Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
21(6): 972-977, Nov./Dec. 2011

# Constituents and antiproliferative activity of extracts from leaves of *Croton macrobothrys*

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*Article*

Received 10 Nov 2010
Accepted 16 Feb 2011
Available online 23 Sep 2011

**Keywords:**
alkaloids
antiproliferative activity
diterpenes
medicinal plants

ISSN 0102-695X
http://dx.doi.org/10.1590/S0102-695X2011005000174

**Abstract:** *Croton macrobothrys* Baill, Euphorbiaceae, is a tree from the Atlantic Forest in Southern Brazil, used in traditional medicine and popularly known as "dragon's blood" and "pau-sangue". Leaf *n*-hexane, dichloromethane and methanol extracts were analyzed by GC/MS and evaluated for their *in vitro* antiproliferative activity on cell lines 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) and UACC-62 (melanoma). The diclorometane extract exhibited activity against all cell lines at the concentration 25 µg/mL, in particular on cell lines NCI-H460 (GI50 0.33 µg/mL) and K5662 (GI50 0.91 µg/mL). Relevant constituents in dichloromethane extract are the alkaloids corydine and salutaridine, as well as the diterpenes geranylgeraniol and crotonin-derived clerodanes.

## Introduction

Over 85000 plant species have been documented as used worldwide in traditional medicine. The World Health Organization (WHO) estimates that almost 75% of the world's population employs plant-based traditional remedies (Liu et al., 2008). Over 60% of the currently used anticancer chemotherapeutics are derived in one way or another from plants (Cragg & Newman, 2009; Tan et al., 2006). The major categories of plant-derived compounds with anticancer properties are alkaloids and terpenoids (Gupta et al., 2005); examples are vincristine and vinblastine from *Catharanthus roseus* (L.) G. Don (Johnson et al., 1963; Carvalhaes et al., 2002) and taxol and docetaxel from *Taxus brevifolia* Nutt. (Wani et al., 1971).

*Croton* is a genus of Euphorbiaceae comprising around 1300 species, widespread in tropical regions of the New and Old Worlds. Several species have a long role in traditional medicine in Africa, Asia and South America (Salatino et al., 2007; Perazzo et al., 2007). Red latex of *C. draconoides* Mull. Arg., *C. lechleri* Müll. Arg., *C. palanostigma* Klotzsch and *C. urucurana* Baill., popularly known as "sangre de drago" (dragon's blood), are used medicinally by indigenous and rural communities (Riina, et al., 2009). In upper Amazonia, *Croton* latex is used to treat tuberculosis and cancer, sometimes combined with other medicinal plants (*e.g.*, uña-de-gato, or *Uncaria tomentosa* (Willd. ex Roem. & Schult.) DC. (Maxwell, 1990). *C. palanostigma* has been used by indigenous people of the region of Pucallpa (Peru) to treat tumors (Jones 2003). The red latex of *C. lechleri* has been shown to have anti-tumor activity (Rossi et al., 2003). Shoots of *C. hieronymi* Griseb. have been shown to have strong activity against lung A-549 carcinoma cells, mouse lymphoma and human colon carcinoma (Catalán et al., 2003). The dichloromethane extract of leaves of *C. zambesicus* Müll. Arg. showed *in vitro* cytotoxicity against human cervix carcinoma cells (Block et al., 2002). Croton species are abundant sources of active substances against cancer, such as diterpenoids (clerodane, furoclerodane and acyclic diterpenes) and alkaloids (*e.g.* taspine) (Salatino et al., 2007).

*Croton macrobothrys* Baill. is a tree from the Atlantic Forest in Eastern Brazil, popularly known as "dragon's blood", "pau-sangue" and "sangue-de-drago-de-folha-miúda" and used in the treatment of several diseases (Caruzo, 2005 and Gouveia et al., 2007). The purpose of this study was to identify relevant constituents of leaf extracts of *C. macrobothrys* and evaluate their *in vitro* antiproliferative activity against several malignant tumor cell lines.

## Materials and Methods

*Plant material*

Leaves of *Croton macrobothrys* Baill, Euphorbiaceae were collected in April 2009, in the reserve of the Atlantic Forest of Paranapiacaba, municipality of Santo André-SP (southeast Brazil). A voucher specimen (Lima 999) was deposited in the Herbarium Maria Eneyda P. K. Fidalgo (SP) of the Institute of Botany (SEMA, São Paulo state).

*Preparation of plant extracts*

Material (30 g) from dried powdered leaves was extracted in Soxhlet sequentially with *n*-hexane, dichloromethane and methanol, for 6 h with each solvent. The extracts were concentrated under reduced pressure and evaporated to dryness under nitrogen flow.

*GC/MS analyses*

Identification of the extract constituents of was performed with 1 µL of 2 mg/mL extracts by gas chromatography/mass spectrometry, using a system Hewlett-Packard 6890/5975B, DB-5ht fused silica capillary column (30 m x 0.32 mm x 0.10 µm) and helium as carrier gas at 1 mL/min. The temperature program of the column started at 150 ºC (1 min), rising 6 ºC/min to 320 ºC; injector and detector temperatures were 250 ºC. Mass spectra were obtained at 70 eV and scans ranged from 40 to 450 daltons at 2 scans/s (modified from Tansakul et al.,1998). Identification of the compounds followed comparisons of mass spectra with NIST library and literature data (Ichimura et al., 1986; Zdero et al., 1992; Pizzoferrato et al. 1993; Pereira et al. 1999; Quintana et al., 2003; Souza et al., 2006).

*Antiproliferative activity assays*

Cell cultures

Cancer cell lines used were human 786-0 (kidney), HT-29 (colon), K562 (leukemia), NCI-ADR/RES (drug resistant ovary), NCI-H460 (lung), MCF-7 (mammary), NCI-PC-3 (prostate), OVCAR-3 (ovary), U251 (glioma), UACC-62 (melanoma) and a normal cell line VERO (kidney epithelial cells of African green monkey). Stock cell cultures were grown in medium containing RPMI 1640, supplemented with 5% of fetal bovine serum. Experimental cultures were supplemented also with peniciline:streptomicine (10 µg/mL:10 UI/mL).

*Antiproliferative assay*

Cells (100 µL cells/well, inoculation density from 3-6x$10^4$ 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 ºC, 5% of $CO_2$ in air for 48 h. Final DMSO concentration did not affect cell viability. Cells were then fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content, 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, $T_0$) and 48 h post-incubation for compound-free (C) and tested (T) cells. Cell proliferation was determined according to the equation $100x[(T-T_0)/C-T_0]$, for $T_0<T\leq C$, and $100x[(T-T_0)/T_0]$, 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, GI50 (concentration causing 50% growth inhibition; Shoemaker, 2006) was determined by means of non-linear regression analysis, using software Origin 7.5 (OriginLab Corporation). The average activity (mean of log GI50) of the extracts tested was also determined using MS Excel software (Fouche et al, 2008).

## Results and Discussion

Retention times and mass spectra data of constituents in the extracts analyzed are given in Table 1. Only one compound (corydine, an alkaloid) was detected in the methanol extract. Major constituents of the *n*-hexane extract are the steroid β-sitosterol and the triterpenoid β-amyrin (Table 1); campesterol and squalene are other relevant constituents, while corydine, geranylgeraniol and a clerodane derivative are minor compounds of the hexane extract. The dichlormethane extract exhibited the widest diversity of constituents. Among the major constituents the acyclic diterpenoid geranylgeraniol was detected and two clerodanes were tentatively identified as crotonin derivatives (Table 1). Corydine and salutaridine (both alkaloids) are important compounds in the dichloromethane extract, while stigmasterol and β-sitosterol are minor constituents.

All compounds detected in the present work have been reported for *Croton* species. Acyclic diterpenes have been found in *C. kerrii* Airy Shaw, *C. stellatopilosus* H. Ogba and *C. sublyratu*s Kurz (Sato et al., 1980; Salatino et al., 2007). Corydine and other isoquinoline alkaloids have been reported as major constituents of

*C. hemiargyreus* Müll. Arg. and *C. echinocarpus* Baill. (Pereira et al., 1999). Salutaridine has been reported as occurring in *C. balsamifer* Jacq. (Chambers et al., 1966) and *C. salutaris* Casar. (Barnes and Soeiro, 1981). β-Amyrin, β-sitosterol, stigmasterol and campesterol were identified in *C. betulaster* Müll. Arg., *C. hieronymi, C. draco* Schltdl. & Cham., *C. cajucara* Benth. and *C. urucurana* Baill. Derivatives of squalene are constituents of *C. hieronymi* Griseb. (Salatino et al., 2007).

Coherent with a wider diversity of compounds, the dichloromethane extract was shown to have higher antiproliferative activity than the other two extracts. Toward most cell lines, GI50 values of the dichloromethane extract are substantially lower than those of the *n*-hexane and methanol extracts (Table 2). Activities were more pronounced against NCI-H460 and K562 cancer cell lines (IG50<1 μg/mL, Table 2). Moderate activity (GI50<10 μg/mL) of the dichloromethane extract was noted against cell lines U251, 786-0, OVCAR-3 and VERO (Table 2).

Extracts of plants from other taxa have shown to exert activity against the same cell lines used in the present work. For example, dichloromethane and

**Table 1.** Relevant constituents of leaf extracts of *Croton macrobothrys* and respective data of GC-MS analyses.

| RT (min) | MW | Fragment ion (intensity, %) | Compound | Relative amount (%) | | |
|---|---|---|---|---|---|---|
| | | | | Hex | DCM | MeOH |
| 8.9 | 290 | 290 (1), 272 (7), 257 (5), 203 (5), 187 (9), 161 (13), 147 (11), 133 (18), 119 (36), 107 (29), 93 (53), 81 (55), 69 (100) | geranylgeraniol | 0.7 | 20.7 | - |
| 16.9 | 341 | 341 (70), 340 (28), 326 (51), 324 (31), 310 (100), 295 (9), 281 (12), 155 (21), 42 (41), | corydine | 0.1 | 8.5 | 2.1 |
| 18.0 | 316 | 316 (2), 222 (27), 107 (28), 95 (63), 93 (34), 81 (98), 69 (100), 55 (29), 43 (48), 41 (43) | clerodane crotonin derivative | - | 18.2 | - |
| 18.5 | 327 | 327 (100), 312 (66), 299 (36), 284 (77), 242 (24), 226 (24), 87(31), 73(49) | salutaridine | - | 2.0 | - |
| 19.6 | 374 | 374 (5), 356 (12), 341 (27), 324 (62), 309 (7), 261 (28), 178 (50), 96 (100), 95 (77), 81 (46) | clerodane crotonin derivative | 0.4 | 29.4 | - |
| 20.6 | 400 | 400 (2), 382 (47), 145 (58), 107 (54), 95 (50), 91 (53), 81 (56), 55 (59), 41 (43), 43 (100) | campesterol | 2.2 | - | - |
| 21.0 | 412 | 412 (2), 354 (18), 351 (12), 300 (14), 271 (22), 255 (26), 159 (44), 105 (46), 91 (47), 83 (70), 81 (72), 79 (43), 43 (100) | stigmasterol | 2.9 | 0.2 | - |
| 21.6 | 414 | 414 (35), 396 (52), 145 (64), 105 (64), 107 (59), 95 (56), 91 (55), 81 (63), 55 (62), 57 (55), 43 (100) | β-sitosterol | 15.4 | 0.9 | - |
| 22.1 | 426 | 393 (1), 359 (1), 279 (2), 218 (100), 203 (34), 189 (20), 95 (34), 81 (31), 44 (30), 93 (29), 69 (29) | β-amyrin | 11.0 | - | - |
| 22.9 | 410 | 395 (6), 269 (16), 229 (21), 207 (50), 189 (45), 175 (35),107 (61), 95 (81), 81 (75), 69 (80), 55 (91),43 (100) | squalene | 2.0 | - | - |

RT: retention time; MW: molecular weight; DCM: dichloromethane; Hex: hexane; MeOH: methanol; -: not detected or trace amounts.

**Table 2.** Antiproliferative activity (GI50, μg/mL) of leaf extracts *Croton macrobothrys* on culture cell linesa.

| | Cell lines | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Material tested | U251 | UACC-62 | MCF-7 | NCI-ADR/RES | 786-0 | NCI-H460 | PC-3 | OVCAR-3 | HT-29 | K562 | VERO | Mean log GI50[c] |
| Doxorubicin[b] | 0.025 | 0.028 | 0.14 | 0.093 | 0.034 | <0.025 | 0.052 | 0.12 | 0.033 | 0.054 | 0.66 | -1.24 P |
| Hexane | 36.53 | 83.32 | 28.09 | 70.21 | 159.60 | 30.71 | 65.21 | 51.41 | 140.08 | 67.87 | >250 | 1.86 I |
| Dichloromethane | 8.90 | 25.38 | 46.41 | 25.59 | 7.47 | 0.33 | 13.34 | 7.54 | 18.66 | 0.91 | 5.06 | 0.89 M |
| Methanol | 27.75 | 29.04 | 11.66 | 26.93 | 28.28 | 6.08 | 28.26 | 24.47 | 16.31 | 7.45 | 120.05 | 1.33 W |

[a]U251: glioma; UACC-62: melanoma; MCF-7: mammary; NCI-ADR/RES: drug resistant ovary; 786-0: kidney; NCI-H460: lung; NCI-PC-3: prostate; OVCAR-3: ovary; HT-29 colon; K562: leukemia; VERO: Kidney epithelial cells of African green monkey. [b]Positive control. [C]NCI's criteria (Foucher et al, 2008): I: Mean log GI50>1.5= inactive; W: weak activity: Mean log GI50>1.10-1.5; M, moderate activity: Mean log GI50>0-1.1; P, potent activity: Mean log GI50<0.

methanol crude extracts of dried leaves of *Aspidosperma tomentosum* Mart., Apocynaceae, displayed antiproliferative activity in a concentration-dependent way against some cell lines used in the present work. The dicloromethane extract presented higher inhibition toward the lung cells (NCI460) (Kohn et al., 2006). The crude dichloromethane extract of *Virola sebifera* Aubl., Myristicaceae, was shown to be highly active, with selectivity toward NCI460 (Denny et al., 2007). The cytotoxicity of the dichloromethane crude extract, obtained from the aerial parts of *Pothomorphe umbellate* (L.) Miq., Piperaceae, was evaluated against nine human cancer cell lines and presented antiproliferative activity against all cell lines, including leukemia (K-652) (Sacoman et al., 2008).

*In vitro* antiproliferative activity of the latex of *Croton lechleri* was determined against leukemia K562 cells line (Rossi et al., 2003). In our experiment, dicloromethane extract of *C. macrobothrys* exhibited high selectivity against the same cell line (GI50 0.91 µg/mL, Table 2). The activity may be accounted for geranylgeraniol, the major component in the extract (Table 1), and corydine, an aporphine alkaloid. Acyclic diterpenoids, such as plaunotol from *C. sublyratus,* have been recently reported as inhibitor of angiogenesis (Kawai et al., 2005; Yoshikawa et al., 2009). Corydine has shown inhibitory activity against several mouse tumor cell lines such as leukemia P388 and L1210, melanoma B16, bladder cancer MBC2, and colon cancer Colon 26 (Kondo et al, 1990; Bruneton, 1999). Other alkaloids from *Croton* species have been shown to be active against cancer; examples are taspine (from *C. lechleri*) and julocrotol, isojulocrotol and julocrotone, from *C. cuneatus* Klotzsch (Salatino et al., 2007). Also clerodanes may be involved in the observed activity: the furoclerodane croblongifolin from *C. oblongifolius* Sieber ex Spreng. was shown to be cytotoxic (Roengsumran et al., 2002) and trans-dehydrocrotonin from *C. cajucara* exhibited anti-tumor effect (Melo et al., 2004). The next step in our investigation is to test antiproliferative activity of fractions and isolated compounds from dichloromethane extracts, obtained from column chromatography.

The use of material from *C. macrobothrys* in popular medicine or phytotherapy requires evaluation of possible undesirable side effects. For example, 25 cases of hepatoxicity were documented among people from Amazonia, which were ascribed to consumption of sacaca (*Croton cajucara*) along 36 months (Soares, 2006). Twenty-one cases corresponded to acute, three to chronic and one to fulminating hepatitis. This issue requires further investigation, because a recent study did not detect significant alterations on hepatic transferases in animals treated with *C. cajucara* (Rodrigues et al., 2010). Nothing is known about toxic effects of *C. macrobothrys*.

The results of the present work, suggesting that leaves of *C. macrobothrys* may contain antiproliferative active compounds, are in agreement with previous evidences, which have shown that *Croton* species are likely sources of substances useful for the development of new drugs (Salatino et al. 2007).

## Acknowledgments

The authors are grateful to Frederick Cancer Research & Development Center of the National Cancer Institute (Frederick, MA, USA) for the kind provision of cell lines, and to Fundação de Amparo à Pesquisa do Estado de São Paulo (08/10595-3 auxílio) (08/09942-0 bolsa), CAPES and CNPq for financial support.

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