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BIOACTIVE FLAVONOIDS FROM *HELIANTHUS ANNUUS* CULTIVARS*

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Key Word Index—*Helianthus annuus*; Compositae; flavonoids; chalcones; flavanones; flavonol; heliannones A–C; allelopathy.

Abstract—Isolation, structure elucidation and allelopathic bioassay of five flavonoids (the flavonol tambulin, the chalcones kukulcanin B and heliannone A, and the flavanones heliannones B and C) from the sunflower cultivar VYP® are described. Heliannones A–C are reported in the literature as natural products, for the first time. The effects of a series of aqueous solutions at 10^{-4}–10^{-9} M of the flavonoids on the root and shoot length of *Lycopersicon esculentum* and *Hordeum vulgare* has been studied. They influence, principally, the shoot growth of seedlings, but germination and radical length can be affected by chalcones. The effects of very similar compounds, kukulcanin B and heliannone A, varied. The reason for this variation could be related to the number and position of free hydroxy groups.

INTRODUCTION

We are carrying out a systematic study of the different chemicals that may be involved in the allelopathic activity of *Helianthus annuus* cultivars. From medium polar active fractions we have isolated five flavonoids, three of which are new. Flavonoids have important and diverse effects across much of the biological spectrum: nectar-guide components in flowers [1], larval-growth inhibition [2], cytotoxicity [3, 4], phytoalexin [5, 6], effects on the oxidative properties of intact plant mitochondria [7, 8], effects upon the enzymatic destruction of indole-3-acetic (auxin) acid through 'IAA oxidasa' [9, 10].

In spite of the large number and wide distribution, only a few flavonoids have been implicated in allelopathy [11]. Recently, Inderjit and Dakshini have related ononin, hesperidin and taxifolin 3-arabinoside with the allelopathic activity of *Pluchea lanceolata* [12, 13]. Chalcones and dihydrochalcones are particularly interesting allelochemicals. 2′,6′-Dihydroxy-4′-methoxychalcone is inhibitory at low concentration (5×10^{-4}–5×10^{-6} M), but stimulatory at even lower concentration (5×10^{-7}–5×10^{-9} M) [14]. Ceratiolin is an inactive dihydrochalcone, but its photo-degradation product, hydrocinnamic acid, is a selective toxin for native Florida grasses and pines [15].

RESULTS AND DISCUSSION

The extract of the fresh leaf aqueous extract of *Helianthus annuus* L. var VYP® with dichloromethane was chromatographed by column chromatography on silica gel using hexane–ethyl acetate mixtures of increasing polarity. Medium polar fractions yielded five flavonoids: two chalcones, two flavanones and one flavonol (Fig. 1). Spectroscopic data of tambulin (**1**) [16], and kukulkanin B (**2**) [17] were identical to those previously reported.

Heliannone A (**3**) was isolated as a gum, *m/z* 300, which together with the ^{1}H NMR data was in agreement with the molecular formula $C_{17}H_{16}O_5$. Mass spectral and UV data were consistent with a methoxy chalcone structure. The ^{1}H NMR spectrum was also in good agreement with the assignments of Mabry *et al.* [18] for substituted chalcones and those reported for kukulkanin A and B. Compound **3** exhibited two methoxy singlets at δ 3.95 and 3.91 and two doublets at δ 7.85 and 7.45 ($J_{\alpha,\beta}$ = 15.5 Hz) corresponding to H-β and H-α, respectively. Peaks for H-2, H-6 and H-3, H-5 appeared as two doublets (2H) and δ 7.57 and 6.88 ($J_{2,3} = J_{5,6}$ = 9 Hz), indicating the presence of a single *para* substituent in ring A. The analysis of its mass fragmentation, with an important peak at *m/z*

* Part 9 in the series 'Allelopathic Studies in Cultivar Species'. For part 8 see ref. [24].

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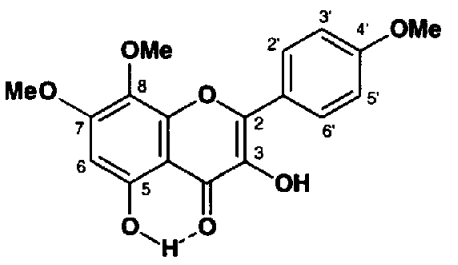

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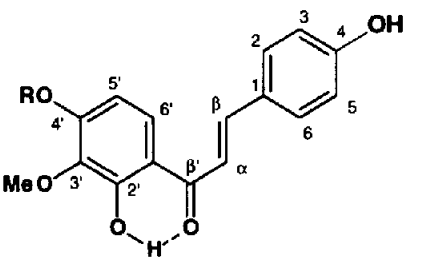

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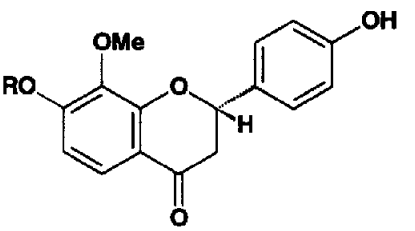
3 R=Me



4 R=Me
5 R=H

Fig. 1. Bioactive flavonoids from *Helianthus annuus* cultivars.

120 due to *p*-hydroxyphenylethene, clearly indicated that **3** had a *p*-hydroxy group in a ring A [19]. It was also found to give a bathochromic shift of 68 nm of band I in the UV spectrum on adding sodium methoxide (433 nm) and sodium acetate (371 and 425sh nm), indicating the presence of a free hydroxyl group at C-4.

Doublets assigned to H-6′ and H-5′ were located at δ 7.67 and 6.52 ($J_{5',6'}$ = 9 Hz), respectively, which indicate an adjacent tetrasubstitution in ring B. A 2,3,6-substitution pattern that would also produce two similar doublets for these two hydrogens was considered unlikely on the basis of both chemical shifts, which were very similar to those reported for **2**, and on biosynthetic grounds. In view of the presence of a signal at δ 13.2 (s, C_2-O<u>H</u>) and the bathochromic shift of band I by 61 nm observed with aluminium chloride that is a clear indication of the presence of a hydroxyl group at C-2′, the methoxyl groups were placed at C-3′ and C-4′. The ^{13}C NMR data were in good agreement with those reported for similar compounds [20].

Heliannones B (**4**) and C (**5**) were isolated as a gum. Mass spectra were very similar in the low mass region, differing in molecular ion values that were 300 for heliannone B and 286 for heliannone C, agreeing with molecular formulae of $C_{17}H_{16}O_5$ and $C_{16}H_{14}O_5$, respectively. Mass spectral and UV data (282 and 324 nm for **4**; 275 and 325 nm for **5**) were consistent with flavanone structures.

^{1}H NMR spectra of **4** and **5** were analogous to those obtained for **3** and **2**, respectively. Compound **4** exhibited two methoxy singlets at δ 3.92 and 3.85, while one methoxy at δ 3.87 was found in **5**. Instead of signals for H-α and H-β, their spectra contained signals corresponding to protons H-2β and H-3β. Peaks for H-2′, H-6′, H-3′ and H-5′ appeared as two doublets (2H) at δ 7.36 and 6.86 for **4** and at δ 7.35 and 6.88 for **5**, again indicating the presence of a single *para* substituent in ring B.

Doublets attributed to H-5 and H-6 were located at δ 7.65 and 6.65 ($J_{5,6}$ = 8.5 Hz) in **4** and δ 7.64 and 6.67 ($J_{5,6}$ = 8.5 Hz) in **5**, indicating adjacent tetrasubstitution on ring A. Compound **5** was found to give a bathochromic shift of 61 nm of band II in the UV spectrum on adding sodium methoxide and sodium acetate, indicating the presence of hydroxyl group at C-7. Compound **4** isomerized to chalcone on adding sodium methoxide, having a band I peak at 433 nm. The addition of sodium acetate did not provoke any shift in UV spectrum that evidenced that there was no free hydroxyl group at C-7. A 7,8-substitution pattern was chosen for these compounds on the bases of the concordance between the reported chemical shift for analogous compounds [21, 22] and those for **4** and **5**, and UV spectra.

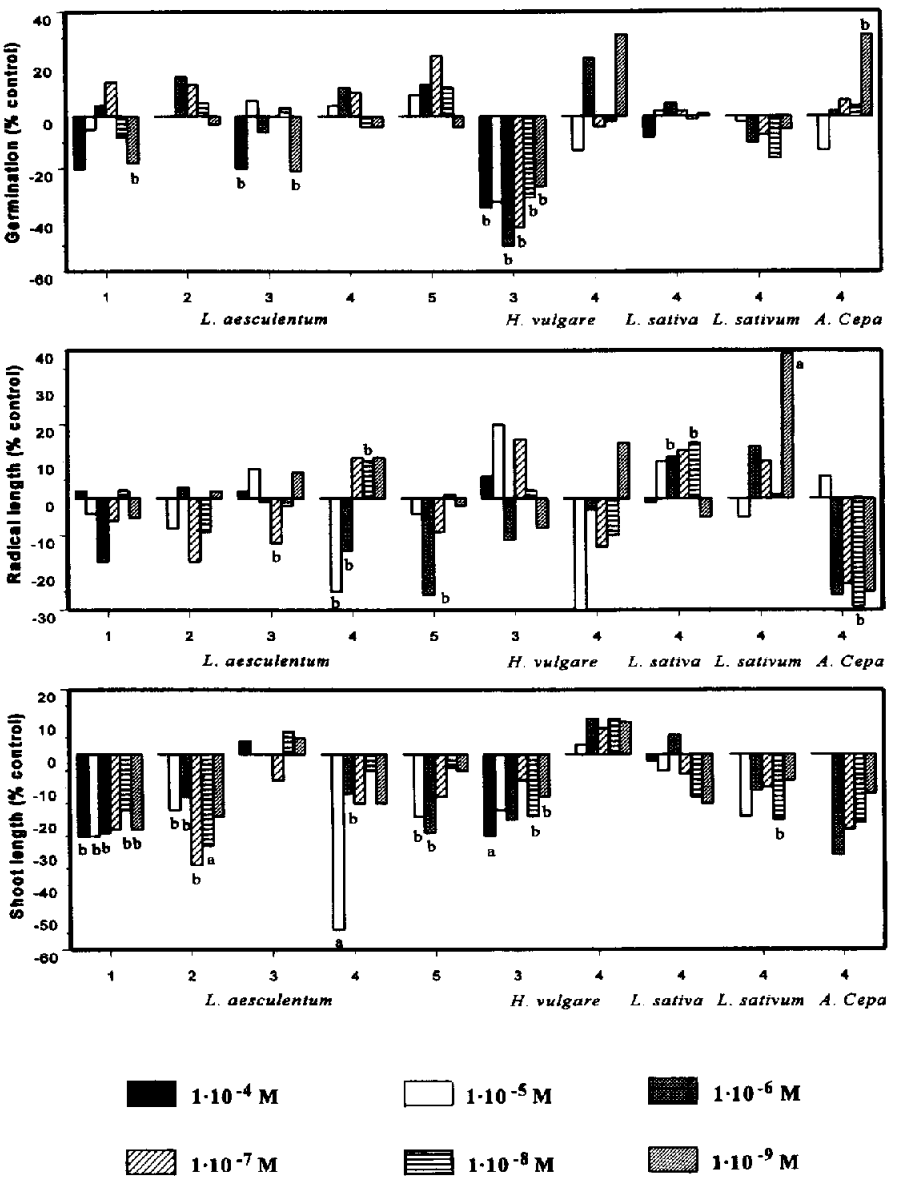


Fig. 2. Selected effects of compounds **1**–**5** on the germination, radical and shoot length of *L. esculentum*, *H. vulgare*, *L. sativa*, *L. sativum* and *A. cepa*. Values are expressed as percentage from the control and are not significantly different with $P > 0.05$ for Mann–Whitney's test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

The structures of **4** and **5** (Fig. 2) were also confirmed from their mass fragmentation, with major peaks corresponding to M^+, $[M-1]^+$, ring A and ring B. The presence of an important peak at *m/z* 120 again indicated that both compounds, **4** and **5**, had *p*-hydroxyl groups in rings B.

Thus heliannone A (**3**), B (**4**) and C (**5**) have been characterized as 2′,4-dihydroxy-3′,4′-dimethoxychalcone, 4′-hydroxy-7,8-dimethoxyflavanone and 7,4′-dihydroxy-8-methoxyflavanone, respectively, and this is the first report in the literature of these natural products. The equilibrium between chalcones and flavanones justifies the presence of the pairs of compounds **2**, **5** and **3**, **4**. This fact could explain the low optical activity observed for the flavanones **4** and **5**, that contain a chiral centre in their structures. Thus, the orientations assigned above for hydrogens attached at C-2 and C-3 are relative, so they do not indicate absolute configurations.

Bioassay results

In order to evaluate the potential allelopathic activity and to obtain information about the specific requirements needed for bioactivity, we have studied the effects of a series of aqueous solutions at 10^{-4}–10^{-9} M of isolated compounds on root and shoot

length of *Lycopersicon esculentum* and *Hordeum vulgare* seedlings (Fig. 2).

The flavonol **1** did not show any effect against germination and radical length of both species, tomato and barley, but inhibited shoot growth of tomato (average −25%) and barley (−22%, 10^{-5} M) (Fig. 2). We can point out that the effect on the dicotyledonous species was maintained with the dilution, with significance $P < 0.05$.

Chalcones **2** and **3** have very similar structures. They differ in the presence of an additional methyl group in **3**; however their activities are quite different. Compound **3** inhibited germination of tomato (−20%, 10^{-4} M) and barely (average −35%) and affected sightly barley shoot growth with an inhibitory profile of activity. On the other hand, **2** only showed inhibitory activity on the shoot growth of tomato (−17%, 10^{-5} M; −13%, 10^{-6} M; −34%, 10^{-7} M; −28%, 10^{-8} M) (Fig. 2).

Flavanones **4** and **5** showed a similar profile of activity, although the activities produced by compound **4** solutions are, in general, higher than those produced by **5**. Compounds **4** and **5** showed inhibitory effects on the shoot (**4**, −54%, 10^{-5} M; **5**, −19%, 10^{-5} M; −24%, 10^{-6} M) and radical length (**4**, −25%, 10^{-5} M; −14%, 10^{-6} M; **5**, −26%, 10^{-6} M) of tomato. The observed effects of **4** and **5** over barley are of small significance. Based on the important value of inhibition over shoot length of tomato, we carried out bioassays of **4** against other species: lettuce, cress and onion. Compound **4** generally had no effect on germination and growth of these species, except stimulatory effect on the radical length of cress (+39%, 10^{-9} M) and onion germination (+31%, 10^{-9} M) in low concentration, as well as inhibitory profile on the radical and shoot length of onion (Fig. 2).

We conclude that flavonoids isolated from *Helianthus annuus*, possibly, play roles in the allelopathic activity of sunflower in addition to those played by other compounds previously reported [23, 24]. They influence, principally, the shoot growth of seedlings, but germination and radical length can be affected by chalcones. It may be noted that the effects of very similar compounds (**2** and **3**) varied. The reason for this variation could be related to the number and position of free hydroxy groups. Another important point is that the observed effects varied with the seed tested. Tomato seeds were found to be more sensitive than the other crop species. This may be due to differences in seed size, seed coat permeability, differential uptake and metabolism [25].

EXPERIMENTAL

Plant material. Leaves of *Helianthus annuus* L. var. VYP[®] commercialized by KOYPE™ (Spain) were collected in September 1990 during the third plant development stage (plants 1.2 m tall with flowers, 1 month before harvest) and were provided by Rancho de la Merced, Agricultural Research Station, Junta de Andalucía, Jerez, Spain.

Extraction and isolation. Fresh leaves (1.7 kg) were soaked in H_2O (wt plant.: solvent volume 1:3) for 24 hr at 25° in the dark. The H_2O extracts were extracted (8×) with 1.0 l of CH_2Cl_2 for each 1.2 l of H_2O and the combined extracts were dried over Na_2SO_4 and evapd *in vacuo* to yield 24 g of crude extract which was sepd by CC on silica gel using *n*-hexane–EtOAc mixts of increasing polarity yielding 192 × 50 ml frs which were reduced to 30 frs after comparison by TLC.

By following the bioactivity exhibited by the low polar frs on *L. sativa* and *Ho. vulgare*. Medium polar frs were chromatographed using silica gel (with N_2 pressure) and eluting with hexane–Me_2CO (7:3) and hexane–Me_2CO (6:4) and rechromatographed using HPLC with a Hibar Si60 (Merck) column, hexane–Me_2CO (6:4) as eluent, with 3 ml min^{-1} flow, yielding **1** (4 mg), **2** (6 mg), **3** (5 mg), **4** (5 mg) and **5** (3 mg).

Heliannone A (**3**): $C_{17}H_{16}O_5$, IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3430 (OH); 1645 (C=O). UV λ_{max} nm (MeOH) 303sh, 365; ($AlCl_3$) 249sh, 345sh, 426; ($AlCl_3$/HCl) 249sh, 342, 408; (NaOMe) 278sh, 433; (NaOAc) 297, 371, 425sh; (NaOAc/H_3BO_3) 298, 366. EIMS (70 eV) *m/z* (rel. int.): 300 $[M]^+$ (38); 181 $[(CH_3O)_2(O)C_6H_2COH]^+$ (21); 180 $[(CH_3O)_2(O)C_6H_2CO]^+$ (61); 152 $[(CH_3O)_2(O)C_6H_2]^+$ (10); 147 $[(HO)C_6H_4C_2H_3CO]^+$ (5); 120 $[(HO)C_6H_4C_2H_3]^+$ (31). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (*d*, $J_{\alpha,\beta}$ = 15.5 Hz; H-β), 7.67 (*d*, $J_{5',6'}$ = 9 Hz; H-6′), 7.57 (2H, *d*, $J_{2,3} = J_{5,6}$ = 9 Hz; H-2, H-6), 7.45 (*d*, $J_{\alpha,\beta}$ = 15.5 Hz; H-α), 6.88 (2H, *d*, $J_{2,3} = J_{5,6}$ = 9 Hz; H-3, H-5), 6.52 (*d*, $J_{5',6'}$ = 9 Hz; H-5′), 3.95 (3H, *s*, C$\underline{H}_3$O) and 3.91 (3H, *s*, C$\underline{H}_3$O). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 194.3 (*s*, C-β′), 164.5 (*s*, C-4′), 161.5 (*s*, C-2′), 158.2 (*d*, C-4), 144.6 (*d*, C-β), 133.4 (*d*, C-6), 131.2 (*d*, C-2), 126.6 (*s*, C-1), 122.3 (*s*, C-1′), 119.4 (*d*, C-α), 116.7 (*d*, C-3 and C-5), 109.1 (*s*, C-3′), 105.5 (*d*, C-5′), 101.3 (*d*, C-6′) and 60.2 (*s*, C_3-OCH_3 and C_4-OCH_3).

Heliannone B (**4**): $C_{17}H_{16}O_5$, $[\alpha]_D^{25}$ +11.2° ($CHCl_3$; *c* 0.1). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3420 (OH); 1650 (C=O). UV λ_{max} nm (MeOH) 282, 324sh; ($AlCl_3$) 281, 325sh; ($AlCl_3$/HCl) 281, 325sh; (NaOMe) 282sh, 433; (NaOAc) 281, 324sh; (NaOAc/H_3BO_3) 282, 324sh. EIMS (70 eV) *m/z* (rel. int.): 300 $[M]^+$ (82); 299 $[M-1]^+$ (7); 181 $[(CH_3O)_2(O)C_6H_2COH]^+$ (18); 180 $[(CH_3O)_2(O)C_6H_2CO]^+$ (78); 151 $[(CH_3O)_2C_6H_2OC_2H_2OH]^+$ (50); 120 $[(HOC_6H_4C_2H_3]^+$ (30). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.65 (*d*, $J_{5,6}$ = 8.6 Hz; H-5), 7.36 (2H, *d*, $J_{2',3'} = J_{5',6'}$ = 8.5 Hz; H-2′, H-6′), 6.86 (2H, *d*, $J_{2',3'} = J_{5',6'}$ = 8.5 Hz; H-3′, H-5′), 6.65 (*d*, $J_{5,6}$ = 8.6 Hz; H-6), 5.44 (*dd*, $J_{2\beta,3\beta}$ = 3.2, $J_{2\beta,3\alpha}$ = 12.4 Hz; H-2β), 3.92 (3H, *s*, C$\underline{H}_3$O) 3.85 (3H, *s*, C$\underline{H}_3$O), 3.03 (*dd*, $J_{3\alpha,3\beta}$ = 17, $J_{2\beta,3\alpha}$ = 12.4 Hz; H-3α) and 2.85 (*dd*, $J_{3\alpha,3\beta}$ = 17, $J_{2\beta,3\beta}$ = 3.2 Hz; H-3β).

Heliannone C (**5**): $C_{16}H_{14}O_5$, $[\alpha]_D^{25}$ +1.2° ($CHCl_3$, *c* 0.1). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3420 (OH); 1652 (C=O). UV λ_{max} nm (MeOH) 275, 325; ($AlCl_3$) 275, 329sh; ($AlCl_3$/HCl) 274, 328sh; (NaOMe) 334; (NaOAc) 336;

($NaOAc/H_3BO_3$) 272, 327sh. EIMS (70 eV) *m/z* (rel. int.): 286 $[M]^+$ (14); 181 $[(CH_3O)_2(O)C_6H_2COH]^+$ (51); 167 $[(HO)(CH_3O)(O)C_6H_2COH]^+$ (66); 166 $[(HO)(CH_3O)(O)C_6H_2CO]^+$ (78); 137 $[(HO)(CH_3O)C_6H_2OC_3H_2OH]^+$ (51); 120 $[(HO)C_6H_4C_2H_3]^+$ (27). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.64 (*d*, $J_{5,6} = 8.6$ Hz; H-5), 7.35 (2H, *d*, $J_{2',3'} = J_{5',6'} = 8.5$ Hz; H-2′, H-6′), 6.88 (2H, *d*, $J_{2',3'} = J_{5',6'} = 8.5$ Hz; H-3′, H-5′), 6.67 (*d*, $J_{5,6} = 8.6$ Hz; H-6), 5.44 (*dd*, $J_{2\beta,3\beta} = 3.2$, $J_{2\beta,3\alpha} = 13.2$ Hz; H-2β), 3.87 (3H, *s*, $C\underline{H}_3O$), 3.03 (*dd*, $J_{3\alpha,3\beta} = 16.7$, $J_{2\beta,3\alpha} = 13.2$ Hz; H-3α) and 2.83 (*dd*, $J_{3\alpha,3\beta} = 16.7$, $J_{2\beta,3\beta} = 3.2$ Hz; H-3β).

Lettuce, cress, tomato, onion and barley seed germination bioassay. Seeds of *Lactuca sativa* var. *nigra*, and *H. vulgare* were obtained from Rancho La Merced, Junta de Andalucía, Jerez, Spain. Seeds of *Lepidium sativum*, *Allium cepa* and *Lycopersicon esculentum* were obtained from FITÓ S.L. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity. Bioassays consisted of germinating 25 seeds for 5 days (3 for germination and 2 for root and shoot growth) for lettuce and onion, 3 days (1 for germination and 2 for root and shoot growth) for cress, 4.5 days (3 for germination and 1.5 for root and shoot growth) for tomato, and 5 barley seeds for 4 days in the dark at 25° in 9 cm plastic Petri dishes containing a 10 cm sheet of Whatman no. 1 filter paper and 10 ml of a test of control soln, except for barley (5 ml). Test solns (10^{-4} M) were prepd as initial solution. Test solns (10^{-5}–10^{-9} M) were obtained by diluting the previous soln. Parallel controls consisted of deionized H_2O. There were three replicates, except for barley (19 replicates), of each treatment, and parallel controls. The number of seeds per replicate, time and temp of germination were chosen in agreement with a number of preliminary experiments, varying the number of seeds, vol. to test soln per dish and the incubation period. All the pH values were adjusted to 6.0 before the bioassay using MES (2-[*N*-morpholino]ethanesulfonic acid, 10 mM).

Statistical treatment. The germination, root and shoot length values were tested by Mann–Whitney's test being the differences between the experiment and the control, significant with a value of $P = 0.01$.

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REFERENCES

1. Harborne, J. B. and Smith, D. M., *Biochemistry Systematics and Ecology*, 1978, **6**, 287.
2. Ellinger, C. A., Chan, B. C., and Waiss, A. C. Jr, *Naturwissenschaften*, 1980, **67**, 358.
3. Wang, Z. Q., Kuo, Y. H., Schnur, D., Bowen, J. P., Liu, S. Y., Han, F. S., Chemg, Y. C. and Lee, R. H., *Journal of Medical Chemistry*, 1990, **33**, 2660.
4. Cushman, M. and Nagarathnam, D., *Journal of Natural Products*, 1991, **51**, 1656.
5. Martin, S. S., *Physiology of Plant Pathology*, 1977, **11**, 297.
6. Ingham, J. L., *Biochemistry Systematics and Ecology*, 1978, **7**, 29.
7. Ravanel, P., Tissut, M. and Douce, R., *Phytochemistry*, 1981, **20**, 2101.
8. Ravanel, P., Creuzet, S. and Tissut, M. *Phytochemistry*, 1990, **29**, 441.
9. Stenlid, G., *Phytochemistry*, 1976, **15**, 911.
10. Lee, T. T., Starratt, A. N. and Jevnikar, J. J., *Phytochemistry*, 1982, **21**, 517.
11. Rice, E. L., *Allelopathy*, 2nd edn, Academic Press, Orlando, FL, 1984.
12. Inderjit and Dakshini, K. M. M., *Journal of Chemical Ecology*, 1991, **17**, 1585.
13. Inderjit and Dakshini, K. M. M., *Journal of Chemical Ecology*, 1992, **18**, 713.
14. Star, A. E., *Bulletin of the Torrey Botanical Club*, 1980, **107**, 146.
15. Fischer, N. H., Williamson, B., Weidenhamer, J. D. and Richardson, D. R., *Journal of Chemical Ecology*, 1994, **20**, 1355.
16. Chatterjee, D., Malakar, D. and Ganguly, D., *Indian Journal of Chemistry*, 1976, **14B**, 233.
17. Domínguez, X. A., Garcia, S., Williams, H. J., Ortiz, C., Scott, I. and Reibenspies, J. H., *Journal of Natural Products*, 1989, **52**, 864.
18. Mabry, T. J., Markham, K. L. and Thomas, M. B., *The Systematic Identification of Flavonoids*, Springer, New York.
19. Mabry, T. J. and Markham, K. R., in *The Flavonoids*, ed. J. B. Harborne, T. J. Mabry and H. Mabry, Chapman and Hall, London, 1975.
20. Agrawal, P. K., in *Carbon-13 NMR of Flavonoids*, ed. P. K. Agrawal, Elsevier, New York, 1989, p. 380.
21. Kemp, M. S., Burden, R. S. and Brown, C., *Phytochemistry*, 1979, **18**, 1765.
22. Garg, S. P., Bhushan, R. and Kapoor, R. C., *Phytochemistry*, 1980, **19**, 1265.
23. Macías, F. A., Varela, R. M., Torres, A. and Molinillo, J. M. G., in *Allelopathy: Procedures and Processes*, ed. K. M. M. Dakshini and Inderjit, Springer, Berlin (in press).
24. Macías, F. A., Molinillo, J. M. G., Torres, A. and Varela, R. M., *Phytochemistry* (in press).
25. Williams, R. D. and Hoagland, R. E., *Weed Science*, 1982, **30**, 206.