Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 6687–6691
Bioorganic & Medicinal Chemistry

Bioassay-guided fractionation of pterocarpans from roots of *Harpalyce brasiliana* Benth

Gardenia C. G. Militão,[a] Sávio M. Pinheiro,[b] Ivana N. F. Dantas,[a] Cláudia Pessoa,[a] Manoel Odorico de Moraes,[a] Letícia V. Costa-Lotufo,[a,*] Mary Anne S. Lima[b] and Edilberto R. Silveira[b]

[a]*Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Caixa Postal 3157, 60430-270 Fortaleza, Ceará, Brazil*
[b]*Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará, Caixa Postal 12 200, 60021-940 Fortaleza, Ceará, Brazil*

Received 23 May 2007; revised 31 July 2007; accepted 6 August 2007
Available online 15 August 2007

Abstract—Pterocarpans, a special kind of isoflavonoids possessing two contiguous benzofuran and benzopyran rings, have been reported as possessing several biological activities. In order to isolate and identify the active principles possibly responsible for the stronger activity of the EtOH extract from roots of *Harpalyce brasiliana* on the antimitotic assay using sea urchin egg development, a bioassay-guided fractionation was performed. Six bioactive pterocarpan derivatives: 4′-dehydroxycabenegrin A-I, leiocarpin, medicarpin, cabenegrins A-I and A-II, and maackiain were isolated from the chloroform fraction of *H. brasiliana* extract. Leiocarpin was the most active on the sea urchin egg assay with IC_{50} values ranging from 0.1 to 1.2 μg/mL, followed by 4′-dehydroxycabenegrin A-I. The isolated compounds were also tested for cytotoxicity against tumor cell lines in cultures, where 4′-dehydroxycabenegrin A-I was the most active, followed by leiocarpin. Additionally, some studies on the structure–activity relationship of these pterocarpans are suggested.

1. Introduction

In the continuing search to discover anticancer agents from the plant kingdom we have investigated *Harpalyce brasiliana* Benth., a shrub popularly designated as 'erva de cobra' (Port. lit.: snake's root), and used by peasant people to treat snake bites. Previous phytochemical studies with the roots of *H. brasiliana* have demonstrated the presence of betulinic acid and 4′-dehydroxycabenegrin A-I.[1,2] More recently, Da Silva et al.[3] demonstrated that 4′-dehydroxycabenegrin A-I, mistakenly called edunol, and its derivatives inhibited myotoxic, proteolytic, and PLA_2 activities of *Bothrops jararacussu* venom. According to them the presence of those compounds justifies the popular use of this plant. In fact, the first active pterocarpans against snake venoms, cabenegrins A-I and A-II, were isolated from a Brazilian folk medicine called '*Específico Pessoa*',[4] whereas the authors believed that a plant, commonly called 'cabeça-de-negro', was the source of the isolated compounds. Nagakawa et al.[4] stated that they were unable to identify the plant source because in South America there were at least ten plants with that name. Just in Brazil at least four plant species are popularly designated as 'cabeça-de negro', *Cayaponia tayuya* (Kell.) Cogn, *Cayaponia espelina* Cogn., *Annona coriacea* (Mart.), and *Wilbrandia* sp.[4–6] Lately, experimental evidences including the isolation of cabenegrins A-I and A-II, supported that *H. brasiliana*, but not one of those species referred to as 'cabeça-de-negro', should be the major plant material used to prepare the beverage.[1,2]

Many other biological activities are described for pterocarpans, including antiviral, cytotoxic, and antimitotic.[7–11] In fact, Falcão et al.[8] evaluated the cytotoxic effect of nine flavonoids isolated from *Platymiscium floribundum* on tumor cell lines, and the pterocarpans were found to be the most active compounds. Lately, these pterocarpans were tested for antimitotic activity using sea urchin egg and the results

Keywords: *Harpalyce brasiliana*; Pterocarpans; Sea urchin egg.
* Corresponding author. Tel.: +55 85 3366 8255; fax: +55 85 3366 8333; e-mail: lvcosta@secrel.com.br

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.08.011

indicated an activity as strong as those observed to doxorubicin and etoposide, also at lower concentrations (nanomolar range). Moreover, the presence of a methoxy group on the C-2 position seemed to be an important structural requirement for cytotoxic activity among these compounds, since 2,3,9-trimethoxypterocarpan was much more active than the other tested compounds in both tumor cells and sea urchin egg assays.[8,9] Further, it was demonstrated that these naturally occurring pterocarpans displayed a strong antiproliferative activity in HL-60, a promyelocytic leukemia cell line, and this activity was related to cell cycle arrest and apoptosis induction.[10,11] All these data point to the importance of pterocarpans as an emerging potential class of anticancer prototypes.

In the present paper, we focus on a bioassay-guided fractionation of the EtOH extract from roots of *H. brasiliana* using the sea urchin eggs, since previous findings demonstrated that these cells are highly sensitive to pterocarpans.[9] Additionally, the cytotoxicity of isolated compounds against human tumor cell lines was evaluated. It is worthwhile to mention that several pterocarpans previously isolated from *H. brasiliana* presented a prenyl group, while the pterocarpans isolated from *P. floribundum* did not.[1,2,8,9] Thus, this is the first study evaluating the cytotoxic activity of prenylated pterocarpans.

2. Results and discussion

The sea urchin egg assay is largely applied in investigations of anticancer and teratogenic properties of secondary metabolites.[12] The sea urchin egg possesses strong sensitivity against toxic agents and the development presents several peculiarities which provide an important tool for discovery of drugs with anticancer potential. The inhibition of mitoses can be related to different events of this process, such as DNA and RNA synthesis, protein synthesis, and mitotic spindle assembly. In some cases, it is possible to analyze these processes one at a time.[13]

Prenylated pterocarpans have long been described as anti-snake venom[4] but the antimitotic properties of these compounds have never been studied. In fact, the antimitotic assay on sea urchin egg can be used as a very sensitive test to detect pterocarpans' anticancer activity since pterocarpans isolated from *Platymiscium floribundum* showed IC_{50} in the nanomolar range, being even more active than chemotherapeutic agents currently used in cancer treatment, such as etoposide and doxorubicin.[9]

A first screening of the EtOH extract from leaves, bark, stem bark, and roots in the sea urchin egg assay showed that all extracts presented antimitotic activity with the root extract causing a total inhibition of the sea urchin egg development at a concentration of 10 μg/mL (Table 1). According to Jacobs et al.[14] if a substance promotes 100% inhibition in this assay at a concentration of 16 μg/mL or less, it can be considered very active and should undergo in vivo evaluation as an anticancer compound. Therefore, only the EtOH extract from roots was further investigated. Liquid–liquid partition of an aliquot of the EtOH extract using hexane, $CHCl_3$, EtOAc, and *n*-BuOH as eluents yielded four fractions and the $CHCl_3$ fraction reached the lowest IC_{50} value (1.1 μg/mL) while the butanol and aqueous fraction were inactive (Table 2).

Table 1. Antimitotic activity on sea urchin egg development of the EtOH extracts from several plant parts of *Harpalyce brasiliana*

Fractions	1° Cleavage IC_{50} (μg/mL)	3° Cleavage IC_{50} (μg/mL)	Blastulae IC_{50} (μg/mL)
HBFE	134.0 107.0–167.8	58.5 49.97–68.42	47.7 40.9–55.7
HBLCE	36.3 26.09–50.46	33.7 31.0–36.5	10.0 8.7–10.4
HBCCE	111.0 80.10–154.6	41.6 36.3–47.7	17.0 15.98–17.92
HBRE	<10	<10	<10

Data are presented as IC_{50} values for first, third cleavages and blastulae. The IC_{50} values (concentration which causes 50% of inhibition) and their 95% confidence interval (CI 95%) were obtained by nonlinear regression. HBFE, EtOH extract from leaves of *H. brasiliana*; HBLCE, EtOH extract from trunk heartwood of *H. brasiliana*; HBCCE, EtOH extract from trunk bark of *H. brasiliana*; HBRE, EtOH extract from roots of *H. brasiliana*.

Bioguided fractionation of the chloroform fraction afforded 4′-dehydroxycabenegrin A-I (**1**), leiocarpin (**2**), medicarpin (**3**), cabenegrins A-I (**4**) and A-II (**5**), and maackiain (**6**).[1,4,15,16] Leiocarpin (**2**) was the most active with IC_{50} values ranging from 0.1 to 1.2 μg/mL followed by cabenegrins A-I (**4**, 0.3–1.8 μg/mL) and A-II (**5**, 1.4–7.3 μg/mL), 4′-dehydroxycabenegrin A-I (**1**, 2.9–17.1 μg/mL), medicarpin (**3**, 2.2–11.6 μg/mL), and maackiain (**6**, 3.8–4.9 μg/mL) (Table 3). The above

Table 2. Antimitotic activity on sea urchin egg development of five fractions of the EtOH extract from roots from *Harpalyce brasiliana*

Fractions	1° Cleavage IC_{50} (μg/mL)	3° Cleavage IC_{50} (μg/mL)	Blastulae IC_{50} (μg/mL)
HBREEp	6.0 2.7–13.0	1.7 1.3–2.2	2.1 1.7–2.7
HBREC	2.7 2.0–3.8	0.9 0.8–0.9	1.1 1.0–1.2
HBREA	6.3 5.3–7.5	4.0 2.8–5.7	3.0 2.4–4.0
HBREB	>100	>100	>100
HBREFA	>100	>100	>100

Data are presented as IC_{50} values for first and third cleavages and blastulae. The IC_{50} values (concentration which causes 50% of inhibition) and their 95% confidence interval (CI 95%) were obtained by nonlinear regression. HBREEp, petrol ether fraction of the EtOH extract from roots; HBREC, chloroform fraction of the EtOH extract from roots; HBREA, ethyl acetate fraction of the EtOH extract from roots; HBREB, butanol fraction of the EtOH extract from roots; HBREFA, aqueous fraction of the EtOH extract from roots.

Table 3. Antimitotic activity of pterocarpans isolated from *Harpalyce brasiliana* on sea urchin egg development

Pterocarpans	1° Cleavage IC_{50} (μg/mL)	3° Cleavage IC_{50} (μg/mL)	Blastulae IC_{50} (μg/ml
4′-Dehydroxycabenegrin A-I (**1**)	15.2 13.5–17.1	4.8 4.2–5.3	3.3 2.9–3.7
Leiocarpin (**2**)	1.1 1.0–1.2	0.4 0.4–0.5	0.2 0.1–0.2
Medicarpin (**3**)	10.5 9.6–11.6	5.3 4.8–5.8	4.1 2.2–7.5
Cabenegrin A-I (**4**)	1.5 1.25–1.8	0.52 0.48–0.58	0.43 0.3–0.6
Cabenegrin A-II (**5**)	5.18 3.6–7.3	2.1 1.8–2.6	1.7 1.39–2.1
Maackiain (**6**)	6.5 5.4–7.8	4.3 3.8–4.9	4.6 4.0–5.2
Positive control	6.28 4.34–9.09	0.34 0.16–0.73	0.54 0.27–1.07

Data are presented as IC_{50} values for first and third cleavages and blastulae. The IC_{50} values (concentration which causes 50% of inhibition) and their 95% confidence interval (CI 95%) were obtained by nonlinear regression. Doxorubicin was used as positive control.

Table 4. Cytotoxic activity of pterocarpans isolated from *Harpalyce brasiliana* against tumor cell lines

Pterocarpans	Cell line CI_{50} (μg/mL)		
	MDA-MB-435	HCT-8	HL-60
4′-Dehydroxycabenegrin A-I (**1**)	4.9 2.1–4.4	8.5 6.1–11.8	3.1 1.9–5.0
Leiocarpin (**2**)	13.7 9.4–20.0	6.9 5.1–9.3	5.5 2.7–11.0
Medicarpin (**3**)	16.5 13.2–20.7	17.6 15.6–19.9	22.3 14.7–33.7
Cabenegrin A-I (**4**)	>25	8.8 6.9–11.2	16.2 4.4–59.2
Cabenegrin A-II (**5**)	16.9 12.8–22.4	10.8 9.2–12.7	>25
Maackiain (**6**)	11.5 4.1–31.8	>25	17.9 11.8–27.1
Positive control	0.48 0.34–0.66	0.01 0.01–0.02	0.02 0.01–0.02

Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression for leukemia (HL-60), melanoma (MDA-MB-435), and colon (HCT-8) cancer cells from three independent experiments. Doxorubicin was used as positive control.

substances showed antiproliferative effects since the first cleavage, with IC_{50} values less than 10 μg/mL, except for compound **1**. These compounds could be affecting the DNA or protein synthesis, and/or microtubule assembly, since all these processes are active at the first cleavage.

The isolated compounds **1**–**6** were further tested for their cytotoxicity using three human cell lines, HL-60 (leukemia), MDA-MB-435 (melanoma), and HCT-8 (colon). In human cells, 4′-dehydroxycabenegrin A-I (**1**) was the most active compound presenting IC_{50} in the range of 3.1–8.5 μg/mL followed by leiocarpin (**2**) (Table 4). However, the compounds isolated in the present study could be considered only weakly active also against tumor cells compared to non-prenylated pterocarpans, such as 2,3,9-trimethoxy-pterocarpan, which present IC_{50} values in the range of 0.1–0.7 μg/mL.[6]

It is interesting to observe that the presence of a prenyl group either at C-2 or C-4 as in **1**, **2**, **4** and **5** increased the antimitotic properties compared to **3** and **6**. In addition, the presence of the methylenedioxy moiety at C-8/C-9 seems not essential for the activity since **3** was also an active compound, despite its lower activity. It seems that the presence of the hydroxy at C-3 is not an important feature since the most active pterocarpan is **2**, which do not possess hydroxyl but a bridged oxygen through a precocene-like moiety. This precocene moiety would be a more important feature than the methylenedioxy moiety at ring D since compounds **1**, **2**, **4**, **5** and the less active, **6**, all possess this moiety. Finally the presence of the hydroxylated prenyl moiety would account for the better activity of **4** and **5** over compound **1**. It is worth noticing that in a previous study, whereas the activity of five known pterocarpans: 3,10-dihydroxy-9-methoxy-pterocarpan, homopterocarpin, 2,3,9-trimethoxy-pterocarpan, vesticarpan, and medicarpin, isolated from the trunk of *Platymiscium floribundum,* was evaluated using the same model, the authors concluded that the C-2 methoxyl substituent was an important pharmacophoric unit.[9] In fact, comparing the present data with those reported by Militao et al.[9] 2,3,9-trimethoxypterocarpan is the most active compound with an IC_{50} in nanomolar range (8–10 ng/mL), being 12.5–120 times more active than leiocarpin (**2**).

Then, it can be concluded that despite the influence of the precocene-like moiety in the activity of prenylated pterocarpans, the C-2 methoxyl substituent plays a much more important role in the activity of these compounds on both sea urchin egg and tumor cell lines.

3. Experimental

3.1. Pterocarpans' isolation

Roots of *Harpalyce brasiliana* (3.5 Kg) were pulverized and extracted with EtOH (3 × 7.0 L) at room temperature. The solvent was removed under reduced pressure to give a dark viscous extract (260.0 g).

Liquid–liquid partition of an aliquot of the EtOH extract (107.3 g) using hexane, $CHCl_3$, EtOAc, and *n*-BuOH as eluents yielded the four correspondent fractions after solvent evaporation. Part of the $CHCl_3$ fraction (12.5 g) was further purified over Sephadex LH-20 by elution with MeOH to give 23 fractions, which were combined in seven resulting fractions according to TLC analysis. Successive flash chromatography of fraction 4

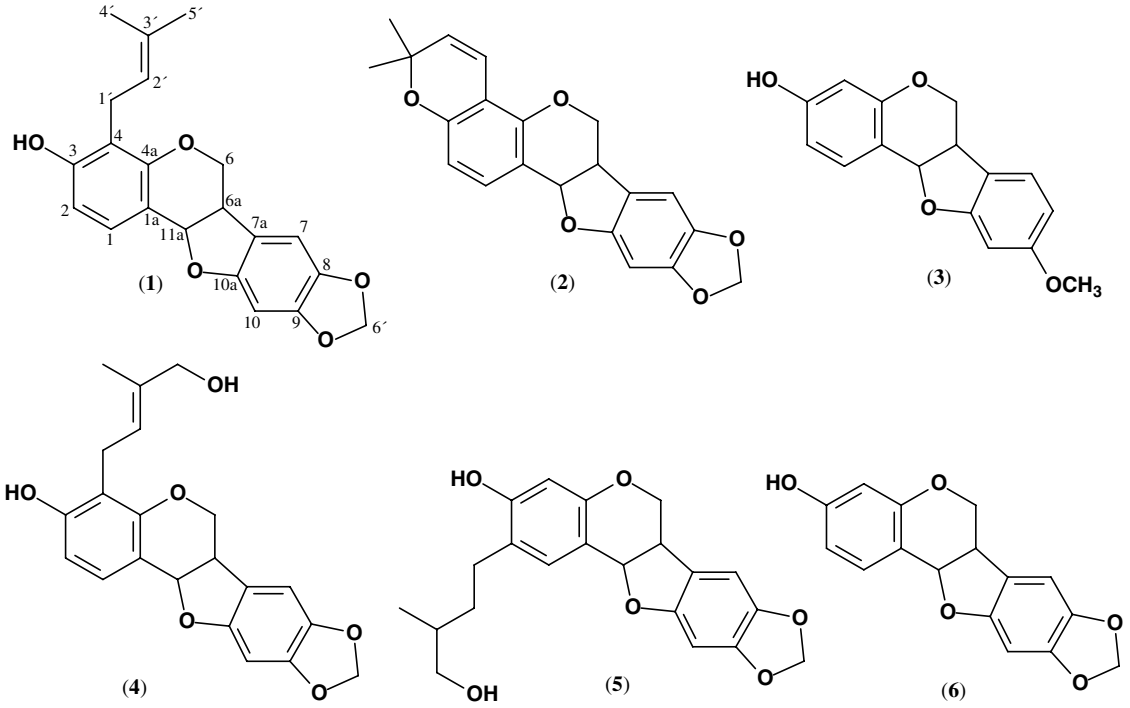

Figure 1. Pterocarpans from *Harpalyce brasiliana*.

(3.34 g) using $CHCl_3$, EtOAc or MeOH as binary mixture of increasing polarity yielded 20 resulting fractions according to TLC analysis. Flash chromatography of subfraction 4 (8–28) (331.8 mg) by elution with hexane, CH_2Cl_2, and MeOH as binary mixture of increasing polarity yielded 4′-dehydroxycabenegrin A-I (**1**) (63.0 mg) and leiocarpin (**2**) (8.6 mg).[1,15] Successive flash chromatography of subfraction 4 (59–88) (193.7 mg) by elution with hexane, $CHCl_3$, and MeOH as binary mixtures of increasing polarity yielded medicarpin (**3**) (25.0 mg).[16] After successive flash chromatography of subfraction 4 (201–225) (490.0 mg) using mixtures of CCl_4, AcOEt, and MeOH of increasing polarity, the cabenegrins A-I (5.5 mg) (**4**) and A-II (5.0 mg) (**5**) were obtained.[4]

Flash chromatography of fraction 5 (989.2 mg) by elution with mixtures of $CHCl_3$, AcOEt, and MeOH of increasing polarity yielded maackiain (25.9 mg) (**6**).[17] The structures of all compounds are shown in Figure 1.

3.2. Antimitotic activity

For the sea urchin egg development assay, specimens of *Lytechinus variegatus* were collected from Pecém beach, São Gonçalo do Amarante, Ceará, Brazil. Gamete elimination was induced by injecting 3.0 mL of a 0.5 M KCl solution into the perivisceral cavity. The eggs were allowed to settle to the bottom of a graduated cylinder filled with filtered seawater. This process was repeated twice to wash off the jelly coat of the eggs. Concentrated sperm was collected with a Pasteur pipette. Egg fertilization was performed by adding 1 mL of a sperm suspension (0.01 mL of concentrated sperm plus 2.49 mL of filtered seawater) to 100 mL of filtered seawater containing the eggs. Fecundation was confirmed by the elevation of the vitellin membrane. Next, the eggs were distributed in 24-well plates, with each well receiving 1 mL of the egg suspension. Five minutes after fertilization, the drugs were added at concentrations ranging from 0.1 to 100 μg/mL. The eggs were incubated at room temperature (26 ± 2 °C) in a final volume of 2 mL. At appropriate times, 200 μL aliquots were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages, and blastulae. One hundred eggs were counted for each concentration of the tested substances in order to obtain the percentage of normal development.

3.3. Cytotoxicity against tumor cell lines

The isolated compounds (0.39–25 μg/mL) were tested for cytotoxic activity against three tumor cell lines (National Cancer Institute, Bethesda, MD): HCT-8 (colon), MDA-MB-435 (melanoma), and HL-60 (leukemia) after 72 h of incubation. Doxorubicin (0.01–0.58 μg/mL) was used as a positive control. The general viability of cultured cells was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product as described by Mosmann.[18]

3.4. Statistical analysis

The IC_{50} and its 95% confidence interval were obtained from data of 3 independent experiments by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.08.011.

References and notes

1. Silva, G. L.; Matos, F. J. A.; Silveira, E. R. *Phytochemistry* **1997**, *46*, 1059–1062.
2. Silva, G. L.; Machado, M. I. L.; Matos, F. J. A.; Braz-Filho, R. *J. Braz. Chem. Soc.* **1999**, *10*, 438–442.
3. Da Silva, A. J.; Coelho, A. L.; Simas, A. B.; Moraes, R. A.; Pinheiro, D. A.; Fernandes, F. F.; Arruda, E. Z.; Costa, P. R.; Melo, P. A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 431–435.
4. Nakagawa, M.; Nakanishi, K.; Darko, L. L.; Vick, J. A. *Tetrahedron Lett.* **1982**, *23*, 3855–3858.
5. Rizinni, C. T.; Mors, W. B.; Pereira, N. A. *Rev. Bras. Farm.* **1988**, *69*, 84–86.
6. Lorenzi, H.; Matos, F. J. A. In *Plantas Medicinais do Brasil. Nativas e Exóticas*; Instituto Plantarum: Nova Odessa, Brazil, 2002; p 12.
7. Engler, T. A.; Lynch, K. O.; Reddy, J. P.; Gregory, G. S. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1229–1232.
8. Falcão, M. J. C.; Pouliquem, Y. B. M.; Lima, M. A. S.; Gramosa, N. V.; Costa-Lotufo, L. V.; Militão, G. C. G.; Pessoa, C.; Moraes, M. O.; Silveira, E. R. *J. Nat. Prod.* **2005**, *68*, 423–426.
9. Militão, G. C. G.; Jimenez, P. C.; Wilke, D. V.; Pessoa, C.; Falcão, M. J. C.; Lima, M. A. S.; Silveira, E. R.; Moraes, M. O.; Costa-Lotufo, L. V. *Planta Med.* **2005**, *71*, 683–685.
10. Militão, G. C. G.; Dantas, I. N. F.; Pessoa, C.; Falcão, M. J. C.; Silveira, E. R.; Lima, M. A. S.; Curi, R.; Lima, T.; Moraes, M. O.; Costa-Lotufo, L. V. *Life Sci.* **2006**, *78*, 2409–2417.
11. Militão, G. C. G.; Bezerra, D. P.; Pessoa, C.; Moraes, M. O.; Ponte, F. A. F.; Lima, M. A. S.; Silveira, E. R.; Costa-Lotufo, L. C. *J. Nat. Med.* **2007**, *61*, 196–199.
12. Jacobs, R. S.; Wilson, L. In *Modern Analysis of Antibiotics*; Aszalor, A., Ed.; Marcel Dekker Inc., 1986; pp 481–493.
13. Fusetani, N. In *Biorganic Marine Chemistry*; Scheur, P. J., Ed.; Springer-Verlarg: Berlin, Heidelberg, 1987; pp 61–92.
14. Jacobs, R. S.; White, S.; Wilson, L. *Fed. Proc.* **1981**, *40*, 26–29.
15. Braz-Filho, R.; Gottlieb, O. R. *Phytochemistry* **1970**, *10*, 2433–2450.
16. Agrawal, P. K. In *Carbon-13 NMR of Flavonoids*; Elsevier: Amsterdam, 1989; p 218.
17. Bedir, E.; Çalis, I.; Aquino, R.; Piacente, S.; Pizza, C. *Phytochemistry* **1999**, *16*, 1541–1544.
18. Mosmann, T. *J. Immunol. Methods* **1983**, *16*, 55–63.