

# Isolation, structural identification and cytotoxic activity of hexanic extract, cyperenoic acid, and jatrophone terpenes from *Jatropha ribifolia* roots

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**Abstract:** The cytotoxicity of a hexanic fraction produced from the ethanolic crude extract, obtained from *Jatropha ribifolia* (Pohl) Baill, Euphorbiaceae, roots was evaluated against ten human cancer cell lines (MCF-7, NCI-ADR/RES, OVCAR-3, PC-3, HT-29, NCI-H460,786-O, UACC-62, K-562, U251) compared with doxorubicine as positive control. Compounds jatrophone and cyperenoic acid were isolated from the hexanic extract and characterized by spectroscopic techniques (NMR of <sup>1</sup>H, <sup>13</sup>C and IR). The *in vitro* antiproliferative activity of jatrophone showed selectivity in a concentration dependent way with Total Inhibition growth of: glioma 0.57 µg mL<sup>-1</sup> (U251), breast cancer 9.2 µg mL<sup>-1</sup> (MCF-7), adriamycin-resistant ovarian cancer 0.96 µg mL<sup>-1</sup> (NCI-ADR/RES), kidney 4.2 µg mL<sup>-1</sup> (786-0), prostate cancer 8.4 µg mL<sup>-1</sup> (PC-3), colon cancer 16.1 µg mL<sup>-1</sup> (HT29) and leukemia 0.21 µg mL<sup>-1</sup> (K-562).

## Article

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## Introduction

Bioactive terpenes have a significant importance for the pharmaceutical industry which covers a wide range of diverse chemical compounds, providing opportunities for synthesis (Wang & Bidigare, 2005). The genus *Jatropha* is recognized as an important source of secondary metabolites, with mainly terpenes that are fairly known to this genus (Can-Aké et al., 2004).

*Jatropha ribifolia* (Pohl) Baill (Euphorbiaceae) is found throughout the Brazilian northeastern region, popularly known as “pinhão-de-purga” (purgin nut). The latex is used by folkmedicine for treatment of snake bites and to treat upper tract decongestions. No previous reports were found describing the phytochemical studies of this species that is considered endemic in the state of Mato Grosso do Sul and known in Naviraí as “minâncora-do-campo” (Souza & Rodal, 2010). Herein we report the isolation and structure elucidation of the compounds jatrophone and cyperenoic acid with *in vitro* antiproliferative activity.

## Materials and Methods

### Plant material

Material was collected at Naviraí, Mato Grosso do Sul, Brazil. Voucher specimen (CGMS 31.481) was deposited at the Herbarium of the Institute of Botany of São Paulo at the University of São Paulo, Brazil.

### Isolation procedures

Fresh roots (1.5 kg) of *Jatropha ribifolia* (Pohl) Baill., Euphorbiaceae, were ground, and extracted with EtOH at room temperature. The solvent was removed under reduced pressure yielding 386 g (25.7%) of crude ethanol extract. The crude extract was further partitioned with *n*-hexane and MeOH-H<sub>2</sub>O (9:1). The crude dried *n*-hexane-soluble fraction (7.75 g) was purified by silica gel column chromatography using gradients of *n*-hexane/EtOAc and EtOAc. Fractions eluted with *n*-hexane:EtOAc (20 and 25%) were grouped and purified by column chromatography yielding jatrophone compound **1** (431.3

mg). Cyperenoic acid was isolated from the crude *n*-hexane extract obtained from freshly picked roots (660 g) further extracted with *n*-hexane under reflux. After removal of the organic solvent under reduced pressure, 8 g of the extract was purified by silica gel column chromatography affording 475.8 mg of compound **2**. The active compounds were monitored by the *in vitro* antiproliferative assay.

### Spectroscopic analysis

The structures determination were based mainly on spectroscopic investigation of  $^1\text{H}$  and  $^{13}\text{C}$  NMR studies including HMBC (hetero nuclear multiple bond correlation) and COSY (proton-proton correlation) experimental. Infrared (IR) spectroscopy was utilized for identifying absorption band and gas chromatography coupled mass spectrometry for the structure mass-charge ( $m/z$ ).

### Biological tests

*In vitro* antiproliferative assay in human cancer cell lines

For the *in vitro* antiproliferative activity screening, ten cell lines were selected from human tumors, designated as: strains K562 (leukemia), MCF-7 (breast), NCI-ADR/RES (ovarian phenotype of resistance to multiple drugs), UACC-62 (melanoma), NCI-H460 (lung), PC 3 (prostate), HT29 (colon), OVCAR-3 (ovarian), 786-0 (kidney) and U251 (glioma), and a normal cell lines: VERO (monkey kidney) later replaced by HaCat (human keratinocyte). All cell lines were provided by the National Cancer Institute (NCI), USA. The experimental procedures were performed according with the literature (Skehan et al., 1990; Monks et al., 1991; Rubinstein et. al., 1990).

Stock solution of the *n*-hexanic extract, jatrophone (**1**) and cyperenoic acid (**2**) compounds ( $100\text{ mg mL}^{-1}$ ) were prepared in dimethylsulfoxide (DMSO). Cell culture DMSO was diluted 400 times in RPMI/FBS/gentamicin, to avoid toxicity. After dilution,  $100\text{ }\mu\text{L}$  of medium containing the extract and compounds to be tested were added to a 96 compartments (except for the control) at concentrations of 0.25, 2.5, 25 and  $250\text{ }\mu\text{g mL}^{-1}$ . The plates were incubated for 24 h at  $37\text{ }^\circ\text{C}$  in a  $5\%$   $\text{CO}_2$  in a humid environment.

Cells were cultured in RPMI-1640 medium ( $10\text{ mL}$ ), supplemented with  $5\%$  fetal calf serum (SFB). The vials were centrifuged ( $2000\text{ g}$ ) by 4 min at  $4\text{ }^\circ\text{C}$ . The supernatant was collected and discarded and precipitated of cells were resuspended in  $5\text{ mL}$  culture medium. After 48 h, the plate T0 was fixed by adding  $50\text{ }\mu\text{L}$  trichloroacetic acid (TCA)  $50\%$  determining the actual amount of cells present at the time the samples were applied. After 48 h

treatment  $50\text{ }\mu\text{L}$  of TCA ( $50\%$ ) was added and incubated for 1 h at  $4\text{ }^\circ\text{C}$ . Then the plates were subjected to four successive washes with water to remove residues of TCA, medium, SFB and secondary metabolites, and then stored at room temperature until complete drying with further addition of  $50\text{ }\mu\text{L}$  of sulforhodamine B (SRB) to  $0.4\%$  (weight/volume) dissolved in  $1\%$  acetic acid, and incubated at room temperature for 30 min. After wards the plates were washed four times consecutively with a solution of  $1\%$  acetic acid for complete removal of residues of SRB. After complete drying the plates at room temperature, the protein bound dye was solubilized by adding  $150\text{ }\mu\text{L}$  of  $10\text{ }\mu\text{M}$  Trizma Base in pH 10.5. The cells were then fixed with  $50\%$  trichloroacetic acid. Cell proliferation was determined by spectrophotometric quantification at  $540\text{ nm}$  using the sulforhodamine B as dyeing reagent. Doxorubicin chloridate ( $0.025$  to  $25\text{ }\mu\text{g mL}^{-1}$ ) was used as positive control (Table 3).

### Date analysis

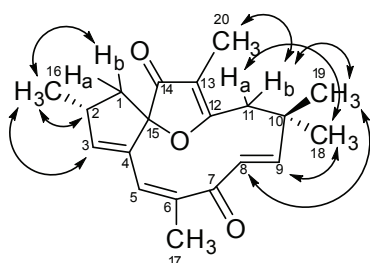
The averages of the absorbance were calculated discounting the value the white and total growth inhibition (TGI) was determined by the equation: if  $T > C$  the drug stimulated the growth and showed not IC; if  $T \geq T_0$  and  $< C$ , the drug was cytostatic and the equation used was  $100 \times [(T - T_0)/(C - T_0)]$ ; is  $T < T_0$  and drug was cytotoxic equation used was  $100 \times [(T - T_0)/(T_0)]$ , being T the absorbance of treated cell, C the cell control and  $T_0$  control cells on day of addition of the drugs, the results was subtracted from  $100\%$  to yield the percentage of growth. The absorbance data were analyzed and compiled in constructing graphs using the Origin 7.5 software correlating the percentage of inhibition or cell death at the concentration of extract and terpenes.

## Results and Discussion

### Isolation and structural identification

The antitumor properties of species of the genus *Jatropha* have been targets of phytochemical studies. No previous reports on *in vitro* antiproliferative activity on human cancer cell lines *Jatropha ribifolia* (Pohl) Baill., Euphorbiaceae, were found. Compound **1** (Figure 1) was obtained as colorless crystals with mp  $142\text{--}144\text{ }^\circ\text{C}$ . The GC/MS spectrum gave an [M-H] ion at  $m/z$  312.1, corresponding to the molecular formula  $\text{C}_{20}\text{H}_{24}\text{O}_3$ . The FTIR spectrum showed absorption at OH  $3449\text{ cm}^{-1}$  indicating the presence of OH group and carbonyl group at  $1697\text{ cm}^{-1}$ . The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy and 2D NMR technique (Table 1) were compared with data reported by Goulart et al., (1993) and Taylor et al. (1983) confirming the structure which is a common compound among the genus *Jatropha*. Compound **2** (Figure 2)

was obtained as colorless crystals with mp 162-164 °C. The GC/MS spectrum gave an [M-H] ion at  $m/z$  234.1 corresponding to the molecular formula  $C_{15}H_{22}O_2$ . The FTIR spectrum showed a broad absorption at  $3430\text{ cm}^{-1}$  due to the presence of OH group and carbonyl group at  $1674\text{ cm}^{-1}$  indicating the presence of carboxylic acid. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy and 2D NMR technique (Table 2) were compared with the data reported by Pertino (2006) for cyperenoic acid, confirming the chemical structure. This is the first time where the presence of this compound was described in this specie. The NOESY spectrums of jatrophone and cyperenoic acid showed the H groups in proximity (Figure 1 and 2) confirming the stereochemical aspects observed by X-ray, for both compounds. (Pertino et. al., 2006; Jacobs et al., 1987).



**Figure 1.** NOESY correlations for jatrophone (1).

**Table 1.** NMR data for jatrophone in  $\text{CDCl}_3$ , with instrument operating at 250 MHz for NMR  $^1\text{H}$  and NMR  $^{13}\text{C}$  and 500 MHz for 2D COSY and HMBC.

Position	$^1\text{H}^b$	$^{13}\text{C}^b$	COSY ( $^1\text{H}$ - $^1\text{H}$ ) <sup>b</sup>	HMBC ( $^{13}\text{C}$ - $^1\text{H}$ )
1	2.15 dd (2.15)	42.461 (42.45)	H-1b, H-2 (H-1b, H-2)	-
	1.78 dd (1.79)		H-1a, H-2, H-3 (H-1a, H-2, H-3)	
2	2.98 m (3.00)	38.342 (38.30)	H-1a, H-1b, H-3 (H-1a, H-1b)	H-16
3	5.79 m (5.77)	123.760 (123.07)	H-1b, H-2 (H-1b)	H-17
4	-	137.078 (137.10)	-	H-1a, H-3, H-5
5	5.79 m (5.77)	147.143 (147.03)	-	H-1a, H-16
6	-	141.764 (141.75)	-	H-8, H-17
7	-	202.041 (201.84)	-	H-9, H-17
8	5.95 d (5.98)	128.722 (128.71)	H-9 (H-9)	-
9	6.46 d (6.48)	159.051 (158.71)	H-8 (H-8)	H-11b, H-18, H-19
10	-	36.642 (36.59)	-	H-11a, H-11b, H-8, H-18, H-19
11	2.90 d (2.91) 2.42 d (2.44)	41.237 (41.25)	-	H-18, H-19
12	-	183.305 (183.12)	-	H-11a, H-11b, H-20
13	-	112.424 (112.36)	-	H-11b, H-20
14	-	203.967 (203.78)	-	H-20
15	-	99.799 (99.75)	-	H-1a, H-3
16	1.09 d (1.09)	18.974 (18.93)	H-2 (H-2)	H-1b
17	1.87 s (1.87)	20.738 (20.68)	-	-
18	1.24 s (1.26)	30.402 (30.38)	H-19 (H-19)	H-11a, H-19
19	1.36 s (1.37)	26.909 (26.89)	H-18 (H-18)	H-11b, H-18
20	1.73 s (1.74)	6.105 (6.02)	H-11b	-

<sup>b</sup>Data in parenthesis from literature (Goulart et al., 1997).

#### In vitro antitumour activity

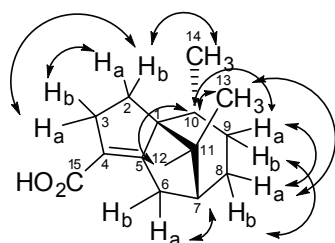
This is the first report exploring the *in vitro* antiproliferative activities effect of *J. ribifolia* extract and two isolated terpenic compounds, jatrophone and cyperenoic acid against ten human cells lines and normal monkey cells (VERO). A range of *J. ribifolia* roots extract and terpenes concentration ( $0.25\text{ }\mu\text{g mL}^{-1}$  to  $250\text{ }\mu\text{g mL}^{-1}$ ) were used to investigate the relative degree of TGI against the following cell line: glioma (U251), melanoma (UACC-62), breast cancer (MCF7), adriamycin-resistant ovarian cancer (NCI-ADR/RES), kidney (786-0), non-small lung cancer (NCI-H460), ovarian cancer (OVCAR-3), prostate cancer (PC-3), colon cancer (HT29), leukemia (K-562) and normal green monkey kidney cells (VERO). Figure 3 shows the curves of the concentration response for the doxorubicin control drug, hexanic extract, cyperenoic acid and jatrophone terpenes against human tumor cells. Table 3 presents the results determined by the *in vitro* antiproliferative assay with human cancer cell lines.

The activity of hexanic extract was efficient for seven cell lines tested, when compared with doxorubicine, with an *in vitro* antiproliferative activity ranging from 1.2 to  $26.5\text{ }\mu\text{g mL}^{-1}$ . The hexanic extract's cytotoxic activity against tumoral cells growth was significantly efficient

**Table 2.** NMR data for cyperenoic acid in CDCl<sub>3</sub>, with instrument operating at 250 MHz for NMR <sup>1</sup>H and NMR <sup>13</sup>C and 500 MHz for 2D COSY and HMBC.

Position	<sup>1</sup> H <sup>b</sup>	<sup>13</sup> C <sup>b</sup>	COSY ( <sup>1</sup> H- <sup>1</sup> H)	HMBC ( <sup>13</sup> C- <sup>1</sup> H)
1	-	68.20 (68.23)	-	H-2a, H-2b, H-10
2	1.78 m (1.78) 1.58 m (1.58)	25.71 (25.74)	H-2b, H-3b H-2a, H-3a, H-14	H-16
3	2.83 m (2.83) 2.75 m (2.75)	36.31 (36.33)	H-2b, H-3b H-2a, H-3a	H-2a, H-2b,
4	-	123.07 (123.19)	-	-
5	-	173.09 (173.10)	-	-
6	2.78 m (2.77) 2.28 brd (2.28)	31.30 (31.34)	H-6b, H-7 H-6a	-
7	2.00 m (2.00)	48.15 (48.20)	H-6a, H-8B	H-9a, H-12, H-13
8	1.91 m (1.92) 1.42 m (1.41)	26.91 (26.96)	H-8b, H-9b H-7, H-8a, H-9a, H-9b	H-9b, H-13
9	1.55 m (1.55) 1.17 m (1.17)	27.86 (27.90)	H-9b, H-8b, H-10 H-8a, H-8b, H-9a	H-10, H-14
10	2.10 m (2.10)	35.96 (36.00)	H-9a	H-2a, H-2b, H-9a, H-9b, H-14
11	-	41.70 (41.73)	-	H-2a, H-10, H-12, H-13
12	0.85 s (0.86)	26.19 (26.22)	-	H-13
13	1.03 s (1.03)	19.26 (19.28)	-	H-12
14	0.88 d (0.90)	17.96 (17.99)	H-2b	H-10, H-9 b
15	0.85 s (0.86)	170.71 (171.05)	-	-

<sup>b</sup>Data in parenthesis from literature (Pertino et al., 2006).



**Figure 2.** NOESY correlations for cyperenoic acid (2).

for seven strains. The necessary concentration for Total growth Inhibition (TGI) for tumoral cells were 1.2 µg mL<sup>-1</sup> (U251), 15.5 µg mL<sup>-1</sup> (MCF-7), 9.5 µg mL<sup>-1</sup> (NCI-ADR/RES), 9.1 µg mL<sup>-1</sup> (786-0), 12.3 µg mL<sup>-1</sup> (PC-3), 26.5 µg mL<sup>-1</sup> (HT29) and 2.4 µg mL<sup>-1</sup> (K-562). The best selectivity was observed with glioma (U251) with 1.2 µg mL<sup>-1</sup>, above this concentration was observed cell death of VERO cells

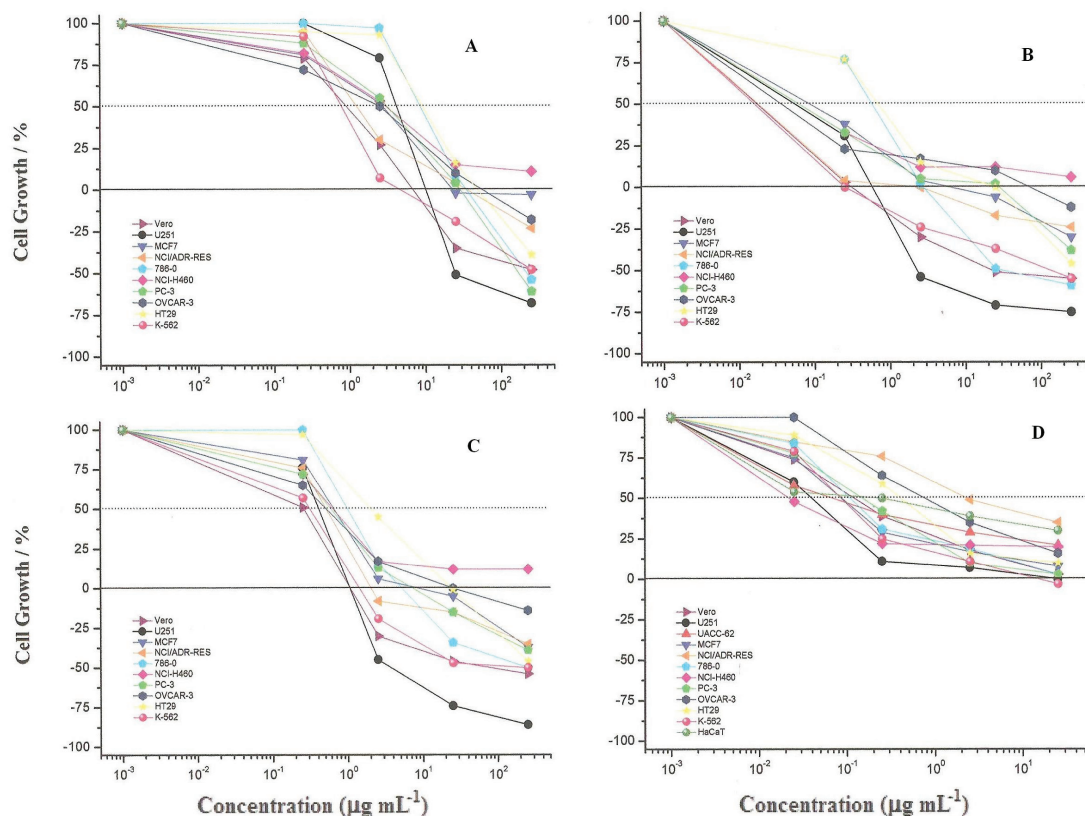
(normal cell) with 1.7 µg mL<sup>-1</sup>. The activity on tumoral cells was also observed for the crude hexane extract due to the presence of jatrophone and cyperenoic acid. The terpenes when isolated also showed significant results in the bioassay under study. The cytotoxic activity for cyperenoic acid against tumoral growth cells was efficient for three tested cells. The TGI for tumoral cells were 11.4 µg mL<sup>-1</sup> (U251), 25.1 µg mL<sup>-1</sup> (PC-3) and 10.5 µg mL<sup>-1</sup> (K-562). For VERO cells 9.5 µg mL<sup>-1</sup> was the concentration determined. Better results were noted for jatrophone (1) with selectivity in a concentration dependent way determined as 0.57 µg mL<sup>-1</sup> (U251), 9.2 µg mL<sup>-1</sup> (MCF-7), 0.96 µg mL<sup>-1</sup> (NCI-ADR/RES), 4.2 µg mL<sup>-1</sup> (786-0), 8.4 µg mL<sup>-1</sup> (PC-3), 16.1 µg mL<sup>-1</sup> (HT-29) and 0.21 µg mL<sup>-1</sup> (K-562) and VERO (normal cells). Doxorubicin drug was efficient to total growth inhibition in concentration 25 µg mL<sup>-1</sup>.

**Table 3.** Efficacy of organic extract and terpenes obtained from roots of *Jatropha ribifolia* and positive control doxorubicin against human tumor cells - Total Growth Inhibition (TGI).

	TGI (µg.mL <sup>-1</sup> )									
	2	m	a	7	4	p	o	h	k	v
Hexanic extract	1.2	15.5	9.5	9.1	>250	12.3	37.5	26.5	2.4	1.7
Cyperenoic acid	11.4	86.9	41.2	41.0	>250	25.1	65.6	57.9	10.5	9.5
Jatrophone	0.57	9.2	0.96	4.2	>250	8.4	55.8	16.1	0.21	0.21
Doxorubicin	25	>25	>25	>25	>25	25	>25	>25	25	>25

2: U251 (glioma, SNC); m: MCF-7 (breast); a: NCI-ADR/RES (ovarian phenotype resistance to multiple drugs); 7: 786-0 (kidney); 4: NCI-H460 (lung); p: PC-3 (prostate); o: OVCAR-3 (ovarian); h: HT-29 (colon); k: K562 (leukemia); v: Vero (monkey kidney epithelial cell).





**Figure 3.** Cytotoxic activity profile of doxorubicin in cultured human tumor cells. Cyperenoic acid (A); jatrophone (B); hexanic extract (C) and doxorubicin (D) in cultured human tumor cells.

Further studies are necessary to better understand the toxic effects toward normal cells. These terpenes were isolated in other Euphorbiaceae species (Pertino et al., 2006), nevertheless no antiproliferative activities test were reported for cell lines mentioned herein. Previous authors evaluated activities against epithelial gastric cell line (AGS) and gastroprotection effect (Pertino et al., 2006; Fröhlich et al., 2010). Cyperenoic acid (2) has been also isolated from different species of plants as *Sandwithia guyanensis* (Jacobs et al., 1987) *Croton crassifolius* (Boonyaratavej et al., 1988) and from *Joannesia princeps* which was evaluated for antifungic activity (Fröhlich et al., 2010). The preliminary results presented here show that jatrophone (1) and cyperenoic acid (2) could be a promising molecule to study for the development of a new cancer treatment.

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#### Authors' contributions

ESF, FAR, and DT (graduation students)

contributed in collecting plant sample and identification and isolation, purification of the extracts of the isolated compounds. PMI contributed to purification and spectroscopic analysis. JEC, ALTGR and MAF contributed to in vitro antiproliferative assay and interpretation. SM and RCLS contributed to EF student orientation and analysis of the results and writing of the manuscript. All the authors have read the final manuscript and approved the submission.

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