SHORT REPORT

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Antiproliferative Activity and Constituents of *Aspidosperma macrocarpon* (Apocynaceae) Leaves

Geanderson Bannwart[1], Cecília M. A. de Oliveira[2], Lucilia Kato[2], Cleuza C. da Silva[1], Ana Lúcia T. G. Ruiz[3], João E. de Carvalho[3] and Silvana M. O. Santin[1*]

[1]*Departamento de Química, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900, Maringá, Paraná, Brazil*
[2]*Instituto de Química, Universidade Federal de Goiás, Campus Samambaia; P.O. Box 131,74001-970, Goiânia, Goiás, Brazil*
[3]*Centro Pluridisciplinar de Pesquisa Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, P.O. Box 6171, 13081-970, Campinas, São Paulo, Brazil.*

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Abstract: *Aspidosperma macrocarpon* belongs to the family Apocynaceae and is endemic to Americas and mainly found from Mexico to Argentina. It is known in Brazil as "guatambu" or "peroba". Crude extracts and their fractions from leaves were assayed against human cancer cells lines: glioma (U251), melanoma (UACC-62), mammary (MCF-7), ovarian expressing the multidrug resistance phenotype (NCI-ADR/RES), lung (NCI-H460), prostate (PC-3), kidney (786-0), ovarian (OVCAR-3), colon (HT-29) and leukemia (K-562). The crude extract (EAM), hexane (HA) and chloroform (CA) fractions were the most active fractions against K-562 with GI50 values low than 1 µg/mL. Also, CA was moderate active against OVCAR-3 and NCI-ADR/RES cells lines. This phytochemical study allowed to identify the known kopsanone, kopsinine, ursolic acid, rutin, 5-*O*-caffeoylquinic acid, and 3,5-*O*-dicaffeoylquinic acid. The kopsanone was also evaluated against human cancer cell lines and showed activity to the U251 and K-562 cell lines, with GI50 values of 20.6 µg/mL and 8.7 µg/mL, respectively.

Keywords: *Aspidosperma macrocarpon*; antiproliferative activity; alkaloids.

1. Plant Source

As part of our continuing search for antiproliferative agents derived from natural herbals resources, and assessment of the efficacy crude drugs used by traditional communities in Brazil, extract and fractions from leaves of *Aspidosperma macrocarpon* were assayed against ten human cancer cell lines. We report here the bioactivity of these extracts, fractions and the alkaloid kopsanone as well as the isolation of secondary metabolites.

Leaves of *Aspidosperma macrocarpon* were collected on May 2010 in the municipality of Goiânia (Goiás, Brazil), and identified by Dr. Heleno Dias Ferreira. A voucher specimen (45524) was deposited in the Herbarium of the Instituto de Biologia, Universidade Federal de Goiás (ICB/UFG).

* Corresponding author: E-Mail: smoliveira@uem.br

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2. Previous Studies

The alkaloids (-)-vincadifformine, ervinceine, kopsanone, kopsinine, kopsanol and 18-epikopsanol have been isolated from seeds and stem bark of *Aspidosperma macrocarpon* [2-3].

3. Present Study

Dried powdered leaves (738.9 g) of *Aspidosperma macrocarpon* were exhaustively extracted by maceration with methanol at room temperature. Evaporation of the solvent yielded the crude extract (**EAM** 139.0 g).

Part of the crude extract (14.2 g) was re-dissolved in MeOH-H_2O 1:1 (50 mL) and HCl 0.1 M (100 mL).The acid solution was partitioned with $CHCl_3$ (5x25 mL) yielded a chloroform fraction (1.35 g). The acid solution fraction was basified with NH_4OH (pH 9.0) and partitioned with $CHCl_3$ (3 x 70 mL) yielding alkaloid fraction (**AF**, 0.57 g). The alkaloid fraction (0.47 g) was fractionated on silica gel column eluted with hexane: $CHCl_3$ (10-100%), $CHCl_3$ and $CHCl_3$: MeOH (10-100%), resulting in 55 sub-fractions. The sub-fractions AM-14 and AM-15 (hexane: $CHCl_3$ 4:6) yielded kopsanone (45.8 mg, **1**) and kopsinine (9,4 mg, **2**).

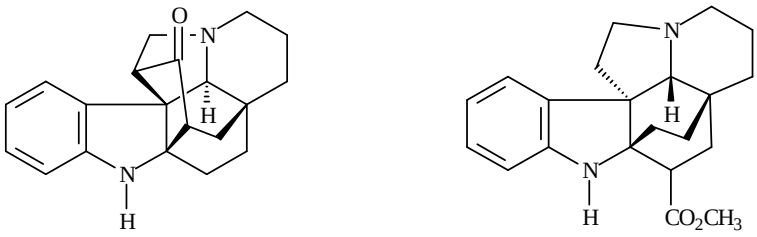

Figure 1. Structures of alkaloids from leaves of *A. macrocarpon.*

Part of the crude extract (11.5 g) was dissolved in MeOH-H_2O 1:1 and partitioned with different solvents to obtain the hexane (**HA**, 1.30 g), chloroform (**CA**, 3.45 g), ethyl acetate (**EA**, 1.58 g) and hydromethanolic (**HMA**, 3.40 g) fractions. The resulting fractions were subjected to conventional purification procedures and leading to isolation of ursolic acid, 5-*O*-caffeoylquinic acid, rutin and 3,5-*O*-dicaffeoylquinic acid.

Cell lines and culture medium: The *in vitro* antiproliferative activity of the extracts and fractions was assessed using ten different human cancer cell lines: U251 (glioma), UACC-62 (melanoma), MCF-7 (mammary), NCI-ADR/RES (ovarian expressing the multidrug resistance phenotype), NCI-H460 (lung), PC-3 (prostate), 786-0 (kidney), OVCAR-3 (ovarian), HT-29 (colon) K-562 (leukemia) and normal cell line HaCaT (human keratinocyte) were kindly provided by Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA. The chemotherapeutic agent doxorubicin was used as a positive control. Stock cultures were grown in a medium containing 5 mL of RPMI 1640 (GIBCO BRL, Life Technologies) and supplemented with 5% of foetal bovine serum. Gentamicine (50 μg/mL) was added to the experimental cultures.

Cytotoxicity assay: Cells in 96 well-plates (100 μL cells/well) were exposed to varying concentrations of samples in DMSO (0.25; 2.5; 25 and 250 μg/mL) and 5% CO_2 in air for 48 h at 37 °C. The final concentration of DMSO did not affect the cell viability. A 50% trichloroacetic acid solution was added and incubated with the cells for 30 min at 4 °C. After washing and drying, the degree of cell proliferation was determined by spectrophotometric quantification (540 nm) of the 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 48h post-incubation for sample-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) [4].

Using the concentration-response curve for each cell line, GI_{50} (concentration causing 50% growth inhibition) was determined by means of non-linear regression analysis, using software Origin 7.5 (Origin Corporation). The average activity (mean of log GI_{50}) of the extracts tested was also determined using MSExcel software [5]. (**Table 1**)

Table 1. Antiproliferative activity (GI_{50}, $\mu g.mL^{-1}$) of leaves crude extract, fractions and kopsanone of *Aspidosperma macrocarpon* on different cancer cell lines.

	U251	UACC-62	MCF-7	NCI-ADR/RES	786-0	NCI-H460	PC-3	OVCAR-3	HT-29	K562	HaCaT	Mean log GI_{50}
Doxo	0,027	0,025	<0,025	0,15	0,051	<0,025	0,12	0,26	0,10	0,049	0,053	<-1,3 P
EAM	67,0	20,4	26,0	20,0	51,2	38,4	61,8	23,5	130,0	0,51	1,5	1,3 W
AF	26,6	27,3	28,6	13,6	29,1	37,1	40,9	9,7	89,8	2,6	<0,25	<1,0 M
HA	24,7	23,3	9,2	8,8	25,1	25,8	25,5	22,7	27,2	0,78	2,9	1,1 M
CA	13,3	38,7	7,6	1,6	3,1	22,0	7,5	1,2	69,4	0,36	9,1	0,8 M
EA	48,5	38,1	49,7	11,5	31,7	83,1	120,6	25,0	88,0	3,0	8,5	1,5 W
HMA	>250	>250	55,6	56,1	>250	>250	>250	147,9	159,6	40,6	>250	>2,2 I
kopsanone	20,6	38,9	54,7	56,0	66,4	96,7	79,4	47,2	56,0	8,7	60,9	1,7 I

Cell lines: U251 (glioma); UACC-62 (melanoma); MCF-7 (mammary); NCI-ADR/RES (ovarian expressing the multidrug resistance phenotype); 786-0 (kidney); NCI-H460 (lung); PC-3 (prostate); OVCAR-3 (ovarian); HT-29 (colon); K562 (leukemia); HaCat (human keratinocyte, normal cell).
NCI´s critera: W, weak activity: $1.5 \geq \log GI_{50} > 1.10$; M, moderate activity: $1.1 \geq \log GI_{50} > 0$; P, potent activity: log GI50 <0. (Foucher et al, 2008).

The crude methanolic extract from leaves (**EAM**) exhibited a high inhibitory effect on cell growth, and was very effective against the UACC-62, MCF-7, NCI-ADR/RES and K-562 cell lines, with GI_{50} values of 20.4, 26.0, 20.0 and 0.51 µg/mL, respectively (Table 1). This crude extract was further separated into five fractions, alkaloid fraction (**AF**), hexane (**HA**), chloroform (**CA**), ethyl acetate (**EA**) and hydromethanolic (**HMA**) fractions, which were assayed against the same cells. **AF** fraction presented high activity in most of the cell lines tested. The highest antiproliferative activities of fraction was found against the NCI-ADR/RES, OVCAR-3 and K-562 cells, with GI_{50} values of 13.6, 9.7 and 2.6 µg/mL, respectively. A significant amount of the alkaloid kopsanone (**1**, 45.8 mg) was isolated from **AF,** along with kopsinine (**2**). These compounds were identified by analysis of their spectroscopic data and comparison with available literature data [6-7]. The alkaloid **1** also was assessed and showed high selectivity and activity against the U251 and K-562 cell lines, with GI_{50} values of 20.6 µg/mL and 8.7 µg/mL, respectively.

HA fraction showed potent activity against MCF-7, NCI-ADR/RES and K-562 cell lines, with GI_{50} values of 9.2, 8.8 and 0.78 $\mu g.mL^{-1}$, respectively. Toward most cell lines, GI_{50} values of the **CA** were lower than those of **HA.** This fraction showed highest activity against MCF-7, NCI-ADR/RES, 786-0, PC-3, OVCAR-3 and K562 cell lines, with GI_{50} values of 7.6, 1.6, 3.1, 7.5, 1.2 and 0.36 µg/mL, respectively. Phytochemical investigation showed the presence of an expressive amount of the ursolic acid triterpene in this fraction, identified by comparison of their spectroscopic data with those previously published [8-9]. In the literature, the ursolic acid is described as very active against a broad range of cancer cell lines [10]. The NCI-ADR/RES, OVCAR-3 and K562 were more sensitive to **EA** fraction than the other cells, with GI_{50} values of 11.5, 25,0 and 3.0 µg/mL, respectively. Studies of **EA** fraction allowed identify the 5-*O*-caffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and rutin, also identified by their spectroscopic data compared with available literature [11-12]. The chlorogenic acids and rutin have been reported as antioxidant and anti-inflammatory agents [13-14] and, as antioxidants, can inhibit carcinogenesis. Recently, the dicaffeoylquinic derivatives have been reported as potent antiproliferative agents against breast carcinoma (MCF-7), myeloid leukemia (HL-60), histiocytic lymphoma (U937), leukemia (HL-60), colon cancer (DLD-1) cell lines [11].

In this study we focused the evaluation of the extracts, fractions and the indol alkaloid from leaves of *A. macrocarpon* against a representative set of human cancer cell lines. The bio-guided

fractionation of the active crude extract showed that the hexane (**HA**), chloroform (**CA**) fractions were the most active fractions against the K-562 with GI_{50} values low than 1 µg/mL. **CA** fraction showed moderate activity against OVCAR-3 and NCI-ADR/RES cells line. The phytochemical study allowed to identify the known kopsanone, kopsinine, ursolic acid, rutin, 5-*O*-caffeoylquinic acid, and 3,5-*O*-dicaffeoylquinic acid, identified by NMR data. The kopsanone was assayed against the U251 and K-562 cell lines, with GI_{50} values of 20.6 µg/mL and 8.7 µg/mL, respectively. Our results are in agreement with previous evidences which have shown that *Aspidosperma* species are likely sources of useful substances for the development of new drugs.

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