

## Larvicidal activity of *Piper tuberculatum* extracts on the tobacco budworm, *Heliothis virescens* Fabr. (Lepidoptera:Noctuidae) under laboratory conditions

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### ABSTRACT

Plant extracts and isolated metabolites have long been a subject of research due to the increased concern for adverse effects of conventional insecticides on human health and environment. The larvicidal activity of the neotropical “matico” *Piper tuberculatum* on the tobacco budworm, *Heliothis virescens* was evaluated by contact bioassays. The secondary compounds were extracted from mature spikes with fruits and seeds of wild plants and in vitro 12-months-old plants of *P. tuberculatum*.  $CH_2Cl_2:MeOH$  (2:1) extracts from mature spikes caused 76.6 and 83.3% mortality when doses of 0.012 mg/ $\mu$ L were applied to *H. virescens* in 76 and 120 h of exposure, respectively, with  $LD_{50}$  0.007 mg/ $\mu$ L and  $LD_{90}$  0.033 mg/ $\mu$ L, in 76 h of exposure. The  $CH_2Cl_2:MeOH$  (2:1) extracts from in vitro plants caused 56.6% mortality when doses of 0.012 mg/ $\mu$ L were also applied in 76 h of exposure with  $LD_{50}$  0.011 mg/ $\mu$ L and  $LD_{90}$  0.046 mg/ $\mu$ L. The potential value of extracts derived from *P. tuberculatum* as efficient insecticides against *H. virescens* is discussed.

**Keywords:**  $CH_2Cl_2:MeOH$  extract, in vitro propagation, insecticide, larval susceptibility, lethal concentration.

### INTRODUCTION

The tobacco budworm or tomato budworm, *Heliothis virescens* (Fabricius) or *Helicoverpa virescens*, is a native species which is distributed throughout the eastern and southwestern United States, though it is also known from California. It also occurs widely in the Caribbean, and sporadically in Central and South America. *H. virescens* is a major pest of many row crops throughout the United States, and larvae typically cause economic damage to tobacco and cotton, specially, in the mid-South of the United States, although their relative abundance can vary from year to year<sup>1,7</sup>. In Peru, *H. virescens* known as “gusano silvador”, “bellotero” or “perforador grande de la bellota del algodónero”, and is a major pest in chickpea, cotton and apple, respectively<sup>11,15,48</sup>.

Since commercialization in 1996, *H. virescens* currently controlled by cotton plants engineered to express *Bacillus thuringiensis* (Bt) Cry1Ac toxin; however, although this technology is highly effective against *H. virescens*, supplemental foliar insecticide applications to control *Helicoverpa zea*, other heliothine frequently associated with *H. virescens*, have been used extensively in Bollgard fields (Bollgard, Monsanto Co.; St. Louis, MO)<sup>52</sup>.

In addition, analysis of midgut proteinases from *B. thuringiensis*-susceptible and resistant *Heliothis virescens* can avoid intoxication by Bt toxins, since serine proteinases are a critical component of Bt toxin mode of action, these differences may contribute to decreased toxicity in the Bt-resistant strains<sup>19</sup>. In Australia, it has been reported that transgenic cotton plants expressing the *cryIAc* gene from *Bacillus thuringiensis* Berliner variety *kurstaki* are less toxic to first-instar *Helicoverpa armigera* after the plant is producing fruit<sup>33</sup>.

The method currently used to control *H. virescens* and other insect pests, is based mainly on the use of organochlorine and organophosphorous insecticides; however, chemical control of insect pests of agricultural crops is plagued with problems such as insecticide resistance and environmental contamination<sup>6,22</sup>. The indiscriminate use of synthetic insecticides has not only caused environmental contaminations but also toxicity to living organisms<sup>29</sup>, indicating the need of developing not-hazardous to the environment, target-specific and biodegradable products.

In that sense, biopesticides, biochemical or microbial, are an important group of naturally occurring, often slow-acting crop protectants that are usually safer to humans and environment than conventional pesticides, and with minimal residual effects<sup>3</sup>. Biochemical pesticides may include plant-derived pesticides that can interfere with the growth, feeding, or reproduction of pests or insect pheromones applied for mating disruption, monitoring or attract-and-kill strategies<sup>8</sup>. In a study of insecticidal activity of eight plants collected from Burkina Faso, these were tested at 250 µg/mL concentration on *Helicoverpa zea* and *Heliothis virescens* larvae in the antifeedant assays against *H. zea* and *H. virescens*, the MeOH extracts of *Cassia nigricans*, *Swartzia madagascariensis* and *Strophantus hispidus* were more effective against *H. zea* as indicated by 74% larval weight reduction as compared to the control, probably by the presence of femodin, the most abundant and active anthraquinone in *C. nigricans*<sup>13</sup>. The essential oils of *Cymbopogon nardus* (citronelal 49.8%) and *Coriandrum sativum* (linalol 76.8%) significantly inhibited the feeding larvae of *Heliothis virescens*, with 90 and 63%, respectively, while the volatile oils of *Plecthanthus amboinicus* (carvacrol 58.1%) and *Mentha spicata* (carvona 24.5%) were effective against *Spodoptera frugiperda* larvae<sup>38</sup>. In this regard, many authors have studied the larvicidal and antifeedant activities of essential oils on Lepidoptera<sup>17</sup>, delayed growth and development of larvae<sup>26</sup> and its deterrent effect on oviposition<sup>30</sup>. Likewise, the stem wood of the Caribbean shrub, *Ryania speciosa* (Flacourtiaceae) contains the alkaloid ryanodine, that acts as a muscle poison; ryania has toxic and growth inhibiting effects against *Heliothis virescens*<sup>53</sup>, and has seen limited use by organic apple and pear growers for control of the codling moth, *Cydia pomonella*<sup>3</sup>.

In addition, about 3% aqueous ethanolic spray formulation derived from the lipophilic extract of *Excoecaria agallocha* (dry leaf) was evaluated against *Helicoverpa armigera* in *Abelmoschus esculentus* (lady's finger) and *Cajanus cajan* (pigeon pea), in field conditions, and this concentration was found to be promising for the control of the insect pest<sup>40</sup>. Similarly, the aqueous extract of individual and mixed form of *Azadirachta indica* seeds kernel and leaves of *Milletia ferruginea*, and *Croton macrostachyus* was tested against African bollworm, *Helicoverpa armigera* in chickpea, *Cicer arietinum*; however, even though Diazinon 60% EC (positive control) was found to be effective by considering the interaction of beneficial in the field botanical preparations are much better particularly NSKE (Neem Seed Kernel Extract) and also suitable to spray under rain fed condition to protect the crop by small farming communities<sup>24</sup>.

On the other hand, microbial pesticides contain a microorganism such as a bacterium, virus, fungus, protozoan or an alga as an active ingredient to control pests, although, the most widely used microbial pesticide is the bacterium *Bacillus thuringiensis* or Bt for control of insect pests on various crops<sup>3</sup>. Interactions among insect-resistant soybean genotypes extracts with populations of the velvetbean caterpillar, *Anticarsia gemmatalis*, susceptible and resistant to its nucleopolyhedrovirus (AgMNPV), used regularly in Brazil as a biological insecticide, were studied. The two resistant genotypes, identified as PI 227687 and PI 274454, contain higher concentrations of rutin (quercetin 3-O-rutinoside) compared to other genotypes, and play an important role in soybean defense against defoliator insects, such as *Heliothis virescens*, *Trichoplusia ni* and *A. gemmatalis*<sup>36</sup>; however, the oxidation of phenolics such as rutin and chlorogenic acid by foliar oxidative enzymes (e.g., peroxidases and polyphenol oxidases) can also decrease the infectivity of NPVs against *H. zea*<sup>12</sup> and *H. virescens* larvae<sup>16</sup>.

In another study, diluted plasma from larval *Heliothis virescens* exhibited a virucidal effect against *Helicoverpa zea* single capsid nucleopolyhedrovirus (HzSNPV) *in vitro*, reducing the TCID<sub>50</sub> ml<sup>-1</sup> by more than 64-fold (from 4.3±3.6 x 10<sup>5</sup> to 6.7±0.6 x 10<sup>3</sup>)<sup>37,45</sup>. Likewise, a recombinant baculovirus expressing ScathL (AcMLF9.ScathL), a cathepsin L-like cysteine protease derived from the flesh fly *Sarcophaga peregrina* that functions in basement membrane (BM) remodeling during insect development, kills larvae of the tobacco budworm, *Heliothis virescens*, significantly faster than the wild-type virus, and was also lethal to adult pea aphids, *Acyrtosiphon pisum* with a similar loss of integrity of the gut and fat body<sup>23</sup>.

Several species of *Piper* (Piperaceae) have been reported in the literature to have insecticidal activity<sup>35</sup>. For example, the Amazonian species, *Piper rotundistipulum*, is locally used as insecticide and fish poison<sup>41</sup>. Crude extracts, essential oils and seed powders of *Piper guineense* are effective against numerous Tropical African insect pests<sup>32</sup>, and *P. guineense* and *P. nigrum* are used as insecticide and molluskicide in several parts of Africa<sup>18</sup>. The Indian species *Piper longum*, *P. betle*, *P. peepuloides* and *P. cubeba* have demonstrated insecticidal activity against mosquitos and flies<sup>28</sup>. Recently, the potential value of extracts and amides derived from *Piper tuberculatum* as insecticides against *Anticarsia gemmatalis* was determined<sup>31</sup>, 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*, *Anopheles pseudopunctipennis* and *Dysdercus peruvianus*<sup>4,27,46,47</sup>.

Chemical studies carried out on Brazilian Piperaceae species have revealed the occurrence of pyrones, lignoids and chromenes besides various amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties<sup>20,35</sup>; 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<sup>10,31,34</sup>. One of these species is *P. tuberculatum*, known as “matico”, “nudillo”, “cordoncillo” or “palo soldado”, which is widely distributed from Brazil to Mexico<sup>27</sup>.

The objective of this research was to investigate the insecticidal activity of extracts from mature spikes, with fruits and seeds, of wild plants and *in vitro* plants of *P. tuberculatum* on third instar larval of *H. virescens*.

## MATERIALS AND METHODS

### Plant material

Spikes with mature seeds of *P. tuberculatum* Jacq. were collected in december 2009 from Cumbil river (Lambayeque, Peru). Botanical identification was performed by Dr. Guillermo E. Delgado-Paredes from Universidad Nacional Pedro Ruiz Gallo (UNPRG) based on taxonomic description realized by Yuncker<sup>54</sup>, and botanic specimen vouchers were deposited at the UNPRG Herbarium (HPR).

### *In vitro* micropropagated plants

The protocol for micropropagation of *P. tuberculatum* has been extensively described in previous studies<sup>27,47</sup>.

### Insects

Larvae of *H. virescens* were collected from “lenteja de palo” or “pigeonpea” (*Cajanus cajan*) during its middle to late reproductive stage, in the caserío Puente El Pavo, Túcume – Lambayeque, and were reared in the Laboratorio de Entomología of the Facultad de Agronomía (UNPRG), under laboratory conditions. Larvae were grouped in six instars (instar I to VI) and introduced in 500 cc plastic vials covered with tulle. Larvae were reared at 24.5 °C and 72.5% RH under a 16:8 (L:D) photoperiod, and fed daily with pigeon pea and geranium leaves (*Pelargonium hortorum*) until reaching the pupal stage. Subsequently, one day old pupae were sexed (genitalia opening in the eighth abdominal segment for the female and ninth abdominal segment for the male), and surface-sterilized with sodium hypochlorite 0.1% (w/v) for 30 sec. The adults were kept in glass jars (25x15 cm) in proportion 6:5 (female:male) and covered with tulle. Inside the glass jars folded papers were conditioned to increase the area of oviposition and placed a branch of geranium in a small jar with water for oviposition. The adults were provided with hooney bee solution (10%) and sterilized water soaked in cotton swabs. After mating and oviposition eggs were removed from the paper and the branch of geranium and placed in incubation until hatching. Third instar larvae of *H. virescens* were fed to repletion with fresh seeds of pigeon pea.

### Extraction of the constituents

25 g of spikes of wild plants of *P. tuberculatum* were oven dried at 40 °C, milled and submerged three times in CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) at room temperature, yielding 9.1% (2.28 g) of extract; likewise, 25 g of *in vitro* micropropagated plants of 12 months-old yielding 10.1% (2.53 g) of extracts with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1). The extracts obtained were evaporated under reduced pressure (45 °C).

### Topical test

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 1800 mg of dry extract in 1.5 mL of methanol-water to obtain 1200 mg/mL concentration solution, and 0.3 mL (300 µL) were prepared for each treatment with application of the following formula:  $C_1 \times V_1 = C_2 \times V_2$ . After 24 h, and using an Eppendorf® 0-10 µL pipette, 10 µL of the solution, containing an aliquot of each of the treatments, was applied directly on the larval mesothorax of *H. virescens*. The plant extract was tested at doses of 0.0, 0.00025, 0.0005, 0.001, 0.002, 0.004, 0.006, 0.008, 0.010 and 0.012 mg/µL for each larva. Thirty larvae were tested per treatment and the experiment was carried out twice. The control insect received a topical application with MeO—water alone. Larval mortality was recorded at 24, 48, 72, 96 and 120 hours post treatments, under the same conditions of temperature and humidity described above. The larvae were considered dead if they displayed no observable response to a mechanical stimulus, i.e. short-time 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<sub>50</sub> and LD<sub>90</sub> values were determined used the software US. EPA Probit Program Version 1.5<sup>49</sup>.

## RESULTS

The response of tobacco budworm to the topical applications of CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) extract from mature spikes of wild plants and CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) extract from *in vitro* plants of *P. tuberculatum* showed a positive relationship between dose and mortality. The responses varied with the time of exposure.

The larval mortality at 76.6% was reached after 72 h when using 0.012 mg/µL of CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) extract from mature spikes; and a mortality of 83.3% was reached with 0.012 mg/µL in 120 h (Table 1). In reference to the *in vitro* plants, the extract obtained with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) generated a 56.6% larval mortality with 0.012 mg/µL in 72 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 general, the LD<sub>50</sub> and LD<sub>90</sub> values decreased when the time of application and evaluation increased (Table 3); likewise, the small variations with respect to time of exposure also suggest a rapid toxic action. Our data confirms that mature spikes and *in vitro* plants extracts from *P. tuberculatum* presented potential insecticide activity; however, in general, topically-applied CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) extract from mature spikes induced the highest mortality of III instar tobacco budworm larvae compared with the *in vitro* plants.

The results also showed an increase of mortality rates of 50% (LC<sub>50</sub>) only after 72 h of application of the dose of 0.012 mg/µL, although lower doses as 0.010 and 0.008 mg/µL showed that the larvae ceased feeding and died after 120 h of application.

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

Treatment (mg/µL/larvae)	Larvae tested (N <sup>o</sup> )	Hours after application/Mortality (%)				
		24	48	72	96	120
0.00	30	0.00	0.00	0.00	0.00	0.00
0.00025	30	0.00	0.00	0.00	0.00	3.33
0.0005	30	3.33	3.33	3.33	3.33	3.33
0.001	30	3.33	6.67	10.0	10.00	10.00
0.002	30	10.00	16.67	26.67	26.67	30.00

0.004	30	10.00	16.67	30.00	30.00	33.33
0.006	30	13.13	20.00	43.33	46.67	46.67
0.008	30	13.13	30.00	53.33	56.67	56.67
0.010	30	13.13	33.33	63.33	70.00	70.00
0.012	30	16.67	36.67	76.67	80.00	83.33

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

Treatment (mg/ $\mu$ L/larvae)	Larvae tested (N <sup>o</sup> )	Hours after application/Mortality (%)				
		24	48	72	96	120
0.00	30	0.00	0.00	0.00	0.00	0.00
0.00025	30	0.00	0.00	0.00	0.00	0.00
0.0005	30	0.00	0.00	0.00	0.00	0.00
0.001	30	0.00	3.33	3.33	3.33	3.33
0.002	30	0.00	6.67	13.33	13.33	16.67
0.004	30	0.00	6.67	13.33	16.67	16.67
0.006	30	3.33	13.33	26.67	26.67	30.00
0.008	30	6.67	16.67	40.00	43.33	43.33
0.010	30	6.67	20.00	50.00	56.67	56.67
0.012	30	10.00	26.67	56.67	63.33	66.67

**Table 3: Components of the probit analysis and LD<sub>50</sub> and LD<sub>90</sub> values for the larvae of tobacco budworm *Heliothis virescens* exposed to two extracts (spikes of wild plants and *in vitro* plants) of *Piper tuberculatum***

Extract	Time after treatment (h)	Slope ( $\pm$ SE)	Lethal concentration (mg/ $\mu$ L)				Significance X <sup>2</sup> (g.l.) <sup>a</sup>
			LD <sub>50</sub>	LD <sub>50</sub> 95% FL	LD <sub>90</sub>	LD <sub>50</sub> 95% FL	
Spikes	72	1.81 ( $\pm$ 0.32)	0.007	0.005–0.009	0.033	0.021–0.085	3.71 ns
	96	1.95 ( $\pm$ 0.33)	0.006	0.005–0.006	0.027	0.018–0.058	3.93 ns
	120	1.96 ( $\pm$ 0.35)	0.006	0.004–0.007	0.026	0.017–0.058	4.34 ns
<i>In vitro</i> plants	72	2.06 ( $\pm$ 0.46)	0.011	0.009–0.02	0.046	0.025–0.194	3.36 ns
	96	2.23 ( $\pm$ 0.46)	0.009	0.008–0.01	0.035	0.021–0.107	3.59 ns
	120	2.15 ( $\pm$ 0.43)	0.009	0.007–0.012	0.036	0.022–0.106	4.72 ns

<sup>a</sup>Significance level: ns = not significant (P>0.05)

## DISCUSSION

The results have demonstrated that CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) extracts from spikes of wild plants and *in vitro* plants showed insecticidal activity against the III instar of *H. virescens* tested at dose ranging from 0.0 to 0.012 mg/ $\mu$ L.

Previous studies of *P. tuberculatum* showed that the results agree with dose reported in the control of several orders of insect species, for example, in the control of third instar larval of *Diatraea saccharalis* (Lepidoptera)<sup>46</sup>, second and third instar larval and adult stage of *Aedes aegypti* and *Anopheles pseudopunctipennis* (Diptera)<sup>4</sup>, third instar larval of *Spodoptera frugiperda* (Lepidoptera)<sup>47</sup> and adults of *Dysdercus peruvianus* (Coleoptera)<sup>27</sup>; however, disagree with the results reported for extracts of leaves and stems of *P. tuberculatum* used in the control of *Aedes atropalpus*<sup>5</sup> and *Anticarsia gemmatilis*<sup>31</sup>. According to Scott *et al.*<sup>42,43</sup> interplant differences related to the efficacy of extracts may be due to the large variability observed with the individual piperamide concentrations, especially 4,5-dihydropiperlonguminine, in leaves. In our study, the spikes extracts used from wild plants showed that the amide content is greater than in leaves and stems<sup>10</sup>, as well as in *in vitro* adult plants (12 months-old) where it is even possible biosynthesis of new secondary metabolites<sup>9</sup>.

According to Bernard *et al.*<sup>5</sup> and Scott *et al.*<sup>43</sup>, the action mechanism and toxicity of the pellitorine, 4,5-dihydropiperlonguminine and others related compounds (piperamides), found in *Piper* species, could be attributed to the presence of the methylenedioxyphenyl ring (MDP) in their structures.

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 also are useful for plants of *Piper* genus as a defense strategy against herbivores<sup>31</sup>.

As previously described for larvae of *Anticarsia gemmatalis*<sup>31</sup> and *Spodoptera frugiperda*<sup>47</sup>, almost immediately following the application of doses of each treatment, larval movement decreased and feeding practically ceased almost immediately following the application of doses of each treatment; and furthermore, typical intoxication symptoms, such as described by Marchine et al.<sup>25</sup> and Sahayaray and Shoba<sup>39</sup>, spasmodic movements, regurgitation and faecal elimination, were observed, thus confirming the acute toxicity of these extracts for tobacco budworm.

In the other hand, there are several studies evaluating the biological activity of crude plant extracts as botanical insecticides against Lepidoptera. For instance, crude methanolic extracts of *Bifora radians* and *Humulus lupulus* incorporated into diet of obliquebanded leafroller, *Choristoneura rosaceana* deterred larval feeding, and *H. lupulus* and *Arctium lappa* extracts were deleterious to larvae, exhibiting toxic, developmental, and antifeedant effects<sup>14</sup>. Crude methanolic extracts of six species of *Trichilia* collected in Costa Rica exhibited toxic activity against the larvae of Asian armyworm, *Spodoptera litura*, and the most active species was *T. americana*<sup>50,51</sup>. Likewise, ethanolic seed extracts of *Annona squamosa* were significantly more active (20-fold) than those of *A. muricata* and both reduced larval growth of *S. litura* and the cabbage looper, *Trichoplusia ni*<sup>21</sup>, while crude extracts of *Melia volkensii* have shown to inhibit larval growth of *T. ni* and the armyworm, *Pseudaletia unipunctata*, and were also the most potent feeding deterrent for third instar *P. unipunctata* and *Plutella xylostella*<sup>2</sup>. In another study, aqueous extract of *Melia azedarach*, *Nerium indicum* and *Azadirachta indica* showed higher mortality of larvae of cabbage butterfly, *Pieris brassicae* (19.6 to 18.5%)<sup>44</sup>.

In the case of *Heliothis* species, antifeedant activity of selected plants extracts, aqueous extracts of individual and mixed form of *Azadirachta indica* (Neem) seeds kernel, leaves of *Milletia ferruginea* (Birbira), and *Croton macrostachyus*, were tested at several concentrations against 4<sup>th</sup> instar larvae of *H. armigera* in the laboratory and under field condition, and all the tested plant extracts showed 100% protection at 5 and 10% concentration, and among the various botanical treatments Neem Seed Kernel Extract (NSKE) and NSKE+BLE (Birbira Leaves Extract) were effective at 2.5% concentration with minimum chickpea (*Cicer arietinum*) pod damage<sup>24</sup>. In another study, about 3% aqueous ethanolic spray formulation derived from the lipophilic extract of milky mangrove tree *Excoecaria agallocha* (dry leaves) was evaluated against *H. armigera* in *Abelmoschus esculentus* (lady's finger) and *Cajanus cajan* (pigeon pea), under field conditions, and on the 9<sup>th</sup> day of the 4<sup>th</sup> spray the larval count in the plot treated with 3% *E. agallocha* formulation drastically came down to 0.23 larva/plant, compared to 1.63 in the ethanol control plot and 1.60 in the unsprayed plot<sup>40</sup>.

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### REFERENCES

1. Adamczyk, J.J. and Hubbard, D., Changes on populations of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in the Mississippi Delta from 1986 to 2005 as indicated by adult male pheromone traps. *The Journal of Cotton Science*, **10**: 155-160 (2006).
2. Akhtar, Y. and Isman, M.B., Comparative growth inhibitory and antifeedant effects of plant extracts and pure allelochemicals on four phytophagous insect species. *J Applied Entomology*, **128**: 32-38 (2004).
3. Akhtar, Y., Yeung, Y.R. and Isman, M.B., Comparative bioactivity of selected extracts from Meliaceae and some commercial botanical insecticides against two noctuidae caterpillars, *Trichoplusia ni* and *Pseudaletia unipuncta*. *Phytochemistry Reviews*, **7**: 77-88 (2008).

4. Bazán-Calderón, J., Ventura-Flores, R., Kato, M.J., Rojas-Idrogo, C. and Delgado-Paredes, G.E., Actividad insecticida de *Piper tuberculatum* Jacq. sobre *Aedes aegypti* L. (Diptera: Culicidae) y *Anopheles pseudopunctipennis* Tehobal (Diptera: Culicidae). *Anales de Biología*, **33**: 135-147 (2011).
5. Bernard, C.B. Krishnamurthy, H.G. Chauvet, D. Durst, T. Philogéne, B.J.R. Sánchez-Vindas, I. Hasbur, C. Poveda, L. San Román, L. Arnason, J.T., Insecticidal defenses of Piperaceae from the neotropics. *Journal of Chemical Ecology*, **21**: 801-814 (1995).
6. Clark, J.M. Yamaguchi, I., Scope and status of pesticide resistance. American Chemical Society. Washington, DC. (2002).
7. Clower, D.F., Changes in *Heliothis* spp. attacking cotton in recent years and how they have affected control. P. 139-141. In Proc. Beltwide Cotton Prod. Res. Conf., St. Louis, Mo. 6-10 Jan. 1980. Natl. Cotton Counc. Am. Memphis, TN. (1980).
8. Copping, L.G. Menn, J.J., Biopesticides: a review of their action, applications and efficacy. *Pest Management Science*, **56**: 651-676 (2000).
9. Danelutte, A.P. Costantin, M.B. Delgado, G.E. Braz-Filho, R. Kato, M.J., Divergence of secondary metabolism in cell suspension cultures and differentiated plants of *Piper cernuum* and *P. crassinervium*. *Journal of the Brazilian Chemical Society*, **16 (6B)**: 1425-1430 (2005).
10. Da Silva, R.V. Navickiene, H.M.D. Kato, M.J. Bolzani V da, S. Méda, C.I. Young, M.C.M., Furlan, M., Antifungal amides from *Piper arboreum* and *Piper tuberculatum*. *Phytochemistry*, **59**: 521-527 (2002).
11. De Tomás, L. Peralta, K., *Heliothis virescens* como plaga del manzano en el valle de Mala. *Revista Peruana de Entomología*, **36**: 89-90 (1994).
12. Felton, G.W. Duffey, S.S., Inactivation of baculovirus by quinones formed in insect damaged plant tissues. *Journal of Chemical Ecology*, **16**: 1221-1236 (1990).
13. Georges, K. Jayaprakasam, B. Dalavoy, S.S. Nair, M.G., Pest-managing activities of plant extracts and anthraquinones from *Cassia nigricans* from Burkina Faso. *Bioresource Technology*, **99**: 2037-2045 (2008).
14. Gökçe, A. Stelinski, L.L. Whalon, M.E. Gut, L.J., Toxicity and antifeedant activity of selected plant extracts against larval obliquebanded leafroller, *Choristoneura rosaceana* (Harris). *The Open Entomology Journal*, **3**: 30-36 (2009).
15. Hambleton, E.J., *Heliothis virescens* as a pest of cotton, with notes on host plant in Peru. *Journal of Economic Entomology*, **37**: 660-666 (1994).
16. Hoover, K. Yee, J.L. Schultz, C.M. Rocke, D.M. Hammock, B.D. Duffey, S.S., Effects of plant identity and chemical constituents on the efficacy of baculovirus against *Heliothis virescens*. *Journal of Chemical Ecology*, **24**: 221-252 (1998).
17. Isman, M.B., Miresmailli, S., Machial, C., Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer products. *Phytochemistry Reviews*, **10**: 197-204 (2011).
18. Ivbijaro, M.F., Bolaji, O.O., Effects of cypermethrin + dimethoate and extracts of *P. guineense* and *Azadirachta indica* on the pests and yield of cowpea, *Vigna unguiculata*. *Journal Agriculture Science*, **115**: 227-231 (1990).
19. Karumbaiah, L., Oppert, B., Jurat-Fuentes, J.L., Adang, M.J., Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae). *Comparative Biochemistry and Physiology, Part B* **146**: 139-146 (2007).
20. Kato, M.J. Furlan, M., Chemistry and evolution of the Piperaceae. *Pure and Applied Chemical*, **79**: 529-538 (2007).
21. Leatemia, J.A., Isman, M.B., Insecticidal activity of crude seed extracts of *Annona* spp., *Lansium domesticum* and *Sandoricum koetjape* against Lepidopteran larvae. *Phytoparasitica*, **32**: 30-37 (2004)
22. Li, H., Oppert, B., Zhu, K.Y., Higgins, R.A., Huang, F. and Buschman, L.L., Transgenic plants expressing *Bacillus thuringiensis* deltaendotoxins. *Entomogy Sinica*, **10**: 155-166 (2003).
23. Li, H., Tang, H., Sivakumar, S., Philip, J., Harrison, R.L., Gatehouse, J.A. and Bonning, B.C., Insecticidal activity of a basement membrane-degrading protease against *Heliothis virescens* (Fabricius) and *Acyrtosiphon pisum* (Harris). *Journal of Insect Physiology*, **54**: 777-789 (2008).

24. Lulie, N. and Raja, N., Evaluation of certain botanical preparations against African Bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) and non target in chickpea, *Cicer arietinum* L. J. *Biofertilizer Biopesticide* 3:130.doi:10.4172/2155-6202.1000130 (2012).
25. Marchini, L.C. Alves, S.B. Nakano, O., Sericultura. Curso de Entomología Aplicada à Agricultura. FEALQ, Piracicaba, Brazil, pp. 705-736 (1992).
26. Marimuth, S., Gurusubramanian, G. and Krishna, S.S., Effect of exposure of eggs to vapours from essential oils on egg mortality, development and adult emergence in *Earias vitella* (F.) (Lepidoptera: Noctuidae). *Biological Agriculture & Horticulture*, **14**:303-307 (1997).
27. Mendoza-Frías, N.L., Monsalve-Asencio, L.J., Rojas-Idrogo, C., Kato, M.J. and Delgado-Paredes, G.E., Insecticidal activity of *Piper tuberculatum* extracts on the cotton stainer bug, *Dysdercus peruvianus* Guérin-Ménéville (Hemiptera: Pyrrhocoridae). *Academic Journal of Entomology*, **6**:153-161 (2013).
28. Miyakado, M. Nakayama, I. Ohno, N., Insecticidal unsaturated isobutylamides: From natural products to agrochemical leads. Pp. 183-187. In: Arnason, JT, Philogène BJR, Morand P. (Eds.). Insecticides of plant origin. ACS Symposium Series 387, American Chemical Society, New York (1989).
29. Nakata, H., Hirakawa, Y., Kawazo, M., Nakabo, T., Arizono, K., Abe, S.I., Kitano, T., Shimada, H., Watanabe, I., Li, W. and Ding, X., Concentrations and compositions of organochlorine contaminants in sediments, soils, crustaceans, fishes and birds collected from Lake Tai, Hangzhou Bay and Shanghai city region. *China Environmental Policy*, **133**: 415-429 (2005).
30. Naumann, K. and Isman, M.B., Evaluation of neem *Azadirachta indica* seed extracts and oils as oviposition deterrents to noctuid moths. *Entomologia Experimentalis et Applicata*, **76**: 115-120, EE. UU (1995).
31. Navickiene, H.M.D., Miranda, J.E., Bortoli, S.A., Kato, M.J., Bolzani V da, S. and Furlan, M., Toxicity of extracts and isobutyl amides from *Piper tuberculatum*: potent compounds with potential for the control of the velvetbean caterpillar, *Anticarsia gemmatilis*. *Pest Management Science*, **63**:399-403 (2007).
32. Ntonifor, N.N., Potentials of tropical African species as sources of reduced-risk pesticides. *Journal of Entomology*, **8**:16-26 (2011).
33. Olsen, K.M. and Daly, J.C., Plant-toxin interactions in transgenic Bt cotton and their effect on mortality of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, **93**:1293-1299 (2000).
34. Palacios, Z.G.F. Delgado, G.E. Moreno, M.C. Kato, M.J. Rojas, C., Actividad antifúngica *in vitro* de extractos crudos de *Piper tuberculatum*. *Revista Peruana de Biología*, **16**: 209-214 (2009).
35. Parmar, V.S. Jain, S.C. Bisht, K.S. Jain, R. Taneja, P. Jha, A. Tyagi, O.D. Prasad, A.K. Wengel, J. Olsen, C.E. Boll, P.M., Phytochemistry of the genus *Piper*. *Phytochemistry*, **46**: 597-673 (1997).
36. Piubelli, G.C., Moscardi, F. and Hoffmann-Campo, C.B., Interactions among insect-resistant soybean genotypes extracts with populations of *Anticarsia gemmatilis* (Lepidoptera: Noctuidae) susceptible and resistant to its nucleopolyhedrovirus. *Anais da Academia Brasileira de Ciências (Annals of the Brazilian Academy of Sciences)*, **81**: 861-871 (2009).
37. Popham, H.J.R., Shelby, K.S., Brandt, S.L. and Coudron, T.A., Potent virucidal activity in larval *Heliothis virescens* plasma against *Helicoverpa zea* single capsid nucleopolyhedrovirus. *Journal of General Virology*, **85**: 2255-2261 (2004).
38. Romeu, C.R. and Veitía, M., Efecto antialimentario de aceites esenciales de plantas aromáticas sobre *Heliothis virescens* y *Spodoptera frugiperda*. *Fitosanidad*, **16**: 155-159 (2012).
39. Sahayaraj, K. Shoba, J., Toxic effect of *Tephrosia purpurea* (Linn.) and *Acalypha indica* (Linn.) aqueous extracts impact on the mortality macromolecules, intestinal electrolytes and detoxication enzymes of *Dysdercus cingulatus* (Fab.). *Asian Journal of Biochemistry*, **7**:112-122 (2012).
40. Santhanam, S.R. Egigu, M.C., Field evaluation of a botanical formulation from the milky mangrove *Excoecaria agallocha* L. against *Helicoverpa armigera* Hübner. In *Abelmoschus esculentus* (lady's finger) and *Cajanus cajan* (pigeon pea). *Asian Pacific Journal of Tropical Medicine*, **7 (Suppl. 1)**: S171-S176 (2014).



41. Schultes, R.F. and Raffauf, R.F., Medicinal and toxic plants of the Indians of northwest Amazonia, Piperaceae. Pp. 362-368. In: Schultes RE, Raffauf RF (Eds.). The Healing forest: medicinal and toxic plants of the northwest Amazonia. Historical, Ethno- & Economic Botany Series. Vol. 2. Dioscoride Press, Portland, Oregon (1990).
42. Scott, I.M., Puniani, E., Durst, T., Phelps, D., Merali, S., Assabgui, R.A., Sánchez-Vindas, P., Poveda, L., Philogéne, B.J.R. and Arnason, J.T., Insecticidal activity of *Piper tuberculatum* Jacq. extracts: synergistic interaction of piperamides. *Agricultural and Forest Entomology*, **4**:137-144 (2002).
43. Scott, I.M., Jensen, H., Scott, J.G. and Isman, M.B. Arnason, J.T. Philogéne, B.J.R., Botanical insecticides for controlling agricultural pests: piperamides and the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera:Chrysomelidae). *Archives of Insect Biochemistry and Physiology*, **54**: 211-225 (2003).
44. Sharma, A. and Gupta, R., Biological activity of some plant extracts against *Pieris brassicae* (Linn.). *Journal of Biopesticides*, **2**: 26-31 (2009).
45. Shelby, K.S. and Popham, J.R., Plasma phenoloxidase of the larval tobacco budworm, *Heliothis virescens*, is virucidal. *Journal of Insect Science*, **6**: 1-12 (2006).
46. Soberón, G.V., Rojas, C., Saavedra, J., Kato, M.J. and Delgado, G.E., Acción biocida de plantas de *Piper tuberculatum* Jacq. sobre *Diatraea saccharalis* (Lepidóptera, Pyralidae). *Revista Peruana de Biología*, **13**: 107-112 (2006).
47. Soberón, G.V., Rojas Idrogo, C., Kato, M.J., Saavedra Díaz, J., Armando, Jr. J. and Delgado Paredes, G.E., Larvicidal activity of *Piper tuberculatum* on *Spodoptera frugiperda* (Lepidoptera: Noctuidae) under laboratory conditions. *Revista Colombiana de Entomología*, **38**: 35-41 (2012).
48. Torres, M. Barandiarán, J., Control químico del “gusano silvador” del garbanzo *Heliothis virescens* F. (Lep.: Noctuidae). *Revista Peruana de Entomología*, **11**: 18-21 (1968).
49. US/EPA (EPA Environmental Protection Agency). Probit program version 1.5., Ecological Monitoring Research Division. Environmental Monitoring Systems Laboratory. U.S. Environmental Protection Agency Cincinnati, OH. USA (2003).
50. Wheeler, D.A., Isman, M.B., Sanchez-Vindas, P.E. and Arnason, J.T., Screening of Costa Rica *Trichilia* species for biological activity against the larvae of *Spodoptera litura* (Lepidoptera: Noctuidae). *Biochemical Systematic and Ecology*, **29**: 347-358 (2001).
51. Wheeler, D.A. Isman, M.B., Antifeedant and toxic activity of *Trichilia americana* extract against the larvae of *Spodoptera litura*. *Entomologia Experimentatlis et Applicata*, **98**: 9-16 (2001).
52. Williams, M.R., Cotton insect loss estimates-1999. P. 884-888. In: Proc. Beltwide Cotton Conf., San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN (2000).
53. Yoshida, A.H. Toscano, C.N., Comparative effects of selected natural insecticides on *Heliothis virescens* (Lepidoptera, Noctuidae) larvae. *Journal of Economic Entomology*, **87**: 305-310 (1994).
54. Yuncker, T.G., The Piperaceae of Brazil II: *Piper* – Group V; *Ottonia*, *Pothomorphe*, *Sarcorhachis*. *Hoehnea*, **3**: 29-284 (1973).