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Insect Growth Regulator (IGR) effects of *Eucalyptus citriodora* Hook (Myrtaceae)

[Efectos tóxicos e IGR de *Eucalyptus citriodora* Hook (Myrtaceae)]

J. Rodrigo SALAZAR[1]*, Patrício TORRES[2], Blanca SERRATO[3], Mariana DOMINGUEZ[4], Julio ALARCÓN[5] & Carlos L. CÉSPEDES[5]*

[1]Facultad de Ciencias Químicas, Universidad La Salle, México D.F., México
[2]Facultad de Ciencias, Instituto de Ciencias Ambientales y Evolución. Universidad Austral de Chile, Valdivia, Chile
[3]Campo Experimental Uruapan, Inifap, Uruapan, Michoacán, México
[4]Departamento de Biología Celular y del Desarrollo, Instituto de Fisiología Celular, UNAM, México
[5]Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bio-Bio, Chillan, Chile
***Contactos | Contacts:* J. Rodrigo SALAZAR - *E-mail address:* juan.salazar@ulsa.mx**
Contactos | Contacts: Carlos L. CÉSPEDES - E-mail address: cespedes.leonardo@gmail.com

Abstract In addition to eucalyptin the methanol extract from leaves of *Eucalyptus citriodora* (Myrtaceae) afforded the known compounds apigenin, chrysin, luteolin, naringenin, quercetin; together with betulinic acid, oleanolic, ursolic acid, and two remaining complex mixtures of unidentified flavonoids and triterpenes. These compounds together with triterpenes mixtures, hexane and ethyl acetate extracts showed antifeedant, insecticidal and insect growth regulatory activities against fall armyworm [*Spodoptera frugiperda* JE Smith (Lepidoptera: Noctuidae)] an important pest of corn, and yellow mealworm [*Tenebrio molitor* (Coleoptera:Tenebrionidae)] a pest of stored grains. The most active compounds were chrysin, eucalyptin, quercetin, luteolin, and betulinic and oleanolic acids and the mixtures of flavonoids and triterpenes (M1 and M2). These compounds and mixtures had IGR activity between 0.2 to 5.0 µg/mL and insecticidal effects between 5.0 and 10.0 µg/mL.The extracts were insecticidal to larvae, with lethal doses between 20-100 µg/mL. These compounds appear to have selective effects on the pre-emergence metabolism of the Lepidoptera, since in all treatments of the larvae of *S. frugiperda* the pupation was shortened and this process showed precociousness in relation to control. Thus, these substances may be useful as potential natural insecticidal agents.

Keywords: *Eucalyptus citriodora*, Myrtaceae, triterpenes, IGR, *Spodoptera frugiperda*, *Tenebrio molitor*.

Resumen: Extracto metanolico de hojas de *Eucalyptus citriodora* (Myrtaceae) proporcionó además de eucalyptin, flavonoides tales como apigenina, crisina, luteolina, naringenina, quercetina; los triterpenos, ácido oleanólico, ácido ursólico betulínico, y dos mezclas complejas de flavonoides y triterpenos no identificados M1 y M2. Los flavonoides, triterpenos y mezclas de triterpenos, extractos de acetato de etilo y hexano mostraron efectos antialimentarios, insecticida y actividad reguladora de crecimiento (IGR) frente al gusano cogollero [*Spodoptera frugiperda* JE Smith (Lepidoptera: Noctuidae)], una plaga importante del maíz y frente al gusano de la harina [*Tenebrio molitor* (Coleoptera: Tenebrionidae)], una plaga de los granos almacenados. Los compuestos más activos fueron crisina, eucalyptin, quercetina, luteolina, ácido betulínico y ácido oleanólico y las mezclas de flavonoides y triterpenos (M1 y M2). Estos compuestos y mezclas mostraron actividad IGR entre 0,2 y 5,0 µg/mL y efectos insecticidas entre 5,0 y 10,0 µg/mL. Los extractos mostraron carácter insecticida para las larvas a dosis letales entre 20-100 µg/mL. Estas muestras parecen tener efectos selectivos sobre el metabolismo de pre-emergencia de los lepidópteros, ya que en todos los tratamientos de las larvas de *S. frugiperda* el tiempo de la pupación se acortó; este proceso muestra precocidad en relación con el control, las substancias ensayadas en este trabajo pueden ser útiles como potenciales agentes insecticidas naturales.

Palabras clave: *Eucalyptus citriodora,* Myrtaceae, triterpenos, IGR*, Spodoptera frugiperda, Tenebrio molitor*

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INTRODUCTION

A great number of secondary metabolites (SM) are produced by plants, for instance species of Myrtaceae family. These SM, which are stored in roots and aerial parts, include alkaloids, flavonoids, phenolics and terpenoids. Some of these compounds occurring into the leaf and stems prevent waterless and probably protect the plant from sunlight. Another interesting ecological role of secondary metabolites is related to defense against phytophagous insects and pathogens (Macias *et al*., 2007).

Our field observations in different regions of Central and South America, had shown us that the known and very common "tree lemon scent eucalyptus" (*Eucalyptus citriodora* Hook) is very resistant to the insect attack, and specially to *Spodoptera frugiperda* and *Tenebrio molitor*, that are insect widely distributed and attack fruits, corn crops and stored grains.

The *Eucalyptus* genus (Myrtaceae), is native to Australia, because of their pronounced resistance to insect attack in nature, we have chosen to investigate the insecticidal activity specifically of *Eucalyptus citriodora* species from the family Myrtaceae that until now has not been considered important as medicinal or agronomic plant. These plants survive under strong environmental stress conditions and are not attacked by insects (Imatomi *et al.,* 2013), and are used as cellulose sources for paper industry.

To the best of our knowledge, very few species of the large genus *Eucalyptus* have been studied phytochemically, there are more than 500 species widely cultivated in various parts of the world (Brooker & Kleinig, 2004). These are commonly called gum trees, as these exude a gum and are known world over for insect-repellant properties. The eucalypt trees are characterized by evergreen foliage that is variably fragrant due to the presence of volatile essential oils. These find an extensive use in perfumery and pharmaceutical industry and their amount and fragrance varies with the species. In Americas, eucalypts was introduced around 1890' and is now one of the major crops of trees for cellulose industries under the forest plantations (FAO, 2001). These species are cultivated for forest industry and are majority red gum (*E. camaldulensis*), lemon-scented gum (*E. citriodora*), Tasmanian blue gum (*E. globulus*), Shining gum (*E. nitens*) and Cider gum (*E. tereticornis*). Among these, *E. citriodora* is a large, quick-growing tree with smooth and white bark and lemon-scented leaves. It is extensively planted and coppiced for the extraction of essential oil that is rich in citronellal and used in perfumery and as flavouring agent. The oil is known to possess a wide spectrum of biological activities including fungicidal (Ramezani *et al.,* 2002), antimicrobial pathogens (Shahnaz & Mohammed, 2013), anticancer (Bhagat *et al.,* 2012), antioxidant (Pal Singh *et al.,* 2012), insecticidal (Isman, 2000), nematicidal (Pandey *et al.,* 2000), toxicity and repellency (Gusmão *et al.,* 2013) and allelopathic properties (Kohli, 1990). With the exception of the protective role in gastric ulcer (Al-Sayed & El-Naga, 2015) and the increasing effects on glucose transporter of triterpenes and flavonoids (Wang *et al.,* 2014), until now nothing is known in relation to the role of flavonoids, phenolics and terpenes from this plant species and its repellency activity of insects with reference to the presence and amount of these chemical constituents and its role as chemical defense properties (Kessler & Baldwin, 2002; Simmonds, 2006; Romanelli *et al.,* 2010; Barbehen & Constabel, 2011; Smith, 2011; Nenaah, 2013). This information can serve as an important resource for exploring its commercial utilization. With this objective, a study was therefore planned to determine the content, composition and insect growth inhibitory activities of secondary metabolites of *E. citriodora*.

In the present study, flavonoids and triterpenes isolated from the leaves of *E. citriodora* were evaluated as insect growth inhibitors. The flavonoids and triterpenes found in this specie have previously been isolated from many plant species; however, no any insecticidal study has been carried out yet (Lamberton, 1964; Horn *et al.,* 1964; Horn & Lamberton, 1964; Zapesochnaya *et al.,* 1984; Gottlieb *et al.,* 1972; Wollenweber & kohorst, 1981; Voirin, 1983; Wollenweber *et al.,* 2000).

Increasing interest in the application of plant secondary metabolites for insect pest management (IPM) has led us to search for new environmentally friendly but biologically active and biodegradable natural products with low mammalian toxicity to avoid some of the deleterious effects on the environment by synthetic pesticides and the origin of resistant strains of insects (Kubo, 1997; Gonzalez & Estevez-Braun, 1998; Céspedes *et al.,* 2000; Céspedes *et al.,* 2001a; Céspedes *et al.,* 2001b; Torres *et al.,* 2003; Céspedes *et al.,* 2004; Cespedes *et al.,* 2005; Cespedes *et al.,* 2008). Some previous studies are focused on nortriterpenoids (limonoids) from the family Meliaceae because of their potent effects on insect pests and low toxicity (Kubo &

Klocke, 1982a; Kubo & Klocke, 1982b; Champagne *et al.,* 1989; Klocke *et al.,* 1989; Carpinella *et al.,* 2002; Carpinella *et al.,* 2003). One of such compounds discovered for us, gedunin, has proven to have excellent insecticidal properties (Céspedes *et al.,* 2000; Céspedes *et al.,* 2004). Another triterpenoids as sterols with insect growth regulator activities are β-ecdysone, ajugasterone C, cyasterone and other phytoecdysteroids that were discovered by Kubo's group (Kubo *et al.,* 1981; Kubo *et al.,* 1983; Kubo & Klocke, 1983; Kubo *et al.,* 1987; Zhang *et al.,* 1992).

The plant species under study in this work showed be very rich in flavonoid and triterpenoid composition. Flavonoids and terpenes have an important biological function as key compounds in the acquirement of cholesterol by insects (Simmonds, 2006). On the other hand, mammals obtain cholesterol either by dietary absorption or by biosynthesis from mevalonate. Since insects have no capacity for *de novo* sterol synthesis, they obtain sterols exclusively from exogenous sources for their growth, development, and reproduction. Many phytophagous and omnivorous insects satisfy their cholesterol requirements by side chain dealkylation of C-24 alkyl group of dietary C_{28} and C_{29} phytosterols (nortriterpenoids) (Svoboda & Feldlaufer, 1991; Ikekawa *et al.,* 1993).

Researchs on the sites and mechanisms of action of allelochemicals responsible for insecticidal or insect growth regulation (IGR) activities indicate that many phenolic and terpenoid compounds are involved. These substances are important enzymatic and metabolic inhibitors (Kubo & Klocke, 1983; Klocke *et al.,* 1989; Hammond & Kubo, 1999; Kubo & Kinst-Hori, 1999; Kubo *et al.,* 2000; Céspedes *et al.,* 2000; Céspedes *et al.,* 2001a; Calderón *et al.,* 2001; Panzuto *et al.,* 2002; Kubo *et al.,* 2003a; Kubo *et al.,* 2003b; Barbehenn & Constabel, 2011). In addition, many metabolites of angiosperms have antifeedant effects on phytophagous insects (Feeny, 1968; Feeny, 1976; Rhoades & Cates, 1976; Swain, 1979). Some of them bind to proteins, acting as nutritional protein precipitating agents, inhibiting insect digestive enzymes (Feng, 1995; Duffey & Stout, 1996; Korth & Dixon, 1997; Tamayo *et al.,* 2000) and thus reducing their digestibility (Feeny, 1976; Rhoades, 1979). We have previously demonstrated that diverse secondary metabolites have different sites of action and different molecular targets, when they interact with enzymes and metamorphosis processes (Céspedes *et al.,* 2000; Kubo, 2000; Céspedes *et al.,* 2001a; Céspedes *et al.,* 2001b; Calderón *et al.,* 2001; Kubo *et al.,* 2003a; Kubo *et al.,* 2003b; Torres *et al.,* 2003).

The aim of this work was to correlate the phytochemical composition of *E. citriodora* with the inhibitory behavior on growth and development of *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) as model systems for the study of pest insects. Our data indicate also that it is possible to correlate some IGR parameters, i.e. physiological activities (e.g., delay of pupation and moulting, emergence, deformities, etc) with the chemical structure and moieties of our compounds; these data are important for insect control and IPM studies (Klocke & Kubo 1982; Kubo & Klocke, 1982a; Berenbaum, 1988; Hedin *et al.,* 1991; Dhadialla *et al.,* 1998; Agarwal *et al.,* 2000; Kessler & Baldwin, 2002). On the other hand, these parameters are accepted as indirect measures of other physiological processes (Camps, 1988; Céspedes *et al.,* 2000; Kubo *et al.,* 2003a; Kubo *et al.,* 2003b; Torres et al., 2003; Céspedes *et al.,* 2004; Macias *et al.,* 2007) affected by the assayed chemicals.

The present paper specifically deals with the effects against to all growth parameters of fall armyworm (FAW) (*S. frugiperda*) and yellow mealworm (*T. molitor*) of isolate compounds from the ethyl acetate (EA-ext) and n-hexane (Hex-ext) extracts of the aerial parts (leaves) of *E. citriodora* [Flavonoids **1**, **2**, **3**, **4**, **5**, **6** and mixture $\mathbf{M_1}$ **(1 - 6** + unidentified flavonoids**)** and triterpenes **7, 8, 9** and mixture $\mathbf{M_2}$ (**7 - 9** + unidentified terpenes)]. Aspects examined were insecticidal and growth regulatory activities, rate of development, time of pupation, adult emergence, and deformities in insects at each of the stages. The effects of these substances were compared to those of gedunin and to *Yucca periculosa* (Me-Yuc) and *Cedrela salvadorensis* MeOH (Me-Ced) extracts, all known growth inhibitors of *S. frugiperda* (Céspedes *et al.,* 2000; Calderón *et al.,* 2001; Torres *et al.,* 2003).

MATERIAL AND METHODS

Plant materials

Leaves of *Eucalyptus citriodora* were collected on the mountains slope between Michoacán, Hidalgo and Queretaro States, Mexico, during springs from years 2003 and 2007. Voucher specimens have been deposited in the Herbarium of INIFAP, Uruapan, Mexico, 17500/-517, and in the Herbarium of the University of Illinois, at Urbana–Champaign, IL,

USA (ILL, Voucher DS-10253/54). The samples were identified by Prof. David S. Seigler, Ph.D. (Emeritus Professor of Plant Biology and Curator of the Herbarium of the University of Illinois at Urbana-Champaign, USA).

Spectral data

IR spectra were recorded on a 750 spectrometer (Nicolet Magna-IR). ^1H-NMR spectra were recorded at 300 and 500 MHz, and ^{13}C-NMR spectra at 75 and 125 MHz, respectively, (Varian VXR-300S and VXR-500S spectrometers). Chemical shifts (ppm) are relative to $(CH_3)_4Si$. $CDCl_3$, MeOD-d_4, and acetone-d_6 (Aldrich Chemical Co.) were used as solvents. Coupling constants are quoted in Hz. EIMS data were determined on a mass spectrometer at 70 eV (JEOL JMS-AX505HA). FABMS were obtained on a mass spectrometer operated with an acceleration voltage of 10 kV (JEOL JMS-SX102A). Samples were desorbed from a nitrobenzyl alcohol matrix using 6 keV Xenon atoms. UV spectra of pure compounds were determined on a Shimadzu UV-160 instrument. Optical rotation was measured on a spectropolarimeter (JASCO DIP-360). Melting points were obtained on a Fisher-Johns hot-plate apparatus and remain uncorrected.

Chemicals and Solvents

All reagents used were analytical or chromatographic grade. Methanol, CH_2Cl_2, $CHCl_3$, KCl, $CuSO_4$, NH_4Cl, $MgCl_2$, silica gel GF_{254} analytical chromatoplates, silica gel grade 60, (70-230, 60 Aº) for column chromatography, *n*-hexane, and ethyl acetate were purchased from Merck-Mexico, S.A., Mexico. Column chromatography was also carried out on Silica Gel G (Merck, Darmstadt, Germany).

Extraction, Isolation of flavonoids and triterpenes

Dried and ground leaves (18.5 kg) of *E. citriodora* was processed, extracted, and purified. This sample was extracted with MeOH and then was concentrated under vacuum; this extract was partitioned throughout water/methanol (1:1) with *n*-hexane, CH_2Cl_2 and ethyl acetate partition, obtaining four extracts. These extracts were used for preliminary bioassay evaluation. The most active extract of each of the samples were Hex and EA extracts, which were tested for insecticidal activity and then submitted to CC using Si-gel (G 60, Merck) as solid phase. Column chromatography elution of both extracts (EA and Hex) was carried out with different solvent systems (hexane: ethyl acetate: methanol; mixtures) afforded mixtures of active fractions **M1** and **M2**, respectively, that were analyzed by TLC and insecticidal assay (Céspedes *et al.,* 2004). Repeated TLC of these fractions (**M_1** and **M_2**) led to the isolation of the secondary metabolites which were purified by prep-TLC. From **M_2** (from Hex-ext) were isolated and identified ursolic **7**, betulinic **8** and oleanolic acids **9,** together other terpenes that remains unidentified. Furthermore, from **M_1** (from EA-ext) were isolated the known flavonoids eucalyptin **1**, quercetin **2**, luteolin **3**, chrysin **4**, apigenin **5** and naringenin **6,** together other flavonoids that remains unidentified. All these compounds were obtained as pure natural products, which were analyzed and characterized completely by their R_f and IR, UV, ^1H NMR and ^{13}C NMR spectral data, and comparison with authentic samples. Additionally, gedunin was used as positive control obtained from previous works (Cespedes *et al.,* 2000).

Compounds **1** to **9** were purified in sufficient amount to perform the bioassays. Analytical TLC was performed on Silica gel 60 F_{254} E. Merck plates and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 ºC.

Bioassays with fall armyworm (S. frugiperda).

Larvae used for experiments were obtained from culture at the Centro de Investigación en Biotecnología at the Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México, maintained under previously described conditions (Aranda *et al*., 1996; Céspedes *et al.,* 2000; Torres *et al.,* 2003). An artificial diet containing 800 mL of sterile water, 10.0 g of agar, 50.0 g of soy meal, 96.0 g of corn meal, 40.0 g of yeast extract, 4.0 g of wheat germ, 2.0 g of sorbic acid, 2.0 g of choline chloride, 4.0 g of ascorbic acid, 2.5 g of p-hydroxybenzoic acid methyl ester, 7.0 ml of Wesson salt mixture, 15.0 ml of Vanderzant vitamin mixture for insects, 2.5 ml of formaldehyde, 0.1 unit of streptomycin, 5.0 g of aureomycin, and 20.0 g of milled ear of corn grain (for 1 kg of diet) were used for the bioassay, which was prepared by the procedure described earlier (Mihm, 1987). 24-Well polystyrene multidishes were filled with the liquid diet, and then left for twenty minutes at room temperature under sterile conditions. The 3.4 ml wells, 17 mm in depth x 15 mm in diameter with a 1.9 cm^2 culture area. All test compounds were dissolved in 95% ethanol and layered on top each well filled with the artificial diet using six concentrations (see Table 1) and a control (1 ml 95% ethanol), allowing evaporation of solvent.

Table 1[a]
Pupation[b], emergence[b] , mortality and LD_{50} parameters of compounds eucalyptin 1, quercetin 2, luteolin 3, chrysin 4, apigenin 5, ursolic acid 7, oleanolic acid 8, betulinic acid 9, mixtures M_1, M_2, Hex and EtOAc extracts from *E. citriodora* on fall armyworm growth bioassay[a].

Doses	Conc. [µg/mL]	Pupation[c] SP [%][d]	Mortality (%)[f]	Emergence (%)[b]	LD_{50}[e] (µg/mL)
Control	0.0	97.2	7.50	100	
1	5.0	11.1	88.9	0	0.91
	10.0	**0**	**100**	0	
2	2.0	1.4	**94.4**	0	
	5.0	1.4	94.4	0	0.77
	10.0	0	**100**	0	
3	2.0	18.4	81.6	1.4	4.11
	5.0	0	**100**	0	
4	2.0	4.0	96.0	0	0.68
	5.0	0	**100**	0	
5	5.0	11.0	89	1.4	1.34
	10.0	1.4	98.6	0	
7	50.0	75.9	24.1	37.9	8.7
	100.0	18.4	81.6	9.2	
8	50.0	49.0	51.0	18.4	5.3
	100.0	33.0	67.0	9.4	
9	50.0	1.4	98.5	0	2.0
	100.0	0	**100**	0	
M_1	10.0	1.4	97.9	0	0.25
	20.0	0	**100**	0	
M_2	100.0	13.3	86.7	1.4	3.5
	200.0	0	**100**	0	
EA ext	20.0	5.6	94.4	0	0.78
	100.0	0	100	0	
Hex ext.	250.0	12.0	88.5	1.1	9.91
	500.0	0	100	0	
Gedunin	10.0	46.7	53.3	9.2	9.8
	25.0	20.0	79.9	1.4	
	50.0	3.4	96.6	0	
MeOH-Yuc	2.0	55.0	45.0	1.4	8.2
	10.0	42.0	58.0	0	

Note. [a]Mean values of three replicates, taken after to complete life cycle,. The values for growth bioassay were from weight only, values taken at 22 ± 1 days before pupation, the criteria followed was to account larvae that formed pupae, the larvae that not formed pupae were counted as died larvae. [b]Values taken after complete pupation. The values for 6 were omitted because were irrelevant and this compound not showed any effect at all assayed concentrations. Average under a Student-Newman-Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% Confidence limits. [d] Percentage with respect to control. SP: Survival Pupation = Number of surviving pupae x 100 / Total larvae for pupation. [e]LD_{50} = Lethal doses for 50% of death

Hex-ext (10.0-30.0 µg/ml) and EA-ext (1.0-10.0 µg/ml) were used, as these extracts showed the highest inhibitory activity in preliminary trials (data not shown). For each concentration used and for the controls, a single *S. frugiperda* neonate first instar larva was placed on the diet mixture in each well for

7 days, thus each experiment contains 72 larvae in total (each plate of 24 wells with three replicates). After 7 days, surviving larvae were measured and weighed and then transferred to separate vials containing fresh stock diet. Larval weight gains and mortality were recorded after 23 days of incubation, as the pupation average is 23 ± 1 days.

Other life cycle measurements were recorded, such as time to pupation, mortality of larvae and adult emergence and deformities. All treatments were carried out in a controlled environmental chamber with an 18L: 6D photoperiod, at 25 ºC day and 19 ºC night temperature regime, and a relative humidity of 80% ± 5%. There were three replications for each assay. Control assays (24-wells) contained the same numbers of larvae, volume of diet, and ethanol as the test solutions (Céspedes *et al.,* 2000; Torres *et al.,* 2003;).

Bioassays with yellow meal worm (T. molitor)

A stock culture of *T. molitor* L. (Coleoptera: Tenebrionidae) larvae was fed with wheat bran in plastic boxes at 24.0 ± 1° C, with a 16:8/L:D photoperiod, and these larvae were maintained into a chamber under these environmental conditions. Bioassays were performed with last instar larvae of *T. molitor* based on live weight (103-160 mg). Serial dilutions over the range of (0.5-2.0) μg/larva of each test material were prepared using test solutions Me_2CO/MeOH (9.5:0.5 v/v) were topically applied to ventral abdominal segments with a microsyringe. Aliquots, each of 2 μL/larva of tested sample were applied; equivalent to 2.0 μg/larva of the assayed compounds for each one of concentrations used. Controls were treated with the solvent alone. All treatments were set up in three replicates of 20 larvae each along with control sets. After treatment insects were placed in Petri dishes (5 cm diameter), with 3g of sterilized wheat bran, a moistened cotton for preserve humidity and held at 24.0 ± 1° C with 16:8 (L:D) photoperiod. The number of larvae that successfully pupated, as well as the duration of the pupal stage (in days) were recorded every 24 h for 30 days (end-point of the experiment).

Acute toxicity

Acute toxicity was determined by topical application and oral injection of compounds to larvae of the last stage (fifth instar) of *S. frugiperda* and *T. molitor*, respectively. The larvae were iced to stop their movement and treated on their abdomens and mouths with each of the test compounds, at concentrations of 1.0 - 20.0 μg/ml, for both insect species. The solvent used was acetone (10.5 μl) which was administered with a microsyringe Hamilton of 25 μl. The control was only treated with 10.5 μl of acetone. After 24 h, survivals were recorded. Ten larvae were used for each concentration, respectively (Calderón *et al.,* 2001; Torres *et al.,* 2003).

Relative growth index and growth index.

The relative growth index (RGI) and growth index (GI) were calculated according to Zhang *et al.* 1993.

Statistical Analyses

Data shown in figures and tables are average results obtained by means of three replicates and independent experiments and are presented as average ± standard errors of the mean (SEM). Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by GLM procedures. Results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance. Differences between treatment means were established with a Student-Newman-Keuls (SNK) test. The GI_{50}, RI_{50} and I_{50} values for each activity were calculated by PROBIT analysis based on percentage of inhibition obtained at each concentration of the samples. I_{50} is the concentration producing 50% inhibition of growth. Complete statistical analysis was performed by means of the MicroCal Origin 6.1 statistical and graphs PC program.

RESULTS AND DISCUSSION

In our screening program designed to discover interesting biological activities from plants, it was found that *E. citriodora* showed insecticidal activity in a preliminary trial. Based on this information, we have carried out several studies of the aerial parts of *E. citriodora*.

From EA-ext was obtained the mixture $\mathbf{M_1}$ that was worked through open column chromate-graphy yielding the known flavonoids eucalyptin **1**, quercetin **2**, luteolin **3**, chrysin **4**, apigenin **5**, and naringenin **6**, together with a complex mixture of other flavonoids that remains unidentified (Figure 1). From the mixture $\mathbf{M_2}$ obtained from the Hex-ext of the leaves of this plant were identified the triterpenes ursolic **7**, betulinic **8,** and oleanolic **9** acids (Figure

2), and the known triterpenes stigmasterol, lupeol, friedelin, β-amirin, together with a complex mixture of other triterpenes that remains unidentified, the chemical structure characterization of triterpenes was made by comparison with references spectral data and authentic samples and standards, complete NMR assignments were in according with those previously reported (Lamberton, 1964; Gottlieb *et al.,* 1972; Voirin, 1983; Zapesochnaya *et al.,* 1984; Peng *et al.,* 1998; Wollenweber *et al.,* 2000; Upasani *et al.,* 2003; Seebacher *et al.,* 2003; Adeyemi *et al.,* 2010; Asnaashari *et al.,* 2010; Park *et al.,* 2010) (Figure 1).

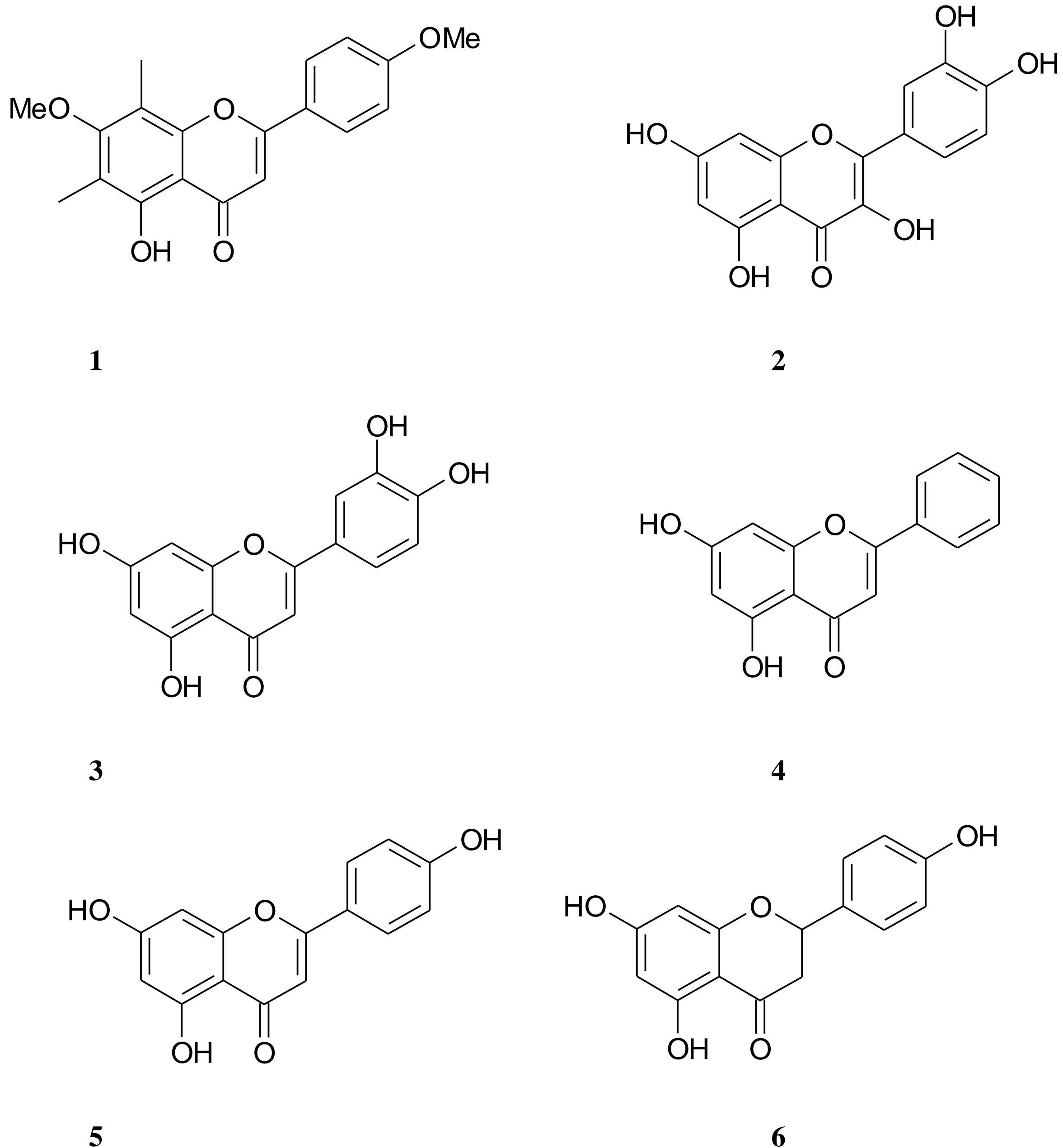

Figure 1
Chemical structures of flavonoids isolated from *E. citriodora*: eucalyptin 1, quercetin 2, luteolin 3, chrysin 4, apigenin 5, naringenin 6.

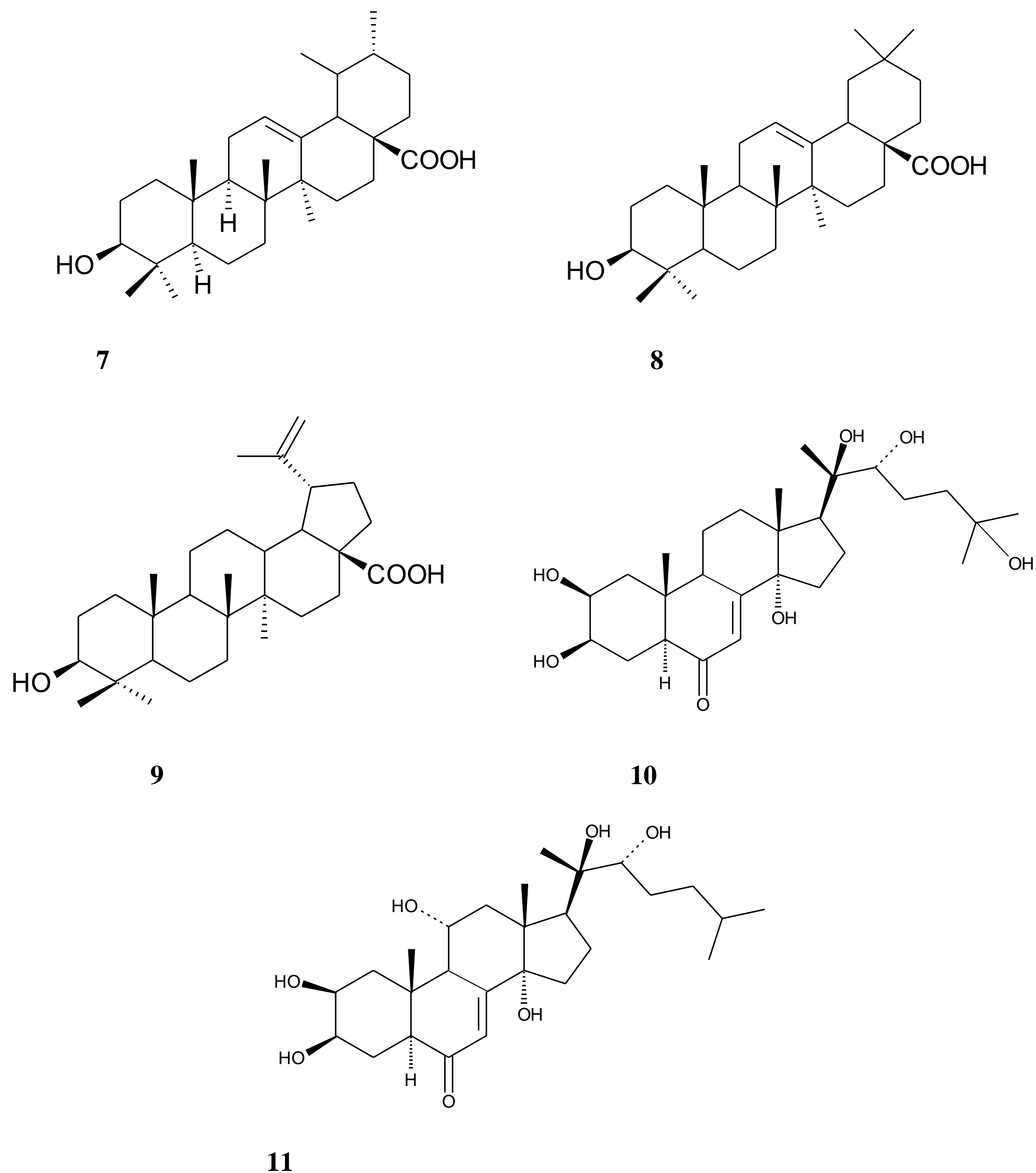

Figure 2
Chemical structures of triterpenes ursolic acid 7, oleanolic acid 8, and betulinic acid 9, β-ecdysone 10 and ajugasterone C 11

Table 2
Acute Toxicity compounds 1-4, 7-9, M1, M2, EA, Hex extracts against larval of last stage of S. *frugiperda* [a]

Compounds	Conc [µg/mL]	% Survival [b]	LD_{50} [c]
Control	0.0	100.0	
1	1.0	3.2 ± 0.32a	**0.27**
	2.0	1.4 ± 0.11b	
	5.0	0	
2	1.0	3.2 ± 0.29a	**0.23**
	2.0	1.4 ± 0.10b	
	5.0	0	
3	1.0	27.0 ± 2.44c	**0.56**
	2.0	10.5 ± 2.30d	
	5.0	3.92 ± 0.66e	
4	1.0	10.5 ± 1.92d	**0.35**
	2.0	1.4 ± 0.12b	
	5.0	0	
5	1.0	35.5 ± 3.92f	**0.71**
	2.0	20.1 ± 2.98c	
	5.0	7.9 ± 1.3d	
7	5.0	20.1 ± 3.02c	**2.45**
	10.0	10.5 ± 1.29d	
8	1.0	53.1 ± 7.36g	**1.39**
	2.0	40.1 ± 4.24f	
	5.0	19.0 ± 2.33c	
	10.0	10.0 ± 1.53d	
9	1.0	75.0 ± 6.54h	**3.73**
	2.0	69.1 ± 5.14h	
	5.0	41.9 ± 4.05f	
	10.0	13.9 ± 2.15d	
M1	1.0	6.8 ± 0.99i	**0.29**
	2.0	1.4 ± 0.05b	
M2	10.0	10.5 ± 1.33d	**3.30**
	20.0	2.8 ± 0.25a	
EA-ext	2.0	3.9 ± 0.59a	**0.65**
	10.0	0.0	
Hex-ext	25.0	20.1 ± 3.55c	**11.4**
	50.0	3.9 ± 0.22a	
MeOH-Yuc	25.0	5.0 ± 0.29a	**8.0**
	50.0	0.0	
Gedunin	25.0	14.1	**10.78**
	50.0	0	

[a] After 24 h, survival of adults was recorded (percent relative to controls). [b] Mean of three replicates. Means followed by the same letter within a column after ± standard error values are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared by concentration to control). [c] The LD_{50} is the lethal dose producing 50% survival

The identified triterpenes, the flavonoids, the mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$, EA-ext and Hex-ext were used in the insecticidal bioassays, but for compounds whose yield was so small that we cannot study its biological activity and only was used for structural determination. Additionally, the range of criteria of admissibility of concentration in the biological assays used was between 1 to 100 µg/mL, those samples whose effects were values higher than 100 µg/mL were considered out of range and were discarded (Isman, 2000; Isman, 2006; Akhtar *et al.,* 2008).

In order to obtain more satisfactory data for insecticidal activity, the bioassay was carried out at lower concentrations with compounds **1, 2, 3, 4**, **5, 7, 8** and **9**, the EA-ext, Hex-ext, and mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$. Gedunin, Me-Yuc, and Me-Ced extracts were used as positive controls.

Insecticidal activity against larvae of S. frugiperda

First was evaluated the effects of MeOH, *n*-hexane, CH_2Cl_2 and ethyl acetate extracts on growth and development of larvae of first instar of *S. frugiperda* and larvae of last instar of *T. molitor*, at concentrations of 50.0, 100.0, and 300 µg/ml (data not showed), being the most active Hex-ext and EA-ext (activity level lower than 100.0 µg/ml), and these extracts were worked in open-column chromatography.

The results on *S. frugiperda* outlined in Tables 1 and 2 shown that EA-ext induce a significative decreasing in the larval feasibility, in similar form Hex-ext produce a decreasing in the number of live larvae. At 21 days the number of larvae and pupae decrease drastically in all treatments, at concentration higher than 10 µg/ml for **1**, **2**, **3**, **4, 5, 7, 8, 9,** and EA-ext and higher than 2.0 and 20 µg/ml for $\mathbf{M_1}$ and $\mathbf{M_2}$, respectively. The percentage of emergence of adults from the pupae was also drastically affected by these substances from *E. citriodora* (compounds, mixtures and extracts) completely blocked adult emergence (only emerged several with abnormalities), as no viable adults emerged from the pupae at this step, included those that no complete its ecdysis and sclerotization, and all these pupae died at those concentrations.

At higher concentrations than 10 µg/ml, the compounds **1-5**, **7-9**, mixtures $\mathbf{M_1}$, $\mathbf{M_2}$, Hex-ext and EA-ext showed toxic effect on this insect larvae, in experiments carried out against larvae of the first instar of *S. frugiperda*, during the first 7 days, the insecticidal effects of these extracts were almost 100% lethal (outlined in Table 1 and 2). Thus **1**, **2**, **3**, **4**, **5**, and the mixture $\mathbf{M_1}$ produced significant larval mortalities (> 90%, at 10.0 µg/ml), whereas the triterpenes **7, 8, 9** and $\mathbf{M_2}$ produced larval mortality at concentrations higher than 10.0 µg/ml. Thus, **1-5**, **9**, $\mathbf{M_1}$ and $\mathbf{M_2}$, EA-ext exhibited 100% larval mortality and showed the highest insecticidal activity. It is important to point out that **1, 2, 4,** $\mathbf{M_1}$ and EA-ext possessing an LD_{50} of 0.91, 0.77, 0.68, 0.25, and 0.78 µg/mL were more active as an insecticide than gedunin or either of the two pattern extracts (Me-Ced and Me-Yuc) used as positive controls (Table 1).

Insect growth inhibitory activity for S. frugiperda.

In additional experiments at lower concentrations (< 1.0 µg/ml), **1-5**, **7-9**, Hex-ext, EA-ext, and the mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ specifically inhibited each larval growth stage (*e.g*., growth (up to 75% of length)(data not showed). Moreover, **1**, **2**, **4**, $\mathbf{M_1}$ and EA-ext produced total inhibition (100%) of growing at 21 days. Interestingly, after 21 days, growth reduction by $\mathbf{M_1}$ and $\mathbf{M_2}$ was clearly significant between 1.0-2.0 and 2.0-10.0 µg/ml ($p < 0.05$), but compounds **1**, **2**, **4**, $\mathbf{M_1}$ and EA-ext showed the highest larval growth inhibition at the same concentrations.

The percentage of larvae that reached pupation decreased drastically with almost all compounds, mixtures and extracts tested in comparison to the controls (except **6**). Thus, **1** (5.0 µg/ml, 11.1%), **2** (2.0 µg/ml, 1.4%), **3** (2.0 µg/ml, 18.4%), **4** (2.0 µg/ml, 4.0%), **5** (5.0 µg/ml, 11.0%), **9** (50.0 µg/ml, 1.4%), Hex-ext (250.0 µg/ml, 12.0%), gedunin (10 ppm 46.7%), Me-Yuc (10 ppm, 42.0%) all showed significant delay of pupation, (Table 1). At 10.0, 25.0, 10.0 µg/ml no larvae survived to pupation with $\mathbf{M_1}$, $\mathbf{M_2}$, and EA-ext, respectively (data not showed). Thus, outstandingly were observed many delays in time to pupation (> 24 days) at 10.0 µg/ml for **1-5**, $\mathbf{M_1}$ (2.0 µg/ml) and $\mathbf{M_2}$ (5.0 µg/ml) (data not showed). Furthermore, **1, 2, 3, 4,** $\mathbf{M_1}$ and EA-ext between 0.2 and 1.0 µg/ml significantly reduced pupal weights, whereas $\mathbf{M_2}$**, 7, 8, 9** and Hex-ext produced the greatest effect on pupal weights between 5.0 – 20.0 µg/ml (data not showed), whereas Me-Ced and Me-Yuc extracts produced greatest effect on pupal weights at 10.0 ppm, as previously reported (Céspedes *et al.,* 2000; Torres *et al.,* 2003).

The percentage of emergence of adults from the pupae was also drastically affected by these substances and showed strongest reductions.

Table 3
GI and RGI of *S. frugiperda* as a function of increased concentrations of compounds 1, 2, 3, 5, 7, 8, 9, mixtures M_1 and M_2, EA, and Hex extracts from *E. citriodora*[a]

Compounds	Concentration (ppm)	GI[b]	RGI[c]
Control		0.95	1.0
1	0.5	0.79	0.83
	1.0	0.69	0.73
	2.0	0.28	0.29
	5.0	0.07	0.07
	10.0	0.04	0.04
2	0.5	0.93	0.98
	1.0	0.916	0.96
	2.0	0.28	0.29
	5.0	0.174	0.175
	10.0	0.027	0.029
3	1.0	0.95	1.0
	2.0	0.83	0.87
	5.0	0.55	0.58
4	1.0	0.28	0.29
	2.0	0.07	0.07
	5.0	0.00	0.00
5	1.0	0.93	0.98
	2.0	0.88	0.93
	5.0	0.87	0.92
	10.0	0.69	0.73
7	5.0	0.95	1.0
	10.0	0.94	0.99
8	1.0	0.93	0.98
	2.0	0.92	0.96
	5.0	0.55	0.58
	10.0	0.28	0.29
9	1.0	0.83	0.87
	2.0	0.55	0.58
	5.0	0.28	0.29
	10.0	0.07	0.07
M2	5.0	0.86	0.90
	10.0	0.79	0.83
	20.0	0.69	0.73
	35.0	0.28	0.29
M1	0.5	0.93	0.98
	1.0	0.55	0.58
	2.0	0.28	0.29
	5.0	0.07	0.07
	10.0	0.00	0.00
EA-ext	2.0	0.83	0.87
	5.0	0.69	0.73
	10.0	0.00	0.00
Hex-ext	25.0	0.33	0.35
	50.0	0.00	0.00

MeOH-Yuc	2.0	0.83	0.87
	10.0	0.27	0.28
MeOH-Ced	25.0	0.79	0.83
	50.0	0.69	0.73
	300.0	0.41	0.44

[a] Mean of three replicates. [b] (GI = Growth Index = Number of surviving larvae / Total larvae used. RGI = GI treated / GI control). [c] $RGI_{treatment} = GI_{treated} / GI_{control}$.

The analysis of the effects on insect fed with substances from E. citriodora revealed a developmental and growth disruption in which the insects died in the range of concentration used (0.5 to 25.0 µg/ml), during the pharate conditions following initiation of molting (apolysis), but before completion of molting (ecdysis).

During a molt, ecdysteroid levels first rise to stimulate onset of apolysis and cuticle synthesis, but then must fall to facilitate the release of eclosion hormone (Truman *et al.,* 1983) and the ecdysis-triggering hormone (ETH) (Zitnan *et al.,* 1996; Zitnan *et al.,* 1999), which act in concert to trigger the insect's ecdysis behavior during the final stages of the molt. Possibly as sterols, the flavonoids and triterpenes here reported act via ecdysteroids effects to result in an inhibition of emergence behavior, or may, alternatively, act directly to inhibit the release of ETH (Hesterlee & Morton, 1996).

Growth inhibition and relative growth index for S. frugiperda

At the lowest concentrations the pupae that emerged show many deformities. Thus, in all treatments, the average time to reach the mean weight of the adult stage relative to control larvae was significantly delayed. The growth index (GI or number of surviving larvae / total larvae used) and relative growth index (RGI or $GI_{treated}$ /$GI_{control}$) showed (Table 3) that the strongest effects were shown at 35.0 µg/ml by $\mathbf{M_2}$ (RGI 0.29), at 25.0 µg/ml by Hex-ext (RGI 0.35), 10.0 µg/ml by **1** (RGI 0.04), **2** (RGI 0.029), **5** (RGI 0.69), **7** (RGI 0.99), **8** (RGI 0.29), **9** (RGI 0.07), at 5.0 µg/ml by **3** (RGI 0.58), $\mathbf{M_1}$ (RGI 0.07), EA-ext (RGI 0.73) and at 2.0 µg/ml by **4** (RGI 0.07). These parameters together with the LD_{50} (the lethal dose producing 50% of dead) values (Tables 1 and 2), corroborated the highest effect that was showed by **1** (0.91 µg/ml), **2** (0.77 µg/ml), 4 (0.68 µg/ml), $\mathbf{M_1}$ (0.25 µg/ml) and EA-ext (0.78 µg/ml), as these substances produced the greatest insecticidal effects.

It is important to note that similar insect growth regulatory effects on *S. frugiperda* (fall armyworm) was showed by phytoecdysteroids from *Ajuga remota* (Labiatae) (Kubo *et al.,* 1981), and on two polyphagous species (*S. littoralis* and *Ostrinia nubilalis*) and a mono-phagous (*Bombyx mori*) (Marion-Poll & Descoins, 2002).

Although there are a disparate literature about biological activities (anti-inflammatory, antioxidant and antimicrobial) of phytoecdysteroids that have previously been reported (Simon & Koolman, 1989; Sláma & Lafont, 1995; Schmelz *et al.,* 1999; Saez *et al.,* 2000; Savchenko *et al.,* 2000; Dinan *et al.,* 2001), there are no reports for insecticidal activity of these sterols. On the other hand, there are reports about the effects on growth and inhibitory effects, on Cyt-P_{450}, glutathione transferase (GSTs) and carboxyesterases (COE) at short- and long-term (Zhang *et al.,* 2012) by flavonols, and the effects on growth (Asnaashari *et al.,* 2010), insecticidal (Adeyemi *et al.,* 2010), and as insect growth regulator (Upasani *et al.,* 2003; Simmonds, 2006; Cespedes *et al.,* 2006; Nenaah *et al.,* 2013) by flavonols and phenylethanoids.

Insect growth inhibitory activity for T. molitor

In relation to *T. molitor* (see Table 4), the EA-extract cause a decrease in the number of larvae that to reach the pupation (15%), while the $\mathbf{M_1}$ have this same action but at minor concentration

(10%). However, as well as Hex-ext, **M_2**, **7**, **8** and **9** showed an acceleration and shortening of the time of pupation and emergence on this insect species, respectively, and many of the pupae were not viable and died (Table 4). Thus, at lower levels (1.0 µg/ml), **1** (5.0% survival), **2** (3%), **3** (10%), **4** (0%) and **5** (12%) exhibited potent emergence toxicity on larvae and pupae of *T. molitor*.

Table 4
Growth Inhibitory Activities on *T. molitor* as a function of increased concentrations of compounds 1, 2, M_2, and MeOH extract from *E. citriodora*[a]

Samples	[µg/ml] Doses	successfully pupation % [c]	Emergence % [d]
Control		98.0 ± 1.55a	95 ± 0.93a
1	1.0	5.0 ± 0.03b	5.0 ± 0.021b
	2.0	0	0
	5.0	0	0
	10.0	0	0
2	1.0	3.0 ± 0.01c	3.0 ± 0.01c
	2.0	0	0
	5.0	0	0
	10.0	0	0
3	5.0	10.0 ± 1.5c	10.0 ± 1.5c
	10.0	5.0 ± 0.03b	5.0 ± 0.03b
4	1.0	0	0
	2.0	0	0
5	1.0	12.0 ± 1.67c	12.0 ± 1.67c
	2.0	5.0 ± 0.03b	5.0 ± 0.03b
7	5.0	0	0
	10.0	0	0
	20.0	0	0
8	5.0	5.0 ± 0.03b	5.0* ± 0.03b
	10.0	5.0 ± 0.08b	5.0 ± 0.08b
	20.0	0	0
9	5.0	0	0
	10.0	3.0 ± 0.01c	3.0 ± 0.01c
	20.0	0	0
M1	1.0	0	0
	2.0	0	0
M2	5.0	5.0 ± 0.03b	5.0 ± 0.03b
	10.0	3.0 ± 0.01c	3.0 ± 0.01c
	20.0	0	0
EA-ext	1.0	0	0
	2.0	0	0
Hex-ext	20.0	5.0 ± 0.03b	5.0*± 0.03b
	30.0	0	0

[a]20 larvae by assay and by triplicate, larvae of last stage, topical application.
[b] Average duration, the criteria used was to measure until emergence of survival pupae, n.d. meaning correspond to pupae that not produce any adult. Means followed by the same letter within a column after ± standard error values are not significantly different in a Student-Newman-Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% Confidence limits.
[c] Percentage with respect to control. [d] The asterisk indicate adults with deformities.

By other hand, in addition to the time of duration of pupal stage that was shorted for those pupae that attained emerged, many pupae did not emerged and that effect was observed to 20.0 µg/ml for **8**, **9** and $\mathbf{M_2}$, respectively and that effect was observed from 5.0 µg/ml for **7** (Table 4).

These results suggest that these compounds and mixtures have some effect on ecdysone receptors (Dinan, 2001). Additionally, it is possible to infer that Hex-ext and M2 accelerate the time of pupation of this insect (data not show). This extract contain a high percentage of triterpenes and sterols (> 30%) and not show a similar activity to $\mathbf{M_1}$. However, this extract showed an acute toxicity on larvae of this insect in function of the number of larvae that to reach the pupal stage. Noteworthy our compounds, mixtures and extracts showed be more potent than stigmasterol, sitosterol, cholesterol and their epoxy, hydroxyl and chloride derivatives, reported previously by Meyer (Meyer *et al.,* 1998), and on other sterols (Miles *et al.,* 1994; Quiroz *et al.,* 2015).

Acute toxicity on larvae of last stage of S. frugiperda and T. molitor.

In order to determine a possible correlation between insect growth regulatory (IGR) and acute toxicity with ecdysis properties of these compounds, oral injection into ten instar of *S. frugiperda* with 2.5 µg/ml of flavonoid samples and 5.0 µg/ml of terpene samples were made, these concentrations were used due to are those that promoted apolysis to the fifth instar but inhibited ecdysis, while oral injection of 2.5 µg/ml of $\mathbf{M_2}$ resulted in only a delay in a normal molt to the fifth instar (Table 2). Doubling the oral dose to 5.0 and 10.0 ppm of both flavonoids and triterpenes, respectively, after 48 and 72 h they induced prothetely in several (30%) of the treated fifth instar larvae as they molted directly to pupae. $\mathbf{M_1}$ and $\mathbf{M_2}$-induced prothetely resulted in pupae roughly half of control and browning (data not showed).

The same bioassay was carried out on last instar larvae of *T. molitor*. At 10 µg/ml, **1-5**, **7-9**, and mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$, and the EA-ext and Hex-ext showed strong acute toxicity with 12.5, 25.0, 35.0 and 10% survival, respectively (data not showed). On other hand all assayed samples at higher levels (30.0 µg/ml), exhibited potent acute toxicity on larvae of *T. molitor* (data not showed).

These observations suggest that acute toxicity and growth inhibition may be due to inhibition of proteinase, ETH and other polyphenol oxidases (PPO) binding with these compounds, as this target was demonstrated for other samples from natural origin (Kubo *et al.,* 1986; Carrizo *et al.,* 1998; Tamayo *et al.,* 2000; Karban & Baxter, 2001).

The sites and mode of action of these compounds and extracts are being investigated and probably correspond to a combination of antifeedant action, as well as midgut phenol oxidase, proteinase, ETH, tyrosinase or other PPOs and cuticle synthesis inhibition, as well, and resultant moulting sclerotization toxicity, as has been found for other similar compounds (Kubo & Kinst-Hori, 1999; Kubo *et al.,* 2000; Kubo, 2000, Kubo *et al.,* 2003a; Kubo *et al.,* 2003b, Taibi *et al.,* 2003; Berghiche *et al.,* 2003) and extracts (Feng *et al.,* 1995).

CONCLUDING REMARKS

Based on these results, we suggest that the insect growth inhibition caused by mixtures of flavonols and triterpenes assayed could due to a strong inhibitory activity produced by a synergistic effect in the mixture composition as in $\mathbf{M_1}$ and $\mathbf{M_2}$. These plant compounds may be considered as efficient insect growth regulators (IGR) as well as possible phytoecdysteroids, as was evidenced by their significant inhibition of molting or apolysis.

The flavonols, triterpenes, Hex-ext, EA-ext and the mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ had all potent insecticidal and growth inhibitory activities. Then, is possible to infer that the position of the hydroxyl group to the flavonols and C-methyl-flavonol as in compound **1** results in significant inhibitory activity and therefore this moiety must play an important role in both the insecticidal and IGR activities of these compounds.

The active triterpenes **7, 8**, and **9** contained relatively lipophilic group at C-4 and C-20, hydroxyl groups at C-3, a carboxylic group at C-28 and (**8** and **9**) a double bond between C-12 and C-13 (Δ^{13})(endo position). On the other hand, β-ecdysone (20-hydroxyecdysone) (20E) **10** and ajugasterone C **11** that have these moieties (functions) between C-7 and C-8 (Δ^7) and at C-6 there is a carbonyl (α-β-congujate) group. These features show a relative good potency of our compounds that in comparison with **10** and **11** show a similar behavior but at higher concentrations than **10** and **11** (Kubo & Klocke, 1983). These results confirm previous findings on structure activity relationships for 20-E and derivatives, namely that the growth inhibitory activity of the respective natural product depends on the number of hydroxyl groups and the presence of a

moderated bulky group at C-17. In comparison to the previous empirically derived SAR studies that it is not clear that the cis-A/B-ring junction, the Δ^{13}-double bond, the 17-carboxy group, the 3-hydroxy and methyl group at C-4, C-19 and C-20 (as in our case) moieties do seem increase the activity, these features need more and deep investigation (Dinan, 2001; Cespedes *et al.,* 2005; Cespedes *et al.,* 2008; Muñoz *et al.,* 2013).

Thus, the effect of compounds **1-5, 7-9,** and mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ on reducing insect growth, increasing/shortening of development time, apolysis ecdysis, molting and mortality of *S. frugiperda* and *T. molitor* were stronger than gedunin, MeOH-Yuc and MeOH extract from *Cedrela salvadorensis* (Meliaceae) (Céspedes *et al.,* 2000; Calderón *et al.,* 2001).

Although chemically distinct, the levels of insecticidal activities of these metabolites and mixtures derived from the plant in study are comparable to that of the known insect growth regulator, gedunin. Based on the present investigations, materials from *E. citriodora* and related species should prove to be valuable sources of interesting biologically active compounds, including insecticides.

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