Natural insecticides from *Ocotea longifolia* Kunth. (Lauraceae) leaf essential oil against *Sitophilus oryzae* L. (Coleoptera: Curculionidae)

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Abstract

Many customer complaints of cereal based foods relate to insect infestations due to the stored product pest *Sitophilus oryzae* L. (Coleoptera: Curculionidae). Pyrethroids are used to treat the grain but new alternative insecticides will be helpful. *Ocotea longifolia* Kunth (Lauraceae) is a native Colombian tree with aromatic leaves that has potential for the extraction of natural insecticides. Leaf essential oil (EO) and its main compounds (α-terpinolene, α-phellandrene and δ-3-carene) were used in fumigation bioassays and lethal concentrations (LC_{50}) in the rice weevil of 271.3 μL/L for the EO and 55.2 μL/L for δ-3-carene, the most toxic product, were obtained. The activity of acetylcholinesterase (AChE) extracted from the insects after the treatments was measured by the Ellman method. In all cases, a dose – response pattern was obtained *in vivo* being EO and α-terpinolene the greater inhibitors of the enzyme. If the application of the volatiles took place *in vitro*, there was a reduction in the AChE activity but in this case δ-3-carene and α-phellandrene were highlighted as potent inhibitors, particularly the former with an IC_{50}=0.45 mM. This is the first report of *Ocotea* EO, α-terpinolene or α-phellandrene reducing the activity of the enzyme; α-phellandrene showed a non competitive inhibition whilst in the other compounds a mixed inhibition was the mode of action.

Keywords: stored product pests, rice weevil, α-terpinolene, α-phellandrene, δ-3-carene, AChE

1. Introduction

The genus *Cinnamomum*, *Laurus* or *Persea* belong to the plant family Lauraceae and yield economically important products such as cinnamon, laurel seasoning leaves or avocado fruits. *Ocotea longifolia* Kunth. is a tree native to Colombia. *Ocotea* comprise about 350 species from tropical regions, their bark and leaves are like other Lauraceae in that they are aromatic and have been used to enhance food flavor or in perfumes.

Takaku et al. (2007) analyzed leaf essential oil of 10 species of *Ocotea* (*O. longifolia* was not included) and they found a great diversity among species with only 9 compounds in common out of the 91 identified; the main products were α-pinene, β-pinene, β-caryophyllene and germacrene D. Prieto et al. (2010) showed that *O. longifolia* leaf essential oil had fumigant activity against *Sitophilus zeamais* Motschulsky (LC_{50} = 280 μL/L) and reported that α-terpinolene, α-phellandrene and δ-3-carene were the main compounds in its oil. In an study of 20 species of the Lauraceae, the ethanolic extracts of *Ocotea minor* were identified as potent inhibitors of the enzyme acetylcholinesterase (AChE) and the authors (Yamaguchi et al., 2011) attributed the activity to the possible presence of alkaloids in the extracts.

The rice weevil (*Sitophilus oryzae* L.) is the insect pest of cereal-derived processed foods that causes the main complaints in Spain due to infestations throughout the commercialization chain. Deltamethrin is the grain protectant used now for its control but plant essential oils

could be an alternative (López et al., 2008) if active substances are identified and the formulation and application technologies for such volatile products are developed.

Park et al. (2003) obtained over 80% mortality in *S. oryzae* applying terpinolene or phellandrene at 0.26 mg/cm^2 in impregnated – paper test but the effects increased in fumigation tests, suggesting that the toxins penetrate the insect body via the respiratory system. Several authors have published that the mode of action of essential oils could be by inhibiting the AChE enzyme (López and Pascual-Villalobos, 2010) although acting as weak inhibitors, such works refer to in vitro assays and sometimes the insecticidal activity and AChE inhibition are not correlated (Abdelgaleit et al., 2009). In vivo tests are required for the validation of in vitro tests (Keane and Ryan, 1999) and to provide a more accurate knowledge on how the volatiles act on the insect.

The objective of our work was to perform in vivo and in vitro tests on the effect of the leaf essential oil of the Colombian plant species *O. longifolia* against the stored product pest *S. oryzae* with the aim to generate new knowledge on the mode of action of such natural insecticidal products.

2. Materials and Methods

2.1. Essential oils and monoterpenoids

Fresh leaves (2 kg) of *O. longifolia* collected in Icononzo (Tolima) in Colombia, were steam distilled to obtain the essential oil (EO) that was used in the bioassays. Prieto et al. (2010) analyzed the oil by GC giving a composition of 74% α-terpinolene, 4.7% α-phellandrene, 3.6% δ-3-carene and minor amounts of α- and β-pinene, γ-terpinene and limonene. Pure compounds for the bioassasys were purchased from Sigma Aldrich.

2.2. Insects

S. oryzae was maintained on polished rice, in the dark, at 24 ± 1°C and 75 ± 5% r.h. Emerged adults (1-6 days old) were used for the experiments.

2.3. Fumigation test

The experimental unit to test for volatile toxicity consisted of 4 mL glass vial with a 2 cm diameter (Whatman nº1) filter paper disk (placed into the top) in which 0.1-13 µL (6.7 – 633.3 µL/L air) of the EO or monoterpenoid was applied. This vial was enclosed inside another (22 mL) together with 10 insects. A control was prepared the same but the application of the products. The bioassays were performed in triplicate in controlled conditions (30 ± 1°C and 75 ± 5 % r.h.). Insect mortality was recorded after 24 h and Probit analysis (Finney, 1971) was run using POLO plus software for $LC_{25,}$ LC_{50} and LC_{75} calculation. Observations on the effects of the treatments in mobility and behaviour of *S. oryzae* were done 8 hours following the bioassay.

2.4. Acetylcholinesterase (AChE) inhibition tests.

Inhibition of AChE by *O. longifolia* EO or the monoterpenoids (α-terpinolene, α-phellandrene or δ-3-carene) was assessed following the colorimetric method described by Ellman et al. (1961), the activity was estimated by Helios Zeta UV-VIS (Thermo Scientific) spectrophotometer at 412 nm and measured at 25ºC for 15 min.

The enzyme was extracted from *S. oryzae* adults. 1.8 g of insects were homogenized in 10 mL of buffer (0.1 M, pH 8) containing 0.1 % (w/v) bacitracine and 0.3% w/v benzamidine. The homogenate was centrifuged at 18,200 g for 90 min at 4ºC and the supernatant containing AChE was filtered through glasswool (to remove excess lipid) and stored at 0ºC.

The *in vitro* assays consisted of the extraction of the insect enzyme first and addition of the inhibitor (EO or monoterpenoid) afterwards. The reaction (3 mL total volumen) took place into the spectrophotometer cuvettes: 1.5 mL of buffer, 1 mL essential oil (50 – 200 ppm) or monoterpene (0.5 – 2 mM) solution, 200 µL of substrate (acetylthiocholine iodide) (1-10 mM), 200 µl of DTNB (10 mM) and 100 µl of enzymatic extract. Each test was replicated twice.

For the *in vivo* assays, fumigant treatments of *S. oryzae* adults (with the EO or monoterpenoid) at LC_{25}, LC_{50} and LC_{75} were done first and then followed by AChE extraction. 900 insects per product and concentration were treated with the volatiles and after 24 h the enzyme was extracted (from the pool of dead and alive insects). The activity of the enzymatic extracts was measured using the Ellman method. As a control an enzymatic extract obtained from 900 untreated insects was used.

3. Results and Discussion

O. longifolia leaf EO shows fumigant activity against *S. oryzae* (Table 1), the LC_{50} was 271.3 µL/L. The monoterpenoids were active at lower doses being δ-3-carene the most toxic (LC_{50}=55.2 µL/L). In previous works with *S. oryzae*, Lopez and Pascual-Villalobos (2008) obtained a similar range of values for other monoterpenoids (camphor, estragole, fenchone and geraniol): LC_{50} = 25.6-151.3 µL/L but for carvone with 2.7 µL/L, that was more active.

Table 1 Lethal concentration (LC) of *O. longifolia* leaf essential oil and its main compounds against *S. oryzae*.

Compound	LC_{50} (µL/L)
O. longifolia essential oil	271.33 (205.47 – 364.11)
α-terpinolene	98.74 (82,04 – 132.26)
δ-3-carene	55.24 (50,46 – 60.55)
α-phellandrene	82.07 (70.98 – 98.80)

Fiducial limits at 95% interval

To investigate the mode of action of *O.longifolia* EO compounds, an in vivo bioassay was performed applying the products to the insects at increasing lethal doses and extracting the enzyme (AChE) afterwards; then its activity was measured (Fig. 1). Increasing doses of essential oil or monoterpene gave higher mortality and a dose response enzymatic inhibition as well. AChE inhibition was greater after EO or α-terpinolene applications but δ-3-carene was the compound in which there was a bigger reduction in activity when LC_{75} instead of LC_{50} was applied.

On the other hand, the results of in vitro assays are shown in Figure 2, in this case the AChE was extracted from untreated insects and then different doses of the inhibitors were applied. All monoterpenoids and the EO reduced the activity of the enzyme, δ-3-carene and α-phellandrene were the more potent inhibitors.

In vivo inhibitory effects of δ-3-carene were less evident than in vitro results. Other authors (Grundy and Still, 1985) reported that increasing exposure times of roaches to pulegone (a potent AChE in vitro inhibitor) lead to progressive development of symptons and insect death but this was not correlated with a decrease in AChE activity in vivo. This is maybe a consequence of the monoterpene being metabolized before it reaches the insect brain or that the insect synthesizes more enzyme after the treatment (Perry et al., 2000).

Comparing in vivo and in vitro effects of the EO and monoterpenoids (Fig. 1, 2), it seems that the inhibitory effects on AChE in vitro are greater that in vivo. The doses, however, do not

allow a fair comparison: in the former LC_{25}, LC_{50} and LC_{75} are applied to the insects while in the latter 0.5 mM, 2 mM (monoterpenoides) or 50 ppm, 100 ppm (EO) are added for the reaction.

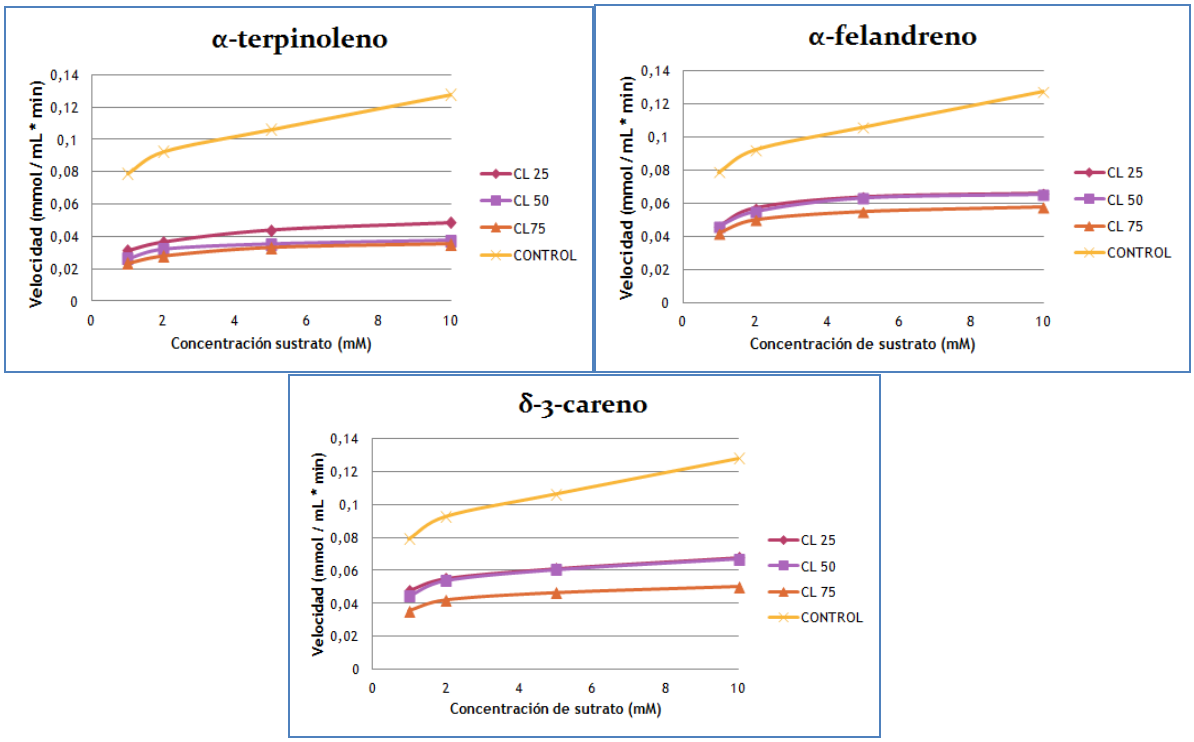

Figure 1 AChE activity (speed vs substrate concentration) after in vivo bioassays (fumigant treatments at LC_{25}, LC_{50} and LC_{75} of *S.oryzae* adults followed by AChE extraction).

Table 2 summarizes the concentrations that reduce the activity by 50%, for instance δ-3-carene has an IC_{50} = 0.45 mM. According to Lopez et al. (2014) other monoterpenoids (linalool, estragole, carvone and fenchone) proved to be less active for electric eel AChE in comparison: IC_{50} = 15.60, 12.67, 5.56 and 7.00 mM respectively. Unpublished data using *S. oryzae* AChE indicated a less inhibitory action IC_{50}=6.66-11.73 mM for these monoterpenoids as refered to the ones we have obtained for *Ocotea* oil; possibly due to that other modes of action (besides inhibition of AChE) are also taking place.

This is the first report of AChE inhibitory activity of α-terpinolene, α-phellandrene and *O. longifolia* leaf EO. Miyasawa and Yamaguji (2005) have published that δ-3-carene was a potent inhibitor of bovine eritrocyte AChE in comparison with other bicyclic monoterpenes. Aazza et al. (2011) reported that δ-3-carene (from the oil of *Cuppressus sempervirens* L.) was more active than carvacrol or thymol in inhibiting the enzyme. Monoterpenes present in the *Ocotea* oil in minor amounts (α and β pinene, γ-terpinolene and limonene) have previously been reported as AChE inhibitors (Aazza et al., 2011; Öztürk, 2012) and might as well be responsible for the results obtained in our work.

Monoterpenes are weak inhibitors (Ferrari et al., 2000; Howes et al., 2003) in comparison with organophosphate or carbamate insecticides, e.g. IC_{50} = 13 ppm for a carbamate (carbaril) whilst 61-307 ppm for the compounds listed in Table 2 (following unit conversion).

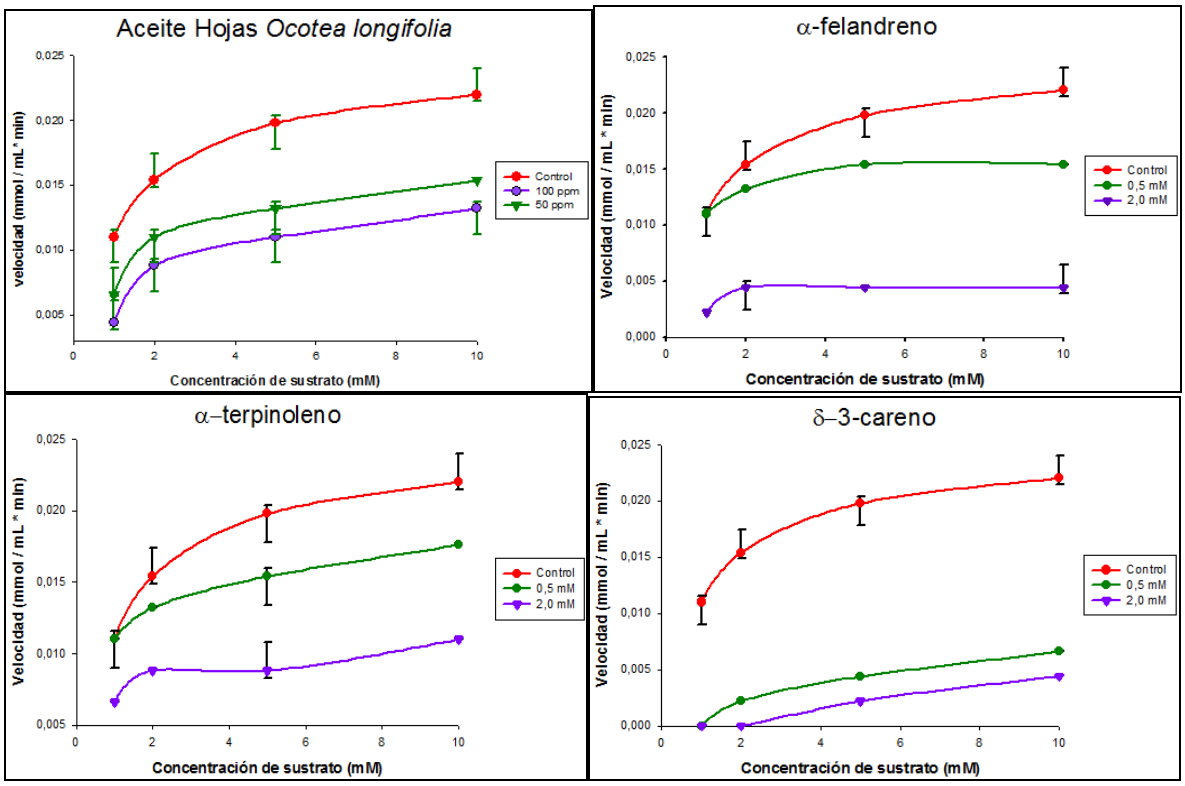

Figure 2 AChE activity (speed vs substrate concentration) of in vitro bioassays at two doses of the inhibitors (the AChE enzyme was extracted from untreated *S. oryzae* adults prior to the assays).

Table 2 Inhibitory concentration (IC_{50}) of AChE from in vitro bioassays.

Compound	IC_{50} (mM)
O. longifolia leaf essential oil	0.68 ± 0.02
δ-3-carene	0.45 ± 0.13
α-terpinolene	2.26 ± 0.37
α-phellandrene	1.30 ± 0.07

To study the effect of EO in behaviour of *S. oryzae*, adults were treated with LC_{50} and after 3 hours their movements were observed. The insects had slow and uncoordinated movements with difficulties to stand up after falling backs. Such observations are coherent with a likely inhibition of the AChE enzyme, one mode of action of *Ocotea* EO and its main compounds according to our results. This enzyme is responsible for ending the transmission of the nervous impulse by hydrolizing acetylcholine, its inhibition causes accumulation of this substance in the synapsis and the insect nervous system suffers from maintaining the post-synaptic membrane in a permanent state of stimulation giving way to convulsions, uncoordinated movements and death (Rattan, 2010).

Other authors have reported the AChE inhibitory activity of *Salvia* species (Mukherjee et al., 2007) but also nicotinic activity (Perry et al., 2000) and suggested that action occurs at various metabolic routes.

Many monoterpenes have a hydrophilic carbonated skeleton suitable to interact with the active site of the AChE (hydrophilic too). The three monoterpenes tested are isomers and

even though skeletons contain the same number of carbons and hydrogens their 3D configuration does not have similarity and this is playing a role in the results.

Figure 3 and Table 3 are useful in understanding the mode of inhibition being caused by *Ocotea* EO and its main compounds. Phellandrene presents a non-competitive inhibition (Fig. 3) since the inhibitor decreases the activity of the enzyme and binds equally to the enzyme whether or not it has already bound the substrate. In this kind of inhibition the V_{max} is reduced whereas the Km is maintained at constant value (Table 3). *O. longifolia* EO, α-terpinolene and δ-3-carene show a mixed inhibition (Fig. 3) because of the inhibitor has a higher affinity for binding the enzyme in one state (once it is bound to the substrate) or the other (unbound). In all of these cases V_{max} falls (Table 3) and for δ-3-carene Km changes.

All products tested were AChE inhibitors but δ-3-carene was highlighted for being more potent (even at 0.45 mM).

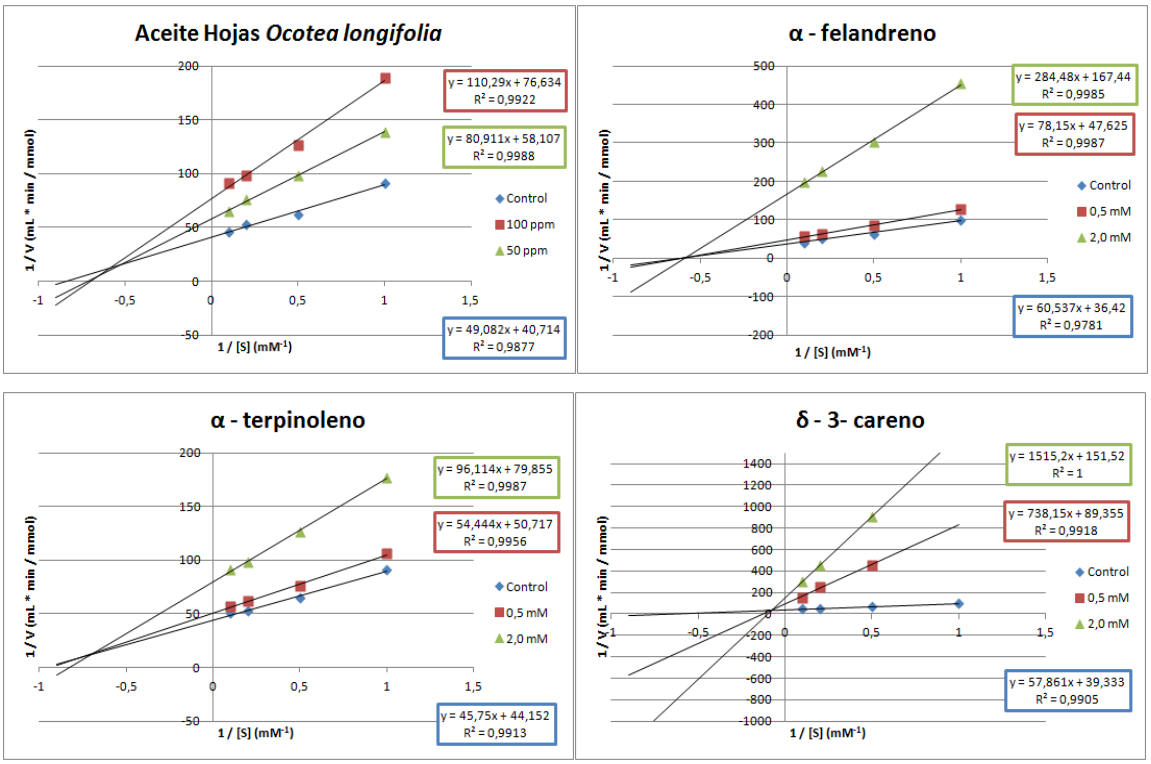

Figure 3 Lineweaver-Burk plots at different doses of the inhibitors (essential oils and monoterpenes).

Table 3 Michaelis-Menten (Km) constant and maximum velocity (V_{max}) values for the inhibitors tested in vitro. The AChE enzyme was extracted from *S. oryzae* adults prior to the assays.

Compound	Dose	K_m (mmol/L)	V_{max} (mmol/ min*mL)
Control	0 ppm	1.206	0.0246
O. longifolia essential oil	50 ppm	1.392	0.0172
	100 ppm	1.439	0.0130
Control	0 ppm	1.662	0.0275
α-phellandrene	68 ppm	1.641	0.0210
	272 ppm	1.699	0.0060
Control	0 ppm	1.036	0.0226
α-terpinolene	68 ppm	1.073	0.0197
	272 ppm	1.204	0.0125
Control	0 ppm	1.471	0.0254
δ-3-carene	68 ppm	8.261	0.0112
	272 ppm	10.000	0.0066

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