (Morelet, 1866) (Schaufelberger and Hostettmann, 1983; Sukumaran, et al., 2002; Araújo, et al., 2010). Besides lethality, the saponins present in *Yucca schidigera* Roezl. and *Hedera helix* Linné inhibit the feeding of the land snail *Helix aspersa* (Müller, 1774) (Mason, et al., 1994).

According to Cunha and Roque (2005), a hemolytic property that disorganizes the membrane can explain the biocidal activity of saponins. These authors suggested that the high power of complexation with steroids can be related to the antifungal activity of this chemical group. Complexation between tannins and proteins is considered to be the base of their biological activity for control of pests (Neto and Caetano, 2005).

The molluscicidal effect of *Solanum* species was verified on *Biomphalaria alexandrina* (Ehrenberg, 1831) (Amer and Manal, 2004) at concentrations similar to those used in the present study. Silva et al., (2005) noted molluscicidal activity of *Solanum species* on *B. glabrata*. However, these authors did not observe activity of *S. paniculatum* in the concentration utilized here. They also mentioned that the glycoalkaloids present in these active species are responsible for the molluscicidal action. Finally, Xavier et al., (2010) demonstrated that *S. lycocarpum* caused reduced survival of *B. glabrata* at concentrations of 5% and 2.5%.

CONCLUSION

Present work is the first report of the effects of exposure to the aqueous extract of *S. paniculatum* on the physiology of *S. octona,* focusing on the effects of this exposure on the snail's energy metabolism. The results show that this extract can be utilized to control these animals, by reducing the hatchability of the eggs laid by snails exposed to the *S. paniculatum* extract, as well as increasing the time necessary for the offspring to reach maturity, indicating that the extract has both spatial and temporal action. Successive applications of the aqueous extracts of these plants can maintain low population levels, preventing population imbalances.

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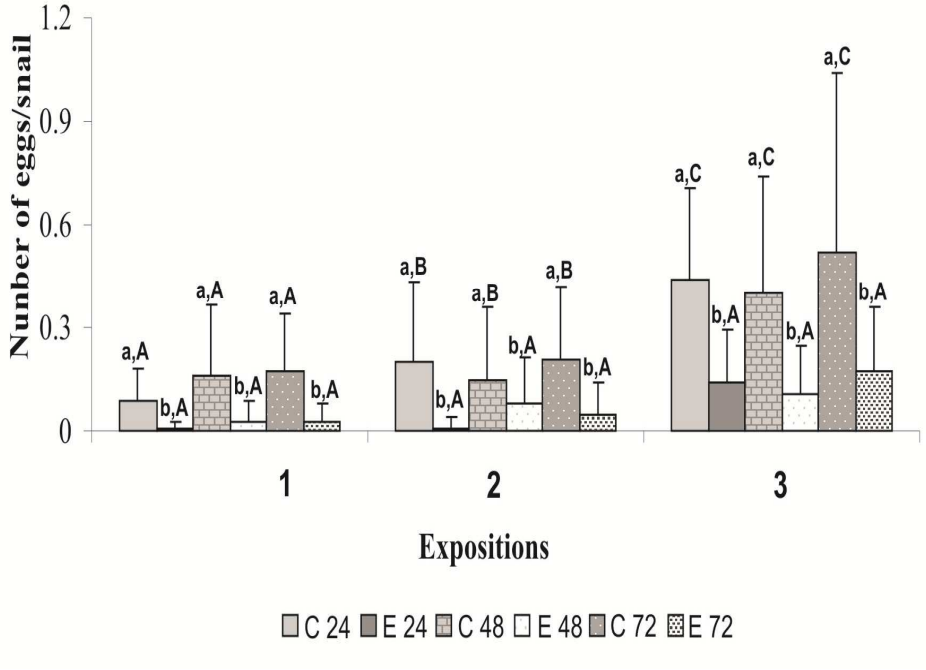

Figure- 1: Fecundity of *Subulina octona*, expressed as mean number ± standard deviation of eggs/snail not exposed (control) and exposed to the LC_{50} of the *Solanum paniculatum* extracts after 24, 48 and 72 hours of exposure, in three successive applications. C24: control for 24 hours; C48: control for 48 hours; C72: control for 72 hours; E24: snails exposed to the LC_{50} for 24 hours; E48: snails exposed for 48 hours and C72: snails exposed for 72 hours.

- *Small letters indicate difference between the treatments; capital letters indicate difference between the applications

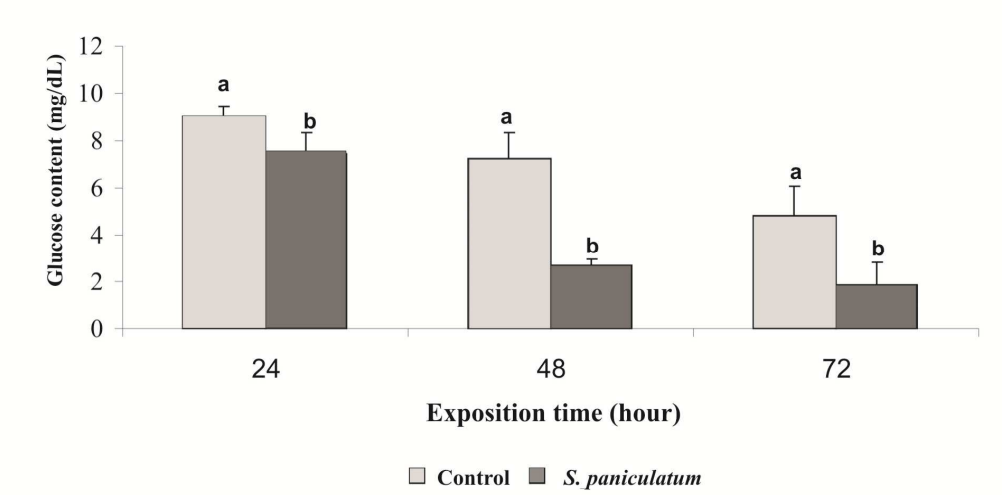

Figure- 2: Glucose concentration, expressed in mg/dL, mean and standard deviation (I), on hemolymph from individuals of *Subulina octona* non exposed (Control) and individuals exposed to LC_{50} of *Solanum paniculatum* for 24, 48 e 72 h.

- [a,b]:Means followed by different letters represents statistics difference.

Table-1: Time to reach sexual maturity of the offspring of *Subulina octona* produced by adult snails exposed to the *Solanum paniculatum* extracts for 24, 48 and 72 hours.

Exposure time	Applications		
	First	Second	Third
24 hours			
Control	$82.7 \pm 5.2^{A, a}$	$85.0 \pm 11.8^{A, a}$	$88.0 \pm 0.0^{A, a}$
S. paniculatum	$120.0 \pm 0.0^{B, a}$	$139 \pm 0.47^{B, a}$	$117 \pm 19.8^{B, a}$
48 hours			
Control	$80.7 \pm 5.4^{A, a}$	$85.7 \pm 11.8^{A, a}$	$85.0 \pm 11.8^{A, b}$
S. paniculatum	$112.3 \pm 81.3^{A, a}$	$163.3 \pm 2.4^{B, b}$	$100.3 \pm 3.8^{A, a}$
72 hours			
Control	$81.3 \pm 6{,}3^{a, b}$	$85.0 \pm 11.8^{A, b}$	$88.0 \pm 0.0^{A, b}$
S. paniculatum	$119 \pm 0.0^{B, a}$	$139.0 \pm 0.0^{B, b}$	$105.0 \pm 3.3^{B, a}$

- A; B = indicate significant difference between the means in different treatments.
- a, b = indicate significant differences between the means in different applications.

Table- 2: Mean number of young snails produced by *Subulina octona* not exposed (control) and exposed to the *Solanum paniculatum* extracts that reached sexual maturity.

Exposure time	Applications		
	First	Second	Third
24 hours			
Control	$10.0 \pm 0.0^{A, a}$	$6.3 \pm 2.4^{A, a}$	$10.0 \pm 0.0^{A, a}$
S. paniculatum	$6.3 \pm 0.0^{B, a}$	$4.0 \pm 3.3^{B, a}$	$3.5 \pm 1.9^{B, a}$
48 hours			
Control	$10.0 \pm 0.0^{A, a}$	$10.0 \pm 0.0^{A, a}$	$10.0 \pm 0.0^{A, a}$
S. paniculatum	$8.0 \pm 0.0^{A, a}$	$4.0 \pm 0.8^{B, b}$	$3.0 \pm 4.2^{A, a}$
72 hours			
Control	$9.7 \pm 0{,}47^{A, b}$	$10.0 \pm 0.0^{A, a}$	$10.0 \pm 0.0^{A, a}$
S. paniculatum	$1.7 \pm 1.7^{B, a}$	$5.5 \pm 1.3^{B, b}$	$3.0 \pm 4.2^{B, a}$

- A; B = indicate significant difference between the means in different treatments.
- a; b = indicate significant differences between the means in different applications.

Table- 3: Shell length at sexual maturity, in mm, expressed as mean ± standard deviation, of young *Subulina octona* snails from the groups not exposed (control) and exposed to the LC_{50} of the aqueous extract of *Solanum paniculatum*.

Exposure time	Applications		
	First	Second	Third
24 hours			
Control	$9.9 \pm 0.8^{A,a}$	$9.9 \pm 0.8^{A,a}$	$10.1 \pm 1.0^{A,a}$
S. paniculatum	$13.0 \pm 2.1^{B,a}$	$13.0 \pm 1.8^{B,a}$	$11.6 \pm 1.2^{B,a}$
48 hours			
Control	$10.4 \pm 1.0^{A,a}$	$10.1 \pm 0.0^{A,a}$	$10.6 \pm 1.0^{A,a}$
S. paniculatum	$13.9 \pm 1.3^{A,a}$	$13.3 \pm 1.3^{B,a}$	$11.7 \pm 1.5^{A,a}$
72 hours			
Control	$10.2 \pm 1,2^{A,a}$	$10.2 \pm 1.1^{A,a}$	$10.3 \pm 1.1^{A,a}$
S. paniculatum	$12.8 \pm 0.9^{B,a}$	$13.9 \pm 1.1^{B,a}$	$3.0 \pm 4.2^{B,a}$

- A; B = indicate significant difference between the means in different treatments.
- a = indicate significant differences between the means in different applications.