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Insecticidal Activity of *Piper tuberculatum* Extracts on the Cotton Stainer Bug, *Dysdercus peruvianus* Guérin-Méneville (Hemiptera: Pyrrhocoridae)

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Abstract: Development of alternative methods for pest management is needed due to the increased concern for adverse effects of pesticides for human health and for the environment. The adulticidal activity of the neotropical *Piper tuberculatum* was evaluated. The acute toxicities to the cotton stainer bug, *Dysdercus peruvianus* Guérin-Méneville, of both extracts of mature spikes and *in vitro* plants of *P. tuberculatum* were evaluated by means of spray treatments. Dichloromethane:methanol (2:1) extracts of mature spikes showed significant levels of adult mortality causing 91.1 and 97.8% mortality when doses of 0.012 mg/µL were applied to the *D. peruvianus* in 24 and 48 h of exposure, respectively, with LD_{50} 0.006 mg/µL and LD_{90} 0,010 mg/µL, in 48 h of exposure. The dichloromethane:methanol (2:1) extract from *in vitro* plants caused 80% mortality when doses of 0.012 mg/µL were applied in 96 h of exposure with LD_{50} 0.007 mg/µL and LD_{90} 0.015 mg/µL, in 96 h of exposure.

Key words: Adult Mortality · *In vitro* Propagation · Insecticide · Lethal Doses · Piperaceae

INTRODUCTION

The genus *Dysdercus* currently contain about 65 species, occurring in the tropical and subtropical parts of Central and South America, Africa, Asia, the Malay Archipelago and Australia. Most of *Dysdercus* species feed on various species belonging to Malvaceae familiy in special from *Gossypium* genus. A detailed review of the literature on the economic importance of members of the *Dysdercus* genus was provided by Schaefer and Ahmad [1].

The cotton stainer bug (*Dysdercus peruvianus* Guérin-Méneville, 1831) causes serious damage by feeding on developing bolls and ripe cotton seeds and staining the fibers [2]. *D. peruvianus*, an insect pest in cotton crops (*Gossypium hirsutum* and *G. barbadense*), cause heavy economic losses, reaching 5 – 30% in Lambayeque, Peru [3]. Others cotton stainer as *D. cingulatus*, commonly known as red cotton bug and *D. maurus*, cotton stainer bug, are also economically important crop pests in several regions of India [4] and Brazil [5], respectively.

The current method used to control the *D. peruvianus* and other insect pests, is based mainly on the use of organochlorine and organophosphorous insecticides. However, the indiscriminate use of synthetic insecticides has caused environmental contaminations and toxicity to living organisms with reduction in populations of beneficial insects, resurgence and eruption of pests and loss of effectiveness due to the selection of resistant populations, indicating the need for the development of not hazardous products to the environment, target-specific and biodegradable [6, 7]. Thus, the development of new biological products such as insecticidal proteins that interfere with the digestive process can be an alternative strategy for the control against *Dysdercus* bugs. For example, canatoxin-like proteins and peptides derived from

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Cannavalia ensiformis have been tested for controlling *D. peruvianus*; however, adults were not susceptible to diets containing higher concentration of canatoxin (0.04%) [8] and the proteolytic enzymes in the gut of the banana weevil, *Cosmopolites sordidus* [9]; likewise, the use of substances from the secondary metabolism of plants with insecticidal activity [10] and several commercial compounds called insect growth regulators or IGRs as andalin, an reproductive inhibidor for the red cotton stainer *D. koenigii* [11].

With the exception of sulphur, botanical insecticides have been used longer than any other type of insecticide [12]. Plant extracts or their potent derivatives such as derris, rotenone, pyrethrum, nicotine, sabadilla, physostigmine, quassin or azadirachtin, have been used as insecticides for decades and still represent an important alternative [13], especially in the control of the several mosquitoes species [14-16]. Lignans and amides produced by Piperaceae species have demonstrated potential insecticidal activity [17-19]; limonoids such as azadirachtin and gedunin, present in Meliaceae and Rutaceae species are recognized for their toxic effects on insects and are used in several insecticide formulations in many parts of the world [20] and neem extracts negatively influenced growth and development of African bollworm *Helicoverpa armigera* [21]; the discovery of insecticide activity of phototoxins present in Asteraceae species has stimulated the interest in this family as part of the search for new plant derived insecticides [22]; several acetogenins, isolated from a Bolivian collection of *Annona montana* (Annonaceae), were evaluated for their antifeedant and toxic effects on *Spodoptera frugiperda* [23] and other acetogenins, from an Argentine collection of *A. cherimolia* and a Bolivian collection of *A. montana*, had their toxicity investigated against the cotton pest *Oncopeltus fasciatus* [24] and ureases, present in *Canavalia ensiformes*, a Fabaceae species, are toxic to insects of different orders (*Callosobruchus maculatus*, *Rhodnius prolixus*, *Dysdercus peruvianus* and others) [8, 25].

Several species of *Piper* (Piperaceae) have been reported in the literature to have insecticidal activity [18]. For example, crude extracts, essential oils and seed powders of *P. guineense* are effective against numerous Tropical African insect pests such as *Callosobruchus maculatus*, *Voandzeia subterranean*, *Tribolium castaneum*, *Anthonomus grandis*, *Dysdercus superstitiousus* and others [26]; in Brazil, essential oils from American species *Piper aduncum* and *P. hispidinervum* were tested against *Tenebrio molitor* larvaes; [27] and essential oils extracted from *P. aduncum* against *Cerotoma tingomarianus* [28]; the toxic and repellent effect of the essential oil of *P. crassinervum* on *Sitophilus zeamais* through methods of contact with grains, was determined [29]. Recently, the potential value of extracts and amides derived from *P. tuberculatum* as insecticides against *Anticarsia gemmatalis* was determined [30] and the dichloromethane and ethanolic extracts of spikes and *in vitro* plants of *P. tuberculatum* have shown insecticidal activity against *Diatraea saccharalis*, *Spodoptera frugiperda*, *Aedes aegypti* and *Anopheles pseudopunctipennis* [31-33].

Many species of the Piperaceae family were reported to contain pyrones, lignoids and chromenes besides various amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties [18, 19]; however, in spite of the large array of secondary metabolites produced by Piperaceae species, to date only lignans and amides have demonstrated potential insecticidal and antifungal activity [30, 34, 35]. One of these species is *P. tuberculatum*, known as “matico”, “nudillo”, “cordoncillo” or “palo soldado” is widely distributed from Brazil to Mexico.

Natural products of plant origin with insecticidal properties have been recently tried for controlling a variety of *Dysdercus* species. Extracts and triterpenes from *Manilkara subsericea* act as potent growth inhibitors of two species of agricultural pest insects, *Oncopelus fasciatus* and *Dysdercus peruvianus* [36]; the biological effects of thallium extract of *Ulva fasciata* and *U. lactuca* were tested against *Dysdercus cingulatus* third instar nymphs [37]; insecticidal effects of canatoxin, a variant form of urease isolated from *Canavalia ensiformis* (Fabaceae) seeds on the cotton stainer bug *Dysdercus peruvianus* was determined [8]; insecticidal effects of *Croton urucurana* extracts and crude resin on *Dysdercus maurus* was evaluated [5]; the fecundity and fertility control of red cotton bug (*Dysdercus cingulatus*) by the extract of *Psoralea corylifolia* was observed [38] and nymphicidal and ovipositional efficacy of seaweed *Sargassum tenerrimum* against *Dysdercus cingulatus* was studied [39].

The objective of this research was to investigate the insecticidal activity of extracts of mature spikes, with fruits and seeds, of wild plants and *in vitro* plants of *P. tuberculatum* on adults of cotton stainer bug *D. peruvianus*.

MATERIALS AND METHODS

Plant Material: Spikes with mature seeds of *P. tuberculatum* Jacq. were collected in December 2009 from Cumbil 1.river (Lambayeque, Peru). Botanical identification was performed by Doctor Guillermo E. Delgado-Paredes from Universidad Nacional Pedro Ruiz Gallo (UNPRG) based on taxonomic description realized by Yuncker (1973) [40]. The botanic specimen vouchers were deposited at same herbarium of the institution (HPR).

***In vitro* Micropropagated Plants:** The culture was initiated from axenic seedlings explants. A total of 50 seeds per flask were thoroughly washed with distilled water for 10-20 min, decontaminated with 70% ethanol (v/v) for 60 s and a 2.5% sodium hypochlorite (w/v) for 20 min and then washed three times with sterile water. Floating seeds were discharded; about 3-10 of them were transferred to glass test tubes containing 15 mL of MS medium [41] and 2% sucrose. Shoot-tip and nodal segments, 1 cm length containing an axillary bud, taken from three-month-olds *in vitro* seedling, were used as explant source. MS medium, supplemented with 0.02 mg/L indolacetic acid (IAA), 0.02 mg/L gibberellic acid (GA_3) and 3% sucrose was used to initiate cultures and were maintained by subculturing every twelve months on a fresh medium containing the same formulation. In all cultures, the same MS medium was supplemented with 100 mg/L m-inositol and 1 mg/L thiamine.HCl. The pH of the medium was adjusted to 5.8 ± 0.1, solidified with "Phytagel" 0.3% prior to autoclaving at 121°C for 20 min. Cultures were maintained at 24-28°C with 80 ± 5% relative humidity under 16-h photoperiod at a light intensity provided by cool white fluorescent tubes, with 5 μmol m^{-2} s^{-1}, for seed germination and 30 mol m^{-2} s^{-1} for clonal propagation.

Insects: Colonies of adult of *D. peruvianus* were collected from *Sidastrum paniculatum* ("escobita de monte"), a Malvaceae species, during its vegetative to early reproductive stage, in the Fundo La Peña, UNPRG – Lambayeque, Peru and were reared in the Laboratorio de Entomología of the Facultad de Agronomía (UNPRG), under laboratory conditions 26 ± 2°C, 85 ± 5% relative humidity and 16:8 h light:dark photoperiod. Colonies of adult were maintained in a chamber which was a cardboard box of 30x20x20 cm, covered with a white tulle in its both lateral and top side.

Extraction of the Constituents: Spikes (25 g) of wild plants of *P. tuberculatum* were oven dried at 40°C, milled and submerged three times in dichloromethane: methanol (2:1) at room temperature, yielding 9.1% (2.28 g) of extract; likewise, *in vitro* micropropagated plants (25 g) yielding 10.1% (2.53 g) of extract with dichloromethane:methanol (2:1). The extracts were evaporated to dryness under vacuum at 40°C.

Spray Treatments: Bioassays were carried out in the Laboratorio de Entomología of UNPRG. The stock solutions of extracts (mature spikes and *in vitro* plants) were prepared by dissolving 300 mg of dry extract in 2 mL of methanol-water to obtain 150 mg/mL concentration and then diluted with 25 mL of distilled water to obtain the tested doses. After 24 h and using a glass sprayer, 1 mL of the solution, containing an aliquot of each one of the treatments was applied directly on the adult of *D. peruvianus*. The plant extract was tested at doses of 0.0, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.010, 0.011 and 0.012 mg/μL. Forty-five adults were tested per treatment and the experiment was carried out twice. The control insects were sprayed with methanol-water alone. Adult mortality was recorded at 24, 48, 72, 96 and 120 h post-treatments, under the same conditions of temperature and humidity described above. The insects were fed on crushed cotton seeds and young branches of "escobita de monte" placed in a plastic container containing distilled water. The insects were considered dead if they displayed no observable response to a mechanical stimulus, *i.e*. short-term pressure applied with a spatula.

A dose-response correlation was obtained using a linear regression model to fit the probit data to the log of the dose of each extract applied. LD_{50} and LD_{90} values were determined used the software US. EPA Probit Program Version 1.5 (2003).

RESULTS

The response of cotton stainer bug to the topical applications with glass sprayer of dichloromethane: methanol (2:1) extract from mature spikes of wild plants and dichloromethane: methanol (2:1) extract from *in vitro* plants of *P. tuberculatum* showed a positive relationship between dose and mortality. The response varied with the time of exposure.

Table 1: Percentage of mortality by dichloromethane:methanol (2:1) extract from spikes of *P. tuberculatum* on *D. peruvianus*

		Hours after application Mortality (%)				
Treatment (mg/µL)	Adults Tested (N°)	24	48	72	96	120
0.0	45	0.00	0.00	0.00	0.00	0.00
0.003	45	4.44	6.67	8.89	11.11	11.11
0.004	45	11.11	20.00	24.44	26.67	26.67
0.005	45	17.78	24.44	28.89	28.89	31.11
0.006	45	37.78	44.44	48.89	51.11	51.11
0.007	45	68.89	68.89	71.11	73.33	73.33
0.008	45	73.33	75.56	77.78	80.00	80.00
0.009	45	75.56	84.44	84.44	84.44	84.44
0.010	45	91.11	91.11	91.11	91.11	91.11
0.011	45	91.11	95.56	97.78	97.78	97.78
0.012	45	91.11	97.78	97.78	97.78	97.78

Table 2: Percentage of mortality by dichloromethane:methanol (2:1) extract from *in vitro* plants of *P. tuberculatum* on *D. peruvianus*

		Hours after application Mortality (%)				
Treatment (mg/µL)	Adults Tested (N°)	24	48	72	96	120
0.0	45	0.00	0.00	0.00	0.00	0.00
0.003	45	4.44	6.67	6.67	6.67	6.67
0.004	45	8.89	11.11	15.56	15.56	15.56
0.005	45	20.00	26.67	31.11	31.11	31.11
0.006	45	17.78	20.00	24.44	24.44	24.44
0.007	45	37.78	42.22	46.67	46.67	48.89
0.008	45	51.11	53.33	60.00	60.00	60.00
0.009	45	55.56	62.22	66.67	66.67	68.89
0.010	45	55.56	60.00	64.44	66.67	68.89
0.011	45	68.89	73.33	75.56	77.78	77.78
0.012	45	66.67	71.11	77.78	80.00	80.00

Table 3: Components of the probit analysis and LD_{50} and LD_{90} values for the adults of cotton stainer bug *Dysdercus peruvianus* exposed to two extracts (spikes of wild plants and *in vitro* plants) from *Piper tuberculatum*

			Lethal concentration (mg/µL)				
				LD_{50} 95% FL		LD_{90} 95% FL	
Extract	Time after treatment (h)	Slope (±SE)	LD_{50}	Lower-upper	LD_{90}	Lower-upper	Significance X^2 (g.l.)[a]
Spikes	24	6.08 (± 0.54)	0.007	0.006-0.007	0.011	0.010-0.012	7.153 ns
	48	6.06 (± 0.53)	0.006	0.006-0.006	0.010	0.010-0.011	4.267 ns
	72	5.32 (± 0.47)	0.006	0.005-0.006	0.010	0.010-0.011	9.618 ns
	96	5.53 (± 0.48)	0.006	0.005-0.006	0.010	0.009-0.011	6.244 ns
	120	5.48 (± 0.48)	0.006	0.005-0.006	0.010	0.009-0.011	5.401 ns
In vitro plants	24	3.99 (± 0.47)	0.009	0.008-0.010	0.018	0.015-0.024	4.498 ns
	48	3.88(± 0.44)	0.008	0.008-0.009	0.017	0.015-0.022	5.688 ns
	72	3.91 (± 0.42)	0.008	0.007-0.008	0.016	0.014-0.020	5.232 ns
	96	4.05 (± 0.43)	0.007	0.007-0.008	0.015	0.014-0.019	4.934 ns
	120	4.11 (± 0.43)	0.007	0.007-0.008	0.015	0.013-0.018	4.995 ns

[a]Significance level: ns = not significant (P>0.05).

The adult mortality at 91% was reached after 24 h when using 0.010 mg/µL of dichloromethane:methanol (2:1) extract from mature spikes; and a mortality of 98% was reached with 0.012 mg/µL in 48 h (Table 1). In reference to the *in vitro* plants, the extract obtained with dichloromethane:methanol (2:1) generated a 80% adult mortality with 0.012 mg/µL in 96 h (Table 2). The mortality of the control group was 0%.

The resultant regression lines for all the extracts appeared to be very similar by showing a relatively fast intoxication process on the insects exposed to *P. tuberculatum* extracts. In a general way, the LD_{50} and LD_{90} values decreased when the time application and evaluation increased (Table 3); likewise, its small variations with respect to time of exposure also suggest a rapid toxic action. The data presented confirm that mature spikes and *in vitro* plants extracts from *P. tuberculatum* presented potential insecticide activity.

DISCUSSION

The results have demonstrated that dichloromethane: methanol (2:1) extracts of spikes from wild plants and *in vitro* plants showed insecticidal activity against the adults of *D. peruvianus* tested at dose ranging from 0.003 to 0.012 mg/µL and extracts of mature spikes showed higher toxicity than extracts from *in vitro* plants.

Previous studies of *P. tuberculatum* showed that the results agree with dose reported in the control of third instar larval of *D. saccharalis* [31] and *S. frugiperda* [32] and second and third instar larval and adult stage of *A. aegypti* and *A. pseudopunctipennis* [33]; however, disagree with the results reported from extracts of aerial parts of *P. tuberculatum* used in control of *Aedes atropalpus* [17] and *Anticarsia gemmatalis* [30]. In this last work, the extracts caused 80% mortality when doses higher than 800.00 µg $insect^{-1}$ of extract of seeds, leaves and stems were administered to the fourth-instar velvetbean caterpillars and the isobutyl amides pellitorine and 4,5-dihydropiperlonguminine showed 100% mortality at doses of 200 and 700 µg $insect^{-1}$, respectively.

According to Bernard *et al*. [17] and Scott *et al*. [42], the action mechanism and toxicity of the pellitorine, 4,5-dihydropiperlonguminine and other related compounds (piperamides), found in *Piper* species, could be attributed at the presence of methylenedioxyphenyl ring (MDP) in their structures. In this context, the comparative effects of five new synthetic juvenile hormone analogues, with MDP ring, were evaluated for their morphogenetic activity against the last instar nymphs of the red cotton bug *Dysdercus koenigii*, showing varying degrees of activity [43]. Additionally, the piperamides present dual biological activities, being neurotoxic, affecting the activity of the central nervous system and also as inhibitors of cytochrome P450 enzymes; these characteristics are too useful to plants of *Piper* genus as a defense strategy against herbivores [30]. As it is known, the inhibition of cytochrome P450 (P450) enzymes is a major mechanism for metabolism-based drug interactions [44].

Typical intoxication symptoms, as described by Sahayaraj and Shoba [37], such as spasmodic movements and faecal elimination, were observed; also, feeding practically ceased or decreased, thus confirming the acute toxicity of these extracts to adults of cotton stainer bug.

In the other hand, several studies have shown insecticidal activity of plants extracts against *Dysdercus* sp. For instance, nymphicidal and ovipositional efficacy of brown seaweed alga *Sargassum tenerrimum* against *D. cingulatus*, an economically important cotton pest in many parts of Asia; the benzene extract showed the best insecticidal activity (LC_{50}=0.009%), higher than a mixture of benzene and chloroform (LC_{50}=0.021%) and chloroform extracts (LC_{50}=0.2481%) and all the extracts reduced the total nymphal developmental period of the pest in a dose-dependent way [39]. In other related work, the biological effects of thallium methanol extract of *Ulva fasciata* and *U. lactuca* were observed against *D. cingulatus* third instar nymphs at different concentrations (100, 200, 400, 600 and 800 ppm); the methanol extract of *U. fasciata* (LC_{50}=313.59 ppm) and *U. lactuca* (LC_{50}= 399.27 ppm) possesses nymphicidal and antiovipositional activity, reduced fecundity, hatchability, adult longevity and relative growth rate [37]. In both works, the doses were slightly lower compared to the doses reported in our work.

Working with vascular plants, Bai and Koshy [46] and Sahayaraj *et al.* [47] reported that *Azadirachta indica* (Meliaceae) leaf and kernel extracts, *Thevetia neriifolia* (Apocynaceae) leaf and seed extract and *Pedalium murex* (Pedaliaceae) affect *D. cingulatus* oviposition. Likewise, *Bougainvillea* sp. (Nyctaginaceae) and *Abrus precatorius* L. (Papillionaceae) interfere with its reproduction [48]; *Tephrosia purpurea* (Fabaceae) and *Acalypha indica* (Euphorbiaceae) showed toxicological effects on the mortality, reproductive organs macromolecules, mineral level in alimentary channel and detoxication enzyme level in the fat body and intestine of *D. cingulatus*, using 1.5, 0.75 and 0.385% of crude extracts [45] and *Streblus asper* (Moraceae) root extracts prolonged *D. cingulatus* mating duration and reduced its fecundity, hatchability and adult longevity [49]. Moreover, insecticidal effect of *Croton urucurana* (Euphorbiacaeae) extracts and crude resin on *D. maurus* showed that the average adult mortality treated topically

with 0.5, 1.0 and 2.0% of the extracts was significantly higher compared to controls [5] and essential oils of *Piper guineense* (Piperaceae) exhibited acute toxicity to the adult cotton stainer *D. superstitiosus* [26]. These results were lightly similar with the result obtained in our work; however, only in the study of Silva *et al*. [5], with *D. maurus*, the concentrations used were significantly higher.

In the case of *D. peruvianus*, canatoxin, a variant form of urease isolated from *Canavalia ensiformis* (Fabaceae) and canatoxin-derived peptide, were evaluated against this pest of cotton culture. The ingestion of both compounds severely affected young forms of the insects, delaying their development or leading to their death; in contrast, adults were insensitive to diets containing higher concentrations of canatoxin [8]. Complementary studies support the view that a differential processing of ingested urease by the insects explains at least in part the lack of toxicity in adults [25]. Extracts of twenty-three native Peruvian and exotic plants were studies against *D. peruvianus*; however, only *Lonchocarpus nicou* (Fabaceae) (LC_{50}=16.62 mg/mL), *Chromolaena laevigata* (Asteraceae) (LC_{50}=26.52 mg/mL) and *Schinus molle* (Anacardiaceae) (LC_{50}=42.64 mg/mL) showed a poor insecticidal activity at 72 h of exposure [50]. In other study, all treatments of insects with *Manilkara subsericea* (Sapotaceae) extracts and triterpenes (α- and â-amyrin acetates) induced mortality, delayed development and inhibited moulting in *D. peruvianus* and also produced body deformities in the few adults that emerged [36].

Although the *in vitro* plant extracts showed lower toxicity than mature spikes extracts, may be an active compound biosynthesis at large scale using the establishment of cellular suspensions [51, 52]. The major advantages of a cell culture system over the conventional cultivation of whole plants are: useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions; cultured cells would be free of microbes and insects; the cell of any plants could easily by multiplied to yield their specific metabolites; automated control of cell growth and rational regulation of metabolite processes would reduce of labor costs and improve productivity [53].

In conclusion, *P. tuberculatum* is an abundant species and is semi-domesticated in Peru where it has been used as a hedge plant. It is for the reasons that these results are very promising in creating new effective and affordable approaches to the control of *Dysdercus peruvianus* and other pests.

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