Research Article

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Antifeedant activity of *Jatropha gossypifolia* and *Melia azedarach* senescent leaf extracts on *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and their potential use as synergists

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Abstract

BACKGROUND: To reduce rates of synthetic insecticide applications, natural product alternatives and synergists are needed. A study has been made of the toxicity of ethanolic senescent leaf extracts (SLEs) of *Jatropha gossypifolia* and *Melia azedarach* on larvae of the noctuid pest *Spodoptera frugiperda*. Their effects as syngergists and inhibitors of several enzyme activities are also reported.

RESULTS: When added to the diet, *M. azedarach* SLE showed lower toxicity than *J. gossypifolia* SLE. However, after 2 weeks on the diet, the *M. azedarach* SLE proved to be lethal to 100% of the larval population. Artificial diets with both SLEs have an antifeedant effect on armyworm larvae. Acute toxicity after topical application in a dipping assay was relatively low for both *J. gossypifolia* and *M. azedarach* SLEs (LC_{50} of 2.6 and 1.4 g L^{-1}, respectively, after 24 h). However, mixtures of the SLEs of *M. azedarach* and *J. gossypifolia* had a strong synergistic effect with cypermethrin. Synergism was higher with the *J. gossypifolia* SLE, perhaps because it contains several natural products with a methylenedioxyphenyl moiety. Both extracts inhibited P450, general esterase and acetylcholinesterase activities *in vitro* and *in vivo*.

CONCLUSION: Both *J. gossypifolia* and *M. azedarach* SLEs are antifeedants to armyworm larvae when present in the food, and also have a synergistic effect with cypermethrin in topical assays. Although the synergistic effect is less than with piperonyl butoxide, both SLEs have some inhibitor activity against detoxification enzymes and acetylcholinesterase. Thus *J. gossypifolia* and *M. azedarach* SLEs may be considered as ecofriendly approaches for the control of *S. frugiperda* in order to reduce cypermethrin usage.

Keywords: *Spodoptera frugiperda*; *Jatropha gossypifolia*; *Melia azedarach*; cypermethrin; piperonyl butoxide; detoxification enzymes; acetylcholinesterase

1 INTRODUCTION

Noctuidae is the largest family in the order Lepidoptera, and it contains some of the most destructive insect pests of agriculture. The fall armyworm *Spodoptera frugiperda* (J.E. Smith) is a major pest of tropical–subtropical origin in the Western Hemisphere. Its larvae feed on a variety of plants, corn, rice, peanuts, sorghum, Bermudagrass and cotton in many countries.[1,2] Almost all agronomic crops in many countries are vulnerable to infestations every year by migrant populations of *S. frugiperda* moths carried by winds, causing outbreaks of this pest species. To control their populations, an integrated pest management scheme is required for efficient, low-residue, cost-effective management of fall armyworm populations. Insecticides applied to control *S. frugiperda* on cotton and corn are primarily directed against the deleterious larval stages. Pyrethroids are currently among the major insecticides used against this insect pest. Cypermethrin is a pyrethroid insecticide, first synthesised in 1974.[3] It is a synthetic chemical similar to the pyrethrins found in pyrethrum extract from the chrysanthemum plant. Cypermethrin is widely used in the control of pests such as termites, household pests and some agricultural pests. Soil microbes rapidly break down cypermethrin, but cypermethrin has high toxicity to bees, fish and other aquatic organisms.[3,4] Thus, efforts to reduce its use can be beneficial to the environment. Although synthetic insecticides such as cypermethrin are fast acting, highly active and cost effective, their use in integrated pest management programmes is made difficult by their wide spectrum of activity, and therefore toxicity to natural enemies. Pyrethroid use has

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also caused major problems of insecticide resistance. Therefore, new tools of insect pest management are needed, and botanical insecticides as well as plant-derived semiochemicals are now actively sought as leads towards more ecofriendly alternatives. Several plant extracts have been evaluated for their activity against important agricultural pests for a few decades in different countries, especially in Thailand. Numerous insecticidal plants and semiochemicals from plants are recorded as good candidates for insect control.[5] *Jatropha gossypifolia*, the bellyache bush, is a tropical plant species of the Euphorbiacea family originating from South America and used in folk medicine.[6] Various medicinal and pesticidal properties have been attributed to this species.[7–9] Different parts of the plant have been examined chemically, and this revealed that it contains many complex lignans and diterpenoids.[10–13] Extracts of chinaberry, *Melia azedarach*, are also known to have various effects on more than 30 species of insects,[14] e.g. growth retardation, reduced fecundity, moulting disorders, morphogenetic defects and changes in behaviour. Like many Meliaceae plants, it contains a variety of insecticidal triterpenoids, such as bakayanin, meliacarpinins 1 to 3 and azadirachtin, as well as antifeedants or growth regulators.[15] However, so far there are no reports on the insecticidal activity of leaf extracts of either *M. azedarach* or *J. gossypifolia* against *S. frugiperda* larvae. Insecticide synergists have been recommended as powerful research tools for diagnosing resistance mechanisms, determining the confirmation of target sites and elucidating metabolic pathways.[16] The first commercial synergist was introduced in 1940 to increase the effectiveness of the botanical insecticide pyrethrum. Since that time it has been well established that synergists increase the effectiveness of insecticides by reducing their metabolism by detoxifying enzymes. Probably the best known and the most used synergistic compound is piperonyl butoxide (PBO), an inhibitor of cytochrome P450 enzymes or microsomal oxidases.[17–21] Apart from acting as insecticides, plant extracts can also demonstrate synergistic activity. This was shown with synthetic pyrethroids against different pests[22,23] and vectors.[24] While most studies focus on fresh plant material, senescent leaves are being used here as the starting material because they constitute a non-destructive collection of readily available plant material that is normally lost to decay. In addition, leaf extracts of *M. azedarach* are reported to have a stronger toxicity to *Aedes aegypti* than the fruit extract,[25] or similar insecticidal and antifeedant activity to *Xanthogaleruca luteola*.[26] Even though in some cases the fruit may have more active ingredients than leaves, collecting senescent leaves may have advantages over collection of the fruit. Here, the toxicity of *J. gossypifolia* and *M. azedarach* senescent leaf extracts and their antifeeding effect against *S. frugiperda* larvae are studied, and the synergistic approach is extended further. The present results emphasise the larvicidal activity and antifeeding activity of *J. gossypifolia* and *M. azedarach* senescent leaf extracts, alone or in combination with cypermethrin, an efficient synthetic pyrethroid against *S. frugiperda* larvae. In addition, the mode of action on acetylcholinesterase (AChE) and on detoxification enzymes such as cytochrome P450 monooxygenases (P450) and esterases is explored.

2 MATERIALS AND METHODS

2.1 Mass rearing of *S. frugiperda*

Larvae of *S. frugiperda* used in the experiments were maintained in an environmental control room at 25 °C, 70% RH and 12 : 12 h light : dark photoperiod. The artificial diet used for the bioassay contained 150 g of agar, 10 g of sorbic acid, 50 mL of sunflower oil, 500 g of chickpea flour, 125 g of yeast, 125 g of wheat germ, 30 g of ascorbic acid, 30 mL of antibiotic and 10 mL of formaldehyde in a total water volume of 6 L.

2.2 Extraction procedure

Senescent leaves of *Melia azedarach* and *Jatropha gossypifolia* were collected from June to July 2009 from the central part of Thailand and air dried. Dried leaves (15 g) were extracted with 95% ethanol in a Soxhlet apparatus for 8 h. Extracts were separated from the solvents with a vacuum rotary evaporator (BUCHI® model R-210/R-215, V-700+ V-850) set at 175 mbar and 40 °C. The extracts were then freeze dried to produce a solidified crude residue. Residues obtained from each material were dissolved in ethanol independently to yield stock solutions of 40 g L^{-1}. Different test concentrations for larval exposure were prepared by further diluting these stocks with 70% ethanol.

2.3 Toxicity assay

Twenty third-instar *S. frugiperda* larvae were individually dipped in 10 mL beakers containing 5 mL of test concentration in 70% ethanol for 1 s. For the control group, the larvae were dipped in 70% ethanol. They were then immediately placed on filter paper. The tested larvae were then moved into each well of a 24-well microplate that had been filled with 1 mL of artificial diet. Experiments were set up and developed for each extract using a parallel control. Mortality observations were noted 24 h and 48 h post-treatment. Cypermethrin (25% EC) was purchased from Fluka®, and piperonyl butoxide (PBO) from Sigma®. Each chemical was diluted in ethanol to obtain a 1 mg L^{-1} stock solution. Different test concentrations were prepared by diluting with 70% ethanol. The toxicity assays against larvae were performed as described above. Mortality data were recorded 24 h and 48 h post-treatment. Finally, for combination-based studies, each concentration of cypermethrin and each concentration of plant extract preparation or of PBO were prepared in a ratio of 1 : 1. Larvicidal efficacy of each formulation was estimated as above, and mortality was noted 24 h and 48 h post-exposure.

2.4 Antifeedant toxicity assay

The antifeeding effect and mortality were estimated through a no-choice assay. For each concentration, use was made of 72 fourth-instar *Spodoptera frugiperda* larvae starved for 25 h before the start of the experiment. Leaf extracts of *Jatropha gossypifolia* and *Melia azedarach* were diluted in 1 mL of 70% ethanol and were then mixed with 40 mL of artificial diet to final concentrations of 0.4–40 g L^{-1}. For the control group, 1 mL of 70% ethanol was mixed with 40 mL of artificial diet. Each extract–diet mix was dispensed in 24-well plates at a volume of 1 mL per well, and larvae were placed individually in each well. The experiment was done under laboratory conditions at 28 °C and 70% RH. Mortality was recorded every day, whereas the weight of larvae was recorded every 3 days.

2.5 Statistical analysis of mortality response

Mortality data obtained for the toxicity bioassays were analysed by probit analysis to obtain regression equations, LC_{50} values with standard errors and 95% confidence limits using Stat Plus program® v.2008. The synergistic factor (SF) for the mixed

formulation was also computed after calculating the LC_{50} for each combination following the method used by Mohan *et al*.,[27] Sarup *et al*.[28] and Kalyansundaram and Das:[29]

$$\text{Synergistic factor (SF)} = \left[\frac{LC_{50}\text{of cypermethrin alone}}{LC_{50}\text{of cypermethrin} + \text{SLE}}\right]$$

A value of SF > 1 indicates synergism, while SF < 1 indicates antagonism.

2.6 Mode-of-action studies

2.6.1 Enzyme extraction method

To measure cytochrome P450 (P450) *in vitro* activities, sixth-instar larvae were starved for 1 h. The midguts were dissected and rinsed, and were then homogenised in buffer A [100 mM phosphate buffer (pH 7.2) containing 1 M of DTT, 100 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 0.5 M of EDTA). The homogenate was centrifuged at 10 000 × *g* for 5 min at 4 °C, and the resulting supernatant was used for esterase activity analysis or further centrifuged at 100 000 × *g* for 1 h at 4 °C. Finally, the pellet was resuspended in buffer B (buffer A + 10% glycerol) and used for P450 activities. For esterase *in vitro* activity assay, fourth-instar larvae were homogenised in 0.5 mL of homogenised buffer [100 mM Phosphate buffer (pH 7.2) and 1% Triton X-100]. The homogenate was centrifuged at 10 000 × *g* for 15 min at 4 °C, and the supernatant was used as an enzyme source. For acetylcholinesterase (AChE) *in vitro* assay, heads of fourth-instar larvae were removed and homogenised in 0.5 mL homogenised buffer [100 mM phosphate buffer (pH 7.2) and 1% Triton X-100]. The homogenate was centrifuged at 10 000 × *g* for 15 min at 4 °C, and the supernatant was used as an enzyme source. For *in vivo* experiments, treated fourth-instar larvae at 6, 12 and 24 h after exposure were kept and starved for 1 h (for P450) or were used immediately (for esterase and AChE) for enzyme extraction. The enzyme extractions were prepared as described above.

2.6.2 Cytochrome P450 monooxygenase (P450) activities

P450 activities were evaluated by measuring 7-ethoxycoumarin-*O*-deethylase (ECOD) activities on microsomal fractions, based on the microfluorimetric method of De Sousa *et al*.[30] For *in vivo* experiments, 7 µL of microsomal proteins was added to 0.1 M phosphate buffer (pH 7.2) containing 7 µL of 0.4 mM 7-ethoxycoumarin (7-EC, Fluka) and 7 µL of an NADPH-regenerating system (100 mM of glucose 6-phosphate + 100 mM phosphate buffer (pH 7.2) + 1 U mL^{-1} glucose 6-phosphate dehydrogenase and 5 mM of NADP) and phosphate buffer (pH 7.2) for a total reaction volume of 100 µL and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 100 µL of stop solution [ethanol/0.1 mM glycine buffer (pH 10.4), 1 : 1 volume $volume^{-1}$], and the amount of 7-hydroxycoumarin (7-OH) produced was measured in a Varian Eclipse spectrofluorometer (370 nm excitation, 455 nm emission). Fluorescence was calibrated with a standard curve of authentic 7-OH (Sigma). For *in vitro* experiments, the same procedure was used, but the microsomal proteins were preincubated for 30 min with 7 µL of solution of the test compound or 70% ethanol for control, before starting the 7-EC dealkylation assay. P450 activities were expressed as pmol of 7-OH per mg of microsomal protein per minute. Three biological replicates per treatment were made. Statistical comparisons of P450 activities were done by means of Tukey's test using the SAS program.

2.6.3 Esterase activities

Esterase activities were measured from the 10 000 × *g* supernatant (see above) according to the method described by van Asperen,[31] with α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA) (Sigma–Aldrich, Germany) used as substrates. For *in vivo* experiments, 3 µL of enzyme was added to 50 µL of 0.25 mM α-NA or β-NA in 0.1 M phosphate buffer and then 0.1 M phosphate buffer (pH 7.2) was added for a total reaction volume of 153 µL, followed by incubation at 30 °C for 30 min. After incubation, the reactions were stopped by the addition of 85 µL of 10 mM Fast Garnett (Sigma) in water and 1.5% sodium dodecyl sulfate. The production of α- or β-naphthol was measured at 527 nm or 505 nm with a 96-microplate reader in comparison with a standard curve of α-naphthol or β-naphthol and expressed as µM of α- or β-naphthol per mg of protein per minute. Three biological replicates per treatment were made. Statistical comparisons of α-NA and β-NA activities were made by means of Tukey's test using the SAS program. For *in vitro* experiments, 3 µL of enzyme was preincubated for 30 min at 30 °C with 17 µL of the treated test compound or 70% ethanol as control and brought to a total volume of 100 µL with 0.1 M phosphate buffer (pH 7.2). The substrate was then added, 50 µL of 0.25 mM α-NA or β-NA in 0.1 M phosphate buffer, and incubated for 30 min at 30 °C. After incubation, the reactions were stopped by the addition of 85 µL of 10 mM Fast Garnett (Sigma) in water and 1.5% sodium dodecyl sulfate. The production of α- or β-naphthol was measured in the same way as above.

2.6.4 Acetylcholinesterase (AChE) activities

For *in vivo* studies, AChE activity was measured with acetylthiocholine (ASCh) (Sigma-Aldrich) as substrate by the spectrophotometric method of Ellman *et al*.[32] using head homogenate as the enzyme source, as described above. The 50 µL of enzyme was incubated for 30 min at 30 °C with 50 µL of TpS [10 mM of DTNB, 0.1 mM of EDTA, 100 mM of ASCh and 100 mM phosphate buffer (pH 7.2)]. The change in absorbance at 412 nm was measured in a microplate reader, and the AChE activity was converted to nM of acetylthiocholine hydrolysed per min ($\varepsilon_{412\,nm} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$). Three biological replicates per treatment were made. Statistical comparison of AChE activities was done by means of Tukey's test using the SAS program. For *in vitro* inhibition experiments, 50 µL of enzyme was preincubated for 30 min at 30 °C with 50 µL of test compounds or 70% ethanol for control, and then 100 mM phosphate buffer (pH 7.2) was added to a 150 µL total volume. After preincubation, 50 µL of TpS solution was added, and the same procedure as in the *in vivo* experiment was followed.

2.6.5 Protein concentration determination

The protein content of each fraction used as enzyme source was determined by the Bradford method before measuring enzyme activities.

3 RESULTS

3.1 Toxicity and antifeeding effects of the *J. gossypifolia* and *M. azedarach* senescent leaf extract (SLE)

Larvae of *S. frugiperda* were first exposed to *M. azedarach* and *J. gossypifolia* SLEs added to the diet in a no-choice assay, and weight gain, development and mortality were recorded. A dose-dependent antifeeding effect was observed that led to significant mortality, starting 3 days after initial exposure, and to

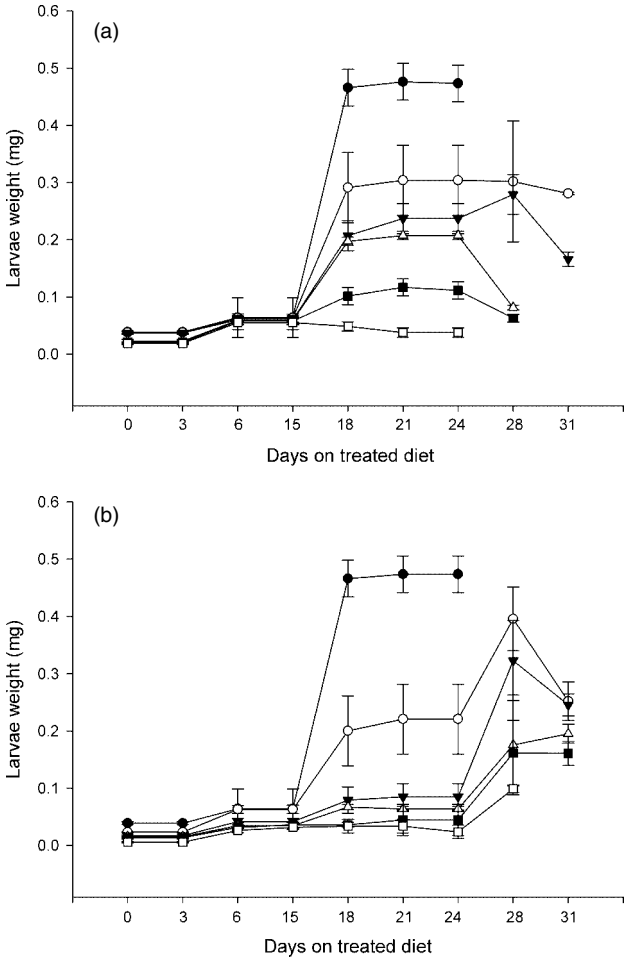

Figure 1. Average weight (± SD) of *Spodoptera frugiperda* after feeding on diet supplemented with (A) *Melia azedarach* SLE [–●–control; –○–120 mg L^{-1}; –▼–400 mg L^{-1}; –△–120 mg L^{-1}; –■–4000 mg L^{-1}; –□–12 000 mg L^{-1}] or (B) *Jatropha gossypifolia* SLE [–●–control; –○–400 mg L^{-1}; –▼–1200 mg L^{-1}; –△–4000 mg L^{-1}; –■–12 000 mg L^{-1}; –□–40 000 mg L^{-1}] in a no-choice assay.

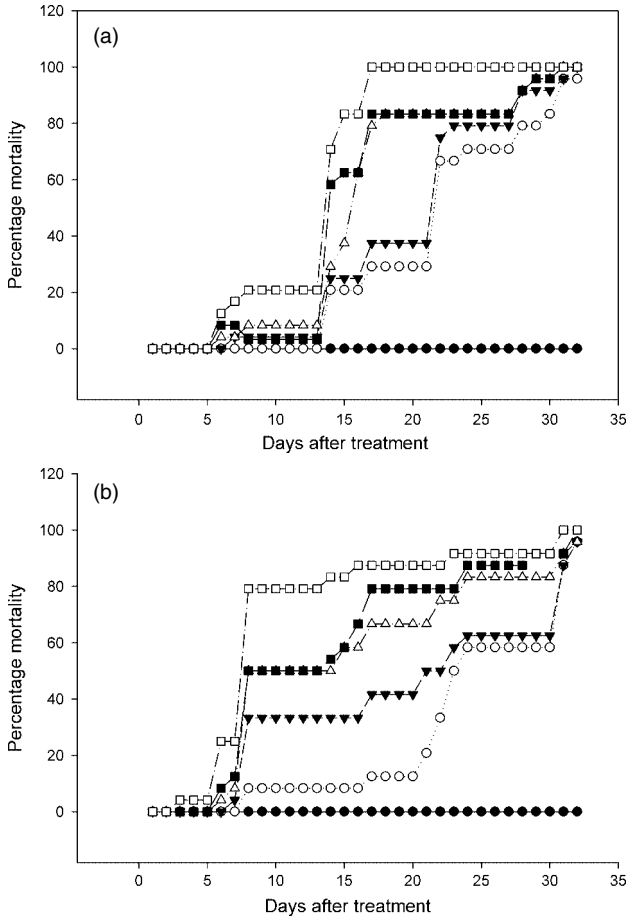

Figure 2. Percentage mortality of *Spodoptera frugiperda* after feeding on diet supplemented with (A) *Melia azedarach* SLE [–●–control; –○–120 mg L^{-1}; –▼–400 mg L^{-1}; –△–120 mg L^{-1}; –■–4000 mg L^{-1}; –□–12 000 mg L^{-1}] or (B) *Jatropha gossypifolia* SLE [–●–control; –○–400 mg L^{-1}; –▼–1200 mg L^{-1}; –△–4000 mg L^{-1}; –■–12 000 mg L^{-1}; –□–40 000 mg L^{-1}] in a no-choice assay.

developmental arrest in the survivors. Weight gain was lower on the *M. azedarach* SLE diet than on the *J. gossypifolia* SLE diet (Figs 1A and B).

This is also reflected in the cumulative mortality curves for each SLE. The *M. azedarach* SLE was more toxic than the *J. gossypifolia* SLE: 100% mortality was observed after 14 days on the *M. azedarach* SLE diet at 12 000 mg L^{-1}, whereas *J. gossypifolia* SLE caused 100% mortality only after 31 days exposure to the highest concentration tested (Figs 2A and B).

The symptoms of toxicity were different on the *M. azedarach* and *J. gossypifolia* SLE diets. On the *J. gossypifolia* SLE diet, *S. frugiperda* did not reach the pupal stage and died as larvae or as larval–pupal intermediates. On the *M. azedarach* SLE diet, larvae died without initiating metamorphosis. The plant extracts may affect different physiological processes in the larvae, and this needs additional studies.

In addition, both SLEs affected the larval development rate, with larvae from the control group reaching the pupal stage faster than all concentrations of both treated groups. This may be caused by the antifeeding effect, which diminished food intake and led to smaller sizes of the larvae (Figs 1A and B). Larvae treated with *M. azedarach* SLE pupated 3 days later than the control larvae, whereas with *J. gossypifolia* the delay was 7 days, with sometimes incomplete pupae, especially at doses higher than 12 000 mg L^{-1}. However, with *M. azedarach* sublethal concentrations, all larvae were able to pupate. After becoming pupae, all control and the complete pupae from both treatments reached the adult stage, with the exception of the larval–pupal intermediates obtained in the *J. gossypifolia* treatments (Fig. 3).

3.2 Contact toxicity of *J. gossypifolia* and *M. azedarach* SLEs and synergism of cypermethrin toxicity

The contact toxicity of the plant extracts was also tested using a dip bioassay. The acute toxicity of the *M. azedarach* and *J. gossypifolia* SLEs was considerably lower than that of cypermethrin, with LC_{50} values at 24 h of 2591 ± 680, 1439 ± 386 and 0.087 ± 0.0225 mg L^{-1} respectively (Table 1), whereas no mortality was observed for the control group. Based on 95% confidence intervals, the *J. gossypifolia* SLE was more toxic than the *M. azedarach* SLE. The combined effect of the plant SLEs with cypermethrin was then tested in comparison with the synergistic effect of PBO. Both plant SLEs showed a synergistic effect on the toxicity of cypermethrin. The highest synergistic factors were observed at concentrations of 60 mg L^{-1} of *J. gossypifolia* SLE (290), 60 mg L^{-1} of *M. azedarach* SLE (72.5) and 2 mg L^{-1} of PBO (435) (Table 1).

Table 1. Synergistic effect of the SLEs of *J. gossypifolia* and *M. azedarach* and PBO on the toxicity of cypermethrin to *S. frugiperda* larvae

Compound	$LC_{50} \pm SD$(mg L^{-1})[a]	SF[b]
Cypermethrin alone	0.087 ± 0.0225 d	–
J. gossypifolia alone	2591 ± 680 a	–
M. azedarach alone	1439 ± 386 b	–
Cypermethrin + 4 mg L^{-1} of *J. gossypifolia*	0.0026 ± 0.0008 h	33.46
Cypermethrin + 12 mg L^{-1} of *J. gossypifolia*	0.0015 ± 0.0005 i	58.00
Cypermethrin + 40 mg L^{-1} of *J. gossypifolia*	0.0007 ± 0.0002 l	124.29
Cypermethrin + 120 mg L^{-1} of *J. gossypifolia*	0.0003 ± 0.0001 m	290.00
Cypermethrin + 4 mg L^{-1} of *M. azedarach*	0.0221 ± 0.004 c	3.94
Cypermethrin + 12 mg L^{-1} of *M. azedarach*	0.0074 ± 0.0012 e	11.76
Cypermethrin + 40 mg L^{-1} of *M. azedarach*	0.0031 ± 0.0005 g	28.06
Cypermethrin + 120 mg L^{-1} of *M. azedarach*	0.0012 ± 0.0002 j	72.50
Cypermethrin + 0.012 mg L^{-1} of PBO	0.0035 ± 0.0012 f	24.86
Cypermethrin + 0.04 mg L^{-1} of PBO	0.0015 ± 0.0006 i	58.00
Cypermethrin + 0.12 mg L^{-1} of PBO	0.0008 ± 0.0007 k	108.75
Cypermethrin + 0.4 mg L^{-1} of PBO	0.0007 ± 0.0003 l	124.29
Cypermethrin + 1.2 mg L^{-1} of PBO	0.0003 ± 0.0001 m	290.00
Cypermethrin + 4 mg L^{-1} of PBO	0.0002 ± 0.000001 n	435.00

[a] Values with the same letter are not significantly different at $P<0.05$ according to Tukey.
[b] SF = synergist factor = (LC_{50} of cypermethrin alone)/(LC_{50} of cypermethrin + SLE).

Figure 3. Toxicity symptoms of *Spodoptera frugiperda* after treatment with 12 g L^{-1} of *Jatropha gossypifolia* SLE. Note that larvae died either as larval–pupal intermediates (left panel) or as larvae (right panel). Larvae treated with the *Melia azaderach* SLE died without initiating metamorphosis.

The SLE from *J. gossypifolia* had a greater synergistic effect than the *M. azedarach* SLE. At 120 mg L^{-1}, *J. gossypifolia* caused 100% mortality when combined with cypermethrin at doses starting from 0.01 mg L^{-1}, whereas 120 mg L^{-1} of *M. azedarach* caused mortality of around 75% only. However, the synergistic effects of the SLEs from both plants were less pronounced than that of piperonyl butoxide, which caused 100% mortality at a concentration of only 1.2 mg L^{-1} with 0.03 mg L^{-1} of cypermethrin (Figs 4–6).

Within 10 min of treatment with cypermethrin alone or in combination with both plant extracts at any concentration ratio, *S. frugiperda* larvae displayed symptoms including hyperactivity and knockdown leading to paralysis and death. Such reactions may provide additional protection to plants by dislodging surviving insects and/or exposing them to increased predation.

3.3 Effects of the SLEs on enzyme activities

The effects of the SLEs were first tested *in vitro* on acetylcholinesterase activity and on two types of detoxification enzyme, general esterases and a typical P450 activity. In each of these assays, the SLE effects were compared with those of PBO. For acetylcholinesterase activity, only the *J. gossypifolia* SLE showed a dose-dependent inhibitory activity, while the *M. azaderach* SLE and PBO were not inhibitory (Fig. 7A). As expected, PBO inhibited 7-EC deethylase activity in a dose-dependent manner, as did the *J. gossypifolia* SLE, while the *M. azaderach* was only inhibitory at the highest concentrations (Fig. 7B). The two esterase activities were only slightly inhibited, with the *M. azaderach* SLE showing highest activity (Figs 7C and D).

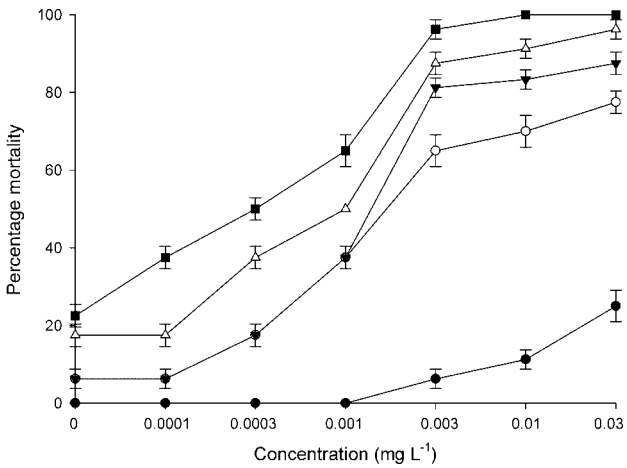

Figure 4. Percentage mortality (± SD) of *S. frugiperda* larvae after topical exposure (dipping assay) to *Jatropha gossypifolia* SLE and cypermethrin mixtures: –●–cypermethrin alone; –○–cypermethrin + 4 mg L^{-1} of *Jatropha gossypifolia* SLE; –▼–cypermethrin + 12 mg L^{-1} of *Jatropha gossypifolia* SLE; –△–cypermethrin + 40 mg L^{-1} of *Jatropha gossypifolia* SLE; –■–cypermethrin + 120 mg L^{-1} of *Jatropha gossypifolia* SLE.

These enzyme activities were also measured 6, 12 and 24 h after topical exposure of the insects to the SLEs in the dipping

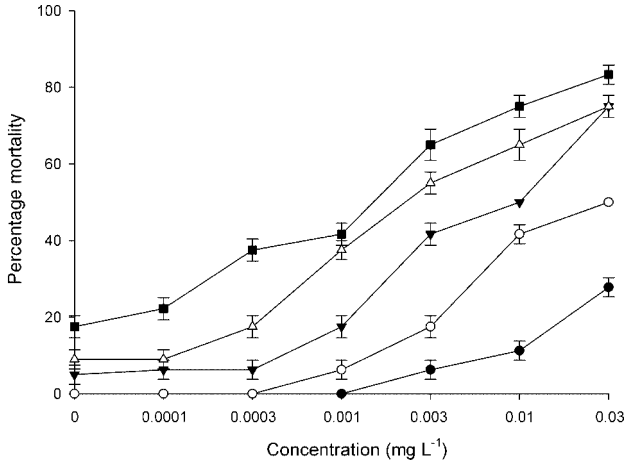

Figure 5. Percentage mortality (± SD) of *S. frugiperda* larvae after topical exposure (dipping assay) to *Melia azaderach* SLE and cypermethrin mixtures: –●–cypermethrin alone; –○–cypermethrin + 4 mg L^{-1} of *Melia azaderach* SLE; –▼–cypermethrin + 12 mg L^{-1} of *Melia azaderach* SLE; –△–cypermethrin + 40 mg L^{-1} of *Melia azaderach* SLE; –■–cypermethrin + 120 mg L^{-1} of *Melia azaderach* SLE.

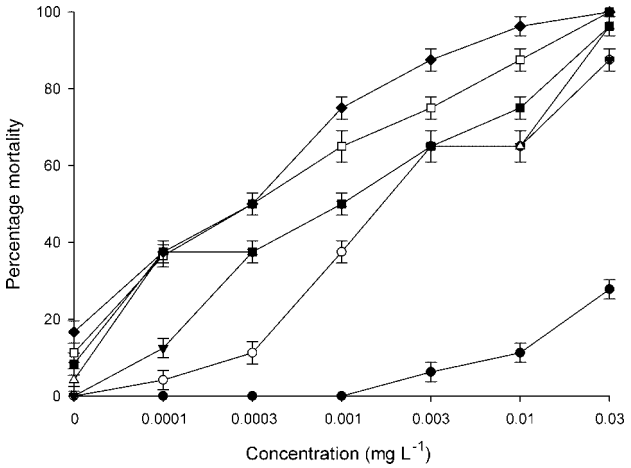

Figure 6. Percentage mortality (± SD) of *S. frugiperda* larvae after topical exposure (dipping assay) to piperonyl butoxide and cypermethrin mixtures: –●–cypermethrin alone; –○–cypermethrin + 0.012 mg L^{-1} of piperonyl butoxide; –▼–cypermethrin + 0.40 mg L^{-1} of piperonyl butoxide; –▼–cypermethrin + 1.20 mg L^{-1} of piperonyl butoxide; –□–cypermethrin + 4 mg L^{-1} of piperonyl butoxide.

assay. This experimental set-up was meant to evaluate the effectiveness of topically applied compounds to reach potential targets *in vivo*, at the doses at which they were shown to be synergistic. The *J. gossypifolia* and *M. azedarach* SLE treatments inhibited acetylcholinesterase activity, but PBO had little effect (Fig. 8A). Similarly, the plant SLEs inhibited P450 activity in a dose-dependent fashion, as did PBO (Fig. 8B). The SLEs were most effective 12 h after treatment, and less so after 6 or 24 h, but the effect of PBO remained more stable over time (results not shown). The two plant SLEs also inhibited general esterase activities (Figs 8C and D), but PBO did not significantly inhibit esterase activities after topical treatment, in contrast to its potent activity against the P450 activity.

4 DISCUSSION

It is shown here that senescent leaf extracts of two plants have an antifeedant effect on larvae of the fall armyworm when provided in the diet in a no-choice assay, and that, in spite of relatively low acute toxicity, these SLEs have a synergistic activity to cypermethrin in acute toxicity tests. The acute toxicity levels of crude *J. gossypifolia* SLE appear to be higher than those obtained by Khumrungsee *et al.*[33] who extracted *J. gossypifolia* senescent leaves with 95% ethanol and diluted the extracts with water for bioassays. They obtained an LC_{50} of 35 000 mg L^{-1} on *Spodoptera exigua* larvae. Furthermore, the SLE seems to show a better control efficacy on another related insect species, *Spodoptera litura* (LC_{50} = 6500 mg L^{-1}).[7] In addition, *J. gossypifolia* SLE prepared in ethanol as described here is more potent than *J. gossypifolia* SLE prepared in ethyl acetate, or than the active ingredient ricinine on *S. exigua* (LC_{50}∼8644 and 3215 mg L^{-1} respectively).[34] Similarly, the toxicity of the *M. azedarach* SLE is higher on *S. frugiperda* than that observed on *S. exigua* (LC_{50}∼9800 mg L^{-1})[35] or on the nematode *Haemonchus contorts* (LC_{50}∼9200 mg L^{-1}).[36] The present results show that *J. gossypifolia* and *M. azedarach* SLEs are good candidates to control *S. frugiperda*, as are those of *Myrtillocatus geometruzans*,[37] which have an insect growth regulatory effect. The present ethanol extracts of *M. azedarach* and *J. gossypifolia* senescent leaves show a control efficacy on *S. frugiperda* equivalent to that obtained in previous research on *Aedes aegypti*,[25] in which the active ingredient(s) may have been azadirachtin or other hydrophilic compounds. Although secondary allelochemicals from plants are sometimes commercially available as single, purified compounds, compound mixtures may be more effective in reducing pest resistance.[38] The present experiments show that both extracts suppressed the larval activity of *S. frugiperda* even at low dose. They suppressed the consumption rate, and the growth rate values declined significantly compared with controls. A similar result has been obtained by Nathan,[39] who found that *M. azedarach* affected the nutritional physiology and enzyme activities of the rice leaffolder *Cnaphalocrocis medinalis*. In addition, previous research found that *M. azedarach* extract can affect NADPH–cytochrome *c* reductase and cholinesterase activity in *Spodoptera frugiperda*.[40] The *J. gossypifolia* SLE exhibited better larvicidal activity against *S. frugiperda* than the *M. azedarach* SLE. However, the acute toxicity of both extracts is not high, and LC_{50} values at 24 h after exposure are only moderate. The prospect of using these extracts as botanical insecticides was then compared with the possibility of using them instead as synergists. The present studies revealed a synergistic activity of the SLE of both plants towards the insecticide cypermethrin, a photostable synthetic pyrethroid. The LC_{50} value of cypermethrin against *S. frugiperda* larvae was reduced from 0.087 to 0.0003 mg L^{-1} when combined with 120 mg L^{-1} of *J. gossypifolia* SLE and to 0.0012 mg L^{-1} when combined with *M. azedarach* SLE (Table 1). The synergistic effect was concentration dependent in both cases (Table 1). This result is similar to those obtained in studies on botanicals as synergists for synthetic chemicals such as *Andrographis paniculata* Neem extract against *Anopheles stephensi* Liston.[41] Similarly, 2500 mg L^{-1} azadirachtin or 2500 mg L^{-1} Pongamia oil with 0.026% endosulfan induced 100% mortality of *Diacrisia oblique*.[42] Although both *M. azedarach* and *J. gossypifolia* SLE have lower synergistic activities compared with the standard synergist, PBO (Table 1), both extracts may provide alternative synergist compounds at a cheaper price (both extracts cost less than \$US 0.5 kg^{-1}) and may contribute to reducing the use of the synthetic insecticide cypermethrin. The

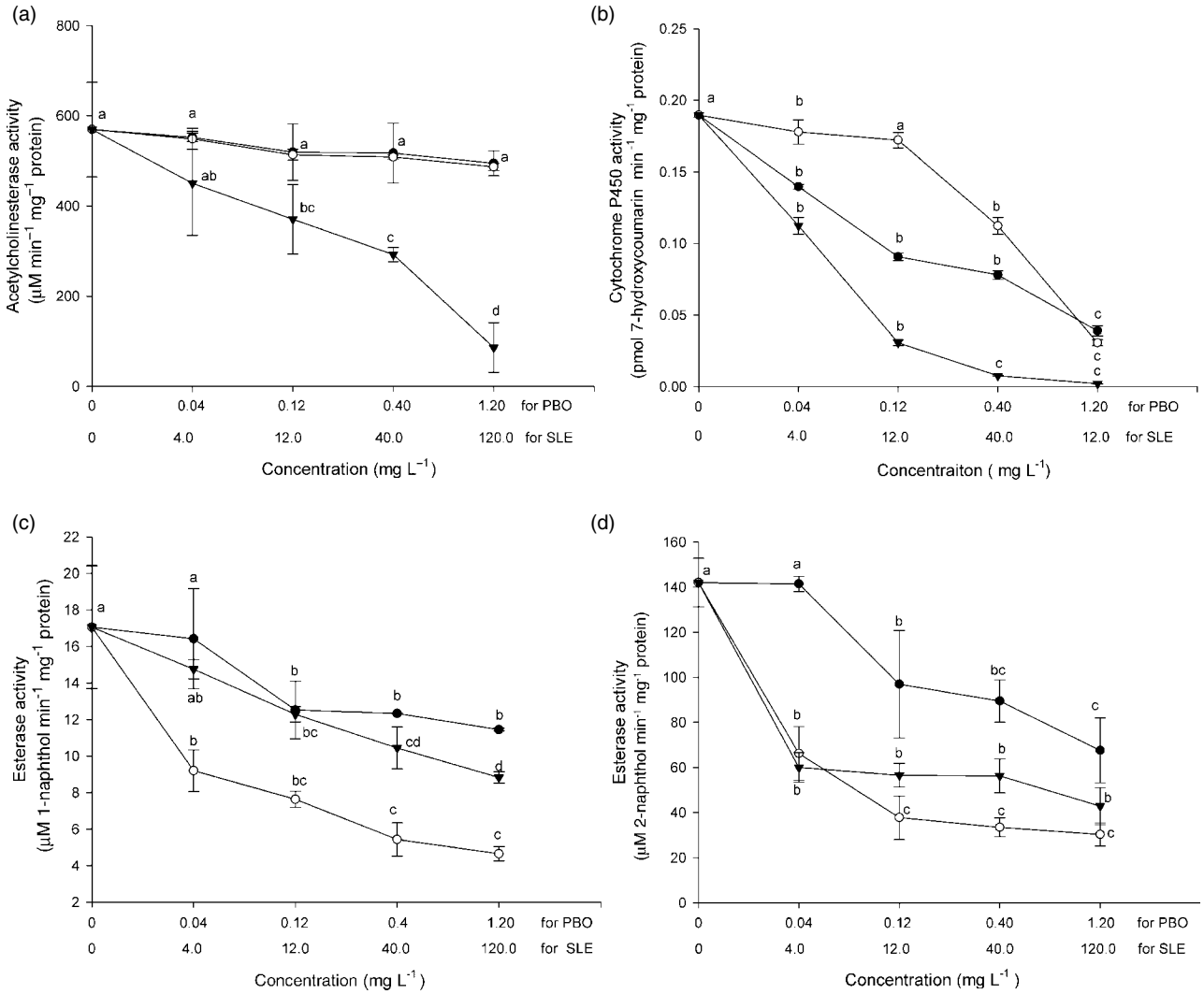

Figure 7. *In vitro* inhibition of enzyme activities by the SLEs and by piperonyl butoxide (PBO): (A) acetylcholinesterase; (B) cytochrome P450 monooxygenase (ethoxycoumarin *O*-deethylation); (C) esterase (α-naphthyl acetate); (D) esterase (β-naphthyl acetate). Mean enzyme activities (± SD) marked by the same letter are not significantly different ($P<0.05$, Tukey's test) for each compound tested: –●–piperonyl butoxide; –○–*Melia azedarach* SLE; –▼–*Jatopha gossypifolia* SLE.

synergism on other insect pests has to be studied because it may not affect other species in the same way. For instance, sesame oil has been reported as being synergistic for pyrethrum insecticides in *Plutella* xylostella,[43,44] but it does not show synergism in *Musca domestica* when combined with pyrethroids.[45] Synergistic activity has been observed for both plant extracts, but the *J. gossypifolia* SLE was most effective. It is likely that this extract contains some factors that can inhibit the detoxification enzyme in *S. frugiperda* larvae, as does PBO. Indeed, lignans such as gossypidien[10] or gossypifan[11] are most likely involved because these lignans have a methylenedioxyphenyl moiety that is typical for inhibitors such as PBO (i.e. the standard synergist and well-known cytochrome P450 inhibitor).[46] Hence, the synergistic effect observed with the *J. gossypifolia* SLE may be due to inhibition of the P450-mediated metabolism of cypermethrin.

It is well known that herbivorous insects use detoxification enzymes, including cytochrome P450 monooxygenases, GST and carboxyl/cholinesterases, to metabolise otherwise deleterious plant secondary metabolites.[47,48] However, such compounds often act as inhibitors of enzymes, such as the methylenedioxyphenyl compounds described above. The present *in vitro* experiments show that the SLEs can act as inhibitors of esterases and P450 enzymes. When the SLEs were applied *in vivo*, and the enzyme activities were measured thereafter, inhibition of esterases and P450 activities was also observed. This suggests that the synergism of cypermethrin observed in the same experimental conditions may be caused by inhibition of its degradation. Interestingly, PBO, which showed some inhibition of esterases *in vitro* at high concentrations, did not lead to significant inhibition after *in vivo* treatment, whereas it was inhibitory of the P450 activity both *in vivo* and *in vitro*. In addition to the inhibition of detoxification activities, it was also shown that acetylcholinesterase, a known target of organophosphate and carbamate insecticides and an essential enzyme involved in neurotransmission, was inhibited by the *J. gossypifolia* SLE. This suggests that the *J. gossypifolia* SLE may have multiple biological activities that contribute to its toxicity to the larvae. Esterases and P450 normally play an important role in allelochemical metabolism and resistance.[47–50] The present results therefore confirm previous studies on the inhibition of P450 and esterases by

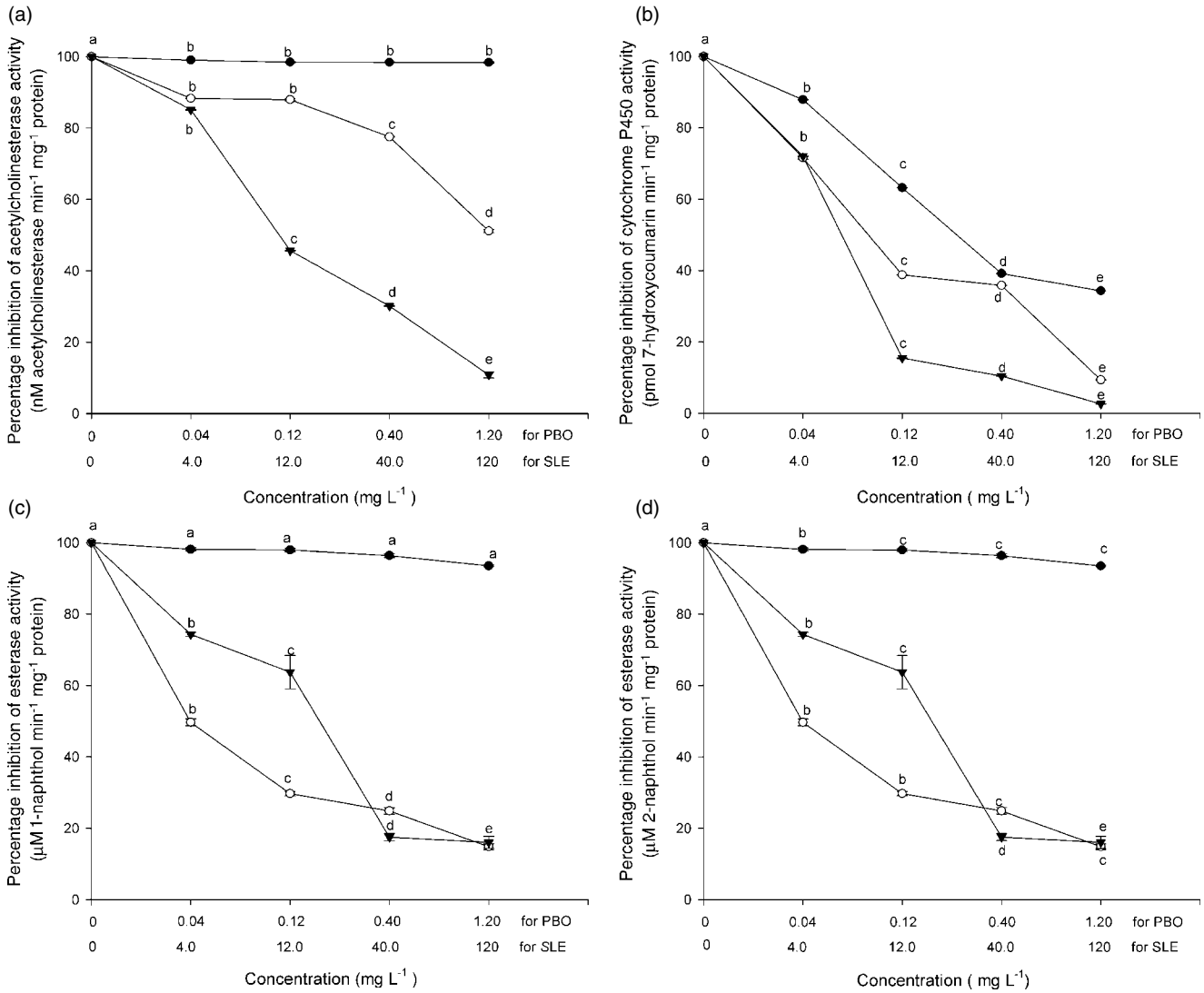

Figure 8. Inhibition of enzyme activities *in vivo* (i.e. after topical exposure) by the SLEs and by piperonyl butoxide (PBO): (A) acetylcholinesterase; (B) cytochrome P450 monooxygenase (ethoxycoumarin-*O*-deethylation); (C) esterase (α-naphthyl acetate); (D) esterase (β-naphthyl acetate). Mean enzyme activities ($\pm$ SD) marked by the same letter are not significantly different ($P<0.05$, Tukey's test) for each compound tested. –●–piperonyl butoxide; –○–*Jatopha gossypifolia* SLE; –▼–*Melia azedarach* SLE.

crude plant extracts, for instance of *M. azedarach* and *Amaranthus viridis* against *Spodoptera exigua* (Hübner),[35] of *Melia toosendan* Sieb. et Zucc. Pron. against *Spodoptera litura* (F.) and *Melanoplus sanguinipes* (F.)[51] and of *Alpinia galanga* against *Bactocera dorsalis*.[52] In conclusion, the results demonstrate the potential of *J. gossypifolia and M. azedarach* SLEs as botanical insecticides on *S. frugiperda*. These SLEs have also been shown to inhibit several enzyme activities that are important to insect survival. In particular, the inhibitory activity on P450 activities may explain the synergistic activity of the plant SLEs towards cypermethirn toxicity. The inhibition of these enzymes by plant allelochemicals may constitute a useful alternative approach for integrated pest management.

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