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Title page with Author Information

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Bioactivity of semisynthetic eugenol derivatives against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae infesting maize in Colombia

Abstract

The anti-acetylcholinesterase, larvicidal, antifeedant activities and general toxicity of 15 semisynthetic eugenol derivatives based on clove oil (including the own oil), were evaluated against the maize armyworm, *Spodoptera frugiperda* (J.E. Smith). Therefore, promising eugenol molecules were classified with larvicidal, anti-acetylcholinesterase and antifeedant activities for controlling this pest. During structure–activity relationship studies and physicochemical profile analysis, it was found that among tested molecules **1-15**, eugenol **1**, prenyl eugenol **4**, isoeugenol **8** and isoeugenol acetate **11** exhibited lethal effects LD_{50} at concentrations < 1 mg/g of insect. On the other hand, eugenol **1**, metallyl eugenol **3**, isoeugenol **8** and isoeugenol acetate **11** showed a good antifeedant activity (CE_{50} =158-209 µg/mL) with a high antifeedant index (70-78 %) at concentration 1000 µg/mL, possessing a weak anti-acetylcholinesterase activity (IC_{50} = 21-31 µg/mL). According to their ecotoxicological profiles (LC_{50} = 2033.1-6303.8 µg/mL on *Artemia salina* larvae), isoeugenol **8** and its acetate derivative **11** could be potential used in control of the growth, feeding, or reproduction of *S. frugiperda* larvae, acting as moderate insecticidal acetylcholinesterase inhibitors and/or antifeedant molecules. Such structure–activity relationship studies could stimulate the identification of lead structures from natural sources for the development of larvicidal and deterrent products against *S. frugiperda* and related insect pests.

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Keywords: *Spodoptera frugiperda;* eugenol derivatives; clove oil; acetylcholinesterase activity; insecticidal activity; antifeedant activity; toxicity on *Artemia salina* larvae

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1. Introduction

The fall armyworm moth *Spodoptera frugiperda* (J. E. Smith), is a polyphagous insect noctuid that feeds on over 60 species of plants, becoming a destructive pest of many agricultural crops of major economic impact such as maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.) and rice (*Oryza sativa* L.) (Sparks, 1979). Larvae can quickly destroy the potential for plant growth by consuming foliage and burrowing into its growing points. To control plant damage requires high volumes of insecticide, resulting in resistance to several kinds of insecticidal compounds (Denholm et al., 1998). In Colombia, *S. frugiperda* directly affects growth and condition of several crops, primarily maize, causing severe damage that consequently generates losses of up to 60% of the production (CAI, 2012). To date, the most common method for controlling this pest depends on the use of conventional insecticides (synthetic insecticides) such as methomyl, carbaryl, cypermethrin, among others. These compounds are very toxic and non-selective, causing enormous damage to the environment and to non-target organisms (Hill, 1983). Among these synthetic agrochemical acetylcholinesterase inhibitors (AChEIs) stand out for their efficiency, but not for their general toxicity. Acetylcholinesterase is a well-validated insecticide target site that has been exploited for many years through the use of organophosphates and carbamates (Thompson and Richardson, 2004). However, their efficacy is consistently weakened by the development of resistance, remarkable in economical important agricultural pest control, including armyworms. It is well known that the rate of resistance development depends on a complex interaction between intrinsic factors connecting to genetic traits, and extrinsic factors such as insecticide management (Denholm and Rowland, 1992).

Therefore, new tools of insect pest management are urgently required. Among these tools, new natural-based insecticides, could lead towards more ecofriendly alternatives. Indeed, recent reports indicates that the use of natural product and natural-derived insecticides continue to increase, whereas sales of organophosphates and carbamates are declining (Dayan et al., 2009). The usefulness of AChEIs as insecticides has also stimulated in recent years many researches towards to find natural products with this activity. Nevertheless, the development of natural AChEIs as leads for the creation of novel insecticidal agrochemicals has not advanced too much (Houghton et al., 2006). Biochemical pesticides may include plant-derived pesticides (botanicals)

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that can interfere with the growth, feeding, or reproduction of pests (Copping and Menn, 2000; Isman, 2005; Isman, 2006). However, some of these compounds are very toxic to mammals.

Among several groups of natural insecticides (pyrethroids, neonicotinoids, avermectins, etc.), essential oils have also shown particular potential as insecticides. Thus, their use for insect control in organic agriculture seems promising. Essential oils, produced by steam distillation of many aromatic plants, have recently received much attention due to their broad spectrum of action. These oils are generally composed of complex mixtures of monoterpenes, biogenetically related phenols and sesquiterpenes (Akhtar et al., 2008). The essential oil obtained by steam distillation of dried flower buds of tropical tree *Eugenia caryophyllus* is part of the class of essential oils that are generally recognized as safe by the FDA (Isman and Machial, 2006). Clove oil contains eugenol as major component (> 92 %) that is an important flavoring agent in the food and cosmetic industry (Cortés-Rojas et al., 2014), and is a powerful insecticide, effective on a wide variety of domestic arthropod pests (Dayan et al., 2009). Moreover, its simpler derivative, methyl eugenol was found to be active against black cowpea aphid *Aphis craccivora*, important polyphagous insect-pest that colonizes, with marked preference for legumes (Tewary et al., 2006). Also, this derivative was recently reported as a new potential botanical insecticide against major insect pests and their natural enemies on rice (Xu et al., 2015). Additionally, O-benzoyl (acetyl) eugenol derivatives and their analogous, demonstrated antileishmanial properties (de Morais et al., 2014) and larvicidal activity against both *Aedes aegypti* larvae (Barbosa et al., 2012; Pandey et al., 2013) and the tobacco armyworm *Spodoptera litura* (Bhardwaj et al., 2010; Hummelbrunner and Isman, 2001). However, to the best of our knowledge, there have not been studies on bioactivity of semisynthetic eugenol derivatives against *Spodoptera frugiperda*, a pest of maize (Zea mays L.)

Taking into account the above stated, in the present study we have synthesized a 15-membered collection of semisynthetic eugenol derivatives **1-15**, based on clove oil (Fig. 1) and sequentially evaluated them against *Spodoptera frugiperda* larvae. Aspects examined include anti-acetylcholinesterase, larvicidal and antifeedant activities. Additionally, toxicity of eugenol derivatives against A*rtemia salina* larvae and physicochemical profile (Lipinski' parameters) for all tested molecules are also presented.

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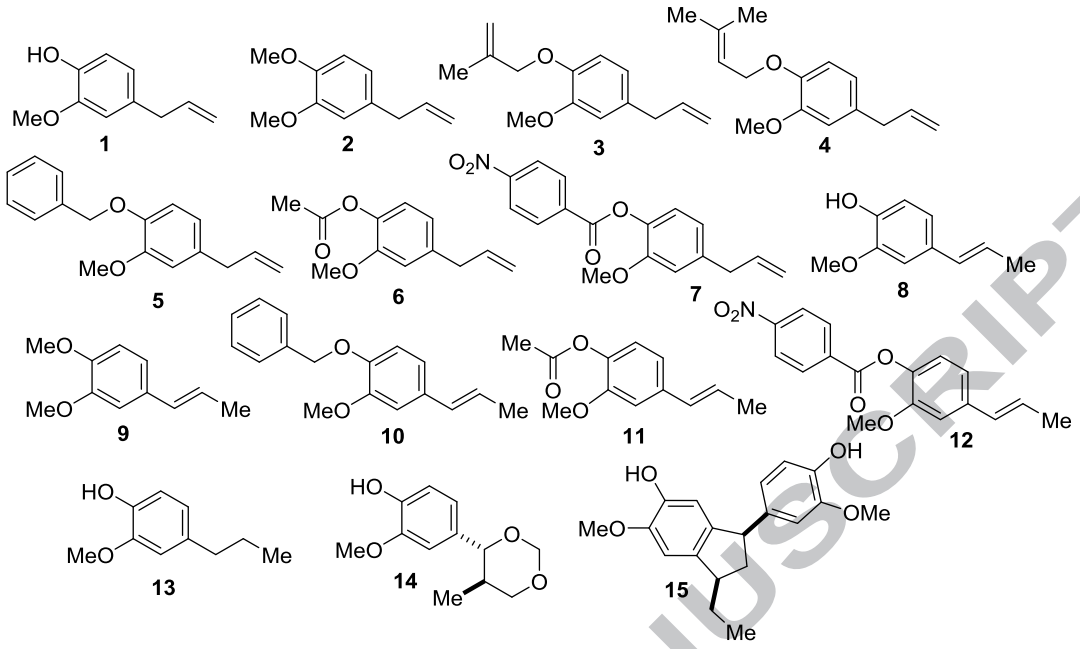

Figure 1 Structures of experimental (iso)eugenol derivatives used in this study.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (ATCh) (≥ 98% purity), acetylcholinesterase from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S), methomyl, cypermethrin and sodium phosphate buffer were obtained from Sigma-Aldrich. 5,5'-Dithiobis-(2-nitro)benzoic acid (DTNB) (99% purity) and sodium hydrogen phosphate (98% purity) were purchased from Alfa Aesar. Di-potassium hydrogen phosphate (98% purity) was acquired from Panreac. Sodium chloride (99.5% purity) was obtained from Mallinckrodt Baker S.A. Tween® 20 for synthesis, solvents dimethyl sulfoxide, absolute ethanol and acetone were acquired from Merck.

Semisynthetic eugenol derivatives **2-7** and isoeugenol derivatives **9-12** were prepared via acetylation or alkylation reactions of commercial eugenol **1** or isoeugenol **8** following described protocols (Pandey et al., 2013; Furniss et al., 1989; Bhagat et al., 1982; Kouznetsov and Merchan-Arenas, 2009). The hydrodistillation of clove oil from dried flower buds acquired at the local market was accomplished following reports (Merchán-Arenas et al., 2011a). Dihydroeugenol (2-methoxy-4-propylphenol) **13**, 2-methoxy-4-(5-methyl-1,3-dioxan-4-yl)-phenol **14** and γ-diisoeugenol **15** were prepared from the isoeugenol as described (Merchán-Arenas et al., 2011b). Eugenol derivatives **2-7** were also obtained directly from clove oil. All

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experimental compounds were purified by column chromatography and were > 95% pure by NMR, GC-MS and C, H, N elemental analyses. Structures of the experimental (iso)eugenol derivatives **1-15** (see, ESI) referred to in this study are shown in the Figure 1.

2.2. Recollection and mass rearing of S. frugiperda

The field populations of the S. *frugiperda* larvae were collected from a crop of corn during harvest time at the municipality of Girón, Santander, Colombia. Larvae were transported to the laboratory in a plastic container with enough plant material to prevent cannibalism. Subsequently, they were placed in plastic containers, (one larva per vessel) and enough food with corn. Adults of all the insect species were used in this study. They were reared in an environment chamber at 25 ± 1 °C with a 16:8 h light:dark photoperiod before they were used for experiments.

2.3. In vitro acetylcholinesterase activity

Acetylcholinesterase (AChE, EC 3.1.1.7, Type VI-S) inhibition was assessed by the Ellman method modifying by scaling microplates, which is based on the reaction of released thiocholine to give a coloured product with a chromogenic reagent (Ellman et al., 1961). The assay was performed in liquid medium, in a 96-well microplate with a final volume of 200 µL. 100 µL of a solution of the reference compound (methomyl), experimental compounds **1-15** or clove oil (at serial concentrations from $1x10^{-3}$ M to 4.88 x 10^{-7} M or from 1000 to 1 ppm for essential oil), dissolved in phosphate buffered saline pH 7.5 and 50 µL of the AChE (0.25 u/mL) were placed.

The assay plate was incubated at 25 °C for 30 min. and was added 100 mL of the substrate solution at pH 7.5, which was composed of DTNB and ATCh. After five minutes from the start of the reaction the absorbance was read at 405 nm in a microplate reader (Biochrom EZ 400 read). Assays were performed in triplicate. The IC_{50} was defined as the concentration required achieving 50% of inhibitory effect. IC_{50} calculations and graphics were performed using SoftMax Pro 5.2 software from Molecular Devices. The AChE inhibition rate (AChEI, %) was calculated by following equation:

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$$\textbf{AChEI (\%) = 100-[(AS-AB)/(AC-AB)] x 100} \quad (\textbf{1})$$

where **AS** is the absorbance of sample, **AB** is the absorbance of blank, **AC** is the control absorbance, to determine the enzyme activity without inhibitor.

2.4. Larvicidal assay

The bioassay of essential oil, and semi-synthetic derivatives **1-15** was evaluated by topical application following by described protocol (Kiran et al., 2006). First, groups of 10 larvae were selected and weighed in order to determine the average weight of each group (between 495 and 505 mg). The experimental compounds and reference insecticide (methomyl) were tested at ratios of 1000, 500, 250, 100, 50 and 1 µg/larva for each compound, using acetone as solvent (Ahmad et al., 2009).

After selecting the group for each concentration of tested compounds **1-15**, they were placed in Petri dishes (one larva per box to avoid cannibalism) (Abbassy et al., 2009). Then, 1µL solution was applied on the back of the larva, leaving a considerable time (Pavela, 2014; Hummelbrunner and Isman, 2001). Assays were performed in triplicate. The mortality rate (%) obtained was analyzed 2, 4, 6, 8, 24, 48 and 72 h after treatment, considering that death of the larva does not respond to mild twinges (Pavela and Vrchotová, 2013). Finally, lethal concentration, at which 50 % of the population dies (LC_{50}), was calculated using following equation:

$$\textbf{Mortality\% = (total died larvae/total larvae) x 100} \quad (\textbf{2})$$

2.5. Antifeedant assay

The antifeedant effect was estimated through a no-choice assay. *S. frugiperda* larvae with average weight of 500 ± 5 mg with five acetone solutions with concentrations of 1000, 500, 250 100 and 50 µg/mL was used. Cypermethrin was employed as a reference compound. Experimental compounds **1-15** were prepared in 1 mL of acetone and 100 µL were added to a disk with a maize average weight 7 ± 1 g, where 100 µL acetone solution of cypermethrin was placed (Simmonds et al., 1996).

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Larvae selected for the bioassay were stored in individual containers without food for six hours before the bioassay. Then, a corn disk with the experimental compounds was given to these larvae. Ten larvae were used for each concentration. Food was weighed every 24 h for 72 h and the percentage of antifeedant index (% **AI**) was calculated with the following equation:

$$\%AI = [(C-T) / (C+T)] \times 100\% \qquad (3)$$

where **C** represents the weight of the diet consumed by the larvae control and **T** is the weight of the diet consumed by the larvae treatment (D'Incao et al., 2012; El-Aswad et al., 2003).

2.6. Toxicity assay on Artemia salina larvae

Assays on brine shrimp lethality larvae (instar II-III) were conducted in 96-well microplates with a final volume of 200 µL following described protocols (Chorus and Bartram, 1999; Carballo et al., 2002). Three replicates were performed for each treatment and control (methomyl and cypermethrin). Two negative controls were used, one only with artificial sea water and methanol. Briefly, dried cysts of *A. salina* were hatched in artificial sea water (1 g cyst/L) at 25 °C under continuous illumination and aeration. After 24 h of incubation, the *A. salina* nauplii were collected and 15 individuals were transferred to each well.

The assay consisted of the exposure of *A. salina* nauplii to different concentrations (1000, 500, 250, 100, 50 and 25 µg/mL) of each eugenol derivatives **1-15** and clove oil. The toxicity was determined after 24 and 48 h of exposure at 25 ± 2 °C in darkness. The number of dead larvae in each well was counted, as well as the total number of brine shrimps after Lugol's fixation. Larvae were considered dead if no internal or external movement was observed during 15 seconds (Mclaughlin et al., 1998). Results are presented as percentage of mortality (Equation 4) and LC_{50} values (Meyer et al., 1982).

$$\text{Mortality\%} = (\text{total died larvae}/\text{total larvae}) \times 100\% \qquad (4)$$

2.7. Statistical analysis

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All experiments were carried out in triplicate. The concentration giving 50% inhibition (IC) was calculated by nonlinear regression with the use of Prism Graphpad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The dose–response curve was obtained by plotting the percentage inhibition versus concentration. The lethal concentration (LC_{50}) is expressed as the standard error of the mean (SEM) of three different experiments in triplicate, the analysis was made using Regression Probit analysis with SPSS for windows version 19.0.

3. Results

3.1. In vitro acetylcholinesterase activity

The IC_{50} results of electric-eel AChE enzyme, presented in Table 1, showed that clove oil of *Eugenia caryophyllus* displayed a moderate enzyme inhibition (IC_{50} = 42.01 µg/mL), eugenol **1** and eugenol acetate **6** (Fig. 1) presented better values of IC_{50} (29.52 and 37.94 µg/mL) than the same oil. Enzyme inhibition of the isoeugenol **8** was practically equal to the eugenol (IC_{50} = 29.78 µg/mL). O-Methyl substitution of these molecules slightly improved inhibition capacity, e.g. methyl eugenol **2** and methyl isoeugenol **9** exhibited IC_{50} 28.04 µg/mL and 26.96 µg/mL values, respectively. Other O-alkyl (acyl) substitutions on the eugenol and isoeugenol rings did not improve anti-acetylcholinesterase activity of compounds **3-7** and **10-12**. Dihydroeugenol **13** showed the best activity with an IC_{50} = 21.91 µg/mL value.

3.2. Larvicidal assay

The LD_{50} analysis of prepared molecules **1-15** (Fig. 1, Table 2) revealed that methomyl, reference broad-spectrum insecticide possessed a strong potential for *S. frugiperda* larvae (LD_{50} = 0.31 mg/g of insect). Clove oil and eugenol **1** and isoeugenol **8** showed LD_{50} 1.02, 0.49 and 0.46 mg/g of insect, respectively. The LD_{50} values of other (iso)eugenol derivatives vary considerably from LD_{50} = 0.88 mg/g to LD_{50} = 4.25 mg/g.

3.3. Antifeedant test

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From antifeedant bioassays data (Table 2), it could be observed differences in the antifeedant activity of tested compounds: eugenol **1** (CE_{50} = 187.73 µg/mL), isoeugenol **8** (CE_{50} 158.51 µg/mL), isoeugenol acetate **6** (CE_{50} = 182.00 µg/mL), methallyl eugenol **3** (CE_{50} = 209.18 µg/mL) exhibited a good antifeedant activity on larvae of *S. frugiperda* and presented the highest antifeedant index (AI = 72-75 %) at the highest concentration tested (1000 µg/mL) (Table 3). For the insecticide reference, cypermethrin showed CE_{50} = 23.83 µg/mL and AI 77.9 %.

3.4. Toxicity assay

Assays on brine shrimp larvae of clove oil and eugenol analogs confirmed that two insecticides references, methomyl and cypermethrin, are very toxic to A*rtemia salina* larvae showing LC_{50} 205.18 and 404.18 µg/mL, respectively. Evaluating the toxicity of semisynthetic eugenol derivatives against *A. salina* larvae, performed in serial concentrations from 500 µg/mL to 0.24 µg/mL, it was found that all compounds exhibited less toxicity (LC_{50} > 1085.1-8488.4 µg/mL) than the toxicity of insecticide references. Methyl- and metallyl-eugenols **2** and **3** were shown to be essentially nontoxic showing LC_{50} 8488.4 and 9478.2 µg/mL, respectively. On the other hand, isoeugenol *p*-nitrobenzoate **12**, eugenol *p*-nitrobenzoate **7** and dihydroeugenol **13** resulted to be the most toxic molecules (LC_{50} 1137.3, 1334.7 and 1085.1 µg/mL, respectively) of tested molecules. Clove oil presented LC_{50} = 4113.98 ppm (µg/mL), being 10-20 times less toxic than insecticide references (Table 2).

4. Discussion

We started our study with *in vitro* determination of anti-AChE activity for prepared eugenol derivatives and clove oil. It because, AChE in insects is an important target for some insecticides, e.g. methomyl that was used as reference insecticide knowing that insecticidal activity is based on the overstimulation of the insect's cholinergic system. Moreover, structure of AChE in the fruitfly *Drosophila melanogaster* was found to be very comparable to vertebrate AChEs in the general charge distribution (Harel et al., 2000; Walsh et al., 2001). Analyzing results on AChE inhibition (Table 1), we could observe that the inhibitory potencies of prepared

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eugenol and isoeugenol derivatives **1-15** (Fig. 1) were usually lower, in the range of IC_{50} = 21.91-98.28 µg/mL, while methomyl exhibited potent anti-AChE activity (IC_{50} = 0.31 µg/mL).

Table 1

Physicochemical properties of eugenol-based molecules **1-15** and clove oil and their inhibition potencies against electric-eel AChE.

	Physicochemical properties[a]				AChE inhibition properties[b]	
Tested comp.	Molecular formula	Molweight	clogP	cLogS	IC_{50} (µg/mL) ± SD	%CV
Clove oil					42.01 ± 2.00	4.70
1	$C_{10}H_{12}O_2$	164.20	2.40	-2.41	29.52 ± 1.34	4.54
2	$C_{11}H_{14}O_2$	178.23	2.87	-2.61	28.04 ± 1.37	4.90
3	$C_{14}H_{18}O_2$	218.30	4.05	-3.01	31.98 ± 0.93	2.90
4	$C_{15}H_{20}O_2$	232.32	4.58	-3.68	32.69 ± 1.16	3.55
5	$C_{17}H_{18}O_2$	254.33	4.64	-4.45	44.22 ± 0.73	1.63
6	$C_{12}H_{14}O_3$	206.24	2.30	-2.74	37.94 ± 1.17	3.09
7	$C_{17}H_{15}NO_5$	313.31	4.18	-4.98	98.28 ± 4.09	4.16
8	$C_{10}H_{12}O_2$	164.20	2.58	-2.57	29.78 ± 0.26	0.86
9	$C_{11}H_{14}O_2$	178.23	3.05	-2.77	26.96 ± 0.78	2.89
10	$C_{17}H_{18}O_2$	254.33	4.62	-4.61	44.65 ± 1.35	3.01
11	$C_{12}H_{14}O_3$	206.24	2.48	-2.90	26.87 ± 0.99	3.72
12	$C_{17}H_{15}NO_5$	313.31	3.86	-5.15	71.30 ± 2.68	3.76
13	$C_{10}H_{14}O_2$	166.22	2.88	-2.48	21.91 ± 0.46	2.13
14	$C_{12}H_{16}O_4$	224.26	0.94	-2.01	37.61 ± 0.63	1.69
15	$C_{20}H_{24}O_4$	328.41	4.64	-4.82	94.37 ± 2.23	2.36
Methomyl	$C_6H_{12}N_2O_2S$	176.23	0.21	-1.56	0.31 ± 0.006	2.00
Cypermethrin	$C_{22}H_{19}Cl_2NO_3$	416.30	6.84	-6.31	nt[c]	--

[a] Performed using ChemDraw 15.0: cLogP (molecular hydrophobicity) - calculated log of 1-octanol/water partition coefficient (neutral form), cLogS -calculated log of aqueous solubility.

[b] IC_{50} values are shown as mean (n > 3) values and are expressed in µg/mL.

[c] Not tested.

However, from obtained results it may be concluded that 1. Clove oil of *Eugenia caryophyllus* as bioinsecticidal model possesses a moderate AChE activity; 2. Substitution of hydroxyl group of the eugenol (isoeugenol) by strong electron-donating group (methoxy, **2** and **9**) enhanced inhibitory potencies. In contrast, presence of the electron-withdrawing groups (acetyl and *p*-nitrobenzoyl) (**6**, **11** and **7**, **12**) deteriorated AChE activity; 3. Simple hydrogenation of the allyl fragment of the eugenol (or isoeugenol) could improve inhibitory

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capacity: being hydrogenated eugenol, the 2-methoxy-4-propylphenol **13** revealed the best activity with an IC_{50} 21.91 µg/mL value.

Taking into consideration that the field of agrochemical discovery could benefit from treatment of pesticide-likeness (agrochemical-likeness) (Tice, 2001; Clarke and Delaney, 2003; Clarke, 2009; Hao et al., 2011; Lamberth et al., 2013) like Lipinski's Ro5 approach for drug research, we tried to correlate calculated molecular properties (clogP, solubility, etc.) and insecticidal activity of tested molecules. As these molecular properties influence considerably absorption and distribution of agrochemicals, we analyzed the results obtained of this initial study. Regarding to the lipophilicity (clogP)-AChE activity relationship of compounds, methomyl, a strong AChEI, shows a hydrophilic character (clogP = 0.21) and good water solubility (clogS = -1.56). Comparing its behavior with that of prepared (iso)eugenol derivatives, we could note that best AChEIs (**2**, **9**, **11** and **13**) with IC_{50} 21-28 µg/mL are moderate lipophilic compounds (2.48 < clogP < 3.05) with clogS (aqueous solubility) values between 2.48-2.90. Despite of we observed a trend between clogP and AChE activity for some molecules groups, compound **14**, which is also a very lipophilic molecule, has poor enzyme inhibition.

Larvicidal activity bioassays of generated eugenol molecules (Table 2) pointed out that some O-derivatives (iso)eugenols (comp. **3-5** and **11**) exhibited lethal effect in 50 % of the population at concentrations < 1 mg/g of insect. The best insecticides activities were showed by propenylphenols, eugenol **1** and isoeugenol **8** with LD_{50} 0.49 mg/g and 0.46 mg/g, while their analogous, dihydroeugenol **13** was a poor insecticide (LD_{50} = 2.02 mg/g of insect), possessing good AChE inhibition properties. Within this group of active and non-active molecules, there are not many differences between their *in silico* hydrophobicity and solubility data. Methomyl, commercial insecticide showed LD_{50} values of 0.31 mg/g of insect. It is interesting to note that the toxicity of the (iso)eugenol acetates were drastically different: whereas eugenol acetate **6** exhibited LD_{50} 2.02 mg/g, isoeugenol acetate **11** showed acceptable toxicity (LD_{50} = 0.91 mg/g), being both moderate AChEIs with similar lipophilicity values (clogP 2.30 and 2.48, respectively) and solubility parameters (clogS -2.74 and -2.90, respectively). Methyl eugenol **2**, with clogP 2.87 and methyl isoeugenol **9** with clogP 3.05 are also moderate AChEIs, but these did not affect *S. frugiperda* larvae development (LD_{50} > 2 mg/g). Prenyl eugenol **4** has clogP 4.58, a higher parameter than methyl eugenol **2** and methallyl eugenol **3**, but its insecticidal potency is good enough (LD_{50} = 0.88 mg/g).

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Table 2

Insecticidal and antifeedant activities of eugenol-based molecules, clove oil and reference agrochemicals.[a,b]

Tested comp.	Insecticidal activity		Antifeedant activity	
	LD_{50} (mg/g of insect)	X^2	CE_{50} (µg/mL)	X^2
Clove oil	1.02 (0.9-1.1)	3.40	489.3 (446.0-532.6)	1.05
1	0.49 (0.45-0.53)	2.38	187.7 (176.6-198.9)	3.60
2	2.02 (1.87-2.17)	2.39	504.5 (498.4-510.5)	0.32
3	1.93 (1.78-2.07)	2.73	209.2 (200.8-217.6)	2.74
4	0.88 (0.83-0.93)	2.02	339.9 (327.2-352.6)	1.04
5	0.90 (0.83-0.97)	2.72	626.4 (600.9-651.9)	1.13
6	2.02 (1.87-2.14)	2.09	712.4 (646.8-778.1)	0.16
7	2.03 (1.87-2.17)	2.55	724.2 (717.4-731.1)	0.17
8	0.46 (0.42-0.49)	2.15	158.5 (149.3-167.8)	3.46
9	2.03 (1.92-2.15)	1.45	536.4 (494.4-578.4)	0.88
10	4.25 (4.03-4.46)	1.77	4226.9 (3901.6-4552.3)	5.14
11	0.91 (0.84-0.97)	3.39	182.0 (172.7-191.3)	2.25
12	2.02 (1.87-2.17)	2.83	388.1 (365.7-410.6)	1.20
13	2.02 (1.87-2.17)	2.09	318.7 (303.5-334.0)	1.77
14	4.24 (4.03-4.46)	2.79	1796.0 (1668.7-1923.4)	4.61
15	2.83 (2.78-2.88)	1.90	2530.1 (2449.3-2611.0)	5.76
Methomyl	0.31 (0.29-0.33)	2.22	nt[c]	--
Cypermethrin	nt[c]	--	23.83 (22.55-25.15)	1.05

[a] Expressed in LD_{50} values as well as CE_{50} values. 95% confidence limits in parentheses.

[b] All means are statistically significant ($P < 0.05$).

[c] Not tested

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After analyzing antifeedant effects (Table 2) of prepared eugenol-based molecules and clove oil on *S. frugiperda* larvae, we could note that various compounds had a moderate to good deterrent effect on *S. frugiperda* larvae (CE_{50} < 340 µg/mL), compared to antifeedant activity of cypermethrin (CE_{50} = 23.85 µg/mL), widely used to control armyworm (Ahmad et al., 2009). The best antifeedant molecules were isoeugenol acetate **11**, eugenol **1**, isoeugenol **8** and methallyl eugenol **3**, all them possessing CE_{50} < 210 µg/mL. Prenyl eugenol **4** and dihydroeugenol **13** showed CE_{50} 339.9 and 318.7 µg/mL, respectively. It should be noted the CE_{50} values of these molecules derived from clove oil are better than that of the clove oil itself (CE_{50} = 489.3 µg/mL). This shows that chemical modifications of our natural product afford potentiation of antifeedant activity. Other eugenol analogous did not act considerably as deterrent molecules (CE_{50} > 500 µg/mL).

Antifeedant index (% **AI**) of tested molecules and clove oil is present in Table 3, in which good antifeedant activity of eugenol **1**, methallyl eugenol **3**, isoeugenol **8**, isoeugenol acetate **11** and dihydroeugenol **13** (AI > 70-78 %) at concentration 1000 µg/mL can be observed. Index AI values obtained are comparable to the antifeedant index of cypermethrin (AI = 77.9 %). It is interesting to note that clove oil, at the same concentration, resulted in less activity (AI = 56.9 %) than semisynthetic molecules. For all derivatives, isoeugenol **8** was the best deterrent compound with 78.5 % index.

Finally, as the brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity, all eugenol-based molecules and clove oil were tested in this assay. Obtained results from this test indicated that in general, all these molecules and clove oil did not present significant toxicity for the *A. salina* nauplii (Table 4). Among these, isoeugenol *p*-nitrobenzoate **12**, eugenol *p*-nitrobenzoate **7** and dihydroeugenol **13**, showed highest values with LC_{50} 1137.3, 1334.7 and 1085.1 µg/mL, respectively. Noteworthy, that all three compounds are almost non-active in both insecticidal and antifeedant tests. Interestingly, dihydroeugenol was found to be more toxic to *A. salina* larvae than eugenol **1** (LC_{50} = 1745.3 µg/mL) and isoeugenol **8** (LC_{50} = 2033.1 µg/mL), which in turn, resulted active against *S. frugiperda* larvae. Structural features are common as all three compounds have a free hydroxyl group. Thus, our SAR analysis suggests that double C=C bond could be responsible for the insecticidal activity and not for the general toxicity in this case.

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Table 3

Antifeedant index (% AI) of clove oil, (iso)eugenols and their semisynthetic analogous **1-15**.

	Concentration, µg/mL					
Tested comp.	1000	500	250	100	50	25
Clove oil	56.9	50.2	42.3	35.4	25.9	15.5
1	75.0	62.8	55.3	44.9	32.3	13.5
2	59.5	48.3	41.2	31.5	25.0	17.1
3	72.4	66.7	56.7	41.1	22.8	11.0
4	63.7	53.8	46.2	37.2	24.9	15.4
5	52.7	47.5	42.5	37.3	28.5	19.0
6	55.3	44.4	36.6	26.7	17.8	nt[a]
7	55.0	45.1	39.4	31.6	23.8	nt
8	78.5	65.9	56.6	45.6	37.3	15.6
9	58.5	47.3	40.1	33.2	20.5	nt
10	35.6	25.7	19.2	10.4	nt	nt
11	74.4	65.4	54.5	42.7	34.5	15.6
12	66.9	52.8	41.5	30.2	14.4	nt
13	72.9	65.0	38.6	26.7	11.7	nt
14	41.9	33.2	20.0	10.5	nt	nt
15	42.5	33.7	23.5	11.6	nt	nt
Cypermethrin	77.9	72.7	66.5	61.0	56.0	49.2

[a] Not tested

On the other hand, toxicity on *A. salina* larvae of O-benzyl eugenol derivative **5** and O-benzyl isoeugenol **10** was slightly decreased (LC_{50} 3756.0 and 3594.7 µg/mL, respectively) comparing with that of (iso)eugenol molecules. Moreover, methyl, methallyl or acetyl substitution in hydroxyl group of eugenol (comp. **2**, **3** and **6**) or isoeugenol (comp. **9** and **11**) has led to decline more general toxicity (LC_{50} 5691.1-9478.2 µg/mL). In these series, isoeugenol derivatives resulted more toxic than eugenol-based molecules. Nevertheless, the most toxic eugenol molecules tested in this work are much less harmful than reference agrochemicals: both methomyl and cypermethrin showed very high toxic properties (LC_{50} 205.2 and 404.2 µg/mL, respectively). It proves the potential of modified eugenol molecules from clove oil as possible insecticides.

In summary, larvicidal activity of eugenol derivatives tends to decrease as follows **1** > **4** > **5** > **3** > **6** ≈ **2** ≈ **7** ≈ **13** and isoeugenol derivatives gave following range **8** > **11** >> **12** ≈ **9** ≈ **13** >> **10**, while antifeedant activity of these series ranged in following orders **1** > **3** > 1**3** > **4** > **2** > **5** > **6** > **7** and **8** > **11** > **13** > **12** > **9** >> **10**, respectively. Regarding general toxicity, their toxic effects tend to increase in following manner **2** > **3** > **6** > **5** > **4** > **1** > **7** (eugenol molecules series) and **9** >

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11 > **10** > **8** > **13** > **12** (isoeugenol molecules series). Such structure–activity relationship studies can promote the identification of lead structures from natural sources and for the development of larvicidal products against *S. frugiperda* and related insect pests.

Table 4

Toxicity of eugenol-based molecules **1-15** and clove oil in *Artemia salina* larvae test[a,b]

Tested comp.	LC_{50} (µg/mL)	LC_{50} (mM)	X^2
Clove oil	4114.0 (3727.8-4500.2)	--	9.39
1	1745.3 (1614.3-1876.2)	10.6 (9.80-11.4)	0.41
2	8488.4 (7956.9-9020.0)	47.7 (47.6-50.7)	3.24
3	9478.2 (9010.3-9946.1)	43.5 (41.7-45.6)	3.68
4	2776.7 (2541.9-3011.4)	12.0 (11.0-13.0)	3.42
5	3756.0 (3527.4-3984.6)	14.8 (13.9-15.7)	0.39
6	7928.2 (7721.3-8135.1)	38.3 (37.3-39.3)	4.90
7	1334.7 (1291.6-1377.8)	4.26 (4.12-4.40)	0.30
8	2033.1 (1980.3-2086.0)	12.4 (12.1-12.7)	0.51
9	5691.1 (5448.3-5933.9)	32.0 (30.6-33.4)	4.11
10	3594.7 (3336.7-3852.7)	14.1 (13.1-15.1)	0.56
11	6303.8 (5782.01-6825.6)	30.5 (28.0-33.0)	3.09
12	1137.3 (1063.6-1210.9)	3.63 (3.40-3.87)	0.65
13	1085.1 (1024.7-1145.5)	6.53 (6.17-6.89)	3.42
14	1629.6 (1508.7-1750.5)	7.27 (6.73-7.81)	1.10
15	34266.1 (31380.3-37151.9)	104.0 (95.21-112.8)	0.38
Methomyl	205.2 (184.9-225.5)	0.71 (0.64-0.78)	0.57
Cypermethrin	404.2 (383.2-425.2)	0.97 (0.92-1.02)	0.62

[a] Expressed in LC_{50} value. 95% confidence limits in parentheses.

[b] All means are statistically significant ($P < 0.05$).

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5. Conclusion

The anti-acetylcholinesterase, larvicidal, antifeedant activities and general toxicity of 15 semisynthetic eugenol derivatives based on clove oil, including the own oil, against the maize armyworm, *Spodoptera frugiperda* (J.E. Smith) were evaluated. These bio-applications have not been reported earlier. Comparing biological results of clove oil and prepared eugenol-based molecules, we could conclude that clove oil and (iso)eugenols are a good choice as models for developing new insecticides against *S. frugiperda*. Chemical manipulation of eugenol (or isoeugenol) core allowed to identify some potential derivatives as insecticides or deterrent agents, among them eugenol **8** and its acetate derivative **11** stand out for their characteristics. Both are small lipophilic molecules with moderate insecticidal acetylcholinesterase inhibitory effects and antifeedant properties that could control the growth, feeding, or reproduction of *S. frugiperda* larvae, that is pest of maize (*Zea mays* L.). Structure–activity relationship studies and physicochemical profile (Lipinski' parameters) analysis can endorse the rapid identification of lead structures from natural sources for the development of larvicidal and deterrent products against *S. frugiperda* and related insect pests.

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