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Insecticidal effects of Moroccan plant extracts on development, energy reserves and enzymatic activities of *Plodia interpunctella*

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Abstract

The aim of this work was to study the effects of methanol extracts of ten plant species used in traditional medicine in Morocco (*Peganum harmala*, *Ajuga iva*, *Rosmarinus officinalis*, *Lavandula stoechas*, *Lavandula dentata*, *Cistus ladanifer*, *Cistus salviaefolius*, *Cistus monspeliensis*, *Centaurium erythraea* and *Launaea arborescens*) on *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) larvae. Firstly, we studied the effects of the ingestion of these extracts at 500 ppm on post-embryonic development parameters. Most plant extracts provoked a notable decrease of larval weight 8 days after treatment (up to 33% weight loss with *C. erythraea*) and caused significant alterations on pupation (ranging from 5% to 85%) and adult emergence (below 2.5% with *R. officinalis, C. erythraea* and *A. iva*). The plant extracts that showed strongest effects on post-embryonic development were selected to test their effects on the following physiological parameters: larval reserve substances (at 500 ppm); and midgut activities of hydrolytic and detoxification enzymes (at 500, 750 and 1,000 ppm). All treatments provoked a significant reduction of protein and carbon hydrate larval contents, the inhibition of proteases and α-amylase activities in a dose depended manner, and the induction of glutathione S-transferase and esterase (using MtB as substrate) activities, whereas the activity of cytochrome P450 monooxygenases and esterases (using 1-NA as substrate) increase or decrease depending on the extract concentration and the plant analyzed.

Additional key words: bioinsecticides; detoxification enzymes; digestive enzymes; reserve substances.

Introduction

The Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) is an important cosmopolitan pest of stored grain and value-added food products with a worldwide distribution. In addition to damaging a commodity by feeding, the larvae spin silken webs causing economic losses by affecting food quality. In Morocco, *P. interpunctella* is considered a major pest during the processing and storage of dried fruits such as dates (Azelmat *et al.,* 2005). Currently, synthetic insecticides and methyl bromide fumigation are used for the treatment of these stored products (Azelmat *et al.,* 2005). However, serious concerns have been raised about health and environmental problems associated with the widespread use of insecticides, including development of insecticide resistant strains (White, 1995; Phillips & Throne, 2010). In addition, methyl bromide has been identified as an ozone-depleting substance and its use restricted in accordance with the Montreal Protocol (Patil *et al.,* 2004). Therefore, there is an urgent need to develop safe, convenient, environmentally friendly and low-cost alternatives.

Alkaloids, terpenoids, phenolics, flavonoids and other secondary metabolites of plants play an important role in plant-insect interaction and are commonly responsible of plant resistance to insects (Isman, 2002). These plant allelochemicals can act as attractive, repellent or toxic agents, as well as growth regulators, affecting physiological processes of insects (Ortego *et al.,* 1995; Khambay *et al.,* 2002; Liang *et al.,* 2003). The insecticidal activity of some plant derivatives has been well established, and the efficacy of a few of these active principles, such as azadirachtin, has resulted in their exploitation as crop protectants (Mordue *et al.,* 2005).

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Plants frequently used in traditional medicine in Morocco (Bellakhdar, 1997) have shown their potential as bioinsecticides. Methanol extracts from seven plant species: *Centaurium erythraea*, *Peganum harmala*, *Ajuga iva*, *Aristolochia baetica*, *Pteridium aquilinum* and *Raphanus raphanistrum* were shown to inhibit the growth of *Tribolium castaneum* larvae (Jbilou *et al.,* 2008). Leaf and seed extracts of *P. harmala* reduced the food intake and delayed larval development of *Schistocerca gregaria* (Abbassi *et al.,* 2003a,b) and *T. castaneum* (Jbilou & Sayah, 2008). Likewise, Papachristos & Stamopoulos (2002) reported that larvae of *Acanthoscelides obtectus* were susceptible to the essential oils from *Lavandula hybrida*, *Rosmarinus officinalis* and *Eucalyptus globulus*. None of these plant extracts have been tested against *P. interpunctella*. However, previous studies have shown that this species is susceptible to different allelochemicals, such as 20-hydroxyecdysone (Rharrabe *et al.,* 2009), azadirachtin (Rharrabe *et al.,* 2008), harmaline (Rharrabe *et al.,* 2007) and harmine (Bouayad *et al.,* 2012).

The mode of action of plant allelochemicals at the enzymatic level is mostly unknown, but some of them appear to disturb the digestive process by targeting insect gut digestive enzymes, such as α-amylases and proteases (Duffey & Stout, 1996). Senthil Nathan *et al.* (2005a,b) showed that ingestion of azadirachtin by *Spodoptera litura* and *Cnaphalocrocis medinalis* caused a decrease on gut enzyme activities. The limonoids 1,7-di-O-acetylhavanensin and 3,7-di-O-acetylhavanensin reduced digestive protease activity in *Leptinotarsa decemlineata* (Ortego *et al.,* 1999) and *S. exigua* (Caballero *et al.,* 2008). Likewise, ingestion of Jatropherol-I induced a decline in midgut protease activities in *Bombyx mori* (Jing *et al.,* 2005). Besides, the walls of the digestive tract in insects have a high content of detoxification enzymes, as a barrier to toxic allelochemicals that they may consume with the diet (Ahmad *et al.,* 1986). A common strategy in insects is to produce these enzymes in response to the ingestion of allelochemicals, suggesting that induction plays an important role in the adaptation of insects to plant diets (Terriere, 1984). However, some plant allelochemicals are known to inhibit the activity of detoxification enzymes (Smirle *et al.,* 1996; Jing *et al.,* 2005), increasing the toxicity of co-occurring plant compounds (Berenbaum & Neal, 1985).

The aim of this study was to evaluate the potential of ten medicinal plants commonly found in Morocco: *P. harmala*, *A. iva*, *R. officinalis*, *C. erythraea, Lavandula stoechas*, *Lavandula dentata*, *Cistus ladanifer*, *Cistus salviaefolius*, *Cistus monspeliensis*, and *Launaea arborescens* for the control of *P. interpunctella*. We assessed the effect of methanol extracts from these plants on post-embryonic development, reserve substances levels and gut enzymatic activities, in order to have some insights about the physiological responses of the insect to the ingestion of the plant extracts.

Material and methods

Insect rearing

P. interpunctella used in this study originated from a laboratory culture reared for many generations on wheat (*Triticum aestivum*) flour as a medium. All stages of the insect were kept under standard conditions at 26-30 °C, 55-65% relative humidity and a photoperiod of 16:8 (L:D). Adults were collected for oviposition and eggs were hatched in 0.25-L glass containers half full of wheat flour. In the present study, 5th instar larvae weighing 3.4 ± 0.2 mg were used in all assays.

Plant materials and extracts preparation

Methanol extracts were prepared from ten plants commonly used in traditional medicine in Morocco. The plants tested were chosen based on previous records of insecticidal activity or because they belong to plant families that are known to be rich in secondary metabolites, being all of them readily available in Tangier Region (NW of Morocco). Seeds of *P. harmala* (Zygophyllaceae) and aerial parts of *A. iva* and *R. officinalis* (Lamiaceae), *L. stoechas* and *L. dentata* (Lamiaceae), *C. ladanifer*, *C. salviaefolius* and *C. monspeliensis* (Cistaceae), *C. erythraea* (Gentianaceae) and *L. arborescens* (Compositae) were collected during February-March period. The plants were identified by Professor Ennabili Abdessalam (Institut National des Plantes Aromatiques et Médicinales de Taounate, Morocco). Plants were rinsed with distilled water, dried in an oven at 38 °C for 72 h, ground to a powder with an electrical blender and stored at room temperature in hermetically sealed plastic bags prior to extraction. Each plant sample (20 g) was extracted twice with 160 mL methanol to gain polar and medium polar metabolites by using ultrasound apparatus for 30 min. All extracts were stored at 4 °C. Before using, extracts were evaporated to dryness, and the residue was weighted

and redissolved in the same solvent at a concentration of 100 mg of crude extract mL^{-1} of methanol.

Bioassays

Methanol extracts were mixed with 5 g of the diet (wheat flour) at a concentration of 500, 750 or 1,000 ppm, depending on the experimental conditions (see below). The solvent was allowed to evaporate at 37 °C during 48 h.

To study the effect of the methanol extracts on post-embryonic development, 25-5th instar larvae were added to each glass vial (diameter 4 cm, height 7 cm) containing diet treated at 500 ppm. A control was prepared in the same way with methanol alone. To verify that the effect was due to plant extracts ingestion and not to a deterrency effect, another experimental group (starved) was generated by transferring larvae into clean glass vials with no food. Ten replicates were set up for the treated, starved and control larvae. Observations including larval weight, cannibalism, pupation and adult emergence were recorded. Mortality was determined by brown colour with no observable movements. Cannibalism was noted by disappearance of body larvae from Petri-dishes. Larvae and pupae were maintained at the conditions described above during the whole experiment.

For biochemical analysis, 5th instar larvae were reared individually for 8 days in diets treated with the different extracts tested. Control and starved larvae were also reared individually for 8 days as described above. For analyses of reserve substances, plant extracts were tested at 500 ppm, and each sample was composed of three cold-anesthetized larvae. For digestive and detoxification enzyme activities, plant extracts were tested at 500, 750 and 1,000 ppm, and larvae were dissected and the midguts individually stored frozen at –20 °C until needed. Ten and 20 replicates per concentration were done for reserve substances and enzymatic activities, respectively.

Biochemical analyses

Protein content was determined according to the method of Bradford (1976). Three larvae were homogenized in 1 mL of Tris-HCl buffer (50 mM, pH 7). The homogenate was centrifuged at 9000 g for 20 min. A volume of 200 µL of extract was mixed with 2 mL of Bradford reactive. The absorbance was measured at 595 nm and protein levels calculated using bovine serum albumin as the standard.

Carbon hydrate content was quantified by the method of Roe (1955) using anthrone as reagent. Three larvae were homogenized in 100 µL of NaCl 125 mM. A volume of 25 µL was mixed with 1 mL of ethanol saturated with sodium sulphate. It was then centrifuged at 1,000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 0.5 mL of 70% ethanol, then centrifuged as before and the supernatant was discarded. The final pellet was heated to drive off residual ethanol and then dissolved in 0.5 mL of 30% potassium hydroxide and heated at 100 °C for 15 min. The digest was allowed to cool and 1 mL of absolute ethanol was added. This was spun down by centrifugation at 1,000 rpm for 10 min. The supernatant was carefully removed and the pellet was dissolved in 0.5 mL of distilled water. Two mL of anthrone reagent (0.05% in sulfuric acid) was then added. After mixing, the samples were heated at 90 °C for 15 min. They were rapidly cooled. The absorbance was read at 620 nm and carbon hydrate levels were calculated by reference to a standard curve using glycogen.

Lipids were extracted according to the method of Van Handel (1965). Three larvae were homogenized in 1 mL of chloroform/methanol (1:1 v/v). The homogenate was centrifuged at 1,000 rpm for 10 min at 4 °C. The supernatant was mixed with 1 mL of chloroform and 0.5 mL of distilled water and centrifuged at 1,500 rpm for 1 h at 4 °C. The aqueous phase was discarded (this operation was repeated twice). The lipid extract was evaporated to dryness. Lipid content was quantified by the method of Zöllner & Krich (1962). The sample was digested with 1 mL of sulphuric acid at 100 °C for 10 min. The tube was cooled and 5 mL of sulphosphovanillin reagent (orthophosphoric acid/0.6% aqueous vanillin solution 4:1) was added to the mixture. After 40 min, the absorbance was measured at 530 nm and lipid level was calculated by reference to a standard curve prepared using cholesterol palmitate.

Proteolytic activities were determined using specific substrates as described by Ortego *et al.* (1996). Trypsin-like activity was assayed using 0.25 mM BApNa (Nα-benzoyl-DL-arginine p-nitroanilide) at pH 10.5 and incubating for 1 h, chymotrypsin-like activity with 0.25 mM SA_2PPpNa (N succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide) at pH 10.5 and incubating for 1 h, elastase-like activity with 0.25 mM SA_3pNa (N-succinyl-alanine-alanine-alanine p-nitroanilide) at pH 10.5 and incubating for 24 h, leucine

aminopeptidase-like activity with 1 mM LpNa (L-leucine p-nitroanilide) at pH 7 and incubating for 1 h, carboxypeptidase A-like activity with 1 mM HPA (hippuryl-phenylalanine) at pH 7 and incubating for 4 h, and carboxypeptidase B-like activity with 1 mM HA (hippuryl-L-arginine) at pH 7 and incubating for 4 h.

Alpha-amylase activity was measured by the method of Valencia *et al.* (2000). A volume of 50 µL of midgut extract was added to 50 µL of 50 mM sodium citrate buffer (NaCl 10 mM, $CaCl_2$ 20 mM), pH 8.5. To the mixture was added 100 µL of 0.5% starch solution. The sample was incubated at 37 °C during 15 min. Then 2.5 mL of iodide (0.5% I_2 and 5% KI) was added. It was centrifuged at 4,000 rpm for 20 min. The absorbance of the supernatant was read at 580 nm. The α-amylase activity was calculated by reference to a standard curve prepared using starch solution.

Esterase activity was determined using 1-NA (1-naphtyl acetate) and MtB (S-methyl thiobutanoate) as substrates. For activity towards 1-NA, 5 µL of midgut extract were added to 160 µL of reaction mixture (pH 7) to give a final concentration of 0.25 mM 1-NA, and the mixture incubated 1 h at 30 °C. The reaction was stopped by adding 80 µL of 0.12% (w/v) o-dianisidine bis (diazotized) zinc double salt (Fast Blue B Salt, FBB) and 2% (w/v) sodium dodecyl sulphate (SDS). The absorbance was read at 600 nm. Specific activity was calculated by reference to the procedure described by Gomori (1953) using a standard curve of 1-naphthol accordingly. Esterase activity towards MTB was measured using a reaction mixture that contained 5 µL of midgut extract, 0.1 M phosphate buffer pH 7.2 mM MtB and 2.5 mM DTNB (5'5 dithio-bis (2-nitrobenzoic acid)). The increment in absorbance at 412 nm was recorded during 15 min at 30 °C to determine the nmol substrate hydrolyzed min^{-1} mg^{-1} protein, using a molar extinction coefficient of 13,600 M^{-1} cm^{-1} (Ellman, 1959).

Glutathione S-transferase activity was measured using 40 µL of midgut extract, 1 mM CDNB (1-chloro-2,4-dinitrobenzene), 5 mM reduced glutathione, and 0.1 M Tris buffer pH 8. The increment in absorbance at 340 nm was recorded during 5 min at 30 °C to determine the nmol substrate conjugated per min per mg protein, using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig *et al.,* 1974).

P450 monooxygenases activity was assayed using 50 µL of midgut extract, 130 µL NADPH generating system at pH 7, and 20 µL of cytochrome C solution, and the mixture incubated 1 h at 30 °C. The reaction was stopped by adding 100 µL of methanol, and then centrifuged at 12,000 rpm for 5 min. The absorbance was read at 550 nm to determine the nmol cytochrome c reduced min^{-1} mg^{-1} protein, using a molar extinction coefficient of 27.6 mM^{-1} cm^{-1} (Masters *et al.,* 1965).

Statistical analysis

Statistical analyses were performed using Statistica (1997) software. Cannibalism, pupation and adult emergence data (percentages) were analyzed by one-way ANOVA, after logit transformation, followed by Dunnett's test for multiple comparisons with respect to the control. Larval weight and biochemical parameters data were analyzed using one-way ANOVA followed by Dunnett's test. The significance was tested at the $p = 0.05$ level.

Results

Effects of plant extracts on post-embryonic development of *P. intepunctella*

At the beginning of the experiment, the average weight of 5th instar larvae was 3.4 ± 0.2 mg. After 10 days, the weight of larvae feeding on control diet reached 7.3 mg (115% weight gain), whereas the weight of starved larvae decreased to 1.1 mg (68% weight loss) (Table 1). During the same period, the weight of larvae feeding on diet treated with the different plant extracts tested was significantly reduced when compared with the control group. In fact, most of the treatments led to a net weight loss: 32%, 18%, 21%, 6%, 33%, 32%, and 12% for larvae treated by *P. harmala*, *C. ladanifer*, *L. dentata*, *R. officinalis*, *C. erythraea, A. iva* and *L. stoechas*, respectively. Extracts of *R. officinalis* and *C. erythraea* induced a marked cannibalism (20-25%) between larvae, similar to that observed for starved larvae (30%) (Table 1). There was no cannibalism between larvae fed the control diet, and for the rest of the treatments was below 5%.

Pupation of control larvae was 90%, whereas it was completely abolished in starved larvae (Table 1). The percentage of pupation varied among treatments (ranging from 5% to 85%), being significantly lower than in the control for all treatments, except for *C. monspeliensis*. The higher reductions in pupation corresponding to larvae treated with *P. harmala* and *C. ladanifer*.

Table 1. Effect of ingestion of diet treated with plant extracts (500 ppm) on post-embryonic development of *P. interpunctella*

Treatment	Larval weight[1] (mg)	Cannibalism[2] (%)	Pupation[3] (%)	Adult emergence[4] (%)
Control	7.3 ± 0.2	0 ± 0.0	90 ± 1.2	90 ± 0.7
P. harmala	2.3 ± 0.1*	5 ± 0.3*	5 ± 0.3*	0 ± 0.0*
C. ladanifer	2.8 ± 0.1*	5 ± 0.4*	5 ± 0.3*	0 ± 0.0*
L. dentata	2.7 ± 0.1*	2.5 ± 0.2*	67.5 ± 1.3*	27 ± 1.0*
R. officinalis	3.2 ± 0.1*	25 ± 0.7*	20 ± 0.9*	2 ± 0.1*
C. salviaefolius	4.3 ± 0.1*	7.5 ± 0.2*	75 ± 0.9*	45 ± 1.4*
C. monspeliensis	4.1 ± 0.1*	5 ± 0.3*	85 ± 1.0	68 ± 1.6*
C. erythreae	2.3 ± 0.1*	20 ± 0.7*	14 ± 0.5*	2 ± 0.1*
A. iva	2.3 ± 0.1*	5 ± 0.1*	20 ± 0.9*	1.5 ± 0.2*
L. stoechas	3.0 ± 0.1*	5 ± 0.3*	65 ± 1.2*	26 ± 1.7*
L. arborescens	3.5 ± 0.1*	5 ± 0.2*	75 ± 1.3*	67.5 ± 2.0*
Starved	1.1 ± 0.1*	30 ± 1.1*	0 ± 0.0*	0 ± 0.0*

Values are the mean ± standard error of [1] larval weight 8 days after treatment, [2] cannibalism 16 days after treatment, [3] pupation 20 days after treatment, [4] adult emergence 26 days after treatment (10 replicates of 25 larvae each). * Significantly different from control (Dunnet test, $p \leq 0.05$).

In the same way, adult emergence was significantly affected by the presence of plant extracts in the larval diet (Table 1). Larval starvation and treatment with *P. harmala* and *C. ladanifer* completely inhibited adult emergence. Strong effects on adult emergence (below 2.5%) were also obtained with larvae treated with *R. officinalis, C. erythraea* and *A. iva*.

Effects of plant extracts on energy reserves and enzymatic activities

The plant extracts that showed strongest effects on post-embryonic development were selected to test their effects on reserve substances levels and enzymatic activities of *P. interpunctella* larvae.

Protein content was severely reduced in larvae fed on treated diets and on starved larvae as compared with the control group; being the strongest effect for larvae treated with *C. erythraea*, *P. harmala* and *C. ladanifer* (Table 2). Plant extracts induced also a decrease in carbon hydrate level, but the reduction was smaller than that observed for starved larvae (Table 2). The lipid content was not affected by the different plants extracts tested, whereas it was significantly reduced in starved larvae (Table 2).

Our results showed that the specific activity for the hydrolysis of BApNa, SA_2PPpNa, SA_3pNa, LpNa, HPA, HA and starch was significantly reduced in starved larvae compared to control larvae, and all extracts tested produced a decrease in a dose depended manner (Table 3). The most remarkable reductions occurred for trypsin-, carboxypeptidase A- and B-, and α-amylase-like activities, with inhibitions over 50% for some plant extracts. The only exception was a significant increase in the hydrolysis of HPA in larvae that have ingested *R. officinalis* at 500 ppm.

On the contrary, different patterns of activity were obtained for the detoxification enzymes after ingestion of plant extracts (Table 3). Esterase activity using 1-NA as substrate increased in starved and in treated larvae at the low (500 ppm) concentration tested, but decreased when the concentration of the plant extracts was 1,000 ppm. However, esterase activity using MtB as substrate increase in starved and in treated larvae at all concentrations tested. Glutathione S-transferase increased in treated larvae, but not in starved. P-450 monooxygenase activity increase only at 500 ppm for

Table 2. Effect of ingestion of diet treated with plant extracts (500 ppm) on protein, carbon hydrate and lipid content of *P. interpunctella* larvae

Treatment	Protein (µg/larvae)	Carbon hydrate (µg/larvae)	Lipid (µg/larvae)
Control	545 ± 21	35.6 ± 4.1	846 ± 25
C. erythreae	117 ± 10*	11.8 ± 0.7*	852 ± 28
P. harmala	121 ± 11*	7.8 ± 0.7*	880 ± 25
C. ladanifer	133 ± 12*	11.3 ± 0.7*	870 ± 25
A. iva	210 ± 15*	12.4 ± 1.0*	843 ± 25
R. officinalis	273 ± 17*	15.8 ± 0.7*	833 ± 18
Starved	187 ± 16*	2.9 ± 0.2*	570 ± 23*

Values are the mean ± standard error of protein, carbon hydrate and lipid content (n = 10). * Significantly different from control (Dunnet test, $p \leq 0.05$).

Table 3. Effect of ingestion of diet treated with plant extracts on midgut hydrolytic and detoxification enzyme activities of *P. interpunctella* larvae

Specific activity[1]	Control	*C. erythreae*			*P. harmala*			*C. ladanifer*			*A. iva*			*R. officinalis*			Starved
		500 ppm	750 ppm	1000 ppm	500 ppm	750 ppm	1000 ppm	500 ppm	750 ppm	1000 ppm	500 ppm	750 ppm	1000 ppm	500 ppm	750 ppm	1000 ppm	
TRY (BApNa)	200±5	156±4*	126±3*	113±4*	174±3*	131±4*	98±4*	160±4*	130±5*	119±4*	188±4	158±3*	65±4*	187±6	157±4*	132±4*	83±3*
CHY (SA_2PPpNa)	955±8	895±8*	810±7*	708±10*	740±7*	680±9*	643±8*	850±7*	781±7*	620±6*	860±6*	782±10*	742±8*	875±7*	741±7*	711±7*	680±8*
ELA (SA_3pNa)	11.6±1.1	7.6±0.5*	6.8±0.4*	6.4±0.5*	7.7±0.5*	7.0±0.6*	6.4±0.3*	8.8±0.4*	7.5±0.6*	7.4±0.3*	8.2±0.4*	8.4±0.4*	7.4±0.3*	8.5±0.5*	7.3±0.3*	7.4±0.3*	4.7±0.4*
LAP (LpNa)	248±8	200±6*	179±5*	153±4*	203±4*	183±5*	165±6*	210±6*	177±4*	161±5*	210±6*	181±7*	170±5*	203±6*	187±7*	172±4*	92±5*
CPA (HPA)	192±4	171±5*	153±6*	146±4*	150±6*	112±5*	79±3*	176±5	150±5*	134±5*	179±4	153±5*	121±6*	224±6*	196±5	178±6	114±4*
CPB (HA)	109±5	85±4*	70±4*	63±3*	80±4*	75±5*	63±3*	62±3*	51±3*	40±3*	105±4	89±3*	73±4*	98±4	73±3*	61±3*	55±3*
α-AMY (starch)	10.0±0.4	6.0±0.2*	6.0±0.6*	5.0±0.5*	6.0±0.2*	3.2±0.3*	2.0±0.2*	6.5±0.4*	5.5±0.5*	3.0±0.2*	6.0±0.2*	4.6±0.3*	4.0±0.2*	4.9±0.1*	5.2±0.4*	4.4±0.5*	7.0±0.5*
EST (1-NA)	647±8	733±9*	682±14*	600±9*	753±8*	710±8*	629±6	724±6*	662±8	600±6*	716±9*	692±6*	612±8*	703±6*	689±11*	648±8	724±6*
EST (Mtb)	23±1	46±2*	52±3*	36±2*	41±2*	55±3*	46±2*	37±2*	42±2*	40±2*	39±2*	43±2*	39±2*	41±3*	48±3*	42±3*	33±2*
GST (CDNB)	352±5	433±8*	549±8*	645±11*	443±8*	531±8*	600±8*	466±7*	583±9*	624±9*	448±7*	526±9*	594±10*	437±8*	523±10*	584±8*	339±8*
P450 (cytochome C)	53±2	66±2*	55±2	42±2*	68±3*	62±2	46±2	63±3*	54±3	50±2	67±3*	58±3	44±2*	62±3	56±3	51±3	47±3

[1] Specific activities as nmoles of substrate hydrolyzed or modified/min/mg protein for: TRY (Trypsin), CHY (Chymotrypsin), ELA(Elastase) LAP (Aminopeptidase), CPA (Carboxypeptidase A), CPB (Carboxypeptidase B), α-AMY (α-amylase), EST (Esterase), GST (Glutathion-S-Transferase), and P450 (P450 Monooxigenase). Values are the mean ± SE (n = 20). * Significantly different from control (Dunnet test, $p \leq 0.05$).

some of the plant extracts tested, whereas a significant inhibition was obtained when the larvae were exposed to *C. erythraeae* or *A. iva* at 1,000 ppm.

Discussion

The present work revealed the insecticidal effect of plants commonly found in Morocco, and corresponding to different botanical families, on *P. interpunctella* larvae. Indeed, the insecticidal activity of methanol extracts from these plants was manifested in larval weight loss, cannibalism between larvae, reduction of pupation and adult emergence. The most active plant species were *P. harmala* and *C. ladanifer*, with which total inhibition of adult emergence was obtained. A strong effect (adult emergence below 2%) was also obtained with *A. iva*, *C. erythreae* and *R. officinalis*. Similar disturbances on *P. interpunctella* larvae have been reported after ingestion of 20-hydroxyecdysone (Rharrabe *et al.,* 2009), azadirachtin (Rharrabe *et al.,* 2008), harmaline (Rharrabe *et al.,* 2007) and harmine (Bouayad *et al.,* 2012). Interestingly, cannibalistic behaviour was observed in starved larvae and in larvae exposed to plant extracts, especially *C. erythreae* and *R. officinalis*. Cannibalism is a common phenomenon in many species of insects (Polis, 1998) that increases in stressful conditions, such as when density of populations is high or food is scarce (Via, 1999). Accordingly, the insecticidal effects of plant extracts on *P. interpunctella* larvae appear to provoke increased cannibalism in comparison with larvae reared on control diet. Likewise, Rharrabe *et al.* (2007) reported that the presence of toxic plant allelochemicals in diet

causes the appearance of this behavior among larvae of *P. interpunctella*.

After plant extract ingestion, a significant reduction of reserve substances in *P. interpunctella* larvae was observed, especially in protein and carbon hydrate contents. Overall, the depletion of energy reserves could be due to a reduction in plant consumption, or to high mobilization of these primary metabolites to compensate the metabolic stress produced by the toxic effects of the plant extracts. A depression of energy reserves has also been reported after ingestion of Jatropherol-I by *B. mori* (Jing *et al.,* 2005), neem oil by *Choristoneura rosaceana* (Smirle *et al.,* 1996), *P. harmala* leaves by *T. castaneum* (Jbilou *et al.,* 2008), and azadirachtin by some insects such as *S. litura* (Huang *et al.,* 2004). Moreover, reductions in energy reserves were reported in *P. interpunctella* after ingestion of harmine (Bouayad *et al.,* 2012), harmaline (Rharrabe *et al.,* 2007), azadirachtin (Rharrabe *et al.,* 2008) and 20-hydroxyecdysone (Rharrabe *et al.,* 2009). Remarkably, we have found that lipid reserves were not affected by ingestion of diets treated with plant extracts, whereas a reduction was observed in starved larvae. In general, the lipid content of the fat body increases continuously during the larval period of holometabolous insects to be used during metamorphosis as well as to provide reserves for the new adult to support flight and embryogenesis (Arrese *et al.,* 2001; Canavoso *et al.,* 2001). Moreover, lipid metabolism provides the energy needed during extended starvation periods (Arrese & Soulages, 2010). However, our results suggest that protein and carbohydrates provided primary sources for energy in *P. interpunctella* larvae under extreme nutritive stress conditions, whereas lipid metabolism was not mobilized. Previous studies showed a reduction of lipid reserves in *P. interpunctella* after ingestion of azadirachtin and 20-hydroxyecdysone (Rharrabe *et al.,* 2008, 2009), but not after ingestion of harmine (Bouayad *et al.,* 2012). Nevertheless, we cannot discard effects on specific classes of lipids (as triacylglycerols, diacylglycerols and phospholipids), since we have only analyzed total lipid content.

In lepidopteran insects, digestion of proteases is mainly based on serine proteases (trypsin-, chymotrypsin- and elastase-like) and exopeptidases (aminopeptidase and carboxypeptidases A- and B-like) (Terra & Ferreira, 1994), whereas carbohydrate hydrolysis is due to α-amylases (Franco *et al.,* 2002). All these enzymatic activities have already been characterized in *P. interpunctella* (Baker, 1986; Oppert *et al.,* 1996). Production of digestive enzymes has been related to food intake in different insect species (Lehane *et al.,* 1996), including larvae of *P. interpunctella* (Bouayad *et al.,* 2008). However, our results showed that α-amylase and all proteolytic activities were reduced in *P. interpunctella* larvae after ingestion of diet treated with plant extracts, compared with control larvae. These results are in agreement with those reported by Senthil Nathan *et al.* (2005a,b), who demonstrated that ingestion of azadirachtin causes a decrease in digestive enzymatic activities in the midgut of *S. litura* and *C. medinalis*. In the same context, Senthil Nathan (2006) revealed that the toxic effects of *Melia azedarach* in *C. medinalis* were mediated through inhibition of digestive enzyme activities. This reduction of activity may be due to several reasons. The first hypothesis is the inhibition of the activity of the hydrolytic enzymes themselves. Indeed, several studies have already shown that plant extracts inhibit *in vitro* the activity of α-amylase and proteases (Duffey & Stout, 1996). However, there are also examples of plant secondary metabolites that inhibit the activity of insect digestive enzymes in vivo, but not *in vitro*, suggesting alternative target sites for these compounds (Liu *et al.,* 2008, 2009). The second hypothesis is that plant secondary metabolites cause cytotoxicity in epithelial cells synthesizing these digestive enzymes. In fact, severe disorganization of proteosynthetic organelles of the midgut epithelial cells of *P. interpunctella* larvae was found after harmaline or 20-hydroxyecdysone ingestion (Rharrabe *et al.,* 2007, 2009). Marked cytotoxicity in intestinal epithelium cells has also been demonstrated in other insects caused by azadirachtin (Nasiruddin & Mordue (Luntz), 1993) and seed extract of *P. harmala* (Jbilou *et al.,* 2008). A third hypothesis may be a reduction of enzymatic activity due to a decrease in food ingestion, since we have found that the activity of digestive enzymes in starved larvae was also reduced significantly. A reduction of enzyme activities has been linked with the lack of food intake in several insect species (Baker *et al.,* 1984; Hill & Orchard, 2005). A significant decrease of α-amylase activity was also described after seven days of starvation in *P. interpunctella* larvae (Rharrabe *et al.,* 2007).

Induction of detoxification metabolic system plays an important role in insects host plants adaptation (Terriere, 1984). For example, Weimin *et al.* (2003) demonstrated that P450 monooxygenases in swallowtail caterpillars that feed on furanocoumarin-containing host plants display higher activities against fura-

nocoumarins than those from species that feed on plants lacking these compounds. However, detoxification enzyme systems may also be inhibited by secondary plant metabolites, such as flavonols, isothiocyanates, polyphenols, hydroxamic acids and terpenoids (Yu, 1983; Brattsten, 1987). Our results show that the activity of detoxification enzymes in *P. interpunctella* larvae was altered after ingestion of plant extracts. Thus, GST activity increased significantly in a dose-dependent manner with all treatments. These enzymes have been reported to be induced by plant allelochemichals in a number of insects (Yu, 1983; Ortego *et al.,* 1999; Caballero *et al.,* 2008) and are considered major elements conferring resistance against insecticides (Oppenoorth *et al.,* 1977; Kao & Sun, 1991; Sun *et al.,* 2001). However, the results obtained with esterase and P450 monooxygenase activities in *P. interpunctella* larvae varied depending on the concentration of the plant extract tested and the substrate used. The increase in esterase activity at low concentrations may be due to a general induction of esterase genes in response to secondary metabolites ingestion, while the inhibition with higher concentrations, when using 1-NA as substrate, could be due to a direct inhibitory effect of these compounds on specific esterase enzymes. The inhibition of esterase activity with increasing plant extract concentrations seem to be in agreement with the results reported by Jing *et al.* (2005) who observed that esterases showed lower activity after ingestion of Jatropherol-I in *B. mori*. Similar results were obtained in *C. rosaceana* after neem oil treatment (Smirle *et al.,* 1996) and in *L. decemlineata* larvae fed with potato leaves treated with limonoids (Ortego *et al.,* 1999). A wide variety of plant chemicals have been shown to induce P-450 monooxygenase activity in insects (Scott *et al.,* 1998). An induction of these enzymes was also observed in *P. interpunctella* larvae treated with some of the plant extracts tested. However, a significant inhibition of P-450 monooxygenase activity was obtained when the larvae were exposed to *C. erythraeae* or *A. iva* at 1,000 ppm. Taking together, these results indicate that *P. interpunctella* larvae respond to the ingestion of plant extracts by the induction of detoxification enzymes, but some of the plant allelochemicals may be able to inhibit some of these enzymes. An equilibrium between induction and inhibition of detoxification enzymes may then occurs that results in an increase or a reduction of the enzymatic activity depending on the concentration of the plant extract ingested. In any case, the detoxification process is not capable to counteract the insecticidal effect of these plant extracts, resulting in the death of the larvae after a few days.

In summary, this work shows the potential of plants commonly found in Morocco for the control of *P. interpunctella*. A remarkable bioinsecticidal effect was obtained with methanol extracts from *P. harmala* and *C. ladanifer* that completely abolished adult emergence. The alteration of several physiological parameters (digestive and detoxification enzymes and energy reserves) provides some insights about the mode of action of the allelochemicals in the plant extracts.

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