

Searching for a non toxic natural insecticide from *Pombalia bigibbosa* (Violaceae) extracts against *Tribolium castaneum* (Herbst)

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Summary

At the moment, there is growing concern about the negative effects on human health, and the environment due to the widespread and indiscriminate use of conventional insecticides. For that reason, obtaining and characterizing new molecules of plant origin with insecticidal activity is, currently, one of the main research focuses for pest control. *Pombalia bigibbosa* (Violaceae), native species to the Argentinian flora, is known in Brazil as "canela-de-veado". In this study the insecticidal activity of dichloromethane, 50 % EtOH, and tannin-free 50 % EtOH extracts of *P. bigibbosa* was evaluated on *Tribolium castaneum*, a pest found in stored grains. The composition of the tannin-free 50 % EtOH extract was analyzed by HPLC. Also, the effects of these extracts on the central nervous system were assessed. The 50 % EtOH and tannin-free 50% EtOH extracts showed significant lethal and sub-lethal effects on *T. castaneum* larvae. Compounds with an HPLC elution profile similar to cyclotides were observed through HPLC analysis. Lastly, these extracts exhibited no effects on the central nervous system.

Búsqueda de un insecticida natural no tóxico a partir de extractos de *Pombalia bigibbosa* (Violaceae) contra *Tribolium castaneum* (Herbst)

Resumen

Actualmente existe una creciente preocupación por los efectos negativos sobre la salud humana y el medio ambiente debido al uso generalizado e indiscriminado de insecticidas convencionales. Por este motivo, la obtención y caracterización de nuevas moléculas de origen vegetal con actividad insecticida es, en la actualidad, uno de los principales focos de investigación para el control de plagas. *Pombalia bigibbosa* (Violaceae), especie nativa de la flora argentina, se conoce en Brasil como "canela-de-veado". En este estudio se evaluó la actividad insecticida de los extractos diclorometano, EtOH 50 % y EtOH 50 % destanificado de *P. bigibbosa* sobre *Tribolium castaneum*, plaga de granos almacenados. También, se analizó mediante HPLC la composición del extracto EtOH 50 % destanificado y se evaluaron los efectos de estos extractos sobre el sistema nervioso central. Los extractos EtOH 50 % y EtOH 50 % destanificado mostraron efectos letales y subletales significativos sobre las larvas de *T. castaneum*. Mediante el análisis por HPLC se observaron compuestos con un perfil de elución similar al de los ciclótidos. Por último, estos extractos no mostraron efectos sobre el sistema nervioso central.

Introduction

There is growing concern about the negative effects on human health, environment, and non-target organisms due to the widespread and indiscriminate use of conventional insecticides (Chen *et al.*, 2021). For example, certain insecticides have negative effects on the nervous, renal, respiratory, and reproductive systems of men and women (Ataei and Abdo-

llahi, 2022; Matich *et al.*, 2021; Ventura *et al.*, 2019; Pavela, 2016).

The potential adverse effects associated with the use of chemical pesticides have also encouraged the research of new pesticides alternatives from natural sources. In this sense, plants are a safe and efficient source of compounds with less toxicity for mammals, low persistence

Palabras clave: *Pombalia bigibbosa* – actividad insecticida – *Tribolium castaneum* – toxicidad sobre sistema nervioso central

Key words: *Pombalia bigibbosa* – insecticidal activity – *Tribolium castaneum* – central nervous system toxicity

in the environment, and biodegradability (Isman 2015, Céspedes 2014). Currently, several studies have confirmed many of the beneficial biological activities attributed to them, such as insecticide, fungicide, and repellent (Broussalis *et al.*, 2010; Tarcaya *et al.*, 2014; Tito Mansilla *et al.* 2018; Pavela and Benelli, 2016). At present, numerous agricultural producers are achieving the re-conversion of their systems through the implementation of agroecological practices and the application of bioinsecticides (Gliessman, 2016). *Pombalia bigibbosa* (A. St.-Hil.) Paula-Souza ex *Hybanthus bigibbosus* (De Paula Souza and Ballard, 2014) (Violaceae), native species to the Argentinian flora, is a 1 to 2 meter-high bush that grows in meadows, at 0 and 1000 m above sea level (Biganzoli and Múlgura de Romero, 2004). This plant is found in the Argentine Provinces of Misiones and Córdoba, and in Brazil and Paraguay (Zuloaga and Morrone, 1999). *P. bigibbosa* is known in Brazil as “canela de veado”, its roots are used in popular medicine as an emetic due to the presence of alkaloids with activity similar to emetine (Mariz De Lyra, 1952). In species of the *Pombalia* genus, flavonoids, alkaloids and cyclotides have been reported (Mariz De Lyra, 1952; Broussalis *et al.*, 2001; Broussalis and Ferraro, 2006; Pinto *et al.* 2018). Various other *Pombalia* species have exhibited insecticidal and antitumoral activities in previous studies (Pinto *et al.* 2018; Broussalis *et al.*, 2010).

Tribolium castaneum Herbst (Coleoptera: Tenebrionidae) the red flour beetle is one of the main pests of stored grains that causes loss of nourishing value, reduction of seed germinative power, weight loss and reduction of grain commercial value (Champ and Dyte, 1976; Clemente *et al.* 2003). The general approach used for decades to control this pest has been the use of chemical fumigants, which have several deleterious effects such as their persistent toxicity in food grains, the subsequent development of resistance in insect populations, lethal effects on non-target organisms and the toxicity to users (Champ and Dyte, 1976; Phankaen *et al.*, 2017; Zhang *et al.*, 2017).

Toxicity studies are important to assess the toxic or deleterious effects that plant derived products could cause in the short, medium, or long term in human health and the environment. In a previous work, the acute toxicity in mice, the sub-acute toxicity in rats, as well as dermal and ocular irritation in rabbits of the 50 % hydroalcoholic extract of *P. bigibbosa* were determined (Cufre *et al.*, 2014).

In this work, the insecticidal effect of the dichloromethane (CH₂Cl₂), 50 % EtOH and the tannin-free 50 % EtOH extracts of *P. bigibbosa* on *T. castaneum* larvae was determined. Due to the promising insecticidal activity of this extract and knowing the neurotoxic effects of synthetic insecticides on human health, the effects on the central nervous system (CNS) of this extract were studied. On the other hand, the active tannin-free 50% EtOH extract was analyzed by HPLC.

Materials and Methods

Plant material

Pombalia bigibbosa (A. St.-Hil.) Paula-Souza (Violaceae) was collected in Posadas, in the Province of Misiones. A voucher specimen is kept at the Herbarium of the Pharmacy Department of the School of Exact, Chemical and Natural Sciences (MNEF 3984), National University of Misiones, Argentina.

Extraction and Bioguided Fractionation

The method employed for obtaining and purifying the extracts was described by Claeson *et al.* (1998) with the aim of obtaining peptide-enriched extracts.

The dried and ground aerial parts of *P. bigibbosa* (30.2 g) were extracted by maceration with CH₂Cl₂ for 1 h under continuous shaking. The plant residue was then macerated in 50 % v/v EtOH (300 ml). To remove the tannins, the ethanolic extract was solubilized in 2 % AcOH and eluted through a polyamide column. The column was eluted first with 2 % AcOH and then with a mixture of 50 % EtOH / 2 % AcOH in order to elute those peptides that were insoluble in 2 % AcOH. Afterwards, the ethanolic tannin-free extract was lyophilized (Broussalis *et al.*, 2001).

Analysis by HPLC

The tannin-free 50 % EtOH extract was analyzed by HPLC. A Varian 9012 device with a Varian 9050 UV-Vis detector and diode array detector (UV-DAD) Varian Polycrom 9065 was used, with a Dynamax RP 18 analytical column (250 mm x 4.6 mm internal diameter) and a binary mobile phase of 0.1 % TFA in water (Solvent A) and 60 % ACN in 0.1 % TFA in water (Solvent B). It was eluted in isocratic gradient (100 % A) for 7 min., then in linear gradient: 100 % A to 50 % A (7 min.) and 50 % A to 100 % B (23 min.). The UV detection was conducted between 200 and 300 nm. The injection volume was 25 µl and the flow rate 1 ml/min.

Insecticidal Activity Bioassay

Insect Rearing

T. castaneum Herbst. (Coleoptera: Tenebrionidae) larvae from an established laboratory colony in the Organic Plant Production Laboratory, Faculty of Agronomy, University of Buenos Aires, Argentina, were employed. *T. castaneum* larvae were reared with an artificial diet (wheat flour, beer yeast, cornstarch 10:1, 5:10) and environmental standard conditions (25 ± 1 °C, 60 ± 5 % RH, in darkness). This laboratory strain is susceptible to all insecticides (Casadio and Zerba, 1996; Clemente *et al.*, 2003).

Ingestion bioassay

The insecticidal activity of the CH₂Cl₂, 50 % EtOH and the tannin-free 50 % EtOH extracts was investigated.

Table 1.- Insecticidal activity of the CH₂Cl₂ extract, the 50% EtOH and the tannin free 50 % EtOH extracts of *P. bigibbosa* against *T. castaneum*

Extract	Concentration (mg/ml)	Mortality (%)	Pupal formation *
Cl ₂ CH ₂	Control	5 ^a	100 ^a
	0.05	5 ^a	100 ^a
	0.50	2.5 ^a	100 ^a
	5.00	5 ^a	100 ^a
50 % EtOH	Control	5 ^a	100 ^a
	0.20	10 ^a	100 ^a
	2.00	29 ^b	65 ^b
	20.0	25 ^b	30 ^c
Tannin free 50 % EtOH	Control	5 ^a	100 ^a
	0.20	22 ^b	100 ^a
	2.00	36 ^b	65 ^b
	20.0	65 ^c	30 ^c

Different letters indicate significant differences ($p < 0.05$)

* Pupal formation 50 days after starting the bioassay (%)

The concentrations used to determine the insecticidal activity were as follows: for the CH₂Cl₂ extract, the concentrations were 0.05, 0.50, and 5.00 mg/ml, while for the 50 % EtOH and 50% tannin-free EtOH extracts, the concentrations were 0.20, 2.00, and 20.0 mg/ml.

Ten grams of the artificial diet were mixed with 1.0 ml of each acetonic solution of *P. bigibbosa* extracts. The solvent was evaporated at room temperature during 24 h. Then, ten neonate larvae of *T. castaneum* were placed in each glass vessel containing a treated artificial diet. All tests were performed in quadruplicate with acetone as a negative control. The bioassay was conducted for nine weeks under standardized conditions (25 ± 1 °C and 60 ± 5 % RH). The mortality at each stage of the life cycle of the red flour beetle as well as the overall mortality was weekly assessed. The sublethal effects expressed as delays in the development of the insect produced by the extracts were also evaluated (Casadio and Zerba, 1996).

Statistical analysis

Bioassays were conducted following a fully randomized design. Lethal effects were statistically analyzed (ANOVA, Tuckey test, $p < 0.05$) (Steel and Torrie, 1993; Norman and Streiner, 1996). Sublethal effects were estimated through Probit ($p < 0.05$) (Finney, 1971).

Effects on Central Nervous System

Animals

Healthy Swiss female and male mice were used following international guidelines and local regulations concerning the care and use of laboratory animals for biomedical research (NIH Publication No 85–23, Revised 1985). The study was approved by the Institutional Animal Care and Use Committee of the

Faculty of Pharmacy and Biochemistry, University of Buenos Aires (Ethics approval: Exp-FFyB 0077054/17). The animals were acclimatized to laboratory conditions for 7 days. Mice were housed in groups of five and were maintained in standard conditions (21 ± 2 °C; 12 h light/dark cycle). The animals were fed with a standard diet and tap water *ad libitum*.

Twenty mice, 10 males and 10 females, were randomly distributed into two groups of 10 animals each (5 males and 5 females). An hour before conducting the tests, group 1 received the 50% ethanolic extract of *P. bigibbosa* (5 g/kg) p.o (treated group) while group 2 received normal saline solution (control group).

Sleep potentiation by pentobarbital

In the assessment of the hypnogenic activity, the treated and the control groups were intraperitoneally administered with a 40 mg/kg dose of pentobarbital. Time elapsed from the administration of pentobarbital to the loss of righting reflex was recorded as the onset of sleep, while the time from the loss of righting reflex (in which the mouse cannot roll back when turned over) to recovery was recorded as the sleeping time in minutes (Vogel and Vogel, 2002).

Spontaneous locomotor activity

The spontaneous motor activity was recorded by means of an activity monitor (Photocell Activity Meter, Animex, LKB, Farad, Sweden). The mice of the treated and control group were placed individually in the monitor. The number of ambulatory trips was recorded for 5 minutes (Kulkarani, 1999).

Exploratory behavior

In the study of the exploratory behavior, the open field with hole-board test was used. The animals of the treated and control group were individually placed in the center of the board.

Table 2.- Effects of 50 % ethanolic extract of *P. bigibbosa* on pentobarbital-induced sleeping time

	Onset of sleep (min)	Duration of sleep (min)
Control (vehicle)	4.30 ± 0.47	24.70 ± 2.08
50 % EtOH extract of <i>P. bigibbosa</i> (5 g/kg)	5.80 ± 0.73	29.60 ± 2.56

Values expressed as mean ± SEM (n = 5 per group)

The number of times the mice dipped their heads into the holes during the 5 min test was automatically counted (d'Isa, Comi, and Leocani, 2021; Kliethermes and Crabbe, 2006).

Statistical analysis

The statistical significance of the differences among groups was assessed by means of ANOVA followed by the Bonferroni multiple comparison test (GraphPad Software, San Diego, CA, U.S.A.). In all cases, it was considered that the $p < 0.05$ values were statistically significant (Siegel, 1956).

Results and discussion

Insecticidal Activity Results

The ethanolic extracts of *P. bigibbosa* have caused significant lethal and sublethal effects on *T. castaneum*, whereas there has been no response with the CH₂Cl₂ extract (Table 1). The tannin-free 50 % EtOH extract has produced significant mortality in all tested concentrations, the 20 mg/ml concentration being the most active one, whereas the 50 % EtOH extract has only been active in the two highest concentrations. Comparing the mortality of the ethanolic extracts, it has been observed that, at 20 mg/ml, the tannin-free 50 % EtOH extract has produced a mortality which was three times higher (65 %) than the 50% EtOH extract at the same concentration (25 %).

Sublethal effects expressed as development delays were observed with both ethanolic extracts (Table 1). The delay in the development of the insect life cycle was determined through the % of pupae formed. The control group completed its life cycle 50 days after the bioassay start, whereas with the 50 % EtOH and the tannin-free 50 % EtOH extracts, the life cycle was completed 70 days afterwards. This was particularly evident with the two ethanolic extracts at 20 mg/ml, 50 days after the test

start, where the percentage of pupae formed was 30 % and 100% for the control group. At the end of the test, insects in the larval stage were found with the ethanolic extracts at the highest concentrations (2 and 20 mg/ml). This prolongation in the life cycle could result in a smaller number of insect generations, leading to a reduction in their population size. Consequently, the economic damage caused by this insect would be reduced.

Evidence of cannibalism was also observed. Larvae, pupae, and partially consumed adults were found, and in some cases, their complete disappearance. Cannibalism is a behavior observed in *T. castaneum* under unfavorable conditions of food scarcity (Hastings and Costantino, 1987). In this case, the atypical behavior in optimal rearing conditions may be attributed to the presence of compounds in the extract that have an antifeedant effect, leading to the observed cannibalism.

HPLC Analysis Results

HPLC analysis of the tannin-free 50% EtOH extract showed the presence of compounds with an HPLC elution profile similar to cyclotides, with retention times (rt) equivalent to or higher than 20 min (Broussalis *et al.*, 2001, 2010; Gruber *et al.*, 2006). Furthermore, the UV spectrum obtained with the UV-DAD detector was the same as the cyclic peptides found in *Pombalia parviflora* (Mutis ex L.f.) Paula-Souza (Broussalis *et al.*, 2001).

Cyclotides are found in plants in small quantities. The presence of the cyclized backbone and the tightness of the cystine knot, forces cyclotides to expose their hydrophobic amino acids on the surface of the peptide instead of hiding them inside the molecule (Craik, Simonsen, and Daly, 2001). This may account for some behaviors of cyclotides during their isolation and purification, such as prolonged retention times (higher than 20 min) in HPLC-RP (Broussalis *et al.*, 2001).

The tannin-free 50 % EtOH extract, enriched in these compounds with a reported insecticidal activity (Jennings

Table 3.- Effects of 50 % ethanolic extract of *P. bigibbosa* on spontaneous motor activity

	Spontaneous motor activity (number of ambulatory trips)
Control (vehicle)	821.70 ± 45.47
50 % EtOH extract of <i>P. bigibbosa</i> (5 g/kg)	908.80 ± 24.81

Values expressed as mean ± SEM (n = 5 per group)

Table 4.- Effects of 50 % ethanolic extract of *P. bigibbosa* on exploratory behavior

	Exploratory behavior (number of head dips)
Control (vehicle)	61.50 ± 2.67
50 % EtOH extract of <i>P. bigibbosa</i> (5 g/kg)	71.80 ± 3.46

Values expressed as mean ± SEM (n = 5 per group)

et al., 2005; Broussalis *et al.*, 2010) has had a higher activity than the 50 % EtOH extract. Comparable results were found in ethanolic extracts of *P. parviflora*, the tannin-free extract showed a percentage of mortality on *Ceratitis capitata* higher than the 50 % EtOH extract (Broussalis *et al.*, 2010).

The promising activity of both ethanolic extracts may be attributed to the presence of these compounds.

Effects on the central nervous system

Sleep potentiation by pentobarbital

After the administration of the 50 % EtOH extract of *P. bigibbosa*, no significant increase in sleep was observed in mice in the hypnogenic activity test (Table 2).

Spontaneous motor activity

After the administration of the 50 % EtOH extract of *P. bigibbosa* there was no decrease in the motor activity in the spontaneous motor activity test (Table 3).

Exploratory behavior

The 50 % EtOH extract did not show a reduction of exploration (reduction of head-dippings) compared with the values obtained with the control group (Table 4).

The principal aim of evaluating the safety of any medicinal plant is to identify the nature and importance of the adverse effects that its use could cause. In previous works, the acute and subchronic toxicity as well as dermal and ocular irritation of the 50% ethanolic extract of *P. bigibbosa* was studied and no signs of toxicity were observed (Cufre *et al.*, 2014). Considering the neurotoxic effects of synthetic insecticides, it is important to evaluate the effects of the active ethanolic extract on the central nervous system. In this way, spontaneous motor activity, the exploratory behavior and hypnogenic activity were performed.

The spontaneous motor activity is a model that has been used in laboratory animals to evaluate the gross behavioral effects of drugs. Agents that suppress this behavior usually do so through central inhibition (Nwinyi and Kwanashie, 2009). In this study, no alterations were observed in spontaneous motor activity, therefore the 50 % ethanolic extract would not present depressant effects on the central nervous system.

The study of exploratory behavior in the board test allows us to determine the sedative or anxiolytic effects of substances (Adzu *et al.*, 2001). The administration of the extract did not produce changes in the exploratory behavior of the animals compared to the control group, so the 50 % ethanolic extract would not present sedative or anxiolytic effects.

Sleep potentiation by pentobarbital assay, allows predicting depressant effects of the central nervous system (Nwinyi and Kwanashie, 2009). The increase in sleep indicates a depressant effect. The administration of the extract did not produce an increase in sleep time compared to the control group, so the 50 % ethanolic extract would not present depressant effects.

The results of the study of the effects on the CNS showed that the 50 % ethanolic extract of *P. bigibbosa* does not produce depressant effects on the CNS.

Conclusions

In the present study, the insecticidal activity of *P. bigibbosa* was studied. The 50 % EtOH and the tannin-free 50 % EtOH extracts have had significant lethal and sublethal effects on *T. castaneum* larvae. In the active extract the presence of cyclotides was observed. Furthermore, the results show that the 50 % EtOH extract does not have effects on the activity of the central nervous system. Taking into account the toxic effects that synthetic insecticides may cause in humans, these results suggest that *P. bigibbosa* could be considered a potential source of secondary metabolites with insecticidal activity for the development of botanical insecticides. This would aid in controlling pests and achieving sustainable systems that ensure the production of quality food.

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