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Antiproliferative effects of abietane diterpenoids isolated from *Hyptis martiusii* Benth (Labiatae)

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Two abietane diterpenes were isolated from a hexane extract of *Hyptis martiusii* roots and identified as carnasol and 11,14-dihidroxy-8,11,13-abietatrien-7-one. These compounds were tested for their antiproliferative effects on tumor cell lines using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide and on the sea urchin egg development. Both compounds displayed cytotoxic activity against tumor cell lines, but only carnasol was able to inhibit the sea urchin egg cleavages.

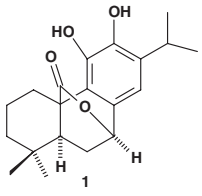
The genus *Hyptis* (Labiatae) includes many species currently used in folk medicine as antifungal, antibacterial, anticonvulsant agents, in various gastrointestinal ailments, to cure fungal diseases and malaria (Correa 1984; Pereda-Miranda et al. 1993). In fact, several phytochemical studies have been performed with *Hyptis* species, leading to the isolation of a great variety of biological activity compounds such as: cytotoxic lignans (Novelo et al. 1993), cytotoxic flavones (Kingston et al. 1979), antimicrobial and cytotoxic dihydro-alpha-pyrones (Pereda-Miranda et al. 1993), cytotoxic and anti-HIV triterpenes (Yamagishi et al. 1988; Kashiwada et al. 1998), insecticidal labdane diterpenes (Fragoso-Serrano et al. 1999).

H. martiusii is distributed in the tropics and subtropics, and is a small shrub that grows in abundance in the Northeast of Brazil, where it is popularly known as "cidreira-do-mato" (Port. Lit.: Wild *Lippia*). Recently, essential oils of leaves and inflorescences of *H. martiusii* were characterized, leading to isolation of 26 compounds for the essential oil from leaves and 27 for the essential oil from inflorescences. The essential oil from leaves and one of its major components, 1,8-cineole, showed pronounced insecticidal effect against the *Aedes aegypti* larvae and *Bemisia argentifolii,* the vectors of Dengue fever and white fly fruit plague, respectively (Araujo et al. 2003).

The present study took place in our ongoing investigations directed toward the discovery of potential antitumor constituents from traditionally used Northeastern Brazilian plants. Here, the results obtained from the evaluation of the cytotoxicity of carnasol and 11,14-dihidroxy-8,11,13-abietatrien-7-one, isolated from *H. martiusii* are reported.

Carnasol was first isolated from *Salvia carnosa* at the beginning of the 1940s, and had its structure established 20 years later (Brieskorn et al. 1964). Recently, carnasol was also isolated from *Hyptis dilatata* (Urones et al. 1998). The other abietane diterpenoid, 11,14-dihidroxy-8,11,13-abietatrien-7-one, was first isolated from *Chamaecyparis obtusa* var. *formosana* (Cupressaceae) (Kuo et al. 1998). Despite the previous chemical characterization of these two diterpenoids from other species, there is no data on the biological activity of these two compounds reported to date.

The compounds were obtained from a *Hyptis martiusii* specimen collected in "Chapada do Araripe" (Araripe's Plateau), Moreilândia County, Pernambuco State, Northeastern Brazil. A voucher specimen (# 25046) was deposited at the Herbário Prisco Bezerra (EAC), Biology Department, Federal University of Ceara, Brazil. The air dried roots (2.0 kg) and aerial parts (3.7 kg) of *Hyptis martiusii* were individually pulverized and extracted with hexane at room temperature. The solvents were removed under reduced pressure to yield a viscous greenish oil (60.1 g) and a dark brown solid (9.85 g) residues, respectively. The hexane extract of the aerial parts (60.1 g) was coarsely partitioned on a Silica gel column chromatography and eluted with hexane, $CHCl_3$, EtOAc and MeOH, to yield four fractions. Carnasol **1** (57.0 mg) (Urones et al. 1998) was obtained from the EtOAc fraction as a colorless solid upon recrystallization from MeOH. Chromatography over silica gel of the hexane extract from the roots (9.85 g) by elution with hexane, $CHCl_3$, EtOAc and MeOH, yielded four fractions. After successive chromatography on silica gel of the $CHCl_3$ fraction, 11,14-dihidroxy-8,11,13-abietatrien-7-one (**2**, 4.0 mg) (Kuo et al. 1998) was obtained as a yellow solid by elution with hexane : AcOEt as binary mixture. The structures of the metabolites were determined by spectroscopy analysis, particularly uni and bidimensional NMR experiments, run and a Bruker DRX 500 spectrometer, and posterior comparison with published data (Urones et al. 1998; Kuo et al. 1998).



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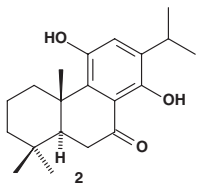

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Since several diterpenes have been shown to be cytotoxic to fertilized sea urchin eggs (Costa-Lotufo et al. 2002), we examined the two isolated diterpenes in this model to know whether it could exert an adverse influence on the embryonic development. This assay is a suitable model for detecting cytotoxic, teratogenic and antineoplastic activities of new compounds, and it has also been extensively used as a model for developmental toxicology evaluation (Jacobs et al. 1981; Costa-Lotufo et al. 2002). Adult sea urchins (*Lytechinus variegatus*) were collected in Pecém beach, northeastern Brazilian coast. The assays were performed as described by Costa-Lotufo et al. (2002) using 24-multiwell plates. The tested drugs were added immediately after fecundation (within 2 min) to get concentrations of 10 and 100 μg/ml. The plates were then shaken on a constant temperature water bath at 26 ± 2 °C.

Table 1: Cytotoxic activity of abietane diterpenoid 1 and 2 on tumor cell lines

Substances	CEM µg/ml (µM)	HL-60 µg/ml (µM)	HCT-8 µg/ml (µM)	MCF-7 µg/ml (µM)	B-16 µg/ml (µM)
1	6.4 (5.4–7.4)	7.3 (6.0–8.9)	11.3 (1.9–67.0)	15.0 (13.4–16.8)	12.0 (10.8–13.3)
2	14.6 (nd)	11.7 (10.1 –13.6)	14.7 (13.6–16.0)	22.3 (18.6–26.9)	13.0 (12.4–13.7)

Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression for leukemias (HL-60 and CEM), breast (MCF-7), colon (HCT-8) and skin (B-16) cancer cells. Experiments were performed in triplicate.

Table 2: Antimitotic activity of abietane diterpenoids 1 and 2 on sea urchin (Lytechinus variegatus) eggs development

Substances	Concentration (µg/mL)	1st cleavage (%)	3rd cleavage (%)	Blastulae (%)
Control	–	85.0 ± 1.5	82.8 ± 2.9	95.0 ± 1.2
1	10	64.7 ± 4.4*	38.0 ± 9.3*	24.7 ± 2.3*
	100	0.7 ± 0.3*	0 ± 0*	0 ± 0*
2	10	95.0 ± 1.5	85.0 ± 1.0	89.0 ± 5.0
	100	93.3 ± 0.3	85.0 ± 3.8	96.3 ± 1.8

* $p < 0.05$, ANOVA followed by Student-Newman-Keuls.
Data are presented as mean ± S.E.M. of the percentage of cleavage inhibition obtained from 3 experiments

At appropriate intervals, aliquots of 200 µl 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 test substance to obtain the percentage of normal cells.

These compounds were also evaluated for the cytotoxicity using MCF-7 (human breast cancer), HCT-8 (human colon cancer), B16 (murine skin cancer), CEM and HL-60 (leukemias cancer) cell lines (Children's Mercy Hospital, Kansas City, MO, USA) using the MTT assay (Mosmann 1983). This assay is based on the reduction of the yellow coloured 3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyl-tetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a purple-blue formazan. The compounds (0.39 to 25 µg/ml) were incubated for 72 h in cell culture media, before the MTT assay.

Both compounds presented cytotoxic activity against tumor cell lines. The IC_{50} values are presented in Table 1. Pure compounds could be considered satisfactory to warrant further studies as an antineoplastic drug when present an IC_{50} lower than 1 µg/ml or 1 µM (Pessoa et al. 2000). The dieterpenes isolated from *H. martiusii* could be considered only weakly cytotoxic, since they present IC_{50} values higher than 1 µg/ml on all tumor cell lines tested.

Table 2 shows the activity of the abietane diterpenes on the sea urchin egg development. Carnasol partially inhibited the cleavages at the smallest tested concentration (10 µg/ml), and at the highest concentration completely abolished the mitotic divisions. On the other hand, the other tested diterpene, 11,14-dihidroxy-8,11,13-abietatrien-7-one, did not induce any alterations in this assay. This data suggested that carnasol also possessed a weak activity on sea urchin, since according to Jacobs et al. (1981), only substances that promote 100% inhibition at a concentration of 16 µg/ml or less, could be considered very active.

The present results show that the abietane diterpenes isolated from *H. martiusii* possess only weak *in vitro* antimitotic activity. Nonetheless, the compounds studied here could be considered as potential lead compounds from which more potent derivatives could be produced by chemical derivatization.

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References

Araujo ECC, Silveira ER, Lima MAS, Andrade Neto M, Andrade IL, Lima MA, Santiago GMP, Mesquita LL (2003) Insecticidal activity and chemical composition of volatile oils from *Hyptis martiusii.* Benth J Agric Food Chem in press.

Brieskorn CH, Fuchs A, Brendenderg JB, McChesney JD, Wenkert E (1964) The structure of carnasol. J Org Chem 29: 1035–1038.

Correa MP (1984) Dicionário de plantas úteis do Brasil e das exóticas cultivadas. Vol. 4., Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal, Brasília, Brasil. p. 707.

Costa-Lotufo LV, Cunha GMA, Farias PAM, Viana GSB, Cunha KMA, Pessoa C, Moraes MO, Silveira ER, Gramosa NV, Rao VSN. (2002) The cytotoxic and embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii* oleo-resin. Toxicon 40: 1231–1234.

Fragoso-Serrano M, Gonzalez-Chimeo E, Pereda-Miranda R (1999) Novel labdane diterpenes from the insecticidal plant *Hyptis spicigera.* J Nat Prod 62: 45–50.

Jacobs RS, White S, Wilson L (1981) Selective compounds derived from marine organisms: effects on cell division in fertilized sea urchin eggs. Fed Proc 40: 26–29.

Kashiwada Y, Wang HK, Nagao T, Kitanaka S, Yasuda I, Fujioka T, Yamagishi T, Cosentino LM, Kozuka M, Okabe H, Ikeshiro Y, Hu CQ, Yeh E, Lee KH (1998) Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. J Nat Prod 61: 1090–1095.

Kingston DG, Rao MM, Zucker WV (1979) Plant anticancer agents. IX. Constituents of *Hyptis tomentosa*. J Nat Prod 496–499.

Kuo YH, Chen CH, Huang SL (1998) New diterpenes from the heartwood of *Chamaecyparis obtusa* var. *formosana.* J Nat Prod 61, 829–831.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 16, 55–63.

Novelo M, Cruz JG, Hernandez L, Pereda-Miranda R, Chai H, Mar W, Pezzuto JM. (1993) Cytotoxic constituents from *Hyptis verticillata*. J Nat Prod 56: 1728–1736.

Pereda-Miranda R, Hernandez L, Villavicencio MJ, Novelo M, Ibarra P, Chai H, Pezzuto JM (1993) Structure and stereochemistry of pectinolides A-C, novel antimicrobial and cytotoxic 5,6-dihydro-alpha-pyrones from *Hyptis pectinata*. J Nat Prod: 583–593.

Pessoa C, Silveira ER, Lemos TGL, Wetmore LA, Moraes MO, Leyva A (2000) Antiproliferative effects of compounds derived from plants of northeast Brazil. Phytother Res 14, 187–191.

Urones J.G., Marcos I.S., Diez D. and Cubilla L.R. (1998), Tricyclic diterpenes from *Hyptis dilatata*. Phytochemistry 48, 1035–1038.

Yamagishi T, Zhang C, Chang JJ, McPhail DR, McPhail, Lee KH (1988) The cytotoxic principles of *Hyptis capitata* and the structures of the new triterpenes hyptatic acid-A and -B. Phytochemistry 27: 3213–3216.