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Antiproliferative activity from *Aldama arenaria* (Baker) E. E. Schill. & Panero

[Actividad antiproliferativa de *Aldama arenaria* (Baker) E. E. Schill. & Panero]

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Abstract: Chloroform extract (CE) and fractions obtained from *Aldama arenaria* roots were evaluated for their *in vitro* antiproliferative activity against 10 human tumor cell lines [leukemia (K-562), breast (MCF-7), ovary expressing a multidrug-resistant phenotype (NCI/ADR-RES), melanoma (UACC-62), lung (NCI-H460), prostate (PC-3), colon (HT29), ovary (OVCAR-3), glioma (U251), and kidney (786-0)]. CE presented weak to moderate antiproliferative activity (mean log GI_{50} 1.07), whereas fractions 3 and 4, enriched with pimarane-type diterpenes [ent-pimara-8(14),15-dien-19-oic acid and ent-8(14),15-pimaradien-3β-ol], presented moderate to potent activity for most cell lines, with mean log GI_{50} of 0.62 and 0.59, respectively. The results showed promising *in vitro* antiproliferative action of the samples obtained from *A. arenaria*, with the best results for NCI/ADR-RES, HT29, and OVCAR-3, and TGI values ranging from 5.95 to 28.71 µg.mL-1, demonstrating that compounds of this class may be potential prototypes for the discovery of new therapeutic agents.

Keywords: *Aldama arenaria*; Antiproliferative activity; Ent-pimara-8(14),15-dien-19-oic acid; ent-8(14),15-pimaradien-3β-ol; Pimarane; *Viguiera arenaria*.

Resumen: El extracto de cloroformo (CE) y las fracciones obtenidas de las raíces de *Aldama arenaria* se evaluaron para determinar su actividad antiproliferativa *in vitro* contra 10 líneas celulares tumorales humanas [leucemia (K-562), mama (MCF-7), ovario que expresa un fenotipo resistente a múltiples fármacos (NCI/ADR-RES), melanoma (UACC-62), pulmón (NCI-H460), próstata (PC-3), colon (HT29), ovario (OVCAR-3), glioma (U251) y riñón (786-0)]. CE presentó actividad antiproliferativa débil a moderada (log GI_{50} medio 1.07), mientras que las fracciones 3 y 4, enriquecidas con diterpenos de tipo pimarane [ent-pimara-8 (14), ácido 15-dien-19-oico y ent-8(14),15-pimaradien-3β-ol], presentaron actividad moderada a potente para la mayoría de las líneas celulares, con un log GI_{50} medio de 0.62 y 0.59, respectivamente. Los resultados mostraron una acción antiproliferativa *in vitro* prometedora de las muestras obtenidas de *A. arenaria*, con los mejores resultados para NCI/ADR-RES, HT29 y OVCAR-3, y valores de TGI que van desde 5.95 a 28.71 µg.mL-1, demostrando que los compuestos de esta clase pueden ser prototipos potenciales para el descubrimiento de nuevos agentes terapéuticos.

Palabras clave: *Aldama arenaria*; Actividad antiproliferativa; Ent-pimara-8(14) ácido 15-dien-19-oico; ent-8 (14), 15-pimaradien-3β-ol, Pimarane; *Viguiera arenaria*.

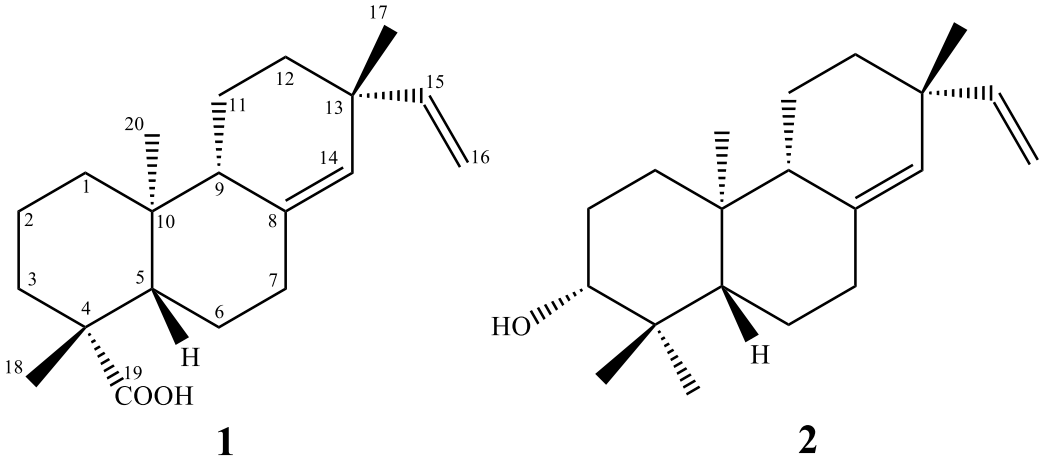
INTRODUCTION

According to the World Health Organization, cancer is the second leading cause of death worldwide and in responsible for approximately 9.6 million deaths in 2018. Globally, about 12 in 6 deaths is due to cancer. Estimates predict that the number of new cancer cases will rise by from 18.1 million to 29.5 million, from 2018 to 2040 (WHO, 2014; WHO, 2019).

A review by Newman & Cragg (2016), demonstrated that during 33 years (1981-2014), 73% of the 1,562 new therapeutic agents approved were inspired to some extent by natural products. Moreover, in the same period, about 87% of all approved therapeutic innovations for cancer treatment were directly or indirectly developed from natural products, highlighting that nature still offers viable and safe options in screening for new drugs (Newman & Cragg, 2016).

Aldama arenaria (Baker) E. E. Schill. & Panero (Asteraceae), classified previously as *Viguiera arenaria* (Baker), is a cespitosis subshrub herb found in *Cerrado* areas and along roadsides in Brazil, mainly in the central-east region of São Paulo State, with fewer occurrences in the southwest of Minas Gerais State (Schilling & Panero, 2002; Magenta, 2006). Reports of its biological activities are still scarce in the literature. The major metabolites described for this species are pimarane-type diterpenes and sesquiterpene lactone (Ambrosio *et al*., 2004; Bombo *et al*., 2017).

Ambrosio *et al*. (2002) reported that *ent*-pimara-8(14),15-dien-19-oic acid (**1**) (Figure Nº 1), inhibited vascular smooth muscle contraction in the carotid of rats, in other work, Hipólito *et al*. (2009) studied the mechanisms involved in the vasorelaxation action of *ent*-8(14),15-pimaradien-3β-ol (**2**) in isolated aorta of male Wistar rats.



1 **2**

Figure No. 1
Structures of *ent*-pimara-8(14),15-dien-19-oic acid (1) and *ent*-8(14),15-pimaradien-3β-ol (2).

In vitro studies showed that extracts of *V. arenaria*, and the diterpenes isolated from them, were active against the protozoan *Trypanosoma cruzi* (Ambrosio *et al*., 2008). Porto *et al*. (2009) described the potential antibacterial activities of the dichloromethane extract and the isolated compounds of *V. arenaria*. Other work reported *in vitro* and *in vivo* activity against the *Schistosoma mansoni* parasite, comparing compound **1** and two derivatives obtained from fungi (Porto *et al*., 2012). Research has also shown the antiprotozoal activities of three *ent*-pimarane compounds isolated from *Aldama discolor* (Nogueira *et al*., 2016). Recent work by our research group has revealed the potential *in vitro* antiproliferative activity of this species (Oliveira *et al*., 2013; Oliveira *et al*., 2014).

The present work evaluates the chemical composition and the *in vitro* antiproliferative activity

of the chloroform extract (CE) and fractions of *Aldama arenaria* against 10 human tumor cell lines.

MATERIALS AND METHODS

Plant material

The plant material was collected at Itirapina Ecological Station, São Paulo State, Brazil (22°14'S, 47°51'W), in its natural state, during the period of dormancy. Identification was carried out by botanist Mara Angelina Galvão Magenta, and the exsiccata was deposited at the Herbarium of Luiz de Queiroz College of Agriculture (ESALQ-USP, number 111847). The study was authorized under CGEN process number 010216/2012-0.

Extraction and isolation

Fresh tuberous and non-tuberous roots (200 g) of *A. arenaria* were ground and extracted in an Ultra-Turrax disperser (IKA®) with 1.0 L of chloroform (3 min, Synth®), followed by filtration and re-extraction of the plant residue with 0.8 L of chloroform (Synth®). The extracts were grouped and then evaporated under vacuum, resulting in the crude chloroform extract (CE, 15,48g – 7,74% m/m).

The CE (2.04 g) was fractionated using dry column chromatography (DCC) with a cellulose acetate support (diameter 1.5 cm), silica gel 60 as the stationary phase (45 cm height, Merck®), and hexane/ethyl acetate (70:30, Synth®) as the mobile phase. After completion of elution, the column was divided into nine 5 cm fractions (F1 to F9), which were filtered using a funnel with a sintered plate and washed with ethyl acetate (Synth®).

The fractions were analyzed using thin layer chromatography (TLC, Merck®), with the same mobile phase used in the column chromatography and anisaldehyde solution as reagent (glacial acetic acid, sulfuric acid, and anisaldehyde, 100:2:1, Synth®). The resulting fractions were grouped according to their similarity and evaporated under reduced pression.

F3-4 (0.52 g) was purified using a classical chromatography column (CCC) with a diameter of 2.0 cm, containing 40 g of silica gel 60 (0.040-0.063 mm, Merck®), employing a gradient of hexane/ethyl acetate (Synth®). This resulted in the collection of 60 samples, which were analyzed using TLC and were then grouped into 17 fractions (FA to FQ). After drying under vacuum in a rotary evaporator, all the fractions were evaluated for *in vitro* antiproliferative activity, and fractions FD and FG were crystallized in methanol, providing compounds 1 and 2, respectively.

Structural characterization of compounds 1 and 2 was carried out by analysis of the data obtained using ^{1}H and ^{13}C NMR, infrared (IR) spectroscopy, melting point measurements, optical rotation measurements, and GC-MS mass spectra. The experimental data were compared with data reported in the literature.

Gas chromatography mass spectrometry (GCMS) analysis

GCMS analysis was performed on a Agilent 6890 N GC System fitted with an HP5-MS (J & W Scientific) capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; maximum temperature, 250ºC) and coupled to a Agilent 5975 MS. Ultra-high purity helium was used as carrier gas at a constant flow of 1,0 $mL.min^{-1}$. The ionizing energy was 70 eV. The sample was prepared in ethyl acetate, and the injected sample volume was 1 µL with a split ratio of 30:1. The injector operated in 250ºC and detector 300ºC. The oven temperature program was 110ºC, and accelerated at a rate of $5°C.min^{-1}$, 280°C (26 min). The compounds were identified by comparing the spectra obtained with mass spectrum library NIST 2011 and data reported in the literature (Adams, 2007).

Nuclear magnetic resonance (NMR) analysis

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 250 instrument at 250 MHz and 62,5 MHz, respectively, using $CDCl_3$ as a solvent and TMS as internal reference.

(1) *ent*-pimara-8(14),15-dien-19-oic acid: ^{1}H NMR: δ = 0.66 (s, 3H, 10-Me), 1.01 (s, 3H, 13-Me), 1.27 (s, 3H, 4-Me), 4.91 (dd, 1H), 4.96 (dd, 1H), 5.16 (s, 1H), 5.72 (dd, 1H). ^{13}C NMR: δ = 184.3, 147.2, 137.9, 128.0, 112.9, 56.1, 50.5, 44.0, 39.2, 39.2, 38.5, 37.9, 35.8, 36.4, 29.3, 29.2, 24.1, 19.6, 19.2, 13.8. Data was compared with the available literature (Matsuo *et al*., 1976).

(2) *ent*-8(14),15-pimaradien-3β-ol: ^{1}H NMR: δ = 0.83 (s, 3H, 4-Me), 1.02 (s, 3H, 4-Me), 1.07 (s, 3H, 10-Me), 1.17 (s, 3H, 13-Me), 3.3 (dd, 1H), 4.89 (dd, 1H), 4.96 (dd, 1H), 5.16 (d, 1H), 5.74 (dd, 1H). ^{13}C NMR: δ = 147.3, 137.9, 128.1, 112.8, 79.2, 54.1,

51.2, 39.0, 38.6, 38.1, 37.1, 35.7, 35.7, 29.4, 28.4, 27.5, 22.1, 19.1, 15.7, 14.7. Data was compared with the available literature (Ansell *et al*., 1993).

In vitro antiproliferative activity test

Ten human tumor cell strains, donated by NCI (National Cancer Institute, USA), were used for *in vitro* antiproliferative activity tests: K-562 (leukemia), MCF-7 (breast), NCI/ADR-RES (ovary expressing a multidrug-resistant phenotype), UACC-62 (melanoma), NCI-H460 (lung), PC-3 (prostate), HT29 (colon), OVCAR-3 (ovary), U251 (glioma), and 786-0 (kidney). A non-tumor epithelial green monkey kidney cell line (VERO, Rio de Janeiro Cell Bank) was also used.

The cells were cultured in RPMI 1640 medium (Gibco®) with 5% fetal bovine serum (FBS, VitroCell®) and stored at 37°C in a humid atmosphere with 5% CO_2. The samples were diluted to 100 $mg.mL^{-1}$ in dimethyl sulfoxide (DMSO, Synth®). These stock solutions were diluted in RPMI 1640 medium with 5% FBS plus 1% penicillin:streptomycin (1000 $U.mL^{-1}$: 1000 $\mu g.ml^{-1}$, Vitrocell® Embriolife®). In the antiproliferative assay, 100 µL of each cell suspension (inoculation density between 3 x 10^{-4} and 7 x 10^{-4} $cel.L^{-1}$) was inoculated into a well of a 96-well plate. After 24 h, the samples were added (100 µL per well) at concentrations of 250, 25, 2.5, and 0.25 $\mu g.mL^{-1}$, in triplicate, and the plates were incubated at 37°C in a humid atmosphere with 5% CO_2 for 48 h (Monks *et al*., 1991). A control plate (T0) containing all the cell lines evaluated was then fixed using trichloroacetic acid (TCA, 50 µL per well, Sigma-Aldrich®), in order to determine the number of cells at the moment the samples were added. At the end of the treatment, the cells were fixed with 50 µL of 50% TCA per well and incubated for 1 h at 4°C. After washing and drying, the total protein content was dyed by addition to each well of 50 µL of sulforhodamine B (SRB, Sigma®) at 0.4% (m/v) in 1% acetic acid (Synth®). After washing to remove the excess dye, followed by drying the plates, the dye linked to cellular proteins was solubilized by the addition to each well of 150 µL of 10 µM Trizma Base solution (pH 10.5, Sigma®). A microplate reader was used to measure the absorbance at 540 nm (Rubistein *et al*., 1990, Skehan *et al*., 1990). As a positive control, doxorubicin (DOX, Eurofarma®) was used at concentrations of 0.025, 0.25, 2.5, and 25 $\mu g.mL^{-1}$ (100 µL/well, in triplicate). The DMSO (maximum final concentration of 0.25%) used for sample dispersion did not affect cell viability. The absorbance readings were used to calculate the cell growth for all the cell lines, at four concentrations of each sample, considering T (the mean cell absorbance), CC (cell control), and T0 (cell control on the day the samples were added). If $T \geq T0 < CC$, the sample was cytostatic and the formula used was: $100x[(T-T0)/CC-T0)]$; if $T \leq T0$, the sample was cytocidic and the formula used was: $100x[(T-T0)]/(T0)]$. The data obtained were used to generate curves correlating cell growth with sample concentration. The results for the *in vitro* antiproliferative activities of CE, the fractions, and the compounds were based on NCI criteria and were evaluated using TGI and GI_{50} values (the concentration required for 100% and 50% inhibition of cell growth), calculated using sigmoidal regression performed with Origin 8 software (Monks *et al*., 1991; Shoemaker, 2006).

RESULTS

The i*n vitro* antiproliferative activities of the *A. arenaria* samples were evaluated considering the TGI, GI_{50}, and log GI_{50} values for ten tumor cell lines, in order to establish an order of priority for study, based on the classification criteria described by Fouche *et al*. (2008). Figure Nº 2 presents cell growth curves as a function of the concentrations of CE and fractions F3, F4, FD, and FG, which were the samples that presented potential antiproliferative activity. The Table No. 1 shown TGI, GI_{50} and Mean log GI_{50} ($\mu g.mL^{-1}$) values for the five most affected cell lines and VERO, calculated for CE, F3, F4, FD (1), FG (2), and FO.

DISCUSSION

This work investigates, for the first time, the in vitro antiproliferative activity of Aldama arenaria against a panel of ten tumor cell lines. These cell lines represent some of the major neoplasms with high mortality rates worldwide, especially lung (2.09 million deaths), breast (2.09 million deaths), and colorectal (1.80 million deaths). These data corroborate the importance of studies aimed at identifying new drug prototypes for cancer treatment, in order reduce mortality, improve the quality of life

of people with this disease, and minimize social and economic losses. The economic impact of cancer is significant and is increasing. Total medical care expenditures for cancer in 2010 were estimated at US$1.16 trillion (Ferlay *et al.*, 2015; WHO, 2014; WHO, 2019).

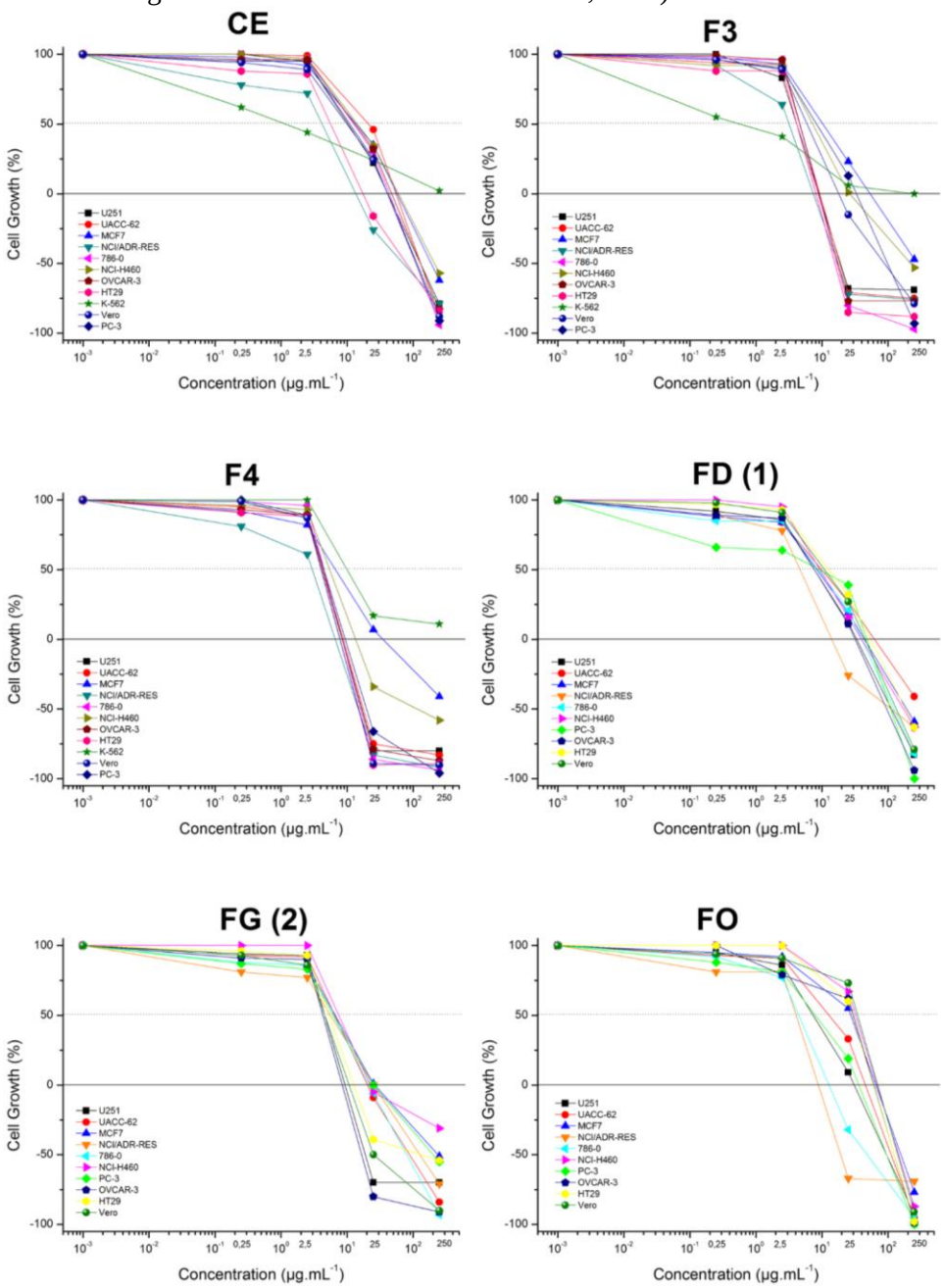


Figure N° 2
Cellular growth curves as a function of concentration
Caption: (CE) Chloroform extract; (F3 and F4) fractions 3 and 4 obtained from dry chromatography column fractionation of CE; (FD, FG, and FO) fractions D, G, and O obtained from classical chromatography column fractionation of F3-4. Human tumor cell lines: U251 (glioma), UACC-62 (melanoma), MCF-7 (breast), NCI/ADR-RES (ovary expressing a multidrug-resistant phenotype), 786-0 (kidney), NCI-H460 (lung), PC-3 (prostate), OVCAR-3 (ovary), HT29 (colon), and K-562 (leukemia); Non-tumor cell line: green monkey kidney fibroblast (VERO)

Table N° 1
TGI, GI_{50} and Mean log GI_{50} ($\mu g.mL^{-1}$) values for the five most affected cell lines and VERO, calculated for CE, F3, F4, FD (1), FG (2), and FO.

Sample	*TGI ($\mu g.mL^{-1}$) of the five most affected cell lines*	*GI_{50} ($\mu g.mL^{-1}$) of the five most affected cell lines*	*Mean log GI_{50}*	*TGI VERO ($\mu g.mL^{-1}$)*
CE	14.90 (NCI/ADR-RES); 19.49 (HT29); 34.91 (PC-3); 35.55 (786-0); 37.36 (U251)	1.14 (K-562); 2.83 (NCI/ADR-RES); 4,84 (HT29); 20.55 (U251); 21.36 (PC-3)	1.07 M	35.56
F3	7.24 (NCI/ADR-RES); 8.32 (HT29); 9.71 (U251); 10.87 (786-0); 11.05 (UACC-62)	0.57 (K-562); 2.68 (NCI/ADR-RES); 2.70 (UACC-62); 3.02 (U251); 3.15 (HT29)	0.62 M	20.55
F4	5.95 (NCI/ADR-RES); 7.61 (HT29); 9.14 (U251); 9.27 (OVCAR-3); 9.77 (UACC-62)	2.59 (NCI/ADR-RES); 3.12 (OVCAR-3); 3.15 (HT29); 3.18 (U251); 3.19 (UACC-62)	0.59 M	7.63
FD (1)	17.86 (NCI/ADR-RES); 28.71 (OVCAR-3); 30.61 (U251); 35.31 (786-0); 39.08 (PC-03)	2.93 (NCI/ADR-RES); 7.05 (OVCAR-3); 7.05 (OVCAR-3); 7.06 (U251); 8.57 (786-0);	1.10 M	40.06
FG (2)	9.32 (OVCAR-3); 11.14 (U251); 15.61 (HT-29); 22.27 (786-0); 22.38 (UACC-62)	2.93 (UACC-62); 3.22 (OVCAR-3); 3.34 (U251); 3.35 (HT-29); 4.09 (NCI/ADR-RES)	0.66 M	12.45
FO	9.47 (NCI/ADR-RES); 14.08 (786-0); 27.95 (U251); 31.11 (PC-3); 36.86 (UACC-62)	3.97 (786-0); 5.64 (NCI/ADR-RES); 13.54 (U251); 15.12 (PC-3); 20.19 (UACC-62)	1.19 W	>50

Caption: (CE) chloroform extract; (F3 and F4) fractions 3 and 4 obtained from dry chromatography column fractionation of CE; (FD, FG, and FO) fractions D, G, and O obtained from classical chromatography column fractionation of F3-4 (grouped). Human tumor cell lines: NCI/ADR-RES (ovary expressing a multidrug-resistant phenotype), HT29 (colon), PC-3 (prostate), 786-0 (kidney), U251 (glioma), UACC-62 (melanoma), OVCAR-3 (ovary) and K-562 (leukemia); TGI: Concentration ($\mu g.mL^{-1}$) required for 100% inhibition of cell growth. GI_{50}: Concentration ($\mu g.mL^{-1}$) required for 50% inhibition of cell growth. Activity criteria: Mean log GI_{50} > 1.5: inactive (I); mean log GI_{50} from 1.1 to 1.5: weak (W); mean log GI_{50} from 0 to 1.1: moderate (M); mean log GI_{50} < 0: potent (P) (Fouche et al., 2008); Positive control: Doxorubicin (mean log GI_{50} -1.04, TGI 20.88 $\mu g.mL^{-1}$)

Most of the biological activity studies of *A. arenaria* reported in the literature have focused on the roots of the plant, which are rich in pimarane-type diterpenes (Hipólito *et al*., 2009, Porto *et al*., 2009). Diterpenes isolated from *Croton zambesicus*, *Petalostigma pubescens, Drypetes perreticulata* and *Mitrephora alba*, presented cytotoxic activity against differents cells lines (Block *et al*., 2002; Block *et al*., 2004; Grace *et al*., 2006; Rayanil *et al*., 2013; Ge *et al*., 2014).

All samples were evaluated against the studied strains and the most promising results are presented here in this article (Table Nº 1).

The fractionation of CE using a dry chromatography column resulted in nine fractions, F1 (45.8 mg), F2 (933.4 mg), F3 (434.2 mg), F4 (117.5 mg), F5 (44.9 mg), F6 (68.6 mg), F7 (85.1 mg), F8 (61.2 mg), and F9 (104.5 mg).

The mean log GI_{50} value for the ten cell lines evaluated was 1.07, confirming the moderate activity of CE. CE (yield of 7.74%) showed low or moderate antiproliferative activity towards seven tumor cell lines, with greatest activities towards the ovary cell line expressing a multidrug-resistant phenotype (NCI/ADR-RES, TGI 14.90 $\mu g.mL^{-1}$) and the colon cell line (HT29, TGI 19.49 $\mu g.mL^{-1}$), Table I. Analysis of CE using GC/MS showed the presence of terpenes, especially diterpenes with molecular weights (*m/z*) of 288 and 302 $g.mol^{-1}$.

Fractions F3 and F4 were more active than CE and the other fractions evaluated, with mean TGI values of 28.88 and 36.06 $\mu g.mL^{-1}$, respectively, maintaining selectivity towards the cell lines NCI/ADR-RES (TGI: 7.24 $\mu g.mL^{-1}$ (F3); 5.95 $\mu g.mL^{-1}$ (F4)) and HT29 (TGI: 8.32 $\mu g.mL^{-1}$ (F3); 7.61 $\mu g.mL^{-1}$ (F4)), combined with increased activity

for all cell lines. The mean log GI_{50} values for F3 and F4 were 0.62 and 0.59, respectively. Their chemical compositions, determined using GC-MS, included two major diterpenes with *m/z* 288 and 302, eluting at retention times of 22.6 min and 26.4 min, respectively. The fragmentation patterns of these compounds showed *m/z* 288, 270, 255, 152, 135 (100%), and 91, and *m/z* 302, 287, 167, 139, 121 (100%), and 91, indicative of an alcohol and a diterpene acid, respectively (Adams, 2007).

The classical column fractionation of F3-4 resulted in 17 fractions (FA to FQ), which were analyzed using TLC and GC-MS. Fractions FA (28.4 mg), FD (177.8 mg), FG (88.7 mg), FL (39.42 mg), FN (5.1 mg), FO (46.9 mg), FP (8.1 mg), and FQ (22.5 mg) were evaluated for their *in vitro* antiproliferative activities. The results revealed greater selectivity among the cell lines, especially in the case of fraction G, for which the mean TGI value was 24.83 $\mu g.mL^{-1}$.

Fractions FA, FP, and FQ were inactive (TGI >50 $\mu g.mL^{-1}$), while fractions FD, FL, and FN presented weak activity (TGI 17.80-50 $\mu g.mL^{-1}$). Fraction FO showed weak activity towards five cell lines, with greatest selectivity towards NCI/ADR-RES (TGI 9.47 $\mu g.mL^{-1}$) and 786-0 (TGI 14.08 $\mu g.mL^{-1}$). Fraction FG showed weak and moderate activities towards U251 (TGI 11.14 $\mu g.mL^{-1}$) and OVCAR-03 (TGI 9.32 $\mu g.mL^{-1}$), respectively. Fractions FD and FG were enriched in compounds with *m/z* 302 and 288, respectively, which after crystallization resulted in the compounds *ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*-8(14),15-pimaradien-3β-ol (**2**). The identities of these compounds were confirmed by ^{1}H and ^{13}C NMR spectral analysis and comparison with data reported in the literature (Matsuo *et al*., 1976; Ansell *et al*., 1993). Fraction FO presented two diterpene compounds with m/z of 288 and 302, eluting at retention times of 22.1 min and 25,1 min, respectively. The fragmentation patterns revealed *m/z* 288, 273, 255, 187, 135 (100%), and 91, the other compounds presented *m/z* 302, 284, 121,105 and 91 (100%) and 79. These compounds differed from compounds **2** and **1**.

Fraction D, containing only *ent*-pimara-8(14),15-dien-19-oic acid (**1**), presented mean TGI of 38.84 $\mu g.mL^{-1}$, which was lower than for CE, but higher than for F3 and F4. In this case, the mean activity and the specific activity towards the NCI-ADR/RES cell line (TGI 17.86 $\mu g.mL^{-1}$) were close to the values observed for CE (Table Nº 1). Compound **1** was first described by Mihashi *et al*. (1969), who isolated it from *Aralia cordata*, and later by Matsuo *et al*. (1976), who isolated it from the methanolic extract of *Jungermannia thermarum*. This compound presents *in vitro* anti-inflammatory activity by inhibiting the induction of inflammatory mediators, shows schistosomicidal activity against *Schistosoma mansoni*, and inhibits the contraction of vascular smooth muscle (Kang *et al*., 2008; Hipolito *et al*., 2009). Another study found that this compound was effective in reducing the genotoxicity induced by doxorubicin and methyl methanesulfonate, indicating its chemopreventive action (Kato *et al*., 2012).

Fraction G, containing *ent*-8(14),15-pimaradien-3β-ol (**2**), showed higher antiproliferative activity (mean TGI 24.83 $\mu g.mL^{-1}$) compared to CE, the source fractions (F3 and F4), or compound **1**. Fraction G was more selective towards the ovarian tumor (OVCAR-3, TGI 9.32 $\mu g.mL^{-1}$), glioma (U251, TGI 11.14 $\mu g.mL^{-1}$), and colon (HT29, TGI 15.61 $\mu g.mL^{-1}$) cell lines. Compound **2** was first isolated from *Erythroxylum cuneatum* and subsequently from *Viguiera arenaria* (Ansell *et al*., 1993).

The *in vitro* antiproliferative activities observed in this study suggest that in addition to the participation of compounds **1** and **2** in the action of fractions F3 and F4, other minor substances may contribute to the antiproliferative effect, especially in the case of the NCI/ADR-RES cell line.

Evaluation of the effects of CE and fractions F3, F4, FD, FG, and FO on the non-tumor cell line (VERO) revealed the same profile observed for the other cell lines (Table No. 1), indicating effects on the proliferation of non-tumor cells. The TGI values observed for CE, FD, and FO (ranging from 35.56 to 59.87 $\mu g.mL^{-1}$) were higher than the value for doxorubicin (20.88 $\mu g.mL^{-1}$), indicative of lower cytotoxicity. On the other hand, F4 and FG showed higher cytotoxicity, with TGI values of 7.63 and 12.45 $\mu g.mL^{-1}$, respectively. This profile followed the same trend found for doxorubicin, suggesting that these compounds might interfere in the proliferation of neoplastic tissue *in vivo*, similarly to other chemotherapeutic drugs widely used in clinical medicine (de Almeida *et al*., 2005).

The findings demonstrated that these pimarane compounds strongly affected tumor cell growth, with cytostatic action towards all cell lines evaluated, revealing prototypes for new molecules active against cancers.

The chloroformic extract and fractions of *A. arenaria* showed promising *in vitro* antiproliferative activity towards virtually all the cell lines tested, with concentration-dependent activity profiles. The fractionation of CE potentiated the antiproliferative action, notably in the case of fractions 3 and 4, which were enriched in *ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*-8(14),15-pimaradien-3β-ol (**2**). Fraction D (containing compound **1**) showed lower activity than fractions F3, F4, and FG (containing compound **2**), but was more selective towards the ovary expressing a multidrug-resistant phenotype cell line (NCI/ADR-RES). Fraction FG (**2**) was especially active towards the ovary (OVCAR-3), glioma (U251), and colon (HT29) cell lines, while fraction FO was more active towards the NCI/ADR-RES and kidney (786-0) cell lines. Among the results obtained, it should be highlighted that the cell lines more susceptible to these samples are related to hormone-dependent cancers, such as those of the ovary and colon.

As a conclusion, the results reported here showed promising *in vitro* antiproliferative action of the fractions obtained from the chloroform extract of the *Aldama arenaria* roots. The *ent*-pimara-8(14),15-dien-19-oic acid and *ent*-8(14),15-pimaradien-3β-ol present in these fractions can be considered as potential prototypes for the discovery of new therapeutic agents.

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REFERENCES

Adams RP. 2007. **Identification of essential oil components by Gas Chromatography/Mass Spectrometry**. Allured Publishing Corporation, USA.

Ambrosio SR, Arakawa NS, Esperandim VR, Albuquerque S, da Costa FB. 2008. Trypanocidal activity of pimarane diterpenes from *Viguiera arenaria* (Asteraceae). **Phytother Res** 22: 1423 - 1415. **https://doi.org/10.1002/ptr.2512**

Ambrosio SR, Schorr K, da Costa FB. 2004. Terpenoids of *Viguiera arenaria* (Asteraceae). **Biochem Syst Ecol** 32: 221 - 224. **https://doi.org/10.1016/s0305-1978(03)00139-x**

Ambrosio SR, Tirapelli CR, Bonaventura D, de Oliveira AM, da Costa FB. 2002. Pimarane diterpene from *Viguiera arenaria* (Asteraceae) inhibit rat carotid contraction. **Fitoterapia** 73: 484 - 489. **https://doi.org/10.1016/s0367-326x(02)00170-3**

Ansell SM, Pegel KH, Taylor DAH. 1993. Diterpenes from the timber of 20 *Erythroxylum* species. **Phytochemistry** 32: 953 - 959. **https://doi.org/10.1016/0031-9422(93)85235-j**

Bombo AB, Filartiga L, Garcia VL, Appezzato-da-Glória B. 2017. Secretory structures in *Aldama* species (Heliantheae–Asteraceae): morphology, histochemistry and composition of essential oils. **Flora** 228: 39 - 49. **https://doi.org/10.1016/j.flora.2017.01.011**

Block S, Baccelli C, Tinant B, van Meervelt L, Rozenberg R, Jiwan JH, Llabrès G, de Pauw-Gillet MC, Quetin-Leclercq J. 2004. Diterpenes from the leaves of *Croton zambesicus*. **Phytochemistry** 65: 1165 - 1171. **https://doi.org/10.1016/j.phytochem.2004.02.023**

Block S, Stévigny C, de Pauw-Gillet MC, de Hoffmann E, Llabrès G, Adjakidjé V, Quetin-Leclercq J. 2002. ent-Trachyloban-3b-ol, a new cytotoxic diterpene from *Croton zambesicus*. **Planta Med** 68: 2 - 4. **https://doi.org/10.1055/s-2002-32903**

De Almeida VL, Leitão A, Barrett Reina LDC, Montanari CA, Donnici CL, Lopes MTP. 2005. Câncer e agentes antineoplásicos ciclo-celular específicos e ciclo-celular não específicos que interagem com o DNA: Uma introdução. **Quim Nova** 28: 118 - 129. **https://doi.org/10.1590/s0100-40422005000100021**

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. **Int J Cancer** 136: 359 - 386. **https://doi.org/10.1002/ijc.29210**

Fouche G, Cragg GM, Pillay P, Kolesnikova N, Maharaj VJ, Senabe J. 2008. *In vitro* anticancer screening of South African plants. **J Ethnopharmacol** 119: 455 - 461. **https://doi.org/10.1016/j.jep.2008.07.005**

Ge YZ, Zhang H, Liu HC, Dong L, Ding J, Yue JM. 2014. Cytotoxic dinorditerpenoids from *Drypetes perreticulata*. **Phytochemistry** 100: 120 - 125. **https://doi.org/10.1016/j.phytochem.2014.01.013**

Grace MH, Jin Y, Wilson GR, Coates RM. 2006. Structures, biogenetic relationships, and cytotoxicity of pimarane-derived diterpenes from *Petalostigma pubescens*. **Phytochemistry** 67: 1708 - 1715. **https://doi.org/10.1016/j.phytochem.2005.09.026**

Hipólito UV, Rodrigues GJ, Lunardi CN, Bonaventura D, Ambrosio SR, de Oliveira AM. 2009. Mechanisms underlying the vasorelaxant action of the pimarane *ent*-8(14),15-pimaradien-3β-ol in the isolated rat aorta. **Eur J Pharmacol** 616: 183 - 191. **https://doi.org/10.1016/j.ejphar.2009.06.010**

Kang OH, Chae HS, Choi JG, Oh YC, Lee YS, Kim JH. 2008. *ent*-pimara-8(14),15-dien-19-oic acid isolated from the roots of *Aralia cordata* inhibits induction of inflammatory mediators by blocking NF-κB activation and mitogen-activated protein kinase pathways. **Eur J Pharmacol** 601: 179 - 185. **https://doi.org/10.1016/j.ejphar.2008.10.012**

Kato FH, Viana NI, Santini CB, de Souza CGG, Veneziani RCS, Ambrósio SR., Tavares DC. 2012. Assessment of the *in vitro* and *in vivo* genotoxic and antigenotoxic effects of pimaradienoic acid in mammalian cells. **Mutat Res** 749: 87 - 92. **https://doi.org/10.1016/j.mrgentox.2012.09.001**

Magenta MAG. 2006. ***Viguiera* Kunth (Asteraceae, Heliantheae) na América do Sul e sistemática das espécies do Brasil.** PhD thesis, Universidad de Sao Paulo, Sao Paulo, Brasil. **https://doi.org/10.11606/T.41.2006.tde-12032008-162008**

Matsuo A, Nakayama M, Hayash S, Yamasaki K, Kasai R, Tanaka O. 1976. (-)-Thermarol, a new *ent*-pimarane-class diterpene diol from *Jungermannia thermarum* (Liverwort). **Tetrahedron Lett** 28: 2451 - 2454. **https://doi.org/10.1016/0040-4039(76)90017-4**

Mihashi S, Yanagisawa I, Tanaka O, Shibata S. 1969. Further study on the diterpenes of *Aralia* spp. **Tetrahedron Lett** 10: 1683 - 1686. **https://doi.org/10.1016/s0040-4039(01)87979-x**

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. **J Nat Cancer Inst** 83: 757 - 766. **https://doi.org/10.1093/jnci/83.11.757**

Newman DJ, Cragg GM. 2016. Natural products as sources of new Drugs from 1981 to 2014. **J Nat Prod** 79: 629 - 661. **https://doi.org/10.1021/acs.jnatprod.5b01055**

Nogueira M, da Costa F, Brun R, Kaiser M, Schmidt T. 2016. *ent*-Pimarane and *ent*-Kaurane diterpenes from *Aldama discolor* (Asteraceae) and their antiprotozoal activity. **Molecules** 21: 1237. **https://doi.org/10.3390/molecules21091237**

Oliveira ASS, Rehder VLG, Ruiz ALTG, Longato GB, Carvalho JE, Oliveira TS, Appezzato-da-Glória B, Imamura PM. 2013. **Atividade antiproliferativa *in vitro* do óleo essencial das folhas e frações de *Aldama arenaria* Baker (sin. *Viguiera arenaria*)**. I Simpósio Iberoamericano de Investigação em Câncer, Campinas, Brasil.

Oliveira ADSS, Rehder VLG, Ruiz ALTG, Longato G, Carvalho JE. 2014. *In vitro* antiproliferative evaluation of underground organs essential oil and fractions of *Aldama arenaria* Baker. **Planta Med** 80: P2O17. **http://doi.org/10.1055/s-0034-1395010**

Porto TS, da Silva Filho AA, Magalháes LG, dos Santo SRA, Furtado NAJC, Arakawa NS. 2012. Fungal transformation and schistosomicidal effects of pimaradienoic acid. **Chem Biodiver** 9: 1465 - 1474. **https://doi.org/10.1002/cbdv.201100336**

Porto TS, Furtado NAJC, Heleno VCG, Martins CHG, da Costa FB, Severiano ME. 2009, Antimicrobial *ent*-pimarane diterpenes from *Viguiera arenaria* against Gram-positive bacteria. **Fitoterapia** 80: 432 - 436. **https://doi.org/10.1016/j.fitote.2009.06.003**

Rayanil KO, Limpanawisut S, Tuntiwachwuttikul P. 2013. *ent*-pimarane and *ent*-trachylobane diterpenoids from *Mitrephora alba* and their cytotoxicity against three human cancer cell lines. **Phytochemistry** 89: 125 - 130. **https://doi.org/10.1016/j.phytochem.2013.01.012**

Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P. 1990. Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. **J Nation Canc Inst** 82: 1113 - 1117. **https://doi.org/10.1093/jnci/82.13.1113**

Schilling EE, Panero JL. 2002. A revised classification of subtribe Helianthinae (Asteraceae: Heliantheae). I. Basal lineages. **Bot J Linn Soc** 140: 65 - 76. **https://doi.org/10.1046/j.1095-8339.2002.00079.x**

Shoemaker RH. 2006. The NCI60 human tumour cell line anticancer drug screen. **Nat Rev Cancer** 6: 813 - 823. **https://doi.org/10.1038/nrc1951**

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. **J Nation Canc Inst** 82: 1107 - 1112. **https://doi.org/10.1093/jnci/82.13.1107**

WHO (World Health Organization). 2014. **Global status report on noncommunicable diseases World Health**, **http://www.who.int**

WHO (World Health Organization). 2019, **Cancer Report**, **http://www.who.int**