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Preliminary phytochemical screening and molluscicidal activity of the aqueous extract of *Bidens pilosa* Linné (Asteraceae) in *Subulina octona* (Mollusca, Subulinidade)

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ABSTRACT

The aim of this study was to establish reference concentrations (LC_{50} e LC_{90}) of aqueous extracts of *Bidens pilosa* on the land snail *Subulina octona*, in order to assess the changes caused by 24 and 48 h exposure to the sublethal concentration regarding species' fecundity, hatchability and in offspring produced after exposure to confirm the presence of tannins, saponins and flavonoids in this plant species. Eggs newly hatched and 30 day-old snails were exposed to sublethal concentration, calculated for adults. The phytochemical analysis confirmed the presence of flavonoids, condensed tannins and saponins in the aqueous extract of *B. pilosa*. The LC_{50} and LC_{90} obtained were 51.4mg/mL and 74.1mg/mL respectively. The exposure to sublethal concentration reduced significantly the hatchability and the survival of the offspring hatched from exposed eggs and also caused a reduction on survival and growth in snails exposed for both time period. The exposure time also caused a reduction at the evaluated parameters.

Key words: Agricultural pest, control, land sn,ail, molluscicide, vegetable extract.

INTRODUCTION

Subulina octona (Bruguière 1789) (Subulinidae) is a land snail widely distributed in Brazil with medical and veterinary importance due to its participation as intermediate host in the life cycle of helminths such as *Platynossomun illiciens* (Braum, 1901) (Digenea, Dicrocoeliidae) and *Aelurostrongylus abstrusus* (Railliet, 1898) (Nematoda, Angiostrongylidae) parasites of the domestic cat (Ash 1962, Maldonado 1945), *Postharmostomum gallinum* (Witenberg, 1923) (Digenea, Brachylaimidae) and *Paratanaisia bragai* (Santos, 1934) (Digenea, Eucotylidae) which are parasites of birds (Alicata 1940, Maldonado 1945), *Angiostrongylus vasorum* (Baillet 1866) (Kamensky 1905) (Nematoda, Angiostrongylidae) which is a parasite of canids (Bessa et al. 2000), *Angiostrongylus cantonensis* (Chen, 1935) (Angiostrongylidae), parasite of rodents and humans (Andersen et al. 1986).

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This species is usually found at damp and shaded places such as gardens and vegetable gardens (Aráujo and Bessa 1993). When it occurs at high densities, the control of this species becomes necessary in order to reduce the damages caused in crops. Besides, *S. octona* is a great experimental model for laboratory studies, due to some characteristics of its life cycle such as fast sexual maturity, short incubation period, high hatchability, high reproductive rates (Bessa and Araújo 1995a, b) and homogeneous growth (D'ávila and Bessa 2005a) making easy the analysis of substances with molluscicidal effect.

The currently used molluscicides are highly toxic to environment (Gasparotto et al. 2005) therefore; the World Health Organization recommends the application of plant derived molluscicides, because of its lower impacts to the environment (WHO 1983).

Studies have confirmed molluscicidal activity of methanolic and chloroformic extracts of 14 plant species of the family Asteraceae collected in Bolivia and Argentina on *Biomphalaria glabrata* (d'Orbigny 1835) (Planorbidae) (Bardon et al. 2007). Hmamouchiu et al. (2000) found molluscicidal activity in the species *Artemisia herba- alba* Asso (Asteraceae) on *Bulinus truncatus* (Audouin 1827) (Bulinidae) in Morocco, and reported the presence of saponins and flavonoids in this plant. These results demonstrate the importance of this plant family in the control of other snail species. The species *Bidens pilosa* Linné (Asteraceae) popularly known as hairy beggarticks, is a herbaceous plant with a short annual cycle (Lorenzi 2000) widely distributed in Brazilian territory, being used in folk medicine (Adegas et al. 2003) for antimicrobial (Rojas et al. 2006, Deba et al. 2008), hepatoprotective (Yuan et al. 2008), diabetes (Chien et al. 2009) and ulcer (Freise 1933) antitumoral (Kumari 2009, Kviecinski et al. 2008) and antioxidant (Krishnaiah et al. 2011).

This species contains chemical compounds such as flavonoids, saponins and tannins (Hoffmann and Holzl 1988, Silva et al. 2011, Valdés and Rego 2001) all of which with proven biocidal activity, including against snails (Filho 2010, Lopes et al. 2011, Schaufelberger and Hostettmann 1983).

The aim of this study was confirm the presence of flavonoids, tannins and saponins in the aqueous extract of *B. pilosa*, calculate LC_{50} e LC_{90} on adult *S. octona* and verify the effect of the LC_{50} in different life stages of *S. octona* exposed to the sublethal concentration for 24 and 48 hours.

MATERIALS AND METHODS

Snails

The eggs, adults (60 day-old), 30 day-old, and newly hatched 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 the Universidade Federal de Juiz de Fora (latitude: 21°45'13"S; longitude: 43°21'19''W; 678 m altitude). These snails were reared in plastic terrariums containing 20 g of moistened sterilized mulch (120°C for 1 hour) and fed *ad libitum* with poultry feed enriched with calcium carbonate (3:1 proportion) according to Araújo and Bessa (1993).

Preparation of the Aqueous Extracts

The aerial parts of *B. pilosa* were collected in January, 2011 in the São Benedito district of the city of Juiz de Fora (S 21°45.014' HO 43°19.684' and 814 m of altitude). One exsiccate was added at the herbarium Leopoldo Krieger at the Universidade Federal de Juiz de for a under register CESJ 58.406. Afterwards, these parts were washed and dried at room temperature for 10 days in an average temperature of 26±5°C. After that, the aerial parts were ground up in a domestic blender and stored in a sterilized recipient in cold storage. The aqueous extracts were produced by cold static soaking in distilled water for 72 hours. After that the suspended solutions were filtrated and the aqueous solution was used for the tests.

Phytochemical Analysis of Aqueous Extract of *B. pilosa*

For the phytochemical analysis, it was prepared a decoction with 5 g of the dried plant in distilled water during 30 minutes followed by simple filtration and reserved for the tests. To identify flavonoids a sodium hydroxide solution was used according to Mouco et al. (2003). The identification of saponins in the aqueous extract and the determination of foaming index were realized according to the WHO protocol (1992) and Farmacopéia Brasileira (2010). To confirm the presence of tannins in the extract of *B. pilosa* an agar solution was made (Merck) at 2.5% in distilled water. To differentiate the class of tannins (condensed or hidrolysable) a colorimetric test with ferric chloride ($FeCl_3$) was realized at 2%. To quantify the condensed tannins it was utilized the Stiasny method (Doat 1978) with three repetitions.

Calculation of the Lethal Concentration (LC_{90}) and Sublethal Concentration (LC_{50}) of *B. pilosa*

The protocol used was adapted from Silva et al. (2012). A pilot test was realized to find the two limit concentrations, a maximal (mortality of 100%) and a minimal (mortality of 10%). For this, 6 concentrations were used: 10, 20, 30, 80, 90 and 100 (mg/mL) and groups of 10 snails for each concentration. The maximal and minimal concentrations obtained for this test were 20 and 100 (mg/mL), respectively and from that limit concentrations more 3 intermediate concentrations of 40, 60 and 80 (mg/mL) were established, totaling 5 concentrations to calculate the LC_{50} and LC_{90}. Thirty adult snails randomly selected and distributed (10 snails/group) were used in plastic terrariums with 9 cm diameter and 6 cm deep. Snails were exposed through dermal contact directly with 5 mL of aqueous extract, for each concentration, 20 mg/mL, 40 mg/mL, 60 mg/mL, 80 mg/ml and 100 mg/mL. The same number of animals were used for control group, and were exposed to the same amount of distilled water. The snails remained exposed for 24 hours and after this, were transferred to similar terrariums containing 50 g of moistened sterilized mulch (120°C for 1 hour) to remain more 24 h to recovery. During the 48 h period of the assays snails were not fed and at the end of recovery period the mortality was verified.

Effects of the LC_{50} on the Offspring Hatched from Eggs Exposed for 24 and 48 Hours

Sixty eggs were distributed in four groups (15 eggs/group) for both 24 and 48 h period. The assays were conduced at room temperature and the relative air humidity was measured every day (21° - 24°C e 82% of relative air humidity). The exposure was carried out by spraying 20 mL of LC_{50} of the extract, previously calculated, on the eggs and control groups with of the same number of eggs were exposed to 20 mL of distilled water during the same time period. Afterwards, the eggs were transferred to terrariums with the same measurements lined with previously sterilized mulch and moistened with tap water. The hatchability analysis was realized every other day, during 30 days.

Survival of the Offspring Originated from Exposed Eggs

Snails hatched from eggs exposed to the LC_{50} of extract were kept in the same terrariums. These newly hatched were fed according Araújo and Bessa (1993) and terrariums moistened with water every three days. Observations for mortality analysis were performed every three days, through direct observation of animals, dead individuals were removed from the terrarium. The reach sexual maturity was performed on the same frequency and was determined by the presence of eggs in the uterus, visible through the transparent shell (Bessa and Araújo 1995b, D'ávila and Bessa 2005b). The mortality and time to reach maturity was checked every third day for 120 days.

Effects of the LC_{50} on Growth, Survival and Reproduction of Newly Hatched and 30 Day Old Subulina Octona Exposed to the Aqueous Extract of *B. pilosa* for 24 and 48 Hours

For this assay were used 40 newly hatched and 30 day old snails with mean size of 2.03±0.23 mm and 5.9±0.57 mm respectively. The snails were distributed in groups (10 individuals/group, with four repetitions) kept in plastic terrariums where 20 mL of LC_{50} the extract were pulverized, previously calculated, and closed with cotton cloth and an elastic rubber to avoid the escape of the snails. Control groups composed by the same number of snails were subjected to the same treatment, but received only distilled water. After the exposure period, snails were transferred to other terrariums and fed as described above. The growth was determined by measurement of shell length every month using a caliper rule Kanon (Mardened Stainless 1/28 in. 1/20 mm). To asses the mortality, analysis were conduced every 3 days, for a period of 90 days by direct observation of snails and the dead ones were removed from terrariums. The reach of sexual maturity was observed with the same frequency and determined by the presence of eggs in the uterus, seen through the shell (D'ávila and Bessa 2005b).

Statistical Analysis

An acute intoxication occurred for sublethal (LC_{50}) and lethal (LC_{90}) concentrations obtained by Probit analysis (significance of 0.05), calculated with the BioStat 2008 software, version 2.5 the same test was applied to built the curves of concentration-response (Finney 1971). The Kruskal-Wallis and the Student-Newman-Keuls ($p<0.05$) were applied to compare the hatching means, mortality and growth of snails exposed to LC_{50} by using the BioEstat software version 5.0. The curves were obtained using the Origin software version 6.0.

RESULTS

Phytochemical Analysis

The aqueous extracts of *B. pilosa* showed a yellowish color that changed into red after the addition of a solution of sodium hydroxide. This coloration is typical of chalcones and aurones, what confirm the presence of flavonoids in the aqueous extracts. The test with Agar solution confirmed the presence of tannins by turbidity in the aqueous extract. The moss green coloration observed after the addition of ferric chloride confirmed the presence of condensed tannins, which was also confirmed by the Stiasny test. The total tannins in extract (%) and total tannins in plant (%) were 11.40 and 3.71 respectively. The presence of saponins was verified and the foaming index was 100.

Calculation of Lethal Concentration and Sublethal Concentration (LC_{50} and LC_{90}) for the Aqueous Extract of *Bidens pilosa*

Both LC_{50} and LC_{90} were calculated using the Probit analysis, resulting in 51.4 mg/mL and 74.1 mg/mL respectively. The ratio between concentrations-response is represented in Figure 1.

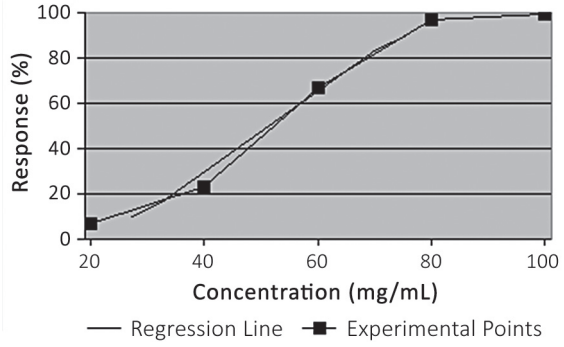

Figure 1 - Ratio concentration-response of *Subulina octona* exposed to the aqueous extract of *Bidens pilosa*. Evaluated response=lethality, expressed as a percentage of dead snails.

Influence of the *Bidens pilosa* Extract on the Hatchability of *Subulina octona*

The hatch means of the groups exposed to the *B. pilosa* extract were significantly lower in comparison to the hatch means of control groups to

the same period (24 h: H=9.3384; p=0.0024; 48 h: H=23.6739; p<0.0001). The hatching percentages of treatments are represented below. The exposure time also influenced the hatch means, and to the higher exposure period it was obtained a lower hatch mean (H=5.2036; p=0.0229) (table I). These results shows that the eggs exposed for a longer period to the LC_{50}, presented more negative effects in hatchability. On the other hand, control groups do not differed among both time intervals (H=0.0625; p=0.8026).

TABLE I
Hatchability of *Subulina octona* offspring originated from eggs exposed to the aqueous extract of *Bidens pilosa* for 24 and 48 hours, during 30 days of observation.

Groups		Hatchability X±SD	Range of variation	Hatchability percentage (%)
Control	24 hours	14.75±0.43[a]	(14-15)	98.3
	48 hours	14.25±1.29[a]	(12-15)	94.6
Exposed	24 hours	9.00±2.12[b]	(6-12)	61.6
	48 hours	1.02±1.63[c]	(1-5)	14.9

[a,b,c] = means followed by different letters are significantly different by Kruskal-Wallis (p<0.05).

Survival of the Offspring Hatched from Exposed Eggs

The survival of snails hatched from eggs exposed to *B. pilosa* extract was signifficantly lower (24 h: H=11.8127; p=0.0027; 48 h: H=15.4655; p=0.0004).

The survival on groups exposed for 24 h was signifficantly higher in comparison to the groups exposed for 48 h (H=8.1699; p=0.0047) once that for these groups all the snails were dead at the end of 45 days of observation (Figure 2). The survival of control groups did not vary on both exposure intervals (H=8.3333; p=0.2612). It was not observed the reach of sexual maturity in exposed and control groups for both exposure intervals during the observation period.

Effects of the LC_{50} on Survival, Growth and Reproduction of Newly Hatched *Subulina octona*:

The survival of exposed groups was lower in comparison to the control groups exposed for the same time period (24 h: H=5.4634, p=0.0209; H= 5.6709, p=0.0209). The exposure time influenced the survival of exposed snails, and for the longer exposure period the survival rates were lower (H= 4.5733, p=0.0433). The exposure time do not influenced the survival of control groups (H=0.35, p=0.5541). These results are presented on Figure 3.

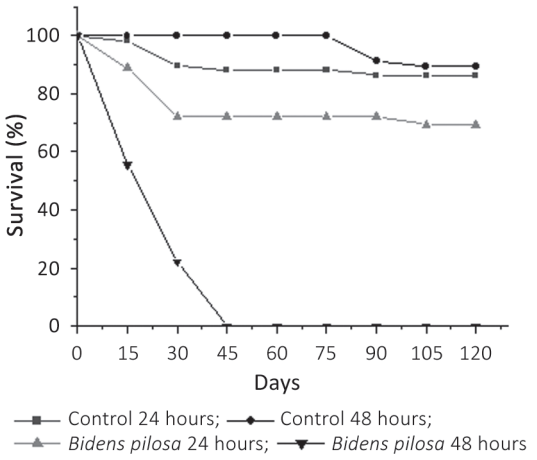

Figure 2 - Survival of the offspring hacthed from eggs exposed to *Bidens pilosa* extract for 24 and 46 hours observed for 120 days.

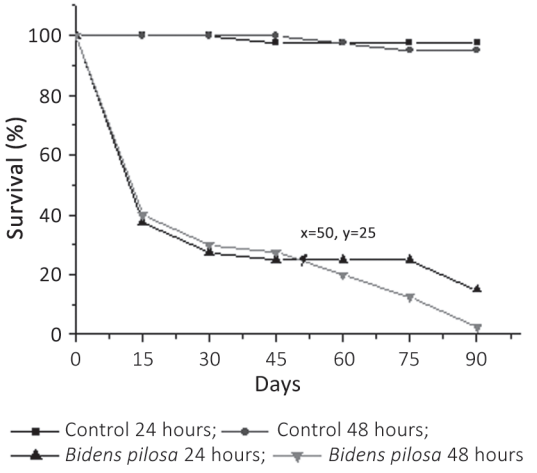

Figure 3 - Survival of newly hatched *Subulina octona*, exposed to the aqueous extract of *Bidens pilosa* for 24 and 48 hours observed for 90 days.

The growth analysis of groups exposed for 48 hours was carried out until the 60º day of observation due to the high mortality on these groups after this

period. The tests demonstrated that the average size of groups exposed for 24 h do not differed from control group at the end of 90 days (H=0.8628, p=0.353). Nevertheless, the exposure time influenced the growth and the higher exposure period showed a lower growth in comparison to control group (H= 8.6729, p=0.0033). The average size obtained for the groups exposed for 24 h was 9.66±0.87 mm and 8.6±1.93 mm for control and exposed groups respectively and to the groups exposed for 48 h the average size was 10.00±1.71 mm for control group and 7.06±1.61mm for exposed group.

In groups exposed for 24 hours, reached sexual maturity at about 81±8.5 days in 40% of snails that survived at the end of 90 days and for control groups exposed for the same period this percentage was from 13% with an average age of 59±4.9 days.

On the other hand, snails exposed for 48 h did not reach maturity until the end of the experiment. In control groups exposed for 48 h 37% of the snails reached maturity at the end of 90 days, with average age of 56.7±3.6 days.

Effects of LC_{50} on Survival, Growth and Reproduction on 30 Day Old *Subulina octona*

The survival in exposed groups was significantly lower in relation to control groups exposed for the same period (24 h: H=5.3976, p=0.0209; 48 h: H=5.6, p=0.0209). Besides, it can be observed that the exposure time also influenced on survival. Snails exposed for 48 hours showed survival means significantly lower (H=4.7573, p= 0.0296). It was not observed significantly difference on survival of control groups exposed for both intervals (H=0.0208, p=0.8852) Figure 4.

These rates demonstrate that the survival of the snails was high on the first 15 days and after this period it was progressively reduced and in the interval between the 30th and the 45th day it increased. This result suggests that the extract have a residual effect in these snails. In another way, groups exposed for 24 hours presented linear decrease on survival.

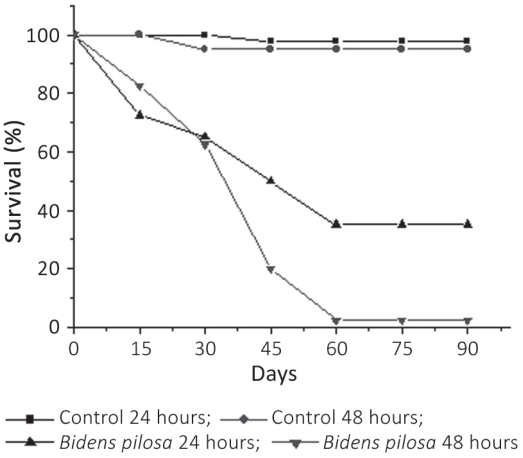

Figure 4 - Survival of 30 day old *Subulina octona*, exposed to the aqueous extract of *Bidens pilosa* for 24 and 48 hours, observed during 90 days.

The measurements of the snails were carried out until the 30th day for groups exposed for 48 h due to the high mortality in these groups after this period. The tests indicates significantly differences in average shell lengths of exposed snails related to control groups (24 h: H=15.2005, p<0.0001; 48 h: H=34.0671, p<0.0001). The average size in exposed groups was 7.96±0.57 mm and 7.18±1.34 mm for the 24 and 48 h exposure time respectively and to control group, this mean was 11.74±2.05 mm for the 24 h interval and 10.83±1.99 in the 48 h interval.

DISCUSSION

The presence of flavonoids, saponnins and tannins in *B. pilosa* can justify the molluscicidal and ovicidal effects showed on this study. Studies have reported the molluscicidal activity of these classes of compounds in other snail species (Bezerra et al. 2002, Cantanhede et al. 2010, Lemmich et al. 1995, Lopes et al. 2011, Marston and Hostettmann 1985, Treyvaud et al. 2000).

According to Schenkel et al. (2004) the ability of saponins to form complexes with steroids, proteins and membrane phospholipids is responsible for a large number of biological properties, especially the action on cell membranes causing their destruction. These properties can

justify their hemolytic effect in snails as was confirmed for *B. glabrata* by Mendes et al. (1993).

The exposure of snails to toxic substances can cause a physiological stress leading to a reduction in carbohydrate reserves (Mello-Silva et al. 2006a, b) that starts the use of proteins as a compensatory process (Schmale and Becker 1977). Studies have shown increased levels of proteins and uric acid in the hemolymph of snails exposed to molluscicides, indicating protein degradation as energy source (Mello-Silva et al. 2006a) however proteins may precipitate after complexation becoming unavailable as an energy source, and may cause death by a lack of energy to maintain homeostasis.

Flavonoids may act by inhibiting the detoxification system of the snail. Silva (2007) found changes in the enzyme cytochrome P450 on the land snail *Cantareus aspersus* (Müller) (Helicidae) exposed to tobacco leaves, *Nicotiana tabacum* (Solanaceae). This enzyme is part of a family of proteins that acts in detoxification process degrading various xenobiotics and turning them into easily excreted molecules (Guecheva and Henrique 2003).

The ineffectiveness of some extracts in snails eggs have been questioned by some authors (Leyton et al. 2005, Magalhães et al. 2003, Souza et al. 1992). According to Lemma and Yau (1974), the low efficiency of molluscicides in eggs is probably due to their high molecular weights that hinder the passing of the molluscicides trough the membrane that involves the egg. Tough the *B. pilosa* extract affected the hatchability of *S. octona*, which suggest that the molecular structure of the active principles of this plant has a more simplified structure that make easy the penetration into the pores of the membrane that involves the embryo. In this way, the *B. pilosa* extract is promising in the control, because it was efficient in different life stages of this snail, including eggs.

The hatching rates obtained in this study for control group was alike to those found by Bessa and Araújo (1995b). According to these authors, the hatches occur between the first and the 15th day after the oviposition, with registered mean viability of the eggs (94.8%) for this snail.

The average mean of hatching in groups exposed for 48 hours was similar to the means found by Ferreira et al. (2009). Those authors tested the ovicidal activity of a caffeine solution at 5 g/L in *S. octona* and obtained an average hatching of 12.5%.

The exposure time influenced the intensity of the effects on different life stages of this species. This result can indicate a stability of these active principles until 48 hours after application indicating the high efficiency of the extract. The negative effect of the *B. pilosa* extract on growth of newly hatched and 30 day-old snails its an important result for the control of this species, once that the shell length is related to the uterus size and therefore with fecundity (D'ávila and Bessa 2005b).

The survivorship curves suggest that the *B. pilosa* extract caused in the 30 day old snails a delayed effect, indicating a strong residual effect on snails in this life stage, that was not observed in newly hatched snails that presented a fast reaction to its toxic effects. These results demonstrate that there is a difference in the snail response to the molluscicide according to the life stage. Souza et al. (1992) verified more sensibility of newly hatched *B. glabrata* to the extract of *Anacardium occidentale* (Anacardiaceae). Nascimento (2006) verified molluscicidal activity of extracts of *Allamanda cathartica* L. (Apocynaceae) in newly hatched *Bradybaena similaris* (Férussac 1821) (Bradybaenidae) while in adults was only checked the repellent action. This distinction can be related to the energy content of individuals at different life stages. During the exposure to molluscicides, the reserves of stored energy can be reduced due to the stress caused, as already verified for *B. glabrata* (Mello-Silva et al. 2006a,b), therefore, reducing its energy resources to the future survival.

For an efficient control of this species it is important for a plant molluscicide to be effective in all life stages of the target snail, being active on

eggs, juvenile phases and adults. Besides, it has also to fit other demands, such as easy access to the plant, simple handling and preparation of the extracts, be toxic in low concentrations, selective and harmless to humans and to the environment (Ministério da Saúde 2008, Mott 1987, Singh et al. 1996, WHO 1965).

Thereby, its necessary more comprehensive studies of molluscicidal compounds in plant species of the Brazilian flora directed to land snails, to consolidate this kind of work.

CONCLUSION

The easy access to this plant, allied to the easy preparation and application of this extract and the absence of toxic effects to humans make the aqueous extract of *B. pilosa* an alternative molluscicide to be applied to control this snail species. Moreover, it is a promising plant for further studies that aim the control of other snail species with epidemiological and agricultural importance.

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RESUMO

O objetivo deste estudo foi estabelecer as concentrações de referência (CL_{50} e CL_{90}) do extrato aquoso de *Bidens pilosa* sobre o molusco terrestre *Subulina octona* bem como avaliar as alterações causadas pela exposição à CL_{50} por 24 e 48 horas sobre a fecundidade, eclodibilidade e na prole produzida após a exposição, além de confirmar a presença de taninos, saponinas e flavonóides nesta espécie de planta. Ovos, jovens recém-eclodidos e com 30 dias de idade do molusco foram expostos a concentração subletal, calculada para adultos. A análise fitoquímica confirmou a presença de flavonóides, taninos condensados e saponinas no extrato aquoso de *B. pilosa*. A CL_{50} e CL_{90} obtidas foram 51,4 mg/mL e 74,1 mg/mL, respectivamente. A exposição a concentrações subletais reduziu significativamente a eclosão e a sobrevivência da prole nascidas de ovos expostos aos dois períodos e também causou uma redução na sobrevivência e crescimento dos moluscos jovens. O maior tempo de exposição também causou uma redução nos parâmetros avaliados.

Palavras-chave: Praga, controle, molusco terrestre, moluscicida, extrato vegetal, agrícola.

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