Pergamon

PII: S0031-9422(98)00110-1

Phytochemistry, Vol. 49, No. 2, pp. 441–449, 1998

Printed in Great Britain
0031-9422/98/$–see front matter

PHYTOTOXIC COMPOUNDS FROM *ESENBECKIA YAXHOOB***†

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

RACHEL MATA,* MARTHA L. MACÍAS, IRMA S. ROJAS, BLAS LOTINA-HENNSEN,*†
RUBEN ALFREDO TOSCANO‡ and ANA LUISA ANAYA§

Departamento de Farmacia, Facultad de Química and Unidad de Investigación de Plantas Medicinales del Instituto de Química; † Departamento de Bioquímica, Facultad de Química; ‡ Instituto de Química; § Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Coyoacán 04510, México D.F., México

(*Received* 23 *September* 1997; *in revised form* 28 *January* 1998)

Key Word Index—*Esenbeckia yaxhoob*; Rutaceae; imperatorin; (24*S*)-24-methyl-dammara-20,25-diene-3β-yl-acetate; 2-tridecanone; 2-pentadecanone; 6,10,14-trimethyl-2-pentadecanone; lupeol; asarinin; *Amaranthus hypochondriacus*; *Echinochloa crusgalli*; *Lactuca sativa*; *Lycopersicum esculentum*; phytogrowth-inhibitory activity; uncoupler; Hill reaction inhibitor; photosynthesis.

Abstract—Investigation of the aerial parts of *Esenbeckia yaxhoob* Lundell (Rutaceae) led to the isolation of a new dammarane-type of triterpene which was characterized by spectral means as (24*S*)-24-methyl-dammara-20,25-diene-3β-yl-acetate. In addition, 2-tridecanone, asarinin, imperatorin, lupeol and hesperidin were obtained. (24*S*)-24-Methyl-dammara-20,25-diene-3β-yl-acetate, 2-tridecanone, asarinin and imperatorin caused significant inhibition of the radicle growth and/or germination of seedlings of *Amaranthus hypochondriacus*, *Echinochloa crusgalli*, *Lactuca sativa* and *Lycopersicum esculentum*. Also, it has been found that imperatorin inhibited ATP synthesis and both phosphorylating and uncoupled electron flow from H_2O to $K_3[Fe(CN)_6]$. On the other hand, this coumarin stimulated the basal electron flow from H_2O to $K_3[Fe(CN)_6]$ and the activity of the light-activated Mg^{2+}-ATPase. These effects were measured in freshly lysed illuminated spinach chloroplasts and allowed to determine that imperatorin acts as an uncoupler and as a Hill reaction inhibitor.

INTRODUCTION

Esenbeckia yaxhoob Lundell (Rutaceae) is a medicinal tree, which grows in different plant communities along the Peninsula of Yucatán in Southeast México, mainly in the subperennial tropical forest. The people of Yucatán refer to the plant as "tankas-ché" and the decoction prepared from the aerial parts of the plant is employed for treating gastrointestinal ailments. To the best of our knowledge, this plant has not been the subject of phytochemical analysis, but the genus *Esenbeckia* has yielded a wide array of limonoids [1, 2], quinolinic and indole alkaloids [1–6, 9], coumarins [1, 3, 6, 7], polyprenols [8], phenylpropanoids [6], lignans [3], triterpenoids [10], phloroglucinol derivatives [8, 11] and flavonoids [12].

In the course of our continuing search for plant-growth inhibiting agents from Mexican plants, we describe in this investigation the isolation and characterization of the phytotoxic principles from the aerial parts of *E. yaxhoob*. In addition, as part of our systematic investigation on the effect of potential allelopathic agents in the process of photosynthesis *in vitro*, we report the activity of imperatorin (**4**), isolated from *E. yaxhoob*, on synthesis of ATP, electron flow and light-activated Mg^{2+}-ATPase in isolated spinach chloroplasts [13–17, *inter alia*].

RESULTS AND DISCUSSION

Bioactivity guided isolation and characterization of (24S)-24-methyl-dammara-20,25-diene-3β-yl-acetate, 2-tridecanone, asarinin, imperatorin, lupeol and hesperidin

The aerial parts of *E. yaxhoob* were extracted with $CHCl_3$–MeOH (1:1). The initial phytotoxic activity

* Authors to whom correspondence should be addressed. Fax No: (525) 6225329; e-mail: rachel@servidor.unam.mx

** Taken in part from the MS theses of M. Macías and S. Rojas.

† Dedicated to Professor Neil Towers on the occasion of his 75th birthday.

Table 1. Effect of the $CHCl_3$–MeOH 1:1 extract, essential oil and isolates from *Esenbeckia yaxhoob* on seed germination

Compound	Concentration μg/ml	% of germination inhibition†			
		A. hypochondriacus	*E. crusgalli*	*L. sativa*	*L. esculentum*
Extract	10	1.7±1.4	11.7±0.2	10.2±0.5	5.7±0.4
	100	21.8±1.0	31.8±1.1	38.1±1.5*	27.3±1.2*
	1000	35.2±0.2*	43.2±0.3*	80.2±0.8*	84.2±1.0*
Essential oil	10	8.0±0.9	20.0±1.4*	21.5±0.3	23.4±0.4
	100	15.0±0.7	33.5±0.3*	65.2±1.0*	49.5±0.1*
	1000	22.5±0.5	88.3±0.9*	86.5±1.1*	82.5±0.8*
1	10	6.3±0.0	5.0±0.5	47.5±0.8*	17.5±0.5
	100	11.2±1.1	11.8±0.8	48.1±0.3*	33.7±1.0*
	1000	13.7±0.5	13.8±0.2	56.3±0.5*	100.0±0.0*
2	10	5.0±0.6	13.7±0.4*	33.7±0.5*	28.0±1.3*
	100	5.0±0.7	50.2±0.7*	58.5±0.1*	39.5±0.8*
	1000	12.5±0.2	70.5±1.1*	81.5±0.9*	81.3±0.9*
3	10	17.8±0.1	13.7±0.1	28.7±1.2*	63.7±0.5*
	100	20.0±0.1	24.7±0.1*	57.5±0.8*	77.5±0.9*
	1000	22.3±0.1	37.5±0.1*	78.7±0.5*	100.0±0.0*
4	10	2.7±0.8	8.7±0.5	20.0±0.9	10.8±0.6
	100	10.0±1.1	8.7±0.9	61.2±1.3*	77.3±0.5*
	1000	10.0±0.5	16.2±0.8	100±0.0*	100.0±0.0*
Control	0	1.5±1.0	1.8±0.5	25.0±0.5	4.7±0.2
2,4-D**	1	25.0±0.8*	92.0±0.8*	47.5±0.5*	8.5±0.5*

† Each value represents the means ± SD of 4 replicates, 20 seedlings per replicate.
** Positive standard control (2,4-diclorophenoxi acetic acid).
* $P \leq 0.05$, Anova, with Duncan's multiple-range test.

of the resulting extract was evaluated on germination and radicle elongation of seedlings of *Amaranthus hypochondriacus* L., *Echinochloa crusgalli* (L.) Beauv., *Lactuca sativa* L. and *Lycopersicum esculentum* Mill. using the Petri dish bioassay (PDPIB) [15]. Tables 1 and 2 summarize the phytotoxic activity of the extract.

The active extract was fractionated by column chromatography over Si gel to yield six primary fractions (FI-FVI). The bioautographic phytogrowth inhibitory bioassay (PBIB) [16, 17] was used at each step for activity-directed fractionation. Extensive chromatography of the phytotoxic frs FI, FII and FIV (see Experimental) allowed the isolation of the new triterpene (24*S*-methyl-dammara-20,25-diene-3β-yl-acetate (**1**), 2-tridecanone (**2**), asarinin (**3**) [18] and imperatorin (**4**) [19, 20]. In addition, lupeol (**5**) [21] and hesperidin (**6**) were isolated from the inactive frs. The spectral properties of compounds **4** and **5**, including IR, ^1H NMR and ^{13}C NMR data, were identical to those previously described in the literature [19–21]. The identification of ketone **2** and flavonoid **6** was performed by comparison with authentic samples.

In the case of compound **3**, the structure was unequivocally assigned to that of asarinin [18] by X-ray crystallography. The molecular structure of **3** is illustrated in Fig. 1 showing the atom numbering used in the X-ray crystal structure determination. Both five-membered rings in the bicyclic structure are *cis* fused and adopt a nearly perfect envelope [22] conformation with the oxygen atoms O-3 and O-7 as a flip and with opposite flipping directions. The planar methylenedioxyphenyl moiety attached to carbon atoms C-2 and C-6 occupy the most stable equatorial position in the bicycle skeleton and again are directed on opposite sides of the molecule.

Compound **1** was found to have the molecular formula $C_{33}H_{54}O_2$ by a combination of mass spectrometry and ^{13}C NMR spectroscopy. Its IR spectrum had absorptions characteristic of ester (1726 and 1210 cm^{-1}) and terminal olefinic (1642 and 824 cm^{-1}) functionalities. The NMR data (see Experimental) was in agreement with a triterpene-type of compound. The spectra contained signals for an acetate (δ_H 1.75; δ_C 21.4 and 171.0), six methyl groups attached to quaternary carbon (δ_H 1.62, 0.94, 0.90, 0.89, 0.86, and 0.75; δ_C 28.0, 18.9, 16.5, 16.3, 15.9, and 15.6), one secondary methyl group (δ_H 1.02, *d*, $J = 6.5$ Hz; δ_C 19.8), two terminal double bonds [δ_H 4.96 (H-21) and 4.92 (H-21′), each *d*, $J = 0.5$ Hz; δ_C 153.3 (C-20) and 107.0 (C-21); δ_H 4.83 (H-26) and 4.81 (H-26′), each *d*, $J = 0.5$ Hz; δ_C 150.0 (C-25) and 109.5 (C-26)], one oxymethine [δ_H 4.68 (*dd*, $J = 13$, 5 Hz, H-3; δ_C 80.9 (C-3)], ten methylenes, five methines and four quaternary carbons. Three of the implied seven degrees of unsaturation, were explained by multiple bonds, two by carbon-carbon double bonds and one by carbon-oxygen double bond, consequently **1** was a tetracyclic triterpene. Furthermore, closer examination of the ^{13}C NMR data revealed that **1** was a dammarane-type triterpene [23, 24, *inter alia*] possessing an extra methyl

Table 2. Effect of the $CHCl_3$–MeOH 1:1 extract, essential oil and isolates from *Esenbeckia yaxhoob* on radicle growth

Compound	Concentration μg/ml	% of radicle growth inhibition†			
		A. hypochondriacus	*E. crusgalli*	*L. sativa*	*L. esculentum*
Extract	10	1.2±0.4	+16.9±1.0	+3.2±1.4	+4.9±0.4
	100	32.1±0.7	30.5±1.1*	14.3±1.4	11.6±0.1
	1000	49.2±0.5*	74.9±0.8*	58.3±0.8*	82.1±0.8*
Essential oil	10	+25.5±1.2	15.7±1.4	+10.4±1.2	19.5±0.4
	100	5.8±1.8	21.8±1.4	+27.5±0.6	38.8±2.1*
	1000	49.8±1.2*	29.2±0.8*	+38.1±1.2	89.4±1.8*
1	10	2.6±1.2	+20.1±1.3	+4.5±1.1	23.3±2.4
	100	6.7±2.2	8.5±2.8	1.2±2.1	30.5±1.8*
	1000	21.4±1.74	27.5±1.9*	4.9±2.5	100±0.0*
2	10	+18.5±2.2	13.3±1.5	+6.4±1.1	28.6±2.4*
	100	2.7±1.74	15.6±0.4	+20.5±1.6	34.8±1.1*
	1000	44.1±1.2*	25.2±0.5*	+23.1±0.9	76.9±2.8*
3	10	8.2±0.4	25.1±0.7*	+20.8±3.2	18.5±2.4
	100	22.1±0.8	26.5±2.4*	+29.8±2.6	40.6±0.4*
	1000	36.2±0.8*	30.8±1.5*	+47.0±2.4	100.0±0.0*
4	10	+17.5±1.74	19.7±0.3	+88.6±1.8	20.1±0.9
	100	25.7±2.2*	53.1±1.7*	+114.6±1.0	75.6±0.9*
	1000	42.1±1.2*	66.1±0.6*	100.0±0.0*	100.0±0.0*
Control	0	0.0±1.9	0.0±0.1	0.0±1.4	0.0±1.3
2,4-D**	1	89.1±1.7*	71.7±1.2*	63.2±2.1*	23.3±2.4*

† Each value represents the means ± SD of 4 replicates, 20 seedlings per replicate.
** Positive standard control (2,4-diclorophenoxi acetic acid).
* $P \leq 0.05$, Anova, with Duncan's multiple-range test.
+ Stimulatory effect.

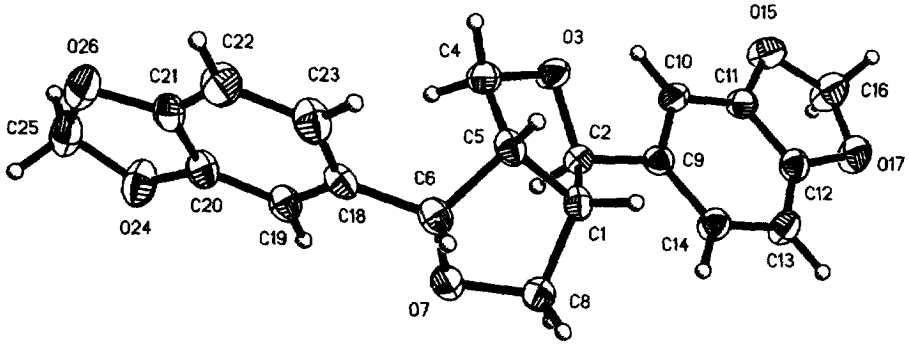


Fig. 1. Stereoscopic view of compound **3**.

group at the side chain. The presence of intense fragment ions at *m/z* 150, 122, 121 and 81 in the mass spectrum of **1** further substantiated the presence of an additional methyl group along the side chain of the triterpenoid.

The dammarane-type of skeleton of **1** was confirmed by the detailed analysis of the HMBC spectrum (Fig. 2), which showed the correlations H-19/C-5, H-28/C-5, H-29/C-5, H-18/C-9, H-19/C-9, and H-30/C-13. The structure of the side chain and the placement of the acetoxy functionality at C-3 were also supported by the HMBC spectrum. Thus, allocation of the $\Delta^{20(21)}$ double bond was based on the correlation observed between both H-21 (δ_H 4.96) and H-21′ (δ_H

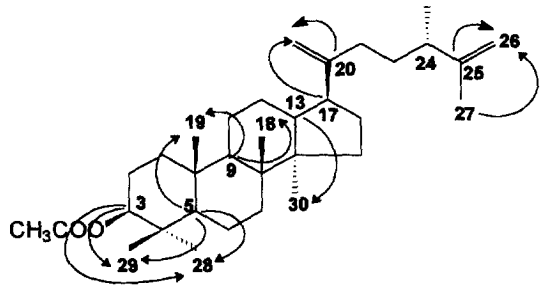


Fig. 2. Key HMBC correlations of compound **1**.

4.92) with C-17 and C-20. On the other hand, the cross peaks observed between the vinylic protons at δ_H 4.83 and 4.81 (H-26 and H-26′) and the carbon signals at δ_C 150.0 (C-25) and 18.9 (C-27) were consistent with the placement of the extra methyl group at C-24. Finally, the disposition of the acetoxy grouping at C-3 was in agreement with the correlations H-29/C-3 and H-28/C-3.

The β configuration of the acetoxy functionality was supported by the large coupling constant ($J = 13$ Hz) between H-3α (δ_H 4.68) and H-2β, which suggested the *trans*-diaxial relationship of both hydrogens. The stereochemical assignment at the stereogenic center C-24 was inferred from the chemical shift value observed for C-31 (δ_C 19.8) in the ^{13}C NMR spectrum. This chemical shift was very similar to that previously described for the methyl group at C-24 of (24*S*)-24-methyl-lanosta-9(11),25-diene-3-one, which possesses a side chain similar to that of **1** [25]. On the basis of the previous discussion, compound **1** was characterized as (24*S*)-24-methyl-dammara-20,25-diene-3β-yl-acetate.

The essential oil obtained by water and steam distillation of the aerial parts also significantly inhibited the radicle growth and germination of the target species (Tables 1 and 2). GC-MS analysis of the essential oil revealed the presence of ketone **2** (84%), 2-pentadecanone (**7**) (14%) and 6,10,14-trimethyl-2-pentadecanone (**8**) (2%). Identification of **2** was made by comparison of the GC mobilities, by study of mass fragmentation and by co-injection with **2** isolated during the course of this study from the $CHCl_3$–MeOH 1:1 extract. Identification of **7** and **8** was made by comparison of their mass spectra with those of Public/NIST library.

Phytogrowth-inhibitory activity of the phytochemicals

Natural products **1**–**6** were evaluated for their ability to inhibit the seed germination and radicle growth of *A. hypochondriacus*, *E. crusgalli*, *L. sativa*, and *L. esculentum*. Tables 1 and 2 summarize the phytotoxic effect of the isolates at three different concentrations. The tested compounds showed different activity profiles and in general were less potent as growth inhibitors than as germination inhibitors. Compounds **2**, **3** and **4** were the most active as germination inhibitors, with *L. sativa* and *L. esculentum* being the most sensitive species. The activity of lignan **3** against *L. esculentum* was comparable to that of the positive control (2,4-D). The 50% inhibitory concentration values (IC_{50}) were 2.1 μg/ml and 3.55 μg/ml, respectively. 2-Tridecanone **2** also inhibited the germination of *E. crusgalli*. In this case the IC_{50} was 3.55 μg/ml. On the other hand, compounds **1**, **2** and **4** were the most potent as radicle growth inhibitors, with *L. esculentum* being the most sensitive species. The three natural products inhibited the germination of *L. esculentum* in a concentration dependent-manner, with higher activity at increasing concentration. The IC_{50} values were 61.6, 79.4 and 39.8 μg/ml, respectively. Coumarin **4** also inhibited the radicle growth of *E. crusgalli*, ($IC_{50} = 144.9$ μg/ml) and *L. sativa*. The inhibitory effect of **4** on the latter species was only observed at the highest concentration tested. The activities displayed by compound **4** on germination and radicle growth of *L. sativa* were similar to that previously described by Macías and coworkers [26]. Finally, compounds **5** and **6** were inactive as both germination and radicle growth inhibitors.

It has been described that 2-tridecanone **2** possesses insecticidal properties against some *Lepidoptera* larvae and aphids [27]. However, to the best of our knowledge its phytotoxic effect has not been previously described; therefore, this compound represents a new structural type of phytotoxin.

Effect of imperatorin on isolated spinach chloroplasts

As with other natural coumarins [28, 29], imperatorin completely inhibited the photosynthetic phosphorylation from water to methylviologen in a concentration-dependent manner, reducing it by 100% at 175 μM (Fig. 2). The IC_{50} was 71.5 ± 1.3 μM. Such an inhibition of ATP synthesis can be explained by three different mechanisms; namely, blockage of the electron transport, inhibition of the phosphorylation reaction itself and uncoupling of ATP synthesis from the electron transport. To investigate if **4** was acting at any of these levels, the effect of various concentrations of this coumarin on non-cyclic electron transport from H_2O to $K_3[Fe(CN)_6]$ was evaluated. Photosynthetic non-cyclic electron transport rates were determined under three different experimental conditions: basal, phosphorylating and uncoupled. The results are shown in Fig. 3. Imperatorin (**4**) stimulated basal electron transport by 430% at 25 μM; however, at higher concentrations the activation decreased, being equal to the control at 125 μM. On the other hand, **4** inhibited phosphorylating electron transport by 26.6% and 7.1%, at 25 and 50μM, respectively. At 75 μM this electron flow was stimulated by 34%. Finally, the uncoupled electron transport rate was only inhibited by 51.6% when compound **4** was added to the assay system at a concentration of 25 μM. At higher concentrations, however, no significant inhibitory effect was observed. These data suggest that coumarin **4** behaves as an uncoupler agent and as a weak Hill reaction inhibitor. The uncoupler activity displayed by compound **4** may be due to an activation of the enzyme Mg^{2+}-ATPase, as do most of the classical uncoupler agents. To test this hypothesis, the effect of imperatorin (**4**) on the enzyme Mg^{2+}-ATPase during the hydrolysis of ATP was studied. The results of this determination are summarized in Table 3. The data indicated that H^+-ATPase activity was activated by 614% when the compound concentrations were increased from 0 to 50 μM. These observations confirm that coumarin **4** acts as an uncoupler agent. The behavior of compound **4** on Mg^{2+}-ATPase is similar

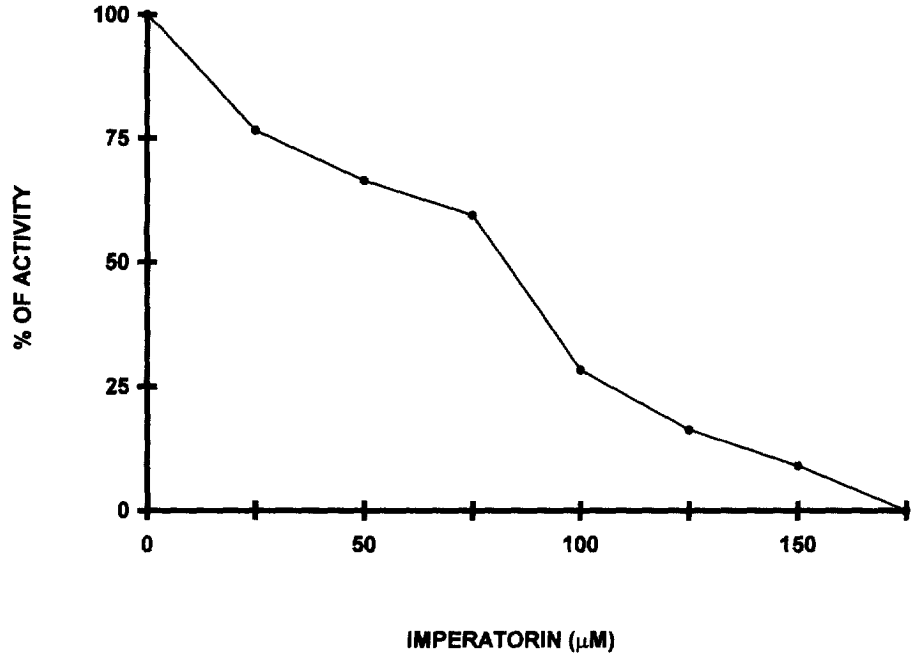


Fig. 3. Inhibitory effect of imperatorin **4** on photophosphorylation, from water to methylviologen. Each cuvette contained 20 μg of chlorophyll per ml in the reaction medium. Other conditions were described in the Experimental. Control value rate was 780 ± 2.3 μMol of ATP h^{-1} mg of chl^{-1}. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts.

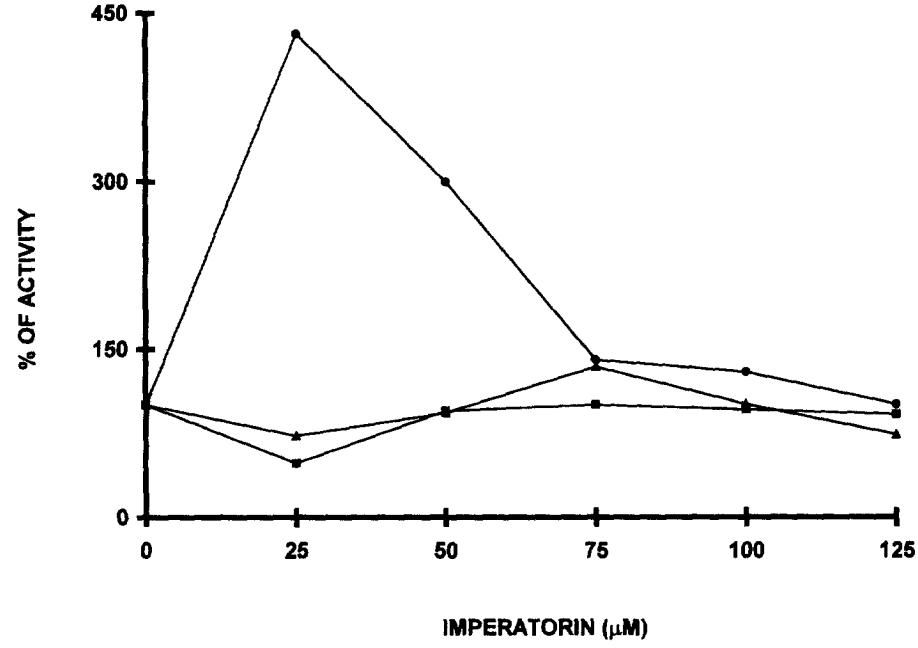
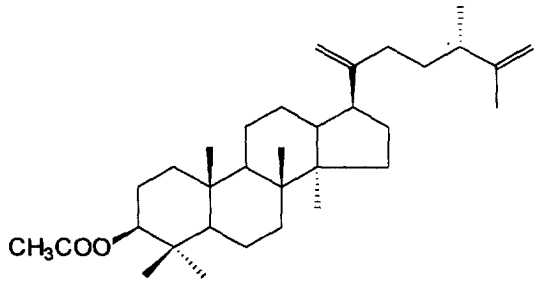
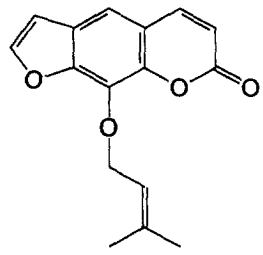


Fig. 4. Non-cyclic electron transport from water to $K_3[Fe(CN)_6]$ as a function of imperatorin **4** concentration in chloroplasts thylakoids isolated from spinach leaves (*Spinaceae oleraceae* L.). Each cuvette contained 20 μg of chlorophyll per ml in the reaction medium. Other conditions are described in the Experimental. Control value rates for basal (●), phosphorylating (▲), and uncoupled (■) electron transport are 292.3 ± 3.2, 438.9 ± 3.1, and 864.8 ± 2.5, respectively, in μeq e^- h^{-1} mg of chl^{-1}. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts.



1



4

Table 3. Effect of imperatorin 4 on ATpase activity. Control value rate was 38 μMol of Pi h^{-1} mg of chl^{-1}. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts

Compound	Concentration	% of activity
Imperatorin 4	0 μM	100±2.1
	25 μM	334.2±1.8*
	50 μM	614.2±1.9*
NH_4Cl	1.1 mM	100±1.5

* $P \leqslant 0.05$, Anova, with Duncan's multiple-range test.

to that found for the classical uncouplers agents such as NH_4Cl, carbonylcyanide *m*-chlorophenylhydrazone and some 4-phenylcoumarins [28–31]. It is important to point out that the potency of imperatorin 4 to activate the Mg^{2+}-ATpase was higher than that of NH_4Cl (Table 2), *p*-trifluoromethoxy(carbonylcyanide)phenyl hydrazone and other coumarins [28–31].

CONCLUSIONS

The results from the present investigation indicated that *E. yaxhoob* contains phytogrowth-inhibitors that could be involved in the allelophatic interactions of the species. The interference of compound 4 with energetic metabolism at the level of photosynthesis as an uncoupler agent and as a weak Hill reaction inhibitor might be partially responsible for its phytogrowth-inhibitory properties and its possible role as an allelopathic agent.

EXPERIMENTAL

General

IR spectra were obtained in KBr on a Perkin-Elmer 599 B spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on Varian VXR-300 S or Varian VXR-500 S spectrometers. Melting points were determined in a Fisher Johns apparatus and are uncorr. Optical rotations were taken on a digital polarimeter JASCO Dip 360. GC-MS analyses were conducted on a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Jeol JMS AX50HA mass spectrometer. The GC column was PAS-1701 tested silicone (25 m × 0.32 mm id). The linear temperature programming was from 150–260°C at a rate of 10° min^{-1}; the carrier gas was He (0.6 psi, 2 ml min^{-1}). The total duration of analysis was 30 min per injection. EI mass spectra were obtained using ionization voltage of 70 eV. Semi-preparative HPLC was performed on a Porasil silica gel column (10 μm, 3.9 id × 300 mm, Waters) at a flow rate of 7 ml min^{-1}. The effluent was monitored with a UV detector at 214 nm. Analytical and preparative TLC were performed on pre-coated silica gel 60 F254 plates (Merck). TLC spots were visualized

by spraying with a 10% solution of $Ce(SO_4)_2$ in 2N H_2SO_4, followed by heating at 110°C. For open CC, silica gel 60 (70–230 mesh, Merck) was used. A Beckman model DU 650 spectrophotometer was used to carry out the spectrophotometric measurements in the photosynthesis experiments.

Plant material

The aerial parts of *E. yaxhoob* were collected in Quintana Roo, México, in February 1995. A voucher specimen (ALA95-8) has been deposited in the National Herbarium (MEXU), Instituto de Biología, UNAM.

Isolation

The air-dried plant material (1.8 kg) was ground into powder and extracted exhaustively by maceration at room temp. with $CHCl_3$–MeOH (1:1). After filtration, the extract was concentrated *in vacuo* to yield 123 g of residue. The active extract was subjected to column chromatography (CC) over Si-gel (1.15 kg) and eluted with a gradient of hexane/$CHCl_3$/MeOH. One hundred and thirty one frs (1 l each) were collected and pooled on the basis of their TLC profiles to yield six major frs (FI–FVI). Bioactivity in the BPIB showed three active pools: FI (2.43 g), FII (1.55 g), and FIV (13.85 g). FI, eluted with hexane/$CHCl_3$ (6:4), was subjected to prep HPLC (see General) to yield **2** (2.04 g, R_t = 7.6 min). From active fr. FII, eluted with hexane/$CHCl_3$ (4:6), crystallized (82 mg) compound **1**. Active fr. FIV (13 g, eluted with $CHCl_3$) was further resolved on a silica gel column (270 g), eluting with a concn gradient of hexane/$CHCl_3$/MeOH, starting with hexane, to afford six secondary frs (FIV-A–FIv-F). According to the BPIB, the phytotoxic activity was concd in a secondary fr. FIV-D; prep. TLC [$CHCl_3$–MeOH (99:1), two developments] of this fr. yielded **3** (81 mg), mp 122–123°C (lit. mp = 121–122°C [32]), and **4** (100 mg), mp 99–100°C (lit. mp = 102°C [31]). Finally, from the inactive frs-FIII and FVI spontaneously crystallized **5** (177 mg), mp 213–215°C (lit. mp = 215–216°C [32]) and **6** (577 mg), mp 256–261°C (lit. mp = 258–262°C [32]), respectively.

GC-MS analysis of the essential oil

The essential oil (50 mg) was obtained from 100 g of the dried plant material by steam distillation. A portion of the oil was directly subjected to GC-MS analysis. 2-tridecanone (**2**). 84%; R_t = 7.60 min; EIMS *m/z* (rel. int.): 198 [M^+ (14)], 183 (5), 155 (4), 140 (12), 138 (10), 85 (15), 71 (35), 58 (100), 43 (55). 2-pentadecanone (**7**). 14%; R_t = 18.18; EIMS *m/z* (rel. int.): 226 [M^+ (15)], 211 (7), 208 (5), 168 (10), 166 (8), 96 (10), 85 (12), 71 (35), 58 (100), 43 (48). 6,10,14-trimethyl-2-pentadecanone (**8**). 2%; R_t = 11.47; EIMS *m/z* (rel. int.): 268 [M^+ (4)], 250 (28), 210 (13), 165 (13), 137 (12), 124 (30), 109 (38), 95 (30), 85 (35), 71 (60), 58 (100), 43 (90).

(24*S*)-24-*methyl-dammara*-20,25-*diene*-3*β*-*yl-acetate* (**1**). Crystalline needles, mp 135–138°C ($CHCl_3$). IR ν_{max} (KBr) cm^{-1}: 1726, 1642, 1252 and 884. ^1H NMR, δ (500 MHz, benzene-d_6): 4.68 (1H, *dd*, *J* = 13, 5 Hz, H-3), 0.94 (3H, *s*, Me-18), 0.75 (3H, *s*, Me-19), 4.96 (1H, *d*, *J* = 0.5 Hz, H-21), 4.92 (1H, *d*, *J* = 0.5 Hz, H-21′), 4.83 (1H, *d*, *J* = 0.5 Hz, H-26), 4.81 (1H, *d*, *J* = 0.5 Hz, H-26′), 1.60 (3H, *s*, Me-27), 0.89 (3H, *s*, Me-28), 0.90 (3H, *s*, Me-29), 0.86 (3H, *s*, Me-30), 1.02 (3H, *d*, *J* = 6.5 Hz, Me-24′). ^{13}C NMR, δ (75 MHz, $CDCl_3$): 38.8 (C-1), 23.7 (C-2), 80.9 (C-3), 37.9 (C-4), 55.9 (C-5), 18.2 (C-6), 35.3 (C-7), 41.0 (C-8), 50.8 (C-9), 37.1 (C-10), 21.4 (C-11), 25.0 (C-12), 45.4 (C-13), 49.4 (C-14), 31.3 (C-15), 29.2 (C-16), 47.5 (C-17), 15.6 (C-18), 16.3 (C-19), 153.3 (C-20), 107.0 (C-21), 32.4 (C-22), 33.7 (C-23), 40.4 (C-24), 150.0 (C-25), 109.5 (C-26), 18.9 (C-27), 28.0 (C-28), 16.5 (C-29), 15.9 (C-30), 19.8 (C-24′), 21.4 ($\underline{C}H_3COO$), 171.0 ($CH_3\underline{C}OO$).

X-ray structure determination of asarinin (**3**). Crystal data: $C_{20}H_{18}O_6$, M_r = 354.3, monoclinic, *a* = 9.620(1), *b* = 5.6234(4), *c* = 15.670(1) Å, β + 103.70(1)°, *V* = 823.7(1) $Å^{-3}$, *Z* = 2, D_{calc} = 1.429 g cm^{-3}, μ(Cu-K_α) = 0.882 mm^{-1}. Space group $P2_1$, from systematic absences: *h*00 when *h* = 2n + 1. Sample dimensions: 0.70 × 0.20 × 0.08 mm. Refined unit cell parameters were derived by least-squares treatment of the diffractometer setting angles of 25 reflections widely separated in reciprocal space. Intensity data (*h*: 0–10, *k*: 0–6, *l*: −16–16, plus Friedel pairs) were recorded on a Siemens P4/Pc diffractometer (graphite-monochromated Cu-K_α radiation λ = 1.54178 Å, ω-scans) with variable scan speed (min. 4.0, max. 60.0° min^{-1}) and scan width 1.00°. From a total 2577 reflections measured, those 2498 reflections with $F > 4.0\sigma(F)$ were retained for the structure analysis and the usual Lorentz and polarization corrections were applied. The crystal structure was solved by direct methods and polarization corrections were applied. The crystal structure was solved by direct methods (SIR92) [33]. The E-map shows all non-hydrogen atoms of the structure and the hydrogen atoms were included at calculated positions. Anisotropic full-matrix least-squares refinement [34] for non-hydrogen atoms with the inclusion of the hydrogen atoms in a ride-on fashion with a fixed isotropic *U* value (0.08 $Å^2$) converged at *R* = 0.042 (*wR* = 0.02). The absolute configuration determination was done by refinement of the Rogers *η*-parameter (η = 1.45(5)) [35]. Non-hydrogen atom positional parameters, bond lengths and angles' anisotropic thermal parameters and hydrogen atom parameters have been deposited at the Cambridge Crystallographic Data Center. Neutral atom scattering factors used in the structure-factor calculation were taken from lit. [36].

Phytogrowth-inhibitory bioassays

The phytogrowth-inhibitory activity of the extract, essential oil and pure compounds was evaluated on

seeds of *Amaranthus hipochondriacus* L., *Echinochloa crusgalli* (L.) Beauv., *Lactuca sativa* L. and *Lycopersicum esculentum* Mill. by using the PDPIB method [15]. In addition, a direct bioautographic bioassay system was employed to guide secondary fractionation and speed up the isolation of active compounds. The data were analyzed by ANOVA ($P < 0.05$). The extract and the essential oil were evaluated at 50, 100 and 200 $\mu g\ ml^{-1}$. The pure compounds were tested at 10, 100 and 1000 $\mu g\ ml^{-1}$. 2,4-D was used as the positive control. The bioassays were performed at 28°C.

Chloroplasts isolation and chlorophyll determination

Chloroplasts thylakoids were isolated from market spinach leaves (*Spinaceae oleracea* L.) as previously described [35, 36] and suspended, unless indicated, in 400 mM sucrose, 5 mM, $MgCl_2$, 20 mM KCl and buffered with 0.03 M Na^+-tricine at pH 8.0. The chlorophyll concentration was measured spectrophotometrically as previously reported [39].

Measurement of ATP synthesis

ATP synthesis was measured as the pH value increase between 8.0 and 8.1 [40], using a combination microelectrode connected to a Coring 12 potentiometer with expanded scale. The pH changes were registered using a Gilson recorder. The reaction medium was 100 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 1 mM ADP, 3 mM KH_2PO_4, 1.0 mM Na^+-tricine and 50 μM methylviologen, at pH 8.0. Methylviologen was added as electron acceptor.

Measurement of non-cyclic electron transport

Photosynthetic noncyclic electron transport activity from water to potassium ferricyanide was determined through reduction of $K_3[Fe(CN)_6]$ which was added as electron acceptor. Reduced $K_3[Fe(CN)_6]$ was measured spectrophotometrically at 420 nm. Basal electron transport was recorded using an equivalent of 60 μg of chlorophyll per 3 ml of medium; the intact chloroplasts were incubated and lysed in 3.0 ml of basal medium (100 mM sorbitol, 5 mM, $MgCl_2$, 10 mM KCl, 1.0 mM Na^+-tricine and 267 μM $K_3[Fe(CN)_6]$ at pH 8.0). Phosphorylating electron transport was measured in the same conditions except that 1 mM ADP and 3 mM KH_2PO_4 were added to the medium. Uncoupled electron transport was tested in the basal medium with the presence of 6.0 mM NH_4Cl. All reaction mixtures were illuminated with actinic light of a projector lamp (GAF 2660) passed through a 5 cm filter of a 1% $CuSO_4$ solution. In each case, a blank experiment was performed with the chloroplasts alone in the reaction medium [40–42].

Mg^{2+}-ATPase isolation and assay

Isolated chloroplasts [31–32] were resuspended in a medium containing 350 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$, at pH 7.6. Mg^{2+}-ATPase activity was measured by the technique reported by Mills *et al.* [43], and released inorganic phosphate was measured as reported by Sumner [44].

Acknowledgements—This work was supported by grants: IN205197 from DGAPA, UNAM, 400313-5-2576 PM and 2358 PN from CONACyT. We wish to thank the following people: M. en C. Isabel Chávez, M. en C. Beatriz Quiroz, Q. Luis Velasco-Ibarra, M. en C. Javier Pérez Flores, M. en C. Rubén Gaviño, M. en C. Héctor R. and QFB Rocio Patiño (Instituto de Química, UNAM) for recording NMR, Mass and UV spectra; Q. Marisela Gutiérrez and Graciela Chávez for registration of IR spectra; QFB. Laura Acevedo Arteaga and Beatriz King Díaz for technical assistance; and Ing. Erwin Castillo Santamaría and Biol. José T. Vivas Cortés (Instituto de Química, UNAM) for their assistance with the computer work. M. Macias and I. Rojas acknowledge the fellowship awarded by CONACyT to carry out graduate studies.

REFERENCES

1. Dreyer, D., *Phytochemistry*, 1980, **19**, 941.
2. Bevalot, F., Fournet, A., Moretti, C. and Vaquete, J., *Planta Medica*, 1984, **50**, 522.
3. Delle, F., Delle, G., De Moraes e Souza, M., Da Salete, M. and Chiappeta, A., *Gazzetta Chimica Italiana*, 1989, **119**, 435.
4. Nakatsu, T., Johns, T. and Kubo, I., *Journal of Natural Products*, 1990, **53**, 1508.
5. Ríos, M. and Delgado, G., *Journal of Natural Products*, 1992, **55**, 1307.
6. Guilhon, G., Baetas, A., Maia, J. and Conserva, L., *Phytochemistry*, 1994, **37**, 1193.
7. Oliveira, M., Santana, G., Conserva, M., Maia, S. and Guilhon, P., *Phytochemistry*, 1996, **44**, 647.
8. Ríos, M. and Delgado, G., *Journal of Natural Products*, 1992, **31**, 3491.
9. Delle, F., Benedetto, R., De Moraes e Souza, M. and Sandor, P., *Gazzetta Chimica Italiana*, 1990, **120**, 387.
10. Dreyer, D., Pickering, M. and Cohan, P., *Phytochemistry*, 1972, **11**, 705.
11. Rios, M. Y. and Delgado, G., *Phytochemistry*, 1992, **31**, 3491-34-94.
12. Kubo, Y., Vieira, P. C. and Fukuhara, K., *Journal of Liquid Chromatography*, 1990, **13**, 2441–2448.
13. Segura-Correa, R., Mata, R., Anaya, A. L., Hernández, B. E., Villena, R., Soriano-García, M., Bye, R. and Linares, E., *Journal of Natural Products*, 1993, **56**, 1567.
14. Calera, M. R., Soto, F., Sánchez, P., Bye, R., Hernández, B., Anaya, A., Lotina-Hennsen, B. and Mata, R., *Phytochemistry*, 1995, **40**, 419.

15. Castañeda, P., García, M., Hernández, B., Torres, B., Anaya, A. and Mata, R., *Journal of Chemical Ecology*, 1992, **18**, 1025.
16. Jiménez, A., Mata, R., Lotina-Hennsen, B., Anaya, A. and Velasco, I., *Journal of Natural Products*, 1996, **59**, 202–204.
17. Castañeda, P., Gómez, L., Mata, R., Lotina-Hennsen, B., Anaya, A. and Bye, R., *Journal of Natural Products*, 1996, **59**, 323–326.
18. Pelter, A., Ward, R., Rao, E. and Sastry, K., *Tetrahedron*, 1976, **32**, 2783–2788.
19. González, A., López Dorta, H., Martín-Hierro, I., Medina, M. and Rodríguez-Luis, F., *Anales de Química*, 1982, **78**, 274.
20. Breitmaier, E. and Voelter, W., *^{13}C NMR Spectroscopy of Organic Compounds. High Resolution Methods and Applications in Organic Chemistry and Biochemistry*. 3rd edn. VCH, Holland, 1994, p. 447.
21. Mahato, S. and Kundu, A., *Phytochemistry*, 1994, **37**, 1517.
22. Cremer, D. and Pople, J., *Journal of American Chemical Society*, 1975, **97**, 1354.
23. Tori, M., Marsuda, R., Sono, M. and Asaka, Y., *Magnetic Resonance in Chemistry*, 1988, **26**, 581.
24. Fattorusso, E., Santacroce, C. and Xaasan, S., *Phytochemistry*, 1985, **24**, 1035.
25. Bates, R., Caldera, S. and Suvannachut, K., *Journal of Natural Products*, 1990, **53**, 209.
26. Macías, F., Galindo, J., Massanet, G., Rodríguez-Luis, F. and Zubía, E., *Journal of Chemical Ecology*, 1993, **19**, 1371.
27. Dimock, M., Kennedy, G. and Williams, W., *Journal of Chemical Ecology*, 1982, **8**, 837.
28. Calera, M. R., Mata, R., Anaya, A. L. and Lotina-Hennsen, B., *Photosynthesis Research*, 1995, **45**, 105.
29. Calera, M. R., Lotina-Hennsen, B., Mata, R. and Anaya, A. L., *Journal of Agricultural and Food Chemistry*, 1995, **44**, 2966.
30. Antony, R. C., *Biochemistry Biophysical Acta*, 1966, **24**, 725.
31. Heytler, P. G. and Prichard, W. W., *Biochemical and Biophysical Research Communications*, 1962, **7**, 272.
32. *Dictionary of Natural Products*, Vols. 3 and 5. pp. 3279, 3698, 5124 and 5811.
33. Altomare, A., Cascarano, G., Giacovazzo, C., Guagliardi, A., Burla, M., Polidori and Camalli, M., *Journal of Applied Crystallography*, 1994, **27**, 435.
34. Sheldrick, G., *SHELXTL/PC User's Manual*. Siemens Analytical X-rays Instruments, Inc., Madison, WI, U.S.A., 1990.
35. Rogers, D., *Acta Crystallographica*, 1981, **A37**, 734.
36. *International Tables for X-ray Crystallography*, Vol. 4. The Kynoch Press, Birmingham, U.K., 1974.
37. Lotina-Hennsen, B., Bernal-Morales, E. L., Jímenez, M. and Aguilar, M., *Z. Naturforsch*, 1991, **46C**, 777.
38. Peña-Valdivia, C. B., Rodríguez-Flores, L., Tuena de Gómez-Puyou, M. and Lotina-Hennsen, B., *Biophysical Chemical*, 1991, **41**, 169.
39. Strain, H. H., Coppe, B. T. and Svec, W. A., *Methods in Enzymology*, 1971, **23**, 452.
40. Dilley, R. A., *Methods in Enzymology*, 1972, **24**, 68.
41. Izawa, S., in *Encyclopedia of Plant Physiology*, ed. A. Trebst and M. Avron, M., 1980, pp. 266–282.
42. Trebst, A., *Methods in Enzymology*, 1972, **24**, 146.
43. Mills, J. D., Mitchell, P. and Schurmann, P., *FEBS Letters*, 1980, **112**, 173.
44. Sumer, S., *Science*, 1974, **100**, 413.