

## Investigación

Bioactive Terpenoids from Roots and Leaves of *Jatropha gaumeri*

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**Abstract.** The methanolic extracts of roots and leaves of *Jatropha gaumeri* showed antimicrobial and antioxidant activity, respectively. The bioassay-guided purification of the root extract resulted in the isolation and identification of 2-*epi*-jatrogrossidione (**1**), a rhamnofolane diterpene with antimicrobial activity, and of 15-*epi*-4*E*-jatrogrossidentadione (**2**), a lathyrane diterpene without biological activity. Similarly, the bioassay-guided purification of the crude leaf extract allowed the identification of  $\beta$ -sitosterol and the triterpenes  $\alpha$ -amyrin,  $\beta$ -amyrin and taraxasterol, as the metabolites responsible for the antioxidant activity. We wish to report here the isolation of the metabolites mentioned above and the complete assignment, not reported to date, of the <sup>13</sup>C NMR signals for **1**.

**Key words:** Antimicrobial activity, antioxidant activity, *Jatropha gaumeri*, Euphorbiaceae, *Bacillus subtilis*, 2-*epi*-jatrogrossidione, 15-*epi*-4*E*-jatrogrossidentadione, rhamnofolane, lathyrane,  $\beta$ -sitosterol,  $\alpha$ -amyrin,  $\beta$ -amyrin, taraxasterol.

**Resumen.** Los extractos metanólicos de la raíz y hojas de *Jatropha gaumeri* mostraron actividad antimicrobiana y antioxidante, respectivamente. La purificación biodirigida del extracto de la raíz resultó en el aislamiento e identificación de 2-*epi*-jatrogrossidione (**1**), un diterpeno tipo rhamnofolano con actividad antimicrobiana contra *B. subtilis*, y de 15-*epi*-4*E*-jatrogrossidentadione (**2**), un diterpeno tipo lathyrano sin actividad en este ensayo. De manera similar, la purificación biodirigida del extracto crudo de las hojas permitió identificar al  $\beta$ -sitosterol y a los triterpenos  $\alpha$ -amirina,  $\beta$ -amirina y taraxasterol como los responsables de la actividad antioxidante. En este trabajo se reporta el aislamiento de los productos mencionados y la asignación completa, no reportada hasta ahora, de las señales de RMN-<sup>13</sup>C de **1**.

**Palabras clave:** Actividad antimicrobiana, actividad antioxidante, *Jatropha gaumeri*, Euphorbiaceae, *Bacillus subtilis*, 2-*epi*-jatrogrossidione, 15-*epi*-4*E*-jatrogrossidentadione, rhamnofolano, lathyrano,  $\beta$ -sitosterol,  $\alpha$ -amirina,  $\beta$ -amirina, taraxasterol.

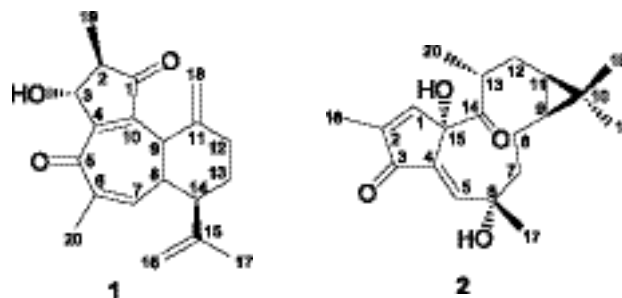
## Introduction

Various medicinal properties have been attributed to plant species of the large genus *Jatropha* (Euphorbiaceae). For instance, the fresh latex of many plants belonging to this genus is used in folk medicine for the treatment of mouth blisters [1], pimples [2], and scabies [3]; similarly, leaf infusions are used to treat ulcers [4], infected wounds [5], and diarrhea [6]; finally, both the leaves and seeds of some *Jatropha* spp. are used as laxatives [7, 8]. Phytochemically, the genus *Jatropha* is recognized as an important source of numerous structural classes of secondary metabolites, including alkaloids [9], diterpenes [10-12], lignans [13], triterpenes [14], and cyclic peptides [15]. A number of biological activities have been detected in natural products from *Jatropha* spp., these include antimicrobial [16] and both antitumor and cytotoxic [17], and tumor-promoting [18] activities.

One of the most frequently used plants in Yucatecan traditional medicine is *Jatropha gaumeri* Greenm., a tree commonly known in Mayan language as "pomolché". The plant grows at sea level in the jungles of Guatemala and Belize, and in the states of Quintana Roo and Yucatan, in Mexico [19]. When cut, the tree exudates a milky resin which is used to alleviate skin rashes and mouth blisters, as well as to treat fever and bone fractures [1, 20].

As part of an ongoing screening project directed towards the search for biologically active metabolites from the

Yucatecan flora [21], we wish to describe herein the bioassay-directed purification of the root and leaf extracts of *J. gaumeri*, which resulted in the isolation and identification of the antimicrobial diterpene 2-*epi*-jatrogrossidione (**1**) and 15-*epi*-4*E*-jatrogrossidentadione (**2**), together with  $\beta$ -sitosterol,  $\alpha$ -amyrin,  $\beta$ -amyrin and taraxasterol.



## Results and discussion

A bioassay-guided purification of the antimicrobial root organic crude extract of *J. gaumeri*, yielded 2-*epi*-jatrogrossidione (**1**), a diterpene with a rare rhamnofolane carbon skeleton previously isolated from the root bark of *J. grossidentata* [22]. A complete assignment of the <sup>13</sup>C NMR spectrum signals for

the structure of **1**, not previously reported in the literature, is detailed in Experimental. 2-*epi*-Jatrogrossidione showed significant antimicrobial activity when tested at 25 µg against *B. subtilis* using the agar-overlay method [21]. The antimicrobial activity of **1** was comparable to that shown by the commercial antibiotic amikacin sulfate when tested at 250 µg.

Inactive 15-*epi*-4*E*-jatrogrossidentadione (**2**) was isolated during the purification of **1**. This lathyrane-type diterpene was previously isolated from the roots of *J. grossidentata* [23].

The methanolic extract obtained from the leaves of *J. gaumeri* showed significant antioxidant activity when tested in both the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the inhibition of the bleaching of β-carotene assays [21]. A bioassay-guided purification of the leaf organic crude extract yielded two main fractions showing secondary metabolites with antioxidant activity; one of the fractions contained a single component which was identified as β-sitosterol by comparing its spectroscopic data with those reported in the literature [24-26] and by direct comparison on TLC with an authentic sample.

The second, less-polar, fraction showed the presence of two main components having very similar  $R_f$  values on TLC; all attempts to separate the components in their natural form proved unsuccessful. Esterification and oxidation of the two-component mixture produced the corresponding acetylated and keto derivatives, which could then be separated by means of PTLC on AgNO<sub>3</sub>-impregnated silica gel plates [27, 28]. Hydrolysis of the acetylated derivatives yielded each of the original components as a single spot on TLC; one of the components was identified by GC/MS as an inseparable mixture of α- and β-amyirin and their identity was confirmed by both comparing their spectroscopic data with those reported in the literature [29, 30] and by direct comparison on TLC with a mixture previously obtained in our laboratory. The second component, obtained in pure form, was identified as the triterpene alcohol taraxasterol by comparing its spectroscopic data with those reported in the literature [29,30].

There are a limited number of reports in the literature on the biological activities of both sterols and triterpenes; β-sitosterol, when tested at 0.02 and 0.04%, apparently exhibits a higher antioxidant activity than that of the well known commercial antioxidants α-tocopherol or butylated-hydroxy-toluene (BHT) [31]. It has also been reported that triterpenes such as α-amyirin, β-amyirin, and taraxasterol have significant antitumor and anti-inflammatory activities in mice [32] and that their capacity to inhibit lipid peroxidation might be due to their high lipophylicity [33].

## Experimental

### General

IR spectra were obtained on a Nicolet Magna Protégé 460 FT-IR instrument, using CHCl<sub>3</sub> (Merck, Uvasol) as solvent. <sup>1</sup>H (400 MHz) and <sup>13</sup>C NMR (100 MHz) spectra were recorded

on a Bruker Avance 400 apparatus using CDCl<sub>3</sub> as solvent and TMS as internal reference. GC-MS analyses were carried out in a Hewlett Packard 5890 gas chromatograph coupled to a HP-5971A mass selective detector [GC conditions: split injection of 1 µL of sample; HP-Ultra 2 column (crosslinked 5% Ph Me silicone; 25 m × 0.32 mm × 0.52 µm film thickness); flow rate: 1 mL/min; oven temperature: 280 to 300 °C; gradient: 5 °C/min; injector: 290 °C; detector: 300 °C]. Analytical TLC was performed on aluminum sheets impregnated with silica gel 60 F<sub>254</sub> (Merck, 0.20 mm thickness). Preparative TLC (PTLC) was performed on glass plates impregnated with silica gel 60 F<sub>254</sub> (Merck, 0.50 mm thickness, 20 × 20 cm). Chromatographic purifications were run using silica gel 60 (Merck, 70-230 mesh) for open column, silica gel 60 GF<sub>254</sub> for VLC, and silica gel 60 (Merck, 200-400 mesh) for flash column. Chromatograms were examined under UV light (365 and 254 nm) using a UV-viewing cabinet (Spectroline, model CX-20) and by spraying with 4% phosphomolybdic acid containing a trace of ceric sulfate in 5% H<sub>2</sub>SO<sub>4</sub>.

### Plant material

Roots and leaves of *J. gaumeri* were collected in April 2001 in an area located 2 Km from Dzemul, Yucatan, Mexico, and identified by one of us (FMP). A voucher specimen (FMay-1883) representing this collection has been deposited in the herbarium of CICY.

### Extraction and isolation

The roots (2.2 Kg) were cut and dried, first at room temperature (five days) and then in an oven at 50-60 °C (72 h); the dry material was ground and extracted three times with MeOH (12 L) at room temperature. The extracts were combined and the solvent eliminated under reduced pressure to yield 206.5 g of crude methanolic extract 1A. The extract was suspended in a mixture of H<sub>2</sub>O/MeOH (3:2, 1 L) and partitioned successively with hexane (2:1, 3×) and ethyl acetate (2:1, 3×) to produce the corresponding low (2A, 36.6 g) and medium (2B, 26.2 g) polarity crude fractions. Testing of both the methanolic crude extract 1A and the resulting crude fractions 2A and 2B for antimicrobial activity using the agar-overlay method [21], indicated that fraction 2A had the strongest activity against *Bacillus subtilis* (ATCC-6633). A bioassay guided purification of fraction 2A (17.8 g) using VLC (7.0 cm height × 5.0 cm width; hexane/AcOEt, gradient elution) yielded nine fractions (3A-I). Fraction 3H (633 mg) was further purified by flash CC (4.0 cm width × 50.0 cm height; CHCl<sub>3</sub>/acetone, 98:2) to afford fractions 4A-G. A final PTLC purification of fraction 4C (290 mg) [hexane/acetone/MeOH, 80:15:5, multiple elution (3×)] resulted in the isolation of **1** (13 mg) and **2** (6.8 mg) in pure form.

The leaves were also air and oven dried, and then manually ground to produce 479 g of plant material which was extracted with methanol (3 × 2 L) at room temperature to yield

38.9 of organic crude extract 5A. The organic crude extract was suspended in a 3:2 mixture of H<sub>2</sub>O/MeOH (500 mL) and successively partitioned with hexane and ethyl acetate to produce the corresponding low (6A, 10.3 g) and medium (6B, 6.0 g) polarity fractions. Both the crude extract and the crude fractions showed antioxidant activity when tested in the inhibition of the bleaching of  $\beta$ -carotene and the reduction of the DPPH assays. The low polarity fraction was subjected to a VLC purification (10.0 cm width  $\times$  5.0 cm height; hexane/AcOEt, gradient elution) to produce 10 major fractions (7A-J). One of the fractions (7F) showed a single component on TLC and GC which was identified as  $\beta$ -sitosterol. Further purification of fraction 7D by flash column chromatography [5 cm diameter  $\times$  20 cm height, hexane/EtOAc/MeOH (95:4:1)] afforded 11 major fractions (8A-K).

**Esterification of fraction 8F.** A mixture of 8F (33 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) was allowed to stir overnight at room temperature. The reaction mixture was poured over water (15 mL) and the resulting suspension was extracted with EtOAc (3 $\times$ , 1:1). The organic layer was successively washed (1:1) with 5% HCl, water, 3% NaOH, and brine and then dried over anhydrous sodium sulfate. Evaporation of the solvent yielded 30 mg of crude acetylated product, which was purified by PTLC using silica gel plates impregnated with 5% AgNO<sub>3</sub> to give two main fractions (9A and 9B), each identified by GC/MS as a mixture of the acetylated derivatives of  $\alpha$ - and  $\beta$ -amyryn (9A) and as the acetylated derivative of taraxasterol (9B).

**Hydrolysis of the acetylated derivatives 9A and 9B.** Each fraction (9A, 8.2 mg and 9B, 10.0 mg) was separately hydrolyzed by stirring overnight with 5 mL of 30% KOH in MeOH at room temperature. The reaction mixture was poured over water (15 mL) and the resulting suspension was extracted with EtOAc (3 $\times$ , 1:1). The organic layer was washed with water and brine and dried over anhydrous sodium sulfate. Evaporation of the solvent yielded the corresponding hydrolyzed product in good yield, *i.e.* 8.0 mg of the  $\alpha$ - and  $\beta$ -amyryn mixture and 10 mg of taraxasterol.

**Oxidation of fraction 8F.** 70 mg of pyridinium chlorochromate (PCC, Corey's reagent) was added to a solution of fraction 8F (20 mg) in CH<sub>2</sub>Cl<sub>2</sub> (3 mL) and the mixture was allowed to stir overnight at room temperature. The reaction mixture was passed through a silica gel bed (70-230 mesh, 3 cm high) and the adsorbent washed with CH<sub>2</sub>Cl<sub>2</sub> to produce 18 mg of crude oxidized product. PTLC purification of the crude product using silica gel plates impregnated with 5% AgNO<sub>3</sub> yielded two main fractions (10A and 10B), each identified by GC/MS as a mixture of  $\alpha$ - and  $\beta$ -amyrenone (10A) and as taraxasterone (10B).

**Antimicrobial assay by the agar overlay method.** *Bacillus subtilis* (ATCC-6633) was used for testing antimicrobial activity. Trypticasein soy agar (Bioxon) was used as solid medium. Inoculum was prepared by suspending the microorganism in sterile water in an approximate concentration of  $1 \times 10^8$

**Table 1.** Spectroscopic data of 2-*epi*-jatrogrossidione (**1**) and 15-*epi*-4*E*-jatrogrossidentadione (**2**).

1				2			
H	$\delta$ m (J) <sup>a</sup>	C	$\delta$ (m) <sup>b</sup>	H	$\delta$ m (J) <sup>a</sup>	C	$\delta$ (m) <sup>b</sup>
2	2.41 dq (7.6, 2.4)	1	209.6 (s)	1	6.86 bs (1.2)	1	151.6 (d)
3	4.88 bs	2	48.3 (d)	5	6.63 bs (0.4)	2	146.7 (s)
7	5.91 dq (5.1, 1.6)	3	76.8 (d)	7a	1.98 m	3	195.9 (s)
8	2.60 dddq (12.0, 12.0, 5.0, 1.5)	4	148.4 (s)	7b	1.70 m	4	137.2 (s)
9	3.18 d (12.0)	5	198.4 (s)	8a	1.68 m	5	144.1 (d)
12a	2.44 ddd (12.0, 5.6, 2.4)	6	141.3 (s)	8b	0.86 m	6	76.2 (s)
12b	2.29 ddd (12.4, 11.6, 4.4)	7	137.2 (d)	9	0.42 br dd (10.0, 8.4)	7	41.6 (t)
13a	1.89 m	8	43.7 (d)	11	0.64 br dd (8.4, 8.0)	8	19.7 (t)
13b	1.47 dddd (12.0, 12.0, 12.0, 5.2)	9	45.4 (d)	12a	1.50 br ddd (14.8, 8.0, 5.2)	9	27.0 (d)
14	2.37 ddd (12.0, 12.0, 4.4)	10	160.5 (s)	12b	1.38 ddd (14.8, 2.4, 2.0)	10	18.1 (s)
16a	4.85 bs	11	148.9 (s)	13	2.89 ddd (6.8, 5.2, 2.4)	11	19.5 (d)
16b	4.82 bs	12	36.4 (t)	16	1.99 d (1.2)	12	29.7 (t)
17	1.59 bs	13	34.3 (t)	17	1.34 s	13	38.3 (d)
18a	4.76 bs	14	51.4 (d)	18	0.99 s	14	211.0 (s)
18b	4.11 bs	15	146.6 (s)	19	0.66 s	15	84.3 (s)
19	1.32 d (7.6)	16	113.4 (t)	20	1.11 d (6.8)	16	11.0 (q)
20	1.85 dd (1.6, 1.6)	17	18.7 (q)	OH	5.29 s	17	29.2 (q)
OH	2.84 bs	18	108.4 (t)	OH	3.29 s	18	28.5 (q)
		19	14.5 (q)			19	15.0 (q)
		20	18.9 (q)			20	17.1 (q)

<sup>a</sup> <sup>1</sup>H NMR spectra were recorded at 400 MHz in CDCl<sub>3</sub>; *J* values in Hz. <sup>b</sup> <sup>13</sup>C NMR spectra were recorded at 100 MHz in CDCl<sub>3</sub>; multiplicity deduced by DEPT and indicated by the usual symbols. The  $\delta$  values are downfield from TMS.

cell/mL. Just before applying the overlay, a portion of the inoculum, with a final concentration of approximately  $1 \times 10^6$  cell/mL, was added to the culture medium kept in a water bath (45 °C). Samples (25 µg) were applied to TLC plates prepared in the laboratory. The TLC plates were placed in a sterile Petri dish and covered with 4.5 mL of inoculum. Once the medium had solidified, the plates were incubated for 15 h at 36 °C. After this time, TLC plates were sprayed with an aqueous solution of methylthiazolyltetrazolium chloride (MTT, 2.5 mg/mL, Fluka) and incubated for another 3 h. Biological activity was observed as clear inhibition zones against the purple background. Amikacin was used as positive control.

**Inhibition of bleaching of  $\beta$ -carotene.** A TLC plate spotted with 100 µg of sample was eluted using a mixture of  $\text{CHCl}_3/\text{MeOH}$  (95:5). After drying, the plate was sprayed with a 0.05% solution of  $\beta$ -carotene (Sigma) in  $\text{CHCl}_3$  and left at room temperature for 12 h until decoloration of the background occurred. Active products remained as orange spots on a white background. Vitamin C (10 µg) was applied to the plates as positive control. All experiments were carried out in duplicate.

**Reduction of DPPH.** A TLC plate spotted with 100 µg of sample was eluted using a mixture of  $\text{CHCl}_3/\text{MeOH}$  (95:5). After drying, the plate was sprayed with a 0.2% solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH, Fluka) in MeOH and left at room temperature for 8 h. Active products appeared as yellow spots against a purple background. Vitamin C (10 µg) was applied to the plates as positive control. All experiments were carried out in duplicate.

**2-*epi*-Jatrogrossidione (1):** Colorless gum;  $R_f$  0.42 [ $\text{CH}_2\text{Cl}_2/\text{acetone}$  (95:5)]; IR  $\nu_{\text{max}}$  (film): 3498, 2925, 2852, 1715, 1649, 1137  $\text{cm}^{-1}$ ;  $^1\text{H}$ - and  $^{13}\text{C}$ -RMN: Table 1; LREIM  $m/z$ : 312  $[\text{M}]^+$ , 284  $[\text{M} - \text{CO}]^+$ , 269  $[284 - \text{CH}_3]^+$ , 241  $[269 - \text{CO}]^+$ , 69, 55.

**15-*epi*-4*E*-Jatrogrossidentadione (2):** IR  $\nu_{\text{max}}$  (film): 3399, 2918, 2851, 1703, 1650, 1455, 1369, 1135, 1107, 979  $\text{cm}^{-1}$ ;  $^1\text{H}$ - and  $^{13}\text{C}$ -RMN: Table 1; LREIM  $m/z$ : 332  $[\text{M}]^+$ , 314  $[\text{M} - \text{H}_2\text{O}]^+$ , 271, 257, 215, 175, 163, 161, 124, 109, 95, 67, 55.

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## References

- Flores, J.S.; Ricalde, R.V. *J. Herbs Spices Med. Plants* **1996**, 4, 53-59.
- Zamora-Martínez, M.C.; Pola, C.N.P. *J. Ethnopharmacol.* **1992**, 35, 229-257.
- Manandhar, N.P. *Econ. Bot.* **1995**, 49, 371-379.
- Gupta, M.P.; Monge, A.; Karikas, G.A.; Lopez de Cerain, A.; Solis, P.N.; De Leon, E.; Trujillo, M.; Suarez, O.; Wilson, F.; Montenegro, G.; Noriega, Y.; Santana, A.I.; Correa, M.; Sanchez, C. *Int. J. Pharmacog.* **1996**, 34, 19-27.
- Giron, L.M.; Freire, V.; Alonzo, A.; Caceres, A. *J. Ethnopharmacol.* **1991**, 34, 173-187.
- Coe, F.G.; Anderson, G.J. *Econ. Bot.* **1996**, 50, 71-107.
- Barrett, B. *Econ. Bot.* **1994**, 48, 8-20.
- Klinar, S.; Castillo, P.; Chang, A.; Schmeda-Hirschmann, G.; Reyes, S.; Theoduloz, C.; Razmilic, I. *Fitoterapia* **1995**, 66, 341-345.
- Staubmann, R.; Schubert-Zsilavecz, M.; Hiermann, A.; Kartnig, T. *Phytochemistry* **1999**, 50, 337-338.
- Denton, R.W.; Harding, W.W.; Anderson, C.I.; Jacobs, H.; McLean, S.; Reynolds, W.F. *J. Nat. Prod.* **2001**, 64, 829-831.
- Brum, R.L.; Cavaleiro, A.J.; Monache, F.D.; Vencato, I. *J. Braz. Chem. Soc.* **2001**, 12, 259-262.
- Adolf, W.; Opferkuch, H.J.; Hecker, E. *Phytochemistry* **1984**, 23, 129-132.
- Das, B.; Anjani, G. *Phytochemistry* **1999**, 51, 115-117.
- Tinto, W.F.; John, L.M.D.; Reynolds, W.F.; McLean, S. *J. Nat. Prod.* **1992**, 55, 807-809.
- Baraguey, C.; Blond, A.; Correia, I.; Poussset, J.L.; Bodo, B.; Auvin-Guette, C. *Tetrahedron Lett.* **2000**, 41, 325-329.
- Naengchomnong, W.; Thebtaranonth, Y.; Wiriyachitra, P.; Okamoto, K.T.; Clardy, J. *Tetrahedron Lett.* **1986**, 27, 2439-2442.
- Evans, F.J.; Taylor, S.E., in: *Progress in the Chemistry of Organic Natural Products*, Vol. 44, Herz, W.; Grisebach, H.; Kirby, G.W., Eds., Springer-Verlag, Wein, **1983**, 1-99.
- Adolf, W.; Opferkuch, H.J.; Hecker, E. *Phytochemistry* **1984**, 23, 129-132.
- Villamar, A.A. *Atlas de las Plantas de la Medicina Tradicional Mexicana*, Vol. 2. Instituto Nacional Indigenista, México, **1994**, 881, 1162, 1186.
- Comerford, S.C. *Econ. Bot.* **1996**, 50, 327-336.
- Sánchez-Medina, A.; García-Sosa, K.; May-Pat, F.; Peña-Rodríguez, L.M. *Phytomedicine* **2001**, 8, 144-151.
- Jakupovic, J.; Grenz, M.; Schmeda-Hirschmann, G. *Phytochemistry* **1988**, 27, 2997-2998.
- Schmeda-Hirschmann, G.; Tschritzis, F.; Jakupovic, J. *Phytochemistry* **1992**, 31, 1731-1735.
- Adler, J.H. *Phytochemistry* **1983**, 22, 607-608.
- Garg, V.K.; Nes, W.R. *Phytochemistry* **1984**, 23, 2919-2923.
- Goad, L.J., in: *Methods in Plant Biochemistry*, Vol. 7, Dey, P.M. and Harborne, J.B., Eds., Academic Press, London, **1991**, 369-434.
- Stahl, E.; Jork, H. *Thin-layer Chromatography. A Laboratory Handbook*. Springer-Verlag, 2<sup>nd</sup> edition, New York, **1969**, 227-229.
- Fried, B.; Sherma, J. *Thin-layer Chromatography: Techniques and Applications*, Vol. 17, Chapter 14. Marcel Dekker, USA, **1982**, 217-220.
- Ogunkoya, L. *Phytochemistry* **1981**, 20, 121-126.
- Conolly, J.D.; Hill, R.A., in: *Methods in Plant Biochemistry*, Vol. 7, Dey, P.M. and Harborne, J.B., Eds., Academic Press, London, **1991**, 331-359.
- Weng, X.C.; Wang, W. *Food Chem.* **2000**, 71, 489-493.
- Akihisa, T.; Yasukawa, K.; Oinuma, H.; Kasahara, Y.; Yamanouchi, S.; Takido, M.; Kumaki, K.; Tamura, T. *Phytochemistry* **1996**, 43, 1255-1260.
- Anton, R.; Beretz, A.; Haag-Berrurier, M., in: *Biologically Active Natural Products*, Chapter 8, Hostettmann, K. and Lea, P.J., Eds., Clarendon Press, Oxford, **1987**, 123-124.