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Antiproliferative activity of methanol extracts of four species of *Croton* on different human cell lines

Jóice P. Savietto,¹ Cláudia M. Furlan,¹ Lucimar B. Motta,¹ Maria Luíza F. Salatino,¹ João E. Carvalho,² Ana Lucia T. G. Ruiz,² Antonio Salatino,¹ Déborah Yara A. C. Santos^{*1}

¹Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, Brazil,

²Divisão de Farmacologia e Toxicologia, CPQBA, Universidade Estadual de Campinas, Brazil.

Abstract: Several species of *Croton* have been described with biological activities, mainly due to diterpenes, alkaloids and/or other secondary metabolites. These activities account for the traditional use of *Croton* species to treat certain diseases in South America, Asia and Western Africa. The crude methanol extracts obtained from leaves and stem bark of *Croton dichrous* Müll. Arg., *C. erythroxyloides* Baill., *C. myrianthus* Müll. Arg. and *C. splendidus* Mart. ex Colla were tested for antiproliferative activity against ten human cancer cell lines. Chemical analyses of all extracts were carried out by GC/MS and HPLC/MS/MS. The leaf extract obtained from *C. erythroxyloides* showed potent activity against PC-3 (prostate) and OVCAR-3 (ovary) cell lines. Lupeol is suggested to be involved in such activity. Tiliroside, an acyl-glycosilated flavonoid ubiquitous in all tested extracts, seems to play an important role in the observed moderate activity of most extracts against the leukemia K562 cell lineage.

Introduction

Several species of Euphorbiaceae are known in many parts of the world for their toxic and/or medicinal properties (Salatino et al., 2007). Such diversity of effects is a reflection of the variety of secondary metabolites which have already been described for this family.

Croton L. is one of the giant genera of angiosperms, comprising over 1200 species of herbs, shrubs, trees and occasionally vines. Recent studies have estimated that approximately two-thirds of the *Croton* species occur in the New World, the other third being distributed across the Old World (van Ee et al., 2011). It is the second largest genus in Euphorbiaceae, with various species used in folk medicine in many regions of the world.

Hypoglycemic, hypolipidemic, anti-estrogenic, anti-tumor and trypanocidal effects have been ascribed to the *trans*-terpenoid dehydrocrotonin obtained from *C. cajucara* (Maciel et al., 2002; Campos et al., 2010). Antiulcerogenic effect in gastric lesion of the hydroalcoholic extract of *C. campestris* leaves has been demonstrated in laboratory assays with mice (Júnior et al. 2013). Both latex and the alkaloid taspine, isolated from *C. lechleri*, show cytotoxic activity (Montopoli et al., 2012), and plaunotol,

an open-chain diterpene found in *Croton* species, acts in synergism with common drugs against *Helicobacter pylori* (peptic ulcer-causing bacteria) (Sasaki et al., 2007) and as antiangiogenic (Kawai et al., 2005).

The biological activities of these compounds account for the traditional use of *Croton* species to treat certain diseases in South America, Asia and Western Africa (Sampson et al., 2000). Cancer is a disease recognized by several hallmarks, one of the main ones being the proliferation of atypical cells (Mantovani, 2009). According to the World Health Organization, cancer is a leading cause of death worldwide. The organization estimates that 84 million people will die from cancer between 2005 and 2015 (WHO, 2007).

The potential use of natural products against cancer has been recognized since 1950 by the National Cancer Institute (USA) (Cragg & Newman, 2005). Some important drugs currently available were discovered by the investigation of secondary metabolites of plants, some examples being taxol, extracted from the stem of *Taxus brevifolia*, Taxaceae (Wani et al., 1971) and vincristine, an alkaloid from the roots of *Catharanthus roseus*, Apocynaceae (Johnson et al., 1963).

The antiproliferative activity of crude extracts of some species of *Croton* have already been described.

Stem bark essential oil obtained from *C. lechleri* showed mutagen-protective efficacy (Rossi et al., 2013) and crude extracts of stems of *C. cajucara* (Maciel et al., 2007) exert antitumor activity against the K562 leukemic cell line. The dichloromethane extract of *C. macrobotryis* leaves exhibited antiproliferative activity against several cell lines, in particular on NCI-H460 (lung) and K562 (leukemia) (Motta et al., 2011).

In this study, we investigated the antiproliferative activity of methanol extracts from four Brazilian native species of *Croton*, and suggest compounds involved in the observed effects. For this, ten human tumor cell lines commonly investigated were used: 786-0 (kidney), HT-29 (colon), K562 (leukemia), NCI-ADR/RES (drug resistant ovary), NCI-H460 (lung), MCF-7 (mammary), PC-3 (prostate), OVCAR-3 (ovary), U251 (glioma), UACC-62 (melanoma).

Materials and Methods

Plant material

Samples of *Croton dichrous* Müll. Arg. (Cordeiro 3066, Campos do Jordão-SP), *C. erythroxyloides* Baill. (Cordeiro 3067, Campos do Jordão-SP), *C. myrianthus* Müll. Arg. (Lima 296, Camará do Sul-RS) and *C. splendidus* Mart. ex Colla (Cordeiro 3041, Santa Barbara-MG) were collected and voucher specimens were deposited in the Herbarium of the Institute of Botany - São Paulo (SP), and in the Herbarium of the Institute of Biosciences, São Paulo (SPF).

Crude extract preparation and analysis

Dried leaves and stems (2 g) were powdered separately, and extracted with methanol under reflux for 1 h (three times). The extracts were pooled and dried under reduced pressure. An aliquot of 10 mg of the dried extract was used for the antiproliferative tests. The samples were submitted to HPLC, HPLC/MS/MS and GC/MS analysis.

HPLC and HPLC/MS/MS

The methanol extracts were analysed by HPLC-DAD (Agilent - HP 1090 II) using a reverse phase column (Zorbax C18 - 4.6 x 250 mm, 5 µm) and a gradient of 0.1% acetic acid (solvent A) and acetonitrile (solvent B) with the following program: 0-5 min 12% of B; 5-8 min 12% - 20% of B; 8-28 min 20% of B; 28-38 min 20% - 50% of B; 38-48 min 50% - 65% of B. The solvent flux was 0.5 mL.min⁻¹, the column temperature was 40 °C and the detection at λ=352 nm (Motta et al., 2009). Samples were also analysed by HPLC/MS/MS (Shimadzu M10AVP/Esquire 3000 Plus,

Bruker Daltonics). The analysis was developed under the same conditions, but the solvent flux of 90 µL.min⁻¹. The mass spectrometer parameters involved skimmer voltage at 4000 V, nebulizer at 27 psi, dry gas at 320 °C with flux of 7,0 L/min. Major compounds identification used MS information and the available private library constructed in the Phytochemistry Lab, Department of Botany, University of São Paulo.

GC/MS analysis

The methanol extracts were submitted to GC/MS (Agilent GC/MS 6859/5975B) employing a DB-5 HT column (30 m x 0,25 mm x 0,25 µm) and He as carrier gas at 1.2 mL.min⁻¹. The injector temperature was 250 °C, and the heating column program was: 150 °C, 1 min, heating ramp of 6 °C.min⁻¹ until 310 °C, keeping this temperature for 3 min. The samples were analysed in the splitless mode. The temperatures of the MS source and the quadrupole were 230 and 150 °C, respectively. The ions were detected in the positive mode, with 2.7 scan.sec⁻¹, in a mass range of 40-450 a.m.u. The electron multiplier voltage was adjusted to 70 eV. The compounds identification followed comparison with NIST 05 MS Library Bundle (Agilent).

Cell cultures

Ten human tumor cell lines were used [786-0 (kidney), HT-29 (colon), K562 (leukemia), NCI-ADR/RES (drug resistant ovary), NCI-H460 (lung), MCF-7 (mammary), PC-3 (prostate), OVCAR-3 (ovary), U251 (glioma), UACC-62 (melanoma)] and were kindly provided by National Cancer Institute at Frederick MA-USA (NCI). Also a non-tumor cell line (VERO, renal, green monkey) was used. Stock cell cultures were grown in medium containing RPMI 1640, supplemented with 5% of fetal bovine serum. Experimental cultures were supplemented also with peniciline:streptomycine (10 µg/mL:10 UI/mL).

Antiproliferative assay

Cells (100 µL cells/well, inoculation density from 3-6 x10⁴ cell/mL) in 96-well plates were exposed to various sample concentrations (0.25 to 250 µg/mL, 100 µL/well) in DMSO/RPMI 1640 at 37 °C, 5% of CO₂ in air for 48 h. Final DMSO concentration (0.2%) did not affect cell viability. Cells, before (T₀) and after 48 h exposition (T₁), were then fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric quantification of cellular protein content at 540 nm, using the sulforhodamine B assay. Doxorubicin (DOX; 0.025, 25 µg/mL) was used as positive control. Three measurements were obtained at the beginning of incubation (time zero, T₀) and 48 h post-incubation for compound-free (T₁) and tested (T) cells. Cell proliferation was determined according to

the equation $100 \times [(T-T_0) / (T_1-T_0)]$, for $T_0 < T \leq T_1$, and $100 \times [(T-T_0)/T_0]$, for $T \leq T_0$ and a concentration-response curve for each cell line was plotted using software Origin 7.5 (OriginLab Corporation) (Monks et al., 1991).

Data analysis

Using the concentration-response curve for each cell line, the GI50 (concentration causing 50% growth inhibition) (Shoemaker, 2006) of each cell line was determined by non-linear regression analysis, using software Origin 7.5. The average activity (mean of log GI50) of the analysed extracts was also determined using MS Excel software (Fouche et al., 2008). The average activity is a parameter used by the National Cancer Institute to classify the test-extract as: I: inactive (mean of log GI50 >1.5); W: weak activity (mean of log GI50 >1.1-1.5); M: moderate activity (mean of log GI50 >0-1.1); and P: potent activity (mean of log GI50 <0).

Results and Discussion

Table 1 presents the results of antiproliferative activity obtained with the methanol extracts of *Croton dichrous* Müll. Arg., *C. erythroxyloides* Baill., *C. myrianthus* Müll. Arg. and *C. splendidus* Mart. ex Colla against human tumor cell lines. Using the National Cancer Institute criteria, which means looking through the mean value of the logarithm of GI50 for all the tumor cell lines used, except the non tumor VERO, only the extract obtained from leaves of *C. erythroxyloides* showed moderate activity (mean of log GI50=1.00) against cancer cells. This extract also presented a high selectivity for PC-3 (prostate, GI50=0.80 $\mu\text{g.mL}^{-1}$) and OVCAR-3 (ovarian, GI50=0.81 $\mu\text{g.mL}^{-1}$) cell lines. Based on the NCI criteria, these individual GI50 values indicate potent activity (log GI50 <0), similar to doxorubicin, an effective drug used in cancer control.

Although an excellent growth inhibitor of cancer cells, doxorubicin also affects the growth of non-tumor cells, for example, the ones from the VERO cell line (Table 1). In this regard, all assayed *Croton* extracts seem to be safer than doxorubicin, since toxicity to healthy cells had been reached only with high concentrations. For example, the extract of *C. erythroxyloides* leaves, the most active against cancer cell lines, reaches an GI50 higher than 250 $\mu\text{g.mL}^{-1}$ for the VERO cell line.

On the other hand, extract of stems of *C. splendidus* had the lowest inhibition potential (mean of log GI50=1.64), while all other six extracts presented at least a weak antiproliferative activity (mean of log GI50 from 1.20 to 1.48) (Table 1). Among leaf extracts, *C. dichrous* extract showed a slightly selectivity for MCF-7 (breast, GI50=16.07 $\mu\text{g.mL}^{-1}$) cell line, *C. splendidus* extract presented some selectivity against H460 (lung,

GI50=6.08 $\mu\text{g.mL}^{-1}$) and K562 (leukemia, GI50=8.00 $\mu\text{g.mL}^{-1}$) cell lines, while *C. myrianthus* was even more selective for MCF-7 (breast, GI50=1.29 $\mu\text{g.mL}^{-1}$) and U251 (glioma, GI50=1.34 $\mu\text{g.mL}^{-1}$) cell lines. Looking at the stem extracts, *C. dichrous* extract showed selectivity for K562 (leukemia, GI50=2.11 $\mu\text{g.mL}^{-1}$) and OVCAR-3 (ovarian, GI50=9.00 $\mu\text{g.mL}^{-1}$) cell line, *C. myrianthus* was more selective for U251 (glioma, GI50=3.34 $\mu\text{g.mL}^{-1}$), K562 (leukemia, GI50=4.05 $\mu\text{g.mL}^{-1}$), MCF-7 (breast, GI50=4.19 $\mu\text{g.mL}^{-1}$) and OVCAR-3 (ovarian, GI50=6.60 $\mu\text{g.mL}^{-1}$) cell lines, and *C. erythroxyloides* extract presented some selectivity against K562 (leukemia, GI50=7.48 $\mu\text{g.mL}^{-1}$), U251 (glioma, GI50=8.51 $\mu\text{g.mL}^{-1}$) and PC-3 (prostate, GI50=9.28 $\mu\text{g.mL}^{-1}$) cell lines.

Manthey & Guthrie (2002) proposed that the activity of extracts with GI50 values lower than 10 $\mu\text{g.mL}^{-1}$ should be regarded as strong. Considering this new perspective, only two extracts, naming the leaf extract of *C. dichrous* and the stem extract of *C. splendidus*, showed no significant activity against the cell strains analyzed (Table 1). The stem extract of *C. myrianthus* also provided promising results toward four cell lines (U251, glioma; MCF-7, breast; OVCAR-3, ovary; K562, leukemia). Five of the eight extracts tested showed antiproliferative activity against K562 (leukemia). Notwithstanding, no extract presented considerable activity against strains UACC-62 (glioma), ADR/RES (resistant ovarian), 786-0 (kidney) and HT-29 (lung) (Table 1).

All methanol extracts were submitted to HPLC and GC/MS analysis. Table 2 shows the major compounds identified. Major compounds detected by HPLC analysis were flavonoids. Apigenin dihexoside was detected in leaf extracts of *C. dichrous*, *C. myrianthus* and *C. erythroxyloides* and tiliroside (kaempferol-*p*-coumaroylglucoside) was ubiquitous. These results suggest that these flavonoids are not key compounds accounting for the better antiproliferative effects of *C. erythroxyloides*, since they are present also in extracts with weak or no growth inhibition activity, sometimes in higher contents. In addition, *C. dichrous*, a species with no promising antiproliferative activity (Table 1), contains significant amounts of these flavonoids.

Apigenin glycosides from chamomile (*Matricaria chamomilla* L.) have been reported as having antiproliferative activity, but much lower in comparison with the corresponding aglycone (Srivastava & Gupta, 2007).

A study conducted with flavonoids from *Citrus* demonstrated loss of antiproliferative activity by different aglycones when they were glycosylated (Manthey & Guthrie, 2002). However, Rao et al. (2007) demonstrated a potent activity of tiliroside against Jurkat (lymphocytic leukemia) and HepG2 (hepatoma) cancer cell lines. Interestingly, for the leukemia K562 lineage, used in our study, there seems to be a correlation between tiliroside

Table 1. Extract concentration ($\mu\text{g.mL}^{-1}$) needed to 50% of growth inhibition of human cancer cell lines.

Samples	Cell lines ^a											Mean log GI50 ^b
	VERO	U251	UACC-62	MCF-7	ADR/RES	786-0	H460	PC-3	OVCAR-3	HT-29	K562	
Doxorubicin ^c	0.66	0.025	0.028	0.14	0.093	0.034	<0.025	0.052	0.12	0.033	0.054	-1.31 P
	Leaf extracts											
<i>C. dichrous</i>	26.43	26.86	22.21	16.07	23.38	60.9	28.72	25.06	27.95	79.63	26.5	1.48 W
<i>C. erythroxyloides</i>	>250	5.32	24.42	17.49	11.66	87.82	174.31	0.80	0.81	21.95	1.86	1.00 M
<i>C. myrianthus</i>	93.37	2.34	44.06	1.29	14.21	31.47	29.59	29.13	10.67	56.15	29.13	1.20 W
<i>C. splendidus</i>	88.03	27.6	26.36	25.32	26.05	30.03	6.08	28.79	28.65	40.35	8.00	1.34 W
	Stem extracts											
<i>C. dichrous</i>	>250	24.83	34.38	17.49	10.05	93.78	33.78	35.71	9.00	82.76	2.11	1.34 W
<i>C. erythroxyloides</i>	191.2	8.51	25.44	29.88	23.36	49.14	16.08	9.28	20.44	64.64	7.48	1.30 W
<i>C. myrianthus</i>	>250	3.34	31.24	4.19	27.36	191.7	212.56	13.88	6.66	27.95	4.05	1.27 W
<i>C. splendidus</i>	133.9	10.25	30.03	69.67	28.3	143.9	49.48	55.87	45.38	199.8	11.06	1.64 I

^aCell lines: VERO: kidney epithelial cells of African green monkey (normal cells - control); U251: glioma; UACC-62: melanoma; MCF-7: mammary; ADR/RES: drug resistant ovary; 786-0: kidney; H460: lung; PC-3: prostate; OVCAR-3: ovary; HT-29: colon; K562: leukemia. ^bNational Cancer Institute criteria (Fouche et al., 2008): I: inactive, mean log GI50>1.5; W: week activity, mean log GI50>1.10-1.5; M: moderate activity, mean log GI50>0-1.1; P: potent activity, mean log GI50<0. ^cPositive control.

content and antiproliferative activity (Table 1). Most stem extracts have over 50% of tiliroside and moderate activity against K562 cell line (Table 2). Even *C. splendidus*, with lower tiliroside content, inhibited the growth of this cell line at a moderate rate. The leaf extract of *C. erythroxyloides* had the highest activity toward leukemia line (GI50=1.86 $\mu\text{g.mL}^{-1}$; Table 1) and the highest concentration of tiliroside (Table 2). A test of the activity of tiliroside against K562 cells would be interesting.

Vitexin appears only in the leaves of *C. splendidus*, which has moderate activity against K562 (log GI50=0.90) and H460 (log GI50=0.78). This compound was isolated from a medicinal plant from cerrado, *Luehea candicans*, Tiliaceae, which showed antiproliferative activity against various cell lines, including K562 (Silva et al., 2012).

The GC/MS analysis unveiled the triterpene lupeol in extracts of *C. erythroxyloides*. As mentioned before, only these extracts showed moderate antiproliferative activity based on NCI criteria (Table 1). In fact, antitumor activity of lupeol has been

demonstrated against several cancer lines, including ovarian (Chaturvedula et al., 2004) and prostate (Prasad et al., 2005). The stem extract of the same species also contains lupeol, although in smaller amounts than in leaves. Nevertheless, its activity against PC-3 is moderate (log GI50=0.97) and higher than the activities of stem extracts from other species.

On the other hand, β -sitosterol was detected in five extracts and, besides its known effect at treatment of benign prostate hyperplasia (Scholtysek et al., 2009), no clear correlation was noted between the presence of β -sitosterol in analyzed *Croton* species and antiproliferative activity against PC-3.

Among the leaf and stem extracts of the four studied species of *Croton*, the leaf extract of *Croton erythroxyloides* seems to be a promising source of substances against at least two different cancer cell lines with the additional benefit of exhibiting low activity against normal cells. This finding turns *C. erythroxyloides* a strong candidate for further in-depth studies about antiproliferative activity.

Table 2. Main compounds from methanol leaf and steam extracts of four species of *Croton*. The values correspond to percentage amounts at each extract.

Rt (min)	Compounds	<i>C. dichrous</i>		<i>C. erythroxyloides</i>		<i>C. myrianthus</i>		<i>C. splendidus</i>	
		L	S	L	S	L	S	L	S
GC analysis									
22.10	β -sitosterol	9.8	22.6	9.1	7.8	6.9			8.2
22.77	lupeol			30.6	8.0				
HLPC analysis									
15.20	apigenin dihexoside	7.5		12.3		15.3			
20.60	vitexin							31.9	
41.49	tiliroside	14.0	56.6	37.7	59.0	27.3	72.5	8.6	38.1

Rt: retention time; L: leaf extract; S: steam extract.

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Authors' contributions

JPS (MS student) contributed in collecting plant sample, running the laboratory work, analysis of the data and drafted the paper. CMF contributed in intellectual discussion during all development of the project and critical reading of the manuscript. LBM contributed in collecting plant sample, in intellectual discussion during all development of the project and critical reading of the manuscript. MLFS contributed in intellectual discussion during all development of the project. JEC contributed with discussion of the results related to antiproliferative assays. ALTGR contributed running the laboratory work of antiproliferative assays, discussion of the results, and critical reading of the manuscript. AS was the main researcher of the project, applied for provision of funds, contributed in intellectual discussion during all development of the project and critical reading of the manuscript. DYACS contributed with JPS orientation at MS thesis, supervised the laboratory work, with intellectual discussion during all development of the project, critical reading of the manuscript and final submission.

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*Correspondence

Déborah Yara A. C. dos Santos
Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo
Rua do Matão, 277. 05508-090 São Paulo-SP, Brazil
dyacsan@ib.usp.br
Tel: +55 11 30918065
Fax: +55 11 30917547