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Insect growth inhibition by tocotrienols and hydroquinones from *Roldana barba-johannis* ☆

Carlos L. Céspedes [a,*], Patricio Torres [b], Juan C. Marín [a], Amira Arciniegas [a], Alfonso Romo de Vivar [a], Ana L. Pérez-Castorena [a], Eduardo Aranda [c]

[a] *Chemical Ecology Lab-2C, Natural Products Department, Chemistry Institute, Universidad Nacional Autónoma de México, Av. Universidad 3000, Coyoacán 04510, México*
[b] *Botany Department, Faculty of Natural Sciences and Oceanography, University of Concepción, Concepción, Chile*
[c] *Biological Control Laboratory, Biotechnology Center, Universidad Autonoma del Estado de Morelos, Cuernavaca, Mexico*

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Abstract

The methanol extract from the aerial parts of *Roldana barba-johannis* (Asteraceae) afforded sargachromenol, sargahydroquinoic acid, and sargaquinoic acid. These natural products and their corresponding acetylated and methylated derivatives showed insecticidal and insect growth regulatory activities against the Fall Armyworm [*Spodoptera frugiperda* J.E. Smith, (Lepidoptera: Noctuidae)], an important insect pest of corn. The most active compounds were sargachromenol and its acetylated derivative; sargahydroquinoic acid and its acetylated derivative; and a mixture of sargachromenol, sargahydroquinoic acid, and sargaquinoic acid (6:3:1) and the acetylated form of this mixture. All these compounds and mixtures had significant inhibitory effects between 5.0 and 20.0 ppm in diets. Most compounds were insecticidal to larvae, with lethal doses between 20 and 35 ppm. In addition, these substances also demonstrated scavenging properties toward 2,2-diphenyl-1-picrylhydrazyl radical in TLC autographic and spectrophotometric assays. These compounds appear to have selective effects on the pre-emergence metabolism of the insect. The results from these compounds were fully comparable in activity to those known natural insect growth inhibitors such as gedunin and methanol extracts of *Cedrela salvadorensis* and *Yucca periculosa*. These substances may be useful as natural insecticidal agents.

Keywords: *Roldana barba-johannis*; Asteraceae; Tocotrienols; Hydroquinones; Insect-growth regulation; *Spodoptera frugiperda*; Fall Armyworm

1. Introduction

Increasing interest in the application of plant secondary metabolites for insect pest management 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 of synthetic pesticides on the environment and the generation of resistant strains of insects (Kubo, 1997; González and Estevez-Braun, 1998). Previous studies have focused on limonoids from the family Meliaceae because of their potent effects on insect pests and low toxicity. One of the compounds discovered, gedunin, has proven to have excellent properties (Céspedes et al., 2000).

Because of their pronounced resistance to insect attack, we have chosen to investigate the insecticidal activity of shrubs from the family Asteraceae (Torres et al., 2003). Species of one such genus, *Roldana*, of the Mexican highlands, are essentially free from insect feeding. These plants have not been considered important as medicinal or agronomic plants, and many are endemic to Mexico. Members of this genus have been segregated from the genus *Senecio* (Robinson and Brettell, 1974). They grow on steep humid mountainsides and in "barrancas" in the shade of the dense shrubby understory of secondary pine-oak forests. They are often found on dark volcanic soils in the states of Chiapas, Durango,

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* Corresponding author. Tel.: +52-55-5622-4447; fax: +52-55-5616-2203.
E-mail address: ccespede@servidor.unam.mx (C.L. Céspedes).

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Guerrero, Hidalgo, Mexico, Morelos, Oaxaca, and Puebla, areas especially rich in endemic vegetation. *Roldana* species are often found in association with *Pinus*, *Quercus*, *Alnus*, *Abies*, *Senecio*, *Solanum*, *Penstemon*, *Physalis*, *Potentilla*, *Eryngium*, *Liquidambar*, *Cupressus*, and *Chiranthodendron* species. These plants survive under environmental stress conditions and are not attacked by insects (Villaseñor et al., 1998).

To the best of our knowledge, only four species of the genus *Roldana* have been studied chemically (Glonti, 1958; González et al., 1973; Bohlmann and Zdero, 1978; Joseph-Nathan et al., 1990; Delgado and García, 1993; Arciniegas et al., 2004). In the present study, the tocotrienols (**1**–**3**) and hydroquinones (**4**–**7**), isolated from the aerial parts of *Roldana barba-johannis* (DC.) H. Rob. & Brettell, were evaluated as insect growth inhibitors. The plastoquinones found in this plant have previously been isolated from terrestrial plants (Lok et al., 1983; Vieira et al., 1983; Delle Monache et al., 1984) and marine algae (Kusumi et al., 1979; Segawa and Shirahama, 1987), and the antioxidant and anti-inflammatory properties of these compounds have been reported (Pérez-Castorena et al., 2002). Plastoquinones have an important biological function as electron transporters of photosystem II (Kruk et al., 1998). In addition, they can function as both constitutive and inducible defense molecules, and play a role in dormancy and growth inhibition of plants (Croteau et al., 2000).

Research on the sites and mechanisms of action of allelochemicals responsible for insecticidal or insect growth regulation (IGR) activity indicates that many phenolic compounds are involved. These substances are important enzymatic and metabolic inhibitors (Hammond and Kubo, 1999; Kubo and Kinst-Hori, 1999; Kubo, 2000; Kubo et al., 2000; Shimizu et al., 2000; Tamayo et al., 2000; Calderón et al., 2001; Kim et al., 2002; Panzuto et al., 2002; Kubo et al., 2003a,b; Torres et al., 2003). In addition, many polyphenolic metabolites of angiosperms have anti-feedant effects on phytophagous insects (Feeny, 1968, 1976; Rhoades and Cates, 1976; Swain, 1979). Some polyphenols or tannins bind to proteins, acting as nutritional protein precipitating agents, inhibiting insect digestive enzymes (Duffey and Stout, 1996; Korth and Dixon, 1997; Tamayo et al., 2000) and thus reducing their digestibility (Feeny, 1976; Rhoades, 1979). Furthermore, many polyphenols are easily oxidized by polyphenol oxidases to produce reactive quinines that polymerize readily (Kessler and Baldwin, 2002). We have previously demonstrated that diverse secondary metabolites have different sites of action and different molecular targets when they interact with enzymes and perturb metamorphosis processes (Céspedes et al., 2000; Céspedes et al., 2001; Calderón et al., 2001; Kubo et al., 2003a,b; Torres et al., 2003).

The aim of this work was to correlate the phytochemical composition of *Roldana* species with the inhibitory behavior on growth and development of *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae). Our data indicate also that it is possible to correlate some antioxidant activities (e.g., scavenging of 2,2-diphenyl-1-picrylhydrazyl radical, DPPH, radicals) with growth and development of insects; these data are important for allelopathic studies (Torres et al., 2003). On the other hand, the parameters above mentioned are accepted as indirect measures of other different physiological processes (Camps, 1988; Galindo et al., 1999; Macías et al., 2000).

The present paper specifically deals with the effects of isolates from the MeOH extract of the aerial parts of *R. barba-johannis* [tocotrienols **1**, **2**, **3**, hydroquinones **4**, **5** and **6**, and mixtures of **1**, **4**, and sargaquinoic acid **7** (**6**:**3**:**1**) (**M**) and the acetylated mixture (**Ma**)], against Fall Armyworm (*S. frugiperda*) and the relationship of these effects to antioxidant activities of the same substances. Aspects examined included insecticidal and growth regulatory activity, rate of development, time of pupation, adult emergence, and deformities in insects at each of the various stages. The effects of substances from this species were evaluated and compared to those of gedunin and to *Yucca periculosa* (**Me-Yuc**) and *Cedrela salvadorensis* (**Me-Ced**) MeOH extracts, all known growth inhibitors of *S. frugiperda* (Céspedes et al., 2000; Calderón et al., 2001; Torres et al., 2003).

2. Results and discussion

In our screening program, designed to discover interesting biological activities of subtropical Mexican plants, it was found that *R. barba-johannis* showed insecticidal activity in a preliminary trial. Based on this information, we have carried out several studies on aerial parts of *R. barba-johannis*.

A mixture of hydroquinone derivatives obtained from the methanolic extract of the aerial parts of this species afforded the tocotrienol sargachromenol **1** (Kusumi et al., 1979), the dihydroplastoquinone sargahydroquinoic acid **4** (Segawa and Shirahama, 1987), and the plastoquinone sargaquinoic acid **7** (Kusumi et al., 1979). These compounds were identified by comparison of their spectral features with those reported in literature (Pérez-Castorena et al., 2002) (Fig. 1).

The transformation of dihydroplastoquinone **4** into plastoquinone **7**, which in turn is converted to sargachromenol **1**, was observed, as previously reported (Kusumi et al., 1979, Segawa and Shirahama, 1987). In fact, it is known that equilibrium among hydroquinones, plastoquinones, and tocopherols exists in vivo (Thomson, 1971). Therefore, compounds **1** and **7** could be considered either as natural products or artifacts. Transformation of compound **4** was limited by acety-

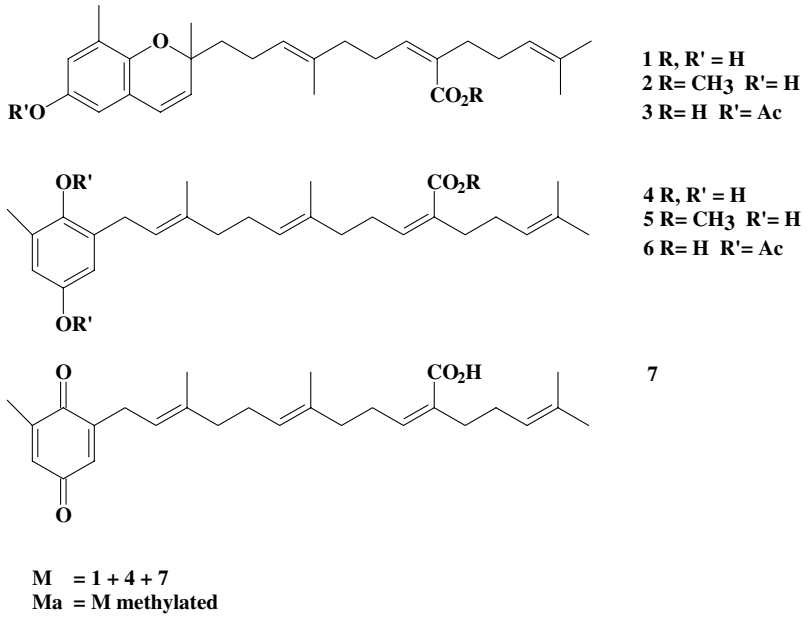

Fig. 1. Structures of compounds **1**–**7**.

lation into compounds **6** and by esterification into compound **5**.

In order to obtain more satisfactory data for insecticidal activity, the bioassay was carried out at low concentrations with compounds **1**–**6**, the mixture **M**, and its acetylated form **Ma**. Gedunin, **Me-Yuc**, and **Me-Ced** were used as positive controls. Because of the small amount of compound **7** present, this substance was not tested.

2.1. Insecticidal activity against larvae

The insecticidal effects of **1**–**6**, **M**, **Ma**, gedunin, **Me-Yuc**, and **Me-Ced** extracts against larvae of the first instar of *S. frugiperda* are outlined in Table 1. Sargachromenol **1** (35.0 ppm), sargahydroquinoic acid **4** (10.0 ppm), and the mixture **Ma** (35.0 ppm) produced significant larval mortalities (>80%), whereas **4** produced 95% larval mortality at 35.0 ppm. On the other hand, **1** (75.0 ppm), **4** (50.0 ppm), the methyl ester of sargahydroquinoic acid **5** (100.0 ppm), **M** (75.0 ppm), and **Ma** (75.0 ppm) all exhibited 100% larval mortality and showed the highest insecticidal activity. It is noteworthy that all larvae died when fed with four different diets, each of which contained 60 ppm of **1**, **3**, **M**, and **Ma**, respectively (data not shown). The 50% lethal dose (LD_{50}) of these compounds and mixtures for larvae at 7 days are presented in Table 6. It is important to point out that sargahydroquinoic acid **4** possessed a LD_{50} of 5.77 ppm and was more active as an insecticide than gedunin or either of the two extracts (**Me-Ced** and **Me-Yuc**) used as positive controls.

2.2. Insect growth inhibitory activity

Sargachromenol **1** (50 ppm), sargachromenol acetate **3** (10 ppm), sargahydroquinoic acid **4** (20 ppm), sargahydroquinoic acid methyl ester **5** (35 ppm), and the mixtures **M** (75 ppm) and **Ma** (75 ppm) specifically inhibited each larval growth stage, e.g., growth (up to 67% of length) when incorporated into diets (Table 1). Moreover, **1** (35 ppm), **3** (35 ppm), **4** (50 ppm), sargahydroquinoic acid acetate **6** (50 ppm), **M** (50 ppm), and **Ma** (50 ppm) produced total inhibition (100%) of weight increase at 21 days (Table 2). Sargahydroquinoic acid methyl ester **5** gave lower inhibition of larval weight gain than **1**, **4**, gedunin, **Me-Yuc**, and **Me-Ced** extracts at a high concentration (75.0 ppm) (Table 2). At 21 days, growth reduction by compound **5** was clearly significant between 20.0 and 35.0 ppm ($p < 0.05$), but compounds **1**, **3**, **4**, **M**, and **Ma** showed the highest larval growth inhibition at the same concentrations (Table 2).

The percentage of larvae that reached pupation decreased with all compounds, mixtures, and extracts tested in comparison to the controls. Thus, **1** (10 ppm, 4.1%), **3** (10 ppm, 9.7%), **4** (35 ppm, 11.1%), **6** (20 ppm, 13.9%), **M** (35 ppm, 16.7%), **Ma** (20 ppm, 13.9%), gedunin (50 ppm 25.0%), **Me-Yuc** (25 ppm, 37.5%), and **Me-Ced** (50 ppm, 25.0%) extracts all showed significant delay of pupation. No larvae survived to pupation with **1** (20 ppm), **3** (20 ppm), **4** (50 ppm), **6** (35 ppm), **M** (50 ppm), **Ma** (35 ppm), and **Me-Yuc** extracts (50 ppm) (Table 3). Significant delays in time to pupation ($\geqslant 24$ days) were observed for **1** (10 ppm), **4** (20 ppm), **M** (35 ppm), and **Ma** (20 ppm). Furthermore, **1**, **4**, **M**, and **Ma** at 25.0 ppm significantly reduced pupal weights (data

Table 1
Growth inhibitory effects of compounds **1**, **2**, **3**, **4**, **5**, **6**, mixtures **M** and **Ma**, **Me-Yuc**, **Me-Ced** extracts, and gedunin on Fall Armyworm growth[a]

Treatment	μg/ml (ppm)	Mean weight gained (mg)[b]	% of weight[c]	Mean long gained (cm)[d]	% of length[c]	Mortality %
Control		101.9 ± 7.1a	100	1.20 ± 0.045	100	5.6
1	5.0	22.5 ± 1.5	22.1	0.80 ± 0.024	66.7	20.8
	10.0	7.2 ± 4.6	7.1	0.59 ± 0.018	59.0	33.3
	20.0	4.9 ± 1.8	4.8	0.58 ± 0.020	48.3	45.8
	35.0	3.5 ± 1.5	3.4	0.57 ± 0.061	47.5	83.36
	50.0	1.3± 1.1	1.3	0.40±0.095	33.3	95.8
	75.0	0.0	0.0	0.0	0.0	100
2	5.0	91.2 ± 2.8 a	89.5	0.7 ± 0.041	58.3	20.8
	10.0	62.5 ± 2.5b	61.3	0.6 ± 0.044	50.0	33.3
	20.0	47.1 ± 1.5b	46.2	0.65 ± 0.049	54.2	41.7
	35.0	43.1 ± 1.2c	42.3	0.51 ± 0.037	42.5	75.0
	50.0	42.1±1.7c	41.3	0.40 ± 0.035	33.3	83.3
	75.0	40.3 ±1.1c	39.5	0.37 ± 0.030	30.8	91.7
	100.0	39.5 ± 0.8c	38.8	0.21 ± 0.022	17.5	95.8
3	5.0	69.9 ± 3.1a	81.7	0.98 ± 2.1 b	68.6	4.2
	10.0	10.9 ±4.0a	53.3	0.64 ± 1.93b	10.7	16.7
	20.0	9.1 ± 2.6b	52.5	0.63 ± 2.2a	8.9	29.2
	35.0	6.8 ± 2.8b	49.2	0.59 ± 1.8b	6.7	46.8
	50.0	3.5± 3.0a	32.5	0.39±2.3a	3.4	79.1
	75.0	3.4 ± 2.5b	29.2	0.35±1.1c	3.3	81.3
	100.0	3.17 ± 2.5b	12.4	0.149± 1.8b	3.1	83.3
4	5.0	48.3 ± 2.1b	47.4	0.61 ± 0.031	50.8	41.7
	10.0	29.9 ± 0.9c	29.3	0.29 ± 0.021	24.2	83.3
	20.0	16.2 ± 0.8c	15.9	0.25 ± 0.022	20.8	91.7
	35.0	5.1 ± 0.3c	5.0	0.12 ± 0.003	10.0	95.8
	50.0	0.0	0.0	0.0	0.0	100
5	5.0	86.6 ± 2.0 b	85	0.7 ± 0.047	58	41.6
	10.0	72.5 ± 2.7a	71	0.6 ± 0.041	50.0	12.5
	20.0	56.6 ± 1.3b	55.5	0.57 ± 0.038	48.0	18.9
	35.0	33.8 ± 1.0b	33.2	0.32 ± 0.018	27.0	26.2
	50.0	25.3 ± 0.6c	24.8	0.24 ± 0.020	20.0	37.5
	75.0	7.0 ± 0.4c	6.9	0.11 ± 0.005	0.92	64
	100.0	0.0	0.0	0.0	0.0	100
6	5.0	76.5 ± 3.0 b	75.1	1.1 ± 0.045	91.7	4.16
	10.0	63.5 ± 2.7b	62.3	1.1 ± 0.051	91.7	5.6
	20.0	60.0 ± 2.8b	58.9	1.0 ± 0.049	83.3	8.0
	35.0	56.3 ± 2.0c	55.3	0.9 ± 0.048	75.0	15.0
	50.0	51.1±1.7b	50.1	0.85 ± 0.047	70.8	25.0
	75.0	40.3 ±1.0c	39.5	0.8 ± 0.051	66.7	44.0
	100.0	38.7 ± 1.6c	37.9	0.7 ± 0.044	58.3	63.0
M	5.0	96.2 ± 2.8a	94.4	0.99 ± 0.054	82.5	41.7
	10.0	61.5 ± 2.4a	60.4	0.89 ± 0.049	74.2	45.8
	20.0	39.8 ± 1.9b	39.1	0.79 ± 0.047	65.8	54.2
	35.0	21.2 ± 0.7c	20.8	0.73 ± 0.043	60.8	63.0
	50.0	10.0±0.3c	9.8	0.69±0.038	57.5	75.0
	75.0	0.0	0.0	0.0	0.0	100
Ma	5.0	98.9 ± 3.7a	97.0	1.1 ± 0.095	91.7	13.6
	10.0	79.8 ± 3.8a	78.3	1.0 ± 0.051	83..3	34.7
	20.0	61.9 ± 3.5b	60.7	0.8 ± 0.021	66.7	22.2
	35.0	39.8 ± 2.9c	39.1	0.7 ± 0.014	58.3	83.3
	50.0	19.9 ± 2.4c	19.5	0.6±0.013	50.0	94.4
	75.0	0.0	0.0	0.0	0.0	100
Me-Yuc	10.0	67.2 ± 1.9a	66	0.73 ± 0.051	61	63
	25.0	28.5 ± 1.1c	28	0.26 ± 0.036	22	81
	50.0	11.2 ± 0.9c	11	0.11 ± 0.005	9	98
Gedunin	10.0	7.2 ± 0.4c	7.1	0.39 ± 0.019	32.5	33
	25.0	3.40 ± 0.2c	3.3	0.24 ± 0.012	20.0	38
	50.0	1.90 ± 0.1c	1.9	0.20 ± 0.010	16.7	70.8

(*continued on next page*)

Table 1 (*continued*)

Treatment	μg/ml (ppm)	Mean weight gained (mg)[b]	% of weight[c]	Mean long gained (cm)[d]	% of length[c]	Mortality %
Me-Ced	10.0	24.3 ± 1.5b	23.8	0.65 ± 0.032	54.2	51.8
	25.0	8.9 ± 0.6c	8.7	0.46 ± 0.023	38.3	78.9
	50.0	6.5 ± 0.4c	6.4	0.40 ± 0.020	33.3	98.6

[a] After 7 days of incubation, means of three replicates.
[b] Means followed by the same letter within a column are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared to control). Means are ±SE. 95% confidence limits.
[c] Percentage with respect to control.
[d] Mean length total increase from eclosion.

not shown), whereas **M** and **Me-Yuc** extract produced the greatest effect on pupal weights at 10.0 ppm, as previously reported (Céspedes et al., 2000, 2001; Torres et al., 2003).

The percentage of emergence of adults from the pupae was also strongly affected by this series of compounds and showed significant reductions upon treatment with **1** (10 ppm, 33.3%), **3** (10 ppm, 14.3%), **4** (35 ppm, 25.0%), **6** (20 ppm, 13.7%), and mixtures **M** (35 ppm, 25.0%) and **Ma** (10 ppm, 21.4%) (Table 3). Furthermore, all compounds and both mixtures drastically reduced the percentage of adult emergence at higher concentrations at which no viable adults emerged from the pupae [**1** (20 ppm), **2** (75 ppm), **3** (20 ppm), **4** (50 ppm), **5** (75 ppm), **6** (35 ppm), **M** (50 ppm), and **Ma** (35 ppm)].

These observations could be correlated with EI_{50} values, i.e., the concentration at which emergence is reduced to 50%, and pI_{50} values. EI_{50} values were calculated as the dose corresponding to the midpoint between complete inhibition (100% of control) and no effect. pI_{50} values correspond to the $\log GI_{50}$ (GI_{50} values are the inhibitory concentrations for reduction of first instar larval growth (weight) rates by 50% in "no choice" tests). The above parameters (inhibition of each larval growth stage, e.g., increase in length of larvae, inhibition of larval weight gain, reduction in pupal weights, percentage of larvae that reached pupation, and percentage of emergence of adults from the pupae) were all strongly affected by the presence of **1** (pI_{50} 0.90), **3** (0.62), **4** (0.69), **M** (0.39), **Ma** (0.71), **Me-Yuc** (0.78), and gedunin (0.40), which indicates the potency of this series of compounds (Table 6).

2.3. Growth inhibition and relative growth index

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 regulatory growth index (RGI or $GI_{treated}/GI_{control}$) showed (Table 4) that the strongest effects were shown by **1** (5 ppm, RGI 0.35), **3** (5 ppm, RGI 0.49), **4** (20 ppm, RGI 0.27), **5** (20 ppm, RGI 0.46), **M** (20 ppm, RGI 0.31), and **Ma** (5 ppm, RGI 0.46). These parameters, together with the LD_{50} (the lethal dose producing 50% attrition) values (Table 5), corroborated the highest effect that was shown by sargachromenol **1** (10 ppm, 97.8% mortality), its acetylated analog **3** (10 ppm, 92.7%), the diacetylated analog **6** (20 ppm, 89.6%), and the acetylated mixture **Ma** (20 ppm, 89.6%), as these substances produced the greatest insecticidal effects (Table 5).

Although the anti-inflammatory activity of some plastoquinones and anti-feedant, anti-fungal, and growth inhibitory activities for chromene compounds (Carrizo et al., 1998; Agarwal et al., 2000; Pérez-Castorena et al., 2002) have previously been reported, there are no reports for insecticidal activity of tocotrienols or plastoquinones on *S. frugiperda*.

The presence of acetyl, hydroxyl or methoxyl groups in an order similar to phenolic compounds seems to be necessary for insecticidal activity (Céspedes et al., 2000; Kubo, 2000; Kubo et al., 2000; Céspedes et al., 2001; Kubo et al., 2003a,b; Torres et al., 2003).

2.4. Acute toxicity on larvae of last stage of S. frugiperda and antioxidant activity

At 5 ppm, sargachromenol **1**, sargachromenol acetate **3**, and mixture **Ma** [an acetylated mixture of **1**, **4**, and the plastoquinone **7** (**6**:**3**:**1**)] showed strong acute toxicity with 26.0%, 36.5%, and 34.4% survival, respectively (Table 5). At higher levels (35 ppm), sargahydroquinoic acid **4** (8.35% survival), sargahydroquinoic acid acetate **6** (0%), and a mixture (**M**) of **1**, **4**, and the plastoquinone **7** (**6**:**3**:**1**) (12.5%) exhibited potent acute toxicity on larvae of the last stage (fifth instar) of *S. frugiperda*. The LD_{50} values of **1** (2.94 ppm), **3** (3.89 ppm), **4** (10.17 ppm), **6** (4.83 ppm), **M** (9.23 ppm), and **Ma** (3.26 ppm) relect this order of activity.

In order to determine a possible correlation between IGR and acute toxicity with antioxidant properties of these phenolic compounds, DPPH radical scavenging test of tocotrienols (**1**–**3**) and hydroquinones (**4**–**7**) were carried out.

In addition, as the presence of phenolic hydroxyl groups increases, the ability of these substances to inhibit radicals derived from DPPH increases (Fig. 2).

Table 2
Fall Armyworm bioassay results on growth of compounds **1**, **2**, **3**, **4**, **5**, **6**, mixtures **M** and **Ma**, **Me-Yuc**, **Me-Ced** extracts, and gedunin on Fall Armyworm growth[a]

Treatment	Concentration (ppm)	Mean weight gained (mg)[b]	%[c]	Mean length gained (mm)	%[c]
Control	0.0	519.4 ± 17.9a	100	45.4 ± 1.6	100
1	5.0	104.9 ± 25.0b	20.1	25.0 ± 4.9	55.0
	10.0	55.2 ± 11.2c	10.6	14.0 ± 2.2	30.8
	20.0	10.9 ± 3.4c	2.09	5.9 ± 1.1	12.9
	35.0	0.0	0.0	0.0	0.0
2	5.0	485.9 ± 14.7a	93.5	38.9 ± 5.9	85.6
	10.0	342.2 ± 12.5a	65.8	27.0 ± 3.5	59.4
	20.0	263.1 ± 10.8a	50.6	19.0 ± 2.8	41.8
	35.0	198.9 ± 9.2b	38.2	17.0 ± 2.4	37.4
	50.0	174.9 ± 10.4b	33.6	17.0 ± 1.1	37.4
	75.0	0.0	0.0	0.0	0.0
3	5.0	269.9 ± 24.9a	51.9	26.0 ± 3.0	57.2
	10.0	246.4 ± 21.1a	47.4	22.0 ± 2.8	48.4
	20.0	56.6 ± 4.1c	30.1	13.0 ± 0.9	28.6
	35.0	0.0	0.0	0.0	0.0
4	5.0	185.2 ± 19.4b	35.6	29.2 ± 7.8	64.3
	10.0	144.2 ± 10.2b	27.7	18.1 ± 3.8	39.8
	20.0	61.0 ± 8.4c	11.7	14.0 ± 2.1	30.8
	35.0	41.0 ± 6.1c	7.8	12.0 ± 1.9	26.4
	50.0	0.0	0.0	0.0	0.0
5	5.0	493.9 ± 20.51a	95.1	41.0 ± 2.1	90.3
	10.0	233.2 ± 58.1a	44.9	27.8 ± 4.1	50.4
	20.0	249.2 ± 22.1a	48.0	17.9 ± 4.9	39.4
	35.0	177.5 ± 18.9b	34.2	17.3 ± 4.0	38.1
	50.0	154.8 ± 15.9b	29.8	17.0 ± 3.1	37.4
	75.0	76.9 ± 5.4c	19.3	13.0 ± 5.0	20.6
	100.0	0.0	0.0	0.0	0.0
6	5.0	243.5 ± 21.2a	46.9	39.9 ± 4.4	87.9
	10.0	140.1 ± 15.0b	27.0	25.3 ± 2.1	55.7
	20.0	49.0 ± 11.0c	9.4	15.9 ± 0.91	35.0
	35.0	22.0 ± 1.19c	4.2	9.3 ± 0.40	20.5
	50.0	0.0	0.0	0.0	0.0
M	5.0	405.0 ± 22.1a	77.9	25.0 ± 3.5	55.0
	10.0	393.1 ± 21.8a	75.6	20.0 ± 3.0a	44.0
	20.0	143.3 ± 15.1b	27.5	15.0 ± 1.9	33.0
	35.0	44.2 ± 6.2c	8.50	9.0 ± 0.5	19.8
	50.0	0.0	0.0	0.0	0.0
Ma	5.0	201.5 ± 24.0a	38.7	23.0 ± 2.7	50.6
	10.0	190.0 ± 18.8a	36.5	18.0 ± 1.8	39.6
	20.0	91.2 ± 10.9 c	17.5	10.0 ± 0.8	22.0
	35.0	22.1 ± 1.8c	4.2	5.5 ± 0.3	12.1
	50.0	0.0	0.0	0.0	0.0
Me-Yuc	10.0	111.0 ± 5.55a, b	23.05	15.4 0.66	35.2
	15.0	45.0 ± 2.25c	9.35	8.1 0.41	18.5
	25.0	23.0 ± 1.15c	4.77	3.0 0.19	6.8
	50.0	0.0	0	0	0
Gedunin	10.0	9.86 ± 0.55c	2.05	5.1 0.22	11.6
	25.0	6.50 ± 0.19c	1.35	3.7 0.18	8.4
	50.0	3.81 ± 0.11c	0.79	2.9 0.11	6.6
Me-Ced	2.0	421.1 ± 22.50a	87.45	31.1 1.20	71.0
	10.0	289.1 ± 14.90a	60.04	22.9 1.11	52.3
	25.0	166.6 ± 7.83a	34.60	15.9 0.98	36.3
	50.0	101.2 ± 4.51b	21.01	12.1 0.67	27.6

[a] Values taken at 21 days before pupation, means of three replicates.
[b] Means followed by the same letter within a column after ±SE 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.

Sargahydroquinoic acid **4** and mixture **M** showed the highest radical scavenging activity with I_{50} values (I_{50} is the concentration producing 50% inhibition of radical DPPH) of 6.14 and 8.92 ppm, respectively (Table 7). Based on these results, we suggest that the insect growth inhibition caused by a mixture of tocotrienols and hy-

Table 3
Activity of compounds **1**, **2**, **3**, **4**, **5**, **6**, mixtures **M** and **Ma**, **Me-Yuc**, **Me-Ced** extracts, and gedunin on pupation and emergences parameters of Fall Armyworm (after 21 days of incubation)[a]

Treatment	Concentration (ppm)	Mean time pupation (days)	Pupation[c] SP (%)[d]	Mean emergence (days)	Emergence (%)[e]
Control		22.0	97.2a	30	100
1	5.0	23.5	34.76b	32	36
	10.0	25	4.16c	36	33.3
	20.0	0	0	0	0
2	5.0	22.0	97.2a	30	74.3
	10.0	22.0	91.7a	30	72.8
	20.0	22.5[b]	70.8a	30	64.6
	35.0	23.5[b]	9.7c	31	42.2
	50.0	23.5[b]	5.6c	31	3.0
	75.0	0	0	0	0
3	5.0	22	48.6b	30	48.5
	10.0	22	9.7c	30	14.3
	20.0	0	0	0	0
4	5.0	22	85.8a	30	48.5
	10.0	23.5b	68.0a	31	44.9
	20.0	24.0b	26.4c	32	42.1
	35.0	25.0b	11.1c	33	25.0
	50.0	0	0	0	0
5	5.0	22.0	91.7a	30	89.1
	10.0	22.0	87.5a	30	88.3
	20.0	22.0	45.8b	30	76.4
	35.0	23.0[b]	34.7b	31	58.2
	50.0	23.0[b]	19.4c	32	45.8
	75.0	0	0	0	0
6	5.0	22.0	62.5b	30	60.0
	10.0	23.0	40.3b	31	38.0
	20.0	23.0[b]	13.9c	32	13.7
	35.0	0	0	0	0
M	5.0	22	95.8a	30	46.4
	10.0	22	54.2b	30	38.5
	20.0	23.5b	30.6b	31	40.9
	35.0	24.5b	16.7c	32	25.0
	50.0	0	0	0	0
Ma	5.0	22	45.8b	30	33.3
	10.0	23	19.4c	32	21.4
	20.0	24	13.9c	33	20.0
	35.0	0	0	0	0
Me-Yuc	10.0	23.5	83.2a	30	20
	15.0	24.0[b]	62.5b	35	20
	25.0	25.0[b]	37.5c	35	18
	50.0	0	0	–	–
Gedunin	10.0	24.0[b]	54.2b	32	15
	25.0	24.5[b]	41.7b	33	13
	50.0	25.0[b]	25.0c	–	–
Me-Ced	2.0	23.5	54.2b	31	15
	10.0	24.5[b]	41.7b	32	13
	25.0	25.0[b]	33.3c	36	8
	50.0	25.0[b]	25.0c	–	–

[a] Means of three experiments.
[b] Means within a column are significantly different from control in a Kruskal–Wallis χ^2 approximation test at $p < 0.005$.
[c] Means followed by the same letter within a column after ±SE 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.
[d] SP, survival pupation = number of survival pupae × 100/total larvae for pupation.
[e] Emergence % = number of adults emerged × 100/total number of pupae.

droquinones could be due to both a strong inhibitory activity and by a synergistic effect in the mixture composition. Plant tocotrienols (**1**–**3**) and hydroquinones (**4**–**7**) may be considered as efficient IGR as well as radical scavengers, as evidenced by their significant inhibition of DPPH activity.

The tocotrienol sargachromenol **1** and the dihydroplastoquinone sargahydroquinoic acid **4** and their

Table 4
Growth index (GI) and regulatory growth index (RGI) of *S. frugiperda* as a function of increased concentrations of compounds **1**, **2**, **3**, **4**, **5**, **6**, mixtures **M** and **Ma**, **Me-Yuc**, **Me-Ced** extracts, and gedunin[a]

Compounds	Concentration (ppm)	GI[b]	RGI[c]
Control		0.99 ± 0.049a	
1	5.0	0.35 ± 0.04b	0.35
	10.0	0.042 ± 0.001c	0.04
	20.0	0.0	0.0
2	5.0	0.97 ± 0.02b	0.98
	10.0	0.92 ± 0.06b	0.93
	20.0	0.71 ± 0.08b	0.71
	35.0	0.097 ± 0.02c	0.10
	50.0	0.06 ± 0.01c	0.06
	75.0	0.0	0.0
3	5.0	0.48 ± 0.06b	0.49
	10.0	0.097 ± 0.02c	0.10
	20.0	0.0	0.0
4	5.0	0.96 ± 0.04b	0.97
	10.0	0.68 ± 0.01b	0.69
	20.0	0.26 ± 0.04c	0.27
	35.0	0.11 ± 0.01c	0.11
	0.0	0.0	0.0
5	5.0	0.92 ± 0.050b	0.93
	10.0	0.87 ± 0.040b	0.88
	20.0	0.46 ± 0.030b	0.46
	35.0	0.35 ± 0.031b	0.35
	50.0	0.19 ± 0.029b	0.196
	75.0	0.0	0.0
6	5.0	0.63 ± 0.050b	1.00
	10.0	0.40 ± 0.03b	1.00
	20.0	0.14 ± 0.08b	0.95
	35.0	0.0	0.0
M	5.0	0.96 ± 0.08b	0.97
	10.0	0.54 ± 0.02b	0.55
	20.0	0.30 ± 0.05c	0.31
	35.0	0.17 ± 0.01c	0.17
	50.0	0.0	0.0
Ma	5.0	0.46 ± 0.03c	0.46
	10.0	0.19 ± 0.04c	0.19
	20.0	0.14 ± 0.01c	0.14
	35.0	0.0	0.0
Me-Yuc	10.0	0.68 ± 0.050b	0.68
	15.0	0.45 ± 0.039b	0.45
	25.0	0.0	–
Me-Ced	2.0	0.99 ± 0.050b	1.00
	10.0	0.69 ± 0.055b	0.70
	25.0	0.59 ± 0.040b	0.60
	50.0	0.39 ± 0.065b	0.40
Gedunin	10.0	0.77 ± 0.060b	0.77
	25.0	0.51 ± 0.040b	0.51
	50.0	0.10 ± 0.010c	0.10

[a] Means of three replicates.
[b] Means followed by the same letter within a column after ± SE 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] $RGI_{treatment} = GI_{treated}/GI_{control}$ (GI, growth index = No. survival larvae/total larvae used. RGI = GI treated/GI control).

Table 5
Acute toxicity of compounds **1**, **2**, **3**, **4**, **5**, **6**, mixtures **M** and **Ma**, **Me-Yuc**, **Me-Ced** extracts, and gedunin against larvae of last stage of S. *frugiperda*[a]

Compounds	Concentration (ppm)	% Survival[b]	LD_{50}[c]
Control	0.0	95.8 ± 5.8	
1	5.0	26.0 ± 9.6	2.94
	10.0	3.13 ± 1.5	
	20.0	0.0	
2	5.0	73.0 ± 6.8	15.52
	10.0	68.8 ± 9.3	
	20.0	53.2 ± 3.5	
	35.0	7.30 ± 6.8	
	50.0	4.17 ± 5.3	
	75.0	0.0	
3	5.0	36.5 ± 3.4	3.89
	10.0	7.30 ± 6.8	
	20.0	0.0	
4	5.0	72.0 ± 2.5	10.17
	10.0	51.1 ± 4.8	
	20.0	19.8 ± 3.2	
	35.0	8.35 ± 0.7	
	50.0	0.0	
5	5.0	68.8 ± 9.3	14.89
	10.0	65.7 ± 6.2	
	20.0	34.4 ± 4.6	
	35.0	26.0 ± 9.6	
	50.0	14.6 ± 1.3	
	75.0	0.0	
6	5.0	46.9 ± 7.2	4.83
	10.0	26.0 ± 9.6	
	20.0	10.4 ± 3.8	
	35.0	0.0	
M	5.0	72.0 ± 2.5	9.23
	10.0	40.7 ± 0.9	
	20.0	22.9 ± 6.4	
	35.0	12.5 ± 2.6	
	50.0	0.0	
Ma	5.0	34.4 ± 4.6	3.26
	10.0	14.6 ± 1.3	
	20.0	10.4 ± 3.8	
	35.0	0.0	
MeOH extrc.	2.0	93.9 ± 4.69b	n. d.
	10.0	78.9 ± 3.95b	
	25.0	70.2 ± 3.51c	
	50.0	69.1 ± 3.45	
Gedunin	10.0	54.7 ± 2.73b	10.78
	25.0	14.1 ± 0.71c	
	50.0	0	

[a] After 24 h, survival of adults was recorded (percent relative to controls).
[b] Means of three replicates. Means followed by the same letter within a column after ± SE 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] The LD_{50} is the lethal dose producing 50% survival.

acetylated derivatives **3** and **6** had both potent insecticidal and growth inhibitory activities. Conversion of the carboxyl group to the methyl ester as in compounds **2** and **5** results in significant loss of activity and this functional group must play an important role in both the insecticidal and IGR activity of these two series of compounds.

These results confirm previous findings on structure activity relationships for chromene derivatives, namely

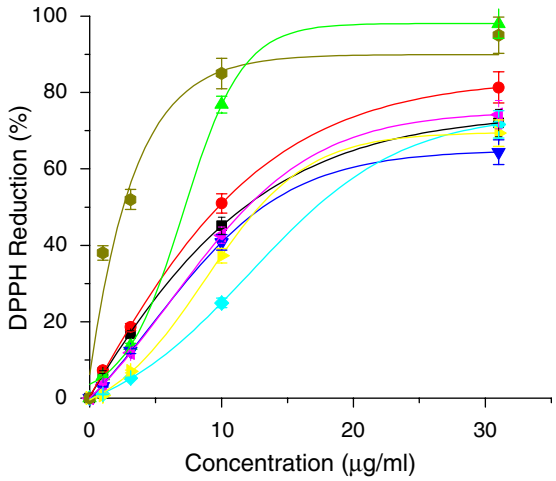

Fig. 2. Radical scavenging activity of **Ma** (■), **M** (●), **4** (▲), **3** (▼), **6** (◆), **1** (◄), tocopherol (►), and caffeic acid (⬢) on radical reduction of DPPH. Measurements at 517 nm, determination after 30 min.

that the growth inhibitory activity of the respective natural product depends on the polarity of the substituents (Carrizo et al., 1998; Agarwal et al., 2000).

The acetoxychromene **3** and diacetoxy sargahydroquinoic acid **6**, in the presence of an methyl substituent in the polyunsaturated aliphatic chain, seem to be the cause of growth inhibitory activity with 30.1% and 9.4% of weight gained at 20.0 ppm, respectively (Table 2).

These observations suggest that acute toxicity and growth inhibition may be due to inhibition of proteinase and other polyphenol oxidases (PPO) binding with these compounds, as this target was demonstrated for other polyphenols from natural origin (Carrizo et al., 1998; Shimizu et al., 2000; Tamayo et al., 2000; Gilly et al., 2001; Karban and Baxter, 2001; Kim et al., 2002.).

In summary, the insecticidal activity of the mixture **M** from aerial parts of *R. barba-johannis* may be due to a synergistic effect shown by phenolic components of the extracts on the test system used during this investigation. Thus, the effect of compounds **1**, **3**, **4**, and **6** and mixtures **M** and **Ma** on reducing insect growth, increasing development time and mortality of *S. frugiperda* is similar to that of gedunin and stronger than the MeOH extract from *C. salvadorensis* (Meliaceae) and *Y. periculosa* (Agavaceae) (Céspedes et al., 2000; Calderón et al., 2001; Torres et al., 2003). The sites and mode of action of these compounds and extracts are being investigated and may be due to a combination of anti-feedant action, as well as midgut phenol oxidase inhibition and resultant moulting sclerotization toxicity, as has been found for other phenolics (Kubo and Kinst-Hori, 1999; Kubo, 2000; Kubo et al., 2000; Kubo et al., 2003a,b) and extracts (Feng et al., 1995).

In addition, the presence of an acetyl group seems to be important for these activities as acetylated compounds **3** and **6** exhibit the most potent activity for emergence of adults from pupae. Furthermore, a great percentage of larvae that could reach pupation decreased with application of **1**, **3**, **6**, and **Ma** compared to

Table 6
Insect growth regulatory activity of the compounds **1**, **2**, **3**, **4**, **5**, **6**, mixtures **M** and **Ma**, from *R. barba-johannis* and **Me-Yuc**, **Me-Ced** extracts, and gedunin against *S. frugiperda* larvae in a no-choice bioassay[a]

Treatment	7 days			21 days			Pupation
	GWI_{50}[b]	GLI_{50}[c]	LD_{50}[d]	GI_{50}[b]	EI_{50}[b]	pI_{50}[e]	pI_{50}[f]
1	5.1	21.3	19.12	8.07	4.32	0.90	3.84
2	24.7	20.6	20.76	14.65	22.60	1.16	24.27
3	5.9	5.6	33.31	4.20	4.59	0.62	6.04
4	6.8	5.8	5.77	4.90	10.54	0.69	14.21
5	31.7	14.7	62.02	18.08	39.91	1.26	23.01
6	57.8	N.D	81.81	24.41	7.07	1.39	7.61
M	4.1	33.4	17.76	2.44	8.92	0.39	13.90
Ma	19.2	33.2	27.51	5.17	3.60	0.71	4.78
Me-Yuc	14.99	15.8	7.18	6.12	5.79	0.78	18.82
Gedunin	2.87	7.16	30.08	2.51	3.95	0.40	14.01
Me-Ced	10.6	16.3	8.22	9.79	1.43	0.99	3.84

[a] The parameters in ppm values.

[b] The GWI_{50} and GI_{50} correspond to the inhibitory concentrations for reduction of 50% of larval growth in weight at 7 and 21 days, respectively, in "no choice" test ($p < 0.05$) and EI_{50} correspond to concentration producing 50% of emergence and were calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by PROBIT analysis and ANOVA ($p < 0.05$) corresponding to the growth inhibition at 7 and 21 days, respectively, under Microcal Origin 6.0.

[c] GLI_{50} correspond to the growth inhibition in length at 7 days, and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by PROBIT analysis and ANOVA ($p < 0.05$) under Microcal Origin 5.1.

[d] LD_{50} is the concentration producing 50% of lethal mortality at 7 days in "no choice" test calculated by PROBIT analysis and ANOVA ($p < 0.05$).

[e] pI_{50} correspond to $\log GI_{50}$.

[f] pI_{50} correspond to concentration producing 50% of pupation, and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA ($p < 0.05$) under Microcal Origin 6.0.

Table 7
Effect of natural compounds **1**, **2**, **3**, **4**, **5**, **6**, gedunin and mixtures **M**, **Ma**, and **Me-Yuc**, **Me-Ced** extracts on DPPH reduction[a], I_{50}[b] values[e]

Compound	I_{50}
Tocopherol	11.86
Caffeic acid	2.13
1	12.83
2	15.85
3	ND
4	6.14
5	17.24
6	ND
M	8.92
Ma	11.89
Me-Yuc	33.53
Me-Ced	n.t.[d]
Gedunin	n.t.

[a] Means of three experiments.
[b] Concentration that produce 50% of scavenging radicals from DPPH.
[c] Not determined.
[d] Not tested.
[e] Values in ppm.

control (at 20.0, 20.0, 35.0, and 35.0 ppm, respectively). This phenomena might be due to proteinase inhibition, tyrosinase or PPO as well.

Although chemically distinct, the level of insecticidal activity of this plant and metabolites and mixtures derived from the plant is comparable to that of the known insect growth regulator, gedunin. Based on the present investigations, materials from *R. barba-johannis* and related species should prove to be valuable sources of interesting biologically active compounds, including insecticides.

3. Experimental

3.1. Plant material

R. barba-johannis (DC.) H. Rob. & Betteli was collected in the region near to Laguna de Zempoala, State of Mexico, Mexico, in March of 1999. A voucher sample (MEXU-862646) was deposited at the Institute of Biology, at National Herbarium UNAM.

3.2. Apparatus

IR spectra were obtained on a Nicolet Magna-IR 750 spectrometer. ^{1}H NMR spectra were recorded at 300 and 500 MHz, and ^{13}C NMR spectra at 75 and 125 MHz, respectively, on Varian VXR-300S and VXR-500S spectrometers. Chemical shifts (ppm) are related to $(CH_3)_4Si$. $CDCl_3$, MeOD-d_4, and acetone-d_6 from Aldrich Chemical Co. were used as solvents. Coupling constants are quoted in Hz. EIMS data were determined on a JEOL JMS-AX505HA mass spectrometer at 70 eV. FABMS were obtained on a JEOL JMS-SX102A mass spectrometer operated with an acceleration voltage of 10 kV. 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. A Spectronic model Genesys 5 spectrophotometer was used to determine biological and DPPH antioxidant activities. Optical rotation was measured on a JASCO DIP-360 spectropolarimeter. Melting points were obtained on a Fisher–Johns hot-plate apparatus and remain uncorrected.

3.3. Chemicals and solvents

All reagents used were either analytical reagent grade or chromatographic grade. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), α-tocopherol, caffeic acid, gallic acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma–Aldrich Química, S.A. de C.V., Toluca, Mexico. Methanol, CH_2Cl_2, $CHCl_3$, NaCl, KCl, NaOH, KOH, *tert*-butanol, *tert*-butyl hydroperoxide, $CuSO_4$, NH_4Cl, $MgCl_2$, acetic anhydride, Silica Gel GF_{254} analytical chromatoplates, Silica gel grade 60, (70-230, 60A°) for CC, *n*-hexane, and ethyl acetate were purchased from Merck-Mexico, S.A., Mexico. CC was also carried out on Silica-gel G (Merck, Darmstadt, Germany).

3.4. Extraction, isolation, and synthesis of tocotrienols (*1*–*3*) and hydroquinone (*4*–*7*) derivatives

Dried and ground leaves, bark, and flowers (470 g) of *R. barba-johannis* were processed, extracted, and purified as described by Pérez-Castorena et al. (2002).

3.5. Bioassays with Fall Armyworm

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 (Céspedes et al., 2000). 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 U 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). Twenty four-well polystyrene multidishes were filled with the liquid diet and then left for 20 min at room temperature under sterile conditions. The 3.4-ml wells measure 17 mm in depth × 15 mm in diameter with a 1.9-cm^2 culture area. All test compounds were dis-

solved in 95% EtOH and layered on top of each well with the artificial diet using up to six concentrations (see Table 1) and a control (1 ml 95% EtOH) allowing evaporation of solvent. Hexane (1.0 ppm) and MeOH extracts (3.5 ppm) 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 well 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 21 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 °C day and 19 °C night temperature regime, and a relative humidity of $80 \pm 5\%$. There were three replications for each assay. Control assays (24-wells) contained the same number of larvae, volume of diet, and EtOH as the test solutions (Céspedes et al., 2000; Torres et al., 2003).

3.6. Acute toxicity on S. frugiperda

Acute toxicity was determined by topical application to larvae of last stage (fifth instar) of *S. frugiperda.* The larvae were iced to stop their movement and treated on their abdomens with each of the test compounds, at concentrations of 1.0, 3.0, 7.0, 10.0, 25.0, and 50.0 ppm. Additional concentrations were used for **Me-Yuc** (15.0 ppm) extract and **Me-Ced** (2.0 ppm) extracts, respectively (Table 6). The solvent used was acetone (10.5 μl) which was poured with a microsyringe (50 μl). The control was only treated with 10.5 μl of acetone. After 24 h survival rate were recorded. Five larvae were used for each concentration, respectively. LD_{50} is the lethal dose producing 50% survival (Calderón et al., 2001; Torres et al., 2003).

3.7. Relative growth index and growth index

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

3.8. Reduction of 2,2-diphenyl-1-picrylhydrazyl (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radical

TLC autographic assay. After developing and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. Plates were examined 30 min after spraying. Active compounds appear as yellow spots against a purple background. In similar manner, TLC plates were sprayed with 0.05% β-carotene solution in $CHCl_3$. Plates were exposed to UV_{254} light until the background becomes discolored (bleached). Active compounds appear as pale yellow spots against a white background.

Spectrophotometric assay (Bors et al., 1992; Cuendet et al., 1997). Solution containing the compound to be tested (50 μl) was added to 5 ml of a 0.004% MeOH solution of DPPH. Quercetin was used as internal reference standard. The absorbance at 517 nm was measured after 30 min and the percent of activity was calculated.

3.9. Statistical analysis

Data shown in figures and tables are average results obtained from three replicates and independent experiments, and separate crocin and DPPH preparations. Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by generalized linear models (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 analyses based on percentage of inhibition obtained at each concentration of the samples. I_{50} is the concentration producing 50% inhibition of radical DPPH. Complete statistical analysis was performed by means of the MicroCal Origin 6.0 statistical and graphs PC program.

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