Acetylcholinesterase and Insect Growth Inhibitory Activities of *Gutierrezia microcephala* on Fall Armyworm *Spodoptera frugiperda* J. E. Smith

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From the aerial parts of *Gutierrezia microcephala* (Asteraceae), four oxyflavones were isolated, namely 5,7,2′-trihydroxy-3,6,8,4′,5′-pentamethoxyflavone (**1**); 5,7,4′-trihydroxy-3,6,8-trimethoxyflavone (**2**); 5,7,2′,4′-tetrahydroxy-3,6,8,5′-tetramethoxyflavone (**3**); 5,2′-dihydroxy-3,6,7,8,4′,5′-hexamethoxyflavone (**4**), and an *ent*-clerodane, bacchabolivic acid (**5**). Compounds **1-5**, the synthetic methyl ester (**6**), *n*-hexane and MeOH extracts were evaluated against the fall armyworm (*Spodoptera frugiperda*). Gedunin, a known insect growth regulator isolated from *Cedrela* spp. was used as a positive control. When tested for activity on neonate larvae into the no-choice artificial diet bioassay, flavone (**1**), clerodane (**5**), its methyl ester (**6**), MeOH and *n*-hexane extracts caused significant larval mortality with MC_{50} of 3.9, 10.7, 3.46, 7.95 and 7.5 ppm at 7 days, respectively, as well as growth reduction. They also increased the development time of surviving larvae and a significant delay in time to pupation and adult emergence. Acute toxicity against adults of *S. frugiperda* was also found, **5**, **6**, gedunin and *n*-hexane extract had the most potent activity with LD_{50} value of 6.59, 15.05, 10.78, and 12.79 ppm, respectively. In addition, MeOH, *n*-hexane extracts, **5**, **6** and gedunin caused acetylcholinesterase inhibition with 93.7, 100, 90.2, 62.0 and 100% at 50.0 ppm, respectively; whereas **1–4** exhibited only moderate inhibitory activity. Compounds **1**, **5** and **6** showed inhibitory activities comparable with gedunin. These compounds could be responsible of the insect growth inhibitory activity of this plant.

Introduction

Until now pesticides of synthetic origin have been widely used, producing a strong impact on the environment with the apparition of resistant strains to this type of compounds. Organic molecules of botanical origin may offer an environmental safe source of compounds for pest management, since they are environmentally friendlier, and an efficient alternative to persistent synthetic insecticides (Kubo, 2000). The increasing interest in the possible application of secondary metabolites to pest management has directed the investigation towards the search of new sources of biologically active natural products with low mammalian toxicity, low persistence in the environment, and biodegradability. These characteristics may enhance their value as botanical pesticide (González *et al.*, 1998).

There is a widespread effort to find new pesticides and this currently has been focused on limonoids from the Meliaceae family due to their potent effects on insect pest and low toxicity. Our interest is centered in the study of possible insecticidal activities of desert shrubs belonging to the Asteraceae family, due to their strong resistance against insect attack observed in nature.

Gutierrezia microcephala A. Gray a member of Asteraceae, commonly known as broomwood, grows in arid regions of the central and north of Mexico and in the southwestern region of the United States (Roitman *et al.*, 1985). There are reports on diverse effects on human health and on

Abbreviations: AChE, acetylcholinesterase; ATC, acetylthiocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); RGI, relative growth index; GI, growth index.

animals of compounds from Asteraceae family (Bittner *et al.*, 1983; Hudson *et al.*, 1993). From several species of *Gutierrezia* (Gao *et al.*, 1985; Zdero *et al.*, 1992; Fang *et al.*, 1985, 1986), flavonoids, labdane-derivatives and other metabolites have been isolated. A phytochemical examination of the aerial parts of *G. microcephala* recollected in north of Mexico (Highway Monterrey-Saltillo, km. 240), was undertaken because its behavior into the ecological habitat, since has been observed that this specie suffers minimal insect-pest attack. In addition, no insecticidal work has been previously carried out on this plant. In the present study four flavonols **1–4** previously reported (Fang *et al.*, 1985), some of them with cytotoxic activity (Cea *et al.*, 1983), and a new compound from this plant, the *ent*-clerodane bacchabolivic acid (**5**) were isolated. The mechanism, by which this plant exhibits its insect growth regulatory effects on fall armyworm (FAW) S*podoptera frugiperda* (growth, pupation and emergence), is unknown. In this context, this work deals with the results of insecticidal activities of the major compounds of *G. microcephala* and their possible mechanisms of action.

Flavonoids are compounds whose occurrence is very high in many plant families. To date, more than 4000 flavonoids are known at present, new ones are reported every month, their biological activities vary considerably, and there are many reports (Forkman and Heller, 1999). The flavonoids are an integral part of the plant kingdom, present in all photosynthesizing cells and one of the major components of Asteraceae (Harborne and Baxter, 1999; Wollenweber, 1997). Their different biological activities including antioxidant, antimicrobial, carcinogenic, cytotoxic, antiinflamatory and mutagenic properties, make them as interesting object of research (Middleton, 1993). In addition, there are reports on the tyrosinase inhibitory activity of flavonols (Kubo *et al.*, 2000) and this information could be of interest for explanation of the allelopathic and insect growth regulatory activities of these compounds.

Materials and Methods

Plant material

1.1 kg of aerial parts (stem, leaf and flowers) from *G. microcephala* was collected in Saltillo, State of Coahuila, in October 1997. A voucher sample was deposited at National Herbarium (MEXU), Instituto de Biología, UNAM. Voucher: M. T. German and P. Tenorio No. 889. Register number: 100.193.

Apparatus

^{1}H-NMR spectra were recorded at 300 and 500 MHz, ^{13}C-NMR at 75 and 125 MHz respectively, on Varian VXR-300S and VXR-500S spectrometers, chemical shifts (ppm) are relative to $(CH_3)_4Si$ as internal reference. $CDCl_3$ and acetone-d_6 from Aldrich Chemical Co. were used as solvents, and coupling constants are quoted in Hz. IR spectra were obtained as KBr pellets on Perkin Elmer 283-B and FT-IR Nicolet Magna 750 spectrophotometers. Electron impact mass spectra were taken on a JEOL JMS-SX102A instrument (70 eV). UV spectra were determined on a Shimadzu UV-160 spectrophotometer; $CHCl_3$ was used as solvent. Optical rotations were measured on a JASCO DIP-360 spectropolarimeter; $CHCl_3$ was used as solvent. Melting points were obtained on a Fisher-Johns hot-plate apparatus and remain uncorrected. Nunc 24-well polystyrene multidishes were purchased from Cole-Parmer. LAB-LINE Chamber model CX14601A, with adjustable Hi-Lo protection thermostats safeguard samples.

A Spectronic model GENESYS 5 spectrophotometer was used to carry out the measurements in the acetylcholinesterase activity. The centrifuge used in this study was B. Braun, model SIGMA 2-15.

Chemicals and solvents

All used reagents were commercially available. Thiamine, sorbate, methyl-paraben, ascorbate, acetic acid, acetaldehyde, acetylcholinesterase (AChE), acetylthiocholine (ATC), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), choline-chloride, calcium pantothenate, niacinamide, riboflavin, folic acid, biotin and vitamin B-12 were purchased from Sigma Chemical Co. Methanol, ethyl acetate, $CuSO_4$, KCl, $NaHCO_3$, $MgCl_2$, NH_4Cl, pyridine, acetic anhydride, Silica-gel GF_{254} analytical chromatoplates, Silica gel grade 60, 70–230, 60 Å, for column chromatography were purchased from Merck. Pre-coated TLC plates SIL G-100 UV_{254}, 1.0 mm, preparative were

purchased from Macherey-Nagel, Düren, Germany.

Isolation and purification of the flavones and ent-clerodane

Milled aerial parts of *G. microcephala* were percolated with three solvents *n*-hexane, acetone and methanol. From the *n*-hexane extract (hex) (45 g) was spontaneously precipitated 7.44 g of white powder with m.p. of 144–147 °C, whose spectrometric and spectroscopic data agree with baccharbolivic acid isolated previously from *Baccharis boliviensis* (Zdero *et al.*, 1989). Esterification of the acid (**5**) with CH_2N_2, afforded the methyl ester (**6**), which was purified by chromatographic procedures. The flavones **1–4** were isolated from the acetonic and methanolic extracts (125.5; 118.0; 85.3; and 55.5 mg, respectively) obtained as yellow crystals. Their spectrometric and spectroscopic data are agree with the compounds isolated previously by Fang *et al.*, 1985.

Bioassays with fall armyworm

Larvae used for the experiments were obtained from the 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 soybean 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, then left for twenty minutes 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 dissolved in 95% ethanol and layered on top of each well with the artificial diet using up to six concentrations (see Table I) and a control (1 ml 95% ethanol) allowing evaporation of solvent. In addition was used 1.0 and 3.5 ppm for hex and MeOH extracts, since these extracts showed the highest inhibitory activity in the preliminary bioassay (data not shown). For each concentration used and control, a single *S. frugiperda* neonate first instar larva was placed on the diet mixture in each well for 7 days. 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, since pupation average is 23 ± 1 days. Other lifecycle measurements were recorded, such as time to pupation, weight of pupae, mortality of larvae and adult emergence and deformities. All treatments were carried out in a controlled environment chamber with an 18L: 6D photoperiod, at 25 °C day and 19 °C 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).

Acute toxicity on Spodoptera frugiperda

Acute toxicity was determined by topical application to larvae of last stage of *S. frugiperda.* The larvae of *S. frugiperda* were iced to stop their movement and treated on their abdomens with each of the test compounds, at concentrations of 2.0, 5.0; 10.0; 25.0 and 50.0 ppm. Additional concentrations (15.0 and 35.0 ppm) were used for compounds **5**, **6** and gedunin (see Table VI). The solvent used was 10.5 µl of acetone injected with 50 µl microsyringe, and control was only treated with 10.5 µl of acetone. After 24 hrs survival were recorded. Five larvae were used for each concentration, respectively. LD_{50} is the lethal dose producing 50% survival.

Inhibition of acetylcholinesterase

An enzyme extract containing acetylcholinesterase (AChE) was obtained according to the method of Grundy and Still (1985). About 100 adults of *Spodoptera frugiperda* were frozen at −20 °C for 7 days. The heads of frozen adults were detached, then milled and homogenized in 20 ml of 0.1 M phosphate buffer at pH 8.0. The crude homogenate was centrifuged at 15,000×*g* for 15 min at 5 °C, and the supernatant was used for the enzyme

Table I. Fall armyworm bioassay results from compounds of *G. microcephala* (after 7 days of incubation)[a].

Treatment	Conc. [ppm]	Mean weight gained[b] [mg]	%[c]	Mean length gained[d] [mm]	%[c,e]	Mortality (%)[f]
Control		81.0 ± 7.2a	100	1.1 ± 0.055	100	7.50
1	2.0	63.6 ± 3.9a	78.5	0.73 ± 0.036	66.4	40.0
	10.0	43.2 ± 2.7b	53.3	0.54 ± 0.027	49.1	58.0
	25.0	22.7 ± 1.4b	28.1	0.43 ± 0.021	39.1	70.0
	50.0	17.0 ± 1.1c	20.1	0.40 ± 0.020	36.4	90.0
2	2.0	65.3 ± 4.1a	80.6	0.95 ± 0.047	86.4	4.80
	10.0	48.8 ± 3.1b	60.3	0.80 ± 0.040	72.7	12.7
	25.0	31.2 ± 1.9b	38.5	0.60 ± 0.030	54.5	23.8
	50.0	21.8 ± 1.3b	26.9	0.45 ± 0.022	40.9	45.8
3	2.0	74.4 ± 4.6a	91.9	0.77 ± 0.038	70.0	17.9
	10.0	57.0 ± 3.5a	70.4	0.54 ± 0.027	49.1	31.0
	25.0	44.8 ± 2.8b	55.3	0.41 ± 0.020	37.3	48.0
	50.0	36.1 ± 2.2b	44.6	0.34 ± 0.017	30.9	78.6
4	2.0	81.2 ± 5.0a	100	1.07 ± 0.053	97.2	2.9
	10.0	79.2 ± 4.5a	90.0	1.01 ± 0.050	91.8	12.7
	25.0	68.8 ± 4.3a	85.0	0.84 ± 0.042	76.4	19.0
	50.0	60.8 ± 3.8a	75.0	0.67 ± 0.029	60.9	27.0
5	2.0	45.5 ± 2.8b	56.2	0.95 ± 0.047	86.4	18
	10.0	11.7 ± 0.7c	14.5	0.70 ± 0.035	63.6	50
	25.0	1.7 ± 0.1c	2.1	0.35 ± 0.017	31.8	98.6
	50.0	0.0	0	0.0	0.0	100
6	2.0	55.9 ± 3.5a	69.0	0.95 ± 0.047	86.4	37
	10.0	16.3 ± 1.0b, c	20.1	0.51 ± 0.025	46.4	81
	25.0	3.2 ± 0.2c	4.0	0.23 ± 0.013	20.9	98.6
	50.0	2.1 ± 0.1c	2.6	0.19 ± 0.007	17.3	98.6
Gedunin	10.0	7.2 ± 0.4c	8.9	0.39 ± 0.019	35.5	33
	25.0	3.40 ± 0.2c	4.2	0.24 ± 0.012	21.8	38
	50.0	1.90 ± 0.1c	2.3	0.20 ± 0.010	18.2	70.8
MeOH ext.	2.0	60.8 ± 3.8a	75.1	0.93 ± 0.046	84.5	45.5
	10.0	24.3 ± 1.5b, c	30.0	0.65 ± 0.032	59.1	51.8
	25.0	8.9 ± 0.6c	11.0	0.46 ± 0.023	41.8	78.9
	50.0	6.5 ± 0.4c	8.0	0.40 ± 0.020	36.4	98.6
n-hex. ext.	2.0	52.8 ± 3.3a	65.2	1.00 ± 0.050	90.9	42.0
	10.0	14.2 ± 0.9c	17.5	0.65 ± 0.040	59.1	54.0
	25.0	4.5 ± 0.3c	5.55	0.40 ± 0.032	36.4	81.0
	50.0	2.5 ± 0.1c	3.1	0.39 ± 0.028	35.5	98.6

[a] Values taken after 7 days of incubation, mean of three replicates. [b] Means (mg) 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 to control). [c] Percentage with respect to control. [d] The reduction of first instar larval length rates (cm) in "no choice" test calculated by ANOVA program ($p < 0.05$). [e] Mean length total increase from eclosion. [f] The MC_{50} is the concentration producing 50% mortality.

Equivalence of ppm to μM

	Compounds (μM)						
ppm	**1**	**2**	**3**	**4**	**5**	**6**	Gedunin
2	4.7	5.5	4.9	4.6	6.3	6.1	4.1
10	23.8	27.7	24.6	23.0	31.6	30.3	20.7
25	59.5	69.4	61.6	57.6	79.1	75.8	51.9
50	119.0	138.9	123.2	115.2	158.2	151.5	103.7

Molecular weights: **1** = 420 g/mol; **2** = 360 g/mol; **3** = 406 g/mol; **4** = 434 g/mol; **5** = 316 g/mol; **6** = 330 g/mol; Gedunin = 482 g/mol.

activity. ATC (cholinesterase substrate) was dissolved in 0.1 M phosphate buffer (pH 8.0). DTNB (3-carboxy-4-nitrophenyldisulfide), Ellman's reagent and a sensitive sulfhydryl reagent (Deakin *et al.*, 1963), 39.6 mg of this compound was dissolved in 10 ml of 0.1 M phosphate buffer at pH 7.0, and 15.0 mg of $NaHCO_3$ was added.

Inhibition of AChE was determined according to the Ellman's procedure (colorimetric method) (Ellman *et al.*, 1961) using both the control (MeOH) and test solutions. The reaction mixture contained 0.2 ml of the enzyme solution and 0.1 ml of DTNB added to 2.4 ml of 0.1 M phosphate buffer (pH 8.0). The reaction mixtures were added to each of the test compounds dissolved in 50 µl of EtOH. The control solution was similarly prepared by the addition of 50 µl of EtOH. Both control and each of the test solutions were preincubated at 25 °C for 10 min. After preincubation, the enzyme reaction was started by the addition of 40 µl of ATC followed by incubation at 25 °C for 20 min. After 20 min, the absorbance at 420 nm was measured spectrophotometrically and compared with that of the control immediately after adding an enzyme to the above reaction mixtures. Reading was repeated for 5 min at 30 sec intervals to verify that the reaction occurs linearly. Blank reaction was measured by substituting saline for the enzyme. AChE activity was calculated with the absorption coefficient 1.56 mmol/min. All experiments were repeated three times and the results were analyzed by SAS ANOVA and GLM procedures and graph by Microcal Origin version 4.1.

Relative growth index and growth index

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

Data analysis

Data for all the live insect bioassays were analyzed by SAS ANOVA and GLM procedures (SAS Institute, 1982) and Microcal Origin version 4.1 ($p < 0.05$), and GWI_{50}, and GLI_{50}, values for each activity were calculated by Probit analysis (Finney, 1971) on the basis of the percentage of inhibition obtained from each one of the concentration of the compounds compared with control. Differences between treatment means were established with a Student-Newman-Keuls (SNK) test.

Results and Discussion

Insecticidal activity against larvae

The insecticidal effects of **1–6**, gedunin, hex and MeOH extract against larvae of first instar of *Spodoptera frugiperda* are outlined in Table I. Compounds **1**, **5**, MeOH and hex extracts at 10.0 ppm concentration, produced significant larval mortalities (> 49%), whereas **6** produced higher mortality (81%) at the same concentration. On the other hand **6**, hex and MeOH extracts showed the highest insecticidal activity producing 98.6% of larval mortality at high concentration (50.0 ppm). It is noteworthy that, when larvae were fed with a diet containing 50.0 ppm of **5** all the larvae died. The 50% lethal concentration (MC_{50}) of larvae at 7 days for these compounds and extracts are outlined in Table IV. It is important to point out that **6**, **1**, hex, MeOH extracts and **5** were more active than gedunin used as control, with MC_{50} of 3.46, 3.9, 7.5, 7.95 and 10.7 ppm, respectively.

Insect growth inhibitory activity

The compounds **1**, **3**, **5**, **6**, gedunin, and hexane and MeOH extracts inhibited specifically each larval stage, *i.e.* growth when incorporated into diets at 25.0 ppm (up 60% of length). On the other hand **5**, **6**, gedunin, hexane and MeOH extracts produced higher inhibition (up 90% of weight) at the same concentration. However, flavonoids **1–4** showed clearly lower larval inhibition than **5**, **6**, gedunin and hexane and MeOH extracts at high concentration (50.0 ppm). Furthermore clerodane **5**, showed the highest inhibition (100% of length and weight) at the same concentration (Table I). At 21 days, this growth reduction was clearly significant between 5.0 and 50.0 ppm. However, only compounds **1**, (**5**) and gedunin showed the highest larval growth inhibition at the same concentrations (Table II).

The percentage of larvae that reached pupation decreased in some tested compounds (**1**, **5**, **6**, gedunin and MeOH and hex extracts) in comparison to control. Thus, **1**, **5**, **6**, gedunin, and hex extracts showed significant delayed of pupation by

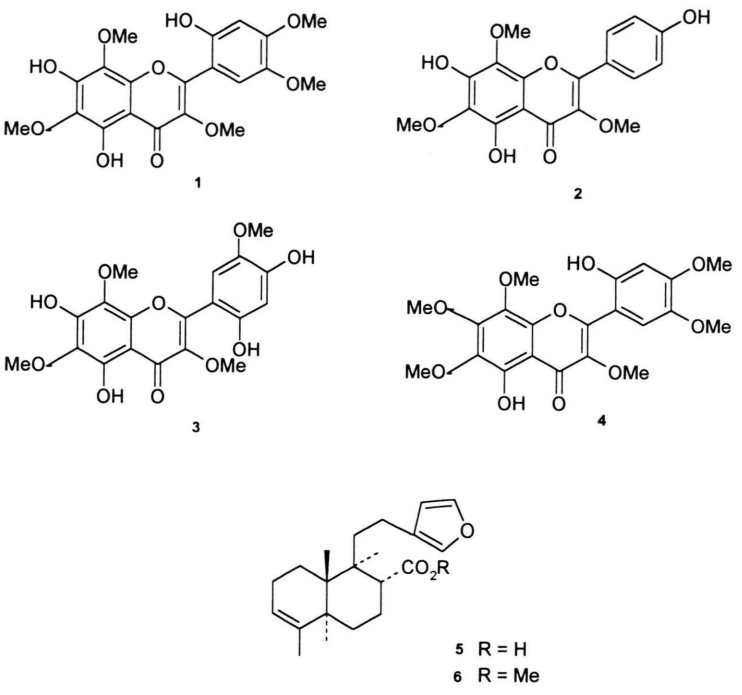

Fig. 1. Chemical structures of flavonoids **1–4** and *ent*-clerodanes **5** and **6**.

12.5, 6.3, 12.5, 4.17 and 12.5% at 50.0, 10.0, 15.0, 50.0 and 50.0 ppm, respectively. The most important effect was observed with **1**, **6** and hex extract at 25.0, 15.0 and 50.0 ppm, which reduced survival pupation by 0.0% in all cases, respectively. Significant delays in time to pupation (25 days) were observed at 10.0, 15.0 and 50.0 ppm for **5, 6** and hex extract, respectively. Furthermore, gedunin and hexane extract, significantly reduced pupae weights at 50.0 ppm, respectively. While, hex extract showed the greatest effect at 50.0 ppm (Table III).

Percentage of emergence, as compared to the pupal stage, showed further reductions with compounds **1**, **5**, **6**, gedunin, and hex extract at 10.0, 10.0, 10.0, 50.0 and 25.0 ppm with 8.3, 8.3, 8.3, 4.17 and 12.5% of emergence, respectively (Table III). However, **1**, **6**, and hex extract drastically reduced the percentage of adult emergence to 0% at 25.0, 15.0 and 50.0 ppm, respectively. These facts could be correlationed with EI_{50} and pEI_{50} values, that parameters showed a strong growth inhibition of compounds **5**, gedunin, **1** and **6** with pEI_{50} of 0.13, 0.22, 0.26 and 0.48 values, respectively, that indicate the potency of **5** and gedunin (Table IV).

Growth inhibition and regulatory growth index

In many of the treatments, mean adult weight was significantly delayed in the average time to reach the adult stage relative to control larvae. GI and RGI clearly showed (Table V) that the stronger effect was shown by **5**, **6** and hex extract, with RGI values of 0.25 at 25.0, 25.0 and 5.0 ppm, respectively. Gedunin also showed a pronounced effect with RGI of 0.51 and 0.10 at 25.0 and 50.0 ppm, respectively. These parameters together with the LD_{50} values (Table VI), corroborated the highest effect that showed the *ent*-clerodane **5**, since it caused the greatest inhibitory effect with 93.2 and 96.4% of growth inhibition in weight at 7 and 21 days, at 15.0 ppm, respectively (Tables I and II). On the other hand, this compound showed lower inhibitory effect in length at 15.0 ppm (45.5 and 72.1%), at 7 and 21 days, respectively (Tables I and II). In addition, this compound, at 25.0 and 50.0 ppm, was noteworthy insecticidal with 98.6 and 100% of mortality, respectively (Table I).

It is important to note that similar insect growth regulatory activity on *S. litura* (common cutworm) was studied by Morimoto *et al.*, 2000. These authors reported that the flavonoids 5-hydroxy-

Table II. Fall armyworm bioassay results from *Gutierrezia microcephala* compounds[a].

Treatment	Conc. [ppm]	Mean weight gained[b] [mg]	%[c]	Mean length gained [mm]	%[c]
Control		478.5 ± 23.92a	100	31.8 ± 1.59	100
1	2.0	23.1 ± 1.155b, c	4.8	23.6 ± 1.18	74.2
	10.0	8.13 ± 0.406c	1.7	12.5 ± 0.63	39.3
	25.0	7.41 ± 0.370c	1.5	8.01 ± 0.40	25.2
	50.0	4.01 ± 0.201c	0.8	7.59 ± 0.37	23.9
2	2.0	316.5 ± 15.82a	66.1	23.7 ± 1.18	74.5
	10.0	123.2 ± 6.16a, b	25.7	12.6 ± 0.63	39.6
	25.0	69.5 ± 3.47b	14.5	9.1 ± 0.45	28.6
	50.0	49.3 ± 2.46b	10.3	8.4 ± 0.42	26.4
3	2.0	353.3 ± 17.66a	73.8	27.76 ± 1.39	87.3
	10.0	111.0 ± 5.55a, b	23.2	18.66 ± 0.93	58.7
	25.0	45.0 ± 2.25b	9.4	10.65 ± 0.53	33.5
	50.0	23.0 ± 1.15b	4.8	6.33 ± 0.32	19.9
4	2.0	393.8 ± 17.05a	82.3	28.4 ± 1.42	89.3
	10.0	197.3 ± 11.80a	41.2	23.9 ± 1.19	75.2
	25.0	114.9 ± 6.90a, b	24.0	21.5 ± 1.07	67.6
	50.0	100.4 ± 4.05b	20.9	20.2 ± 1.01	63.5
5	2.0	27.2 ± 1.36b, c	5.70	16.5 ± 0.83	51.9
	10.0	8.4 ± 0.42c	1.76	9.3 ± 0.46	29.2
	15.0	3.6 ± 0.18c	0.75	8.9 ± 0.44	27.9
6	2.0	318.9 ± 15.95a	66.6	24.5 ± 1.22	77.0
	10.0	43.7 ± 2.18b	9.1	8.7 ± 0.43	27.3
	25.0	9.11 ± 0.45c	1.9	7.1 ± 0.35	22.3
	50.0	8.5 ± 0.43c	1.8	6.5 ± 0.32	20.4
Gedunin	10.0	4.91 ± 0.25c	1.0	4.9 ± 0.24	15.4
	25.0	3.50 ± 0.18c	0.7	3.5 ± 0.17	11.0
	50.0	2.10 ± 0.11c	0.4	3.0 ± 0.15	9.4
MeOH	2.0	410.1 ± 20.51a	85.7	27.8 ± 1.39	87.4
	10.0	279.1 ± 13.96a	58.3	22.1 ± 1.10	69.5
	25.0	176.6 ± 8.83a	36.9	18.5 ± 0.92	58.1
	50.0	110.2 ± 5.51b	23.0	15.6 ± 0.78	49.0
n-hex. ext.	2.0	390.4 ± 19.52a	81.6	26.3 ± 1.24	82.7
	10.0	251.3 ± 12.56a	52.5	20.3 ± 1.05	63.8
	25.0	152.2 ± 7.61b	31.8	15.4 ± 0.77	48.4
	50.0	109.7 ± 5.48b	22.9	14.9 ± 0.74	46.8

[a] Values taken at 21 days before pupation, mean of three replicates. [b] 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] Percentage with respect to control.

3,6,7,8,4′-pentamethoxyflavone; 5-hydroxy-3,6,7,8-tetramethoxyflavone; 5,6-dihydroxy-3,7-dimethoxyflavone and 4,4′,6′-trihydroxy-2′-methoxychalcone are insect antifeedant flavonoids against the common cutworm (*Spodoptera litura*), these flavonoids were detected in small amounts in *Gnaphalium affine* (Asteraceae), their ED_{50} are between 1.1×10^{-7} to 2.5×10^{-8} mol/cm^2. These values are not comparable with our bioassay. However, is possible to infer that the substitution of polymethoxy flavones induce an increase in the activity of these flavones. There are not insecticidal reports, of *ent*-clerodane-type diterpenes, only antifeedant activity has been reported (Simmonds *et al.*, 1999), the presence of a furan ring seems be necessary for insecticidal activity as in limonoids containing this chemical group (Céspedes *et al.*, 2000).

Acute toxicity on larvae of last stage of S. frugiperda

Flavonoids **1–4** showed moderated acute toxicity with a range of 45.0 to 77.0% of survival at 50.0 ppm, respectively (Table VI). However, **5**, **6**, gedunin and hexane extract showed a potent acute toxicity of 9.5, 7.0, 0.0 and 17.9% of survival on larvae of last stage of *S. frugiperda* at 50.0 ppm, respectively. The LD_{50} values of **5**, **6**, gedunin, and

Table III. Activity of **1–6**, gedunin and MeOH and *n*-hexane extracts from *G. microcephala* on pupation and emergences parameters of fall armyworm (after 21 days of incubation)[a].

Treatment	Conc. [ppm]	Mean time pupation [days]	Pupation SP [%][e]	Mean weight pupae [mg][c]	Mean emergence [days]	Emergence [%][f]	Male [%]	Female [%]
Control		22.0	88.2	309.5 ± 15.47a	33	77.50	35	42.5
1	2.0	22.0	60.6	268.2 ± 11.43a	31	28.3	20.	8.3
	10.0	22.5	22.8	180.9 ± 9.78b	33	8.3	8.3	–
	25.0	24.0[b]	16.8	122.7 ± 8.79b	–	0.0	–	–
	50.0	24.5[b]	12.5	104.5 ± 5.22b	–	0.0	–	–
2	2.0	21.5	92.3	281.7 ± 14.08a	31	79.5	39.7	39.7
	10.0	22.5	89.2	245.3 ± 12.25a	31	78.1	26.0	52.1
	25.0	23.0	87.4	243.2 ± 12.15a	32	77.9	26.0	51.9
	50.0	23.0	83.0	240.1 ± 12.01a	33	72.3	24.1	48.2
3	2.0	21.5	88.1	299.5 ± 14.97a	32	81.5	20.4	61.1
	10.0	22.0	82.1	273.1 ± 13.65a	33	77.9	19.5	58.4
	25.0	22.0	80.3	233.7 ± 11.68a	32	75.4	18.8	56.6
	50.0	22.5	77.2	202.1 ± 10.10a	33	72.1	18.0	54.1
4	2.0	22.0	88.0	289.6 ± 14.48a	32	80.1	20.0	60.1
	10.0	22.0	67.7	260.1 ± 13.00a	32	77.5	19.4	58.1
	25.0	22.0	55.6	229.2 ± 11.46a	33	72.1	18.0	54.1
	50.0	22.5	52.1	208.9 ± 10.44a	33	59.8	14.9	44.9
5	2.0	22.0	52.3	205.3 ± 10.26a	32	16.7	8.3	8.3
	10.0	25.0[b]	6.3	109.9 ± 5.49b	35	8.3	8.3	–
6	2.0	21.5	68.3	227.6 ± 11.38a	33	16.7	8.3	8.3
	10.0	24.0[b]	25.7	150.8 ± 7.54b	36	8.3	8.3	–
	15.0	25.0[b]	12.5	148.8 ± 7.44b	–	0.0	–	–
Gedunin	10.0	22.5	49.8	111.5 ± 5.57b	34	16.7	8.3	8.3
	25.0	23.0	24.2	67.1 ± 3.35c	35	15.6	5.2	10.4
	50.0	24.0[b]	4.17[d]	55.1 ± 2.75c	36	4.17[d]	4.17	–
MeOH	2.0	21.5	78.4	235.9 ± 1179a	33	33.3	11.1	22.2
	10.0	22.0	56.2	148.2 ± 7.41b	33	30.5	10.2	20.3
	25.0	23.5	29.5	124.3 ± 6.21b	34	20.8	10.4	10.4
	50.0	24.5[b]	18.2	119.2 ± 5.54b	34	16.7	–	16.7
n-hexane	2.0	22.0	65.7	141.2 ± 7.06b	33	20.8	13.9	6.9
	10.0	23.0	39.2	114.7 ± 5.73b	34	16.7	16.7	–
	25.0	24.0[b]	19.3	76.4 ± 3.82c	35	12.5	12.5	–
	50.0	25.0[b]	12.5	35.1 ± 1.75c	35	0.0	–	–

[a] Mean of three experiments. [b] Means within a column are significantly different from control in a Kruskal-Wallis chi-squared approximation test at $p < 0.005$. [c] 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). [d] These values correspond to one survival larva. [e] SP: Survival Pupation = Number of survival pupae × 100/Total larvae for pupation. [f]% = Number of adults emerged × 100/Total Number of pupae.

hexane extract were 6.59, 15.05, 10.78 and 12.79 ppm, respectively. In order to determine the site of inhibition on the insect growth regulatory activity (IGR) and the acute toxicity, the effect of flavones **1–4**, *ent*-clerodane **5** the methylester **6**, hexane and MeOH extracts, and gedunin on acetylcholinesterase activity was studied.

Inhibition of acetylcholinesterase

Inhibition of AChE was carried out according to the colorimetric method of Ellman *et al.* (1961), to investigated the mode of action of acute toxicity. Inhibitions of AChE of **1–6**, gedunin, MeOH and hexane extracts are outlined in Table VII. In similar form to the acute toxicity **5**, **6**, MeOH, hexane extracts and gedunin showed the greatest inhibitory effect with 90.2, 62.0, 93.7, 100 and 100% at 50.0 ppm, respectively; whereas, flavonoids **1–4** showed lower inhibitory effects by 35.9, 27.5, 25.9 and 17.8% at 50.0 ppm, respectively. The *ent*-clerodanes **5**, gedunin and hexane extract at minor concentrations (25.0 ppm) showed stronger activity level than MeOH extract and **6** (Fig. 2). However,

Table IV. Insect growth regulatory activity of the flavonols and diterpene from *G. microcephala* and authentic flavonoids, clerodane and gedunin against *S. frugiperda* larvae in a no-choice bioassay[a].

Treatment	7 Days			2 Days		Pupation
	GWI_{50}[b]	GLI_{50}[c]	MC_{50}[d]	EI_{50}[b]	pI_{50}[e]	PI_{50}[f]
1	9.7 (23.1)	5.28 (12.6)	3.9 (9.2)	0.55 (1.3)	0.26	3.46 (8.2)
2	13.5 (37.5)	27.3 (75.8)	n.d.	4.68 (13.0)	0.67	n.d.
3	330.3 (74.6)	8.14 (20.0)	27.8 (68.5)	4.46 (10.9)	0.64	n.d.
4	n.d.	n.d.	n.d.	6.91 (15.9)	0.84	n.d.
5	3.1 (9.8)	3.1 (9.8)	10.7 (33.8)	0.74 (2.3)	0.13	2.11 (6.6)
6	4.0 (12.1)	8.36 (25.3)	3.46 (10.5)	3.05 (9.2)	0.48	4.62 (14.0)
Gedunin	2.71 (5.6)	5.9 (12.3)	31.9 (66.2)	0.60 (1.2)	0.21	9.96 (20.7)
MeOH extrac	5.5	14.45	7.95	13.85	1.14	12.4
Hexane extrac	3.2	12.82	7.5	10.37	1.01	5.91

[a] The parameters in ppm values. In parenthesis the μM equivalence.
[b] The GWI_{50} and EI_{50} correspond to the growth inhibition in weight at 7 and 21 days, respectively, 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 4.1.
[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 the computer program ANOVA ($p < 0.05$) under Microcal Origin 4.1.
[d] MC_{50} is the concentration producing 50% mortality.
[e] pI_{50} correspond to $-\log EI_{50}$.
[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 4.1.

MeOH extract and **6** showed higher inhibitory activity than flavonoids **1–4**, above 25.0 ppm (Fig. 2). In addition, both extracts, gedunin and

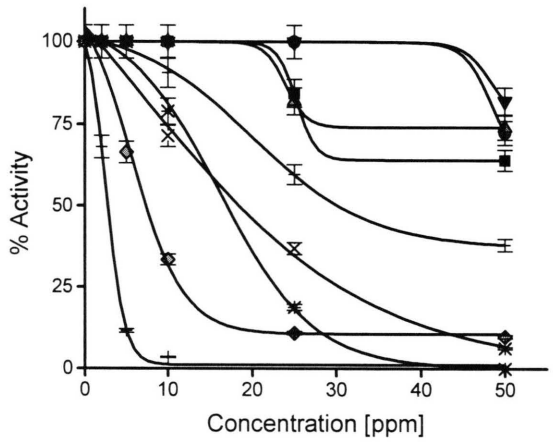

Fig. 2. Dose-dependent inhibition of acetylcholinesterase activity by major *G. microcephala* compounds, gedunin, hex and MeOH extracts. Each value represents mean ± S. E. ($n = 5$). The inhibition efficacy was expressed as a percentage of enzyme activity inhibited compared with the control value (100%) of compound **1** (■), **2** (●), 3 (▲), **4** (▼), **5** (◆), **6** (+), MeOH extract (×), *n*-hexane extract (⚹), and gedunin 7 (—). Acetylcholinesterase activity of the 100% control correspond to 34.55 μmol ATC split/mg protein × min.

ent-clerodanes **5** and **6** inhibited AChE activity in a dose-dependent manner (Fig. 2) confirming that *ent*-clerodanes compounds and extracts are the active inhibitors of acetylcholinesterase in *Gutierrezia microcephala*.

In addition, in clerodanes the presence or absence of a methyl ester group increases or decreases respectively the strength of these compounds on inhibition of AChE. We suggested that inhibitory activity of hexane extract be caused not by one strong inhibitor, but by a synergic effect. Inhibition of AChE activity by terpenoids has been reported on related insecticidal effects (Gracza, 1985). Therefore, the plant terpenes may be considered as AChE antagonists (Miyazawa, *et al.*, 1997; Keane and Ryan, 1999).

The bacchabolivic acid and its methyl ester showed to have more potent insecticidal inhibitory activity. It is obvious that the nature of the ester substituent at C-8 plays an important role for the insecticidal activity of the *ent*-clerodanes. The most active compound **5** contained a small and relatively hydrophilic acid group at C-8, whereas compound **6** with a bulky and more lipophilic ester group exhibited a little minor activity level. These results confirm previous findings on quantitative structure activity relationship of clerodanes deriv-

atives, namely that the antifeedant activity of the respective natural product depends on the polarity of ring B and on the size of the ester substituents (Rodríguez *et al.*, 1999).

Table V. GI and RGI of *S. frugiperda* as a function of increased concentrations of **1–6** and MeOH and *n*-hexane extracts from *G. microcephala* and gedunin[a].

Compounds	Concentration [ppm]	GI[b]	RGI[c]
Control		0.99 ± 0.045a	
1	2.0	0.99 ± 0.050b	1.00
	10.0	0.84 ± 0.085b	0.85
	25.0	0.75 ± 0.031b	0.75
	50.0	0.69 ± 0.055b	0.70
2	2.0	0.99 ± 0.050b	1.00
	10.0	0.84 ± 0.085b	0.85
	25.0	0.83 ± 0.055b	0.84
	50.0	0.80 ± 0.047b	0.81
3	2.0	0.99 ± 0.050b	1.00
	10.0	0.94 ± 0.040b	0.95
	25.0	0.92 ± 0.046b	0.93
	50.0	0.89 ± 0.044b	0.90
4	2.0	0.99 ± 0.050b	1.00
	10.0	0.94 ± 0.040b	0.95
	25.0	0.92 ± 0.046b	0.93
	50.0	0.89 ± 0.044b	0.90
5	2.0	0.59 ± 0.040b	0.60
	10.0	0.39 ± 0.065b	0.40
	25.0	0.25 ± 0.035c	0.25
	50.0	0.15 ± 0.038c	0.20
6	2.0	0.75 ± 0.031b	0.75
	10.0	0.59 ± 0.040b	0.60
	25.0	0.25 ± 0.035c	0.25
	50.0	0.15 ± 0.028c	0.15
MeOH extract	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
Hexane extract	5.0	0.25 ± 0.015c	0.25
	10.0	0.03 ± 0.015c	0.03
	25.0	0.00	0.00
	50.0	0.00	0.00
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] Mean of three replicates. [b] 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] $RGI_{treatment} = GI_{treated}/GI_{control}$.

With respect to the flavonoids with the presence of an extra methoxy substituent in the A ring seems to be the cause of growth inhibitory activities as shown by compounds **1** and **2** with a 20.1 and 26.9% of weight gained at 50.0 ppm, respectively; and 36.4 and 30.9% of length gained at 50.0 ppm, respectively (Table I), in similar form to polymethylated flavonoids from *Gnaphalium affine*, where the introduction of a methyl ether excluding the B-ring in the flavonoids structure increased the antifeedant activity (Morimoto *et al.*, 2000).

Table VI. Acute toxicity compounds **1–6**, gedunin and hex, MeOH extracts against larval of last stage of *S. frugiperda*[a].

Compounds	Concentration [ppm]	% Survival[b]	LD_{50}[c]
Control		0.0	100.0
1	2.0	95.1 ± 4.75	36.65
	10.0	78.1 ± 3.90	
	25.0	59.3 ± 2.96	
	50.0	45.0 ± 2.25	
2	2.0	98.0 ± 4.90	n.d.
	10.0	91.5 ± 4.55	
	25.0	85.3 ± 4.25	
	50.0	77.2 ± 3.85	
3	2.0	98.2 ± 4.90	n.d.
	10.0	90.0 ± 4.50	
	25.0	75.1 ± 3.75	
	50.0	62.3 ± 3.10	
4	2.0	99.0 ± 4.95	n.d.
	10.0	90.3 ± 4.50	
	25.0	79.1 ± 3.95	
	50.0	71.0 ± 3.55	
5	2.0	79.5 ± 3.80b	6.59
	10.0	41.2 ± 2.00b	
	25.0	15.9 ± 0.85b	
	50.0	9.50 ± 0.42c	
6	10.0	65.0 ± 3.25b	
	15.0	51.0 ± 2.55b	
	25.0	27.8 ± 1.39b	
	50.0	7.0 ± 0.35c	
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	
n-hexane extrc.	2.0	89.9 ± 4.49	12.79
	10.0	60.7 ± 3.03	
	25.0	27.3 ± 1.36	
	50.0	17.9 ± 0.89	
Gedunin	10.0	54.7 ± 2.73b	10.78
	25.0	14.1 ± 0.71c	
	50.0	0	

[a] After 24 hrs, 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.

These facts show that acute toxicity and growth inhibition observed may be due to the inhibition of acetylcholinesterase. Since, this target was dem-

onstrated also for the *neo*-clerodane teuscorolide which act as a feeding deterrent against Colorado Potato Beetle (*Leptinostarsa decemlineata*) larvae, whereas the antifeedant activity of teucrin-A, teucvin, and eriocephalin is likely associated with a toxic mode of action (Ortego *et al.*, 1995); but scutalpin-B, with a deterrent mode of action, did not have any significant effect on these enzymatic processes (Ortego *et al.*, 1999).

Table VII. Inhibitory Activity of compounds **1–6**, gedunin, *n*-hexane and MeOH extract against acetylcholinesterase activity[a].

Compounds	Concentration [ppm]	% Inhibition[c]
Control		[b]
1	2.0	[b]
	10.0	[b]
	25.0	15.5 ± 4.23
	50.0	35.9 ± 3.21
2	2.0	[b]
	10.0	[b]
	25.0	[b]
	50.0	27.5 ± 3.63
3	2.0	[b]
	10.0	[b]
	25.0	17.9 ± 0.89
	50.0	25.9 ± 1.29
4	2.0	[b]
	10.0	[b]
	25.0	[b]
	50.0	17.8 ± 4.11
5	2.0	[b]
	10.0	66.5 ± 1.68
	25.0	89.1 ± 0.55
	50.0	90.2 ± 0.49
6	2.0	[b]
	10.0	9.3 ± 4.54
	25.0	40.3 ± 2.98
		62.0 ± 1.90
	50.0	
MeOH extrac.	2.0	[b]
	10.0	28.5 ± 3.29
	25.0	63.1 ± 1.84
	50.0	93.7 ± 0.32
Hexane extrac.	2.0	[b]
	10.0	21.0 ± 3.95
	25.0	81.2 ± 0.94
	50.0	100
Gedunin	10.0	88.4 ± 0.58
	25.0	96.5 ± 0.17
	50.0	100

[a] After incubation for 20 min, changes in absorbance at 420 nm were recorded and compared with control. [b] No effect was observed. [c] Means of three replicates each value represent ± S. E. (n = 5).

In summary, the insecticidal activity of hexane extract from aerial parts of *G. microcephala* may be due to a synergic effects shown by the clerodanic components of the mixture in the test system used in this investigation. Comparison of insecticidal activities of clerodane-type compounds from Mexican *Salvia* species (Labiatae) *S. lineata*, *S. melissodora*, *S. keerlii*, *S. ryacophilla* showed its potent antifeedant activity against *Spodoptera littoralis* (Simmonds *et al.*, 1996), from this plants 1(10)-dehydrosalviarin was isolated (Esquivel *et al.*, 2000), which showed a potent antifeedant effect at 1.1 nmol/disk against the adult western corn root worm (*Diabrotica virgifera virgifera* Le Conte) (Mullin *et al.*, 1994). These facts are indicative of the potency of the hexane extract from *G. microcephala*.

Thus, the effect of the compounds **5**, **6**, MeOH and hexane extracts on reducing insect growth, increasing development time and mortality of *S. frugiperda* is similar to that of clerodane-type such as 1(10)-dehydrosalviarin and more potent than eriocephalin, teucrin-A, teuscorolide and teucvin (Ortego *et al.*, 1995). The mode of action of these compounds is being investigated and may be due to a combination of antifeedant action as midgut esterase inhibition and postdigestive toxicity, as found for other terpenoids (Champagne *et al.*, 1992; Nakatani, *et al.*, 1994) and extracts (Feng, *et al.*, 1995). In addition, the presence of a furanyl group seems to be important for these activities as showed for the most potent compounds in this study **5** and **6**. Furthermore, a great inanition observed may be due to the inhibition by acetylcholinesterase as well.

The activity of this desert plant and their metabolites and hexane and MeOH extracts is comparable to the insect growth regulator gedunin, which suggests potential for further development of these materials.

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